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PCR-DGGE Analysis of Microbial Communities Associated with *Campylobacter* spp. on Equipment Surfaces at Two Pig Processing Facilities

by

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ABSTRACT

The objectives of this research were to use molecular methods to investigate the diversity of the bacterial population in two meat processing plants. Analysis of PCR-DGGE profiles revealed that neither the type of surface material (stainless steel or silicon) nor the composition of the microbial community impacted the presence of *Campylobacter* spp. However, it appeared that there may be common microbial species present in all samples where *Campylobacter* spp. were isolated. No biological niche could be identified for *Campylobacter* spp. in pork processing facilities. The PCR-DGGE analysis of the microbial communities obtained with culture methods showed diverse microbial populations at each sampling site with some common microbial species present consistently at all locations from the kill floor to the cutting room. Certain sampling sites involved in the processing of the same area of carcasses appeared to have very limited diversity in the microflora although in general the microbial composition on all sampling sites was not affected by the size or the types of meat contact surfaces.

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ABBREVIATIONS

BLAST	basic local alignment search tool
Bp	base pair
CFC	cephaloridine, fucidin, and cetrimide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DGGE	denaturing gradient gel electrophoresis
DMSO	Dimethyl sulfoxide
D_{sc}	Dice's co-efficient
EDTA	ethylenediaminetetraacetic acid
GBS	Guillain-Barré Syndrome
HACCP	Hazard Analysis Critical Control Point
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani Medium
mCCDA	modified charcoal cefoperazone deoxycholate agar
MRS	de Man, Rogosa and Sharpe agar
NCBI	National Center for Biotechnology Information
PCA	Plate Count Agar
PCR	polymerase chain reaction
TAE	tris-acetate EDTA
U	Unit
UPGMA	unweighted pair-group method using mathematical average
VBNC	Viable but non-culturable
VRBGA	Violet Red Bile Glucose Agar
X-Gal	5-bromo-4chloro-3-indolyl-beta-D-galactopyranoside

1. INTRODUCTION AND REVIEW OF THE LITERATURE

Campylobacter spp. continue to be the number one cause of foodborne illness but how these fastidious bacteria survive processing conditions and refrigerated storage in high enough numbers to cause significant numbers of cases of foodborne illness is poorly understood. Little is known about their ability to survive in a food processing environment or how they might interact with other organisms that may be present in the same environment.

1.1 Characteristics of *Campylobacter* spp.

Campylobacter spp. are Gram negative organisms belonging to the family of *Campylobacteraceae* and have a typical spiral or corkkscrew shape when cells are young or actively growing. When cells are harvested at late exponential to stationary phase, *Campylobacter* cells generally form a coccoid shape (Ng *et al*, 1985; Hazeleger *et al*, 1995). *Campylobacter* spp. can be either bi- or uni-polar flagellated and motile (Hänel *et al*, 2008**)**. These microorganisms are obligate microaerophiles and generally grow well under low oxygen conditions (10% CO_2, 6% O_2). Kaakoush *et al* (2007), however, demonstrated that some species of *Campylobacter* were able to adapt to an oxygen tension of 21% and that different species of *Campylobacter* strains have different levels of oxygen tolerance. At high cell densities, *C. jejuni* grew equally well in atmospheres with oxygen concentrations between 5 and 19% compared to growth under microaerophilic conditions. On the other hand, at low cell densities *C. jejuni* only grew under

microaerophilic conditions. Quorum sensing within bacterial populations of *Campylobacter* spp. has been studied previously and it appears that quorum sensing affects the ability of *Campylobacter* spp. to initiate the expression of virulence factors and survival in environmental conditions (Khanna *et al*, 2006; Jeon *et al*, 2005). *Campylobacter* spp. are oxidase and catalase positive. While both enzymes are important in counteracting the lethal effect of oxygen and its reduction products, superoxide dismutase was shown to be more important for *C. jejuni* to survive aerobically and for optimal colonization of the chicken gut (Purdy *et al*, 1999).

Campylobacter spp. are thermotolerant and they grow optimally at temperatures ranging from 37 to 42ºC. Many species of *Campylobacter* are naturally competent and are able to incorporate genetic elements from their environments. At 42ºC, which is the internal body temperature of avian species, compared to an ambient temperature, Kim *et al* (2008) found that more *C. coli* could be transformed to acquire genes responsible for the resistance of erythromycin and nalidixic acid. To date, there have been an increasing number of reports of the emergence of antibiotic resistance in *Campylobacter* spp. in food animals and the food production chain (Luber *et al*, 2003; Wilson, 2003; Rodrigo *et al*, 2007; Sahin *et al*, 2008). The use of antibiotics in food animals irresponsibly and the ability of *Campylobacter* spp. to become naturally competent to acquire genes responsible for antibiotic resistance may result in difficulty treating *Campylobacter* infections in the future.

1.2 Epidemiology of *Campylobacter* infections

Campylobacter spp. have been identified as the leading cause of gastroenteritis in many countries including Canada (PHAC, 2002). The species that are frequently isolated from patients who suffered from *Campylobacter* infections are *C. jejuni* and *C. coli* (Frost *et al*, 2002; Gürtler *et al*, 2005). The Public Health Agency of Canada (PHAC) in its Canada Communicable Disease Report revealed that from 1992 to 1995, there was an increase in cases of campylobacteriosis across Canada. The number of infected individuals who required hospitalization (inpatient and outpatient) was lower than the number associated with infections of *Escherichia coli* and *Salmonella* spp. The number of deaths was 15.91 per 1000 infected individuals compared to 34 per 1000 for salmonellosis, and 39.2 per 1000 for *E. coli* infections (PHAC, 2003). Apart from causing gastroenteritis in patients, *Campylobacter* infection can sometimes result in a medical complication known as Guillain-Barré syndrome (GBS; McCarthy and Giesecke, 2001). The Norwegian epidemiological study conducted by McCarthy and Giesecke reported that from 1987 to 1995, the rate of GBS development among 29,563 hospitalized Norwegian patients as a result of *Campylobacter* infection was 30.4 per 100,000 patients. The data in Canada for the development of GBS as a result of *Campylobacter* infections, however, is not known.

Campylobacter outbreaks are usually localized and sporadic involving a small number of individuals. However, there have been numerous reports of massive outbreaks involving several individuals who were exposed to the same food sources (Lehner *et al*, 2000; Frost *et al*, 2002; Richardson *et al*, 2007; Heuvelink

et al, 2009). Outbreaks due to contaminated drinking water sources have been previously reported in Walkerton, ON, Canada (PHAC, 2002), Finland (Kuusi *et al*, 2005) and Wales (Richardson *et al*, 2007) where all outbreaks were a result of the consumption of water sources contaminated by *Campylobacter* spp. originating from pasture land located nearby to water sources. Danish researchers revealed that some *Campylobacter* spp. isolated from poultry, broiler chickens, and cattle shared the same genetic fingerprint as those identified in sporadic outbreaks in different households, confirming that *Campylobacter* spp. in meats is a potential source of sporadic outbreaks (Manfreda *et al*, 2003; Nielsen *et al*, 2006).

In spite of the high number of annual *Campylobacter* infections, *Campylobacter* infection is highly seasonal (Nylen *et al*, 2002; Meldrum *et al*, 2005; Tam *et al*, 2006; Jozwiak *et al*, 2006). In most European countries including Denmark, Finland, Scotland, Sweden and Wales (Britain), the number of reported *Campylobacter* infections peaked during the period from early June to late July based on a combination of studies conducted for several years (Nylen *et al*, 2002). The authors argued that the peak of reported *Campylobacter* infections was due to mainly three factors: (1) human behavior/life style change due to seasonal variations which expose people to *Campylobacter*; (2) prevalence of *Campylobacter* due to seasonal change; and (3) combinations of (1) and (2). Tam *et al* (2006), based on data obtained from 1989 to 1999 in England, reported that

with a 1ºC increase in temperature, there was a 5% increase in the number of reported *Campylobacter* infections up to a maximum threshold of 14^{o}C.

Epidemiological studies of campylobacteriosis in Manitoba revealed three main risk factors for increased exposure of human population to *Campylobacter* infections: (1) consumption of contaminated food products (a more centralized food production system has been identified as the reason for the increased number of *Campylobacter* infections among the population residing within Manitoba); (2) exposure to local factors including farm animals and contaminated water; and (3) infection due to foreign travel (Green *et al*, 2006). Similarly, an epidemiological study conducted in Norway indicated that the increase of *Campylobacter* infections is more likely to occur because of eating foods that were cross-contaminated by raw poultry products, drinking of contaminated water, occupational exposure to animals, eating undercooked pork, and barbecues (Kapperud *et al*, 2003). Moreover, based on the epidemiological study of infants and children aged between 0 to 35 months from 1996 to 1997 in Australia, Tenkate and Stafford (2001) concluded that children less than 3 years of age are at risk of becoming infected by *Campylobacter* if puppies and chickens were kept as pets in their households.

The epidemiology of *Campylobacter* infections appears to be varied from one country to another and the occurrence of campylobacteriosis may well depend on multiple factors including, but not limited to, changing of seasons and exposure to

contaminated farm animals. In most cases, outbreaks were sporadic and involved a small number of individuals who ingested contaminated food products. The highly seasonal patterns of *Campylobacter* infections should result in better control measures to reduce the infection rate of campylobacteriosis.

1.3 *Campylobacter* spp. in the food production chain

Statistics of *Campylobacter* infections reported internationally between 1998 to 2007 show that foods that have been implicated as vectors of transmission include meat products (seafood, beef, pork, chicken, and turkey), produce, dairy products, multi-ingredient foods, bakery items and eggs (Greig and Ravel, 2009). Among these, dairy products account for at least 36.4% outbreaks caused by foodborne *Campylobacter* worldwide, while 29.3% were due to the consumption of chicken. In addition, *Campylobacter* infections due to the consumption of dairy products happened more often in the United States, while outbreaks involving the consumption of chicken happened more often in European countries (Greig and Ravel, 2009).

Two of the most common *Campylobacter* spp. associated with commercially available red and white meats are *C. jejuni* and *C. coli*. (Rivoal *et al*, 1999; Nielsen *et al*, 2006; Inglis *et al*, 2003; Manfreda *et al*, 2003; Miwa *et al*, 2003; 2004; Bohaychuk *et al*, 2006; Jozwiak *et al*, 2006; Lindmark *et al*, 2006; Son *et al*, 2007). *C. coli* is an ubiquitous inhabitant in the grastrointestinal tract of pigs and has been frequently isolated from gastrointestinal tracts and fecal material of

pigs (Pearce *et al*, 2003; Madden *et al*, 2007; Varela *et al*, 2007). Madden *et al* (2007) revealed that individual pigs can carry a very diverse subpopulation of *C. coli* genotypes within different compartments of the gastrointestinal tract and cautioned about the genotyping of *C. coli* for epidemiological studies. On the other hand, *C. jejuni* is predominantly isolated from poultry sources (Bohaychuk *et al*, 2006; Hansson *et al*, 2007; Nielsen *et al*, 2006; Manfreda *et al*, 2003; Rivoal *et al*, 1999). Both *C jejuni* and *C. coli* have been frequently isolated from bovine sources and their environments (Minihan *et al*, 2004; Inglis and Kalischuk, 2003) although *C. lanienae* also has also been frequently isolated (Inglis and Kalischuk, 2003). Varela *et al* (2007) argued that the lower prevalence of *C. jejuni* in pigs could have been due to the fact that pigs carry a larger amount of *C. coli*, thus making the detection of *C. jejuni* in the presence of larger amount of *C. coli* more difficult. Further, the authors also argued that differences in slaughter practices may have contributed to the under detection of *C. jejuni* in pigs and pork.

Farm animals including pigs, birds and cattle can become infected or contaminated by *Campylobacter* spp. through various environmental sources including contaminated farm equipment, feces of infected animals, insects, wild birds, soil, and water (Minihan *et al*, 2004 ; Kemp *et al*, 2005; Rivoal *et al*, 2005; Bull *et al*, 2006; Hansson *et al*, 2007). Physical barriers between the environments and barns are important in preventing the organisms from contaminating and infecting farm animals (Hansson *et al*, 2007). However, in poultry barns, when

transmitted within farm animals, *Campylobacter* spp. can subsequently spread among poultry flocks through horizontal transmission (Callicott *et al*, 2006).

During transportation of animals, contamination among infected and uninfected animals can happen when these animals from different lots are mixed or placed in close proximity. In the slaughter house or processing facilities, equipment surfaces can become contaminated through the spillage of fecal material from contaminated animals, and subsequently contaminate incoming carcasses that may be *Campylobacter* spp. negative (Rivoal *et al*, 1999; Newell *et al*, 2001; Lindmark *et al*, 2006; Lienau *et al*, 2007; Hansson *et al*, 2007). Based on molecular typing methods, Rivoal *et al* (1999) and Miwa *et al* (2003) demonstrated that cross-contamination between batches coming from different flocks can happen and it can be a risk factor for the presence of *Campylobacter* spp. on poultry meat. In addition, Lindmark *et al* (2006) revealed that poultry carcasses are usually contaminated by more than one genotype of *Campylobacter* spp.

To reduce the contamination of meat products prior to shipment to retail outlets, commercial meat processing plants have employed different methods to reduce the bacterial load present on the carcass surface. Blast chilling of carcasses (minimum surface temperatures of 0.0ºC on the round and 1.0ºC on the loin) used in commercial pork processing plant was determined to be effective in destroying *Campylobacter* spp. on the surfaces of carcasses (Nesbakken *et al*, 2008). In

commercial chicken slaughterhouses where carcasses were rinsed with acidified (citric acid) sodium chloride solution (900 mg/kg sodium chlorite, pH 2.5 to 2.6), the number of carcasses found to harbor *Campylobacter* spp. after carcass rinsing was reduced by 90% (Sexton *et al*, 2007). Although various methods used in meat processing facilities have proven to be effective, there is evidence to suggest that meats remain one of the primary sources of *Campylobacter* spp. (Nielsen *et al*, 2006; Manfreda *et al*, 2003; Ragimbeau *et al*, 2008; Callicott *et al*, 2008; Greig and Ravel, 2009).

In addition to meats and dairy products, *Campylobacter* spp. have previously been isolated from various types of raw produce and are responsible for at least 4.7% of foodborne outbreaks worldwide involving produce (Greig and Ravel, 2009). Nicholson *et al* (2005) demonstrated that *Campylobacter* spp. could survive up to 32 days in a dairy slurry that was used as fertilizer on farm-land and continued to survive for more than a month after the application onto farm-land. Moreover, Brandl *et al* (2004) reported that *C. jejuni* could survive on radish roots and in the spinach rhizosphere for at least 23 days and 28 days, respectively, when these were stored at 10ºC. The ability of *Campylobacter* spp. to survive in manure and on farm-land, as well as on raw produce may contribute to the cross-contamination of raw produce at farms and retail outlets. Consumption of raw produce that has been contaminated and harbor the survival of *Campylobacter* can cause food infections if this raw produce are not cleaned and heated appropriately.

Once contaminated foodstuffs are in the consumer household, mishandling can further lead to the cross-contamination of other foods including produce, which is often eaten raw (Mattick *et al*, 2003; Luber *et al*, 2006). Pathogens, including *C. jejuni*, could be transferred from contaminated water onto dishes and sponges, which will further contaminate kitchen surfaces and other dishes when contaminated sponges are used for wiping and cleaning. Washing dishes with a dish-washing machine with water at 60ºC and subsequent drying (air dried or towel dried) have been shown to reduce numbers of *Campylobacter* spp. to an undetectable level (Mattick *et al*, 2003; Kusumaningrum *et al*, 2003). In addition, food heated sufficiently should destroy the organisms. Whyte *et al* (2006) demonstrated that pan frying liver to reach internal temperature of 70 to 80ºC for 2 to 3 min is sufficient to destroy both *C. jejuni* and *C. coli*. Even though various recommendations have been suggested by the Public Health Agency of Canada to the general public to reduce the risk of foodborne disease at household levels, *Campylobacter* infections as a result of consuming contaminated foodstuffs continue to be a problem.

1.4 Survival kinetics of *Campylobacter* spp. in food systems

The processing of food including canning, drying and acidification is deemed to be hostile for the survival of *Campylobacter* spp. Pasteurization, acidification to pH lower than 4.9, dehydration to lower a_w, or addition of salt to a concentration of 2% should destroy the bacteria. Collectively, the enhanced or reduced survival of *Campylobacter* spp. on different food systems was postulated to be attributed

to various independent factors or combinations of several factors including bacterial strain (Koidis and Doyle, 1983; Guillou *et al*, 2008), the presence of endogenous food microflora (F. Nattress, personal communication), presence of food inorganic compounds such as ion porphyrins (Koidis and Doyle, 1983) or mineral content (Guillou *et al*, 2008), endogenous food organic compounds such as proteins and fatty acids (Lee *et al*, 1998; Birk *et al*, 2006), and packaging regimes and storage conditions (Dykes and Moorhead, 2001; Moore and Madden, 2001; Bhaduri and Cottrell, 2004; Boysen *et al*, 2007). To date, the mechanism for survival of *Campylobacter* spp. in various food systems is poorly understood.

Campylobacter spp. are fastidious and do not generally grow in food or under typical conditions for food storage. For example, foods that are rich in glucose may not readily support the growth of *Campylobacter* spp., since the organisms do not ferment or oxidize glucose due to the lack of 6-phosphofructokinase, a key enzyme involved in glycolytic pathway in glucose metabolism (Velayudhan and Kelly, 2002). Instead, amino acid or the intermediates of tricarbocylic acid are utilized as the carbon source to support growth and to maintain physiological activity (Hazeleger *et al*, 1998). However, the ability to utilize these metabolites can be species and strain dependent (Mohammed *et al*, 2004). Mohammed *et al* (2004) categorized strains of *Campylobacter* spp. tested into three distinct metabolic categories: group one was able to metabolize α-ketoglutarate, succinate, fumarate and aspartic acid; group two was not able to metabolite α -ketoglutarate and group three was unable to oxidize succinate, fumarate and aspartic acid.

Birk *et al* (2006), in their study to test the survival of *C. jejuni* at -18ºC in two different food model systems, found that *C. jejuni* survives up to 30 days at -18ºC in both chicken skin and chicken juice. However, there was higher reduction in numbers in the skin model, which lead to the conclusion that the presence of protective factors in chicken juice (probably due to the presence of certain proteins) was responsible for the better survival of *C. jejuni* at lower temperatures. Lee *et al* (1998) suggested that the presence of organic compounds such as proteins, fatty acids, and oils on chicken skin inhibits the formation of ice crystals, and thus reduces the lethal effect of ice crystal formation within bacterial cell and enhances the survival of *C. jejuni*. On the other hand, Moore and Madden (2001) reported that *C. coli* survives better in chilled liver slices and in autoclaved liver homogenates than in raw liver homogenates stored at 4, 15 and 37ºC. The authors suggested that the antagonistic effect of raw livers may have come from the various enzymes that have lethal effect on the survival of *C. coli* in raw liver.

Independent of the components of any simulated food system, *Campylobacter* spp. have been shown to survive better at lower temperatures, such as refrigeration temperatures, than at higher temperatures, such as room temperature (Moore and Madden, 2001; Kärenlampi and Hänninen, 2004). Researchers showed that *Campylobacter* spp. survived better at 4ºC than at 37ºC in simulated food systems (Dykes and Moorhead, 2001; Chan *et al*, 2001). The minimal growth temperature of *Campylobacter* spp. can be strain dependent, although in general all strains cease growth or have reduced growth rates when the temperature is reduced to a few degrees below the minimal temperature for

growth. Nonetheless, strains of *C. jejuni* were physiologically active (oxygen consumption, catalase activity, ATP generation, protein synthesis) at 4ºC and were still able to perform respiration and generate ATP. Under refrigeration temperatures, this organism is fully motile and exhibits chemotaxis and aerotaxis and moves towards favorable conditions (Hazeleger *et al*, 1998). It appeared that chemotaxis is temperature-dependent (Khanna *et al*, 2006). Despite this, the ability of *C. jejuni* to survive at 4ºC for prolonged times may be dependent on the origin of the strain tested. Chan *et al* (2001) demonstrated that clinical isolates of *C. jejuni* showed greater viability in Mueller-Hinton liquid broth at 4ºC compared to poultry isolates.

Campylobacter spp. can survive under modified atmosphere packaging conditions. *C. jejuni* was found to have enhanced survival when oxygen was excluded from the package (chicken fillet stored at 5°C; 70%/30% $N_2/C0_2$; Boysen *et al*, 2007). Processors using a modified atmosphere for chicken packaging may want to consider inclusion of oxygen to help reduce the chance of survival of *Campylobacter* spp.

Generally, *Campylobacter* spp. are heat sensitive organisms. The D_{55} for *C. jejuni* is 1 min (Sorqvist, 1989) and the D_{60} for *C. fetus* is less than 1 min (Gill and Harris, 1982). At 56ºC in phosphate saline buffer, *C. coli* N139 exhibited first order death kinetics over a period of 210 min. On the other hand, when the temperature was raised to 61.2ºC, first order death kinetics was observed for 40 s

and there was a tailing effect from 40 s up to 2 min, resulting in the survival of *C. coli* N139 (Moore and Madden, 2000). This has implications for food preparation which may require mild heat and short time treatments such as pasteurization. Klanĉnik *et al* (2006) reported that cells of *C. jejuni* subjected to 55ºC for 3 min reverted from the culturable spiral shape to non-culturable spiral and coccoid form. The authors argued that 3 min of heat stress was not significant enough for the expression of heat shock and starvation genes (*dnaK*, *htpG*, *groEl*), virulence (*flaA*) and housekeeping genes (16s rRNA, *rpoD*). However, when the duration of heat stress was increased from 3 min to 10 to 20 min, transcription of *groEL* and *rpoD* was increased. In short, strains of *Campylobacter* spp. have different sensitivity to heat and some have a heat shock response which may have significant implications in food processing.

The pH of food systems affects the survival kinetics of *C. jejuni* (Chaveerach *et al*, 2003; Kärenlampi and Hänninen, 2004; Alter *et al*, 2006). Kärenlampi *et al* (2004) demonstrated that clinical and poultry isolates of *C. jejuni* had higher death rates when inoculated onto strawberries (pH 3.36 at the beginning of trial decreased to pH 3.26 after storage of 72 h) when compared to other produce with a pH close to neutral including iceberg lettuce, cantaloupe, cucumber and grated carrot. Alter *et al* (2006) demonstrated that *C. jejuni* did not survive the fermentation and ripening stages of German style fermented turkey sausages after 12 to 24 h of fermentation when the pH and water activity had decreased to lower than pH 6.0 and 0.973, respectively. Chaveerach *et al* (2003) demonstrated that

acidic conditions (formic acid at pH 4.0) were lethal to *C. jejuni* and *C. coli* and at pH 4.0, the organisms reverted to a viable but non-culturable (VBNC) state. *C. jejuni* has also been shown to have the ability to adapt to mild pH stresses (Murphy *et al*, 2003). When inoculated at pH 5.5 or exposed to aerobic conditions for 5 hours, the conditions had been shown to induce adaptive tolerance responses in *C. jejuni*. Consequently, the adapted organisms, when compared to uninduced strains, have 100 to 500-fold increases in survival rate when subjected to lethal pH of 4.5 (Murphy *et al*, 2003).

1.5 Survival of *Campylobacter* spp. in the presence of mixed microbial populations

The synergistic or antagonistic interactions of multi bacterial species in various environmental and food systems have been demonstrated previously. Mixed populations of bacterial species interact among themselves and with other communities through various mechanisms.

Sanders *et al* (2007) demonstrated that *C. jejuni* was able to form biofilms on stainless steel surfaces with or without the presence of poultry meat natural microflora at 37 ºC. However, when inoculated in the presence of the natural microflora of poultry meat, *C. jejuni* had enhanced survival on stainless steel after 24 h. On a separate note, Trachoo *et al* (2002) demonstrated that *C. jejuni* had enhanced attachment and survival at 12 and 23ºC over 7 days, when inoculated onto a biofilm formed by *Pseudomonas* spp. and two other unknown Gram positive bacteria isolated from the chicken house. The authors also showed that *C.*

jejuni could revert into a VBNC state within the biofilms and would remain undetected if conventional culturing methods were used for the detection of *Campylobacter* spp. The authors argued that chemotaxis behaviour of *C. jejuni* allows the organism to move to the interior of a biofilm allowing the bacteria to be protected from the lethal effect of oxygen.

Koidis and Doyle (1983) reported that seven out of eight strains of *C. jejuni*, which originated from human, bovine, porcine and avian sources, survived well in ground beef in the presence of large endogenous microflora when stored at 4ºC aerobically for 14 days (less than a 1.2 $\log_{10}$ reductions was achieved throughout the 14 day storage experiment for 7 of 8 strains). Throughout the experiment, aerobic counts ranged from the initial count of 10^4 to10^6 cfu/g on day 0 to 10^9 cfu/g on day 14, while the pH increased from 5.7 to 6.2 on day 0 to 6.5 to 6.9 on day 14. Koidis and Doyle (1983) suggested that the survival of *C. jejuni* in meat with natural microflora was attributed to the presence of inorganic compounds such as ion porphyrin. Dykes and Moorhead (2001), in their experiment to determine the effect of vacuum and carbon dioxide packaging regimes on the survival of *C. jejuni* (originating from cattle and sheep feces) on naturally contaminated primal beef cuts stored at -1.5°C, found that the cell count of aerobic bacteria and two *Campylobacter* strains tested did not reduce significantly in number ($p > 0.05$) over 41 days of storage. The authors suggested that the packaging conditions could be the reason of enhanced survival of the *Campylobacter* strains tested. Moore and Madden (2001) reported that

Lactobacillus plantarum at 8.0 $\log_{10}$ cfu/ml did not affect the survival and viability of *C. coli* on porcine liver stored at 4°C (cell count of *C. coli* reached 8.5 $\log_{10}$ cfu/ml over 7 days). However, since no negative control (sterile ground beef, steak and porcine liver) was included in all three experiments conducted by Koidis and Doyle (1983), Dykes and Moorhead (2001) and Moore and Madden (2001), it is not possible to deduce whether the changes in pH or the presence of a meat associated microflora would have any effect on the survival of *C. jejuni* during storage experiments.

Nattress *et al* (2009), in their experiment to determine the survival of *C. jejuni* in the presence of an endogenous meat microflora on vacuum packaged pork stored at -1.5 and 4ºC over a period of one month, concluded that the *Campylobacter* strain tested (isolated from pork processing plant) had a higher death rate on sterile pork when compared to that inoculated onto pork with endogenous meat microflora. They concluded that the endogenous meat microflora or their metabolites had a positive impact on the survival of *C. jejuni* on vacuum packaged pork during storage.

1.6 Responses of *Campylobacter* spp. to environmental challenges

Culturability and viability of *Campylobacter* spp. can be affected by environmental stresses such as oxidative stress, temperature changes, starvation, heat shock or acid stress (Hazeleger *et al*, 1995; Chynoweth *et al*, 1998; Tangwatcharin *et al*, 2006; Mihaljevic *et al*, 2007). When exposed to

environmental stresses, such as those found in the various environments where *Campylobacter* spp. are isolated, the organisms have a range of responses from physiological changes [such as the up or down-regulation of genes (Stintzi, 2003; Bras *et al*, 1999)], to morphological changes or to induction of adaptive tolerance responses (Chynoweth *et al*, 1998; Murphy *et al*, 2006). To survive in different hostile environments including the gastrointestines of human and animal hosts, *Campylobacter* spp. must have acquired several survival mechanisms. Bras *et al* (1999) had identified a two-component regulatory system named *RacR-RacS* in *C. jejuni* which is important for temperature-dependent growth and colonization in the chicken intestine tract. The *dnaJ* gene, which is responsible for avian colonization and heat shock response, was adjacent to *RacR* gene. The *dnaJ* gene is postulated to be under the transcriptional control of the *RacR-RacS* signal transduction system, which is responsible for temperature-associated adaptive responses (Bras *et al*, 1999). The proposed *RacR-RacS* two-component regulatory system is required for the differential expression of genes at 37 and 42ºC. Stintzi (2003) reported that when the temperature was increased from 37 to 42ºC, there was a dramatic increase in the up-regulation of genes including chaperones, chaperonins and heat shock proteins while genes encoding proteins involved in membrane structure modification were either up or down regulated. Overall, there was at least a 20% change in the up or down regulation of genes in *C. jejuni* over a 50 min period (Stintzi, 2003). Based on the results, Stintzi (2003) concluded that there was a short growth arrest after the temperature change to allow the organism to reshuttle their energy toward survival and adaptation to the new growth

temperature. Besides adaptation to temperature, *C. jejuni* has the ability to adapt to oxidative stress in food and stream water (Chynoweth *et al*, 1998). Chynoweth *et al* (1998) reported that aerobically grown *C. jejuni* can be subcultured under aerobic conditions for repeated times and that when these organisms are inoculated on sterile chicken mince at 5 and 25ºC and incubated aerobically, they survive as well as those which are grown and incubated under microaerophilic conditions.

When exposed to environmental stresses, *C. jejuni* changes its shape from culturable spiral form to a coccoid-shape morphology, a stage where the cells become VBNC (Hazeleger, 1995; Kelly *et al*, 2001; Tangwatcharin *et al*, 2006; Klancnik *et al*, 2006). Kelly *et al* (2001) reported that *C. jejuni* cultured for an extended period during the stationary phase exhibits unusual decreasing and increasing pattern of heat resistance that coincided with the changes in viable count. This observation is thought to be a direct result of the existence of subpopulations of different morphological forms within a single strain culture and it has been suggested that this is a strategy to promote survival in some strains of *C. jejuni* (Kelly *et al*, 2001). The observations and the relatedness to the morphological forms of the population of *C. jejuni* is not known. Ng *et al* (1985) reported that *C. jejuni* exhibited different morphological forms within different parts of a colony: young and actively growing cells were mainly spiral shape whereas older cells were generally coccoid and could very well be in a degenerative stage where the cells have become inactive. Cells undergoing an

intermediate stage between coccoid and spiral shape morphology appeared to a form ring shape, which looked like a donut. Prolonged starvation (15 h) induced greater impairment of the culturability and viability of the clinical and food isolates of *C. jejuni* when compared to short term starvation (5 h), heat shock and oxidative stress (Mihaljevic *et al*, 2007). Short-term starvation also increased the resistance to heat at 55ºC for 3 min (Klancnik *et al*, 2006). When exposed to starvation in nutrient rich and nutrient poor environment, *C. jejuni* remaind viable for the longest time at 4ºC compared to at higher temperatures (Hazeleger *et al*, 1995).

The morphological change has been arguably a survival state. Sublethal injury including (outer) membrane damage has been shown to precede loss of viability (Kelly *et al*, 2001; Tangwatcharin *et al*, 2006). Tangwatcharin *et al* (2006) and Tholozan *et al* (1999) reported that cells in the VBNC stage had significantly lower internal potassium content and membrane potential compared to culturable cells. In addition, there were no significant changes in total protein, intracellular ATP level and membrane protein profile in cells that are in VBNC state (Hazeleger *et al*, 1995). Thus, the VBNC stage has been arguably a degenerative form where cells are damaged and have lost the ability to remain metabolically active. The loss of culturability and conversion to the VBNC state due to exposure to environmental stresses is important because it has been shown that *Campylobacter* spp. at both states have reduced adhesion and invasion properties (Milhaljevic *et al*, 2007). In contrast, *Campylobacter* spp. that has lost

culturability by reverting to the VBNC state following starvation or environmental stress in simulated food systems can gain culturability following passage into a biological system such as embryonated eggs and rat guts (Saha *et al*, 1991; Cappelier *et al*, 1999; Chaveerach *et al*, 2003; Guillou *et al*, 2008). Hänel *et al* (2008) demonstrated that upon passage through the chicken gut, *C. jejuni* had an altered genetic fingerprint, suggesting genomic instability of *C. jejuni* upon colonization in the chicken gut and that one isolate showed enhancement of adherence to eukaryotic cells, decrease of motility and changes from spiral shape to rod shape (Hänel *et al*, 2008). *C. jejuni* that has regained culturability upon passage through a biological system also has been shown to regain virulence factors such as adhesion properties (Saha *et al*, 1991; Cappelier *et al*, 1999).

There has not been any conclusive evidence to suggest that all strains of *Campylobacter* spp. that have reverted from the VBNC state to the viable state can become more invasive. Due to the nature that these organisms are able to survive in foods and adapt to various environmental challenges that are found in food and food storage systems, the presence of *Campylobacter* spp. in food and the food supply chain can be critical since the infectious dose can be as low as 500 cells (Robinson, 1981; Kothary and Babu, 2001) and the fatality rate can be high (PHAC, 2002).

1.7 Denaturing gradient gel electrophoresis (DGGE) as a molecular tool for the study of microbial diversity

The study of microbial communities provides an insight into the complexity of the structural diversity of different bacterial populations in an ecological niche. In the past, cultivation methods with different enrichment media coupled with standard microbiological techniques have been used to identify microbial species in an environment. With the increasing availability of 16S rDNA sequence information in public domain such as the BLAST database at NCBI, there has been an increasing use of 16S rDNA genes in the identification of microbial species. A 16S rDNA sequence is present in all bacterial species and consists of both conserved and variable regions that are unique in each bacterial species (Chakravorty *et al*, 2007; Schmalenberger *et al*, 2001; Klappenbach *et al*, 2000). The variable regions within the 16S rDNA of bacterial species are divided into 9 hypervariable regions (V1 to V9) with each hyperviable region being more suitable for distinguishing among certain bacterial groups (Chakravorty *et al*, 2007). For example, Chakravorty *et al* (2007) reported that the V2 to V3 hypervariable region is best suitable for distinguishing bacterial species to the genus level compared to the V4, V5, V7 and V8 regions. In bacteria, the average copy number of 16S rRNA genes per genome is reported to be 4.1 (Klappenbach *et al*, 2000). However, certain variable regions within the 16S rRNA gene have fewer copy numbers. For example, there were only 2.2 copies per organism within the regions of V2 to V3. For the V4 toV5 region, the copy number per organism is 1.7, whereas the V6 to V8 region has 2.3 copies per organism (Schmalenberger *et al*, 2001). Primers for PCR-DGGE can be designed to

specifically amplify regions of 16S rDNA that are different enough for species identification. A mixture of 16S rDNA molecules generated with a pair of universal 16S primers can be separated on a DGGE gel with different denaturing concentration (Ercolini, 2004).

Denaturing gradient gel electrophoresis has been used extensively in the study of microbial diversity in various ecological niches including food systems (Bonetta *et al*, 2008; Ercolini *et al*, 2003; Li *et al*, 2006). The principle of DGGE has been discussed previously (Ercolini, 2004). Briefly, the technique is based on the melting characteristics of same length double stranded DNA molecules across a gradient of denaturant formed with formamide and urea. When DNA molecules migrate across polyacrylamide gels with different concentrations of denaturant during electrophoresis, DNA molecules high in AT content tend to melt before DNA molecules rich in GC content due to the stronger hydrogen bonding between the GC nucleotides. The melting characteristics result in the reversion of double stranded DNA molecules to single stranded DNA and the migration across a polyacrylamide gel is retarded. To allow better separation of double stranded DNA molecules, a GC-clamp is often 'cloned' into the 5' end of PCR products to be separated on a DGGE gel.

1.8 Research objectives

The objectives of this research were:

- to use culture independent and culture dependant tools to determine the effect of the diversity of the microflora on the distribution of *Campylobacter* spp. on meat contact surfaces in two pork processing facilities;
- to determine the effect of the type of surface material (stainless steel vs. silicon) and area of swabbed equipment surfaces on the diversity of microflora on meat contact surfaces; and
- to determine the most common microbial species on meat contact surfaces throughout the production chain.

2. Materials and Methods

Samples were collected from two federally inspected pork processing facilities – A and B. Methods for sample collection and analysis are described separately for each facility.

2.1 Sampling and analysis of samples obtained from Facility A

Samples (34 in total) from various equipment surfaces (Table 2.1) were collected approximately 5 to 6 h after the start of processing on two separate occasions. The continuous production begins with carbon dioxide stunning at the kill floor, and ends at chiller where meat was packaged, chilled, and stored. Samples were collected aseptically with Solar-Cult® pre-moistened sterile cellulose sponge (4.2 cm X 8.9 cm, Solar-Biologicals, Ottawa, ON) by swabbing approximately 100 cm^2 of large surfaces (*i.e.* conveyor belt) or the entire exterior of surfaces where it was difficult to obtain a large surface swab (*i.e.* hook). Samples were stored in individual sterile pouches and transported on ice immediately to the laboratory for cultivation. Upon receipt (within 2 h), 50 ml of sterile buffered 1% peptone water (Don Whitley Scientific, West Yorkshire, UK) was added into the pouch and the sample was massaged for 1 min in a stomacher (Lab-Blender, Bury St. Edmunds, UK).

2.1.1 Sample preparation for culture independent analysis

For culture independent analysis, 24 ml of the cell suspension in buffered peptone water was placed into a sterile 50 ml polypropylene tube (Fisher-Scientific,

Edmonton) and centrifuged at 5311 *x g* for 30 min to form a cell pellet. Pelleted cells were washed twice with 2 ml of sterile TN150 buffer (5.59×10^{-3} M Tris-Cl, 4.4×10^{-3} M Sigma 7-9, and 1.49×10^{-1} M sodium chloride; centrifugation at 5311 *x g* for 15 min) and 1 ml of the suspension in TN150 buffer was transferred into a 1.5 ml eppendorf tube and stored at -20°C until further analysis.

Table 2.1: Processing steps and sites where surface samples were obtained at hog slaughter Facility A.

Stage of process	**Sample number** [a]	
	Sampling 1	**Sampling 2**
Kill floor		
CO_2 anesthetizing	NS[b]	NS
Dehairing	NS	NS
Polishing	1-1, 1-2	2-1, 2-2, 2-3
Evisceration (offal pan)	1-3, 1-4	2-4, 2-5, 2-6, 2-7 [c]
Carcass rinsing	NS	NS
Cutting room		
Carcass dropping	NS	NS
Carcass splitting		
- Knife sharpener	1-5	2-8, 2-9, 2-10
Rib flattening	NS	NS
Skin removal	1-6	2-11, 2-12
Trimming- ribs, sirloin, belly, ham, shoulder	1-7, 1-8	2-13, 2-14, 2-15
Skinning and removal of excess parts	1-9, 1-10	2-16, 2-17, 2-18, 2-19
- Hook for liver hanging		2-20, 2-21, 2-22
Floor drain	NS	2-23, 2-24
Packaging, chilling and storage		

[a] all sampling sites are direct contact surfaces
[b] NS – No sample was taken
[c] Boxes indicate that samples were collected from equipment surfaces with an area of at least 100 cm^2. Others are equipment surfaces with a surface area less than 50 cm^2.

2.1.2 Sample preparation for culture dependent analysis

Bolton Selective Enrichment broth (50 ml; Oxoid, Basingstoke, Hampshire, UK) supplemented with 25 ml of Laked Horse Blood (Oxoid) was added to the pouches with the remaining sample suspension in buffered peptone water. The cellulose sponge was retained to maximize the probability of culturing any *Campylobacter* spp. that could have embedded within the cellulose matrices. Samples were incubated at 39°C for 48 h in a BBL GasPak™ jar with microaerophilic conditions generated with BBL™ CampyPak™ Plus (Difco, Becton Dickinson, Sparks, MD). After incubation, samples were massaged by hand for approximately 1 min to ensure equal distribution of bacterial cells before one loopful of broth was plated onto modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid). After 24 h incubation at 39°C in microaeropilic conditions as described above, the mCCDA was washed with 5 ml of TN150 and 1ml of the suspension was transferred into an eppendorf tube and stored at -20°C until further analysis. Several colonies on duplicate mCCDA plates were picked and streaked onto Karmali agar (Oxoid) and incubated at 39°C for 24 h in microaerophilic conditions. After incubation, several colonies were picked and suspended in sterile Milli-Q water. DNA was extracted as outlined below.

2.1.3 DNA extraction

Selected bacterial suspensions obtained from culture independent analysis were subjected to different DNA extraction methods to test the efficiency of each

method. Cell suspensions (1 ml) were divided into four portions for DNA extraction described as follows:

Method A: Extraction was performed with the Qiagen DNEasy Blood Tissue Kit (Qiagen Sciences, Mississauga, ON) according to procedures supplied by the manufacturer.

Method B: Phenol Chloroform Method. Cells were lysed by bead beating with 0.1 nm zirconium beads (300 mg) for 3 min at 5311 *x g* and disrupted cells were placed on ice. Bacterial cell walls and debris were pelleted by centrifugation at 12,000 *x g* for 5 min and the supernatant was transferred into a 1.5 ml microfuge tube. DNA was extracted with phenol chloroform–isoamyl alcohol [25:24:1 v/v (volume/volume), pH 8.0] method described by Sambrook and Russell (2001).

Method C: DNA, extracted as described for method B, was precipitated with ethanol, eluted through a DNeasy Mini Spin Column (Qiagen Sciences) and washed as per manufacturer's instructions to remove any residual phenol-chloroform and PCR inhibitors that might be dissolved within the aqueous phase.

Method D: DNA was extracted with the Promega Magnesil DNA Extraction kit (Promega, Madison, WI) following the manufacturer's instructions.

The quality and yield of extracted DNA was measured with a spectrophotometer (Nanodrop, Thermo Scientific, Wilmington, DE). Preliminary results (Table 2.2) indicated that Method B yielded samples with the highest concentration and quality of DNA. Based on this, Method B was used to extract DNA from cell suspensions obtained from culture independent and culture dependent methods

(cell suspensions obtained from mCCDA). On occasion when the DNA was not amplifiable by optimized PCR conditions (see below), DNA samples were purified with the membrane based column as outlined in Method C.

Table 2.2: Yield and quality of DNA obtained from selected samples by different DNA extraction methods

Method	Sample	ng/µl[a]	A260[b]	A280[c]	260/280[d]
A	1-1	4.39	0.088	0.070	1.26
	1-2	4.73	0.095	0.064	1.47
	1-3	101.77	0.237	0.150	1.58
B	1-1	11.87	0.606	0.323	1.88
	1-2	30.31	0.685	0.338	2.03
	1-3	34.27	2.035	1.001	2.03
C	1-1	5.20	0.104	0.084	1.24
	1-2	18.33	0.367	0.190	1.93
	1-3	16.42	0.328	0.200	1.64
D	1-1	6.64	0.133	0.015	8.79
	1-2	6.73	0.135	0.109	1.24
	1-3	54.96	1.099	0.549	2.00

All of the following descriptions were adapted from Thermo Scientific, 2008.
[a] sample concentration in ng/µl.
[b] absorbance of the sample at 260 nm.
[c] sample absorbance at 280 nm.
[d] The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ca.1.8 is generally accepted as "pure" for DNA. If the ratio is lower than 1.8, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

2.1.4 PCR conditions for the amplification of 16S rDNA for DGGE analysis

Selected DNA samples extracted with method A and method B (Sample 1-1, 1-2, and 1-3) were subjected to PCR amplification with primer pair HDA1-GC and HDA2 (Table 2.3) for the amplification of the V2-V3 regions of the eubacterial 16S rDNA. The PCR reaction mix (total volume of 50 µl) contained 1X reaction buffer, 750 µM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dATP,

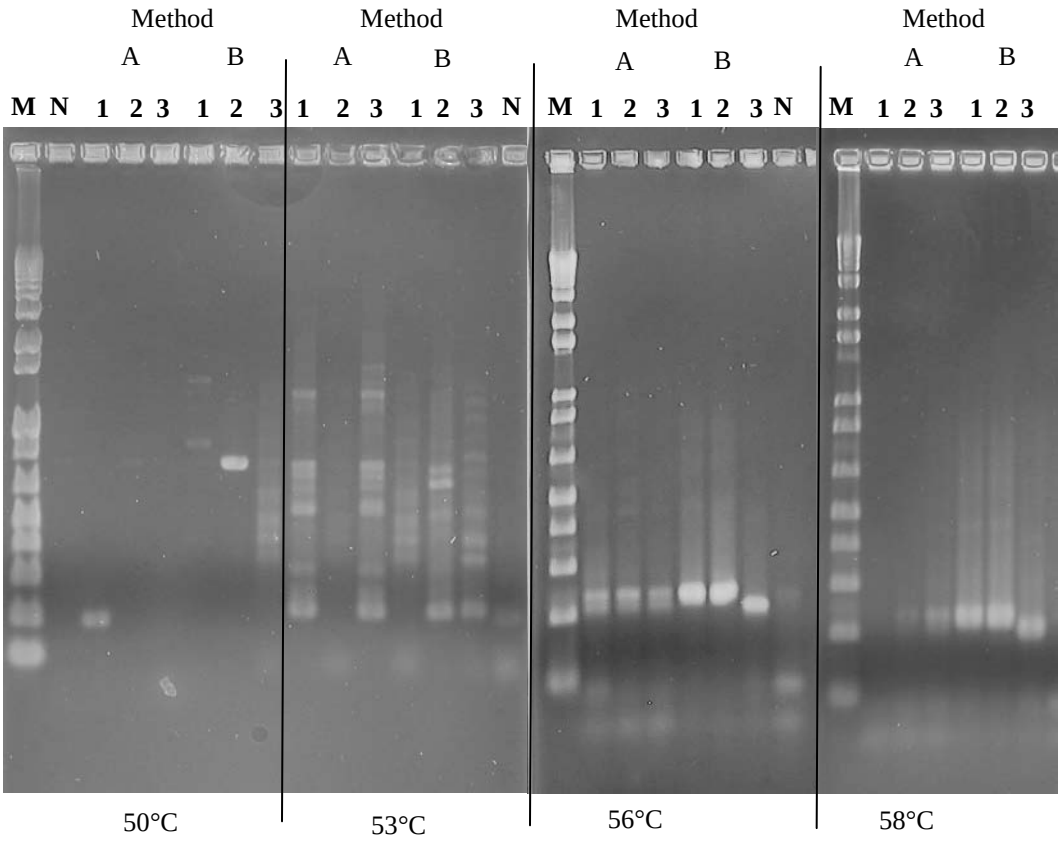
dTTP, dGTP, dCTP), 20 pmol of each primer, 2.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 4 % Dimethyl sulfoxide (DMSO, Fisher) and 1 µl of template DNA. To determine the optimal annealing temperature for the PCR reactions, four annealing temperatures were used (50, 53, 56 and 60°C). The thermal cycling was carried out in a Gene AMP® PCR System (model 9700; Applied Biosystems, Foster City, CA) with an initial denaturation step of 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 50, 53, 56 or 60°C for 1 min and elongation at 72°C for 1 min, followed by a final elongation step at 72°C for 7 min. Preliminary results (Figure 2.1) indicated that an annealing temperature at 56°C yielded visible bands on a 1.5% agarose gel [after staining for 15 min in ethidium bromide solution (Bio-Rad, Hercules, CA), destaining for 15 min in water and photographing with a camera under UV (Alpha Innotech Corporation, San Leandro, CA)] at correct size of about 200 bp for all samples tested. For PCR reactions performed at 50 and 53°C, multiple bands appeared on the agarose gel after staining, indicating sub optimal PCR conditions, while temperature at 58°C resulted in primers not being able to anneal to the target template, resulted in DNA samples not being amplified on lane A1. Based on this, DNA obtained from culture independent and culture dependent methods (cell suspension from mCCDA) was subjected to the PCR reactions described above with annealing temperature of 56°C.

Table 2.3: Primer sequences used in the PCR assays

PCR target and gene	Primer	Sequence	Size (bp)	Reference
Partial eubacterial 16S	HDA1-GC	**CGCCCGGGGCGCGCCCCGGGCGGG GCGGGGGCACGGGGGG**ACTCCTACG GGAGGCAGCAG (GC clamp in bold)	~200	Walter *et al* (2000)
	HDA2	GTATTACCGCGGCTGCTGGCAC		
Almost complete eubacterial 16S	1492R	ACGGYTACCTTGTTACGACTT	~1500	Reysenbach *et al* (2000)
	27F	AGAGTTTGATCMTGGCTCAG		
Campylobacter spp. genus 16S rDNA	C412F	GGATGACACTTTTCGGAGC	~816	Linton *et al*
	C1228R	CATTGTAGCACGTGTGTC		
Campylobacter coli ceu gene	MDCOL2Lower	TGATTTTATTATTTGTAGCAGCG	~462	Denis *et al* (1999)
	COL3Upper	ATTTGAAAATTGCTCCAACTATG		
Campylobacter jejuni mapA gene	MDmapA1Upper	CTATTTTATTTTTGAGTGCTTGTG	~589	Denis *et al* (1999)
	MDmapA2Lower	GCTTTATTTGCCATTTGTTTTATTA		
Sequencing primers	T7	TAATACGACTCACTATAGGG	sequencing	Promega
	Sp6	GATTTAGGTGACACTATAG3		
Epsilobacter 16S rDNA (primary nested PCR)	Epsilo-16S-1F	TAATACGACTCACTATAGGG	~540	Petersen *et al* (2007)
	Epsilo-16S-1R	ATTTAGGTGACACTATAG		

Epsilobacter 16S rDNA (secondary nested PCR)	Epsilo-16S-2R Epsilo-16S-1FGC	CAG CAA CGC CGC GTG GAG GAT CCG TCT ATT CCT TTG AGT TTT AAT C TCT ACG GAT TTT ACC CCT ACA CC CGG CCG CCC GTC CCG CCG CCC CCG CCC CGC CGC GGC CGC CAG CAA CGC CGC GTG GAG GAT	~350	Petersen *et a*. (2007)

Table 2.3 (Continued)



M: 1 Kb base pair DNA ladder
N: Negative control contained all reagents except DNA template, which had been substituted with sterile Milli-Q water.

Figure 2.1: Gel electrophoresis patterns of PCR products generated with primers pairs HDA1-GC/HDA2 at different annealing temperatures.

On occasion when DNA was not amplifiable by the PCR protocol described above, although the sample was purified with Method C, the sample was also subjected to nested PCR. The first nested PCR amplified the almost complete bacterial 16S rDNA fragments with primers 1492R and 27F (Table 2.3). The reaction mixtures (total volume of 25 µl) contained 10X reaction buffer (final concentration 1X), 750 µM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 20 pmol of each primer, 2.5 U of *Taq* DNA polymerase (Invitrogen)

and 1 µl of bacterial DNA. The thermal cycling was carried out in a Gene AMP® PCR System (model 9700; Applied Biosystems) with an initial denaturation step of 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min and elongation at 72°C for 1 min, followed by a final elongation step at 72°C for 7 min. The template from the first step of semi-nested PCR was diluted 10 fold and used in a subsequent PCR that contained the primer pair HDA1-GC and HDA2 with the conditions as described above.

2.1.5 Identification of *Campylobacter* spp. with multiplex PCR

DNA isolated from cell suspension of several colonies enumerated on Karmali agar was subjected to multiplex-PCR, as described by Denis *et al* (1999) with modifications, with three set of primers: 412F/C12228R, MDmapA1Upper/ MDmapA2Lower, and COL3Upper/MDCOL2Lower (Table 2.3), specific for *Campylobacter* spp., *C. jejuni* and *C. coli*, respectively. The reaction mixtures (total volume of 20 µl) contained 10X reaction buffer (final concentration 1X), 200 µM of each deoxynucleoside triphosphate, 750 µM $MgCl_2$, 10 µM of each primer and 0.25U of Taq polymerase. The PCR conditions were: 94°C for 5 min; 30 X (94°C for 45 s, 50°C for 45 s, and 72°C for 45 s), followed by a final elongation step at 72°C for 7 min.

2.1.6 Denaturing gradient gel electrophoresis (DGGE) analysis

PCR products (generated with primer pair HDA1-GC and HDA2) obtained from culture independent and culture dependent methods (cell suspension obtained from mCCDA agar) were subjected to analysis with DGGE. DGGE analysis was

performed with Dcode Universal mutation detection system (Bio-Rad) as described by Walter *et al* (2000), with modifications. The DGGE gel contained 6% (vol/vol) polyacrylamide (made with 37.5:1 bis-acrylamide solution, Bio-Rad) and had a denaturing gradient of 22% to 55% generated with both urea (Sigma-Aldrich, Belgium) and formamide (Fisher Scientific, Edmonton, AB) in the direction of electrophoresis as described by Guan *et al*. (2008). The electrophoresis was carried out at a constant temperature of 60°C in a 1.25X Tris-acetate EDTA (TAE) buffer for 3.5 h at 150V. Gels were stained with ethidium bromide (Fisher Scientific) for 20 min, de-stained in distilled water for 20 min, and photographed with an UV detector (Alpha Innotech Corporation).

2.1.7 Similarity analysis

PCR-DGGE profiles generated from the culture independent method were normalized and analyzed with BIONUMERICS (Applied Maths, Austin, TX). The similarity of band patterns was calculated and dendograms were constructed with the Dice coefficient and unweighted pair-group method using mathematical average linkages (UPGMA) clustering. For band matching, the following comparison settings were used: 0% optimization, 1.0% position tolerance and 0.0% position change. To test if the size of meat contact surfaces affected the distribution of background microflora and whether the distribution pattern of background microflora affected the distribution of *Campylobacter* spp. on meat contact surfaces, dice coefficient similarity values were calculated based on the following parameters: 1) surface area of >100 cm^2 versus <50 cm^2; and 2)

Campylobacter spp. positive samples versus negative samples, and 3) combinations of 1) and 2).

2.1.8 Identification of bands on DGGE gel and DNA sequence analysis

To identify the microflora that grew in Bolton Selective Enrichment broth and on mCCDA, individual bands on PCR-DGGE profiles of culture dependent method were identified as described by Knarreborg *et al* (2002) with modifications. Briefly, bands were excised and purified with QIAEX II Gel Purification Kit (Qiagen) and re-amplified as described above with primer pair HDA1/HDA2 without the GC clamp. The PCR products were cloned into pGEM-T vector systems (Promega) that were later transformed into competent cells of *Escherichia coli* DH5α. Cells were made competent as described by Sambrook and Russell (2001). Transformed *E. coli* DH5α cells were selected on LB agar (Difco) containing 100 µg/ml ampicillin, 40 µl of 2% X-Gal solution (Promega) and 40 µl of 0.2 M IPTG for blue/white screening. Selected transformants were grown in 5 ml of LB/ampicillin broth overnight and the plasmid DNA was isolated as described by Sambrook and Russell (2001). Prior to sequencing, clones were amplified with T7 and SP6 primers (Table 2.3) and separated on a 1.5% agarose gel to check for the presence of the correct insert. The PCR reaction mix contained 1X reaction buffer, 750 µM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 20 pmol of each primer, 2.5U of *Taq* DNA polymerase (Invitrogen), and 1 µl of template DNA. The PCR conditions were: 94°C for 5 min; 30 X (94°C for 45 s, 45°C for 45 s, and 72°C for 45 s), followed

by final extension at 72°C for 7 min. Excess nucleotides and primers were removed in a 8 µl enzymatic reaction (PCR products, 5 µl; calf intestinal alkaline phosphatase, 1 unit/µl; and, exonuclease I, 10 unit/µl) at 37°C for 15 min. To inactivate enzymes, the mixture was held at 80°C for 15 min. Cleaned up PCR products were sequenced with chain-termination method (Sanger sequencing reaction). Briefly, the sequencing reaction was performed in a Gene AMP® PCR system (Applied Biosystems) with the following conditions: 94°C for 5 min, followed by 30 x (96°C for 10 s, 50°C for 5 s, and 60°C for 4 min), and kept at 10°C until sequence analysis. The reaction mix (total volume of 10 µl) contained 1.0 µl PCR products, 6.18 µl sterile MQ water, 0.32 µl of primer SP6/T7 (10 µM), 0.5 µl of BigDye V3.1 sequencing reagent (Applied Biosystems) and 5X reaction buffer (Applied Biosystems). After the sequencing reaction, DNA was subjected to analysis with a DNA analyzer (model 3730, Applied Biosystems). The DNA sequence obtained was subjected to BLAST (Basic Local Alignment Search Tool) analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2 Sampling and analysis of samples obtained from Facility B

2.2.1 Production processes and sampling plan

This study was carried out over three visits to a federally inspected hog slaughter plant. During one visit, samples were collected after sanitation but before processing. During the other two visits, samples were collected during processing. The continuous production process begins with carbon dioxide stunning, dehairing, polishing, evisceration and carcass rinsing at the kill floor and ends at

chiller where carcasses are packaged, chilled and stored (Table 2.4). Before chilling, carcasses are trimmed in the cutting room where carcasses are split, boned, skinned, and cut. The meat is either boxed or vacuum packaged. Carcasses and different parts of the animal come into direct contact with equipment surfaces in the cutting room when meat sits on the conveyor belts, carts, cutting boards or when knives are used to remove trim.

Table 2.4: Process steps in pork processing Facility B and sites where samples were collected

Stage of process	Sample number [a]
Kill floor	
CO_2 anesthetizing	1
Dehairing	2
Polishing	3
Evisceration	4 5
Carcass rinsing	NS[b]
Cutting room	
Carcass dropping	6 7
Carcass splitting	8 9 10
Rib flattening	11 12 13
Skin removal	14 15 16
Trimming- ribs, sirloin, belly	17 18 19 20
Trimming- ham, shoulder	21 22 23 24 25
Skinning and removal of excess parts	26
Packaging, chilling and storage	NS

[a] All sampling sites are direct meat contact surfaces.

[b] NS indicates that no sample was taken at that processing step.

[c] Box indicates silicon surfaces. Other surfaces are stainless steel.

2.2.2 Sample collection and enumeration

Microflora present on equipment surfaces (Table 2.4) were sampled by swabbing the selected site with Solar-Cult® pre-moisturized sterile cellulose sponge

(approximately 12 g, 4.2 cm X 8.9 cm, Solar-Biologicals). Samples were stored in sterile pouches and transported on ice immediately to the laboratory for subsequent analysis. Upon receipt (no more than 2 h after sampling), 108 ml of sterile buffered peptone water (Don Whitley Scientific) was added to each pouch to obtain 10^{-1} dilution, and the pouch was massaged for 1 min in a stomacher (Lab-Blender). To estimate the number of bacteria on sites where samples were obtained, serial dilutions were made and 100 µl aliquots were spread onto duplicate prepoured plates of Plate Count Agar (PCA, Difco) which were incubated at 25°C for 72 h; MRS agar (Oxoid) which were incubated at 25°C for 5 days in an atmosphere flushed with a mixture of gas (4.96% CO_2, 9.96% H_2 and a balance of N_2); *Pseudomonas* CFC media (Oxoid) which were incubated aerobically at 25°C for 48 h and Violet Red Bile Agar (Oxoid) supplemented with 10g/L glucose (VRBGA), which were incubated at 37°C for 48 h in an atmosphere flushed with a mixture of gas (4.96% CO_2, 9.96% H_2 and a balance of N_2). These media are designed to enumerate aerobic bacteria (PCA), lactic acid bacteria (MRS), *Pseudomonas* spp. (CFC) and total *Enterobacteriaceae* (VRBGA) (Baird *et al*, 1987). After incubation, the number of colonies on plates with between 30 to 300 colonies was counted and the numbers of bacteria per 100 cm^2 of surface samples were calculated.

2.2.3 Culture dependent analysis

To determine the profile of the organisms cultured on each microbiological medium, the cultures were collected from the surfaces of each plate. Initially,

culture suspensions were collected from the surface of plates at each dilution for each sample. Preliminary analysis (methods described below) showed that samples from all dilutions were necessary to ensure the greatest chance of collecting DNA from all microflora present (Figure 2.2). PCR products amplified from DNA obtained from cell suspension at a higher dilution yielded more bands on DGGE profile compared to that obtained at lower dilution. For example, PCR-DGGE of DNA obtained from sample 17 at a 10^{-1} dilution showed 4 distinct bands, whereas only 2 bands were visible at 10^{-2} and 10^{-3} dilutions. For sample 23, band 1 at 10^{-3} appears more clearly compared to the other bands at the same positions at 10^{-1} and 10^{-2} dilutions. Based on this, bacterial cell suspensions were obtained by washing plates from each dilution with 2 ml of TN150 buffer and 1 ml of the combined suspension was transferred into a 1.5 ml eppendorf tube and stored at -20°C until DNA extraction and culture dependent PCR-DGGE analysis.

2.2.4 Culture independent analysis

For culture independent analysis, 50 ml of the remaining sample suspension in buffered peptone water was transferred into a sterile tube, centrifuged at 5311 *x g* for 30 min to form a cell pellet. Cell pellets were washed twice with 2 ml of TN150 buffer and centrifuged at 5311 *x g* for 15 min between washings. The resulting pellet was suspended in 1.0 ml of TN150 buffer and stored at -20°C until PCR-DGGE analysis.

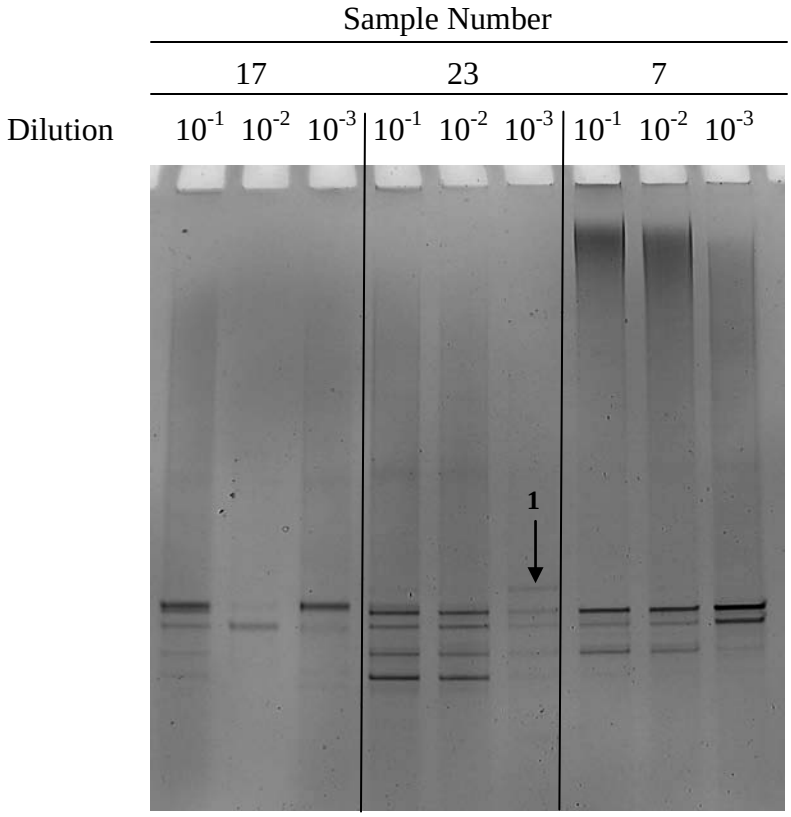


Figure 2.2: Results of PCR-DGGE of DNA extracted from cell suspensions collected from the surface of three dilutions plated onto PCA for 3 samples collected during sampling 3. Biomass of enumerated samples at all dilutions is needed to represent all bacterial cells that grew on the media. For example, band 1 at 10^{-3} for sample 23 appeared to be very weak at 10^{-1} and 10^{-2} dilutions.

2.2.5 Isolation and confirmation of *Campylobacter* spp.

To selectively isolate *Campylobacter* spp., 10 ml of the original sample suspension in buffered peptone water was added into 90 ml of Bolton Selective Enrichment broth (Oxoid) and incubated at 39°C for 48 h under microaerophilic conditions generated with BBL™ CampyPak™ Plus (Difco). Bolton Selective Enrichment broth was prepared by adding 25 ml of laked horse blood (Oxoid) into 50 ml of Bolton broth. After incubation, 10 ml of the Bolton Selective

Enrichment broth was transferred to a sterile conical tube, centrifuged at 5311 *x g* for 45 min, and the cell pellet was washed and resuspended in TN150 buffer as described in 2.1.1, and stored at -20°C until PCR-DGGE analysis. An aliquot of 100 µl of the Bolton Selective Enrichment broth was spread onto Karmali agar (Oxoid) and incubated under microaerophilic conditions for 48 h. Colonies that represented the morphology typical of *Campylobacter* spp. were picked and streaked onto mCCDA (Oxoid) and incubated under microaerophilic conditions (described previously) at 39°C for 24 h. After incubation, several colonies were picked and suspended in sterile Milli-Q water. DNA was extracted with Qiagen DNeasy Blood and Tissue Kit (Qiagen Sciences) for culture dependent PCR-DGGE analysis.

2.2.6 DNA extraction

Cell suspensions obtained from culture dependent, culture independent methods and from Bolton Selective Enrichment broth were thawed on ice and subjected to DNA extraction as outlined in Section 2.1.3 Method B.

2.2.7 PCR confirmation of the presence *Campylobacter* species

DNA obtained from several colonies isolated on mCCDA (Oxoid) was subjected to *Campylobacter* genus specific PCR with primer pair C412 and C1228R (Table 2.3) as described by Inglis and Kalischuk (2003), with modifications. The PCR reaction mix (total volume of 25 µl) contained 1X reaction buffer, 750 µM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 20 pmol of each primer, 2.5U of

Taq DNA polymerase (Invitrogen), 1 µl of internal control (0.005 ng), 4 % DMSO (Fisher Scientific) and 1 µl of template DNA. The internal control was constructed through oligonucleotide based deletional mutagenesis as described by Inglis and Kalischuk (2003) using DNA extracted from *Campylobacter jejuni* ATCC 700819 as the template DNA. Amplification was done in Gene AMP® PCR System (model 9700; Applied Biosystems) and the conditions were: 94°C for 5 min; 30 cycles of 94°C for 45 s, 50°C for 1 min and 72°C for 1 min, followed by 72°C for 7 min.

2.2.8 Preparation of PCR products from Bolton Broth

Total DNA isolated from Bolton selective enrichment broth was subjected to nested PCR with Epsilo-16S-1F/Epsilo-16S-1R and Epsilo-16S-1FGC/Epsilo-16S-2R primer pairs (Table 2.3). The primary and secondary nested PCR reactions (25 µl and 50 µl, respectively) each contained 1X reaction buffer, 750 µM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 40 pmol of each primer, 2.5U of *Taq* DNA polymerase (Invitrogen, US), 4 % DMSO (Fisher) and 1 µl of template DNA. The thermal cycling for primary and secondary nested PCR were carried out in a Gene AMP® PCR System (model 9700; Applied Biosystems) with an initial denaturation step of 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 62°C for 2 min and elongation at 72°C for 1 min, followed by final elongation step at 72°C for 7 min. PCR products were stored at 4°C until used in DGGE analysis.

2.2.9 PCR for the amplification of partial eubacterial 16S rDNA

PCR products were amplified from total DNA isolated from culture dependent and culture independent analysis with primer pair HDA1-GC and HDA2 (Table 2.3). The PCR reaction mix and conditions are outlined in Section 2.1.4. Samples for DGGE analysis included PCR products from DNA obtained from cultures grown on individual media (PCA, MRS, VRBGA, CFC), pooled PCR products from individual media that were concentrated with cold ethanol, centrifuged at 2700 *x g* for 20 min, and re-suspended with 50 µl of sterile Milli-Q water, and PCR products from DNA extracted directly from the original sample suspension (culture independent analysis).

2.2.10 DGGE Analysis

DGGE analysis of PCR products was performed with a Dcode Universal Mutation Detection System (Bio-Rad). PCR products generated with primer pair Epsilo-16S-1FGC/Epsilo-16S-2R were separated on an 8% polyacrylamide gel containing a 15 to 30% denaturaning gradient. PCR products obtained with primer pair HDA1-GC/HDA2 were analyzed on a 6% gel containing a 22 to 55% denaturaning gradient as outlined in Section 2.1.6. For the normalization of gels and gel to gel comparison, a reference standard was made by pooling PCR products from selected DNA samples and the reference PCR products were loaded into the left and right lanes of the polyacrylamide gel.

2.2.11 Normalization of gels and similarity analysis

Selected PCR-DGGE profiles obtained from culture dependent (PCA, MRS, VRBGA, and CFC) and culture independent analyses of DNA obtained from one sample were aligned to check for the presence of non-culturable microflora. Results (Figure 2.3) indicated that PCR-DGGE of culture independent method yielded less bands compared to PCR-DGGE of culture dependent method. Therefore, PCR products (from DNA isolated from PCA, MRS, VRBGA, CFC) were pooled together from one sample as outlined in Section 2.2.9 to represent total culturable microflora and used for subsequent similarity analysis. Similarity analysis and comparison of DGGE fingerprints were performed using BIONUMERICS (Applied Maths) as described in Section 2.1.7. Dice coefficient similarity (D_{sc}) values were calculated based on the following parameters: 1) stainless steel meat contact surfaces versus silicon meat contact surfaces; 2) *Campylobacter* spp. positive samples versus *Campylobacter* spp. negative samples.

2.2.12 Identification of bands and DNA sequence analysis

Selected DNA samples obtained from different media were subjected to PCR with primers HDA1 (without the GC clamp) and HDA2 (Table 2.3) as described in Section 2.1.4. PCR products were cloned into pGEM-T vector systems (Promega) as described in Section 2.1.8. Correct inserts were screened on a DGGE gel (denaturing gradient of 22 to 55%) together with the original sample as described in Section 2.1.6 to eliminate clonal isolates. Only PCR products that migrated as a

single band at the same position as the original sample were selected for sequencing. For PCR products generated with primer pair Epsilo-16S-2R/Epsilo-16S-1FGC, selected bands were identified as outlined in Section 2.1.8 with the primers being replaced with primer pair Epsilo-16S-2R/Epsilo-16S-1FGC. DNA Sequencing was performed in a DNA Analyzer (model 3730; Applied Biosystems) as described in Section 2.1.8.

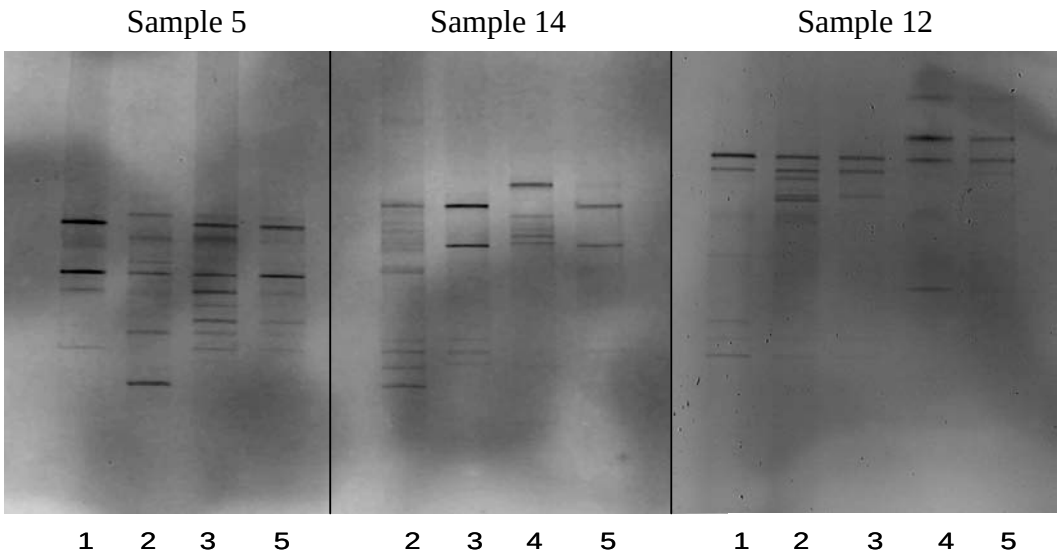


Figure 2.3: PCR-DGGE fingerprints of samples obtained from culture dependent and culture independent methods for samples obtained during sampling 3 at Facility B. Gels were not normalized.
Lane 1, VRBGA; 2, PCA; 3, CFC; 4, MRS; 5, culture independent method.

3. Results

3.1 Sampling and analysis of samples obtained from Facility A

A total of 34 samples were obtained during two visits to Facility A by swabbing the equipment surfaces that come into direct contact with meat. Samples obtained were either approximately 50 or 100 cm^2.

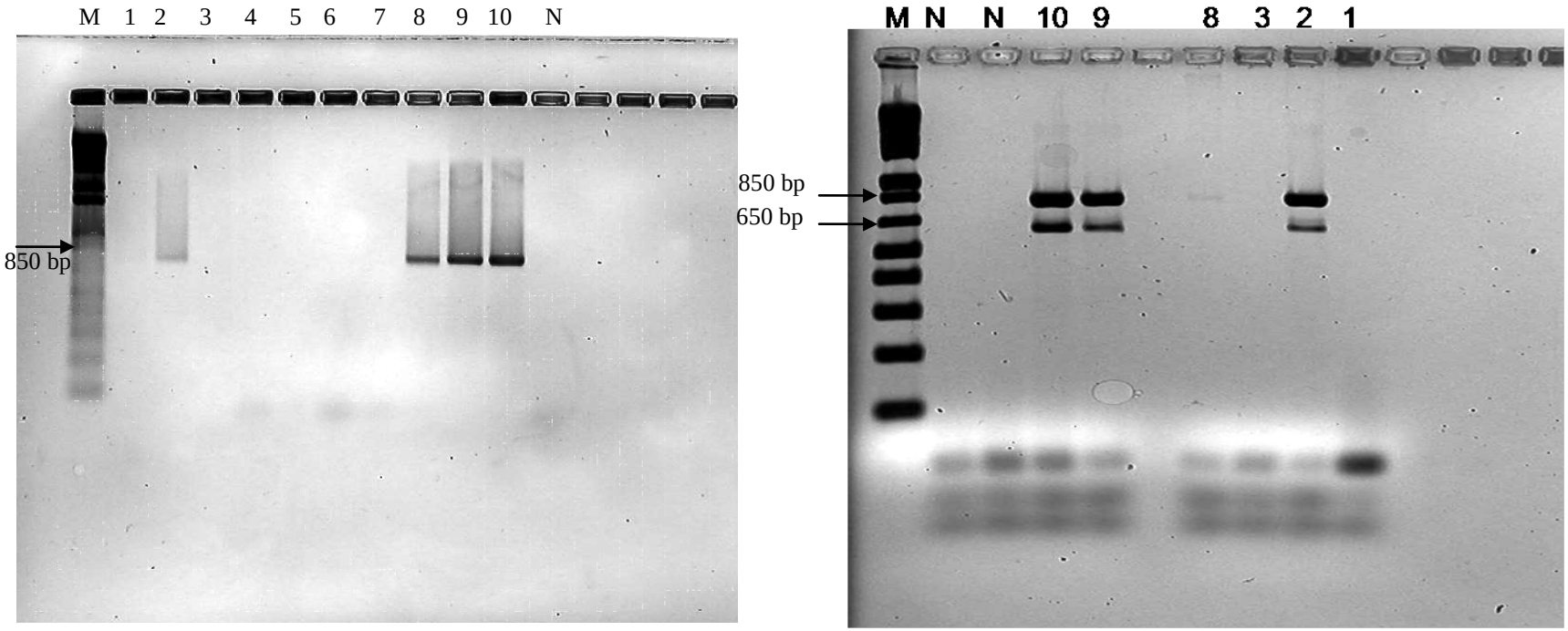
3.1.1 Identification of *Campylobacter* spp. on Karmali media

To determine the presence of *Campylobacter* spp. at each of the sampling sites, multiplex PCR was performed on DNA extracted from the biomass collected from Karmali agar. All PCR reactions were performed a minimum of two times to confirm results. Of the 34 samples, 14 were confirmed positive for the presence of *Campylobacter* spp. (Figure 3.1A and B). Of these 14 samples, 3 samples were positive for the presence of *C. coli* and 3 others were positive for the presence of *C. jejuni*, while the remaining 8 were positive for *Campylobacter* spp. The left panel of Figure 3.1A shows the results of a *Campylobacter* genus specific PCR. Samples collected during sampling 1 were also subjected to multiplex PCR (Figure 3.1A, right panel). Samples with bands at 816 bp were presumed to be positive for the presence of *Campylobacter* spp., whereas samples with bands at 462 bp and 589 bp were presumed to be positive for the presence of *C. coli* and *C. jejuni*, respectively. In Figure 3.1A (right panel), sample 8 was presumed to be positive for *Campylobacter* spp. (band at 816 bp), whereas sample 10 was presumed to be positive for *C. jejuni* (bands at 816 bp and 589 bp). For sample 12 collected during sampling 2 (Figure 3.1B; left panel), the gel for the results of multiplex PCR showed 2 bands with molecular weights higher than 816 bp. When

the multiplex PCR was repeated for sample 12, only one band was present at about 800 bp, indicating that sample 12 was positive for the presence of *Campylobacter* spp.

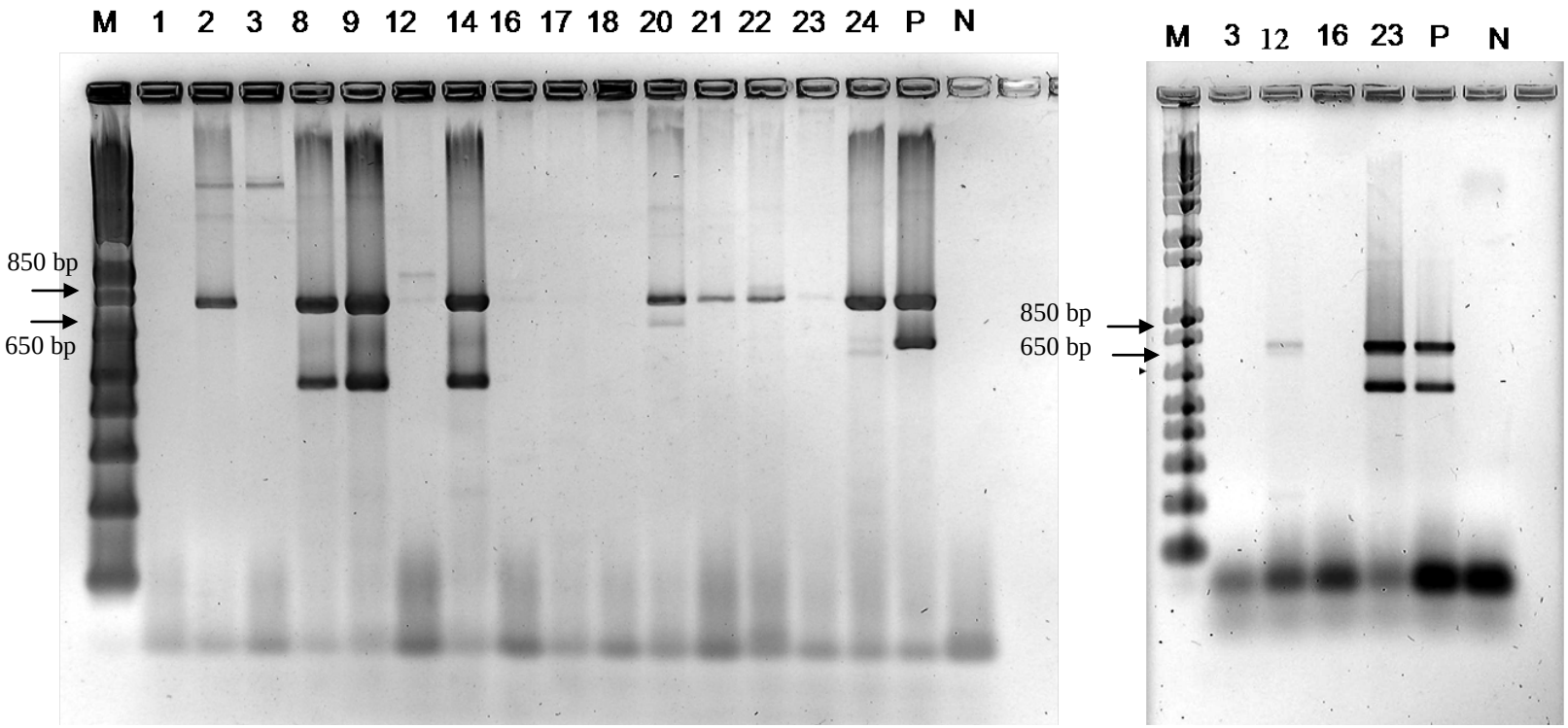
3.1.2 PCR-DGGE profiles of DNA isolated from mCCDA media

To determine what other microbial species were present on equipment surfaces, the biomass was collected from the surface of mCCDA plates and the DNA was extracted for analysis with PCR-DGGE. Selected bands were sequenced and compared to the type strain sequence available at NCBI (BLAST). The results of the PCR-DGGE are shown in Figure 3.2A and B and the identification of the bands that are labeled in Figure 3.2 are shown in Table 3.1. Through morphological observations, of the total colonies present on mCCDA agar plates, less than 2% of the colonies represented the morphology typical of *Campylobacter* spp. Most bacterial species that were identified with PCR-DGGE and sequenced were Gram negative bacteria with the exception of *Lactobacillus salivarius*. Some samples that were positive for the presence of *Campylobacter* spp. as determined by multiplex PCR (Figure 3.1) did not show any visible bands that were identified as *Campylobacter* spp. on the PCR-DGGE gel (Figure 3.2; numbers on all gels correspond to the same sampling site). For example, for sample 8 collected during sampling 2, which was presumed to be positive for the presence of *Campylobacter* spp. based on the results of multiplex PCR, only 1 band (band K1 on Figure 3.2B) was visible on the PCR-DGGE gel. The band is more than 98% similar to either *Caulobacter* spp. or *Brevundimonas* spp.



M = 1.5 kb DNA ladder; N = negative control. Numbers on the top of the gels correspond to the sampling sites listed in Table 2.1 for sampling 1. Bands at 816 bp, 462 bp, and 589 bp are presumed to be positive for *Campylobacter* spp., *C. coli* and *C. jejuni*, respectively.

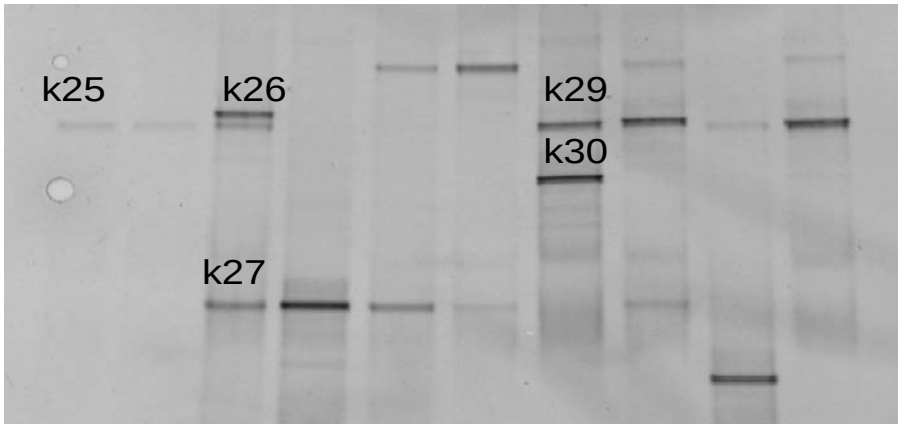
Figure 3.1A: Results of PCR for the detection of *Campylobacter* spp. in samples collected during sampling 1. The panel on the left shows results of the genus-specific PCR for the identification of the presence of *Campylobacter* spp., whereas the panel on the right shows results from the multiplex-PCR.



M =1.5 kb DNA ladder; N=Negative control; P= positive control, *C. jejuni* ATCC 700819. Numbers on top of the gels correspond to sampling sites listed in Table 2.1 for sampling 2. Bands at 816 bp, 462 bp, and 589 bp are presumed to be positive for *Campylobacter* spp., *C. coli* and *C. jejuni*, respectively.

Figure 3.1B: Results of multiplex-PCR for the detection of *Campylobacter* spp. in samples collected during sampling 2. The panel on the left shows a gel for samples where colonies were isolated from Karmali agar. The panel on the right shows a duplicate gel for four of the DNA samples that showed faint bands on the gel on the left.

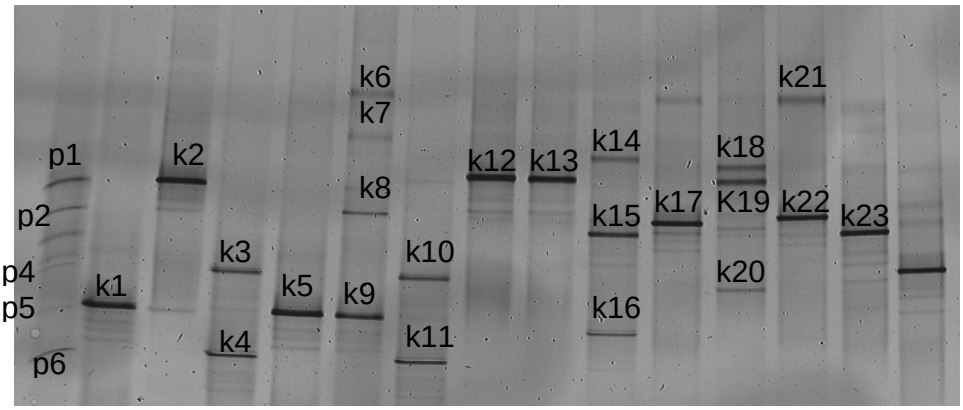
A



1-1 1-2 1-3 1-4 1-5 1-6 1-7 1-8 1-9 1-10

Sampling Site

B



2-2 2-8 2-9 2-10 2-11 2-12 2-14 2-16 2-17 2-18 2-20 2-21 2-22 2-23 2-24

Sampling site

Figure 3.2: PCR-DGGE profiles of DNA isolated from cells collected from mCCDA agar. Samples were collected during sampling 1 (Panel A) or sampling 2 (Panel B) and enriched in Bolton Broth before plating on mCCDA agar. Gels are not normalized. Identification of corresponding bands for sampling 1 and 2 are shown in **Table 3.1**

Table 3.1: Identification of microbial species recovered from mCCDA agar. Corresponding bands (see Figure 3.2A and B) were excised from DGGE gel, amplified with PCR, cloned, and sequenced. Sequence identification was done by comparing to the type strain sequence available in Genebank database (BLAST).

Sampling site	Band number	Identification/ Accession number	% Similarity
1-1	K25	*Myroides odoratimimus* (EU035775.1)	100
1-3	K26	*Lactobacillus salivarius* (FJ581418.1)	100
	K27	*Lactobacillus salivarius* (FJ611792.1)	99
1-7	K29	*Pseudomonas* spp. (AB456678.1)	100
	K30	*Acinetobacter* spp. (FJ64662.1)	100
2-2	P1	*Acinetobacter* spp. (AM989136.1)	100
	P2	*Proteus mirabilis* (FJ581028.1)	100
	P4	*Proteus mirabilis* (FJ655896.1)	100
	P5	*Campylobacter* spp. (AY554143.1)	95
2-8	K1	*Caulobacter* spp. (FJ685991.1), *Brevundimonas* spp. (AM989006.1)	98
2-9	K2	*Acinetobacter baumanii* (FJ609697.1)	98
2-10	K3	Multiple species[a]	99
	K4	*Campylobacter* spp. (AY554143.1)	89
2-11	K5	*Caulobacter* spp. (FJ685991.1), *Brevundimonas* spp. (AM989006.1)	100
2-12	K6	*Pseudomonas* spp. (AM117452.1)	98
	K7	*Arcobacter cryaerophilus* (U25805.1)	97
	K8	*Pseudomonas* spp. (AB456678.1)	100
2-14	K10	Multiple species[a]	99
	K11	*Campylobacter* spp. (AY554143.1)	95
2-16	K12	*Myroides odoratimimus* (DQ648614.1), *Flavobacterium* spp. (DQ679480.1)	99
2-17	K13	*Myroides odoratimimus* (DQ648614.1), *Flavobacterium* spp. (DQ679480.1)	99
2-18	K14	Multiple species[a]	99

	K15	*Myroides* spp. (EF151230.1), *Flavobacterium* spp. (DQ679480.1)	98
	K16	*Myroides odaratimimus* (EU311214.21)	98
2-20	K17	*Pseudomonas* spp. (AB456678.1)	100
2-21	K19	*Myroides odoratimimus* (DQ648614.1), *Flavobacterium* spp. (DQ679480.1)	99
	K20	Multiple species[a]	99
2-22	K21	*Pseudomonas* spp. (DQ127528.1)	99
2-23	K23	*Myroides* spp. (EU035775.1), *Flavobacterium* spp. (DQ301500.1)	98

[a] *Phyllobacterium* spp. (AM989040.1), *Ochrobacterium* spp. (FJ361192.1), *Rhizobium* spp. (EU183346.1), *Aminobacter* spp. (FJ225240.1), *Shinella* spp. (EU430055.1), *Sinorhizobium* spp. (FJ225264.1), *Brucella abortus* (CP000888.1), alpha proteobacterium (EF634293.1)

Table 3.1 (Continued).

3.1.3 Similarity analysis of PCR-DGGE profiles generated from DNA obtained using a culture independent method

Cluster analysis of PCR-DGGE fingerprints generated from samples collected during sampling 1 and 2 at Facility A were constructed to determine if the distribution of the microflora on the equipment surfaces was affected by the surface area of the samples taken and whether the distribution of the microflora on these surfaces had any impact on the presence of *Campylobacter* spp. The PCR-DGGE fingerprints from the culture independent analysis for sampling 1 and 2 are shown in Figure 3.3 and Figure 3.4, respectively. Cluster analysis of PCR-DGGE profiles obtained from sampling one and two at facility A showed that a few of the PCR-DGGE profiles of samples obtained from sites of close proximity (processing of same area of carcasses) could be clustered together (Figures 3.3 and 3.4). For example, from sampling 1 (Figure 3.3), samples 1-3 and 1-4 and samples 1-6 and 1-7 were clustered with more than 80% similarity. From sampling 2 (Figure 3.4), the PCR-DGGE profiles of the microflora obtained from sites 2-16, 2-17, and 2-18 were more than 90% similar. In addition, cluster analysis of PCR-DGGE profiles for sampling 2 produced two large clusters with similarity of approximately 75 and 66%. Many other individual sampling sites could be clustered into smaller clusters with similarity values greater than 80%, which indicated the presence of highly similar microflora among a number of meat contact surfaces. In both sampling 1 and 2, neither the size of the area swabbed nor the presence of *Campylobacter* spp. impacted how the samples were clustered. However, *Campylobacter* spp. were isolated from more samples with a

surface area of <50 cm^2 (12 out of 14).

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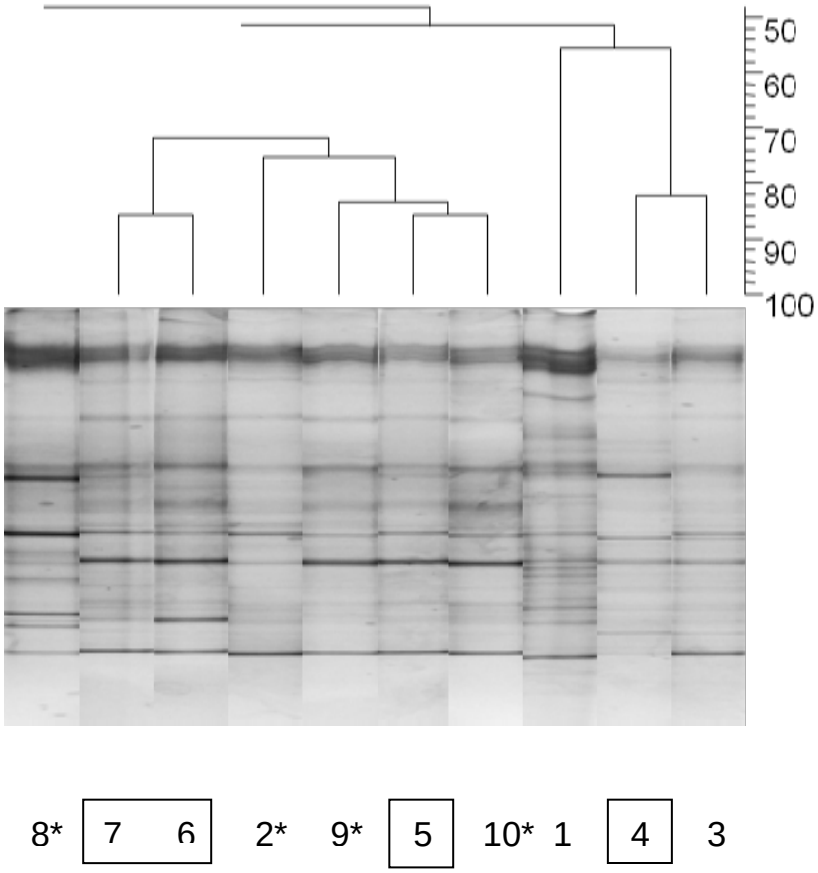


Figure 3.3: PCR-DGGE profiles obtained from PCR products generated from culture independent analysis of samples obtained from equipment surfaces during Sampling 1 at Facility A. The dendogram was constructed with UPGMA clustering algorithm. Sampling sites correspond to the sites listed in Table 2.1. Closed boxes indicate sampling sites with a large surface area (> 100 cm^2), the remainder were those with a surface area smaller than 50 cm^2. * indicates samples that were positive for the presence of *Campylobacter* spp.

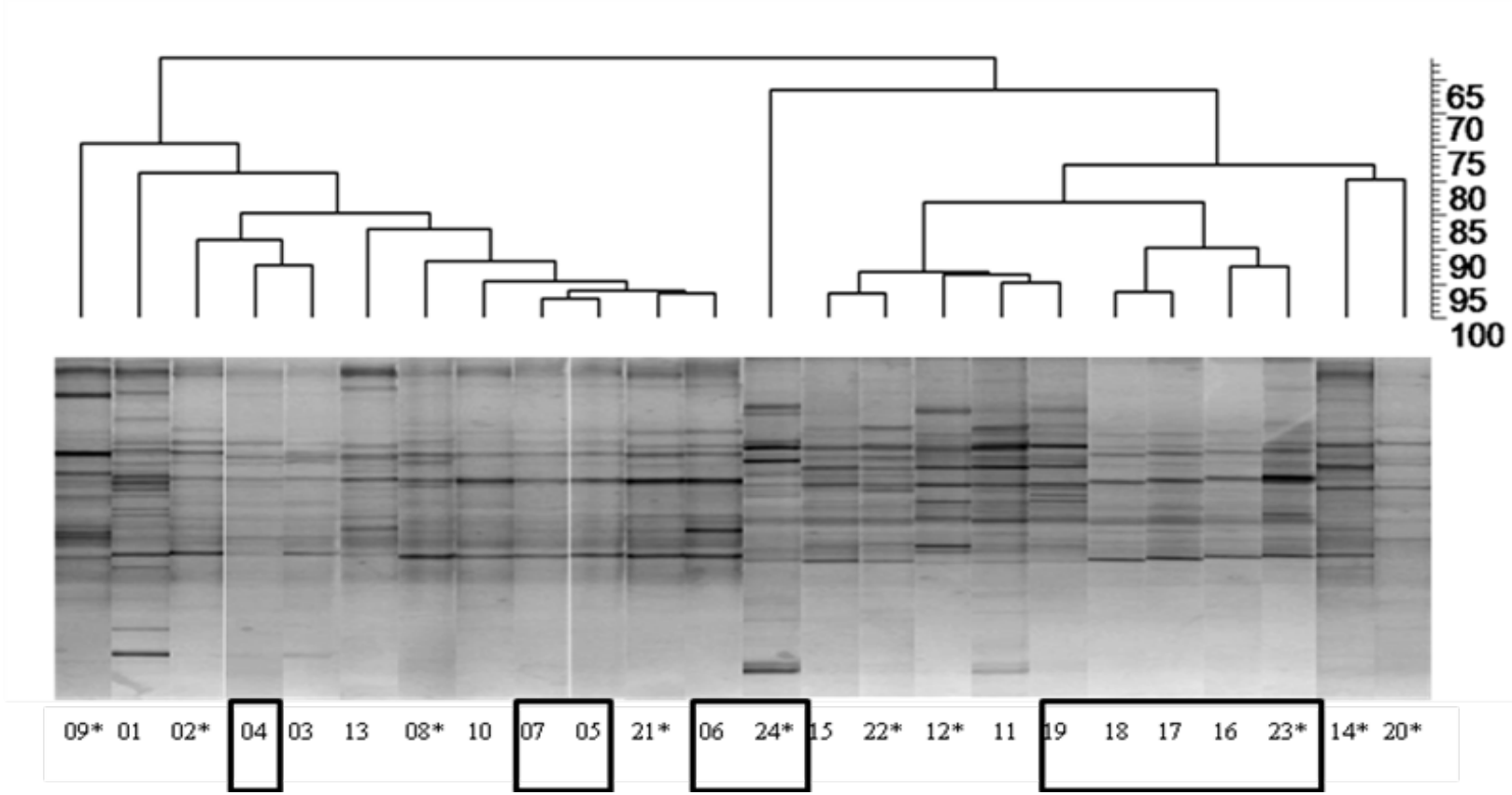


Figure 3.4: PCR-DGGE profiles obtained from PCR products generated from culture independent analysis of samples obtained from equipment surfaces during Sampling 2 at Facility A. The dendogram was constructed with UPGMA clustering algorithm. Sampling sites correspond to the sites listed in Table 2.1. Closed boxes indicate sampling sites with a large surface area (> 100 cm^2), the remainder were those with a surface area smaller than 50 cm^2. * indicates samples that were positive for the presence of *Campylobacter* spp.

Dice coefficient values (D_{sc}) comparing the effect of surface area of the samples on the distribution of the diversity of the microflora and the impact of the diversity of the microflora on the presence of *Campylobacter* spp. are presented in Table 3.2. The average D_{sc} value within the group of samples collected during sampling 1 from a surface area >100 cm^2 or within the group of samples from a surface area of <50 cm^2 were approximately 10% lower than that obtained for similar samples collected during sampling 2. The low D_{sc} values for intergroup similarity indicate that there was considerable variation in the PCR-DGGE profiles between the groups of samples that were of different surface area or between the groups of samples that were positive or negative for the presence of *Campylobacter* spp. To determine if the surface area of the sample impacted whether *Campylobacter* spp. could be isolated, the average D_{sc} values were calculated based on these two parameters. The difference between samples that were positive for *Campylobacter* spp. and <50 cm^2 and that which were negative for *Campylobacter* spp. and <50 cm^2 did not differ more than 5%.

Table 3.2: Intergroup and intragroup similarity (average dice coefficient) of PCR-DGGE profiles for samples obtained from different surface areas or samples that were positive or negative for *Campylobacter* spp. (Facility A)

Sample Group	Sampling 1		Sampling 2	
	Intragroup similarity	Intergroup similarity	Intragroup similarity	Intergroup similarity
Surface area >100 cm^2	62.1	58.5	74.4	72.2
Surface area <50 cm^2	57.3		69.9	
Campylobacter spp. positive	64.3	58.1	67.9	71.8
Campylobacter spp. negative	60.6		74.2	
Surface area <50 cm^2 and *Campylobacter* spp. positive	64.3	-[a]	69.9	-
Surface area <50 cm^2 and *Campylobacter* spp. negative	63.6	-	67.2	-

[a], intergroup similarity comparison is omitted for comparison of the combinations of 2 parameters

3.2 Sampling and analysis of samples obtained from Facility B

3.2.1 Enumeration of bacteria

A total of 65 samples were collected from Facility B at three different sampling times.

Cell counts obtained for total plate count, lactic acid bacteria, *Enterobacteriaceae* and *Pseudomonas* spp. at the three different sampling times are shown in Table 3.3. At the first sampling, samples were collected after sanitation but before the beginning of production processes. At this time, total aerobic counts, determined on PCA, ranged from below detectable levels to $\log_{10}$ 3.6 cfu/10 cm^2. Cell counts for other bacteria groups including presumptive lactic acid bacteria

(determined on MRS agar), *Enterobacteriaceae* (determined on VRBGA), and *Pseudomonas* spp. (determined on CFC agar) were generally lower than $\log_{10}$ 3.0 cfu/10 cm^2. At samplings 2 and 3, samples were collected during processing and cell counts obtained during both visits ranged from below detection limits to $\log_{10}$ 6.0 cfu/10 cm^2. For some specific sites, cell counts varied between sampling 2 and 3. For example, the sample obtained from site 11 at the second sampling had a total plate count of $\log_{10}$ 5.1 cfu/10 cm^2, whereas at the third sampling, the same site had a total plate count of $\log_{10}$ 2.8 cfu/10 cm^2. The cell counts obtained from samples collected on the kill floor and the cutting room generally differed by about $\log_{10}$ 1.0 cfu/10 cm^2.

Table 3.3: Counts of total aerobic bacteria, lactic acid bacteria, *Enterobacteriaceae* and *Pseudomonas* spp. obtained from swabs of equipment surfaces collected during three visits (S1, S2, and S3) to Facility B. Results are expressed as $\log_{10}$ cfu/10cm^2.

Sampling Sites	Total aerobic bacteria			Lactic acid bacteria			*Enterobacteriaceae*			*Pseudomonas* spp.		
	S1[a]	S 2	S 3	S 1	S 2	S 3	S 1	S 2	S 3	S 1	S 2	S 3
1	-[b]	5.9	NS[c]	-	5.2	NS	-	3.8	NS	-	2.6*	NS
2	2.8	4.8	NS	0.3*	4.2	NS	-	3.1	NS	-	3.4	NS
3	4.4	NS	NS	-	NS	NS	0.3*	NS	NS	-	NS	NS
4	-	2.9*	3.3	-	-	-	-	1.6*	2.5*	-	-	2.0*
5	0.3*	2.8*	3.1	-	-	-	-	-	1.8*	2.4	-	2.3
6	2.1*	3.3	NS	0.3*	-	NS	-	1.9*	NS	-	2.2*	NS
7	1.7*	2.2	3.0	-	-	-	0.3*	-	-	1.8*	-	1.0*
8	-	2.7	2.2	-	-	-	-	0.7*	-	-	1.8*	-
9	NS	NS	3.6	NS	NS	1.9*	NS	NS	2.4	NS	NS	3.5
10	NS	NS	3.3	NS	NS	1.8*	NS	NS	-	NS	NS	1.5*
11	-	5.1	2.8	-	3.1*	2.0*	-	2.8*	-	2.4	4.1	1.3*
12	2.5	2.2*	2.6	0.8*	2.5*	1.7*	1.6*	-	-	-	2.1*	0.6*
13	0.3*	3.0*	2.9	-	1.0*	2.2	0.3*	-	2.8*	-	2.5*	2.6
14	0.7*	3.5	3.5	-	2.4*	2.9	2.6	2.4*	2.3	-	-	2.6
15	-	3.1*	3.3	-	2.4*	2.4	-	-	1.8*	-	2.4*	2.2*
16	-	-	4.0	-	2.4*	2.8	-	-	2.8	1.5*	2.4*	4.0
17	1.4*	3.1	4.0	-	2.3*	2.7	-	-	2.7	-	3.0*	3.7
18	3.1	4.0	4.2	1.2*	3.6	2.7	0.7*	1.0*	2.5	2.6	2.9*	3.4
19	-	3.2	3.3	-	3.1	2.5	-	-	2.0*	0.3*	2.2*	2.9
20	NS	3.6*	3.2	NS	-	2.4	NS	-	2.3	NS	-	3.2
21	3.6	2.9*	4.8	1.5	2.0*	3.9	0.3*	2.0*	3.3	2.7	2.6*	4.0
22	0.5*	3.2	4.5	0.2	2.5*	3.7	-	-	3.2	2.3	2.4*	4.1

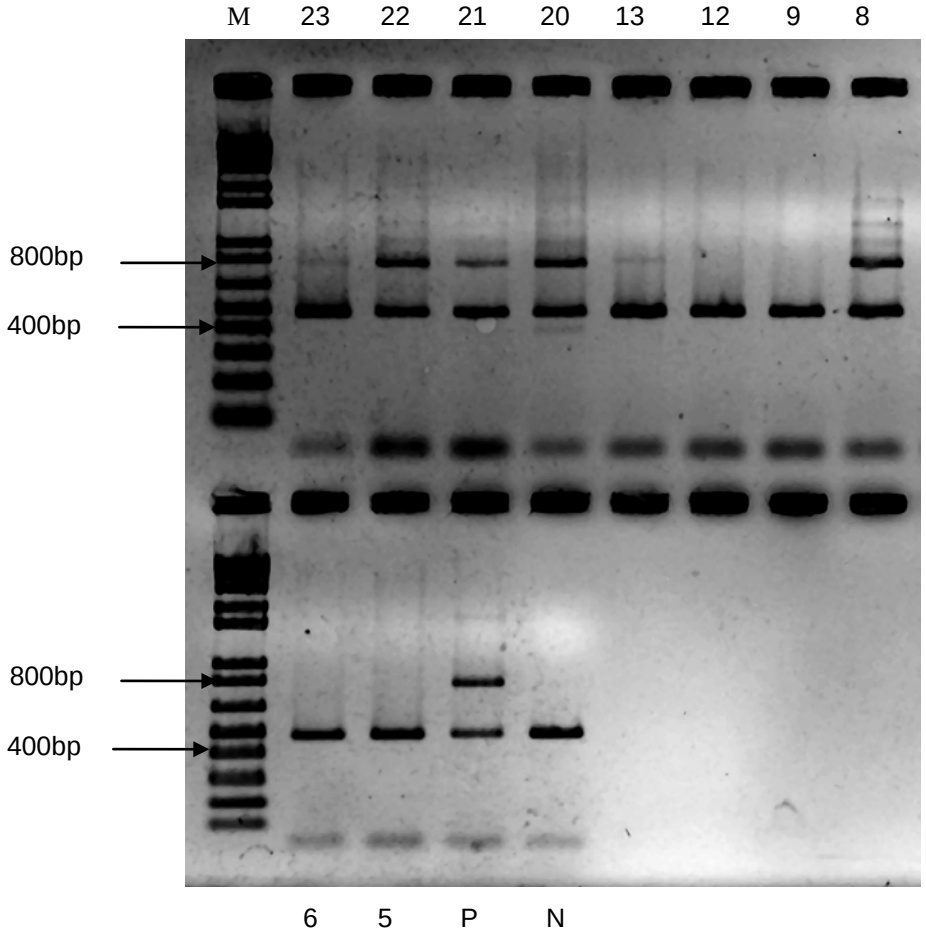
23	-	3.4	3.0	0.4	-	2.7	1.3*	-	1.5*	2.7	-	2.4
24	NS	NS	4.7	NS	NS	2.8	NS	NS	3.6	NS	NS	4.8
25	1.8*	3.6*	4.3	-	-	3.6	-	-	2.4*	-	3.0*	3.4
26	-	4.6	3.8	-	3.0*	3.1	-	3.4*	-	-	4.5	2.3*

[a], S1- Sampling 1. See Table 2.4 for the identification of sampling sites.
[b], - no colonies detected on media.
[c], NS- no sample taken due to limited access during processing.
*, below limit for reliable count (<30 colonies on agar plate). Biomass was collected for PCR-DGGE analysis.

Table 3.3 (Continued)

3.2.2 Identification of *Campylobacter* spp. using the culture dependent method

To determine which samples were positive for the presence of *Campylobacter* spp., the DNA isolated from colonies picked from mCCDA agar was subjected to PCR with genus specific primers for *Campylobacter* spp. An internal control was included in every PCR reaction to avoid false negative results. A representative gel showing the PCR products of DNA from colonies isolated on mCCDA agar are shown on Figure 3.5. Samples with bands at 816 bp were presumed to be positive for *Campylobacter* spp. Twelve samples were confirmed positive for the presence of *Campylobacter* spp. One sample, 5 samples and 6 samples collected during sampling 1, 2 and 3, respectively, were positive for *Campylobacter* spp. To determine if other groups of Epsilon-bacterial could have grown in the Bolton broth, the total DNA was extracted from the Bolton broth and subjected to nested PCR prior to DGGE analysis. Data indicated that not all samples that were positive for *Campylobacter* spp. with genus specific PCR were positive for *Campylobacter* spp. when analyzed by PCR-DGGE (data not shown). Epsilon-proteobacteria that were present included *Arcobacter cryaerphilus* and *Arcobacter butzleri*.



M = 1.5 kb DNA ladder; P= positive control with *C. jejuni*; N = negative control, DNA template was replaced with sterile MQ water; Samples 23, 22, 21, 20, 13 and 8 are *Campylobacter* spp. positive. Band at 400 bp is the internal control

Figure 3.5: Representative gel electrophoresis patterns of PCR products of DNA from colonies isolated on mCCDA obtained using *Campylobacter* genus specific primers showing samples that were positive for the presence of *Campylobacter* spp. for sampling 3

3.2.3 DGGE profiles of culture independent method and biomass collected from different media

PCR-DGGE of DNA obtained from the culture independent method was compared to that obtained from culture methods, and it has been established that culture independent method yielded less bands compared to culture dependent methods. Due to this problem, subsequent PCR-DGGE analysis was performed with DNA obtained from culture dependent methods only.

The PCR-DGGE profiles of bacterial populations recovered from meat contact surfaces and grown on different selective and non-selective media before and during processing are shown in Figures 3.6 and 3.7, respectively. The microbial ecology of meat contact surfaces after sanitation and during processing was characterized by the complexity of the PCR-DGGE profiles. PCR-DGGE profiles of microbial species recovered on meat contact surfaces after sanitation (Figure 3.6) yielded fewer bands compared to samples obtained during processing (Figure 3.7). When samples were collected after sanitation, there were very few lactic acid bacteria, *Enterobacteriaceae* or *Pseudomonas* spp. detected; however, when samples were collected during processing all groups of organisms were detected. The identification of bacterial DNA in each band after cloning, sequencing and BLAST analysis of the partial eubacterial 16S rDNA is shown in Table 3.4. Clones from the bands that migrated with a band detected in the original sample were selected and sequenced. For samples obtained after sanitation (Sampling 1), the PCR-DGGE analysis of the biomass obtained from all four different media showed that *Pseudomonas* spp. and *Escherichia coli* were prevalent on the meat

contact surfaces after sanitation. For samples obtained during processing (Sampling 3), DNA representing *Enterobacteriaceae* were identified on the PCR-DGGE profiles recovered from all four different media where the organisms formed the prevalent bacterial groups on meat contact surfaces from kill floor to the cutting room. Prevalent bacterial groups on MRS medium included mostly Gram positive organisms including *Enterococcus* spp., *Streptococcus parauberis, Micrococcus* spp., *Carnobacterium* spp., *Staphylococcus equorum* and *Bacillus* spp. However, *Enterobacteriaceae* (*Escherichia coli*, *Shigella* spp., *Escherichia fergusonii*, *Serratia* spp.*)* and *Flavobacteriaceae* were also identified in the samples plated on MRS agar. In the PCR-DGGE profiles of DNA obtained from cultures grown on VRBGA, *Enterobacteriaceae* formed the prevalent bacterial group. However, two bands were present in samples obtained throughout the processing chain that represented *Pectobacterium carotovorum*/*Serratia* spp. and *Pseudomonas* spp. Several bands belonging to *Pseudomonas* spp. were identified on the PCR-DGGE profiles of DNA obtained from bacterial cultures grown on *Pseudomonas* CFC agar. These bands (for example bands 35, 26, and 33) had more than 98% of sequence homology to different species of *Pseudomonas* spp. in the Genebank database. Band 35 had 100% similarity to *Pseudomonas* spp. (*P. stutzeri, P. mendocina, P. lubricans, P. anguilliseptica, P. pseudoalcaligens*). Band 26 was 98% similar to *Pseudomonas* spp. (*P. putida, P. mosselii, P. plecoglossicida, P. oryzihabitants, P. cinnamophila,* and *P. metavorans*) and band 33 was 98% similar to *Pseudomonas* spp. (*P. fluorescens, P. putida, P. corrugate, P. gingeri, P. ludensis*). On plate count agar, *Acinetobacter* spp. (*A. johnsonii, A.*

iwoffii), *Psychrobacter* spp., and *Brevundimonas* spp./*Caulobacter* spp. were among the prevalent bacterial groups that were present consistently on all different sites throughout processing. Bands representing *Chryseobacterium* spp. and *Stenotrophomonas* spp. were present in samples obtained from sites on the kill floor. Bands representing *Acinetobacter* spp. (*A. xiamenensis*) were consistently present on DGGE profiles recovered from *Pseudomona*s CFC and MRS agars.

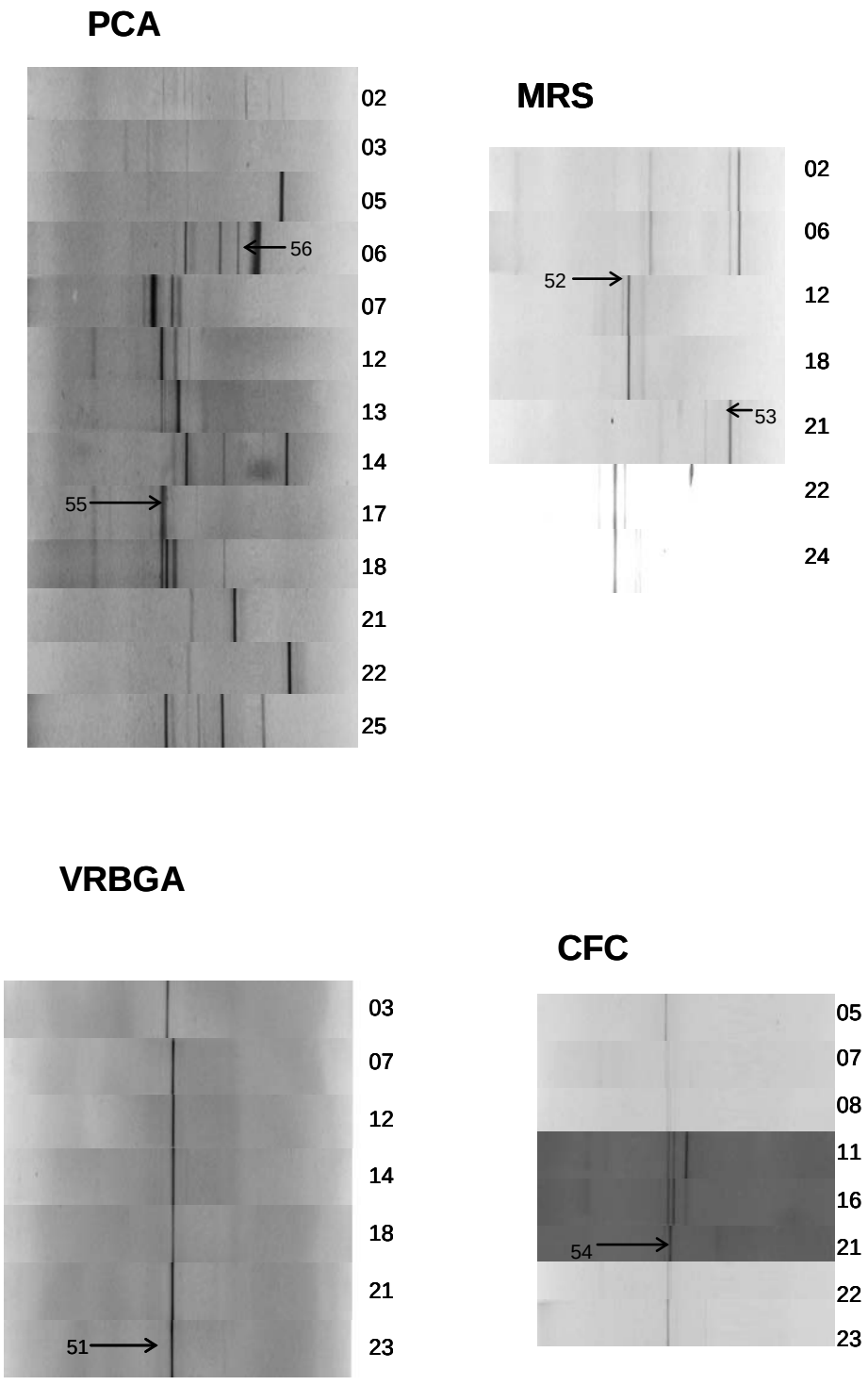


Figure 3.6. PCR-DGGE profiles generated from culture dependent isolation of DNA obtained from different culture media for samples recovered from different sampling sites during from Sampling 2 (during processing) in Facility B. The gels shown were normalized with BIONUMERICS by including two reference standards on both sides of each gel during electrophoresis. Numbers on the right side of gel indicate sampling site. Numbers on the gels indicate bands selected for sequencing.

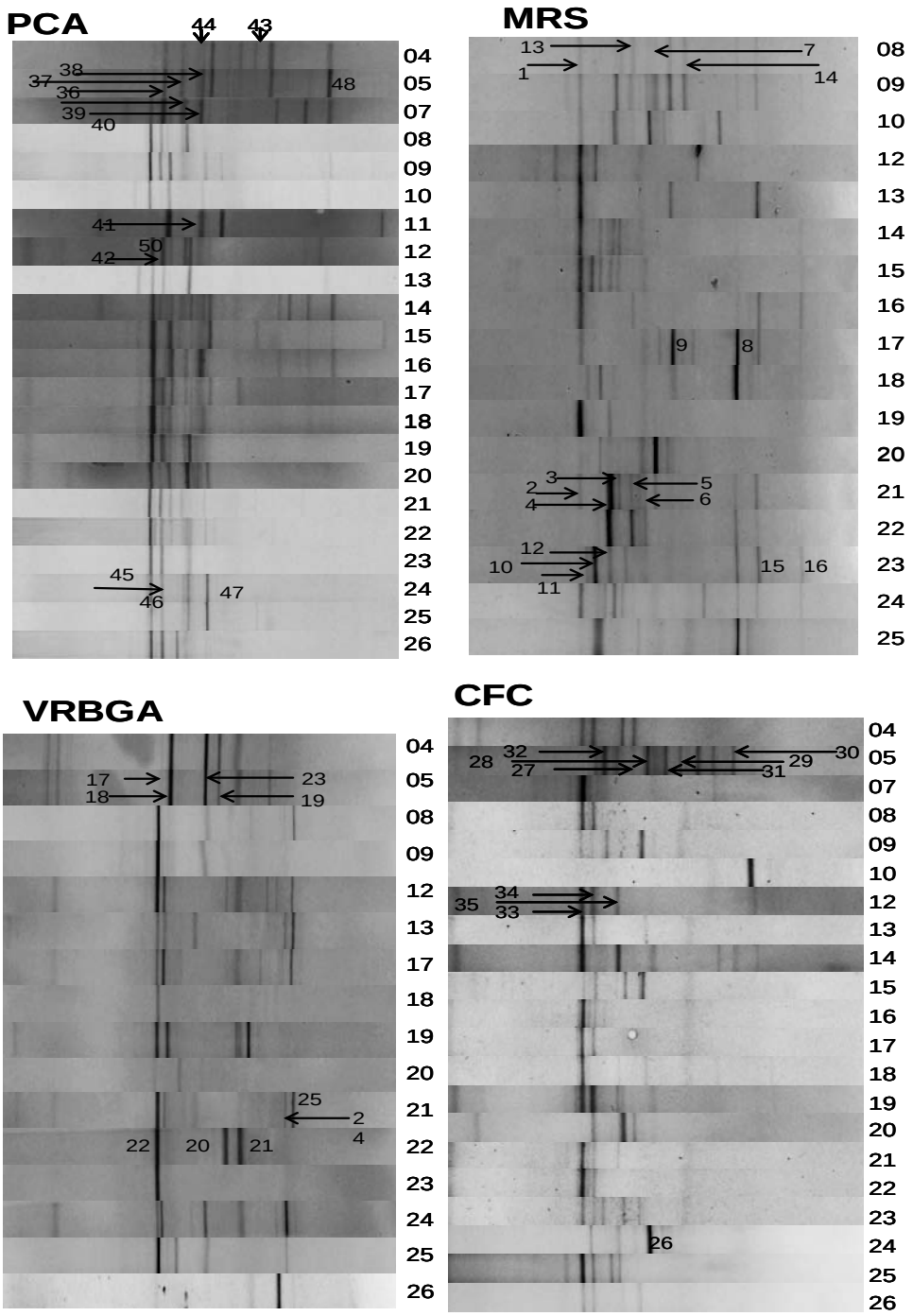


Figure 3.7. PCR-DGGE profiles generated from culture dependent isolation of DNA obtained from different culture media for samples recovered from different sampling sites during from Sampling 3 (during processing) in Facility B. The gels shown were normalized with BIONUMERICS by including two reference standards on both sides of each gel during electrophoresis. Numbers on the right side of gel indicate sampling site. Numbers on the gel indicate bands selected for sequencing.

Table 3.4: Identification of microbial species associated with samples obtained during sampling 1 and 3 at Facility B and plated on different microbiological media based on the sequencing of partial 16S rDNA

Sampling	Media	Band Number[a]	Closest relative (Source)	% identity
1	VRBGA	51	*Pseudomonas* spp. (EU747696.1)	100
	MRS	52	*Pseudomonas* spp. (EU747696.1)	100
		53	*Escherichia coli* (CU651637.1) ; *Shigella* spp. (FJ594947.1) ; *Escherichia albertii* (EU926634.1)	98
	CFC	54	*Pseudomonas putida* (EU833944.1); *Pseudomonas citronellosis* (AF489938.1)	89
	PCA	55	*Pseudomonas clemancea* (AM419155.2)	89
		56	*Acinetobacter* spp. (FJ542809.1)	100
3	MRS	1,2	*Enterococcus* spp. (EU438983.1)	98
		3, 6, 7	*Acinetobacter xiamenensis* (EF030545.1)	99
		4	*Streptococcus parauberis* (FJ009631.1)	100
		5, 13	*Acinetobacter* spp. (EF204261.1)	99
		8	*Escherichia coli* (EU849161.1); *Shigella* spp. (FJ594947.1) ; *Erwinia rhapontici* (EU490593.1)	98
		9	*Shigella* spp. (FJ594947.1)	100
		10	*Wautersiella falsenii* (FM162560.1); *Empedobacter* spp. (EU794729.1) ; *Flavobacterium* spp. (AY363052.1)	98
		11	*Staphylococcus equorum* (EU855190.1)	100
		12	*Staphylococcus* spp. (FJ380997.1)	99
		14	*Obseumbacterium proteus* (FJ492810.1); *Citrobacter freundii* (EU545403.1) ; *Hafnia* spp. (EU159563.1); *Enterobacter* spp. (AB428448.1) ; *Kluyvera ascorbata* (AM933755.1)	99
		15	*Carnobacterium s*pp. (FJ151401.1)	100
		16	*Micrococcus* spp. (AB478094.1)	99

3	VRBGA	17, 22	*Pseudomonas* spp. (FJ515303.1)	99
		18	*Pseudomonas* spp. (FJ539118.1)	100
		19	*Pectobacterium carotovorum* (FJ527462.1); *Serratia* spp. ((FJ231172.1)	98
		20	*Obseumbacterium proteus* (FJ492810.1); *Hafnia* spp. (EU159563.1); *Enterobacter* spp. (AB428448.1); *Enterobacteriaceae bacterium* (DQ822752.1)	96
		21	*Hafnia* spp. (FJ394920.1); *Kluyvera intermedia* (AB435598.1); *Enterobacter* spp. (EU047557.1); *Obsesumbacterium proteus* (DQ223874.1); *Buttiauxella* spp. (DQ822728.1); *Raoultella* spp. (DQ812970.1); *Enterobacteriaceae bacterium* (DQ822716.1)	100
		23	*Shigella* spp. (FJ594947.1); *Escherichia albertii* (EU926634.1); *Escherichia coli* (EU849161.1); *Pectobacterium* spp. (EU496611.1); *Erwinia rhaponticia* (EU490593.1); *Brenneria* spp. (EU490604.1)	100
		24	*Citrobacter freundii* (FJ542329.1); *Salmonella typhi* (DQ480723.1)	100
		25	*Pseudomonas fluorescens* (EU543578.1)	100
	CFC	26, 31	*Pseudomonas* spp. (FJ515304.1)	98
		27	*Acinetobacter* spp. (FJ494778.1)	98
		28	*Pseudomonas* spp. (FJ608777.1); *Klebsiella* spp. (FJ555520.1); *Escherichia hermanii* (FJ544365.1)	
		29	*Erwinia* spp. (AJ494778.1); *Pseudomonas* spp. (FJ515304.1); *Hafnia* spp. (FJ394920.1); *Buttiauxella* spp. (EU159562.1); *Enterobacter* spp. (EU047557.1); *Raoultella* spp. (DQ812970.1); *Kluyvera intermedia* (AB435598.1)	98
		30	*Pseudomonas* spp. (FJ605176.1); *Xanthomonas* spp. (FJ600362.1); *Stenotrophomonas* spp. (FJ493144.1)	99
		32	*Pseudomonas* spp. (FJ608777.1); *Klebsiella* spp. (FJ555520.1); *Escherichia hermanii* (FJ544365.1)	99
		33, 34	*Pseudomonas* spp. (FJ539118.1)	99
		35	*Pseudomonas* spp. (EU747696.1)	100
		36, 47	*Acinetobacter* spp. (EU438969.1)	100

Table 3.4 (Continued)

3	PCA	37, 39	*Acinetobacter* spp. (FM865882.1); *Acinetobacter johnsonii* (EU275352.1)	100
		38, 40, 41, 42, 44	*Acinetobacter* spp. (FJ542809.1); *Acinetobacter iwoffii* (FJ544339.1)	100
		43	*Stenotrophomonas* spp. (EU438979.1)	100
		45	*Psychrobacter* spp. (FJ546058.1)	99
		46	*Pseudomonas* spp. (FJ515304.1)	100
		48	*Brevundimonas* spp. (FJ535474.1) ; *Caulobacter* spp. (FJ605177.1)	100
		49	*Chryseobacterium* spp. (EF204449.1)	100
		50	*Pseudomonas* spp. (EU747696.1)	100

[a]Band numbers correspond to numbers indicated on DGGE gels shown in Figures 3.5 and 3.6.

Table 3.4 (Continued)

3.2.4 Similarity analysis of PCR-DGGE of total culturable microflora obtained from different media enumerated from samples collected during sampling 2 and 3.

To determine if the distribution of the microflora was affected by the type of surface material or if the background microflora affected the prevalence of *Campylobacter* spp., the biomass from different media (PCA, CFC, MRS, and VRBGA) was collected and pooled together. The extracted DNA samples were subjected to PCR-DGGE analysis, and the resulting fingerprints were analyzed with Bionumerics. Cluster analysis of PCR-DGGE profiles obtained from sampling two and three showed that only a few PCR-DGGE profiles from sampling sites of close proximity (processing of same area of carcasses) could be clustered together (Figures 3.8 and 3.9) and clustering was not consistent between sampling times. For example, for samples obtained during sampling 2, the microflora recovered from site 11 and site 17 (the silicon conveyor belts) used during trimming of the ham and shoulder, were more than 90% similar. However, during sampling 3, the PCR-DGGE profiles of the microflora recovered from the same sites were only 40.0% similar.

In general, the average D_{sc} value among samples collected during sampling 3 were higher than that for samples collected during sampling 2. In addition, the average D_{sc} values between samples collected from silicon and stainless steel type surface materials for each sampling 2 and 3 differ by less than 5%. To test whether the presence of microbial communities had any impact on the occurrence of *Campylobacter* spp. on the same meat contact surfaces, D_{sc} values of samples tested positive for *Campylobacter* spp. from sampling two and three were

calculated. The average D_{sc} value among samples that were *Campylobacter* spp. positive for both sampling two and three were higher than that for samples which were negative for the presence of *Campylobacter* spp. The differences were 8% for sampling 3 and more than 20% for sampling 2.

Table 3.5: Intergroup and intragroup similarity (average dice coefficient) of PCR-DGGE profiles for samples obtained from different surface types or samples that were positive or negative for *Campylobacter* spp. (Facility B)

Sample Group	Sampling 2		Sampling 3	
	Intragroup similarity	Intergroup similarity	Intragroup similarity	Intergroup similarity
Silicon	47.1	41.1	55.5	57.4
Stainless steel	44.0		59.23	
Campylobacter spp. positive	59.0	44.7	62.3	57.6
Campylobacter spp. negative	39.7		54.3	

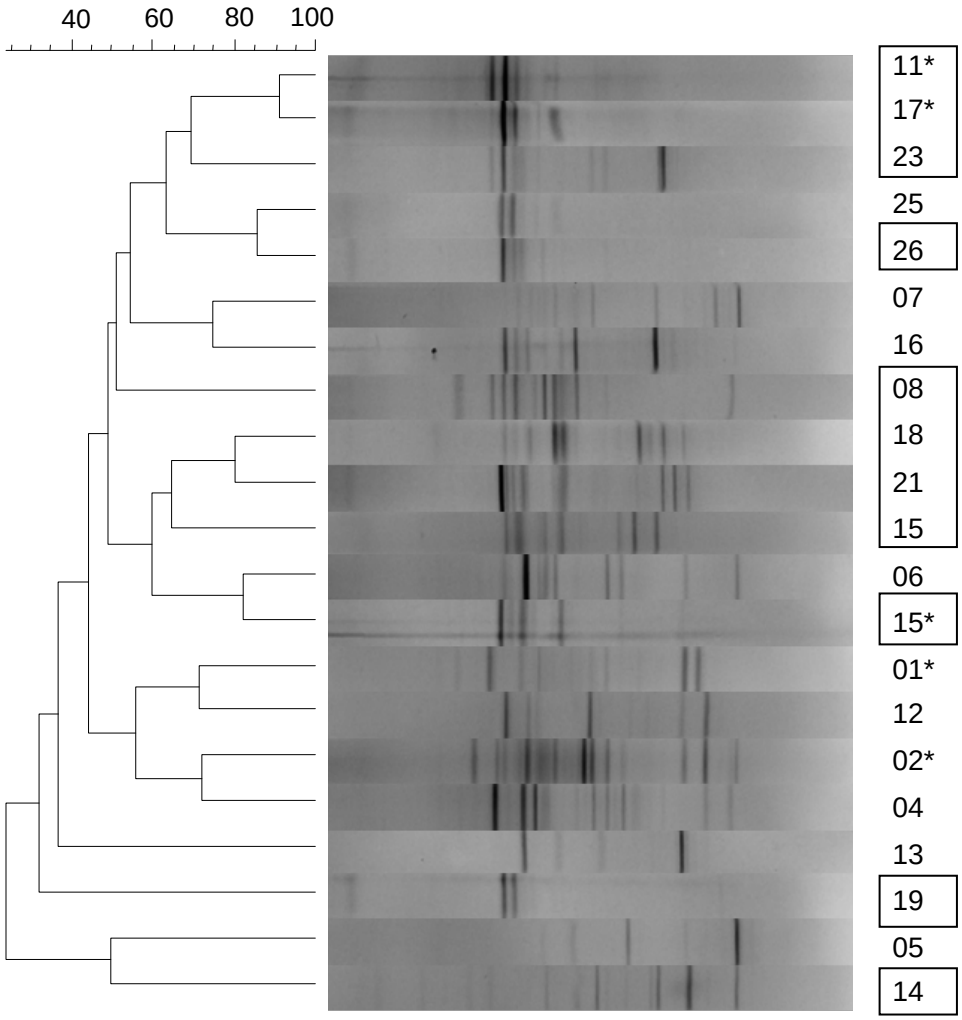


Figure 3.8: Cluster analysis of PCR-DGGE profiles of pooled DNA from all media used for cultivation of sponge samples from Sampling 2 at Facility B. The dendogram was constructed with UPGMA clustering algorithm. Boxes indicate sampling sites that were silicon; * indicates samples that were positive for the presence of *Campylobacter* spp.

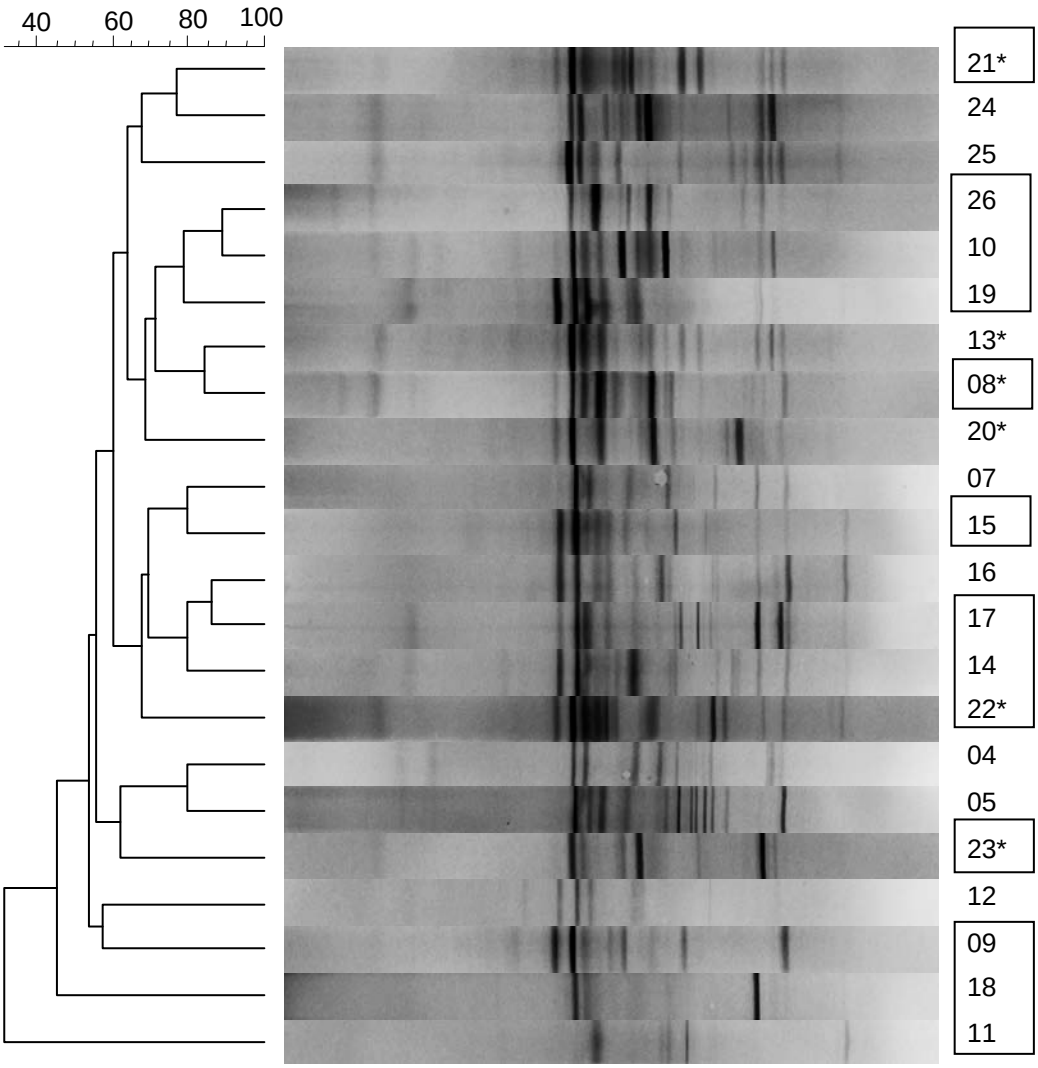


Figure 3.9: Cluster analysis of DGGE profiles of pooled DNA from all media used for cultivation of sponge samples from Sampling 3 at Facility B. The dendogram was constructed with UPGMA clustering algorithm. Boxes indicate sampling sites that were silicon; * indicates samples that were positive for the presence of *Campylobacter* spp.

4. Discussion

Samples obtained from Facilities A and B were initially subjected to culture independent analysis to determine the diversity of the microbial community on meat contact surfaces. However, some samples of DNA obtained from the biomass in swabs of meat contact surfaces in Facility A could not be amplified after direct PCR with the HDA1-GC/HDA2 primer pair. As a result, when samples were collected at Facility B, the bacterial load on the equipment surfaces was also determined. In general, meat contact surfaces with cell counts below the detection limits failed to yield enough DNA for direct PCR amplification. No cell counts were obtained for samples collected at Facility A. However, DNA extracted from culture independent samples collected at Facility A could be amplified with nested PCR. Some samples collected from Facility B after nested PCR did not produce amplicons that were sufficient for separation on a DGGE gel, suggesting that the bacterial load on meat contact surfaces at Facility A may have been higher than that at Facility B.

In general, the cell counts from meat contact surfaces in Facility B were highly variable among locations and among sampling times. The variation in cell counts of samples could have been caused by differences in the initial bacterial load on the incoming pigs and whether the carcass or muscle tissue came in direct contact with the surface that was sampled. The variability in the counts obtained from different sampling sites could have been due to the redistribution of contamination. Gill *et al*. (2000) and Pearce *et al*. (2004) collected surface

samples from pig carcasses and found that the cell count on meat surfaces varied after each processing step, indicating that certain processes such as scalding effectively reduced the counts while other processes such as polishing increased the cell count, as a result of the build-up of microbial species on the equipment surfaces. Bacterial accumulation on equipment surfaces can potentially contaminate incoming meat (Rivoal *et al*, 1999; Lindmark *et al*, 2006; Lienau *et al*, 2007; Hansson *et al*, 2007). Upmann and Reuter (1998) found that surfaces with direct contact with fresh cut pork during processing generally had more consistent surface counts than a rough cutting board and transport containers. Cutting boards and transport containers had the highest counts before the start of processing. The saw blade also had higher counts before the start of processing, compared to during processing when counts have become more consistent. In the current study, the type of surface (silicon vs. stainless steel) could not be related to the bacterial load on surfaces. This may have been due to the small number of samples used in the current study and the variability in the microbial load found on the same surface at different sampling times. .

In the current study, *Campylobacter* spp. were isolated from various surfaces throughout the processing facilities and no consistent pattern of isolation was detected. *Campylobacter* spp. were isolated from the drains, polisher, offal pans, conveyor belts, head table, knife sharpeners, and cutting boards. Out of 99 samples taken in the current thesis research, a total of 26 were confirmed positive for the presence of *Campylobacter* spp. Other researches have also reported the

inconsistent pattern of the isolation of *Campylobacter* spp. from equipment surfaces (Pearce *et al*, 2003; Steinhauserova *et al*, 2005). Pearce *et al* (2003) were able on two separate occasions to isolate *Campylobacter coli* from slaughter equipment (2 positive out of 42 samples collected from equipment surfaces) and a cutting board (1 positive out of 30 samples collected from equipment surfaces) in a swine slaughter and processing facility. Steinhauserova *et al*. (2005), in a study of the prevalence of thermophilic *Campylobacter* spp. in pig slaughter facilities in the Czech Republic, were able on occasion to isolate *Campylobacter* spp. from the dehairing machines and conveyor belts, which is similar to what was found in the current study. Although Pearce *et al* (2003) reported that *Campylobacter* spp. were more likely to be isolated from certain areas of the carcasses such as the neck area than the belly and ham, our results indicated that no specific equipment or meat contact surface involved in the processing of hog carcasses could be associated with the isolation of *Campylobacter* spp. One sample obtained after sanitation and prior to production at Facility B was positive for *Campylobacter* spp. Based on observations at the facility, it appeared that the sampling site was covered with detritus, such as pieces of feces and hairs, and this could have contributed to the survival of *Campylobacter* spp. on the equipment surface at Facility B. This explanation is supported by the conclusions of De Cesare *et al* (2003) who indicated that organic material could support the survival and persistence of *C. jejuni* on stainless steel surfaces. However, it is also important to note that Peyrat *et al*. (2008) determined that *C. jejuni* was able to survive the cleaning and disinfection processes in four poultry slaughterhouses that had been

sanitized with either foam containing alkaline-chlorinated molecules, neutral detergent, or quaternary ammonium compounds combined with glutaraldehyde or poly(hexamethylene biguanide) chlorohydrate. Sanitation personnel and HACCP coordinators at meat processing facilities need to ensure that proper cleaning procedures are sufficient to remove any detritus, including feces, and biofilms on meat contact surfaces. In addition, proper selection of a sanitizer (including rotation of sanitizers) is important to reduce the risk of the survival of meatborne pathogens such as *Campylobacter* spp. on equipment surfaces.

In the present study, when enrichment in Bolton broth and subsequent plating onto mCCDA was used for the isolation of *Campylobacter* spp., this method was not exclusively selective for *Campylobacter* spp. When this combination was used, at least 98% of the microflora on some of the mCCDA plates was not morphologically similar to *Campylobacter* spp. To determine which organisms can grow in conjunction with *Campylobacter* spp. in Bolton broth and non-blood charcoal based media selective for *Campylobacter* spp., the total DNA from samples enriched in Bolton broth and plated on mCCDA for samples obtained at Facility A was extracted and examined with PCR-DGGE. Various Gram negative bacteria including *Brevundimonas* spp., *Myroides* spp., *Arcobacter* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Proteus* spp. and *Flavobacterium* spp. were among some of the organisms that could grow concurrently in the medium with *Campylobacter* spp. under the conditions used in this study. Oyarzabal *et al* (2005), in research to evaluate the efficiency of different types of media used in

the enumeration of *Campylobacter* spp. from poultry carcass rinses, reported that major contaminants were found on medium supposedly selective for *Campylobacter* spp. (CAMPY, mCampy-Cefex, Campy-Cefex, Karmali, mCCDA and Campy-Line). All of these types of media contain at least one or more antibacterial compounds that are active against either Gram negative or Gram positive bacterial species. For example, mCCDA medium contains cefoperazone and amphotericin (similar to mCampy-Cefex), Karmali agar contains cefoperazone, vancomycin and cycloheximide (similar to Campy-Cefex with the exclusion of vancomycin), while CAMPY contains the combination of antibacterial compounds found in both Karmali and mCCDA. The authors reported that among some of the prevalant contaminants on all types of media tested included *Acinetobacter baumanii* (CAMPY and Karmali), *A. Iwoffi* (CAMPY and Karmali), *Pseudomonas spp*. (CAMPY and Campy-cefex) and *Staphylococcus hominis* (CAMPY and Campy-Cefex). All of these bacterial species also grew on mCCDA (after enrichment in Bolton broth) used in this study with the exception of *S. hominis*, although other bacterial species, such as *Myroides* spp., were identified. Oyarzabal *et al* (2005) also suggested that the selectiveness of each type of media may not be solely dependent on the antibacterial compounds that are present, but also depends on the other nutrients that are included in each medium (for example laked horse blood in Campy-Cefex and sodium pyruvate in Karmali agar). Pearce *et al* (2003) discussed the discrepancies between direct plating onto Campy-Line or Campy-Cefex and pre-enrichment in Bolton Broth before plating onto Campy-Line or Campy-Cefex in

the enumeration of *Campylobacter* spp. from samples collected from pig slaughter facilities. On two occasions Pearce *et al* (2003) isolated *Campylobacter* spp. from equipment surfaces only when an enrichment technique was used, and suggested that low numbers of *Campylobacter* spp. may not be detected with direct plating technique. In contrast, they were able to isolate *Campylobacter* spp. from carcasses when direct plating techniques were used; however, when the same samples were enriched in Bolton broth before plating onto Campy-Cefex and Campy-Line agar, the authors were not able to isolate *Campylobacter* spp. The authors suggested that pre-enrichment may have allowed other background microflora to grow at the expense of *Campylobacter* spp, making the detection of *Campylobacter* spp. impossible. Oyarzabel *et al* (2005) and Pearce *et al* (2003) both pointed out the importance of choosing the proper conditions for the enumeration of *Campylobacter* spp. from meat and meat processing facilities. In this study, samples were pre-enriched in Bolton broth at 39°C (to allow for the growth of both thermophlic and non-thermophilic *Campylobacter* spp.) before being plated onto mCCDA or Karmali agar, and if *Campylobacter* spp. was present in low amount, the pre-enrichment step should have allowed *Campylobacter* spp. to grow to substantial numbers to allow detection. The pre-enrichment step may have accounted for the presence of a large amount of background microflora on mCCDA (98%). Many contaminating bacterial species can have a colony morphology that is similar to that of *Campylobacter* spp. on either mCCDA or Karmali agar. To eliminate the possibility of picking colonies that represented non-*Campylobacter* spp., and to avoid false negative results, all

colonies that represented typical morphology of *Campylobacter* spp. were picked from the agar plates and the DNA isolated from all colonies was subjected to identification with PCR. When samples were obtained from Facility B, the total DNA from Bolton broth was also extracted and examined with PCR-DGGE to determine which other epsilon bacteria could grow with *Campylobacter* spp. The effect of the presence of large amount of background microflora on the detection of *Campylobacter* spp. was observed when DNA originated from samples from Facility B were grown in Bolton broth and subjected to analysis with PCR-DGGE. PCR-DGGE profiles of samples presumed to be positive for the presence of *Campylobacter* spp. based on PCR identification with DNA extracted from biomass collected from Karmali agar did not produce any visible bands that were identified to be *Campylobacter* spp. This suggests that the presence of large proportion of a background microflora could interfere with the detection of *Campylobacter* spp. In this study, *Arcobacter cryaerphilus* and *Arcobacter butzleri* were among epsilonbacteria that grew in conjunction with *Campylobacter* spp. in Bolton broth. *Arcobacter* spp. have been isolated from porcine sources (Driessche *et al*, 2004) and retail meats (Rivas *et al*, 2004) and have been implicated as an emerging foodborne pathogen that is capable of causing human and animal diseases (Prouzet-Mauléon *et al*, 2006). It is not surprising to find *Arcobacter* spp. associated with a medium that is supposedly "selective" for *Campylobacter* spp. *A. butzleri* were initially classified as "*Campylobacter butzleri*" but was assigned to *Arcobacter* based on DNA–rRNA hybridization results (Vandamme *et al*. 1992).

The microbial populations associated with surfaces in meat processing facilities have a diverse array of bacterial species associated with them (Brightwell *et al*, 2006). In the current study, a diverse microbial community was identified through culture dependent analysis of the populations isolated on different selective media. PCR-DGGE profiles of samples obtained from Facility B after the cleaning and sanitation process but before production yielded distinctive bands belonging to *Pseudomonas* spp. and *Enterobacteriaceae*. In Facility B, meat contact surfaces were left to air dry after sanitation procedures at a temperature lower than ambient and it is unknown how fast the surfaces dried but it is unlikely that the drying occurred rapidly. Fuster-Valls *et al* (2008) demonstrated that wet stainless steel surfaces could support the survival of *Pseudomonas aeruginosa*, *Enterobacter clocoa*, and *Staphylococcus aureus* compared to surfaces that were rapidly air-dried. In addition, these organisms are able to form biofilms on a stainless steel surface within 26 h after contamination (Fuster-Valls *et al*, 2008). *Pseudomonas* spp. and *Enterobacteriaceae* can be sensitive to desiccation, but due to the presence of moisture on meat contact surfaces, these organisms may have been able to survive overnight at Facility B. Chang *et al* (2007) demonstrated that the moisture on surfaces could be retained within biofilms formed by *Pseudomonas* spp., which is important for the organism to survive water-limiting conditions and osmotic shock. In addition, bacterial species within a biofilm matrix on a stainless steel and rubber type surfaces have decreased susceptibility to various disinfectants (Ronner and Wong, 1993). tThis may also explain the presence of *Pseudomonas* spp. and

Enterobacteriaceae on sanitized equipment surfaces at Facility B. The formation of biofilms may have allowed multiple bacterial species to attach or deposit themselves within the biofilm matrices and survive for an extended time under nutrient limited environments. For example, Liu and Li (2008) demonstrated that biofilms formed by *P. aeruginosa*, depending on the amount and structural components, could allow the attachment and survival of *Escherichia coli* on porous materials used in the packing of a filtration system. The survival of multiple bacterial species on meat contact surfaces could have been a result of multiple factors including extended drying period (Fuster-Vall *et al*, 2008), presence of organic materials (De Cesar *et al*, 2003), formation of biofilms (Ronner and Wong, 1993) and type of surface materials (Arnold and Silvers, 2000). At Facility B the residues of organic material and extended drying times overnight may have allowed for the formation of biofilms by bacterial communities, and all these factors in combination could have contributed to the survival of *Pseudomonas* spp. and *Enterobacteriaceae* on meat contact surfaces.

Common culturable bacteria that were present consistently on meat contact surfaces throughout the production chain in Facility B during processing were *Enterococcus* spp., *Psychrobacter* spp., *Micrococcus* spp., *Carnobacterium*, *Staphylococcus equorum*, *Streptococcus parauberis*, *Acinetobacter* spp., *Enterobacteriaceae, Pseudomonas* spp., *Brevundimonas* spp./*Caulobacter* spp. and *Flavobacteriaceae*. Most of the microbial communities that have been identified consistently or inconsistently on meat contact surfaces during processing in this study have been indicated as part of the meat natural microflora

in studies done by Li *et al* (2006) and Olsson *et al* (2003). Olsson *et al* (2003), based on the construction of a clone library of the microflora of fresh pork with culture independent methods, reported that the dominant species on fresh pork were *Acinetobacter* spp. (36.5%), followed by *Staphylococcus*/*Macrococcus* (17.3%), *Pseudomonas* spp. and *Moraxella* spp. This is similar to the findings in the current study, with the exception of *Moraxella* spp. since no distinct band representing this group of organisms was identified on DGGE gels. Since meat can acquire some of the microflora through direct contact with equipment surfaces, it is possible that the natural microflora of meat reflects the proportions of microbiota on meat contact surfaces. In a study to determine the microbial diversity on an intralox conveyor belt in a lamb boning room, Brightwell *et al* (2006) reported that the most common microbial species were *Pseudomonas* spp. and *Sphingomonas* spp. In the present study, no consistent band belonging to *Sphingomonas* spp. was observed on any of the PCR-DGGE gels. In contrast, *Enterobacteriaceae* were identified on all types of media (PCA, MRS, CFC and VRBGA), suggesting that these organisms are capable of growing under the conditions used in this study or that these organisms were present in a higher proportion compared to other bacterial groups. Microorganisms, including *Pseudomonas* spp., *Carnobacterium* spp. (member of lactic acid bacteria) and *Enterobacteriaceae,* have been determined to be the agents of spoilage of refrigerated pork (Borch *et al*, 1996). Bacterial groups including lactic acid bacteria and *Enterobacteriaceae* may be of special interest for vacuum packaged meat stored at refrigeration temperature since these organisms could influence the

shelf life of the product (Knox *et al*, 2008). However, the growth rate of these organisms on vacuum packaged pork stored at refrigeration temperature may be dependent on the initial pH of the muscle tissues. For example, Knox *et al* (2008) determined that muscle tissues at higher pH (>6.0) had higher *Enterobacteriaceae* counts compared to that at lower pH during storage trial â€.4Other aerobic bacteria including *Pseudomonas* spp. and *Streptococcus* spp. are not likely to grow on meat stored in a vacuum package. *Streptococcus parauberis* has been isolated from bovine milk **(**Pitkälä *et al*, 2008; McDonald *et al*, 2005) and poultry products stored in modified atmosphere packaging (Koort *et al*, 2005) and it has been identified as a causative agent of bovine mastitis (McDonald *et al*, 2005). *Staphylococcus equorum* can be part of the natural microflora of fresh meat and has been developed to be used as a starter culture in the production of fermented sausage (Talon *et al*, 2008).

Similarity analysis of PCR-DGGE fingerprints of samples obtained from Facilities A and B revealed that there was a diverse microbial community on meat contact surfaces at both facilities. The diverse microbial communities could be due to the differences in production practices adopted in Facilities A and B. For example, at Facility A, more than 3000 hogs are processed per day and in Facility B 300 hogs are processed daily. The volume of hogs processed daily in both facilities could have contributed to the diversity of the microflora on meat contact surfaces. The microbial diversity on the incoming herd also could have affected the diversity of the microbiota on meat contact surfaces. In general, the

composition of the microbiota on meat contact surfaces at a few sampling sites were quite similar since some of the larger clusters could be divided into individual smaller clusters with similarity values of more than 75%. The present study demonstrated that certain processing steps involved in the processing of specific areas of the carcasses appeared to have a more homogeneous microbial composition than that which was present at other processing steps. For example, 4 samples from sites in Facility A involved in the removal of head and tongue were clustered together as a group with a similarity of about 86%, suggesting that the equipment had a homogeneous microbial population.

Campylobacter spp. are fastidious and are not likely to grow in the processing environment. The factors affecting the survival of *Campylobacter* spp. in meat processing facilities are largely unknown. The present study attempted to determine if the diversity of the microflora on meat contact surfaces could have impacted the presence of *Campylobacter* spp. To achieve the objective, average D_{sc} values within or between groups of samples were computed. A similar technique using average D_{sc} values has been demonstrated previously (Guan *et al*, 2008). In general, and for the purposes of this research, an average D_{sc} value within a group of samples that was low (i.e. 50%) was interpreted as an indication that the microflora was very diverse or that there were very few common species within the group of samples. Such comparisons allow for the preliminary assessment of any factors (i.e. sample size or surface type) that could have impacted the diversity of the microflora that was detected. Cluster analysis of

PCR-DGGE fingerprints constructed with samples obtained from both Facilities A and B indicated that *Campylobacter* spp. could be isolated from either stainless steel or silicon surface materials and that the type of surface did not affect the presence of *Campylobacter* spp. Pearce *at el* (2003) reported that *Campylobacter* spp. was more likely to be isolated from certain area of carcasses. The authors suggested that the prevalence of *Campylobacter* spp. on specific areas of a carcass (higher prevalence in the neck area than in the ham area) could be due to production practices. For example, during production, hogs are hung upside down and this allows water used for carcass rinsing, which could be contaminated with *Campylobacter* spp., to accumulate on the neck area. Production practices could be responsible for the accumulation of a diverse microflora associated with *Campylobacter* spp. on carcasses which are later transferred onto meat contact surfaces (either stainless steel or silicon surface materials). Within each of the groups of samples that were positive for the presence of *Campylobacter* spp. there was little similarity in the composition of the microflora (intragroup D_{sc} values were less than 70%). When the PCR-DGGE fingerprints of samples that were positive or negative for the presence of *Campylobacter* spp. were compared (intergroup comparison), the average D_{sc} values for each sampling time were generally low (<72%). In general, the microbial composition between the samples that were *Campylobacte*r spp. positive and those that were negative was not very similar. However, it is worthy to note that the D_{sc} values for *Campylobacter* spp. positive samples collected from Facility B were higher than that for samples that were negative for the presence of *Campylobacter* spp. This may indicate that the

composition of the microbial population on meat contact surfaces where *Campylobacter* spp. have been isolated was more similar compared to the microflora on meat contact surfaces that were negative for the presence of *Campylobacter* spp.; or that surfaces where *Campylobacter* spp. have been isolated had a more consistent microflora; however, more work would have to be done to confirm this since we did not observe a similar pattern for samples collected at Facility A.

Bacteria that may have an association with *Campylobacter* spp. on meat contact surfaces at Facility B included *Pseudomonas*, *Enterobacteriaceae*, *Staphylococcus* and *Acinetobacter* spp. Other bacteria that were consistently present on all surfaces included *Enterococcus*, *Carnobacterium*, *Streptococcus parauberis*, *Micrococcus*, *Psychrobacter* and *Breuvindumonas* spp. and these may also be important in the ecology of *Campylobacter* spp. in meat processing facilities since all of these organisms are among the common species that were found on meat contact surfaces. The bacterial species that were detected conjunction with *Campylobacter* spp. in Bolton broth could have had an impact on the survival of *Campylobacter* spp. in the environment. These organisms include *Myroides* spp., *Lactobacillus salivarius*, *Proteus mirabilis*, and *Arcobacter cryaerophilus*. To date, there has been no published research that discusses the survival of *Campylobacter* spp. in the presence of these organisms in the environment. The current research has demonstrated that *Campylobacter* spp. can be associated with certain microflora in the meat processing facilities.

However, more research will have to be performed with pure or mixed cultures to determine if the survival of *Campylobacter* spp. in a processing environment is influenced by the presence of these other organisms.

Since there is a possibility that the diversity of microbiota on meat contact surfaces could affect the presence of *Campylobacter* spp., it was the aim of this project to determine if the distribution of the microbiota could be impacted by the size or type of contact surfaces and subsequently affect the prevalence of *Campylobacter* spp. on meat contact surfaces. At Facility A, neither the diversity of the microflora nor the presence of *Campylobacter* spp. on meat contact surfaces was affected by the size of area swabbed on equipment surfaces (>100cm^2 or <50 cm^2). D_{sc} values for samples collected during sampling 1 for surface area >100cm^2 or <50 cm^2 indicated that both groups of samples were generally not that similar. If the size of the equipment surface that was swabbed had any effect on the microbial diversity, we would have expected to observe a higher D_{sc} value for samples with either a surface area of >100cm^2 or of <50 cm^2 (intragroup comparisons). Generally, the similarity values for both intergroup and intragroup were higher for samples collected during the second sampling. In all cases, since the D_{sc} values were very close for both intragroup and intergroup comparisons, it can be concluded that size of the equipment surface that was swabbed had little impact on the similarity of the microflora recovered from meat contact surfaces. In addition, the microflora recovered from the individual surfaces was quite diverse and no relationship between type of surface and

similarity in microbial populations could be established from these results. The importance of the type of surface materials has been demonstrated previously. Arnold and Silver (2000) found that surface material (stainless steel, polyethylene, or conveyor belt) did not affect the ability of the microflora typically found on poultry to form biofilms in a poultry processing facility except on surfaces made of picker-fingers rubbers. The results of Arnold and Silver (2000) study may indicate that the choice of the type of surface material in meat processing plants may have a direct effect on the survival of potential pathogens in meat processing plants. However, this was not demonstrated in the current research.

Culture independent analysis of microbial communities can complement culture dependent analysis (Edenborn and Sexstone, 2007). In studies to determine the microbial diversity of soil, Edenborn and Sexstone (2007) found that culture independent analysis did not result in the same PCR-DGGE profiles as the culture dependent analysis and that both were needed to ensure a complete representation of the microbial community. In the current study, the unsuccessful amplification of certain bands with the culture independent method for samples collected at Facility B may be due to the difficulty in recovering all DNA from the total nonculturable microflora during DNA extraction with a phenol choloroform method. It is possible that the culture dependent method may have allowed any microbial species that were present in a low abundance to grow to sufficient cell numbers to permit the isolation of enough DNA for detection in a PCR-DGGE

profile. Primers HDA1-GC and HDA2 used in this study were designed to amplify the V2 to V3 variable region of the bacterial conserved 16S rRNA gene and have been used in the past to study various microbial communities (Ercolini *et al*, 2003; Knarreborg *et al*, 2002). However, sequence homology of primer pair HDA1-GC/HDA2 has also been found in 18S rDNA region of various species of yeast, fungi and protozoa (Lopez *et al*, 2003, Huws *et al*, 2007) but not *Pediococcus* sp. Strain PC800 (Lopez *et al*, 2003). The amplification of certain eukaryotic DNA has a masking effect on the amplification of bacterial DNA. However, in the current study it is very unlikely that non-16S rDNA, such as that from *Saccharomyces* or protozoan species, were amplified at the expense of eubacterial 16S rDNA since nested PCR was performed to troubleshoot samples that could not be amplified with direct PCR with HDA1-GC/HDA2. The first cycle of the nested PCR amplified the almost complete bacterial 16S rDNA fragments with primer pair 1492R/27F and would eliminate the amplification of non-eubacterial 16S rDNA such as those from *Saccharomyces* and protozoa. The effect of template DNA ratio of a dominant bacterial species versus a poorly represented bacterial species can affect the detection of bacterial species that are present in low abundance in a mixed population (Zhang *et al*., 2005). All of these may account for some of the differences observed between culture dependent and culture independent methods used in the current study for samples collected at Facility B. Due to sequence similarity within the V2 to V3 region of the 16S rDNA of the members of *Enterobacteriaceae* and *Pseudomonadaceae,* certain bands can not be assigned at the species level. This is especially true for bands

that were identified as *Enterobacteriaceae*. These results are supported by the findings of Chakravorty *et al* (2007), who indicated that V2-V3 region of the 16S rDNA is not variable enough to distinguish among members within the family of *Enterobacteriaceae*. To illustrate from the current research, band 8 (Facility B, Table 3.4) was identified as 16S rDNA belonging to *Escherichia coli* (EU849161.1), *Shigella* spp. (FJ594947.1) and *Erwinia rhapontici* (EU490593.1). *Erwinia rhapontici* is usually associated with the development of plant disease, and therefore it is unlikely to be associated with the microflora of meat or meat contact surfaces in a processing facility. In addition, the 16S rDNA of *E. coli* and *Shigella* spp. can be more than 99% identical (Wang *et al*, 1997). Other examples where multiple genus of bacteria were identified were evident in the analysis of the sequences of the PCR products generated with the primer pair HDA1-GC/HDA2 used in this study. Obviously, this primer pair does not generate sequences of sufficient length to distinguish between genera of the bacteria found in this study. Future research should include a clone library with full length 16S rDNA.

The PCR-DGGE method has been used extensively in the study of microbial communities in various environments and food systems. In this study, we have demonstrated that PCR-DGGE is a robust and less labour intensive way to study microbial ecology on meat contact surfaces in meat processing plants. Although culture independent methods yielded less DNA for subsequent PCR-DGGE analysis, samples obtained from culture dependent methods allowed the

construction of PCR-DGGE profiles to represent the total culturable microflora for the analysis with PCR-DGGE. The construction of clusters based on Dice's similarity coefficient allowed the comparison of microbial communities found in the presence or absence of *Campylobacter* spp. and on different types of surface materials.

5. Conclusions

Campylobacter spp. are fastidious and are not likely to grow in a food processing environment. Factors that impact the survival of *Campylobacter* spp. in meat processing plants to allow the organism to contaminate food at any point in the production chain are largely unknown. However, it is known that the association and persistent survival of a particular bacterial species within an ecological niche is usually a result of interactions of such bacterial species with the biotic and abiotic components. To study whether the presence of *Campylobacter* spp. on meat contact surfaces can be impacted by background microflora or the type of meat contact surfaces (stainless steel or silicon), environmental swab samples from two pig processing facilities were collected and subjected to culture dependent and culture independent analysis.

PCR-DGGE was used to analyze the non-culturable microflora components of samples collected from Facility A. Samples from Facility B were subjected to both culture independent and culture dependent methods before analysis with PCR-DGGE. Methods used for the processing of samples collected from Facility B were modified based on the results obtained from processing samples collected at Facility A. The culturable microflora obtained from Facility B represented the major bacterial groups that are usually associated with meat and pig microflora.

PCR-DGGE analysis of the non-culturable microflora or culturable microflora obtained from Facilities A and B both revealed that the composition of the microbial community on meat contact surfaces did not affect the presence of *Campylobacter* spp. However, there may be specific bacteria that are associated

with *Campylobacter* spp. At Facility A, where a higher volume of hogs is processed each day, the size of equipment surfaces that were swabbed did not affect the composition of the microbial community nor did it affect the detection of *Campylobacter* spp. on such surfaces. Similarly, at Facility B, the type of equipment surfaces did not affect the composition of the microbial community and the presence of *Campylobacter* spp. on such surfaces. However, based on the PCR-DGGE analysis of the culturable microflora of samples collected from Facility B, several common microbial species were found to be present at all locations from the kill floor to cutting room. The occurrence and survival of *Campylobacter* spp. in the presence of such bacterial species in the environment, however, is not known.

This research has demonstrated that *Campylobacter* spp. could be inconsistently isolated from all types of equipment surfaces in pig processing plants. No specific niche was identified for *Campylobacter* spp. in pork processing facilities. However, the detection of *Campylobacter* spp. in a sample collected before the beginning of production suggested that *Campylobacter* spp. are able to survive regular sanitation procedures in commercial pig processing facilities. The presence of detritus on surfaces could have protected the organism from desiccation and the lethal effect of sanitizers; in addition, *Enterobacteriaceae* and *Pseudomonas* spp. have been isolated from such surfaces. It is known that *Pseudomonas* spp. are capable of forming biofilms on stainless steel surfaces overnight and that biofilms can provide ‘protective matrices’ for *Campylobacter* spp. There may be other interactions between *Campylobacter* spp. and other

bacterial groups on equipment surfaces and that could aid in the survival of *Campylobacter* spp. in processing facilities.

The outcome of this research is critical for future research on the ecology of *Campylobacter* spp. in meat processing facilities. Knowledge of the microbial community associated with *Campylobacter* spp. that may allow it to survive on meats and in processing facilities will allow development of targeted interventions that could reduce the risk from this meatborne pathogen.

5. References

Alter T, Bori A, Hamedi A, Ellerbroek LP, Fehlhaber K. 2006. Influence of inoculation levels and processing parameters on the survival of *Campylobacter jejuni* in German style fermented turkey sausages. Food Microbiology 23:701-707.

Arnold JW and Silvers S. 2000. Comparison of poultry processing equipment surfaces for susceptibility to bacterial attachment and biofilm formation. Poultry Science 79:1215-1221.

Birk T, Rosenquist H, Brondsted L, Ingmer H, Bysted A, Christensen BB. 2006. A comparative study of two food model systems to test the survival of *Campylobacter jejuni* at -18°C. Journal of Food Protection 69:2635-2639.

Bohaychuk, V M, Gensler GE, King RK, Manninen KI, Sorensen O, Wu JT, Stiles ME and McMullen LM. 2006. Occurrence of pathogens in raw and ready to eat meat and poultry products collected from the retail marketplace in Edmonton, Alberta, Canada. Journal of Food Protection 69:2176-2182.

Bonetta S, Bonetta S, Carraro E, Rantsiou K and Cocolin L. 2008. Microbiological characterisation of Robiola di Roccaverano cheese using PCR-DGGE. Food Microbiology 25(6):786-92.

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

Boysen L, Knochel S and Rosenquist H. 2007. Survival of *Campylobacter jejuni* in different gas mixtures. FEMS Microbiology Letters 266, 152-157.

Bhaduri S and Cottrell B. 2004. Survival of cold-stressed *Campylobacter jejuni* on ground chicken and chicken skin during frozen storage. Applied and Environmental Microbiology 70:7103-7109.

Brandl MT, Haxo AF, Bates AH and Mandrell RE. 2004. Comparison of survival of *Campylobacter jejuni* in the phyllosphere with that in the rhizophere of spinach and radish plants. Applied and Environmental Microbiology 70: 1182-1189.

Bras AM, Chatterjee S, Wren BW, Newell DG and Ketley JM. 1999. A novel *Campylobacter jejuni* two-component regulatory system important for temperature-dependent growth and colonization. Journal of Bacteriology 181: 3298-3302.

Brightwell G, Boerema J, Mills J, Mowat E and Pulford D. 2006. Identifying the bacterial community on the surface of Intralox™ belting in a meat boning room

by culture dependent and culture independent 16S rDNA sequence analysis. International Journal of Food Microbiology 109:47-53.

Bull SA, Allen VM, Domingue G, Jørgensen F, Frost JA, Ure R, Whyte R, Tinker D, Corry EL, Gillard-King J, Humphrey TJ. 2006. Sources of *Campylobacter* spp. colonizing housed broiler flocks during rearing. Applied and Environmental Microbiology 72:645-652.

Callicott KA, Friðriksdo´ttir V, Reiersen J, Lowman R, Bisaillon J, Gunnarsson, E, Hiett KL, Needleman DS, Stern NJ. 2006. Lack of evidence for vertical transmission of *Campylobacter* spp. in chickens. Applied and Environmental Microbiology 72:5794-5798.

Callicott KA, Harðardóttir H, Georgsson F, Reiersen J, Friðriksdo´ttir V, Gunnarsson E, Michel P, Bisaillon J, Kristinsson KG, Briem H, Hiett K L, Needleman DS, Stern NJ. 2008. Broiler *Campylobacter* contamination and human campylobacteriosis. Applied and Environmental Microbiology 74:6483-6494.

Cappelier JM, Minet J, Magras C, Colwell RR and Federigh M. 1999. Recovery in embryonated eggs of viable but nonculturable *Campylobacter jejuni* cells and maintenance of ability to adhere to HeLa Cells after resuscitation. Applied and Environmental Microbiology 65:5154-5157.

Chakravorty S, Helb D, Burday M, Connell N and Alland D. 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. Journal of Microbiological Methods 69(2):30-39.

Chan KF, Tran HL, Kanenaka RY and Kathariou S. 2001. Survival of clinical and poultry-derived isolates of *Campylobacter jejuni* at low temperature (4°C). Applied and Environmental Microbiology 67:4186–4191.

Chang WS, Mortel MVD, Nielsen L, Nino Guzman GND, Li X, Halverson LJ. 2007. Alginate production by *Pseudomonas putida* creates a hydrated microenvironment and contributes to biofilm architecture and stress tolerance under water-limiting conditions. Journal of Bacteriology 189: 8290-8299.

Chaveerach P, Huurne AAHM, Lipman LJA and Knapen FV. 2003. Survival and resuscitation of ten strains of *Campylobacter jejuni* and *Campylobacter coli* under acid conditions. Applied and Environmental Microbiology 69:711-714.

Chynoweth RW, Hudson JA and Thom K. 1998. Aerobic growth and survival of *Campylobacter jejuni* in food and stream water. Letters in Applied Microbiology 27: 341-344.

Denis M, Soumet C, Rivoal K, Ermel G, Blivet D, Salvat G and Colin P. 1999. Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C. coli*. Letters in Applied Microbiology 29:406-410.

De Cesare A, Sheldon BW, Smith KS and Jaykus LA. 2003. Survival and persistence of *Campylobacter* and *Salmonella* species under various organic loads on food contact surfaces. Journal of Food Protection 66:1587-1594.

Dykes GA and Moorhead SM. 2001. Survival of *Campylobacter jejuni* on vacuum or carbon dioxide packaged primal beef cuts stored at -1.5ºC. Food Control 12:553-557.

Driessche EV, Houf K, Vangroenweghe F, Nollet N, Zutter LD, Vandamme P, Hoof JV. 2004. Occurrence and strain diversity of *Arcobacter* species isolated from healthy Belgian pigs. Research in Microbiology 155:662-666.

Edenborn SL and Sexstone AJ. 2007. DGGE fingerprinting of culturable soil bacterial communities complements culture independent analyses. Soil Biology and biochemistry 39:1570-1579.

Ercolini D. 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. 2004. Journal of Microbiological Methods 56:294-314.

Ercolini D, Hill PJ and Dodd CER. 2003. Bacterial Community Structure and Location in Stilton Cheese. Journal of Applied and Environmental Microbiology 69:3540-3548.

Frost JA, Gillespie IA, O'Brien SJ. 2002. Public health implications of Campylobacter outbreaks in England and Wales, 1995-1999: epidemiological and microbiological investigations. Epidemiology and Infection 128:111-118.

Fuster-Valls N, Hernandez-Herrero M, Marin-de-Mateo M, Rodriguez-Jerez JJ. 2008. Effect of different environmental conditions on the bacterial survival on stainless steel surfaces. Food Control 19:308-314.

Gill CO and Harris LM. 1982. Survival and growth of *Campylobacter fetus* subsp. *jejuni* on meat and in cooked foods. Applied and Environmental Microbiology 44: 259-263.

Gill CO, Dussault F, Holley RA, Houde A, Jones T, Rheault N, Rosales A, Quessy S. 2000. Evaluation of hygienic performances of the processes for cleaning, dressing and cooling pig carcasses at eight packing plants. International Journal of Food Microbiology 58:65-72.

Green CG, Krause DO, Wylie JL. 2006. Spatial analysis of *Campylobacter* infection in the Canadian province of Manitoba. International Journal of Health Geographics 5:2.

Greig J D and Ravel A. 2009. Analysis of foodborne outbreak data reported internationally for source attribution. International Journal of Food Microbiology 130:77-87.

Guan LL, Nkrumah JD, Basarab JA and Moore SS. 2008. Linkage of microbial ecology to phenotype: correlation of rumen microbial ecology to cattle's feed efficiency. FEMS Microbiology Letters 288:85-71.

Guillou S, Leguerinel I, Garrec N, Renard MA, Cappelier JM, Federighi M. 2008. Survival of *Campylobacter jejuni* in mineral bottled water according to differences in mineral content: Application of the Weibull model. Water Research 42:2213-2219.

Gürtler M, Alter T, Kasimir S, Fehlhaber K. 2005. The importance of *Campylobacter coli* in human campylobacteriosis: Prevalence and genetic characterization. Epidemiology and Infection 133:1081-1087.

Hänel I, Borrmann E, Müller J, Müller W, Pauly B, Liebler-Tenorio EM, Schulze F. 2008. Genomic and phenotypic changes of *Campylobacter jejuni* strains after passage of the chicken gut. Veterinary Microbiology 136:121-129.

Hansson I, Vågsholm I, Svensson L and Olsson Engvall E. 2007. Correlations between *Campylobacter* spp. prevalence in the environment and broiler flocks. Journal of Applied Microbiology 103:640- 649.

Hazeleger WC, Wouters JA, Rombouts FM, Abee T. 1998. Physiological activity of *Campylobacter jejuni* far below the minimal growth temperature. Applied and Environmental Microbiology 64:3917-3922.

Hazeleger WC, Janse JD, Koenraad PMFJ, Beumer RR, Rombouts FM, Abee T. 1995. Temperature-dependent membrane fatty acid and cell physiology changes in coccoid forms of *Campylobacter jejuni*. Applied and Environmental Microbiology 61:2713-2719.

Heuvelink AE, Heerwaarden CV, Zwartkruis-Nahuis A, Tilbirg JJHC, Bos MH, Heilmann FGC, Hofhuis A, Hoekstra T, Boer ED. 2009. Two outbreaks of campylobacteriosis associated with the consumption of raw cows' milk. International Journal of Food Microbiology 134:70-74

Huws SA, Edwards JE, Kim EJ, Scollan ND. 2007. Specificity and sensitivity of eubacterial primers utilized for molecular profiling of bacteria within complex microbial ecosystems. Journal of Microbiological Methods 70:565-569.

Inglis GD and Kalischuk LD. 2003. Use of PCR for direct detection of *Campylobacter* species in bovine feces. Applied and Environmental Microbiology 69 :3435-3447.

Jeon B, Itoh K, Ryu S. 2005. Promoter analysis of cytolethal distending toxin genes (cdtA, B, and C) and effect of a luxS mutation on CDT production in *Campylobacter jejuni*. Microbiology and Immunology 49:599-603.

Jozwiak A, Reichart O and Laczay P. 2006. The occurrence of *Campylobacter* species in Hungarian broiler chickens from farm to slaughter. Journal of Veterinary Medicine 53:291-294.

Kaakoush NO, Miller WG, De Reuse H, and Mendz GL. Oxygen requirements and tolerance of *Campylobacter jejuni*. Research in Microbiology 158:644-650.

Kärenlampi R and Hänninen ML. 2004. Survival of *Campylobacter jejuni* on various fresh produce. International Journal of Food Microbiology 97:187-195.

Kapperud G, Espeland G, Wahl E, Walde A, Herikstad H, Gustavsen S, Tveit I, Natas O, Bevanger L, Digranes A. 2003. Factors associated with increased and decreased risk of *Campylobacter* infection: A prospective case-control study in Norway. American Journal of Epidemiology 158:234-242.

Kelly FA, Park FS, Bovill R and Mackey MB. 2001. Survival of *Campylobacter jejuni* during stationary phase: evidence for the absence of a phenotypic stationary-phase response. Applied and Environmental Microbiology 67:2248-2254.

Kemp R, Leatherbarrow AJH, Williams NJ, Hart A, Clough HE, Turner J, Wright EJ, French NP. 2005. Prevalence and genetic diversity of *Campylobacter* spp. in environmental water samples from a 100-square-kilometer predominantly dairy farming area. Applied and Environmental Microbiology 71:1876-1882.

Khanna MR, Bhavsar SP and Kapadnis BP. 2006. Effect of temperature on growth and chemotactic behavior of *Campylobacter jejuni*. Letters in Applied Microbiology 43:84-90.

Kim J, Kim J, Kathariou S. 2008. Differential effects of temperature on natural transformation to erythromycin and nalidixic acid resistance in *Campylobacter coli*. Applied and Environmental Microbiology 74:6121-6125.

Klanĉnik A, Botteldoorn N, Herman L, Mozina SS. 2006. Survival and stress induced expression of *groEL* and *rpoD* of *Campylobacter jejuni* from different growth phases. International Journal of Food Microbiology 112: 200-207.

Klappenbach JA, Dunbar JM, Schmidt TM. 2000. rRNA operon copy number reflects ecological strategies of bacteria. Applied and Environmental Microbiology 66:1328-1333.

Knarreborg A, Simon MA, Engberg RM, Jensen BB and Tannock GW. 2002. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. Journal of Applied and Environmental Microbiology 68:5918-5924.

Knox BL, Van Laack RLJM, Davidson PM. 2008. Relationships between Ultimate pH and microbial, chemical, and physical characteristics of vacuum-packaged pork loins. Journal of Food Science 73:104-110.

Koidis P and Doyle PM. 1983. Survival of *Campylobacter jejuni* in fresh and heated red meat. Journal of Food Protection 46:771-774.

Koort J, Coenye T, Vandamme P and Björkroth J. 2005. *Streptococcus parauberis* associated with modified atmosphere packaged broiler meat products and air samples from a poultry meat processing plant. International Journal of Food Microbiology 106:318-323.

Kothary MH and Babu US. 2001. Infective dose of foodborne pathogens in volunteers: A review. Journal of Food Safety 21:49-73.

Kusumaningrum HD, Riboldi G, Hazeleger WC, Beumer RR. 2003. Survival of foodborne pathogens on stainless steel surface and cross-contamination to foods. International Journal of Food Microbiology 85:227-236.

Kuusi M, Nuorti JP, Hanninen ML, Koskela M, Jussila V, Kela E, Miettinen I, Ruutu P. 2005. A large outbreak of campylobacteriosis associated with a municipal water supply in Finland. Epidemiology and Infection 133:593-601.

Lee A, Smith SC and Coloe PJ. 1998. Survival and growth of *Campylobacter jejuni* after artificial inoculation onto chicken skin as a function of temperature and packaging conditions. Journal of Food Protection 61:1609-1614.

Lehner A, Schneck C, Feierl G, Pless P, Deutz A, Brandl E, Wagner M. 2000. Epidemiologic application of pulsed-field gel electrophoresis to an outbreak of *Campylobacter jejuni* in an Austrian youth centre. Epidemiology and Infection 125:13-16.

Li MY, Zhou GH, Xu XL, Li CB and Zhu WY. 2006. Changes of bacterial diversity and main flora in chilled pork during storage during PCR-DGGE. Food Microbiology 23:607-611.

Lienau J, Ellerbroek L and Klein G. 2007. Tracing flock-related *Campylobacter* clones from broiler farms through slaughter to retail products by pulsed-field gel electrophoresis. Journal of Food Protection 3:536-542.

Lindmark H, Diedrich C, Andersson L, Lindqvist R, Olsson Engvall E. 2006. Distribution of *Campylobacter* genotypes on broilers during slaughter. Journal of Food Protection 69:2902-2907.

Linton D, Owen RJ and Stanley J. 1996. Rapid identification by PCR of the genus *Campylobacter* and five *Campylobacter* species enteropathogenic for man and animals. Research in Microbiology 147:707-718.

Liu Y and Li J. 2008. Role of *Pseudomonas aeruginosa* biofilm in the initial adhesion, growth and detachment *of Escherichia coli* in porous media. Environmental Science and Technology 42:443-449.

Lopez I, Ruiz-Larrea F, Cocolin L, Orr E, Phister T, Marshall M, VanderGheynst J, Mills DA. 2003. Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69:6801-6807.

Luber P, Brynestad S, Topsch D, Scherer K, Bartelt E. 2006. Quantification of *Campylobacter* species cross-contamination during handling of contaminated fresh chicken parts in kitchens. Applied and Environmental Microbiology 72:66-70.

Luber P, Wagner J, Hahn H, Bartelt E. 2003. Antimicrobial resistance in *Campylobacter jejuni and Campylobacter coli* strains isolated in 1991 and 2001-2002 from poultry and humans in Berlin, Germany. Antimicrobial Agents and Chemotherapy 47:3825-3830.

Madden RH, Moran L and Scates P.2007. Diversity of *Campylobacter coli* genotypes in the lower porcine gastrointestinal tract at time of slaughter. Letters in Applied Microbiology 45:575-580.

Manfreda G, De Cesare A, Bondioli V and Franchini A. 2003. Ribotyping characterization of *Campylobacter* isolates randomly collected from different sources in Italy. Diagnostic Microbiology and Infectious Disease 47:385-392.

Mattick K, Durham K, Domingue G, Jørgensen F, Sen M, Schaffner DW, Humphrey T. 2003. The survival of foodborne pathogens during domestic washing-up and subsequent transfer onto washing-up sponges, kitchen surfaces and food. International Journal of Food Microbiology 85:213- 226.

McCarthy N and Giesecke J. 2001. Incidence of Guillain-Barré Syndrome following infection with *Campylobacter jejuni*. American Journal of Epidemiology 153:610-14.

McDonald, WL, Fry BN and Deighton MA. 2005. Identification of *Streptococcus* spp. causing bovine mastitis by PCR–RFLP of 16S–23S ribosomal DNA. Veterinary Microbiology 111:241-246.

Meldum RJ, Griffiths JK, Smith RMM, Evans MR. 2005. The seasonality of human *Campylobacter* infection and *Campylobacter* isolates from fresh, retail chicken in Wales. Epidemiology and Infection 133:49-52.

Mihaljevic RR, Sikic M, Klancnik A, Brumini G, Mozina SS, Abram M. 2007. Environmental stress factors affecting survival and virulence of *Campylobacter jejuni.* Microbial Pathogenesis 43:120-125.

Minihan D, Whyte P, O'Mahony M, Fanning S, McGill K, Collins D. 2004. *Campylobacter* spp. in Irish Feedlot Cattle: A longitudinal study involving pre-harvest and harvest phases of the food chain. Journal of Veterinary Medicine 51: 28-33.

Miwa N, Takegahara Y, Terai K, Kato H, Takeuchi T. 2003. *Campylobacter jejuni* contamination on broiler carcasses of C. jejuni-negative flocks during processing in a Japanese slaughterhouse. International Journal of Food Microbiology 84:105-109.

Mohammed KAS, Miles RJ, Halablab MA. 2004. The pattern and kinetics of substrate metabolism of *Campylobacter jejuni* and *Campylobacter coli*. Letters in Applied Microbiology 39:261-266.

Moore EJ and Madden HR. 2000. The effect of thermal stress on *Campylobacter coli*. Journal of Applied Microbiology 89:892-899.

Moore EJ and Madden HR. 2001. Survival of *Campylobacter coli* in porcine liver. Food Microbiology 18:1-10.

Murphy C, Carroll C and Jordan KN. 2003. Induction of an adaptive tolerance response in the foodborne pathogen, *Campylobacter jejuni.* FEMS Microbiology Letters 223:89-93.

Murphy C, Carroll C and Jordan KN. 2006. Environmental survival mechanisms of the food borne pathogens *Campylobacter jejuni*. International Journal of Food Microbiology 100:623-632.

Nattress, F, Sampathkumar, B., Gill CO and McMullen LM. 2009. Survival of *Campylobacter jejuni* in the presence of natural microflora on meats and in meat

processing environments. Interim report 2006F078R submitted to the Alberta Livestock Industry Development Fund, March 2009.

Nesbakken T, Eckner K and Rotterud O. 2008. The effect of blast chilling on occurrence of human pathogenic *Yersinia enterocolitica* compared to *Campylobacter* spp. and numbers of hygienic indicators on pig carcasses. International Journal of Food Microbiology 123:130-133.

Newell DG, Shreeve JE, Toszeghy M, Domingue G, Bull S, Humphrey T, Mead G. 2001. Changes in the carriage of *Campylobacter* strains by poultry carcasses during processing in abattoirs. Applied and Environmental Microbiology 67: 2636-2640.

Ng LK, Sherburne R, Taylor DE, Stiles ME. 1985. Morphological forms and viability of *Campylobacter* species studied by electron microscopy. Journal of Bacteriology 164:338-343.

Nicholson FA, Groves SJ and Chambers BJ. 2005. Pathogen survival during livestock manure storage and following land application. Bioresource Technology 96:135-143.

Nielsen EM, Fussing V, Engberg J, Nielsen NL, Neimann J. 2006. Most *Campylobacter* subtypes from sporadic infections can be found in retail poultry products and food animals. Epidemiology and Infection 134:758-767.

Nylen G, Dunstan F, Palmer SR, Anderson Y, Bager F, Cowden J, Feierl G, Galloway Y, Kapperud G, Megraud F, Molbak K, Peterson LR, Ruutu P. 2002. The seasonal distribution of *Campylobacter* infection in nine European countries and New Zealand. Epidemiology and Infection 128:383-390.

Olsson C, Ahrne S, Petterson B and Molin G. 2003. The bacterial flora of fresh and chill-stored pork: analysis by cloning and sequencing of 16S rRNA genes. International Journal of Food Microbiology 83:245-252.

Oyarzabal OA, Macklin KS, Barbaree JM and Miller RS. 2005. Evaluation of agar plates for direct enumeration of *Campylobacter* spp. from poultry carcass rinses. Applied and Environmental Microbiology 71:3351-3354.

Pearce RA, Bolton DJ, Sheridan JJ, McDowell DA, Blair IS, Harrington D. 2004. Studies to determine the critical control points in pork slaughter hazard analysis and critical control point systems. International Journal of Food Microbiology 90: 331-339.

Pearce RA, Wallace FM, Call JE, Dudley RL, Oser A, Yoder L, Sheridan JJ, Luchansky JB. 2003. Prevalence of *Campylobacter* within a swine slaughter and processing facility. Journal of Food Protection 66:1550-6.

Petersen RF, Harrington CS, Kortegaard HE and On SLW. 2007. A PCR-DGGE method for detection and identification of *Campylobacter, Helicobacter, Arcobacter* and related Epsilobacteria and its application to saliva samples from humans and domestic pets. Journal of Applied Microbiology 103:2601-2615.

Peyrat MB, Soumet C, Sanders MP. 2008. Recovery of *Campylobacter jejuni* from surfaces of poultry slaughterhouses after cleaning and disinfection procedures: Analysis of potential source of carcass contamination. International Journal of Food Microbiology 124:188-194.

Pitkälä A, Koort J and Björkroth J. 2008. Identification and antimicrobial resistance of *Streptococcus uberis* and *Streptococcus parauberis* isolated from bovine milk samples. Journal of Dairy Science 91:4075-4081.

Prouzet-Mauléon V, Labadi L, Bouges N, Ménard A, Mégraud F. 2006. *Arcobacter butzleri*: underestimated enteropathogen. Emerging Infectious Diseases 12:306-309.

Public Health Agency of Canada. 2001. Material Safety Data Sheet-Infectious Substances. Url http://www.phac-aspc.gc.ca/msds-ftss/msds29e.html. Accessed on 15 May 2009.

Public Health Agency of Canada. 2002. Comparison *of Campylobacter, Salmonella* and *E. coli* Human Cases. Url http://www.phac-aspc.gc.ca/publicat/ccdr-rmtc/98vol24/24s5/24s5g_e.html. Accessed on 15 may 2009.

Public Health Agency of Canada. 2002. Waterborne outbreak of gastroenteritis associated with a contaminated municipal supply, Walkerton, Ontario, May- June, 2000. Url http://www.phac-aspc.gc.ca/publicat/ccdr-rmtc/00vol26/dr2620eb.html. Accessed on 20 Febuary2009.

Purdy D, Cawthraw S, Dickinson JH, Newell DG, Park SF. 1999. Generation of a superoxide dismutase (SOD)-deficient mutant of Campylobacter coli: evidence for the significance of SOD in *Campylobacter* survival and colonization. Applied and Environmental Microbiology 65:2540-2546.

Ragimbeau C, Schneider F, Losch S, Even J and Mossong J. 2008. Multilocus sequence typing, pulsed-field electrophoresis and fla short variable region typing of clonal complexes of *Campylobacter jejuni* strains of human, bovine and poultry origins in Luxembourg. Applied and Environmental Microbiology 24:7715-7722.

Reysenbach A, Longnecker K and Kirshtein J. 2000. Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a mid-Atlantic ridge hydrothermal vent. Applied and Environmental Microbiology 66:3798-3806.

Richardson G, Thomas D, Smith RMM, Nehaul L, Ribeiro CD, Brown AG, Salmon RL. 2007. A community outbreak of *Campylobacter jejuni* infection from a chlorinated public water supply. Epidemiology and Infection 135:1151-1158.

Rivas L, Fegan N, Vanderlinde P. 2004. Isolation and characterization of *Arcobacter butzleri* from meat. International Journal of Food Microbiology 91:31-41.

Rivoal K, Denis M, Salvat G, Colin P, Ermel G. 1999. Molecular characterization of the diversity of *Campylobacter* spp. isolates collected from a poultry slaughterhouse: analysis of cross-contamination. Letters in Applied Microbiology 29:370-374.

Rivoal K, Ragimbeau C, Salvat G, Colin P, Ermel G. 2005. Genomic diversity of *Campylobacter coli* and *Campylobacter jejuni* isolates recovered from free-ranged broiler farms and comparison with isolates of various origins. Applied and Environmental Microbiology 10:6216–6227.

Robinson DA. 1981. Infectious dose of *Campylobacter jejuni* in milk. British Medical Journal 282:1584.

Rodrigo S, Adesiyun A, Asgarali Z and Swanston W. 2007. Antimicrobial resistance of *Campylobacter* spp. isolated from broilers in small poultry processing operations in Trinidad. Food Control 18:321-325.

Ronner AB and Wong ACL. 1993. Biofilm development and sanitizer inactivation of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and Buna-n rubber. Journal of Food Protection 56:750–758.

Saha SK, Saha S and Sanyal SC. 1991. Recovery of injured *Campylobacter jejuni* cells after animal passage. Applied and Environmental Microbiology 57:3388-3389.

Sahin O, Plummer PJ, Jordan DM, Sulaj K,Pereira S, Robbe-Austerman S, Wang L, Yaeger MJ, Hoffman LJ and Zhang Q. 2008. Emergence of a tetracycline-resistant *Campylobacter jejuni* clone associated with outbreaks of ovine abortion in the United States. Journal of Clinical Microbiology 46:1663-1671.

Sambrook J and Russell DW. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory Press, New York.

Sanders SQ, Boothe DH, Frank JF and Arnold JW. 2007. Culture and detection of *Campylobacter jejuni* within mixed microbial populations of biofilms on stainless steel. Journal of Food Protection 6:1379-1385.

Schmalenberger A, Schwieger F, Tebbe CC. 2001. Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. Applied and Environmental Microbiology 67:3557-3563.

Sexton M, Raven G, Holds G, Pointon A, Kiermeier A, and Sumner J. 2007. Effect of acidified sodium chlorite treatment on chicken carcasses processed in South Australia. International Journal of Food Microbiology 115:252-255.

Son, I, Englen MD, Berrang ME, Fedorka-Cray PJ, and Harrison MA. 2007. Prevalence of *Arcobacter* and *Campylobacter* on broiler carcasses during processing. International Journal of Food Microbiology 113:16-22.

Sorqvist S. 1989. Heat resistance of *Campylobacter* and *Yersinia* strains by three methods. Journal of Applied Bacteriology 67:543-549.

Steinhauserova I, Nebola M and Mikulicova M. 2005. Prevalence of thermophilic *Campylobacter* spp. in slaughtered pigs in the Czech Republic, 2001-2003. Veterinarni Medicina 50:171-174.

Stintzi A. 2003. Gene expression profile of *Campylobacter jejuni* in response to growth temperature variation. Journal of Bacteriology 185:2009-2016.

Talon R, Leroy S, Lebert I, Giammarinaro P, Charornac J, Latorre-Moratalla M, Vidal-Carou C, Zanardi E, Conter M and Lebecque A. 2008. Safety improvement and preservation of typical sensory qualities of traditional dry fermented sausages using autochthonous starter cultures. International Journal of Food Microbiology 126:227-234.

Tam CC, Rodrigues LC, O'Brien SJ and Hajat S. 2006. Temperature dependence of reported *Campylobacter* infection in England, 1989-1999. Epidemiology and Infection 134:119-125.

Tangwatcharin P, Chanthachum S, Khopaibool P, Griffiths WM. 2006. Morphological and physiological responses of *Campylobacter jejuni* to stress. Journal of Food Protection 69:2747-2753.

Tenkate TD and Stafford RJ. 2001. Risk factors for *Campylobacter* infection in infants and young children: a matched case control study. Epidemiology and Infection 127:399-404.

Thermo Scientific. 2008. Nanodrop 1000 Spectrophotometer v3.7 User's Manual. 5-2 and 5-3.

Tholozan JL, Cappelier JM, Tissier JP, Delatre G and Federighi M. 1999. Physiological characterization of viable but nonculturable *Campylobacter jejuni* cells. Applied and Environmental Microbiology 65:1110-1116.

Trachoo N, Frank JF and Stern NJ. 2002. Survival of *Campylobacter jejuni* in biofilms isolated from chicken houses. Journal of Food Protection 65:1110-1116.

Upmann M and Reuter G. 1998. The surface count on equipment and premises and the handling of hygiene in a meat cutting plant for pork. Food Science & Technology. Fleischwirtschaft 78(9):971-974

Vandamme P, Vancanneyt M, Pot B, Mels L, Hoste B, Dewettinck D, Vlaes L, Vandenborre C, Higgins R, Hommez J, Kersters K, Butler JP and Goossens H. 1992. Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. International Journal of Systematic Bacteriology 42: 344-356.

Varela NP, Friendship RM and Dewey CE. 2007. Prevalence of *Campylobacter* spp. isolated from grower-finishers pigs in Ontario. Canadian Veterinary Journal 48:515-517.

Velayudhan J and Kelly D. 2002. Analysis of gluconeogenic and anaplerotic enzymes *in Campylobacter jejuni*: an essential role for phosphoenolpyruvate carboxykinase. Microbiology 148:685-694.

Wang R, Cao W and Cerniglia CE. 1997. Phylogenetic analysis and identification of *Shigella* spp. by molecular probes. Molecular and Cellular Probes 11:427-432.

Walter J, Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Loach DM, Munro K, and Alatossava T. 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Applied and Environmental Microbiology 66:297–303.

Whyte P, McGill K, Cowley D,Madden RH, Moran L, Scates P, Carroll C, Whyte R, Hudson JA and Graham C. 2006. *Campylobacter* in chicken livers and their destruction by pan frying. Letters in Applied Microbiology 43:591-595.
Wilson IG. 2003. Antibiotic resistance of *Campylobacter* in raw retail chickens and imported chicken portions. Epidemiology and Infection 131:1181-1186.

Zhang L, Danon S, Grehan M, Lee A and Mitchell H. 2005. Template DNA ratio can affect detection by genus-specific PCR-denaturing gradient gel

electrophoresis of bacteria present at low abundance in mixed populations. Helicobacter 10:80-82.