BACTERIAL LEVELS IN SASKATCHEWAN RETAIL GROUND BEEF

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ABSTRACT

This thesis describes the results of three studies that used different measures of bacterial numbers in retail ground beef (n=309) collected across different locations in Saskatchewan within a one-year period (May 2011 – May 2012). The measurements were compared among three sample categories: 1 - ground beef displaying government inspection information on the label legend (n=126), 2 - originating from facilities licensed by local health regions and thus not subjected to government inspection (n=80), or 3 - processed and repackaged at the retail level thus carrying no government inspection information on the label (n=103).

The first study reports baseline levels of bacteria in Saskatchewan retail ground beef as measured by traditional (total aerobic plate count (TAPC) and total *E. coli* plate count (TEPC)) and culture-independent methods (estimate of total bacterial load (TBL) by real-time quantitative polymerase chain reaction). After accounting for season and whether the samples were fresh or frozen at purchase, the lowest TAPC ($\log_{10}$ 4.9 culture forming units per gram (cfu/g); 95% CI $\log_{10}$ 4.7 to $\log_{10}$ 5.1 cfu/g), TEPC ($\log_{10}$ 0.58 cfu/g; 95% CI $\log_{10}$ 0.39 to $\log_{10}$ 0.77 cfu/g), and TBL in frozen ground beef ($\log_{10}$ 4.5 target copies per gram (tc/g); 95% CI $\log_{10}$ 4.0 to $\log_{10}$ 4.9 tc/g) were observed in samples originating from federally regulated or provincially licensed facilities.

In the second study, presence of known *Enterobacteriaceae* virulence factors (*stx1*, *stx2*, and *eae*) was detected by polymerase chain reaction (PCR) and compared between samples originating from three different regulatory and inspection environments as well as collected during different seasons of the year, and purchased fresh or frozen. One hundred and twelve out of all tested samples (n=308) were positive for the presence of at least one

virulence marker with *stx*1 identified in 107 samples, *stx*2 - in 8, and *eae* - in 26. No significant associations were found between the virulence markers presence and sample category, state or season of purchase.

The third study investigates the presence and diversity of *Campylobacter* spp. organisms in the same pool of 309 retail beef samples as detected by molecular methods. Fifty samples (16.2%) tested positive for *Campylobacter* genus-specific DNA in conventional PCR and 49 samples (15.9%) tested positive for at least one *Campylobacter* species DNA presence in real-time qPCR, but the crude agreement between the two methods was less than 50%. *C. coli* DNA presence was observed in 14 samples (4.5%), *C. curvus* – in 11 (3.6%), *C. fetus* – in 6 (1.9%), *C. hyointestinalis* – in 24 (7.8%), *C. jejuni* – in 12 (3.9%), *C. rectus* – in 6 (1.9%), and *C. upsaliensis* – in 9 (2.9%). There was no difference in the frequency of *Campylobacter* identified among the three sample categories, fresh and frozen, or samples purchased during the cold or warm season.

These studies provide data on prevalence of bacteria in retail ground beef offered for sale in Saskatchewan and compare differences between samples presented to the consumer as originating from federally regulated or provincially licensed facilities, locally licensed facilities, or repackaged and processed directly at a retail outlet. The information on baseline levels of bacteria in retail ground beef and the comparisons among different categories can be used in prioritising food safety improvement efforts in Saskatchewan.

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ORIGINAL CONTRIBUTION

The original concept of this study was created by Dr. Cheryl Waldner. I was involved in developing the study design and writing funding proposals. All but a few of the early samples were collected by me as an integral part of the field CIPARS sampling.

Most of the conventional microbiology work (sample processing, preparation for cryopreservation, aliqouting, TAPC and TEPC) was performed at a commercial laboratory (SunWest Food laboratory Ltd., Saskatoon, SK). Sixty five samples were processed and tested by me in the epidemiology laboratory at the WCVM.

All molecular microbiology work (total bacterial DNA extraction, real-time qPCR with universal bacterial 16S rRNA target, detection of *stx1*, *stx2*, and *eae* targets in conventional PCR, detection of *Campylobacter* genus specific DNA in conventional PCR, and detection of *Campylobacter* species-specific DNA in real-time qPCR) was performed by me at the Molecular Microbiology Research Laboratory of the Western College of Veterinary Medicine under supervision of Drs. Janet Hill, Bonnie Chaban, and Champika Fernando.

Validation of the bacterial virulence markers (*stx1*, *stx2*, and *eae*) detection reaction was performed by me at Dr. Wolfgang Koester's laboratory at the Vaccine and Infectious Diseases Organization (VIDO) under supervision and with assistance of Neil Rawlyk.

E. coli isolates preparation for antimicrobial resistance testing as well as an effort of additional *E. coli* recovery from the study sample pool were undertaken at the University of Guelph, ON under supervision of Nicol Janecko. These data are not included in the thesis.

All the data entry, processing and statistical analysis were performed by me under the guidance and supervision of Dr. Cheryl Waldner.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
°C	Degrees Celsius
µl	Microlitre
CA	California
CFIA	Canadian Food Inspection Agency
cfu/g	Culture forming units per gram
CI	Confidence interval
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
g	Gram
GA	Georgia
GAP	Good Agricultural Practices
GDP	Good Distribution Practices
GMP	Good Manufacturing Practices
ISO	International Standards Organization
LFZ	Laboratory for Foodborne Zoonoses
MD	Maryland
MeSH	Medical subject headlines
ml	Millilitre
MO	Missouri
n	Number
NH	New Hampshire
NY	New York
ON	Ontario
OR	Odds ratio
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PHAC	Public Health Agency of Canada
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal RNA
TAPC	Total aerobic plate count
TBL	Total bacterial load
tc/g	Target copies per gram
TEPC	Total *E. coli* plate count
USA	United States of America
USDA	United States Department of Agriculture
VBNC	Viable but not culturable

1. INTRODUCTION

Food safety is an important issue in modern public health, agriculture, food processing, public perception, and politics. Changing patterns of food production, distribution and consumption, a growing population of immune compromised people due to medical conditions or advanced age, and the emergence of new pathogens all contribute to the burden of foodborne illnesses in Canada and world-wide*(1, 8, 12, 16)*.

Consumers are becoming more interested in the safety of their food and are willing to pay a premium for products perceived as safer *(9, 14)*. Trends for consumption of locally produced foods, organic products, and foods raised in humane ways are all exploiting the idea of enhanced safety. Yet to make an informed choice, quite often the only real source of the food safety related information available to the consumer is the label legend on a retail package.

Ground beef has been historically one of the most scrutinized commodities from the food safety perspective *(2-4, 15)*. Government meat inspection is one of the long standing traditional safeguards to public health *(10, 13, 14)*. While a substantial proportion of ground beef available to Canadian consumers at the retail level is produced, packaged, and shipped directly from federally regulated meat processing facilities, there are a number of other supply channels *(11)*. For example, there are provincially licensed raw meat processing plants which are not required to have the Hazard Analysis and Critical Control Point (HACCP) systems required in federally regulated facilities. Provincial meat inspection programs are diverse and not uniformly applied

throughout Canada *(7)*. Products packaged in either a federally regulated or provincially licensed facility can be identified by information on the product label legend.

Requirements are even more diverse at the local level. For example, in Saskatchewan the operation of small abattoirs is licensed by local health regions and these licenses do not require inspection of either the live animal or meat product *(11)*. In these situations there is only the vendor's local reputation to serve as a measure of product safety assurance.

Regardless of the regulatory or licensing environment of the facility where the animal was slaughtered, final processing and custom packaging is commonly carried out at retail establishments creating the potential for the product to be exposed to additional sources of microbial contamination *(5, 6)*. Different types of meat are often handled by the same person introducing the risk of cross-contamination. Thorough cleaning of the equipment is not always completed between processing different meats. Temperature violations are possible as not all retail facilities refrigerate their meat cutting rooms. Though most retailers are supplied from federally regulated or provincially licensed meat processing facilities, product source information cannot be displayed on repackaged product.

Despite the potential differences in food safety risk associated with the scenarios described above, there is very little research to date that provides evidence to inform policy discussions comparing the risks and benefits of these different options for product handling and processing. In the present study, an opportunity was identified to compare the potential differences in bacterial levels in Saskatchewan retail ground beef originating from different supply and inspection channels as represented by the information on the label legend.

After initial review of a pertinent literature, the first chapter of the thesis describes the work done to establish a current baseline of bacterial levels in retail ground beef offered for sale in Saskatchewan. Three different techniques were employed to evaluate bacteria present in ground beef available to consumers:

- total aerobic plate count was used to enumerate the viable bacterial population in the study samples associated with overall hygienic history of the product handling, storage, and potential temperature abuse;
- total *E. coli* plate count was used to enumerate the most important fecal contamination indicator organism and consequently potential presence of pathogens; and,
- real-time quantitative polymerase chain reaction with *16S* rRNA universal bacterial target was used as a culture-independent alternative for bacterial numbers evaluation to include non-culturable flora in the estimates.

The second chapter of the thesis describes analysis to detect the presence of specific known bacterial virulence factors implicated in foodborne illnesses. Detection of three targets was performed by conventional polymerase chain reaction:

- lysogenic phages *stx1* and *stx2* known to be carried by different pathogenic *Enterobacteriaceae;*
- attachment and effacement protein intimin coding gene *eae* known to be carried by a number of groups of enteric pathogens.

The third and final chapter of the thesis evaluates the presence of *Campylobacter spp.* in Saskatchewan retail ground beef using a culture independent technique. Also the presence of

seven different *Campylobacter* species (*C. coli, C. curvus, C. fetus, C. hyointestinalis, C. jejuni, C. rectus, and C. upsaliensis*) was investigated employing real-time polymerase chain reaction with species-specific *cpn60* targets.

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2. LITERATURE REVIEW

2.1. Introduction

The Canadian food supply is rated as one of the safest in the world *(25)*. At the same time the Public Health Agency of Canada estimates that each year about four million Canadians experience domestically acquired foodborne illnesses *(62)*. These high numbers indicate that there is still a room for food safety improvements *(37)*.

Microbial contamination of raw meat is an important factor contributing to foodborne illness and zoonotic diseases *(27, 47, 54, 59, 63, 64)*. Improper household handling of raw meats during preparation and inadequate cooking are responsible for many of the foodborne illness cases in Canada each year *(26)*. The inclusion of trimmings from numerous carcasses, extensive handling, and intense mechanical processing of ground meat results in bacterial contamination levels up to 100-fold higher than in original intact cuts *(22, 54, 60)*.

Ground beef is one of the most commonly consumed meats in Canada *(48)* despite consumer awareness of potential risks *(55)*. In most cases the only information available to the consumer on product safety is the product label legend *(8)*. Public opinion surveys show that the government meat inspection information on the package provides consumers with a certain degree of confidence in the meat safety assurance *(25, 36)*.

In all jurisdictions in Canada, including Saskatchewan, there are more than one supply channel offering ground beef to consumers *(44)* and there are differences between regulatory and licensing requirements established at the federal, provincial, and local levels. Ground beef

processed and packaged at a federally regulated or provincially licensed facility usually has an establishment number and either a federal meat inspection logo (Figure 2.1) or a provincial inspection logo (Figure 2.2) on the label legend. Ground beef packaged at a locally licensed facility in Saskatchewan is not subjected to inspection, consequently there is no inspection logo on the label legend, but the business name and address of the production facility are usually indicated (Figure 2.3). And finally, there is no production facility and inspection information displayed on the label legend when grinding and re-packaging occurs "in-house" at the retail outlet (Figure 2.4). The label typically contains the retail chain identification only.

The information on the label reflects the provenance or history of the product. It is logical to hypothesise differences in the potential for exposure to a variety of risk factors that could affect product safety. This creates a natural question from the consumer's perspective of whether is there are differences in food safety risk among ground beef labelled as originating in a federally regulated or provincially licensed facility compared to product that is from a facility where there is no inspection of the animal and or final product, or product bearing no definitive inspection information *(44)*.

To assess the scope of available information pertinent to this question, a review of the recent literature was performed with the following objectives:

1. To overview the meat inspection history in Canada.
2. To identify jurisdiction, requirements, and legislative basis for federal meat inspection in Canada.
3. To identify the mandate, requirements, and legislative basis for provincial meat inspection in Saskatchewan.

4. To identify the mandate, requirements, and legislative basis for local abattoirs licensing by health regions in Saskatchewan.
5. To identify legislative basis for regulations and regulatory requirements for retail meat sales in Saskatchewan.
6. To identify meat inspection development trends in Canada and internationally.
7. To identify the potential impact of government meat inspection constraints, regulatory and economic burden on the agricultural business in Saskatchewan and changing social landscape.

The literature search for the project was done in the period from August 8 to August 24, 2011 with major revision and updated search in the period from March 14 to March 20, 2012 and again during August 9 to 22, 2013. During entire project length, ongoing material collection occurred via subscriptions to specialized and public periodical informational resources (ProMed mail http://www.promedmail.org/, Weekly Mortality and Morbidity Report http://www.cdc.gov/mmwr/, and Canadian Broadcasting Corporation http://www.cbc.ca/news/health/). The search was performed using the University of Saskatchewan library web portal. The primary search was done on the Web of Knowledge (Thomson Reuters, New York, NY, USA) research platform version 5, that incorporates simultaneous access to key scientific resources: Web of Science, Chinese Science Citation Database, Current Contents Connect, Dervent Innovations Index, BIOSIS Citation Index, BIOSIS Previews, Biological Abstracts, CABI, CAB Abstracts, Global Health, Inspec, Medline, Food Science and Technology Abstracts, Zoological Record, Journal Citation Reports, FSTA – Food Science Technology Abstracts, and Essential Science Indicators. The National Centre for Biotechnology Information (National Library of Medicine, Bethesda, MD, USA) Medical

Subject Headlines (MeSH) definition database keywords were used to construct search parameters for some parts of the literature search. Google Scholar (Google Inc., Mountain View, CA, USA) (http://scholar.google.ca/) search engine and Wikipedia (Wikimedia Foundation Inc., San Francisco, CA, USA) (http://www.wikipedia.org/) free online encyclopedia were used for routine retrieval of general information and terms clarification.

Literature search results were handled using EndNote Web (Thomson Reuters, New York, NY, USA) reference management software. Literature search keywords an MeSH terms are presented in Appendix A.

2.2. The history of meat inspection

Meat inspection is one of the cornerstones of food safety. Historical observational evidence of meat related infectious disease transmission led to the development of customs, beliefs, and later rules on animal slaughter and preparation of meat for human consumption *(29)*.

The first evidence of animal slaughter regulations were described as early as 200 A.D. in Hebrew religious texts *(28)*, though there are some similar artefacts found in much earlier ancient Egyptian manuscripts. Later, the Roman Empire developed a code of practice for both slaughter house operations and retail meat sales *(33)*.

In many instances cultural and religious beliefs evolved as a surrogate of meat hygiene code. For example there are taboos on pork consumption in both Islam and Judaism prescribed by food rules (*kosher* and *halal* respectively)*(29)*. These different religions readily agree on proclaiming swine as "unclean" and complete prohibition of pork consumption. Free roaming pigs of ancient times were vectors of a variety of zoonotic diseases including deadly

trichinellosis. This taboo based on empirical findings received scientific justification only many centuries later by fundamental works of Virchow *(53)*.

Later on, meat hygiene and safety regulations evolved along with establishment of other state institutions. Elements of compulsory meat inspection can be identified in various countries as each society attained sufficient structural organisation and enforcement abilities *(43)*. The first Medieval system of compulsory slaughter inspection was documented in Augsburg (Germany) in 1276 *(42)*. As early as in XIII century self –governing guilds in Italian cities required butcher shops to renew their licenses annually and provide means for meat inspection. *(9)*. Prototypes of modern meat inspection were established in many countries in the 19th century, and Australia was the first to effectively enforce a national abattoir licensing and meat inspection system in 1840 *(40)*.

The first efforts to establish state meat inspection in Canada were made as early as 1706 when meat inspection law was passed in New France creating a pioneer precedent in North America *(28)*. The *Act to Regulate the Packing, Curing and Inspection of Beef and Pork in Upper Canada* of 1805 was extended to all jurisdictions of the Dominion of Canada in 1873 after confederation. However, Montreal was the only place in Canada by 1884 that had meat inspectors *(28)*.

By the end of 19th century industrialization of the meat packing industry and rapid acceleration of the international meat trade created the need for standardized government meat inspection both in North America and Europe *(39)*. In 1899, the International Veterinary Congress produced a resolution on meat inspection encouraging legislative processes in all countries involved in international meat trade *(49)*.

2.3. Federal meat inspection

The Meat and Canned Foods Act and associated regulations took effect in Canada on September 3, 1907 and was the basis to create the Meat Inspection Division of the Health of Animals branch of the federal Department of Agriculture *(28)*. Under these regulations, all establishments involved in export and interprovincial trade were mandated to conduct ante- and post-mortem inspection of slaughtered cattle, sheep, swine, goats, game, and poultry. Basic sanitation requirements were prescribed by the regulations as well *(28)*.

The Meat and Canned Foods Act with changes and amendments was the legislative basis for meat inspection in Canada for over 50 years until *The Meat Inspection Act* came into effect in 1959. *The Meat Inspection Regulations* were subsequently adopted in 1990 *(8, 10)*. By 1957 more than 75% of all cattle, swine, and sheep slaughtered commercially in Canada were subjected to federal meat inspection *(28)*. The combined efforts of the Meat Inspection Division and Contagious Diseases Division of the Health of Animals Branch of the Department of Agriculture led to significant progress in the control of two important zoonotic diseases: bovine tuberculosis and bovine brucellosis *(28)*.

Increasing complexity of the market and trade relations were recognized in a series of government reports between 1970 and 1985 and ultimately led to creation of the Canadian Food Inspection Agency (CFIA) in 1997*(35)*. Currently, the *Food and Drugs Act*, 1985 is the main legislative document used by CFIA to regulate food safety including specific requirements on inspecting and labelling foods *(7)*.

As of August 2013, there were no facilities in Saskatchewan holding a federal license for cattle slaughter*(20)*. There were three cattle processing establishments participating in the

Federal/Provincial/Territorial Interprovincial Meat Trade Pilot Project: Drake Meat Processors - Drake, SK; Western Prime Meat Processors - Weyburn, SK; Diamond 7 - Lloydminster, SK *(18)*. A total of 17 medium-sized provincial abattoirs in Canada were selected to participate in the project that started in 2011 with objective to transition towards meeting federal requirements for interprovincial trade.

Federal meat inspection procedures are outlined by the Meat Programs system of technical requirements and include:

- registration and inspection of slaughter, processing and storage establishments;
- delivery and renewal (or conditional renewal) of operator's license;
- HACCP system and control programs verification;
- ante and post mortem inspection;
- sampling and laboratory testing for residues;
- recipe and labeling registration;
- fair labeling and recall verification;
- inspection of imported meat products;
- inspection of meat products for Canadian and export markets; and
- export certification.

The mandatory requirement to develop a HACCP system at every federally registered establishment was introduced in 2005 *(24)*.

In summary, the federal meat inspection program is based on the comprehensive legislative foundation and requires the development and implementation of a HACCP system at participating facilities *(35, 44)*.

2.4. Provincial meat inspection in Saskatchewan

Besides the federal mandate to insure meat safety and safeguard public health, the *Constitution Act* of 1867 provides powers to other levels of government in this area. Sections 91, 92, and 95 of the act stipulates concurrent jurisdiction of federal and provincial governments over public health and agriculture *(1, 35)*. However, on the provincial level practical implementation of the constitutional responsibilities regarding meat inspection did not occur till the 1960s*(28)*.

As of today, there are three provincial acts and corresponding regulations governing the animal slaughter and meat processing in Saskatchewan *(44)*:

- *The Public Health Act*, 1994, *The Sanitation Regulations*, 1964, and *The Public Health Officers Regulations*, 1997, which provides powers to public health inspectors to conduct inspections and defines basic requirements for safe food *(2, 11, 12)*;
- *The Diseases of Animals Act*, 1966 and *The Regulations Governing the Inspection of Meat in Domestic Abattoirs*, 1968, which outlines standards for the humane treatment of animals, procedures before, during, and after slaughter, the disposal of carcasses and waste, and the safe handling of meat products *(3, 5)*;
- *The Animal Products Act*, 1978, giving inspectors the power to search, seize, inspect, or detain animals or animal products *(4)*.

Saskatchewan Department of Agriculture oversees the inspection of the animal slaughter plants, slaughter, and carcass processing under the Saskatchewan Domestic Meat Inspection Program *(56)*. This program provides voluntary inspection of abattoirs and meat processing plants selling their products within the province of Saskatchewan. If a slaughter plant participates in the program, then it is not required to be locally licensed and undergo inspection by the Ministry of Health as described below *(44)*. Under the Domestic Meat Inspection Program participating establishments request ante-mortem slaughter animal and post-mortem carcass inspection which is currently provided through a contract between the Saskatchewan Ministry of Agriculture and the Canadian Food Inspection Agency. As of August 2013, this agreement is set to expire December 31, 2013 *(44)*. Currently, 11 slaughter facilities are provincially inspected for compliance with *The Regulations Governing the Inspection of Meat in Domestic Abattoirs*, 1968 and related standards *(44, 50)*. In the 2011 – 2012 year these facilities processed over 5000 cattle *(44)*.

In summary, the provincial meat inspection program in Saskatchewan is voluntary, includes basic ante- and post-mortem animal inspection, but does not impose on participants the requirement to develop a HACCP system.

2.5. Local animal slaughter and meat processing regulations in Saskatchewan

Saskatchewan Ministry of Health overseas the inspection of slaughter plants that operate under a local health region license. Under *The Public Health Act*, 1994 and *The Sanitation Regulations*, 1964, section 22, this licensing is mandatory for all slaughter plants that are not inspected under the Domestic Meat Inspection Program or Federal Inspection *(2, 11)*. The same regulations specify that meat sold commercially must come from an inspected slaughter facility.

However, it is not mandated to perform ante-mortem inspection of the slaughtered animals or post-mortem inspection of carcasses or meat. Only minimal sanitation guidelines are stipulated by licensing requirements and only annual inspection is required *(52)*. Consequently, commercial trade of uninspected meat is legally permitted via this channel in Saskatchewan *(44)*. There are also no requirements to collect information on volumes of meat processed and sold by these establishments and therefore the information on this subject is not available. Public health inspectors with regional health authorities inspected 76 Saskatchewan meat processing establishments in 2011 – 2012 *(51)*. No information is available on number of animals processed at these facilities *(44)*.

In summary, Saskatchewan local health region licensing provides only registration of the facilities involved in animal slaughter and meat processing, but no actual product inspection.

2.6. Retail meat sales regulations in Saskatchewan

Regulation of raw meat and processed meat products sales in Saskatchewan falls under three jurisdiction levels. On the federal level, retail meat sales are subject to the *Food and Drugs Act (7)*, the *Consumer Packaging and Labelling Act (6)*, and the *Consumer Packaging and Labelling Regulations (17)*. For commercial sale of meat within the province regulations are imposed only on the production facility, but not the product. It is mandatory to inspect the plant where the animal is slaughtered, but it is not mandatory to inspect the meat produced *(44, 52)*.

On the municipal level, the larger population centers in Saskatchewan (Regina, Moose Jaw, and Saskatoon) previously had by-laws prohibiting sale of uninspected meat. Under the increasing pressure of the consumers interested in buying locally produced foods these regulations were abolished as of August 2013; currently no such by-laws are in effect *(41)*. There

are no regulations in Saskatchewan pertinent to meats sold directly from the producer to the final consumer referred to as “farm gate sales”; therefore, this practice is permitted. Consequently, there is no requirement for meat from farm gate sales to be inspected.

Comparison of the legislative approaches towards raw meat sales regulations in different jurisdictions of Canada is presented in Table 2.1.

There are no other regulatory requirements to retail meat packaging, labelling, and sales in Saskatchewan other than stipulated by the *Consumer Packaging and Labelling Act* on the federal level and the *Public Health Act* on the provincial level *(6, 11)*. Consequently, there are two different regulatory bodies involved in supervision of compliance with the legislation: Canadian Food Inspection Agency (CFIA) is responsible for enforcement of the *Consumer Packaging and Labelling Act* and local Health Regions are in charge of the *Public Health Act* and the Food Safety Regulations under it *(13)*.

Most of the responsibilities on ensuring food safety in retail meat sales in Saskatchewan are under the authority of the local health regions. Considering the finite resources and a broad range of tasks assigned to each health region, inspection and enforcement efforts are prioritised on the basis of public health risks. A retail facility with a meat department performing cutting and processing is inspected based on its safety rating from once a month to once every twelve months *(19)*. Inspection requirements are outlined in the Sanitation Regulations and the Food Safety regulations with emphasis on premises cleanliness, pest control, hot and cold water supply, sewage management, ventilation, lighting, appropriate usage of designated areas, equipment cleanliness, refrigeration, and food sourcing records *(13)*.

2.7. Future development trends of meat inspection

The early history of meat inspection identifies consumer protection and control of infectious diseases as the major justifications for the economic and organizational burden *(21, 49)*. The model for meat inspection developed during late 1800s and early 1900s was based on scientific works defining public health threats and control strategies for tuberculosis and brucellosis *(43, 45)*. However, from the early days meat inspection has also been used as a political tool and non-tariff barrier in international trade *(39)*.

Today most of the major meat-associated zoonoses of historical importance in North America and many developed countries around the globe are virtually eliminated or well-controlled in domestic livestock; production standards and quality control systems (HACCP, ISO), as well as good business practices guidelines (GAP, GMP, GDP), are providing mechanisms and incentives to safeguard food quality and safety; and comprehensive agreements govern international trade *(31, 34)*. However, new challenges to meat inspection on the organizational side have arisen with changes in animal production practices, industrialization and centralization of meat processing, and globalization of international food supply and associated distribution chains *(57)*.

No lesser are the challenges on the technical side of meat inspection. Traditional reliance on the "poke and smell" organoleptic assessment methods have limited use for the detection of emerging pathogens in meat *(31)*. Massive outbreaks and recalls related to *E. coli* O157 and other pathogens in meats dramatically changed approaches to meat inspection and shifted emphasis to application of rapid laboratory methods *(65)*. The current approach to ground beef inspection is based on the Good Manufacturing Practices (GMP) and Hazard Analysis Critical Control Point (HACCP) principles *(14)*.

A separate set of challenges to meat inspection is presented by rising demands for organically produced, ethnic, local, and specialty foods *(30)*. Traditional meat inspection practices are difficult to apply to these market models and each of these categories possesses a variety of unique risks to food safety *(58)*. For example, increased immigration from Africa created a significant population in Canada willing to pay premium for bushmeat (game animals meat from tropical forests), which has resulted in the emergence of a niche market. There are reports from the last 10 years of regular trading occurring in Toronto and Montreal. Since this activity is not legal, the true magnitude of the problem is unknown; however, there are estimates that more than 25 million kilograms of bushmeat in smuggled into the USA alone annually *(32)*.

Future development and modernization of meat inspection and food safety regulations require sound assessment of current risks as well as analysis of potential emerging risks *(57, 58)*. There have been efforts to reduce both the financial and administrative burden of state meat inspection programs at various times in different jurisdictions throughout the world. The first documented precedent was set in Great Britain in 1954 *(21)* and later developments in the USA *(9)* showed that the consequences of these actions can be highly controversial and poorly predictable. In a recent example, the Canadian federal government has decided that it will no longer provide inspection services within provincial programs *(44)*. Proposed changes in the USA are intended to experiment with privatisation of the government meat inspection system through the Food Safety Modernization Act. This act allows for third party certification and full inspection cost recovery by commercial inspection providers *(61)*. Canada has a parallel initiative referred to as "Safe food for Canadians" which is in the very earliest stages of legislative development *(16)*.

In summary, current meat inspection practices face numerous challenges. Advancements

in infectious disease control, industrial animal agriculture, and corporate business practices eliminated many of the original problems government meat inspection was historically addressing. At the same time, increasing complexity of global and local food markets, rising food safety standards, growing immune-compromised populations, and the emergence of new foodborne pathogens require a reliable and robust government presence to safeguard public health.

2.8. Changing social landscape of Saskatchewan and the challenge of food security

There is a marked decreasing trend in rural populations in Canada. Currently only about 1 in 5 Canadians (18.9%) live outside major centres, but the situation in Saskatchewan is different. Almost 35% of Saskatchewan residents live in rural areas although the trend to urbanization is continuing *(23, 46)*. One of the results of lower rural population numbers has been a decreasing number of meat processing facilities serving local rural markets *(44)*. Difficulties with supplying fresh meat to smaller communities are exacerbated by the inability of many communities to sustain a retail grocery store *(38)*. Additionally, a growing proportion of the population in these communities are seniors, whose capacity to travel to larger centers can be limited. Access to a local butcher shop might provide better food security, supply higher nutritive value fresh meats, and ultimately result in better overall food safety by reducing the amount of long shelf life products consumed *(38)*.

The large First Nations population of Saskatchewan also presents unique opportunities and challenges to ensure the safety of traditional foods. For example, growing number of elderly First Nations people are moving into residential care facilities. Many retain their tastes for wild meats *(38)*, but legislated meat inspection for these products is problematic from a number of

perspectives. First of all, the locations of these care facilities are usually remote from major centres where inspection staff are stationed *(30)*. Secondly, there are no central processing and distribution facilities for meat which can only be harvested locally *(30)*. If an acceptable meat inspection process was available local butcher shops would be able to provide these much needed services *(38)*.

Support of a full-time operating butcher shop by a small population base is problematic. Small business volume limits equipment upgrading and expenditures including those for sanitation and food safety *(44)* therefore additional burdens related to meat inspection in small-scale animal slaughter and processing facilities should be carefully evaluated *(38)*. Small facilities are disadvantaged by challenges related to the economy of scale and uncertain business volumes. Even relatively small additional operating expenses can impact the viability of the business, and losing a butcher shop for a rural community can potentially result in decreasing food security and negative impacts to local livestock operations *(15, 30)*.

2.9. Conclusions and rationale for this study

This review describes the current legislative basis, organizational structure, and jurisdictional division among the three levels of government for meat inspection in Saskatchewan. There are substantial differences among meat regulations at the federal, provincial, and local government levels in Canada. Retail meat sales in Saskatchewan and specifically requirements for re-packaging and re-processing of ground beef at the point of sale are not closely regulated.

Despite the differences in provenance and inspection history of retail ground beef available to Saskatchewan consumers through different marketing channels, there is no

information in the available literature comparing food safety attributes, such as microbial levels, among the different consumer options. These data are needed for critical evaluation of existing animal slaughter, meat processing, and retail marketing practices in Saskatchewan in order to ensure the availability of safe food and the promotion of local animal agriculture.

Based on the identified knowledge gaps, this study was designed to address the following research objectives:

- To establish a current baseline of bacterial levels in Saskatchewan retail ground beef;
- To compare bacterial levels in retail ground beef produced and packaged under different inspection regimes;
- To describe the occurrence of *Enterobacteriaceae* virulence markers in Saskatchewan retail ground beef;
- To compare the frequency of *Enterobacteriaceae* virulence markers in retail ground beef produced and packaged under different inspection regimes;
- To describe the occurrence of *Campylobacter* spp. in Saskatchewan retail ground beef.

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Figure 2.1 Retail ground beef package with federal government inspection information on the label legend

Figure 2.2 Retail ground beef package with provincial meat inspection information on the label legend

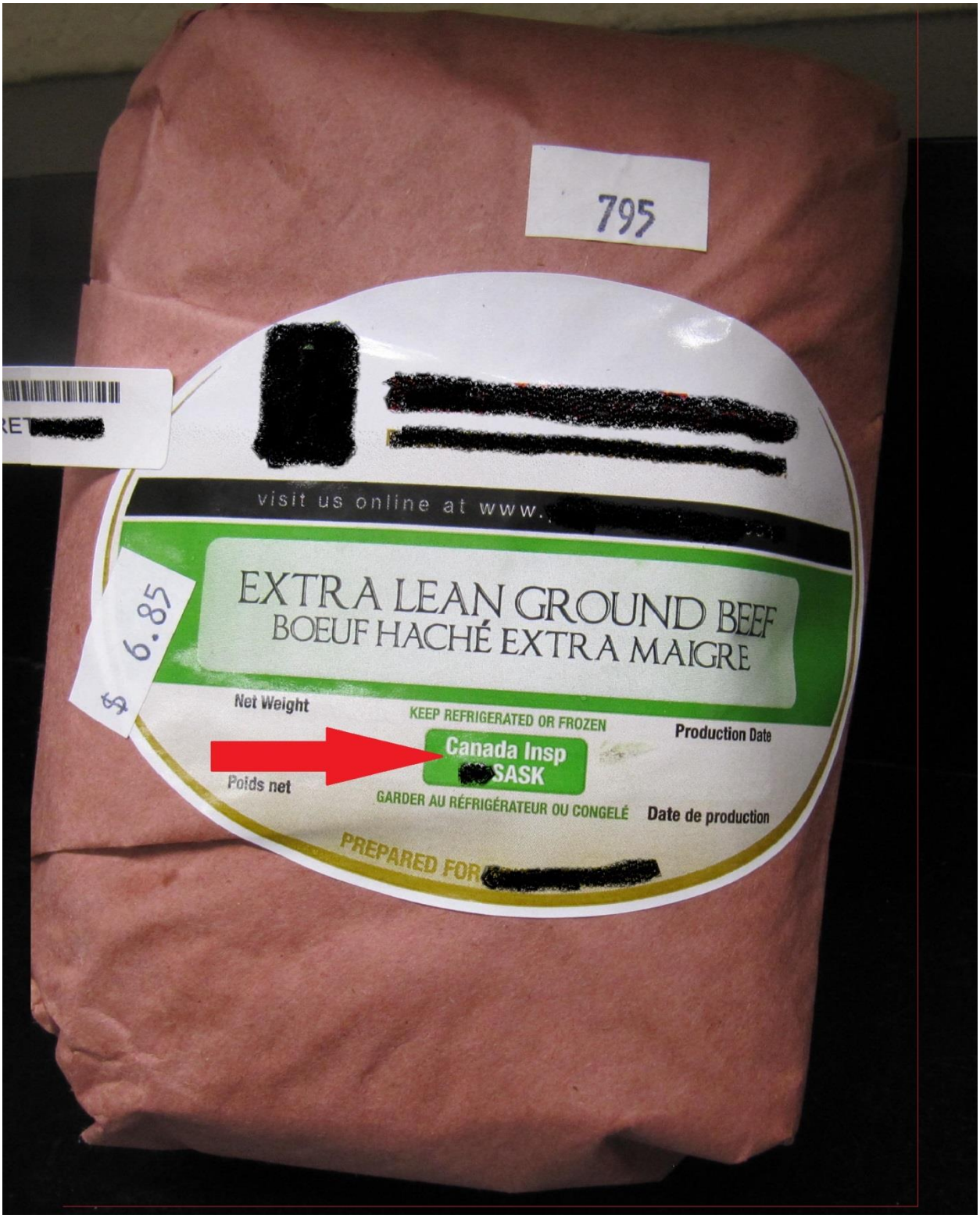

Figure 2.3 Retail ground beef produced and packaged at a locally licensed facility

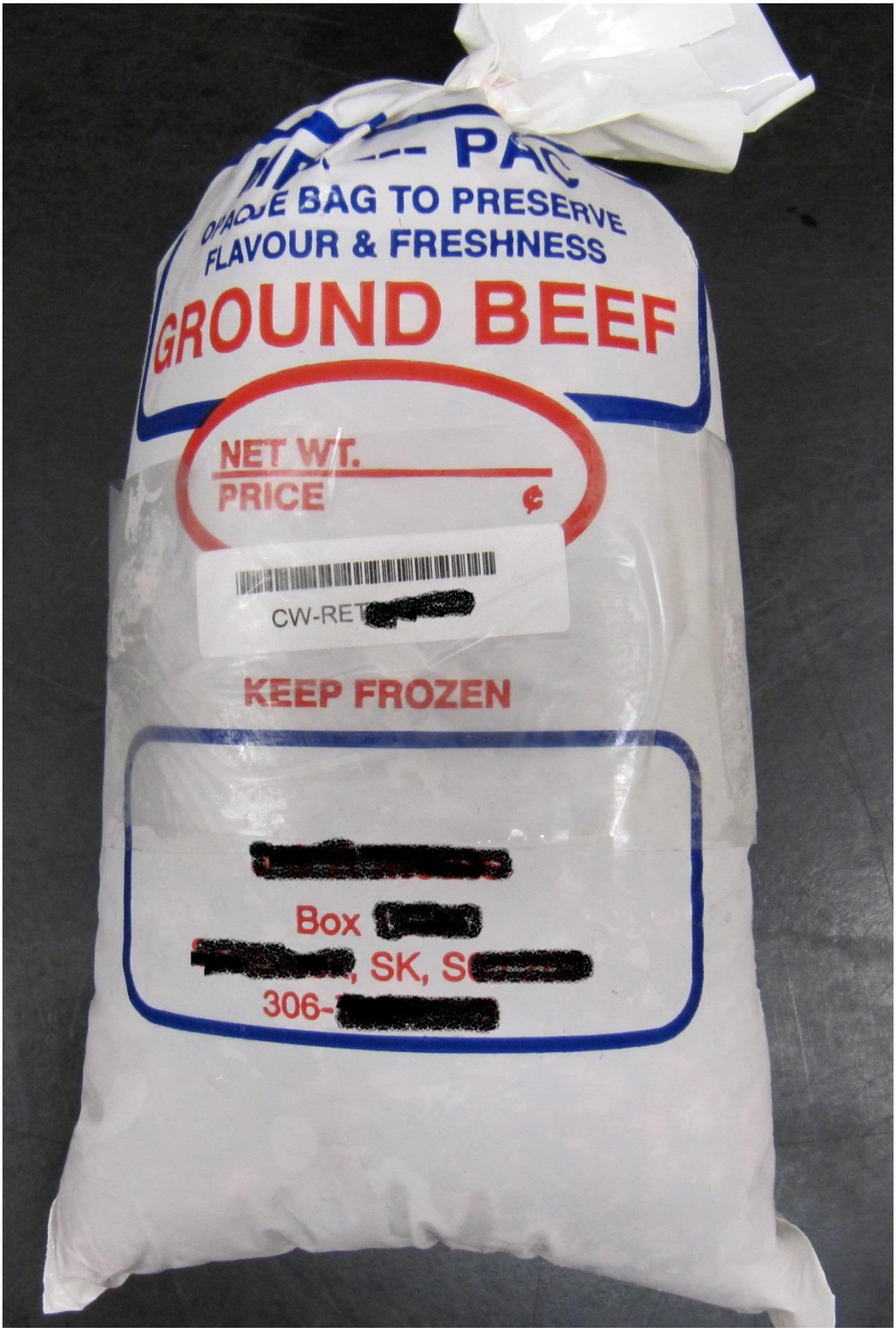

Figure 2.4 Ground beef repackaged at a retail outlet

Table 2.1 Varied approaches to regulating raw meat inspection and sales in Canadian jurisdictions (adopted from Lysyk, 2012)

	British Columbia	Alberta	Saskatchewan	Manitoba	Ontario	Quebec	New Brunswick	Prince Edward Island	Nova Scotia	New Found Land and Labrador
Ministry of Agriculture		X	X	X	X	X			X	X
Ministry of Health	X		X				X	X		
Inspect all meat before sale	Yes except remote locales	Yes	No	Yes except poultry	Yes	Yes	No	Yes cold carcass	Yes except farm gate	No
Allow farm gate sales	No	No	Yes	No except poultry	No	No	No	No	Yes	Yes

2.11. Appendix A. List of Medical Subject Headlines (MeSH) terms and keywords used for literature search

Food contamination

Food inspection

Food microbiology

Foodborne diseases

Foodborne illnesses

Foodborne zoonoses

Ground beef

Meat inspection

Meat microbiology

Retail meat

Small abattoir

3. COMPARISON OF BASELINE BACTERIAL LEVELS IN RETAIL GROUND BEEF ORIGINATING FROM DIFFERENT REGULATORY, PROCESSING AND PACKAGING ENVIRONMENTS

This chapter presents baseline bacterial levels in retail ground beef as enumerated by three different laboratory methods. The measures were analysed and compared among three established study groups. Measures of bacterial levels investigated in this study were intended to evaluate general cleanliness and potential history of storage temperature abuse, but alone were not sufficient to evaluate potential public health risks.

Trokhymchuk, A., C. Waldner, S. P. Gow, C. Chaban, and J. E. Hill. 2013. Comparison of baseline bacterial levels in retail ground beef originating from different regulatory, processing and packaging environments. *J. Food Prot.* Accepted for publication.

Trokhymchuk and Gow organized samples collection. Trokhymchuk and Waldner performed data analysis and manuscript writing, organized conventional microbiology laboratory tests. Trokhymchuk, Chaban and Hill performed molecular laboratory tests.

3.1. Introduction

In a recent survey, approximately 97% of the Canadian meat-buying population reported eating beef with average annual per capita consumption between 16 and 28 kg *(23, 25)*. There were no available data regarding consumption of specific beef cuts for Canada. However, Taylor et al. estimated that more than 50% of all beef in the United States is sold as hamburger *(29)*, which is a recognized consumer favourite at both retail and restaurants *(21)*.

Bacterial contamination of beef can occur during slaughter and initial processing associated with transfer of organisms present on the hide, in the gastrointestinal tract, and in the feces of the animal *(27)*. Subsequent exposure to storage temperatures above 4°C and contamination during downstream processing and repackaging steps can further increase bacterial numbers in raw meat *(8)*. Bacterial numbers found in ground beef compared to intact cuts can be increased by 2 to 3 $\log_{10}$ per gram of meat with as a result of the inclusion of external trim, mixing of material from a large number of carcasses, and intensive mechanical processing *(27)*.

Human exposure to bacteria in beef can occur through cross contamination of other food products or consumption of raw or undercooked meat. Despite the media attention received as a result of numerous widely publicized *E. coli* O157:H7 outbreaks starting from 1993 *(12)*, Taylor et al. showed that at least 16% of American consumers consciously choose not to fully cook their hamburger to optimize texture and taste *(29)*. The USDA also reported that in 5% of cases, consumers unintentionally fail to adhere to cooking times and temperatures necessary for bacteria inactivation *(30)*. According to a

United States-based study by the EcoSure group, in up to 38 % of cases, consumers failed to cook ground beef to an internal temperature of 71° C (160° F) *(13)*.

The safety of raw meat products is a responsibility shared among livestock producers, slaughter facilities, processors, retailers, and regulators. In Canada, participation in the federal meat inspection program is mandatory for slaughter and processing facilities involved in interprovincial or international trade *(10)*. Provincial legislation applies to abattoirs and raw meat processing facilities that only supply intra-provincial markets *(20)*.

Under Saskatchewan (SK) legislation, there are three options to regulate the trade of raw meat produced within the province. First, farm-gate sales of meat are allowed in Saskatchewan and there are no formal requirements or regulations for non-processed meat sold directly from a producer to a consumer if transaction occurs at the farm. For commercial animal slaughter and meat processing, an abattoir can choose to participate in the Domestic Meat Inspection program, which includes ante- and post-mortem inspection of slaughtered animals, but does not mandate Hazard Analysis and Critical Control Points (HACCP) system development *(20)*. The remaining commercial slaughter and processing facilities are licensed by local health regions. Under the licensing process, facilities should be inspected annually by the health region, but the process does not include inspection of the slaughtered animals or meat products *(20)*. Consequently, retail stores in Saskatchewan can offer meat for sale from federally regulated facilities, provincially licensed establishments, or locally licensed small abattoirs.

The package label can provide the consumer with surrogate provenance information on the regulatory or licensing environment for slaughter and processing. There is no comparable information on the transportation, storage, or conditions of repackaging history of the product after it leaves a raw meat processing facility. Meat packaged in a federally regulated or provincially licensed raw meat production facility can be identified by a standardized marking on the label referred to as the legend. Meat from local suppliers might have identifying information from the company on the product label, but this is not mandatory. There is no federally regulated or provincially licensed environment information permitted or product source information required on product reprocessed or repackaged at a retail outlet *(1)*. Differences in the regulatory and licensing requirements for meat intended for retail sale within the province of Saskatchewan have resulted in questions about the potential for variation in retail product safety *(20)*.

The presence of specific pathogens and the antimicrobial resistance of target organisms in Canadian retail ground beef have been investigated *(6, 26, 31, 34)*. However, to date no studies have examined whether measures of bacterial load vary among the different sources of retail samples or whether additional contamination results from retail-level handling and packaging practices in Canada. Previous studies in 1975, 2001, and 2005 of baseline bacterial levels in Canadian retail ground beef only included samples from federally regulated facilities *(2, 11, 14)*. Although we identified one study of baseline bacterial levels in beef carcasses from provincially licensed abattoirs *(5)*, information about bacterial levels in locally-produced ground beef and bacterial contamination following additional retail handling has not been reported in Canada.

The primary objectives of this study were to collect information describing baseline bacterial levels in retail ground beef offered for sale in Saskatchewan and to assess potential contamination differences among retail ground beef samples processed and packaged in facilities operated under different regulatory and licensing environments. Samples of retail ground beef originating from federally regulated or provincially licensed slaughter and raw meat processing facilities were compared to ground beef from slaughter and processing facilities operated under license from the local health region, and also to ground beef with no inspection or source information on the label legend suggesting the product was reprocessed or repackaged before retail sale.

3.2. Materials and methods

3.2.1. Sample collection

Small packages of ground beef (0.4-1.0 kg) (n=309) were purchased from 158 different sources, including large chain retail grocery stores, independent small grocery stores, butcher shops, and individuals representing the farm gate and 'freezer trade' in Saskatchewan. All samples were collected from May 2011 to May 2012 *(Table 3.1)*. The sampling plan was based on a strategy developed by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) *(7)*. Samples were collected from 17 of the 18 Saskatchewan Census Divisions (CD) and reflected the product available to consumers at each location. Division No.18 La Ronge, representing the northern part of the province, was not included due to the difficulty and expense associated with sample collection. The number of sampling trips to each CD and samples collected from each were proportional to the population size.

3.2.2. Sample categorization

Using information available on the ground beef package at the time of sale and, where possible, confirmed by the retailer, all samples were divided into three groups to reflect the provenance information regarding processing environment and product source *(Table 3.1)*.

The first group of samples included those packaged in a federally regulated or provincially licensed raw meat production facility, as indicated by the federal inspection symbol and establishment information or by the provincial facility information on the product label legend. Potential differences between these two categories included typically larger scale of production at federally regulated establishments and no requirement for a HACCP system in provincially licensed facilities. The decision to combine samples from both into one study category was based on the consideration of one common feature – mandatory ante-mortem slaughter animal inspection and post-mortem carcass inspection in Saskatchewan is performed by the CFIA inspectors.

The second group of samples originated from locally licensed facilities and included ground beef packaged by small abattoirs operating under a local health region license, consequently, not subjected to ante- or post-mortem inspection by provincial or federal inspectors.

The third group included samples with no provenance information available to the consumer regarding the inspection environment and included those samples with no inspection or source information on the label legend; that is, no label legend, no

identification of the local processor, or no information about the regulatory or licensing requirements at the site where the product was processed and packaged. We concluded that samples that had no label legend information, and were not from a federally regulated, provincially licensed, or locally licensed facility were in most cases repackaged by the retailer based on direct observations during the purchase of samples.

3.2.3. Sample handling and processing

Immediately after purchase, samples were labelled and placed up to eight in a cooler with one ice pack if the daily high temperature was below 20° C or two ice packs if the daily high temperature exceeded 20° C to maintain temperatures from 1 to 4° C during transportation to the laboratory. Commercial temperature loggers (Temp 100, MadgeTech Inc., Warner, NH USA) were used to monitor transit conditions for each of the 27 sample shipments. Frozen samples were thawed overnight at 4°C in a refrigerator before processing.

Each ground beef package was aseptically opened and five - 5g samples were collected from different areas of the package using a sterile metal spoon. The subsamples were then homogenized with 225 mL of buffered peptone water (acc. to ISO 6579) in a stomacher bag with a filter insert (Fisherbrand® FILTRA-BAG, Fisher Scientific, Whitby, ON, Canada) using a laboratory mixer (BagMixer®400VW, Interscience, Rockland, MA, USA) at 6 strokes per second for 60 seconds. The resulting filtered homogenate was used to inoculate total aerobic count plates, total *E. coli* count plates, and aliquots were prepared for subsequent DNA extraction for quantitative real-time polymerase chain reaction (qPCR) with universal bacterial 16S RNA gene primers.

3.2.4. Total aerobic plate count (TAPC)

Total aerobic bacterial count evaluation was performed according to MFHPB-33 "Enumeration of total aerobic bacteria in food products and food ingredients using 3M™ Petrifilm™ aerobic count plates" per the manufacturer's specifications (3M™ Petrifilm™ Aerobic Count plates 6400/6406, 3M Canada Inc., London, ON, Canada) *(32)*. Plates were incubated at 35° C for 48 hours. The lower detection limit of the TAPC was 10 cfu/g *(32)*.

3.2.5. Total *E. coli* plate count (TEPC)

Total generic *E. coli* count evaluation was performed according to MFHPB-34 "Enumeration of *E. coli* and coliforms in food products and food ingredients using 3M™ Petrifilm™ *E. coli* count plates" per the manufacturer's specifications (3M™ Petrifilm™ *E. coli* Count plates 6404/6414, 3M Canada Inc., London, ON, Canada) *(33)*. Plates were incubated at 35° C, observed after 24 hours for presence of blue colonies with gas and additionally incubated for another 24 hours. The lower detection limit of the TEPC was 10 cfu/g *(33)*.

3.2.6. Estimation of total bacterial load (TBL) by qPCR with 16S RNA gene universal bacterial primers

Total bacterial DNA extraction was performed from 0.6 mL of ground beef homogenate prepared as described above, using a commercial kit (DNeasy® Blood & Tissue Kit, QIAGEN Sciences, MD, USA) per the manufacturer's specifications. Oligonucleotide primers and reaction conditions for total bacterial load evaluation were used as described by Lee at al. with modifications reported by Chaban et al.*(9, 19)* A Bio-Rad iCycler iQ5 thermal cycler and iQ™ SYBR® Green Supermix (both from Bio Rad Laboratories, Inc., Hercules, CA, USA) were used for the reaction.

3.2.7. Statistical analysis

Laboratory results were entered into a commercial database (Microsoft Access) and transformed to $\log_{10}$ of analytical unit per gram of ground beef for the culture-based tests (TAPC and TEPC). TBL calculation was based on the starting number of target copies estimated per qPCR reaction. Samples that returned no growth on total *E. coli* count plates or where the bacterial levels were below the detection limit of the test (<10 cfu/g) were coded as having a count of 1 colony forming unit (cfu/g) to facilitate $\log_{10}$ transformation.

All analyses were completed using commercial software (SPSS ver. 21, IBM, Armonk, NY, USA). Multivariable linear regression models compared TAPC, TEPC, and TBL across the three study categories after accounting for season of collection and whether the samples were fresh or frozen at the time of purchase. Two-way interactions

were examined between all significant main effects in the model, and were included if $P<0.05$. All results are reported as differences among sample categories with 95% confidence intervals (95% CI).

3.3. Results

3.3.1. Total aerobic plate count (TAPC)

The TAPC values for all samples (n=309) ranged from $\log_{10}$ 1.7 to $\log_{10}$ 8.9 culture forming units per gram of ground beef (cfu/g), with a median $\log_{10}$ 6.0 cfu/g, 5th percentile $\log_{10}$ 2.9 cfu/g, and 95th percentile $\log_{10}$ 7.4 cfu/g *(Fig. 3.1)*.

The effect of sample state (fresh or frozen) at the time of purchase on TAPC varied with the season of purchase (warm, May 1 to October 31; cold, November 1 to April 30) (P=0.01) *(Table 3.2, Fig. 3.2)*. After accounting for state, season, and their interaction, differences in TAPC among all study groups were significant (P<0.001) *(Table 3.2, Fig. 3.2)*. TAPC was lower in samples from federally regulated or provincially licensed facilities (mean, $\log_{10}$ 4.9 cfu/g; 95% CI $\log_{10}$ 4.7 to $\log_{10}$ 5.1 cfu/g) than samples from locally licensed facilities ($\log_{10}$ 5.6 cfu/g; 95% CI, $\log_{10}$ 5.3 to $\log_{10}$ 5.8 cfu/g). Samples from federally regulated or provincially licensed facilities also had lower TAPC than samples with no inspection or source information on the label legend ($\log_{10}$ 6.3 cfu/g; 95% CI, $\log_{10}$ 6.1 to $\log_{10}$ 6.6 cfu/g).

3.3.2. Total *E. coli* plate count (TEPC)

The TEPC values for all samples (n=309) ranged from below the detection limit of the test to $\log_{10}$ 4.2 cfu/g per gram of ground beef; the 5th percentile and the median TEPC were both <10 cfu/g and the 95th percentile was $\log_{10}$ 3.2 cfu/g *(Fig. 3.1).* TEPC was above the detection limit or > 10 cfu/g in 44.7% (138/309) of the samples.

After accounting for product state at the time of purchase (*P*=0.045) and season of purchase (*P*=0.33) *(Table 3.3, Fig. 3.3),* the TEPCs of samples from federally regulated or provincially licensed facilities were significantly lower than samples with no inspection or source information on the label legend (*P*=0.01) and samples from locally licensed facilities (*P*=0.002), the latter two of which were not significantly different from one another (*P*=0.57) *(Table 3.3).* TEPC was lowest in ground beef from federally regulated or provincially licensed facilities ($\log_{10}$ 0.58cfu/g; 95% CI $\log_{10}$ 0.39 to $\log_{10}$ 0.77 cfu/g) followed by samples with no inspection or source information on the label legend ($\log_{10}$ 0.96 cfu/g; 95% CI $\log_{10}$ 0.73 to $\log_{10}$ 1.2 cfu/g), and then by samples from locally licensed facilities ($\log_{10}$ 1.1 cfu/g; 95% CI $\log_{10}$ 0.82 to $\log_{10}$ 1.3 cfu/g).

3.3.3. Total bacterial load (TBL) estimated by qPCR with 16S RNA gene universal bacterial primers

The TBL values for all samples (n=309) ranged from $\log_{10}$ 1.7 to $\log_{10}$ 11.5 target copies per gram of ground beef (tc/g). The median TBL was $\log_{10}$ 5.9 tc/g, the 5th percentile was $\log_{10}$ 2.6 tc/g, and the 95th percentile was $\log_{10}$ 8.8 tc/g *(Fig. 3.1).*

The effect of both study group (P=0.012) and the season of purchase (P=0.004) varied with the state of the sample at the time of purchase *(Table 3.4, Fig. 3.4)*. Fresh ground beef from federally regulated or provincially licensed facilities ($\log_{10}$ 5.8 tc/g; 95% CI $\log_{10}$ 5.4 to $\log_{10}$ 6.2 tc/g) had a significantly lower TBL (P=0.038) than fresh samples with no inspection or source information on the label legend ($\log_{10}$ 6.4 tc/g; 95% CI $\log_{10}$ 6.1 to $\log_{10}$ 6.8 tc/g).

Similarly, frozen ground beef from federally regulated or provincially licensed facilities ($\log_{10}$ 4.5 tc/g; 95% CI $\log_{10}$ 4.0 tc/g to $\log_{10}$ 4.9 tc/g) had a significantly lower TBL than fresh samples from federally regulated or provincially licensed facilities (P=0.001) ($\log_{10}$ 5.8 tc/g; 95% CI $\log_{10}$ 5.4 to $\log_{10}$ 6.2 tc/g), fresh samples from locally licensed facilities (P=0.001) ($\log_{10}$ 6.1 tc/g; 95% CI $\log_{10}$ 5.3 to $\log_{10}$ 6.9 tc/g), frozen samples from locally licensed facilities (P<0.001) ($\log_{10}$ 6.4 tc/g; 95% CI $\log_{10}$ 5.9 to $\log_{10}$ 6.8 tc/g), fresh samples with no inspection or source information on the label legend (P<0.001) ($\log_{10}$ 6.4 tc/g; 95% CI $\log_{10}$ 6.1 to $\log_{10}$ 6.8 tc/g), and frozen samples with no inspection or source information on the label legend (P=0.003) ($\log_{10}$ 6.0 tc/g; 95% CI $\log_{10}$ 5.1 to $\log_{10}$ 7.0 tc/g) *(Table 3.4, Fig. 3.4)*.

3.3.4. Relationship between TAPC, TEPC, and TBL

The results of all three methods were significantly correlated (P<0.001). Spearman's pairwise correlation coefficient for TBL vs. TAPC was 0.542, TBL vs. TEPC was 0.403, and TAPC vs. TEPC was 0.377. For every one unit or 1 $\log_{10}$ tc increase in TBL, TAPC increased by 0.382 $\log_{10}$ cfu (95% CI 0.317 to 0.448, P<0.001). For every

one unit or 1 $\log_{10}$ tc increase in TBL, TEPC increased by 0.225 $\log_{10}$ cfu (95% CI 0.166 to 0.284, P<0.001).

3.4. Discussion

Information available to the consumer about a beef product's slaughter, processing, and handling provenance is often limited to what is on the product label at the time of purchase. Many Canadian consumers are not aware that legislation varies among provinces in Canada and that not all retail product for local sale is subject to the same slaughter and processing regulations. New Brunswick, Newfoundland and Labrador, and Saskatchewan have licensing options for local meat supply safety control that do not require animal or product inspection *(20)*. This is the first study identified by the author to examine the potential for differences in microbial contamination based on the information available to the consumer at the time of purchase with respect to product source and subsequent processing.

The objective of the sampling plan was to represent the consumer experience when sourcing retail ground beef in Saskatchewan. Obtaining locally produced ground beef was a challenge due to the large geographical area of the province and regions of low population density. A small number of samples in this study were purchased from individual farmers via farm gate sales or from other individuals in the 'freezer trade' market; however, samples purchased through these channels were identified as being processed by locally licensed facilities. Categorization of locally produced samples in many cases depended on personal communication with the vendor, so reliability of the information was limited by the retailer's knowledge of product source and integrity.

The laboratory techniques implemented in this study were selected based on several factors. TAPC with 3M™ Petrifilm™ plates is one of the most widely used methods for evaluating the cleanliness of food and production processes in the food processing industry.*(22, 32)* In addition, TEPC with 3M™ Petrifilm™ plates is a simple method for evaluating the most important indicator group of fecal contamination *(22)*. Quantifying the total microbial DNA load by qPCR with a universal bacterial target is a well-established and reliable technique to estimate the total historical bacterial presence in a sample *(9, 19)*. We found that the results of all three methods were correlated and that TBL was a significant predictor of both TAPC and TEPC.

Baseline levels of bacteria from ground beef in this sample collection were comparable to previous Canadian and international studies *(11, 14, 24, 28)*. TAPCs observed more than 20 years ago were typically 1 to 3 $\log_{10}$ higher than more recent studies, including this one *(17)*. These findings might be attributed to the introduction of mandatory HACCP systems at federally regulated facilities, significant improvements in overall production hygiene, and better refrigeration. Conversely, decreases in the extant of direct and indirect fecal contamination as measured by number of indicator organisms (e.g., by TEPC) appears to be less pronounced, with observations similar to this study reported by Canadian studies in 1975 and 2005 *(11, 14)*.

Overall, ground beef clearly labelled as produced and packaged at a federally regulated or provincially licensed facility had the lowest observed measures of bacterial counts and historical bacterial load based on the three reported testing protocols. This finding is in agreement with the results of a comparable Swedish study investigating

bacterial contamination from ground beef produced by large industrial and small traditional facilities *(15)*.

The TAPCs reported for ground beef produced by locally licensed facilities were lower than for product with no inspection or source information on the label legend. The difference suggests the potential for additional contamination during re-packaging as well as growth of spoilage organisms associated with time and temperature abuse. However, there were no significant differences between product from locally licensed facilities and product with no inspection or source information on the label legend for either TEPC or TBL based on qPCR. TEPC is an indicator of fecal contamination, and the highest risk of fecal contamination is during slaughter and carcass dressing. TBL was reported as the simple $\log_{10}$ value. This relatively crude measurement scale was limited in sensitivity to changes of at least 0.23 $\log_{10}$ cfu TEPC or 0.38 $\log_{10}$ cfu TAPC resulting from potential additional exposure and bacterial growth during retail handling.

The techniques for assessing bacterial counts and historical load used in this study were intended to evaluate the numbers of indicator organisms present using relatively rapid and inexpensive methodologies; they were not designed or intended to determine the presence of particular pathogens. Known limitations to these culturing methods include the awareness that some bacterial species and cells that are in a viable but non-culturable state cannot be cultured on 3M™ Petrifilm™ media *(4)*, as well as the lower detection limits of the techniques that are based on a small amount of initial sample material and a significant number of serial dilutions. The qPCR method provides additional information on the cumulative history of product microbiome *(19)*, and was

useful for enumerating bacteria present in very high numbers, but could not distinguish between viable and inactivated organisms.

Other studies have examined the presence of specific pathogens in Canadian and international retail ground beef *(6, 8, 18, 26, 27, 30, 31, 34)*. Identifying specific bacteria of public health importance or screening for known bacterial virulence factors in samples from this collection would have provided additional information on the public health risks associated with either product source or retail handling. A number of other product factors, including transportation time (product offered for sale in major centres vs. remote communities), different retail chains (wholesale warehouses vs. small retailers or national chains vs. local chains), product grade (fat content), or product source (produced from mixed trim vs. specified cuts) could also be evaluated with a larger sample size to gain a more comprehensive picture of factors that could potentially influence bacterial levels in retail product.

Efforts to enhance food safety have the potential to result in marked decreases in some foodborne illnesses, as demonstrated by experience in the United States *(16)*. However, consumers continue to question the safety of their food and recent trends show the desire for more locally produced food. For example, the National Restaurant Association (USA) names "locally sourced meats" as the No.1 trend on its "What's Hot in 2013" list *(3)*. Locally sourced meat is also a potential option for improving access to fresh food for rural and remote communities in Canada, where transport times make it difficult and expensive to obtain fresh or even frozen meat from large, more centrally located processing facilities. However, policy makers face the challenge of upholding an

appropriate regulatory process for small volume processors in the face of evolving market conditions and ever increasing consumer demands. The findings of this study suggest that additional work is necessary to explore the potential differences in the risk to public health associated with both the initial processing environment and the final processing and packaging of raw meat products.

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Figure 3.1 Box plots summarizing the distribution of total aerobic plate count (TAPC), total *E. coli* plate count (TEPC), and total bacterial load (TBL) values for ground beef based on season of purchase, fresh or frozen state at the time of purchase, and source information available at the time of purchase (n= 309) (*outliers are represented as circles and color coded according to respective study categories)

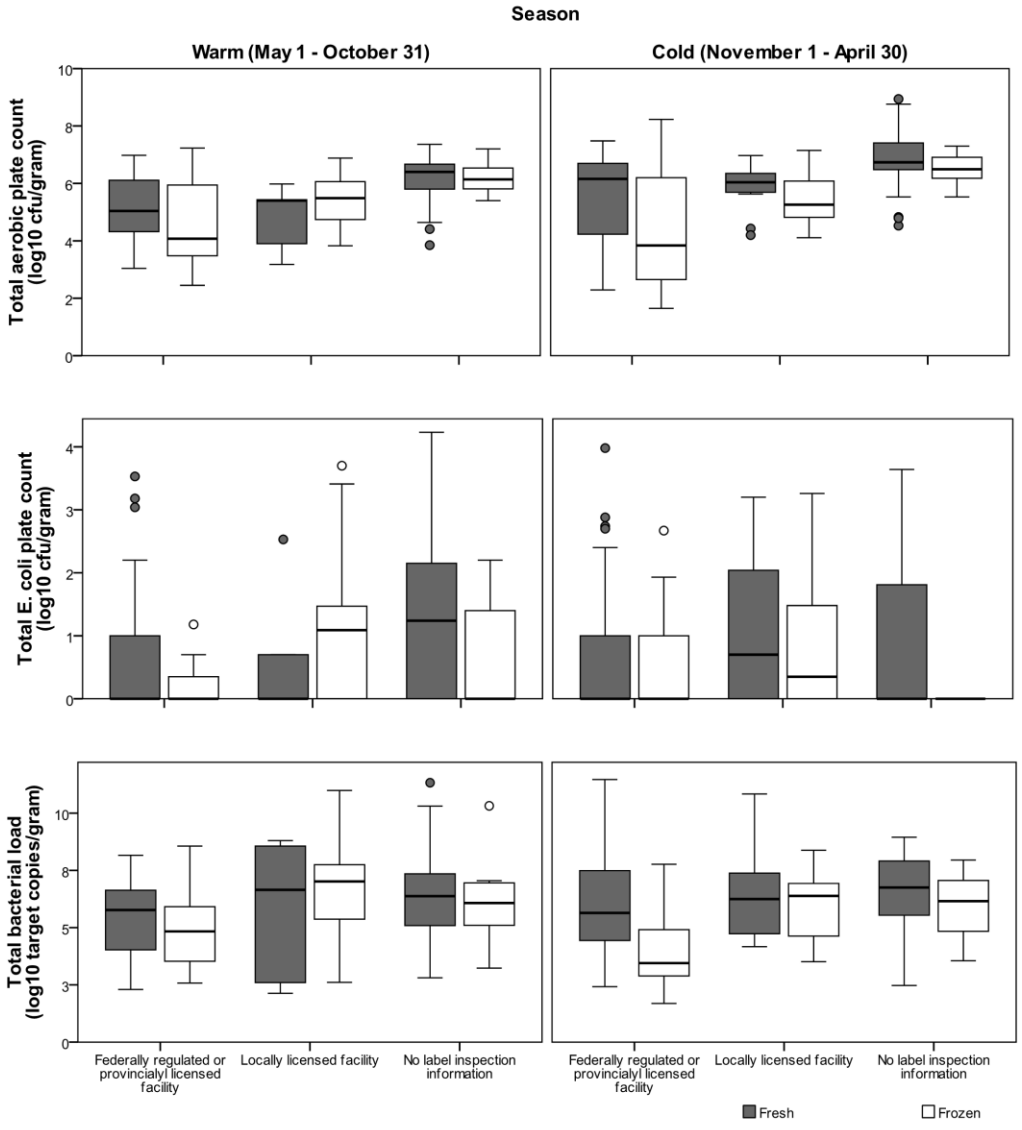

Figure 3.2 Mean total aerobic plate counts (TAPC) (log10 cfu/g) (95% confidence intervals) predicted by the final regression model including study group, sample state (fresh or frozen), season of sample purchase (warm, May 1 to October 31; cold, November 1 to April 30)

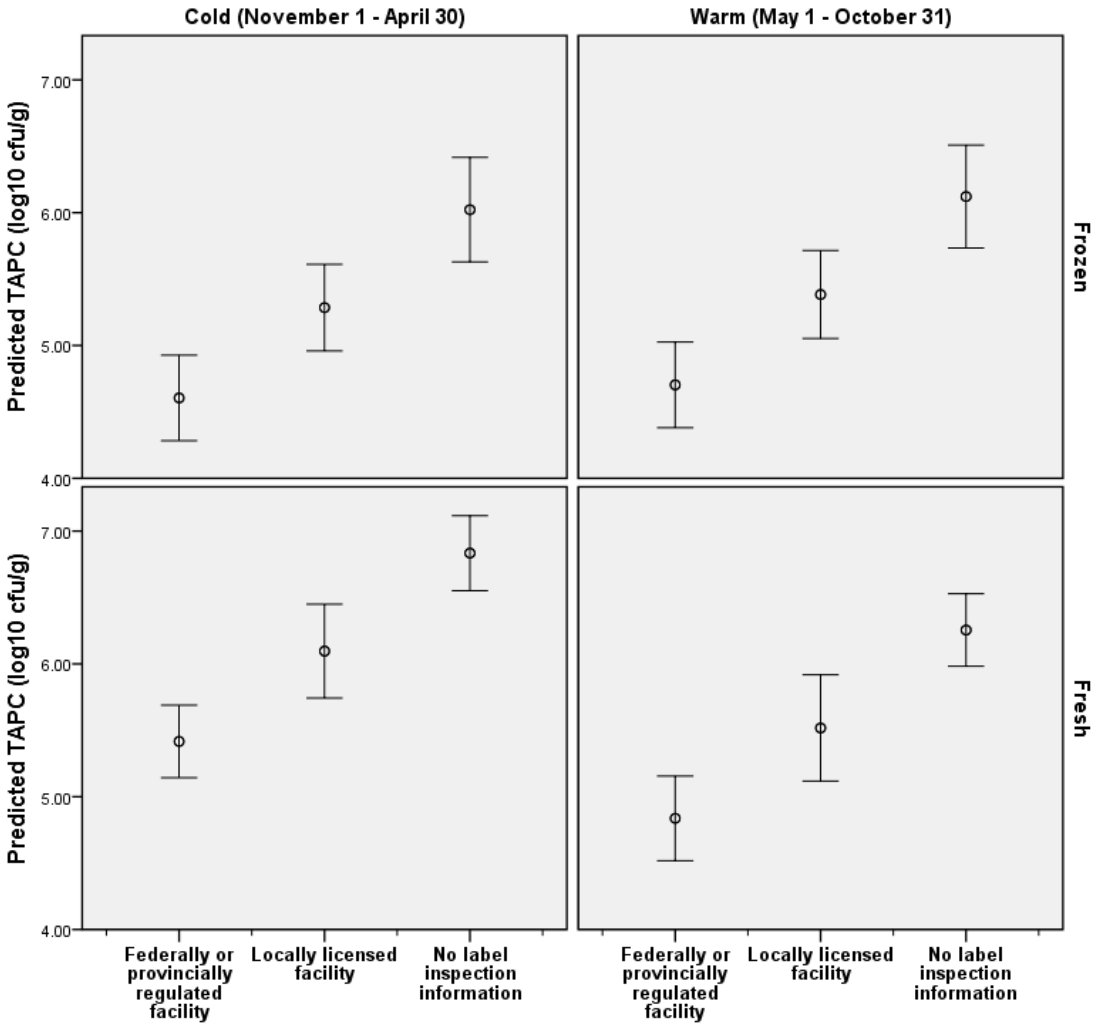

Figure 3.3 Mean total *E. coli* plate counts (TEPC) (log10 cfu/g) (95% confidence intervals) predicted by the final regression model including study group, sample state (fresh or frozen), and season of sample purchase (warm, May 1 to October 31; cold, November 1 to April 30), and interaction between state and season (n= 309)

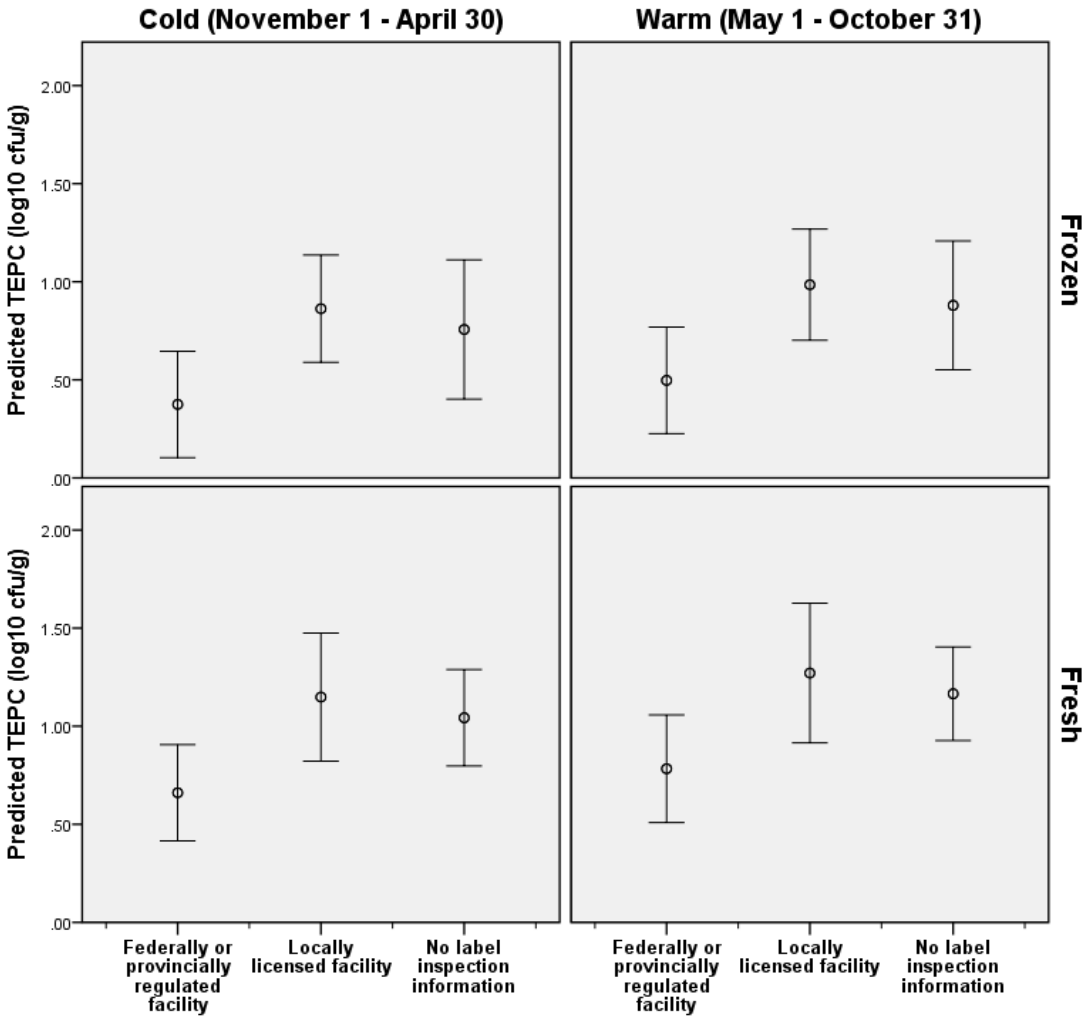

Figure 3.4 Mean total bacterial load (TBL) (log10 tc/g) (95% confidence intervals) predicted by regression model including study group, sample state (fresh or frozen), season of sample purchase (warm, May 1 to October 31; cold, November 1 to April 30), and interactions between sample state and study group as well as sample state and season of sample purchase (n= 309)

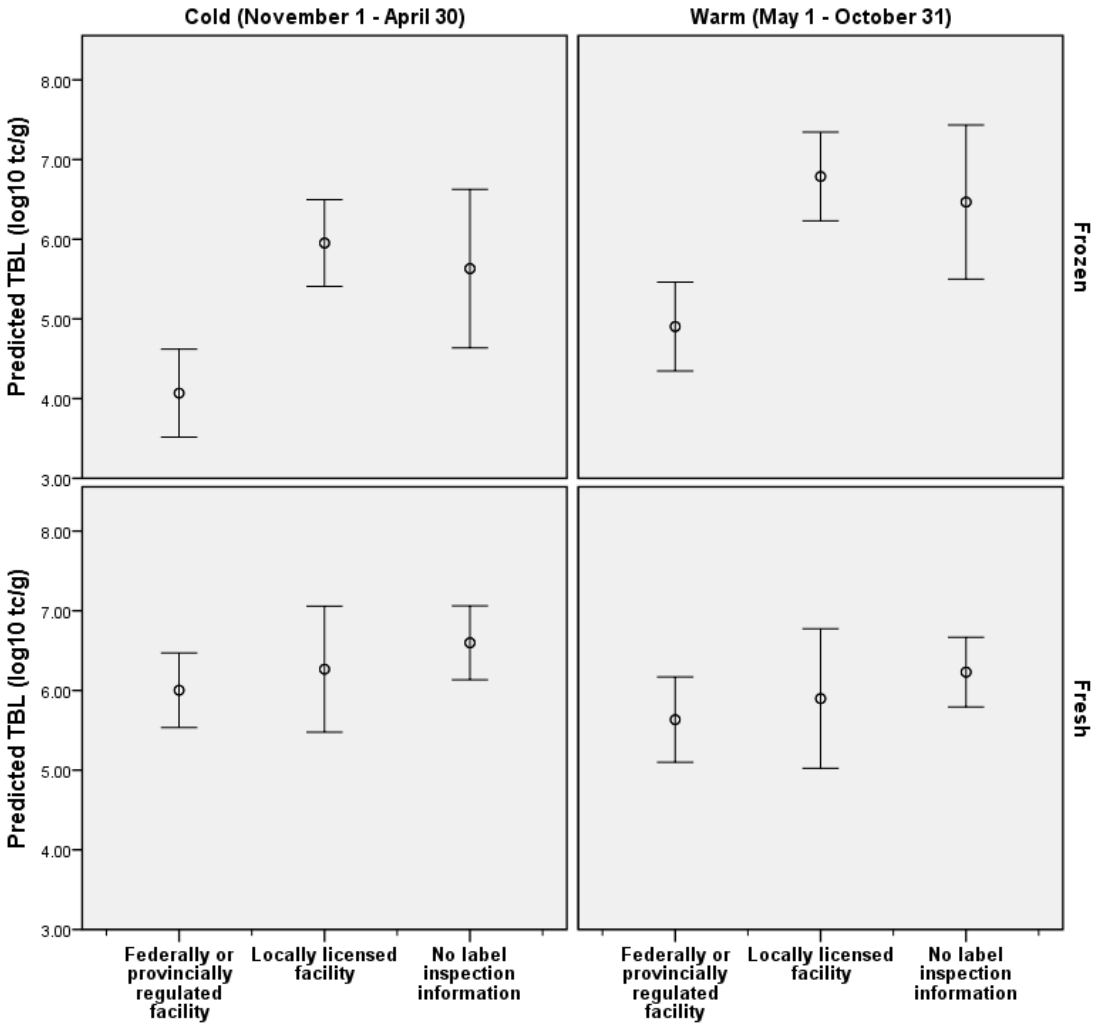

Table 3.1 Summary of product source information, season of purchase, and product state at the time of purchase for retail ground beef samples

		Collection season			
		Warm (May 1-Oct 30)		Cold (Nov 1-Apr 30)	
Sample categories	Total	Fresh	Frozen	Fresh	Frozen
Federally regulated or provincially licensed facility*	125	26	28	42	29
Locally licensed facility	78	5	28	15	30
No label inspection information	106	50	8	42	6
Total	309	81	64	99	65

* samples from federally regulated facilities (n=92), samples from provincially licensed facilities (n=33)

Table 3.2 Summary of absolute differences in total aerobic plate count (TAPC) (log_{10} colony forming units per gram) among samples from federally regulated or provincially licenced facilities, locally licensed facilities, and no inspection or source information on the label legend after accounting for the interaction between season of collection and sample state at the time of purchase* (n = 309)

Summary of pairwise comparisons among different source types and among state and season interactions		Mean difference in TAPC (log_{10} cfu/g)	95% CI		*P* value
			Lower (log_{10} cfu/g)	Upper (log_{10} cfu/g)	
Federally regulated or provincially licensed facility	Locally licensed facility	-0.68**	-1.01	-0.35	**<0.001**
	No label inspection information	-1.42	-1.73	-1.10	**<0.001**
Locally licensed facility	No label inspection information	-0.74	-1.12	-0.35	**<0.001**
Fresh/ Warm season	Frozen/ Warm season	0.13	-0.27	0.54	0.52
	Fresh /Cold season	-0.58	-0.92	-0.24	**0.001**
	Frozen/ Cold season	0.23	-0.18	0.64	0.27
Frozen/ Warm season	Fresh/Cold season	-0.71	-1.09	-0.34	**<0.001**
	Frozen/ Cold season	0.10	-0.29	0.49	0.62
Fresh/ Cold season	Frozen/ Cold season	0.81	0.43	1.19	**<0.001**

The final model included fixed effects for source type, state, season, and state season interaction.

** Example interpretation: The mean (95% confidence intervals) of TAPC for samples from federally regulated or provincially licensed facilities is 0.68 log_{10} cfu/g lower than for samples from locally licensed facilities. Differences and their respective 95% CI are derived from model based estimates of the mean value for the category in column 2 subtracted from the mean value estimated for categories listed in column 1 after correcting for differences due to state, season, and state* season interactions.

Table 3. 3 Summary of differences in total *E. coli* plate count (TEPC) (log_{10} colony forming units per gram) among samples from federally regulated or provincially licenced facilities, locally licensed facilities, and no inspection or source information on the label legend after accounting for season of collection and sample state at the time of purchase (n = 309)

Summary of pairwise comparisons among different source types, between states and between seasons		Mean difference in TEPC (log_{10} cfu/g)	95% CI		*P* value
			Lower (log_{10} cfu/g)	Upper (log_{10} cfu/g)	
Federally regulated or provincially licensed facility	Locally licensed facility	-0.49**	-0.80	-0.17	**0.002**
	No label inspection information	-0.38	-0.68	-0.09	**0.011**
Locally licensed facility	No label inspection information	0.11	-0.26	0.47	0.57
Fresh	Frozen	0.29	0.01	0.57	**0.045**
Warm season	Cold season	0.12	-0.12	0.37	0.33

*The final model included fixed effects for source type, state, and season.

** Example interpretation: The mean (95% confidence intervals) of TEPC for samples from federally regulated or provincially licensed facilities is 0.49 log_{10} cfu/g lower than for samples from locally licensed facilities. Differences and their respective 95% CI are derived from model based estimates of the mean value for the category in column 2 subtracted from the mean value estimated for categories listed in column 1 after correcting for differences due to state and season.

Table 3.4 Summary of absolute differences in total bacterial load (TBL) (log_{10} target copies per gram) among samples from federally regulated or provincially licenced facilities, locally licensed facilities, and no inspection or source information on the label legend after accounting for the interactions between both study group and then season of collection with sample state at the time of purchase (n = 309)

Summary of pairwise comparisons among different source type and state interactions and among state and season interactions		**Mean difference in TBL (log_{10} tc/g)**	**95% CI**		***P* value**
			Lower (log_{10} tc/g)	Upper (log_{10} tc/g)	
Federally regulated or provincially licensed facility/Fresh	Federally regulated or provincially licensed facility/Frozen	1.33**	0.70	1.96	**<0.001**
	Locally licensed facility/Fresh	-0.26	-1.15	0.62	0.56
	Locally licensed facility/Frozen	-0.55	-1.18	0.07	0.084
	No label inspection information/Fresh	-0.60	-1.16	-0.03	**0.038**
	No label inspection information/Frozen	-0.23	-1.26	0.79	0.66
Federally regulated or provincially licensed facility/Frozen	Locally licensed facility/Fresh	-1.60	-2.51	-0.68	**0.001**
	Locally licensed facility/Frozen	-1.88	-2.53	-1.23	**<0.001**
	No label inspection information/Fresh	-1.93	-2.52	-1.34	**<0.001**
	No label inspection information/Frozen	-1.56	-2.60	-0.52	**0.003**
Locally licensed facility/Fresh	Locally licensed facility/Frozen	-0.29	-1.20	0.63	0.54
	No label inspection information/Fresh	-0.33	-1.21	0.54	0.46
	No label inspection information/Frozen	0.03	-1.19	1.26	0.96
Locally licensed facility/Frozen	No label inspection information/Fresh	-0.04	-0.63	0.54	0.88
	No label inspection information/Frozen	0.32	-0.72	1.36	0.55
No label inspection information/ Fresh	No label inspection information/Frozen	0.27	-0.75	1.30	0.60
Fresh/	Frozen/Warm season	-0.13	-0.79	0.53	0.70

Warm season	Fresh /Cold season	-0.37	-0.90	0.17	0.18
	Frozen/Cold season	0.70	0.03	1.38	**0.04**
Frozen/Warm season	Fresh/Cold season	-0.24	-0.85	0.38	0.45
	Frozen/Cold season	0.84	0.22	1.45	**0.008**
Fresh/Cold season	Frozen/Cold season	1.07	0.45	1.70	**0.001**

*The final model included fixed effects for source type, state, season, source type*state and state*season interactions.

** Example interpretation: The mean (95% confidence intervals) of TEPC for fresh samples from federally regulated or provincially licensed facilities is 1.33 $\log_{10}$ tc/g higher than for frozen samples from federally regulated or provincially licensed facilities. Differences and their respective 95% CI are derived from model based estimates of the mean value for the category in column 2 subtracted from the mean value estimated for categories listed in column 1 after correcting for differences due to season and state*season interactions.

4. *E. COLI* VIRULENCE MARKERS IN SASKATCHEWAN RETAIL GROUND BEEF ORIGINATING FROM DIFFERENT PROCESSING AND PACKAGING ENVIRONMENTS

General measurements of bacterial levels in retail ground beef were investigated in Chapter 3 and statistical analysis revealed significant differences among study groups. The question whether differences in general bacterial levels translate into differences in public health risks associated with ground beef consumption is of great importance. Chapter 4 presents the investigation of three common Enterobacteriaceae virulence factors measured using culture independent methods.

This chapter will be submitted for publication as a research note. The copyright of this chapter will belong to the journal it will be published in.

Trokhymchuk, Waldner, and Gow organized samples collection. Trokhymchuk and Waldner performed data analysis and manuscript writing, organized conventional microbiology laboratory tests. Trokhymchuk, Chaban and Hill performed molecular laboratory tests. Trokhymchuk and Koester performed tests validation.

4.1. Introduction

Estimates based on surveillance information from the early 1990s indicated that as many as 81% of all foodborne illnesses cases, 82% of related hospitalizations, and 64% of corresponding deaths were attributable to unknown etiological agents (1). Reports as recent as 2011 provide similar figures: 80% of all foodborne illnesses cases, 56% of related hospitalizations, and 56% of attributable deaths are still caused by unrecognized agents (2). Thus, considerable uncertainty persists despite more than a decade of intensive research and advancements in the collection of both passive and active foodborne disease surveillance data.

Bacteria are the most extensively studied causative agents of foodborne illnesses, yet our understanding of these organisms' ecology, virulence, and pathogenicity is far from complete. Reliance on culturing techniques for diagnostics combined with the inability to reproduce suitable growth conditions for many species are major factors limiting diagnostic success. Indeed, less than 10% of known bacteria species can be cultured (3). Furthermore, there are indications that there are many bacterial species yet to be described (4).

Advancements in molecular microbiology offer an alternative approach. While the complexity of prokaryotic taxonomy in many cases does not allow for practical application of diagnostic molecular techniques to identify species (5), detection of particular genetic markers of interest is a rapidly developing area (6). Among the variety of bacterial virulence markers with implicated roles in foodborne disease pathogenesis, Shiga-like toxins type 1 (*stx1*), Shiga-like toxins type 2 (*stx2*), and intimin adherence proteins (*eae*) are commonly recognized (7). While *Escherichia coli* O157:H7 has been traditionally postulated as the principal carrier of public health importance for these virulence markers, a variety of non-O157 Shiga toxin-producing *E.*

coli strains (STEC) and a growing number of other bacterial species have also been shown to carry these genes and demonstrate pathogenic abilities. Reports of *Hafnia alvei* carrying *eae* (8), *Citrobacter spp.* carrying *eae* and *stx2* (9-12), *Escherichia albertii* carrying *eae* (13), and *Shigella spp.* carrying both *stx* groups (14) provide support for a metagenomic approach to determine bacterial virulence in foodborne diseases pathogenesis (6).

The consumption of undercooked ground beef is well recognized as food safety risk due to the numerous foodborne illness outbreaks associated with *E. coli* O157:H7 and other STECs (15-18). *E. coli* bacterial virulence markers *stx1*, *stx2*, and *eae* in ground beef have received extensive research attention (19-22). Evolution in scientific understanding and the advancement of various molecular tools now allow for the direct detection of these virulence markers, thus bypassing many of the limitations of culture-based techniques (6, 19). These advances have resulted in the development of a molecular diagnostic method approved for ground beef inspection purposes by the Food Safety and Inspection Services of the United States Department of Agriculture (23).

Ground beef offered for sale to Saskatchewan consumers originates from different supply channels: federally regulated meat processing facilities, provincially licensed establishments, abattoirs operating under local health region licensing, or farm gate sales (24). Bacterial levels measured in ground beef produced under different packaging and processing environments can be significantly different (25). This leads to the question of whether there is a potential public health risk associated with different retail sources and the associated differences in bacterial load. While most of the microflora found in ground beef consists of benign psychrotrophic bacteria

that develop during meat storage, there is also a risk associated with the presence of pathogenic organisms (26).

There are no previous reports of the application of a metagenomic approach and direct bacterial virulence marker detection techniques to retail ground beef originating from different supply channels in Western Canada. Thus, the objectives of this work were to: (i) investigate the presence of *stx1*, *stx2*, and *eae* bacterial virulence markers by direct molecular microbiology methods, (ii) assess any differences in the distribution of bacterial virulence markers in Saskatchewan retail ground beef originating from different processing and packaging environments, and (iii) evaluate the association of these virulence factors with bacterial load measured in the ground beef samples. To accomplish these objectives, we first had to optimize the direct molecular methods for application to the ground beef matrix and determine whether we could adapt multiplex polymerase chain reaction (PCR) for simultaneous detection of all three targets in this complex environment without initial culture enrichment.

4.2. Materials and methods

4.2.1. Sample collection

Small packages of ground beef (0.4-1.0 kg) (n=309) were purchased from 158 different sources between May 2011 and May 2012. These included large chain retail grocery stores, independent small grocery stores, butcher shops, and individuals representing the farm gate and "freezer trade" in Saskatchewan. The sampling plan was based on a strategy developed by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), Public Health Agency of Canada, retail surveillance (27). Samples were collected from 17 of the 18 Saskatchewan Census Divisions (CD) and reflected the assortment of ground beef varieties

available to consumers at each location. Division No.18 La Ronge, which represents the remote northern part of the province, was not sampled. The number of sampling trips to each CD and samples collected in each CD were proportional to the population size.

4.2.2. Sample categorization

Using information available on the ground beef package at the time of sale and, where possible, confirmed by the retailer, all samples were divided into three groups to reflect the product source and processing history (Table 4.1). The first group of samples included those packaged in a federally regulated or provincially licensed raw meat production facility, as indicated by the federal inspection symbol and establishment information or by the provincial facility information on the product label legend. The second group included samples packaged by small abattoirs operating under a local health region license and farm gate samples that had also been processed at facilities operating under a local health region license. The third group were samples with no available inspection history and included those with no definitive data on the label (i.e., no label legend, no identification of the local processor, and/or no information about the regulatory or licensing requirements at the site where the product was processed and packaged). Most of these samples had been repackaged by the retailer. Samples were also categorized by physical state at the time of purchase (fresh or frozen) and season of purchase ("Warm" – May 1 to October 31 or "Cold" – November 1 to April 30).

4.2.3. Sample handling and processing

Immediately after purchase, samples were labelled and placed in a cooler with an ice pack to maintain proper temperature during transportation to the laboratory. Commercial temperature

loggers (Temp 100, MadgeTech Inc., Warner, NH, USA) were used for monitoring transit conditions of each of 27 sample shipments. Frozen samples were thawed overnight at 4 °C in a refrigerator before processing.

Each ground beef package was aseptically opened and a 25 g subsample collected from five different parts of the package using a sterile metal spoon. The subsamples were homogenized with 225 mL of buffered peptone water (acc. to ISO 6579) in a stomacher bag with filter insert (Fisherbrand® FILTRA-BAG, Fisher Scientific, Whitby, ON, Canada) using a laboratory mixer (BagMixer®400VW, Interscience, Rockland, MA, USA) at 6 strokes per second for 60 seconds. Total bacterial DNA extraction was performed on 0.6 mL of the ground beef homogenate using a commercial kit (DNeasy® Blood &Tissue Kit, QIAGEN Sciences, MD, USA) as per the manufacturer's specifications.

4.2.4. Total aerobic plate count (TAPC)

Total aerobic organism counts were evaluated according to MFHPB-33 "Enumeration of total aerobic bacteria in food products and food ingredients using 3M™ Petrifilm™ aerobic count plates", as per the manufacturer's specifications (3M™ Petrifilm™ Aerobic Count plates 6400/6406, 3M Canada Inc., London, ON, Canada) (28).

4.2.5. Total *E. coli* plate count (TEPC)

Total generic *E. coli* counts were evaluated according to MFHPB-34 "Enumeration of *E. coli* and coliforms in food products and food ingredients using 3M™ Petrifilm™ *E. coli* count plates", as per the manufacturer's specifications (3M™ Petrifilm™ *E. coli* Count plates 6404/6414, 3M Canada Inc., London, ON, Canada) (29).

4.2.6. Viable *E. coli* isolation

Isolation of confirmed viable *E. coli* was performed by at the Canadian Research Institute for Food Safety (CRIFS), University of Guelph (Guelph, ON) on MacConkey agar per a standardized protocol (27).

4.2.7. Estimation of total bacterial load (TBL) by qPCR with 16S rRNA gene universal bacterial target primers

Aliquots of total bacterial DNA extract were obtained from the initial sample preparation stage. Oligonucleotide primers and reaction conditions for evaluation of total bacterial load were used as described by Lee at al.(30). A Bio-Rad iCycler iQ5 thermal cycler and iQ™ SYBR® Green Supermix (both from Bio Rad Laboratories, Inc., Hercules, CA, USA) were used as a platform for the reaction.

4.2.8. Direct detection of *stx1*, *stx2*, and *eae* targets by multiplex PCR

Aliquots of total bacterial DNA extract were obtained from the initial sample preparation stage. Primers for selected targets were used as reported by Gannon et al. (31). Experimental work to identify reaction temperature, primer concentration, and template concentration was undertaken, but attempts to optimize a 3-plex PCR for ground beef matrix were ultimately unsuccessful. Subsequently, the conditions for the study matrix were experimentally optimized for separate reactions with each pair of primers and included initial denaturation at 94 °C for 10 min; 35 cycles of 15 s denaturation at 94 °C, 15 s annealing at 65 °C and 75 s extension at 72 °C,

and final extension for 5 min at 72°C. Reactions were performed using an Eppendorf™ Mastercycler® thermocylcler (Eppendorf AG, Hamburg, Germany) with two units of Invitrogen™ Platinum® Taq Polymerase (Life Technologies Inc., Burlington, ON, Canada). Reaction products were visualized using 1% agarose gel with ethidium bromide.

4.2.9. Identifying sensitivity limits of the reaction for the study matrix

A ground beef sample with relatively low bacterial counts was identified from the pool of samples collected for the study. Total aerobic plate count and total *E. coli* count evaluated by 3M Petrifilm™ methods for the sample were 60 and <10 (below the method detection limit) colony forming units per gram (cfu/g) of ground beef, respectively. The calculated quantity of bacterial DNA present in this sample, as identified by real-time PCR with 16S rRNA gene universal bacterial primers, was $\log_{10}$ 1.7 target copies per gram (tc/g) of ground beef. No DNA targets were detected in the sample by conventional PCR with *stx1*, *stx2*, and *eae* primers.

An overnight culture of *E. coli* O157:H7 strain 141, which is known to carry *stx1*, *stx2*, and *eae* genes, in lysogeny broth (LB) with an optical density of 1.684 and an approximate concentration of log 9 bacterial cells per mL was obtained from the Vaccine and Infectious Disease Organization (Saskatoon, SK Canada) and used for sample inoculation. Twenty aliquots of 4.5 mL of ground beef homogenate were prepared as described above. The first aliquot was inoculated with 0.5 mL of *E. coli* O157:H7 strain 141 and 19 subsequent 1:10 serial dilutions were made. 3M Petrifilm™ plates were used to evaluate total aerobic plate counts and total *E. coli* counts of the serial dilutions of inoculated ground beef. Total DNA was extracted from the inoculated ground beef as described above and evaluated by PCR for the presence of *stx1*, *stx2*, and *eae* targets.

4.2.10. Statistical analysis

Results were entered into a Microsoft Access database from laboratory reports and $\log_{10}$ transformed per gram of ground beef for the culture-based tests (TAPC and TEPC). Samples that returned no growth for total *E. coli* count plates and where contamination was below the sensitivity limit of the test were coded as having a count of 1 colony forming unit (cfu)/g to facilitate $\log_{10}$ transformation. Calculation of TBL was based on the starting number of target copies estimated per qPCR reaction and $\log_{10}$ transformed per gram of ground beef.

All analyses were completed using IBM SPSS Statistics, version 21 (IBM corp., Armonk, NY, USA). Unconditional univariate analysis using binary logistic regression was performed to evaluate potential relations between the investigated outcome (bacterial virulence markers presence) and other potential risk factors (sample category, sample state at collection, season of sample collection, confirmed viable *E. coli* isolation, TAPC, TEPC, TBL). Analysis of the association between TAPC, TEPC, and TBL and the presence of bacterial virulence markers were completed considering the bacterial levels first as continuous values and then after categorizing each variable to evaluate the linearity assumption. Multivariable logistic regression models were built if more than one variable had $P<0.20$ based on unconditional analysis to identify significant risk factors ($P<0.05$) and evaluate the potential for confounding.. Two-way interactions were evaluated between all significant risk factors and included if $P<0.05$. Standardized residuals were graphed to detect and evaluate the influence of any outliers.

4.3. Results

4.3.1. Multiplex PCR for simultaneous detection of *stx1, stx2,* and *eae*

Previously published research used as a guide for the reaction setup described a 3-plex multiplex PCR with simultaneous detection of *stx1*, *stx2*, and *eae* targets in DNA extract from isolated *E. coli* colonies (31). However, the original published results could not be replicated with this complex sample matrix. Attempts to optimize PCR conditions for simultaneous detection of all three targets of interest (*stx1*, *stx2*, and *eae* genes) in a total DNA extract from a non-enriched ground beef matrix were not successful. A variety of different product sizes were consistently generated by the PCR despite efforts to optimize reaction conditions and starting component concentrations. Expected compared to observed PCR product sizes were 732 base pairs (bp) vs. 500 to 1200 bp with *stx1* primers, 779 bp vs. 400 to 1100 bp with *stx2* primers, and 890 bp vs. 200 to 1500 bp with *eae* primers. The overlap of products sizes effectively prohibited meaningful interpretation of multiplex reaction data (Figure 4.1).

4.3.2. Detection sensitivity for *E. coli* virulence genes

Total aerobic and total *E. coli* plate counts indicated expected levels of bacterial presence in the serial dilutions of inoculated ground beef homogenate (Figure 4.2). PCR results indicated reliable detection of all three targets up to a concentration of 550 cfu/g. Intermittent detection of *stx1* and *stx2* occurred at inoculate concentrations as low as $\log_{10}$ (-1) cfu/g. Intermittent detection of *eae* was observed at inoculate concentrations as low as $\log_{10}$ (-9) cfu/g (Figure 4.1).

4.3.3. PCR results

Positive PCR results for at least one of the reaction targets were observed for 112 of 308 tested samples (note: total bacterial DNA extract from one of the original 309 samples was not available) (Table 4.2). Of these 112 samples, 107 tested positive for *stx1*, 8 for *stx2*, and 26 for *eae*. There was a marked pattern of variability in visualized PCR product sizes. For analysis purposes, PCR reaction products were categorized by expected size (732 bp for *stx1*, 779 bp for *stx2*, 890 bp for *eae*), smaller than expected size, and larger than expected size (Table 4.2).

4.3.4. Statistical analysis

There were no differences in the likelihood of detecting *stx1* in samples originating from locally licensed facilities (OR 1.00, 95% CI 0.55 to 1.72) or ground beef without clear inspection labelling (OR 1.36, 95% CI 0.79 to 2.37) when compared to ground beef from federally regulated or provincially licensed facilities (Table 4.3). Also when compared to samples from federally regulated or provincially licensed facilities, there were no differences in the odds of detecting *stx2* (OR 0.41, 95% CI 0.07 to 2.51) or *eae* (OR 0.70, 95% CI 0.24 to 2.01) in samples from locally licensed facilities or in the odds of detecting *stx2* (OR 0.56, 95% CI 0.09 to 3.43) or *eae* (OR 0.60, 95% CI 0.23 to 1.54) in samples without clear inspection labelling (Table 4.3).

In addition there were no associations between the detection of *stx1*, *stx2*, and *eae E. coli* virulence markers and sample state (fresh vs. frozen) at sample collection, season of sample collection, or TACP or total TECP (Table 4.3).

Total bacterial load was significantly associated with the presence of *stx2* (P=0.02) when considered as a continuous variable, but not when categorized. The odds of detecting *stx2* in

ground beef decreased 1.65 times (95% CI 1.07 to 2.54) with each successive $\log_{10}$ tc/g increase in TBL; however, this relationship was not monotonic when TBL was categorized. Total bacterial load was not a significant predictor for *stx1* and *eae*.

Similarly there was no significant association between isolation of *E. coli* on MacConkey agar and detection of *stx1*, *stx2*, and *eae E. coli* virulence markers (Table 4.3).

4.4. Discussion

The presence of Shiga-toxin producing *E. coli* (STEC) is one of the primary food safety concerns related to ground beef. The world-wide prevalence of STEC in ground beef varies dramatically, from as low as 0.01% to as high as 54.2% (32). Some studies utilizing culture-independent techniques for STEC detection report even higher marker prevalence of up to 70% in meats (33). The findings that 34.7% of samples were positive for *stx1*, 2.6% were positive *stx2*, and 8.4% were positive for *eae* suggests that raw ground beef remains a potential food safety risk if not handled and prepared appropriately.

There is no simple association between the detection of specific virulence markers role and the detection of specific types of STEC organisms. Most virulent *E. coli* strains can carry either combination of *stx1* and *stx2*, or all three genes investigated in this study (34); however, there are reports of pathogenic strains carrying none of these genes (35). None of the samples were positive for all three investigated bacterial virulence markers. Only three samples were positive for both *stx1* and *stx2*, the combination with the highest virulence probability (6). Nine samples were positive for *stx1* and *eae* and none for *stx2* and *eae.* Although similar bacterial virulence marker distributions have been reported (33), in most cases these profiles are distinct for different geographical locations and likely reflect the uniqueness of the local microbiome.

Despite the existence of a well-established molecular diagnostic protocols for STEC detection (23), we deliberately chose not to use techniques that required pre-enrichment of samples. A pre-enrichment step would have resulted in preferential growth of some groups of organisms while potentially obscuring the identification of others. The intent of this analysis was to describe the microbiome composition of the ground beef available to the consumer at the time of purchase; therefore, the bacterial DNA extract was obtained from non-enriched samples. A metagenomic approach was used instead to screen for virulence factors of interest in public health.

Understanding bacterial virulence marker distribution and their presence in bacterial communities contaminating ground beef is a new area of research. This study found no difference with respect to the presence of ground beef bacterial virulence markers from product produced and packaged in facilities under federal regulations/provincial licensing, local health region licensing, or without any origin information on the label legend. However, given the relatively low frequency of detection and small sample size in each category, the power of this analysis was limited. Similarly, there was no difference in the likelihood of detecting virulence markers with respect to whether the product was fresh or frozen at the time it was offered for sale or by season of purchase. While also potentially limited by study power, it was interesting that the isolation of viable *E. coli* from the samples was also not associated with the distribution of bacterial virulence markers. This finding provides additional incentive to consider a metagenomic approach in assessing public health implications of ground beef bacterial contamination.

Notably, high total bacterial load was associated with a decreased risk of identifying the *stx2* marker. This might indicate competitive exclusion of bacteria capable of carrying *stx2*, which could occur due to overwhelming growth of spoilage flora, though non-monotonic character of this relationship makes it very likely that this finding was not biologically relevant.

Detection limits for the presence of *stx1*, *stx2*, and *eae* genes in the bacterial flora contaminating ground beef in this study were consistent at $\log_{10}3$ cfu/g of inoculated organism (*E. coli* O157:H7 strain 141), though some significant signal detection was observed at much lower concentrations. Obtaining positive PCR reaction products at estimated inoculate concentrations of $\log_{10}$ (-9) cfu/g can be attributed to the detection of a single target copy and is a strong indication of high reaction sensitivity. Comparable studies using minor groove probes report sensitivity limits as low as 30 cfu/g (19). Our observations indicate that improved methodologies for the extraction of total bacterial DNA from ground beef is a simple way to substantially increase direct *stx1*, *stx2*, and *eae* gene detection efficiency. However, relying on molecular virulence marker detection as the primary tool for identification of pathogenic strains of *E. coli* is problematic (7), and this is reflected in the complexity of results interpretation in the present study.

The observed variety in PCR product sizes might represent the known diversity in the *stx1*, *stx2*, and *eae* virulence genes investigated; for example, at least 10 subtypes of Shiga-like toxins have been observed in more than 100 Shiga-toxin producing *E. coli* strains (36). There is a growing awareness of potential horizontal genetic information transfer between different bacterial species, including phage-based *stx1* and *stx2* as well as plasmid-based *eae* genes. These virulence markers have also been reported in organisms other than *E. coli*, such as *Enterobacter*

cloacae producing Shiga-like toxin (37) and intimin presence in *Hafnia alvei* (10). The complexity of the ground beef matrix and previous reports on PCR inhibition provide reason for cautious interpretation of study findings (19). Utilization of complex matrices for molecular diagnostics, especially when the investigated substances are very rich in potential PCR inhibitors, is becoming more feasible with advancements in both the stability of the key reaction components and the precision of available instruments. Focusing on detecting virulence factors rather than the carrying organisms might be an alternative approach to assess foodborne disease risks associated with ground beef.

This study had two major limitations. First, the small sample size resulted in an insufficient number of positive samples for meaningful statistical analysis of the distribution of particular subtypes of *stx1*, *stx2*, and *eae E. coli* virulence markers across the study categories. Second, the specificity of the primers chosen for this study should be further evaluated based on the variety of products sizes that were generated and complicated the interpretations of results. Further work to understand the diversity of *stx1*, *stx2*, and *eae* gene-carrying bacteria potentially present in ground beef should include sequencing and attribution analysis of the observed variety of PCR products.

4.5. Acknowledgments

Validation of the bacterial virulence markers (*stx1*, *stx2*, and *eae*) detection reaction was performed by me at Dr. Wolfgang Koester's laboratory at the Vaccine and Infectious Diseases Organization (VIDO) under supervision and with assistance of Neil Rawlyk.

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Figure 4.1 Observed product sizes and sensitivity of conventional PCR detecting *stx1*, *stx2*, and *eae* virulence markers in ground beef experimentally inoculated with serial dilutions of the overnight culture of *E. coli* O157:H7 strain 141

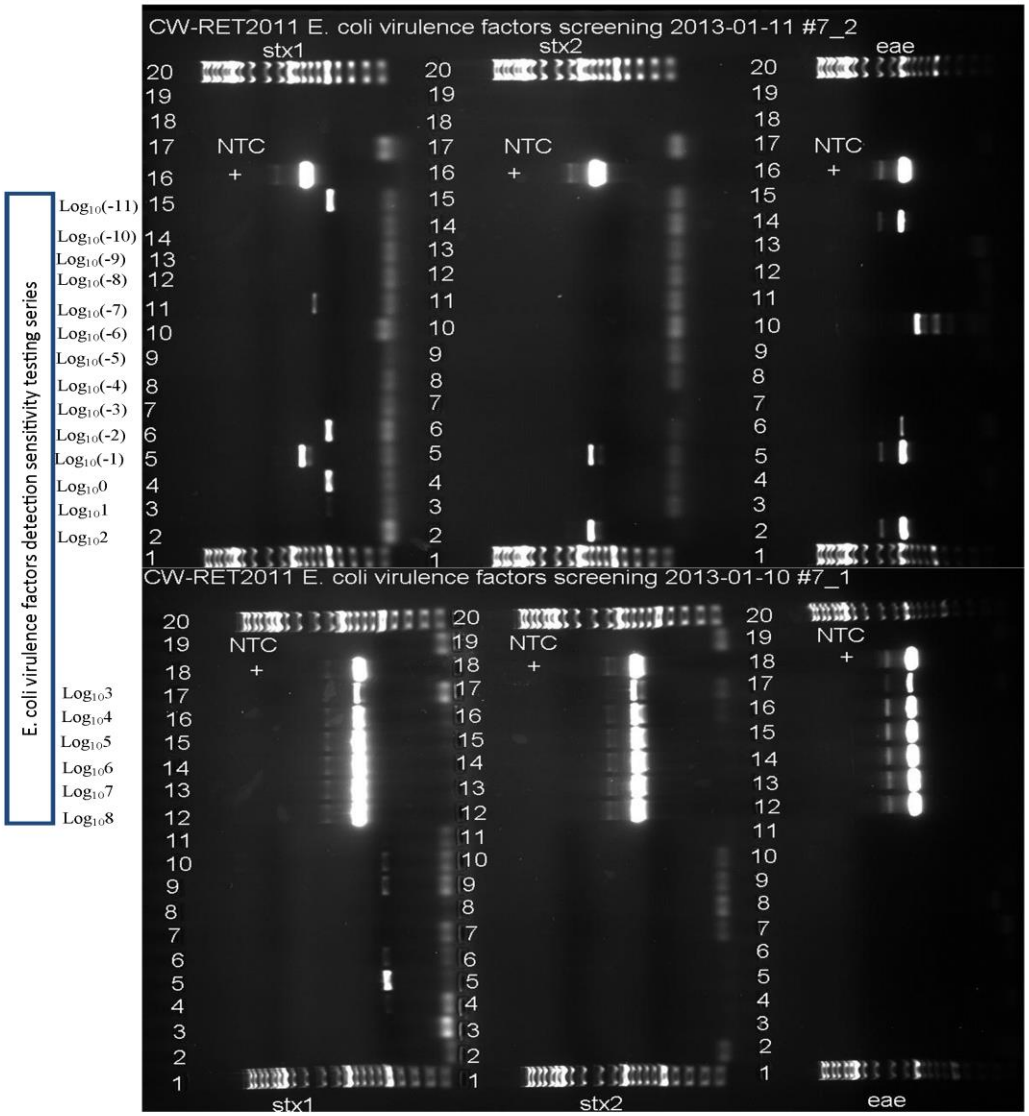

Figure 4.2 Serial dilution curve for *E. coli* O157:H7 strain 141 inoculated on 3M PetrifilmTM

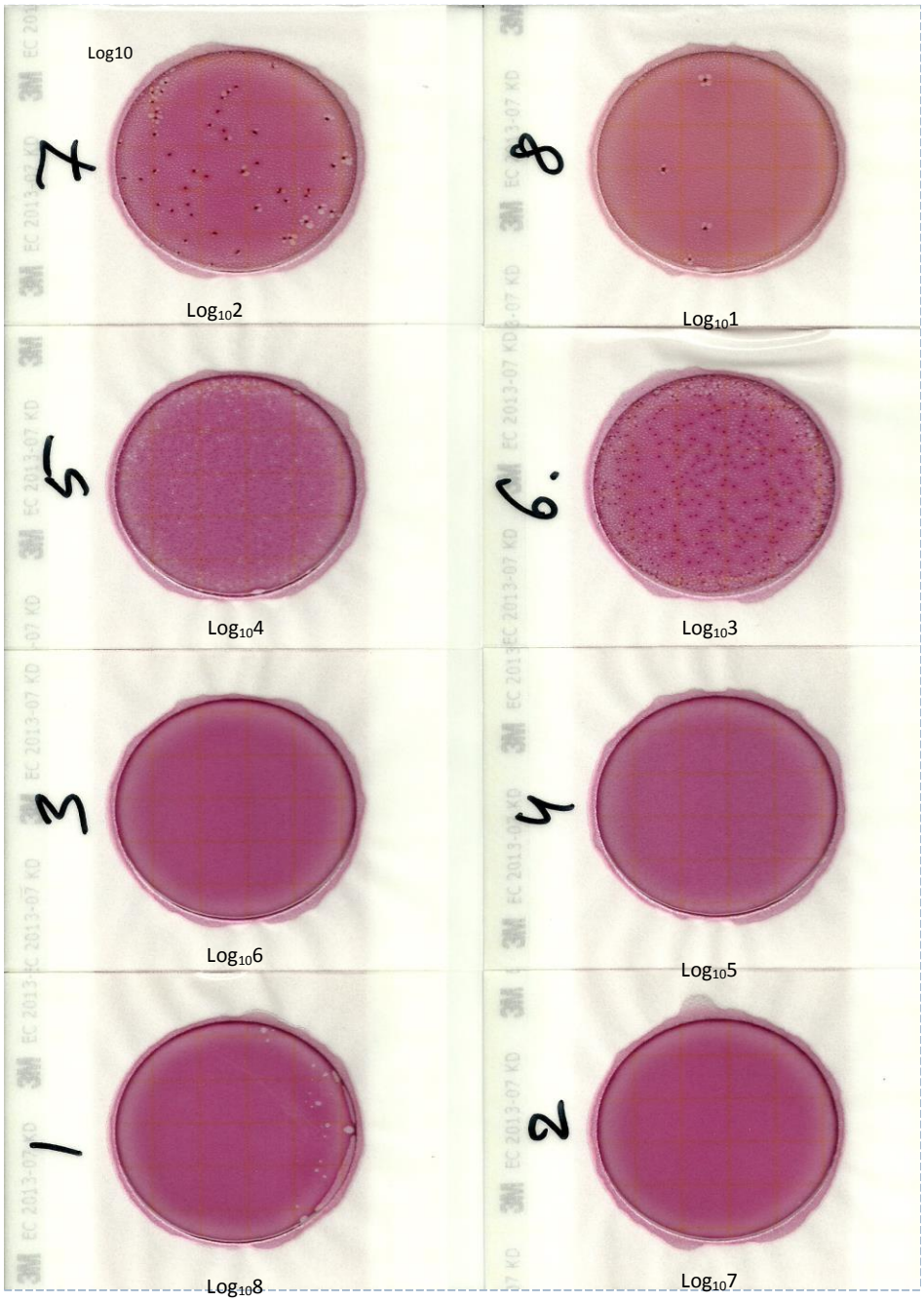

Table 4.1 Summary of product source information, season of purchase, and product state at the time of purchase for the retail ground beef samples included in the study

Sample categories	Total	Collection season			
		Warm (May 1 – October 30)		Cold (November 1 – April 30)	
		Fresh	Frozen	Fresh	Frozen
Federally regulated or provincial licensed facility	125	26	28	42	29
Locally licensed facility	78	5	28	15	30
No label inspection information	106	50	8	42	6
Total	309	81	64	99	65

Table 4.2 Observed size and prevalence of *stx1*, *stx2*, and *eae* bacterial virulence markers detected by direct PCR in Saskatchewan retail ground beef originating from different processing and packaging environments (n=308)

Study group	Samples positive for at least one target	Samples positive for more than one target	Samples positive for *stx1/stx2*	Samples positive for *stx1/eae*	Samples positive for *stx1*	*stx1* product size			Samples positive for *stx2*	*stx2* product size			Samples positive for *eae*	*eae* product size		
						Normal (≈732 bp)	Small (<732 bp)	Large (>732 bp)		Normal (≈779 bp)	Small (<779bp)	Large (>779 bp)		Normal (≈890 bp)	Small (<890 bp)	Large (>890 bp)
Federally regulated or provincially licensed	**48 (38.7%)**	**5 (4%)**	**0 (0%)**	**5 (4%)**	**46 (37.1%)**	**5 (4%)**	**40 (32%)**	**1 (0.8%)**	**2 (1.6%)**	**1 (0.8%)**	**2 (1.6%)**	**0 (0%)**	**8 (6.4%)**	**3 (2.4%)**	**3 (2.4%)**	**2 (1.6%)**
Locally licensed	**30 (38.5%)**	**4 (5.1%)**	**2 (2.6%)**	**2 (2.6%)**	**29 (37.2%)**	**5 (6.4%)**	**20 (25.6%)**	**6 (7.7%)**	**3 (3.8%)**	**3 (3.8%)**	**1 (1.3%)**	**0 (0%)**	**7 (8.9%)**	**3 (3.8%)**	**2 (2.6%)**	**2 (2.6%)**
No inspection information on label legend	**34 (32.1%)**	**3 (2.8%)**	**1 (0.9%)**	**2 (1.9%)**	**32 (30.2%)**	**4 (3.8%)**	**26 (24.5%)**	**4 (3.8%)**	**3 (2.8%)**	**0 (0%)**	**1 (1.3%)**	**2 (2.6%)**	**11 (10.4%)**	**4 (5.1%)**	**8 (10.2%)**	**1 (1.3%)**
Total	**112 (36.4%)**	**12 (3.9%)**	**3 (1%)**	**9 (2.9%)**	**107 (34.7%)**	**14 (4.5%)**	**86 (27.8%)**	**11 (3.6%)**	**8 (2.6%)**	**4 (1.3%)**	**4 (1.3%)**	**2 (0.6%)**	**26 (8.4%)**	**10 (3.2%)**	**13 (4.2%)**	**5 (1.6%)**

Table 4.3 Summary of the associations between Saskatchewan retail ground beef source, packaging and processing information available at purchase, product state and season of purchase, measured bacterial levels, and the presence of *E. coli* virulence markers (n=308)

Potential risk factors	*E. coli* virulence factors (*P*-values)		
	stx1	*stx2*	*eae*
Study category (federally regulated/provincial licensed, local licensed, no label legend or source information)	0.48	0.63	0.56
State (fresh vs. frozen)	0.44	0.80	0.65
Season (warm vs. cold)	0.82	0.23	0.73
Log_{10} TAPC (cfu/g)*	0.36	0.63	0.31
Log_{10} TAPC categorized (low (<5 cfu/g), medium (5-6 cfu/g), high (>6 cfu/g))	0.64	0.55	0.24
Log_{10} TEPC (cfu/g)	0.61	0.27	0.81
Log_{10} TEPC categorized (low (<1 cfu/g), high (>1 cfu/g))	0.41	0.10	0.86
TBL (log_{10}tc/g)**	0.95	0.02	0.97
TBL categorized (low (<5 log_{10}tc/g), medium (5-7 log_{10}tc/g), high (>7 log_{10}tc/g))	0.73	0.09	0.67
E. coli isolation (yes/no)	0.48	0.09	0.73

* colony forming units per gram

** target copies per gram

5. PREVALENCE AND DIVERSITY OF *CAMPYLOBACTER* SPP. IN SASKATCHEWAN RETAIL GROUND BEEF

The investigation of three common Enterobacteriaceae virulence factors reported in Chapter 4 addressed just one aspect of potential public health risk associated with ground beef consumption. The results of the investigation of Campylobacter spp. as another important public health hazard are reported in Chapter 5.

This chapter will be submitted for publication. The copyright of this chapter will belong to the journal it will be published in.

Trokhymchuk, Waldner, and Gow organized samples collection. Trokhymchuk and Waldner performed data analysis and manuscript writing, organized conventional microbiology laboratory tests. Trokhymchuk, Chaban and Hill performed molecular laboratory tests.

5.1. Introduction

Campylobacter spp. are recognized as important bacterial contributors to the global burden of gastrointestinal illness *(4, 31, 56, 61)*. In addition, there is growing evidence implicating an association between *Campylobacter* spp. with human autoimmune neuropathic disorders, such as Guillain-Barré syndrome, Miller-Fisher syndrome *(17, 55, 59)*, and Reiter's arthritis, as well as celiac disease *(68)*. Despite the large volume of research conducted during the last 30 years, *Campylobacter* spp. remain some of the least studied bacteria, with limited information available on their ecology, virulence factors, and pathogenicity mechanisms *(5, 38)*.

Recognition of the public health importance of *Campylobacter* spp. was documented as early as 1880 by Theodor Esherich, who first described the organism observed by direct microscopy in the intestinal mucus of children affected with diarrhoea *(11, 41, 63)*. Similar organisms were reported to cause foodborne illnesses outbreaks, abortions, and septicaemia *(41, 56)*. Besides a unique shape and movement patterns, a common feature of these pathogens was how difficult they were to culture. Evidence from medical and veterinary research in both outbreak settings and individual cases led to taxonomical definition of the genus in 1963 by Sebald and Véron *(11)*. The first successful isolation of the organism from feces by advanced filtering technique was reported by Dekeyser and Butzler in 1972 *(11)*, and a simple culturing method for *Campylobacter* was subsequently developed in 1975 by Skirrow *(62)*. Even now, the fastidious nature of these organisms is a limitation to its successful diagnosis *(50)*.

Traditionally, consumption of chicken was considered the most common risk factor for human campylobacteriosis; however a growing number of reports are examining the role of cattle in *Campylobacter* spp. infections *(26, 33-35)*. Observations of a high prevalence of human

campylobacteriosis in intensive cattle production areas and similarities in the genetic makeup of cattle and human clinical isolates suggest the existence of epidemiological links between the *Campylobacter* spp. reservoir in cattle and human illnesses *(5, 29)*. The popularity of ground beef in North America and challenges with educating the public about undercooking and cross-contamination makes this exposure vehicle an important consideration for public health.

Campylobacter is a genus of Gram-negative, spiral, motile, and microaerophilic bacteria included in the *Campylobacteriaceae* family. While there are a number of disagreements in the genus taxonomy, 23 species and eight subspecies of *Campylobacter* are recognized at present: *C. avium*, *C. canadensis*, *C. coli*, *C. concisus*, *C. cuniculorum*, *C. curvus*, *C. fetus* (*C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*), *C. gracilis*, *C. helveticus*, *C. hominis*, *C. hyointestinalis* (*C. hyointestinalis* subsp. *hyointestinalis* and *C. hyointestinalis* subsp. *lawsonni*), *C. insulaenigrae*, *C. jejuni* (*C. jejuni* subsp. *doylei* and *C. jejuni* subsp. *jejuni*), *C. lanienae*, *C. lari* (*C. lari* subsp. *lari* and *C. lari* subsp. *concheus*), *C. laridis*, *C. mucosalis*, *C. peloridis*, *C. rectus*, *C. showae*, *C. sputorum*, *C. troglodytis*, and *C. upsaliensis (16, 39, 45).* At least 12 among these have recognized pathogenic potential for humans and many more species are observed to be opportunistic pathogens (Table 5.1). Despite this fact, most of the public health study to date has focused on *C. jejuni* and *C. coli*.

Campylobacter have a preference for microaerobic (<5% O_2) and capnophillic conditions (3-5% CO_2), do not possess specialized survival mechanisms such as spore-formation, and are sensitive to drying. However, under certain instances they can survive outside the environment of the host's intestinal tract for prolonged periods of time *(64)*. Moist conditions with temperature near 4°C are optimal for extended *Campylobacter* survival, but freezing with large

initial organism numbers can result in the long-term survival of some cells *(56)*. Thus, typical retail temperatures for ground beef offer almost ideal conditions for *Campylobacter* spp. Even though the number of viable organisms declines over the time, *Campylobacter* can persist throughout the shelf life of fresh ground beef if initial levels of contamination are sufficient *(69)*. Similarly, *Campylobacter* spp. can be isolated from frozen ground beef that had high initial levels of contamination *(7)*.

The complexity of *Campylobacter* spp. isolation and the absence of unanimous agreement on molecular detection technique standards have led to significant variability in reports on the prevalence of this organism in retail ground beef. In addition, differences exist in the geographic distribution of *Campylobacter* both in animal hosts and as a causative agent for foodborne illnesses. In one study, as many as 46% of retail ground beef samples from Alberta tested positive for the presence of *Campylobacter* spp. DNA; however, no successful isolation was achieved *(28)*. Comparable studies from other locations in North America and worldwide that employed culture, molecular, and biochemical methods reported prevalence levels from 0 to 20% *(6, 7, 25, 54, 69)*.

Bacterial levels in ground beef are measured as a cumulative indicator of production hygienic practices and sanitation standards, but are not considered as a reliable predictor of the presence of pathogenic organisms *(65)*. However, a very substantial proportion of foodborne illnesses cannot be attributed to known pathogens, leaving questions with respect to the definition and recognition of bacterial pathogenicity *(60)*. Strict application of classical epidemiological postulates that require isolation of a pathogenic organism as a proof of its role in the epidemiological process can limit progress towards a more inclusive understanding of the

role of bacterial pathogens in human foodborne illnesses. This is especially true for bacteria, including many *Campylobacter* species, that are not readily cultured under known laboratory conditions or when culturing success can be extremely variable due to the fastidious nature of the organisms.

A number of *Campylobacter* organisms have been recognized as emerging pathogens during the last two decades, though understanding of the true public health importance of these organisms is still developing *(40).* Despite some awareness of *Campylobacter* diversity, information on species other than *C. coli*, *C. jejuni*, and *C. fetus* is limited. Culturing requirements for the majority of *Campylobacter* species remains poorly understood and specific isolation protocols for species other than the aforementioned three do not yet exist *(49).* Successful detection of a variety of *Campylobacter* species, either in ground beef or cattle feces, has been achieved by molecular techniques. Inglis and Kalischuk report on the diversity of *Campylobacter* spp. associated with cattle, including the presence of *C. lanienae, C. coli*, *C. fetus*, *C. hyointestinalis*, and *C. jejuni (33, 49).* Hannon et al. report the presence of *C. jejuni*, *C. coli*, and *C. hyointestinalis* DNA in ground beef *(28),* and Acik et al. report the identification of *C. lari* in cattle *(2).* Sophisticated pre-enrichment procedures in combination with a universal culturing approach (Cape Town protocol) and molecular speciation allowed Lynch et al. to successfully recover 10 *Campylobacter* species (*C. coli*, *C. concisus*, *C. curvus*, *C. fetus*, *C. helveticus*, *C. jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei*, *C. lari*, *C. mucosalis*, *C. sputorum*, and *C. upsaliensis*) both experimentally and from retail ground beef *(50).* A number of reports identify *C. lanienae* as the most frequently isolated *Campylobacter* organism from cattle feces *(33, 34).*

While a number of attempts have been made to evaluate the diversity in *Campylobacter* spp. contaminating retail ground beef in North America, only a few of the most well-studied species were typically investigated. The influence of the season and whether the ground beef is fresh or frozen at purchase on the presence of the organism has not been reported. Similarly, the processing environment and effect of re-packaging have not been evaluated as potential risk factors for the prevalence and diversity of *Campylobacter* spp. in retail ground beef. Ground beef offered for sale to Saskatchewan consumers originates from different supply channels: federally regulated meat processing facilities, provincially licensed establishments, abattoirs operating under local health region licensing, and farm gate sales *(51)*. These facilities also typically differ with respect to production volumes and technological capabilities. At least one study published to date has documented differences in different bacterial counts in their products *(30)*.

Thus, the primary objectives of this study were to investigate the prevalence of *Campylobacter* spp. DNA by conventional polymerase chain reaction (PCR) in retail ground beef offered for sale in Saskatchewan and to identify the presence of DNA from individual *Campylobacter* species (*C. coli*, *C. curvus*, *C. fetus*, *C. hyointestinalis*, *C. jejuni*, *C. rectus*, *C. upsaliensis*) using *cpn*60-based real-time qPCR. Secondary objectives were to assess potential differences in the prevalence of *Campylobacter* in ground beef offered for sale during cold and warm seasons, compare *Campylobacter* prevalence in ground beef offered for sale fresh and frozen, investigate any association between the presence of *Campylobacter* spp. and *E. coli* counts as well as total aerobic bacterial counts, and finally to compare the prevalence of *Campylobacter* spp. in ground beef originating from different production and retail environments.

5.2. Materials and methods

5.2.1. Sample collection

Small packages of ground beef (0.4-1.0 kg) (n=309) were purchased from 158 different sources between May 2011 and May 2012. These included large chain retail grocery stores, independent small grocery stores, butcher shops, and private transactions representing the farm gate and "freezer trade" in Saskatchewan. The sampling plan was based on a strategy developed by the Canadian Integrated Program for Antimicrobial Resistance Surveillance of the Public Health Agency of Canada (CIPARS) for retail surveillance *(12)*. Samples were collected from 17 of the 18 Saskatchewan Census Divisions (CD) and reflected the assortment of ground beef varieties available to consumers at each location. Division No.18 La Ronge, which represents the remote northern part of the province, was not sampled. The number of sampling trips to each CD and samples collected in each CD were proportional to the population size.

5.2.2. Sample categorization

Using information available on the ground beef package at the time of sale and, where possible, confirmed by the retailer, all samples were divided into three groups to reflect the product source and processing history (Table 5.2). The first group of samples included those packaged in a federally regulated or provincially licensed raw meat production facility, as indicated by the federal inspection symbol and establishment information or by the provincial facility information on the product label legend. The second group included samples packaged by small abattoirs operating under a local health region license and farm gate samples that had also been processed at facilities operating under a local health region license. The third group were samples with no available inspection history and included those with no definitive data on

the label (i.e., no label legend, no identification of the local processor, and/or no information about the regulatory or licensing requirements at the site where the product was processed and packaged). Based on our observations, these samples had been repackaged by the retailer. Samples were also categorized by physical state at the time of purchase (fresh or frozen) and season of purchase ("Warm" – May 1 to October 31 or "Cold" – November 1 to April 30).

5.2.3. Sample handling and processing

Immediately after purchase, samples were labelled and placed up to eight in a cooler with one ice pack if the daily high temperature was below 20° C or two ice packs if the daily high temperature exceeded 20° C. Commercial temperature loggers (Temp 100, MadgeTech Inc., Warner, NH, USA) were used for monitoring transit conditions of each of 27 sample shipments. Frozen samples were thawed overnight at 4 °C in a refrigerator before processing.

Each ground beef package was aseptically opened and a subsample of 25 g collected from five different parts of the package using a sterile metal spoon. The subsamples were homogenized with 225 mL of buffered peptone water (acc. to ISO 6579) in a stomacher bag with filter insert (Fisherbrand® FILTRA-BAG, Fisher Scientific, Whitby, ON, Canada) using a laboratory mixer (BagMixer®400VW, Interscience, Rockland, MA, USA) at 6 strokes per second for 60 seconds. Total bacterial DNA extraction was performed from 0.6 mL of the ground beef homogenate using a commercial kit (DNeasy® Blood &Tissue Kit, QIAGEN Sciences, MD, USA) as per the manufacturer's specifications.

5.2.4. Total aerobic plate count (TAPC)

Total aerobic organism counts were evaluated according to MFHPB-33 "Enumeration of total aerobic bacteria in food products and food ingredients using 3M™ Petrifilm™ aerobic count plates", as per the manufacturer's specifications (3M™ Petrifilm™ Aerobic Count plates 6400/6406, 3M Canada Inc., London, ON, Canada) *(70)*.

5.2.5. Total *E. coli* plate count (TEPC)

Total generic *E. coli* counts were evaluated according to MFHPB-34 "Enumeration of *E. coli* and coliforms in food products and food ingredients using 3M™ Petrifilm™ *E. coli* count plates", as per the manufacturer's specifications (3M™ Petrifilm™ *E. coli* Count plates 6404/6414, 3M Canada Inc., London, ON, Canada) *(71)*.

5.2.6. Estimation of total bacterial load (TBL) by qPCR with 16S rRNA universal bacterial target primers

Aliquots of total bacterial DNA extract were obtained from the initial sample preparation stage. Oligonucleotide primers for evaluation of total bacterial load were used as described by Lee et al.*(44)*, and reaction conditions as reported by Chaban et al. (13) . A Bio-Rad iCycler iQ5 thermal cycler and iQ™ SYBR® Green Supermix (both from Bio Rad Laboratories, Inc., Hercules, CA, USA) were used as a platform for the reaction.

5.2.7. Detection of *Campylobacter* genus-specific DNA by conventional PCR

Aliquots of total bacterial DNA extract were obtained from the initial sample preparation stage. Primers for the *Campylobacter* genus-specific 16S rRNA gene target were used as

reported by Linton et al. *(47)*. Reaction conditions were optimized for use on the ground beef matrix.

To test reaction sensitivity limit on the study matrix, 900 µL of ground beef homogenate was inoculated with 100 µL of freshly prepared suspension of *C. fetus venerealis* in phosphate buffered saline (PBS) at a concentration of 5.7×10^7 CFU/mL. Six serial dilutions were made to obtain a bacterial concentration in the inoculated ground beef homogenate in a range from $\log_{10}$ 6 to $\log_{10}$ 0 cfu/mL. Total bacterial DNA extraction was performed as described above. The extracts from serial dilutions of the inoculated ground beef homogenate, uninoculated ground beef homogenate, PBS containing $\log_{10}$ 6 cfu/mL of *C. fetus venerealis*, and pure PBS as a control were tested for the presence of *Campylobacter* genus DNA by conventional PCR.

5.2.8. Detection of *Campylobacter* species-specific DNA by real-time qPCR

Aliquots of total bacterial DNA extract were obtained from the initial sample preparation stage. Primers for specific *cpn*60 targets of the *Campylobacter* species of interest (*C. coli, C. curvus, C. fetus, C. hyointestinalis, C. jejuni, C. rectus, C. upsaliensis*) were used as reported by Chaban et al. *(14)*. Reaction conditions were optimized for use on the ground beef matrix.

Escherichia coli JM109 carrying pGEM-T Easy plasmid (Invitrogen) with the *cpn*60 universal target construct for each of the investigated *Campylobacter* species were obtained from the Molecular Microbiology Research Laboratory of the Western College of Veterinary Medicine, University of Saskatchewan (Dr. Janet E. Hill). Overnight cultures in Luria-Bertani broth containing ampicillin were used for plasmid DNA extraction and preparation of positive controls and assay standards.

5.2.9. Statistical analysis

All data were entered into a Microsoft Access database. Raw data from real-time qPCR were analyzed using iQ5 optical system software (Bio-Rad Laboratories Limited, Mississauga, ON, Canada).The results of culture-based tests (TAPC and TEPC) were $\log_{10}$ transformed per gram of ground beef. Samples that returned no growth for total *E. coli* count plates and where contamination was below the sensitivity limit of the test were coded as having a count of 1 colony forming unit (cfu)/g to facilitate $\log_{10}$ transformation. Calculation of TBL was based on the starting number of target copies estimated per qPCR reaction and $\log_{10}$ transformed per gram of ground beef.

All analyses were completed using IBM SPSS Statistics, version 21 (IBM corp., Armonk, NY, USA). Unconditional binary logistic regression was used to evaluate potential associations between potential risk factors (sample category, sample state at collection, season of sample collection, TAPC, TEPC, TBL) and the detection of both *Campylobacter* genus-specific DNA and individual *Campylobacter* species DNA. Analyses of the association between TAPC, TEPC, and TBL and the detection of *Campylobacter* genus-specific or species-specific DNA were completed by first considering the bacterial levels as continuous values and thereafter categorizing each variable to evaluate the linearity assumption. Multivariable logistic regression models were built if more than one variable had $P<0.20$ based on unconditional analysis to identify significant risk factors ($P<0.05$) and evaluate the potential for confounding. Two-way interactions were evaluated between all significant risk factors and included if $P<0.05$. Standardized residuals were graphed to detect and evaluate the influence of any outliers.

5.3. Results

5.3.1. Detection sensitivity for *Campylobacter* genus-specific PCR

The presence of DNA specific to the *Campylobacter* genus was evaluated based on visualization of bands of an expected size on the agarose gel. To evaluate the assay's performance, ground beef was spiked with known quantities of *Campylobacter* culture (*C. fetus venerealis*) and processed in the same fashion as study samples. The assay's sensitivity limit was sufficient to detect target DNA in all extracts from inoculated ground beef homogenate from an inoculated concentration of $\log_{10}$ 6 cells/ml to $\log_{10}$ 0 cells/ml and from culture in PBS alone (Figure 5.1).

5.3.2. PCR results for 16S RNA *Campylobacter* genus-specific target

Out of the 309 total samples included in the study, 50 (16.2%) tested positive for *Campylobacter* genus-specific DNA using the conventional PCR for *Campylobacter* at the genus level. The distribution of test results by ground beef source/processing history group, state, and season of purchase is presented in Table 5.3.

5.3.3. Real-time qPCR results for *cpn*60 *Campylobacter* species-specific targets

DNA from all seven *Campylobacter* species investigated was detected in the pool of study samples (n=309) by real-time qPCR for individual species of *Campylobacter*. Forty-nine samples (15.9%) were identified as positive for the presence of at least one of investigated targets. In total, 14 samples (4.5%) were positive for *C. coli*, 11 (3.6%) for *C. curvus*, 6 (1.9%) for *C. fetus*, 24 (7.8%) for *C. hyointestinalis*, 12 (3.9%) for *C. jejuni*, 6 (1.9%) for *C. rectus*, and 9 (2.9%) for *C. upsaliensis*.

Presence of DNA from more than one *Campylobacter* species was detected in 24 samples (7.8%), including 17 samples (5.5%) positive for DNA from two species, three samples (0.9%) positive for three species, two samples (0.6%) positive for four species, and one sample (0.1%) positive for five species.

Of the 50 samples identified as positive in the *Campylobacter* genus-specific assay, 27 samples (54%) did not test positive for any of the seven *Campylobacter* species investigated. Conversely, 26 samples (53%) out of 49 that were positive in real-time qPCR species-specific assays returned no product in the genus-level assay.

5.3.4. Statistical analysis

Results from the genus- and species-level *Campylobacter* testing were compared to the collected information about the sample's processing and packaging, fresh or frozen state, season of purchases, and measured levels of bacteria (Table 5.4). Only the presence of *C. hyointestinalis* DNA was significantly associated with bacterial levels in retail ground beef, though only when TAPC and TBL were analysed as continuous variables (P=0.01 and P=0.01, respectively). This observed association was found not significant when TAPC and TBL were presented as categorical variables for analysis.

5.4. Discussion

The prevalence of *Campylobacter* spp. DNA in retail ground beef offered for sale in Saskatchewan was 16.2% in this study, which is similar to reported baseline contamination rates in the USA (0-20%) *(69)*. This is considerably lower than the 46% previously reported for retail ground beef distributed by large grocery chains in Alberta in 2009.*(28)* Most retail ground beef offered for sale in Saskatchewan is from federally regulated establishments that use the same processors and distribution centers that provide product to major Alberta retailers. Differences in *Campylobacter* detection rates between our study and this previous one could be accounted for by potential ground beef processing changes between sampling periods, differences in sample sources (large chain retailers vs. assortment representing all segments of the market), and different laboratory processing and analysis techniques.

This is the first work to address the diversity of *Campylobacter* species in ground beef from a variety of processing and packaging environments. We identified the presence of DNA from all seven of the investigated *Campylobacter* species, including *C. coli*, *C. curvus*, *C. fetus*, *C. hyointestinalis*, *C. jejuni*, *C. rectus*, and *C. upsaliensis*. While the results of the PCR assays used in this analysis do not allow us to evaluate organism viability and potential associated public health risks, previous research clearly demonstrate that ground beef stored under normal retail conditions presents a favourable environment for *Campylobacter* survival.*(28, 50, 54)*

C. hyointestinalis was the most prevalent of the species observed (48% of species-level positives and present in 7.7% of all study samples). This observation is distinct from previous reports, where Lynch et al. reported *C. jejuni* to be the most prevalent *Campylobacter* species in ground beef *(50)*, Kalischuk et al. (2003) reported the most common *Campylobacter* in Alberta

cattle was *C. lanienae (34)*. Hannon et al. reported that *C. coli* had a 26.8% prevalence in ground beef from large retailers in Alberta *(28)* and that *C. jejuni* had the highest prevalence in feedlot cattle feces *(27)*. Differences in sampling and methodology might explain some of the deviations in observed individual species prevalence; however, these results support the complexity of *Campylobacter* ecology and epidemiology and the need to look beyond considering only *C. jejuni* and *C. coli* in food safety assessments. The unexpectedly high prevalence of *C. hyointestinalis* is a possible public health concern given reports describing the potential pathogenicity of this species *(9, 10, 18)*.

Interestingly, there were several instances where the genus-species and species-specific assay results were incongruent. The first situation was where samples were positive by the *Campylobacter* genus-specific PCR were not positive for any of the seven investigated species. This is most likely an indication of an even richer *Campylobacter* diversity in retail ground beef than surveyed for in this study. Comparable works have identified the presence of as many as 10 different species in retail meats *(50)*. The second situation was the presence of samples negative by the *Campylobacter* genus-specific PCR, but positive for *Campylobacter* species-specific DNA in qPCR. This situation is most likely explained by a higher sensitivity of the qPCR assays as compared to the conventional PCR assay. Differences in assay gene targets (16S rRNA gene vs *cpn*60 gene), assay reagents (conventional vs SYBR Green master mixes) and even programs and thermocyclers used could all contribute to this differential detection limit effect. *(36)*.

There were no significant differences in the presence of *Campylobacter* genus DNA or individual *Campylobacter* species DNA among retail ground beef assigned to the three study categories representing source and processing history, between fresh and frozen state at the time

of purchases, or between ground beef offered for sale during warm and cold seasons. Similarly, there were no associations between most measures of bacterial levels in retail ground beef and *Campylobacter* presence. The significant association between bacterial levels and *C. hyointestinalis* presence was not monotonic and was unlikely to be biologically relevant.

Sample size restricted the power of this analysis to examine differences across all of the risk factors, particularly for individual *Campylobacter* species. While the application of direct molecular techniques to the complex ground beef matrix provided an attractive alternative to culturing, there were considerable limitations in the laboratory methods. The challenge of extracting total bacterial DNA from a large volume of complex organic material with variable composition could result in inconsistent results, especially from ground beef grades with different fat content. Molecular techniques are not universally accepted for *Campylobacter* detection because of the uncertainty related to genus taxonomy and general limitations of molecular bacterial identification *(1)*. Moreover, the inability to distinguish between viable and inactivated organisms restricts a public health interpretation of the results.

The next steps in this work should include screening for additional *Campylobacter* species to explain samples positive for genus-specific DNA presence but negative for any of the investigated species. Utilization of more advanced molecular microbiology techniques, such as nested PCR with highly specific primers, might also allow for higher detection sensitivity. Finally, sequencing of the obtained PCR products would allow for reliable confirmation of the taxonomic identification of the detected organisms.

The findings of this study suggest the potential for high prevalence of DNA from *Campylobacter* species in Saskatchewan retail ground beef with rich taxonomic diversity.

However, the prevalence of viable pathogens is more important from a public health perspective. Further investigation can contribute to better understanding and control of foodborne illnesses related to ground beef consumption.

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5.6. References

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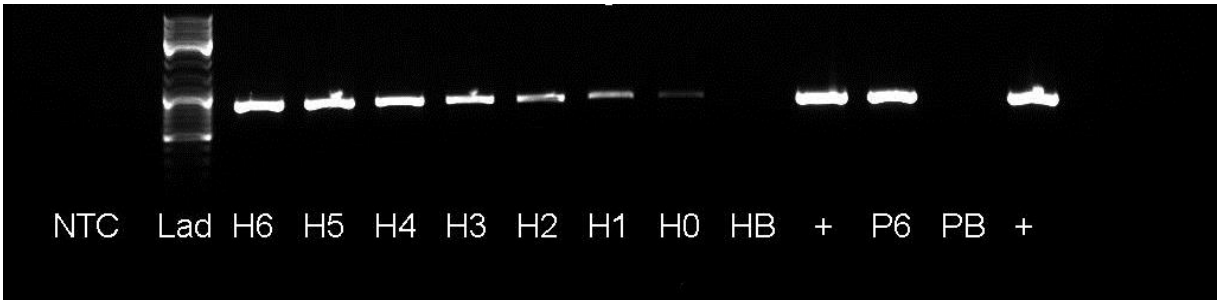
Figure 5.1. Conventional PCR assay results targeting *Campylobacter* genus-specific 16S RNA gene. (1% agarose gel visualized with ethidium bromide under UV light)



* NTC - no template control

** Lad - molecular markers ladder

*** H6-H0 - inoculated ground beef homogenate in descending decimal dilution series

**** HB – not inoculated ground beef homogenate

***** + - positive control (purified *Campylobacter fetus* genomic DNA)

****** P6 - buffer solution inoculated with $\log_{10}$ 6 *Campylobacter fetus*

******* PB – pure buffer

Table 5.1. *Campylobacter* species for which their main epidemiological characteristics are known

Campylobacter species	Original isolation source	Pathogenicity	Observed presence in cattle or ground beef
C. coli	Pigs, birds, environment surface waters	Gastrointestinal illnesses, septicaemia	Yes (2, 33, 35, 50)
C. concisus	Human oral cavity	Gastrointestinal illnesses, periodontal diseases	Yes (50)
C. curvus	Human oral cavity	Liver abscesses (72), gastroenteritis (1)	Yes (50)
C. fetus	Cattle, sheep, goats	Bacteremia, meningitis (52)	Yes (33, 35, 50)
C. gracilis	Human oral cavity	Periodontitis, brain abscesses, infected wounds, pulmonary infections (37)	No
C. helveticus	Domestic and wild animals	No (32)	Yes (50)
C. hominis	Human feces (42)	Opportunistic pathogen (46)	No
C. hyointestinalis	Swine (22)	Gastrointestinal illness (18)	Yes (34, 35, 57)
C. insulaenigrae	Sea mammals (21)	Enteritis, septicaemia (15)	No
C. jejuni	Human feces	Leading cause of human enteric illness	Yes (2, 35, 50)
C. lanienae	Abattoir workers (48)	No (32)	Yes (2, 24, 35)
C. lari	Birds and mammals	Gastrointestinal illness (53)	Yes (2, 50)
C. laridis	Seagulls (58)	Gastroenteritis, septicaemia (3, 67)	No
C. mucosalis	Swine (43)	Enteritis (20)	Yes (50)
C. rectus	Human oral cavity	Periodontal disease (23)	No
C. showae	Human oral cavity (19)	Opportunistic pathogen (66)	No
C. sputorum	Cattle, sheep	Opportunistic pathogen	Yes (50)
C. troglodytis	Chimpanzees (39)	Unknown	No
C. upsaliensis	Dogs and cats	Enterocolitis (8)	Yes (50)

Table 5.2. Summary of product source information, season of purchase, and product state at the time of purchase for the retail ground beef samples included in the study

Sample categories	Total	Collection season			
		Warm (May 1 – Oct 30)		Cold (Nov 1 – Apr 30)	
		Fresh	Frozen	Fresh	Frozen
Federally regulated or provincial licensed facility	125	26	28	42	29
Locally licensed facility	78	5	28	15	30
No label inspection information	106	50	8	42	6
Total	309	81	64	99	65

Table 5.3. Presence of *Campylobacter* genus DNA in retail ground beef samples by study group, state, and season of purchase as identified by conventional PCR with 16S RNA targeting primers (n=309)

	Federal or provincial inspected facility				Locally licensed facility				No label inspection information			
	Cold season		Warm season		Cold season		Warm season		Cold season		Warm season	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
Campylobacter genus DNA:												
positive	8	5	4	5	2	6	1	2	6	2	8	1
negative	34	24	22	23	13	24	4	26	36	4	42	7
Total tested	42	29	26	28	15	30	5	28	42	6	50	8
% positive	19.0	12.8	18.2	21.7	15.4	25.0	25.0	7.7	16.7	50.0	19.0	14.3

Table 5.4. Statistical significance (p-values) of investigated factors for detection of *Campylobacter* species in the retail ground beef offered for sale in Saskatchewan (α=0.05) (n= 309)

	Study group	State	Season	TAPC (continuous)	TAPC (categorical)	TEPC (continuous)	TEPC (categorical)	TBL (continuous)	TBL (categorical)
Genus positive (PCR)	0.80	0.97	0.45	0.66	0.84	0.32	0.18	0.48	0.64
C. jejuni	0.50	0.24	0.83	0.78	0.90	0.67	0.42	0.99	0.27
C. coli	0.79	0.64	0.81	0.74	0.41	0.47	0.49	0.78	0.43
C. upsaliensis	0.38	0.87	0.41	0.78	0.55	0.55	0.51	0.57	0.82
C. curvus	0.84	0.13	0.20	0.84	0.99	0.17	0.09	0.80	0.66
C. fetus	0.86	0.68	0.34	0.82	0.98	0.37	0.29	0.80	0.82
C. rectus	0.98	0.68	0.51	0.83	0.36	0.79	0.79	0.45	0.64
C. hyointestinalis	0.69	0.20	0.34	0.01	0.14	0.56	0.58	0.01	0.09
Total real time qPCR positive	0.76	0.44	0.35	0.74	0.13	0.64	0.73	0.31	0.24

TAPC – total aerobic plate count, TEPC – total *E. coli* plate count, TBL – total bacterial

6 CONCLUSIONS

6.1 Introduction

This thesis describes the investigation of bacterial levels in retail ground beef offered for sale in Saskatchewan.

Foodborne illnesses attributed to ground beef consumption, especially related to infections with *E. coli* O157:H7 and other verotoxigenic *E. coli* (VTEC), are important public health concerns *(35)*. Furthermore, human *Campylobacter* infections in Western Canada were found to have epidemiological links with cattle production and beef consumption *(6, 16-18, 36)*.

Ensuring the safety of ground beef offered for sale to consumers is a joint responsibility of animal producers, meat processors, retailers, and government. However, the legislative and organisational approaches to meat inspection and food safety vary in different jurisdictions in Canada and at a different levels of government *(29)*. Potential differences in ground beef bacterial levels resulting from variability in regulatory requirements have not been previously investigated in Canada.

The objectives of this study were to: 1) provide a current baseline describing bacterial counts in retail ground beef offered for sale in Saskatchewan and compare the bacterial levels among product processed and packaged under three different regulatory environments, 2) investigate the presence of *stx1*, *stx2*, and *eae Enterobacteriaceae* virulence markers, and 3) describe the prevalence and diversity of *Campylobacter* organisms.

Measures of bacterial levels were compared across the study categories based on surrogate provenance information as available to consumer on retail ground beef packaging. This concluding chapter summarizes the key findings of the project, examines the strengths and limitations of the methods used, and identifies further research questions and opportunities.

6.2 Key findings of the study

A geographically stratified sample of retail ground beef packages (n=309) was collected from Saskatchewan during a one year period from May 2011 to May 2012. Using information available on the ground beef package at the time of sale, all samples were divided into three groups to reflect the product source and processing history. The first group of samples (n=125) included those packaged in a federally regulated or provincially licensed raw meat production facility, as indicated by the federal inspection symbol and establishment information or by the provincial facility information on the product label legend. The second group (n=78) was comprised of ground beef from locally licensed facilities and included samples packaged by small abattoirs operating under a local health region license as identified by vendor's information. The third group (n=106) included samples with no inspection history available to consumer and included those with no definitive data on the label; that is, no label legend, no identification of the local processor, and no information about the regulatory or licensing requirements at the site where the product was processed and packaged. Most of these samples had been repackaged by the retailer.

Three different laboratory techniques were applied to evaluate bacterial levels in the study samples: 1) total aerobic plate count (TAPC) which enumerated all viable bacteria capable of

growing under unrestricted aerobic conditions what provided information on general sample cleanliness and potential history of storage temperature abuse; 2) total *E. coli* plate count (TEPC) which enumerated generic *E. coli* and is commonly interpreted as an indicator of fecal contamination; and 3) culture independent real-time quantitative PCR with universal bacterial target enumerating presence of bacterial DNA (total bacterial load (TBL)) which estimated the total number of bacteria of all types that were or had been present in the sample.

6.2.1 Total aerobic plate count results

The TAPC values for all samples (n=309) ranged from $\log_{10}$ 1.7 to $\log_{10}$ 8.9 culture forming units (cfu) per gram of ground beef, with a median $\log_{10}$ 6.0 cfu/g, 5th percentile $\log_{10}$ 2.9 cfu/g, and 95th percentile $\log_{10}$ 7.4 cfu/g. The effect of sample state (fresh or frozen) at the time of purchase on TAPC varied with the season of purchase (warm, May 1 to October 31; cold, November 1 to April 30). After accounting for state, season, and their interaction, differences in TAPC among all study groups were significant. TAPC was lower in samples from federally or provincially licensed facilities than samples from locally licensed facilities as well as from samples with no label legend or source information.

6.2.2 Total *E. coli* plate count results

The TEPC values for all samples (n=309) ranged from 0 (below the detection limit) to $\log_{10}$ 4.2 cfu/g per gram of ground beef; the 5th percentile and the median TEPC were both 0 cfu/g and the 95th percentile was $\log_{10}$ 3.2 cfu/g. After accounting for product state at the time of purchase and season of purchase, the TEPCs of samples from federally regulated or provincially licensed facilities were significantly lower than samples with no inspection information on the

label and samples from locally licensed facilities, the latter two of which were not significantly different from one another.

6.2.3 Culture independent real-time quantitative PCR with universal bacterial target enumerating presence of bacterial DNA results

The total bacterial load (TBL) values for all samples (n=309) ranged from $\log_{10}$ 1.7 to $\log_{10}$ 11.5 target copies per gram of ground beef (tc/g). The median TBL was $\log_{10}$ 5.9 tc/g, the 5th percentile was $\log_{10}$ 2.6 tc/g, and the 95th percentile was $\log_{10}$ 8.8 tc/g. The effect of both study group and the season of purchase varied with the state of sample at the time of purchase. Fresh ground beef from federally regulated or provincially licensed facilities had a significantly lower TBL than fresh samples with no inspection information on the label. Similarly, frozen ground beef from federally or provincially licensed facilities had a significantly lower TBL than fresh samples from federally or provincially licensed facilities, fresh samples from locally licensed facilities, frozen samples from locally licensed facilities, fresh samples with no inspection information on the label, and frozen samples with no inspection information on the label.

6.2.4 Detection of *Enterobacteriaceae* virulence factors

To evaluate the potential presence of *Enterobacteriaceae* of public health importance, samples were screened for *stx1*, *stx2*, and *eae* virulence factors encoding genes using conventional PCR.

Positive PCR results for at least one of the reaction targets were observed for 112 of 308 tested samples. Of these 112 samples, 107 tested positive for *stx1*, 8 for *stx2*, and 26 for *eae*. There was a marked pattern of variability in visualized PCR product sizes. There were no

differences in the likelihood of detecting *stx1* in samples originating from locally licensed facilities or ground beef without clear inspection labelling when compared to ground beef from federally regulated or provincially licensed facilities. Also when compared to samples from federally regulated or provincially licensed facilities, there were no differences in the odds of detecting *stx2* or *eae* in samples from locally licensed facilities or in the odds of detecting *stx2* or *eae* in samples without clear inspection labelling.

In addition there were no associations between the detection of *stx1*, *stx2*, and *eae* virulence markers and sample collection state (fresh vs. frozen), season of sample collection, TAPC, or TEPC.

TBL was significantly associated with the presence of *stx2* when analyzed as a continuous variable, but not when categorized. The odds of detecting *stx2* in ground beef decreased 1.65 times with each successive $\log_{10}$ tc/g increase in TBL; however, this relationship was not monotonic when TBL was categorized and could not be meaningfully interpreted. Total bacterial load was not a significant predictor for *stx1* and *eae* presence.

Similarly there was no significant association between successful isolation of *E. coli* on MacConkey agar and detection of *stx1*, *stx2*, and *eae* virulence markers.

6.2.5 Detection of *Campylobacter spp.* genus-specific DNA by conventional PCR and identification of seven *Campylobacter* species by real-time PCR

The presence of *Campylobacter spp.* was assessed by conventional PCR and of the 309 total samples included in the study, 50 (16.2%) tested positive for *Campylobacter* genus-specific DNA using a conventional PCR for *Campylobacter* at the genus level.

DNA from all seven investigated *Campylobacter* species were detected in the pool of study samples (n=309) by real-time PCR. Forty-nine samples (15.9%) were identified as positive for the presence of at least one of investigated target. In total, 14 samples (4.5%) were positive for *C. coli*, 11 (3.6%) for *C. curvus*, six (1.9%) for *C. fetus*, 24 (7.8%) for *C. hyointestinalis*, 12 (3.9%) for *C. jejuni*, six (1.9%) for *C. rectus*, and nine (2.9%) for *C. upsaliensis*.

The presence of DNA from more than one *Campylobacter* species was detected in 24 samples (7.8%), including 17 samples (5.5%) positive for DNA of two species; three samples (0.9%) positive for three species, two samples (0.6%) positive for four species, and one sample (0.1%) positive for five species.

A total of 27 samples (54%) out of 50 identified as positive in the *Campylobacter* genus-specific assay did not test positive for any of the seven *Campylobacter* species investigated. Conversely, 26 samples (53%) out of 49 that were positive in real-time qPCR species-specific assays returned no product in the genus-level assay.

Test results from the genus- and species-level *Campylobacter* testing were compared to the collected information about the sample's source, fresh or frozen state, season of purchases and *E. coli* isolation results. Only the presence of *C. hyointestinalis* DNA was significantly associated with bacterial levels in retail ground beef as represented by TAPC and TBL when analyzed as continuous variables.

6.3 Sampling strengths and limitations

The collection of retail ground beef samples was made possible through the collaboration with the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) of the Public Health Agency of Canada. Utilising CIPARS logistic support secured efficient procurement of samples but at the same time imposed some restrictions. All the sampling trips were designed as two Census Divisions runs starting Monday. Sample submission was scheduled for Wednesday morning to optimise the timing of the laboratory testing work flow.

Thirty-nine percent of all samples were purchased on Monday and stored in a plastic cooler with ice packs till the submission to the processing laboratory on Wednesday morning. 49% of samples were purchased on Tuesday. Some samples were purchased on other days of the week (Table 6.1). Automatic temperature loggers (Temp 100, MadgeTech Inc., Warner, NH, USA) were used to record the transport temperatures for each of 27 shipments. Samples temperatures at laboratory reception arrival varied from -7.1 to 9° C with a median of 3.4° C and interquartile range of 1.1 to 6.5° C. A graph of the change in sample temperature in transit is presented on Figure 6.1. Even though these temperatures are still too low for the majority of bacteria to grow, there potentially could have been instances of temperature violation since at certain times some samples were exposed to temperatures above the recommended (4° C) *(5)*. The time of exposure to plastic cooler storage conditions for samples purchased on Monday was significantly longer then for samples purchased on Tuesday. Samples collected on Tuesdays and Wednesdays had the shortest time from collection to submission. However, retail storage conditions are known to fluctuate as well, and in some instances temperature of a retail meat display might be significantly higher than recommended *(14).*

It is unknown whether the difference in the proportions of samples from each study category collected on different days could have potentially led to biases in the assessment of bacterial numbers and composition. However, there was a significantly higher percentage of samples collected on Tuesday or Wednesday from the sample group with no source information on the label as compared to those samples from federally regulated or provincially licensed facilities (P=0.04) (Table 6.1). If anything, this difference should have biased the results so that samples from the unknown inspection group had a lower TAPC than those from the federally regulated or provincially licensed facilities because samples on Tuesdays were submitted to the lab the following morning. However, in this study, the samples with no source information on the label that were likely reprocessed and/or repackaged at the retail level had consistently higher bacterial numbers than those from federally regulated or provincially licensed facilities. Time from sample collection to laboratory submission alone could not have explained the differences observed in this study.

Only 39 out of 76 abattoirs and butcher shops that held a local health region license in Saskatchewan in 2011 were sampled for this study due to logistics limitations *(34)*. Samples were not collected from the Census Division No 18 La Ronge. This division is the largest in the province representing 46% of Saskatchewan territory, but with a population of 36557 (Census 2011) it only represents 3.5% of the total number of people living in Saskatchewan *(4)*. Most of the subdivisions in this Census Division are represented by northern villages, northern hamlets, and First Nations reserves. Considering remote locations of these settlements, their small size, scarce infrastructure, and specific food supply model that significantly differs from the rest of the province, the decision was made to exclude this Census Division from the sampling plan.

6.4 Limitations of primary sample processing

Due to the workflow organisation in the processing laboratory, all the samples in the cohort were processed on Wednesday in order to complete tests reading on Friday. Primary samples preparation included obtaining a 25 g representative subsample from a package of ground beef. While this is a basic task for packages of fresh ground beef, it was more complicated for frozen product. Samples of ground beef purchased frozen on Monday thawed during the storage in transit, but for some sample purchased frozen on Tuesday there was insufficient thawing time. As a result, some frozen ground beef samples purchased on Tuesday might not have been subsampled comparably to the majority of study samples.

6.5 Strengths and limitations of total aerobic plate count (TAPC)

3M Petrifilm^{TM} total aerobic plate count (TAPC) is industry-wide universally recognized technique that allows enumeration of all bacteria culturable under non-restrictive aerobic conditions *(38)*. The simplicity of the methodology and minimal potential for user errors or deviations from the protocol recommended by the manufacturer facilitate consistent and comparable testing results across different laboratories *(32)*.

However, the wide range in numbers of bacteria that might be present in ground beef created challenges. As per approved protocol *(38)*, ground beef homogenate in the initial concentration of 1:10 was subjected to five additional decimal dilutions. The lower detection limit of the test allowed enumeration of bacterial concentrations as low as 10 culture forming

units per gram of ground beef (cfu/g). The upper detection limit of the test did not exceed 10^7cfu/g since it was not possible to count colonies when the total number was greater than approximately 200 per plate.

We did not observe any samples with TAPC less than 10 cfu/g, so it did not create any concerns. But there were six samples reported with TAPC in the range of 10^7cfu/g that might have actually exceeded the upper detection limit of the test.

6.6 Strengths and limitations of total *E. coli* plate count (TEPC)

We used 3M Petrifilm™ TEPC as a widely recognized and universally accepted method for primary detection of fecal contamination indicator organisms in variety of substrates. It is reported to provide consistent results with minimal user influence, thus allowing for realistic comparison across time and laboratories *(39)*.

For this approved protocol, the lower calculated detection limit of the TEPC is 10 cfu/g *(39)*. Many of the ground beef samples tested for the study (171 out of 309) returned TEPC below the detection limit of the test, creating the potential for information loss due to limitations in the test lower detection limit.

3M Petrifilm™ total *E. coli* count plates method uses modified violet red bile agar (VRBA) with the addition of β-glucuronidase activity indicator to differentiate *E. coli* from other coliforms *(7, 39)*. Some strains of *E. coli* (including O157) lack the gene coding this enzyme (uidA) or β-glucuronidase activity might vary depending on culturing conditions. In previous reports, β-glucuronidase activity was not detected in between 11% and 35% of *E. coli* isolates in

pure cultures *(10, 19)*. This creates a substantial limitation of the method, especially considering that the true prevalence of β-glucuronidase negative *E. coli* in bacterial populations contaminating ground beef is not known. This limitation deserves a special consideration since there are many β-glucuronidase negative *E. coli* strains (EHEC, VTEC) that present a serious risk to public health *(23)*.

6.7 High numbers of psychrophilic bacteria found in study samples

There was an abnormal pattern observed in appearance of incubated TEPC plates for 14 ground beef samples (Figure 6.2). Large numbers of red gas-producing colonies indicated presence of an organism from the *Enterobacteriaceae* family in numbers up to 10^7cfu/g (upper detection limit for the test). This organism was preliminary identified as *Hafnia alvei* by API 20 biochemical test panel (bioMerieux, Inc., Hazelwood, MO USA). Work on the definitive confirmation of the organism's in question taxonomy by phage typing as recommended by an expert (Dr. Michael Janda, Microbial Diseases Laboratory, Richmond, CA USA – personal communication) has not been performed.

There are a numerous reports on *Hafnia alvei* as a candidate emerging food borne pathogen *(1, 2, 12, 20, 24, 31, 37)*. Close similarities between *Hafnia alvei* and enteropathogenic *E. coli* make this organism potentially important from a public health perspective. There is also the potential for horizontal gene transfer *(3)*.

The most notable characteristic of *Hafnia alvei* is its ability to grow under wide variety of conditions and in very diverse environments. Minimal growth temperature for some strains of

Hafnia alvei has been reported to be as low as 0.2° C *(22)*. This organism has been reported as one of the bacteria responsible for spoilage of irradiated ground beef stored at 4° C *(13, 22)*.

Such a unique combination of characteristics (ability to grow under normal refrigeration conditions, resistance to irradiation, ability for horizontal gene transfer, and potential candidacy as a foodborne pathogen) warrants further investigation on microbiology and epidemiology of *Hafnia alvei.*

6.8 Limitations of the samples preparation for molecular testing

In order to perform molecular testing for the targets of interest, total bacterial DNA must first be extracted from the ground beef matrix. The extraction technique principle is based on the enzymatic digestion of all solid organic substance of the sample. This is followed by the capture of DNA molecules which naturally carry negative electrical charge by positively charged sorbent resin.

As per manufacturer's specifications, not more than 50 mg of sample material by mass and not more than 200 µl by volume can be used for the total bacterial DNA extraction in DNA Blood and Tissue kit (Biorad Canada, London, ON). This created a challenge since study samples used for this purpose contained approximately 100 mg of ground beef in 1 ml of volume of homogenate with buffered peptone water. To be able to proceed to total bacterial DNA extraction we must reduce the amount of ballast material and sample volume.

As a first step in sample preparation we used low-speed centrifugation for 3 minutes at 800 rpm and 4°C to separate fat and large tissue fragments present in ground beef homogenate.

This decision was based on the assumption that bacterial cells are predominantly located in a liquid phase of the homogenate. We were not able to find any published information to either support or refute this assumption. If the assumption is not valid and bacterial cells in reality do have an affinity either to fat particles or tissue fragments, this manipulation would be counterproductive.

During the second step of sample preparation, supernatant was transferred to a new tube and centrifuged for 10 min at high speed (10000 rpm) and room temperature. The resulting supernatant was discarded and the pellet used for total DNA extraction.

Even though results of our molecular tests indicate that this total bacterial DNA extraction strategy was successful, the small total volume of sample material utilised for testing limits the sensitivity of all tests based on this methodology.

6.9 Strengths and limitations of total bacterial load estimate by quantitative real-time PCR

The enumeration of total bacterial DNA using quantitative real-time PCR with a universal bacterial target was first described by Lee et al in 1996 *(27)*. For the purposes of this study we used the method with modification suggested by Chaban et al*(9)*. This culture-independent method allows for estimation of all bacterial DNA historically present in the sample, thus circumventing the problem of satisfying diverse culturing requirements of different types of bacteria. Consequently, it detects all bacteria including those in a viable but non-culturable state (VBNC). Even though VBNC phenomenon was described for many non-spore forming organisms as a survival mechanism *(10)*, the true prevalence of bacteria in this state has not been

described and remains unknown. Additionally, there is an opinion that many bacterial species have not yet been described due to limitations in currently available culturing technology *(40)*.

An important technical limitation of this method is the extreme sensitivity to bacterial contamination during the molecular bench work. Even smallest numbers of organisms introduced during the preparation and set-up of the real-time PCR with universal bacterial target could potentially skew the obtained estimation of total bacterial DNA present in the sample.

The fundamental systematic limitation of this methodological approach is its inability to distinguish between DNA from viable bacterial cells and DNA from deactivated organisms. Potential solutions to this problem could include using specialized nucleic acid dyes to distinguish between DNA originating from viable intact cells and DNA from deactivated bacteria *(26)*. Application of propidium monoazide or similar agents for the purpose of total viable bacterial load estimation in such a rich organic substrate as ground beef would be a potentially important direction of further work based on the presented study and would generate a more direct assessment of the potential risks to public health.

6.10 Strengths and limitations of comparing TAPC, TEPC, and TBL

The three methods utilized in this study are fundamentally distinct. While TAPC and TEPC are culture-based methods, TBL was estimated utilising a completely culture-independent methodology. Biologically, all organisms capable of growing under bile salts restrictive influence of TEPC medium are also able to grow under non-restrictive aerobic conditions of

TAPC, but not vice versa. Consequently, measurements of bacterial levels estimated by TEPC can be viewed as a subset of measurements of bacteria concentrations estimated by TAPC.

Estimation of TBL utilizing completely culture-independent methodology results in obtaining an inclusive measure of bacterial levels. Consequently, TAPC estimation can be viewed a subset of TBL.

6.11 Strengths and limitations of direct molecular detection of *stx1, stx2,* and *eae Enterobacteriaceae* virulence markers in ground beef matrix

Focusing on detection of *Enterobacteriaceae* virulence markers instead of identification of particular organisms potentially allows more efficient and epidemiologically sound way of the food safety risk assessment. There are a number of considerations in support of this hypothesis.

First of all, due to limitations of current culture-based laboratory methods, attempts to identify causative organisms for a significant proportion of foodborne illnesses are not successful. Recent estimation indicates that even after adjustment for underreporting and surveillance system deficiencies, as much as 60% of all foodborne illnesses in Canada are caused by unspecified agents *(36).*

Secondly, there is an increasing scientific awareness of horizontal gene transfer mechanisms and accumulating evidence of frequent occurrence of this phenomenon in *Enterobacteriaceae (15).* Hypothetically, prevalence and characteristics of certain mobile genomic elements in the bacterial community can be currently underappreciated factors influencing the virulence and pathogenicity traits of individual species.

And finally, the ability to obtain test results in a very short time by utilization of completely culture-independent techniques gives a tremendous advantage in time-sensitive situations.

On the down side, there are number of limitations associated with this approach. First, the analytical sensitivity of this technique is limited by the efficiency of total bacterial DNA extraction from the tested matrix and efficiency of the PCR detecting the target. Secondly, even considering 100% extraction efficiency and 100% PCR efficiency, simple estimates show that since we were using 0.1 g of matrix for total bacterial DNA extraction (a) and 1% of obtained eluate to run the PCR reaction(b), to insure presence of at least one copy of target DNA introduced in to PCR ($n = 1$), the minimal concentration of target DNA in original matrix ($N_{\min}$) must be $N_{\min} = \frac{n}{a*b} = \frac{1}{0.1*0.01} = \frac{1}{0.001} = 1000$ copies/g. The lower detection limit than that obtained by culture based techniques requires dramatic improvements before it can be considered as a functional diagnostic tool.

Secondly, development of the scientific understanding of bacterial virulence is still in progress even for the most studied organisms like *E. coli (30)*. Paradoxically, there are numerous *E. coli* strains that carry *stx1/stx2* genes but are not virulent, and many of the *E. coli* strains capable of causing gastrointestinal illnesses do not possess the targeted in this study virulence markers *(23)*. In our work we report variability in sizes of products generated by PCR targeting *eae*, *stx1*, and *stx2* coding genes. This observation leaves many questions open: we are not able to confirm either it is a result of genetic diversity within *E. coli* population, or it is a result of similar genes detection in other bacterial species. Consequently, it is even more problematic to assess potential public health implications based on this information.

Another common shortcoming of the culture-independent molecular techniques is the inability to discriminate between the signal from viable and inactivated bacteria present in tested samples.

6.12 Strengths and limitations of direct molecular detection of *Campylobacter* genus-specific and *Campylobacter* species-specific DNA in ground beef matrix

Campylobacter spp. are recognized as the leading causative agents of foodborne bacterial infections in Canada *(36)*. At the same time reports on their prevalence in meats vary widely. A number of papers report either the complete absence or very low prevalence of *Campylobacter spp*. in meats based on conventional culturing techniques *(17, 25)*. Failure to culture *Campylobacter spp.* is commonly attributed to the fastidiousness of these organisms. Even though culturing protocols for *C. coli* and *C. jejuni* are relatively well established, success rate varies with user experience, investigated matrices, and sample quality. There are no universally recognized culturing protocols for other species belonging to genus *Campylobacter* making their routine isolation not feasible.

To circumvent the difficulties in culturing *Campylobacter spp*. both genus-level and species–level identification were performed in this study utilising completely culture-independent techniques. The prevalence of *Campylobacter* measured in Saskatchewan retail ground beef was in line with comparable Canadian and international studies *(17, 21, 28)*. We were also able to demonstrate the presence of several *Campylobacter* species with no established culture protocols. These finding are important to increasing awareness about the presence of a variety of *Campylobacter* species in ground beef and the potential public health implications.

Shortcomings of the completely culture-independent method include low sensitivity and the inability to distinguish between the signal obtained from viable and from inactivated bacteria *(33)*.

6.13 Limitations of the study findings interpretation and identification of biases

Food safety is a multifaceted subject of tremendous complexity. In this study we attempted to focus on a very narrow question regarding the ability of a consumer to judge at least to some degree food safety attributes of Saskatchewan retail ground beef based on the information available on the packaging label. In order to fulfill this objective, we consciously allowed a number of simplifications as a mean to maintain the scope and volume of the study within manageable limits, but potentially introducing a number of biases.

First of all, there is a potential danger of sampling bias introduced by the utilization of Canadian Program for Antimicrobial Resistance Surveillance (CIPARS) infrastructure and logistical support for samples procurement. While the main objective of the sampling for this study was collection of representative sample from three identified retail ground beef categories, the goal of CIPARS sampling is different – to collect a representative sample of meats consumed in Saskatchewan. Since ground beef produced and packaged at federally inspected or provincially regulated facilities comprise the largest volume of the Saskatchewan market, this resulted in challenges in acquiring the planned number of samples representing ground beef produced and packaged at locally licensed abattoirs.

Secondly, there is a possibility of information bias introduction as a result of differences in the way information on samples provenance was obtained. For the ground beef produced and packaged at federally regulated or provincially licensed facilities information was obtained directly from the label legend, consequently it was easily identifiable and accurate. Collection of information required to identify the provenance of retail ground beef samples placed into other two study categories required consideration of numerous indirect indications and communication with a vendor. Reliance on the latter information sources might potentially introduce the risk of sample misclassification.

A number of other information and misclassification biases were discussed with respect to the limitations of each of the analytical techniques. While all of the techniques had limitations there is no evidence of differences in how these biases would have impacted the measurements from the different study groups. Given that most of the measurement challenges in this study were non-differential, any bias would have most likely been towards the null and would have minimized the observed differences among the study groups.

Two potentially confounding factors, state and season at the time of purchase, were identified and accounted for by multivariable regression analysis. However, there were other variables that might have differed across the three study categories and defined a certain degree of differences found in bacterial levels across the three study categories.

First of all, retail outlet size or ownership type has not been accounted for in this study. Even though retail meat regulatory requirements are universal and must be followed independently of organisational structure and scale of a business, there are many differences between international or large national retail chains (Wal-Mart, Loblaw's), local retail chains

(Co-op), and independent or smaller franchised retailers. Product sourcing, supply chain logistic schemes, internal policies, and budgets dedicated to hygiene and food safety all might have potential influences on the measures investigated in this study.

Secondly, distance of a sample source from major centres might potentially influence the bacterial levels. There are longer transit times for both merchandise being delivered to a remote retail outlet as well as sample shipped to the processing lab. However, as it was discussed above, there was no evidence that the bias was in the same direction as the observed differences between the study categories.

Thirdly, country of sample origin has not been accounted for in this study. According to Statistics Canada, about 20% of beef consumed in Canada is imported *(41).* Significant proportion of Canada beef imports is coming from Australia and New Zealand. Due to reliance of these countries' economies on exports, their internal requirements and standards of cattle slaughter and beef processing are very stringent. As a result, reported bacterial levels in Australian and New Zealand produced meats are very low *(8).* At the same time bacterial levels in beef imported from Uruguay were reported to be much higher *(8).* Hypothetically, meat from any country granted entry by the Canadian Food Inspection Agency can be used at any level of meat processing or retail facilities in Canada, so we are not able to identify whether country of origin would potentially introduce a differential bias in to this study.

6.14 Future research questions and opportunities generated by this study

There are many factors throughout the production chain that determine bacterial levels in retail ground beef. A diagram reflecting levels of complexity of these predictors is presented in Figure 6.3. Detailed investigation and analysis of these factors was beyond the scope of this work, though would be of great potential interest for further research based on the questions raised by this project.

This study was designed as a pilot project to investigate bacterial levels in Saskatchewan retail ground beef. Besides answering the main questions outlined in the study objectives, there were a number of issues identified that are requiring further research.

First of all, it has been identified that the retail ground beef reprocessed and repackaged directly at the point of sale possesses significantly higher bacterial levels then product identified as processed and packaged at a federally regulated or provincially licensed facilities. Future studies should investigate the risk factors associated with re-processing and re-packaging to inform policies to minimize bacterial levels in repakaged product available to the consumer.

Secondly, identification of a high numbers of psychrophilic *Enterobacteriaceae* (especially *Hafnia alvei*) in ground beef creates a number of questions regarding their potential pathogenicity. More importantly mobile genetic elements encoding virulence factors and antimicrobial resistance might be transferred to other bacterial species from *Hafnia alvei*.

Thirdly, considering overall successful application of the direct molecular techniques to answer a number of questions in this study, improving sensitivity of this methodology via

optimising the total bacterial DNA extraction and developing a methodology to process larger volumes of matrix into a single extract would be of great value for food safety research.

Fourthly, developing a practical methodology to distinguish between viable and inactivated bacteria within the framework of culture-independent molecular testing applied to rich organic matrices of food samples would increase the validity of bacterial level interpretation as it relates to public health implications.

And finally, the prevalence and diversity of *Campylobacter* spp. in retail ground beef needs further attention. Since we were targeting only 7 out of at least 23 *Campylobacter* species currently recognized, a full investigation and description of the true diversity and prevalence of these organisms in retail ground beef is important to better understand the public health risks associated with ground beef.

6.15. Conclusions

In this study we investigated the bacteria present in retail ground beef from Saskatchewan markets using a variety of different analytical tools. Measures of the product bacterial load were compared among three different supplies of retail ground beef based on the information available to consumers. This analysis also considered differences between the warm and cold seasons and fresh and frozen state of ground beef at the point of sale. The presence of *Enterobacteriaceae* virulence markers and *Campylobacter* spp. DNA were evaluated as indicators of potential importance for public health. This pilot study can be used to inform future work on identification of particular pathogens and assessment of public health risk associated with retail ground beef.

6.16 References

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Figure 6.1 Example of retail ground beef samples transit temperature changes (for samples purchased on Monday, March 26, 2012)

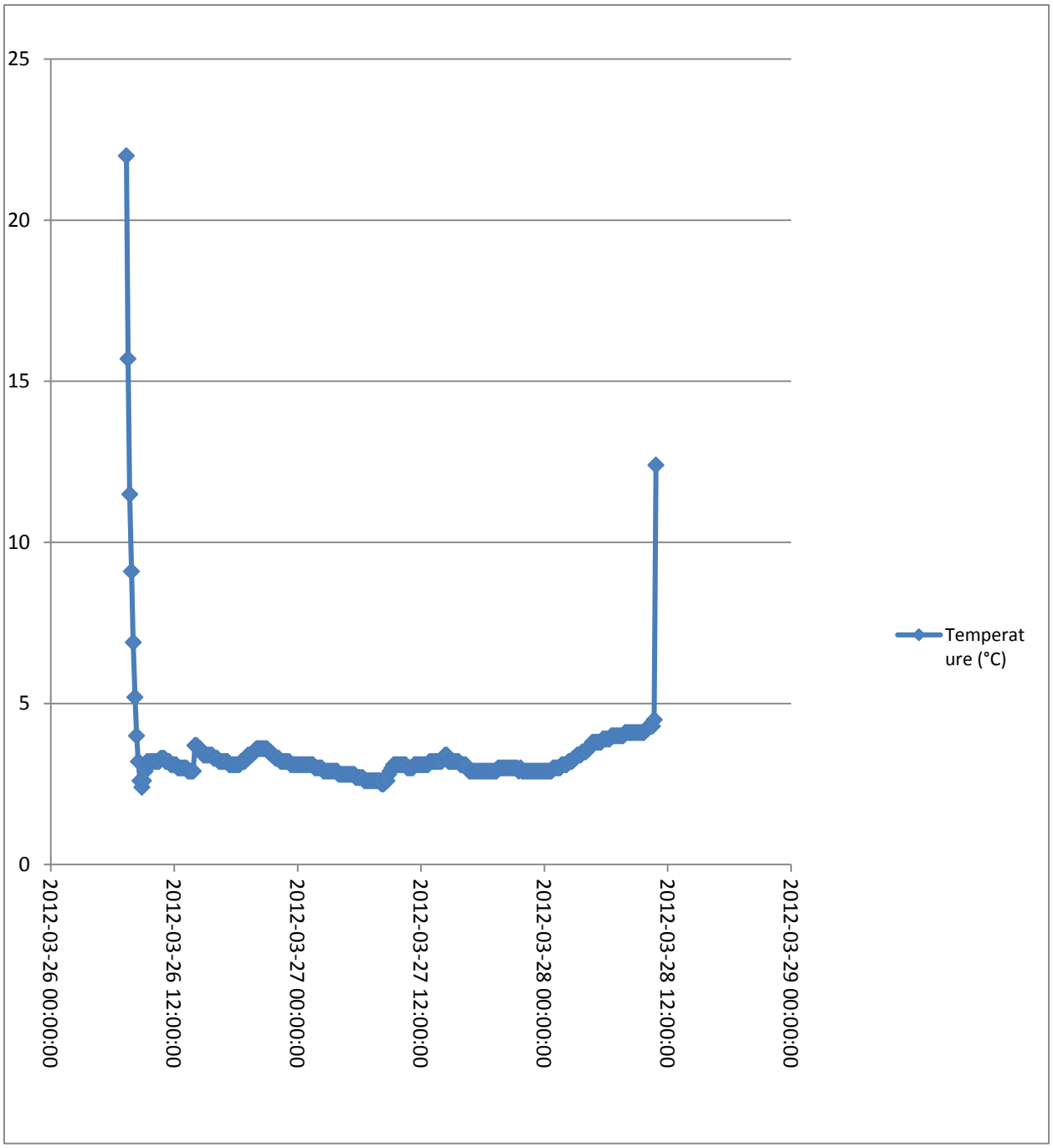
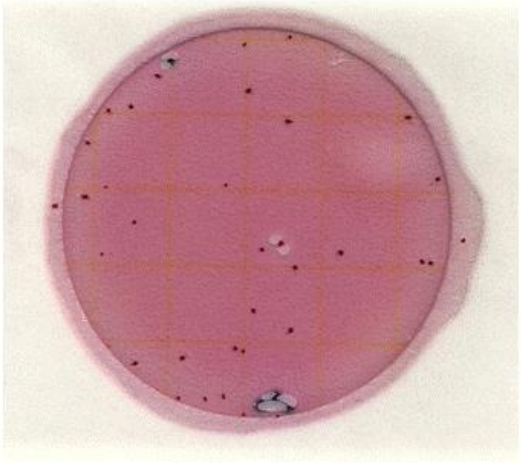
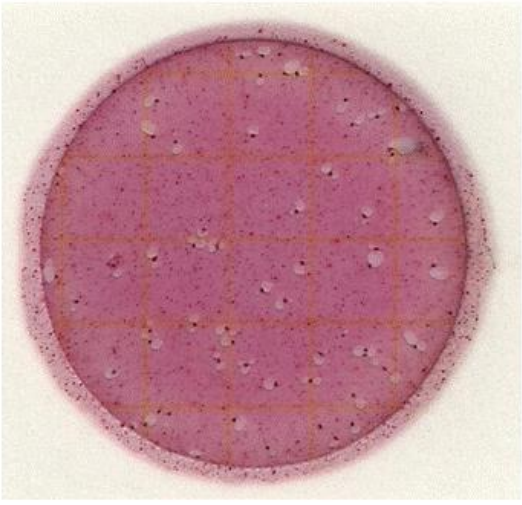


Figure 6.2 TEPC plates with observed growth of *Escherichia coli* and *Hafnia alvei*



Total *E. coli* count plate with two confirmed *E. coli* colonies (blue colonies with gas)



Total *E. coli* count plate with numerous colonies identified as *Hafnia alvei* by API 20 test panel

Figure 6.3 Levels of factors influencing bacterial counts in retail ground beef (compilated from Blackburn, C. de W. and P. J. McClure, ed. 2009)

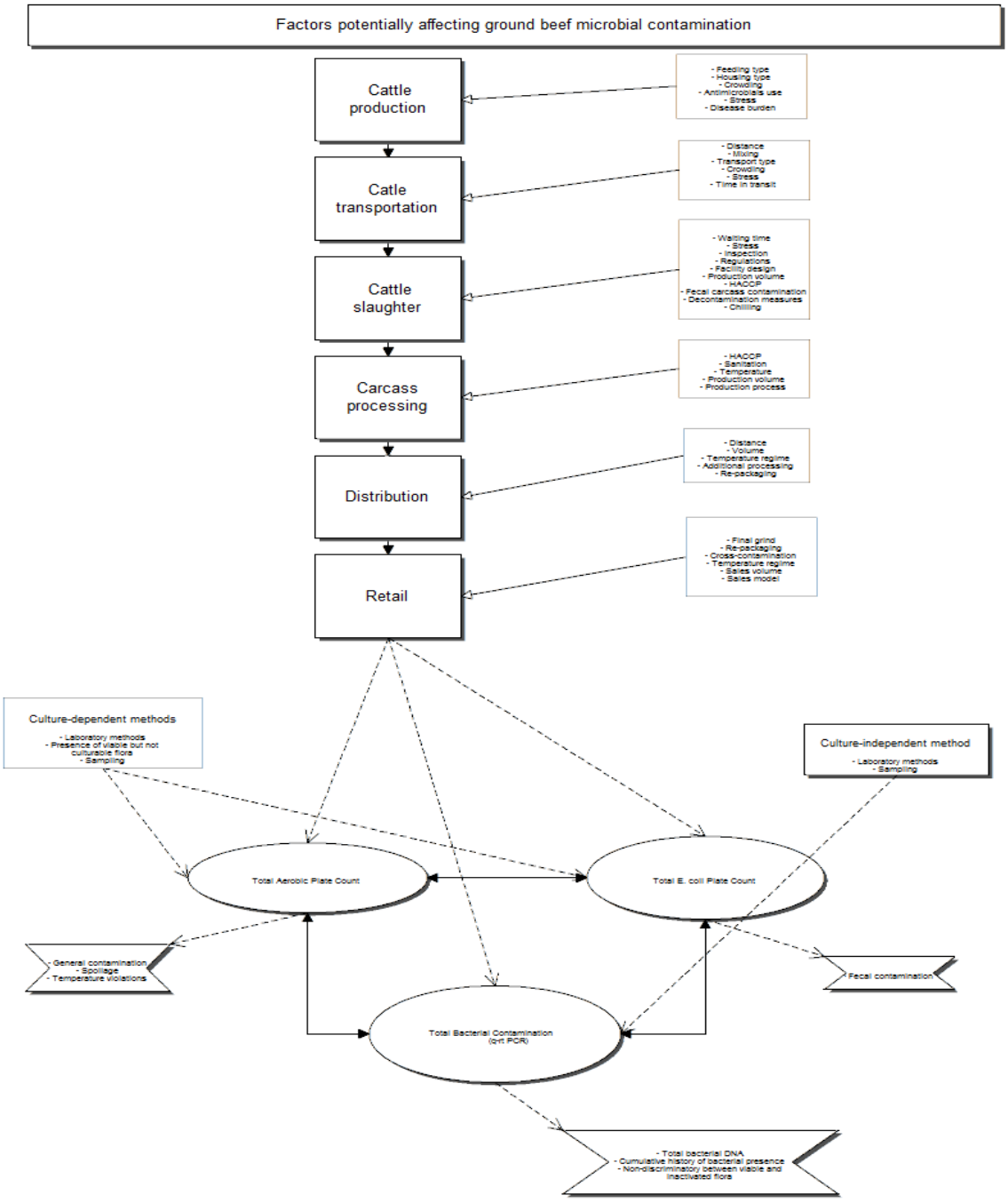


Table 6.1 Frequency of ground beef samples collected for each day of the week

	Federally or provincially regulated	%	Locally licensed	%	Unknown	%	Total	%
Sunday	17	13%	6	8%	1	1%	24	8%
Monday	50	40%	34	43%	38	37%	122	39%
Tuesday	45	36%	31	39%	51	50%	127	41%
Wednesday	13	10%	7	9%	9	9%	29	9%
Thursday	0	0%	2	3%	1	1%	3	1%
Friday	0	0%	0	0%	0	0%	0	0%
Saturday	1	1%	0	0%	3	3%	4	1%
Proportion of samples collected on Tuesday and Wednesday	46%		47%		58%		50%	
Total	126		80		103		309	