BACTERIAL STARTER CULTURES AND PROBIOTICS AS RESERVOIRS OF ANTIBIOTIC RESISTANCE TRANSMISSIBLE TO ZOONOTIC PATHOGENS

By

Roniele Peixoto Cordeiro

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Submitted to the Faculty of Graduate Studies

In Partial Fulfilment of the Requirements For the Degree of

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FACULTY OF GRADUATE STUDIES

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Roniele Peixoto Cordeiro

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

Master of Science

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The Lord told me:
My grace is enough; it's all you need.
My strength comes into its own in your weakness.
I just let Christ take over!
And so the weaker I get, the stronger I become.

2 Corinthians 12:9-10

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DEDICATION

To my husband and my parents.

ORGANIZATION OF THE THESIS

This thesis is divided into five chapters and includes one manuscript that was sent for publication which is included as chapter 3. These chapters were standardized for presentation in thesis format.

The first chapter gives a brief introduction about the use of antimicrobial drugs in the animal production and the potential risk associated with such practice for the emergence and spread of antibiotic resistance. General and specific objectives are described in the last section of this chapter.

The second chapter presents a comprehensive literature review with a more detailed examination of the use of antibiotics in animal agriculture and how it may account for increases in antibiotic resistance of bacteria. The risk associated with the use of starter cultures in fermented products, because these organisms may serve as reservoirs of resistant genes transferable to pathogenic bacteria, is also within the scope of this chapter.

Chapter three was submitted for peer review and publication in the Journal of Food Protection. Authorship is by R. P. Cordeiro, T. Du, M. R. Mulvey, D. O. Krause and R. A. Holley, and this paper is entitled " Susceptibility of meat starter cultures to antibiotics used in food animals in Canada."

Chapter four summarizes the main conclusion of this study. Finally, chapter five presents some recommendations for future research. Appendices A-F contain detailed description of the microbiological breakpoints and the minimum inhibitory concentration for those antimicrobials studied as well as the incidence of multiple antibiotic resistances.

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ABSTRACT

The animal production industry is an important component of food production and has experienced rapid growth in the last few years. The economies of scale have led to the intensification of livestock production in developed countries, where a large number of animals are raised in a small area. Antibiotic use in veterinary medicine has become indispensable to the growth of the animal food industry because of the close proximity of a large numbers of animals at these facilities, which increases the potential for spreading diseases. Antibiotics are used in livestock to treat sick animals, to prevent infections, and to improve feed utilisation. However, antibiotic resistance has been raised as a major concern associated with the widespread use of antibiotics in medicine and veterinary practice and as growth promoters in animal husbandry. Attention has been drawn to the possible role of bacteria used as meat starter cultures to serve as reservoirs for antibiotic resistance genes and the possibility they may transfer these genes to zoonotic pathogens. Coagulase-negative staphylococci (CNS) and lactic acid bacteria (LAB) are the main microorganisms used as starter cultures in meat fermentations. This study examined whether bacterial starter cultures could serve as reservoirs of antibiotic resistance transmissible to zoonotic bacteria by making both phenotypic and genotypic assessments. Thirty of the most common bacterial starter cultures used in food and feed were screened for their resistance to several antimicrobial drugs registered in Canada for veterinary use. Antibiotic susceptibility tests were performed by broth microdilution using Iso-Sensitest broth (90% v/v) and deMan-Rogosa-Sharpe broth (10% v/v), while polymerase chain reaction (PCR) was used to investigate the presence of genetic determinants of antibiotic resistance. The results showed that all 30 isolates exhibited resistance to at least 3 antimicrobials regardless of antimicrobial class while 17% or 30% of

strains were resistant to antibiotics in 3 or 6 different classes, respectively. Among the strains tested, the highest incidence of resistance noted was to carbadox (100%), sulfamethazine (83%), monensin (83%), chlortetracycline (80%), and vancomycin (80%). The incidence of antimicrobial resistance was higher among *Pediococcus pentosaceus* and lower for *Staphylococcus carnosus* strains, suggesting that the latter might be safer than the former when used as starter cultures, from an industrial point of view. Genetic determinants for the lincosamide, macrolide, and tetracycline antimicrobials were not found using PCR. However, the absence of genetic determinants did not invalidate the phenotypic results since the resistance observed may have been encoded by a gene not included in the tests or occurred by a mechanism related to structural/physical features of resistant cells. Expanding the number of genes which were screened in the genotypic tests would increase the degree of certainty that a genetic element was or was not involved in encoding resistance.

CHAPTER 1

1. Introduction

As the world's population continues to rise, demand for livestock-derived food has substantially increased, leading to a major transformation of global animal food production (Otte et al., 2007). It was estimated that the consumption of meat and milk in developing countries would grow by 2.8 and 3.3% per year between 1990 and 2020, while the growth rates in the developed world would be between 0.6 and 0.2% (Delgado et al., 2001). To satisfy this demand, industrial food animal production systems have experienced dramatic changes that include the raising of food-producing animals in confined animal-feeding operations (CAFOs). In the US, CAFOs are operations that hold at least 1,000 animal units (AUs; 1 AU = 1000 pounds body weight) stabled or confined for at least 45 days during any 12 month period (Otte et al., 2007). In 2001, there was more livestock in high-density areas than a decade ago in Canada, more specifically, 16.3% of Canada's livestock was located in high-density areas with a concentration of more than 70 AU's for every square km of farmland (Beaulieu and Bédard, 2003). To maintain the viability of these operations where animals are confined at high stock densities, the use of antibiotics in animal production has become a common practice to mitigate the spread of diseases.

Many antibiotics have appeared on the market since the discovery of penicillin by Alexander Fleming in 1928 for treatment of human and animal diseases or for use as growth promoters, and improve feed efficiency (Kapil, 2005). For more than 50 years, antibiotics have been used as growth promoters in animal production in the US and other countries (Dibner and Richards, 2005). However, the use of antibiotics in animal production raises some concerns. The major risk posed by the use of antibiotics is the development of resistance by pathogenic bacteria, which leads to unsuccessful disease treatment (Mathur and

Singh, 2005). Today, diseases caused by antibiotic resistant bacteria are being linked to CAFO practices, including methicillin-resistant *Staphylococcus aureus* (MRSA), a disease responsible for over 18,000 deaths each year in the US (Wood, 2008). According to the FDA, two million Americans acquire bacterial infections during their hospital stay every year and 70% of the infections are resistant to at least one antibiotic (Abbott, 2007). In Canada, around 13% of all isolates from intensive care units (ICU) were found to be resistant to multiple antibiotics (Zhanel et al., 2008).

The adaptation of bacteria to antibiotics is an impressive phenomenon of biological evolution provoked by mankind that has been observed in the last 60 years (Blázquez et al., 2002). Bacteria become resistant due to the overexposure to a large arsenal of antibiotics used to treat human and animal diseases. This antibiotic pressure has led to the emergence and spread of resistant genes amongst bacteria. For many years, studies have focused on the evolution of antibiotic-resistant food borne pathogens (Phillips et al., 2004; White et al., 2002). However, a broader view of antibiotic resistance would include resistance genes of pathogenic and non-pathogenic bacteria and even those genes with the potential to function as resistance genes, which would encompass the full pan-microbial genome (Wrigth, 2007). The pool encompassing all these possible origins is called the resistome. Inside this broad domain, it has been recently hypothesized that commensal bacteria may serve as reservoirs of antibiotic resistance genes which may be transferred to pathogenic bacteria (Ammor et al., 2007), but little information exists regarding the presence of antibiotic resistance genes in bacterial starter culture strains and their potential to transfer these resistance genes to pathogens (Mathur and Singh, 2005).

Lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS) are commonly used as starter cultures to improve the quality and safety of fermented products (Rantsiou and

Cocolin, 2008). More recently, some strains of LAB have also been listed as good candidates as probiotic starter cultures that may be used in meat products (Leroy et al., 2006). Despite these desirable technological traits, safety issues concerning the use of these organisms in fermented foods that are not heat treated before consumption should be addressed by demonstrating the absence of acquired resistance factors. There have been few studies regarding acquired antibiotic resistance in LAB (Mathur and Singh, 2005) and CNS (Resch et al., 2008) used as starter cultures, which make it difficult to assess the safe use of these strains for human consumption.

The aim of this research was to examine whether or not bacterial starter cultures may serve as reservoirs of antibiotic resistance transmissible to zoonotic bacteria. The specific objectives were:

1. To screen 30 of the most common bacterial starter cultures used in food and feed for their resistance to a panel of the most common antimicrobial drugs registered in Canada for use in feed as nutritional supplements or growth promotion in cattle, calves, swine and poultry;

2. To examine resistant cultures for additional resistance to structurally-related antibiotics used in human clinical medicine; and

3. To perform a molecular investigation of the occurrence of genes involved in any phenotypic resistance observed.

CHAPTER 2

2. Literature review

2.1 Fermented meat products

Meat plays an important nutritional role in the human diet. Meat products are sources of protein, fatty acids, vitamins, minerals, energy and water, and are used in the synthesis of protein, fat and membranes in the body (Higgs et al., 2002). However, factors such as a high water activity (aw = 0.96 - 0.97), favourable pH (5.6 - 5.8), and availability of nutrients, contribute to the susceptibility of meat to microbial spoilage (Hammes et al., 1990).

The perishable nature of meat led to the development of preservation methods such as salting, drying, and fermentation. Fermentation of meat products began about 1500 BC, when people learned that the mixing of salt and sugar into ground meat, followed by a holding period, was beneficial for the preservation of meat and resulted in a product acceptable to the palate (Moore, 2004). A classical application of fermentation in meat production is the processing of fermented sausage which can be classified as either dry or semi-dry. While the latter is heated at 60-68°C during smoking and have an A_w of 0.9-0.95, dry products may not be smoked, are not heat processed, and have an A_w of less than 0.9 (Bamforth, 2005).

The characteristics of the final sausage are the result of biochemical, microbiological, physical, and sensorial transformations occurring in the meat formulation during the ripening of these products (Dalmış and Soyer, 2008). These transformations included a reduction in pH, a change in the initial microflora, the reduction of nitrates to nitrites, the formation of nitrosomyoglobin, the solubilization and gelation of myofibrillar and sarcoplasmic proteins, plus proteolytic, lipolytic and oxidative phenomena, and dehydration (Casaburi et al., 2007). Commonly, sausage production utilizes curing ingredients, spices, and cultured

microorganisms in the fermentation process (Essien, 2003). While the natural occurring microflora is sometimes used by artisanal manufacturers, the use of starter cultures consisting of single or multiple-species combinations of LAB and staphylococci are more common in developed countries (Ricke et al., 2007). Starter cultures are largely used for production of fermented food in order to guarantee safety and standardize properties such as flavor, texture, and appearance (Essid et al., 2009).

2.2 Microorganisms in fermented meat

2.2.1 Starter cultures

Starter cultures can be defined as a microbial preparation of a large numbers of cells of at least one microorganism which is added to a raw material for developing the desired metabolic activity in the fermentation substrate (Leroy and Vuyst, 2004). These organisms are intentionally added in large quantity (10^7-10^8CFU/g) to ensure that a desired fermentation takes place (Resch et al., 2008). The conversion of carbohydrates to metabolites such as lactic acid, alcohol, acetic acid, or CO_2 is the primary activity of the starter culture in these fermentations (Hansen, 2002). Coagulase-negative staphylococci and LAB are microorganisms commonly used as starter cultures in meat fermentations (Bonomo et al., 2009), to develop texture, color, and flavor (Hammes et al., 1990). In addition, these microorganisms have a positive effect on the safety of the fermented products due to the suppression of the pathogenic flora by acidification or by the production of antimicrobials. Studies have shown that the use of a starter that produces bacteriocin and causes a rapid pH decrease may eliminate both *Escherichia coli* O157:H7 and *Listeria monocytogenes* during the manufacturing of dry sausages. Lahti et al. (2001) reported a 5 $\log_{10}$ unit reduction in counts of *E. coli* O157:H7 in sausage fermented and stored for 49 days using starter cultures of *Staphylococcus carnosus* with *Lactobacillus curvatus*. The authors also found that the

concentrations of *L. monocytogenes* in the high-inoculum (5.10 and 5.21$\log_{10}$ CFU/g) sausages decreased to below 2.0 $\log_{10}$ CFU g^{-1} within 21 and 35 days, when using starter cultures containing *Staphylococcus xylosus* with bacteriocin-producing *Pediococcus acidilactici* and *Lactobacillus bavaricus*. Benkerroum et al. (2003) studied the behaviour of *L. monocytogenes* in raw sausages in the presence of a bacteriocin-producing lactococcal strain as a protective culture. Their results showed that the reduction of *L. monocytogenes* counts was greater in samples fermented with the bacteriocin-producing *Lactococcus lactis* than in those fermented with the non-bacteriocin-producing *Lactococcus lactis*.

Coagulase-negative staphylococci play an important role in the development of color and flavor in fermented meat products. The most important technological characteristic of CNS is the ability to reduce nitrate to nitrite leading to the formation of nitrosomyoglobin (Rantsiou and Cocolin, 2008) which promotes the desired red color. The organoleptic quality of meat products is also dependent on the lipolytic and proteolytic activities of CNS (Casaburi et al., 2007), and catalase activity that prevents lipid oxidation (Barriére et al., 2001). Although *S. carnosus* and *S. xylosus* are the starter cultures most commonly used in meat fermentations (Simonová et al., 2006), *S. xylosus* seems to be the dominant CNS species in many fermented sausages. Bonomo et al. (2009) reported that the microflora in traditional fermented sausage (37 strains) was found to be dominated by 17 strains of *S. xylosus* (45.9%). Similarly, Fiorentini et al. (2009) found that *S. xylosus* with satisfactory technological traits such as nitrate reductase, catalase and lipase activity was the dominant species in naturally fermented sausage, which would possibly make this species a good choice for starter culture applications. However, the incidence of resistant strains and the frequency of antibiotic resistance within strains is notably lower for *S. carnosus* than for *S. xylosus*. Martín et al. (2006) conducted a study to characterize the molecular, technological and safety features of Gram-positive and catalase-positive cocci from slightly fermented

sausages. While *S. xylosus* showed resistance to 16 of 19 antibiotics, *S.carnosus* was resistant to only 5 of the antibiotics tested. Resch et al. (2008) studied the antibiotic resistance of CNS used in starter cultures and found that *S. xylosus* isolates (130/137) exhibited resistance to < 7 antibiotics, whereas only a few strains of *S. carnosus* (13/103) showed some antibiotic resistance. Hence, from a industrial standpoint, strains of *S. carnosus* seem to be a safer choice than those of *S. xylosus* for use as starter cultures in fermented meat.

Lactic acid bacteria are Gram-positive microorganisms that include *Lactococcus, Enterococcus, Oenococcus*, *Pediococcus, Streptococcus, Lactococccus*, and *Lactobacillus* species (Makarova et al., 2006). Microorganisms which belong to the LAB group are catalase-negative, non-sporeforming cocci, coccobacilli or rods with less than 55 mol% G + C content in their DNA (Stiles and Holzapfel, 1997). In LAB, there are two major fermentation pathways of glucose which are: (i) homofermentative, where the pyruvate is reduced exclusively to lactic acid; and (ii) heterofermentative, where alternative end products such as acetic acid, ethanol and carbon dioxide are produced alongside lactic acid (Bamforth, 2005). Regarding biosafety of industrial LAB cultures, there is a growing concern about the possible role of LAB as vectors for antibiotic resistance genes (Danielsen and Wind, 2003).

2.2.2 Safety and health aspects of microorganisms used in fermented meat

The safety assessment of the microorganisms used as starter cultures in fermented meat is relevant from a consumer's safety point of view. Consequently, several food safety authorities have developed safety models to enhance consumer confidence. The most widely known program is GRAS (Generally Recognized as Safe) status afforded by the US Food and Drug Administration (FDA), which lists microorganisms considered safe only for specific uses (Chamba and Jamet, 2008). In Europe, a similar concept is called "Qualified Presumption of Safety" (QPS) and this involves a safety assessment of a defined taxonomic

group (e.g. genus or group of related species) made based on four pillars: establishing identity, the body of scientific knowledge available to support use, presence of acquired antibiotic resistance factors, and possible pathogenicity and end use (EFSA, 2007).

Lactic acid bacteria have a long history of safe use in the processing of fermented food (Essid et al., 2009; Temmerman et al., 2003). Besides the technological and safety properties associated with starter cultures in fermented products, potential health and nutritional benefits derived from some species of LAB have also been discussed (Buckenhiiskes, 1993), which characterized some of these bacteria as being probiotic. Although probiotic functions can sometimes be confounded with those of starter cultures, probiotics have the distinctive property of providing a wide variety of health benefits to the host (Farnworth, 2008). Some of the claimed effects attributed to probiotics include prevention and treatment of diarrhea, treatment of irritable bowel syndrome (IBS), treatment of inflammatory bowel disease, and prevention of colon cancer (Santosa et al., 2006).

Although probiotic cultures are primarily used in dairy products such as fermented milk and yoghurt (Bernardeau et al., 2008), in the last few years, strains of LAB species have been also included in other food products such as fermented meats (Ruiz-Moyano et al., 2008; Klingberg et al., 2005). Although many meat products are heated before consumption, thus killing probiotic bacteria, dry sausages are processed by fermenting without heat (Bamforth, 2005), which make this product a potential carrier for probiotic cultures. Another important feature for a probiotic strain to exert its beneficial effect on the host is its ability to survive passage through the host's digestive tract (Maragkoudakis et al., 2006). Ruiz-Moyano et al. (2008) screened LAB for potential probiotic use in dry fermented sausages. Of the *in vitro* investigations used to predict the survival of a strain in conditions present in the gastrointestinal tract, exposure to a pH of 2.5 was a highly discriminating factor with only 51

of 312 pre-selected strains resistant after 1.5 h of exposure. Strains of *Lactobacillus sake* and *Pediococcus acidilactici* from meat starter culture mixtures were pontentially probiotic in meat products because of their survival capacity under acidic exposure simulating gastrointestinal conditions (Erkkilaä and Petäjä, 2000). Pennacchia et al. (2004) attempted to select potentially probiotic *Lactobacillus* strains directly from fermented meat products. In their study, at least 20 *Lactobacillus* strains were capable of surviving the pH of the stomach and the environment of the intestine, which suggests they have potential as probiotics.

Some authors argue that the marketing potential for probiotics in fermented sausage as a "health food" could be compromised because of its perceived image as meat and its controversial nutrient profile with the presence of nitrate, salt, and fat (Vuyst et al. 2008). Given these difficulties, careful selection must be made if health claims are to be proposed in the application of LAB as potential probiotics in fermented meats. Factors justifying *in vitro* attachment, antimicrobial effects, and immune stimulation must be taken into account. Furthermore, even though some bacterial strains have been recognized as safe, even bacterial strains with a probiotic claim as well as those used as starter cultures may serve as antimicrobial resistance reservoirs (Resch et al., 2008; Charteris et al., 2001), which may be overlooked by GRAS status (Temmerman et al., 2003), since it does not address antibiotic resistance as a criterion.

2.3 Antimicrobial use in animal agriculture

2.3.1 Animal production industry

The animal production industry contributes a significant proportion of easily digested complete proteins to the human diet. The increased standard of living and the consequent expansion in consumer demand for animal products has led to the "livestock revolution", where the annual rate of growth for meat production has been estimated to be 1.8% for the

period 1993-2020 (Delgado et al., 1999). However, this growth is not globally uniform. According to Gerber et al. (2005), annual growth rate between 1982 and 1994 was 5.4 and 1.1% for developing and developed countries, respectively. The economies of scale have led to the intensification of livestock production in developed countries. For example, the cattle feeding sector in the United States and the United Kingdom has changed from semi-intensive to a quite intensive approach based on specialized management since the end of World War II (Mintert, 2003; Hooda et al., 2000). In Denmark, the total production of pork increased from 0.8 to over 1.8 x 10^6 tonnes between 1980 and 2000, while the number of pig producers dropped from 70000 to around 15000 (WHO, 2002). In Canada, overall animal production has increased, despite a decline in the number of farms during the last several decades, and this had led to the intensification of livestock production (Beaulieu and Bédard, 2003). In today's numbers, animal herds in the United States and Canada total approximately 96 and 13.9 million cattle and calves, respectively (NASS, 2009).

2.3.2 Benefits of antibiotic use in animal production

The term antibiotic was first used to define naturally occurring chemical substances which are produced by microorganisms and which have the capacity to suppress the growth of bacteria (JETACAR, 1999). Antibiotic use in veterinary medicine has become indispensable to the growth of the animal food industry. Food animals are raised in confined animal feeding operations defined in the US as CAFOs. The close proximity of a large numbers of animals at these facilities and the potential for spreading diseases has made the use of veterinary drugs necessary to sustain these operations (Sarmah et al., 2006). Antibiotics are widely used in livestock production for three purposes: i) therapeutic use to treat sick animals; ii) prophylactic use to prevent infection in animals; and iii) as

antimicrobial growth promoters (AGP's) to improve feed utilisation and production (Barton, 2000).

Antibiotics can be classified differently based on the type of antibacterial activity. Usually, antibiotics that stop bacterial growth are called bacteriostatic, while antibiotics that cause bacterial cell death are classified as bactericidal (Walsh, 2003). *In vitro* bacteriostatic/bactericidal data may provide information on the potential action of antibacterial agents, which predict a favourable clinical outcome. Another criterion to categorise antibiotic drugs addresses the range of susceptible bacterial groups. For instance, tetracycline, phenicols, and fluoroquinolones are broad-spectrum drugs since they are effective against many different pathogens; in turn, penicillins, glycopeptides, aminoglycosides are examples of narrow spectra drugs as they are active against a specific bacterial group (Guardabassi and Courvalin, 2006). Different methods of administration are used and methods followed are determined by drug stability, target organisms and animal health challenge. Antibiotics used for therapeutic treatment are delivered over a short period by feed or drinking water at doses exceeding the minimal inhibitory concentration of the known or suspected pathogen (Barton, 2000). Prophylactic treatment involves higher doses for a very short period (i.e. single dose) and lower doses for long periods (i.e. weeks or years), often given in feed or water to a group of animals (JETACAR, 1999). Antibiotics used as growth promoters are administered as feed additives at subtherapeutic levels over extended periods to entire herds and flocks, which results in improved physiological performance (Phillips et al., 2004). Although the mechanisms of AGP action are unclear, possible modes of action are metabolic, nutritional, or through disease control effects (Cromwell, 2002). While some effects are associated with alterations of the normal intestinal microbiota, these can result in more efficient digestion of feed and metabolism of nutrients (Dennis et al.,

1981), others are mediated through pathogen and disease suppression and immune system stimulation (Phillips et al., 2004).

The performance benefits from antibiotic use in animals have been documented in the literature. Cromwell (2002) demonstrated the efficacy of antibiotics in improving the rate and efficiency of growth in young pigs by summarizing data from more than a 1000 experiments conducted in the US between 1950 and 1985. Results showed that antibiotics improved growth rate and the efficiency of feed utilization by an average of 16.4% and 6.9%, respectively. It was noteworthy that these results were mostly from controlled experiments performed at universities and other research centers, and that antibiotic performance at the farm level was likely to be more beneficial with growth rates from 25 to 30% and feed efficiency improvement from 12 to 15%. In Denmark, the removal of AGPs from feed for weaner pigs showed significant negative consequences in the form of reduced gain and higher mortality (Callesen, 2002). A decrease in daily gain (20 g) and a corresponding increase in the pigs' age at 30 kg, plus a 0.7% increase in postweaning mortality were observed.

Benefits of growth promoters have also been noted in the feedlot industry. Growth promotant use has generally been reported to improve average daily growth and feed conversion efficiency (FCE) in the range of 1-10% (CAFA, 1997). In Australia, for example, an improvement in FCE in the range of 4-11% was observed through the use of AGP's (JETACAR, 1999). Moreover, the efficacy of ionophore antibiotics for growth promotion in ruminant livestock was reported by Armstrong (1983). These data showed that beef cattle fed monensin-supplemented diets at levels of 25-33mg/kg had 4% lower feed intake and had an overall improvement in feed conversion efficiency of 8.7%.

The gains attributed to the use of growth promoters have led to an increase in their use. Annually, approximately 70% of 16 million kg of antimicrobial compounds used in the US are used for non-therapeutic purposes (Mellon et al., 2001). In Europe, the consumption of veterinary antimicrobials by animals increased by 5.2% from 115.2 tonnes in 2006 to 121.1 tonnes in 2007 (DANMAP, 2007). In Canada, even though the use of antimicrobials as growth promoters is very similar to that in the US, there are no comprehensive estimates of antimicrobial consumption in animal production (Sarmah et al., 2006).

2.4 Antibiotic resistance (AR)

Antibiotic resistance is a major concern associated with the widespread use of antibiotics in medicine and veterinary practice and as growth promoters in animal husbandry. The threat is associated with the increased observation of antibiotic resistant bacteria, some of which are resistant to multiple antibiotics, and this may result in unsuccessful disease treatment. Antibiotics that were once effective against certain bacteria are becoming ineffective for treatment of infections. For instance, some strains of *Escherichia coli*, a common cause of urinary tract infection, now exhibit resistance to members of six drug families including the more recently recommended fluroquinolones (Levy and Marshall, 2004). Moreover, there is an economic impact associated with infections caused by resistant organisms that render therapy more precarious and costly. According to Vandijck et al. (2008), the daily antimicrobial costs per infected patient at the Ghent University hospital with multidrug-resistant bloodstream infection (BSI) was 50% higher than those caused by organisms not multidrug-resistant.

Phillips et al. (2004) inferred that most of the antibiotic resistance problem in humans has arisen from antibiotic use in humans. The extent of the occurrence of AR bacteria in hospital settings has been recently documented in the literature (Klevens et al., 2007).

According to Friedman and Whitney (2008) in the US, 55% of all antibiotics prescribed for acute respiratory tract infections in the outpatient setting are probably not needed. Most of the inappropriate prescription of antibiotics results from the fact that physicians consider that patients expect to receive an antibiotic when they come to the office. Hecker et al. (2003) found that a total of 576 (30%) of the 1941 days of antimicrobial therapy prescribed for nonintensive care inpatients were considered unnecessary.

It is not only the medical use of antimicrobial agents that represents a risk factor for development of antimicrobial resistant bacteria in humans, but also there are indications that the use of antimicrobial agents in veterinary medicine and livestock production play an important role in the emergence and dissemination of resistant bacteria. Such concern is directly related to the potential impact on human health, since certain antibiotics used for treatment or growth promotion in animals are also used in human medicine (Table 2.1). Garofalo et al. (2007) screened samples of chicken and pork meat plus 20 faecal samples from the same animals for the presence of genes encoding resistance to clinically important antibiotics. Resistance genes to macrolides and tetracyclines were found to be the most prevalent in the specimens tested. The authors suggested that antibiotic resistance genes were highly prevalent in food-associated bacteria and that AR contamination was likely related to husbandry rather than processing techniques. Gevers et al. (2000) investigated the incidence of tetracycline resistant lactic LAB in ready-to-eat modified atmosphere packed (MAP) sliced meat products including fermented dry sausage, cooked chicken breast meat and cooked ham. Their results showed that only fermented dry sausage contained a high level of tetracycline resistant LAB ($5x10^1$ - $2.23x10^4$ CFU/mL). The authors inferred that the low level of resistant LAB in the remaining products might have been due to the heat treatment used during the production process that eliminated most of the viable bacteria naturally present on the raw meat. Mayrhofer et al. (2007) assessed the antibiotic susceptibility of *Bifidobacterium*

thermophilum and *Bifidobacterium pseudolongum* isolated from cattle and hog feces taken at farms and at slaughter. Overall, fecal isolates from hogs showed higher resistance than bacteria from cattle feces. Resistance to clinically important antibiotics such as tetracycline, clindamycin and erythromycin was observed.

2.4.1 Modes of antibiotic action and mechanisms of bacterial resistance

Antibiotics have played a considerable role in the treatment of bacterial infections in humans and the efficient production of food animals. These substances can be used in combinations of two or more compounds with actions on the main bacterial targets which are: (i) the inhibition of protein synthesis; (ii) the inhibition of cell wall synthesis; and (iii) inhibition of DNA replication (Walsh, 2003) (Figure 2.1). Antibiotics that exert their bacteriostatic or bactericidal action by inhibiting protein biosynthesis belong to the aminoglycoside, macrolide, lincosamide, streptogramin, and tetracycline classes, while those that act on cell wall biosynthesis include the β-lactam, bacitracin, and glycopeptide antibiotics. Besides, quinolone and rifamycin antibiotics block DNA replication (JETACAR, 1999).

Bacteria have exhibited various mechanisms to protect themselves from antibiotic agents (Figure 2.1). The most widespread mechanisms of antibiotic resistance are (i) enzymatic inactivation of the drug, (ii) modification or replacement of the drug target, and (iii) active drug efflux (Mulvey and Simor, 2009). The most common mechanism of resistance to β-lactam antibiotics is the production of enzymes that degrade the drugs, namely β-lactamase (Aarestrup and Schwarz, 2006). The ability of *Campylobacter jejuni* and *Campylobacter coli* isolates to produce β-lactamase has conferred intrinsic resistant to β-lactams (Tajada et al., 1996). Modification of the target site is the main mechanism of

bacterial resistance to glycopeptides. Resistance of *Lactobacillus* species to the glycopeptide vancomycin is attributed to the synthesis of modified cell wall glycopeptides containing D-Ala-D-lactate rather than the D-Ala-D-Ala dipeptide (Klein et al., 2000), which is a property shared with some other Gram-positive bacteria such as *Pediococcus* species (Danielsen et al., 2007). While such resistance is not usually considered a risk factor because it is chromosomally encoded and non-transmissible to other bacteria, vancomycin resistance is of greater importance in *Staphylococcus* and *Enterococcus* species, in which the resistance involves the horizontal exchange of genetic determinants by a plasmid or transposon (Teuber et al., 1999). For antibiotics targeting the bacterial ribosome, structural modifications of the binding sites are usually due to methylation by genetically acquired methylases (Guardabassi and Courvalin, 2006). Recently, the erythromycin ribosomal methylase gene *erm*B has been found in the plasmid of *L. plantarum* (Feld et al., 2009). Active efflux is a mechanism by which antibiotic resistance is manifested in bacteria. There is a diversity of tetracycline resistance genes which can be associated with an efflux pump such as *tet*(A), (B), (C), (D), (E), (G), (I), (M), and (K) (Ng. et al., 2001), and these are responsible for the extrusion of antibiotic from within cells to the external environment. The emergence of tetracycline resistance genes in LAB species has been reported by some authors. Aquilanti et al. (2007) documented the occurrence of genes involved in resistance to tetracyclines [*tet*(M), *tet*(O), *tet*(K)] in LAB isolated from swine and poultry meat samples. The prevalence of *tet*(M) was observed among tetracycline resistant *Lactococcus* and *Lactobacillus* isolates, and this gene was plasmid-encoded.

2.4.2 Acquisition and transfer of antibiotic resistance genes

Resistance to antibiotics can be classified as either intrinsic or acquired. Intrinsic resistance (also called "natural resistance") is similar in all strains of a specific bacterial

group. Conversely, acquired resistance is a trait associated with only some strains of a particular bacterial group (EFSA, 2008). Development of the latter type of resistance involves modification in the genetic composition of the organism which may occur by either mutation in the bacterial chromosomal DNA or acquisition of new genetic material (Blázquez et al., 2002). It is the development of resistance from the acquisition of new a genetic element that is the major threat to animal and human health because it can result in the spread of resistance (Kapil, 2005). Horizontal gene transfer (Figure 2.2) between bacteria of the same or different species can occur via bacteriophage-mediated transfer (transduction), transfer of free DNA into competent recipient cells (transformation), or by contact between donor and recipient cells (conjugation) (Alekshun and Levy, 2007). While transformation and phage transduction mediate narrow host range transfers, conjugation appears to mediate all broad host range DNA transfer (Salyers and Shoemaker, 1994).

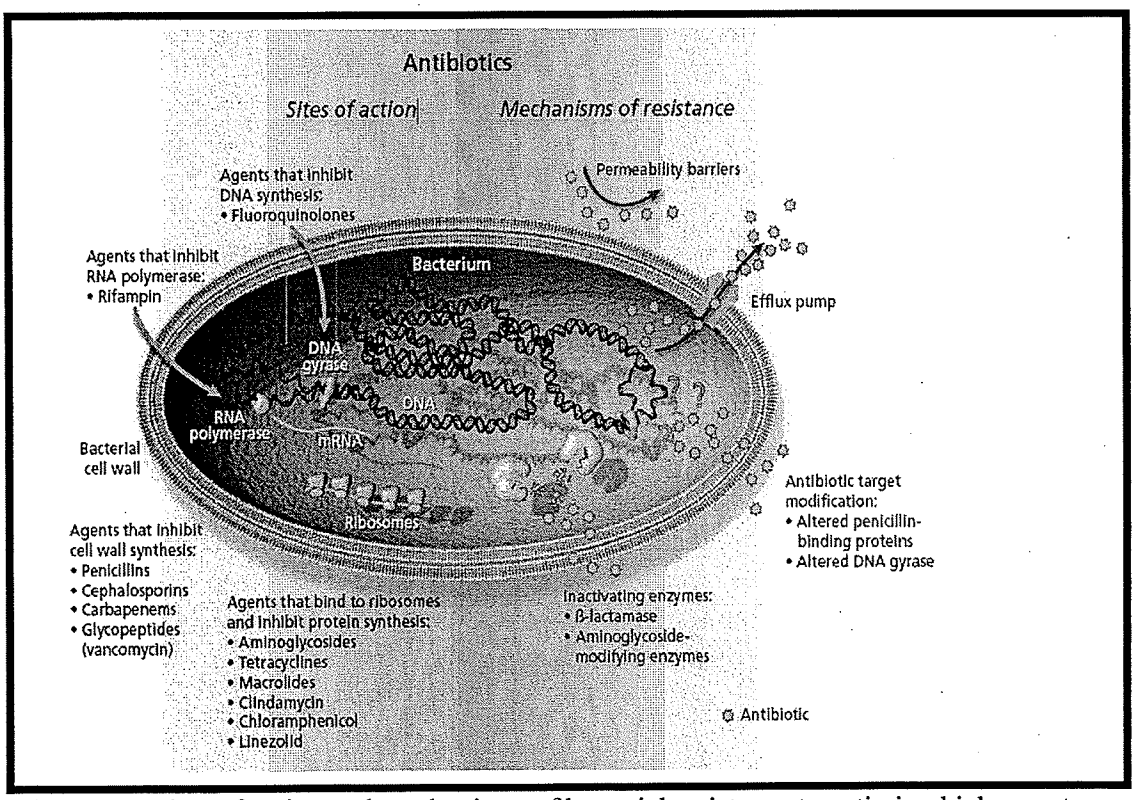

Figure 2.1: Sites of action and mechanisms of bacterial resistance to antimicrobial agents. Source: Mulvey and Simor (2009).

Table 2.1 Antimicrobials registered for use in animals[a] and humans in Canada

Antimicrobial class and drug	Therapy	Growth promotion	Disease Prevention	Drugs in same class registered for human therapy
Aminoglycosides				
Gentamicin	Pi, C, T, Ch		Ch, T	Gentamicin, Neomycin, Streptomycin
Neomycin	Br, Brl, C, Sw, T	C	Br, Brl, C,	
Streptomycin	C, Pi		Sw, Pi	
Macrolides				
Erythromycin	C, Pi, Sw, Br	Br, Brl	Ch, T, Sw	Erythromycin, Azithromycin
Tylosin		Sw	C, Sw, Ch	
Penicillins				
Ampicillin	C, Sw			Ampicillin
Penicillin G potassium	T, Sw	Ch, T	T	PenicillinG potassium
Tetracyclines				
Chlortetracycline	Ch, T, Sw, C	Ch, C, Sw	Sw, Ch, T, C	Tetracycline hydrochloride
Oxytetracycline	C, Ch, T, Sw	Sw, Ch, T, C	T, Ch, C, Sw	
Tetracycline hydrochloride	Ch, T, Sw, C		Ch, T	

[a] C: cattle, Sw: swine, Ch: chicken, T: turkey, Br: breeder, Brl: broiler, Pi: piglets
Source: adapted from Health Canada (2009)

Horizontal transfer of resistance genes occurs via transfer of a small segment of the chromosome, either as a plasmid or a conjugative transposon. Plasmids replicate independently of the host chromosome, whereas transposons are mobile genetic elements that can exist in plasmids or integrate into other transposons of the host's chromosome (Alekshun and Levy, 2007).

Few studies have provided conclusive evidence of the horizontal transfer of resistance genes among LAB and probiotic bacteria. Gevers et al. (2003b) investigated the potential of tetracycline resistant *Lactobacillus* isolates to transfer the *tet*(M) gene to other Gram positive bacteria. Their findings indicated that the R-plasmid of the investigated strains had different conjugation abilities since some plasmids were transferable to the genera *Enterococcus* and *Lactococcus*, some to *Enterococcus* only, while others could not be transferred to any of the recipient strains used. Also, no transconjugants were obtained after mating the *Lactobacillus* isolates with *S. aureus* as the recipient strain. Quoba et al. (2008) studied the antimicrobial resistance of LAB used as probiotics and starter cultures and the ability of LAB to transfer resistance genes to other bacteria. In their study, the horizontal transfer of the resistance gene [*erm*(B)] for erythromycin occurred from *L. reuteri* to *E. faecalis* under laboratory conditions, but not from *L. reuteri* to *E. faecium.* Thus, the role of LAB in the dissemination of resistance genes to other bacteria is still not completely understood.

2.4.3 Dissemination of antibiotic resistance

The excessive use of antibiotics can result in the emergence of bacterial resistance where resistant organisms have a survival advantage under the selective pressure of antibiotics (Kapil, 2005). According to Teuber et al. (1999), the spread of antibiotic resistance has multiple dimensions (Figure 2.3) such as (i) translocation of a resistance gene from one place in the bacterial genome (plasmid or chromosome) to another; (ii) horizontal

spread of resistance genes from one bacterium to another of the same species or across species and genus borders; (iii) spread of resistant bacteria from animal to animal and from animal to the environment; (iv) spread from animals to humans by direct contact or via food; (v) worldwide spread by export/import of live animals and products; and (vi) spread of antibiotic-resistant bacteria in hospital settings as well as through community transmission.

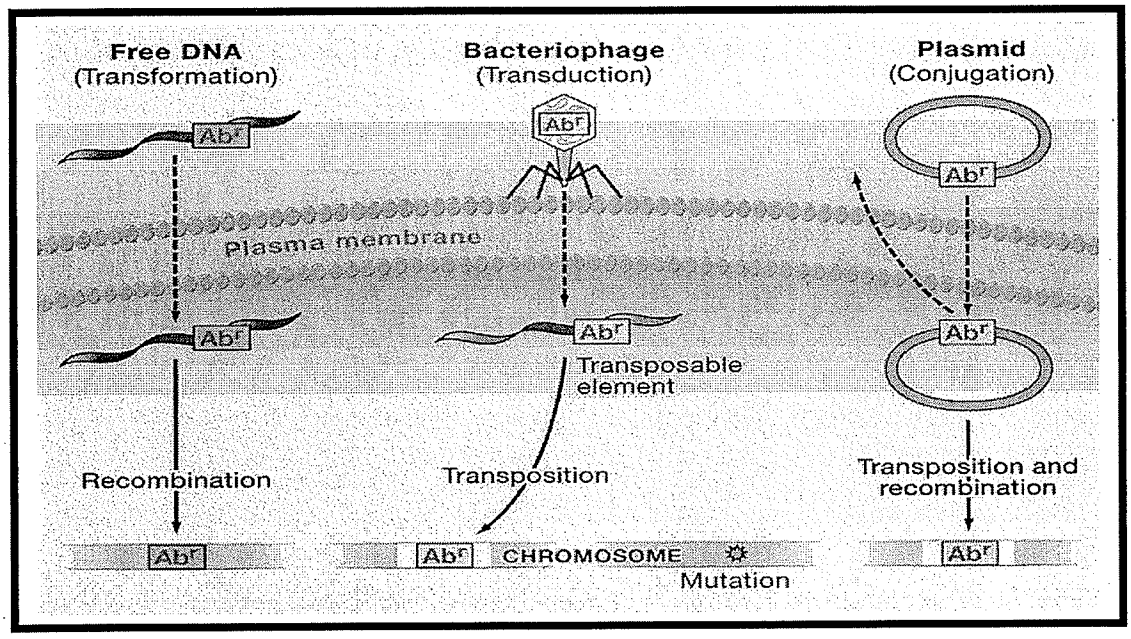

Figure 2.2: Acquisition of antibiotic resistance. Source: Alekshun and Levy (2007)

The dissemination of resistant bacteria associated with the use of antibiotics in food animals is a widely known concern. In recent years studies have recognized the food chain as one of the main routes of transmission of antibiotic resistance between animal and human bacterial populations (Garofalo et al., 2007). The importance of foods from animal source in the direct transmission of zoonotic bacteria including antibiotic resistant organisms, from animals to human has been well reported in various studies (Johnson et al., 2007; Varma et al., 2006). Furthermore, the role of commensal bacteria as reservoirs of AR and their ability to supply AR genes to other non-pathogenic bacteria have been largely documented

(Danielsen et al., 2007; Gevers et al., 2003b). However, the magnitude to which LAB starter cultures and probiotics may harbour resistance genes transferable to zoonotic pathogens merits more investigation (Mathur and Singh, 2005).

Some antibiotic resistance genes have already been found on plasmids and transposons of LAB suggesting that such traits may be disseminated horizontally between strains. Aquilanti et al. (2007) determined the contributions of non-pathogenic microflora to the occurrence and spread of antibiotic resistance genes in the food chain. Their findings indicated that strains of lactobacilli and lactococci isolated from raw and processed meat products harboured the *tet*(M) gene. Of note, their molecular investigation confirmed that the *tet*(M) gene can be located on plasmids, and this gene showed genotypic similarities with the *tet*(M) genes found in human pathogenic species. The sequence and analysis of an erythromycin resistant plasmid was done by O'Connor et al. (2007). In addition to the *erm*(B) gene, a streptomycin resistant *aadE* gene was also identified which was identical to a plasmid encoded *aadE* gene found in *Campylobacter jejuni*. As a result, the authors supported the hypothesis that the horizontal transfer of gene(s) from a Gram-positive to a Gram-negative bacteria could and did occur. Devirgillis et al. (2009) characterized a group of tetracycline-resistant *L. paracasei* isolates at the molecular level. The authors provided evidence that tetracycline resistance was due to the presence of the conjugative transposon Tn*916*, carrying the *tet*(M) gene and it was capable of horizontal, interspecies transfer to the opportunistic pathogen *Enterococcus faecalis*. However, the low conjugation frequency of *L. paracasei* which carried the *tet*(M) gene in the conjugative transposon Tn*916* indicated that there was a reduced risk of horizontal transfer to pathogenic species within the human gut microflora.

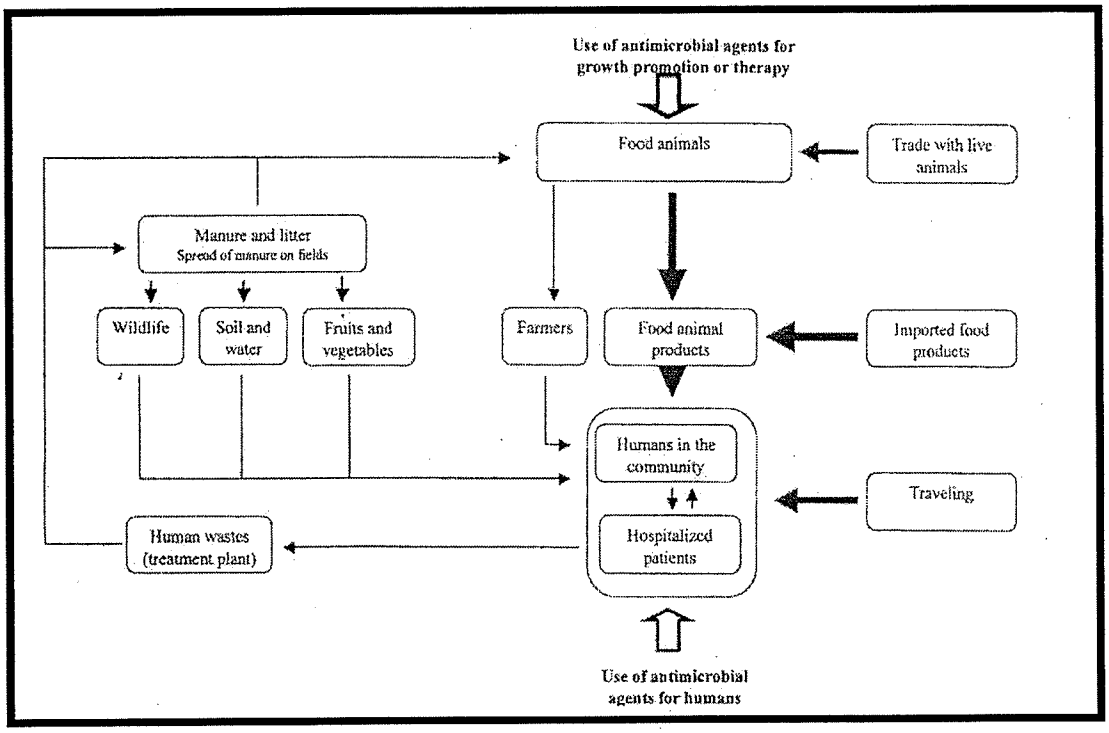

Figure 2.3: Potential routes of dissemination of antimicrobial resistant bacteria and resistance genes. Source: Aarestrup (2006).

2.5 Methods for assessment of antibiotic resistance

2.5.1 Phenotypic characterization of antibiotic resistance

Phenotypic tests cannot guarantee the presence or absence of resistance genes, but they will indicate the likelihood of transferable resistance genes in the strain (Danielsen et al., 2003). Analysis of the minimum inhibitory concentration (MIC) may help differentiate between susceptible and resistant bacteria, and can identify intrinsic and acquired forms of resistance, plus serve as a starting point to study the molecular mechanisms responsible for the spread of resistance (Ammor et al., 2008). According to EFSA (2008), resistance can be intrinsic to the taxonomic group where all strains within this group show phenotypic resistance to an antibiotic. In contrast, when a bacterial strain demonstrates higher resistance to specific antibiotic than the other strains of the same taxonomic unit, the presence of

acquired resistance is indicated and additional information is needed on the genetic basis of the antibiotic resistance. However, comprehensive interpretation of MIC values among meat starter cultures has been difficult due to the variety of techniques used to assess antibiotic susceptibility (Flórez et al., 2008; Doming et al., 2007; Kastner et al., 2006). These differences have included the choice of a suitable medium, variations of incubation time and temperature, and these may lead to conflicting outcomes and poor reproducibility of breakpoint values (which describe the antibiotic concentrations above which an organism is considered resistant) for the same genera or species (Egervärn et al., 2007).

Dilution methods and the Epsilometer test (Etest) are favoured over the disk diffusion test for the best evaluation of the biosafety of industrial meat starter cultures since quantitative MIC information can be obtained which is preferred for classification into resistant and susceptible phenotypes (Klare et al., 2007). Although the E-test has been found to give reliable and reproducible results when tested against LAB, substantial growth toward the inside elliptical inhibition zones with some antimicrobial agents was noted by different authors (Mayrhofer et al., 2007; Danielsen and Wind, 2003) Moreover, the E-test can be significantly more cost prohibitive when a large number of drug-strain combinations are to be studied (Wiegand et al., 2008; Miller et al., 1994).

The performance of broth microdilution tests has been reported by a number of authors (Zonenschain et al., 2009; Aquilanti et al., 2007; Holley and Blaszyk, 1998). The use of small volumes of reagents and the large numbers of bacteria that can be tested against a panel of antimicrobial agents are the major advantages of the microdilution method. Usually, a polystyrene dish with 96-wells is filled with 50 to 100μL of broth and a serial two-fold dilution of the antimicrobial agent is performed before the addition of the inocula to each well (Vigil et al., 2005). Although Muller-Hinton broth supplemented with Ca^{++} and Mg^{++} is

the medium recommended for susceptibility testing of common isolates (CLSI, 2002), evaluation of a new broth medium which is a mixed formulation of Iso-Sensitest broth (ISO) and deMan-Rogosa-Sharpe broth (MRS) for microdilution antibiotic susceptibility testing has been shown to support growth of LAB (Klare et al., 2005). It is noteworthy that standardizing the density of inocula in an antibiotic susceptibility assay is critical for the generation of reliable and reproducible results. An inoculum of 3 to 5 x 10^5 CFU/mL is recommended to assess the antibiotic susceptibility of LAB using mixed media formulations (90% ISO + 10% MRS) (Egervärn et al., 2007; CLSI, 2002).

2.5.2 Molecular assessment of antibiotic resistance

Disagreement between phenotypic resistance found in LAB using susceptibility tests and genotypic analysis by PCR have been reported by some authors (Alonso-Rodríguez et al., 2009; Aquilanti et al., 2007; Klare et al., 2007). Guidance from the breakpoint defined for a given antibiotic may allow the identification of resistance or susceptibility in some borderline strains, especially where no resistance genes can be detected by molecular methods (Hummel et al., 2007). Since it is unlikely that all possible antimicrobial resistance mechanisms are known (Kushiro et al., 2009), genetic analysis alone has limitations when evaluating whether resistance is intrinsic or acquired. Therefore for a safe assessment of the starter cultures used in fermented meat, the use of genetic techniques combined with phenotypic methods is necessary.

Molecular methods allow the detection of known genes conferring resistance to a given antibiotic. Since its introduction in the mid-1980s, the polymerase chain reaction (PCR) has become very popular, and it is now a widely used molecular tool due to its rapidity and simplicity. Disadvantages of the method include the ease for sample contamination and the need for specific information about the nucleotide sequence of the target DNA (Klug and

Cummings, 2003). PCR-based detection has been a tool widely used to explore the genotypic basis of phenotypic resistance in LAB (Huys et al., 2008; Rojo-Bezares et al., 2006; Gevers et al., 2003a).

The PCR procedure involves a repeated sequence of three thermal cycles: denaturation, annealing and extension. Each reaction requires deoxynucleotides, which provide energy and nucleosides for the synthesis of DNA, DNA polymerase, primers, template, and a buffer containing magnesium. Of note, the choice of the primer sequences determines specificity, and consequently the success of the PCR (Aarts et al., 2006). In the first step, heating at 90-95°C denatures and separates the double-stranded DNA into single strands. To start the second step, the temperature of the reaction is lowered to between 50°C and 70°C, where the primers will bind to the denatured DNA. In the final step, DNA synthesis is carried out at temperatures between 70° and 75°C, where the Taq polymerase extends the primers, making a double-stranded copy of the target DNA (Klug and Cummings, 2003). Further, the PCR product is analysed using agarose gel electrophoresis. The purpose of the gel is to investigate the DNA produced for quantification or isolation of a particular fragment. Smaller fragments migrate faster on the gel and so move farther in the same amount of time. These fragments of DNA are compared with a marker DNA of known size. The DNA is visualised in the gel by the addition of ethidium bromide, a fluorescent dye used for staining nucleic acids (Aarts et al., 2006).

Assessment and molecular characterization of genetic determinants in LAB using PCR has been extensively reported in the literature. The presence of genes involved in resistance to tetracycline and macrolide-lincosamide-streptogramines was determined by Zonenschain et al. (2009) using PCR. Garofalo et al. (2007) detected antibiotic resistance genes in LAB specimens from chicken and pork meat using a molecular approach based on

PCR amplification of bacterial DNA directly extracted from specimens. Hummel et al. (2007) determined antibiotic resistances in LAB and verified these at the genetic level. They screened strains by PCR for known resistance genes and were able to determine the presence of *cat* genes in 15 of 46 strains that phenotypically were not resistant to chloramphenicol.

CHAPTER 3

Resistance of meat starter cultures to antibiotics used in food animals in Canada

3.1 Abstract

Lactic acid bacteria (LAB) are extensively used in the food industry for fermentation processes. However, it is possible that these bacteria may serve as a reservoir for antibiotic resistance genes that can be transferred to pathogens, giving rise to public health concerns. Animal operations that use antimicrobials as growth promotants have been linked to the origin of resistance due to the selective effect of low levels of antimicrobial used in this management strategy. The objective of this study was to determine the antimicrobial susceptibilities and mechanisms of resistance for 30 isolates of meat starter cultures commonly used in dry sausage fermentations to 20 antimicrobial agents. Susceptibility tests were performed by broth microdilution using Iso-Sensitest broth (90% v/v) and deMan-Rogosa-Sharpe broth (10% v/v). The results showed that all 30 isolates exhibited resistance to at least 3 antimicrobials regardless of antimicrobial class while 17% or 30% strains were resistant to antibiotics in 3 or 6 different classes, respectively. The incidence of antimicrobial resistance was higher among *Pediococcus pentosaceus* and lower for *Staphylococcus carnosus* strains. Genetic determinants for the lincosamide, macrolide, and tetracycline antimicrobials were not found using PCR. Phenotypic resistance in the absence of known resistance genes found here suggests that other mechanisms or genes might have contributed to the negative results. Further studies are needed to explore the genetic mechanisms underlying the prevalence of antibiotic resistance in *Pediococcus* species.

3.2 Introduction

The emergence and spread of antimicrobial resistant bacteria is an increasing public health problem worldwide. Clinical use and misuse of prescribed antimicrobials for humans and the extensive use of antimicrobials for therapeutic or prophylactic purposes, as well as for growth promotion in animal husbandry (Mathur and Singh, 2005) contribute to this trend. Concerns associated with antimicrobial use and resistance have been frequently reported (Ammor et al., 2007), while the incidence of multi-drug resistant bacteria that are difficult to treat in hospitals is also observed (Resch et al., 2008). Bacterial resistance to antimicrobials can be intrinsic or acquired (i.e. through mutation or gene acquisition), respectively. Intrinsic and mutational resistances are unlikely to be disseminated, and strains showing these origins of resistance are still acceptable for food consumption. Conversely, the presence of acquired genes coding for antimicrobial resistance poses the greatest risk for horizontal dissemination of resistance, particularly when carried by mobile genetic elements (EFSA, 2008).

Lactic acid bacteria (LAB), which typically encompass Gram-positive organisms belonging to the genera *Lactococcus, Lactobacillus, Pediococcus, and Leuconostoc* (Mathur and Singh, 2005), are largely used in the production of fermented foods. Selected LAB strains and some non-pathogenic staphylococci are used as meat starter cultures to improve the quality and safety of the final product and standardize the production process (Resch et al., 2008; Leroy et al., 2006). However, there is concern that starter cultures may act as reservoirs of antimicrobial resistance genes which might be transferred to commensal or pathogenic bacteria (Hummel et al., 2007). According to Mathur and Singh (2005), fermented dairy products and fermented meats that are not heat-treated before consumption provide a vehicle for antimicrobial resistant bacteria, and serve as a direct link between the indigenous animal microflora and the human gastrointestinal tract. Although some studies on the

antimicrobial susceptibility of *Lactobacillus* and coagulase-negative *Staphylococcus* (CNS) species have been published (Resch et al., 2008; Ammor et al., 2008), data regarding *Pediococcus* species are sparse in the literature.

Monitoring of antimicrobial resistance genes can be used to provide better understanding of the prevalence, risk, and spread of antimicrobial resistance. Food authorities in Europe have undertaken an initiative to establish safety system models similar to the 'Generally Regarded As Safe' (GRAS) category of the US Food and Drug Administration (FDA), which in Europe lists microorganisms considered safe only for specific uses. However, the potential risk for transfer of resistance genes from LAB to pathogenic bacteria has not been fully addressed (Mathur and Singh, 2005). In order to do that, a well-established MIC (minimum inhibitory concentration) breakpoint level should be devised for separating susceptible and resistant bacteria. These breakpoints describe the antibiotic concentrations above which an organism is considered resistant. However, MIC results may differ for a particular susceptibility test, as they may be affected by growth medium, composition of the atmosphere, inoculum size, and incubation time (Egervän et al., 2007; Klare et al., 2005). To date, no standard for susceptibility testing has been defined for LAB and CNS (Resch et al., 2008; Klare et al., 2007; Holley and Blaszyk, 1998). Thus, useful comparison and interpretation of MIC results across different studies is limited.

In the present work, broth microdilution with a mixed formulation of Iso-Sensitest broth (90% v/v) and deMan-Rogosa-Sharpe broth (10% v/v) was used to assess the level of resistance of 30 meat starter cultures to 20 antimicrobial agents used in Canada. The objectives of this study were i) to generate, by means of phenotypic susceptibility tests, a consistent breakpoint dataset for common meat starter cultures which can be compared to those used by the medical community, and ii) to characterize the genetic determinants

associated with resistances found for antimicrobials commonly used in human clinical medicine belonging to the macrolide, lincosamide, and tetracycline classes.

3.3 Materials and Methods

3.3.1 Bacterial strains and culture conditions

Thirty bacterial isolates belonging to 3 different genera were tested. Commercial starter cultures which are added in combination (*Staphylococcus* plus *Lactobacillus* or *Pediococcus*) to the formulation of fermented sausage (Holley and Blaszyk, 1998) were obtained from the Institute Rosell Inc., Canada; Rudolph Muller, Germany; Trumark Inc., Canada; Diversitech, Canada; and Quest International, Canada. The isolates tested were *Pediococcus pentosaceus* (n=10), *Pediococcus acidilactici* (n=6), *Lactobacillus plantarum* (n=4), *Lactobacillus curvatus* (n=4), and *Staphylococcus carnosus* (n=6). Routinely, isolates of *P.pentosaceus, P. acidilactici*, *L. plantarum* , and *L. curvatus* were grown aerobically on deMan-Rogosa-Sharpe agar (MRS; Oxoid) at 35°C, whereas *Staphylococcus* spp. were grown on Tryptone Soya agar (TSA; Oxoid). From these plates, 3 to 5 isolated colonies were plated onto either MRS broth (*Pediococcus* spp. and *Lactobacillus* spp.) or TSB (*S. carnosus*) and incubated overnight at 35°C. The bacterial density was then adjusted using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, England) at 600 nm in order to achieve a concentration near 7.4 log CFU mL^{-1}. The cultures were further diluted in sterile 0.1% peptone water and each well was inoculated with 50 µL of bacterial suspension to obtain a final concentration of approximately 5×10^5 CFU mL^{-1} (CLSI, 2002).

3.3.2 Antimicrobial drugs

Nineteen antimicrobials currently registered in Canada for use in food animals plus vancomycin were used in this study (Health Canada, 2009). The latter was included because

of its relevance in terms of emerging resistance (i.e. vancomycin-resistant enterococci) in North America (Deshpand et al., 2007; Nichol et al., 2006) and because it had been used as a growth promotant until recently in Canada (Holley and Blaszyk 1998), thus being important from an historical resistance-induction standpoint. Antimicrobials (powders) of known potencies were obtained from a commercial source (Sigma-Aldrich Canada Ltd., Oakville, ON). The ranges of antibiotic concentration used were 0.007-16μg mL^{-1} (ERY: erythromycin), 0.015-32μg mL^{-1} (CLI: clindamycin), 0.03-64μg mL^{-1} (GEN: gentamicin, NEO: neomycin, TYL: tylosin, PEN: penicillin, and BAC: bacitracin), 0.06-128μg mL^{-1} (STR: streptomycin, MON: monensin, NAR: narasin, SAL: salinomycin, OXY: oxytetracycline, CHO: chlortetracycline, and AMP: ampicillin), 0.125-256μg mL^{-1} (LIN: lincomycin, TET: tetracycline, CAR: carbadox, VAN: vancomycin, and TRI: trimethoprim), and 0.25-512μg mL^{-1} (SUL: sulfamethazine). Twelve antimicrobials were dissolved in distilled water and filter sterilized through 0.20 μm syringe filter units (Fisher Scientific, Edmonton, AB). Small amounts of solubility mediators were required for the remaining antimicrobials. Ethanol (25% v/v) was used as a solvent for MON, NAR, and TET; acetone (25% v/v) was used for SAL and SUL; 0.1N NaOH solution was used for CAR and CHO; and dimethyl sulfoxide (25% v/v, DMSO) for TRI. The solvent concentration in the starting well for ethanol, acetone, and DMSO was no more than 2.5%. The effect of solvents on the growth of bacteria was examined in susceptibility tests.

3.3.3. Antimicrobial susceptibility testing and MIC determination

Sterile 96-well microtitre plates (Falcon no. 3072, Becton Dickinson and Co., Franklin Lakes, New Jersey, USA) were used for the assay. Each well was filled with 50 μL of double-strength 90/10 Iso-Sensitest/MRS broth. The first wells were filled with 50 μL of antimicrobial solutions and serial two-fold dilutions were made to the desired concentration

with the last well being discarded. Wells were then inoculated with 50 µL of bacterial suspension of each culture giving a total volume of 100 µL. Plates were covered and incubated statically overnight at 35°C. Following incubation, 40 µL of p-iodonitrotetrazolium violet (p-INT) was added to each well and microtitreplates were additionally incubated for 2 h (Eloff, 1998). The trials were conducted in triplicate and control checks were performed according to the Clinical and Laboratory Standards Institute (CLSI, 2002) with modification. The minimum inhibitory concentration (MIC) was considered to be the lowest sample antimicrobial concentration at which no red color (signifying no metabolic activity) appeared. The MIC for different species was determined according the European Food Safety Authority (EFSA, 2008; EFSA, 2005). In an attempt to set a breakpoint for those antimicrobials not listed there, breakpoint values established by CLSI (2002), and the Danish Integrated Antimicrobial resistance Monitoring and Research Programme (DANMAP, 1998) were also used. When no microbiological breakpoint was found in any of these sources, breakpoint values used were derived from clinical performance of the antimicrobial class because they are structurally, and often functionally related. Finally, the breakpoint value for carbadox used was suggested by Huber (1982). Isolates with MIC values above these breakpoints were considered resistant.

3.3.4 Determination of resistance genes

Total genomic DNA was extracted according to Wierzbowski et al. (2005) with slight modification. In the present study, the cell suspensions were grown overnight in either MRS broth (LAB species) or TSB (*Staphylococcus*). Polymerase chain reaction (PCR) assays were used to determine the presence of genes involved in macrolide and lincosamide (*ermA, ermB, ermC*), and tetracycline [(*tet*(M), *tet*(O), *tet*(S), *tet*(K), *tet*(L), *tet*(Q)] resistance. The target antimicrobial resistance genes were detected by PCR assay using primer sets described

elsewhere (Quoba et al., 2008). The positive control organisms used in this study were from the National Microbiology Laboratory, Health Canada (Winnipeg, MB, Canada) and their sources are presented in Table 3.1. PCR reaction volumes were 25µl and contained 12.5µL Multiplex PCR Mastermix (Qiagen, Mississauga, ON, Canada), 0.5µL of each primer, 2µL bacterial DNA and 9.5µL water. All PCR amplifications were carried out in a Gene Ampl PCR system 97000 (Applied Biosystems, Foster City, CA, USA) using the following temperature program: initial denaturation at 94°C for 10 min, 30 cycles at 94°C for 30 sec, annealing temperature for individual primers at 45-55°C according to Quoba et al. (2008), an extension at 72°C for 1 min and a final extension step at 72°C for 7 min. The amplification products were subjected to gel electrophoresis in a 1.5% agarose gel at 120 V for 60 min (Owl Separation Systems Inc., model D2, Portsmouth, NH, USA), followed by ethidium bromide staining (Quoba et al., 2008).

3.4 Results

3.4.1 Antimicrobial resistant phenotypes

Antimicrobial breakpoints for the 30 cultures were established by microdilution using Iso-Sensitest broth (90% v/v) and deMan-Rogosa-Sharpe broth (10% v/v). Table 3.2 lists the distribution of MIC values and applicable breakpoints for the 5 bacterial groups for which antimicrobial resistance was found. Appendices A-E give the detailed range of MIC values and applicable breakpoints for the isolates, which were grouped together for similar antimicrobials in the different classes. The incidence of single as well as multiple antibiotic resistances is shown in Appendix F. All 30 strains exhibited resistance to at least 3 classes of antimicrobials. Resistance to 5 or 8 antimicrobials was shown by 27% and 13% of meat starter strains, respectively. The incidence of antimicrobial resistance varied from species to species and was high for *P. pentosaceus*, but substantially lower for *S. carnosus*. Among the

30 starter strains tested, the highest incidence of resistance was shown to carbadox (100%), sulfamethazine (83%), monensin (83%), chlortetracycline (80%), and vancomycin (80%). None of the isolates tested were classified as resistant to gentamicin, neomycin, streptomycin, clindamycin, or penicillin, which have application in human therapy.

Resistance to lincomycin was more frequent among *P. pentosaceus*, *L. curvatus,* and *L. plantarum* isolates. All LAB isolates tested were resistant to monensin, except for one strain of *P. pentosaceus* and 4 strains of *P. acidilactici.* The remaining ionophore antimicrobials (narasin and salinomycin) were uniformly effective against all isolates tested. Likewise, bacitracin was effective against 27/30 meat starter cultures, with 3 isolates of *P. pentosaceus* being resistant. Resistance to trimethoprim was observed for all 10 isolates of *P. pentosaceus* and 4 isolates of *P. acidilactici.* All *S. carnosus* and *L. curvatus* isolates were susceptible to trimethoprim, and only one isolate of *L. plantarum* showed resistance to this antibiotic. Twenty-eight of the isolates tested were susceptible to ampicillin. Resistance to this antibiotic was exhibited by only two isolates of *L. plantarum* with MICs of 4 $\mu g\ mL^{-1}$ and 32 $\mu g\ mL^{-1}$, respectively. It was noteworthy that resistance to tetracycline, oxytetracyline, and chlortetracycline was shown by the majority of *P. pentosaceus*, whereas all *P. acidilactici* and *S. carnosus* were only resistant to chlortetracycline. With *L. curvatus*, only one isolate exhibited resistance to the 3 antimicrobials belonging to the tetracycline class.

3.4.2 PCR detection of antibiotic resistance genes

All isolates were tested for the presence of genes which confer resistance to macrolide, lincosamide, and tetracycline antimicrobials. All 30 isolates investigated showed negative PCR results for resistance genes. From the genomic DNA of *P. pentosaceus, P. acidilactic,* and *L. curvatus* isolates showing resistance to the 3 tetracycline antimicrobials, neither the genes encoding ribosomal protection proteins [*tet* (M), *tet*(O), tet(S), *tet*(Q)] nor

genes encoding the tetracycline efflux pumps [*tet*(K), *tet*(L)] could be amplified. Likewise, these genes were not found in the *L. curvatus* and *S. carnosus* showing only resistance to chlortetracycline. The DNA from *P. pentosaceus* showing low resistance to erythromycin at 2 $\mu g\ mL^{-1}$ could not amplify any of the erythromycin ribosomal methylase (*erm*) genes tested. Even DNA from the three *P. pentosaceus* isolates with multiple resistances to macrolide-lincosamide antimicrobials could not amplify the *erm* genes tested. Amplicons for *erm* genes were not detected in the *Lactobacillus* strains that showed resistance (4 $\mu g\ mL^{-1}$) to only one lincosamide antibiotic. The susceptibility of *S. carnosus* strains to macrolide, lincosamide, and tetracycline antimicrobials was confirmed by the absence of relevant genetic determinants in these organisms.

5. Discussion

Pediococci showed the largest percentage of isolates resistant to the selected antimicrobials used among all the species tested, suggesting that they may be prone to harbor resistance genes; nonetheless, information regarding resistance to antimicrobial agents in pediococci is limited. This possibility raised food safety concerns since *P. pentosaceus* and *P. acidilactici* are largely used in the fermentation of meats (Simpson et al., 2002). Among the two *Pediococcus* species tested, only *P. pentosaceus* exhibited cross-resistance to antimicrobials used as animal growth promotants belonging to the lincosamide and macrolide classes. Cross-resistance observed between these two classes of antimicrobials may be due to the modification of the target site by the erythromycin ribosomal methlylase (*erm*) genes (Roberts et al., 1999). Despite the resistance shown in the broth microdilution test, none of *P. pentosaceus* tested in this study were found to harbor *erm* genes. Hummel et al. (2007) found similar results using other antimicrobials, where resistance was not confirmed by genotypic tests. The MIC observed here for *P. pentosaceus* was only one dilution higher than the

breakpoint value for both lincosamide and macrolide antimicrobials tested (Table 3.2). It is possible that the resistance characterized by the susceptibility test was not genetically encoded since the concentrations of breakpoint and MIC values were very much alike. This result may suggest that a small difference between cut-off and MIC values should be viewed with caution for these antimicrobials.

Resistance to tetracycline by *tet*(M) also confers resistance to oxytetracycline and chlortetracycline (Chopra and Roberts, 2001). In the susceptibility test, *P. pentosaceus* species seemed to harbor the *tet*(M) gene since the majority of these isolates were resistant to the 3 antimicrobials belonging to the tetracycline class. For *P. acidilactici* isolates, resistance was found only to chlortetracycline. However, genetic determinants for tetracycline resistance were not found in any *Pediococcus* isolates for which phenotype resistance was either present or absent. None of the *P. pentosaceus* and *P. acidilactici* isolates harbored the ribosomal protection proteins [*tet*(M), *tet*(O), tet(S), *tet*(Q)] or genes encoding the tetracycline efflux pumps [*tet*(K), *tet*(L)].

Difference between the genotypic and phenotypic analysis has been reported by other authors (Ammor et al., 2008; Quoba et al., 2008). The results presented here agree with those of Tankovic et al. (1993) where 34 isolates of *P. acidilactici* and *P. pentosaceus*, most from clinical isolates, were resistant to tetracycline. However, hybridization with *tet* probes [*tet*(M), *tet*(O), tet(K), *tet*(L)] showed that the gene conferring tetracycline resistance in *Pediococcus* was not homologous to determinants frequently responsible for acquired resistance to this antibiotic in Gram-positive cocci. The findings from the present study could mean that the phenotypic resistance found may be either encoded by other *tet* genes not tested here or occur by other mechanisms not involving resistance genes, such as point mutation (Hummel et al., 2007). Kobashi et al. (2007) tested 19 different *tet* genes in *Pediococcus*

isolates from poultry feces and found resistance to tetracycline encoded by 4 genes [*tet*(B), *tet*(H), *tet*(J), *tet*(W)] that were not tested here because these are less commonly reported.

When a bacterial isolate demonstrates higher resistance to a given antibiotic than the other isolates of the same taxonomic unit, acquired resistance is a possible explanation, but additional information is needed to confirm whether there is a genetic basis for the resistance (EFSA, 2008). Resistance to bacitracin was observed in 3 of 10 isolates of *P. pentosaceus* (MIC $\geq$ 64μg mL^{-1}), which suggests the presence of acquired resistance among isolates of this species. For sulfamethazine and carbadox, high natural resistance was observed, but information about resistance to these antimicrobials in *Pediococcus* is scarce (Holley and Blaszyk, 1998). A high level of resistance to vancomycin was shown by all isolates of *P. pentosaceus* and *P. acidilactici* (MIC >256 μg mL^{-1}). These findings are in agreement with those of Tankovic et al. (1993) who demonstrated that all isolates of *Pediococcus* tested were highly resistant to this glycopeptide with an MIC_{90} value of >1,024 μg mL^{-1}. Moreover, resistance to tylosin was also exhibited by *P. pentosaceus* isolates. These results are relevant because: i) the rate of infection by vancomycin-resistant enterococci has increased in Canada (Ofner-Agostini et al., 2008); ii) unexpected persistence of vancomycin resistance in enterococci in pigs following the withdrawal of avoparcin as a growth promoter was attributed to the continued use of tylosin as a replacement for avoparcin in pigs in Denmark (Aarestrup et al., 2001).

Since EFSA has not established breakpoints for the action of vancomycin against *L. plantarum* and *L. curvatus,* the breakpoint values applied by DANMAP (1998) for *Enterococcus* and *Staphylococcus* were used in the present investigation. As a result, all isolates of *Lactobacillus* species were assessed as resistant to vancomycin. This result is consistent with other studies where intrinsic resistance to vancomycin has been reported among *Lactobacillus* species since they have D-Ala-D-lactate in the peptidoglycan instead of

the dipeptide D-Ala-D-Ala (Danielsen et al., 2007; Mathur and Singh, 2005). In contrast to the pediococci species, resistance to tetracycline was infrequently observed in the *Lactobacillus* species tested in this study. These findings differ from data published elsewhere (Ammor et al., 2008; Quoba et al., 2008) where *Lactobacillus* isolates tested were tetracycline resistant.

It is recognized that some species of coagulase-negative staphycococci (CNS) contribute to flavor production and color stability of fermented sausages (Leroy et al., 2006). However, antimicrobial resistance in CNS from food is a cause for concern because of the potential for transfer of this resistance to clinically important bacteria or for transfer of resistance genes from animals to humans (Martín et al., 2006). Among *Staphylococcus* species, isolates of *S. carnosus* and *S. xylosus* are widely used in starter cultures for meat fermentations (Ordóñez et al., 1999). While all species of the *S. carnosus*-group are members of the phylogenetic *S. simulans*-group, in which only non-pathogenic staphylococci are included, all species of the *S. xylosus*-group belong to the phylogenetic *S. saprophyticus*-group, which contains an important opportunistic pathogen in human urinary tract infections (Götz et al., 2006). It has been speculated that *S. carnosus* is less likely related to the incidence of antimicrobial resistances in food-associated CNS because of the close phylogenetic relationship with non-pathogenic *Staphylococcus* species (Resch et al., 2008). Indeed, in the present study, *S. carnosus* isolates were infrequently resistant to the 20 antimicrobials tested. The present findings are in line with those of Resch et al. (2008) who assessed resistance to 21 antimicrobials of CNS associated with food and used as starter cultures. Their results, interpreted according to the CLSI (2002) standards, showed that *S. carnosus* were susceptible to the clinically important antimicrobials erythromycin, clindamycin, gentamicin, neomycin, streptomycin, and vancomycin. With the exception of clindamycin, these results are also in agreement with results from a study done by Martín et

al. (2006) on the prevalence of antibiotic resistant staphylococci isolated from slightly fermented sausages. In the latter study, the authors followed the breakpoints recommended by the CLSI (2002). Moreover, tetracycline resistance does not seem to be a common trait in *S. carnosus* since all isolates tested in this study had negative results for the phenotypic and genotypic assessment of resistance for tetracycline antibiotic classes. Similarly in *S. carnosus*, tetracycline resistance was found only in one of 27 and one of 11 isolates by Resch et al. (2008) and Martín et al. (2006), respectively. In addition, the resistant strain found by Resch et al. (2008), yielded a negative result for PCR amplification of the *tet*(K) gene.

In the present investigation the isolates of *S. carnosus* tested were classified as susceptible to erythromycin according to the breakpoint value of 4 μg ml^{-1}, as defined by DANMAP (1998) for staphylococci species. Nonetheless, staphylococci could be considered resistant if the MICs were compared to the breakpoints proposed by the EFSA (2008) for Gram-positive bacteria to erythromycin (0.5 μg ml^{-1}). However, the genes encoding resistance to erythromycin could not be detected in any of the six *S. carnosus* strains tested. Based on the present results, the breakpoints for resistance used for Gram-positive bacteria defined by EFSA (2008) should be reviewed and be made more genera-specific. Until this is done, caution should be used in the interpretation of resistance or susceptibility to antimicrobials, given that even a slight difference in the breakpoint may affect the final decision.

The identification, screening and separation of isolates harboring resistance genes from those that do not can be used to prevent the dissemination of resistance genes via fermented meat products. Effective monitoring of resistance genes would be possible with data from a standardized susceptibility testing method. In this study, a consistent breakpoint dataset was generated for 30 meat starter cultures to 20 antimicrobials used in food animals.

The phenotypic resistances to macrolide, lincosamide, and tetracycline antimicrobials observed were verified by microdilution tests, but genes encoding these resistances could not be found using PCR. It is possible that the negative genetic results found here were obtained because tests used screened for only the most common genetic determinants of resistance. Thus, these bacteria may contain other mechanisms or genes that contributed to the observed resistant phenotypes. Future research should focus on the genetic mechanisms underlying the phenotypic resistance by analysing for a broader range of antibiotic resistance genes in a larger number of starter cultures, particularly for *Pediococcus* species.

Table 3.1: Reference strains used as positive controls

Gene	Organism	Reference
erm(A)	*Staphylococcus aureus* RN1389	M. Mulvey[a]
erm(B)	*Streptococcus pyogenes* 02C1061 AC1 (pAC1)	M. Mulvey[a]
erm(C)	*S. aureus* RN4220 pE194	M. Mulvey[a]
tet(M)	*Escherichia coli* pJ13	Ng et al.
tet(K)	*E. coli* pAT102	Ng et al.
tet(L)	*E. coli* pVB.A15	Ng et al.
tet(S)	*E. coli* pAT415	Ng et al.
tet(O)	*E. coli* pUOA1	Ng et al.
tet(Q)	*E. coli* pNFD13-2	Ng et al.

[a] Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, CA

Table 3.2: Distribution of MIC values for 5 groups of meat starter cultures

Organism	Number of isolates with antimicrobial MIC (µg/mL)												
Antimicrobial	≤0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	>512
P. pentosaceus													
Tylosin (1)[a]		4		2	4 r[b]								
Erythromycin (1)				6	4 r								
Lincomycin (1)	3			5	2 r								
Oxytetracycline (8)								8 r	2 r				
Monensin (16)								1	9 r				
Bacitracin (≥64)						3	1	2	1	3[c] r			
Carbadox (25)										10[c] r			
Sulphamethazine (512)													10 r
Tetracycline (8)										10[c] r			
Chlortetracycline (8)							1	9 r					
Vancomycin (16)													10 r
Thimethoprim (8)										10 r			
P. acidilactici													
Oxytetracycline (8)						4	1	1 r					
Monensin (16)							2	2		2 r			
Carbadox (25)											1 r	5 r	
Sulphamethazine (512)													6 r
Tetracycline (8)					1	3	1	1 r					
Chlortetracycline (8)								6 r					
Vancomycin (16)													6 r
Thimethoprim (8)						1					1 r	3 r	
L. curvatus													
Oxytetracycline (8)			1	1			1	1 r					
Lincomycin (1)	2				1 r	1 r							
Monensin (16)											4 r		
Carbadox (25)										1 r	1 r	2 r	
Sulphamethazine (512)													4 r
Tetracycline (8)	2						1	1 r					
Chlortetracycline (8)							1	3 r					
Vancomycin (16)												4 r	
L. plantarum													
Lincomycin (1)	1					2 r			1 r				
Monensin (16)										3 r	1 r		
Carbadox (25)												4 r	
Sulphamethazine (512)													4 r
Vancomycin (16)												4 r	
Ampicillin (2)					2	1 r			1 r				
Thimethoprim (8)	3										1 r		
S. carnosus													
Monensin (16)											6 r		
Carbadox (25)										3 r	1 r	2 r	
Sulphamethazine (512)									4 r		1 r	1 r	
Chlortetracycline (8)									2 r	4 r			

[a] Antimicrobial breakpoint (µg/mL)
[b] Resistance was identified
[c] MIC was > 64µg/mL

CHAPTER 4

4. Conclusions

- Minimum inhibitory concentrations (MICs) for individual bacterial species studied were established even though no breakpoint values have been published officially for these organisms. Despite the small number of strains tested, this information may be used to define resistance or sensitivity when screening strains for the absence of potential transferrable resistance genes. The distribution of MIC values within genera may help to differentiate between natural and acquired resistance, which is important to know before use of single bacterial isolates for food manufacture.

- Phenotypic and genotypic tests yielded different results, with molecular assessments indicating the absence of genetic determinants of antibiotic resistance. While phenotypically some organisms were antibiotic resistant, the absence of genetic determinants did not invalidate the phenotypic result since the resistance may have been encoded by a gene not tested for or was due to some undescribed mechanism. These results may suggest problems associated with safety determinations of starter strains used in fermented products, where it is difficult to establish that antibiotic resistance is not transferable.

- Phenotypic results suggested that meat starter cultures may harbor resistance genes for antibiotics used in animal veterinary and human medicine even though the genetic determinants could not be confirmed in this study by PCR. This is plausible since only a limited number of genes was investigated for those antibiotics to which resistance was observed.

- From a phenotypic point of view, the assessment of antibiotic resistance suggested that the use of *Staphylococcus carnosus* strains would be safer for application in fermented meat products than *Pediococcus pentosaceus* because the latter organisms appeared to show antibiotic resistance. The combined use of *S. carnosus* and *P. pentosaceus* strains as starter cultures in fermented meat involves some risk that antibiotic resistance may be transferred from pediococci to the staphylococci.

- Antibiotics registered in Canada for growth promotion belonging to the tetracycline, sulphonamide, and ionophore classes seemed to have contributed to the level of antibiotic resistance among LAB because these are the antibiotics for which more resistance was observed. The antibiotics gentamicin, neomycin, streptomycin, clindamycin, and penicillin were shown to be more effective since they inhibited growth of all the strains tested.

CHAPTER 5

5. Recommendations for future research

- Further study is needed to expand the number of meat starter culture strains for investigation of antibiotic resistance. The number of strains used in this study was only a starting point and results suggest that these bacteria can be a reservoir of resistance. Evaluation of this possibility would increase the certainty about the safety of the starter cultures used in the meat industry in Canada.

- Different broth media used in the phenotypic test could be tested. To date, there is no standard procedure for assessment of minimum inhibitory concentration (MIC) of antimicrobials toward LAB. Tests of other growth media than those evaluated in the present study may help to develop methodology that may be adopted as a standard procedure for testing resistance in LAB.

- Expanding the number of genes examined in the genotypic test would increase the degree of certainty regarding the presence of elements encoding resistance. This aspect would also cast some light on resistance mechanisms and its eventual origin. These investigations should be performed on *Pediococcus* strains because of their importance in the meat processing industry since the literature supports the possibility of resistance genes being present even in the absence of confirmatory results from phenotypic tests.

- Determination of the molecular mechanisms underlying phenotypic resistance to those antibiotics where genetic determinants could not be found would be important for a better understanding of how antibiotic genes are maintained and spread through bacterial populations.

- Further work could also focus on the possible transferability of resistance genes from commensal to zoonotic pathogens. Evaluation of this risk is a key aspect for understanding the spread of resistance via the food chain and for the safety assessment of meat starter cultures and probiotic bacteria of industrial value.

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APPENDICES

Appendix A: Distribution of MICs of the aminoglycoside antibiotic class

Drug	Organism	MIC Range ($\mu g\ mL^{-1}$)	Breakpoint concentration[a] ($\mu g\ mL^{-1}$)
Gentamicin	*P. pentosaceus*	0.5-1	16
	P. acidilactici	1-4	16
	L. curvatus	0.125-1	16
	L. plantarum	0.125-8	16
	S. carnosus	≤0.03-0.06	8
Neomycin[b]	*P. pentosaceus*	0.25-4	16
	P. acidilactici	0.5-1	16
	L. curvatus	0.25-2	16
	L. plantarum	≤0.03-4	16
	S. carnosus	≤0.03	8
Streptomycin	*P. pentosaceus*	4-16	64
	P. acidilactici	8-64	64
	L. curvatus	2-8	64
	L. plantarum	2-32	64
	S. carnosus	0.25-8	16

[a] Microbiological breakpoints as defined by the EFSA (2008), except for *S. carnosus* which follow DANMAP (1998).

[b] Breakpoint values used were derived from clinical performance of the antimicrobial class.

Appendix B: Distribution of MICs of the lincosamide and penicillin antibiotic classes

Drug	Organism	MIC Range ($\mu g\ mL^{-1}$)	Breakpoint concentration[a] ($\mu g\ mL^{-1}$)
Lincomycin[b]	*P. pentosaceus*	0.125-2	1
	P. acidilactici	≤0.125-0.125	1
	L. curvatus	≤0.125-4	1
	L. plantarum	≤0.125-32	1
	S. carnosus	0.5-2	4
Clindamycin	*P. pentosaceus*	≤0.015	1
	P. acidilactici	≤0.015	1
	L. curvatus	≤0.015	1
	L. plantarum	≤0.015-0.06	1
	S. carnosus	≤0.015-0.125	4
Ampicillin	*P. pentosaceus*	1-2	4
	P. acidilactici	1	4
	L. curvatus	≤0.06-0.125	4
	L. plantarum	2-32	2
	S. carnosus	≤0.06	0.5
Penicillin[b]	*P. pentosaceus*	0.125-1	4
	P. acidilactici	0.125-0.5	4
	L. curvatus	≤0.03-1	4
	L. plantarum	≤0.03-0.25	2
	S. carnosus	≤0.03	0.25

[a] Microbiological breakpoints as defined by the EFSA (2008), except for *S. carnosus* which follow CLSI (2002).

[b] Breakpoint values used were derived from clinical performance of the antimicrobial class.

Appendix C: Distribution of MICs of the ionophore antibiotic class

Drug	Organism	MIC Range ($\mu g\ mL^{-1}$)	Breakpoint concentration[a] ($\mu g\ mL^{-1}$)
Monensin	*P. pentosaceus*	16-32	16
	P. acidilactici	8-64	16
	L. curvatus	128->128	16
	L. plantarum	64->128	16
	S. carnosus	>128	16
Narasin[b]	*P. pentosaceus*	≤0.06	8
	P. acidilactici	≤0.06-0.06	8
	L. curvatus	≤0.06	8
	L. plantarum	≤0.06	8
	S. carnosus	≤0.06	8
Salinomycin	*P. pentosaceus*	≤0.06	8
	P. acidilactici	≤0.06-0.06	8
	L. curvatus	≤0.06	8
	L. plantarum	≤0.06	8
	S. carnosus	1-2	8

[a] Microbiological breakpoints as defined by the DANMAP (1998), except when indicated otherwise.

[b] Breakpoint values used were derived from clinical performance of the antimicrobial class.

Appendix D: Distribution of MICs of the macrolide and tetracycline antibiotic classes

Drug	Organism	MIC Range ($\mu g\ mL^{-1}$)	Breakpoint concentration[a] ($\mu g\ mL^{-1}$)
Erythromycin	*P. pentosaceus*	1-2	1
	P. acidilactici	0.25-0.5	1
	L. curvatus	0.06-0.5	1
	L. plantarum	0.125-0.25	1
	S. carnosus	1	4
Tylosin[b]	*P. pentosaceus*	0.25-2	1
	P. acidilactici	0.25-1	1
	L. curvatus	0.25-0.5	1
	L. plantarum	0.125-0.5	1
	S. carnosus	0.125-2	4
Tetracycline	*P. pentosaceus*	>64	8
	P. acidilactici	2-16	8
	L. curvatus	≤0.125-16	8
	L. plantarum	1-8	32
	S. carnosus	≤0.125	8
Oxytetracycline[b]	*P. pentosaceus*	16-32	8
	P. acidilactici	4-16	8
	L. curvatus	0.5-16	8
	L. plantarum	1-16	32
	S. carnosus	≤0.06	8
Chlortetracycline[b]	*P. pentosaceus*	8-16	8
	P. acidilactici	16	8
	L. curvatus	8-16	8
	L. plantarum	16	32
	S. carnosus	32-64	8

[a] Microbiological breakpoints as defined by the EFSA (2008) except for *S. carnosus* which follow DANMAP (1998).

[b] Breakpoint values used were derived from clinical performance of the antimicrobial class.

Appendix E: Distribution of MICs for miscellaneous antibiotics

Drug	Organism	MIC Range ($\mu g\ mL^{-1}$)	Breakpoint concentration[a] ($\mu g\ mL^{-1}$)
Sulfamethazine[b]	*P. pentosaceus*	>512	≥512
	P. acidilactici	>512	≥512
	L. curvatus	>512	≥512
	L. plantarum	>512	≥512
	S. carnosus	32-512	≥512
Trimethoprim[c]	*P. pentosaceus*	64	8
	P. acidilactici	4-256	8
	L. curvatus	≤0.125-2	8
	L. plantarum	≤0.125-128	8
	S. carnosus	≤0.125	8
Carbadox[d]	*P. pentosaceus*	>64	25
	P. acidilactici	128-256	25
	L. curvatus	64->256	25
	L. plantarum	256->256	25
	S. carnosus	64->256	25
Vancomycin	*P. pentosaceus*	>256	16
	P. acidilactici	>256	16
	L. curvatus	256->256	16
	L. plantarum	>256	16
	S. carnosus	<0.125-2	16
Bacitracin	*P. pentosaceus*	4->64	64
	P. acidilactici	4-32	64
	L. curvatus	2-32	64
	L. plantarum	32	64
	S. carnosus	16-32	64

[a] Microbiological breakpoints as defined by the DANMAP (1998), except when indicated otherwise. [b] Microbiological breakpoints as defined by CLSI (2002) for the sulphonamide antibiotic class. [c] Microbiological breakpoints as defined by the EFSA (2005).

[d] Microbiological breakpoints suggested by Huber (1982).

Appendix F: Incidence of (multiple) antibiotic resistances of 30 meat starter culture strains [a]

Antibiotic	*P. pentosaceus*										*P. acidilactici*						*L. curvatus*				*L. plantarum*				*S. carnosus*						% Resistant strains
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	1	2	3	4	1	2	3	4	1	2	3	4	5	6	
Gentamicin																															0
Neomycin																															0
Streptomycin																															0
Lincomycin			+			+											+		+		+	+	+								23
Clindamycin																															0
Erythromycin						+	+	+		+																					13
Tylosin			+		+	+		+																							13
Ampicillin																						+		+							7
Penicillin																															0
Tetracycline	+	+	+	+	+	+	+	+	+	+						+	+														40
Oxytetracycline	+	+	+	+	+	+	+	+	+	+						+	+														40
Chlortetracyline	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+	+					+	+	+	+	+	+	80
Sulfamethazine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+					83
Monensin	+	+		+	+	+	+	+	+	+	+					+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	83
Narasin																															0
Salinomycin																															0
Bacitracin			+			+		+																							10
Trimethoprim	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+								+							53
Carbadox	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Vancomycin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							80

[a]: + indicates antibiotic resistance