ISOLATION, IDENTIFICATION AND APPLICATION OF ANTIMICROBIAL INTERVENTIONS AGAINST GAS-PRODUCING SPOILAGE ORGANISMS ASSOCIATED WITH RAW BEEF PRODUCTS.

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Raw beef products provide a complex niche for colonization and development of a variety of microorganisms that might be present during slaughter and further processing. Lactic acid bacteria (LAB) are generally-recognized-as-safe (GRAS) for human consumption and play an important role in food fermentation and preservation based on the production of lactic acid. Some strains of LAB are known to produce various types of protein inhibitors (i.e., bacteriocins, Bac^{+}) which may be used as antimicrobial agents against susceptible microorganisms. The objective of our study was to isolate and identify gas-producing organisms (Gas^{+})associated with package bloating of vacuum-packaged fresh beef products, examine incoming raw materials and niches in the meat processing workplace, and identify possible antimicrobial solution interventions to reduce their occurrence in products on retail supermarket shelves. Samples taken from incoming package purge, environmental swabs or the processed meat samples themselves, were enriched in MRS broth and then inoculated into MRS-Durham tubes and incubated for the visual presence of gas production. Gas^{+} isolates were definitively identified by 16S rRNA PCR and sequencing. Several gas-producing strains were used to inoculate lean beef discs for testing the efficacy of various commercially available antimicrobials (Zesti AM-5, BioVia-CDV, Danisco NovaGARD NR-100, Durafresh 2012, Durafresh 5924), organic acids, and combinations of laboratory-generated bacteriocin (Bac^{+}) preparations from bacteriocinogenic LAB. The dominant Gas^{+} organisms were found to be Leuconostoc mesenteroides. These organisms were shown to be resistant to nalidixic acid and vancomycin which allowed us to generate selective media to enumerate them during antimicrobial assays on raw beef. Gas-producing organisms are intrinsically supplied to further raw beef processors from their incoming raw beef suppliers and may present problems to their retail products during refrigerated storage. Our study attempted to identify antimicrobial interventions that will reduce or prevent the survival/growth of the Gas^{+} organisms that could be present on finished raw beef products.

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CHAPTER I

INTRODUCTION

Meat is a great source of nutrients that provides a favorable environment for the growth of various microflora. The high water content and the presence of important nutrients are the important factors that facilitate the growth of microorganisms. Raw or cooked meat has been associated with the growth of both pathogenic and non-pathogenic microorganisms. Although intact meat cuts are considered internally sterile, the microflora associated on the surface of meat develops due to contamination during slaughter occurring from animal hides, intestinal contents, equipment, and workers, and may increase due to extended storage time and temperatures (U.S. National Research Council, 1985).

The pathogenic microorganisms are the cause of several foodborne illness outbreaks in the United States and in other parts of the world. Non-pathogenic microorganisms may also cause distortions in the color and flavor of meat (Borch et al., 1996). A major problem for the food industry and consumers is the loss of food due to spoilage. Food spoilage is characterized by any change in a food product that makes it unacceptable to the consumer from a sensory point of view (Nychas et al., 1998). Spoilage may be physical damage to the food, chemical changes (oxidation, color changes), off-flavors and off-odors, or gas and slime formation due to microbial growth and

metabolism in the product. In the United States, loss from the spoilage of fresh and processed meat products is over $5 billion per year (Ray et al., 1992). Temperature abuse at different stages is one of the main problems that cause food spoilage. Temperature abuse may occur at any stage from production to consumption (Hutton et al., 1991). Other challenges are the occurrence and survival of psychrotrophic and mesophilic bacteria. It is difficult to prevent contamination however; the rapid growth of potential spoilage and pathogenic microflora can be significantly reduced or delayed. This creates a need for proper handling and processing of food products as meat and other food commodities are interested in producing better food products with longer shelf-life and little or no spoilage.

The initial mesophilic bacterial count on meat is about 10^2 -10^3 cfu/cm^2 or gram, consists of a large variety of species (Blickstad et al., 1981; Jackson et al., 1992). Only 10% of the bacteria initially present are able to grow at refrigeration temperatures and the fraction causing spoilage is even lower than that. For cooked/processed meats, most of the vegetative cells are killed from heating the meat products to a temperature of 65-75 ^{0}C, and recontamination that occurs after heat treatment will influence shelf-life (Borch et al., 1996). The shelf-life of meat (from a microbial sense) is the storage time until spoilage. Commercial shelf-life of retail products is predicted on time frames until spoilage occurs. The point of spoilage is when a product reaches a certain maximum acceptable bacterial level or has an unacceptable level of off-odor, off-color, or off-appearance. Microbial shelf-life often depends on the numbers and types of microorganisms initially present and their growth characteristics at the particular storage temperatures (Holley, 1997b; Mol et al., 1971).

During storage, environmental factors such as temperature, gaseous atmosphere, pH and NaCl concentration play a major role in affecting the growth rate and activity of certain bacteria. The shelf-life of refrigerated meat and meat products may vary from days to several months depending on the product and the processing it has received (Blickstad and Molin, 1983b; Gill

and Molin, 1991). One common type of spoilage is characterized by an accumulation of gas and purge in the package leading to a sour or decayed odor. Other signs of spoilage include slime formation, color and odor change, or discoloration.

It is of major concern for processors to develop methods to control spoilage, extend product shelf-life, and insure a safe food supply. Food industries are committed to fulfill the consumer's need for fresh, high-quality and convenient meals. Increased use of refrigeration in combination with vacuum-packaging (VP) or modified atmospheric packaging (MAP) is a popular method for industries to inhibit the growth of spoilage bacteria, thus extending the shelf life of meat (Vitale et al., 2014). However, all bacteria are not controlled by these techniques which have two major disadvantages. First, the low temperatures and reduced oxygen tension can provide a selective advantage to certain bacteria which may lead to spoilage of fresh and processed meats (Ray et al., 1992). Second, the bright red color of fresh or unprocessed meat is distorted in the absence of oxygen which can create a negative perception of freshness among consumers (Brody, 1989). Build-up of potential spoilage and pathogenic microflora is controlled by the use of antimicrobials and nitrites as reported in the literature (Ghaly et al., 2010). Antibiotic use is not permitted because of the risk of development of resistant pathogen strains that may pose hazardous to consumers. The use of nitrite has also been questioned because of the formation of nitrosamines but is allowed at regulatory levels (Ghaly et al., 2010). They are formed from the interaction of nitrite and the amines present in the meat which are potential carcinogens. Therefore, because of their potential occurrence it is important to develop strategies to identify and control spoilage causing organisms.

Molecular methods such as random amplified polymorphic DNA (RAPD) (Ventura et al., 2000), specific polymerase chain reaction (PCR) (Macian et al., 2004; Scarpellini et al., 2002; Yost and Nattress, 2000), pulsed field gel electrophoresis (PFGE) (Bjorkroth et al., 1996), and ribotyping (Bjorkroth and Korkeala 1996b) have been applied to the identification of spoilage

organisms. Multiplex PCR and RAPD analysis demonstrated that in vacuum-packed beef, a mixed community of *Lactobacillus sakei*, *Lactobacillus curvatus*, and *Leuconostoc* spp. was dominated by a single *Leuconostoc* strain (Yost and Nattress, 2002). Denaturing gel electrophoresis has also been applied for the rapid detection of bacteria responsible for spoilage in meat processing plants and in meat products (Takahashi et al., 2004). Sequencing of the 16S rRNA gene of cultured LAB, together with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) whole-cell protein pattern analysis, has been used for grouping and identification of LAB and other spoilage organisms in food (Hong et al. 2014).

Naturally produced biopreservatives may play a vital role in controlling spoilage related organisms. The use of antimicrobial compounds produced by lactic acid bacteria (LAB) has garnered much attention in recent years. Since LAB are associated with meat and meat products, they may inhibit the microflora, ensuring and improving shelf-life stability and product safety (Osmangaoglu, 2002). Antimicrobial metabolites, such as bacteriocins produced by the LAB, are being tested as alternative preservatives for use on meats. These antimicrobial metabolites include products like organic acids, hydrogen peroxide, diacetyl, and bacteriocins (Ray and Daeschel, 1992). LAB are present in microbial populations found on vacuum-packaged meat products (Dykes et al., 1995; Holley, 1997a and 1997b; Yang and Ray, 1994a). In addition to LAB, several commercial antimicrobials like AFTEC 3000, Danisco NovaGARD NR 100, DuraFresh, Zesti AM-5, etc. are currently used in various food industries. Some of these antimicrobials contain natural preservatives and others contain organic acids that control spoilage. Organic acids such as lactic acid, citric acid, acetic acid, nitrites, have shown to delay spoilage effectively (Abugroun et al., 1993; Miller et al., 1993; Ouattara et al., 1997b), but the mode of their application in meat systems is critical for success.

The objectives of this study were:

1. To detect gas-producing microorganisms from the vacuum packaged retailed raw beef products, incoming raw materials and equipment surfaces in an Oklahoma commercial meat manufacturing facility.

2. To identify the gas-producing organism by 16S rRNA PCR and sequence analysis.

3. To examine antimicrobial interventions that can be sprayed on raw beef to inhibit gas-producing leuconostocs, and prevent gas formation in finished raw beef products.

4. To make suggestions that may help reduce problems associated with gas-producing spoilage microorganisms in the local processing facility.

CHAPTER II

REVIEW OF LITERATURE

2.1 The microflora of meat

Meat is a major nutrient source which creates an ideal ecosystem for many microorganisms by providing all necessary growth requirements. The factors affecting the growth of microflora of meat are water activity, pH, temperature and nutrient content (Frazier and Westhoff, 1988). Many microorganisms gain access to these nutrients during slaughter and subsequent handling. The organisms found on hides and the surfaces of carcass meats usually originate from water, soil, vegetation, air, or from the skin of the animal (Egan, 1983). Factors determining the storage life of meat are: types of microorganisms present, extent of initial contamination, storage temperature, and packaging material. The interaction of all ecosystem factors ultimately determines the microbial quality of meat products (Frazier and Westhoff, 1988).

The predominant types of bacterial genera reported on raw meats are *Brochothrix thermosphacta, Carnobacterium* spp., *Enterobacteriaceae, Lactobacillus* spp., *Leuconostoc* spp., *Pseudomonas* spp. and *Shewanella putrefaciens* (Jay et al., 2003).The predominant organisms growing and multiplying on the surface of the meat are influenced by storage temperature and will vary according to the meat species. The pseudomonads are present primarily on aerobically

refrigerated meats and are involved in aerobic spoilage (Jay et al., 2003). During processing, meat carcasses or primal cuts are held near 0°C to reduce the rate of biochemical and microbial change. Hence, *Pseudomonas* spp. overcome the mesophilic surface contaminants. Nychas et al. (1998) found that meats like beef, pork and lamb stored under vacuum or enriched atmospheric conditions were dominated by LAB and *B. thermosphacta* rather than pseudomonads.

Off-odors and off-flavors are the main defects in meat but, discoloration and gas production can also occur. *B. thermosphacta, Carnobacterium* spp., *Lactobacillus* spp., *Leuconostoc* spp., and *Weissella* spp. are associated with the spoilage of refrigerated meat products, causing the defects such as sour off-flavors, discoloration, gas production, slime production and decrease in pH (Borch et al., 1996). Garcia-Lopez et al. (1998) found species of psychotrophic *Enterobacteriaceae* in chilled meats. Pathogens such as *Campylobacter jejuni* (Palumbo, 1986), *Yersinia enterocolitica* (Stern and Pierson, 1979), and *Listeria monocytogenes* (Farber and Peterkin, 1991) have also been isolated from meats at different stages of production and processing.

Hayes et al. (2003) found *Enterococcus* spp. to be among the most dominant bacteria on 971 out of the 981 meat samples (chicken, turkey, pork and beef) collected from 263 grocery stores in Iowa. *Enterococcus faecalis* was identified in 54% of isolates and 38% were identified as *Enterococcus faecium*, 3.4% as *Enterococcus hirae*, 2.4% as *Enterococcus durans*, 0.8% as *Enterococcus casseliflavus*, 0.4% *Enterococcus gallinarum*, and 1% were unindentified. All of beef samples contained enterococci with 65% of isolates identified as *Enterococcus faecium*, 17% as *Enterococcus faecalis*, 14% as *Enterococcus hirae*, 2% as *Enterococcus durans* , 0.7%, as *Enterococcus casseliflavus*, 0.4% *Enterococcus gallinarum*, and 0.9% unindentified.

2.2 Growth phases in bacterial cultures

The rate of bacterial growth in a culture varies greatly with time (Buchanan and Fulmer, 1928). In general, there may be no appreciable initial increase in the numbers of bacteria (Lane-Claypon, 1909; Ledingham and Penfold, 1914).

Buchanan (1928) differentiated seven such phases as follows:

1. Initial stationary (lag) phase: During this phase, the number of bacteria remains constant. The duration of this phase would be altered due to differing experimental conditions and different organisms used.

2. Phase of positive growth acceleration: The cells begin to divide and the average rate of increment in numbers rises with time. This phase extends over a period of time because all the cells do not germinate (for spores) or actively start growing at the same time. When all the viable cells of the original inoculum have germinated or started growing, they continue to divide regularly, and the rate of increase is then constant; this gives rise to the third phase. The term "lag phase" has been defined as covering the period which occurs between "the time of seeding and the time at which maximum rate of growth begins" (Chesney, 1916).

3. Logarithmic growth phase: During this phase, the rate of increase per organism remains constant.

4. Phase of negative growth acceleration: In this phase, the rate of increase decreases but the number of bacteria increases.

5. Maximum stationary phase: In this phase, the number of bacteria remains constant.

6. Phase of accelerated death: During this phase, the number of bacteria decreases slowly followed by a rapid change.

7. Logarithmic death phase: Here, the rate of decrease per organism remains constant, perhaps due to inhibition produced by the culture itself.

2.3 Bacteria associated with spoilage

2.3.1 Off odors and off colors

Putrid, cheesy, and sulphury off-odors are the type of spoilage observed with aerobically stored meat (Dainty and Mackey, 1992). Ethyl esters are produced by *Pseudomonas* spp. in the early stages of spoilage. Sulfur containing compounds, such as hydrogen sulfide formed by *Enterobacteriaceae*, add to putrid and sulfur odors (Edwards and Dainty, 1987). *Enterobacteriaceae* spp. and *Lactobacillus* spp. create cheesy odors due to the formation of acetoindiacetyl and 3-methylbutanol (Borch and Molin, 1989; Dainty and Mackey, 1992). The off-odor in modified atmospheric packaged meats is characterized as cheesy and rancid in which LAB, *Pseudomonas* spp., and *B. thermosphacta* are responsible for spoilage (Jackson et al., 1992). The spoilage potential by *B. thermosphacta* and LAB is increased due to the presence of oxygen, as it helps to form end-products such as acetoin and acetic acid (Borch and Molin, 1988).

In vacuum and anaerobic MAP meat, sour and acid are the spoilage characteristics that are specifically associated with LAB and *Leuconostoc* spp., in which, lactic acid and acetic acid are the end-products (Dainty and Mackey, 1992).

2.3.2 Discoloration

Three typical meat colors are purple red, bright red, and brown. These colors of meat may change to green depending on the storage. Greening is typically associated with meat having high

pH, but may also occur in normal pH meat. Sulfmyoglobin and hydrogen peroxide are responsible for greening in meat (Lloyd and Mauk, 1994). Sulfmyoglobin is formed from the reaction of myoglobin with hydrogen sulfide. Hydrogen sulfide and hydrogen peroxide are produced by certain microorganisms under specific storage conditions (Lloyd and Mauk, 1994).

Under aerobic conditions, green discoloration may be induced by hydrogen peroxide-producing LAB on cooked, cured meat products (Peirson et al., 2003). Green color defects of unprocessed, raw meats have been associated with hydrogen peroxide producing *Leuconostoc* spp. and *Lactobacillus* spp. (Feiner, 2006b). Moreover, green discoloration on aerobically stored beef has also been linked to specific strains of *Lactobacillus sakei* and *Carnobacterium maltaromaticum* (Leisner et al., 1995). Hydrogen sulfide is formed by *Lactobacillus sakei* only when glucose and oxygen availability are limited (Egan et al., 1989) whereas sulfmyoglobin is not formed in anaerobic atmospheres (Borch and Agerhem, 1992).

Color deterioration, also referred to as browning of meat, is the typical limiting factor for fresh beef shelf life as it loses consumer appeal (Lawrie, 1998). Vihavainen and Björkroth (2007) found that the discoloration that developed on commercial, value-added beef steaks was clearly greenish. During experiments with beef steaks inoculated with *Leuconostoc gasicomitatum* RSNS1b and *Leuconostoc gelidum* RSNL1b, green discoloration developed in 3 days. Greenish discoloration did not follow when the steaks were inoculated with single cultures of *Lactobacillus sakei* RSNL1a, *Leuconostoc gasicomitatum* RSNU1d, or *Clostridum divergens* RSNU1h. In addition to discoloration, the steaks inoculated with *L. gasicomitatum* RSNS1b and RSNU1d, *L. gelidum* RSNL1b or *C. divergens* RSNU1h developed an unpleasantly strong buttery/diacetyl-like odor. They also stated that *L. gasicomitatum* and *L. gelidum* strains may cause rapid spoilage of high-oxygen MAP, value-added beef. They concluded that the vacuum-packaged meat enhanced with glucose and sugar containing ingredients, and packed under oxygen-containing

MAP is likely to make value-added meat susceptible to spoilage, characterized by greening and strong buttery odors.

2.3.3 Slime formation

Ropy slime formation associated with vacuum-packed meat products is caused by homofermentative *Lactobacillus* sp. and *Leuconostoc* sp. (Dykes et al., 1994; Korkeala et al. 1988). Slime is caused by *Lactobacillus sakei, Leuconostoc religiosum, Leuconostoc carnosum, Leuconostoc gelidum, Leuconostoc mesenteroides* subsp. *dextranicum,* and *Leuconostoc mesenteroides* subsp. *mesenteroides* (Dykes et al., 1994). Ropy slime-producing *Lactobacillus sakei* and *Leuconostoc amelibiosum* were recovered from processing rooms in meat plants where the meat products were handled after heat processing (Makela et al., 1992).

Slime formation is an early indication of spoilage, often observed before the sell-by date (Korkeala et al., 1988). Korkeala et al. (1988) found that sausage from one manufacturer in Finland had *Leuconostoc mesenteroids* subsp. that caused ropy slime in the meat. The population of pseudomonads to the level of 10^{7-8} CFU/g has been attributed to slime and off-odor formation in meat (Nychas et al., 2008). Russell et al. (1996) stated that a favorable pH for the growth of spoilage bacteria that causes slime in meat is in the range of 5.5-7.0 and it does not require sucrose for its growth. Slime formation, structural components, degradation, off odors, and appearance change were found in meat as a result of microbial growth within this pH range.

2.3.4 Gas production

Commercial raw or cooked meat products are packed under vacuum or in a MAP which helps in maintaining their sensory qualities for long storage times under refrigerated conditions.

Composition of the gaseous-phase changes during storage and the bacterial microbiota undergoes selection towards, CO_2-tolerant, slow growing species such as pshychrotrophic leuconostocs and lactobacilli (Hammes and Hertel, 2003; Nissen et al., 2001).

Gas formation in meat is commonly referred as bloating in meat or blown pack spoilage and is caused by the abundant production of gases formed by spoilage organisms (Adam et al., 2010). Bloating by LAB has mainly been associated with highly acidic foods such as fermented vegetables; however, vacuum packed meat products are also highly affected (Fleming, 1982). *L. sakei* and *L. curvatus* are found to be among the most common bacteria responsible for gas-production in meat products (Bjorkroth and Korkeala, 1996a). Shaw and Harding (1989) found that leuconostocs were the dominant microbial population on meats stored in vacuum packs or under modified gas atmospheres containing carbon dioxide that contributed to spoilage. Shaw and Harding (1989) isolated *Leuconostoc mesenteroides* subsp. *mesenteroides* from these products, and also identified two new species, *Leuconostoc gelidum* and *Leuconostoc carnosum. L. carnosum* and *L. gelidum* are the other common species occurring in vacuum or modified atmosphere-packaged cold-stored meat products (Bjorkroth and Korkeala, 1996b; Shaw and Harding, 1989; Yang and Ray, 1994).

Leuconostoc sp. produce gas (CO_2) during normal glucose fermentation (Bjorkroth et al., 2000). *L. mesenteroides* have heterofermentative metabolism and produce gas from the glucose present in meat whereas some *Lactobacilli* are facultatively homofermantative (Hammes and Hertel, 2003), thus being able to produce CO_2 via the phosphogluconate pathway from gluconate and pentoses. This supports the main role of *L. mesenteroides* in the bloating spoilage by the production of CO_2.

Bromberg et al. (2003) investigated the microbiological cause for the bloating of meat. Their results implicated that temperature abuse during storage and the presence of LAB were the

main cause. Some other genera, such as *Clostridium* and *Enterobacteriaceae*, can multiply in vacuum-packaged meat causing deterioration and blowing of the package at different refrigeration temperatures (Brightwell et al., 2007). Psychrophilic and psychrotrophic species of *Clostridium* such as *C. estertheticum* subsp. *estertheticum* (Spring et al., 2003), *C. estertheticum* subsp. *laramiense*, *C. algidicarnis*, *C. frigidicarnis*, *C. gasigenes,* and *C. algidixylanolyticum* that are able to grow at refrigeration temperatures have been identified as causative agents of blowing vacuum packages (Broda et al., 2002).

Chaves et al. (2012) indicated that LAB and *Enterobacteriaceae* isolated from chilled vacuum-packaged beef had high potential in producing large amounts of gas and caused "blown pack" spoilage. Further, Chaves et al. (2012) stated that not only the presence of psychrotrophic *Clostridium* sp. but also the presence of LAB and *Enterobacteriaceae* are challenging for the microbiological stability of chilled vacuum-packaged beef products. Hence, more studies need to be performed to understand the conditions of blown-pack associated microorganisms. Additionally, effective intervention strategies or control measures should be developed to avoid or control the spoilage.

Hanna et al. (1979) showed that meats that suffered temperature abuse in processing and storage were observed with gas production when vacuum packed at a later time. However, it is known that distension or blowing problems can occur without temperature abuse (Dainty et al., 1989; Kalychayanand et al., 1989). This is a challenge and a great risk for industries related with the production, processing, and marketing of meats.

2.4 Control of spoilage

2.4.1 Organic acids

Safe food preservation in the meat industry has grown to be a more complex issue due to the need of longer shelf life and greater assurance of the safety of meat products. Many antimicrobial chemicals have been used to control microbial growth on the surface of meat and meat products. The use of organic acids and their salts as antimicrobial dips and sprays have been useful in controlling the microbial growth and hence, prolonging the shelf life of fresh meats (Anderson and Marshall, 1989; Abugroun et al., 1993; Castillo et al., 2001; Mustapha et al., 2002; Serdengecti et al., 2006). However, due to evaporation, neutralization, and diffusion, the preservatives were not able to stay on the surface of the food and may have little impact beyond their initial application (Siragusa and Dickson, 1992; Torres et al., 1985).

2.4.1.1 Fatty acids

Fatty acids are found to have antibacterial and antifungal activities against many food spoilage microorganisms (Shelef et al., 1980; Russel, 1991). In a study conducted by Ouattara et al. (1997), lauric acid, a saturated fatty acid was found to exhibit the greatest inhibitory effect against *C. piscicola*, *L. curvatus*, and *L. sakei.* Hydrophobic groups in saturated fatty acids have been shown to have an effect on antibacterial activity (Branen et al., 1980), but an increase in their hydrophobicity with longer chain length might reduce their solubility in aqueous systems. Thus, due to the interaction with hydrophobic proteins or lipids on the bacterial cell surface, hydrophobic groups may be prevented from reaching sufficient concentration (Wang and Johnson, 1992). Lauric acid has been found to have the best balance between hydrophobic and hydrophilic groups (Branen et al., 1980; Kabara et al., 1977). Lauric acid has been used commercially in the form lauric arginate, which has been proven to be an effective food grade

antimicrobial agent. *L. monocytogenes* were reduced by at least 1.0 log_{10} CFU/ham within 24h at 4°C when a 5% solution of lauric arginate was sprayed on the surface of hams (Luchansky et al., 2005).

2.4.1.2 Lactic acid

Lactic acid has the ability to reduce pH levels, exert feedback inhibition, and interfere with proton transfer across cell membranes. These antimicrobial activities inhibit microorganisms like *Clostridium botulinum* (Cassen, 1994). The salt of lactic acid (lactate) is used in the meat industry as an antimicrobial agent (Davison et al., 2005). Lactic acid overall concentration up to 5% has been permitted in the raw meat by the USDA.

It has been reported that a combination of sodium chloride (1%, w/w) and sodium lactate (2%, w/w) enhanced microbial stability in refrigerated fresh ground pork (Tan and Shelef, 2002). Mbandi and Shelef (2002) found that the growth of strains of *Listeria* and *Salmonella* were delayed during aerobic storage at 5-10°C for up to 60 days when sodium lactate (2.5%) and sodium diacetate (0.2%) were added to ready-to-eat meat. Greer and Dilts (1995) reported that several pathogenic microorganisms were inhibited and did not grow on lean pork tissue during 15 days of storage at 4°C when immersed in 3% lactic acid solution.

Gill et al. (2004) applied 2% and 4% lactic acid as a spray to beef trimmings to control natural flora of the distal surfaces of pieces of brisket from chilled beef carcasses. The treatments with 4% lactic acid were more effective than the others and showed reductions on aerobes, coliforms, and *E. coli*. Prasai et al. (1997) found that 1.5% lactic acid sprayed on beef subprimals improved the microbiological quality of meat after 14, 28, 56, 84, and 126 days of vacuum storage compared to the unsprayed or untreated samples. Kotula et al. (1994) observed a larger log reduction compared to the non-treated control beef samples when 1.2% lactic acid was

applied as a spray on retail beef cuts. Due to the efficiency of lactic acid, it has become the most commonly used organic acid applied prior to evisceration for sanitizing whole carcasses in the meat industries (Davison et al., 2005).

The use of LAB as an inoculum is a newly developed approach for food preservation (Davison et al., 2005). LAB are a group of Gram-positive, catalase-negative bacteria that produce various types of compounds such as bacteriocin, organic acid, diacetyl, and hydrogen peroxide during lactic acid fermentation (Mandal et al., 2008). They are generally-recognized-as-safe (GRAS) for human consumption, and play an important role in food fermentation and preservation based on the production of lactic acid (Tahara and Kanatani, 2006). Their ability to promote food preservation is linked to the fact that they cause a decrease in pH as a consequence of lactic acid production and the production of a number of additional antimicrobial agents (such as bacteriocins and non-proteic organic compounds). A combination of these factors limits the proliferation of undesirable microorganisms (spoilage- or pathogenic- microorganisms). LAB therefore undoubtedly play a large role in promoting food safety. Some strains of LAB are known to produce various types of bacteriocins, which may also act as the alternative agent in therapeutic use.

Bacteriocins have been characterized in both Gram-negative and Gram-positive bacteria. The colicins of *E. coli* are among the most studied (Lazdunski, 1988). The colicins constitute a diverse group of antibacterial proteins, which kill closely related bacteria by various mechanisms such as inhibiting cell wall synthesis, permeabilizing the target cell membrane, or by inhibiting RNase or DNase activity. Many hypotheses of the mechanisms involved in the inhibition phenomenon (acidification, bacteriocin production, H_2O_2 production) have been tested and reported in the scientific literature. Though extensive reports exist on bacteriocinogenic activities of LAB, only a few substances have been well characterized and the target for their activity been defined. The most well-known and best characterized bacteriocin is nisin, with a molecular

weight (M.W) of 3500 Da. Barefoot and Klaenhammer (1983) screened 52 strains of *Lactobacillus acidophilus* for production of bacteriocins whereby a majority produced bacteriocin-like compounds inhibitory to different lactobacilli.

2.4.1.3 Acetic acid

Acetic acid has been found to be an effective agent in controlling food spoilage bacteria (Davison et al., 2005). Tinney et al. (1997) found that beef treated with 2% acetic acid spray was observed to incur a 1-log reduction of inoculated *E.coli* and *Salmonella* spp. Similarly, populations of other bacteria, like *Enterobacteriaceae*, were found to be reduced when combinations of organic acids (1.5% acetic, 2% lactic and 1.5% propionic) were sprayed on to sheep/goat carcasses (Dubai et al., 2004). Anderson et al. (1989) conducted a study where beef cores were dipped in 0%, 1%, 2%, and 3% acetic acid at 25 ºC, 40 ºC, 55 ºC, 70ºC, respectively. They found that 3% acetic acid at 70ºC to be the most effective treatment in reducing total aerobic plate counts followed by *Enterobacteriaceae* counts.

A modified form of acetic acid used in the meat industry is peroxyacetic acid (PA). PA is a powerful oxidizer often used as a carcass wash in processing plants (King et al., 2005). Under laboratory conditions, a solution of 0.02% PA has shown to achieve 1.0-1.4 log reductions in *E. coli* O157:H7 inoculated onto beef carcass tissue (King et al., 2005). PA is approved for use by USDA for washing, rinsing, cooling, or processing fresh beef carcasses at maximum concentrations of 0.020% for PA and 0.0075 % for hydrogen peroxide.

2.4.1.4 Citric acid

Citric acid (CA) is a natural preservative and a weak organic acid. It can add an acidic or sour taste to foods and soft drinks. It exists in small amounts in a variety of fruits and vegetables. The mold, *Aspergillus niger* is mostly used for the production of CA in commercial operations (Kirimura et al., 2000). Cultures of *A. niger* are inoculated on sucrose containing medium to produce citric acid. *A. niger*, is used to ferment a carbohydrate source such as molasses. Many industrial applications of CA are based not only upon its acidic properties, but also upon its powerful sequestering action with various transient metals, such as iron, copper, nickel, cobalt, chromium and manganese (Kirimura et al., 2000). CA shows sufficient antimicrobial activity against bacterial spoilage as it has an inhibitory effect due to its ability to diffuse through the cell membranes, via the weak, non-dissociated form of the acid (Mroz, 2005). Hence, CA decreases the ionic concentration within the bacterial cell membrane of the exterior cell wall of bacterial organisms. This leads to an accumulation of the acid within the cell cytoplasm, acidification of the cytoplasm, disruption of the proton motive force, and inhibition of substrate transport. Due to these effective inhibitory characteristics, it has been used extensively in poultry and feed industries in combinations with other organic acids (Vasseur et al., 1999).

2.4.1.5 Others

Nitrites, also known as curing agents are used in meat preservation industry in the form of salts such as sodium nitrite or potassium nitrite and they help in stabilizing red meat color, curing meat flavor, or retarding rancidity (Jay, 2005). Nitrites have an antimicrobial effect against the growth of the toxin producing *Clostridium botulinum*, *Staphylococcus aureus, Yersinia enterocolitica* and many other pathogenic spoilage organisms which grow under anaerobic environments in vacuum packages (Ray, 2004; Sindelar and Houser, 2009). Nitrites inhibit

microrganisms in food by reacting with alpha-amino groups of the amino acids at low pH levels, blocking sulfhydryl groups thus, limiting sulfur nutrition of the organism, reacting with iron-containing compounds, ultimately restricting the use of iron by bacteria and interfering with the membrane permeability which limits the transport across cells (Cassen, 1994; Ray, 2004). Despite its usefulness, use of nirites as food additive has been controversial and may form carcinogenic nitrosamines with prolonged exposure (Ghaly et al., 2010).

Sodium sulfite is an efficient antimicrobial agent against aerobic Gram-negative bacilli, molds and yeasts in meat and meat products (Dave and Ghaly, 2011; Ray, 2004). Sulfites are used as antimicrobial agents in specified comminuted products such as fresh sausage as they are effective in controlling *Enterobacteriaceae* including pathogenic *Salmonellae* (Banks and Board, 1982). Antimicrobial activity is the result of the undissociated sulfurous acid which enters the cell, reacts with thiol groups of proteins, enzymes, and cofactors which gives rise to the antimicrobial activity. Yeast cells are attacked by sulfite because sulfite reacts with cellular adenosine triphosphate (ATP) and blocks the cystine disulfide linkages (Davidson et al., 2005). Sulfur dioxide and salts like potassium bisulfite, potassium metabisulfite, sodium bisulfite, sodium metabisulfite, and sodium sulfite (collectively known as sulfites) are not allowed to be used as meat preservatives in the U.S. because they degrade thiamine and people have been shown to have allergenic reactions (Cassen, 1994).

Ascorbic acid (vitamin C), sodium ascorbate, and D-isoascorbate (erythorbate) have also been used as meat preservatives. Their antioxidant properties oxidize reactive oxygen species producing water. Antimicrobial activity of sulfites and nitrites is enhanced by ascorbic acid (Jay, 2005). Ascorbate and erythorbate reduced nitrosamine formation by a level of 550 ppm when they were used in combination with nitrite. Raevuori (1975) observed that the addition of 500 mg sodium erythorbate/kg of meat and 200 mg sodium nitrite/kg of meat prevented the growth of *Bacillus cereus* spores in sausages kept at 20°C for 48 h.

Sorbic acid and its salts are widely used throughout the world as meat preservatives for inhibiting bacterial and fungal growth (Davison et al., 2005; Feiner, 2006). A concentration of 0.3% sorbates in food is high enough to inhibit microorganisms (Feiner, 2006). The sorbic acid depresses internal pH and inhibits microorganisms. Davison et al. (2005) stated that sorbates interfere with the bacterial spore germination, inhibiting the activity of several enzyme systems, thus interfering with substrate and electron transport mechanisms. Tompkin et al. (1974) found that the growth of *Salmonella aureus* and *Clostridium botulinum* was inhibited by sorbate (0.1%wt/wt). In their study, sorbate was applied on cooked uncured sausage and incubated at 27°C. Osthold et al. (1981) found that the spraying a solution containing a mixture of potassium sorbate, sodium acetate, and sodium chloride increased the shelf-life of beef carcasses upto 4 days at 15°C. Ahmed et al. (2003) sprayed meats from freshly slaughtered sheep and goat carcasses with a solution containing a mixture of potassium sorbate (2.5%), sodium acetate (2.5%), and sodium citrate (2.5%) and found the treatment inhibited *Bacillus* spp. to minimum levels. Hence, the lag phase of all organisms, including psychrotrophs, was extended throughout refrigerated storage at 5-7°C.

2.4.2 Essential oils, natural extracts and salts

Ouattara et al. (1997) found that the tested essential oils, clove, cinnamon, pimento, and rosemary were found to have antimicrobial activity. Gas-positive organisms like *C. piscicola*, *L. curvatus*, and *L. sakei* were inhibited by those oils. Rosemary oil was found to be as effective as other essential oils. The presence of 0.10% camphor was suspected to be responsible for the inhibitory activity. Zaika (1988) reported the antimicrobial activity of many spices and categorized their activities as strong, medium, or weak and found that the cinnamon, clove,

pimento, thyme, oregano and rosemary had strong and stable inhibitory effect against the spoilage organisms.

Sodium chloride (NaCl) is a source of osmotic stress in food or growth media as it decreases water activity (Doyle, 1999). Lower NaCl concentration of 2% to a higher concentration of 20% can inhibit various spoilage and pathogenic microorganisms (Urbain, 1971; Praphailong and Fleet, 1997). They found that salt-sensitive microorganisms like pseudomonads and Enterobacteriaceae do not grow in reduce water activity (Aw) of 0.97 with the addition of 4% (w/w) sodium chloride (Borch et al., 1996). However, salt tolerant microorganisms such as LAB and yeasts can still grow at that level of water activity.

The combination of NaCl and other microbial agents have a great inhibitory impact which has been discussed by Tan and Shelef (2002). They reported that a combination of NaCl and sodium lactate was more effective than lactate alone in delaying the onset of meat spoilage. Moreover, the use of NaCl in combination with sodium lactate was found to maintain the chemical quality, reduce microbial growth, and extend the shelf life of ground beef during refrigerated storage (Sallam and Samejima, 2004). Proliferation of aerobic bacterial plate count, psychrotrophic bacterial count and lactic acid bacterial count was found to be delayed by the use of NaCl and extended the shelf life for up to 24 days (Kenawi et al., 2009). However, prooxidant activity of NaCl, which accelerates the development of lipid oxidation, could be one of the limiting factors for its use (Decker and Xu, 1998).

CHAPTER III

METHODOLOGY

3.1 Bacterial cultures, handling, and growth conditions

All cultures were grown in MRS broth by inoculation from a single colony on streak plates (new isolate) or with a 1% inoculum from thawed frozen working stocks maintained at -20°C. After inoculation, MRS broth tubes were incubated at 30°C for 12-18 hours, and then transferred a second time before use. Frozen culture stocks were prepared from similarly grown fresh cultures by centrifuging (6000 rpm) 9 ml of culture and resuspending the pellet in 2 ml of sterile MRS broth (containing 10% glycerol) and storing at -80°C for long term storage.

3.2 Local beef processor

This study represents the efforts to assist a local meat processor who was experiencing excessive problems from microbially-generated gassing and bloating of vacuum-packaged raw meat products (bacon-wrapped beef filets). Our assistance was in the form of a) testing samples brought to our lab for gas-producing bacteria, b) making monthly trips to their plant to personally retrieve and test samples, c) make identifications of the gas-producing (Gas^{+}) isolates, and

d) perform antimicrobial spray treatment of Gas^+-inoculated raw beef discs to evaluate inhibition or growth over 28 days of shelf-life. Prior to our initiation of monthly visits to the meat processing facility, they brought a variety of samples including samples of incoming raw beef, purge from incoming beef, environmental swabs, and samples of finished product showing signs of gas production. During monthly trips to the local meat processor, we identified specific areas from the two different large working areas in the manufacturing facility that should be tested during our visits (Fig.1).

a) The receiving, trimming, tenderization, marination room

b) The pack-out room where marinaded product was vacuum-packaged

At the local meat processor, the incoming fresh meat, ingredients, packaging stuffs, and returned (rejected, spoiled) meat products are received and stored in different areas of the facility as meat products are stored in a storage cooler at a temperature of $\leq 40^oF$, while non-meat packaging materials and labels are stored in dry storage. The incoming meat products such as chuck tenders and filet mignons are used for further processing in the receiving, trimming, tenderization, and marination room where the working temperature is maintained at $\leq 50^oF$ (Fig 1). Each of the meat pieces are removed from vacuum-packaged bags, dipped in lactic acid, and then subjected to the skinner machine where the workers remove the fascia over the muscle tissue. The product then continues onto a conveyor belt through a splitter table where workers further trim the large tenders/loins into smaller sections and further processed by passage through two blade tenderizing machines. The tenderized meat is then trimmed, flattened and sectioned into filets. The sectioned meat piece is then marinated by vacuum tumbling.

The meat pieces are then brought to a second room of the processing facility. In this processing facility (or ‘Pack-out’ room), marinaded meat pieces are wrapped with bacon, and then passed through Pre-pack-off equipment where R-565 equipment is used to vacuum-package

the finished product. This room is maintained at ≤ 50ºF. Every finished meat product is handled by a worker (with gloves) before vacuum-packaging. Vacuum-packaged and labeled products are then stored in the chiller at a temperature of ≤ 40ºF prior to shipping. Products undergo metal detection tests and rework is performed for the required products. Finished vacuum-packaged products are packed into shipping boxes, labeled, stored, and shipped to the clients.

3.3 The collection of samples

The receiving, trimming, tenderization, and marination rooms consisted of different working areas (Fig.1). During the un-bagging operation, 10 ml of purge was retrieved with a sterile pipette from the incoming meat packages purchased from beef suppliers. The Skinner table itself, and the gloves of the worker at the skinner table were swabbed with sterile gloves using Whirl-Pak Pre-Moistened Speci-Sponge Bags (B01423, Nasco, Fort Atkinson, WI). The targeted spots were swabbed throughout a maximum area (not restricted to certain measurements in order to track the challenge organisms). The same procedure was followed to swab other areas in the room such as the whizard table, gloves of workers at the whizard table, whizard conveyor, splitter table, gloves of workers at the splitter table, tenderizer belt, portioner belt, and gloves of workers at the portioner belt.

The pack-out room consisted of targeted areas such as the bacon-wrapped build table, bacon-wrap build conveyor, bacon-wrapper gloves, Pre-Pack-off equipment, and Pre-Pack-off gloves which were swabbed in the same way as described above (Fig.1). The final vacuum-packaged products of bacon-wrapped filets were collected, massaged, and purge was pulled out from them.

Similarly, swollen and recovered retail vacuum-packaged, refrigerated raw beef products were collected and maintained under refrigeration (4ºC ± 1ºC). Incoming ingredients such as seasoning blend, sodium tripolyphosphate, and salts were also sampled during our testing program.

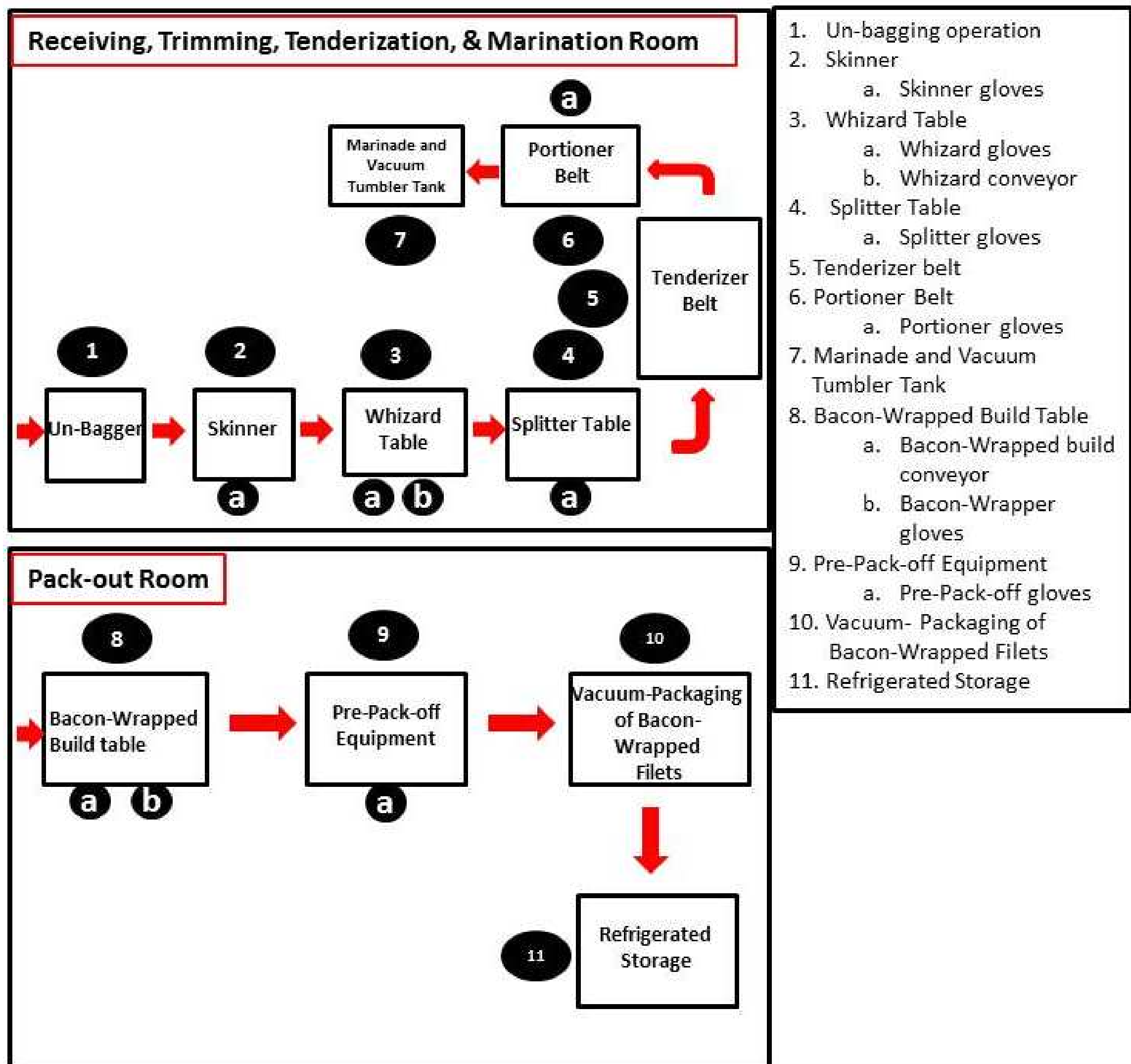


Figure 1. Schematic representation of sample collection areas within the local meat processing facility.

3.4 Testing of samples and isolation of gas-producing organisms

The incoming packages of beef and finished vacuum-packaged products were physically massaged and aseptically opened to remove product and then 1ml purge was retrieved with a calibrated pipette and placed into 9 ml of sterile MRS broth and incubated at 30ºC for 24 hours for enrichment. For environmental surface and swab samples, swabbed hydrated sponges were added to 25 ml of sterile MRS broth and incubated at 30ºC for 24 hours.

In order to determine the presence of Gas^+ bacteria, 1 ml of each overnight enriched sample was transferred into a sterile tube containing 9 ml MRS broth containing an inverted Durham tube. The MRS-Durham tubes were then incubated at 30ºC for 24 hours.

Any MRS-Durham tubes showing gas production (Fig. 8) were then diluted with 0.1% buffered peptone water (BPW), and plated on MRS agar incubated at 30ºC for 48 hours in order to get isolated colonies. Approximately six isolated colonies were selected from each Gas^+ plating and transferred into the 9 ml MRS-Durham tubes to confirm gas production by the clonal isolate. Isolated Gas^+ organisms from each sample or swab were then cultured and stored as a frozen stock in our culture collection as described earlier for subsequent analysis.

3.5 Identification of gas producing organisms

In order to determine the identity of the Gas^+ isolates, the polymerase chain reaction (PCR) was used to amplify 16S rRNA-related DNA sequences, examined by agarose gel electrophoresis to verify the quality of the amplified products, and then submitted to the OSU DNA core facility for sequencing.

Prior to sequence analysis, DNA extraction was initiated by inoculating overnight grown Gas^+ isolates into tubes containing MRS broth and incubated at 30ºC. A volume of 1 ml of

overnight culture was then transferred into sterile microcentrifuge tubes and centrifuged at 12,000 rpm for 1 minute. Centrifugation was followed by discarding the supernatant and the pellets were washed twice in 0.5 ml of sterile, deionized water. The final pellet was resuspended in 100 µL of 10 µM Tris buffer (pH 7.4). Resuspended pellets were transferred to new microcentrifuge tubes containing acid-washed silica beads (VWR brand 800M, VWR International, LLC, Radnor, PA) so that the liquid covered the top of the beads. Bead-pellet mixtures were then put on ice for 3 minutes and subsequently transferred to a pulsing vortex with attached shaker head for 3 minutes followed by another cycle of icing, shaking, and icing. Tubes containing the beads and the cells were finally centrifuged at 12,000 rpm for 2 minutes. Approximately, 50-100 µl of supernatant containing extracted DNA was transferred to a new microcentrifuge tube and then stored at -20ºC. The concentration of DNA was then measured using a NanoDrop®ND-1000 spectrophotometer.

The amplification of DNA from Gas^{+} isolates was done by PCR using universal 16S ribosomal RNA primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 1391R (5'-GACGGGCGGTGTGTRCA-3'). The PCR reaction mix consisted of 1 µl total DNA extract obtained as explained above, 5 µl of 5X GoTaq® PCR Buffer (Promega, Madison, WI), 2.5 µl of 15 mM MgCl2 solution, 2 µl of 5 mM dNTP mix, 1.25 µl of 10 µM 515F primer, 1.25 µl of 10 µM 1391R primer, and 0.25 µl of 5 U/µl GoTaq® Flexi DNA Polymerase (Promega). The final concentration of primers used was 500 nM in a final volume of 25 µL of each reaction. Reaction mixtures were placed into 0.2 ml PCR tubes and then subjected to thermal cycling using a PTC-200 Peltier Thermal Cycler (MJ Research, St. Bruno, Quebec, Canada) with the following thermal cycles: initial denaturation at 95ºC for 4 minutes, followed by 30 cycles of 94ºC for 1 minute (denaturation), 60ºC for 45 sec (annealing), 72ºC for 1 minute (extension), followed by a final extension cycle at 72ºC for 4 minutes, and a final hold at 4ºC. All PCR reactions were run with a negative control (no added template DNA) and a positive control (template DNA from

previous runs). PCR reactions were accompanied by agarose gel electrophoresis and DNA sequence analysis of PCR reaction run in both directions.

The amplified 16S rRNA sequences were purified via the GenCatchTM Advanced PCR Extraction Kit (Epoch Life Sciences, Missouri City, TX). The entire PCR reaction was transferred and mixed with 500 µL of supplied PX Buffer in a sterile microcentrifuge tube. Buffered PCR reaction mixtures were transferred to the top of Spin Columns (Epoch Life Sciences, Missouri City, TX) which rested in a collection tube. The mixture was centrifuged at 5000 rpm for 1 minute, followed by discarding of the filtrate. The DNA retained by the Spin Columns was washed with the supplied WN Buffer and then WS buffer by adding 500 µL to the column and centrifuging at 5000 rpm for 1 minute. The filtrate was again discarded between and after washing. Washed columns were spun down at 13,000 rpm for 3 minutes to dry. Dried columns were placed into new microcentrifuge tubes and 25 µL of Elution Buffer was added to the center of the column membranes and allowed to sit at room temperature for 3 minutes. Eluted DNA was finally collected by centrifuging the column at 13,000 rpm for 2 minutes and the eluate was then stored at -20°C.

Purified DNA recovered from PCR reactions was submitted to the Dept. of Biochemistry and Molecular Biology Recombinant DNA/Protein Resource Facility (Oklahoma State University) for DNA sequencing using an automated DNA sequencer via “BigDyeTM”-terminated reactions analyzed on an ABI Model 3700 DNA Analyzer. ABI sequence files were analyzed using MEGA5 software (Tamura et al., 2011). Both forward and reverse sequences were compared, aligned, and trimmed. Consensus sequences between the forward and reverse amplimers were analyzed using NCBI’s Nucleotide BLAST and identities obtained were then recorded and likelihood tree for rRNA sequences were drawn using MEGA5 (Fig. 6).

3.6 Meat preparation and inoculation

For all beef inoculation and spray treatment experiments, beef roasts were purchased from a local Walmart store (Stillwater, OK). Sample discs of 20.25 cm^2 (2-inch diameter) were prepared from the roasts using a 8512 Univex-Max slicer (Univex Corp., Salem, NH.) to obtain 1/4 – inch beef slices, and a stainless steel coring cylinder with a 2-inch diameter to obtain beef discs (Fig. 2). Meat samples were sliced, stored frozen, and then defrosted prior to use for experiments. Meat discs were inoculated with freshly grown overnight culture of two predominant Gas^+ isolates (*Leuconostoc mesenteroides* 2a and *Leuconostoc lactis* 107) that were mixed in equal volumes, serially diluted to a 10^4 CFU/ml culture dilution, and inoculated with 100 µl spread evenly across the surface of each meat disc with a sterile gloved finger. After inoculation, samples were left at 5°C for 30 minutes to allow for bacterial attachment.

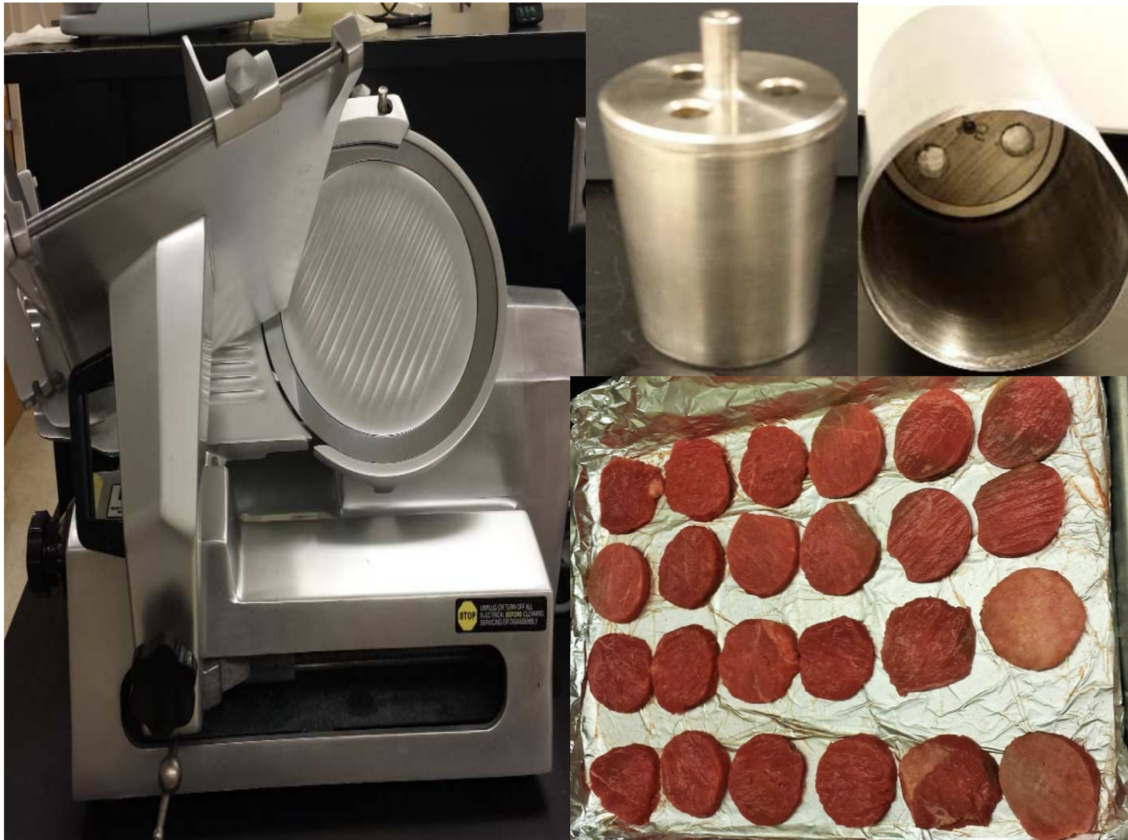


Figure 2. 8512 Univex-Max slicer, stainless steel coring cylinder and beef sliced into 2 diameter discs.

Figure 3. Handheld spray bottles for manual spray application of treatments.

3.7 Spray system

Although we had an automated commercial spray system available for spray treatment, it required high volumes of solutions to fill the reservoir and purge the system after the water wash. Therefore, manual, hand-held spray bottles were used to spray the antimicrobials (Fig. 3). The use of the manual, hand held spray bottles prevented the wasting of the antimicrobial solution to be sprayed on the meat discs. The hand-held spray bottles were calibrated before, and during use, by determining spray amounts using an analytical top-loading balance. Antimicrobial solutions, including water controls, were sprayed on the inoculated discs so that the discs received a volume of 48 ml of the solution which was equivalent to the volume sprayed by our commercial air-assisted, automatically timed, fine mist spray system in which the treatment solution was supplied by a pump and reservoir while being expelled by a pressurized air source (40 psi) for a designated time of 20 sec.

3.8 Antimicrobial treatment solutions

A variety of antimicrobial solutions were obtained directly from various commercial sources, or from the local meat processor for testing against the Gas^+ challenge strains.

3.8.1 Danisco NovaGARD NR 100

Danisco NovaGARD NR 100, a natural antimicrobial powder (manufactured by DuPont Nutrition Biosciences) was used as one of the treatment solutions. It was received in a closed container of 2.2 lbs., with the storage instructions 'to be stored at below 25°C/77°F'. Nisin (1.25%), and natural rosemary extract were the major ingredients, and NaCl was the carrier present in the antimicrobial sample. In order to achieve 1% (w/v) usage level, 10 gm of the

antimicrobial was measured and dissolved in 990 ml of Distilled (DI) water to make a final volume of 1000 ml.

3.8.2 Bio Via CDV

Bio Via CDV (manufactured by DuPont), was labeled as an experimental sample as received (proprietary ingredient details not disclosed to public). In order to make 1000 ml of required solution, 1.5 % (w/v) of the antimicrobial was dissolved in 985 ml of DI water.

3.8.3 Durafresh 2012

Durafresh 2012 (Kerry Brand) powder was received (proprietary ingredient details not disclosed to public). In order to prepare to prepare 1000 ml of final solution, 1.5% (w/v) was weighed, and mixed in 985 ml of DI water.

3.8.4 ZESTi AM-5

ZESTi AM-5, 5 gal pail (Kerry Brand) liquid (proprietary ingredient details not disclosed to public) was received and 1000 ml of 100% concentrate was removed for spray application.

3.8.5 Durafresh 5924

Durafresh 5924 (Kerry Brand) (proprietary ingredient details not disclosed to public), 50-lb bag of powder was received. In order to make 1000 ml of the required solution, 1.5% (w/v) was weighed and mixed with 985 ml of DI water.

3.8.6 Water

Water was used as a control for the entire experiment. Volume of 1000 ml of DI water was used as one of the solutions.

3.8.7 Lactic acid

Lactic acid 88% F.G. (Birko Corporation; Denver, CO) was received and stored in a cool, dry place. The received container was labeled as 'complies with 21 CFR Sections 182 or 184', and 'Generally recognized as safe by the FDA'. In order to make 1000 ml of required solution (5%, v/v), lactic acid was measured and mixed with DI water to make 1000 ml of working stock solution.

3.8.8 Acetic acid

Acetic acid (Glacial, certified A.C.S. PLUS; Lot No. 012099) manufactured by Fisher Scientific, was received and stored in a cool place. In order to make 1000 ml of required solution, an appropriate amount was measured and mixed with DI water to make a 5% (v/v) working stock.

3.8.9 Citric acid

Citric acid monohydrate certified A.C.S. (Lot No. 044571) with a formula weight of 210.14, was used to make a desired treatment solution. In order to make 1000 ml of required solution, an appropriate amount was measured and mixed with DI water to make a 5% (w/v) working stock.

3.8.10 Organic acids mixture

Mixture of the above mentioned organic acids (lactic acid, citric acid and acetic acid) was used as the treatment solution. The organic acid solutions were prepared in the same manner as described above and were mixed to make the desired solution of 1000 ml.

3.8.11 CytoGuard STAT-N-PLUS and CytoGuard LA 20

CytoGuard STAT-N-PLUS and CytoGuard LA 20 (A&B Ingredients; Fairfield, NJ) (Lauric arginate blend, other proprietary ingredient details not disclosed to public) were received from the supplier. A solution of 95% (950 ml) of STAT-N-PLUS (v/v), and 5% (50 ml) of LA 20 (v/v) were mixed to obtain the desired solution in 1000 ml.

3.8.12 Chef's mix with Bestate

Chef's mix, obtained from a local meat processing company, was used as another antimicrobial spray. Bestate is a blend of lactate plus diacetate. The compositions listed were 91.5% water, 2% fresh flavor, 3% Zesti AM-5, 1% sodium phosphate, and 2% Ultra-Pure Bestate-P4218. Final volume of 1000 ml of mix was used.

3.8.13 Chef's mix without Bestate

The compositions listed for Chef's mix without bestate were 94% water, 2% fresh flavor, 3% Zesti AM-5, and 1% sodium phosphate. Final volume of 1000ml of the supplied solution was used.

3.8.14 AFTEC 3000

AFTEC 3000 (buffered sulphuric acid) was received as an already diluted working stock solution. It was sprayed as received (pH 1.0).

3.8.15 Bacteriocins from bacteriocin-producing LAB

In order to determine the combination of bacteriocins to be used as one the antimicrobial treatment solutions, against the Gas^+ challenge organisms, agar spot assay were performed. Bacteriocin-producing (Bac^+) LAB cultures isolated from various food samples in Dr. Muriana's food microbiology lab (Robert M. Kerr Food and Agricultural Products Center, OK) were cultured in sterile MRS broth and incubated at 30°C for 24 hours. Overnight cultures were centrifuged at 8000 rpm at 4°C for 12 minutes. The decanted Bac^+ supernatants were pasteurized at 80°C for 15 minutes. Pasteurized bacteriocins were allowed to cool down until use. Indicator strains of Gas^+ Organisms (*L. mesenteroides* 2a and *L. lactis* 107), cultured in sterile MRS broth and grown overnight were added (separately) in soft MRS overlay agar (0.75% agar). Overlayed agar was allowed to set for 10 minutes. A volume of 10 µl of bacteriocin was spotted on the MRS agar base plate containing the indicator layer. Spots were allowed to dry for a while and were incubated at 30°C for 24 hours. Cultures of Bac^+ LAB were selected based on the ability of their bacteriocins to form the strong zones of inhibition on the individual Gas^+ challenge strains (Fig. 9).

LAB cultures selected for bacteriocin combination treatment were namely; *Lactococcus lactis* RDSH-3, *Lactococcus lactis* BSP, *Lactococcus lactis* SL-1, *Lactococcus lactis* FL-1, *Lactococcus lactis* FS-162, *Lactococcus lactis* ASPG-3, *Lactococcus lactis* FLS-1, and *Lactococcus lactis* FS-95 (Table. 2). Cultures were inoculated at 1% levels into MRS broth from thawed frozen stocks, incubated at 30°C for 24 hours, and then reinoculated into 125 ml MRS

broth for a second transfer before use. The overnight cultures were centrifuged at 8000 rpm at 4ºC for 12 minutes, decanted, and supernatants were pasteurized at 80ºC for 15 minutes, and mixed together to give a final volume of 1000 ml which was used as a bacteriocin-containing spray treatment solution.

3.9 Antibiotic disc assay of Gas$^+$ isolates selected as test organisms

Isolated Gas$^+$ organisms: *Leuconostoc mesenteroides* 2a and *Leuconostoc lactis* 107 were selected for determining antibiotic resistance using a disc assay procedure (Fig. 9). The Gas$^+$ organisms (*L. mesenteroides* 2a and *L. lactis* 107) were cultured in MRS broth and added to soft overlay agar (MRS). The seeded overlay agar was overlaid with various antibiotic discs (Becton, Dickinson and Company, Sparks, MD). Plates with antibiotic discs were incubated at 30ºC for 24 hours and then evaluated for susceptibility (Fig. 10).

Several antibiotics were chosen for which both Gas$^+$ challenge strains were resistant to include in plating media during spray treatment and shelf-life testing experiments to exclude the appearances of other indigenous contaminants of raw beef discs.

3.10 Antimicrobial spray treatment of beef inoculated with Gas$^+$ isolates

Beef discs (20.25 cm^2, 2-inch diameter) were inoculated with challenge organisms, *Leuconostoc mesenteroides* 2a and *Leuconostoc lactis* 107 that were mixed in equal volumes, serially diluted to a 10^4 CFU/ml culture dilution, and inoculated with 100 µl spread evenly across the surface of each meat disc with a sterile gloved finger. These organisms were selected as they were the most prevalent Gas$^+$ isolates at this time. Beef samples were then placed at 5ºC for 30 minutes to allow for bacterial attachment. Inoculated beef discs were sprayed with the different

antimicrobials, or water as control solution, which were prepared as explained above. After spray treatment, they were allowed to sit for 30 sec to drain the excess liquid. After treating all beef discs, two individually-treated beef discs were placed in each sample bag. Triplicate sample bags, each with two discs, were prepared for day 0 (i.e., the starting day), and days 3, 7, 14, and 28, respectively. The sampling bags were vacuum-packaged using a Hobart's H-Series vacuum-packaging equipment (Howard Corporation; Troy, OH) (Fig. 4). The vacuum-packed samples were refrigerated at 4°C for days 3, 7, 14, and 28 days prior to sample plating. On the day of plating, the bags were cut opened and 40.5 ml of Dey-Engley (DE) Neutralizing broth was added (i.e., 1 ml=1 cm^2) and stomached for 2 minutes (60 sec on each side of the bag) using a Seward 400 laboratory stomaching blender (Tekmar Company; Cincinnati, OH). A volume of 1 ml liquid sample was pulled out and diluted accordingly. The samples were then plated on MRS agar plates containing Nalidixic Acid (10 µg/ml) and Vancomycin (10 µg/ml), and incubated at 30°C for 48 hours. The same procedure was followed for 3, 7, 14, and 28 days samples (Fig. 5). After incubation, plates were enumerated using a Darkfield colony counter.

Figure 4. Vacuum-packaging using a Hobart's H-Series vacuum-packaging equipment.

3.11 Antimicrobial and water treatment with handheld (manual) spray bottle

Leuconostoc strains were grown and prepared as explained above. Cultures were initially used at high concentrations of 10^7 cfu/cm^2 and then lowered 4 log levels to result in approximately 10^3 cfu/cm^2 on the discs in order to use more practical starting levels. Samples of beef discs were inoculated and allowed for bacterial attachment, as stated above. Samples were then divided according to the treatments: uninoculated-untreated sample, inoculated samples (but no treatment), inoculated samples (with treatment; antimicrobials or water).

All treatment samples were performed in triplicate replication. Samples were sprayed with the manual hand held sprayer. The manual sprayer was calibrated to deliver the same volume as the air-assisted automatic spray system (i.e., 47.5 ml in 15 sec). Treated samples were processed in the same way as mentioned earlier.

3.12 Statistical analysis

Trials were performed in triplicate replications consisting of paired samples within each replication. Samples were serially diluted and plated in duplicate for each analysis. Experimental results were analyzed using a repeated measures one-way analysis of variance (ANOVA) to determine the level of significance between each treatment. Pairwise multiple comparisons were completed using the Holm-Sidak method. All statistical analysis was performed using SigmaPlot (ver. 12.5) (Systat Software, San Jose, CA) at a p-value of 0.05.

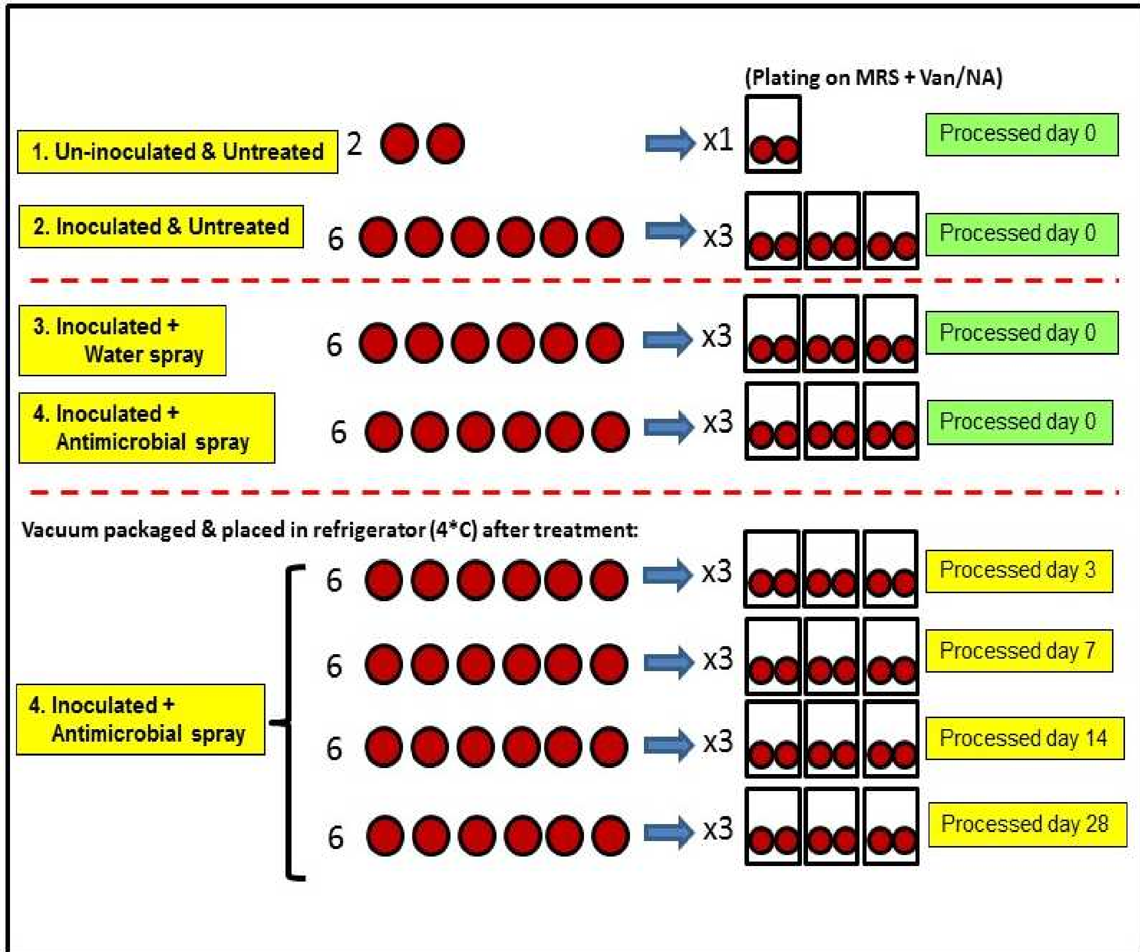


Figure 5. Antimicrobial treatment plating scheme on beef discs inoculated with gas-producing isolates.

CHAPTER IV

RESULTS AND DISCUSSION

Results and discussion will be presented in two sections. First section will contain the isolation and identification of gas producing organisms and the second section will include the results of the different antimicrobial interventions against the selected Gas^+ isolates.

4.1 Isolation and identification of gas-producing organisms

Gas-producing microorganisms present in the enrichment samples were confirmed by the observation of gas bubbles in the inoculated MRS-Durham tubes (Fig. 8). Repeating the MRS-Durham tube inoculation process, using isolated colonies obtained from streaking the gas containing test sample allowed us to isolate the specific Gas^+ organism. Different colonies were selected based on different morphology characteristics. The identity of Gas^+ isolates was determined by utilizing universal 16S ribosomal RNA (rRNA) primers in PCR reactions with extracted DNA.

The identification results revealed that, out of a total of 552 samples collected, 51 samples were Gas$^+$ (Table 1). Gas-positive isolates were: *Leuconostoc* spp. (76.47%) and *Lactobacillus* spp. (11.76%); they represented a combined total of 88.23% of the total 51 Gas$^+$ isolates. Zhang (1992) isolated 97% of 76 total isolates as *Leuconostoc* spp. and *Lactobacillus* spp. from vacuum-packaged, cooked, cured pork products. Other authors (Holzapfel, 1992; Makela et al. 1992) indicated that the predominant species associated with the spoilage of vacuum-packaged meats are leuconostocs and other LAB.

Out of the 51 Gas$^+$ isolates, 25 isolates (49.01%) were identified as *Leuconostoc mesenteroides*. Similarly, 12 isolates (23.52%) were *Leuconostoc lactis*, 4 of the isolates (7.84%) were *Lactobacillus sakei*, 3 of the isolates (5.88%) were *Weissella confusa,* 3 of the isolates (5.88%) were *Weissella viridescens* and 2 of the total isolates were each *Leuconostoc citreum* (3.92%), and *Lactobacillus curvatus* (3.92%), respectively (Fig. 7). *Leuconostoc mesenteroides* and *Leuconostoc lactis* were the major bacteria isolated and the most probable cause of swelling of the package (Table 1). Holzapfel (1992) and Makela et al. (1992) also indicated that leuconostocs and LAB were associated with the spoilage and gas production in vacuum-packaged meats.

Tested Samples	Total Number of Samples	Total Number of Gas-Positives	Identified Microorganisms
Purge from Incoming raw meat	198	21	*Leuconostoc mesenteroides* Lot 2013154 *Leuconostoc mesenteroides* M-6 *Leuconostoc mesenteroides* M-17 *Leuconostoc mesenteroides* M-18 *Leuconostoc mesenteroides* M-19 *Leuconostoc mesenteroides* 5 *Leuconostoc mesenteroides* 9-1 *Leuconostoc mesenteroides* 9-3 *Leuconostoc mesenteroides* 9-5 *Leuconostoc lactis* 107 *Leuconostoc lactis* 337 *Leuconostoc lactis* 340 *Leuconostoc lactis* 401 *Leuconostoc lactis* 406 *Leuconostoc lactis* 407 *Leuconostoc lactis* 2 *Leuconostoc citreum* 216 *Leuconostoc citreum* 329 *Weissella viridescens* M-21 *Weissella viridescens* 6 *Lactobacillus curvatus* 5
Incoming dry ingredients	17	2	*Leuconostoc mesenteroides* M-8 *Leuconostoc mesenteroides* M-9
Swab from the working units inside the production and storage facility (unbagging, skinning, splitting, tenderizing room and pack-out room)	282	17	*Leuconostoc mesenteroides* 25 *Leuconostoc mesenteroides* 8 *Leuconostoc mesenteroides* 9 *Leuconostoc mesenteroides* 2 *Leuconostoc mesenteroides* 7-7 *Leuconostoc mesenteroides* 7-9 *Leuconostoc mesenteroides* 9-7 *Leuconostoc lactis* 109 *Leuconostoc lactis* LG *Leuconostoc lactis* AP *Leuconostoc lactis* 7-10 *Leuconostoc lactis* 9-4 *Weissella viridescens* BPG *Weissella confusa* BC *Weissella confusa* TB *Lactobacillus curvatus* 6(8) *Lactobacillus sakei* 6(20)
Finished vacuum packaged product	55	11	*Leuconostoc mesenteroides* 1a *Leuconostoc mesenteroides* 2a *Leuconostoc mesenteroides* 3b *Leuconostoc mesenteroides* 4(2)B *Leuconostoc mesenteroides* 7(11) *Leuconostoc mesenteroides* BB *Leuconostoc mesenteroides* 13 *Lactobacillus sakei* 3(a) *Lactobacillus sakei* 7(2) *Lactobacillus sakei* D *Weissella confusa* 4a

Table 1. Total number of samples collected and the identification of the Gas^{+} isolates.

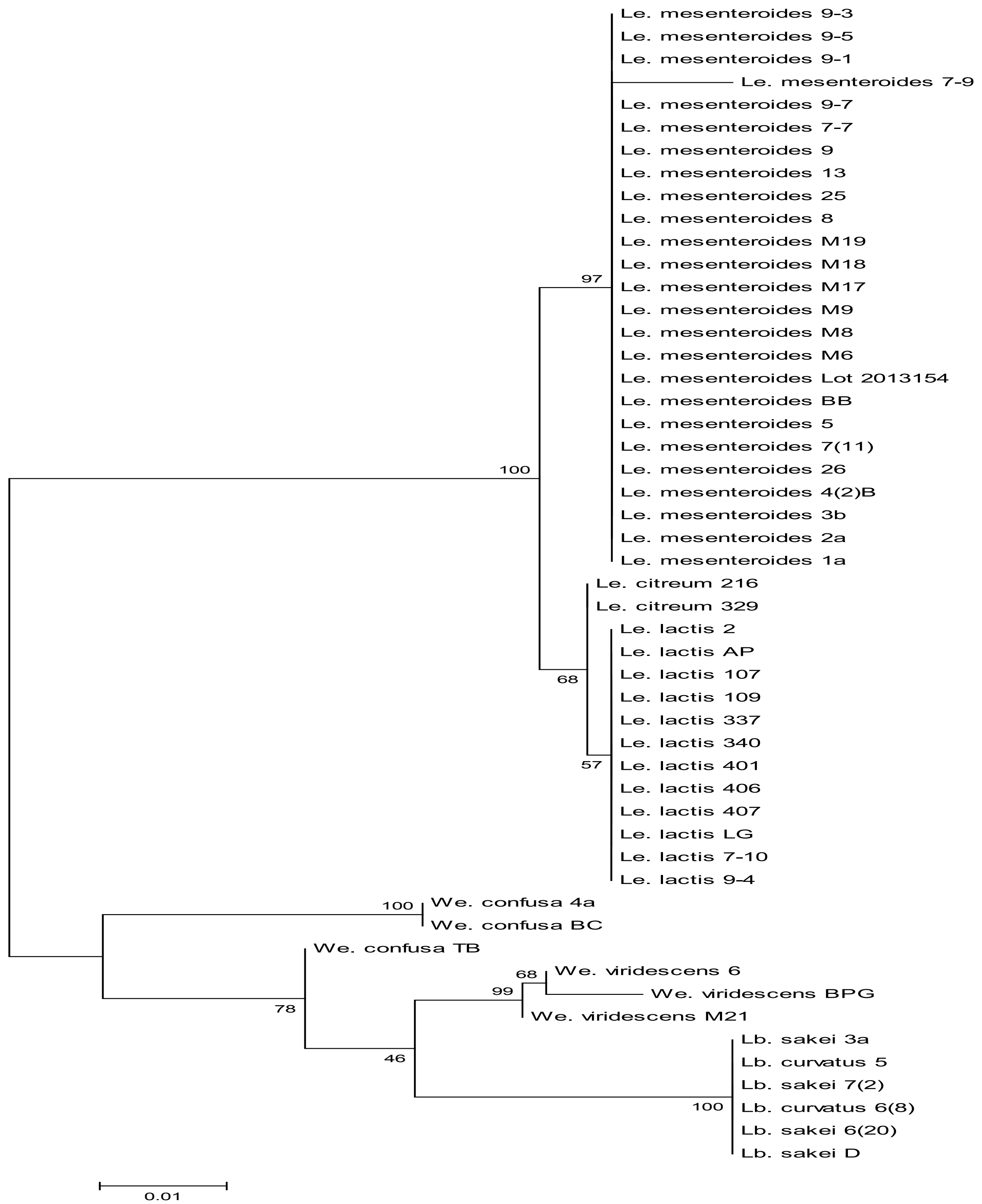
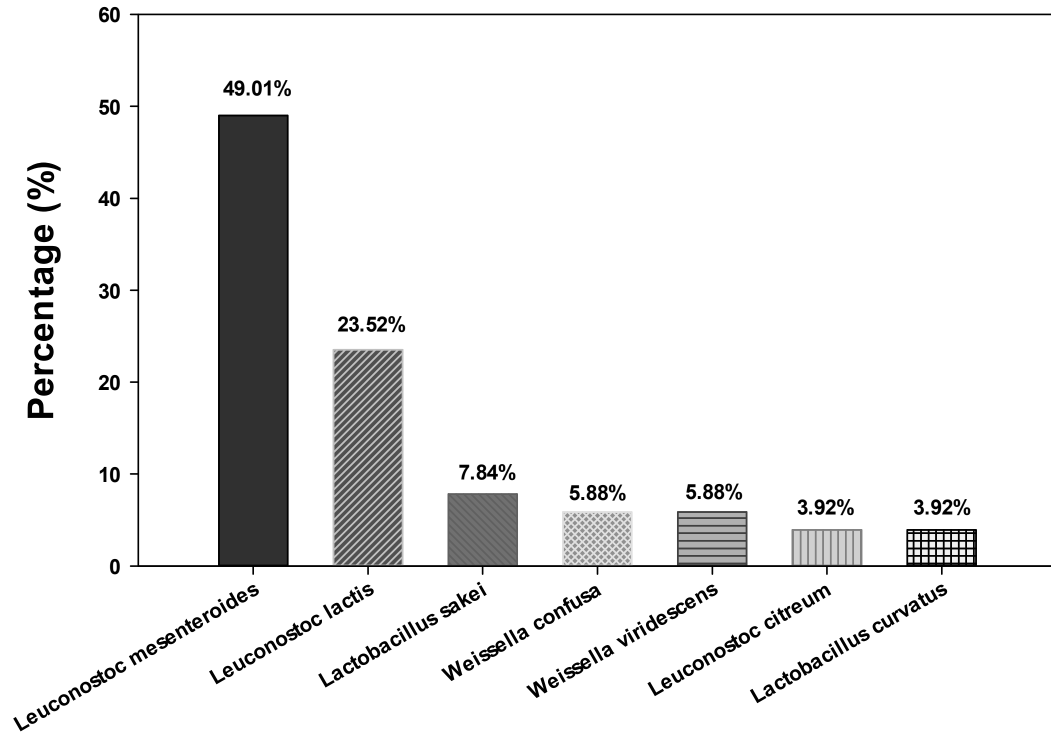


Figure 6. Maximum likelihood tree for rRNA sequences for gas-producing isolates.

Isolated and Identified Bacteria Responsible for Gas-Production



Major bacterial isolates

Figure 7. Major bacterial isolates identified from the local meat manufacturing facility. % number of isolates identified from genera of the total number of isolates (51).

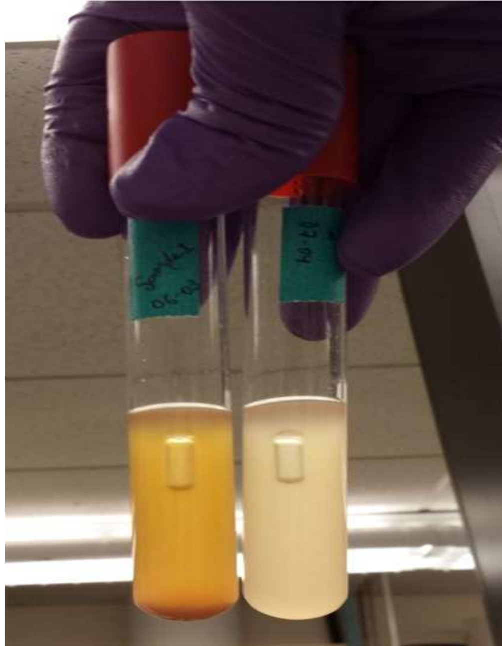


Figure 8. MRS-Durham tube (Right) and TSB-Durham tube (Left) showing gas production that indicates the presence of Gas-positive organisms.

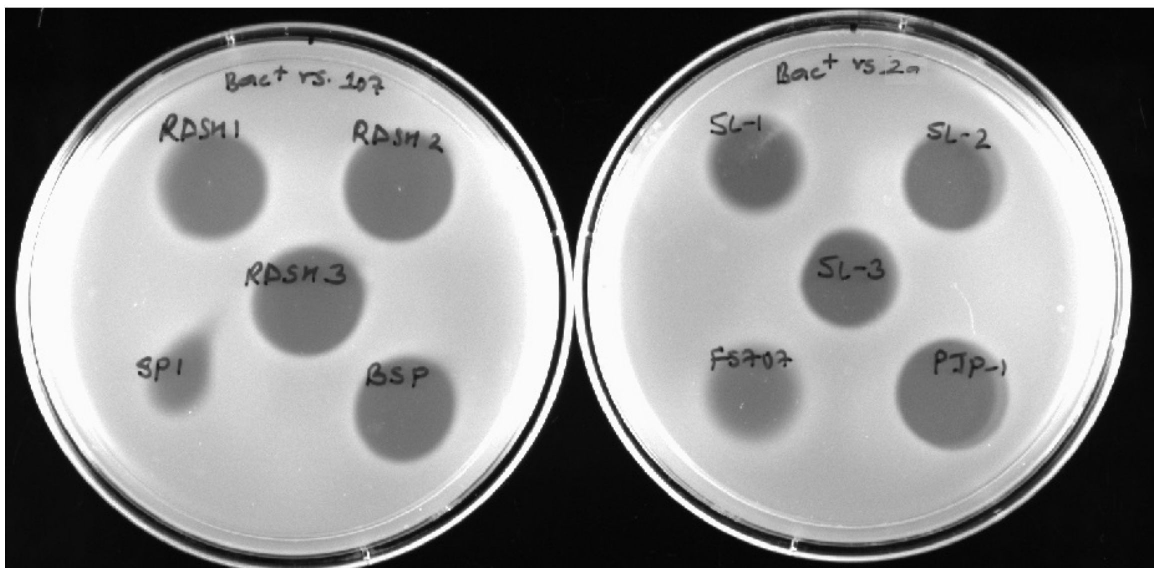


Figure 9. Bacteriocin spot assay showing zones of inhibiton against Gas-positive organisms (*L.mesenteroides* 2a *and L. lactis* 107).

Organism	Bac+ Preps	*Leuconostoc mesenteroides* 2a	*Leuconostoc lactis* 106
Lactococcus lactis	RDSH 1	+++	+
Lactococcus lactis	RDSH 2	+++	++
Lactococcus lactis	RDSH 3	+++	++
Lactococcus lactis	FLM 1	+++	+
Lactococcus lactis	SP 1	+++	+
Lactococcus lactis	ASPG 1	+++	+
Lactococcus lactis	ASPG 2	+++	+
Lactococcus lactis	ASPG 3	+++	++
Serratia plymuthica	POT	--	--
Lactococcus lactis	BSP	+++	++
Lactococcus lactis	YO-1	+++	+
Lactococcus lactis	YO-2	+++	+
Lactococcus lactis	YO-3	+++	+
Lactococcus lactis	SL-1	+++	++
Lactococcus lactis	SL-2	+++	++
Lactococcus lactis	SL-3	+++	+
Lactococcus lactis	PJP-1	+++	+
Enterococcus durans	FS 707	++	+
Enterococcus faecium	FS 56-1	--	+++
Leuconostoc mesenteroides	BFS-1	--	--
Lactococcus lactis	FLS-1	+++	++
Lactococcus lactis	FS95	+++	--
Lactococcus lactis	FS91-1	+++	--
Lactobacillus curvatus	FS47B	--	+
Lactobacillus curvatus	BJ-21	--	+
Lactobacillus curvatus	FS36-1	--	--
Enterococcus thailandicus	FS92	+	--
Lactobacillus curvatus	Beef 2L-1	--	--
Lactobacillus curvatus	Beef 2L-2	--	--
Lactococcus lactis	FL-1	+++	+
Lactococcus lactis	FL-2	+++	+
Lactococcus lactis	FS-162	+++	+
Enterococcus faecium	JCP-9	--	+
Enterococcus faecium	JCPB-5	--	++
Lactobacillus ingluviei	FS-60	--	--
Enterococcus faecium	Thyme2	--	--
Lactococcus lactis	BJ-23	+	+++

Table 2. Response of Gas$^+$ organisms (*L. mesenteroides* 2a and *L. lactis* 107) to Bac$^+$ preparations in agar spot assays (+++ indicates very strong zone of inhibition, ++ indicates strong zone, + indicates mild zone whereas, – indicates no zone of inhibition).

4.2 Agar spot assay of bacteriocin producing LAB

Out of the total LAB cultures tested, bacteriocins produced by 8 LAB cultures showed strong zone of inhibition against the selected Gas^{+} isolates (*L. mesenteroides* 2a and *L. lactis* 107) (Fig. 9). LAB cultures that were selected on the basis of agar spot assay were *Lactococcus lactis* RDSH-3, *Lactococcus lactis* BSP, *Lactococcus lactis* SL-1, *Lactococcus lactis* FL-1, *Lactococcus lactis* FS-162, *Lactococcus lactis* ASPG-3, *Lactococcus lactis* FLS-1, and *Lactococcus lactis* FS-95 (Table. 2). These combinations were further used for spray treatment application.

4.3 Antibiotic disc assay of Gas^{+} isolates selected as test organisms

Several Gas^{+} isolates that were selected as potential challenge organisms as inoculants for beef challenge studies with various antimicrobials were tested against an array of antibiotics (Fig. 10). We were able to identify that both strains were resistant to several of the same antibiotics, allowing us to add them to plating media when enumerating survivors of antimicrobial treatments. The assay revealed that both *Leuconostoc* strains used in the following experiments were resistant to nalidixic acid and vancomycin (Fig. 10). We then compared plating levels of *Leuconostoc mesenteroides* 2a and *Leuconostoc lactis* 107 on MRS. MRS containing nalidixic acid (10 µg/ml) and vancomycin (10 µg/ml) to determine their susceptibility in selective media.

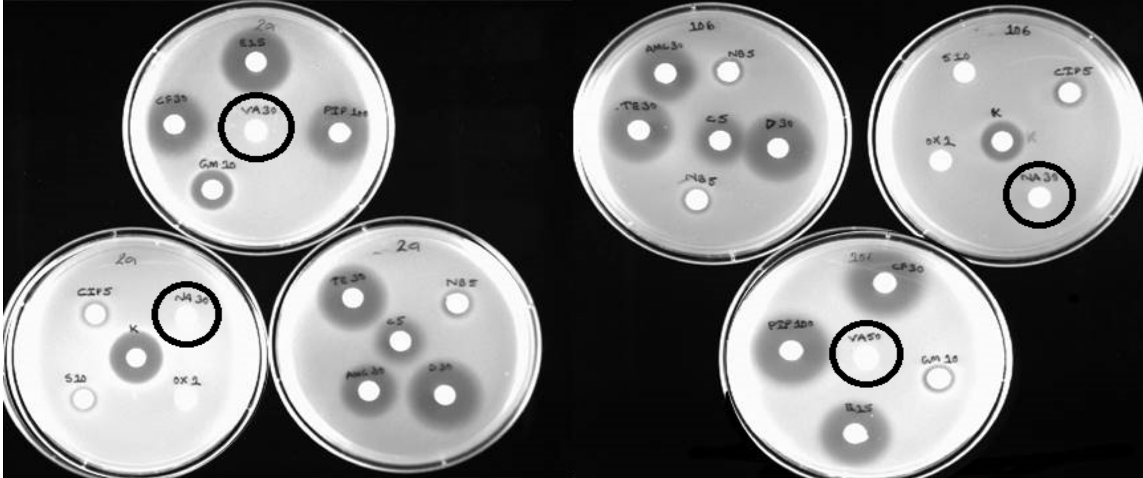


Figure 10. The Gas$^+$ strains were tested with the antibiotic susceptibility disc assay which showed that both *L. mesenteroides* 2a and *L. lactis* 107 were resistant to nalidixic acid and vancomycin.

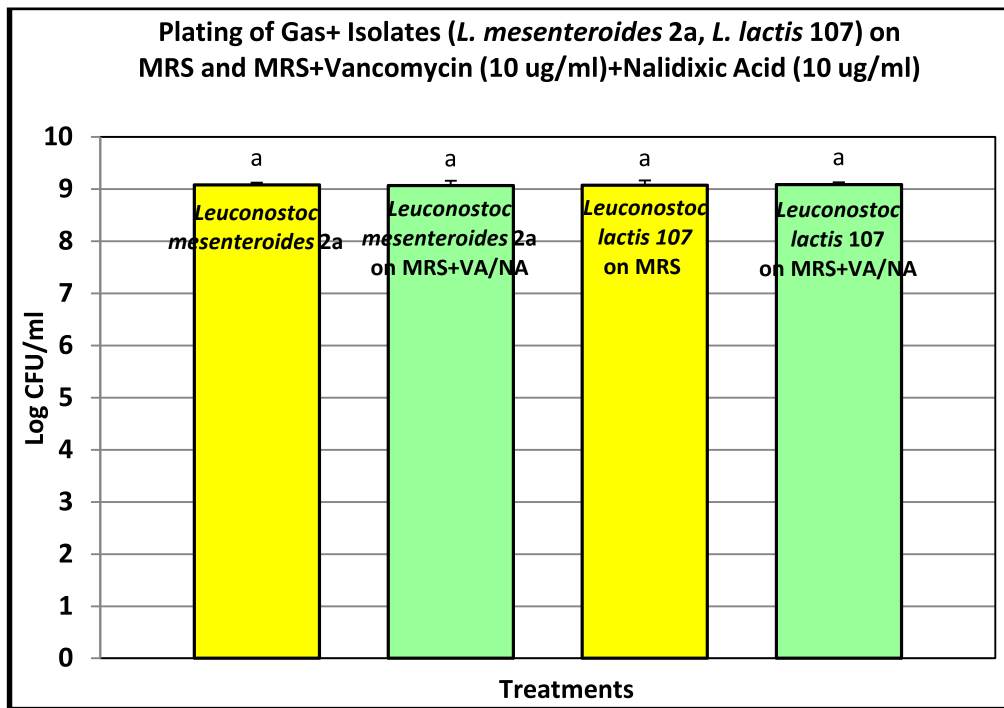


Figure 11. Plating of Gas$^+$ strains resistant on MRS agar, and on MRS agar containing Vancomycin and Nalidixic acid (each at 10 μg/ml), to confirm that the antibiotics do not affect growth levels of these organisms. Same lower case letters represents no significant difference ($P > 0.05$).

4.4 Spray treatment with antimicrobials

Meat discs inoculated with the Gas^+ isolates were treated with different antimicrobials to compare effectiveness of treatments in controlling the growth of those isolates. All antimicrobial treatments were divided into two categories: a) antimicrobial treatment of different commercial brands, and b) organic acids.

Effectiveness of these antimicrobials was determined by the survivor graph plots over the extended time period of day 0, 3, 5, 7, 14, and 28. Log-reductions at the point of application or suppression of growth during shelf-life helped in analyzing the significant difference ($P < 0.05$) of the treatments when compared with other treatments.

4.4.1 Antimicrobial treatment of different commercial brands of antimicrobials

Antimicrobial treatments of different commercial brands of antimicrobials were compared in this study. These brands include those manufactured by a) DuPont, b) Kerry, c) an assortment of antimicrobials from other manufacturers (CytoGUARD, Chef's mix with Bestate, Chef's mix w/o Bestate and AFTEC 3000), and d) various organic acids (lactic acid, acetic acid, citric acid, organic acids mix, and our bacteriocins mixture).

4.4.1.1 DuPont brand

Two commercial antimicrobials manufactured by DuPont (Solae Bio Via CDV and Danisco NovaGARD NR 100) were used and compared with each other and with untreated inoculated controls and water treated samples (Fig. 12). Initial log-reductions and growth

performance of Gas^+ isolates over the 28 day time period determined the effectiveness of these treatments.

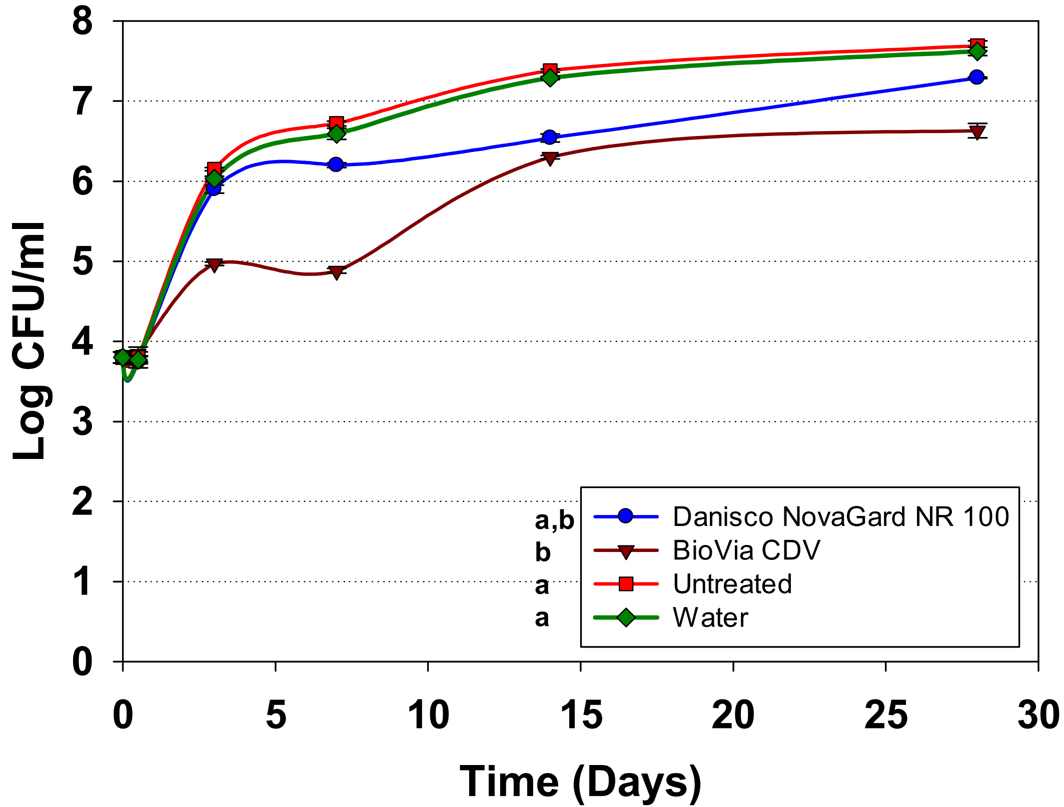


Figure 12. The effect of beef discs inoculated with with *L. mesenteroides* 2a and *L. lactis* 107, and sprayed with DuPont brand antimicrobials and stored for upto 28 days at 4ºC. The trials included inoculated beef discs that were a) untreated, or sprayed with b) water, c) Danisco NovaGARD NR100, or d) Bio Via CDV. All trials were performed in triplicate replication of paired samples and data points represent the means (standard deviations are represented by the standard error bars). Treatments that share the same lower case letters (to the left of the legend) are not significantly different (P> 0.05); treatments that do not share the same lower case letters are significantly different (P< 0.05).

Results obtained from day 0 (1st day) spray treatment with 1.5% Bio Via CDV antimicrobial showed 1.18, 1.84, 1.08, and 1.06-log differences on days 3, 5, 7, 14, and 28 respectively compared to untreated control (Fig. 12). The growth curve showed maximum log-differences between 5-10 days of storage. Pairwise multiple comparisons showed that the Bio Via CDV treatment was significantly different ($P < 0.05$) than control and water treatment (Fig. 12). However, the treatment was not found to be significantly different ($P > 0.05$) than the Danisco NovaGARD NR 100 treated samples (Fig. 12). Danisco NovaGARD NR 100 was not found to be significantly different ($P > 0.05$) compared to the untreated control and water treatment. Results also indicated that 1.5% Bio Via CDV was effective in controlling the growth of Gas^+ organisms until 5-10 days of treatment when compared to Danisco NovaGARD NR 100 (Fig. 12) and probably the best one among the DuPont brand antimicrobials.

Danisco NovaGARD NR 100 consists of E234 Nisin (1.25%) and natural rosemary (an antioxidant) extract as the major ingredients. These ingredients are considered as an effective antimicrobial agent against various microorganisms (Mustapha et al., 2002). However, limitations in the stability of these ingredients and mode of action against Gas^+ organisms over the time period might be a factor that limits the effectiveness of Danisco NovaGARD NR 100.

4.4.1.2 Kerry brands

Commercial antimicrobials manufactured by Kerry included: DuraFresh 2012, Durafresh 5924, and Zesti AM-5. The objective of the study was to compare the effectiveness of these antimicrobials in controlling or inhibiting Gas^+ organisms over the time period of 28 days to see which antimicrobials may be best suited for suppressing growth of Gas^+ organisms causing bloating of vacuum-packaged beef filets.

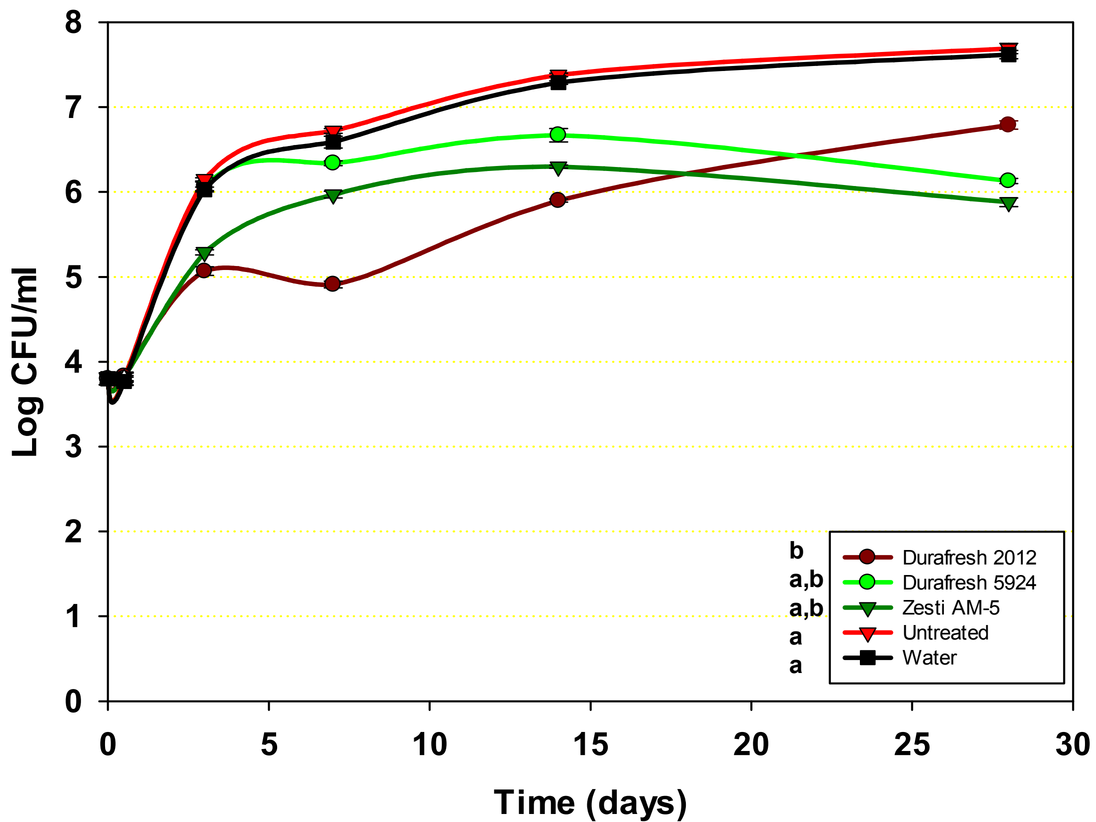


Figure 13. The effect of beef discs inoculated with *L. mesenteroides* 2a and *L. lactis* 107, and sprayed with Kerry brand antimicrobials stored for 28 days at 4ºC. Treatments included inoculated beef discs that were a) untreated, or sprayed with b) water, c) Durafresh 2012, d) Durafresh 5924, or e) Zesti AM-5. All trials were performed in triplicate replication of paired samples and data points represent the means (standard deviations are represented by the standard error bars). Treatments that share the same lower case letters (to the left of the legend) are not significantly different (P> 0.05); treatments that do not share the same lower case letters are significantly different (P< 0.05).

The results obtained from these spray treatments indicated that Durafresh 2012 was significantly different (P< 0.05) compared to the untreated control and water treatment (Fig. 13). Durafresh 2012, Durafresh 5924, Zesti AM-5, untreated, and water treatment were not significantly different (P> 0.05) compared to each other (Fig. 13). However, Durafresh 5924 and

Zesti AM-5 showed slight reduction of Gas^+ after 25th day when compared to Durafresh 2012, untreated, and water treatment respectively. Durafresh 2012 which was initially working better among the other antimicrobials in this group (as it showed maximum log-reductions from 5-10 days of storage), could not hold the same suppressive effect until 28 days.

Durafresh 2012 showed maximum growth differences from 5-10 days of storage; it can be assumed that it might have components that acted as an antimicrobial agent which reduced the growth of Gas^+ organisms to some extent when compared to the control treatment and others. The increase in the growth curve of Durafresh 2012 after 15 days of storage might be due to the uncontrolled growth of the inoculated Leuconostocs or the instability of the antimicrobial component in Durafresh 2012. Since, the proprietary ingredients of these antimicrobials were not disclosed; it cannot be predicted about their ingredients components that might be responsible for the antimicrobial activity or their mode of action upon storage.

4.4.1.3 Others

Antimicrobials of other commercial brands or homemade mixes (Chef's Mix) used in this study included CytoGuard STAT N PLUS and CytoGuard LA 20 (combined and named as CytoGuard), Chef's mix (with Bestate), Chef's mix (without Bestate), and AFTEC 3000.

The results showed that CytoGuard was significantly different ($P < 0.05$) when compared to AFTEC 3000, untreated, and water treatment (Fig. 14). Similarly, Chef's mix (with Bestate) and Chef's mix (without Bestate) were significantly different ($P < 0.05$) than untreated control and water treatment but were not significantly different ($P > 0.05$) compared to other treatments (Fig.14).

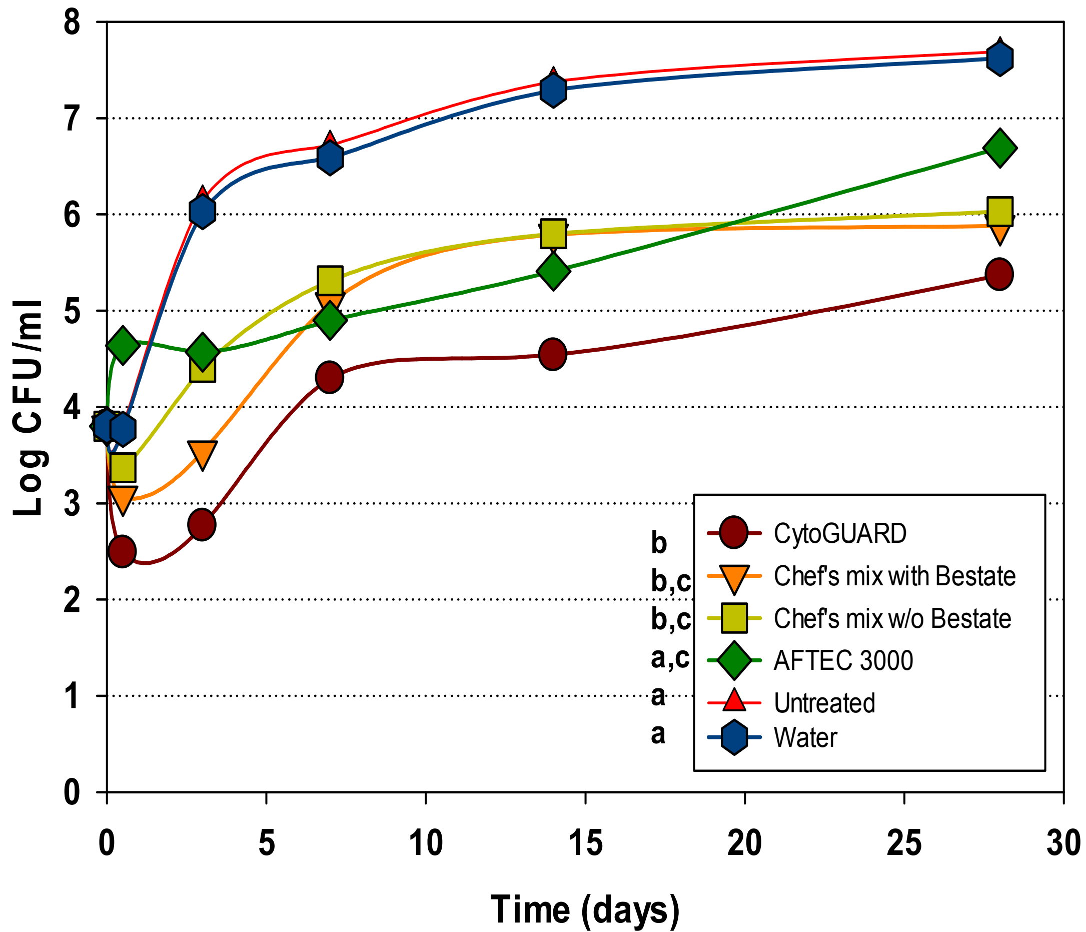


Figure 14. The effect of beef discs inoculated with *L. mesenteroides* 2a and *L. lactis* 107, sprayed with antimicrobials and stored for 28 days at 4°C. Treatments of inoculated beef discs included: a) no treatment, b) water spray, c) CytoGUARD, d) AFTEC 3000, e) Chef's mix with Bestate, and f) Chef's mix w/o Bestate. All trials were performed in triplicate replication of paired samples and data points represent the means (standard deviations are not shown to prevent clutter). Treatments that share the same lower case letters (to the left of the legend) are not significantly different ($P > 0.05$); treatments that do not share the same lower case letters are significantly different ($P < 0.05$).

CytoGuard has lauric arginate (LA 20) as one of its major components. Lauric arginate (LAE) is an antimicrobial compound that has been found to be effective at reducing pathogenic organisms in wide range of food products including meat and poultry products. Martin et al. (2009) found out that 22-ppm LAE gave more than 1-log reduction of *L. monocytogenes* surface inoculated onto frankfurters within 12 hr. The combination of either 1.8%/0.13% or 2.1%/0.15% potassium lactate/sodium diacetate (L/D), respectively, in combination with 22 ppm LAE caused more than a 2-log reduction in 12 hr. Storage studies revealed that complementary interactions of L/D and LAE also extended the shelf life at refrigerated temperature. This combination initially reduced *L. monocytogenes* by 2 logs and suppressed growth to less than 2 logs even at the end of the 156-day storage life for frankfurters. Our intervention had CytoGuard with 5% LAE-20 and the treatment showed a 1.31-log difference on day-0, 3.38-log difference on day-3, 2.29-log difference on day-7, 2.75-log difference on day 14 and 2.25-log difference on day-28 when compared to the untreated control. In this study, CytoGuard showed the maximum difference and lowest level of challenge organisms from day 0 to day 28 in comparison to the other treatments.

4.4.1.4 Organic acids

Different organic acids used in this study were acetic acid (5% v/v), lactic acid 5% (v/v) and citric acid 5% (w/v), and a mixture of all (5% each) of them. A combination of bacteriocins produced by LAB, prepared as described in methodology, was also used as one of the treatment solutions. These treatments were compared with untreated control, water treatment and among each other to determine and compare the effectiveness in inhibiting the growth of Gas^+ that helps in extending the shelf-life of the meat without any gas production.

Acetic acid treatment was significantly different ($P< 0.05$) than untreated control, water treatment, and the bacteriocins mixture treatment, whereas it was not significantly different (P>

0.05) than other treatments (Fig. 15). Acetic acid was found to be most effective treatment among others as it showed highest log-difference compared to all the treatments and 1.25-log difference on day 0, 2.59-log difference on day 3, 3.26-log difference on day 7, 4.05-log difference on day 14, and 3.7-log difference on day 28 compared to the untreated control. Citric acid treatment was significantly different ($P< 0.05$) than untreated control and water treatment but was not significantly different ($P> 0.05$) than other treatments (Fig. 15). Organic acid mix treatment was significantly different ($P> 0.05$) than untreated control but was not significantly different ($P< 0.05$) than rest of the other treatments (Fig. 15).

A similar study done by Anderson et al. (1989) showed that the beef cores dipped in 0%, 1%, 2%, and 3% acetic acid at 25ºC, 40ºC, 55ºC, and 70ºC respectively was found to control meat spoilage organisms. They found that 3% acetic acid at 70ºC to be the most effective treatment. The effectiveness was on total aerobic plate count followed by *Enterobacteriaceae* count.

Bacteriocin combination was expected to be an effective antimicrobial agent but our results showed that the other organic acids were much better in inhibiting the Gas$^+$ organisms. Instability of bacteriocins (due to storage temperature and time) and possible weaker effect when applied on meat might be a factor for not being able to suppress the Gas$^+$ organisms although the agar spot assay of bacteriocins showed impressive results against the Gas$^+$ isolates (Fig. 9).

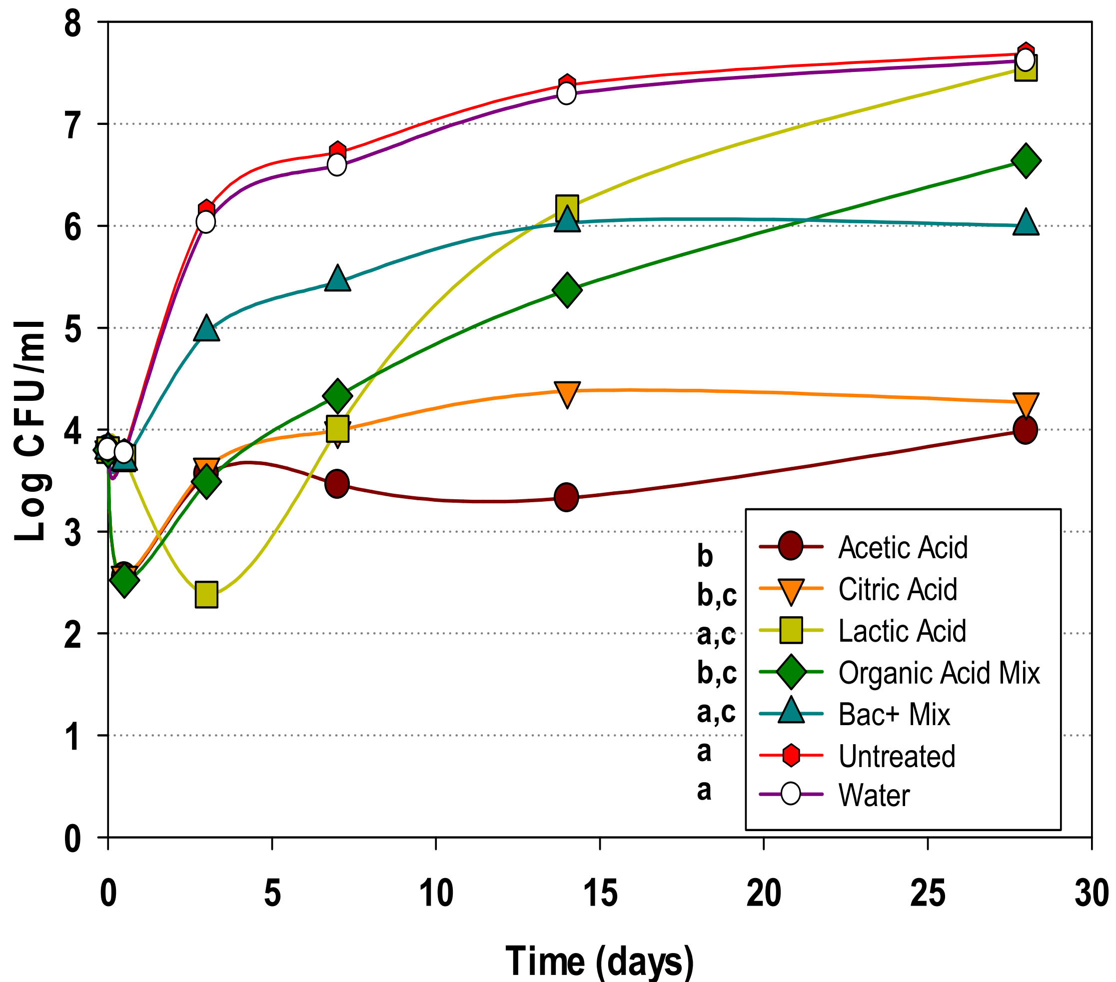


Figure 15. The effect of beef discs inoculated with *L. mesenteroides* 2a and *L. lactis* 107, and sprayed with organic acids (stored for 28 days at 4ºC). The trials included inoculated beef discs that were a) untreated, or sprayed with b) water, c) lactic acid (5% v/v), d) acetic acid (5% v/v), e) citric acid (5% w/v), f) organic acid mix (mixture of lactic acid 5%, acetic acid 5%, and citric acid 5%), or d) Bacteriocins (Bac^{+}) mix. All trials were performed in triplicate replication of paired samples and data points represent the means (standard deviations are not shown to prevent clutter). Treatments that share the same lower case letters (to the left of the legend) are not significantly different ($P > 0.05$); treatments that do not share the same lower case letters are significantly different ($P < 0.05$).

This study also showed citric acid as an effective treatment throughout the storage time period. Del Rio et al. (2007), examined the effects of dipping treatments (15 minutes) in potable water in comparison to solutions of 12% trisodium phosphate (TSP), 1,200 ppm acidified sodium chlorite (ASC), 2% citric acid (CA), and 220 ppm peroxyacids (PA) various pathogenic bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella Enteritidis*, *Escherichia coli*, and *Yersinia enterocolitica*) were inoculated onto chicken legs and stored for 0, 1, 3, and 5 days at 1-3°C. All chemical solutions reduced microbial populations as compared with the control (untreated) samples. Similar bacterial loads ($P > 0.05$) were observed on water-dipped and control legs. Average differences with regard to control samples were 0.28 to 2.41- log difference with TSP. 0.33 to 3.15-log difference with ASC, 0.82 to 1.97-log difference with CA, and 0.07 to 0.96-log difference with PA. CA and ASC were the most effective antimicrobial compounds against Gram-positive and Gram-negative bacteria, respectively.

4.4.2 Comparison of commercial antimicrobial interventions

The objective of this study was to compare effectiveness of all commercial antimicrobials used. Antimicrobials from the same and different manufacturers were compared against each other and with the untreated control and water treatment.

It was found that CytoGuard was significantly different ($P < 0.05$) than untreated control, water treatment, Danisco, Durafresh 5924, Zesti AM-5, Bio Via CDV, Durafresh 2012, and AFTEC 3000 (Fig. 16). CytoGuard showed a huge drop in microbial counts (especially at day 1) when compared to the other commercial antimicrobials from day 0-28, and showed the maximum reduction from day 0-5 (1.4 log CFU/ml) (Fig. 16). Chef's mix (with Bestate) was significantly different ($P < 0.05$) than untreated control, water treatments, and Danisco treatment compared to other treatments ($P > 0.05$) (Fig. 16).

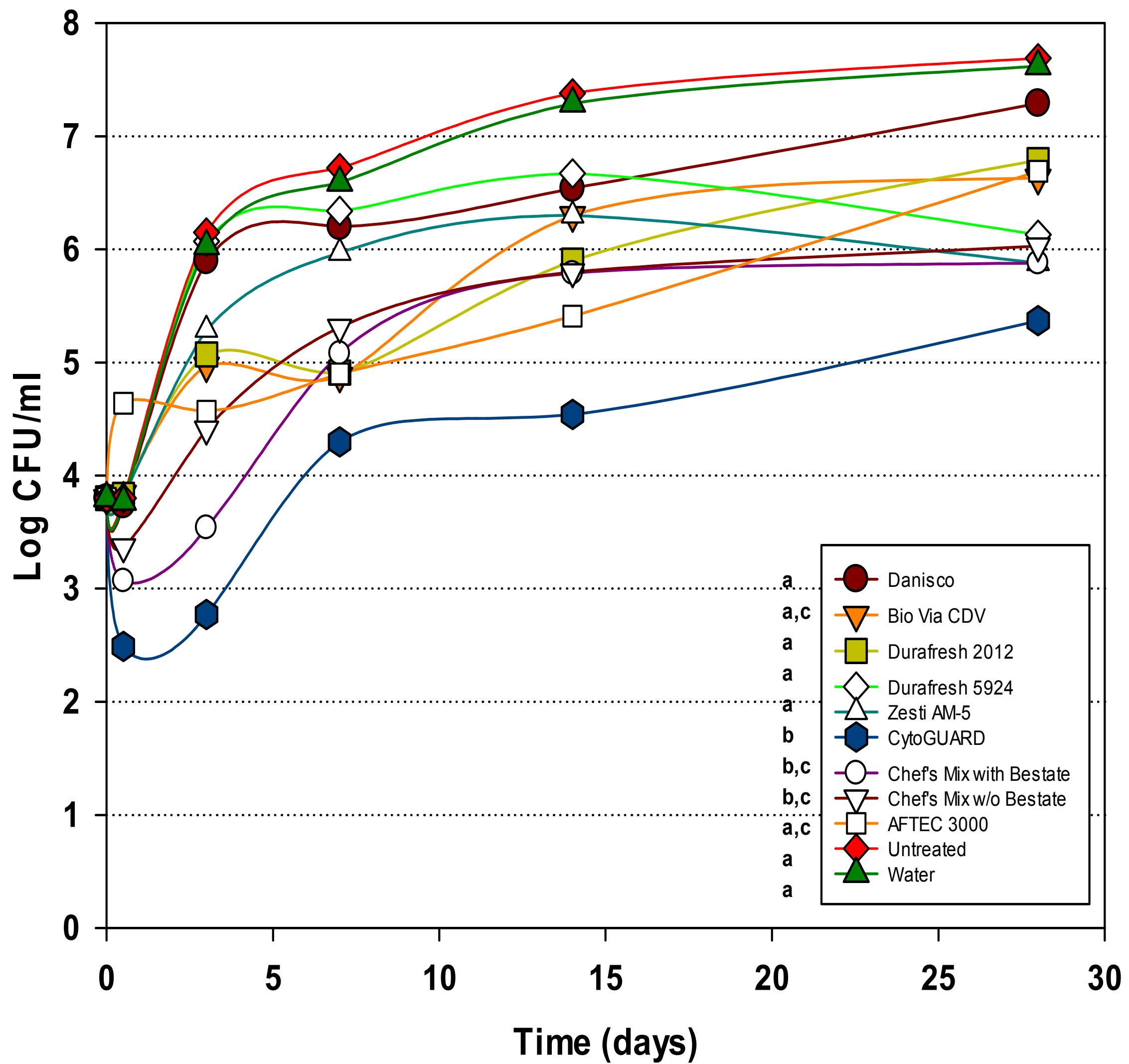


Figure 16. The effect of beef discs inoculated with *L. mesenteroides* 2a and *L. lactis* 107, and sprayed with commercial antimicrobials (stored for 28 days at 4ºC). The trials included inoculated beef discs that were a) untreated, or sprayed with b) water, c) Danisco NovaGARD NR100, d) Bio Via CDV, e) Durafresh 2012, f) Durafresh 5924, g) Zesti AM-5, h) CytoGUARD, i) Chef's mix with Bestate, j) Chef's mix w/o Bestate, or k) AFTEC 3000. All trials were performed in triplicate replication of paired samples and data points represent the means (standard deviations are not shown to prevent clutter). Treatments that share the same lower case letters (to the left of the legend) are not significantly different (P> 0.05); treatments with different lower case letters are significantly different (P< 0.05).

Chef's mix followed the same pattern of survivor curve as CytoGuard treatment but had higher microbial counts than CytoGuard throughout the same time period (day 0-28). Chef's mix (with Bestate) and Chef's mix (without Bestate) showed no significant difference ($P > 0.05$) when compared with each other (Fig. 16). However, there was a larger decrease in the microbial counts with the Chef's mix with Bestate treatment from day 1 through day 7, compared to the Chef's mix without Bestate treatment. The same effectiveness was not seen beyond day 14. Zesti AM-5 was significantly different ($P < 0.05$) than CytoGuard (Fig. 16) but it showed higher microbial counts than CytoGuard and most of the other antimicrobials.

Our research suggests that acetic acid and citric acid served as effective organic acid treatments among all the other antimicrobials sprayed on beef discs inoculated with Gas^+ isolates, *L. mesenteroides* 2a and *L. lactis* 107, stored for upto 28 days at 4ºC. Acetic acid was the best inhibitor of Gas^+ organisms followed by citric acid. Based on these results, it can be suggested that acetic acid was the most efficient antimicrobial agent in controlling the growth of these organisms. Highest reduction on the microbial counts from days 0-28 (stored at 4ºC) suggests that it is stable and has the potential to inhibit or suppress the overgrowth of gas-producing organisms for a longer period of time. Similarly, citric acid, although not as effective as acetic acid, also showed higher suppression of microbial counts compared to other commercial antimicrobials and organic acids. It can be used as an alternative to acetic acid as it showed almost the same pattern in controlling the targeted Gas^+ organisms. Lactic acid, which is popularly used in meat industries as a microbial dip or spray was not effective on gas-producers during the shelf-life period in our study. Lactic acid showed the highest effect in reducing the microbial counts from day 0-day 5; however, the Gas^+ organisms quickly recovered and reached levels as high as the untreated controls at day 28.

Other popular commercial antimicrobials like AFTEC 3000, Zesti AM-5, Durafresh 2012, Durafresh 5924 were not found to be effective against gas-producers. Though, some of them showed initial reduction in the microbial counts but could not inhibit Leuconostocs for longer period of time. Some of the antimicrobials like Danisco NovaGARD NR 100 and AFTEC 3000 were unable to suppress gas-producing organisms and were gradually exceeded by these organisms. However, their microbial counts did not exceed the untreated control. Antimicrobial like CytoGuard was better than the other commercial antimicrobials in terms of log-reduction of gas-producing organisms. However, it was not better than acetic acid and citric acid. Instability of ingredient components of these antimicrobials over prolonged time of storage and inability to show the lethal effect on specific gas-producing Leuconostocs might be some of the reasons for their ineffectiveness.

CHAPTER V

CONCLUSIONS

Our research proposes that acetic acid and citric acid may be able to serve as an efficient antimicrobial agent for meat manufacturing and processing plants. They can be applied in combinations or separately as a pre-processing dip or spray on the meat. Similarly, processing equipment, food contact surfaces, and other inert surfaces found in processing environments can be exposed to these organic acids to reduce the meat spoilage issues such as bloating or gas production in vacuum-packaged meats. However, their possible side effects on meat due to prolonged exposure (change in pH, color, flavor and texture), corrosion of the equipments, etc. might be some of the factors that is necessary to be considered.

Additional research should be done in order to understand the antimicrobial potential of these two products upon exposure to other pathogens and other spoilage organisms. Perhaps a combination treatment of acetic acid, citric acid, and CytoGuard may provide a synergistic reaction by combining their antimicrobial modes of action. Meat industries use different concentrations of these acids (upto 5%) for antimicrobial purpose at different stages of meat processing (Anderson et al., 1989; Kotula et al., 1994; Gill et al., 2004).

Their direct application or intervention on meat before vacuum packaging and storage, in larger scale should be intensively studied and applied which will help in reducing the loss incurred by the spoilage of fresh and processed meat products.

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