1-1-2017

Synergistic Effect Of Natural Antimicrobials Produced From Food On Foodborne Pathogens And Effect Of Meat Production Method On Quality And Shelf Life Of Meat

Mahdi Hassan Al-Ali
Wayne State University,

SYNERGISTIC EFFECT OF NATURAL ANTIMICROBIALS PRODUCED FROM FOOD ON FOODBORNE PATHOGENS AND EFFECT OF MEAT PRODUCTION METHOD ON QUALITY AND SHELF LIFE OF MEAT

by

MAHDI HASSAN HUSSAIN AL-ALI

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2017

MAJOR: NUTRITION AND FOOD SCIENCE

Approved By:

_____

Advisor   Date

_____

_____

_____

_____

DEDICATION

This dissertation is dedicated to

MY FAMILY

For their love, support, and patience

TABLE OF CONTENTS

CHAPTER 4: EFFECT OF MEAT PRODUCTION METHOD ON SHELF LIFE OF MEAT DURING REFRIGERATED STORAGE

LIST OF TABLES

LIST OF FIGURES

OVERVIEW AND SPECIFIC AIMS

The increased usage of foods formulated with chemical preservatives such as nitrites, benzoates, sulfites, sorbates, salt and other chemical compounds has raised consumer health concerns (Murdock et al. 2006). As a result, the use of natural antimicrobial preservatives have been preferred by the food industry due to consumers' demands (DuFour *et al*. 2003). Nisin, lactoferrin and lysozyme are three natural compounds that have received considerable attention in recent years (Murdock and Matthews 2002). Properties of these three sources of natural antimicrobials can be used to improve microbial safety of foods. The combination of natural antimicrobials could possibly increase the effectiveness of the antimicrobials proving to be better than if they were used alone against foodborne pathogens. Synergistic or additive effects between the antimicrobials may permit the use of relatively low amounts of each antimicrobial and thereby reduce cost of the antimicrobial treatment while improving the antimicrobial efficiency.

Nisin is produced by *Lactococcus lactis* subsp. *lactis*. It is effective against gram-postive bacteria and with reduced or no effectiveness against gram-negative bacteria (Delves-Broughton et al, 1996). Nisin mechanism of action is to bind the cell membrane and form pores that destroy membrane integrity. Pore formation leads to leakage of K^+ ions and ATP, depletion of the proton motive force, and depolarization of transmembrane potential, resulting in cell death (Millette *et al.*, 2004). It has also been reported that nisin interferes with cell wall biosynthesis by binding with lipid II, a peptidoglycan precursor (Bauer and Dicks, 2005).

Lysozyme, a lytic enzyme found in foods such as milk and eggs. Like nisin, lysozyme's activity is also limited to gram-positive bacteria because gram-negative cell walls are protected

by an outer membrane (Proctor and Cunningham, 1988). Lysozyme's mechanism of action based on hydrolysis of the β (1–4) glycosidic linkages in bacterial peptidoglycan within the cell wall (Cunningham et al., 1991).

Lactoferrin, an iron-binding glycoprotein, is natural antimicrobial component of milk and other external secretions such as tears and saliva (Murdock and Matthews 2002); (Farnaud and Evans 2003). Lactoferrin exerts an antimicrobial effect against a wide range of gram-negative and gram-positive bacteria, fungi, and parasites (Shimazaki 2000; Masschalck *et al*. 2001). Its actions can be classified into several different modes of action: by sequestering free iron, thereby restricting the growth of gram-positive and gram-negative bacteria; interfering with bacterial membrane function; and by binding bacterial lipopolysaccharide, thereby impairing bacterial cell wall/membrane function (IUCCI *et al*. 2007).

The lack of activity by nisin and lysozyme against gram-negative organisms is because of their inability to penetrate the cell wall. However, in the presence of lactoferrin, general chelator, has the ability to release lipopolysaccharide (LPS) and permeabilize the gram-negative cell wall. This permits nisin and lysozyme to have access to the inner membrane thus providing a novel system for control of gram-negative pathogens such as *E. coli* O157:H7 (Ellison, 1994, and Branen and Davidson, 2004).

There are different types of meat classifications based on the method of animal slaughtering and processing. Halal meat is produced without stunning prior to slaughter, resulting in a rapid and complete bleeding of animals (Eliasi and Dwyer, 2002); (Grandin and Regenstein, 1994). Studies have shown that halal slaughter results in significantly lower residual hemoglobin than conventional slaughter with stunning (non-Halal) (Nakyinsige *et al*, 2014). The

residual blood left in the carcass as a result of improper bleeding may decrease the shelf life; hence the quality of the meat product due to higher hemoglobin levels. Hemoglobin is an important component of blood which is powerful promoter of lipid oxidation and growth of microorganisms (Alvarado *et al* 2007). Also, glucose in the blood serves as substrate favorable for microbial growth such as *Pseudomonas* bacteria (Warriss, 2001). In addition, blood also contains a large number of white blood cells that produce hydroxyl radicals, superoxides, and hydrogen peroxide that enhance lipid oxidation (Gabig & Babior, 1981). This approach may improve the quality and extend the shelf life of meat.

Storage of meat is critical because it is highly perishable food. It contains sufficient nutrient needed to support the growth of microorganisms (Magnus, 1981). During storage, even with proper refrigeration, the meat ultimately undergoes deteriorative changes including microbial spoilage, oxidative changes in pigments and lipids, and weight loss (Urbain and Campbell, 1987). Nakyinsige et al, (2014) reported that one of the meat quality attributes obtained in the carcass with less blood is extending the shelf life and providing a safer meat for human consumption.

The objective of this research is to investigate the following aims:

Specific Aim 1: To test the synergistic effect of combinations of different natural antimicrobials that produced from food (Lactoferrin, Lysozyme and Nisin) on positive and negative gram foodborne pathogens (*Salmonella Typhimurium*, *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus*) *in-vitro* and *in-vivo* (meat).

Specific Aim 2:   To determine the molecular characterization of toxin genes and antibiotic resistance of *S. aureus* isolated from meat.

Specific Aim 3:   To determine the microbial content and physiochemical quality indicators differences between fresh halal and non-halal beef.

Specific Aim 4:   To test effect of meat production method (halal and non-halal) on shelf life of beef during refrigerated storage at 4°C.

CHAPTER 1   THE SYNERGISTIC EFFECT OF NATURAL ANTIMICROBIALS PRODUCED FROM FOOD LACTOFERRIN, LYSOZYME AND NISIN ON FOODBORNE PATHOGENS

 Abstract

The aim of this study was to evaluate the synergistic effect of three natural antimicrobial that are produced from food (lactoferrin from milk, lysozyme from eggs and nisin from *Lactococcus lactis*) on foodborne pathogens that include *S. aureus*, *E. coli, salmonella typhimurium* and *listeria monocytogenes in-vitro* and *in-vivo* (meat). Minimal inhibitory concentration (MIC) for each antimicrobial was measured by broth micro dilution method in cation-adjusted Mueller-Hinton II broth (CAMHB) whereas Synergy between antimicrobial was calculated using the fractional inhibitory concentration (FIC) index which was measured using the checkerboard method.

Antimicrobial activity of lactoferrin, lysozyme and nisin individually *in-vitro* showed that MICs for nisin against tested bacteria were the lowest while lysozyme's MICs were relatively less. FICs for antimicrobial combinations showed that five combinations exhibited synergistic effect as the following: lactoferrin with nisin against *S. aureus, L. monocytogenes*, and *E. coli*. Lactoferrin with lysozyme exhibited synergistic effect against *E. coli* and *S. aureus*. In addition, four combinations showed additive effect as following: lactoferrin with nisin against *L. monocytogenes*, *S. aureus* and *E. coli*, and lactoferrin with lysozyme against *L. monocytogenes*. Using antimicrobial combinations against tested pathogens bacteria demonstrated improved (MIC), compared to the parent compounds.

Antimicrobial activity in food model-meat (*in-vivo*) showed that lactoferrin, lysozyme and nisin individually and in combinations had significant effect (p<0.01) on growth of gram-positive bacteria, *L. monocytogenes* and *S. aureus* compared to control. On the other hand, nisin and lysozyme individually had less effect on the growth of gram-negative bacteria, *S. typhimurium*

and *E. coli*. Conversely, when nisin or lysozyme used in combinations with lactoferrin proved enhanced nisin and lysozyme activity against *E. coli*.

According to results of the present study, we concluded that using natural antimicrobial combinations of lactoferrin with lysozyme, lactoferrin with nisin and lysozyme with nisin can synergistically function against foodborne pathogens in meat industry and may be a novel system to control foodborne pathogens and offer unique properties.

Introduction

The control of microbial pathogens in food is a significant concern because of increase in foodborne illnesses cases around the world in spite of the various methods available to control pathogen and spoilage bacteria in food. The Center for Disease Control and Prevention estimated that foodborne diseases causes approximately 76 million illnesses, 325,000 hospitalizations and 5000 deaths in the United States each year (Mead et al., 1999). There are numerous antimicrobials in plants, animals, and microorganisms where they often take part in defense mechanisms. Nisin, lactoferrin and lysozyme are three natural compounds that have received considerable attention in recent years (Murdock and Matthews, 2002). Properties of these three sources of natural antimicrobials can be used to improve microbial safety of foods. Synergistic effect of these natural antimicrobials in meat and food industry may be a novel system to control pathogenic bacteria and offer unique properties. These such properties increase the effectiveness of the antimicrobials, proving to be more effective rather than if they were used alone against foodborne pathogens. Moreover, these antimicrobial synergistically decrease the amount and cost for antimicrobial treatment and eliminate the harmful chemical effects while improving the efficacy.

Nisin is produced by *Lactococcus lactis* subsp. *lactis*. Its effective against gram-positive bacteria and with reduced or no effectiveness against gram-negative bacteria (Delves-Broughton *et al,* 1996). Its mechanism of action is to bind the membrane and form pores that destroy the membrane integrity. Pore formation leads to leakage of K^+ ions and ATP, depletion of the proton motive force, and depolarization of transmembrane potential resulting in cell death (Millette *et al.*, 2004). It has also been reported that nisin interferes with cell wall biosynthesis by binding with lipid II, a peptidoglycan precursor (Bauer and Dicks, 2005).

Lysozyme is a lytic enzyme found in foods such as milk and eggs. Like nisin, lysozyme's activity is also limited to gram-positive bacteria because gram-negative cell walls are protected by an outer membrane (Proctor and Cunningham, 1988). Its mechanism of action is the hydrolysis of the β (1–4) glycosidic linkages in bacterial peptidoglycan of cell wall (Cunningham *et al.,* 1991).

Lactoferrin, an iron-binding glycoprotein, is natural antimicrobial component of milk and other external secretions such as tears and saliva (Murdock and Matthews 2002; Farnaud and Evans 2003). Lactoferrin exerts an antimicrobial effect against a wide range of gram-negative and gram-positive bacteria, fungi, and parasites (Shimazaki 2000; Masschalck *et al.* 2001). Its actions can be classified into several different modes of action: by sequestering free iron, thereby restricting the growth of gram-positive and gram-negative bacteria; by interfering with bacterial membrane function; and by binding bacterial lipopolysaccharide, thereby impairing bacterial cell wall/membrane function (IUCCI *et al.* 2007).

The lack of activity by nisin and lysozyme against gram-negative organisms is because of their inability to penetrate the cell wall. However, in the presence of lactoferrin, a general

chelator, has the ability to release lipopolysaccharide (LPS) and permeabilize the gram-negative cell wall allowing nisin and lysozyme to have access to the inner membrane and thus providing a novel system for control of gram-negative pathogens, such as *E. coli* O157:H7 (Ellison 1994; Branen and Davidson 2004).

This chapter is aimed to measure the synergistic effect of three natural antimicrobials, Lactoferrin (from milk), lysozyme (from egg) and nisin (from Lactococcus lactis bacteria) on foodborne pathogens that include *S. aureus*, *E. coli, salmonella typhimurium* and *listeria monocytogenes in-vitro* and *in-vivo* (meat).

Nisin:

Nisin was discovered in 1928 by Rogers and Whittier. It was produced by Lactococcus lactis subsp. lactis, which is a heat-stable protein (Holzapfel et al, 1995) and was named bacteriocins. It was produced as a protection mechanism to some lactic acid bacteria (LAB) (Juncioni de Arouz et al., 2009). Many companies have been producing nisin as antimicrobial against different types of bacteria since the 1960's synthetically or naturally by using some bacterial culture in fermented dairy (Bailey and Hurst, 1971; Lee and Kim, 1985). Since then, it has effectively been used as preservative in dairy and meat products (Reunanen and Saris 2004; Samelis et al. 2005). Food and Drug Administration (FDA) approved nisin as GRAS (Generally Regarded as Safe) for use in some dairy product and salad dressing (Anonymous 2000; Cleveland et al. 2001).

Nisin is a single-chained molecule consist of a 34 amino acid with molecular weight 3500Da. It possess two important properties cationic and hydrophobic. The cationic properties because the mixture of three lysine residues and one or more histidine residues (Chandrapati &

O'Sullivan, 1998). It has important functional properties such as acid tolerance and thermal stability at low pH due to it containing distinguished amino acid (De Vuyst & Vandamme, 1992). In addition, Nisin presents amphipathic properties because its N-terminal region contain hydrophobic residues and the C-terminal region contain hydrophilic residues.
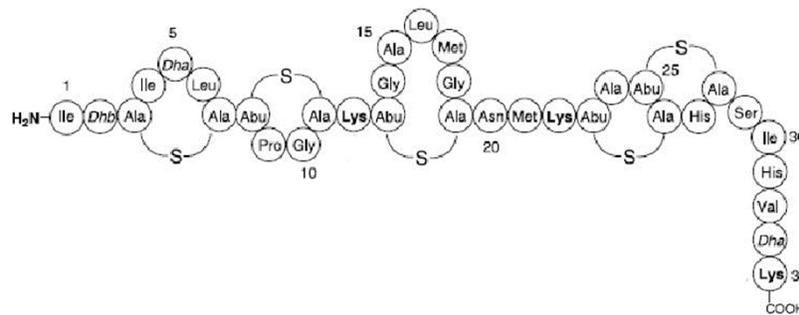
Figure 1.1.   Structure of Nisin

There are two types of nisin depending on type of the amino acid residue in position 27, Nisin A, a natural variant produced by L. lactis subsp. lactis ATCC 11454 and the amino acid histidine is located at position 27 while Nisin Z is produced by other species of L. lactis and the amino acid, asparagine, is located at position 27. This change give a good characteristic of diffusion in the nisin Z but there is no differences in antimicrobial activity. However, asparagine contains a side chain with a higher polarity than histidine allowing Nisin Z to be better soluble at a pH of 6 or above.

Nisin bacteriostatic function is due to its ability to bind the cytoplasmic membrane and form pores that damage the membrane (Abee et al., 1995; Kuwano et al., 2005). This induces cell death due to loss of different important cell compounds such as ATP, some ions like K^+, and the proton motive force (Sahl, 1991; Bruno and Montville, 1993; Millette et al., 2004). Also, many studies have shown that the antimicrobial actions of nisin may be attributed to interferes with

cell wall biosynthesis by binding with lipid II, an important compound in a peptidoglycan layer (Mantovani and Russell, 2001; Bauer and Dicks, 2005).
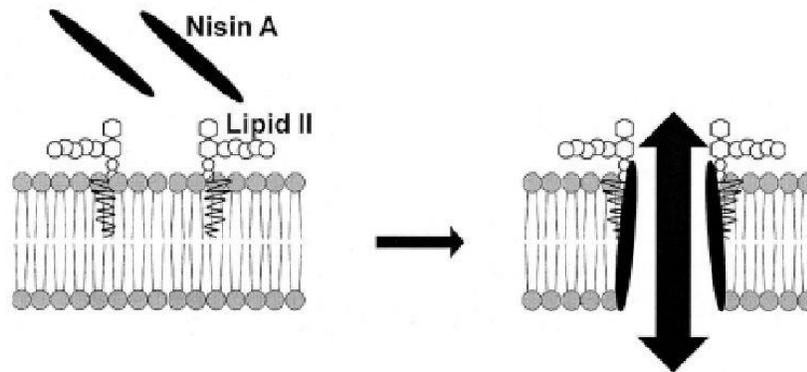
Figure 1.2.   Antimicrobial Mechanism of Nisin

Many studies indicated that cell resistance to nisin depend on cell content of lipid II .The high levels of lipid II the high resistance to nisin (Brotzel et al., 1998).

Lactoferrin

Lactoferrin, a single-chained molecule with a molecular weight of 78 kDa, is a member of the transferrin protein family contains two lobes with four domains that assist the reversible binding of two iron ions (Odell et al. 1996; Ye et al. 2000). Lactoferrin is a natural bio-preservative that is found in many mammalian secretions, such as milk, tears, saliva (Odell et al. 1996; Ye et al. 2000). It is also thought to be one of the most powerful antimicrobial agents in milk (Bellamy et al. 1993; Chantaysakorn and Richter 2000).   The possible use of bovine lactoferrin for decontamination on the surface of beef carcasses, and subsequently its use as a natural food preservative is gaining importance (Al-Nabulsi and Holley 2006).
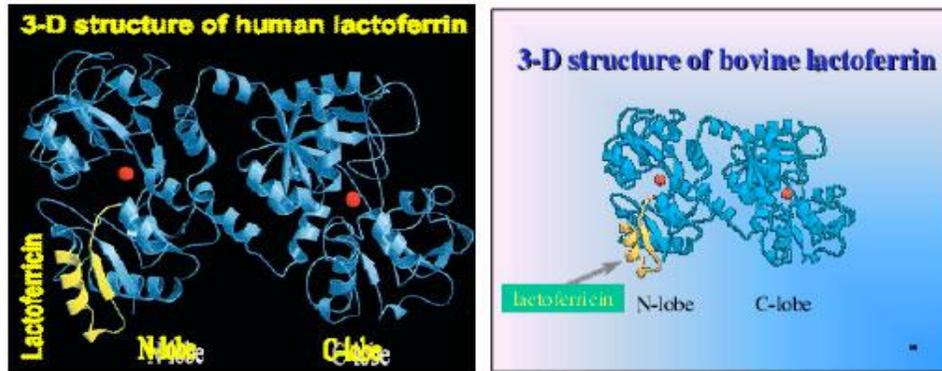
Figure 1.3. Three dimensional structures of diferric human Lactoferrin and bovine lactoferrin.

Bacteriostatic function of lactoferrin is due to its ability to take up the Fe^{3+} ion, limiting use of this nutrient by bacteria at the infection site and inhibiting the growth of these microorganisms as well as the expression of their virulence factors, lactoferrin bactericidal function has been attributed to its direct interaction with bacterial surfaces. The positively charged N-terminus of lactoferrin prevents the interaction between LPS and the bacterial cations (Ca^{2+} and Mg^{2+}), causing a release of LPS from the cell wall, an increase in the membrane's permeability and ensuring damage to the bacteria. In 1988 it was shown that lactoferrin damages the external membrane of gram-negative bacteria through an interaction with lipopolysaccharide (LPS).
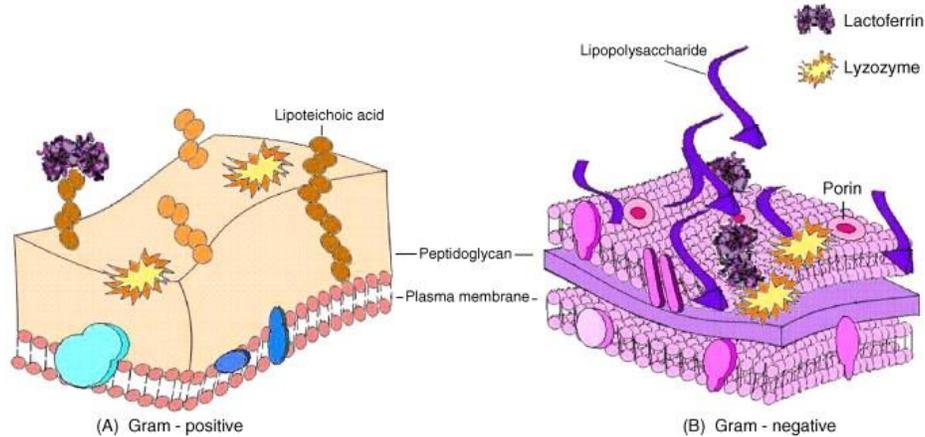
Figure 1.4. Mechanism of antibacterial action of lactoferrin. (A) Gram-positive bacteria: LF is bound to negatively charged molecules of the cell membrane such as lipoteichoic acid, neutralizing wall charge and allowing the action of other antibacterial compounds such as lysozyme. (B) Gram-negative bacteria: LF can bind to lipid A of lipopolysaccharide, causing liberation of this lipid with consequent damage to the cell membrane.

It's mechanism of action against gram-positive bacteria is based on binding due to its net positive charge to anionic molecules such as lipoteichoic acid, resulting in a reduction of negative charge on the cell wall and thus favoring contact between lysozyme and the underlying peptidoglycan over which it exerts an enzymatic effect. Attachment-inhibiting mechanisms are unknown, but it has been suggested that lactoferrin oligo-amino acid glycan's bind bacterial adhesins, preventing their interaction with host cell receptors.

Today, it can be obtained as native lactoferrin, isolated from the milk and colostrum of several mammals, or as recombinant lactoferrin (rLF) generated from bacterial, fungal and viral expression systems. The expression of this protein has also been attained in higher organisms such as plants and mammals.

Lysozyme:

Lysozyme is a lytic enzyme that is found in foods such as milk and eggs with hydrolytic

activity. It is a single polypeptide chain consisting of 129 amino acids with a molecular weight between 14, 300 - 14, 600 Daltons. The majority of commercially prepared lysozyme is purified from egg whites. Lysozyme is naturally occurring that is produced by many animals, and humans. It exhibits activity against a cellular structure specific to bacteria (Proctor & Cunningham, 1988). Lysozyme has a small number of applications in the food industry with the major usage involving the prevention of late blowing in semi-hard cheeses which is caused by the fermentation of lactate by butyric acid bacteria, primarily Clostridium tyrobutyricum (Cunningham et al, 1991; Branen and Davidson 2004).
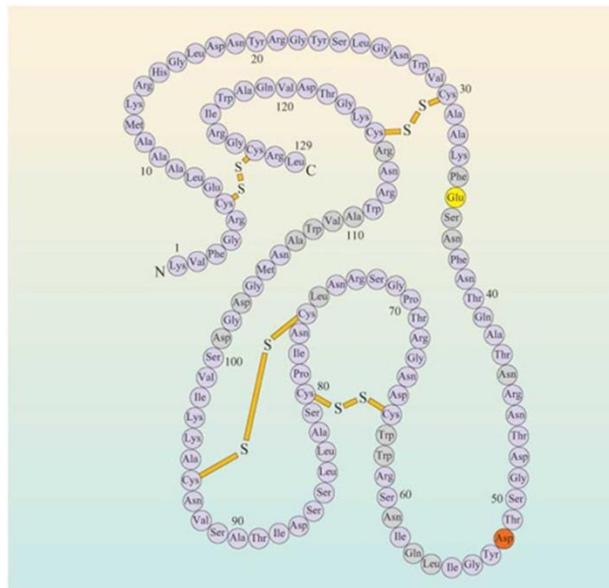
Figure 1.5. Structure of lysozyme

Lysozyme has been demonstrated to be active throughout pH 4 to pH 10 (Davies et al., 1969). However, high ionic strength (>0.2 M salt) was shown to have an inhibitory effect on lysozyme activity (Davies et al., 1969; Chang and Carr, 1971). The lysozyme molecule is cross-linked with four disulfide bonds, which are significant for its enzymatic activity. In order for lysozyme to remain enzymatically active, at least two disulfide bonds must remain intact.

However, if three or four disulfide bonds are broken within the lysozyme structure, lysozyme loses its activity. The activity spectrum of lysozyme is limited to specific gram-positive bacteria (McKenzie and White, 1991). They have been proven quite ineffective against gram-negative bacteria due to the outer membrane barrier that surrounds and protects the peptidoglycan layer. Among gram-negative bacteria, some genera such as Salmonella and Shigella are the most sensitive, whereas E. coli, Vibrio and Proteus are relatively resistant to lysozyme activity. Considering these results, the antimicrobial actions of lysozyme may be attributed to the direct and indirect interaction as well as bacteriolytic actions that destroy cell wall of microorganisms.

The susceptibility of gram-negative organisms to lysis by lysozyme can be increased by the use of detergents and chelators (Shively and Hartse, 1964). EDTA, a chelator, can have the antimicrobial effect by limiting the availability of cations and can act to destabilize the cell membranes of bacteria by complexing di-valent cations which act as salt bridges between membrane macromolecules, such as lipopolysaccharides (Shelef & Seiter, 1993).

Antimicrobial mechanism of lysozyme against bacteria is based on the hydrolysis of the β 1-4 glycosidic bonds in the peptidoglycan layer of the bacterial cell wall between N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM). Phillips (1973) studied hydrolysis of peptidoglycan by lysozyme. He hypothesized that lysozyme attaches to the bacterial cell wall by interacting with six of the residues connected to NAM. The attachment changes the conformation of the NAM structure, allowing the Glu 35 residue of the lysozyme molecule to donate a hydrogen atom to the oxygen atom in the glycosidic bond, resulting in cleavage of the bond.
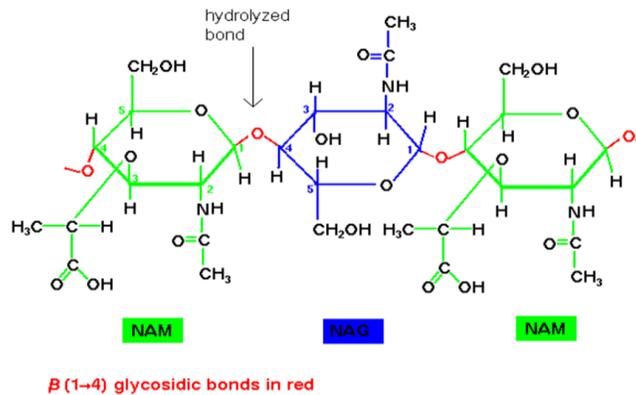
Figure 1.6. β 1-4 glycosidic bonds in the peptidoglycan layer of the bacterial cell wall

Materials and methods

A) **Bacterial cultures:**

The cultures used in this study were *Escherichia coli* (ATCC 25922), *Listeria monocytogens* (ATCC 19114), *Salmonella Typhimurium* (ATCC 14028) and *Staphylococcus aureus* (ATCC 29213). All cultures were obtained from American type culture collection (ATCC) Thermo Fisher Company. Cultures were maintained on tryptic soy agar (TSA) slants and were transferred each month to maintain viability. Test inoculums were prepared by transferring 24-hour old cultures via a cotton swab to 5 ml of 0.85% saline. The saline suspension was adjusted to an optical density of 0.1 for each bacteria which is corresponds to 0.5 McFarland standard (1×10^8 Cfu/ml). Once standardized, 50 µl of the saline suspension was transferred to 10 ml of cation-adjusted Mueller-Hinton II broth (CAMHB).

B) **Antimicrobials:**

Nisin from *Lactococcus lactis* (ssp. *lactis*) was obtained from Sigma-Aldrich (N5764). Nisin was solubilized in 0.02 M HCl with heating (60 – 70 °C) to aid solubilization. Lactoferrin was

obtained from Sigma-Aldrich (L9507). Lactoferrin was dissolved in 0.05 M phosphate buffer (pH 7.5). Lysozyme was obtained from thermo scientific company.

C) Determination of minimum inhibitory concentration (MIC):

The MIC for each antimicrobial was tested by broth micro dilution procedure according to the National Committee of Clinical Laboratories Standards (NCCLS) guidelines (Jorgensen, 1993).The concentration of each antimicrobial was diluted in CAMBH to 10.000 µg/ml and 100 µl of each antimicrobial was added into the first row of a 96-well plate and 50 µl of CAMHB was added to each subsequent row. Then each antimicrobial was serially diluted to obtain final concentration of 5000, 2500, 1250, 625, 312, 156, 78, 39 µg/ml. To each well, 50 µl of standardized inoculum was added, giving a bacterial concentration of $5x10^5$ CFU/ml. A positive control (no antimicrobial) and negative control (no inoculum) were included in each 96-well plate. Plates were incubated for 24 hours at 37°C and observed after 24 hours. MIC was tested as the lowest concentration showing inhibition of bacterial growth.

D) Determination of synergy:

Synergy between antimicrobial was tested using the checkerboard method (Moody, 1992; Schelz et al.2006). Antimicrobial (A) was diluted along the x-axis, while antimicrobial (B) was diluted along the y-axis. The final volume in each well was 100 µl, including 50 µl of antimicrobial dilution and 50 µl of bacteria standardized in CAMHB. Plates were incubated at 37°C for 24 hours. The fractional inhibitory concentration (FIC) indices were calculated as $FIC_A + FIC_B$, where FIC_A and FIC_B are the respective MIC of antimicrobial A and B. Therefore FICs were calculated as:

$$FICA = \frac{\text{MICA Combination}}{\text{MICA Alone}} \qquad FICB = \frac{\text{MICB Combination}}{\text{MICB Alone}}$$

The combination was considered synergistic if the sum of the FICs were equal to or less than 0.5. If the values were between 0.5 – 1.0, 1.0 – 4.0 or higher than 4.0, they were consider additive, indifferent or antagonistic respectively.

E) **Treatment of beef sample:**

Experimental meat samples were aseptically collected from various markets situated in Detroit, Michigan. The procedure of (Kim et al, 2007) was followed to prepare and process the meat samples that were divided into similar pieces and weighed approximately 5 g. The meat sample were then sterilized individually by irradiation using a UV chamber (GS Gene Linker UV chamber-BIO RAD) and inoculated with the bacteria standardized in CAMHB. Furthermore, meat samples were treated with a twofold concentration of the individual *in-vitro* MIC of the antibacterial that expressed synergism. Then meat pieces were stored in refrigerator temperature (4°C) in 60 mm Petri dishes. Samples were prepared for day 0, day 2, day 4 and day 6 for each bacterial treatment. Bacterial count for each bacteria were carried out where 45 ml of 0.1% sterilized peptone water was mixed vigorously for 60 seconds at 230 rpm with each sample by stomacher bag. 1 ml of the solution was taken and serially diluted from 10^{-1} to 10^{-5}. Then 1 ml of each dilution was inoculated to duplicate sterile plate of TSA and incubated at 37°C for 24 hours. After the incubation period, colonies were counted by colony counter. The bacterial count was multiplied by the dilution factor then transformed to Log CFU/g.

F-Statistical analysis:- All experiments were conducted in triple ANOVA (Analysis of Variance) and Tukey's test were used (IBM SPSS statistics 23).

Table 1.1. Minimal inhibitory concentrations (MIC) for nisin, lysozyme and lactoferrin against foodborne pathogens bacteria

Foodborne Pathogens	Nisin (µg /ml)	Lysozyme (µg /ml)	Lactoferrin (µg /ml)
L.monocytogene ATCC 19114	312	625	1250
E. Coli ATCC25922	625	1250	1250
S. aureus ATCC 29213	312	625	1250
Salmonella Typhimurium ATCC 14028	1250	2500	1250

Table 1.2. Fractional inhibitory concentration (FIC) for nisin, lysozyme and lactoferrin against foodborne pathogens (*in-vitro*).

Foodborne Pathogens	Nisin + Lactoferrin	Lysozyme + Lactoferrin	Nisin + Lysozyme
L. Monocytogene ATCC19114	0.49 S	0.99 A	0.74 A
E. Coli ATCC25922	0.37 S	0.49 S	0.99 A
S. aureus ATCC29213	0.31 S	0.49 S	0.74 A
Salmonella Typhimurium ATCC 14028	1.24 I	1.24 I	1.50 I
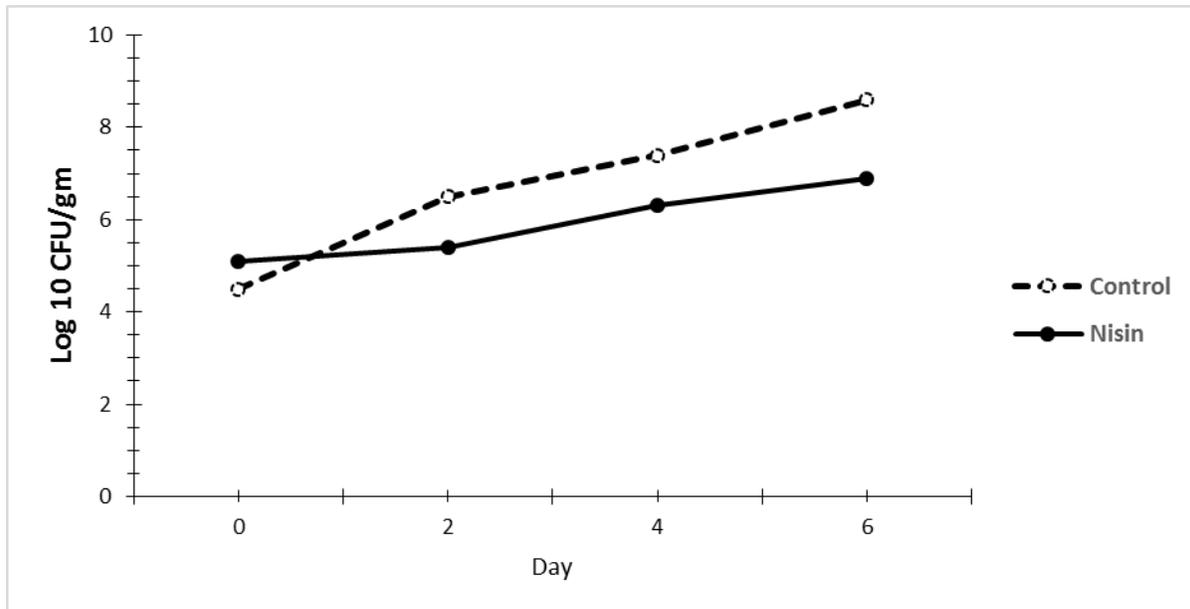
Nisin at 625 µg/ml significantly reduced *L.monocytogens* by 2.2 Log in day 6

Figure **1.7.** Antimicrobial effect of nisin at 625 µg /ml on *L. monocytogens* ATCC 19114 growth in beef in comparison to control at 4 °C.A data represent the mean values of *L. monocytogenes* ATCC 19114 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *L. monocytogenes* a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of p≤0.05.
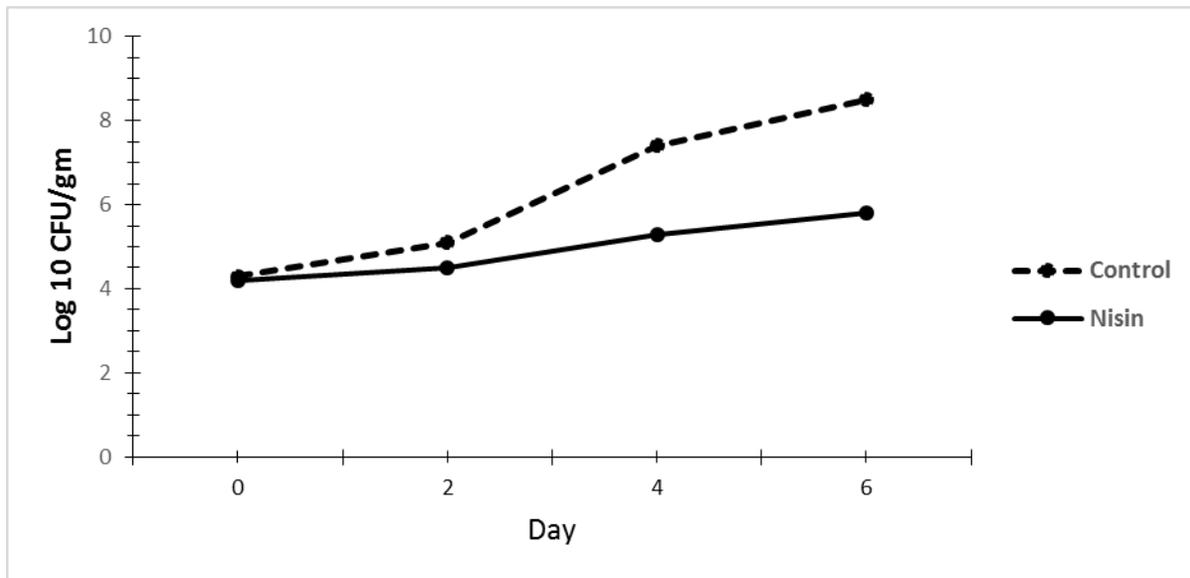
Nisin at 625 µg/ml significantly reduced *S. aureus* by 2.6 Log in day 6

Figure **1.8.** Antimicrobial activity of nisin at 625 µg /ml on *S. aureus* ATCC 29213growth in beef in comparison to control at 4°C.A data represent the mean values of *S. aureus* ATCC 29213a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *S. aureus* ATCC 29213a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of $p \leq 0.05$.
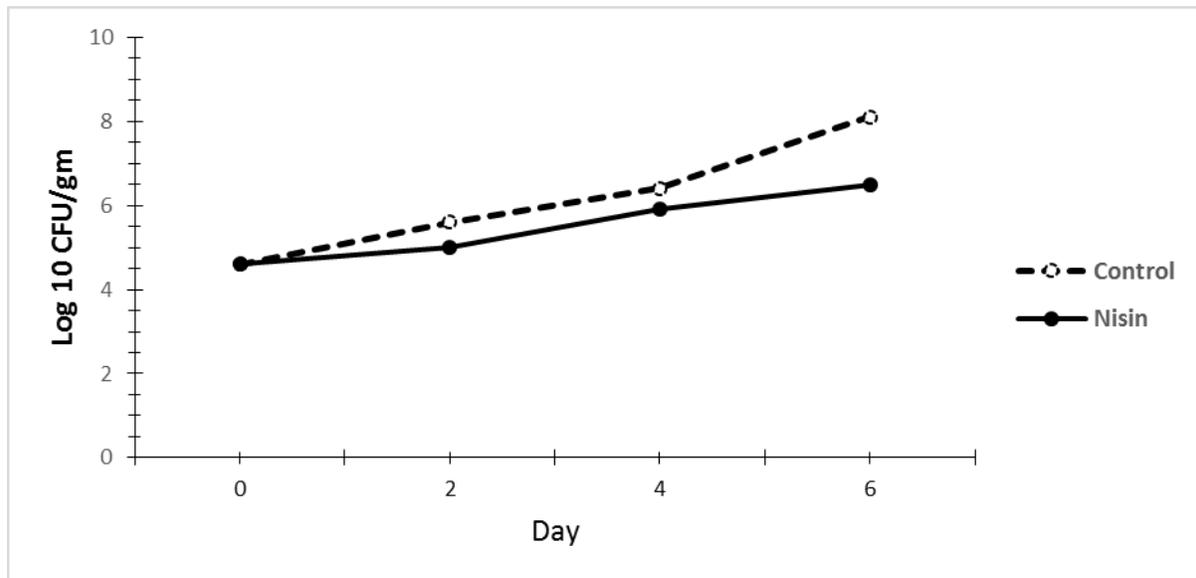
Nisin at 1250 µg/ml significantly reduced *E. coli* by 1.6 Log in day 6

Figure **1.9.** Antimicrobial activity of nisin at 1250 µg /ml on *E. coli* ATCC 25922growth in beef in comparison to control at 4 °C.A data represent the mean values of *E. coli* ATCC 25922 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *E. coli* ATCC 25922 a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of p≤0.05.
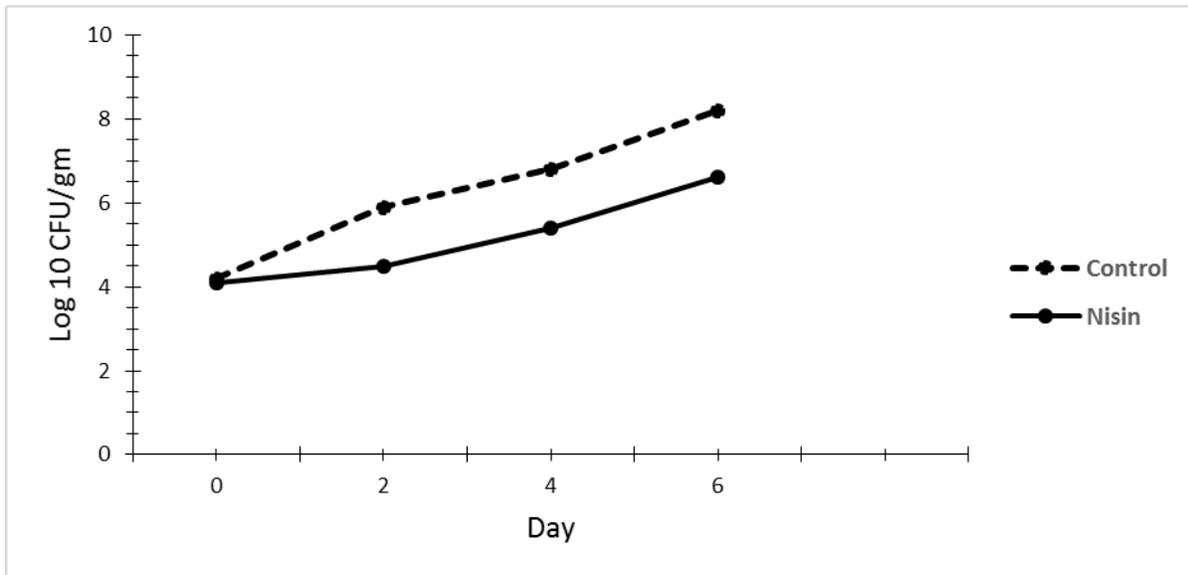
Nisin at 2500 µg/ml significantly reduced *Salmonella Typhimurium* (ATCC 14027) by 1.5 Log in day 6.

Figure **1.10.** Antimicrobial activity of nisin at 2500 µg /ml on *Salmonella Typhimurium* ATCC 14028 growth in beef in comparison to control at 4 °C.A data represent the mean values of *Salmonella Typhimurium* ATCC 14028 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *Salmonella Typhimurium* ATCC 14028 a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of $p \leq 0.05$.
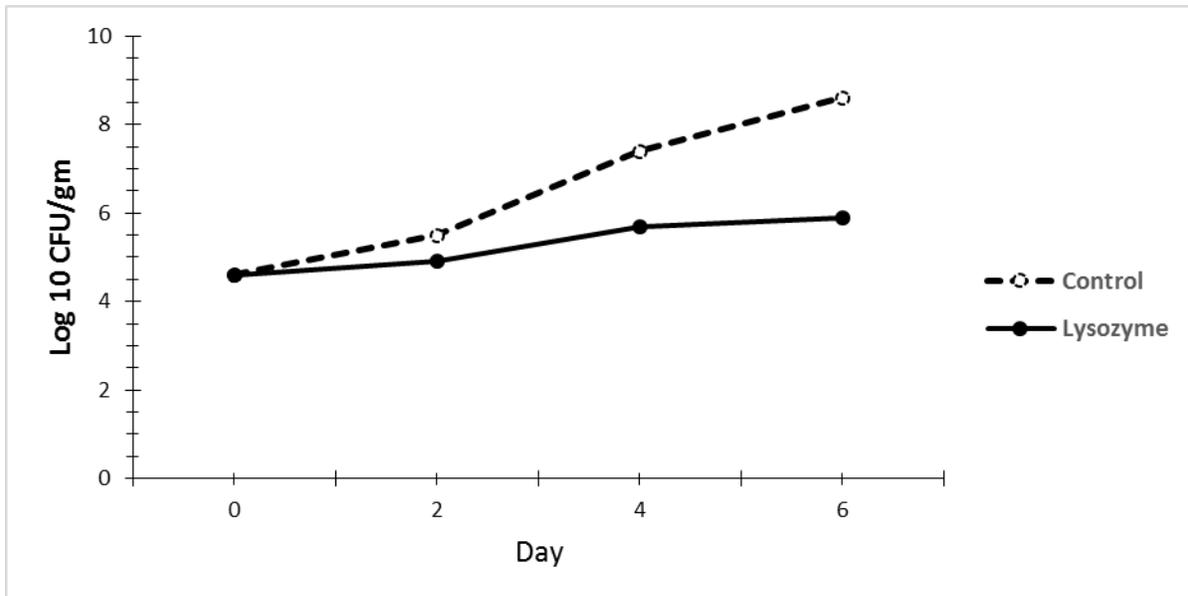
Lysozyme at 1250 µg/ml significantly reduced *L.monocytogens* (ATCC 19114) by 2.7 Log in day 6

Figure 1.11. Antimicrobial activity of lysozyme at 1250 µg /ml on *L. monocytogens* ATCC 19114 growth in beef in comparison to control at 4 °C.A data represent the mean values of *L. monocytogenes* ATCC 19114 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *L. monocytogenes* a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of $p \leq 0.05$.
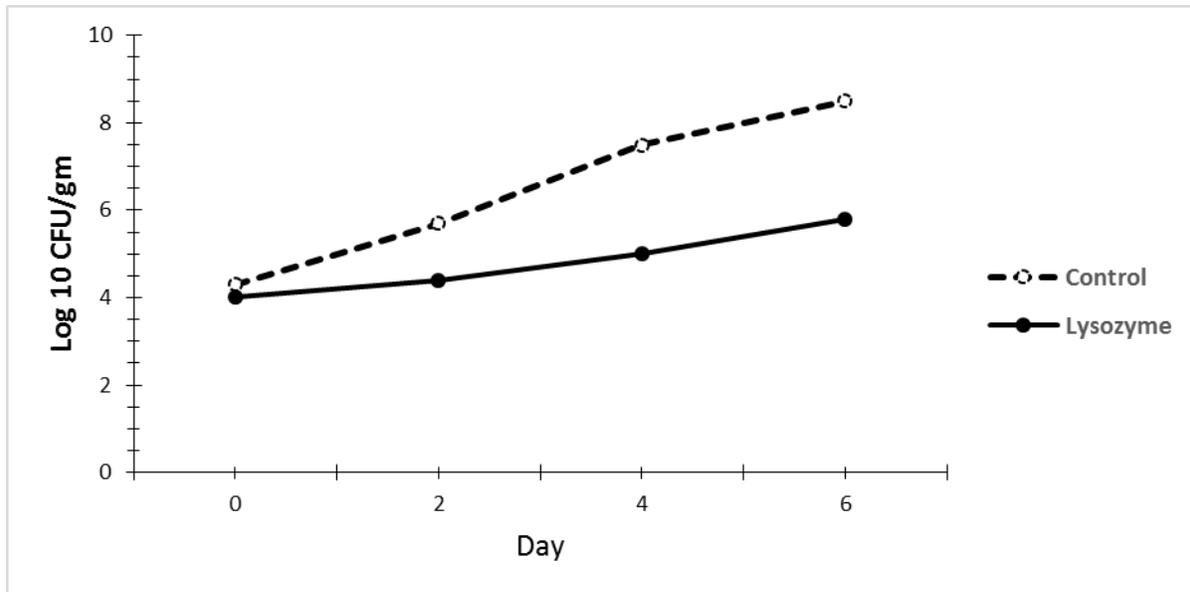
Lysozyme at 1250 μg/ml significantly reduced *S. aureus* (ATCC 29213) by 2.4 log in day 6

Figure 1.12. Antimicrobial activity of lysozyme at 1250 μg /ml on *S. aureus* ATCC 29213growth in beef in comparison to control at 4 °C.A data represent the mean values of *S. aureus* ATCC 29213a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *S. aureus* ATCC 29213a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of p≤0.05.
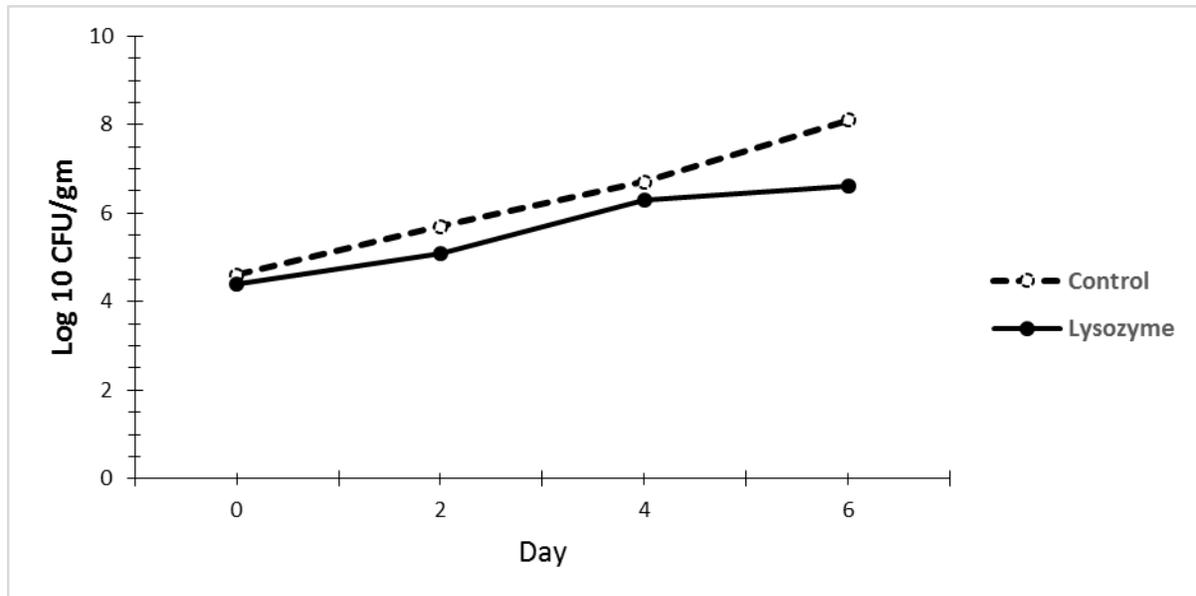
Lysozyme at 2500 µg/ml significantly reduced *E. coli* (ATCC 25922) by 1.3 Log in day 6

Figure 1.13. Antimicrobial activity of lysozyme at 2500 µg /ml on *E. coli* ATCC 25922 growth in beef in comparison to control at 4 °C.A data represent the mean values of *E. coli* ATCC 25922 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *E. coli* ATCC 25922 a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of p≤0.05.
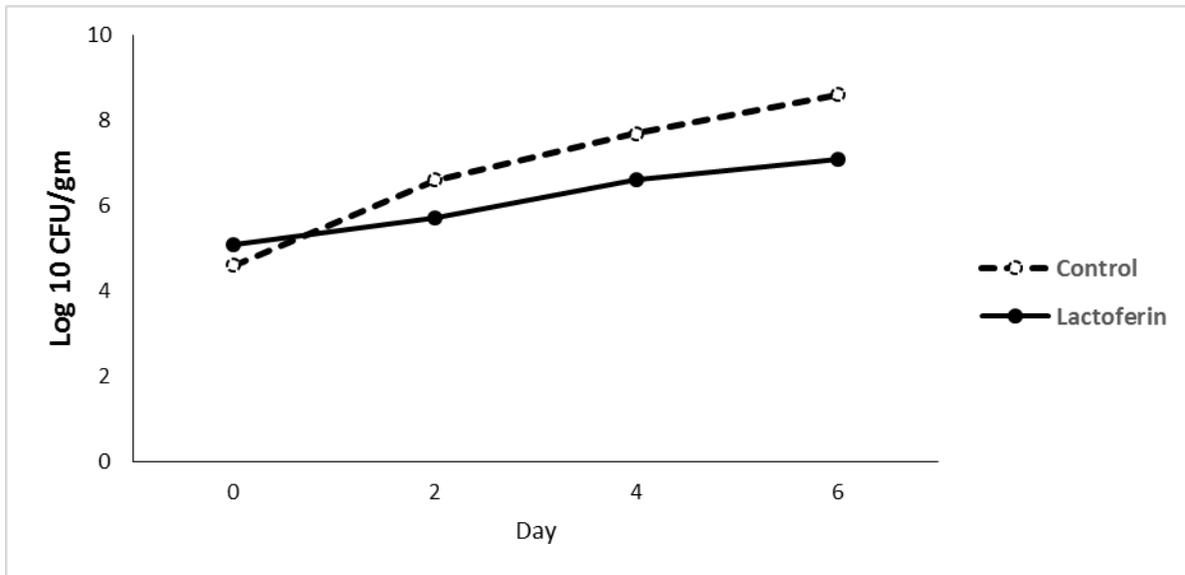
Lactoferrin at 2500 µg/ml significantly reduced *L.monocytogens* (ATCC 19114) by 2.0 Log in day 6

Figure 1.14. Antimicrobial activity of lactoferrin at 2500 µg /ml on *L. monocytogens* ATCC 19114 growth in beef in comparison to control at 4 °C. A data represent the mean values of *L. monocytogenes* ATCC 19114 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *L. monocytogenes* a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of p≤0.05.

Lactoferrin at 2500 μg/ml significantly reduced *S. aureus* (ATCC 29213) by 2.4 Log in day 6

Figure 1.15. Antimicrobial activity of lactoferrin at 2500 μg /ml on *S. aureus* ATCC 29213growth in beef in comparison to control at 4 °C.A data represent the mean values of *S. aureus* ATCC 29213a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *S. aureus* ATCC 29213a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of p≤0.05.
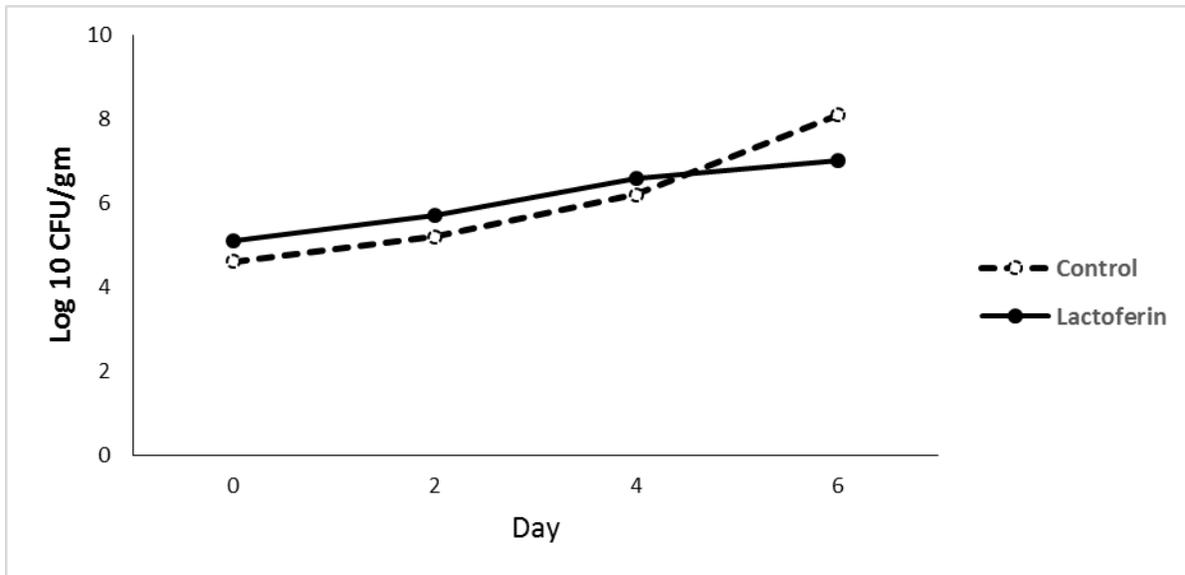
Lactoferrin at 2500 μg/ml significantly reduced *E. coli* (ATCC 25922) by 1.6 Log in day 6

Figure 1.16. Antimicrobial activity of lactoferrin at 2500 **μ**g /ml on *E. coli* ATCC 25922 growth in beef in comparison to control at 4 °C.A data represent the mean values of *E. coli* ATCC 25922   a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *E. coli* ATCC 25922 a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of p≤0.05.

Nisin at 625 µg/ml and Lactoferrin at 2500 µg/ml significantly reduced *L.monocytogens* (ATCC 19114) 3.2 Log in day 6

Figure 1.17. Antimicrobial activity of nisin at 625 µg /ml and lactoferrin at 2500 µg/ml on *L. monocytogens* ATCC 19114 growth in beef in comparison to control at 4 °C. A data represent the mean values of *L. monocytogenes* ATCC 19114 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *L. monocytogenes* a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of p≤0.05.
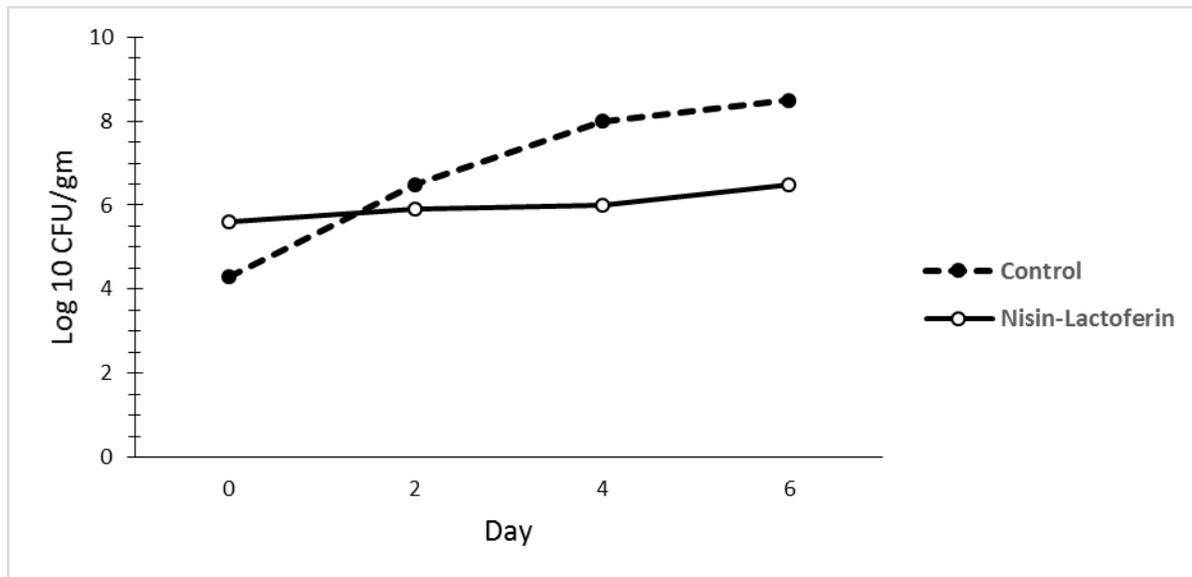
Nisin at 625 μg/ml and lactoferrin at 2500 μg/ml significantly reduced *S. aureus* by 3.4 Log in day 6

Figure 1.18. Antimicrobial activity of nisin at 625 μg /ml and lactoferrin at 2500 μg /ml on *S. aureus* ATCC 29213growth in beef in comparison to control at 4 °C.A data represent the mean values of *S. aureus* ATCC 29213a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *S. aureus* count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of $p \leq 0.05$.
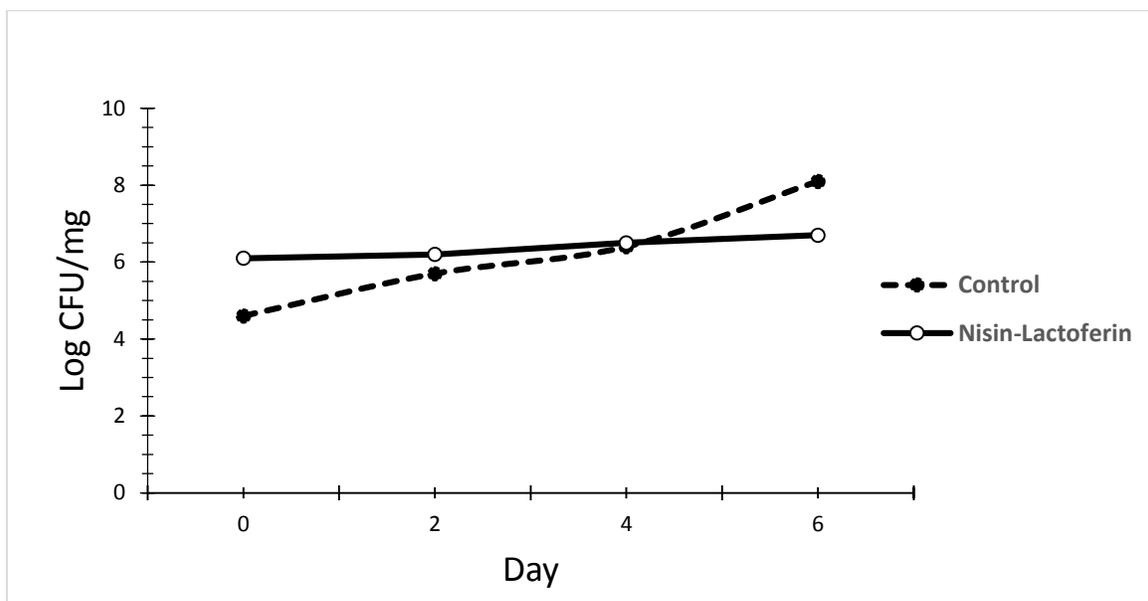
Nisin at 1250 μg/ml and lactoferrin at 2500 μg/ml significantly reduced *E. coli* (ATCC 25922) by 2.8 Log.

Figure 1.19. Antimicrobial activity of nisin at 1250 μg /ml and lactoferrin at 2500 μg /ml on *E. coli* ATCC 25922 growth in beef in comparison to control at 4 °C.A data represent the mean values of *E. coli* ATCC 25922 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *E. coli* a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of p≤0.05.
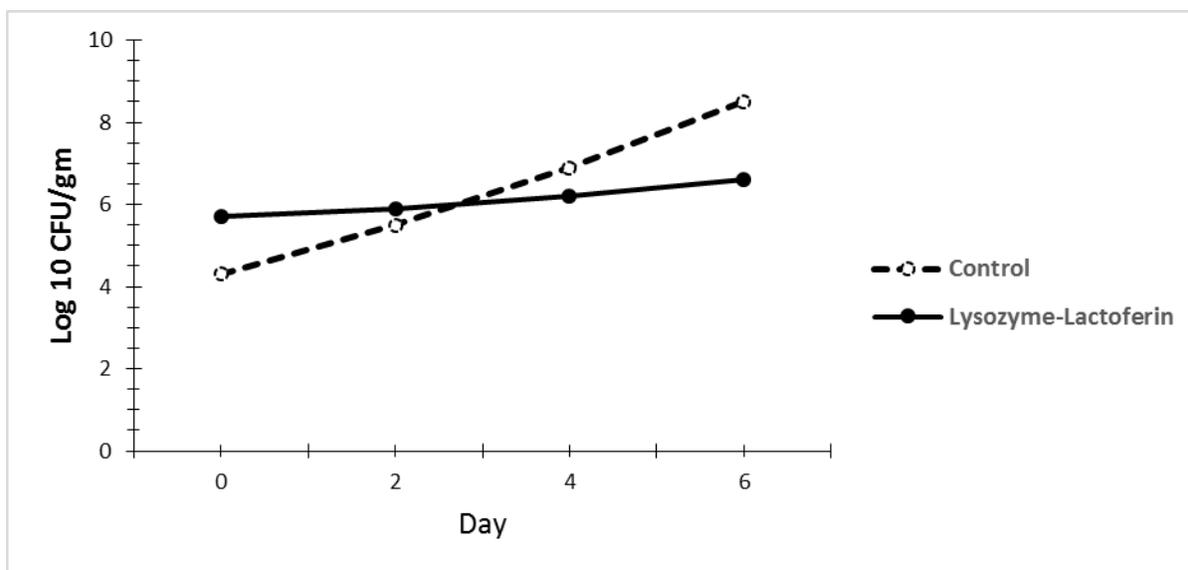
Lysozyme at 1250 µg/ml and Lactoferrin at 2500 µg/ml significantly reduced *S. aureus* (ATCC 29213) by 3.3 Log in day 6

Figure 1.20. Antimicrobial activity of lysozyme at 1250 µg /ml and lactoferrin at 2500 µg /ml on *S. aureus* ATCC 29213 growth in beef in comparison to control at 4 °C.A data represent the mean values of *S. aureus* ATCC 29213 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *S. aureus* ATCC 29213 a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of $p \leq 0.05$.

Lysozyme at 2500 µg/ml and Lactoferrin at 2500 µg/ml significantly reduced *E. coli* (ATCC 25922) by 2.5 Log in day 6

Figure 1.21. Antimicrobial activity of lysozyme at 2500 µg /ml and lactoferrin at 2500 µg /ml on *E. coli* ATCC 25922 growth in beef in comparison to control at 4 °C. A data represent the mean values of *E. coli* ATCC 25922 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *E. coli* ATCC 25922 a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of $p \leq 0.05$.
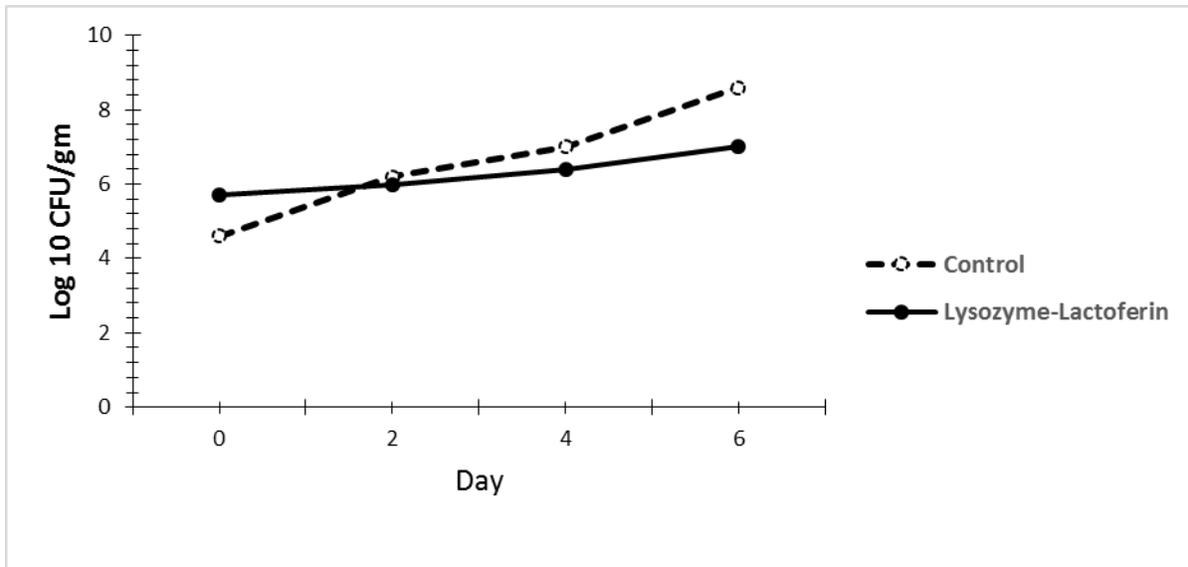
**Lysozyme at 1250 μg/ml and Lactoferrin at 2500 μg/ml significantly reduced *L. monocytogenes* (ATCC 19114) by 2.8 Log in day 6**

Figure 1.22. Antimicrobial activity of lysozyme at 1250 μg /ml and lactoferrin at 2500 μg /ml on *L. monocytogens* ATCC 19114 growth in beef in comparison to control at 4 °C. A data represent the mean values of L. monocytogenes ATCC 19114 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *L. monocytogenes* a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of $p \leq 0.05$.

**Nisin at 625 µg/ml and Lysozyme at 1250 µg/ml significantly reduced _L. monocytogens_ ATCC 19114 by 2.9 Log in day 6**

Figure 1.23. Antimicrobial effect of nisin at 625 µg /ml and lysozyme at 1250 µg /ml on _L. monocytogens_ ATCC 19114 growth in beef in comparison to control at 4 °C.A data represent the mean values of _L. monocytogenes_ ATCC 19114 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in _L. monocytogenes_ a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of $p \leq 0.05$.
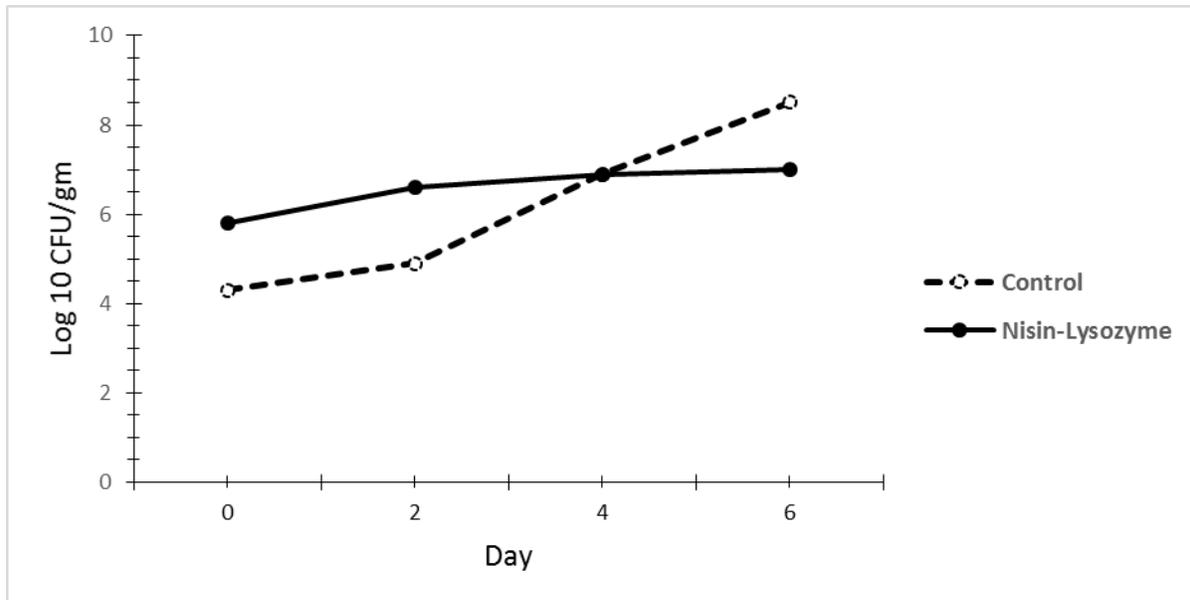
**Nisin at 625 µg/ml and lysozyme at 1250 µg/ml significantly reduced *S. aureus* (ATCC 29213) by 3 Log in day 6.**

Figure 1.24. Antimicrobial effect of nisin at 625 µg /ml and lysozyme at 1250 µg /ml on *S. aureus* ATCC 29213growth in beef in comparison to control at 4 °C.A data represent the mean values of *S. aureus* ATCC 29213 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *S. aureus* ATCC29213 a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of p≤0.05.

Nisin at 1250 µg/ml and lysozyme at 2500 µg/ml significantly reduced *E. coli* (ATTCC 25922) by 1.9 Log in day 6

Figure 1.25. Antimicrobial effect of nisin at 1250 µg /ml and lysozyme at 2500 µg /ml on *E. coli* ATCC 25922 growth in beef in comparison to control at 4 °C. A data represent the mean values of *E. coli* ATCC 25922 a count obtained from meat samples, and are expressed in logarithmic colony forming unit per gram (CFU/gm). As seen from figure there is significant differences in *E. coli* ATCC 25922 a count in comparison to control were established by using ANOVA (Analysis of Variance) at a significant level of p≤0.05.

Results and discussion

The use of natural antimicrobial preservatives has been preferred in the food industry because of consumers' demands (Dufour *et al*. 2003). Lactoferrin, lysozyme and nisin are three natural compounds that have received considerable attention in recent years (Murdock and Matthews, 2002). Properties of these three sources of natural antimicrobials can be used to improve microbial safety of foods. Combining these natural antimicrobials could possibly increase the effectiveness of the antimicrobials proving to be better than if they were used alone against foodborne pathogens.

Antimicrobial activity of lactoferrin, lysozyme and nisin *in-vitro*

Using antimicrobial combinations provide different effects against foodborne pathogens. Synergistic effect when combined effect is greater than the sum of the effects with two agents independently (Barry, 1976). Additive effect is when the combined effect is equal to the sum of the individual effects while antagonism is when the effect of one antimicrobial is reduced in the presence of another antimicrobial.

Minimal inhibitory concentration (MIC) for individual antimicrobial, MIC for combinations and the fractional inhibitory concentration (FIC) were measured to evaluate type of combination effect *in-vitro* (synergistic, additive, indifference and antagonism).

Minimal inhibitory concentrations (MIC) of natural antimicrobials nisin, lysozyme and lactoferrin against tasted bacteria in cation-adjusted Mueller-Hinton II broth (CAMHB) (*in-vitro*) were shown in table (1-1). Nisin, lysozyme and lactoferrin showed different antimicrobial activities against the tested strains based on calculated MICs. MICs for nisin were the lowest compare to other antimicrobials where MICs against *L. monocytogenes, E. coli, S. aureus* and

Salmonella typhimurium were (312, 625, 312 and 1250 μg /ml) respectively. Antimicrobial activity of nisin is due to its ability to bind the cytoplasmic membrane and form pores that damage the membrane (Kuwano et al., 2005) causing cell death due to loss different important cell compounds such as ATP, some ions like K^+, and the proton motive force (Millette et al., 2004). Also, many studies have shown that the antimicrobial actions of nisin may be attributed to interfere with cell wall biosynthesis by binding with lipid II, which is important compound in a peptidoglycan layer (Bauer and Dicks, 2005).

Lysozyme's MICs were relatively less against tested bacteria compare to nisin where MICs for lysozyme against *L. monocytogenes*, *E. coli, S. aureus* and *Salmonella typhimurium* were (625, 1250, 625 and 2500 μg /ml) respectively. Antimicrobial activity of lysozyme is based on the hydrolysis of the β 1-4 glycosidic linkages between *N*-acetylmuramic acid and *N*-acetyl glucosamine in bacterial peptidoglycan, Peptidoglycan is the major component of the cell wall of both Gram positive and Gram negative bacteria (Cunningham et al., 1991).

Lactoferrin had highest MICs among the other antimicrobials. However, using it in different combinations with another antimicrobial, such as nisin and lysozyme, lactoferrin as general chelator that has the ability to release lipopolysaccharide (LPS) and permeabilize the gram-negative cell wall, nisin and lysozyme could then have access to the inner membrane thus providing a novel system for control of gram-negative pathogens, such as *E. coli* (Ellison, 1994); (Branen and Davidson, 2004). In the other hand, although lactoferrin had highest MIC among the other antimicrobials in the present study, this finding was agreement with previous studies for gram-positive bacteria while for gram-negative some studies have reported that lactoferrin MIC against E. coli O157:H7 was higher (Murdock and Matthews 2002). This may be

attributed to composition of media where in most previous studies a typical medium (TSB) was used, which contains high levels of divalent cations. TSB can reduce or eliminate the inhibitory activity of lactoferrin (Bellamy et al**.** 1992) (Jones et al**.** 1994). In addition, divalent cations may be impaired the ability of lactoferrin to permeabilize the gram-negative cell wall by binding to the area of the lactoferrin molecule associated with the antimicrobial activity;(Dionysius and Milne, 1997) ; (Branen and Davidson, 2000).

Different combinations between lactoferrin with nisin, lactoferrin with lysozyme and lysozyme with nisin and their fractional inhibitory concentration (FIC) against tested bacteria in cation-adjusted Mueller-Hinton II broth (CAMHB) (*in-vitro*) were shown in table (1-2). Five combinations exhibited synergistic effect as following (nisin with lactoferrin) against *L. monocytogenes, E. coli* and *S. aureus*, and (lysozyme with lactoferrin) against *E. coli* and *S. aureus*. The FIC for these combinations were less than 0.5 which indicated that were synergy (Amsterdam, 1996). In addition, four combinations showed additive effect as following (nisin with lysozyme) against *L. monocytogenes, E. coli*, and *S. aureus* and (lysozyme with lactoferrin) against *L. monocytogenes*. The FIC for these combinations were between (0.5–1.0) which indicated that were additive effect (Amsterdam, 1996).

Using specific combinations such as nisin with lactoferrin, lysozyme with lactoferrin and nisin with lysozyme against tested pathogens bacteria demonstrated improved minimal inhibitory concentrations (MIC), compared to the parent compounds. Three combinations showed indifference effect against *Salmonella typhimurium* where FIC for all combinations were between (1.24-1.50) which indicate that were indifferent effect. Another studies showed that combination between lactoferrin and nisin did not inhibit growth of Salmonella Stanley (Murdock

et al, 2006). The differences in activity of these combinations against negative bacteria may be attributed to differences in outer membrane or LPS structure in cell wall of bacteria (Branen and Davidson, 2004). No combination showed antagonism against tested strains.

Antimicrobial activity of lactoferrin, lysozyme and nisin in food model-meat (*in-vivo*)

To evaluate antimicrobial activity of lactoferrin, lysozyme and nisin against tested bacteria in food model (*in-vivo*), combinations that exhibited synergistic and additive effect were selected to treat beef samples. Antimicrobial activity of these antimicrobial individually and their combinations against tested bacteria in food model were shown in Figures (1-7)-(1-25).

Our results in food model showed that lactoferrin, lysozyme and nisin individually had significant effect (p<0.01) on growth of gram-postive bacteria (*L. monocytogenes* and *S. aureus*) compared to control. Lactoferrin, lysozyme and nisin individually were effective in reducing count of *L. monocytogenes* by (2, 2.7 and 2.2) Log CFU/gm respectively, while for *S. aureus* by (2.4, 2.4, and 2.6) Log CFU/respectively. This finding was in general agreement with previous studies (Cleveland et al**.** 2001); (Murdok et al, 2006). In addition, combinations of (nisin with lactoferrin) and (lysozyme with lactoferrin) had significant effect (p<0.01) on growth of gram-postive bacteria. Combinations of (nisin with Lactoferrin) and (lysozyme with lactoferrin) were effective in reducing a count of *L. monocytogenes* by (3.2, 2.8) Log CFU/gm respectively (Figure s 1.17, 1.22) and they effect in reducing a count of *S. aureus* by (3.4, 3.3) log CFU/gm respectively, (Figure s 1.18, 1.20).This consistent with *in-vitro* results which were showed synergistic effect for these combinations against *L. monocytogenes* and *S. aureus* (Table 1-2). This finding also is agreement with previous studies, (Branen and Davidson, 2004) reported that lactoferrin

enhanced the activity of nisin against L. monocytogenes, when combined with lactoferrin, 50% less nisin was required to totally inhibit L. monocytogenes**.**

In the other hand, nisin and lysozyme individually had less effect on the growth of gram-negative bacteria *S. typhimurium* and *E. coli* as shown in Figure s 1.9, 1.10 and 1.13. This may be attributed to the outer membrane barrier that surrounds and protects the peptidoglycan layer (McKenzie and White, 1991). However, when nisin or lysozyme used in combination with lactoferrin can be an effective approach to control gram-negative bacteria. The susceptibility of gram-negative organisms to lysis by lysozyme or nisin can be increased by the use of detergents and chelators (Shively and Hartse, 1964). In the presence of lactoferrin as general chelator that has the ability to release lipopolysaccharide (LPS) and permeabilize the gram-negative cell wall, nisin and lysozyme could then have access to the inner membrane thus providing a novel system for control of gram-negative pathogens, such as *E. coli* (Ellison, 1994) ; (Branen and Davidson 2004).

These data support the hypothesis that lactoferrin and nisin or lysozyme can synergistically function to inhibit the growth of foodborne pathogens. Our results indicated that Using combinations (nisin, lactoferrin),(lysozyme, lactoferrin) and (nisin, lysozyme) against foodborne pathogens in meat industry may be a novel system to control foodborne pathogens and offer unique properties such as increase the effectiveness of the antimicrobials proving to be better than if they were used alone against foodborne pathogens and thereby decrease the amounts and cost of each antimicrobial treatment and eliminate the harmful chemical effects while improving the antimicrobial efficiency.

REFERENCES

- Abee, T., Krockel, L., and Hill, C. 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. Int J Food Microbiol. 28: 169-185.

- AL-NABULSI, A.A. and HOLLEY, R.A. 2006. Enhancing the antimicrobial effects of bovine lactoferrin against E. coli O157:H7 by cation chelation NaCl and temperature. J. Appl. Microbiol. 100, 244–255.

- Alvarado, C.M., Richards, S. O'Keefe, H. Wang 2007.The effect of blood removal on oxidation and shelf life of broiler breast meat.Poultryscience,86(1) (2007),pp.156-161.

- Anonymous (2000) Federal Register. 65, 3121–3123

- APHA; American Public Health Association (1992): Compendium of Methods for the Microbiological Examination of Foods, 3rd ed. APHA, Washington, DC, USA.

- Bailey, F.J. and Hurst, A. 1971. Preparation of a highly active form of nisin from Streptococcus lactis. Can. J. Microbiol. 17:61-67

- Bauer, R., and Dicks, L.M. 2005.Mode of action of lipid II-targeting lantibiotics. Int. J. Food Microbiol. 101:201-216

- Bellamy, W.R., Wakabayashi, H., Takase, M., Kawase, K., Shimamura, S. and Tomita, M. (1993) Role of cell-binding in the antibacterial mechanism of lactoferricin B. J Appl. Bacteriol 75, 478–484

- Branen, J.K. and Davidson, P.M. (2004) Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylene diamine tetra acetic acid and lactoferrin. Int J Food Microbiol 90, 63–74.

- Brotz, H. Josten, M. Wiedemann, I. Schneider, U. . Gotz, F. Bierbaum, G. Sah., H.G. 1998 Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. Mol. Microbiol, 30 (1998), pp. 317-327.

- Bruno, M. E. and Montville, T. J.1993. Common mechanistic action of bacteriocins from lactic Acid bacteria. Appl Environ Microbiol. 59:3003-10

- Chantaysakorn, P. and Richter, R.L. (2000) Antimicrobial properties of pepsin-digested lactoferrin added to carrot juice and fltrates of carrot juice. J Food Prot. 63, 376–380.

- Chandrapati, S. O'Sullivan, D.J. 1998.Procedure for quantifiable assessment of nutritional parameters influencing Nisin production by *Lactococcus lactis* subsp. *Lactis*. Journal of Biotechnology, 63 (1998), pp. 229-233

- Chang, K.Y. Carr, C.W. 1971. Studies on the structure and function of lysozyme: I. The effect of PH and cation concentration on lysozyme activity. Biochim. Biophys. Acta, 229 (1971), pp.469-503.

- Cheeseman, G. C. and Berridge, N. J. 1957. An improved method of preparing nisin. Biochem. J. 65:603-608.

- Cleveland, J., Montville, T.J., Nes, I.F. and Chikindas, M.L. (2001) Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Micro. Anonymous (2000) Federal Register. 65, 3121–3123.obiol 71, 1–20.

- Cunningham. F.E, Proctor. V.A, Goetsch.S.J.1991. Egg-white lysozyme as a food preservative: an overview World's Poult. Sci. J., 47 (1991), pp. 141–163

- Davies, R.C. Neuberger, A. Wilson, B.M. 1969. The dependence of lysozyme activity on PH and ionic strength. Biochim. Biophys. Acta. 178 (1969), pp.294-305.

- Delves-Broughton. J, Blackburn P, Evans. R.J, Hugenholtz.J.1996. Applications of the bacteriocin, nisin Antonie van Leeuwenhoek, 69 (1996), pp. 193–202

- Delves-Broughton. J, Blackburn P, Evans. R. J, Hugenholtz.J.1996. Applications of the bacteriocin, nisin Antonie van Leeuwenhoek, 69 (1996), pp. 193–202

- De Vuyst, L. Vandamme, E.J. 1992.Influence of the carbon source on nisin production in *Lactococcus lactis* subsp. *Lactis* batch fermentations. Journal of General Microbiology, 138, pp. 571-578

- Dionysius, D.A. and Milne, J.M. (1997) Antibacterial peptides of bovine lactoferrin: Purification and characterization. *Journal of Dairy Science* 80, 667–674.

- Dufour, M., Simmonds, R.S. and Bremer, P.J. 2003 .Development of a method to quantify in vitro the synergistic activity of natural antimicrobials .Int. J. Food Microbiol. 85, 249–258.

- Eliasi, J.R., Dwyer, J.T., 2002. Kosher and halal: religious observances affecting dietary intakes. J. Am. Diet. Assoc. 101:911–913.

- Ellison, R.T. 1994.The effects of lactoferrin on gram-negative bacteria. Adv. Exp. Med Biol. 357, 71–90.

- Farnaud, S. and Evans, R.W. 2003. Lactoferrin – a multifunctional protein with antimicrobial properties. Mol. Immunol. 40, 395–405.

- Gabig and Babior.1981.The killing of pathogens by phagocytes. Annual Review of Medicine, 32 (1) (1981), pp. 313–326.

- Grandin, T., Regenstein, J.M., 1994. Religious slaughter and animal welfare: a discussion for meat scientists. Meat Focus International. CAB Intern., Wallingford, Oxon, UK, pp 115-123.

- Holzapfel, W.H., Geisen, R., Schillinger, U., 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int. J. Food Microbiol. 24, 343–362

- IUCCI, Patrignani, L., Vallicelli, F., Guerzoni, M., and Lanciotti, R. 2007. Effects of high pressure homogenization on the activity of lysozyme and lactoferrin against Listeria monocytogenes. Food Control 18, 558–565.

- Jorgensen, J.H.1993. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard: NCCLS document M7-A3.NCCLS; 1993.

- Jones, E.M., Smart, A., Bloomberg, G., Burgess, L. and Millar, M.R. (1994) Lactoferricin, a new antimicrobial peptide. *J Appl Bacteriol* **77**, 208–214

- Juncioni de Arauz L, Faustino Jozala A, Gava Mazzola P, and Vessoni penna Ch. Nisin biotechnological production and application: A review. Food Science & Technology 2009; 1-9.

- Kim J.S, Kim Y.2007.The inhibitory effect of natural bioactives on the growth of pathogenic bacteria. Nutrition research and practice 2007, 1:273-278.

- Kuwano, K., Tanaka, N., Shimizu, T., Nagatoshi, K., Nou, S., and Sonomoto, K. 2005. Dual antibacterial mechanisms of nisin Z against gram positive and gram-negative bacteria. Int. J. Antimicrob Agents. 26:396-402.

- Lee, S. H., and Kim, H. U. 1985. Studies on the antibiotic nisin produced by Streptococcus lactis IFO12007. Korean J. Anim. Sci. 27:480-483.

- Magnus, P., 1981. Meat Composition. Food Science and Technology, 4th edition. Gohumunary Pub. London. Pp-108-215.

- Makenzie, H.A. White, F.H.1991. Lysozyme and alpha-lactlbumin: structure, function and interrelationships. Adv. Prot.Chem.41:173-315.

- Mantovani, H.C., and Russell, J.B. 2001. Nisin resistance of Streptococcus bovis. Appl. Environ. Microbiol. 67:808–813

- Masschalck, B., Van houdt, R. and Michiels, C.W. 2001.High pressure increases bactericidal activity and spectrum of lactoferrin, lacto- ferricin and nisin. Int. J. Food Microbiol. 64, 325–332.

- Mead, P.S.; Slutsker, L.; Dietz, V.; McCaig, L.F.; Bresee, J.S.; Shapiro, C.; Griffin, P.M.; Tauxe, R.V. Food-Related Illness and Death in the United States. Emerg. Infect. Dis. 1999, 5, 607–625.

- Millette, M, Smoragiewicz, W., and Lacroix, M. 2004.Antimicrobial potential of immobilized Lactococcus lactis subsp. lactis ATCC11454 against selected bacteria. J. Food Prot. 67:1184-1189.

- Moody, J.1992. Synergism testing: broth microdilution checkerboard and broth macrodilution methods. Clinical microbiology procedures handbook Washington, DC: American Society for Microbiology, 5:22.

- Murdock, J., Cleveland, K.R., Matthews, M.L., Chikindas.2006. The synergistic effect of nisin and lactoferrin on the inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:H7

- Murdock, C.A. and Matthews, K.R. 2002.Antibacterial activity of pepsin digested lactoferrin on food borne pathogens in buffered broth systems and ultrahigh temperature milk with EDTA. J. Appl. Microbiol.

- Nakyinsige, K., Fatimah, A.B., Aghwan, Z.A., Zulkifli, I., Goh, Y.M., Sazili, A.Q. 2014. Bleeding Efficiency and Meat Oxidative Stability and Microbiological Quality of New Zealand White Rabbits Subjected to Halal Slaughter without Stunning and Gas Stun-killing. Asian-Australas J. Anim. Sci.; 27 (3):406-13.

- Odell, E.W., Sarra, R., Foxworthy, M., Chapple, D.S. and Evans, R.W. (1996) Antibacterial activity of peptides homologous to a loop region in human lactoferrin. FEBS Lett 382, 175–178

- Proctor .V.A and Cunningham. F.E, 1988 V.1988.The chemistry of lysozyme and its use as a food preservative and a pharmaceutical Crit. Rev. Food Sci. Nutr., 26 (1988), pp. 359–395

- Reunanen, J. and Saris, P.E.F. 2004. Bioassay for nisin in sausage: A shelf life study of nisin in cooked sausage. Meat Sci. 66, 515– 518.

- Roberts, D. and M. Greenwood, 2003.Practical Food Microbiology.3rd Edn., Blackwell Publishing Ltd., Oxford, UK

- Sahl, H.G. 1991. Pore formation in bacterial membranes by cationic lantibiotics. In Nisin and Novel Lantibiotics, Jung, G. and Sahl, H.G., Eds., Escom, Leiden. The Netherlands. 347-358.

- Samelis, J., Bedie, G.K., Sofos, J.N., Belk, K.E., Scanga, J.A. and Smith, G.C. 2005. Combinations of nisin with organic acids or salts to control L. monocytogenes on sliced pork bologna stored at 4°C in vacuum packages. Lebensm.-Wiss. Technol. 38, 21–28.

- Schelz Z, Molnar J, Hohmann, J.2006 Antimicrobial and antiplasmid activities of essential oils. Fitoterapia, 77:279-285.

- Shimazaki, K. 2000. Lactoferrin: A marvelous protein in milk. Anim. Sci. J. 71, 329–347.

- Shelef LA, Seiter J (1993) Enhancement of the activity of novobiocin against *Escherichia coli* by lactoferrin. J Dairy Sci. 82:492–499

- Shively JM, Hartsell SE (1964) Bacteriolysis of the Pseudomonads: I. Agents potentiating lysis. Can J Microbiol 10:905–909

- Urbain, W. M, and J .F. Campbell. 1987. Meat Preservation. In the Science of Meat and Meat Products. 3rd Edn. (J.F. Price, and B.S. Schweigert, Eds) pp. 371-412. Food and Nutrition Press, Inc. Westport, CN.

- Warriss, P. W. 2001 Postmortem changes in muscles and its convection into meat. Meat science, an introductory text, pp. 100 – 161. CABI Publishing, Wallingford, UK

- Ye, X.Y., Wang, H.X., Liu, F. and Ng, T.B. (2000) Ribonuclease, cell-free translation-inhibitory and superoxide radical scavenging activities of the iron-binding protein lactoferrin from bovine milk. Int J Biochem C. Biol. 32, 235–241.

CHAPTER 2: MOLECULAR CHARACTERIZATION OF TOXIN GENES AND ANTIBIOTIC RESISTANCE OF STAPHYLOCOCCUS AUREUS ISOLATES FROM MEAT

Abstract

Staphylococcal heat-stable enterotoxins and antibiotic resistance are important public health issues in the world. The aim of this research is to investigate the presence and characterization of staphylococcal enterotoxin genes by polymerase chain reaction and test the antibiotic susceptibility of the *Staphylococcus aureus* isolates from meat. 96 and 63 positive *Staphylococcus aureus* isolates were recovered from 142 meat samples and were subjected to prevalence of the enterotoxin genes and antimicrobial susceptibility tests against fourteen antibiotics. Our study indicated that 61 (42.9%) meat samples were positive and confirmed to be *S. aureus* by genotypic and biochemical identification. Enterotoxin genes profile indicated that 80.9% of the tested strains contained either one or more enterotoxin genes. A high percent (60.3%) of SEA gene was detected. The most commonly combinations detected were sea sei, sei she, sea she and sea sei she with 23.8, 17.4, 14.2 and 11.11% respectively. No isolates harbored three SE genes, that is, seb, sec, or see. Mec A gene was positive in 71.4% of *S. aureus* isolates. All isolates carrying the mec A gene showed positive MRSA phenotypes. Antibiotic resistance profile indicated that some isolates showed high resistance to specific antibiotic such as penicillin (71.8%), ampicillin (70.8%), tetracycline (36.4%) amoxicillin (35.4%) and oxacillin (26%). There was no apparent resistance to some antibiotic such as nitrofurantoin, vancomycin and ciprofloxacin. A small percentage of the isolates demonstrated resistance to rifampicin, amikacin and chloramphenicol (2%) for each, gentamycin and clindamycin (3.1%) for each and erythromycin (7.2%). High percentage of multidrug resistant *S. aureus* (48.9%) was detected.

Results from this research indicate that high levels of staphylococcal enterotoxin genes, antibiotic resistance and multidrug resistant were detected in meat. Therefore, these findings are alarming and require the need to prevent the presence of *S. aureus* strains and SEs production in meat.

Introduction

Toxigenic *Staphylococcus aureus* contamination in meat is a major problem in public health due to the production of heat-stable enterotoxins as well as It expresses many array of virulence factors contribute to the ability of *S. aureus* to cause infection include various enzymes, adhesion proteins, cell-surface proteins, factors that help the bacteria to evade the innate immune defense, and antibiotic resistance mediate survival of the bacteria and tissue invasion at the site of infection (Zecconi and Scali, 2013).

S. aureus has the ability to grow, and produce staphylococcal enterotoxins (SE), the causative agent of staphylococcal food poisoning (SFP), over an extensive range of temperature, pH, sodium chloride concentration and water activity **(**Adams and Moss, 2008).The bacteria can be killed through heat treatment of the food, but the enterotoxins are very heat resistant. Thus, although the bacteria are eliminated, the toxins will remain and can cause SFP (Le et al, 2003). The consumption of chicken and beef meat was implicated in a large number of outbreaks of staphylococcal food poisoning in humans in different countries (Argudı´n et al., 2010). However, the real incidence of SFP is underestimated (Smyth et al., 2004). According to Centers for Disease Control (CDC) on food-borne diseases is that SEs affect approximately 80 million individuals in the US, alone, resulting in 325,000 hospitalizations and more than 5,000 deaths (Mead et al, 1999). The genes encoding the different enterotoxins are carried and disseminated by different mobile genetic elements, i.e., prophages, plasmids, pathogenicity islands (SaPIs), enterotoxin

gene cluster (**egc**) and the staphylococcal cassette chromosome (SCC) (Altboum et al, 1985). Staphylococcal enterotoxin SE toxins have been identified on the basis of the antigenic type, which include the classical SEs (SEA, SEB, SEC, SED, SEE), newer SEs (SEG, SEH, SEI, and SEJ), and more recent ones (SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, SEU and SEV). Enterotoxin genes are not distributed uniformly among different *S. aureus* strains in different areas. Genetic variation among enterotoxin genes occurs in these strains (18).

Antibiotic resistance is an important public health issue in many countries because of the extended use and misuse of antibiotics in different fields such as agriculture, stock-farming and in the treatment of human disease, therefore, the number of bacteria that are resistant to antimicrobial agents is rapidly increasing. Isolates from food have shown a considerable increase in resistance against most antibiotics (Valsangiacomo et al, 2000).

Meat is an important vector for the transfer of antibiotic resistances from animals to humans where many Isolates from meat have shown a considerable increase in resistance against most antibiotics (Yu¨cel et al, 2005) and against methicillin (Kitai et al., 2005). **S. aureus** has been reported to frequently show multiple antimicrobial resistance patterns (Enright, 2003) and produce of heat-stable enterotoxins (Zecconi and Scali, 2013). Therefore, the aim of this chapter is to investigate the presence and characterization of staphylococcal enterotoxin genes by polymerase chain reaction and test the antibiotic susceptibility of the Staphylococcus aureus isolates from meat.

Staphylococcus aureus

Staphylococcus aureus are gram-positive of approximately 1 clusters, non-sporeforming, catalase-positive, oxidase-negative, and non-motile cocci. It is a facultative anaerobe forming

yellow colonies on rich medium and causing a hemolysis on blood agar plates (Morandi et al, 2009). Optimum growth of S. aureus occurs when the water activity (a_w) is above 0.99 (Huang et al., 2001); (Portocarrero et al., 2002). It is mesophilic, which generally can grow from 7˚C to 47.8˚C, with 35-37˚C being the optimal temperature for growth (FDA, 2012); (Notermans and Heuvelman, 1983) and at pH values ranging from 4.2 to 9.3 (Narmanno et al, 2005). *S. aureus* belongs to the genus of Staphylococcus which includes more than 30 species. *S. aureus* can be differed from other species of Staphylococcus by various biochemical characteristics, such as the production of coagulase, heat-resistance nuclease (TNase) and hemolysis (Arbuthnott et al., 1990). It expresses many array of virulence factors contribute to the ability of *S. aureus* to cause infection include various enzymes, toxins, adhesion proteins, cell-surface proteins, factors that help the bacteria to evade the innate immune defense, and antibiotic resistance mediate survival of the bacteria and tissue invasion at the site of infection (Zecconi and Scali, 2013). Moreover, certain toxins can cause specific disease entities. The chief function of these enzymes is to turn host components into nutrients that the bacteria may use for growth.
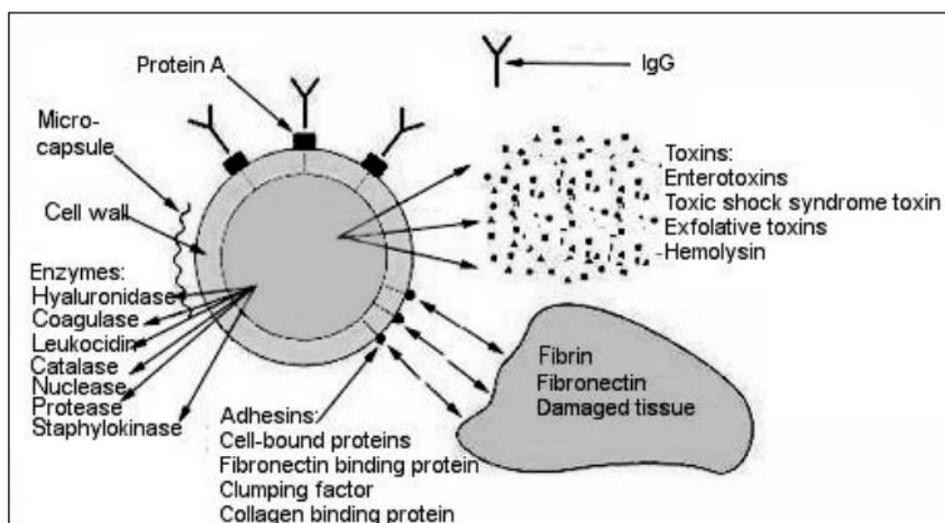
Figure 2.1. Virulence factors of *S. aureus*

The primary habitat of this microorganism is the mucosa of the nasopharynx and the skin of humans and animals (da Silva, 2010) Despite its pathogenicity, *S. aureus* is also harbored in the nares of about 20 to 30% of healthy people, while about 60% of the population harbors the microorganism intermittently (Normanno, 2007).

S. aureus has the ability to grow, and produce staphylococcal enterotoxins (SE), the causative agent of staphylococcal food poisoning (SFP), over an extensive range of temperature, pH, sodium chloride concentration and water activity **(**Adams and Moss 2008).The bacteria can be killed through heat treatment of the food, but the enterotoxins are very heat resistant. Thus, although the bacteria are eliminated, the toxins will remain and can cause SFP (Le et al, 2003).These toxins are for the most part produced by *S. aureus* although other species have also been shown to be enterotoxigenic. It has been reported that the levels of *S. aureus* usually need to reach 5 - 6 log CFU/g in food to produce detectable amount of enterotoxin (Castillejo-Rodriguez et al., 2002); (Fujikawa and Morozumi, 2006). The threshold amount of enterotoxin for causing illness in humans is not known. However, information from food poisoning outbreaks (Bergdoll,1990);(Evenson et al,1988) and human challenge studies (Dangerfield,1973) indicates that individuals experiencing illness probably consumed at least 100 ng of enterotoxin A, the serotype most frequently involved in foodborne staphylococcal illness (Casman et al,1969).

Staphylococcal food poisoning is an acute intoxication resulting from the ingestion of food containing the enterotoxin produced by certain strains of *S. aureus*. It has a short incubation period that The illness starts suddenly, 2-6 h after eating the contaminated food, with major symptoms of acute nausea, vomiting and abdominal pain often followed by diarrhea (Gilbert, 1974). The severity of the illness depends on the amount of food ingested, the amount of toxin

in the ingested food and the general health of the victim (Smyth et al, 2004).The high incidence of staphylococcal food poisoning is due to the insufficient pasteurization/decontamination of originally contaminated product source (Scherrer et al, 2004) or its contamination during preparation and handling by individuals who are carriers of the organism. Also, since *S. aureus* grows over a wide range of temperatures and pH, the bacteria may grow in a wide assortment of foods.

Staphylococcal enterotoxins

Exotoxins produced by staphylococcus include staphylococcal enterotoxins (SEs), toxic shock syndrome toxin 1 (TSST-1), exfoliative toxins (ETs), and an alpha-toxin. Staphylococcal enterotoxins are short, water-soluble, single-chain proteins with molecular weights of 26,000 to 29,000. They are very stable and are resistant to heat and proteolytic enzymes, such as trypsin and pepsin, which allows them to transit intact through the digestive tract (Bennett, 2001). They are pyrogenic and share some other important properties that include the ability to induce emesis and gastroenteritis as well as their noted super antigenicity. The different SE serotypes are similar in composition and biological activity but are different in antigenicity and identified serologically as separate proteins (Bennett, 2001).

According to Centers for Disease Control (CDC) on food-borne diseases is that SEs affect approximately 80 million individuals in the US, alone, resulting in 325,000 hospitalizations and more than 5,000 deaths (Mead et al, 1999). At least 20 serologically distinct staphylococcal super antigens have been described that include SEs (A through V) and toxic shock syndrome toxin-1 (TSST-1). SEA, SED, and SEE share 70–90% sequence homology, while only 40–60% with SEB, SEC, and TSST-1 (Al-Daccak et al, 1998) (Balaban et al, 2000). Toxin formation is not likely at

temperatures lower than 10˚C or at water activities below 0.85 (FDA, 2011). Therefore, exposure of food products contaminated with *S. aureus* to temperatures between 10°C and 21.1°C for more than 12 hours or above 21.1°C for more than 3 hours could result in enterotoxin formation in the products (FDA, 2011). The genes encoding the different enterotoxins are carried and disseminated by different mobile genetic elements, i.e., prophages, plasmids, pathogenicity islands (SaPIs), enterotoxin gene cluster (**egc**) and the staphylococcal cassette chromosome (SCC) (Altboum et al, 1985).

Staphylococcal enterotoxin (SE toxins) have been identified on the basis of the antigenic type, which include the classical SEs (SEA, SEB, SEC, SED, SEE), newer SEs (SEG, SEH, SEI, and SEJ), and more recent ones (SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, SEU and SEV) (Thomas et al., 2006, 2007); (Ono et al., 2008), have been designated members of the SE family on the basis of their sequence similarity to classical SEs (Jarraud et al , 2001)*.* In addition, some of strains of *S. aureus* produce one or both of two immunologically distinct ETs, A (ETA) or B (ETB). These toxins are associated with impetiginous staphylococcal scalded skin syndrome and exhibit unique super antigenic activity (Monday et al, 1999).

Staphylococcal enterotoxin (SEA) is one of the most important gastroenteritis causing agents. In some areas, more than 50% of food poisoning (FP) is caused by staphylococcal enterotoxin A (SEA) (Di Giannatale et al, 2001). SEA is responsible for approximately 80% of the cases of food poisoning outbreaks in the USA. SEB, while it is associated with food poisoning, has been studied for potential use as an inhaled bioweapon (Ler et al, 2006). Because it can easily be aerosolized; it is very stable; and it can cause widespread systemic damage, multi organ system failure, and even shock and death when inhaled at very high dosages. SEB is responsible

for 10% of the cases (Atanassova et al, 2001). SED is the second most common staphylococcal toxin associated with food poisoning worldwide, and only very small amounts of this toxin were needed to induce food poisoning (Bergdoll et al, 1981). SEF staphylococcal toxin was discovered in 1980. This toxin was renamed TSST-1 because of a lack of evidence of emetic activity in monkeys (Su et al, 1997).TSST-1 causes toxic shock syndrome. TSST-1 is exhibited super antigenic activity

Antibiotic resistance of *staphylococcus aureus*

Antibiotic resistance is a significant problem and a major public health concern because of its effect on the rapid spread of threatening diseases and infections and the inability to control them, whereas bacteria evolves and forms ways of counteracting the antibiotics, the antibiotics become ineffective and harmful and fatal bacteria is able to thrive in multiple environments. There has been amerced increase in the number of species that have acquired resistance to antibiotics, as well as an increase in the kinds of antibiotics (Alalem, 2008). **S. aureus** is perhaps the pathogen of greatest concern because of its intrinsic virulence, its ability to cause a diverse array of life-threatening infections, and its capacity to adapt to different environmental conditions (Lowy, 1998) ;(Waldvogel, 2000). The mortality of **S. aureus** bacteremia remains approximately 20–40% despite the availability of effective antimicrobials (Mylotte, 1987). **S. aureus** is now the leading overall cause of nosocomial infections and, as more patients are treated outside the hospital setting, is an increasing concern in the community (CDC NNIS System, 2001); (Diekema et al, 1999).

Before the advent of antibiotics, invasive *S. aureus* disease was a significant cause of mortality; however penicillin was used to treat *S. aureus* infections initially. shortly afterwards,

resistance emerged when strains acquired a genetic element coding for β-lactamase production, and now more than 80 % of all *S. aureus* strains are resistant to penicillin. Staphylococcal resistance to penicillin is mediated by **blaZ**, the gene that encodes β-lactamase. This predominantly extracellular enzyme, synthesized when staphylococci are exposed to β-lactam antibiotics, hydrolyzes the β-lactam ring, rendering the β-lactam inactive. **BlaZ** is under the control of two adjacent regulatory genes, the antirepressor **blaR1** and the repressor **blaI** (Kernodle, 2000).The posterior drug to be introduced for treating infections with *S. aureus* was the semisynthetic, penicillinase-resistant penicillin named oxacillin or methicillin, but shortly after its introduction the first isolate with resistance was detected (Winn Washington, 2006). Methicillin resistance requires the presence of the chromosomally localized **mec A** gene (Kernodle, 2000); (Chambers, 1997). **Mec A** is responsible for synthesis of penicillin-binding protein 2a (PBP2a) (Hartman and Tomasz, 1984); (Song et al, 1987). PBPs are membrane-bound enzymes that catalyze the transpeptidation reaction that is necessary for cross-linkage of peptidoglycan chains (Ghuysen, 1994). PBP2a substitutes for the other PBPs and because of its low affinity for all β-lactam antibiotics, enables staphylococci to survive exposure to high concentrations of these agents. Thus, resistance to methicillin confers resistance to all β-lactam agents, including cephalosporin.

With the emergence of resistance to the penicillinase-resistant penicillin, the glucopeptide agent vancomycin became the treatment of choice for infections with MRSA, and in 1996 the first isolate with intermediate vancomycin resistance was detected in Japan (Winn Washington, 2006). So far, this has not emerged to be a major concern, but the resistance has been detected in different parts of the world and needs to be monitored. MRSA is now a leading

cause of nosocomial infections worldwide and has emerged as a community-associated pathogen (Chambers and Deleo, 2009). MRSA strains are inherently cross-resistant to virtually all beta-lactam antibiotics, the most effective and widely used class of antimicrobials.

Bacteria interactions with antibiotics are depend on the structure of the cell itself. *Staphylococcus aureus* contains a thick cell wall made up of peptidoglycan and in this case are 50 percent by weight. The peptidoglycan is made up of both N-acetyl glucosamine and N-acetylmuramic acid, which are linked with tetrapeptide chains. The cell wall of *S. aureus* was found to contain peptidoglycan along with teichoic acids and proteins (Umeda et al, 1987). Teichoic acids are bound to the peptidoglycan through phosphodiester bonds. The acids then extend out where proteins are bound and form the outer most layer of the cell. Antibiotics work by binding to the proteins and inhibiting cell wall formation by inhibiting the cell enzyme. In this case, *Staphylococcus aureus* contains β-lactamase enzyme and β-lactam antibiotics such as penicillin (e.g. penicillin G, ampicillin and methicillin), cephalosporin and carbapenems, as well as monolactams and β-lactamase inhibitors are able to bind to this enzyme. Some antibiotic have an antibacterial effect by inhibiting protein synthesis. Representatives of this group include the aminoglycosides, tetracycline, macrolides and chloramphenicol which interfere with ribosome function. Also, there are antibiotics that inhibit DNA synthesis, including quinolones, fluoroquinolones and sulfonamides. The cell itself contains prophages, plasmids, and transposons all within a 2800 bp circular chromosome (Lowy and Franklin, 1998). This is where mechanisms of antibiotic resistance are found and transferred between cells through horizontal gene transfer.

Resistance Mechanisms

Mechanisms of antimicrobial resistance can be divided into four general categories (Quale et al, 2003) (Figure s, 2.2. and 2.3.):

1.   Enzymes that destroy or modify the antimicrobial substrate

2.   Target site alteration like alteration of DNA gyrase, a target of fluoroquinolones.

3.   Bypass pathways that substitute for a metabolic pathway.

4.   Barrier to penetration or efflux pumps that exclude the agent.
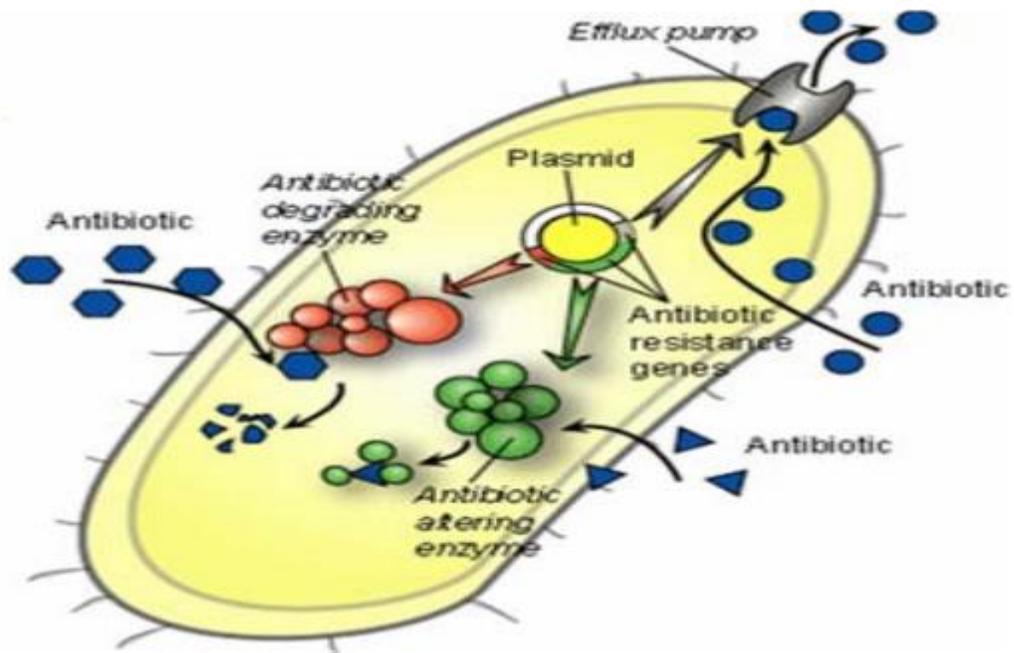
Figure 2.2.Mechanisms of antimicrobial resistance
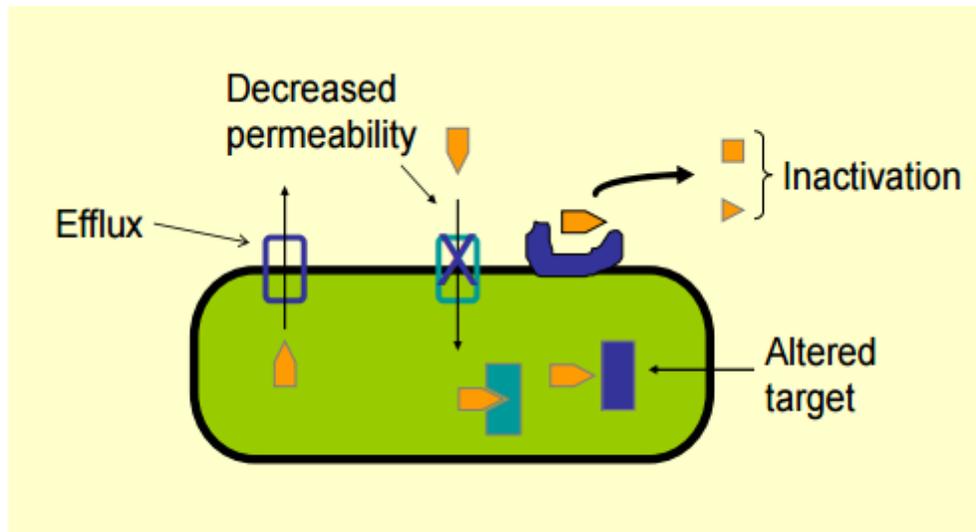
Figure 2.3. Biochemical Mechanisms of antimicrobial resistance

Materials and methods

Meat Sample Collection

Samples of raw meat (beef, lamb and chicken) were collected from different places in Michigan. The collected samples were immediately transported in insulated ice containers to the laboratory for microbial analysis.

Isolation and identification of *S. aureus*

Meat samples were added to 10 ml of buffered peptone water (BPW) in sterile plastic bags. 10 mL was then transferred from the bag and added to 10 mL of enrichment broth of Trypticase Soy Broth (Merck, Darmstadt, Germany) with 10% sodium chloride and 1% sodium pyruvate, then incubated at 37 °C for 24 h. The enrichment broth was streaked onto Baird-Parker agar plates (Merck, Darmstadt, Germany) containing an egg-yolk tellurite emulsion (Merck, Darmstadt, Germany), then incubated at 37°C for 24 to 48 h (Pu et al., 2009). Colonies exhibiting characteristic morphology of *S. aureus* (black colonies surrounded by 2 to 5 mm clear zones) were

randomly selected and subjected to gram stain, tests for catalase reaction, coagulase enzymes, DNase activity and finally genotypic confirmation through PCR detection of *S. aureus* 16s rRNA (Staphylococcus genus-specific, 228 bp) (Lovseth et al, 2004) and nuc (*S. aureus* species-specific, 279 bp) genes (Brakstad et al., 1992). All isolates were stored at - 80C in TSB plus 20% (v/v) glycerol for further use. Working cultures were prepared by streaking directly from the cryo vials onto Try tone Soy Broth (TSB) and incubating at 37.

DNA extraction

Extraction of DNA was done using Qiagen DNeasy blood &tissue kit. Extraction was completed according to manufacture protocol.

Identification of *S. aureus* by PCR (Nuc gene test)

Following the DNA extraction, presumptive S. aureus isolates were identified to the species level by polymerase chain reaction (PCR) detection of thermonuclease gene (nuc, S. aureus specific) (Sharma et al. (2000). Each polymerase chain reaction (PCR) contained 5µL PCR Buffer 10x ,4µL MgCl2 50 mM, 1 µM dNTP mix 10 mM , 1 U Taq DNA Polymerase ,10 pmol of each primer, and 1µL DNA. The final volume was adjusted to 16µL by adding 3.5µL sterile ultra-pure water. The mixes were submitted to a program performed on a thermo cycler with an initial denaturation step at 94°C for 4 min, 35 amplification cycles each with 20 seconds at 94°C; 30 seconds at 62°C; 20 seconds at 72°C followed by an additional extension step of 5 minutes at 72°C. Positive and negative controls were included in each PCR run. Then all isolated strains were identified by PCR were visualized after electrophoresis on 2% agarose gel and the product size was estimated using a DNA ladder.

Detection of enterotoxin genes

The primers used to detect enterotoxin genes and 16s rRNA were described by (Johnson et al. 1991) and (Monday and Bohach, 1999) and are listed in table (2.2). The PCR reaction mixture contained 20 ng of template DNA, 1 U of *Taq* DNA polymerase, 250 µM of each dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, and 1.5 mM MgCl2. The mixes were submitted to a program performed on a thermo cycler with an initial denaturation step at 95°C for 5 min, 30 amplification cycles each with 1 min. at 95°C; 1 min. at 53°C; 2 min. at 72°C followed by an additional extension step of 5 min. at 72°C. Then all isolated strains were identified by PCR were visualized after electrophoresis on 2% agarose gel and the product size was estimated using DNA molecular weight ladder.

Detection of *MEC A* gene

The primer used to detect the *mec A* gene was described by (Vannuffel et al, 1995) and is listed in (table2.2). PCR amplification was performed on a thermo cycler with an initial denaturation step at 94°C for 5 min, 30 amplification cycles each with 30 sec. at 94°C; 30 sec. at 60°C; 30 sec. at 72°C followed by an additional extension step of 5 min. at 72°C. PCR products were visualized after electrophoresis on 2% agarose gel and the product size was estimated using DNA molecular weight ladder.

Antimicrobial susceptibility testing

Antimicrobial susceptibility tests for *S. aureus* were performed using agar dilution method described in the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2007). Suspensions in sterile Ringer's solution equivalent to 0.5 McFarland Standard were prepared from an overnight culture on TSA plates (sigma), then cultured on Muller Hinton Agar (MHA) (sigma) and incubated at 35 °C for 18–24h. The isolates were tested with a panel of fourteen

antimicrobials: penicillin, ampicillin, vancomycin, nitrofurantoin, erythromycin, chloramphenicol (Sigma) and oxacillin, rifampicin, gentamicin, tetracycline, amikacin, clindamycin, amoxicillin, and ciprofloxacin. The MIC was determined in Muller Hinton agar (MHA) (sigma) plus 2% w/v of NaCl in the case of oxacillin, in cation-adjusted MH for penicillin and ampicillin and in MH to test the other antibiotics investigated. Inhibition zones were measured and interpreted as recommended by the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2010). *S. aureus* ATCC 29213 was plated as a control. For each antibiotic susceptibility determination, at least two independent experiments were performed.

Statistical analysis

A chi-square test (IBM SPSS Statistics 23) was used to compare the prevalence of each gene among *S. aureus* isolates between different types of meat

Table 2.1.Biochemical and genotypic identification of *S. aureus* in meat

Sample	No. Samples	No.(%) of positive *S. aureus* samples	Confirmation tests for positive *S. aureus* isolates				
			Gram stain	Catalase Reaction	Coagulase test	16s rRNA	Nuc gene
Beef	55	24 (43.6)	+	+	+	+	+
Lamb	46	20 (43.4)	+	+	+	+	+
Chicken	41	17 (41.4)	+	+	+	+	+
Total	142	61 (42.9)					

Table2.2.PCR primers for detection of enterotoxin genes (*ESs*), *Nuc*, *16srRNA* and *Mec A* genes

Toxin Gene	Primer	Oligonucleotide sequences 5'−3'	Size (bp)	Reference
sea	F	CCTTTGGAAACGGTTAAAACG	127	Johnson et al,1991
	R	TCTGAACCTTCCCATCAAAAAC		
seb	F	TCGCATCAAACTGACAAACG	477	Johnson et al,1991
	R	GCAGGTACTCTATAAGTGCCTGC		
sec	F	CTCAAGAACTAGACATAAAAGCTAGG	271	Johnson et al,1991
	R	TCAAATCGGATTAACATTATCC		
sed	F	CTAGTTTGGTAATATCTCCTTTAAACG	318	Johnson et al,1991
	R	TTAATACTATATCTTATAGGGTAAACATC		
see	F	CAGTACCTATAGATAAAGTTAAAACAAGC	178	Johnson et al,1991
	R	TAACTTACCGTGGACCCTTC		
seg	F	CGTCTCCACCTGTTGAAGG	327	Monday et al,1999
	R	CCAAGTGATTGTCTATTGTCG		
seh	F	CAACTGCTGATTTAGCTCAG	360	Monday et al,1999
	R	GTCGAATGAGTAATCTCTAGG		
sei	F	CAACTCGAATTTTCAACAGGTAC	465	Monday et al,1999
	R	CAGGCAGTCCATCTCCTG		
sej	F	CATCAGAACTGTTGTTCCGCTAG	142	Monday et al,1999
	R	CTGAATTTTACCATCAAAGGTAC		
16s rRNA	F	GTAGGTGGCAAGCGTTATCC	228	Johnson et al,1991
	R	CGCACATCAGCGTCAG		
mecA	F	GAA ATG ACT GAA CGT CCG AT	399	Vannuffel et al, 1995
	R	CTG GAA CTT GTT GAG CAG AG		
Nuc	F	CTGGCATATGTATGGCAATTG	397	Johnson et al,1991
	R	AATGCACTTGCTTCAGGACC		

68
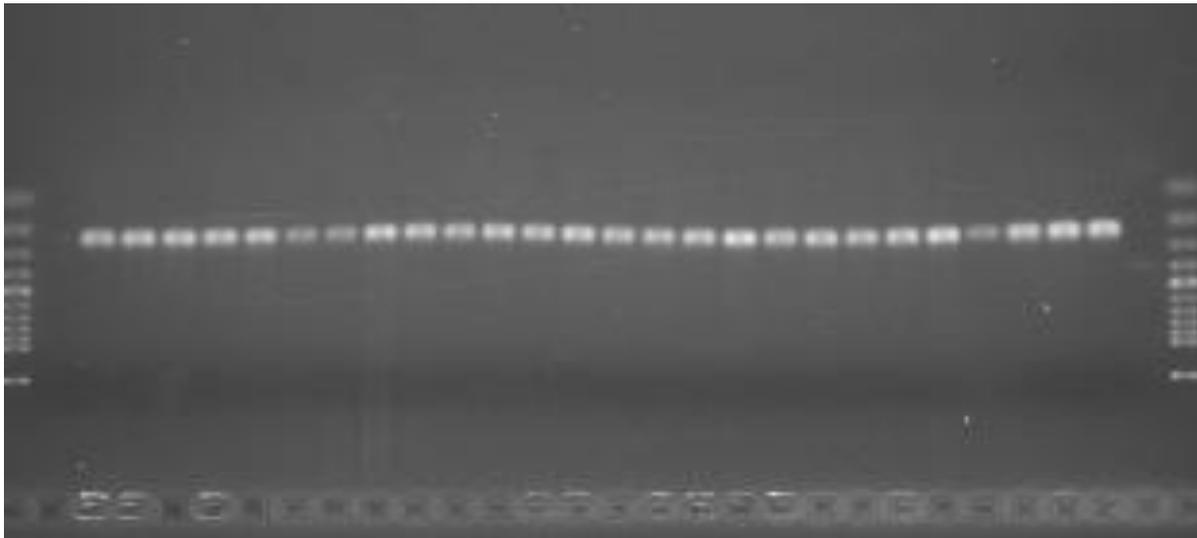
Figure 2.4. *S. aureus* 16s rRNA in beef samples. Lane 1 and 30 contain DNA ladder 100kBP. Lane 2 contains negative control
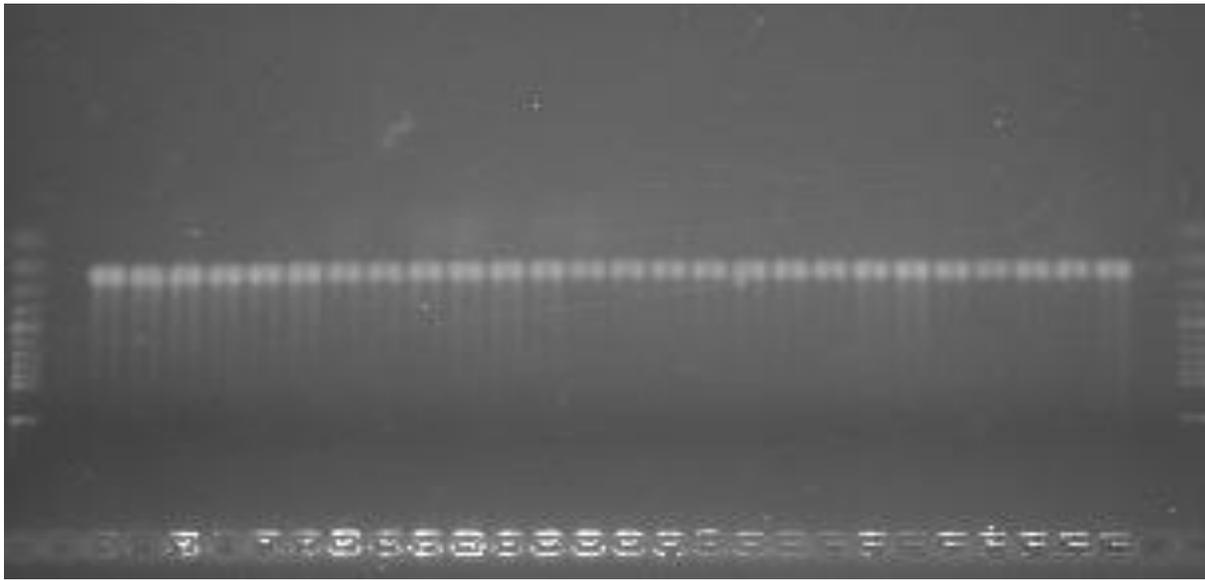
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Figure 2.5. *S. aureus* **16s rRNA in chicken samples**. Lane 1 and 30 contain DNA 100kBP ladder. Lane 2 contains negative control. Lane 29 contains NUC gene as positive (+) control.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
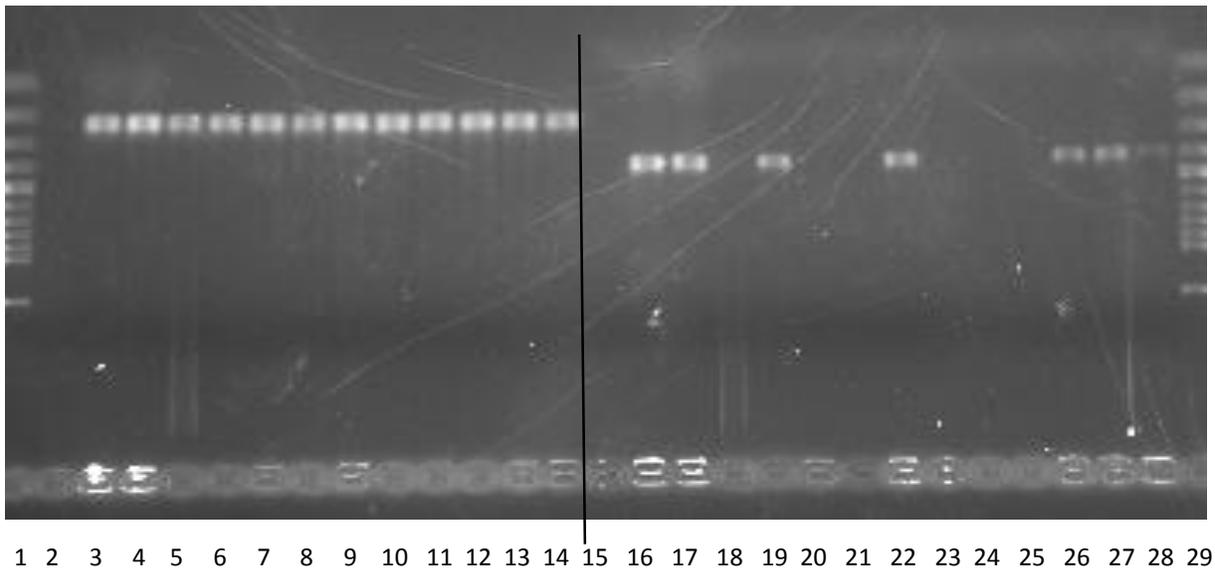
Figure 2.6. *S. aureus* **16s rRNA – nuc gene in lamb samples.** Lane 2 – 14 confirms presence of 16SrRNA gene in all Lamb samples. Lane 1 and 29 contain DNA 100 kBp ladder. Lanes 2 and 15 contain negative (-) control. Lanes 16 – 28 contains Lamb samples (1-12); bands indicate presence of *S. aureus* specific nuc gene for samples 1-12 in lamb.
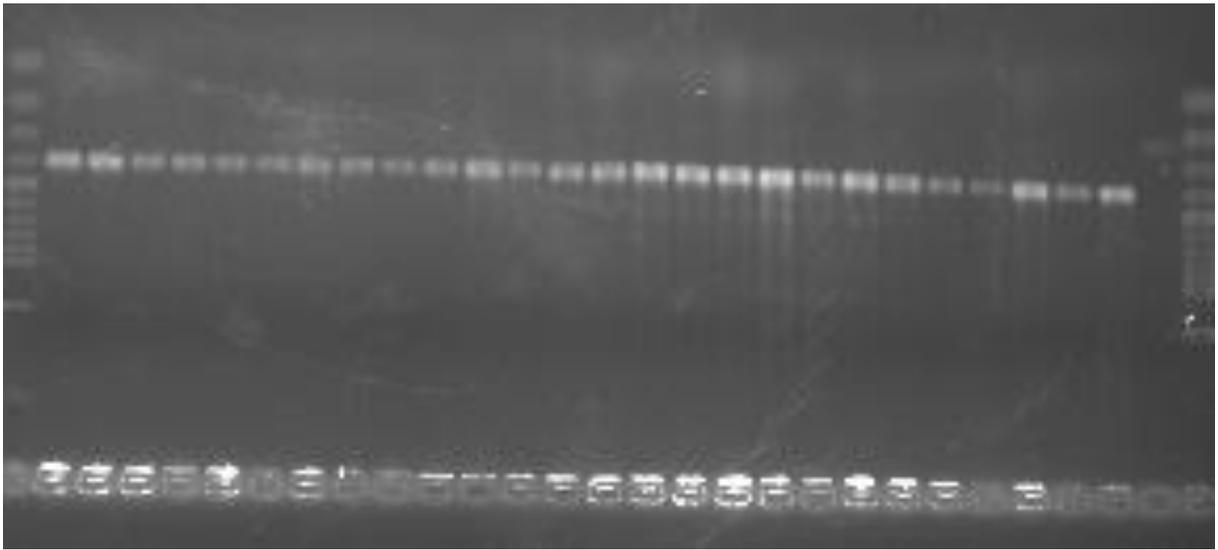
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29

Figure 2.7. *S. aureus* nuc gene in chicken samples. Lane 2 through 27 contains chicken 1 to 25. Lane 1 and 29 contains 100 kBp DNA ladder. Lane 28 contains sample 1 for chicken for 16SrRNA gene as positive control.
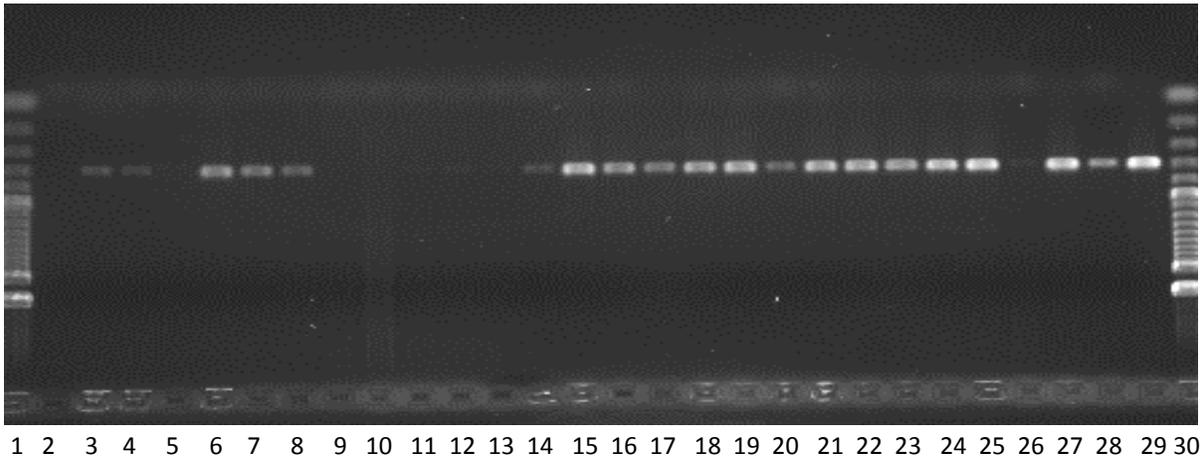
Figure 2.8. *S. aureus* **nuc gene in beef samples**. Lane 2 to 28 contain Beef sample 1 through 26
Lane 1 and 30 contain DNA ladder 100kBP. Lane 2 contains negative control. Lane 29 contains
sample 1 for chicken for nuc gene as positive control.

1 2 3   4   5   6   7   8   9   10 11 12 13 14   15 16   17 18 19 20   21 22   23 24 25   26 27   28   29 30
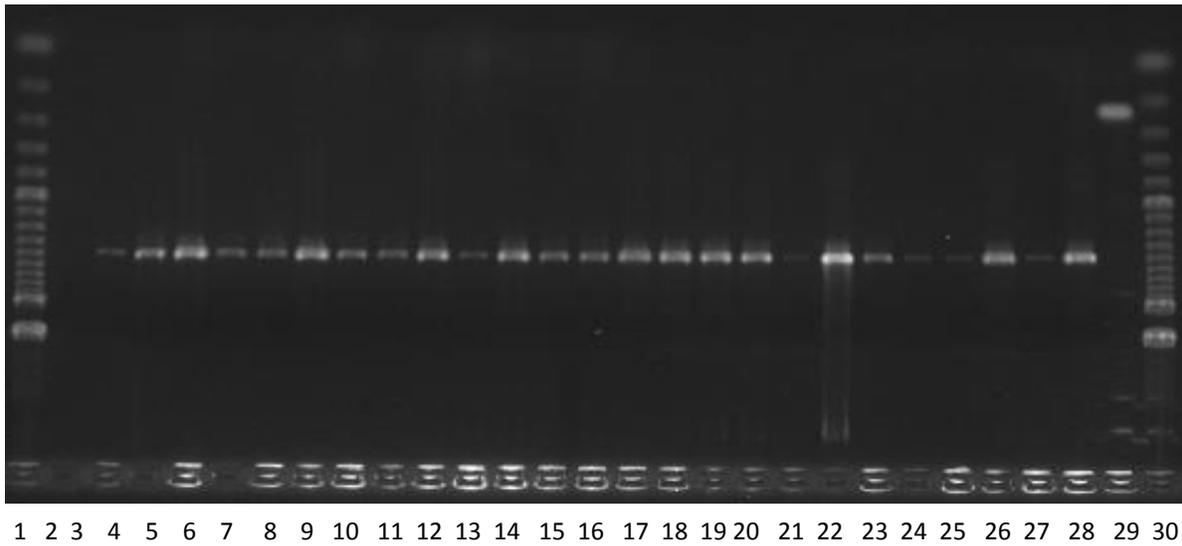
Figure 2.9. *S. aureus* **MecA gene in chicken sample 1 to 25.** Lanes 3 through 27 contains chicken samples. Lane 1 and 29 contains 100 kBp DNA ladder. Lane 2 contains negative control and land 28 contains positive control with sample no.1 of chicken for 16SrRNA gene. * Bands indicate presence of MecA gene in each sample
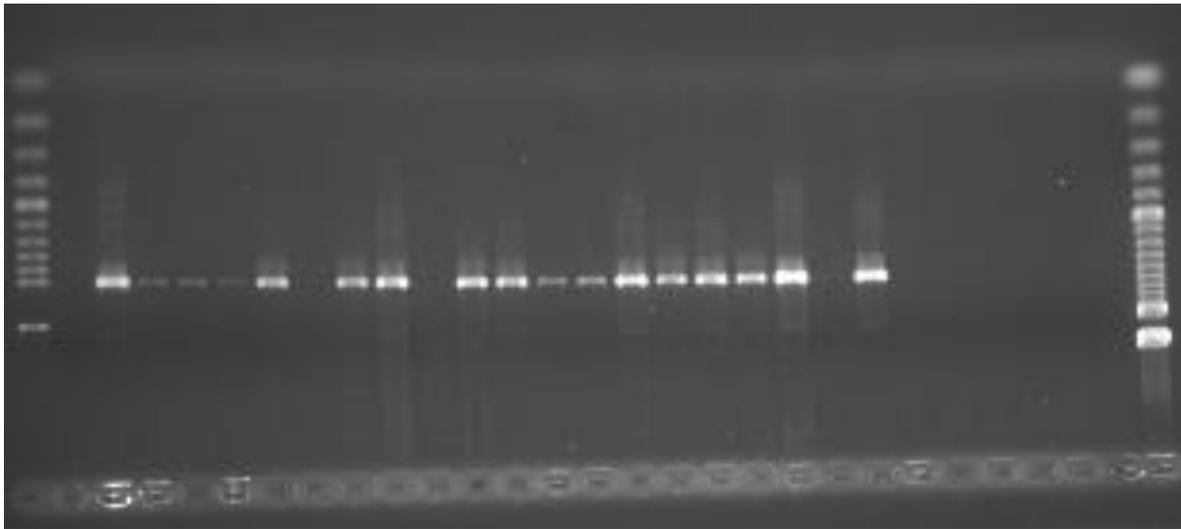
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29

Figure 2.10. *S. aureus* **mecA gene in beef samples 1 - 26.** Lanes 3 through 28 contain beef samples. *Bands indicate presence of mecA gene. Lane 2 contains negative control. Lanes 1 and 29 contains 100kBp DNA ladder.
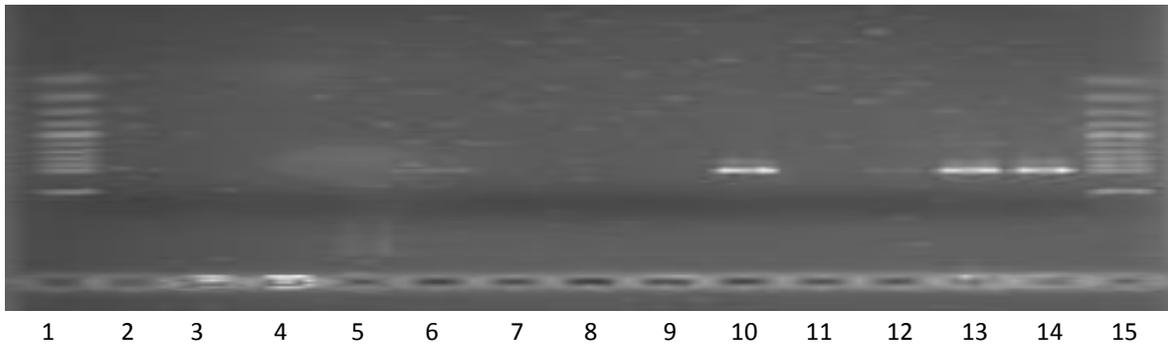
Figure 2.11. *S. aureus* mecA gene in lamb samples. Lanes 3 through 15 contains lamb samples 1 to 12. Bands indicate presence of mec A gene. Lanes 1 and 15 contains 100kBp DNA ladder.

1 2   3   4   5   6   7   8   9   10   11 12 13 14 15   16   17 18 19 20 21 22 23 24 25 26 27 28 29
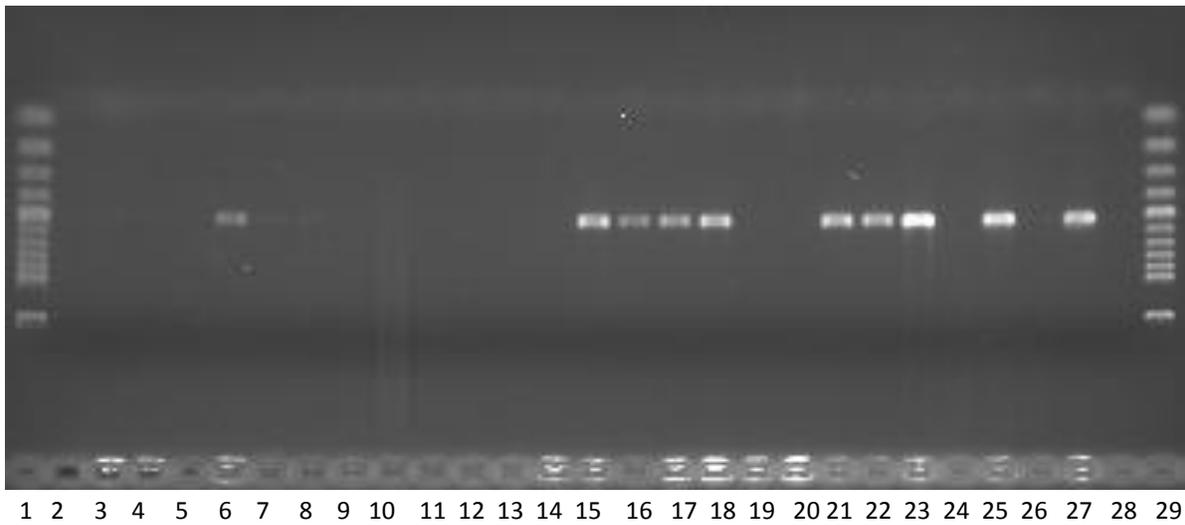
Figure 2.12. *S. aureus* sea gene in beef samples. Lanes 3 through 27 contains chickens samples 1 to 25. Bands indicate presence of *S.aureus* enterotoxins seA gene. Lanes 1 and 29 contains 100kBp DNA ladder. Lane 2 contains negative control.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
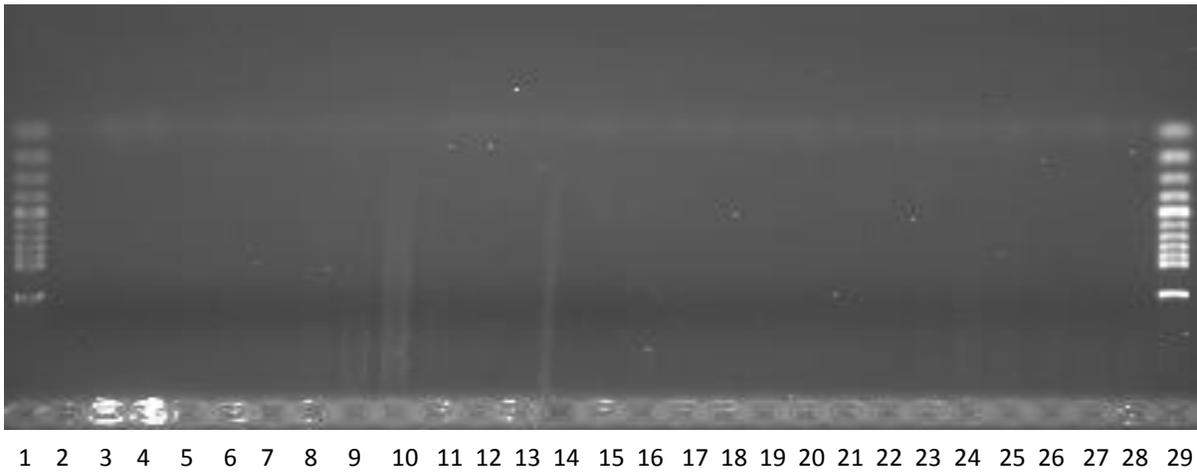
Figure 2.13. *S. aureus* **seb gene in beef samples.** No amplification of PCR products for seB gene of all beef samples. Absence of bands indicate no presence of enterotoxin seB. Lane 1 and 29 are 100kBp DNA ladder. Lane 2 contains negative control.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
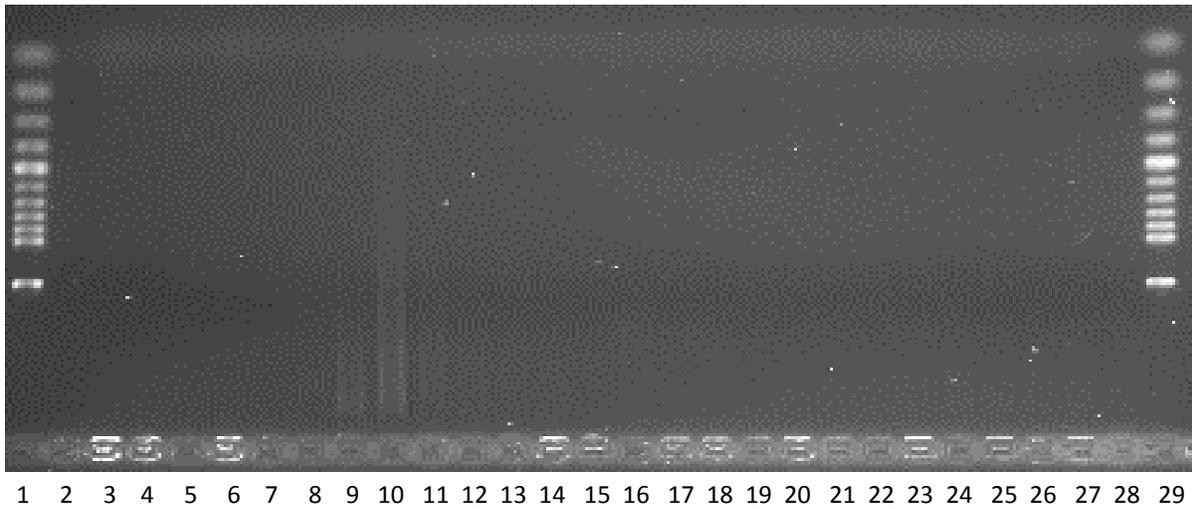
Figure 2.14. *S. aureus* sec gene in beef samples. No amplification of PCR products for seC gene of all beef samples. Absence of bands indicate no presence of enterotoxin seC. Lane 1 and 29 are 100kBp DNA ladder. Lane 2 contains negative control.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
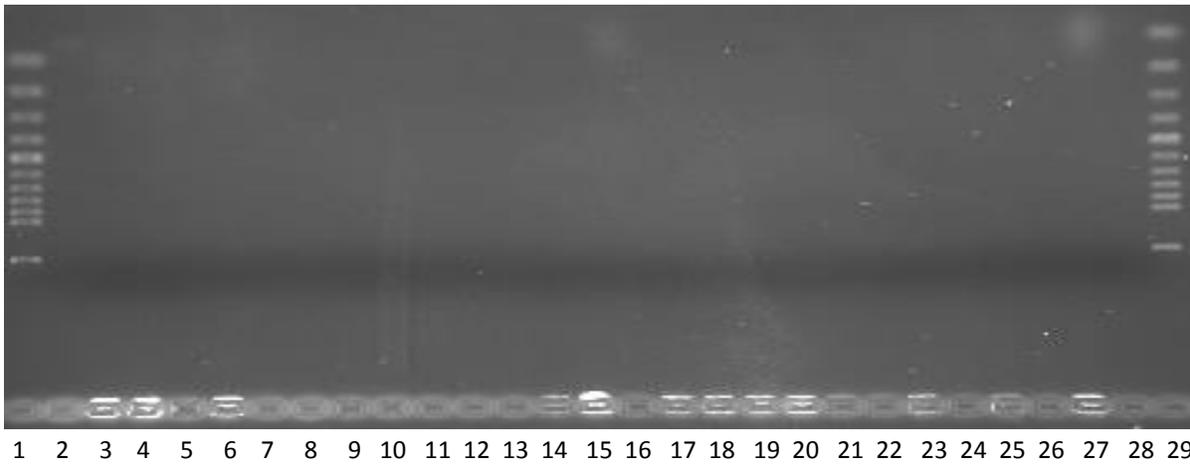
Figure 2.15. *S. aureus* sed gene in beef samples. No amplification of PCR products for seD gene of all beef samples. Absence of bands indicate no presence of enterotoxin seD. Lane 1 and 29 are 100kBp DNA ladder. Lane 2 contains negative control.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
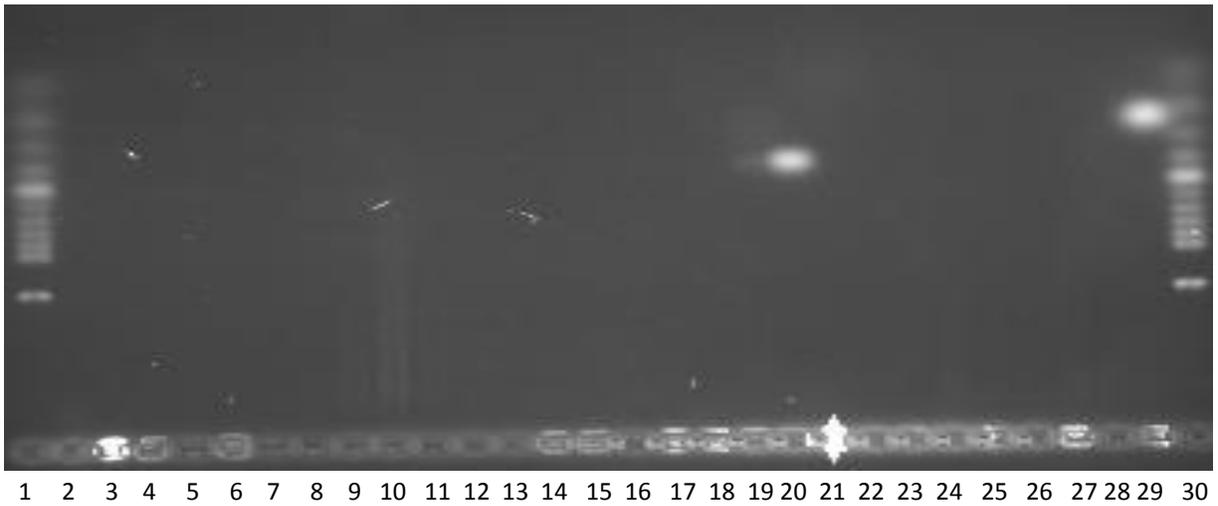
Figure 2.16. *S. aureus* see gene in beef samples. Lane 1 and 30 contains 100kBp DNA ladder. Lane 29 contains beef sample 1 for 16srRNA gene as positive control and lane 2 contians negative control. Absence of bands indicate no presence of enterotoxin seE.
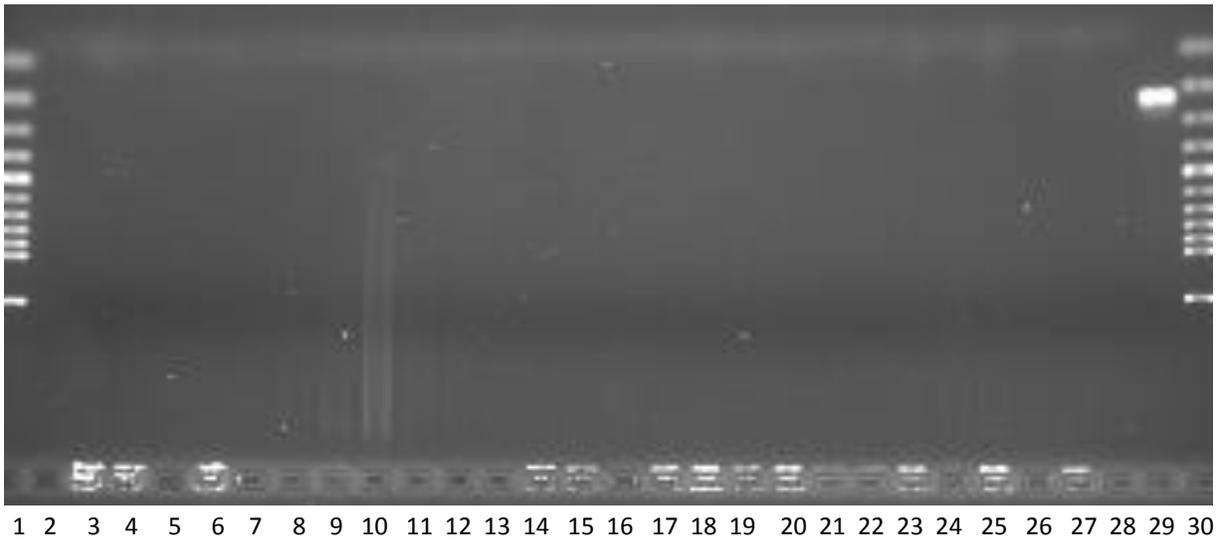
Figure 2.17. *S. aureus* seg gene in beef samples (1-25).   Lane 1 and 30 contains 100kBp DNA ladder. Lane 29 contains beef sample 1 for 16srRNA gene as positive control and lane 2 contians negative control. No amplification of PCR products for seG gene of all beef samples. Absence of bands indicate no presence of enterotoxin seG.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
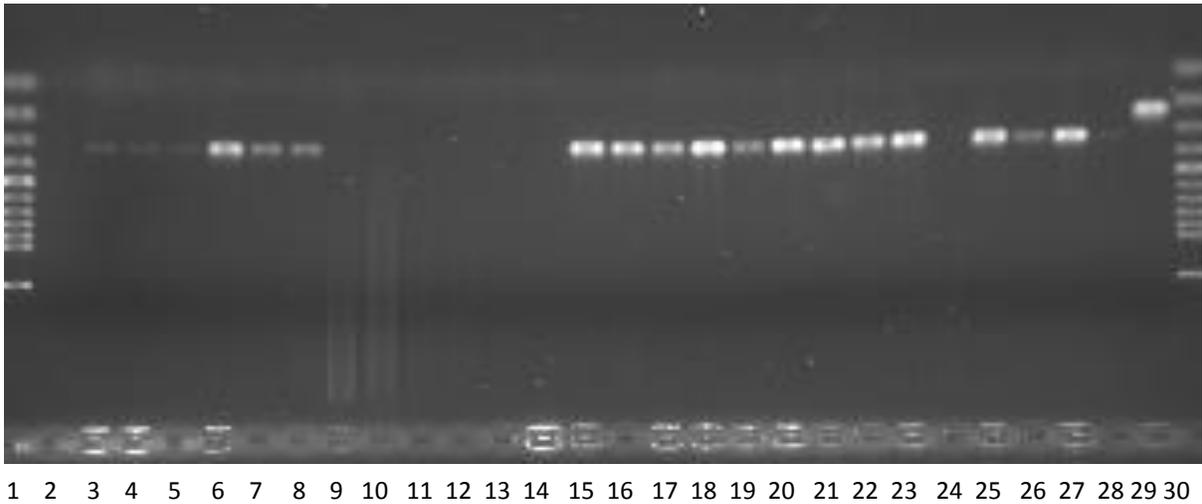
Figure 2.18.   *S. aureus* **seh gene in beef samples.** Lane 3 through 28 contain amplified PCR products for beef samples 1 to 26. Band indicates presense of seH gene. Lane 2 contains negative control and lane 29 contains amplified beef sample 1 for 16SrRNA gene as positive control. Lande 1 and 30 contain 100kBp DNA ladder.

1 2   3   4   5   6   7   8   9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Figure 2.19. *S. aureus* **sei gene in beef samples 1 to 26.** Lane 3 to 28 contain PCR amplified product for beef sample 1 to 26 target seI gene. Band indicates presense of enterotoxis seI. Lane 2 contains negative control and lane 29 contains amplified beef sample 1 with 16srRNA gene as positive control. Lane 1 and 30 contain 100kBp DNA ladder.

1 2 3 4 5 6 7   8   9 10 11 12 13   14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
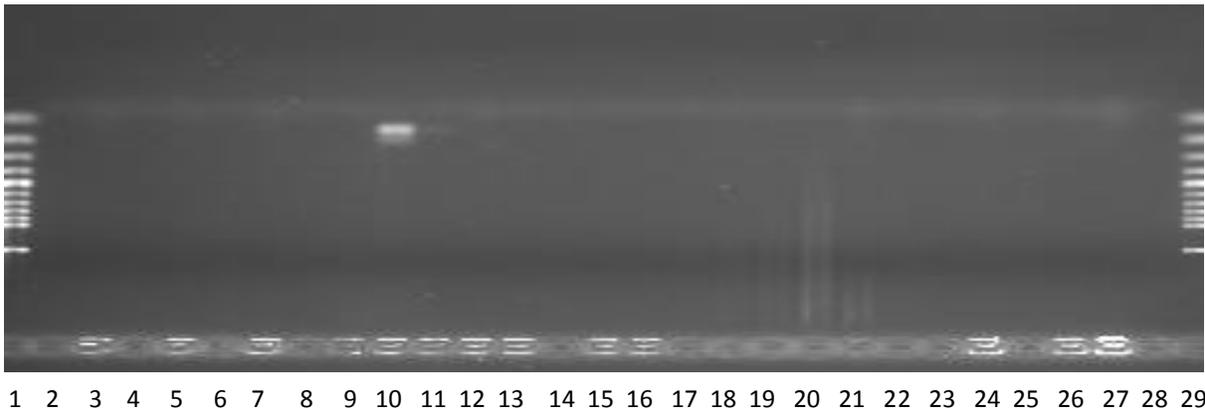
Figure 2.20. S. aureus sej gene in beef samples 1- 26. Lane 3 to 28 contain PCR amplified product for beef sample 1 to 26 target seJ gene. Band indicate presence of amplified product of enterotoxin gene seJ. Lane 2 contains negative control. Lanes 1 and 29 contains 100kBp ladder.

85

1 2   3 4   5   6   7 8 9 10 11 12 13 14 15
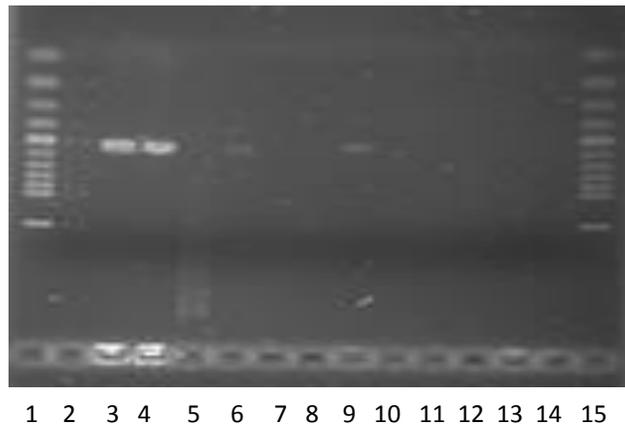
Figure 2.21. *S. aureus* sea gene in lamb samples. Lane 3 through 14 contains PCR amplified product of lamb samples 1 – 12 for target seA gene. Band indicates the presence of enterotoxin gene seA. Lane 1 and 29 contain 100kBp DNA ladder. Lane 2 contains negative control.
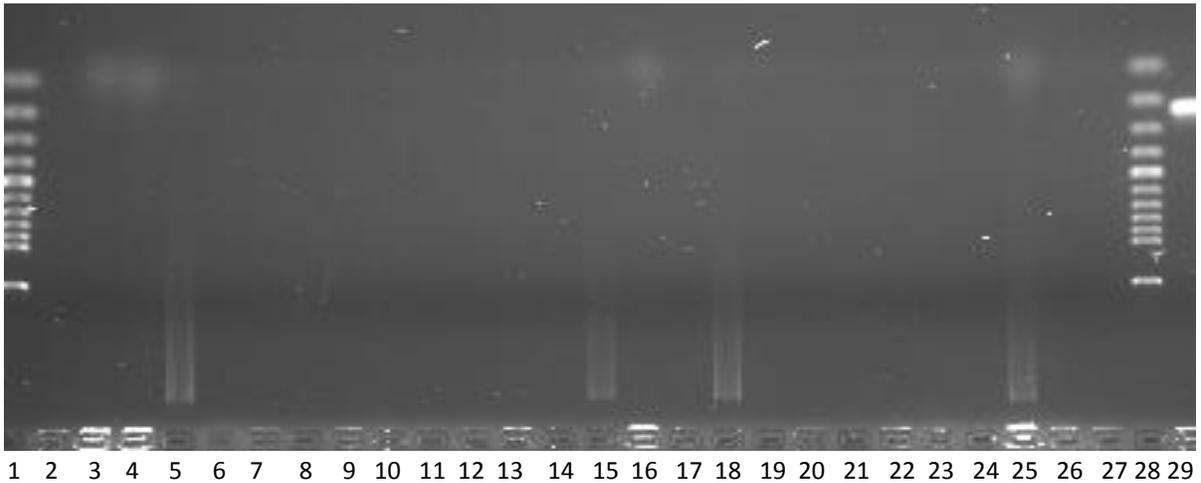
Figure 2.22. *S. aureus* **seb gene 1-12 (left), sec gene (right) 1-12 in lamb samples.** Lanes 2 through 14 contains PCR amplified products for lamb samples 1 to 12 target gene seB. Lanes 16 through 27 contain PCR amplified products for lamb samples 1 to 12 target gene seC. No band indicates absence of enterotoxin gene seB. Lanes 1 and 28 contain 100kBp DNA ladder. Lane 2 contains negative control and lane 29 contain PCR amiplified product of lamb sample 1 for nuc gene as positive control.

1 2 3 4 5   6 7   8 9 10 11 12 13 14 15   1   2 3 4 5   6 7   8 9 10 11 12 13 14 15
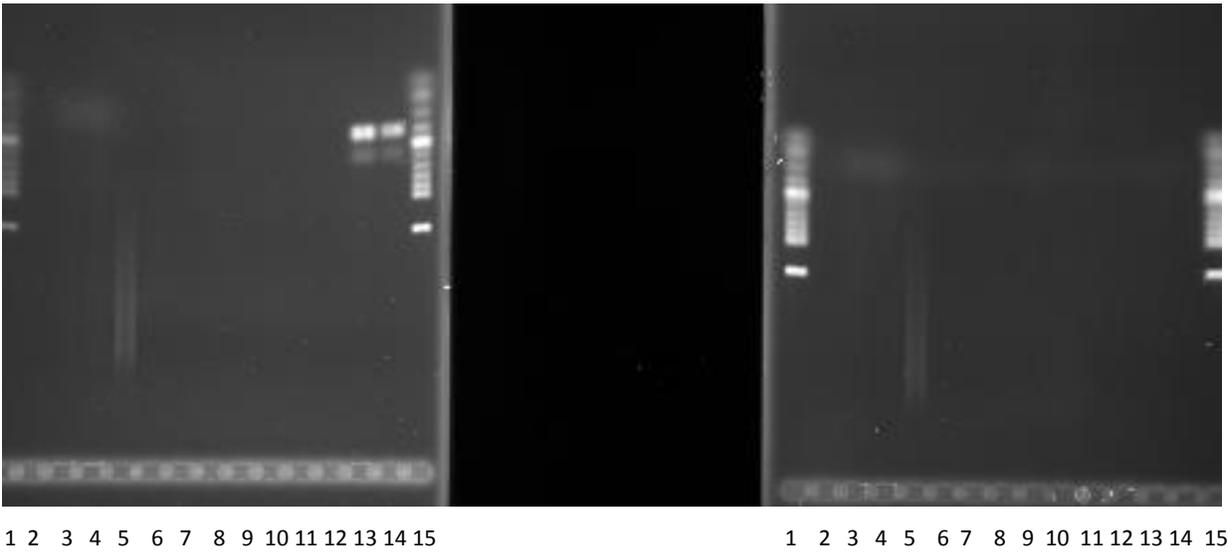
Figure 2.23. *S. aureus* sed gene 1-12 (left), *S. aureus* see gene 1-12 in lamb samples. Lanes 3 to 14 contains PCR amplified products for lamb samples 1 to 12 for target gene seD (left) and seE (right). Band indicate presence of enterotoxin gene seD (left) or seE (right). Lanes 1 and 15 contain 100kBp DNA ladder. Lanes 2 contain negative control.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 617 18 19 20 21 22 23 24 25 26 27 28 29 30
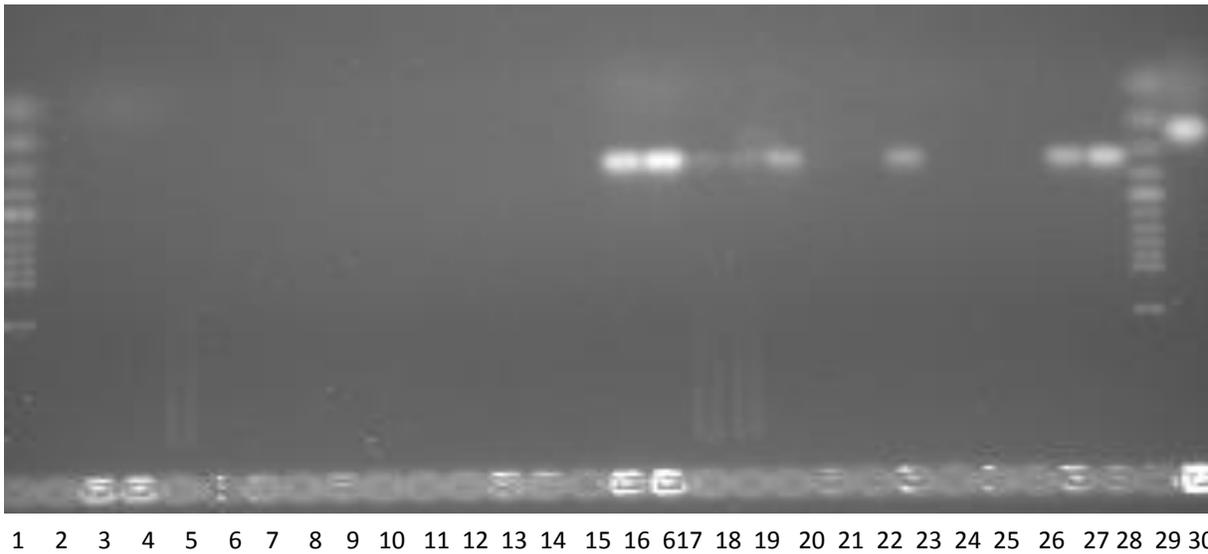
Figure 2.24. *S. aureus* **see gene 1-12 (left) - seg gene (right) 1-12 in lamb samples.** Lanes 3 through 14 and 16 through 28 contain PCR amplified products of lamb samples 1 to 12 for target genes seE and seG, respectively. Lane 1 and 29 contain 100kBp DNA ladder. Lane 2 and 15 contain negative control and lane 30 contains amplified product of lamb samples 1-12 for target 16SrRNA gene.
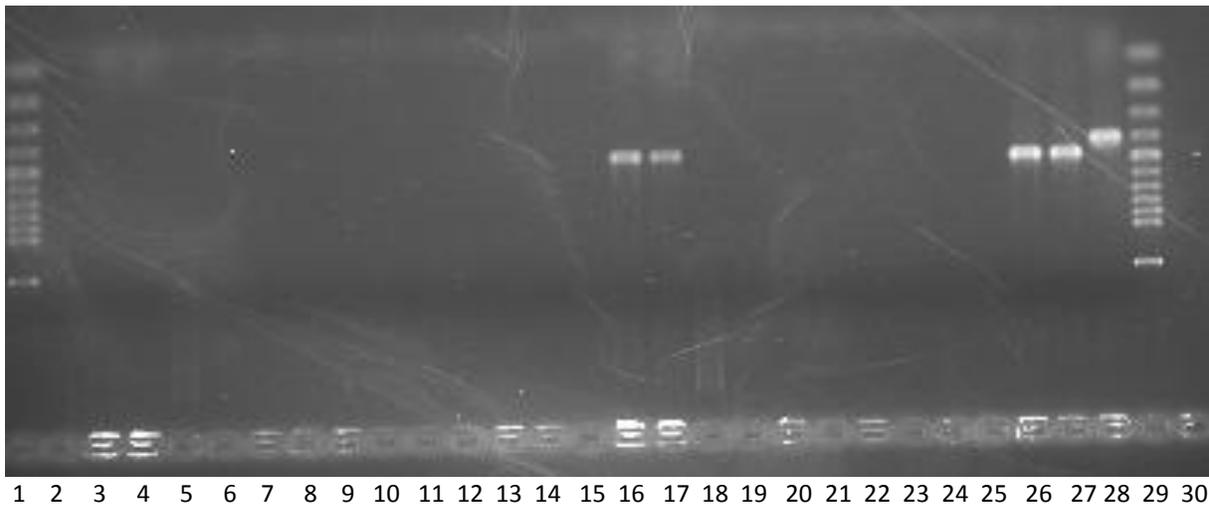
Figure2.25. *S. aureus* **seh gene (left) 1-12-sei gen 1-12 in lamb samples.** Lanes 3 through 14 and 16 through 28 contain PCR amplified products of lamb samples 1 to 12 for target genes seH and seI, respectively. Lane 1 and 29 contain 100kBp DNA ladder. Lane 15 and 30 contain negative control and lane 28 contains amplified product of lamb samples 1 for target nuc gene.
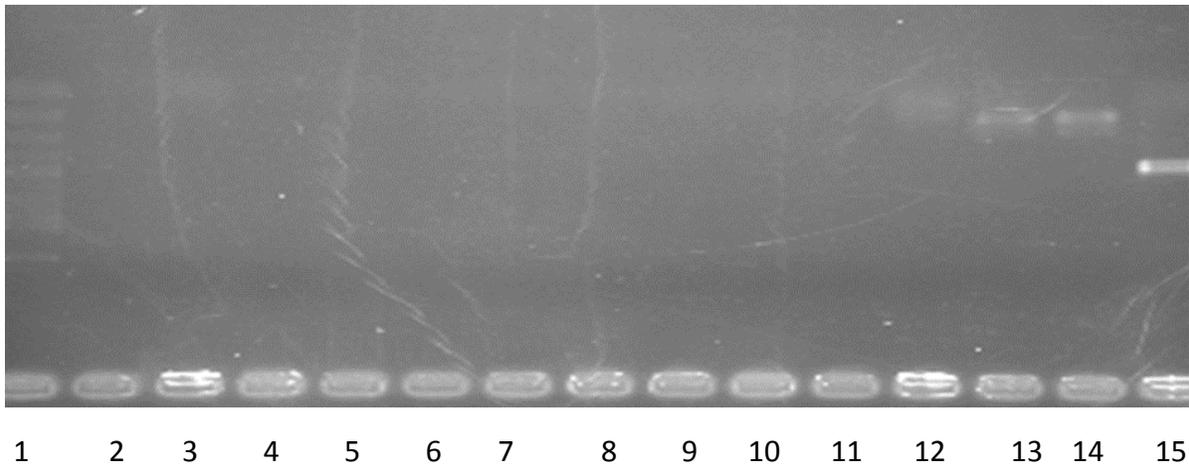
Figure 2.26. *S. aureus* **sej gene in lamb samples.** Lanes 3 through 14 contain amplified PCR product for lamb samples 1 to 12 for target seJ gene. Band indicates presence of enterotoxin seJ in specific samples. Lane 1 contains 100kBp DNA ladder. Lane 2 contains negative control and lane 15 contains amplified PCR product of lamb sample 1 for target nuc gene.
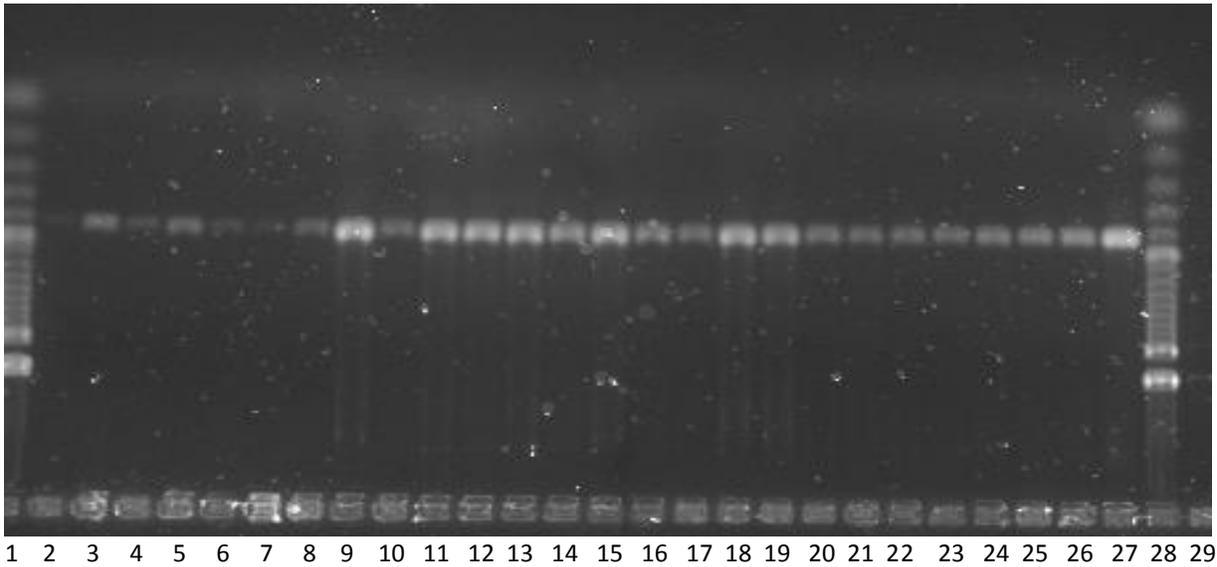
Figure 2.27. *S. aureus* sea gene in chicken samples. Lanes 2 through 26 contain amplified PCR product of chicken samples 1 to 25 and target seA gene. Band indicates presence of enterotoxin gene seA in specific samples. Lanes 1 and 28 contain 100kBP DNA ladder. Lane 29 contains negative control and lane 27 contains amplified PCR product of chicken sample 1 to 25 and target seA gene.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
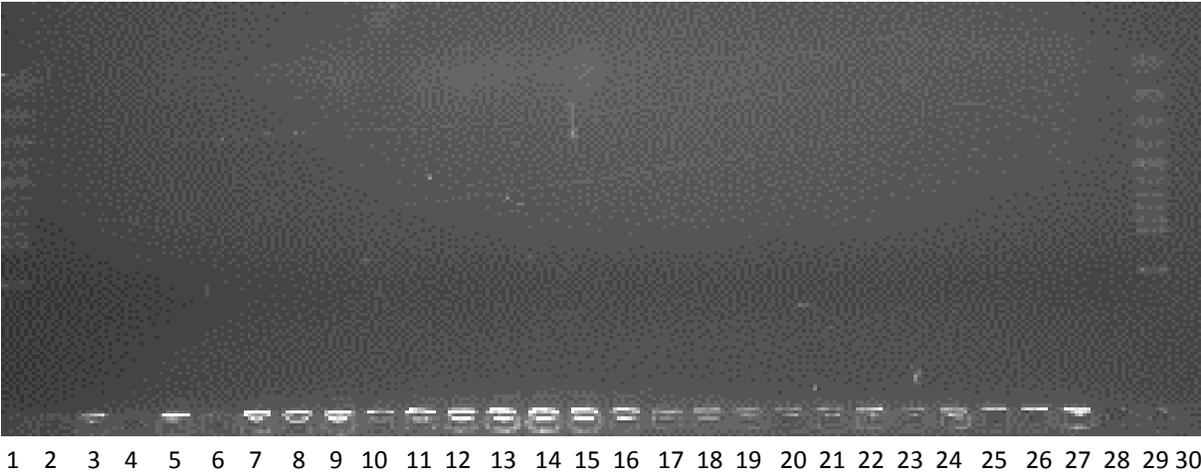
Figure 2.28. *S. aureus seb* gene in chicken samples. Lanes 3 through 28 contains amplified PCR products of chicken samples 1 to 25 and target seB gene. No band indicates absence of enterotoxin seB in specific samples. Lane 1 and 29 contain 100kBp DNA ladder. Lane 2 contains negative control and lane 30 is blank.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
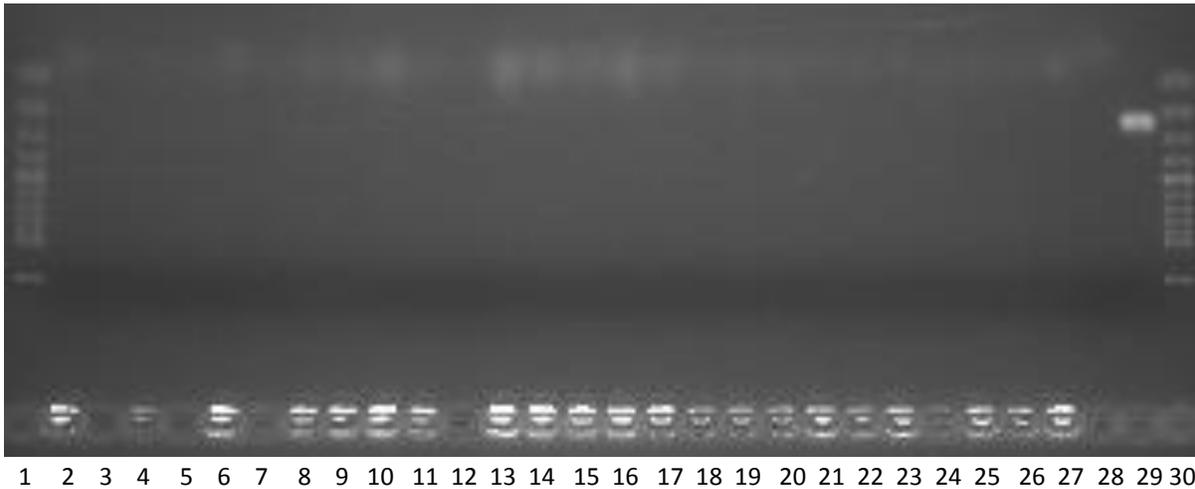
Figure 2.29. *S. aureus sec* gene in chicken samples. Lanes 3 through 28 contains amplified PCR products of chicken samples 1 to 25 and target seC gene. No band indicates absence of enterotoxin seC in specific samples. Lane 1 and 30 contain 100kBp DNA ladder. Lane 2 contains negative control and lane 29 contains amplified PCR product of chicken sample 1 and target 16SrRNA gene as positive control.
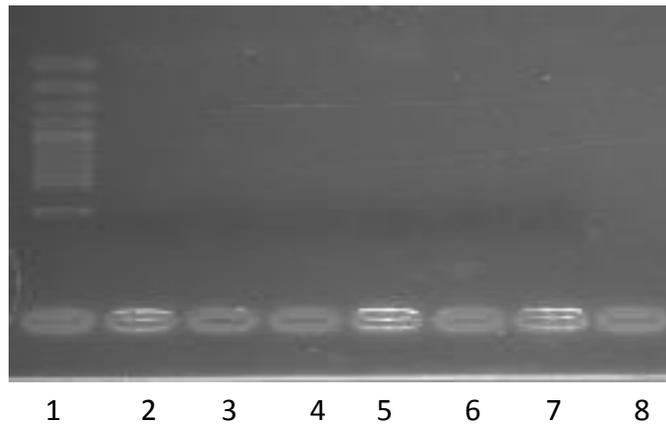
Figure 2.30. *S. aureus* **sed gene 1-6 in chicken samples. Lanes 3 through 8 contain amplified PCR product of chicken samples 1 to 6 and seD gene. No band indicates absence of enterotoxin seD gene. Lane 1 contains 100kBp DNA ladder and lane 2 contains a negative control.**
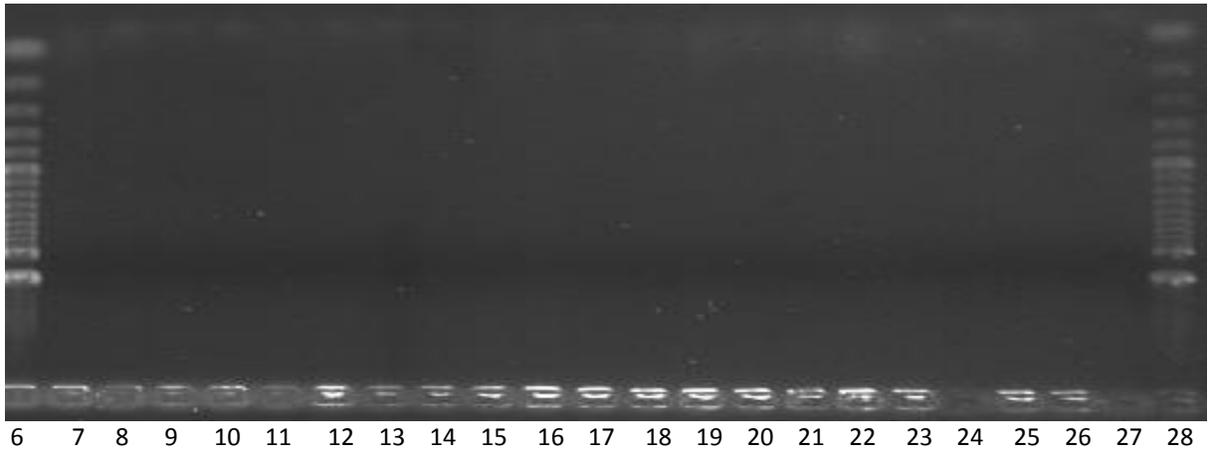
Figure 2.31. *S. aureus* sed gene 6-25 in chicken samples. Lanes 8 through 27 contains amplified PCR product of chicken sample 6 to 25 and target seD gene. No band indicates absence of enterotoxin seD gene. Lane 6 and 28 contains 100kBp DNA ladder. Lane 7 contains negative control.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
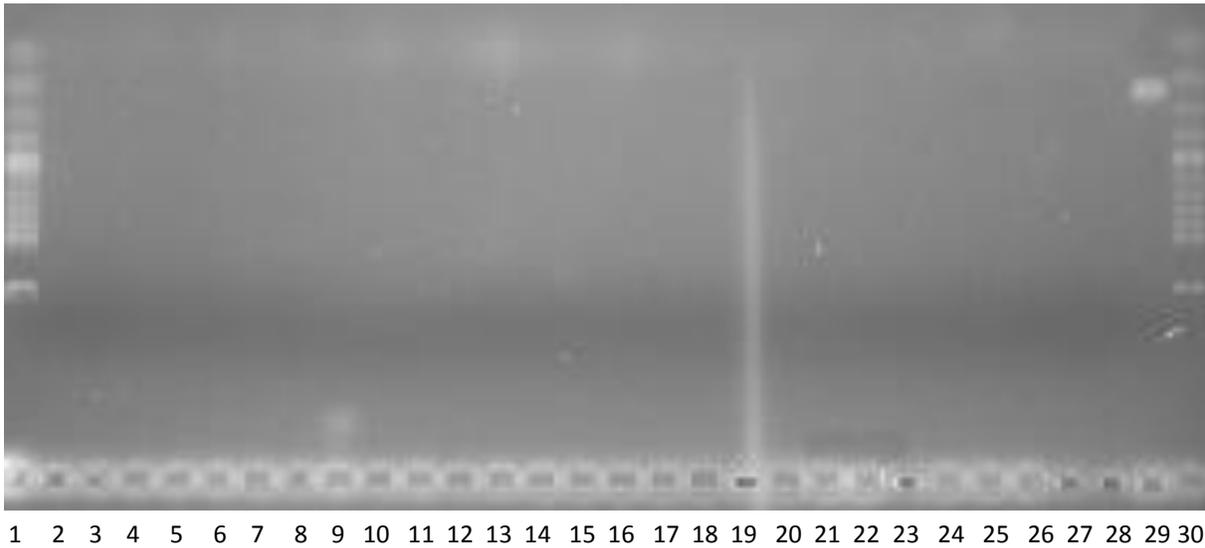
Figure 2.32. *S. aureus* see gene in chicken samples. Lane 3 through 28 contains amplified PCR product of chicken samples 1 to 25 and target seE gene. No band indicated absence of enterotoxin seE in specific samples. Lane 1 and 30 contians 100kBp DNA ladder. Lane 2 contians negative control and lane 29 contains amplified PCR product of chicken sample 1 and target 16SrRNA gene as positive control.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
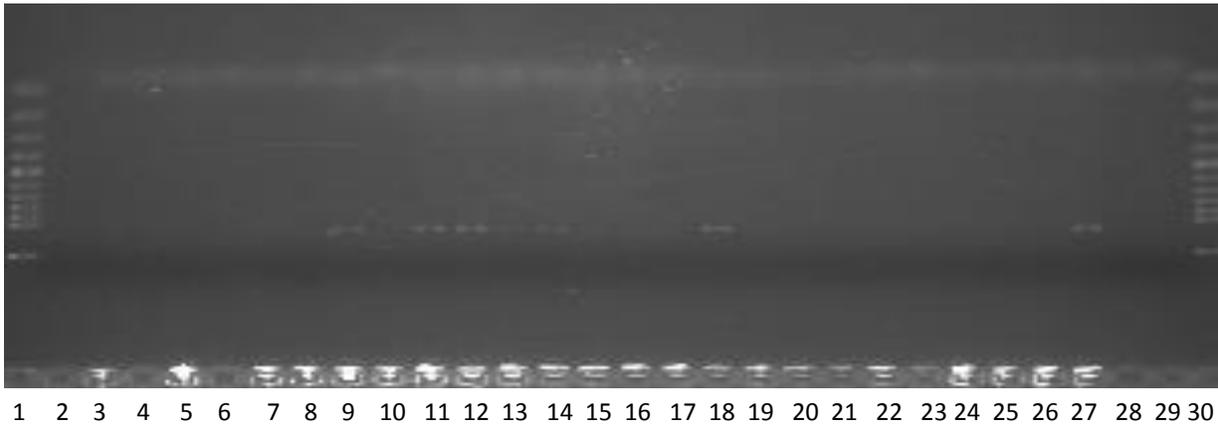
Figure 2.33. *S. aureus* **seg gene in chicken samples.** Lane 3 through 28 contains amplified PCR product of chicken sample 1 to 25 and target seG gene. Band indicates presence of enterotoxin seG gene in specific samples. Lane 1 and 30 contians 100kBp DNA ladder. Lane 2 contians negative control and lane 29 contains blank.
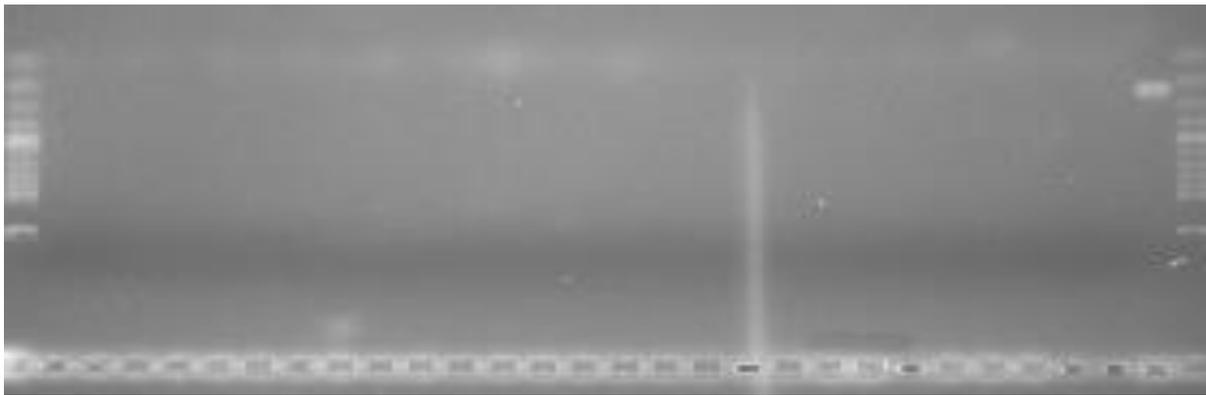
1 2 3   4 5 6   7   8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Figure 2.34. *S. aureus* **seh gene in chicken samples.** Lane 3 through 28 contains amplified PCR product of chicken samples 1 to 25 and target seH gene. No band indicated absence of enterotoxin seH in specific samples. Lane 1 and 30 contians 100kBp DNA ladder. Lane 2 contians negative control and lane 29 contains amplified PCR product of chicken sample 1 and target 16SrRNA gene as positive control.
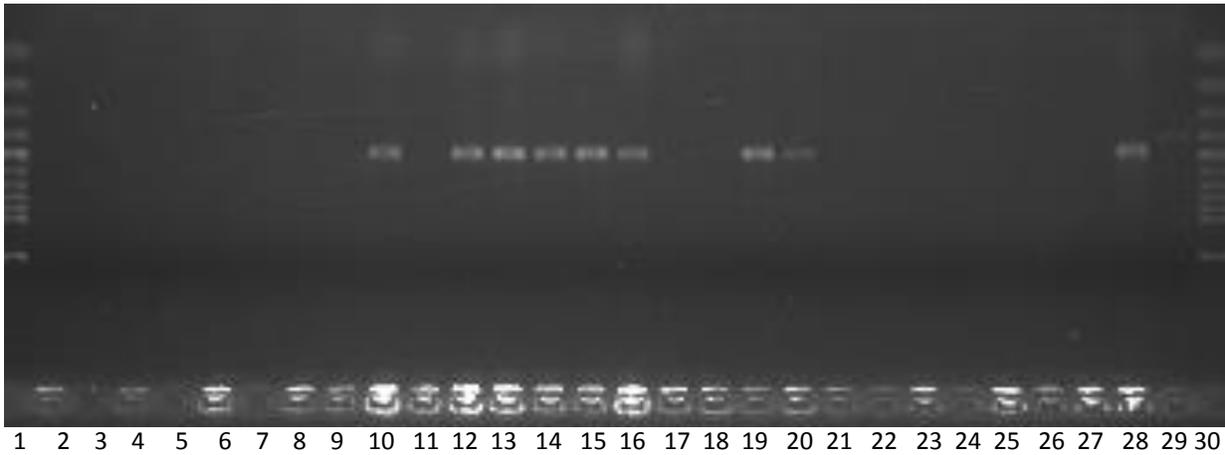
Figure 2.35. *S. aureus* **sei gene in chicken samples.** Lanes 3 through 28 contains amplified PCR products of chicken samples 1 to 25 and target seI gene. Band indicates presence of enterotoxin seI in specific samples. Lane 1 and 30 contain 100kBp DNA ladder. Lane 2 contains negative control and lane 29 contains amplified PCR product of chicken sample 1 and target nuc gene as positive control.
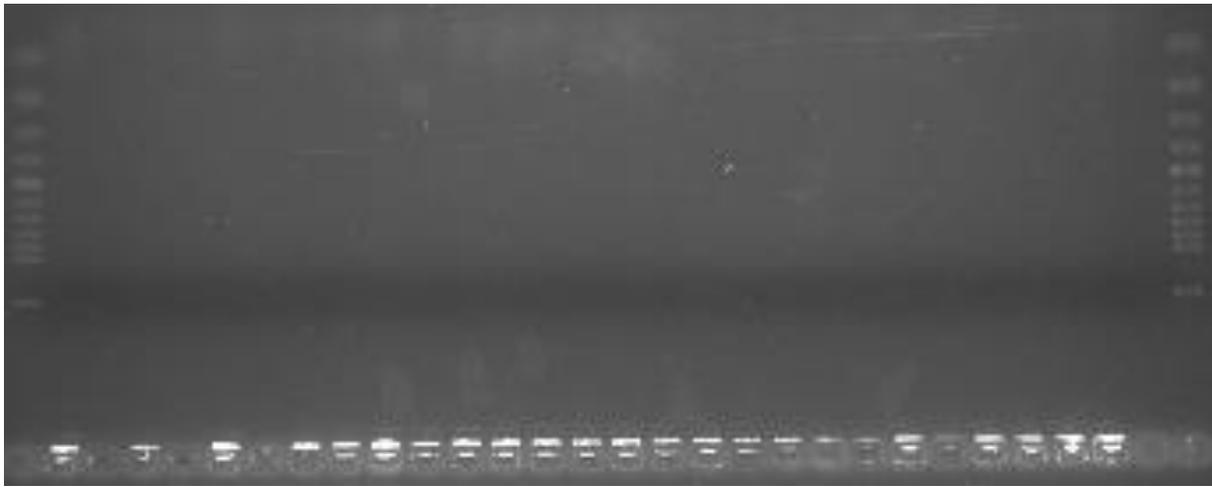
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Figure 2.36. S*. aureus* sej gene in chicken samples. Lane 3 through 28 contains amplified PCR product of chicken samples 1 to 25 and target seJ gene. No band indicated absence of enterotoxin seJ in specific samples. Lane 1 and 30 contians 100kBp DNA ladder. Lane 2 contians negative control and lane 29 contains blank.

Table2.3.Distribution of enterotoxin genes among *S. aureus* isolates from meat.

Gene	No.(%) of *S. aureus* isolates n=63			Total
	S.aureus isolates (beef) n=26	S.aureus isolates (lamb) n=12	S.aureus isolates (Chicken) n=25	
Sea	10 (15.5%)	4 (6.3%)	25(39.6%)	39 (61.9%)
Seb	0	0	0	0
Sec	0	0	0	0
Sed	0	2 (3.1%)	0	2 (3.1%)
See	0	0	0	0
Seg	0	6 (9.5%)	0	6 (9.5%)
Seh	17 (26.9%)	4 (6.3%)	0	21 (33.3%)
Sei	11 (17.4%)	0	8 (12.6%)	19 (30.1%)
Sej	0	2 (3.1%)	0	2 (3.1%)
Sea + sei	7 (11.1%)	0	8 (12.6%)	15 (23.8%)
Sej + seh	0	2 (3.1%)	0	2 (3.1%)
Sei + seh	11 (17.4%)	0	0	11 (17.4%)
Sed + seg	0	2 (3.1%)	0	2 (3.1%)
Sea + seh	9 (14.2%)	0	0	9 (14.2%)
Sed + seh	0	2 (3.1%)	0	2 (3.1%)
Sed + sej	0	2 (3.1%)	0	2 (3.1%)
Sea+ sei + seh	7 (11.1%)	0	0	7 (11.11%)
Sed + seg + sej + seh	0	2 (3.1%)	0	2 (3.1%)

Table2.4. Antimicrobial resistance of *S. aureus* isolates from meat.

Antibiotic	No.(%) of *S. aureus* isolates n=96			
	Beef n=33	Lamb n=35	Chicken n=28	Total
Ampicillin	23 (69.6%)	26 (74.2%)	19 (67.8%)	68 (70.8%)
Gentamicin	2 (6%)	0	1 (3.5%)	3 (3.1%)
penicillin	25 (75.7%)	23 (65.7%)	21 (75%)	69 (71.8%)
Oxacillin	7 (21.2%)	11 (31.4%)	7 (25%)	25 (26%)
Ciprofloxacin	0	0	0	0
Erythromycin	3 (9%)	2 (5.7%)	2 (7.1%)	7 (7.2%)
Tetracycline	14 (42.4%)	12 (34.2%)	9 (32.1%)	35 (36.4%)
Chloramphenicol	1 (3%)	0	1 (3.5%)	2 (2%)
Rifampicin	1 (3%)	0	1 (3.5%)	2 (2%)
Vancomycin	0	0	0	0
Amikacin	1 (3%)	1 (2.8%)	0	2 (2%)
Amoxicillin	4 (12.1%)	6 (17.1%)	4 (14.2%)	34 (35.4%)
Clindamycin	1 (3%)	1 (2.8%)	1 (3.5%)	3 (3.1%)
Nitrofurantoin	0	0	0	0
Multidrug resistance	15 (48.4%)	19 (54.2%)	13 (46.4%)	47 (48.9%)

Results and discussion

1-Prevalence of *S. aureus* in different types of meat

Previous studies showed that *S. aureus* is the most important Staphylococcus species causing food-borne illness (Jablonski & Bohach, 2001). Prevalence of *S. aureus* in meat samples were shown in table (2.1), Our results showed that of the 142 samples, 61 (42.9%) samples were positive for *S. aureus* were confirmed to be *S. aureus* by genotypic identification when the gene *nuc* and the target 16s rRNA were observed, and by biochemical identification when gram stain, catalase reaction, coagulase test were positive. 63 *S. aureus* isolates were recovered from the 61 *S. aureus* –positive samples (1–2 isolates per sample) for enterotoxin genes detection. Also, a total 96 *S. aureus* isolates including (33), (35) and (28) from beef, lamb and chicken, respectively were used for antibiotic resistance test. The presence of *S. aureus* in foods commonly indicates contamination that may be directly introduced into the food by workers who have skin lesions containing *S. aureus,* or by sneezing or coughing (Jay, 1986). Other contamination sources of *S. aureus* are soil, water, dust and air (Arbuthnott, 1990).

Previous studies showed that *S. aureus* is the most important Staphylococcus species causing food-borne illness (Jablonski & Bohach, 2001). Our study showed that prevalence of *S. aureus* (42.9%) in the meat samples was high. (Waters et al, 2011) reported that prevalence of *S. aureus* in meat samples in five cities in USA was (47%). In contrast, much lower prevalence of *S. aureus* was also reported, such as (8%) in Korea (Heo et al., 2008). Beef samples showed the highest prevalence (41.1%) of *S. aureus* contamination while in chicken samples showed the lowest prevalence (27.4%) table (2.1.).

2-Enterotoxin genes of *S. aureus* in meat

Staphylococcal food Poisoning (SFP) is one of the most common causes of food-borne illness due to the widespread occurrence of *S. aureus* and to the ability of many strains to produce one or more Staphylococcal enterotoxin (SEs). The heat stable toxins produced by bacterial pathogens do not lose their biological activity by cooking and their presence in meat at elevated levels is a food safety concern (Le Loir et al., 2003). A genotyping by PCR was performed to detect classical enterotoxin genes (*sea* to *see*) and newly enterotoxin genes (*seg* to *sej*) for total of 63 *S. aureus* isolates were recovered from the 61 *S. aureus* –positive samples (1–2 isolates per sample), including (12,26,25) isolates from lamb, beef and chicken respectively. Our results showed that 80.9% of the tested strains contained either one or more enterotoxin genes, which is agreement with (85%) reported in USA (Pu et al, 2011).However, it was lower than 59.8% reported in an Italy (Normanno et al., 2007) and 62% reported in Korea (Oh et al., 2007),In addition, the percent of *S. aureus* isolates that harbored two to four SE genes in the present study was 36.5%, which is lower than 66% reported in USA (Pu et al, 2011) and agreement with 25.6% reported in an Italy (Normanno et al., 2007).A high percent of beef isolates (60%) contained enterotoxigenic *S. aureus* than that of chicken and lamb. Our study showed that the two most commonly detected Es genes in meat were sea (61.9%) and seh (33.3%) followed by sei (30.1), seg (9.5%), sed and sej (3.17% each). No isolates harbored three other SE genes, that is, *seb*, *sec*, or *see*. A high percent of *sea* gene was detected in present study was consistent with reports that *sea* gene is the most common enterotoxin found during food and food poisoning outbreaks worldwide(Balaban and Rassoly, 2000); (Choi, 2000).The most commonly SE gene combination was (*sea- sei*) occurring in (23.8%) of *S. aureus* isolates followed by (sei, seh), (sea, seh), and (sea,

sei, he) were occurring in (17.4),(14.2),and (11.11) respectively. Mec A gene was positive in 71.4

of *S. aureus* isolates. All isolates containing the mec A Agene showed positive MRSA phenotypes.

Results from this research indicate that high levels of staphylococcal enterotoxin genes were

detected in meat. Therefore, these findings are alarming and require the need to prevent the

presence of *S. aureus* strains and SEs production in meat.

3- Antibiotic resistance of *S. aureus* isolates from different types of meat

Food is an important factor for the transfer of antibiotic resistances. Such transfer can

occur by means of antibiotic residues in food, through the transfer of resistant food-borne

pathogens or through the ingestion of resistant strains of the original food microflora and

resistance transfer to pathogenic microorganisms (Khan et al., 2000**)**; (Pesavento et al., 2007). *S.*

aureus strains are known to be frequently resistant to antibiotic therapy due to their capacity to

produce an exopolysaccharide barrier and because of their location within micro abscesses,

which limit the action of drugs (Gundogan et al., 2006). Antimicrobial susceptibility tests for *S.*

aureus isolates against different antibiotics are shown in table (2.4). Our results indicated that *S.*

aureus isolates showed different antimicrobial resistance levels where the percent of resistance

for a total of (96) *S. aureus* isolates to fourteen antimicrobials were as follow: ampicillin (70.8%),

penicillin (71.8%), tetracycline (36.4%), amoxicillin(35.4%),oxacillin (26%), erythromycin (7.2%),

clindamycin (3.1%), gentamycin (3.1%), Chloramphenicol(2%), amikacin (2%), rifampin(2%)

,vancomycin (0%), ciprofloxacin (0%), and nitrofurantoin (0%) . Our results showed that there

are three groups of isolates based on resistance to antibiotic where some isolates showed high

resistant to specific antibiotic specially the ones that are generally used as initial line of treatment

such as penicillin 69 (71.8**%),** ampicillin 68 (70.8%), tetracycline 35 (36.4**%)** and amoxicillin 34

(35.4**%**). Another group was showed that no resistance to some antibiotic such as nitrofurantoin, vancomycin and ciprofloxacin, they are susceptible, thus giving us some way of treating any infection caused by the same strains of the *S. aureus* isolates. A small percentage of the isolates demonstrated resistance to rifampicin, amikacin and chloramphenicol (2%) for each, to gentamycin and clindamycin were (3.1%) for each and to erythromycin was (7.2%). The isolates were collected from lamb samples demonstrated the most sensitive to the tested antibiotics among tested samples.

Multidrug resistant strains of *S. aureus* are a risk factor for the public health**.** A methicillin susceptibility test showed that (26%) *S. aureus isolates* were resistant to methicillin. The percent of multidrug resistance of *S. aureus* isolates (resistance to three or more classes) were (48.9%) (Table2.2). A high percent of multidrug resistant *S. aureus* was detected in our study is alarming. It raises concerns about inappropriate practices including the use of antimicrobials as growth promotors in food animal production and the frequent use of antimicrobials in poultry husbandry. Genes coding for antimicrobial resistance can move through horizontal gene transfer to clinical pathogenic strains and contribute to the creation of superbugs. Results from this research indicated that high levels of staphylococcal enterotoxin genes, antibiotic resistance and multidrug resistant were detected in meat. Therefore, these findings are alarming and require the need to prevent the presence of *S. aureus* strains and SEs production in meat.

REFERENCES

- Akineden, O., C. Annemu¨ller, A. A. Hassan, C. La¨mmler, W. Wolter, and M. Zscho¨ck. 2001. Toxin genes and other characteristics of Staphylococcus aureus isolates from milk of cow with mastitis. Clin. Diagn. Lab. Immunol. 8:959–964.

- Altboum Z, Hertman I, Sarid S. Penicillinase plasmidlinked genetic determinants for enterotoxins B and C1 production in Staphylococcus aureus. Infect Immun. 1985; 47:514–521.

- Al-Daccak, R.; Mehindate, K.; Damdoumi, F.; Etongue-Mayer, P.; Nilsson, H.; Antonsson, P.; Sundstrom, M.; Dohlsten, M.; Sekaly, R.P.; Mourad, W. Staphylococcal Enterotoxin D Is a Promiscuous Superantigen Offering Multiple Modes of Interactions with the MHC Class II Receptors. J. Immunol. 1998, 160, 225–232.

- Arbuthnott, J., Coleman, D., & Azavedo, J. S. d. (1990). Staphylococcal toxins in human disease. Journal of Applied Microbiology, 69(S19), 101S-107S.

- Argudín, M.A.; Mendoza, M.C.; Rodicio, M.R. Food poisoning and Staphylococcus aureus enterotoxins. Toxins 2010, 2, 1751–1773.

- Atanassova, V.; Meindl, A.; Ring, C. Prevalence of Staphylococcus aureus and Staphylococcal Enterotoxins in Raw Pork and Uncooked Smoked Ham—a Comparison of Classical Culturing Detection and RFLP-PCR. Int. J. Food Microbiol. 2001, 68, 105–113. Toxins 2010, 2 21928.

- Balaban, N.; Rasooly, A. Staphylococcal Enterotoxins. Int. J. Food Microbiol. 2000, 61, 1–10

- Bennett, R. W. 2001. Staphylococcus aureus, p 201-220. Ronald G. Labbe and Santos Garcia. Guide to Foodborne Pathogens, John Wiley and Sons, Inc, New York, NY.

- Bergdoll, M.S.; Crass, B.A.; Reiser, R.F.; Robbins, R.N.; Davis, J.P A New Staphylococcal Enterotoxin, Enterotoxin F, Associated with Toxic-Shock-Syndrome Staphylococcus aureus Isolates. Lancet 1981, 1, 1017–1021.

- Bergdoll, M.S. 1990. Staphylococcal food poisoning. In Foodborne Diseases. D.O. Cliver (Ed.) Academic Press, Inc. San Diego, CA. p. 86-106.

- Brakstad, O.G.; Aasbakk, K.; Maeland, J.A. Detection of Staphylococcus aureus by polymerase chain reaction amplification of the nuc gene. J. Clin. Microbiol. 1992, 30, 1654–1660

- Castillejo-Rodriguez AM, Gimeno RM, Cosano GZ, Alcala EB, Perez MR. Assessment of mathematical models for predicting Staphylococcus aureus growth in cooked meat products. J Food Prot. 2002; 65:659 – 665.

- Casman, E.P., R.W. Bennett, A.E. Dorsey, and J.A. Issa. 1967. Identification of a fourth staphylococcal enterotoxin – enterotoxin D. J. Bacteriol. 94:1875-1882

- CDC NNIS System: National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1992–April 2001, issued August 2001. Am. J. Infect. Control. 2001; 29: 400–421.

- Chambers HF. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clin. Microbiol. Rev. 1997;10:781–791

- Chambers, H.F., Deleo, F.R., 2009. Waves of resistance: Staphylococcus aureus in the antibiotic era. Nat Rev Microbiol 7, 629-641.

- CLSI, Clinical and Laboratory Standards Institute, 2007. Performance Standards for Antimicrobial Susceptibility Testing; Fifteenth Informational Supplement. CLSI document M100-S15. Clinical and Laboratory Standards Institute, Wayne, PA.

- Dangerfield, H.G. 1973. Effects of enterotoxins after ingestion by humans. Presented at the 73 rd Annual Meeting of the American Society for Microbiology. May 6-11, Miami Beach, FL.

- Da Silva MP, dos Passos C, Casarin LS, Serraglio L, Tondo EC. Evaluation of growth and transfer of Staphylococcus aureus from poultry meat to surfaces of stainless steel and polyethylene and their disinfection. Food Control. 2010; 21 (3):298–301.

- Diekema DJ, et al. Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. **Clin. Infect. Dis.**2001;32(Suppl. 2):S114–S132

- Di Giannatale E, Prencipe V, Tonelli A, Marfoglia C, Migliorati G. Characterisation of Staphylococcus aureus strains isolated from food for human consumption. Vet Ital. 2011; 47 (2):165–73.

- Enright, M.C. 2003. The evolution of resistant pathogen – the case of MRSA**.**Curr. Opin. Pharmacol., 3 (2003), pp. 474-479

- Evenson, M.L., M.W. Hinds, R.S. Berstein, and M.S. Bergdoll. 1988. Estimation of human dose of staphylococcal enterotoxin A from a large outbreak in staphylococcal food poisoning involving chocolate milk. Int. J. Food Microbiol.7:311-316.

- FDA. (2011). Fish and Fishery Products Hazards and Controls Guidance. Center for Food Safety&AppliedNutrition http://www.fda.gov/food/guidanceregulation/guidancedocumentsregulatoryinformatio n/seafood/ucm2018426.htm.Accessed:October 20, 2013

- FDA. (2012).Bad Bug Book: Foodborne Pathogenic Microorganisms and Natural Toxins (2 ed.). Rockville, MD. http:// www.fda.gov/download/Food/Foodborne illness contamination/UCM297627.Pdf. Adams MR, Moss MO. Bacterial agents of foodborne illness—Staphylococcus aureus. Food Microbiology. Cambridge: Royal Society of Chemistry; 2008.

- Fujikawa H, Morozumi S. Modeling Staphylococcus aureus growth and enterotoxin production in milk. Food Microbiol. 2006; 23:260 –267. Doi: 10.1016/j.fm.2005.04.005.

- Gilbert, R. (1974). Staphylococcal food poisoning and botulism. Postgraduate Medical Journal, 50(588), 603-611.

- Ghuysen JM. Molecular structures of penicillin-binding proteins and beta-lactamases. Trends Microbiol.1994;2:372–380

- Gundogˇan, N., Citak, S., Turan, E., 2006. Slime production, DNAse activity and antibiotic resistance of Staphylococcus aureus isolated from raw milk, pasteurized milk and ice cream samples. Food Control 17, 389–392.

- Gundogan, N., Citak, S., Yucel, N., Devren, A., 2005. A note on the incidence and the antibiotic resistance of Staphylococcus aureus isolated from meat and chicken samples. Meat Sci. 69, 807–810.

- Hartman BJ, Tomasz A. Low-affinity penicillin-binding protein associated with beta-lactam resistance in Staphylococcus aureus. J. Bacteriol. 1984;158: 513–516.

- Heo, H. J., B. K. Kyung, D. H. Bae, C. K. Park, and Y. Ju Lee. 2008. Antimicrobial resistance of Staphylococcus aureus isolated from domestic and imported raw meat in Korea. Korean J Vet Res. 48:75-81

- Huang, S. L., Weng, Y. M., & Chiou, R. Y. Y. (2001). Survival of Staphylococcus aureus and Escherichia coli as affected by ethanol and NaCl. Journal of Food Protection, 174, 64(4), 546-550.

- Iandolo, J. J. 1989. Genetic analysis of extracellular toxins of Staphylococcus aureus. Annu. Rev. Microbiol. 43:375–402.

- Jablonski LM, Bohach GA (2001) Staphylococcus aureus. In: Doyle MP, Beuchat LR, Montville TJ (eds) Food Microbiology: Fundamentals and Frontiers, 2nd edn. ASM Press, Washington, pp 411–434

- Jarraud, S., M. A. Peyrat, A. Lim, A. Tristan, M. Bes, C. Mougel, J. Etienne, F. Vandenesch, M. Bonneville, and G. Lina. 2001. Egc, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in Staphylococcus aureus. J. Immunol. 166: 669–677.

- Kernodle, D.S. 2000. Mechanisms of resistance to β-lactam antibiotics. In Gram-positive pathogens. V.A. Fischetti, R.P. Novick, J.J. Ferretti, D.A. Portnoy, and J.I. Rood, editors. American Society for Microbiology. Washington, DC, USA. 609–620.

- Kerouanton A, Hennekinne JA, Letertre C, Petit L, Chesneau O, Brisabois A, and De Buyser ML. Characterization of Staphylococcus aureus strains associated with food poisoning outbreaks in France. Int J Food Microbiol 2007; 115:369–375.

- Khan, S.A., Nawaz, M.S., Khan, A.A., Cerniglia, C.E., 2000. Transfer of erythromycin resistance from poultry to human clinical strains of Staphylococcus aureus. J. Clin. Microbiol. 38, 1832–1838

- Kitai, S. Shimizu,A. Kawano,J. Sato,E. Nakano, C. Uji, T. Kitagawa,H. Characterization of methicillin-resistant *Staphylococcus aureus* isolated from retail raw chicken meat in Japan.Journal of Veterinary and Medical Science, 67 (2005), pp. 107-110

- Ler, S.G.; Lee, F.K.; Gopalakrishnakone, P. Trends in Detection of Warfare Agents. Detection Methods for Ricin, Staphylococcal Enterotoxin B and T-2 Toxin. J. Chromatogr. A 2006, 1133, 1–12.

- Le Loir Y, Baron F, Gautier M. Staphylococcus aureus and food poisoning. Genet Mol Res. 2003;2: 63–76.

- Lovseth, A.; Loncarevic, S.; Berdal, K.G. Modified multiplex PCR method for detection of pyrogenic exotoxin genes in staphylococcal isolates. J. Clin. Microbiol. 2004, 42, 3869–3872.

- Lowy, F.D.(1998). *Staphylococcus aureus* infections. N Engl J Med; 339:520-32.

- Mead, P.S.; Slutsker, L.; Dietz, V.; McCaig, L.F.; Bresee, J.S.; Shapiro, C.; Griffin, P.M.; Tauxe, R.V. Food-Related Illness and Death in the United States. Emerg. Infect. Dis. 1999, 5, 607–625.

- Monday, S. R., G. M. Vah, W. A. Ferens, C. D. Deobald, J. V. Rago, P. J. Gahr, D. D. Monie, J. J. - Iandolo, S. K. Chapes, W. C. Davis, D. H. Ohlendorf, P. M. Schlievert, and G. A. Bohach. 1999. Unique superantigen activity of staphylococcal exfoliative toxins. J. Immunol.

- Morandi, S.; Brasca, M.; Andrighetto, C.; Lombardi, A.; Lodi, R. Phenotypic and Genotypic Characterization of Staphylococcus Aureus Strains from Italian Dairy Products. Int. J. Microbiol. 2009, 501362:1–501362:7, doi: 10.1155 /2009/501362.

- Mylotte JM, McDermott C, Spooner JA. Prospective study of 114 consecutive episodes of Staphylococcus aureus bacteremia. Rev. Infect. Dis**. 1987;9:891–907

- Narmanno, G., A. Firinu, G. Virgilio, G. Mula, A. Dambrosio, A. Poggiu, L. Decastelli, R. Mioni, A. Scuota, G. Bolzoni, E. Di Giannatale, A. P. Salinetti, G. La Salandra, M. Bartoli, F. Zuccon, T. Pirino, S. Sias, A. Parisi, N. C. Quaglia, and G. V. Celano. 2005. Coagulase-positive staphylococci and Staphylococcus aureus in food products marketed in Italy. Int. J. Food Microbiol. 98:73–79.

- Normanno G, La Salandra G, Dambrosio A, Quaglia NC, Corrente M, Parisi A, et al. Occurrence, characterization and antimicrobial resistance of enterotoxigenic Staphylococcus aureus isolated from meat and dairy products. Int J Food Microbiol. 2007; 115 (3):290–6.

- Notermans, S., & Heuvelman, C. J. (1983). Combined effect of water activity, pH and sub-optimal temperature on growth and enterotoxin production of Staphylococcus aureus. Journal of Food Science, 48(6), 1832-1835.

- Ono HK, Omoe K, Imanishi K, Iwakabe Y, Hu DL, Kato H, Saito N, Nakane A, Uchiyama T, and Shinagawa K. Identification and characterization of two novel staphylococcal enterotoxins, types S and T. Infect Immun 2008;76:4999–5005

- Pesavento, G., Ducci, B., Comodo, N., Nostro, A.L., 2007. Antimicrobial resistance profile of Staphylococcus aureus isolated from raw meat: a research for methicillin resistant Staphylococcus aureus (MRSA). Food Control 18, 196–200.

- Portocarrero, S. M., Newman, M., & Mikel, B.(2002). Staphylococcus aureus survival, staphylococcal enterotoxin production and shelf stability of country-cured hams manufactured under different processing procedures. Meat Science, 62(2), 267-273.

- Pu, S.; Wang, F.; Ge, B. Isolation and characterization of methicillin-resistant Staphylococcus aureus strains from Louisiana retail meats. Appl. Environ. Microb. 2009, 75, 265–267.

- Quale J, Bratu S, Landman D, Heddurshetti R. Molecualr epidemiology and mechanisms of carbapenem resistance in Acinetobacter baumannii endemic in New York City Clin Infect Dis 2003;37:214-20.

- Scherrer, D.; Corti, S.; Muehlherr, J.E.; Zweifel, C.; Stephan, R. Phenotypic and Genotypic Characteristics of Staphylococcus Aureus Isolates From Raw Bulk-Tank Milk Samples of Goats and Sheep. Vet. Microbiol. 2004, 101, 101–107.

- Sharma NK, Rees CE, Dodd CE. Development of a single-reaction multiplex PCR toxin typing assay for Staphylococcus aureus strains. Appl Environ Microbiol. 2000; 66 (4):1347–53.

- Smyth CJ, Smyth DS, Kennedy J, Twohig J, Bolton DJ. Staphylococcus aureus: from man or animal—an enterotoxin iceberg? In: Maunsell B, Sheridan J, Bolton DJ, editors. EU-RAIN .Padua, Italy: Teagasc— The National Food Centre; 2004.

- Song MD, Wachi M, Doi M, Ishino F, Matsuhashi M. Evolution of an inducible penicillin-target protein in methicillin-resistant Staphylococcus aureus by gene fusion. FEBS Lett. 1987;221:167–171

- Su, Y. C. and A. C. L. Wong. 1997. Current perspective on detection of staphylococcal enterotoxins. J. Food Prot. 60:195–202.

- Thomas D, Chou S, Dauwalder O, and Lina G. Diversity in Staphylococcus aureus enterotoxins. Chem Immunol Allergy 2007; 93:24–41.

- Thomas DY, Jarraud S, Lemercier B, Cozon G, Echasserieau K, Etienne J, Gougeon ML, Lina G, and Vandenesch F. Staphylococcal enterotoxin- like toxins U2 and V, two new staphylococcal superantigens arising from recombination within the enterotoxin gene cluster. Infect Immun 2006; 74:4724–4734.

- Vannuffe, P.1995. Specific detection of methicillinresistant *Staphylococcus* species by multiplex PCR. *J. Clin. Microbiol.* **33,** 2864–2867 (1995).

- Valsangiacomo C, Dolina M, Peduzzi R, Jaggli M (2000). Antimicrobial susceptibility of Staphylococcus aureus isolates from hospitalized patients and dairy food (fresh cheese): a survey over a decade in southern Switzerland. Clin. Microbiol. Infect. 6(7):395-396

- Vasconcelos, N. G. Staphylococcal enterotoxins: molecular aspects and detection methods. Journal of Public Health and Epidemiology 2, 29–42 (2010). 62:4550–4559.

- Umeda, A, Y Ueki, and K Amako. "Structure of the Staphylococcus Aureus Cell Wall Determined by the Freeze-Substitution Method." Journal of Bacteriology 169.6 (1987): 2482–2487

- Waldvogel, F.A. 2000. Staphylococcus aureus (including staphylococcal toxic shock). In Principles and practice of infectious diseases. G.L. Mandell, J.E. Bennett, and R. Dolin, editors. Churchill Livingstone. Philadelphia, Pennsylvania, USA. 2069–2092.

- Waters AE, Contente-Cuomo T, Buchhagen J, Liu CM, Watson L, Pearce K, et al. Multidrug-Resistant Staphylococcus aureus in US Meat and Poultry. Clin Infect Dis. 2011; 52 (10):1227–30.

- Winn Washington, A.S., Janda, W. Koneman, E. Procop, G. Schreckenberger, P. Woods, G. 2006. Koneman's Color Atlas and Textbook of Diagnostic Microbiology Lippincott Williams & Wilkins, Philadelphia

- Yucel N, Citak S, Onder M (2005). Prevalence and antibiotic resistance of Listeria species in meat products in Ankara, Turkey. Food Microbiol .22:241-245

- Zecconi, A., Scali, F., 2013. Staphylococcus aureus virulence factors in evasion from innate immune defenses in human and animal diseases. Immunol Lett 150, 12-22.

CHAPTER 3: EFFECT OF MEAT PRODUCTION METHOD ON MICROBIAL CONTENT AND PHYSIOCHEMICAL QUALITY INDICATORS IN FRESH MEAT

Abstract

There are different types of meat based on the method of animal slaughter and processing. Halal meat is produced without stunning prior to slaughter, resulting in a rapid and complete bleeding of animals. Studies have shown that halal slaughter results in significantly lower residual hemoglobin than conventional slaughter with stunning (non-halal).The aim of this chapter is to evaluate the microbial content and quality indicators differences between fresh halal and non-halal beef. Meat samples were collected from different slaughter houses in Michigan. Microbiological analyses of meat samples showed that halal meat samples had significantly lower ($P < 0.05$) microbial count than non-halal meat. This includes APC (aerobic plate count), Psychrotrophic bacteria, total coliform, Pseudomonas spp., and Enterobacteriaceae. However, no significance differences were observed for *E. coli*, Yeast and mold, staphylococcus aureus, and fecal coliform. Physiochemical analyses showed that quality indicators for meat such as lipid oxidation, drip loss, and Heme iron content were significantly lower ($P < 0.05$) in halal meat than non-halal meat. However, no significant differences were observed for PH and thaw loss. Results from this research indicate that slaughter method significantly affects the meat quality. We show that halal meat processing may be advantageous in reducing bacterial colonization, increasing the usable shelf life, and improving the quality of the meat.

Introduction

Halal food market is increasing and its consumers are estimated at 1.5 billion (Riaz and Chaudry, 2004). The amount of money spent on halal food and drinks represents 16.6% of the global expenditure on food and drinks. This equates to approximately $1.1 trillion and it is estimated that by the year 2018, the value could reach $1.6 trillion (Thomson Reuters and Dinar Standard, 2013). Due to the apparent economic benefits of the halal market, many food businesses in the west have entered into the trade of halal food products. Nestle, one of the world's major food manufacturers now holds halal certification for an estimated 150 manufacturing sites globally (Thomson Reuters and Dinar Standard, 2013).

Meat is one of the most easily spoiled foods because it contains a lot of nutrient, high water content with dissolved substances and appropriate PH needed to encourage the growth of different microorganisms. Some of meat spoilage symptoms come from the growth of microorganisms to unacceptable levels while the other due to the chemical changes such as physical damage, lipid oxidation and color change. There are different types of meat based on the method of animal slaughter and processing. Halal meat is produced without stunning prior to slaughter, resulting in a rapid and complete bleeding of animals (Eliasi and Dwyer, 2002; Grandin and Regenstein, 1994). Studies have shown that halal slaughter results in significantly lower residual hemoglobin than conventional slaughter with stunning (non-halal) (Nakyinsige et al, 2014).There is a correlation between meat quality and blood. The more blood retained, the poorer the meat quality (Gregory, 2008; Strappini et al, 2009). Residual blood left in the carcass because of improper bleeding may decrease the shelf life and hence the quality of the meat product because hemoglobin which is an important component of blood is a powerful promoter

of lipid oxidation and growth of microorganisms (Alvarado et al, 2007). Also, glucose in the blood serves as substrate favorable for microbial growth such as Pseudomonas bacteria (Warriss, 2001). In addition, blood also contains a large number of white blood cells that produce hydroxyl radicals, superoxides, and hydrogen peroxide that enhance lipid oxidation (Gabig & Babior, 1981).

This approach may improve the quality and extend the shelf life of meat. Halal and non halal meat studies involved goat, chicken and rabbit, no data have been published on the differences between microbial content and physiochemical characteristics in beef. Therefore, the objective of this chapter is to evaluate the microbiological and physiochemical differences between fresh halal and non-halal beef.

Microorganism in meat

A-Pseudomonas

Pseudomonads are gram-negative rods, strict aerobes and use glucose as primary substrate for growth. Erichsen and Molin (1981) reported that Pseudomonas species were the dominant bacteria group on normal and high pH beef as well as in some cases, Pseudomonas can constitute up to 96% of the population (Asensio et al, 1988).Pseudomonas is the primary genus involved in the spoilage of refrigerated meats that are stored in air (Bailey et al, 1979) where there are three major species of pseudomonas (Ps. fragi, Ps. fluorescens and Ps. lundensis) isolated from fresh and spoiled meat (Liao, 2006). When the glucose is available as substrate for growth, pseudomonas will consume it first without produce offensive byproducts. But when the glucose fails, the pseudomonads turn to protein degradation (Young et al, 1988) and evolution of ammonia by de-amination of amino acids under aerobic conditions. Most of species of

pseudomonas are produced bacterial enzymes like proteases and lipases therefore the spoilage caused by pseudomonas when growth in meat slimy, putrid odors and breakdown of animal tissues (Liao, 2006).

B-Lactic acid bacteria

Lactic acid bacteria are gram-postive organisms, strictly fermentative where they produce lactic acid as a result of glucose fermentation, facultative anaerobic and non-spore forming bacteria (Stanbridge & Davies, 1998). Although many species of lactic acid bacteria are spread in meat include Lactobacillus, Lactococcus, Leuconostoc, Carnobacterium, and Weissella (Schillinger & Holzapfel, 2006). But some of these such as Lactobacillus spp., Carnobacterium spp. and Leuconostoc spp have significant role in the spoilage of refrigerated raw meat (Labadie, 1999) Symptoms of meat spoilage by these bacteria are slime formation, discolorations, sour flavor and off odor, (Schillinger & Holzapfel, 2006), but they do not produce malodourous substances (Dainty et al, 1975).

C-Enterobacteriaceae

Enterobacteriaceae is large family consist of about 150 species (Baylis, 2006), gram-negative, facultative anaerobic, rod shaped and non-spore forming bacteria. They use glucose as substrate for growth and produce some deleterious compound (Gill, 1986) ;(Lambert et al, 1991). There are different genera of the Enterobacteriaceae such as Klebsiella, Enterobacter, Citrobacter, Hafnia, Kluyera (less commonly), Serratia, Proteus have found on raw beef, lamb, pork, and poultry products(Garcia-Lopez et al, 1998). But the most important species of the Enterobacteriaceae that responsible of the meat spoilage are Serratia liquefaciens, Hafnia alvei and Enterobacter (Pantoea) agglomerans (Samelis, 2006). Stanbridge & Davies (1998)

indicated that H. alvei and S. liquefaciens grown in meat produce malodorous diamines as well as they cause a green discoloration because of the growth these two bacteria.

D-*Escherichia coli*

Escherichia coli are a gram-negative, non-spore forming, motile or stationary straight rod (1-4 μm). It is a mesophilic, facultative anaerobe that is normally inhabitant in the intestines of humans and other warm-blooded animals, mammals and birds. *E. coli* is an indicator organism and its presence in food or water is generally indicative of fecal contamination. E. coli strains that produce Shiga toxin are called Shiga toxin-producing *E. coli* (STEC) because of the strong similarity between the toxin they produce and the toxin produced by Shigella dysenteriae type 1 (Calderwood et al., 1996).

E-Total coliforms

Coliforms are gram-negative, rod-shaped facultative anaerobic bacteria. Identification criteria used are production of gas from glucose (and other sugars) and fermentation of lactose to acid and gas within 48 h at 35°C (Hitchins et al., 1998). One of the most common applications of coliform bacteria as indicator organisms is in their association with hygienic conditions and overall quality, especially concerning heat processed foods. Coliforms at normal levels found in foods are killed by most heat processing conditions therefore their presence in a food generally indicates an inadequate heat process or post-processing contamination.

F-Fecal coliforms

Fecal coliforms are coliforms that ferment lactose with gas production within 48 h at 45.5°C. This test was developed to differentiate between fecal and non-fecal contamination.

Fecal coliforms are considered more directly associated with fecal contamination from warm-blooded vertebrates than are other members of the coliforms.

Physiochemical quality indicators of meat

1-Lipid oxidation

Lipid oxidation is one of the major causes of quality deterioration in raw and cooked meat products during refrigeration and storage (Raharjo and Sofos, 1993). The free radical intermediate **f**rom lipid oxidation can decompose Heme, causing loss of color. Buckley et al., 1989, reported that an increase of lipid oxidation would cause a decrease of flavor, color, texture, nutritional value and acceptability in meat as well as sometimes formation of carcinogenic substances (Ahn, 1992) ;(Shahidi, 1994). Malonaldehyde, which is a degradation product of lipid oxidation, has been criticized as a carcinogenic factor in food (Kurechi, 1980)**.** There is also increasing evidence to indicate that lipid oxidation takes place primarily at the cellular membrane level and not in the triglyceride fraction. Therefore, lipid oxidation has been reported in both lean and fatty meat (Thanonkaew et al, 2006).Most researchers believe that the presence of transition metals, notably iron, is pivotal in the generation of species capable of abstracting a proton from an unsaturated fatty acid (Kanner, 1994).Ferrous ion will cause fission of O-O bonds to form very active alkoxy radicals for the propagation reaction, whereas the ferric ion can form both peroxy and alkoxy radicals (Ingold, 1962). There are three steps of lipid oxidation processing consists of initiation, propagation, and termination and these steps are usually used to explain the autoxidation system: initiation is the formation of free alkyl radicals; propagation is the chain reaction of free alkyl radicals and peroxy radicals; and termination is the formation of nonradical products (Min, 1998)

Autoxidation

Initiation — RH(Unsaturated fatty acids) ↓ Metals, UV → R˙

Propagation ↓ O₂ → ROO˙ (Peroxy radical)

RH → R˙ → ROOH (Hydroperoxides)

Termination R˙ or ROO˙ → ROOR, R-R

Breakdown products: ketones, aldehydes, alcohols, etc
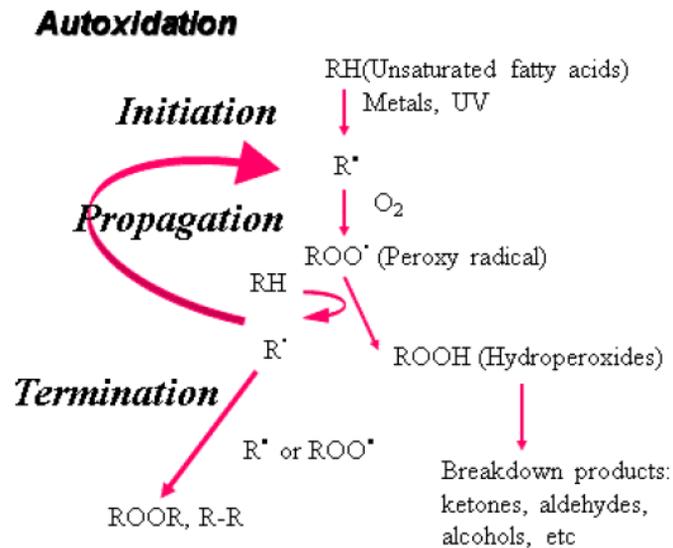
Figure **3.1. Autoxidation of lipid**

RH=Unsaturated fatty acid; R. =Alkyl radical; ROO. =Peroxy radical; RO. =Alkoxy radical; ROOH = Hydroperoxide.

Studies have shown that halal slaughter without stunning resulted in significant lower residual hemoglobin than non-halal slaughter with stunning (K. Nakyinsige et al, 2014); (Griffith's et al, 1985). Residual blood left in the carcass as a result of improper bleeding may decrease the shelf life and hence the quality of the meat product because hemoglobin which is an important component of blood is a powerful promoter of lipid oxidation (Alvarado et al, 2007). Blood also contains a large number of white blood cells that produce hydroxyl radicals, superoxides, and hydrogen peroxide that enhance lipid oxidation (Gabig & Babior, 1981).

2-Heme iron in beef

There are two forms of iron can be found in meat, Heme iron as a component of myoglobin and hemoglobin in the muscle and non-heme iron. Heme iron is absorbed by the enterocyte as an intact molecule and has few factors that interfere with its absorption in the

intestinal lumen (Wheby, 1981), therefore, it is highly bioavailable and significantly than non-heme iron (Hallberg, 1981). Myoglobin, a heme-protein, gives red color to fresh beef. In an aerobic condition deoxygenated redox form of Mb (deoxymyoglobin, DMb) with heme iron present in the ferrous state (Fe^{2+}) binds with oxygen to form oxymyoglobin (OMB). Oxidation of (OMb) or deoxymyoglobin (DMb) results in the formation of metmyoglobin (MMb) with concomitant oxidation of heme iron from ferrous (Fe^{2+}) to ferric redox state (Fe^{3+}). Increasing of MMb results in meat discoloration, which effects on meat quality (Mohan et al, 2009). Hemoglobin is a globular protein and consists of four subunits, as shown in Figure 3.2. Each protein subunit is an individual molecule that joins to its neighboring subunits through intermolecular interactions. In hemoglobin, each subunit contains a heme group, which is displayed using the ball-and-stick representation in Figure 3.2 each heme group contains an iron atom that is able to bind to one oxygen (O_2) molecule. Therefore, each hemoglobin protein can bind four oxygen molecules.
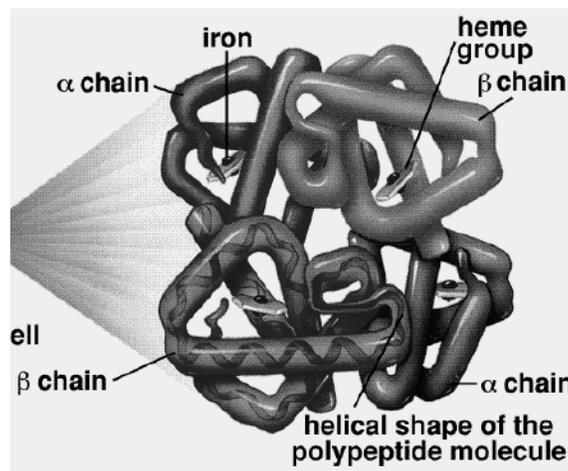
Figure **3.2. Hemoglobin molecule**

Studies have shown that halal slaughter results in significantly lower residual hemoglobin than conventional slaughter with stunning non-Halal (Nakyinsige et al, 2014).
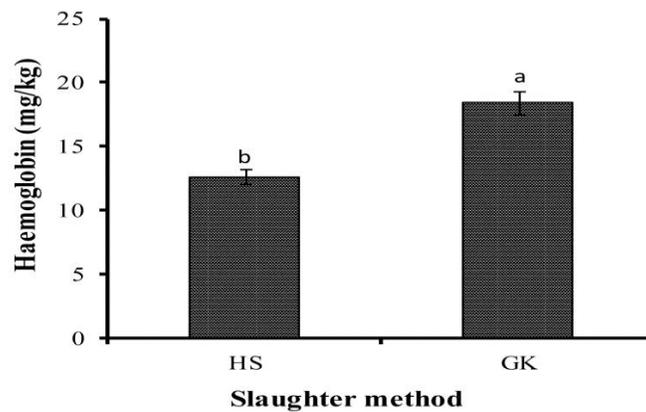
Figure **3.3. Effect of slaughter method on hemoglobin content**

Red meat and processed meat intake is associated with a risk of colorectal cancer, a major cause of death in affluent countries. Epidemiological and experimental evidence supports the hypothesis that heme iron present in meat promotes colorectal cancer. This meta-analysis of prospective cohort studies of colon cancer reporting heme intake included 566,607 individuals and 4,734 cases of colon cancer (Nadia et al, 2011).

Materials and methods

1. **Sample collection:**

Meat samples were collected from different slaughter houses in Michigan. The collected samples were immediately transport in insulated ice containers to the laboratory for microbiological and physiochemical analyses.

2. Microbiological analysis

Samples (25 g) of beef were weighed aseptically, added to (225mL) of 0.1% sterilized peptone water and homogenized in a stomacher for 60 s at room temperature. Decimal dilutions in 0.1% sterilized peptone water were prepared and duplicate 1mL samples of appropriate dilutions were poured on Petri dish then added the specific media for each type of bacteria for the following tests (APHA,1992):-

a) Aerobic plate Count

Pouring method was carried out according to APHA (1992) where 1 ml of the selected dilutions were added onto duplicate sterile Petri dish plates then added plate count agar (Sigma-Aldrich) and incubate at 35°C for 48 hr. Colonies between "25-250" were counted and the total aerobic colony counts were then expressed as log CFU/ gram.

b) Psychrotrophic bacteria

Psychrotrophic bacteria determination were carried out plate count agar (Sigma-Aldrich) and then incubated at 7°C for 7 days.

c) *Staphylococcus aureus*

Baird-Parker agar plates (Merk, Germany) were incubated for 48h at 35°C. Typical *S. aureus* colonies (Black colonies with white margins surrounded by clear zones) were counted.

d) Total coliform and fecal coliform bacteria

Violet red bile lactose agar (Sigma-Aldrich) was poured into 1ml of inoculum. The plates were then incubated at 35°C for count of total coliforms and at 44°C for count of fecal coliforms for 48 h. All typical colonies (red colonies) were counted.

e) Total yeast and mold

Total yeast and mold were counted on Potato dextrose agar(Sigma-Aldrich) , acidified with tartaric acid to pH 3.5 and incubated at 25°C for 5 d. Yellowish-white colonies were identified as yeasts, whereas mold growth were recognized by its mycelial appearance.

f) Lactic acid bacteria

Lactic Acid Bacteria (LAB) counts were determined using the double-layer deMan Rogosa Sharpe (MRS) agar (Sigma-Aldrich) and then incubated at 30°C for 72 h.

g) Enterobacteriaceae

Violet Red Bile Glucose agar (VRBG) (Oxoid, Basingstoke, UK) were used for the enumeration of Enterobacteriaceae, overlaid with the same medium and then incubated at 37 ˚C for 24 h.

h) *Escherichia coli*

Escherichia coli bacteria were enumerated using Eosin Methylene Blue (EMB) agar (Sigma-Aldrich) and then incubated at (37°C for 24h). Colonies of *E. coli* on EMB agar were round with a typical metallic sheen.

i) *Pseudomonas spp.*

Pseudomonas spp. were determined on pseudomonas agar base (Oxoid, Basingstoke,UK) supplemented with Pseudomonas CFC supplement (Oxoid, Basingstoke,UK) and then incubated at 25°C for 48 h.

j) *Listeria monocytogenes*

Pre-enrichment were carried out in (Buffered listeria enrichment broth base) supplemented with (Listeria selective enrichment supplement) and incubated at 30°C for 7 days followed by inoculation onto (Oxford agar) supplemented with (Oxford listeria selective supplement) then incubated at 37°C for 48h.

k)   Presumptive *salmonella spp.*

(25g) of beef were added to (225ml) of (Buffered peptone water) and incubate overnight at 37°C then 1ml of this suspension were inoculated into (Tetrathionate Broth). The TET tubes then were incubated at 37°C for 24 h and after incubation the samples were streaked onto (Xylose Lysine Tergitol (XLT4) agar) and then incubated under the same conditions. Suspected colonies were passed onto (Tryptic Soy Agar) and incubated under the same conditions.

3.   Physiochemical analysis

a) Determination of Heme iron content

Heme iron content of meat was determined according to the method of Cheng & Ockerman (2004). Ground sample (2g) was mixed with 9ml of acid acetone (90% acetone, 8% deionized water and 2% HCl v/v/v). The mixture was mashed with a glass rod and allowed to stand for 1h at room temperature. The mixture was filtered with a Whatman No. 42 filter paper and the absorbance of the filtrate was read at 640nm against an acid acetone used as blank. Heme iron content was calculated as follows;

Heme iron content (ppm) = Total pigment (ppm) X 0.0822

Where total heme pigment (ppm) = A640 X 680

The heme iron content was expressed as mg/100g of wet sample.

b) pH measurement

PH of meat samples were measured according to (Naveena and Mendiratta, 2001) meat samples (10 gram) were homogenized with 50 ml of distilled water then filtered through whatman No.1 filter paper. The PH of filtrate samples were measured using digital PH meter (WtW 2f40-11420D.Germany) calibrated at pH 4.0and 7.0 equipped with a pH electrode.

c)   Thaw loss Percentage

Thaw loss was calculated as [weight of sample before freezing minus sample weight after thawing] x 100/ sample weight before freezing.

d) Drip loss

Drip loss was calculated as [sample weight minus sample weight after 24 hours refrigeration] x 100/ sample weight.

e) Lipid oxidation measurement

Thiobarbituric acid reactive substances (TBARS) were determinate according to the method of Schmedes and Holmer (1989). Ground meat samples (10 g) were mixed with 25 ml of trichloroacetic acid (TCA) solution (200 g/l of TCA in 135 ml/l phosphoric acid solution) and homogenized in a blender for 30 s. After filtration, 2 ml of the filtrate were mixed with equal amount of aqueous solution of TBA (3 g/l) in a test tube. The tubes were incubated at room temperature in the dark for 20 h; then the absorbance was measured at 532 nm using UV-vis spectrophotometer (model UV-1200, Shimadzu, Kyoto, Japan). Lipid oxidation was expressed as thiobarbituric acid reactive substances (TBARS) expressed as mg Malonaldehyde/kg sample.

4. Statistical analysis

Data were represented the means of experiments. The means were compared using Student T test (IBM SPSS statistics 23)

Figure 3.4. Aerobic plate count in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). As seen from the figure there is significant differences in aerobic plate count were established by using independent measures t-tests at a significance level of p≤0.05.
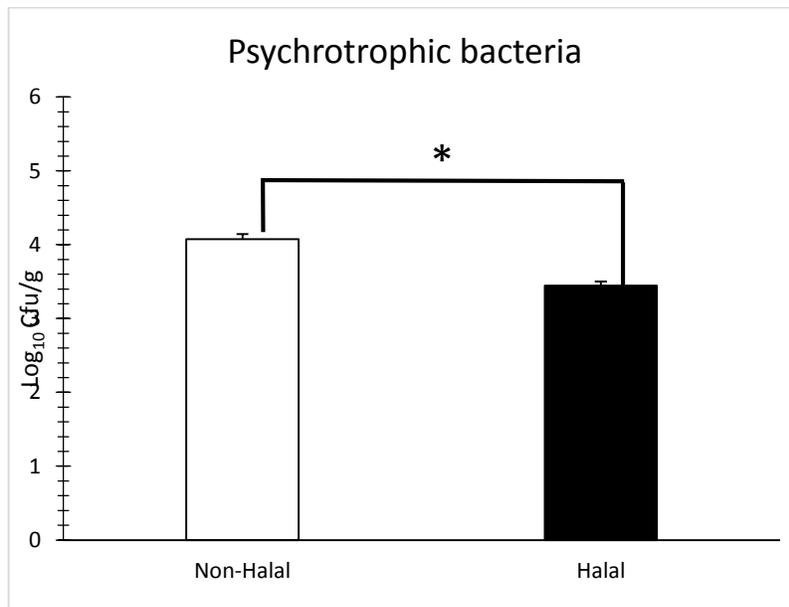
Figure 3.5 Psychrotrophic bacteria in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non- halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). As seen from the figure there is significant differences in Psychrotrophic bacteria count were established by using independent measures t-tests at a significance level of p≤0.05.
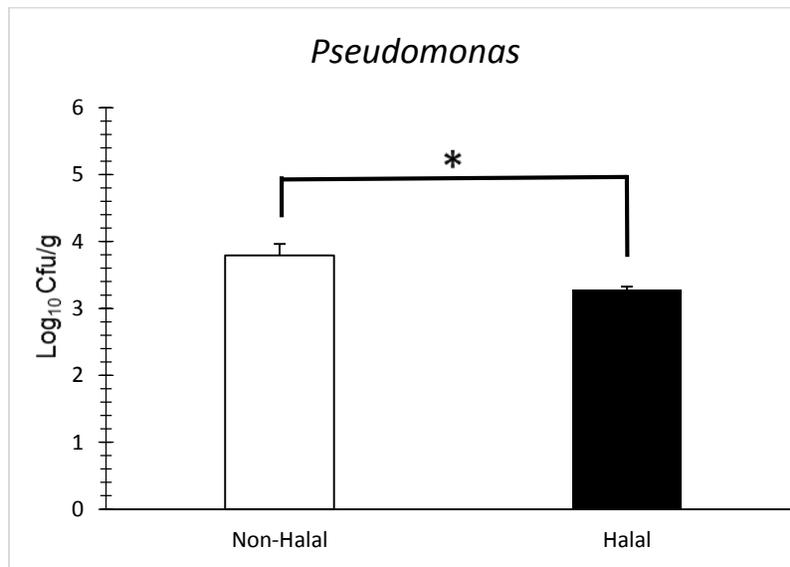
Figure 3.6 *Pseudomonas* in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). As seen from the figure there is significant differences in *Pseudomonas* bacteria count were established by using independent measures t-tests at a significance level of p≤0.05.
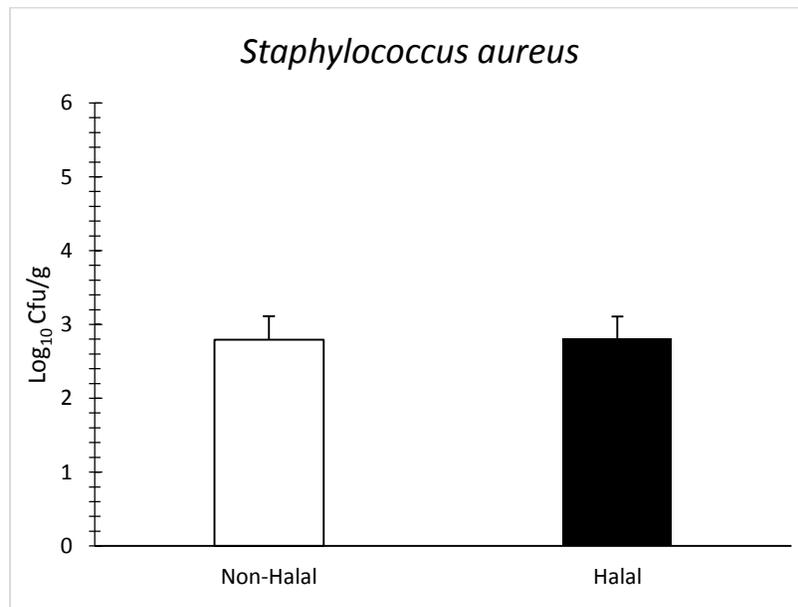
Figure 3.7. *S. aureus* bacteria in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). As seen from the figure there is no significant differences in *S. aureus* bacteria count were established by using independent measures t-tests at a significance level of p≤0.05.
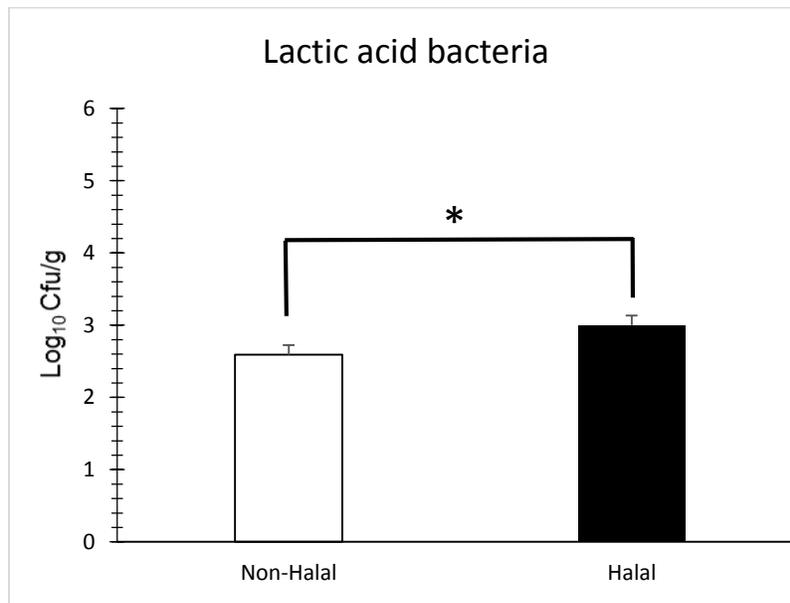
Figure 3.8 Lactic acid bacteria in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). As seen from the figure there is significant differences in Lactic acid bacteria count were established by using independent measures t-tests at a significance level of p≤0.05.
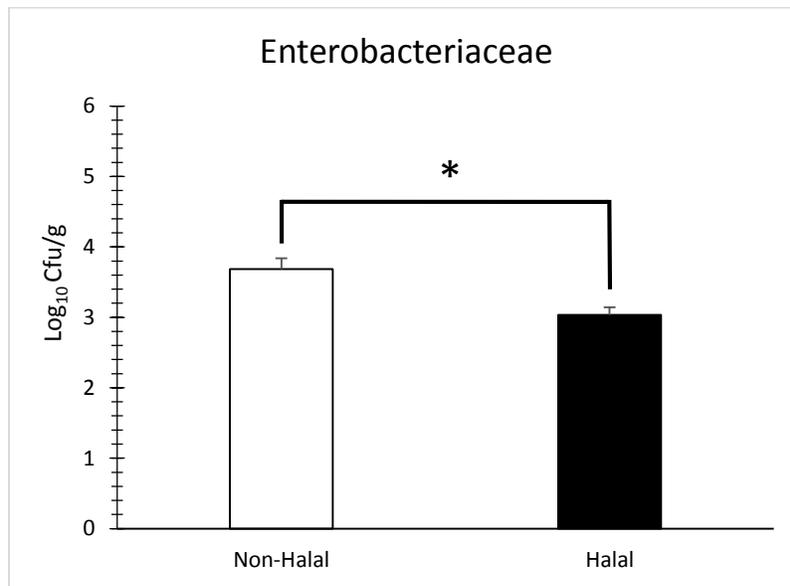
Figure 3.9 Enterobacteriaceae in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). As seen from the figure there is significant differences in Enterobacteriaceae were established by using independent measures t-tests at a significance level of p≤0.05.
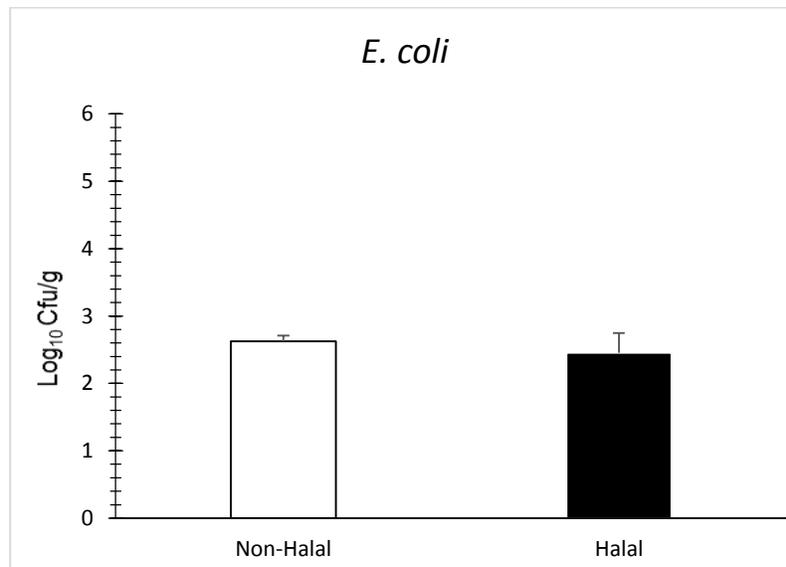
Figure 3.10 *E. coli* in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). As seen from the figure there is no significant differences in Enterobacteriaceae were established by using independent measures t-tests at a significance level of p≤0.05.
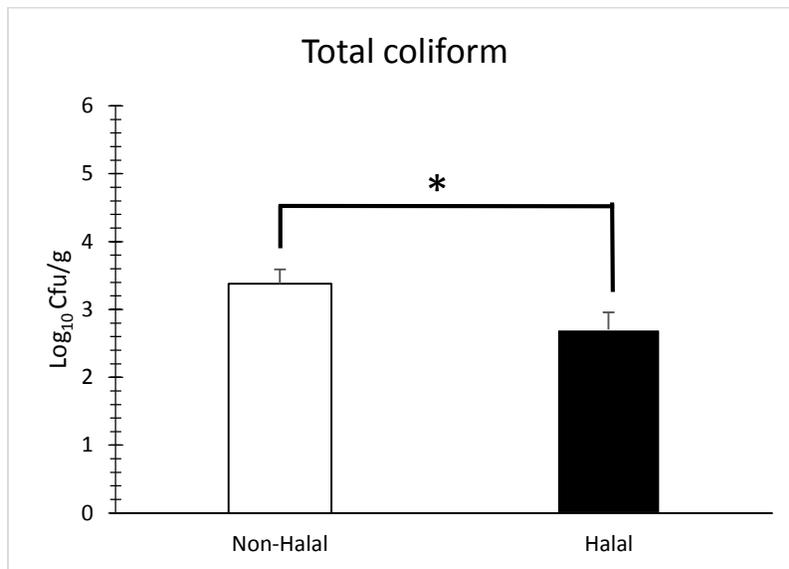
Figure 3.11.Total coliform in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). As seen from the figure there is significant differences in total coliform were established by using independent measures t-tests at a significance level of p≤0.05.

138
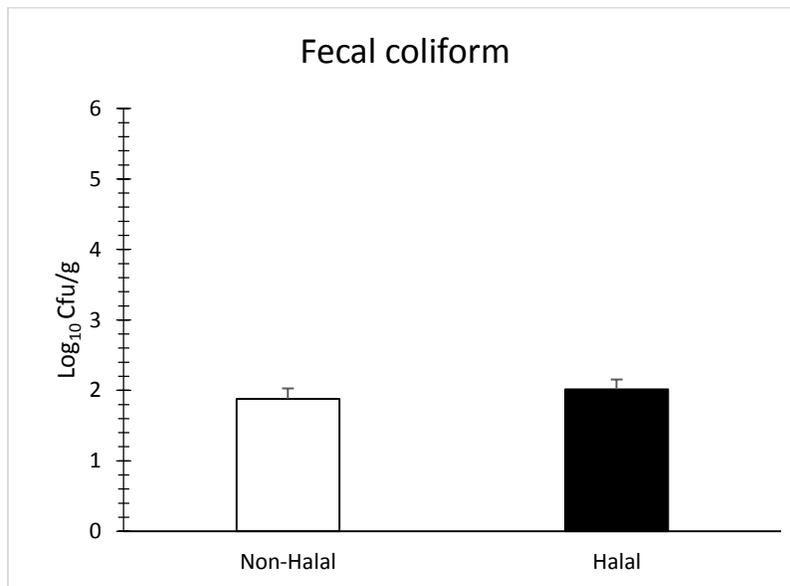
Figure 3.12. Fecal coliform in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). As seen from the figure there is no significant differences in fecal coliform were established by using independent measures t-tests at a significance level of p≤0.05.
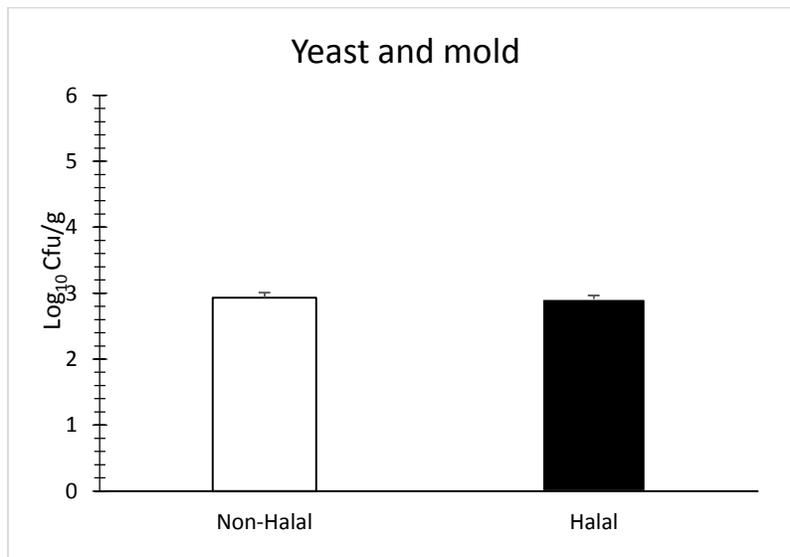
Figure 3.13 Yeast and mold in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). As seen from the figure there is no significant differences in yeast and mold were established by using independent measures t-tests at a significance level of $p \leq 0.05$.
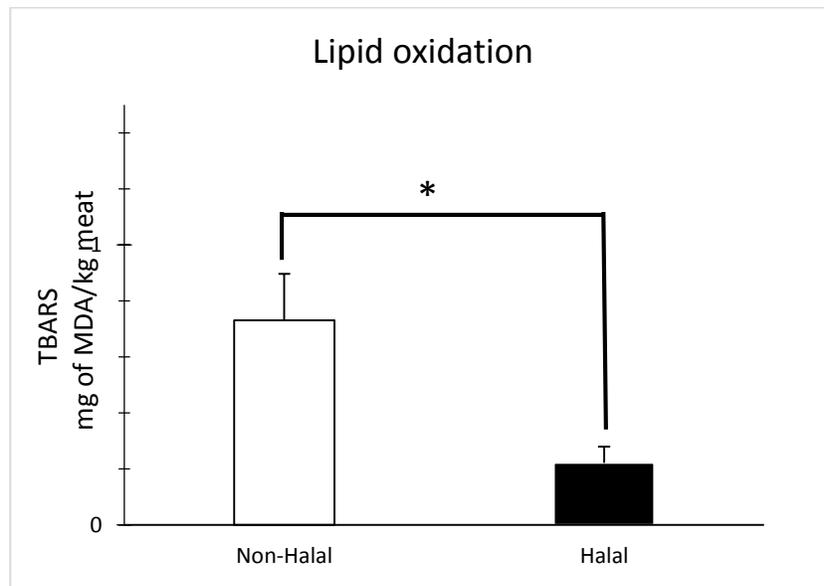
Figure 3.14 Lipid oxidation fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in mg of MDA/kg meat. As seen from the figure there is significant differences in lipid oxidation were established by using independent measures t-tests at a significance level of $p \leq 0.05$.
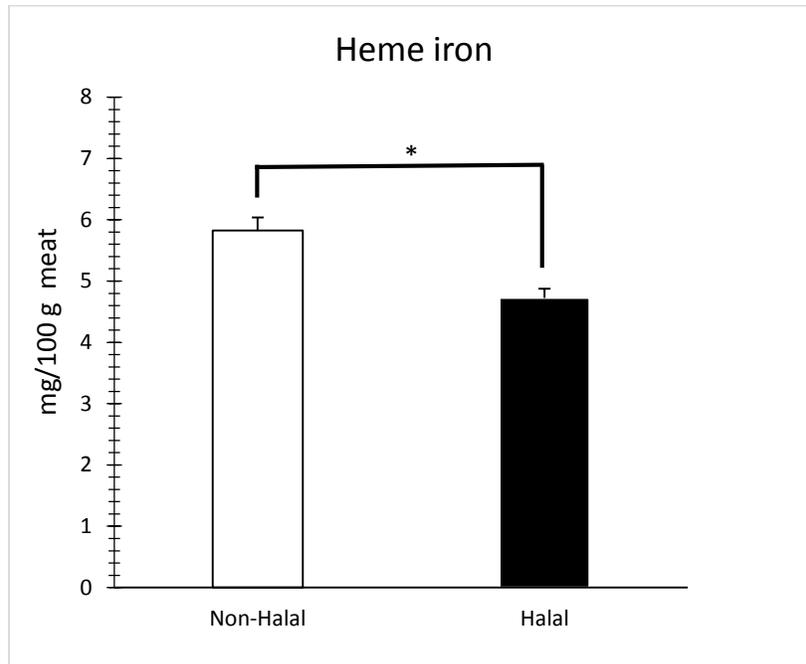
Figure 3.15. Heme iron in fresh halal and non-halal beef.
 A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in mg/100g meat. As seen from the figure there is significant differences in heme iron were established by using independent measures t-tests at a significance level of $p \leq 0.05$.

142
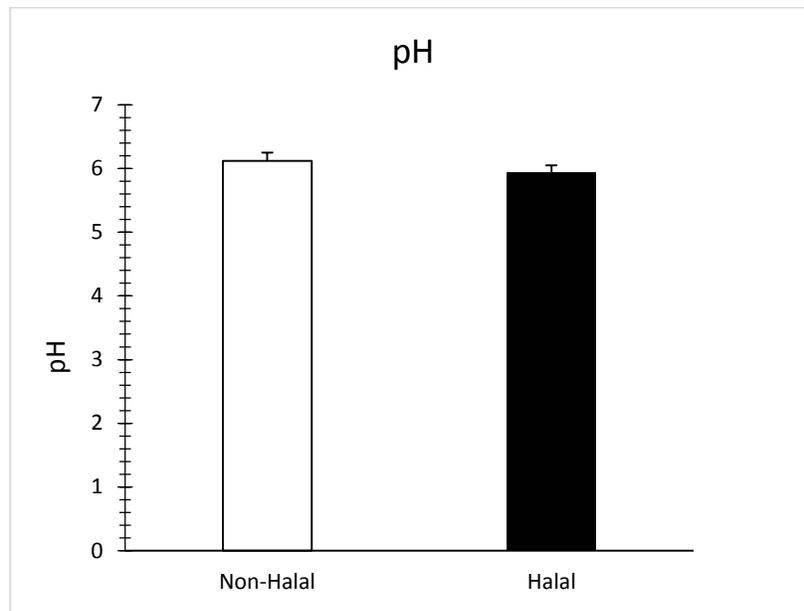
Figure 3.16 pH in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef sample. As seen from the figure there is no significant differences in pH were established by using independent measures t-tests at a significance level of p≤0.05.
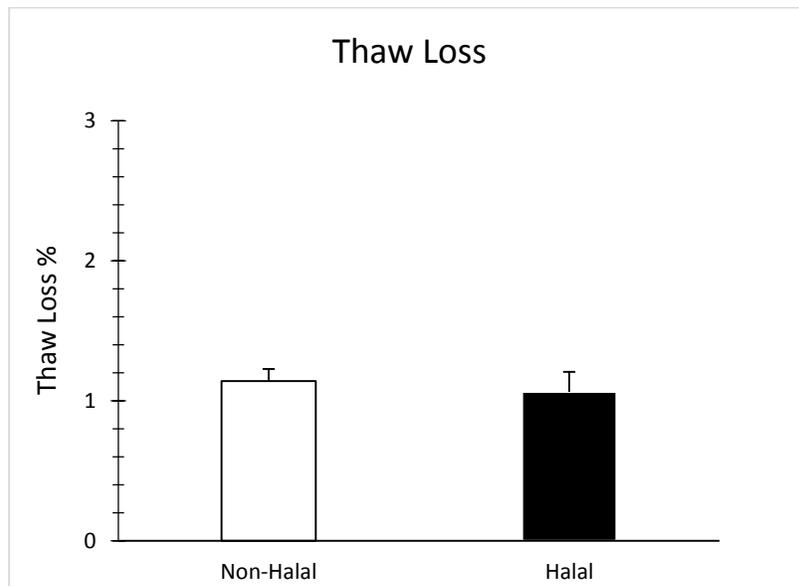
Figure 3.17 Thaw loss in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in thaw loss%. As seen from the figure there is no significant differences in lipid oxidation were established by using independent measures t-tests at a significance level of p≤0.05.
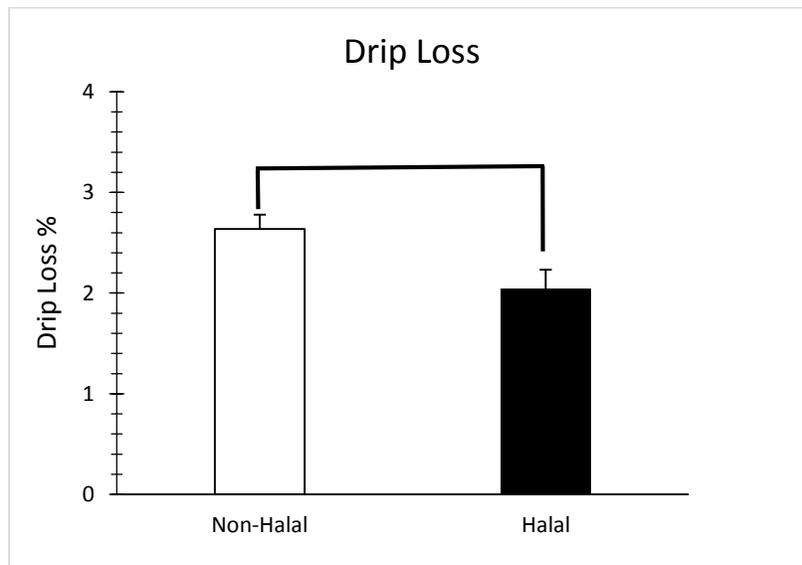
Figure 3.18 Drip loss in fresh halal and non-halal beef.
A data represent the mean values obtained from fresh halal and non-halal beef samples, and are expressed in drip loss%. As seen from the figure there is significant differences in drip loss were established by using independent measures t-tests at a significance level of p≤0.05.

Results and discussion

1. **Bacterial content in fresh halal and non-halal beef**

a) **Aerobic plate count**

Aerobic plate counts are a widely accepted measure of the general degree of microbial contamination and the hygienic conditions of processing plants (DAAHP).The aerobic plate count in fresh halal and non-halal beef were shown in (Fig. 3.4). Fresh halal beef that was produced without stunning resulted in significantly lower (P < 0.05) aerobic plate count than non-halal beef. Comparatively, the aerobic plate count in halal and non-halal beef were 3.59, 4.19 respectively. The lower microbial content in the halal beef could be attributed to the high blood loss because of method of slaughter. Many researchers reported a correlation between meat quality and blood. The more blood retained, the poorer the meat quality (Gregory, 2008; Strappini et al, 2009). The high nutritive value of blood and suitable temperature, pH, water activity and relative humidity influence the degree of deterioration in meat (Lerner, 2009). De Oliveira Roca (2002) showed that bleeding should be efficient to guarantee meat quality. In turn, this reduces the risk of carcass contamination with blood, which serves as a perfect medium for bacteria growth (RMAA, 2011). The level of aerobic plate count in halal and non-halal beef at week 0 was acceptable as indicated by (Insausti et al. 2001) and (Jeremiah, 2001), that spoilage occurs when the level of total viable count reach 7–8 log Cfu/g. These findings are in agreement with the findings of (Addeen et al, 2014) and (Mustafa et al, 2014) in chicken who found that aerobic plate count of broiler chicken was significantly higher in non-halal than halal meat.

In general, aerobic plat count in halal and non-halal beef was consistent with the results obtained by (Hinton et al, 1998) in England, (McEvoya et al, 2004) and (Gill et al, 1998)where they

found the mean of APC in beef ranged from log 2·45 to 4·29, log 2.8 – 4.30and log0.9- 4.9 Cfu cm(2) respectively. And slightly higher than (Vanderlinde et al, 1998) in Australia where they found the mean of the APC was log 3.13 CFU/cm^2.

b) Psychrotrophic bacteria

Psychrotrophic bacteria have ability to grow at low temperatures but have optimal and maximal growth temperatures above 15 and 20 °C, respectively (Moyer and Morita, 2007). Bacteria developing on meat at chill temperatures are regarded as psychrotrophic. Some psychrotrophic bacteria are gram-postive, such as lactic acid bacteria, and the other are gram-negative bacteria, such as *pseudomonas* and Enterobacteriaceae (Gill and Newton, 1978). Species of pseudomonas are especially responsible for the spoilage of meat stored at chill temperatures (Jay et al, 2003). In this study, we compared between account of psychrotrophic bacteria in fresh halal and non-halal beef as shown in (Fig. 3.5). Fresh halal beef that was produced without stunning resulted in significantly lower (P < 0.05) psychrotrophic bacteria than non-halal beef. The count of Psychrotrophic in fresh halal and non-halal beef were log 3.4, log 4.07 respectively. The development of organoleptic spoilage is related to microbial consumption of meat nutrients, such as sugars and free amino acids and the release of undesired volatile metabolites (Ercolini et al, 2009). In chill-stored meat these activities may be performed at low temperatures by psychrotrophic bacteria, compromising the sole effect of temperature as affecting preservation. This characteristic makes these microbes especially significant with regard to meat spoilage and safety, given that the storage of meat at cold temperatures is a routine practice during production, transportation, processing and post-purchase (Beales, 2004), (Russel, 2002).

c) *Pseudomonas*

Pseudomonas species were the dominant bacteria group on normal and high pH beef (Erichsen and Molin, 1981) as well as in some cases, *Pseudomonas* can constitute up to 96% of the population (Asensio et al, 1988). (Fig. 3.6) shows the count of *pseudomonas* bacteria in fresh halal and non-halal beef. Fresh halal beef that was produced without stunning resulted in significantly lower ($P < 0.05$) pseudomonas bacteria than non-halal beef. The count of pseudomonas in fresh halal and non-halal beef were log 3.27, log 3.79 respectively. This may be due to ability of *pseudomonas* bacteria to use glucose in the blood as substrate for microbial growth (Warriss, 2001). Studies have shown that halal slaughter without stunning resulted in significantly higher blood loss than non- halal slaughter with stunning (Nakyinsige et al, 2014). In general, *pseudomonas* bacteria count in beef that produced in both slaughters are in line with the report of (Nel et al,2004) which indicated that count of *pseudomonas* in beef from the deboning room was log 4.0 Cfu/g and (Goulas et al ,2005) which reported that *Pseudomonas* in beef was Log 3.5 Cfu/g.

d) *Staphylococcus aureus*

Staphylococcus aureus produces a heat-stable toxin in foods and causes staphylococcal food poisoning (SFP) by ingesting food that contains staphylococcal enterotoxin. Staphylococcal food poisoning (SFP) is responsible for a third of the foodborne diseases worldwide (Normanno, et al., 2007). The effect of slaughter methods on prevalence of *S. aureus* bacteria in beef were shown in (Fig. 3.7). The count of *S. aureus* bacteria in halal and non-halal beef were log 2.81, log 2.79 respectively. There were no significant differences for *S. aureus* between fresh halal and non-halal beef. *S. aureus* bacteria count in beef that produced in both slaughters in this study

were less than the results that obtained by (Nela et al, 2004) which indicated that count of *S. aureus* in beef from the deboning room was log 3.7 Cfu/g and higher than the results that obtained by (Goulas et al, 2005) which reported that *S. aureus* in beef trimmings at United States and Canada was log 1.0 Cfu/g.

e) Lactic acid bacteria

Lactic acid bacteria are produced lactic acid for of glucose fermentation and some species have significant role in the spoilage of refrigerated raw meat (Labadie 1999). The spoilage characteristics are slime formation, discolorations, sour flavor and off odor, (Schillinger & Holzapfel 2006). Halal meat samples had significantly higher lactic acid bacteria count than non-Halal meat ($P < 0.05$) as shown in (Fig.3.8). Lactic acid bacteria count in fresh halal and non-halal beef were log 3.53, log 2.59 respectively. Lactic acid bacteria count in beef that produced in both slaughters in this study were higher than the results that obtained by (Goulas et al, 2005) which indicated that count of lactic acid bacteria in beef was log 2.2 Cfu/g, and less than the results that obtained by (Katikou et al. (2005), Serdengecti et al, 2006) where they reported that count of lactic acid bacteria of beef samples varied from log 4.72 to log 5.63 Cfu/cm2 with average of log 5.19. However, the levels of lactic acid bacteria for fresh beef samples in both slaughters were acceptable as indicated by (Nortjé and Shaw, 1989), that spoilage occurs when the lactic acid bacteria count reaches log 7 Cfu/g.

f) Enterobacteriaceae

Enterobacteriaceae bacteria are used glucose as substrate for growth and produce some deleterious compound (Gill, 1986); Lambert et al., 1991). Some species such as H. alvei and S. liquefaciens produce malodorous diamines as well as they cause a green discoloration when

they grow in meat (Stanbridge & Davies, 1998).The Enterobacteriaceae count in fresh halal and non-halal beef were shown in fig. 3.9. Fresh halal beef that was produced without stunning resulted in significantly lower ($P < 0.05$) Enterobacteriaceae than non-halal beef. Comparatively, the Enterobacteriaceae in halal and non-halal beef were log 3.03, log3.68 respectively. This may be attributed to ability of Enterobacteriaceae bacteria to use glucose in the blood as substrate for microbial growth (Warriss, 2001). Studies have shown that halal slaughter without stunning resulted in significantly higher residual blood loss than non halal slaughter with stunning (K. Nakyinsige et al., 2014). The levels of Enterobacteriaceae for fresh beef samples in both slaughters were acceptable as indicated by Insausti et al. (2001) and Jeremiah (2001), that spoilage occurs when the levels of Enterobacteriaceae count reach 7–8 log Cfu/g. Enterobacteriacea count in beef that produced in both slaughters in this study within the range that obtained by (McEvoya et al,2004 who found that Enterobacteriaceae counts on beef carcasses after different stages of processing ranged (log 1.48-4.25), however, less than those that obtained by (Nel et al,2004) who reported that count of Enterobacteriaceae in meat from the deboning room was Log 5.6.

g) *E. coli*

The effect of slaughter methods on prevalence of *E. coli* bacteria in beef was shown in (Fig 3.10). The count of *E. coli* bacteria in halal and non-halal beef were log 2.81, log 2.79 respectively. There were no significant differences for *E. coli* between fresh halal and non-halal beef. This could be due to apply the same procedures to control these bacteria during handling the meat as well as following the hygiene practice by workers. *E. coli* bacteria count in beef that produced in both slaughters in this study were less than

the results that obtained by (Nela et al, 2004) which indicated that count of *E. coli* in beef from the deboning room was log 3.6 Cfu/g and higher than the results that obtained by (Scanga et al,2000) which reported that *E. coli* in beef trimmings at United States and Canada was log 1.2 Cfu/g, and within the range that obtained by (Eisel et al, 1997) who found that *E. coli* counts on beef carcasses after different stages of processing ranged log 1.0 - 3.21 Cfu/g

h) Total coliform

Total coliform in fresh halal and non-halal beef were shown in (Fig.3.11). Fresh halal beef that was produced without stunning resulted in significantly lower (P < 0.05) coliform than non-halal beef. Comparatively, the coliform in halal and non-halal beef were log 2.7, log 3.38 respectively. These findings are in agreement with the findings of (Mustafa et al, 2014) in chicken who found that coliform count of broiler chicken was significantly higher in non-halal than halal meat. Coliform count in beef that produced in this study were higher than the results that obtained by (Stopforth et al, 2006) which indicated that count of coliform bacteria in beef was ranged from log 1.1 to 1.8 Cfu/g, and within range that obtained by (McEvoya et al, 2004) which reported that total coliform count from sites on beef carcasses after different stages of processing were ranged from log 1.81-4.93.

i) Fecal coliform

In this study also we evaluated the effect of slaughter method on prevalence of fecal coliform in beef. (Fig. 3.12) shows the count of fecal coliform bacteria in fresh halal and non-halal beef. Fecal coliform in halal and non-halal beef were log 2.01, log 2.88 respectively. There were no significant differences for fecal coliform between fresh halal and non-halal beef. This

could be due to apply the same procedures to control these bacteria during handling the meat as well as following the hygiene practice by workers.

j) Yeast and mold

The effect of slaughter methods on prevalence of yeast and mold in beef was shown in (Fig. 3.13). The count of yeast and mold in halal and non-halal beef were log 2.87, log 2.92 respectively. There were no significant differences for yeast and mold between fresh halal and non-halal beef. This could be due to apply the same procedures in environment of slaughter such as ventilation where spores of yeast and mold can be spread through the air.

K) L. monocytogenes and Salmonella

The results of present study indicated that we did not detect salmonella and *L. monocytogenes* bacteria in halal and non-halal beef samples. Paul et al, 1998 reported that a small percentage (0.59%) of Australian beef carcasses were found positive for L. monocytogenes, 0.22% were positive for Salmonella spp.

2. Physiochemical analyses

a) Lipid oxidation

Lipid oxidation is one of the major causes of quality deterioration in raw and cooked meat products during refrigeration and storage (Raharjo and Sofos, 1993). Lipid oxidation is measured as malondialdehyde which is formed by oxidation of polyunsaturated fatty acids with two or more double bonds. MDA-induced DNA damage is mutagenic in bacterial, mammalian, and human cells (Nadia et al, 2011). The effect of slaughter methods on lipid oxidation in beef was shown in (Fig. 3.14). The lipid oxidation in halal and non-halal beef were 0.73, 0.22 mg/MDA respectively. There were significant differences for lipid oxidation

between fresh halal and non-halal beef. This could be due to halal slaughter without stunning resulted in significant lower residual hemoglobin than non-halal slaughter with stunning (Nakyinsige et al, 2014); (Griffiths et al, 1985). Residual blood left in the carcass because of improper bleeding may decrease the shelf life and hence the quality of the meat product because hemoglobin which is an important component of blood is a powerful promoter of lipid oxidation (Alvarado et al., 2007). Blood also contains a large number of white blood cells that produce hydroxyl radicals, superoxides, and hydrogen peroxide that enhance lipid oxidation (Gabig & Babior, 1981). These values were consistent with the results for heme iron in this study where were (4.72), (5.82) mg for halal and non-halal beef respectively. Transition metal ions, primarily Fe serve as key catalysts for lipid oxidation in muscle (Kanner, 1994).

b) Heme iron

Heme iron content in fresh halal and non-halal beef were shown in Figure (3.15). Fresh halal beef that was produced without stunning resulted in significantly lower (P < 0.05) heme iron than non-halal beef. Comparatively, heme iron in halal and non-halal beef were (4.72), (5.82) respectively. This could be due to halal slaughter without stunning resulted in significant lower residual hemoglobin than non-halal slaughter with stunning (Nakyinsige et al, 2014); (Griffiths et al, 1985). These values were consistent with the results for lipid oxidation and some of microbiological analyses such as aerobic plate count, psychrotrophic bacteria, pseudomonas, lactic acid bacteria , coliform and Enterobacteriaceae. Hemoglobin which is an important component of blood is a powerful promoter of lipid oxidation and growth of microorganisms (Alvarado et al, 2007).

Red meat and processed meat intake is associated with a risk of colorectal cancer, a major cause of death in affluent countries. Epidemiological and experimental evidence supports the hypothesis that heme iron present in meat promotes colorectal cancer. This meta-analysis of prospective cohort studies of colon cancer reporting heme intake included 566,607 individuals and 4,734 cases of colon cancer (Nadia et al, 2011). Therefore, halal slaughter could lower heme iron and hence improve the quality of meat and avoid health problems such as colorectal cancer.

c) PH

The effect of slaughter methods on pH in beef were shown in (Fig. 3.16). The pH of halal and non-halal beef were 5.94 and 6.12 respectively. There were no significant differences for pH between fresh halal and non-halal beef. This could be due to use same treatment with animals before slaughtering.

d) Thaw loss

The effect of slaughter methods on thaw loss in beef was shown in (Fig. 3.17). The thaw loss in halal and non-halal beef were (1.06), (1.14) respectively. There were no significant differences for thaw loss between fresh halal and non-halal beef.

e) Drip loss

Drip loss in fresh halal and non-halal beef were shown in (Fig. 3.18). Fresh halal beef that was produced without stunning resulted in significantly lower ($P < 0.05$) drip loss than non-halal beef. Comparatively, the drip loss in halal and non-halal beef was (0.22), (0.73) respectively. These findings are in agreement with the findings of (D'Agata et al, 2010) who found that meat from beef slaughtered by conventional method (non-halal) showed significantly higher drip loss than those from halal method.

REFERENCES

- Addeen, A., Benjakul, S., Wattanachant, S., Maqsood, S. 2014.Effect of Islamic slaughtering on chemical compositions and post-mortem quality changes of broiler chicken meat. International Food Research Journal 21(3): 897-907

- Ahn, D.W., Wolf, F.H., Sim, J.S., Kim, D.H. (1992) Packaging cooked turkey meat patties while hot reduces lipid oxidation. J Food Sci Publ Inst Food Technol 57: 1075-1077.

- Alvarado, C.M., Richards, S. O'Keefe, H. Wang 2007.The effect of blood removal on oxidation and shelf life of broiler breast meat.Poultryscience,86(1) (2007),pp.156-161.

- APHA; American Public Health Association (1992): Compendium of Methods for the Microbiological Examination of Foods, 3rd ed. APHA, Washington, DC, USA.

- Asensio, M.A., Ordóñez, J. A., Sanz, B. 1988. Effect of carbon dioxide and oxygen enriched atmospheres on the shelf-life of refrigerated pork packed in plastic bagsJ. Food Protect., 51 (1988), pp. 356-360

- Bailey, J.S., Reagan, J.O., Carpenter, J.A. and Schuler, G.A. (1979a). Microbiological condition of broilers as influenced by vacuum and carbon dioxide in bulk shipping packs. Journal of Food Science. 44: 134-136

- Baylis, C. L. (2006). Enterobacteriaceae. In Food spoilage microorganisms, pp. 213-286. Edited by C. de W. Blackburn. Cambridge: Woodhead Publishing Limited, Abington Hall, Abington

- Beales, N. (2004) Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: a review. Compr Rev Food Sci F 3:1–20.

- Buckley, D.J, Gray, J.I., Ashgar, A., Price, J.F., Crackel, R.L. (1989) (Effects of dietary antioxidants and oxidized oil on membral lipid stability and pork product quality. J Food Sci 2 Publ Inst Food Technol 54: 1193-1197.

- Calderwood,S.B., Acheson, D.W.K., Keusch, G.T., Barrett,T.J.,Griffin,P.M., Strockbine,N.A., Swaminathan,B., Kaper,J.B., Levine,M.M., Kaplan,B., Karch,H., OBrien, A.D.,Ohrig,T.G.,

- Carolina, V., Daniel, L., Manuel, O., María, M., Fernando, P. 200. Total Iron and Heme Iron Content and their Distribution in Beef Meat and Viscera. Biol Trace Elem Res (2009) 132:103–111.

- Carrasco, E., Morales-Rueda, A., García-Gimeno, R. M. (2012). Crosscontamination and recontamination by Salmonella in foods: A review. Food Research International, 45(2), 545-556. doi: 10.1016/j.foodres.2011.11.004

- Cheng, J.H., Ockerman, H.W.2004. Effect of ascorbic acid with tumbling on lipid oxidation of precooked roast beef.J.Muscle Food.15:83-93.

- Dainty, R. H., Shaw, B. G., de Boer, K. A., Scheps, E. S. J. (1975). Protein changes caused by bacterial growth on beef. J Appl Bacteriol 39, 73-81.

- D'Agata, M., Russo, C., Preziuso, G. 2010. Effect of Islamic ritual slaughter on beef quality. Italian Journal Animal.Sci. vol. 8 (Suppl. 2), 489-491.

- De Oliveira Roca, 2002. Humane slaughter of bovine. Embrapa: First virtual global conference on organic beef–cattle production. (2002) (September, 02 to October 15, 2002)

- Department of Agriculture, Animal Health and Product. 2004. Animal and animal origin foods. No. 624-04. *Off. Bull.* 5214:727–745.

- Eisel W. G., Linton R. H., Muriana P. M.: A survey of microbial levels for incoming raw beef, environmental sources, and ground beef in a red meat processing plant. Food Microbiol. 1997, 14, 273-282.

- Eliasi, J.R., Dwyer, J.T., 2002. Kosher and halal: religious observances affecting dietary intakes. J. Am. Diet. Assoc. 101:911–913.

- Ercolini D., Russo, F., Nasi, A., Ferranti, P., and Villani, F., 2009. Mesophilic and psychrotrophic bacteria from meat and their spoilage potential in vitro and in beef.

- Erichsen, I., Molin, G.1981. Microbial flora of normal and high pH beef stored at 4 °C in different gas environmentsJ. Food Prot., 44 (1981), pp. 866-869

- Gabig and Babior.1981.The killing of pathogens by phagocytes.Annual Review of Medicine, 32 (1) (1981), pp. 313–326.

- Garcia-Lopez, M. L., Prieto, M., Otero, A. (1998). The physiological attributes of Gramnegative bacteria associated with spoilage of meat and meat products. In The Microbiology of Meat and Poultry, pp. 1-34. Edited by A. Davies, R., Board. London: Blackie Academic and Professional

- Gardner, G.A., A.W. Carson, A.W., Patton, J. 1967. Bacteriology of prepackaged pork with reference to the gas composition within the pack. J. Appl. Bacteriol.

- Gill,C.O. Newton, K.G. 1978.The ecology of bacterial spoilage of fresh meat at chill temperatures.Meat Science, 14 (1978), pp. 43–60

- Gill, C.O., Newton, K.G. 1978.The ecology of bacterial spoilage of fresh meat at chill temperatures. Meat Science, 14 (1978), pp. 43–60

- Gill, C. O. (1986). The control of microbial spoilage in fresh meats. In Advances in Meat Research, Meat and Poultry Microbiology, pp. 49-88. Edited by A. M. Pearson, T. R. Dutson, Westport: AVI Publishing Company Inc.

- Gill, C. O. (1998). Microbiological contamination of meat during slaughter and butchering of cattle, sheep and pigs. In The microbiology of meat and poultry, pp. 118-157. Edited by A. Davies, R. Board, London: Blackie academic and professional.

- Goulas, A.E. Chouliara, I. Nessi, E. Kontominas.M.G. Savvaidis, I.N. 2005. Microbiological, biochemical and sensory assessment of mussels (**Mytilus galloprovincialis**) stored under modified atmosphere packaging.Journal of Applied Microbiology, 98 (2005), pp. 752-760

- Grandin, T., Regenstein, J.M., 1994. Religious slaughter and animal welfare: a discussion for meat scientists.Meat Focus International. CAB Intern., Wallingford, Oxon, UK, pp 115-123.

- Gregory, N. G. (2008). Animal welfare at markets and during transport and slaughter. Meat Science, 80(1), 2–11.

- Griffiths, GL., McGrath. M., Softly, A., jones, C. 1985 Blood content of breiler chicken carcases prepared by different slaughtermethods. *The VeterinaryRecord II* 7: 382-385

- Hallberg, L. 1981. Bioavailability of dietary iron in man. Ann. Rev. Nutr. 1:123

- Hafiz, A., Hassan, Z., Abdul Manap, M. 2015. Effect of Slaughtering Methods on Meat Quality Indicators, Chemical Changes and Microbiological Quality of Broiler Chicken Meat during Refrigerated Storage. Journal of Agriculture and Veterinary Science: pp12-17.

- Hinton, M.H., Hudson, W.r., Mead, G.C. 1998.The bacteriological quality of British beef 1. Carcasses sampled prior to chilling. Meat science.Vol.50, Issue2, October 1998, Pages 265-271.

- Hitchins, A.D., Feng, P., Watkins, W.D., Rippey, S.R., Chandler, L.A.1998. E.coli and the coliform bacteria in : FDA bacterio-logical Analytical Manual,eighth ed.,Revision A.AOAC International,Gaithersburg,MD,pp.4.01-4.29

- Holzapfel, G. 1998.The Gram-positive bacteria associated with meat and meat products. The Microbiology of Meat and Poultry, Blackie Academic and Professional, London (1998), pp. 35–84.

- Ingold, K. V. (1962). Metal catalysis. In Symposium on Foods: Lipids and their Oxidation. Ed. H. W. Schultz, E. A. Day & R. O. Sinnhuber. AVI, Westport, CT, p. 93.

- Insausti, K,.Breiain,M.J.,Purroy,A.,Alberti,P.,Gorraiz,C.,Alzueta,M.J.,Shelf life of beef from local Spanish cattle breeds stored under modified atmosphere.Meat sci.2001;57:273-281

- Jay, J. M., Vilai, J.P., Hughes, M.E. 2003. Profile and activity of the bacterial biota of ground beef held from freshness to spoilage at 5-7°C. Int. J. Food Microbiol. 81:105-111

- Jeremiah, L.E. 2001. Packaging alternatives to deliver fresh meats using short- or long-term distribution. Food Res Int. 2001; 34:749–772.

- Kanner, J. (1994). Oxidative processes in meat & meat products: Quality implications. Meat Science, 36(1), 169–189.

- Katikou, P., Ambrosiadis, I., Georgantelis, D., Koidis, P., Georgakis, S.A. (2005). Effect of Lactobacillus-protective cultures with bacteriocinlike inhibitory substances' producing

ability on microbiological, chemical and sensory changes during storage of refrigerated vacuum-packaged sliced beef. J. Appl. Microbiol., 99, 1303-1313.

- Kurechi, T., Kikugawa, K., Ozawa, M. (1980). Effect of malondialdehyde on nitrosamine formation. Food Cosmet Toxicol 18: 119-122. 6. Pearson AM, Gray JI, Wolzak AM, Horenstein NA (1983) Safety

- Labadie, J., 1999. Consequences of packaging on bacterial growth. Meat is an ecological niche. Meat Sci. 52, 299-305.

- Lambert,A.D., Smith,J.P., Dodds,K.L.1991.Shelf life extension and microbiological safety of fresh meat - a review.Food Microbiology, 8 (1991), pp. 267–297

- Lerner, P.T. 2009. Evaluation of haemoglobin and myoglobin in Poultry slaughtered by stunning and kosher slaughter. Folia Vet. 2009; 53:25–27.

- Liao, C. H. (2006). 19-Pseudomonas and related genera. In C.d. W. Blackburn (Ed.), Food spoilage microorganisms (pp. 507e540). Woodhead Publishing.

- McEvoy, J.M., Sheridan, J.J., Blair, I.S., McDowell, D.A., 2004. Microbiol contamination of beef carcasses in relation to hygiene assessment based on criteria used in EU Decision 2001/47/EC. Int. J. Food Microbiol.

- Min, D.B. (1998) Food Lipid. A course note. Food Science & Technology, Ohio State University, Columbus, OH, p: 57-71.Gill and Newton, 1978

- Mohan, A.Hunt, M.C.Barstow, T.J.Houser, T.A. Bopp,C. Hueber,D.M. Effects of fibre orientation, myoglobin redox form, and postmortem storage on NIR tissue oximeter measurements of beef longissimus muscle. Meat Sci. **87**(11), 3751–3763 (2009)

- Molla, B., Alemayehu, D., Salah, W. 2003. Sources and distribution of Salmonella serotypes isolated from food animals, slaughterhouse personnel and retail meat products in Ethiopia: 1997-2002. Ethiopian Journal of Health Development 17: 63-70.

- Moyer, C.L., Morita, R.Y. (2007) Psychrophiles and psychrotrophs. **In**: Morita RY (ed.) Encyclopedia of Life Sciences. John Wiley & Sons Ltd, Chichester.pp1–6.

- Mustafa, I.S.M., Abdelgadir, M.A., Sulieman, A.E.2014. Impact of *Halal* and Non-halal Slaughtering on the Microbiological Characteristics of Broiler Chicken Meat and Sausages. Food and Public Health 2014, 4(5): 223-228.

- Nadia, M., Bastide, F. H., Pierre, F., Denis, E.C.2011.Heme Iron from Meat and Risk of Colorectal Cancer: A Meta-analysis and a Review of the Mechanisms Involved.Cancer prevention research. 4(2).2011.

- Nakyinsige, K., Fatimah, A.B., Aghwan, Z.A., Zulkifli,I., Goh, Y.M., Sazili, A.Q. 2014. Bleeding Efficiency and Meat Oxidative Stability and Microbiological Quality of New Zealand White Rabbits Subjected to Halal Slaughter without Stunning and Gas Stun-killing. Asian-Australas J Anim Sci.; 27 (3):406-13.

- Naveena, B. M., Mendiratta, S.K. 2001.Tenderization of spent hen meat using ginger extract. British Poultry Science, 42, 344–350.

- Nel,S., Lues, J.F.R., Buys, E.M., P Venter,P. 2004.Bacterial populations associated with meat from the deboning room of a high throughput red meat abattoir.Meat science vol.66.Issue 3.pages 667-674

- Normanno,G.,Corrente,M.,Salandra,G., Dambrosio,A., Parisi,A., Greco,G., Bellacicco,A.L., Celano,G.V.2007. Methicillin-resistant Staphylococcus aureus (MRSA) in foods of animal origin product

in Italy of food .International journal of food microbiology.Volume 117.Issue 2, 30 June, pages 219-222

- Nortje, G.L., Shaw, B.G. 1989. The effect of ageing treatment on the microbiology and storage characteristics of beef in modified atmosphere packs containing 25% CO_2 plus 75% O_2. Meat Science, 25 (1) (1989), pp. 43–58

- Raharjo, S., Sofos, J. N.1993.Methodology for measuring malonaldehyde as a product of lipid peroxidation in muscle tissues: a review. Meat Sci. 1993, 35, 145-169.

- Red Meat Abattoir Association (RMAA), 2011. Animal handling practical guidelines for abattoirs.

  http://www.rmaa.co.za/2011/conference/AnimalHandlingSPCAforCongress201105.05.2 011 (2011) (Accessed 25 April 2015)

- Riaz, M.N., Chaudry, M.M. (2004). Halal food production. Boca Raton, Florida: CRC Press.

- Rodriguez, P., Velarde, A., Dalmau, A., Llonch, P. (2012). Assessment of unconsciousness during slaughter without stunning in lambs. Animal Welfare, 21(S2), 75–80.

- Russell, N.J. (2002) Bacterial membranes: the effects of chill storage and food processing. An overview. Int J Food Microbiol 79:27–34.

- Samelis, J. (2006). Managing microbial spoilage in meat industry. In Food spoilage microorganisms, pp. 213-286. Edited by C. de W. Blackburn, Cambridge: Woodhead Publishing Limited

- Scanga,J.A., Grona,A.D., Belk,K.E., Sofos,J.N., Bellinger,G.R., G.C Smith,G.C.2000. Microbiological contamination of raw beef trimmings and ground beef.Meat science volume 56.Issue 2. October 2000.pages145-152

- Schillinger, U., Holzapfel, W. H. (2006). Lactic acid bacteria. In Food spoilage microorganisms, pp. 213-286. Edited by C. de W. Blackburn, Cambridge: Woodhead Publishing Limited.

- Schmedes, A, Holmer, G. A. 1989. New thiobarbituric acid (TBA) method for determination of free malonaldehyde (MDA) and hydroperoxides selectivity as a measure of lipid peroxidation. Journal of American Oil Chemistry Society.1989; 66:813–817

- Serdengecti, N., Ibrahim, Y., Gokoglu, N. (2006). Effects of sodium lactate, sodium acetate and sodium diacetate on microbiological quality of vacuum-packed beef during refrigerated storage. J. Food Saf.2, 62-71. 24. Stiles, M.E. (1996).

- Shahidi F (1994) Assessment of lipid oxidation and flavor development in meat and meat products. In "Flavor of meat and meat products", Shahidi, F (Eds). Blackie Academic & Professional, Landon, UK, p: 253.

- Stanbridge, L.H., Davies, A.R.1998. The microbiology of chill stored meat. The microbiology of Meat and Poultry, Blackie Academic and Professional, London (1998),pp.174-219

- Stopforth, J.P.Lopes, M.Shultz, J.E. Samadpour, M.2006. Microbiological status of fresh beef cuts. J Food Prot 2006 Jun;69(6):1456-9

- Strappini, A. C., Metz, J. H. M., Gallo, C. B., Kemp, B. (2009). Origin and assessment of bruises in beef cattle at slaughter. Animal, 3(05), 728–736.

- Takeda, Y., Tarr, P. I.Wachsmuth, I.K. 1996. Proposed new nomenclature for SLT (VT) familyAmerican Society for Microbiology News, 62 (1996), pp. 118–119

- Thanonkaew,S.,Benjakul,W.,Visessanguan,E.A.,Decker.The effect of metal ions on lipid oxidation,colour and physiochemical properties of cuttlefish (Sepia pharaonis) subjected to multiple freeze-thaw cycles .Food Chemistry,95(2006)pp.591-599

- Thomson Reuters & Dinar Standard (2013). State of the global Islamic economy. http://www.dinarstandard.com/state-global-islamic-economy-report-2013 (accessed, 24/02/2016).

- Vanderlinde, P.B., Murray, J., Shay, B.1998. Microbiological quality of Australian beef carcass meat and frozen bulk packed beef .Journal of food protection. 61(4):437-43

- Warriss, P. W. 2001 Postmortem changes in muscles and its convection into meat. Meat science, an introductory text, pp. 100 – 161. CABI Publishing, Wallingford, UK

- Wheby, M.S., Spyker, D.A. (1981) Hemoglobin iron absorption kinetics in the iron decient dog. American Journal of Clinical Nutrition 34, 1686 ± 1693.

- Young, L.L., Reviere, R. D., Cole. A. B 1988. Fresh red meats: A place to apply modified atmospheres. Food Technol. 42(9) :6

CHAPTER 4: EFFECT OF MEAT PRODUCTION METHOD ON SHELF LIFE OF MEAT DURING REFRIGERATED STORAGE

Abstract

Storage of meat is critical because during storage even with proper refrigeration, the meat ultimately undergoes deteriorative changes including microbial spoilage, oxidative changes. There are different types of meat based on the method of animal slaughter and processing. Halal meat is produced without stunning prior to slaughter, resulting in a rapid and complete bleeding of animals. The aim of this research is to determine the effect of type of slaughter (halal and non- halal) on microbiological, physiochemical characteristics and shelf life of beef during storage. Microbiological analyses of beef samples during storage showed that halal beef had significantly lower ($P < 0.05$) microbial count than non-halal meat. This includes APC (aerobic plate count), psychrotrophic bacteria, *S. aureus*, *pseudomonas*, lactic acid bacteria, and *Enterobacteriaceae*. However, no significance differences were observed for yeast and mold, *E. coli*, total coliform and fecal coliform. Physiochemical analyses showed that quality indicators for meat such as lipid oxidation, drip loss, and heme iron content were significantly lower ($P < 0.05$) in halal meat than non-halal meat. However, no significant differences were observed for thaw loss and pH. Results from this research indicate that slaughter method significantly affects the meat quality during storage. We show that halal meat processing may be advantageous in reducing bacterial colonization, increasing the usable shelf life, and improving the quality of the meat.

Introduction

Storage of meat is critical because it is highly perishable food since it contains sufficient nutrient needed to support the growth of microorganisms. The growth of spoilage bacteria

reduces shelf-life of meat and the annual economic loss from spoilage of fresh meat and meat products in the United States is estimated at approximately 5 billion dollars (Ray et al. 1992). In addition, high initial bacterial loads in fresh meat indicate lower standards of slaughter and plant hygiene. Despite proper refrigeration, meat ultimately undergoes deteriorative changes upon long-term storage (Urbain and Campbell, 1987). Some of meat spoilage symptoms from growth of microorganisms to unacceptable levels while the other due to the chemical changes such as physical damage, lipid oxidation and color change. The different microbial groups that will potentially contribute to meat spoilage depend on the storage conditions applied and their competition. The development of such microbial association is reported to significantly affect the type of spoilage (Agapi et al, 2012). Many researchers reported a correlation between meat quality and blood. The more blood retained, the poorer the meat quality (Gregory, 2008); (Strappini et al, 2009). The shelf-life of meat depends on the numbers and types of microorganisms mainly bacteria, initially present and their subsequent growth as well as residual blood left in the meat. Decreasing the initial numbers of the bacteria which grow during storage to form the spoilage flora will extend the time required before they reach numbers sufficient to cause spoilage (Gill,1996).There are different types of meat based on the method of animal slaughter and processing. Halal meat is produced without stunning prior to slaughter, resulting in a rapid and complete bleeding of animals (Eliasi and Dwyer, 2002); (Grandin and Regenstein, 1994). Residual blood left in the carcass as a result of improper bleeding may decrease the shelf life and hence the quality of the meat product because hemoglobin which is an important component of blood is a powerful promoter of lipid oxidation and growth of microorganisms (Alvarado et al, 2007). Studies have shown that halal slaughter results in significantly lower

residual hemoglobin than conventional slaughter with stunning (non-halal) (Nakyinsige et al, 2014). Therefore, choosing the proper meat processing may be increasing the usable shelf life and improve the quality of meat during storage.

This chapter was aimed to determine the effect of meat production method (halal and non-halal) on shelf life, microbiological and physiochemical quality indicators of beef during refrigerated storage for three weeks.

Shelf life of meat

Shelf life is the period between packaging of the product and its use that the product properties remain acceptable to the product user, with shelf life properties being appearance, texture, flavor, color, and nutritive value (Singh & Singh, 2005).The point of spoilage may be defined by a certain maximum acceptable bacterial level, or an unacceptable off-odor or appearance. The shelf-life depends on the numbers and types of microorganisms, mainly bacteria, initially present and their subsequent growth (Elisabeth et al,1996).The shelf life of fresh beef is highly depended on many factors such as pH, water activity, microbial contamination including pathogens, lipid oxidation and color changes which if they controlled, potential shelf life can be achieved (McMillin, 2008).

Even with proper refrigeration, the meat ultimately undergoes deteriorative changes including microbial spoilage, oxidative changes in pigments and lipids, and weight loss (Urbain and Campbell, 1987). The shelf life of fresh meats (non-vacuum) is therefore, approximately 14 days (Huffman, 1974), with only 3-6 days retail life (Bartkowski et al., 1982). The number of bacteria and the composition of the microflora will determine the shelf-life of the product (Gustavsson and Borch, 1993). As storage time continues, the number of bacteria increases and

the nature of the dominant bacteria is determined by storage temperature. On meat stored at 20°C, *Pseudomonas* species still dominated the microflora but when the temperature was increased to 30°C Acinetobacter and Enterobacteriaceae dominated the microflora (Gill and Newton, 1980). Decreasing the initial numbers of the bacteria which grow during storage to form the spoilage flora will extend the time required before they reach numbers sufficient to cause spoilage and, as growth is exponential, that exponential decreases are required for incremental increases in the storage life (Gill, 1996).The rate of spoilage varies according to the species of microflora present, the characteristics of the meat, processing methods, product composition, and the environment in which the meat is stored (Ellis et al., 2002); (Nychas et al., 2008).Most spoilage bacteria found in meat are saprophytic Gram-negative and include aerobic and facultative anaerobic psychrotrophic strains (*Pseudomonas* and related genera), while Gram-positive (LAB, Micrococcus) can also be found in high numbers (Samelis, 2006). However, few species dominated spoilage.

Spoilage of meat

Food spoilage usually refers to the deterioration of quality in food products due to the growth of contaminating microorganisms, although non-microbial activity, such as the activity of endogenous enzymes, can also contribute to food spoilage. The main defects of spoilage are sensory changes, such as off-odors and off-flavors, slime production, texture change, discoloration and gas production. Food spoilage processes determine the shelf life of food products, as the products can only be stored until a maximum unacceptable level of off-odor/off-flavors develop (Borch et al., 1996).

The spoilage process is complicated as it involves many bacteria species and during the development of a microbial population there is a succession process occurring (Ingram, 1962). Deteriorative changes during meat storage are affected by metabolic reactions from biological membrane disruption (Stanley, 1991) and biochemical oxidative processes (Xiong & Decker, 1995). Deterioration of quality may include discoloration, off-flavor and off-odor development, nutrient loss, texture changes, pathogenicity, and progression of spoilage factors (Skibsted et al., 1994). Despite the heterogeneity of the initial contaminating microbial flora, aerobic refrigerated meat storage selects *Pseudomonas spp.*, particularly *P. fluorescens*, *P. putida* and *P. fragi*, as the dominant spoilage flora in proteinaceous raw foods (Ternstrom et al., 1993). *Pseudomonas fragi* is recognized as one of the principal agents of meat spoilage (Labadie, 1999) and very frequently isolated from fresh and spoiled meat products (Ercolini et al., 2007); (Ercolini et al., 2009). Holzapfel, 1998, reported that a shift of the microbial populations has been observed under different storage temperatures. Psychrotrophic bacteria which belong to microbial genera of both Gram positive, such as LAB, and Gram negative bacteria, such as Pseudomonas spp. and Enterobacteriaceae could be developing in meat at chill temperatures. It is generally agreed that spoilage defects in meat become evident when the surface spoilage bacteria reach approximately 7 log CFU/cm2 (Ingram and Simonsen, 1980); (ICMSF, 1998). Off-odors are first detected during aerobic spoilage when populations reach approximately7 log CFU/cm2 and muscle tissue surface becomes sticky at 8 log CFU/cm2 which is indicative of early slime formation (Ingram and Dainty, 1971).

Spoilage of chilled fresh meats can also be caused by breakdown of proteins during bacterial growth. Psychrotrophic *Pseudomonas spp*. cause protein degradation (Young et al.,

1988), and evolution of ammonia by de-amination of amino acids under aerobic conditions (Gardner, 1985). Certain Enterobacteriaceae are also capable of decarboxylating amino acids and produce putrid odors (Gill and Harrison, 1988). Discoloration of meat can also be due to the growth of hydrogen sulfide-producing bacteria. Hydrogen sulfide reacts with myoglobin to form sulfmyoglobin and choleglobin which cause surface greening (Fernandez and Pierson, 1985).

Halal meat is produced without stunning prior to slaughter, resulting in a rapid and complete bleeding of animals (Eliasi and Dwyer, 2002); (Grandin and Regenstein, 1994). As improved bleeding can improve the quality of the meat during storage (Ali et al., 2007).This approach may improve the quality of fresh and extend the shelf life of meat during storage.

Materials and methods

1. **Sample collection:**

Meat samples were collected from different slaughter houses in Michigan. The collected samples were immediately transport in insulated ice containers to the laboratory for microbiological and physiochemical analyses.

2. **Microbiological analysis**

Samples (25 g) of beef were weighted aseptically, added to (225mL) of 0.1% sterilized peptone water and homogenized in a stomacher for 60 s at room temperature. Decimal dilutions in 0.1% sterilized peptone water were prepared and duplicate 1mL samples of appropriate dilutions were poured on Petri dish then added the specific media for each type of bacteria for the following tests (APHA, 1992):-

a) **Aerobic plate Count**

Pouring method was carried out according to APHA (1992)where 1 ml of the selected dilutions were added onto duplicate sterile Petri dish plates then added plate count agar (Sigma-Aldrich) and incubate at 35°C for 48 hr. Colonies between "25-250" were counted and the total aerobic colony counts were then expressed as log CFU/ gram.

b) Psychrotrophic bacteria

Psychrotrophic bacteria determination were carried out plate count agar (Sigma-Aldrich) and then incubated at 7°C for 7 days.

c) *Staphylococcus aureus*

Baird-Parker agar plates (Merk, Germany) were incubated for 48h at 35°C. Typical *S. aureus* colonies (Black colonies with white margins surrounded by clear zones) were counted.

d) Total coliform and fecal coliform bacteria

Violet red bile lactose agar (Sigma-Aldrich) was poured into 1ml of inoculum. The plates were then incubated at 35°C for count of total coliforms and at 44°C for count of fecal coliforms for 48 h. All typical colonies (red colonies) were counted.

e) Total yeast and mold

Total yeast and mold were counted on Potato dextrose agar(Sigma-Aldrich) , acidified with tartaric acid to pH 3.5 and incubated at 25°C for 5 d. Yellowish-white colonies were identified as yeasts, whereas mold growth were recognized by its mycelial appearance.

f) Lactic acid bacteria

Lactic Acid Bacteria (LAB) counts were determined using the double-layer deMan Rogosa Sharpe (MRS) agar (Sigma-Aldrich) and then incubated at 30°C for 72 h.

g) Enterobacteriaceae

Violet Red Bile Glucose agar (VRBG) (Oxoid, Basingstoke, UK) were used for the enumeration of Enterobacteriaceae, overlaid with the same medium and then incubated at 37 ˚C for 24 h.

h) *Escherichia coli*

Escherichia coli bacteria were enumerated using Eosin Methylene Blue (EMB) agar (Sigma-Aldrich) and then incubated at (37°C for 24h). Colonies of *E. coli* on EMB agar were round with a typical metallic sheen.

i) *Pseudomonas spp.*

Pseudomonas spp. were determined on pseudomonas agar base (Oxoid, Basingstoke,UK) supplemented with Pseudomonas CFC supplement (Oxoid, Basingstoke,UK) and then incubated at 25°C for 48 h.

3. Physiochemical analysis

a) pH measurement

The pH of meat samples were measured according to (Naveena and Mendiratta, 2001) meat samples (10 gram) were homogenized with 50 ml of distilled water then filtered through whatman No.1 filter paper. The pH of filtrate samples were measured using digital pH meter (WtW 2f40-11420D.Germany) calibrated at pH 4.0and 7.0 equipped with a pH electrode.

b) Thaw loss Percentage

Thaw loss was calculated as [weight of sample before freezing minus sample weight after thawing] x 100/ sample weight before freezing.

c) Drip loss

Drip loss was calculated as [sample weight minus sample weight after 24 hours refrigeration] x 100/ sample weight.

d) Determination of Heme iron content

Heme iron content of meat was determined according to the method of Cheng & Ockerman (2004). Ground sample (2g) was mixed with 9ml of acid acetone (90% acetone, 8% deionized water and 2% HCl v/v/v). The mixture was mashed with a glass rod and allowed to stand for 1h at room temperature. The mixture was filtered with a Whatman No. 42 filter paper and the absorbance of the filtrate was read at 640nm against an acid acetone used as blank. Heme iron content was calculated as follows;

Heme iron content (ppm) = Total pigment (ppm) X 0.0822

Where total heme pigment (ppm) = A640 X 680

The heme iron content was expressed as mg/100g of wet sample.

e) Lipid oxidation measurement

Thiobarbituric acid reactive substances (TBARS) were determinate according to the method of Schmedes and Holmer (1989). Ground meat samples (10 g) were mixed with 25 ml of trichloroacetic acid (TCA) solution (200 g/l of TCA in 135 ml/l phosphoric acid solution) and homogenized in a blender for 30 s. After filtration, 2 ml of the filtrate were mixed with equal amount of aqueous solution of TBA (3 g/l) in a test tube. The tubes were incubated at room temperature in the dark for 20 h; then the absorbance was measured at 532 nm using UV-vis spectrophotometer (model UV-1200, Shimadzu, Kyoto, Japan). Lipid oxidation was expressed as thiobarbituric acid reactive substances (TBARS) expressed as mg Malonaldehyde/kg sample.

4. Statistical analysis

Data were represented the means of experiments. The means were compared using student T test (IBM SPSS statistics 23).

Figure 4.1 Aerobic plate count in halal and non-halal beef during refrigerated storage.
A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). Significant differences in aerobic plate count during refrigerated storage were established by using independent measures t-tests at a significance level of p≤0.05.
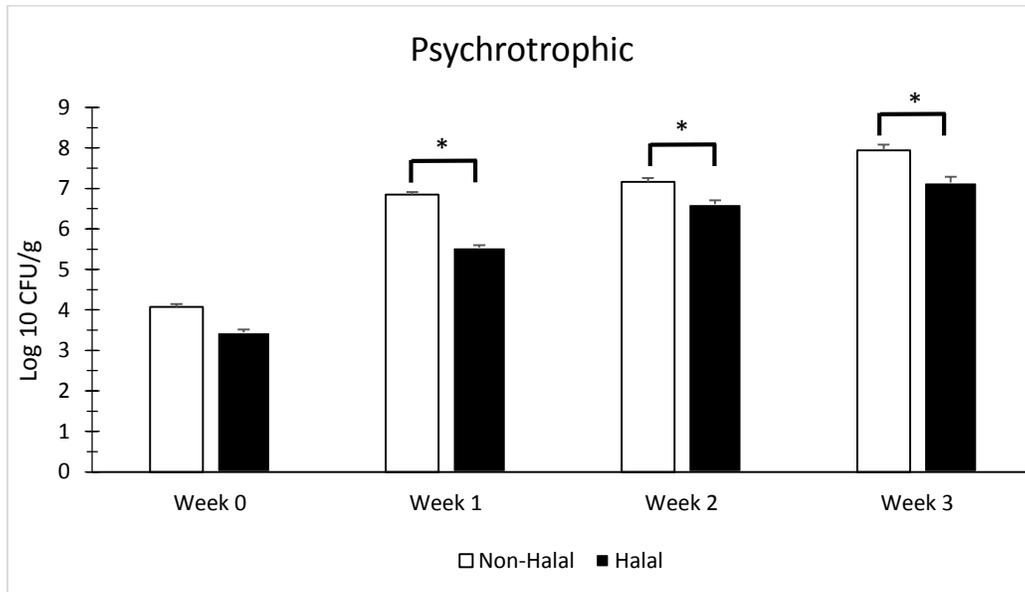
Figure 4.2 Psychrotrophic bacteria in halal and non-halal beef during refrigerated storage.
A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). Significant differences in psychrotrophic bacteria during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of p≤0.05.
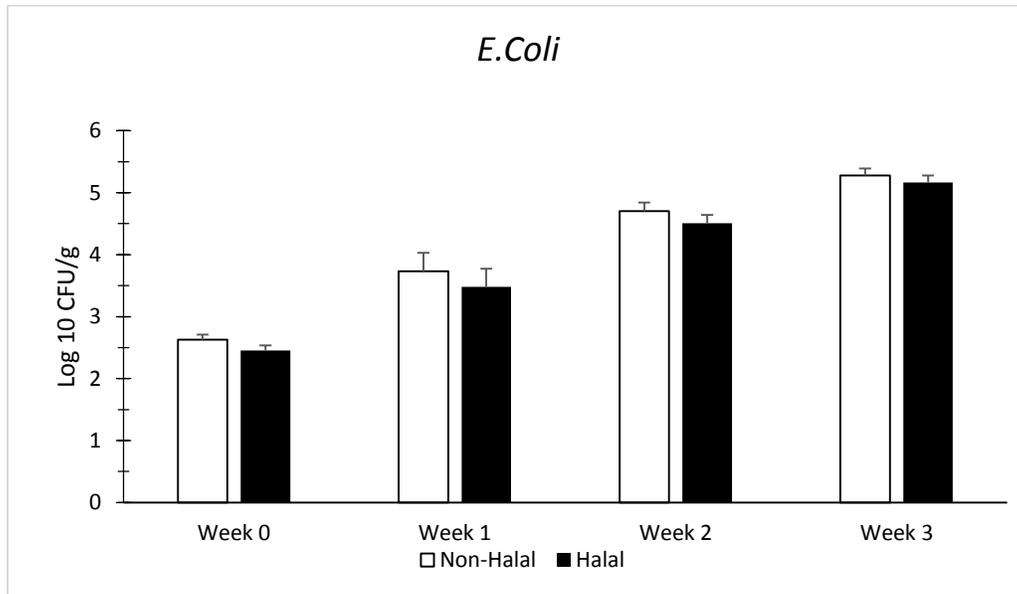
Figure 4.3 *E. coli* bacteria in halal and non-halal beef during refrigerated storage.
A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). No Significant differences in *E. coli* bacteria during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of p≤0.05.
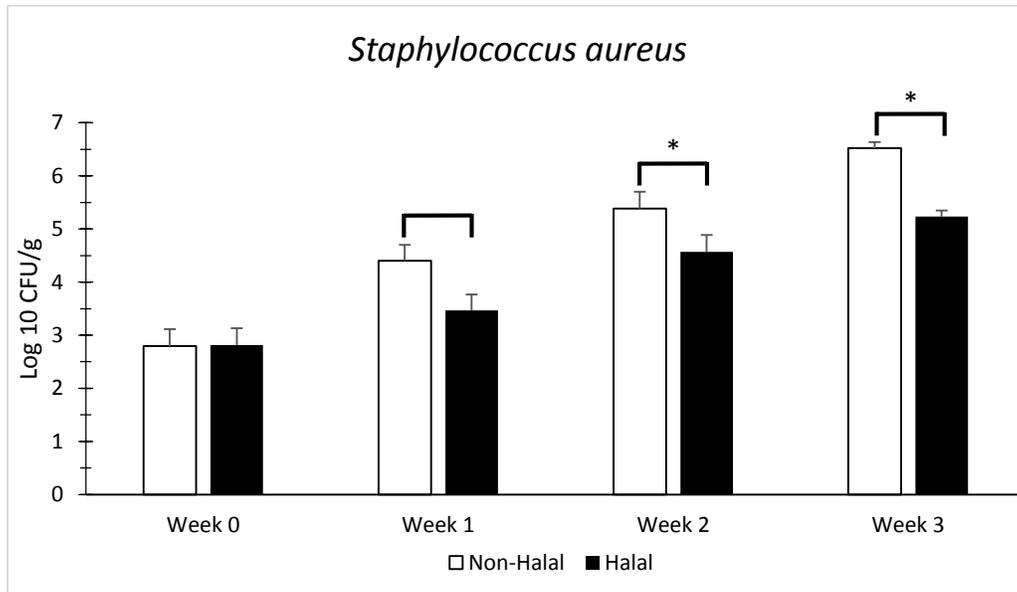
Figure 4.4 *S. aureus* in halal and non-halal beef during refrigerated storage.
A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). Significant differences in *S. aureus* bacteria during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of p≤0.05.
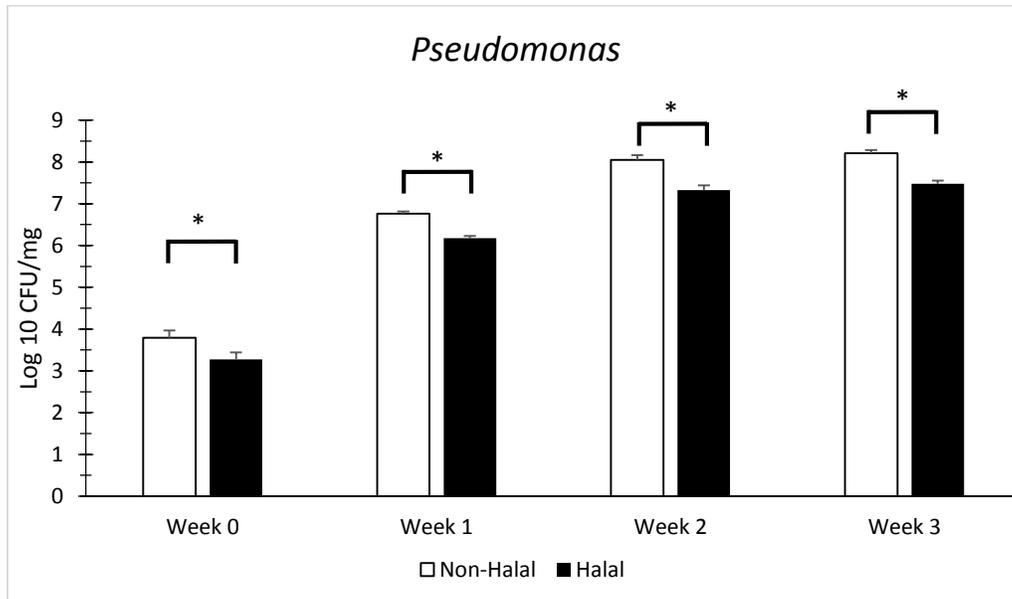
Figure 4.5 *Pseudomonas* bacteria in halal and non-halal beef during refrigerated storage. A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). Significant differences in *pseudomonas* bacteria during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of p≤0.05.
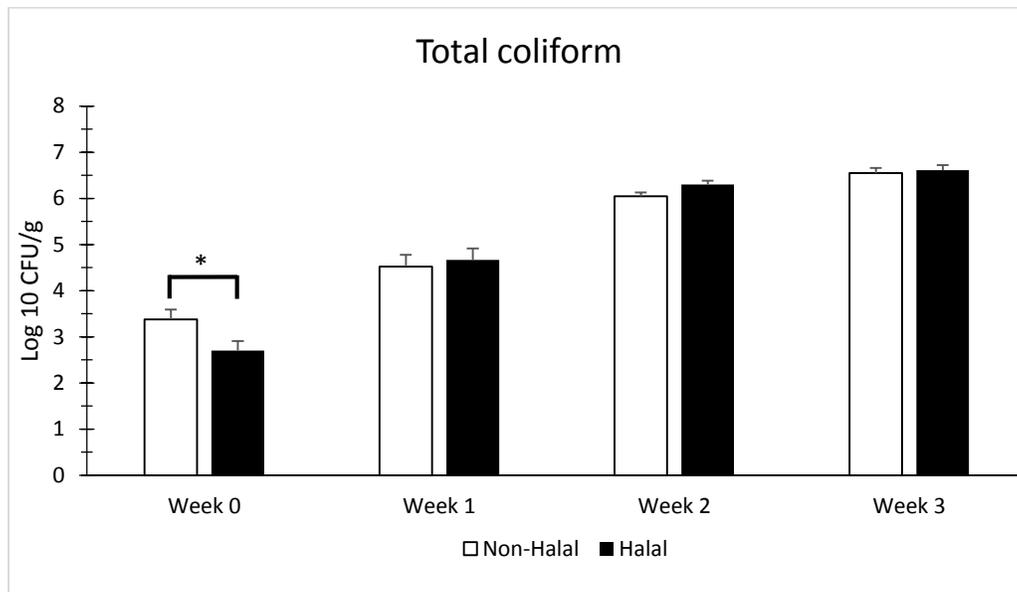
Figure 4.6 Total coliform in halal and non-halal beef during refrigerated storage.
A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). No Significant differences in total coliform bacteria during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of p≤0.05.
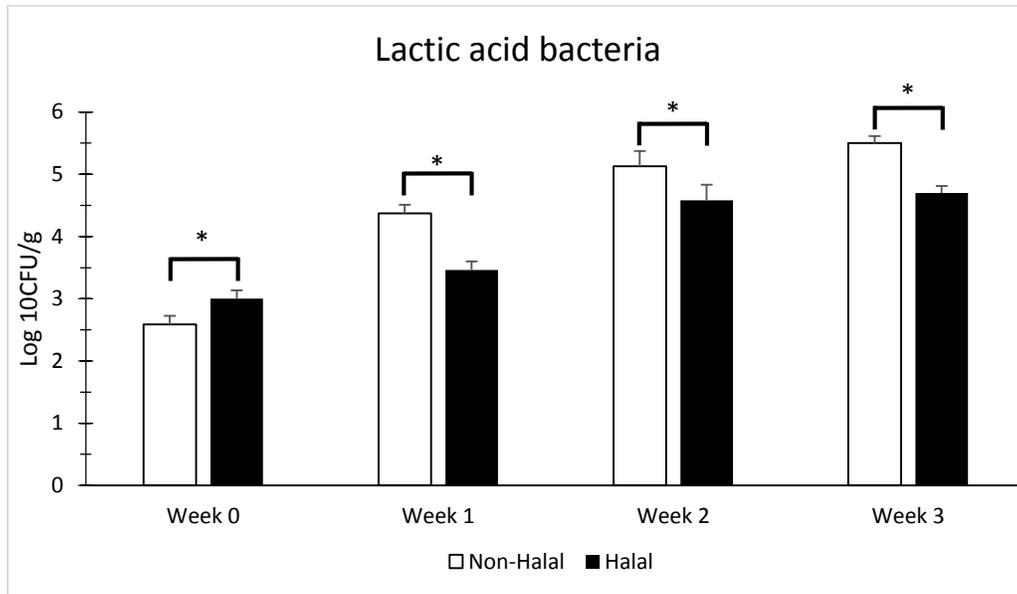
Figure 4.7 Lactic acid bacteria in halal and non-halal beef during refrigerated storage.
A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). Significant differences in Lactic acid bacteria during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of p≤0.05.
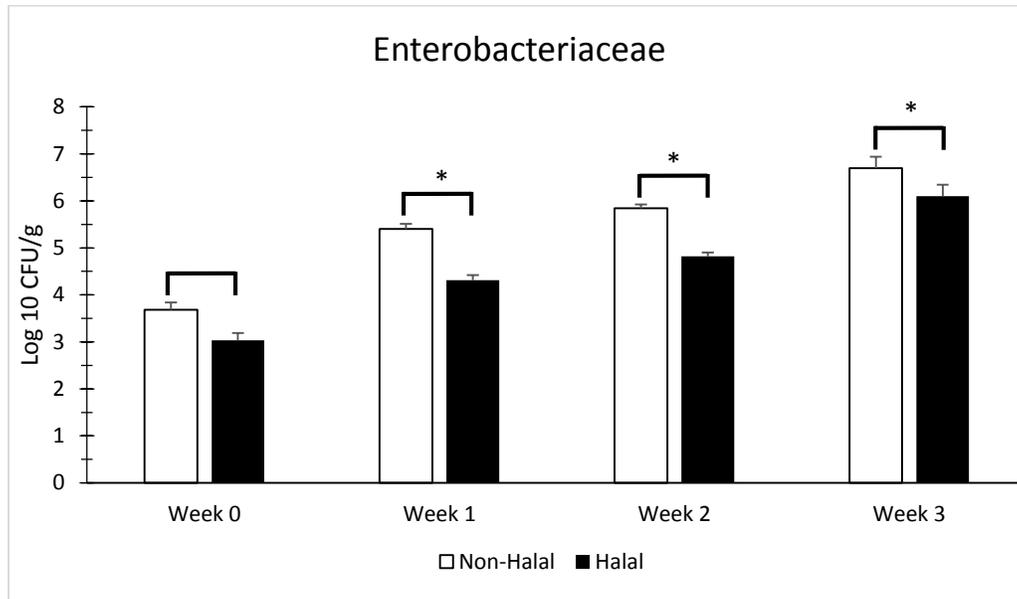
Figure 4.8 Enterobacteriaceae bacteria in halal and non-halal beef during refrigerated storage. A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). Significant differences in Enterobacteriaceae bacteria during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of $p \leq 0.05$.
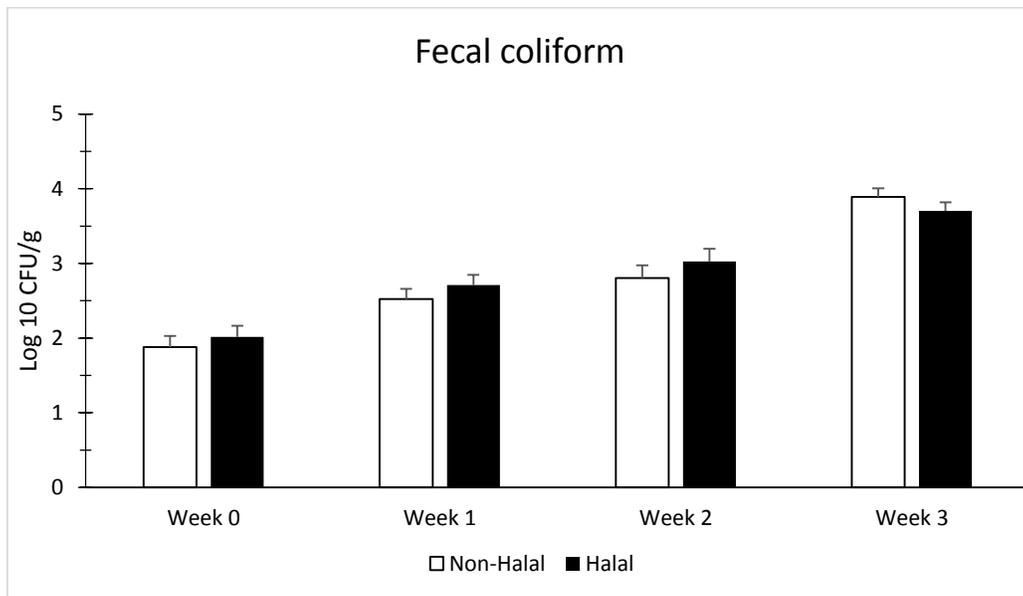
Figure 4.9 Fecal coliform bacteria in halal and non-halal beef during refrigerated storage.
A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). No Significant differences in fecal coliform bacteria during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of $p \leq 0.05$.
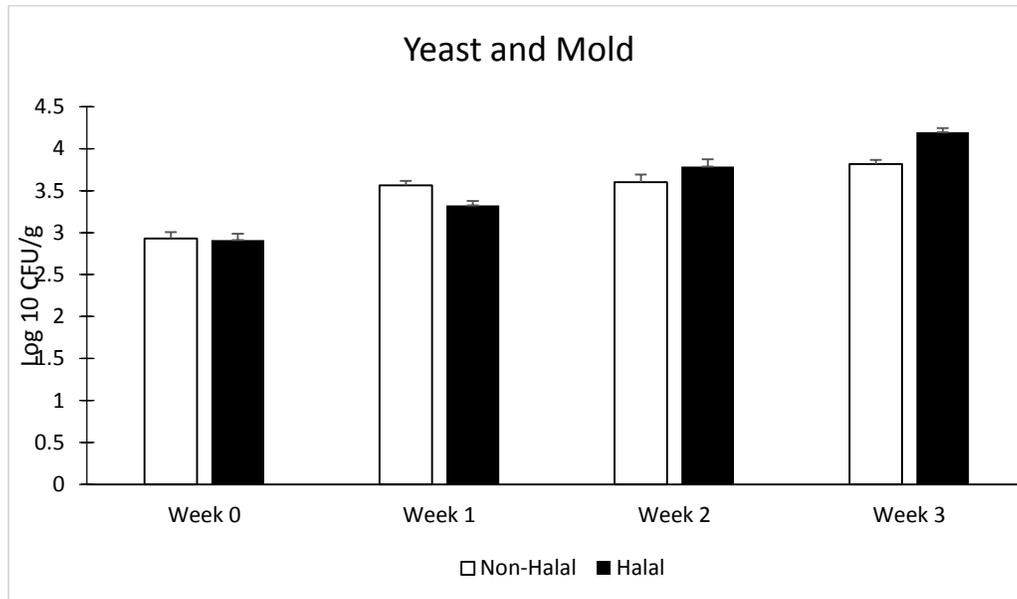
Figure 4.10 Yeast and mold in halal and non-halal beef during refrigerated storage.
A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in logarithmic colony forming unit per gram (CFU/g). No Significant differences in yeast and mold during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of p≤0.05.
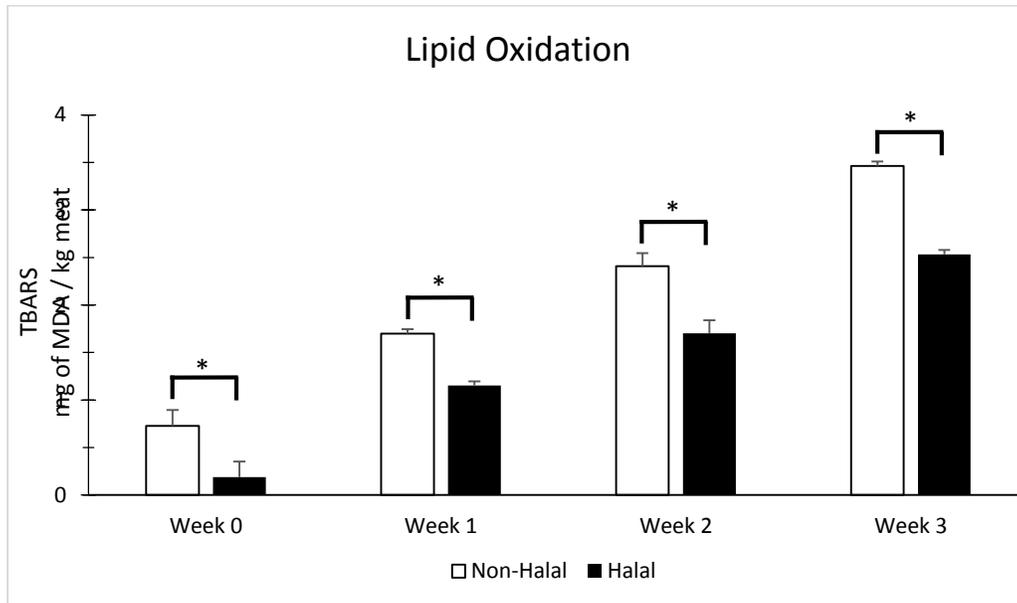
Figure 4.11 Lipid oxidation in halal and non-halal beef during refrigerated storage.
 A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in (mg of MDA/kg meat). Significant differences in lipid oxidation during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of p≤0.05.
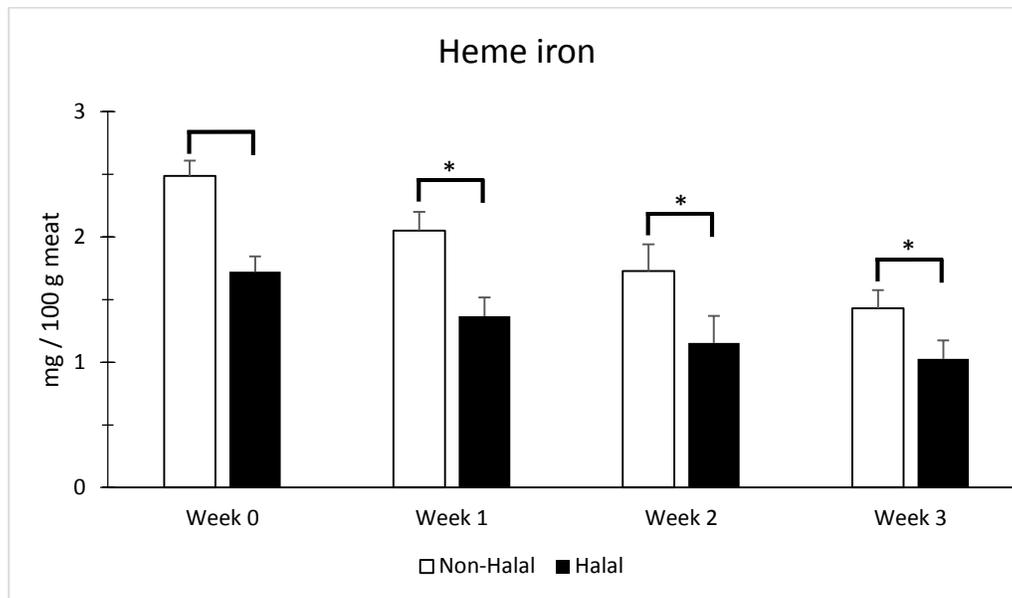
Figure 4.12 Heme iron in halal and non-halal beef during refrigerated storage.

A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in (mg/100g) meat. Significant differences in heme iron content during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of $p \leq 0.05$.
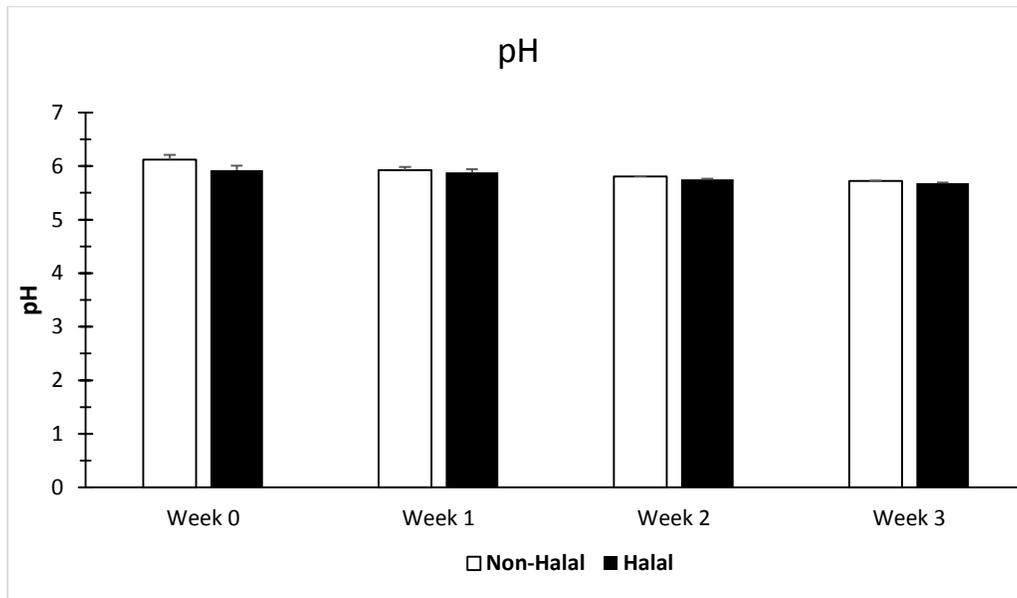
Figure 4.13 pH in halal and non-halal beef during refrigerated storage.
A data represent the mean values obtained from halal and non-halal beef samples. No significant differences in pH during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of p≤0.05.
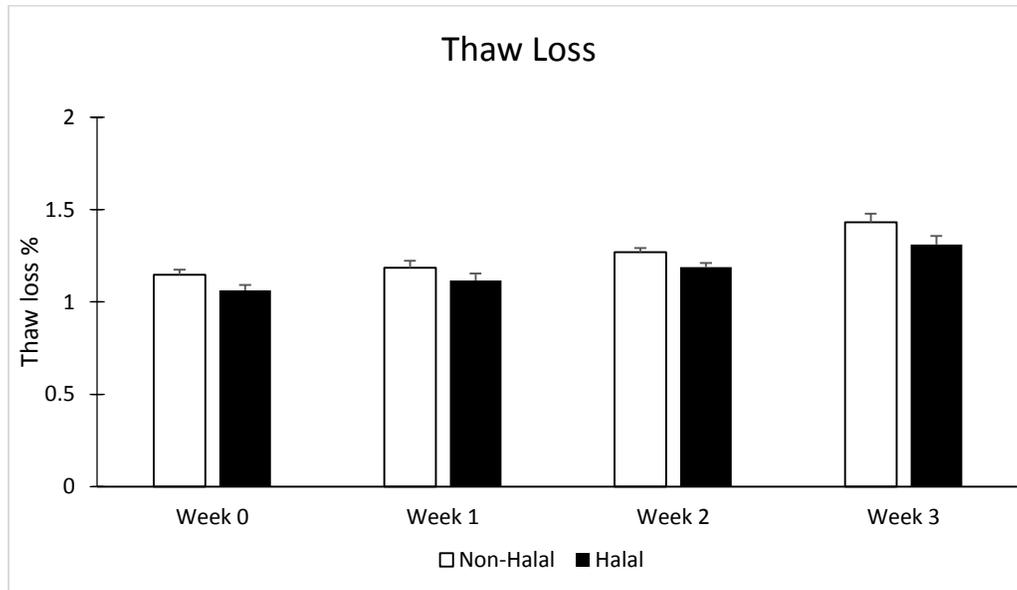
Figure 4.14 Thaw loss in halal and non-halal beef during refrigerated storage.
A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in (Thaw loss). No Significant differences in thaw loss during refrigerated storage for three weeks were established by using independent measures t-tests at a significance level of p≤0.05.
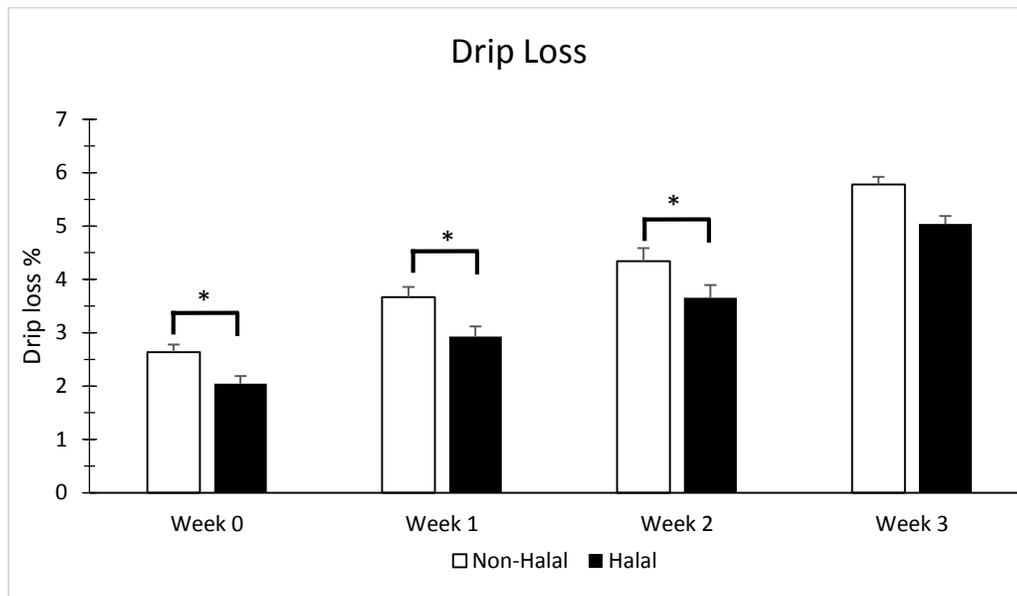
Drip Loss

Figure 4.15 Drip loss in halal and non-halal beef during refrigerated storage.
A data represent the mean values obtained from halal and non-halal beef samples, and are expressed in (drip loss). Significant differences in drip loss during refrigerated storage for the first two weeks but there was no significant difference for third week were established by using independent measures t-tests at a significance level of $p \leq 0.05$.

Results and discussion

1. **Bacterial content in halal and non-halal beef during refrigerated storage**

a) **Aerobic plate count**

Aerobic plate counts are a widely accepted measure of the general degree of microbial contamination and the hygienic conditions of processing plants (DAAHP).The aerobic plate count in halal and non-halal beef during refrigerated storage for three weeks were shown in Figure (4.1). The aerobic plate count in halal and non-halal beef were for first, second and third week were (5.46, 6.41), (6.35, 7.48), (6.70, 8.10) respectively. Halal beef that was produced without stunning resulted in significantly lower (P < 0.05) aerobic plate count than non-halal beef during refrigerated storage three weeks. The lower aerobic plate count in the Halal beef could be attributed to the high blood loss because of method of slaughter. Many researchers reported a correlation between meat quality and blood. The more blood retained, the poorer the meat quality (Gregory, 2008); (Strappini, et al, 2009). Aerobic plate count in both halal and non-halal were increased during refrigerated storage at all three weeks. This may be attributed to high nutritive value of blood, suitable temperature, pH, and water activity of meat and relative humidity influence the degree of deterioration in meat (Lerner, 2009). The level of aerobic plate count in halal beef at week 3 was acceptable as indicated by (Insausti et al., 2001) and (Jeremiah, 2001), that spoilage occurs when the level of total viable count reach 7–8 log Cfu/g. Therefore, choosing the proper meat processing may be increasing the usable shelf life and improve the quality of meat during storage. These findings are in agreement with the findings of (Addeen et al., 2014) and (Mustafa et al, 2014) in chicken who found that APC of broiler chicken was significantly higher in non-halal than halal meat during storage.

b) Psychrotrophic bacteria

Psychrotrophic bacteria have ability to grow at low temperatures but have optimal and maximal growth temperatures above 15 and 20 °C, respectively (Moyer and Morita, 2007). Bacteria developing on meat at chill temperatures are regarded as psychrotrophic. Some psychrotrophic bacteria are gram-positive such as lactic acid bacteria, and the other are gram-negative bacteria, such as *Pseudomonas spp*. and Enterobacteriaceae (Gill and Newton, 1978). Species of Pseudomonas are especially responsible for the spoilage of meat stored at chill temperatures (Jay et al, 2003). The psychrotrophic bacteria in halal and non-halal beef during refrigerated storage for three weeks were shown in Figure (4.2). Non-Halal beef was significantly higher (P < 0.05) psychrotrophic bacteria than halal beef during refrigerated storage for three weeks. The higher psychrotrophic bacteria in non-halal beef could be attributed to the low blood loss because of method of slaughter where in some cases, *Pseudomonas* can constitute up to 96% of the population (Asensio et al, 1988). *Pseudomonas* has ability to use glucose in the blood as substrate for microbial growth (Warriss, 2001). Also, as storage time increases, the psychrotrophic count also increased for both slaughter methods. This showed preference of bacteria to grow at low temperature. The increase in the psychrotrophic count for both methods of slaughter may be due to a shift of the microbial populations during storage period. A shift of the microbial populations has been observed under different storage temperatures. Psychrotrophic bacteria which belong to microbial genera of both gram-postive, such as LAB, and gram-negative bacteria, such as **Pseudomonas** spp. and **Enterobacteriaceae** could be developing in meat at chill temperatures (Gill and

Newton, 1978) and (Holzapfel, 1998). More accurately, species of **Pseudomonas** are particularly involved in the spoilage of meat stored at chill temperatures (Ercolini et al., 2007).

c) *E. coli*

The effect of slaughter methods on prevalence of *E. coli* bacteria in beef during refrigerated storage were shown in Figure (4.3). There were no significant differences in *E. coli* between halal and non-halal beef during refrigerated storage for all three weeks. This could be due to apply the same procedures in both slaughters to control this bacteria during handling the meat as well as following the hygiene practice by workers. The count of *E. coli* bacteria in halal and non-halal beef for first, second and third week were (3.48, 3.73), (4.50, 4.70), (5.16, 5.27) respectively. As storage time increases, the *E. coli* count also increased for both slaughter methods for three weeks. This may be due to content of meat of sufficient nutrient needed to support the growth of microorganisms (Magnus, 1981).

d) *Staphylococcus aureus*

Staphylococcus aureus produces a heat-stable toxin in foods and causes staphylococcal food poisoning (SFP) by ingesting food that contains staphylococcal enterotoxin. Staphylococcal food poisoning (SFP) is responsible for a third of the foodborne diseases worldwide (Normanno, et al., 2007). Figure (4.4) shows the effect of slaughter methods on prevalence of S*. aureus* bacteria in beef during refrigerated storage for three weeks. The *S. aureus* in halal and non-halal beef for first, second and third week were (3.46, 4.40), (4.57, 5.38), (5.23, 6.52) respectively. At week 0, *S. aureus* was not significantly different for the two slaughter methods. However, at weeks 1, 2, and 3 of refrigerated storage, halal beef had lower ($p < 0.05$) growth of S. aureus than

non-halal beef. In general, increased growth of *S. aureus* with storage time was observed in meat samples from both slaughters. This increase in count was due to slow adaptation for growth by **S. aureus** at lower temperature (Kneepan et al, 2013). Results from present study were agreement with (Nakyinsige et al, 2014) who observed that lower amount of residual blood in the carcass of rabbits subjected to halal slaughter caused lower bacteria count. In chicken, (Ali et al, 2011) also reported that higher blood loss in halal slaughter was associated with lower bacteria count in minced meat at 48 h postmortem. Therefore, choosing the proper meat processing such as halal beef may be increasing the usable shelf life and improve the quality of meat during storage.

e) Pseudomonas

Pseudomonas species were the dominant bacteria group on normal and high pH beef (Erichsen and Molin, 1981) as well as in some cases, *Pseudomonas* can constitute up to 96% of the population (Asensio et al, 1988). Figure (4.5) shows the count of *pseudomonas* bacteria in halal and non-halal beef during refrigerated storage for three weeks. Halal beef had significantly lower ($P < 0.05$) *pseudomonas* bacteria than non-halal beef during refrigerated storage at all three weeks. This may be attributed to high amount of blood in non-halal beef and ability of *pseudomonas* bacteria to use glucose in the blood as substrate for microbial growth (Warriss, 2001). The *pseudomonas* bacteria in halal and non-halal beef were for first, second and third week were (6.17, 6.76), (7.32, 8.04), (7.48, 8.21) respectively. Like aerobic plat count and psychrotrophic, as storage time increases, the *pseudomonas* count also increased for both slaughter methods for three weeks. This may be due to content of meat of sufficient nutrient needed to support the growth of microorganisms (Magnus, 1981). Species of **Pseudomonas** are

particularly involved in the spoilage of meat stored at chill temperatures (Ercolini et al., 2007). Therefore, choosing the proper meat processing may be increasing the usable shelf life and improve the quality of meat during storage.

f) Total coliform

Total coliform in halal and non-halal beef during refrigerated storage for three weeks were shown in Figure (4.6). Total coliform in halal and non-halal beef for first, second and third week were (4.66, 4.52),(6.30, 6.04), (6.61, 6.54) respectively. Halal beef that was produced without stunning resulted in significantly lower (P < 0.05) coliform than non-halal beef during refrigerated storage for three weeks. The lower total count in the halal beef could be attributed to the high blood loss because of method of slaughter. Many researchers reported a correlation between meat quality and blood. The more blood retained, the poorer the meat quality (Gregory, 2008); (Strappini et al, 2009). Total coliform in both halal and non-halal were increased during refrigerated storage for all three weeks. This may be due to content of meat of sufficient nutrient needed to support the growth of microorganisms (Magnus, 1981).

g) Lactic acid bacteria

Lactic acid bacteria are produced lactic acid because of glucose fermentation and some species have significant role in the spoilage of refrigerated raw meat (Labadie 1999). The spoilage characteristics are slime formation, discolorations, sour flavor and off odor, (Schillinger &Holzapfel, 2006). Lactic acid bacteria in halal and non-halal beef during refrigerated storage for three weeks were shown in Figure (4.7). At week 0, halal beef was significantly higher (P < 0.05) growth of lactic acid bacteria. However, at weeks 1, 2, and 3 of refrigerated storage, halal beef had lower (p < 0.05) growth of lactic acid than non-halal beef. The lower lactic acid bacteria in

the halal beef could be attributed to the high blood loss as a result of method of slaughter. Many researchers reported a correlation between meat quality and blood. The more blood retained, the poorer the meat quality (Gregory, 2008); (Strappini, Metz et al, 2009). In general, increased growth of lactic acid bacteria with storage for three weeks was observed in meat samples from both slaughters. This may be attributed to high nutritive value of blood, suitable temperature, pH, and water activity of meat and relative humidity influence the degree of deterioration in meat (Lerner, 2009). Increase growth of lactic acid bacteria can explain pH decrease during storage period. The level of lactic acid bacteria in halal and non-halal beef at all three weeks was acceptable as indicated by (Insausti et al. 2001) and Jeremiah (2001), that spoilage occurs when the level of lactic acid bacteria reach 7 log Cfu/g.

h) Enterobacteriaceae

Enterobacteriaceae bacteria are used glucose as substrate for growth and produce some deleterious compound (Gill, 1986), (Lambert et al., 1991). Some species such as *H. alvei* and *S. liquefaciens* produce malodorous diamines and cause a green discoloration when they grow in meat (Stanbridge & Davies, 1998). Enterobacteriaceae bacteria in halal and non-halal beef during refrigerated storage for three weeks were shown in Figure (4.8). Non-halal beef that was resulted in significantly higher ($P < 0.05$) Enterobacteriaceae bacteria than halal beef during refrigerated storage for all three weeks. The Enterobacteriaceae bacteria in halal and non-halal beef for first, second and third week were (4.31, 5.40), (4.82, 5.84), (6.10, 6.69) respectively. The higher Enterobacteriaceae in the non-halal beef could be attributed to high amount of blood in non-halal beef and ability of Enterobacteriaceae bacteria to use glucose in the blood as substrate for microbial growth (Warriss, 2001). Many researchers reported a correlation between meat

quality and blood. The more blood retained, the poorer the meat quality (Gregory, 2008); (Strappini et al, 2009). Enterobacteriaceae bacteria in both halal and non-halal were increased during refrigerated storage at all weeks. This may be attributed to content of meat of sufficient nutrient needed to support the growth of microorganisms (Magnus, 1981). The level of Enterobacteriaceae bacteria in halal and non-halal beef at all three weeks was acceptable as indicated by (Insausti et al., 2001) and (Jeremiah, 2001), that spoilage occurs when the level of total viable count reach 7–8 log Cfu/g. Therefore, choosing the proper meat processing may be increasing the usable shelf life and improve the quality of meat during storage.

i) Fecal coliform

In this study also we evaluated the effect of slaughter method on prevalence of fecal coliform in beef. Figure (4.9) shows the count of fecal coliform bacteria in halal and non-halal beef during refrigerated storage for three weeks. There were no significant differences for fecal coliform between halal and non-halal beef during storage period. This could be due to apply the same procedures to control this bacteria during handling the meat as well as following the hygiene practice by workers (Grohs et al, 2000).

j) Yeast and mold

The effect of slaughter methods on prevalence of yeast and mold in beef during refrigerated storage for three weeks was shown in fig. (4.10). There were no significant differences in yeast and mold between halal and non-halal beef during refrigerated storage for all three weeks. This may be due to apply the same procedures in environment of slaughter such as ventilation where spores of yeast and mold can be spread through the air. Also, as storage

time increases, the yeast and mold count also increased for both slaughter methods during storage for three weeks.

2- Physiochemical analysis

a) Lipid oxidation

Lipid oxidation is one of the major causes of quality deterioration in raw and cooked meat products during refrigeration and storage (Raharjo and Sofos, 1993). Lipid oxidation is measured as malondialdehyde (MDA) which is formed by oxidation of polyunsaturated fatty acids with 2 or more double bonds. MDA-induced DNA damage is mutagenic in bacterial, mammalian, and human cells (Nadia et al, 2011). The effect of slaughter methods on lipid oxidation in beef was shown in fig. (4.11). Halal beef that was produced without stunning resulted in significantly lower (P < 0.05) lipid oxidation than non-halal beef during refrigerated storage for three weeks. This could be due to halal slaughter without stunning resulted in significant lower residual hemoglobin than non-halal slaughter with stunning (Nakyinsige et al, 2014); (Griffiths et al, 1985). Residual blood left in the carcass because of improper bleeding may decrease the shelf life and hence the quality of the meat product because hemoglobin which is an important component of blood is a powerful promoter of lipid oxidation (Alvarado et al, 2007). Also, Blood also contains a large number of white blood cells that produce hydroxyl radicals, superoxides, and hydrogen peroxide that enhance lipid oxidation (Gabig & Babior, 1981). Lipid oxidation in both slaughters was increased during refrigerated storage at all three weeks. This may be due to heme breakdown during storage and hence the released non-heme iron can catalyze lipid oxidation. These values were consistent with the results for heme iron content in this study where were (1.36, 2.05),

(1.15, 1.72), (1.02, 1.43) for halal and non-halal beef respectively. Transition metal ions, primarily Fe serve as key catalysts for lipid oxidation in muscle (Kanner, 1994).

b) Heme iron

Heme iron content in halal and non-halal beef were shown in Figure (4.12). Halal beef that was produced without stunning resulted in significantly lower ($P < 0.05$) heme iron than non-halal beef. This could be due to halal slaughter without stunning resulted in significant lower residual hemoglobin than non-halal slaughter with stunning (Nakyinsige et al, 2014); (Griffiths et al, 1985). Also, as storage time increases, the heme iron was decreased for both slaughter methods for three weeks. This could be due to breakdown of heme and release non-heme iron (Benjakul and Bauer, 2011) and hence it can catalyze lipid oxidation during the storage. Transition metal ions, primarily Fe serve as key catalysts for lipid oxidation in muscle (Kanner, 1994). These values were consistent with the results for lipid oxidation and some of microbiological analyses such as aerobic plate count, psychrotrophic bacteria, pseudomonas , lactic acid bacteria , coliform and Enterobacteriaceae.

Red meat and processed meat intake is associated with a risk of colorectal cancer, a major cause of death in affluent countries. Epidemiological and experimental evidence supports the hypothesis that heme iron present in meat promotes colorectal cancer. This meta-analysis of prospective cohort studies of colon cancer reporting heme intake included 566,607 individuals and 4,734 cases of colon cancer (Nadia et al 2011). Therefore, halal slaughter could lower heme iron and hence improve the quality of meat and avoid health problems such as colorectal cancer.

c) pH

The effect of slaughter methods on pH in beef was shown in Fig (4.13). There were no significant differences for pH between halal and non-halal beef during refrigerated storage for three weeks. This could be due to use same treatment with animals before slaughtering. Also, as storage time increases, the pH was decreased for both slaughter methods for three weeks. This could be due to increase growth of lactic acid bacteria during storage period and produce lactic acid.

d) Thaw loss

The effect of slaughter methods on thaw loss in beef was shown in Figure (4.14). There were no significant differences for thaw loss between halal and non-halal beef during refrigerated storage for three weeks. In addition, as storage time increases, the thaw loss was slightly increased for both slaughter methods for three weeks.

e) Drip loss

Drip loss results in an unsightly appearance and tougher, drier meat. Exudate water contains ca. two-thirds of the protein concentration of whole meat, so drip loss is a costly waste of animal protein (van Laack and Solomon, 1994). Drip loss in halal and non-halal beef was shown in Figure (4.15). At week 1and 2, halal beef that was produced without stunning resulted in significantly lower (P < 0.05) drip loss than non-halal beef while there was no significant different at week 3. As storage time increases, the drip loss increases for both slaughter methods. The increase in drip loss with storage time may be attributed to degradation of muscle proteins caused by the spoilage mechanisms. These findings are in agreement with the findings of (Addeen et al. 2014) in chicken, and (DAgata et al, 2010) in beef who found that drip loss was significantly higher in non-halal than halal meat during storage.

REFERENCES

- Alvarado, C., Richards, M., O'Keefe, S., Wang, H. 2007.The effect of blood removal on oxidation and shelf life of broiler breast meat. Poultry Science, 86 (1) (2007), pp. 156–161.

- Ali, A. S. A., Lawson, M.A., Tauson, A., Jensen, J.F., and Chwalibog, A. 2007. Influence of electrical stunning voltages on bleed out and carcass quality of broiler chickens. Arch. Geflügelk. 71:35-40.

- Ali, A.M., Abdalla, H.O., Mahgoub, I.M. 2011. Effect of slaughtering method on the keeping quality of broiler chickens'meat. Egypt Poultry Science, 31 (2011), pp. 727–736

- Asensio, M.A., Ordóñez, J.A., Sanz, B. 1988. Effect of carbon dioxide and oxygen enriched atmospheres on the shelf-life of refrigerated pork packed in plastic bags J. Food Protect., 51 (1988), pp. 356-360

- Addeen, A., Benjakul, S., Wattanachant, S., Maqsood, S. 2014.Effect of Islamic slaughtering on chemical compositions and post-mortem quality changes of broiler chicken meat. International Food Research Journal 21(3): 897-907

- Agapi I. Doulgeraki a , Danilo Ercolini b, Francesco Villani b , George-John E. Nychas Spoilage microbiota associated to the storage of raw meat in different conditions International Journal of Food Microbiology.

- APHA; American Public Health Association (1992): Compendium of Methods for the Microbiological Examination of Foods, 3rd ed. APHA, Washington, DC, USA.

- Bartkowski, L, F.D. Dryden, and J.A. Marchello. 1982. Quality changes of beef steaks stored in controlled gas atmospheres containing high and low levels of oxygen. J. Food Prot. 45:41.

- Benjakul, S. and Bauer, F. 2011. Biochemical and physicochemical changes in catfish (Silurus granis Linne) muscle as influenced by different freeze-thaw cycles. Food Chemistry 72: 207-217.

- Borch, E. Kant- Muemans, M.L. Blixt, Y. 1996.Bacterial spoilage of meat and cured meat products. International Journal of Food Microbiology, 33 , pp. 103-120

- Cheng, J.H. and Ockerman, H.W.2004. Effect of ascorbic acid with tumbling on lipid oxidation of precooked roast beef. J. Muscle Food.15:83-93.

- Department of Agriculture, Animal Health and Product. 2004. Animal and animal origin foods. No. 624-04. *Off. Bull.* 5214:727–745.

- D'Agata, M., Russo, C., Preziuso, G. 2010. Effect of Islamic ritual slaughter on beef quality. Italian Journal Animal. Sci. vol. 8 (Suppl. 2), 489-491

- Ercolini D., Russo, F., Nasi, A., Ferranti, P., and Villani, F., 2009. Mesophilic and psychrotrophic bacteria from meat and their spoilage potential in vitro and in beef.

- Elisabethm B., Kant-Muermans, M-L.and Blixt, Y., 1996. Bacterial spoilage of meat and cured meat product. Int. J. Food Microbiol. 33, 103-120

- Ellis, D. I., D. Broadhurst, D. B. Kell, J. J. Rowland, and R. Goodacre. 2002. Rapid and quantitative detection of the microbial spoilage of meat by Fourier Transform Infrared Spectroscopy and machine learning. J. Appl. Envron. Microbiol. 68:2822-2828.

- Eliasi, J.R., Dwyer, J.T., 2002. Kosher and halal: religious observances affecting dietary intakes. J. Am. Diet. Assoc. 101:911–913.

- Erichsen,I., Molin,G.1981.Microbial flora of normal and high pH beef stored at 4 °C in different gas environments J. Food Prot., 44 (1981), pp. 866-869

- Ercolini, D., Russo, F., Blaiotta, G., Pepe, O., Mauriello, G., and Villani, F., 2007. Simultaneous detection of Pseudomonas fragi, P. lundensis and P. putida from meat by a multiplex PCR assay targeting the car A gene. Appl Environ Microbiol 73:2354-2359

- Fernandez, C.F., and M.D. Pierson. 1985. Enumeration of hydrogen sulfideproducing bacteria from anaerobically packaged pork. J. Food Prot. 48: 982.

- Gabig and Babior.1981.The killing of pathogens by phagocytes.Annual Review of Medicine, 32 (1) (1981), pp. 313–326.

- Gill, C .O. Extending the Storage Life of Raw Chilled Meats .1996. Agriculture and Agri-Food Canada Research Centre, 6000 C and E, Trail Lacombe Alberta, Canada

- Gill, C.O., and Harrison, J.C.L. 1989. The storage life of chilled pork packaged under carbon dioxide. Meat Sci. 26:313.

- Grandin, T., Regenstein, J.M., 1994. Religious slaughter and animal welfare: a discussion for meat scientists. Meat Focus International. CAB Intern., Wallingford, Oxon, UK, pp 115-123.

- Grohs, B.M, Kliegel, N. Kunz B (2000) Bacteria grow slower: Effects of spice mixtures on extension of shelf life of pork. Fleischwirtschaft 80(9):61–63

- Gill, C.O., Newton, K.G.1978.The ecology of bacterial spoilage of fresh meat at chill temperatures. Meat Science, 14 (1978), pp. 43–60

- Gill, CO. and Newton, K, G, (1980). Growth of bacteria on meat at room temperatures. Journal of Applied Bacteriology. 49:315-323.

- Gregory, N. G. (2008). Animal welfare at markets and during transport and slaughter. Meat Science, 80(1), 2–11.

- Griffiths, GL., McGrath. M., Softly, A., jones, C. 1985 Blood content of breiler chicken carcasses prepared by different slaughter methods. *The Veterinary Record II* 7: 382-385

- Gustavsson, P, and Borch, E, (1993), Contamination of beef carcasses by psychrotrophic Pseudomonas and Enterobacteriaceae at different stages along the processing line. International Journal of Food Microbiology. 20: 67-83

- Huffman, D.L. 1974. Effect of gas atmosphere on microbial quality of pork. J. Food Sci. 39:723.

- Holzapfel, 1998.The Gram-positive bacteria associated with meat and meat products**.** The Microbiology of Meat and Poultry, Blackie Academic and Professional, London (1998), pp. 35–84.

- Ingram, M., and Simonsen, B. 1980. Meat and meat products. In Microbial Ecology of Foods. Vol 2 (J.H. Si Hiker, Chairman) p. 333. Academic Press, New York, NY.

- Ingram, M. (1962), Microbiological principles in pre-packaged meats. Journal of Applied Microbiology. 25: 259- 281

- Ingram, M. and Dainty, R.H. 1971. Changes caused by microbes in spoilage of meats. J. Appl. Bacteriol. 34:2.

- Insausti, K,.Breiain,M.J.,Purroy,A.,Alberti,P.,Gorraiz,C.,Alzueta,M.J.,Shelf life of beef from local Spanish cattle breeds stored under modified atmosphere. Meat sci.2001;57:273-281

- ICMSF, International Commission on Microbiological Specification of Foods. 1998. Microorganisms in foods: Microbial ecology of food commodities. Blackie Academic and Professional, London, England.

- Jay, J. M., Vilai, J.P., Hughes, M.E. 2003. Profile and activity of the bacterial biota of ground beef held from freshness to spoilage at 5-7°C. Int. J. Food Microbiol. 81:105-111

- Jeremiah, L.E. 2001. Packaging alternatives to deliver fresh meats using short- or long-term distribution. Food Res Int. 2001; 34:749–772.

- Kandeepan,G.,Anjaneyulu,A.S.R.,Kondaiah,N.,Mendiratta,S.K.,Rajkuma,R.S.2013.Evaluat ion of quality and shelf life of buffalo meat keema at refrigerated storage. Journal of Food Science and Technology . December 2013, Volume 50, Issue 6, pp 1069–1078

- Kanner, J. (1994). Oxidative processes in meat & meat products: Quality implications. Meat Science, 36(1), 169–189

- Labadie, J., 1999. Consequences of packaging on bacterial growth. Meat is an ecological niche. Meat Sci. 52, 299-305

- Lambert, A.D., Smith, J.P., Dodds, K.L.1991.Shelf life extension and microbiological safety of fresh meat - a review. Food Microbiology, 8 (1991), pp. 267–297

- Lerner, P.T. 2009. Evaluation of hemoglobin and myoglobin in Poultry slaughtered by stunning and kosher slaughter. Folia Vet. 2009; 53:25–27.

- Magnus, P., 1981. Meat Composition. Food Science and Technology, 4th edition. Gohumunary Pub. London. Pp-108-215.

- Maria D'Agata, Claudia Russo, Giovanna Preziuso. 2010. Effect of Islamic ritual slaughter on beef quality. Italian Journal of Animal Science · January 2010. vol. 8 (Suppl. 2), 489-491,

- Moyer, C.L., Morita, R.Y. (2007) Psychrophiles and psychrotrophs. **In**: Morita RY (ed.) Encyclopedia of Life Sciences. John Wiley & Sons Ltd, Chichester. pp1– 6.

- McMillin, K.W., 2008. Where is MAP Going? A review and future potential of modified atmosphere packaging for meat. Meat. Sci. 80, 43-65

- Mustafa, I.S.M., Abdelgadir, M.A., Sulieman, A.E.2014. Impact of *Halal* and Non-halal Slaughtering on the Microbiological Characteristics of Broiler Chicken Meat and Sausages. Food and Public Health 2014, 4(5): 223-228

- Nadia, M., Bastide, F. H., Pierre, F., Denis, E.C.2011.Heme Iron from Meat and Risk of Colorectal Cancer: A Meta-analysis and a Review of the Mechanisms Involved. Cancer prevention research. 4(2).2011.

- Nychas, G. E., Skandamis, P.N., Tassou, C.C., and Koutsoumanis, K.P. 2008. Meat spoilage during distribution. Meat Sci. 78:77-89.

- Normanno, G., Corrente, M., Salandra, G., Dambrosio, A., Parisi, A., Greco, G., Bellacicco, A.L., Celano,G.V.2007. Methicillin-resistant **Staphylococcus aureus** (MRSA) in foods of animal origin product in Italy of food .International journal of food microbiology. Volume 117.Issue 2, 30 June, pages 219-222

- Nakyinsige K, Fatimah AB, Aghwan ZA, Zulkifli I, Goh YM, Sazili AQ. 2014. Bleeding Efficiency and Meat Oxidative Stability and Microbiological Quality of New Zealand White Rabbits Subjected to Halal Slaughter without Stunning and Gas Stun-killing. Asian-Australas J Anim Sci.; 27 (3):406-13.

- Naveena, B. M. and Mendiratta, S.K. 2001.Tenderization of spent hen meat using ginger extract. British Poultry Science, 42, 344–350.

- Ray, B., Kalchayanand, N. and Field, R.A. (1992) Meat spoilage bacteria: are we prepared to control them? National Provisioner. 206, 22.

- Raharjo, S., Sofos, J. N.1993.Methodology for measuring malonaldehyde as a product of lipid peroxidation in muscle tissues: a review. Meat Sci. 1993, 35, 145-169.

- Riette L.J .M. van Laack, M. Solomon, B. 1994.Biochemistry of Lean Muscle Tissue as Related to Water-Holding Capacity. Reciprocal Meat Conference Proceedings, Volume 47, 1994.

- Singh, R. K., & Singh, N., 2005. Quality of packaged foods. In J. H. Han (Ed.), Innovations in food packaging (pp. 24– 44). Amsterdam: Elsevier Academic Press.1-39.

- Samelis, J. (2006). Managing microbial spoilage in meat industry. In Food spoilage microorganisms, pp. 213-286. Edited by C. de W. Blackburn, Cambridge: Woodhead Publishing Limited.

- Schillinger, U., Holzapfel, W. H. (2006). Lactic acid bacteria. In Food spoilage microorganisms, pp. 213-286. Edited by C. de W. Blackburn, Cambridge: Woodhead Publishing Limited

- Stanbridge, L.H., Davies, A. R. 1998. The microbiology of chill stored meat. The microbiology of Meat and Poultry, Blackie Academic and Professional, London (1998),pp.174-219

- Schmedes, A., Holmer, G. A. new thiobarbituric acid (TBA) method for determination of free malonaldehyde(MDA) and hydroperoxides selectivity as a measure of lipid peroxidation. Journal of American Oil Chemistry Society.1989; 66:813–817.

- Strappini, A. C., Metz, J. H. M., Gallo, C. B., & Kemp, B. (2009). Origin and assessment of bruises in beef cattle at slaughter. Animal, 3(05), 728–736.

- Stanley, D. W., 1991. Biological membrane deterioration and associated quality losses in

- Skibsted, L. H., Bertelsen, G., & Qvist, S., 1994. Quality changes during storage of meat and slightly preserved meat products. In Proceedings 40th international congress of meat science and technology (S-II.MP1 pp. 1–10). 28 August-2 September 1994, The Hague, Netherlands.

- Ternstrom, A., Lindberg, A.M. and Molin, G., 1993.Classification of the spoilage flora of raw and pasteurized bovine milk with special reference to Pseudomonas and Bacillus. J Appl Bacteriol 75, 25-34.

- Urbain, W. M, and J .F. Campbell. 1987. Meat Preservation. In the Science of Meat and Meat Products. 3rd Edn. (J.F. Price, and B.S. Schweigert, Eds) pp. 371-412. Food and Nutrition Press, Inc. Westport, CN.

- Van Laack, R.L.J.M., and Solomon, M.B. 1994. Biochemistry of lean muscle tissue as related to water-holding capacity. Reciprocal Meat Conf. Proc. 47: 91-97.

- Warriss, P. W. 2001 Postmortem changes in muscles and its convection into meat. Meat science, an introductory text, pp. 100 – 161. CABI Publishing, Wallingford, UK

- Xiong, Y. L., & Decker, E. A., 1995. Alterations in muscle protein functionality by oxidative and antioxidative processes. Journal of Muscle Foods, 6, 139–160.

- Young, L. L., R.D. Reviere, and A.B. Cole. 1988. Fresh red meats: A place to apply modified atmospheres. Food Technol. 42(9):65.

ABSTRACT

SYNERGISTIC EFFECT OF NATURAL ANTIMICROBIALS PRODUCED FROM FOOD ON FOODBORNE PATHOGENS AND EFFECT OF MEAT PRODUCTION METHOD ON QUALITY AND SHELF LIFE OF MEAT

by

MAHDI HASSAN HUSSAIN AL-ALI

December 2017

Advisor: Dr. Ahmad R. Heydari

Major: Nutrition and Food Science

Degree: Doctor of Philosophy

Meat is highly perishable food since it contains sufficient nutrients needed to support the growth of microorganisms which is effect of nutritional value and spoilage of meat. There are many methods are available to control food pathogens in meat. Using natural antimicrobial may be is a good option because it is provide many benefits. This study showed the synergistic effect of individual and combinations of lactoferrin, lysozyme and nisin *in-vitro* and *in-vivo* (in meat) had significant effect ($P < 0.05$) on reducing the number of foodborne pathogens in meat. Toxigenic *Staphylococcus aureus* contamination in meat is a major problem in public health due to the production of heat-stable enterotoxins. Also, *S.aureus* expresses an many array of virulence factors contribute to the ability of *S. aureus* to cause infection, our results showed that high percentage (42.9) of *S. aureus* in meat and 80.9 % of *S. aureus* isolates contained at least one enterotoxin gene. The three most common enterotoxin genes in meat were sea gene (61.9%) followed by seh gene (33.3%) and sei gene (30.1). Some of *S. aureus* isolates contained more than one enterotoxin gene. In addition, our results indicated that *S. aureus* isolates from meat showed different antimicrobial resistance levels against fourteen antibiotics. Also, *S. aureus* multidrug

resistant were high in meat. There are different types of meat based on the meat production method. Halal meat is produced without stunning prior to slaughter, resulting in a rapid and complete bleeding of animals. Our results showed that halal meat production method resulted in significantly lower residual heme iron than conventional method with stunning (non-halal method). Microbiological analyses of fresh meat samples showed that halal meat production method had significantly lower ($P < 0.05$) microbial count than non-halal meat method. Storage of meat is critical because during storage even with proper refrigeration, the meat ultimately undergoes deteriorative changes including microbial spoilage, oxidative changes. Our results indicated that meat production method significantly affects the meat quality not only for fresh meat but also during storage. Microbiological analyses of meat samples during storage for three weeks at 4°C showed that halal meat method had significantly lower ($P < 0.05$) microbial count than non-halal meat. Physiochemical analyses showed that quality indicators for meat such as lipid oxidation, drip loss, pH, and heme iron content were significantly lower ($P < 0.05$) in halal meat than non-halal meat during storage. Halal meat processing may be advantageous in reducing bacterial colonization, increasing the usable shelf life, and improving the quality of the fresh and stored meat.

AUTOBIOGRAPHICAL STATEMENT

Mahdi Al-Ali

I received my Bachelor's degree in Food Technology in 1985 from Baghdad University, Iraq. My rank was the first student in the department and the fifth student in college. I earned my Master's degree in Food Science and Biotechnology in 1989 at Baghdad University. I started working in Babylon University, as a lecturer. I taught many undergraduate courses for seven years then I transferred to Al-qassim green university, as lecturer, where I still faculty member till now. In 2012 I joined the PhD program in Dr. Ahmad Heydari's lab, Department of Nutrition and Food Science at Wayne State University. I published many publications and presentations. Recently I participated in the following graduate research symposiums

1- Graduate and postdoctoral research symposium, Wayne state University, March 8,2017

2- Midwest graduate research symposium, Toledo University, March 25,2017