Impact of carcass chill time on the microbiology of horse meat

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

FOOD SCIENCE AND TECHNOLOGY

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Abstract

Globally, Canada is the third largest producer of horse meat, having an annual production of approximately 700,000 tonnes. Canadian regulatory standards require carcasses harvested for meat to have the warmest part of the carcass cooled to 7°C before meat can be harvested. Other processes can be approved if scientific evidence of the safety of the meat is provided. This research evaluated the microbiological condition of horse meat harvested at an internal temperature of 13°C. Temperature profiles of horse carcasses were created to determine the chill times required to reach internal temperatures of 13°C, 8°C and <7°C, with 17, 26, and 30 h, respectively, to be optimal chill times for operational purposes in the facility. The process hygiene of the abattoir was comparable to what is found in federally regulated beef plants with counts of 3.25 log CFU/1000 cm^2 total aerobic bacteria and 0.54 log CFU/1000 cm^2 *Enterobacteriaceae*. *Semimembranosus* muscles harvested from horse carcasses after 17 (13°C), 26 (8°C) or 30 h (<7°C) of chilling at 2°C had no significant difference in bacteria counts among chill times with approximately 4.5 log CFU/1000 cm^2 of total aerobic bacteria, 2.2 log CFU/1000 cm^2 of lactic acid bacteria, and *Enterobacteriaceae* were below the detection limit. Chill time had no effect on the total aerobic bacteria or lactic acid bacteria during the first 60 d of storage; however, after 90 d of storage, steaks from *semimembranosus* muscles harvested after 17 h of chilling had lower bacteria counts than those harvested at 30 h. *Enterobacteriaceae* counts on steaks were below the detection limit on most steaks. Metagenomic analysis of the microbial DNA from steaks stored for 90 d revealed an abundance of *Pseudomonas* and *Serratia.* Analysis of culturable bacteria from plate count agar similarly determined that *Pseudomonas* and *Serratia* were present in high abundance, whereas the culturable microbiota obtained from the all-purpose tween agar had a high abundance of *Enterobacteriaceae*. All data support the

hypothesis that horse meat can be harvested at 13°C with no negative microbiological hygiene or safety issues when compared to horse meat harvested at <7°C.

Preface

This thesis on the microbiology of horse meat is an original work by Brian Walker. Dr. Lynn McMullen, Dr. Heather Bruce, and M.M. Rahman contributed to the experimental design and M.M. Rahman assisted with execution of the experiments.

Dedication

To my wife,

Thank you for your patience, love, and support.

Acknowledgements

I would like to thank the funding sources: Alberta Livestock and Meat Association (ALMA), NSERC Create – Meat Education and Training Network, and the federal abattoir whose funding made it possible for me to pursue a Master of Science, study meat science and microbiology, develop skills necessary valued by the meat industry, and make a meaningful contribution the Canadian economy with my research. Thank you to the federal abattoir for supporting me in this research project, allowing me to run free in your plant and provide the best personnel you have available when I needed them.

I am especially grateful for all the lab mates on main campus and at Agri-Food Discovery Place who provided me with guidance, assistance, training, and moral support throughout my studies. A special thanks to Danielle for patiently training me in the lab as a hopeless new student, encouraging me to try again on weekends when everything had gone wrong, and laughing about our experience with racial segregation. Thank you to Mahbubur and Bimol who helped with sample processing, and Mahbubur for being there with me through months of sampling.

I would like to thank Dr. Bruce for teaching me meat science, helping me throughout this experiment, catching a few serious errors that I missed, demonstrating to me how to create an experimental design by creating the experimental design for this experiment, and most importantly always being supportive and encouraging throughout the whole experiment.

I am forever in the debt of Dr. McMullen who took a chance on me despite me having minimal lab experience. She is one of the best teachers and mentors I have ever had the pleasure to train under. Over my years of study, no matter how busy she was, she always found the time to support me when my plans would fall apart. When teaching, she has a special ability to blend

logos with pathos; uniformly creating an understanding of the scientific principles and an emotional connection to why the principles matter. Having Dr. McMullen as a mentor has without question, been the best part of my post-secondary education.

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List of Abbreviations

ATP – Adenosine Triphosphate

APT – All-Purpose Tween

CFIA – Canadian Food Inspection Program

CFU – Colony Forming Unit

CVMP – Committee for Medicinal Products for Veterinary Use

EID – Equine Identification Document

E. coli – *Escherichia coli*

FAO – Food and Agriculture Organization of The United Nations

HACCP – Hazard Assessment Critical Control Points

MAP – Modified Atmosphere Packaging

OTU – Operational Taxonomic Unit

PCA – Plate Count Agar

RDP – Ribosomal Database Project

Spp. – Species

TFI – Temperature Function Integration

VRBGA – Violet Red Bile Glucose Agar

Chapter 1: Introduction and Literature Review

1.1 Global horse meat value-chain

Analysis of the global horse meat market was completed by Belaunzaran et al. (2015) using data from the Food and Agriculture Organization of the United Nations (FAO). The FAO has not released new data since that analysis was done. Belaunzaran et al. (2015) found that equine meat production accounted for less than 0.25% of the total global meat production with an overall production of 700,000 tonnes per year of horse meat. When viewed by continent Asia, America, and Europe produced 46%, 30%, and 18% of global production of equine meat, respectively; but China, Kazkastan and Mexico are the biggest producers (Belaunzaran et al., 2015). Asia, as the largest producer, has almost no export or imports, while North and South America export the majority of equine meat to Europe and Japan (Belaunzaran et al., 2015; Gill, 2005).

Canada was the third largest horse meat exporter in 2013, falling behind Belgium and Argentina and closely matched to Mexico (Figure 1). Statistics Canada reported that Canada exported an average of $80 million (12, 744 tonnes) of fresh, chilled, or frozen equine meat each year from 2013 through 2016 (Statistics Canada, n.d.). Exports were below average in 2016 with only $76.5 million (10,347 tonnes) of equine meat sold; however, the price per kg has been on a steady rise from $5.6/kg in 2013 to $7.0/kg in 2016. Horse meat production in Canada steadily decreased after 2013 when 14,488 tonnes of meat were produced. In 2016, only 10,347 tonnes were produced, which is a 29% decrease from 2013 (Statistics Canada, n.d.). No horse meat is imported into Canada (Food and Agriculture Organization of the United Nations., n.d.) but horse meat is eaten in the province of Quebec.

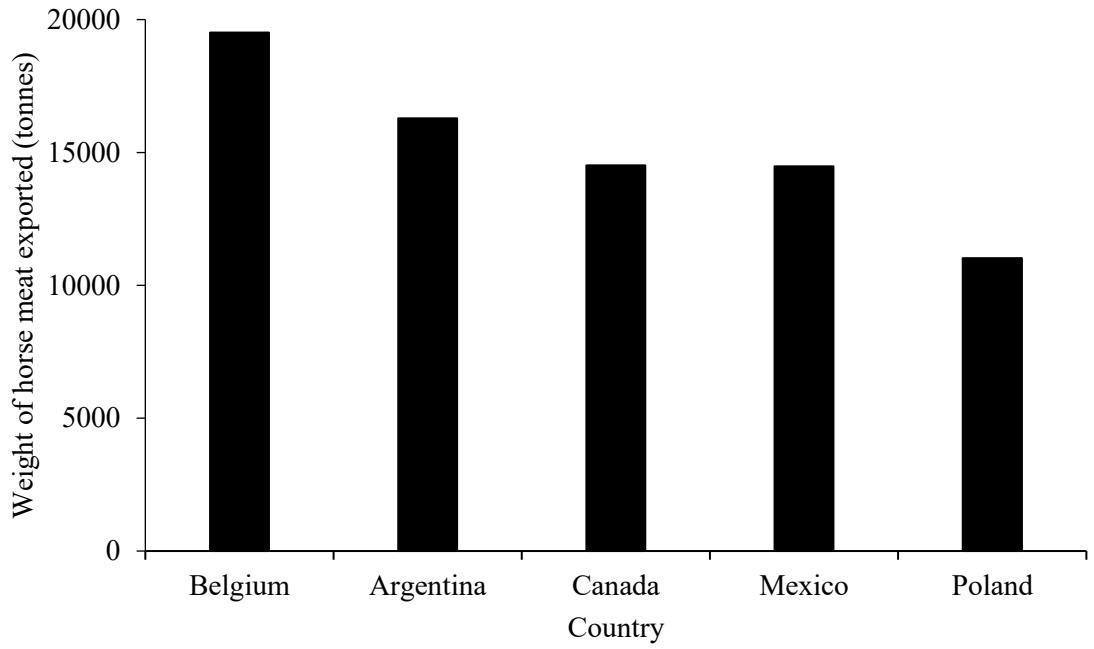

Figure 1: Quantity of horse meat exported in 2013 from the top global exporters (Food and Agriculture Organization of the United Nations., n.d.).

In Canada, abattoirs that export products out of the country require federal inspection and licensing. Currently four abattoirs are licensed to export horse meat: Bouvry Exports Calgary Ltd and Canadian Premium Meats in Alberta and Les Viandes De La Petite-Nation Inc. and Viande Richelieu Inc./Richelieu Meat Inc in Quebec (Canadian Food Inspection Agency, n.d.). Combined, the four plants employ approximately 500 people (Canadian Meat Council, 2013). In 2016, these plants slaughtered a total of 53,673 horses, which is below the 10 year average of 80,941 (Agriculture and Agri-Food Canada, n.d.) . The majority (85%) of the horse meat produced in these plants is exported with the majority of the remaining horse meat sold in

Quebec (Canadian Meat Council, 2013). Some smaller provincially regulated plants also slaughter horses but only sell their meat within the province.

In 2016, Canada exported horse meat to 12 countries. Japan, France, Belgium, Switzerland, and United States accounting for 35.9, 22.4, 15.9, 10.2 and 10.6% of the entire market, respectively (Statistics Canada, n.d.). Prior to 2016, Kazakhstan was one of the main countries for export of Canadian horse meat. Export to France, Belgium, and Switzerland from 2013 to 2016 was stable but Belgium and Switzerland decreased purchase of Canadian horse meat in 2016 (Figure 2). In 2016 the United States imported 1092 tonnes of horse meat from Canada.

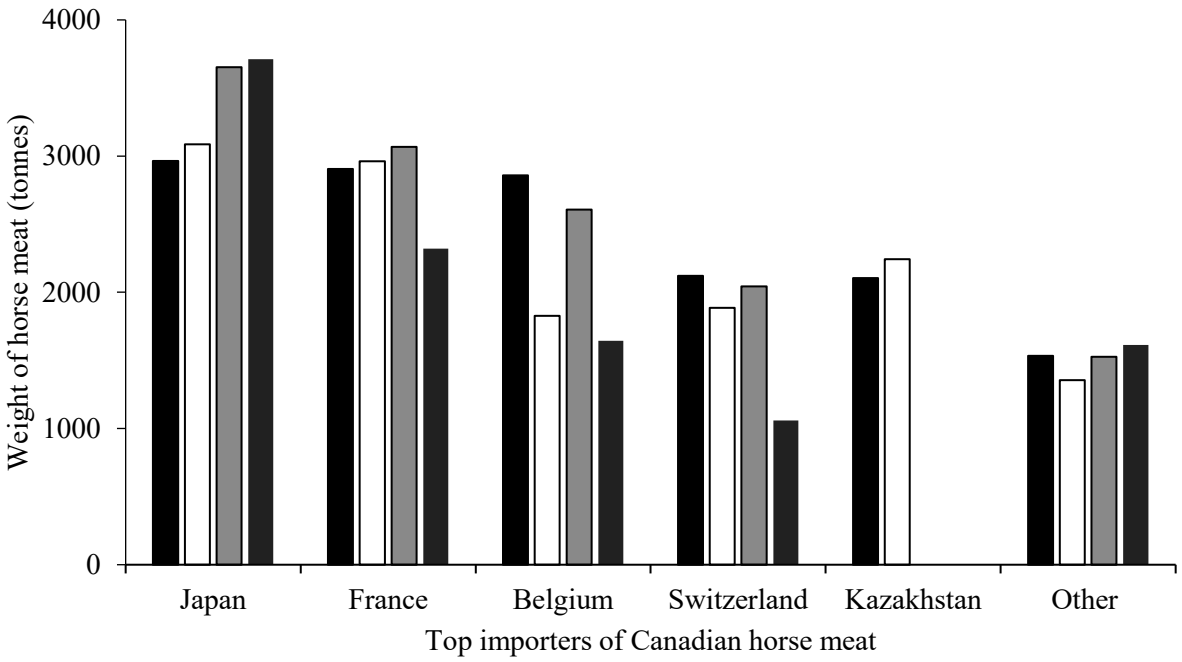

Figure 2: Quantity (tonnes) of Canadian horse meat exported to the largest trading partners in 2013 (■), 2014 (□), 2015 (▩), and 2016 (■) (Statistics Canada, n.d.).

On average, Alberta exported 8,786 tonnes of horse meat from 2006-2013 with the top 5 importing countries being Japan, France, Switzerland, Belgium, and Kazakhstan (Government of Alberta, 2017). Approximately 30% of horse meat exported from Alberta is sent to Japan, 20% to France, and 10% to Switzerland. Prior to 2015 Alberta sent 10-20% of its horse meat to Kazakhstan but as of 2015, Kazakhstan imported no horse meat from Alberta or Canada (Figure 3). The two abattoirs in Alberta primarily export to Japan, France and Switzerland, where as the Quebec abattoirs export to Belgium, Switzerland, and France.

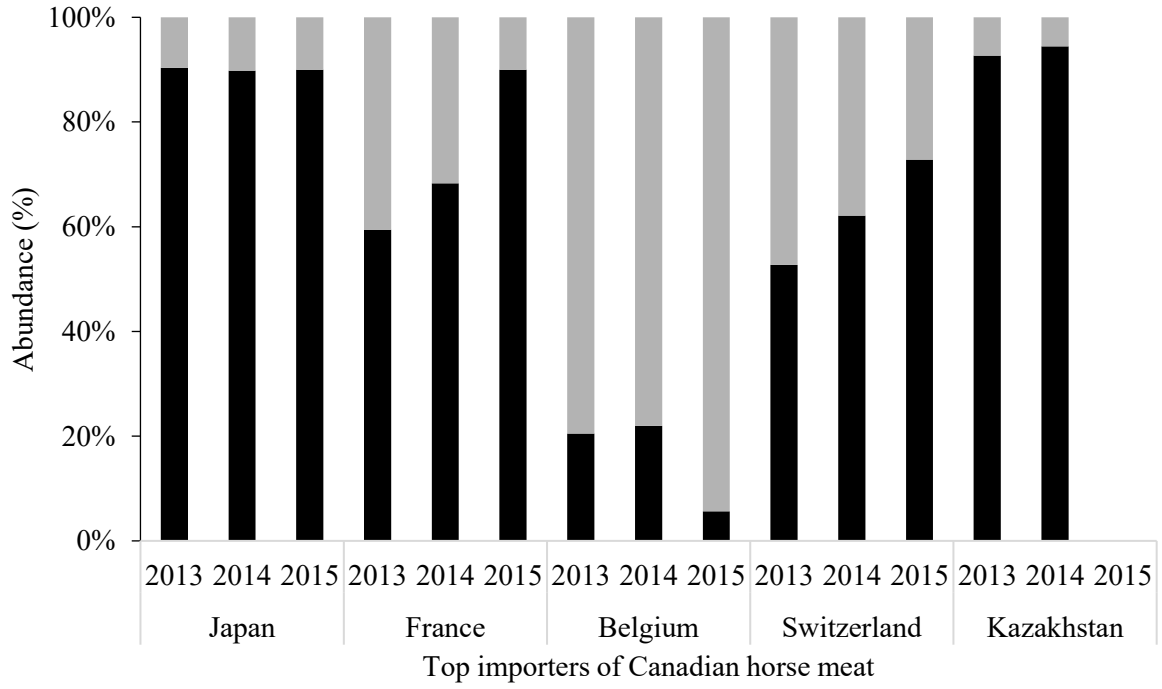

Figure 3: Canadian export of horse meat to top 5 countries over 3 years in Alberta (■) and Quebec (□). Quebec values were calculated from total exports subtracted by Alberta exports (Government of Alberta, 2017; Statistics Canada, n.d.).

Japan enjoys horse meat as horse sushi called basashi, in soups, and fried. Horse meat can blacken within 5 days, and be considered spoiled by the consumer. The meat destined for Japan is often hot boned to ensure a fresher product reaches the market. Japan produces 40-50% of their own horse meat and imports 50-60% from Canada (Table 1) but this does not account for live animals which are imported for slaughter.

Table 1: Production, import and export of horse meat in Japan from 2011 to 2013 in tonnes.

	Amount produced, imported or exported (tonnes) per year		
	2011	2012	2013
Produced	3367	3745	3745[a]
Imported	4512	4436	4438
Exported	0	0	0
Total	7879	8181	8183

[a] an estimation from FAO. (Food and Agriculture Organization of the United Nations., n.d.)

Horse milk and meat consumption is traditional in Kazakhstan where not utilizing the meat from horses is disrespectful to the animal. Kazakhstan was a major importer until 2015 when they stopped importing horse meat. Kazakhstan imported 95% of their horse meat from 2011 to 2013 (Table 2). In 2013 Canada sent 2105 tonnes of horse meat to Kazakhstan which accounted for

44% of their imports and 2.2% of their total accumulation (Food and Agriculture Organization of the United Nations., n.d.; Statistics Canada, n.d.). Prior to 2015 Kazakhstan hired Canadian professionals and bought breeding horses to start their own horse meat market, causing a dramatic decrease in exports of Canadian horse meat to Kazakhstan.

Table 2: Production import and export of horse meat in Kazakstan from 2011 to 2013 in tonnes.

	Amount produced, imported or exported (tonnes) per year		
	2011	2012	2013
Produced	75456	84994	89233
Imported	3680	4848	4758
Exported	0[a]	0[a]	1
Total	79136	89842	93990

[a] estimate by the FAO. (Food and Agriculture Organization of the United Nations., n.d.)

1.2 Safety of horse meat

Horse meat has chemical and microbiological hazards with which that the industry needs to contend. The main chemical hazard of concern is phenylbutazone, a nonsteroidal anti-inflammatory drug used to treat pain, fever, and rheumatoid arthritis in horses (European Food

Safety Authority European Medicine Agency, 2013). The Canadian Food Inspection Agency (CFIA) has a zero tolerance policy for its use in food-producing animals (Canadian Food Inspection Agency, n.d.). Many of the horses slaughtered in Canada for human consumption were not initially raised for food, and concerns arise that these animals have been treated with phenylbutazone during their lifetime. This is even more likely with horses from the racing industry where phenylbutazone can be used to enhance racing performance. The Committee for Medicinal Products for Veterinary Use (CVMP) has not established an acceptable daily intake of phenylbutazone from food sources for humans as there was a lack of research on the side effects from regular low dose consumption. As a preventive measure, the CVMP has banned the use of phenylbutazone in animals produced for human consumption. Phenylbutazone has the same side effects as nonsteroidal anti-inflammatory drugs: perforation, ulceration, and bleeding of the upper gastrointestinal tract, inhibited blood clotting, and blood disorders including aplastic, leukopenia, and agranulocytosis. Deaths from these side effects have been associated with pharmaceutical usage of phenylbutazone (Lees and Toutain, 2013). Lees & Toutain (2013) estimated that concentration of phenylbutazone from a therapeutic dose in horse after 24 h is below parts per billion in the muscle and rapidly decreases. If the maximum amount of phenylbutazone in horse meat harvested within 24 h of dosing a horse is considered, a frequent consumer of horse meat would not consume the daily therapeutic dose of phenylbutazone, although this does not account for highly sensitive individuals who might react to small dosing (Lees and Toutain, 2013).

CFIA requires that every horse slaughtered in a federal plant has an Equine Information Document (EID), and a record of all vaccinations and medications for the previous 6 months. A new policy by the European Union issued in 2017 requires horses destined for meat slaughter to

have a 6-month residency in the country of slaughter. CFIA will not issue export permits unless this requirement is met (Canadian Food Inspection Agency, n.d.). Since 2002 CFIA tests for phenylbutazone using the United Nations Food and Agriculture Organization and the World Health Organizations scientific standards for testing of phenylbutazone to keep meat safe (Canadian Food Inspection Agency, n.d.). Importing countries also test for phenylbutazone content in the meat.

The horse meat industry has two microbiological hazards of most concern - *Salmonella* spp. and *Trichinella* parasites. Parasites are out of the scope of this thesis; however, it is pertinent to mention that many outbreaks of trichinellosis have been associated with the consumption of fresh horse meat that was undercooked or raw (Ancelle et al., 1998; Boireau et al., 2000; Liciardi et al., 2009; Murrell et al., 2004).

Contamination of carcasses with mesophilic bacterial pathogens typically come from contaminated hides prior to slaughter or leakage of intestines during the slaughter and dressing process (Blagojevic et al., 2012, 2011; Madden et al., 2004). Contamination is difficult to control as many factors influence the risk of an animal or carcasses being contaminated. These include the weather, slaughter house throughput, and on-line monitoring of fecal contamination (Barco et al., 2015). Many slaughter facilities use interventions to decrease the amount of bacterial contamination on a carcass, including spray-cooling, steam pasteurization, hot water wash, and organic acid sprays; however, the efficacy of interventions varies among studies (Barco et al., 2015). The abattoir used in this study only subjected carcasses to a cold-water wash, which in studies of beef carcasses has been shown to distribute bacteria over the entire carcass instead of reducing the quantity of viable contamination on beef carcasses (Barboza de Martinez et al., 2002; Madden et al., 2004).

The mesophilic pathogens in meat are killed when meat is properly cooked, thus reducing the risk of foodborne illness. In Japan horse meat is often consumed raw; however, the number of reported illnesses from bacterial pathogens on horse meat is limited. Horses are recognized as carriers of *Salmonella* spp. (Alberghini et al., 2014). Salmonellosis occurs most commonly from the consumption of contaminated foods and under cooked poultry, and raw eggs are often linked to cases of salmonellosis; however, any food that has been contaminated with *Salmonella* can cause salmonellosis. Salmonellosis can also occur from exposure to infected individuals or animals, both of which could be asystematic carriers, showing no symptoms of illness (Government of Canada, n.d.). Data on the presence of *Salmonella* on slaughter horses is limited. Alberghini et al. (2014) sampled the mucosa and intestinal contents of 350 horses at an Italian abattoir finding *Salmonella* spp. in only 5 samples. Other studies have also found *Salmonella* in horses (Anderson and Lee, 1976; Hofer et al., 2000; Lyytikäinen et al., 2000). Yin et al. (2016) sampled 145 horse meats at market places in China and found *Salmonella* on 2% of the horse meat. Other researchers have also found *Salmonella* on horse meat (Anderson and Lee, 1976). An outbreak of salmonellosis was reported in France in 2003 where 14 individuals were infected with *Salmonella* Newport and the source of the contamination was traced to imported horse meat with unknown country of origin and the meat had been eaten either raw, as ground meat, or as a steak (Espié et al., 2005).

Horse meat does not appear to be a primary source of pathogenic *Escherichia coli* as no reports of foodborne illness from *E. coli* in association with consumption of horse meat are available in the literature. Gill and Landers (2005) evaluated bacterial contamination on horse carcasses and meat at a North American abattoir and found numbers of coliforms and *Escherichia coli* below their detection limit of 1 CFU/100 cm^2, while counts of *Enterobacteriaceae* on Spanish horse

carcasses were 2 log CFU/g (Gómez and Lorenzo, 2012). As all of the abattoirs in Canada that slaughter horse meat also slaughter other red meat animals including bison, elk, and beef, the possibility exists that horse meat could become contaminated with pathogenic *Escherichia coli* from these other animal species (Canadian Food Inspection Agency, n.d.).

The risk of consumers being infected with *Salmonella* spp. or pathogenic *Escherichia coli* is reduced by use of a Hazard Analysis and Critical Control Points (HACCP) program that establishes monitoring criteria and corrective actions for potential hazards in meat. The process control in an abattoir is monitored following a consistent sampling schedule evaluated by statistical analysis. The previous performance of an abattoir is used as an indicator of a hygienic process. In Canada, the process monitoring program provides guidelines on sampling that allows each federally regulated abattoir to develop a standard for their individual plant, equipment, and program. This system monitors changes in chilling regimes and is based on counts of *E. coli* on carcasses. Although total aerobic counts are often used to evaluate plant hygiene, Gill et al. (1996) found a poor correlation between total aerobic counts and counts of *E. coli* indicating that monitoring should be done on *E. coli* or coliforms for HACCP programs.

European Union regulators require processors to monitor the microbiological condition of carcasses after dressing but prior to chilling for indictor bacteria, total aerobic bacteria and *Enterobacteriaceae* to assess the hygiene of the slaughter processes and presence of fecal contamination on the carcass (Andreoletti et al., 2012; European Commission, 2005). These regulatory requirements help to keep horse meat safe for consumption.

1.3 Meat Spoilage

Meat spoilage is a change in the organoleptic characteristics of fresh meat to a condition that is not desirable for human consumption, typically involving discoloration, odors, slime, or a decreased pH and acidic flavor, all of which can be undesirable to consumers. The spoilage conditions are often caused by bacteria growth on the meat surface though lipid oxidation and autolytic enzyme activity can both contribute to meat spoilage (Nychas et al., 2008). The meat spoilage characteristics are strongly affected by antemortem treatment of animals, process hygiene during meat harvest and packaging, storage conditions, and packaging systems (Nychas et al., 2008).

Muscles under the hide are considered to be sterile, but during the harvest the meat is contaminated from the environment in which it is harvested. In abattoirs, this includes the air, water, meat contact surfaces, personnel and the equipment they use. Good manufacturing processes, prerequisite programs, HACCP plans, and antimicrobial interventions all work together to limit the amount of bacterial contamination in the final product. The number of bacteria are decreased to reduce the risk of the presence of pathogenic bacteria, and to maintain product quality and shelf life as many spoilage conditions result from bacterial activity.

Consumers often use the color of meat as a determinant of spoilage. Meat color comes primarily from the pigment myoglobin. Myoglobin can exist in many states and the state of the iron moiety in myoglobin determines meat colour. Deoxymyoglobin has a purple color to the meat, upon exposure to oxygen the meat ‘blooms’ converting into oxymyoglobin, which has a bright red color that many consumers associate with a quality and fresh meat. With continual exposure to oxygen, the oxymyoglobin will oxidize to metmyglobin, a brown-grey variant of the myoglobin

molecule that many consumers view as non-fresh meat. Myoglobin can also develop a green color when it reacts with H_2O_2 or H_2S produced from bacteria. Odors and slime are produced on the surface of meat by some spoilage bacteria leading to meat spoilage. Some lactic acid bacteria produce acids that lower the pH of the meat resulting in a sour or acidic flavor that can be considered to be spoilage. When meat is stored aerobically and aerobic spoilage bacteria reach or exceed cell counts of 10^6 log CFU/cm^2, meat is typically considered spoiled (Jones, 2004; Nychas et al., 2008).

Bacterial spoilage of meat can be largely controlled by altering the storage temperature or atmosphere of the meat surface. Lower temperatures will select for the growth of psychotropic bacteria, vacuum packaging selects for the growth of anaerobic and facultative anaerobic bacteria, greatly limiting the variety of bacteria growing on the meat (Nychas et al., 2008). Vacuum packaging is very effective at reducing spoilage of meat. The growth of aerobic bacteria, *Enterobacteriaceae* and *Pseudomonas* spp. can be reduced on horse meat over 14 days of storage at 2°C with the use of vacuum packaging (Gómez and Lorenzo, 2012; Lorenzo and Gómez, 2012). Similarly, when horse meat is stored under vacuum at 2°C the red color, off-odor, and appearance all remain acceptable when evaluated by semi-trained (Lorenzo and Gómez, 2012). In contrast, horse meat stored in packages made with film with a high oxygen transmission rate, in packages with a high O_2 modified atmosphere, or in packages with a very low concentration of O_2, became unacceptable to panelists after 10 d of storage (Gómez and Lorenzo, 2012). The use of vacuum package may improve the safety of the meat as *Enterobacteriaceae* concentrations decrease when beef is stored for 70 days (Youssef et al., 2014).

The control of microbial parameters to improve the storage life and decrease spoilage of horse meat is paramount to the industry. Spoilage of meat has an economic impact on the industry and any strategies that impact the growth of spoilage bacteria should be used to minimize this impact.

1.4 Effect of carcass chilling on meat quality and safety

During the first 24 h post-mortem, structural changes along with biochemical processes affect the quality of the meat (Andreoletti et al., 2014). If a carcass is chilled too quickly, dropping below 10°C in 10 h, the sarcoplasmic reticulum can leak calcium, which can stimulate permanent contraction of the sarcomere making the meat tough (Damodaran et al., 2007). The meat industry commonly uses electrical stimulation on red meat animals such as beef and lamb to improve meat quality (Damodaran et al., 2007). Cold shortening occurs when a pre-rigor muscle is cooled quickly, likely from a decreased function of calcium pumps in the sarcoplasmic reticulum (Ertbjerg and Puolanne, 2017). Electrical stimulation reduces the impact of cold shortening on susceptible muscles fibers by exhausting ATP reserves before muscles cool to the temperature at which calcium pumps loose function. Red muscle fibers have an increase susceptibility of red muscle fibers to cold shortening due to their primarily aerobic metabolism, as they have high amounts of mitochondria which is thought to contribute calcium to the sarcoplasmic reticulum, and their calcium receptors in sarcoplasmic reticulum are less able to reabsorb calcium, unlike white muscle fibers (Buege and Marsh, 1975; Damodaran et al., 2007; Ertbjerg and Puolanne, 2017).

The effect of carcass chilling on microbial load is controversial; however, it is commonly used as a control point in HACCP systems as a means to limit the growth of mesophilic pathogenic bacteria. Cooling caresses with only cold air dries the carcasses surface reducing the number of

indicator organisms (Liu et al., 2016). In an attempt to classify the hygienic performance of carcass cooling processes, Gill and Bryant (1997) evaluated the carcass cooling process at two abattoirs and found that cooling processes generally reduce total aerobic, coliform, and *E. coli* counts. However, calculations demonstrated that an average proliferation of *E. coli* during chilling would be 3.2 and 1.1 generations at each of the facilities. When evaluating the effectiveness of various interventions in beef processing plants, Bacon et al. (2000) found that after chilling, total aerobic, coliform, and *E. coli* counts were reduced. A study of the effect of chilling on total aerobic bacteria and *Enterobacteriaceae* present on sheep carcasses from 5 Irish abattoirs found that total aerobic bacteria and *Enterobacteriaceae* counts increased on 58% and 26% of carcasses, respectively, and decreased or remained the same on the rest of the carcasses (Lenahan et al., 2010). This variation in bacterial growth could result from variables, other than time that are not measured, particularly any variable that is known to give *E. coli* an advantage. This includes nutrient availability, presence and abundance of competitive and symbiotic organisms, and water activity. *E. coli* grows slow at low water activities, which may impact its growth on carcasses where spray chilling is not used and the carcass surface dries reducing the potential growth (Gill et al., 1991).

The risk of microbial growth is present when meat is harvested a higher temperature. Appropriate preventative measures proper packaging and rapid cooling of the meat after harvesting can minimize the risk of microbial growth, especially that of mesophilic bacterial pathogens.

1.5 Regulatory requirements for carcass chilling

The regulation for chilling of carcasses states, “Before the product is cut, the internal temperature (warmest part) of the carcass is 7°C or less” (Canadian Food Inspection Agency, n.d.). The scientific justification for this regulation is based on the risk of contamination of meat with mesophilic pathogens. In an intact carcass from a healthy animal, muscle tissue is not exposed to the environment and is considered sterile. However, each cut made into a carcass or piece of meat increases the risk of contamination with mesophilic bacterial pathogens including *Salmonella* and *Escherichia coli*. These bacteria grow slowly or not at all when temperatures are below 7°C; therefore, if the warmest part of the carcass is at or below 7°C at the time of cutting and contamination with mesophilic pathogenic bacteria occurs, those pathogens will not be able to grow (Gill et al., 1991; Morey and Singh, 2012) . The primary concern with boning meat at a higher internal temperature is that the mesophilic pathogens will be able to grow to numbers that can cause foodborne illness. Gill et al. (1991) developed a method to empirically determine the growth of *E. coli* on meat during cooling using the temperature history of the meat and the growth characteristics of pathogens of concern. The Temperature Function Integration method (TFI) is a calculation of bacterial proliferation based on temperature history of a carcass side in a cooler that has been used to establish carcass chill and boning temperature requirements on beef (Gill et al., 1991). Temperature histories measured from the aitch-bone pocket are used to estimate the amount of growth of bacterial pathogens that can occur during carcass cooling (Gill et al., 1991).

The regulations are set in conjunction with trading partners standards to assist meat producers in meeting the regulations of the trading partners. The European Union requires the core temperature of the meat to be below 7°C; however, the European Food Safety Authority recently

allowed meat producers to use custom cooling regimes, providing more flexibility for producers (Andreoletti et al., 2014; European Commission, 2004).

1.6 Measuring microbial populations on meat

Traditional methods for evaluation of the hygiene of meat have relied on the ability to culture microorganisms from either swabs or tissues that are excised from the surface of a carcass or a muscle. The hygienic process in the abattoir and how meat has been packaged and stored strongly influences the quantity and identity of the culturable microbiota on the meat or carcass (Gill and Bryant, 1997). To determine if meat is safe for consumption, the presence of pathogenic organisms must be determined. This can be concluded with indicator organisms such as coliforms or *Enterobacteriaceae* (Gill et al., 1996) but culturing these groups of organisms does not identify specific pathogenic species that may be present, providing an indicate as to the presence of these organisms. To culture specific pathogens, the laboratory work required is extensive often and often specific pathogens are not present in sufficient numbers to enumerate (Zhao et al., 2014).

With the advent of molecular technologies, it is possible to identify bacterial genera present on meat without culturing. Rapid Amplification of Polymorphic DNA (RAPD) is one such technology that allows rapid evaluation of the relatedness of pathogen contamination from various locations or sources within a processing plant. Aslam et al. (2004) collected samples from carcass hides, dressed carcasses, carcasses leaving the cooler, meat cuts, and trimmings and these samples were analyzed using RAPD method to determine the main sources of contamination within a beef processing plant. The metagenomic technique using shotgun sequencing has limited application for detection of pathogenic bacteria strains as the resolution

of the technique will only allow identification of genus; however, Yang et al. (2016) used shotgun sequencing to characterize bacteria pathogens through a beef production system. As molecular technologies become more advanced with improved databases, rapid pathogen detection on meat may be possible.

1.7 Objective and hypothesis

The goal of this research is to determine if horse meat can be harvested from carcasses whose internal temperature is above the regulatory standard of 7°C without adversely affecting the microbiological quality and safety of the meat. The hypothesis is that the harvest of horse meat from carcasses prior to the internal temperature reaching 7°C does not negatively impact the microbiological condition of horse meat that is stored for up to 90 days.

Chapter 2: Materials and Methods

The abattoir that participated in the current study harvests meat from 100 to 200 head of horses per day. Depending on the supply of horses, the abattoir also harvests meat from other species including bison, elk, and bovine. Horse carcasses are dressed on a rail system, typically taking 45 min from slaughter to reach the cooler. Carcass sides are manually spaced and sorted on the rail and cooled overnight at 2°C until the warmest part of the carcass reaches < 7°C. Small carcass sides (< 125 kg) can reach this temperature in 16 h where large carcass sides (> 180 kg) can take more than 24 h to reach that temperature. Once chilled, carcasses are quartered and cut into primal cuts, which are vacuum packaged and stored at 4°C until shipping.

2.1 Evaluation of cooling rate of horse carcasses

To determine the times for rapid harvest of horse meat, the rate at which horse carcasses are cooled during normal processing in a federally inspected abattoir was determined. Nine right sides of horse carcasses with weights of 120-130, 148-160, or >179 kg were selected, with three from each weight range. A thermocouple was inserted through the obturartor foramen of the aitch bone and the internal temperature of the carcass side in the center of the hip was monitored. This followed the standard procedure of the processor on measuring internal temperatures of carcasses. TinyTag View 2 Data loggers (Thermistor probe TV-4020 Gemini Data Loggers Ltd., West Sussex, UK) recorded the internal temperature every 30 s for 16 h as the carcasses progressed through the standard chilling process. Carcasses were manually organized and spaced by company employees in the 2°C cooler as needed for future processing. The carcasses were chilled overnight, and boned the next morning. Data were averaged within each weight group and used to determine chill treatments.

2.2 Evaluation of carcass hygiene

The right side of 30 carcasses heavier than 150 kg and free from excessive trimming were selected at random over four weeks. Sani-sticks (LABPLAS, Sainte-Julie, Quebec, Ca.) were used to swab two areas (61 cm anteriorly and 43 cm ventrally) on the rump and on the brisket. The total area swabbed on each carcass was 5922.6 cm^2. Each area was swabbed both vertically and horizontally with medium pressure, switching sponge sides between areas. After swabbing, the sponge was placed in a sterile bag, squeezed to allow absorption of NE Buffer, and packed on ice for transport to the lab (6 h). Upon arrival at the laboratory, sponges were hand massaged for 2 min before plating. Numbers of total aerobic bacteria, *Enterobacteriaceae,* and *Escherichia coli*/coliforms were enumerated using Aerobic count petrifilm (3M Health Care, St. Paul, MN, USA) incubated at 23°C for 72 h, Violet Red Bile glucose agar (VRBGA; Oxoid LTD., Basingstoke, Hampshire, England) incubated at 37°C for 24 h, and *Escherichia coli*/coliform petrifilm (3M Health Care, St. Paul, MN, USA) incubated at 37°C for 24 h, respectively. Prior to pouring the VRBGA, molten media was held at 45°C in a Thermo Scientific Neslab EX7 water bath with Digital Plus control (Thermo Fisher Scientific, Newington, NH, USA). After samples were spread onto the VRBGA, 3-5 mL of agar were poured onto the plates to form an over-lay. The number of all colonies grown on *Escherichia coli*/coliform petrifilm were recorded; blue colonies that produced gas indicated *Escherichia coli*, blue and red colonies that produced gas were considered to be coliforms. Isolated colonies from the *Escherichia coli*/coliform petrifilm and the VRBGA were chosen for identification. Counts were converted to log (CFU/cm^2) and averaged to determine microbial load.

2.3 Effect of chill time on meat hygiene

To determine the impact of carcass chill time on the hygiene of the *semimembranosus* muscle, chill times of 17, 26 or 30 h were chosen based on data collected as part of the carcass cooling rate experiment described above. The selection of 30 h was based on regulatory requirements for large carcasses to reach an internal temperature of 7°C prior to boning. To determine the impact of high internal temperature on meat hygiene, carcasses were chilled to internal temperatures of 8°C (26 h) or 13°C (17 h). A total of 36 carcasses over 179 kg with no trimming on the hind quarters were selected over 4 weeks and randomly assigned to chilling treatments (Appendix 1). The weight of the carcass sides for each treatment were recorded to evaluate differences in the mean weight between treatments.

The *semimembranosus* muscle was selected as the test muscle because it is located deep in the hip where carcass temperature decreases the slowest and it has an exposed portion that allowed the pH of the muscle to be measured while still on the carcass. A pH meter (Accumet AP71, Fisher Scientific, Mississauga, ON) and pH probe with a temperature probe attached were calibrated with pH 4, 7, & 10 standards at the start of measurements. The internal temperature of each carcass was measured with a thermocouple as described above. A scalpel blade (Integra York PA, Inc., York, PA, USA) was used to make three 2.5 cm deep incisions into the exposed proximal part of the *semimembranosus* muscle. Theses incisions were used to measure the pH and temperature of the meat 2.5 cm below the surface. The pH probe and thermocouples were rinsed with distilled water and placed into adjacent incisions, and after 30 s measurements were recorded. Temperature and pH were measured when the carcass entered the cooler (1 h post mortem) and at 3 h postmortem. New incisions were made for the second measurement.

After chilling, the *semimembranosus* muscle was harvested and vacuum packaged in a Cyrovac bag® B6620 (Sealed Air, Charlotte, NC, USA) by an experienced company employee. The Cryovac® bags had an oxygen transmission rate of 3-6 $cm^3/m^2/24$ h at 0% relative humidity and 4.4°C and were 0.05 mm thick. All samples were stored overnight at 4°C before transport on ice to Edmonton in a Coleman Extreme Cooler (Wichita, KS, USA). Once reaching the laboratory, the meat was stored overnight at 0°C. Three days post mortem, the *semimembranosus* muscle was weighed in the bag, packages were opened aseptically and the entire surface area of the *semimembranosus* muscle was swabbed with a Sani-stick for enumeration of microbial populations. The swab was placed in NE buffer and stored at 4°C until processing. After removal of the primal cut, the bag was weighed and the weight of the bag was subtracted from the total weight to determine the weight of the meat.

After samples for microbial analysis were taken, the pH and temperature of each *semimembranosus* was measured. Three incisions were made into the proximal part of the muscle, adjacent to the incisions made while the muscle was still on the carcass. The pH was determined as described above. The *semimembranosus* muscle was cut into 11 steaks, individually vacuum packaged (Model C200; Multivac, Kansas City, MO) in 76.2 µm thick, nylon and polypropylene coextruded vacuum bags (Unipac Packaging Products Ltd., Edmonton, Canada) with an oxygen transmission rate of 52 $cm^3/m^2/24$ h at 0 % relative humidity, and adjacent streaks were grouped and four groups were randomly assigned to storage for 3, 30, 60 or 91 d at 0°C (Figure 4). The remaining three steaks were used for collagen crosslink analysis, myoglobin analysis and fiber typing by other researchers.

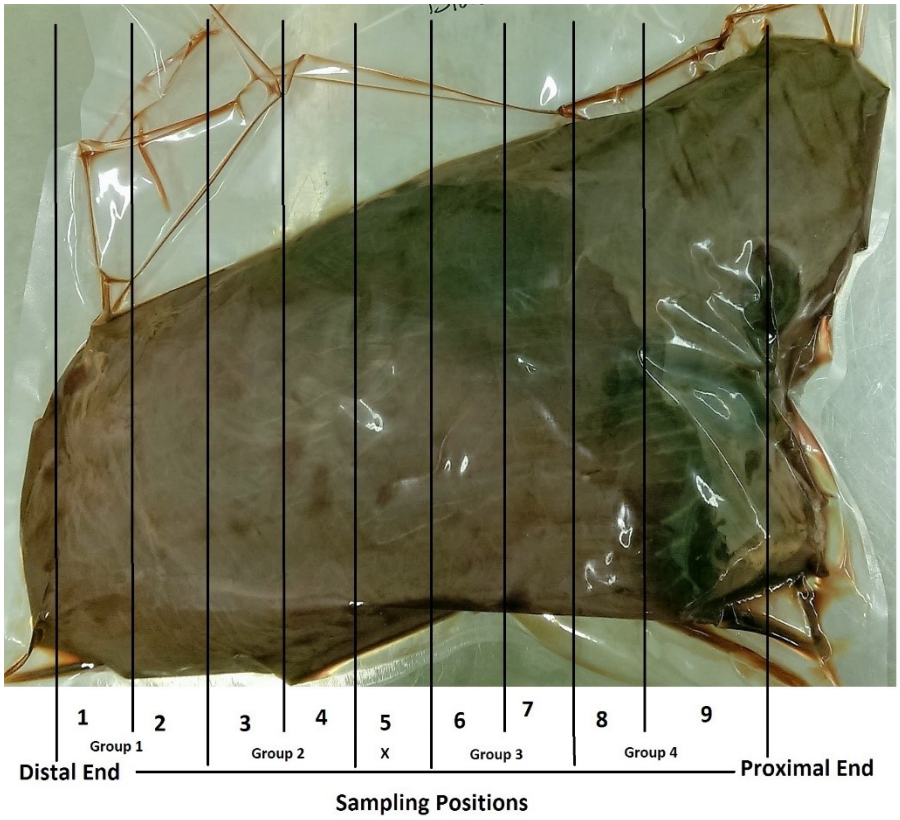

Figure 4: Layout of cutting and grouping patter of steaks cut from *Semimembranosus* muscle. The first steak from each grouping was used for microbiological testing.

At each storage time, samples were removed from storage for microbial analysis including total aerobic bacteria, presumptive lactic acid bacteria and *Enterobacteriaceae*. The steaks were aseptically removed from the vacuum package and the outer 2 mm of the steak removed using sterile tweezers and a scalpel blade. The pH of the steak was measured as described above. The trim was placed in a stomacher bag (Fisherbrand; A&R Belley Inc., Sherbrooke, Quebec, CA) and refrigerated at 4°C until all samples were ready for analysis (< 1 h). The samples were weighed, 100 mL of sterile 0.1% peptone water was added and the contents were stomached for 1 min in a Colworth Stomacher 400 (A.J. Seward, Edmunds, UK). Samples (0.1 mL) were

spread onto Plate Count Agar (Difco, Becton, Dickinson and Company, Sparks, MD, USA) and All Purpose Tween agar (ATP; Difco) to determine total aerobic counts and presumptive lactic acid bacteria, respectively. PCA plates were incubated aerobically at 23°C for 72 h and APT plates were incubated anaerobically at 23°C for 48 h in anaerobic jar with GasPak™ EZ Sachets (Becton, Dickinson and Company, Mississauga, ON). Numbers of *Enterobacteriaceae* were determined using pour plates with VRBGA that were incubated 18-24 h at 37°C prior to enumeration. All samples were plated on each agar in duplicate. All counts were converted to log (CFU/cm^2 or CFU/g) prior to statistical analysis.

2.4 Statistical Analyses

The temperature readings from carcasses chilling were averaged by weight groups. All bacterial counts were transformed to log CFU/cm^2 and converted to 100 or 1000 cm^2 as appropriate. Cell counts from carcass hygiene tests were averaged. Where the carcass side or *Semimembranosus* muscle was the sole experimental unit, a one-way analysis of variance (ANOVA) using the MIXED procedure of the University Edition of Statistical Analysis Software (SAS) was performed to determine if differences among the means. This analysis was used on the dependent variables: bacterial load on the SM muscle, weight of carcass sides, pH at 1 h, 3 h, and 48 h, and Semimembranosus muscle temperature at 1 h and 3 h postmortem comparing the means among chill treatments, blocked by sampling days.

For tests where both the steaks and the *Semimembranosus* muscles are experimental units, a split pot design was used with the *Semimembranosus* muscle being the experimental unit of the main plot and the steaks were the experimental unit of the subplot. A two-way analysis of variance (ANOVA) using the MIXED procedure of SAS was performed to determine if differences

among the means. The independent variables were the chill treatments (17, 26, and 30 h) and the aging periods (3, 30, 60, and 90 d) were the sources of variation with blocking being performed by sampling days. Tukey's test was used to determine differences among treatments.

2.5 Metagenomic Analysis of horse meat microflora

To determine the general diversity of bacteria present on horse meat after 90 d of storage, metagenomic analysis of DNA isolated from meat samples and from the colonies present on PCA and APT agar plates was done.

DNA was extracted from masticated meat after stomaching and from the PCA and APT plates that were incubated for an additional 5 days at 23°C after enumeration. After plating the masticated meat onto PCA and APT plates the masticated meat was filtered, and the liquid from 9 masticated steaks from to the same sampling day at the abattoir were combined to increase the amount of DNA and microbiological diversity in the sample. The combined samples were then centrifuged at 4000 x g for 5 min, the supernatant collected, and centrifuged at 4000 x g for 20 min. The pellet was stored for 5 days at -20°C prior to DNA extraction. The APT and PCA plates were enumerated after 48 h and 72 h of incubation; they were incubated an additional 5 days at 23°C. The colonies were collected with a sterile metal spatula into a 2 mL microcentrifuge tube, combining samples from each of the 9 steak excisions into one that represented the sampling day at the abattoir. DNA extraction was done on 220 mg of sample following the QIamp DNA Stool kit (Qiagen Inc., ON) protocol with the following modifications. Prior to cell lysis, samples were heated to 95°C for 15 min. To pellet the InhibitEX, samples were centrifuged at 4000 x *g* for 10 min. To suspend the DNA, 100 µl of

DNAase free water were added rather than 200 µl of Buffer AE. The DNA was stored in aliquots at -20°C until sent for sequencing.

The universal primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') (Turner et al. 1999) and reverse primer 1064R (5'-CGACRRCCATGCANCACCT-3') was used to amplify the V5 and V6 regions of the bacterial 16s rRNA gene. For the pair-end sequencing, two indexes 8bp in length were applied to each sample for differentiation.

Sequences were preprocessed by following the pair-end protocol of the MICCA (Albanese et al., 2015) pipeline. Sequencing quality was checked with FastQC (Andrews, 2010). Paired-ends were merged with an overlap length of 100 and a maximum allowed mismatch of 33 (Rognes et al., 2016). To filter the sequences by quality, a maximum error rate of 0.25% with a length of 280 was used allowing >92% of sequences from all samples to pass quality filtering with a lower error rate (Rognes et al., 2016). Sequences were clustered into operational taxonomic units (OTUs) by open-reference cluster using a default similarity level of 97% and aligned against the Ribosomal Database Project database (RDP) (Wang et al., 2007).

Chapter 3: Results

3.1 Cooling profiles of horse carcass sides

The cooling profiles for carcass sides of 125, 150 and >182 kg are shown in Figure 5. The horse carcass sides that weighted >182 kg cooled faster than the sides that weighed 150 kg (Figure 5); however, the smallest carcasses sides (125 kg) cooled the fastest. At all times, cooling curves had a consistent downward trend. All the sides of carcasses reached an internal temperature <7°C after 30 h of chill time. The larger carcasses sides (>182 kg) reached an internal temperature of 13°C after 17 h, and an internal temperature of 8°C after 24 h. These chill times were the basis for the following experiments.

3.2 Evaluation of carcass hygiene

To determine the general hygiene of carcasses, microbiological analysis of swabs from 32 carcasses was completed throughout a year. Counts for total aerobic bacteria, *Enterobacteriaceae,* coliforms, and *Escherichia coli* were generally low (Table 3) and often below 15 colonies on a plate, except total aerobic counts. There was considerable variability among counts obtained from the carcasses. *Enterobacteriaceae*, coliforms or *E. coli* were isolated from less than 50% of carcasses (Table 4). Of the 32 carcasses tested, only two and three of the VRBGA and *Escherichia coli*/coliform plates, respectively, had 15 or more colonies; none of the *Escherichia coli*/coliform petrifilm plates had more than 15 *Escherichia coli* colonies.

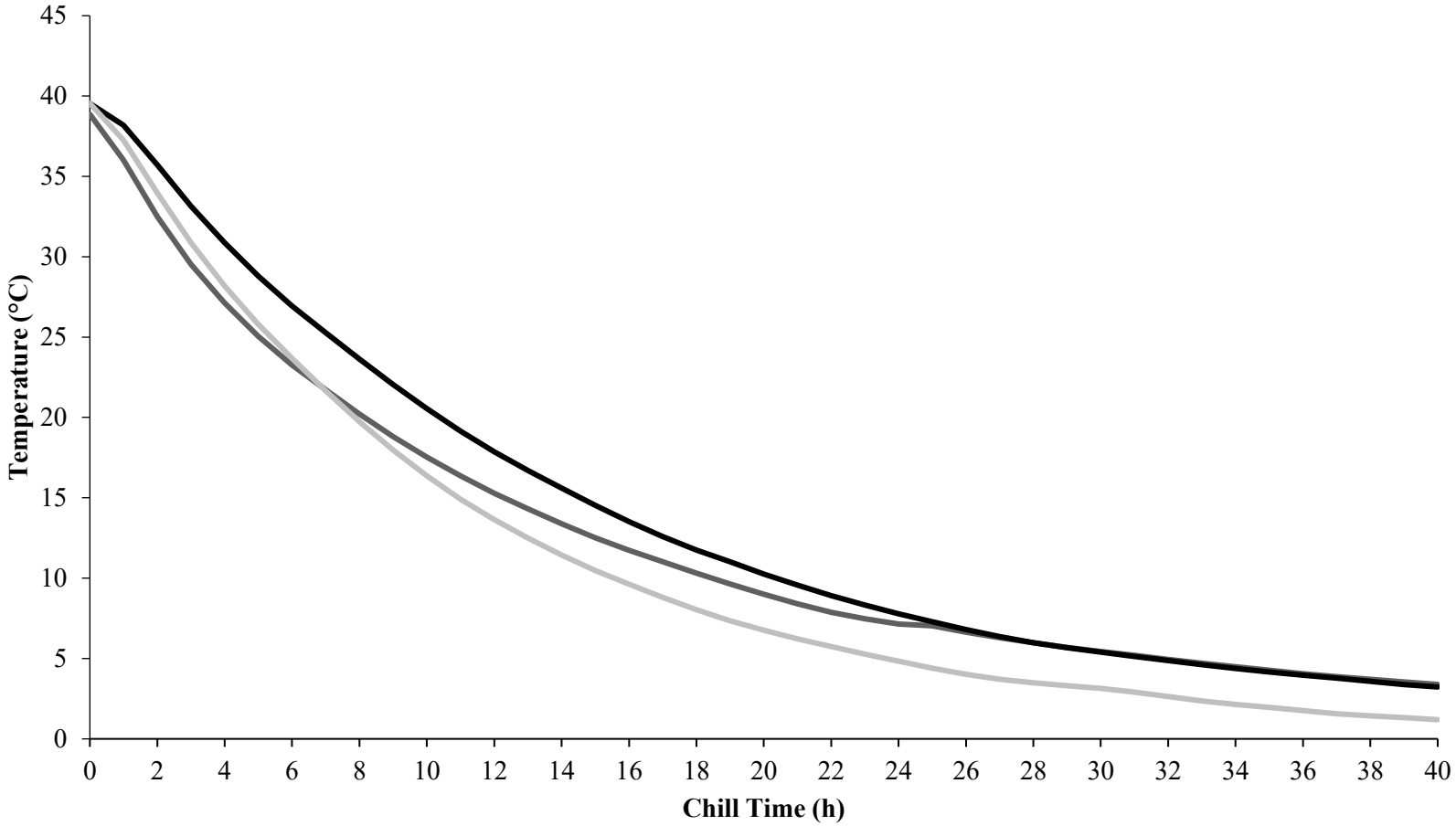

Figure 5: Mean internal temperature profiles of horse carcass sides weighing 182 (—), 150 (—), and 125 (—) kg chilled at 2°C for 40 h. Thermocouples were inserted through the obturartor foramen of the aitch bone (n=3 for each carcass weight).

Table 3: Mean log counts of aerobic bacteria, *Enterobacteriaceae, Escherichia coli,* and coliforms enumerated on aerobic count Petrifilm, Violet Red Bile Glucose Agar, and *Escherichia coli*/coliform Petrifilm, respectively. Data are means ± standard deviation of counts obtained from swabs of 32 horse carcass sides gathered over one year.

Bacteria	Count (log CFU/1000 cm^2)
Total aerobic bacteria	3.25±0.35
Enterobacteriaceae	0.54±1.03
Coliforms	0.51±0.80
Escherichia coli	0.13±0.26

Table 4: *Enterobacteriaceae,* coliform, and *Escherichia coli* counts from swabbing of horse carcasses plated on VRBGA and *E. coli*/Coliform Petrifilm. Data at 48 h were obtained from harvested muscles that were vacuum packaged and stored at 0°C (n=36).

Bacteria	Max Count (log CFU/1000 cm^2)	No. of Positives	% Positive	Mean count of positives (log CFU/1000 cm^2)
Enterobacteriaceae	3.2	10	40%	1.5±1.2
Coliforms	2.9	11	36%	1.2±0.8
Escherichia coli	0.7	6	20%	0.5±0.3

3.3 Post mortem pH and temperature change of *semimembranosus* muscles

The mean ± standard error internal temperature of the carcasses 1 h post mortem used in this study were 39±0.12 measuring in the deepest part of the hip through the foramen of the aitch bone. There was no significant difference among the weight of the carcass sides assigned to each chill treatment (Table 5). The temperature and pH of the *semimembranosus* muscle was measured 2.5 cm deep into the muscle. After 1 and 3 h post mortem, there was no significant difference in mean temperature of the muscle among treatments (Table 6). There was no significant difference in the pH measured 1, 3, and 48 h post mortem (Figure 6). The section of the muscle that was evaluated was on the surface, and so was exposed to the abattoir environment while on the carcass. The mean subsurface temperature and pH decrease from 1 to 3 h postmortem was 12.7°C and 0.13, respectively. After harvest, the pH decreased by an average of 1.18 during 48 h of storage and chill time prior to harvest of muscles did not affect the pH of the *semimembranosus* muscle.

Table 5: Hot weight (kg) of carcass sides assigned to chill treatment. Values are means ± standard errors of 36 carcasses assigned to 3 groups over 4 sampling days (n=12).

Chill Treatment	Carcass side weight (kg)
17 h	193.5±3.93
26 h	195.5±4.33
30 h	185.2±1.73

Table 6: Mean ± standard error of the subsurface temperature of *semimembranosus* muscles measured at 1 and 3 h post mortem (n=36).

Chill Time (h)	Temperature 1 h post mortem (°C)	Temperature 3 h post mortem (°C)
17	31.4±0.35	19.1±0.7
26	31.8±0.66	18.4±1.1
30	30.5±0.59	17.9±0.6

3.4 Effect of chill time on microbial load of primal muscle.

Counts of total aerobic, *Enterobacteriaceae,* and lactic acid bacteria on the *semimembranosus* muscle from carcasses chilled for 17, 26, or 30 h were determined to assess the effect of carcass chill time on meat hygiene. There were also no significant differences in bacterial load among chill treatments (Figure 7). There was high variability in the quantity of *Enterobacteriaceae* among carcasses with 39% of carcasses having 1 or more CFU and an average of 0.4±0.3 log CFU/1000 cm^2 on carcasses where more than 1 colony was observed on the plates. For the majority of the samples, *Enterobacteriaceae* counts were below the detection limit of 1 log CFU/1000 cm^2.

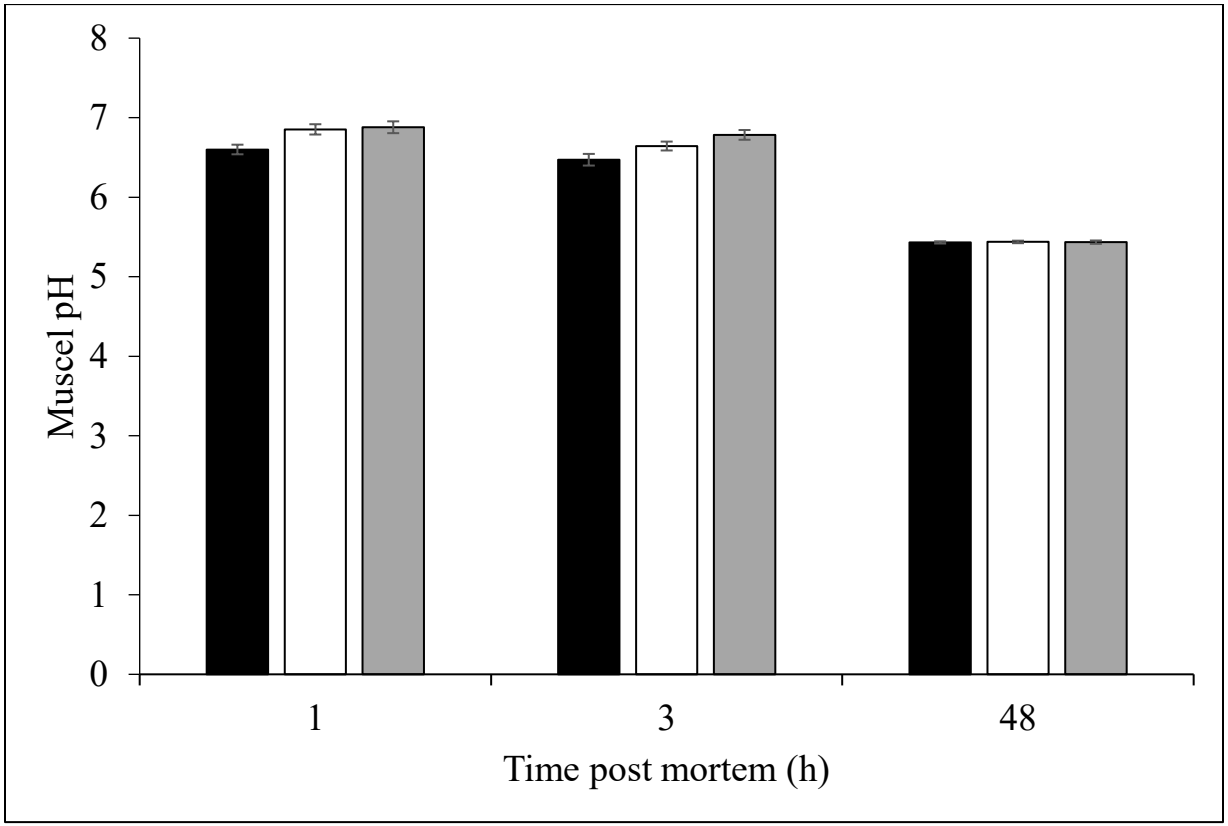

Figure 6: Mean subsurface pH of *semimembranosus* muscles measured at 1, 3, and 48 h post mortem. Data at 48 h were obtained from harvested muscles that were vacuum packaged and stored at 0°C (n=36). Values are mean ± standard error by assigned chill treatment 17 (■), 26 (□) or 30 (▣) h. Times 1 and 3 h postmortem are before the chill treatment has been applied.

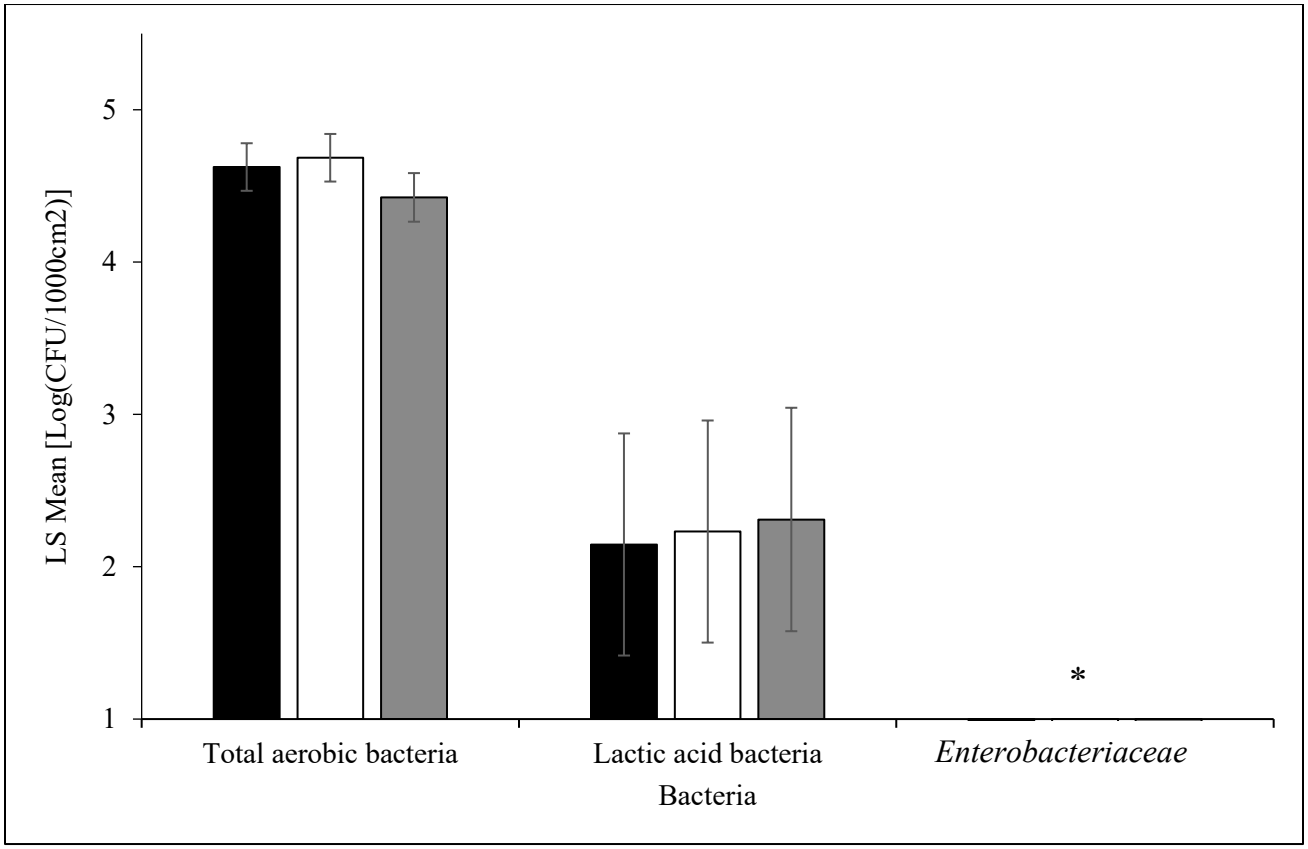

Figure 7: Mean log counts and standard errors for total plate count, *Enterobacteriaceae* and lactic acid bacteria from swabs of *semimembranosus* muscles harvested at from carcasses chilled for 17 (■), 26 (□) or 30 (▣) h. *Counts of *Enterobacteriaceae* were below the detection limit of 1 log (CFU/1000 cm^2) for most samples. There were no significant differences among means for each bacterial count (n=12).

3.5 Bacterial load changes over 90 days of storage.

The effect of carcass chill time on the total aerobic bacteria, lactic acid bacteria and *Enterobacteriaceae* on vacuum packaged steaks stored for 90 d was determined at 30 d intervals. There were no significant differences in microbial counts of steaks among chill treatments during 60 d of storage (Figure 8). At 90 d of storage, the total aerobic bacteria on steaks from carcasses chilled for 30 h were higher than that of steaks from carcasses chilled for 17 h (Figure 8). Counts of lactic acid bacteria were low throughout storage but after 90 d, lactic acid bacteria on steaks from carcasses chilled for 30 h were higher than those on steaks from carcasses chilled for 17 or 26 h. Counts of *Enterobacteriaceae* were below log 1 CFU/g throughout storage, regardless of carcass chill time (Figure 8).

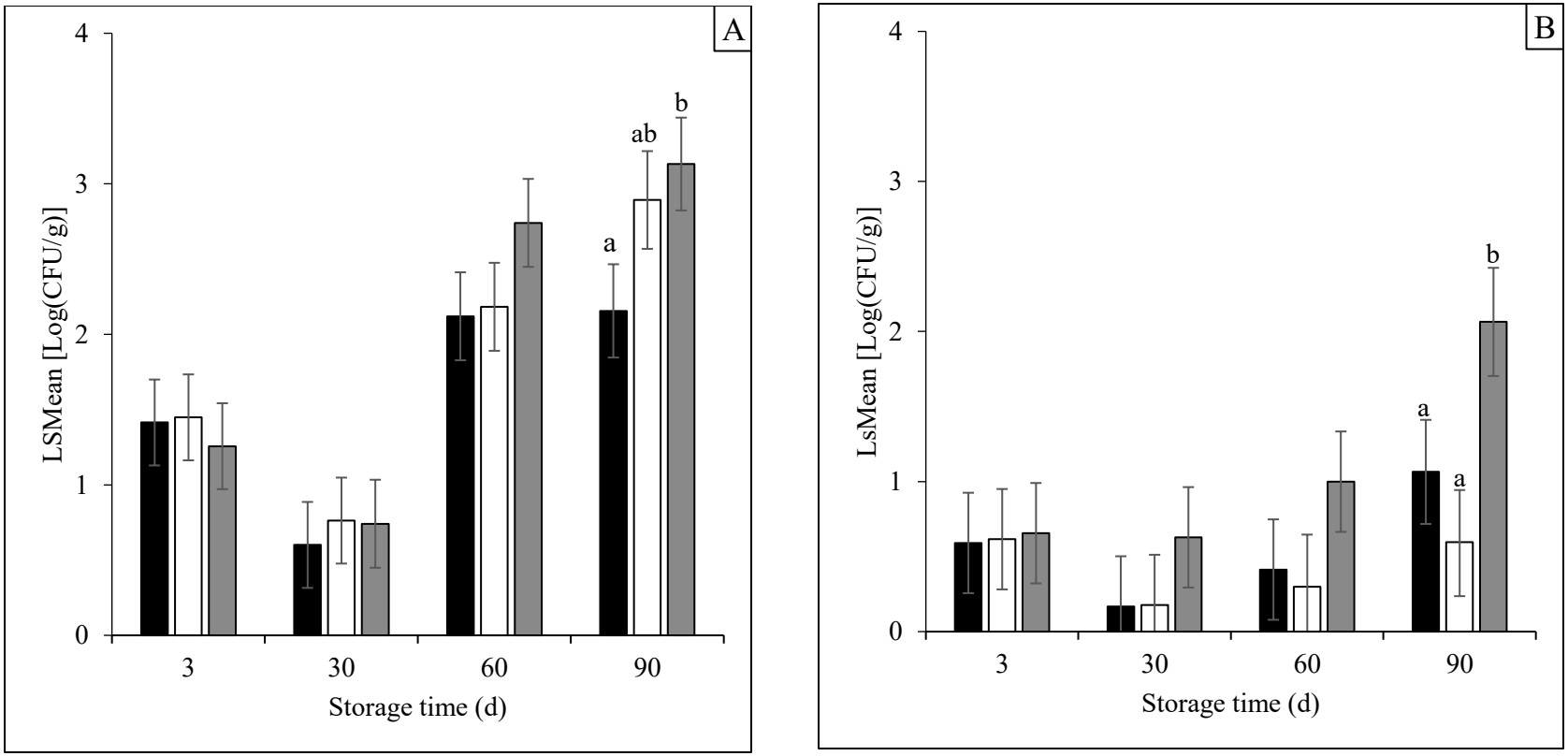

Figure 8: Mean log counts and standard errors of total aerobic (A), and lactic acid bacteria (B) of vacuum packaged horse steaks stored for up to 90 d at 0°C. Steaks were from carcasses that had been chilled for 17 (■), 26 (□) or 30 (▩) h after slaughter (n=12).

3.6 Metagenomic analysis of cultured plates on APT, PCA, and meat samples.

Initially horse carcasses were swabbed after dressing but before chilling. After DNA extraction, the quantity of DNA was too low for sequencing. Instead the samples were collected from the steaks after 90 d of storage. To increase DNA concentration and increase the coverage of each sample sequenced, the liquid from 9 masticated steak excisions which were sampled on the same day, were combined into a single sample. To gain the extra coverage, the DNA obtained from colonies on the APT and PCA plates was combined in the same manner. Metagenomic analysis of the DNA obtained from horse steaks stored for 90 days showed that 82% of bacteria were from the proteobacteria phylum, 18% from Firmicutes, and small fractions from another phylum or unclassified in the RDP database (Figure 9). The microbiota of the meat samples was more diverse than the cultured microbiota from the agar plates.

The microbiota of the meat samples had phylums from Actinobacteria, Candidatus Saccharibacteria, Fusobacteria, and Cyanobacteria; however, combined these phyla of bacteria consisted of less than 1% of the relative abundance. The proteobacteria consisted of 35% *Serratia,* 15% unclassified *Enterobacteriaceae* and 47% *Pseudomonas.* Sample 3 had the largest proportion of *Serratia.* Of the Firmicutes, 22% of the samples were *Lactococcus* and 75% aligned to an unclassified *Lactobacillales* order. Three percent of the *Firmicutes* were *Bacillus* (Figure 10).

Sequence analysis of the cultured aerobic microbiota revealed that the culturable microbiota consisted of 8% *Firmicutes* and 92% *Proteobacteria.* Of the *Firmicutes* 4% and 87% were *Lactococcus* and *Bacillus* respectively, with 8% as unclassified *Lactobacillales*; the

Proteobacteria had 67% *Pseudomonas*, 10% *Serratia,* 10% *Stenotrophomonas*, with 13% of the group as unclassified *Enterobacteriaceae* (Figure 11).

Sequence analysis of the culturable microbiota for sample 2 obtained from APT plates indicated that the microbiota had a high percentage of *Pseudomonas.* Seventeen percent of the sequences aligned to *Firmicutes* with the remaining 82% aligning to *Proteobacteria*. Of the Firmicutes 74% were *Bacillus* with 26% being an unclassified *Lactobacillales.* Of the *Proteobacteria* 89% were unclassified *Entrobacteriaceae* with only 10% being classified as *Serratia* (Figure 12).

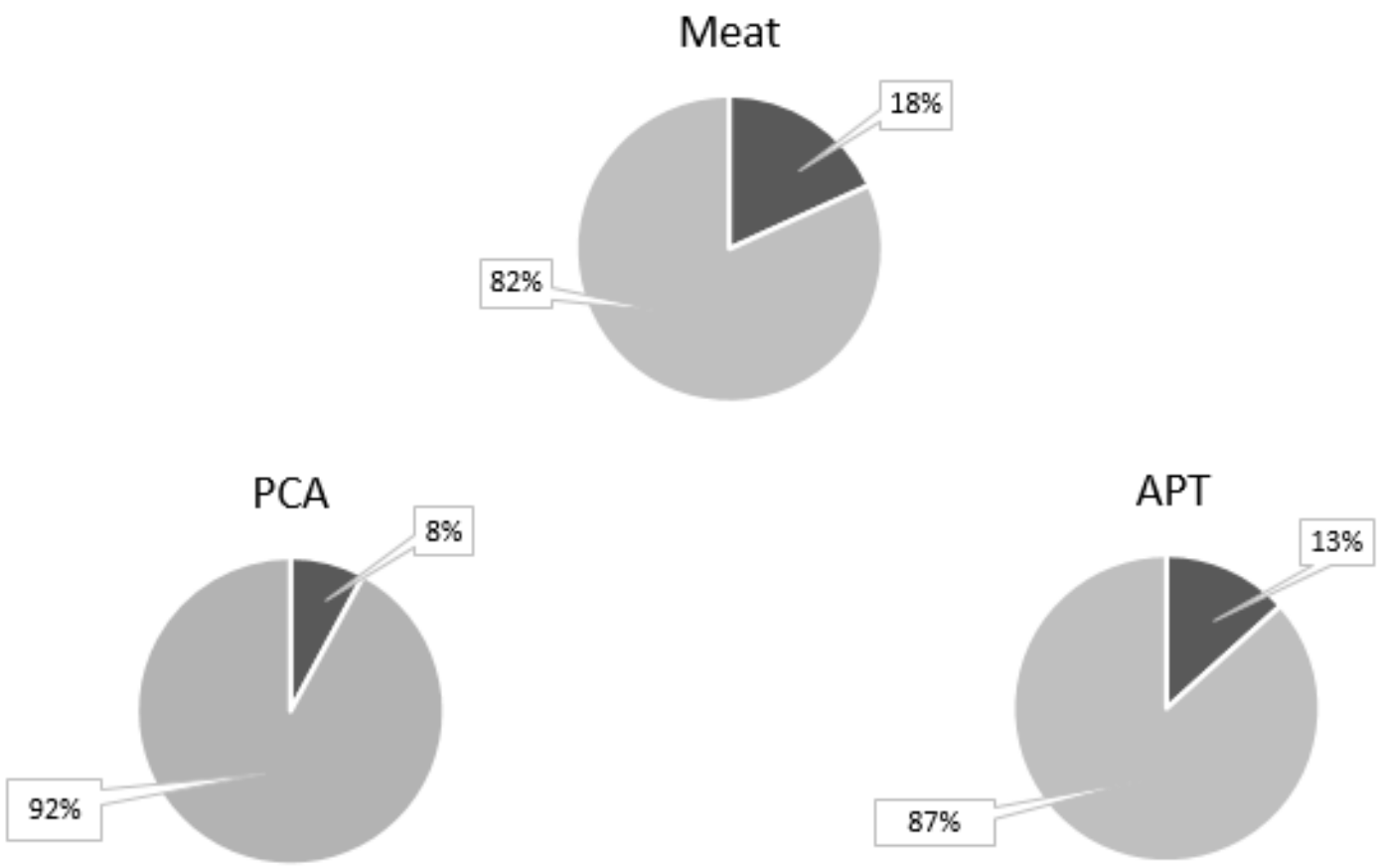

Figure 9: Relative abundance (%) of phyla, Proteobacteria (□) and Firmicutes (■) present in the microbiota of vacuum packaged horse meat stored for 90 d and the cultured microbiota obtained from the DNA recovered from plating the same meat samples on PCA and APT agar (n=3).

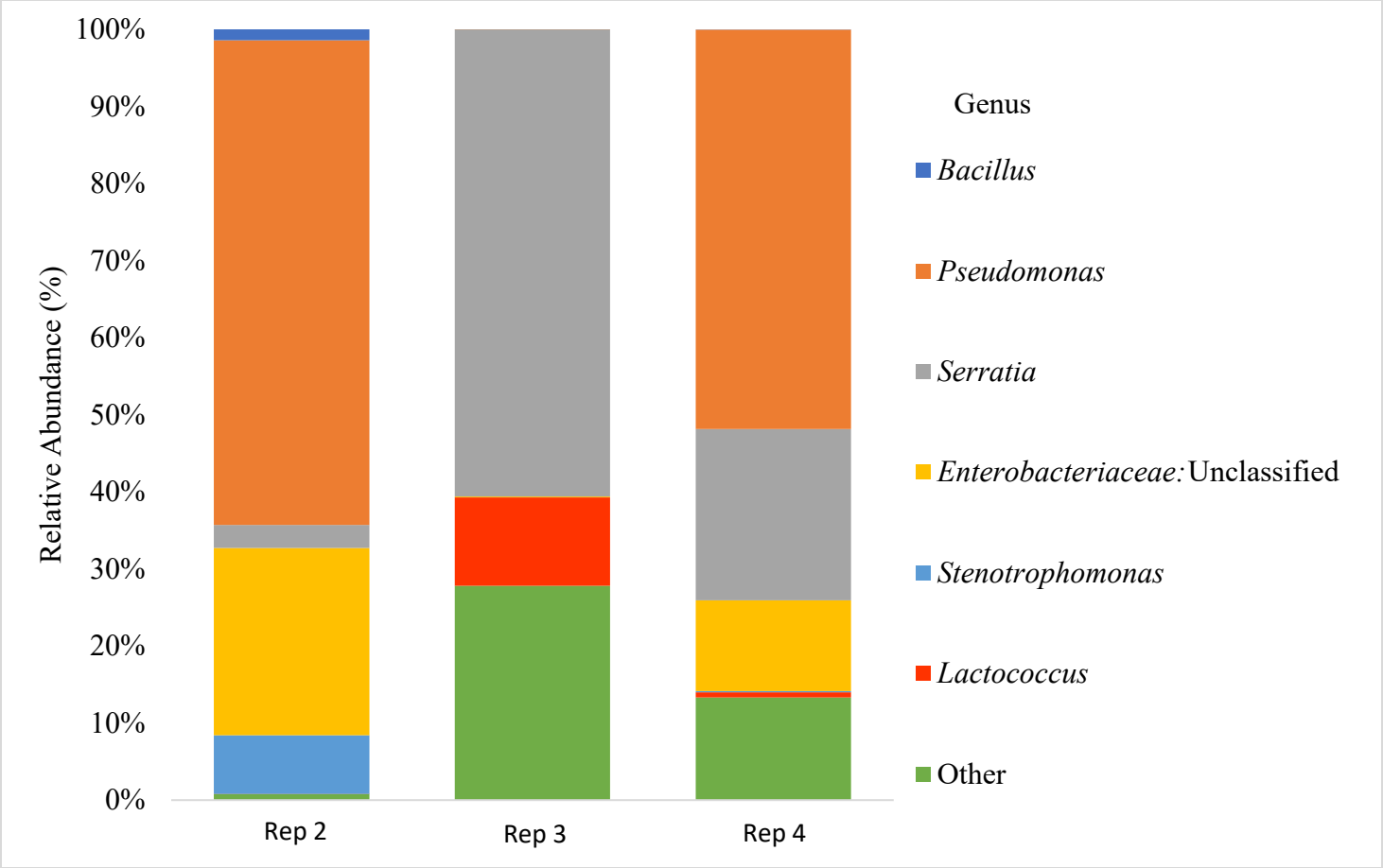

Figure 10: Metagenomic analysis of the microbiota of vacuum packaged horse meat after 90 days of storage at 0°C. Sequences were obtained using illumina MISeq and aligned to the RDP database. Each sample represents a sampling day at the abattoir and is a combination of DNA obtained from the cultured microbiota of 9 steaks sampled on that day. Other category is due to a high number of unclassified samples. (n=3).

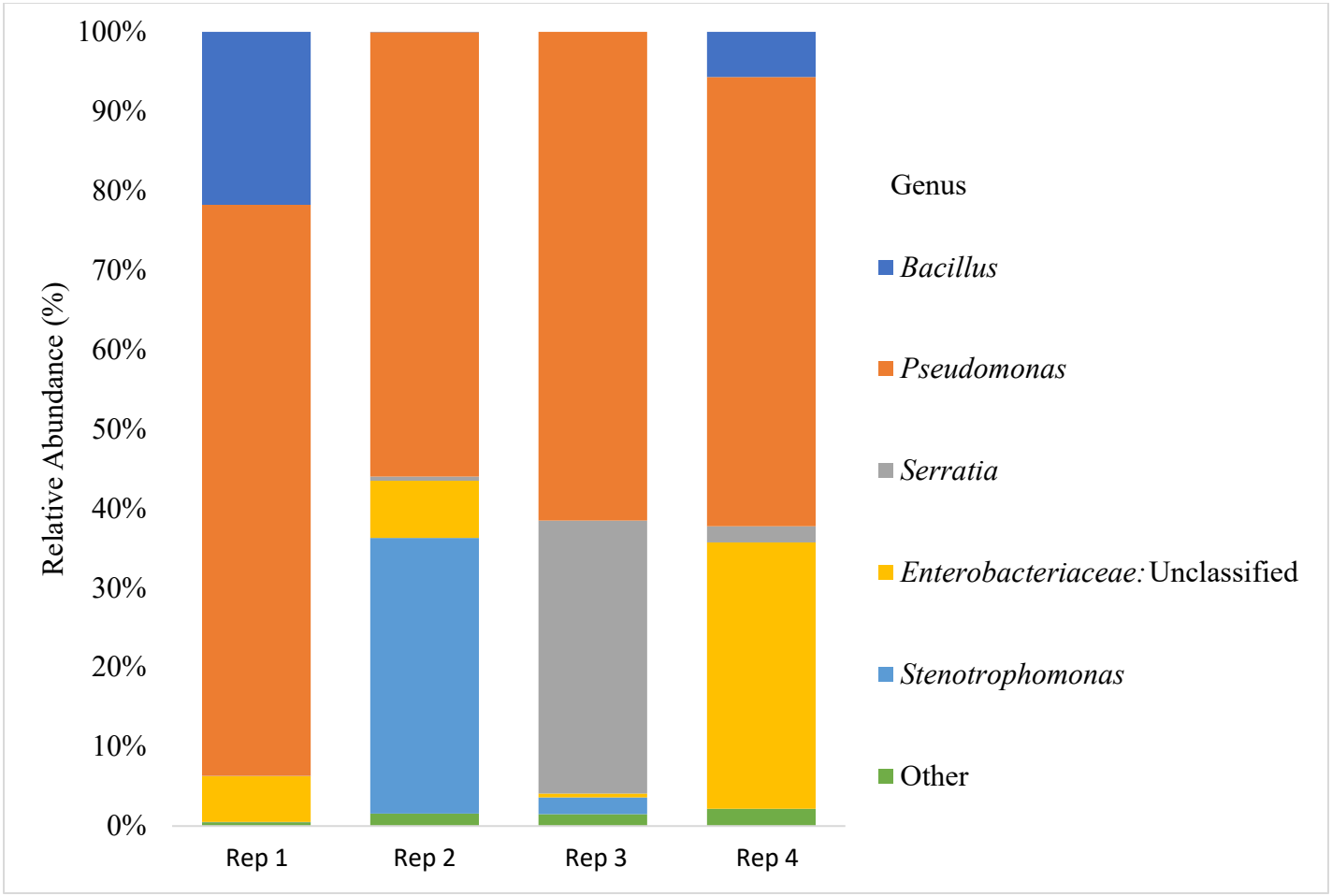

Figure 11: Metagenomic analysis of cultured microbiota of horse meat samples stored for 90 d at 0°C and plated onto PCA. Sequences were obtained with illumina MISeq and aligned to the RDP database. Each sample represents a sampling day at the abattoir and is a combination of DNA obtained from the cultured microbiota of 9 steaks sampled on that day (n=4).

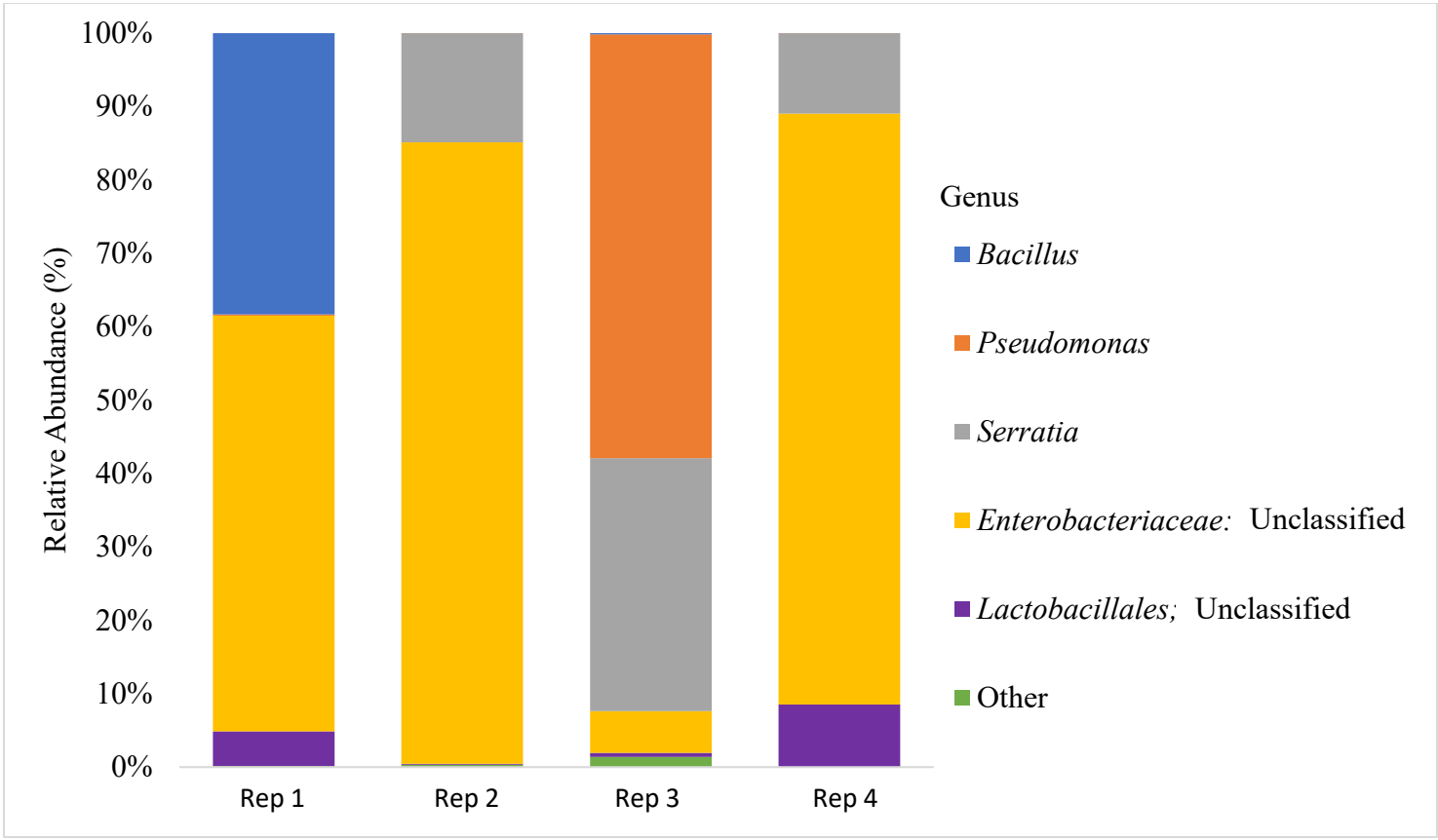

Figure 12: Metagenomic analysis of cultured microbiota of horse meat samples stored for 90 d at 0°C and plated onto PCA. Sequences were obtained with illumina MISeq and aligned to the RDP database. Each sample represents a sampling day at the abattoir and is a combination of DNA obtained from the cultured microbiota of 9 carcasses sampled on that day (n=4).

Chapter 4: Discussion and Conclusions

The aim of this study was to determine if meat can be harvested from horse carcasses at an internal temperature above 7°C without compromising the microbial quality and safety of the meat. CFIA requires that prior to harvest the warmest part of the carcass must be below 7°C (Canadian Food Inspection Agency, n.d.). The results of this study indicated that meat can be harvested at a temperature above 7°C without compromising microbial quality and storage life.

4.1 Evaluation of carcass hygiene.

The hygiene of carcasses needed to be evaluated as high initial bacterial load is one of the most important factors that influence the safety and storage life of meat products (Lambert et al., 1991). To assess the potential change in processing procedures to reduce the time to meat harvest (i.e. harvest at a high internal temperature), baseline data on current microbiological condition of carcasses was collected. Temperatures were measured in the aitch-bone pocket as it is the part of the carcass that cools the slowest, allowing the greatest opportunity for growth of pathogenic bacteria (Gill et al., 1991). The choice of method for determination of carcass hygiene is an important consideration. Gallina et al. (2015) does not recommend the use of sponges for horse carcasses sampling as they found only a 42% recovery rate of total aerobic bacteria compared to excision techniques; however, Gill and Jones (2000) found no difference in total aerobic counts between excision and sponge swabbing techniques.

The microbiological counts of the horse carcasses sampled in the current study were lower than that found on beef carcasses harvested in the US in the early 2000's (Bacon et al., 2000). Gill et al. (1998) evaluated beef carcasses at 10 abattoirs and determined that for beef 1 ± 0.5 log

CFU/cm^2 of coliform or *E. coli* counts and 3 ± 0.5 log CFU/cm^2 of total aerobic counts can be considered as hygienic performance criteria. Yang et al (2012) reported that the total aerobic bacteria on beef carcasses harvested in a large beef processing facility that uses chemical interventions was 2.2 log CFU/cm^2, which was similar to that found in the current study for horse carcasses. Reid et al (2017) swabbed 30 beef carcasses in a commercial abattoir when the carcasses entered the cooler and found them to have 2 and 0.4 log cfu/cm^2 of total aerobic bacteria and total *Enterobacteriaceae,* respectively. The microbiological condition of the horse carcasses swabbed in this study matched that which was previously reported for horse carcasses at a North American abattoir (Gill, 2005). Previous researchers swabbed horse carcasses after either 2 h of cooling or prior to boning and enumerated samples with hydrophobic grid membrane techniques to find approximately 1.5 log CFU/cm^2 total aerobic count, and *Enterobacteriaceae* and *E. coli* counts below the level of detection (Gill, 2005). After swabbing 84 horse carcasses, Gallina et al. (2015) found an average of 2.1 and -0.3 log CFU/cm^2 of total aerobic bacteria and *Enterobacteriaceae,* respectively, using cellulose sponges. If hygiene issues were detected at the abattoir used in the current study, these would have had to be addressed prior to continuing with the study on the impact of chill time on horse meat microbiology as there is a risk that any changes in hygienic condition could change the microbial quality and storage life of the meat harvested for experiments. This was not the case as counts of total aerobic bacteria were low and counts of *Enterobacteriaceae*, coliforms, and *Escherichia coli* were below the detection limit. These low numbers were maintained and possibly reduced as the abattoir allowed the meat surface to dry during carcass cooling, which has been shown to reduce bacteria counts (Gill and Jones, 1997). The good hygiene of the carcasses supports the hypothesis that the meat can be harvested above an internal temperature of 7°C. Harvesting meat

above an internal temperature of 7°C poses a risk of the meat having an internal temperature in the danger zone (4 – 60°C) for sufficient time to facilitate a higher rate of growth of bacteria on the meat. The initial bacteria counts were so low that any extra time the meat temperature is in the danger zone would not likely affect the safety or shelf life of the horse meat.

4.2 Cooling profile of horse carcasses.

A proper carcass cooling routine causes the surface of the carcass to make a continuous decent to less than 7°C within 24 h after dressing (Canadian Food Inspection Agency, n.d.). This slows growth of mesophilic microbial contaminants, especially pathogenic *Enterobacteriaceae,* but keeps the carcass warm enough to allow chemical and enzymatic processes essential to meat quality to continue. The cooling routine of the industry partner was evaluated by tracking the internal carcass temperatures over the first 16 h the carcasses were in the cooler. This information was needed to determine the carcass chill times and temperatures to target to meet the goals of the industry partner. The largest carcasses cooled faster than the medium sized carcasses as the larger carcasses were positioned closer to cooler fans with more space between them allowing more efficient cooling. Finding the larger carcasses cooled faster than smaller carcasses is not unexpected as research has shown beef carcasses can cool at different rates when measuring the temperature in the aitch bone pocket (Gill and Bryant, 1997). Spacing of carcasses, air flow, and the location of the carcasses in the cooler are all important variables affecting the rate at which carcasses cool.

4.3 Post mortem pH and temperature change of horse carcasses.

The pH and temperature of the *semimembranosus* muscle was measured 1 and 3 h after knocking, respectively. This correlates with entry into the cooler and 2 h after the carcass had been in the cooler, this allowed an assessment of potential meat defects such as dry, dark, firm (DFD) or pale, soft, and exudative (PSE). Meat with a higher pH (> 6.0) provides an environment where bacteria can grow faster (Lambert et al., 1991). During the first 3 h post mortem, there was a substantial drop in the temperature of the exposed portion of the *semimembranosus* muscle; however, only a slight drop in muscle pH was detected. This is likely from the measurement been taking 1" below the muscles surface and where the carcasses were positioned. The carcasses were positioned on the rail that was next to fans and the wind speed could have cause the temperature to drop rapidly slowing the cellular metabolism and lactic acid production of the muscle. After harvest and 48 h of storage, the *semimembranosus* muscle reached a terminal pH of 5.4, which is slightly lower than the 5.5-5.7 that Lorenzo et al. (2014) reported. When comparing different chill times, there was no significant difference in terminal pH of the *semimembranosus* muscle or the steaks. The lack of differences between the pH of the *semimembranosus* muscles harvested after different chill times provides an element of support of the hypothesis that horse meat can be harvested above the regulatory standard of an internal temperature < 7°C without a change in quality or safety as spoilage bacteria as the influence of pH on bacteria growth will be equivalent.

4.4 Effect of chill time on microbial load of primal muscle.

The hygiene of the *semimembranosus* muscle among the three chill treatments was evaluated to identify initial microbial loads and to determine if those loads changed if muscles were harvested

prior to the carcass reaching an internal temperature of 7°C. Gill and Landers (2005) evaluated the microbial load on carcass sides immediately after dressing and after 2 h of cooling; meat harvested from carcasses immediately after dressing had 1.90 log cfu/cm^2 and meat harvested after 2 h of cooling had 1.49 log cfu/cm^2. Having less than 1 log difference in total aerobic counts after dressing and after 2 h of cooling is of no practical importance. Similarly, the current study revealed no practical difference in microbial load on the *semimembranosus* muscles that were harvested after 17, 26, and 30 h of cooling as the results were all within 1 log cfu/cm^2. The low microbial load was expected as only a small portion of the *semimembranosus* muscle is exposed and susceptible to contamination during cooling. On muscles with a very low initial microbial load, the change during storage may be limited. Reid et al. (2017) evaluated the change of total aerobic bacteria, total *Enterobacteriaceae*, and *Pseudomonas* spp. on 30 beef carcasses in a commercial slaughter plant and found the change in the average bacteria count to be -0.4, 0.1, 0.1 after 26 h and 0.8, 0.5, and 0.7 log CFU/cm^2 after 96 h, respectively. *Enterobacteriaceae* counts are considered to be a good predictor of the presence or absence of *Salmonella* spp. as the absence of *Enterobacteriaceae* on beef carcasses indicates the absence of *Salmonella* spp. 98% of the time (Ruby and Ingham, 2009). The effectiveness of carcass chilling to maintain carcass hygiene is controversial with studies showing increasing, decreasing, and unchanged total aerobic and *Enterobacteriaceae* counts (Bacon et al., 2000; Gill et al., 2003; Lenahan et al., 2010). In the current study, factors that may influence contamination were controlled and the only differences would have been from contamination on the small exposed portion of the muscle. The lack of differences in the total aerobic bacteria, lactic acid bacteria, and *Enterobacteriaceae* counts among the difference treatments indicates that harvesting meat at shorter chill times had no difference in microbial hygiene or safety of horse meat.

4.5 Changes in bacterial load during storage.

The microbial load of horse steaks was measured every 30 d for a 90 d to determine if shorter carcass chill times would adversely affect the microbiological condition of steaks during storage. No difference in the growth of total aerobic bacteria, lactic acid bacteria, or *Enterobacteriaceae* was found on the steaks that were harvested after different chill times. After 3 days of storage at 0°C there was no difference between chill treatment for either total aerobic or lactic acid bacteria loads; however, after 90 d of storage the steaks that were from muscles harvested after 17 h of chilling had lower counts of total aerobic bacteria and lactic acid bacteria than the samples chilled for 30 h. These results indicated that the harvest of horse meat at shorter chill times does not have adverse effects on the microbiological quality of the meat. The change in total aerobic bacteria, *Enterobacteriaceae,* and lactic acid bacteria counts from 3 and 30 d of storage were similar to results of Gómez and Lorenzo (2012) who detected a decrease in total aerobic bacteria, constant *Enterobacteriaceae* counts, and an increase in lactic acid bacteria on horse meat stored under vacuum for 14 d at 2°C. *Enterobacteriaceae* counts likely decreased because the low temperature and anaerobic conditions during storage gave the competitive advantage to lactic acid bacteria, some of which can produce antimicrobial compounds (Lambert et al., 1991).

4.6 Metagenomic analysis of cultured microbiota and meat samples.

In this study, a metagenomic analysis using 16s rRNA sequencing was done on meat samples, and on the microbiota that was cultured on APT and PCA plates from horse meat that had been stored for 90 d at 0°C. This was done to identify the microflora and potential spoilage organisms or pathogens that may be present after refrigerated storage. Reports in the literature on the metagenomics of horse meat are lacking. Phylum level analysis indicated that on the microbiota

from meat samples, and in the cultured microbiota from APT and PCA plates, gram negative "Proteobacteria" was the most abundant phylum with gram positive "Firmicutes" being the only other phylum detected in the cultured microbiota. The meat samples contained more diversity with *Actinobacteria*, *Cyanobacteria*, *Fusobacteria*, and *Canditatus* s*accharibacteria* detected but at less than 1% of the total relative abundance. These phyla are likely contaminants from the environment, likely in soil or water where they are commonly found. A 16s rRNA metagenomic analysis of meat processing environment found similar results where fresh beef and pork samples contained primarily "Proteobacteria" consisting mainly of *Pseudomonas* and *Enterobacteriaceae* (Stellato et al., 2016). These are the phyla most suited for growth at 0°C on horse meat stored for 90 d as most bacterial contamination on meat comes from the abattoir environment (De Filippis et al., 2013; Hultman et al., 2015). Meat samples were most abundant in *Pseudomonas*, and *Serratia* species, which are commonly found on meat stored under refrigeration and are both associated with spoilage (Carrizosa et al., 2017; Pennacchia et al., 2011). The total aerobic bacteria cultured on PCA had a high abundance of *Pseudomonas* and *Serratia,* but also contained a high proportion of *Bacillus*. The APT plates were expected to have a high abundance of lactic acid bacteria; however, there was a much higher abundance of *Enterobacteriaceae.* Similarly, other researchers have found meat with long shelf life to be low in lactic acid bacteria (Youssef et al., 2014). Sample 3 had a high abundance of *Pseudomonas,* an aerobic bacterium, which may have been the result of a leak in the seal of the vacuum package.

4.7 Study Limitations and Conclusion

In retrospect, there are some changes to this experiment that would have provided better data to support the hypothesis. An evaluation of the spoilage organisms present on the carcass sides, along with a metagenomic analysis profiling those organisms would have been useful when comparing to results of the steaks after 90 days of storage. More data on the change in muscle pH and temperature prior to the harvest of the *semimembranosus* muscle would have been useful. For the experiment on chill times, it would have been useful to have the actual temperature of the carcasses prior to harvest of the *semimembranosus* muscle. This measurement would have also allowed harvest of muscles at a set temperature instead of using an estimate of temperature based on time from the start of chilling. Additional subsurface temperatures and pH measurements at the time the *semimembranosus* muscle was harvested could provide some key insight into the impact of pH on the microbiology of the meat used in this study. The sampling of the microbiota of the *semimembranosus* muscle may have reduced microbial populations sufficiently to impact the microbiology of the vacuum packaged steaks during storage. This also would have simplified the experimental design.

This study demonstrated that shorter carcass chill times did not impact the microbiology of horse meat when evaluating the load of total aerobic bacteria, lactic acid bacteria, and *Enterobacteriaceae* on both the *semimembranosus* muscle and steaks. Meat harvested from horse carcasses after 17 h of chilling, at an estimated internal temperature of 13°C, had no detrimental effect on the microbial load when compared to horse meat harvested to the current regulatory standard with an internal temperature below 7°C. The primary pathogen to consider is *Salmonella enterica* and the current regulatory standard requires that the meat be cold when removed from the carcass so that if *Salmonella* contamination occurs, the growth will be limited

unless temperature abuse occurs. The recommendation from this study would be for abattoir standard operating procedures to package meat in a manner that optimizes cooling of the surface of the harvested meat to 7°C and not allow the surface temperature of the meat to increase.

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Appendix 1 – Table of random assignment of chill treatment to carcasses prior to harvest of the semimembranosus muscles.

	Trip 1								
Group ID	**Sample ID**								
	101	**102**	**103**	**104**	**105**	**106**	**107**	**108**	**109**
1	3	90	60	30	30	60	90	3	30
2	30	3	90	60	60	90	3	30	3
3	60	30	3	90	90	3	30	60	90
4	90	60	30	3	3	30	60	90	60
	Trip 2								
Group ID	**Sample ID**								
	201	**202**	**203**	**204**	**205**	**206**	**207**	**208**	**209**
1	60	90	3	30	90	30	60	3	3
2	90	60	30	3	30	90	3	60	60
3	3	30	60	90	3	60	90	30	30
4	30	3	90	60	60	3	30	90	90
	Trip 3								
Group ID	**Sample ID**								
	301	**302**	**303**	**304**	**305**	**306**	**307**	**308**	**309**
1	90	30	60	3	3	60	30	90	60
2	60	3	90	60	90	30	3	60	90
3	30	60	30	90	30	3	90	30	3
4	3	90	3	30	60	90	60	3	30
	Trip 4								
Group ID	**Sample ID**								
	401	**402**	**403**	**404**	**405**	**406**	**407**	**408**	**409**
1	90	60	30	3	30	60	90	3	90
2	3	90	60	30	3	90	60	30	30
3	60	30	3	90	60	3	30	90	60
4	30	3	90	60	90	30	3	60	3