EFFECT OF DIFFERENT ATMOSPHERES FOR ISOLATION, MOLECULAR CHARACTERIZATION, PATHOGENICITY GENE SCREENING, AND CONTROL OF *CAMPYLOBACTER* SPP. FROM PROCESSED BROILER MEAT

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VITA

Kauser (Syeda) Hussain, daughter of Dr. Syed Madoom Hussain and Shahzadi Hussain; older sister of Aquila Hussain was born on the 18th of August, 1979, in the city of Hyderabad, in the state of Andhra Pradesh, India. She graduated with a Bachelor of Science degree from Osmania University in June, 1999. She pursued her studies to attain her Master of Science degree in May, 2001 from the Department of Genetics, Osmania University. She earned the Council of Scientific and Industrial Research – Junior Research Fellow scholarship in June 2001 and subsequently the Senior Research Fellow scholarship in October 2003. She worked as a Research Fellow in the *E.coli* genetics lab of Dr. J. Gowrishankar at the Center for DNA Fingerprinting and Diagnostics, Hyderabad for three years. Motivated by her sister's move for further studies to the United States of America, she decided to continue pursuing a research career in the USA. In 2004 she joined the *Pseudomonas* laboratory of Dr. Sang-Jin Suh as a Graduate Teaching Assistant at Auburn University, AL. In 2005, she joined the Microbiology lab of Dr. Omar Oyarzabal at the Department of Poultry Science, Auburn, Alabama as a Graduate Research Assistant to pursue her Ph.D.degree. She wishes to continue research in the fields of Microbiology, Genetics and Molecular Biology.

DISSERTATION ABSTRACT

EFFECT OF DIFFERENT ATMOSPHERES FOR ISOLATION, MOLECULAR CHARACTERIZATION, PATHOGENICITY GENE SCREENING, AND CONTROL OF *CAMPYLOBACTER* SPP. FROM PROCESSED BROILER MEAT

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Our first experiments involved the studies of the effects of Oxyrase®, an enzyme based membrane system that selectively reduces oxygen from the medium, during the growth and isolation of *Campylobacter* spp. The effect of Oxyrase was tested without adding the microaerobic gas mixture for Bolton broth and an agar plate medium (modified Campy-Cefex). Based on chi-square analysis, no significant difference was found between enrichment broth cultured under microerobic conditions and enrichment broth that had Oxyrase.

Comparison between plate media, however, showed that plates supplemented with Oxyrase had the lowest recovery rate ($P < 0.05$). The percentage of similar group of organisms after denaturing gradient gel electrophoresis (DGGE) analysis was 85% and of the different group of organisms was 15%. The analysis of *Campylobacter* isolates collected from broth with and without Oxyrase for restriction fragment length polymorphism (RFLP) showed similar band patterns for some strains and different band patterns for other strains.

The second set of experiment examined if incubation under microaerobic conditions was indispensable for *Campylobacter* spp. to grow in Bolton broth. These experiments were also done with naturally contaminated broiler meat. Microscopy results from the two enrichment broth media showed no difference between the sub-samples for the growth and isolation of *Campylobacter* (chi square = 0.21). These results suggest that retail broiler meat enriched in Bolton broth may not need the addition of microaerobic gas mix for *Campylobacter* to multiply. DGGE showed the presence of different microbial niches in both the broth media. RFLP analysis showed band similarity for some samples and band differences for others.

The third set of studies involved the molecular characterization of *Campylobacter* strains from carcasses rinses from four commercial processing plants. Three plants (A, B and C) had similar percentages of *Campylobacter* positive carcass rinses: 78%, 78% and 62%, respectively. Plant D, however, showed the lowest percentage (33%) of *Campylobacter* positive carcasses. The mean log CFU/ml was similar for all the four plants and was less than 1 CFU/ml. Pulsed-field gel electrophoresis (PFGE) analysis

revealed a considerable diversity in the band patterns among the isolates when the rinse of a single broiler carcass was direct-plated on different plate media. These results confirmed the presence of more than one strain of *Campylobacter* spp. on a single carcass. *Campylobacter coli* from different plants showed similar PFGE patterns.

Next set of studies involved the screening of *C. jejuni* isolates for the presence of pathogenecity genes. Multiplex PCR assays were used to test for the presence of *flhB,* flagellar protein biosynthesis and export gene; *flgB,* flagellar basal body protein synthesis gene; *flgE2,* flagellar hook protein gene; *docA,* colonization and infectivity gene; and *cdtA,* a gene whose product makes the eukaryotic cells to be blocked in either G2 or early M phase. Multiplex PCR assays were successfully designed for the following gene combinations, *fliM and docA; flgE*$_{2,}$ *cdtA* and *flhB; and cadF and flgB.* Thirty-six *C. jejuni* isolates from one processing plant were positive for the presence of *flhB, flgB, flgE2* and *cdtA* genes, but were negative for *docA* gene. However, 58 *C. jejuni* isolates from another poultry processing plant were positive for all these genes, including *docA.*

In the last set of experiments, the effects of lemon based marinades were studied. Retail poultry meat positive for the presence of *Campylobacter* was used in these studies. The marinades that were used for the study included mesquite, baja chipotle, herb and garlic and real lemon. In inoculation trials, reduction in numbers of *Campylobacter* at the end of marination in combination with refrigeration at 0.5 h 6 h and 24 h were observed. In the case of naturally occurring *Campylobacters* on retail broiler meat, there was significant reduction in their numbers with all the marinades (baja chipotle, herb and garlic and real lemon) when marinated for 4 h under refrigeration was performed.

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CHAPTER I: INTRODUCTION

Campylobacter jejuni and *Campylobacter coli* are Gram negative, motile bacteria that multiply under microaerobic conditions. Campylobacteriosis is a common intestinal disorder in humans, and the infection is endemic worldwide, with highest incidence of this disease in summer. In the U.S., over 2.5 million persons are estimated to be affected annually. Nearly 80% of these infections are due to food-borne transmission (Mead *et al.*, 1999). The reservoirs of *Campylobacter* spp. are domestic and wild animals. Outbreaks of campylobacteriosis can be caused by the handling and consumption of uncooked poultry meat, although the largest outbreaks have been associated with contaminated water and unpasteurized milk (Fahey *et al.*, 1995; Palmer *et al.*, 1983; Vogt *et al.*, 1982; Vogt *et al.*, 1984) . Thus, the most important way to control campylobacteriosis depends on the prevention of the transmission from animal reservoirs to humans.

It is also very important to differentiate the strains in order to identify the source of contamination and to determine routes of transmission. This valuable information is useful to accurately detect outbreaks and control *Campylobacter* infections. Various molecular typing techniques, such as pulsed-field gel electrophoresis (PFGE) (Michaud *et al.*, 2001; Yan *et al.*, 1991), multi locus sequence typing (MLST) (Dingle *et al.*, 2001), and restriction fragment length polymorphism (RFLP) (Harrington *et al.*, 2003; Nachamkin *et al.*, 1993), have been used to assess the genomic variations of *Campylobacter* spp.

There is also a need to develop a quick method for screening the presence or absence of *Campylobacter* spp. that may carry pathogen city genes and that may be of higher concern for food safety. The polymerase chain reaction (PCR) technique is a quick method that can be used to identify an isolate and to screen for the presence of pathogen city genes.

Campylobacter spp. require low oxygen concentrations, which has been estimated to be approximately 5%. This is usually achieved by replacing the atmosphere within the enrichment broth or the plate media with a gas mix consisting of 5% O_2, 10% CO_2, and 85% N_2 (Veron and Chatelain, 1973; Kiggins and Plastridge, 1956). However, there is no current information of how high the oxygen concentration should be for campylobacters to grow and to isolate them from retail broiler meat.

The main reason for the use of marinades is to tenderize the meat. The antibacterial effects, as preservatives of the food to extend shelf life, are thus secondary functions. It is because of their low pH, high NaCl concentration, and various spices that marinades may have an antimicrobial effect (Perko-Makela, 2000). Few studies have assessed the effects of marination on the survival of *C. jejuni* on broiler meat stored at 4^oC (Perko-Makela, 2000).

The objectives of our studies were:

a) Evaluation of Oxyrase® for the isolation of *Campylobacter* spp. from retail broiler meat and microbial population analysis of enrichment media by denaturing gradient gel electrophoresis (DDGE) and *Campylobacter* strain comparison using restriction fragment length polymorphism (RFLP).

b) Evaluation of enrichment media without the microaerobic gas mixture for isolation of *Campylobacter* spp. from retail broiler meat.

c) Comparison of *Campylobacter* spp. prevalence in commercial processing plants and molecular characterization of isolates using pulsed-field gel electrophoresis (PFGE) analysis.

d) Development of multiplex polymerase chain reaction (mPCR) assays for the screening of virulence genes of *C. jejuni* isolated from processed broilers.

e) Evaluation of the efficacy of commercial, lemon-based marinades for the reduction of *C. jejuni* and *C. coli* on retail broiler meat.

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CHAPTER II: LITERATURE REVIEW

2.1. The History of *Campylobacter*

Theodor Escherich observed and described non-culturable spiral-shaped bacteria in 1886 (Escherich, 1989). The very first successful isolation of *Campylobacter* was done around the beginning of this century. McFadyean and Stockman in 1913 isolated Vibrio-like bacteria from aborted ovine fetuses (McFadyean, 1913) and proposed the name *Vibrio fetus* (Smith, 1919). Vinzent and coworkers in the late 1940s described *V. fetus* strains isolated from blood cultures from women who underwent abortions (Vinzent, 1949; Vinzent, 1947). Later Florent in 1959 showed two similar vibrios caused fertility problems in cows and ewes (Florent, 1957). Today these two similar vibrios are known as *Campylobacter fetus* subsp. *venerealis* and *Campylobacter fetus* subsp. *fetus*. In 1963 Sebald and Veron proposed a new genus, *Campylobacter* came into being (Sebald & Veron, 1963). Ten years after that Veron and Chatelain published a taxonomy describing four distinct species in the genus *Campylobacter*: *Campylobacter fetus, Campylobacter jejuni, Campylobacter coli* and *Campylobacter sputorum* (Veron, 1973).

2.2. *Campylobacter* – General Description

The phylum *Protobacteria*, class *Epsilonproteobacteria*, order *Campylobacterales*, family *Campylobacteraceae*, genus *Campylobacter* includes 18 species and sub-species.

The different species and sub-species can be identified by various phenotypic and biochemical tests (Table 2.1).

The *Campylobacteraceae* family members consist primarily of commensals or parasites in domestic animals and humans. *Campylobacter* is known to colonize the gastrointestinal tracts of a variety of host species like birds and other meat producing animals like pig and cattle. *Campylobacter* is a part of the normal gut flora in many animals. Though some of them are commensals *C. jejuni* and *C. coli* are pathogens of humans and some of the domestic animals. *Campylobacter* is a thermotolerant spiral, S-shaped, gram negative, non-spore forming rod. It is 0.2 to 0.8 μm wide and 0.5 to 5 μm long. It has a single polar unsheathed flagellum at one or on either ends of the cell which facilitates in its typical corkscrew movement. Old cultures usually form spherical or coccoid bodies. They are slow growing microaerobic organisms that require the oxygen concentration to be 5% to grow. It takes around 24 – 48 h at 42^{o}C for them to grow. They are intolerant to freezing and drying.

The genome size is 1.6 – 1.7 Mb except in the case of *C.upsaliensis* where it is 2.0Mb. The G-C content is around 30% (Owen, 1981) . Extrachromosomal elements like bacteriophages and plasmids have been found in *Campylobacter* spp. (Bacon et al., 2000; Bradbury et al., 1983)

2.3. *Campylobacter* and Food – Campylobacteriosis

C. jejuni and *C. coli* are the major causes of the diarrheal disease campylobacteriosis which is acute bacterial enteritis. In United States an estimated 2.4 million cases of campylobacteriosis per year are **recorded** (Allos & Blaser, 1995; Allos,

2001; Altekruse *et al.*, 1999). Infections are higher in summer and early fall in comparison to the rest of the year (Allos & Blaser, 1995). *C. jejuni* infections are seen in all age groups but infants and young adults seem more susceptible (Allos & Blaser, 1995). Symptoms include cramps and abdominal pain, diarrhea, malaise, myalgia, headache, fever, and/or the presence of blood and leukocytes in stool. Patients range from asymptomatic to severely ill and the symptoms last several days to more than a week.

Campylobacteriosis is principally a food borne disease (Figure 2.1) and is spread by handling and consumption of contaminated poultry meat (Butzler & Oosterom, 1991). *Campylobacter* infections are sporadic events, while some of the larger outbreaks have been associated with consumption of unpasteurized milk and/ or contaminated water (Fahey *et al.*, 1995; Vogt *et al.*, 1982; Vogt *et al.*, 1984).

Long term infections of *C. jejuni* lead to Guillian-Barre' syndrome (GBS) (Nachamkin *et al.*, 1998), an acute demyelinating polyneuropathy. Guillian, Barre', and Strohl in 1961 first described this syndrome defined by flaccid paralysis (Guillain, 1916). GBS is an autoimmune-mediated disorder of the peripheral nervous system. This condition leads to rapid muscle weakness which leads to weakness of limbs and of the respiratory muscles.

2.4. *Campylobacter* – Virulence Determinants

Campylobacter virulence determinants and their role in disease is one of the subjects important to research. The availability of the whole genome sequence of *Campylobacter* (Parkhill *et al.*, 2000) and the development of new ways to genetically manipulate this organism (Golden *et al.*, 2000) have contributed a lot to understanding

virulence factors and their role in causing the disease (Figure 2.2). Some of the important factors are discussed below:

Flagella and Motility

The ability of *campylobacter* to be a motile bacterium because of the presence of a polar flagellum plays an important role in its colonization and subsequently its disease causing ability. The *C. jejuni* flagellum is made of a basal body, hook, and filament. Both in *Campylobacter jejuni* and *Campylobacter coli* the flagellar filament is comprised of FlaA and FlaB proteins. Studies have shown that the *flaA* (Nuijten *et al.*, 1990; Nuijten *et al.*, 1991) is expressed at much greater levels than *flab* (Hendrixson et al., 2001). Wassenaar et al. in there studies found that *Campylobacter* expressing *flaA* gene facilitated maximal colonization (Wassenaar *et al.*, 1993). Other researchers have also shown that motility is important and promotes colonization of *Campylobacter jejuni* in animal hosts (Nachamkin *et al.*, 1993; Pavlovskis *et al.*, 1991). In another study it was observed that the flagellar structure played an essential role in internalization process of *C. jejuni* and it was independent of motility (Grant *et al.*, 1993).

Adhesins and Adherence

Molecules that are surface exposed and that facilitate a pathogen's attachment to the host cell receptors are called adhesins. *C. jejuni* has been found to synthesize adhesins. Some studies showed *C. jejuni* binding to cultured cells at levels comparable to metabolically active organisms (Konkel & Cieplak, 1992). Four outer membrane proteins (omps) playing an important role in mediating *C. jejuni* binding to host cells using

ligand-binding assay with molecular masses of 28, 32, 36, and 42 kDa were identified by De Melo and Pechere (de Melo & Pechere, 1990). The 28 kDa omp, was termed as PEB1 and mediates the binding of *C. jejuni* to epithelial cells (Pei *et al.*, 1998). A 37 kDa omp from *C. jejuni* was cloned and partially characterized by Konkel et al (Konkel *et al.*, 1997). This is termed as CadF, and it mediates the binding of *C. jejuni* to fibronectin (Fn) which is a component of extracellular matrix of the host cell. Studies have shown that CadF is conserved among all *C. jejuni* and *C. coli* isolated tested to date (Konkel *et al.*, 1999a).

Secretion

Campylobacter invasion antigens (Cia proteins) are a set of proteins secreted by *Campylobacter jejuni.* To date only one secreted protein CiaB has been completely identified (Konkel *et al.*, 1999b). The *ciaB* gene encodes a protein of mass 73 kDa. Confocal microscopic studies of *C. jejuni*-infected cells have shown the translocation of CiaB protein into the host cell cytoplasm. Some preliminary studies by Konkel et al have shown that Cia proteins may be secreted via the flagellar type III secretion apparatus.

Cytolethal Distending Toxin (CDT)

Johnson and Lior in 1988 first reported the production of CDT by *Campylobacter* isolates. CDT is produced by number of *Campylobacter* strains like *C. jejuni*, *C. coli*, *C. lari*, *C. fetus*, and *C. upsaliensis* (Johnson & Lior, 1988; Mooney *et al.*, 2001). Actively proliferation eukaryotic cells die when treated with CDT. Variable sensitivity to CDT in different cell lines has been reported, which may be because of differences in their

surface receptors (Pickett & Whitehouse, 1999). The CDT toxin was first cloned by Pickett et al (Pickett *et al.*, 1996). The toxin was found to be encoded by three adjacent genes, *cdtA*, *cdtB*, *cdtC* (Pickett *et al.*, 1996). CDT is heat-labile and sensitive to trypsin treatment (Johnson & Lior, 1988). Even though CdtB is the active subunit of the toxin (Lara-Tejero & Galan, 2000) all the three components of the toxin are important for its activity and delivery (Hickey *et al.*, 2000; Lara-Tejero & Galan, 2001). The effects of CDT on a eukaryotic cell are as follows, progressive cell distention by causing cells to irreversibly arrest in G_2/M phase of cell cycle; CDT prevents the dephosphorylation of CDC2 which is a catalytic subunit of the cyclin-dependent kinase which causes the cells to arrest in G_2 phase (Lara-Tejero & Galan, 2000; Whitehouse *et al.*, 1998).

2.5. *Campylobacter* Enrichment and Growth Conditions

The presence of other bacteria in a sample is always a competition for *Campylobacter* spp. which is a fastidious, slow growing organism that require complex media to grow (Corry *et al.*, 1995). That is why it is important to include an enrichment step when trying to isolate *Campylobacter* from a sample that contains other micro-organisms. Most of the *Campylobacter* species require a microaerobic atmosphere containing approximately 5% O_2, 10% CO_2, and 85% N_2 for optimal recovery.

Several types of liquid enrichment media have been developed for isolation of *Campylobacter* from poultry meat (Corry *et al.*, 1995; Doyle, 1981; Hunt, 2001) . Typically enrichment broths are composed of rich, basal medium, such as Brucella broth or nutrient broth (Bolton & Robertson, 1982; Corry *et al.*, 1995), antimicrobials, and addition of lysed horse or sheep blood. Buffered Peptone Water (BPW) the recommended

medium by U. S. Department of Agriculture Food Safety and Inspection Services for collection of microbiological samples from broiler carcasses with rinse method (Anonymous, 1996) has been successfully used as an enrichment medium for isolation of *campylobacter* from retail broiler samples (Oyarzabal *et al.*, 2007).

Primary plating on selective media in combination with a filtration method is the optional method for recovering *Campylobacter* species from food, environmental and clinical samples. To isolate *C.jejuni* and *C.coli* a number of selective media are recommended (Table 2.2) which include the blood-containing Skirrow medium, the Campylobacter-cefoperazone-vancomycin-amphotericin (CVA) medium, the blood-free charcoal cefoperazone deoxycholate agar (CCDA), charcoal based selective medium (CSM). Most of these selective media have one or more antimicrobial agents, mainly cefoperazone, as the primary inhibitor of enteric bacterial flora. The incubation temperature is usually 42^{o}C; this allows the growth of *C.jejuni* and *C.coli* on selective media. *Campylobacter* spp. produce grey, flat, irregular, swarming colonies, on freshly prepared plate media.

2.6. Oxyrase

Several oxygen-quenching agents are traditionally added to agar plates for the isolation of *Campylobacter* spp. These agents neutralize the toxic effects of oxygen radicals, and include blood or alkaline hematin (Border *et al.*, 1974) charcoal (Hutchinson & Bolton, 1984), iron salts and norepinephrine to brucella agar (Bowdre *et al.*, 1976) and ferrous sulfate, sodium metabisulfite, and sodium pyruvate (FBP supplement) (George *et al.*, 1978), among others. Oxyrase®, an enzyme system that

selectively removes oxygen from its surrounding environment, has also been tested for the isolation of *Campylobacter* spp. from food samples that have been artificially inoculated with *Campylobacter* (Wonglumsom *et al.*, 2001). This enzyme is a sterile suspension of *Escherichia coli* membrane fragments and functions as an oxygen scavenger (Adler *et al.*, 1981) in liquid and solid media. The cytochrome electron transport system located within the cytoplasmic membrane appears to be responsible for the oxygen-quenching activities of the membranes fragment (Schnaitman, 1970); (Adler *et al.*, 1981). The Oxygen combines with the protons in the matrix of the mitochondrion and gets reduced to water (Figure 2.3).

2.7. Detection and Characterization by Various Molecular Techniques

At both the phenotypic and genotypic levels the diversity within species of *Campylobacter* has been well defined. The widely employed phenotypic classification method is serotyping. The two accepted and well-established methods are the Penner scheme (Penner & Hennessy, 1980) which is a passive hemagglutination technique based on heat-stable (HS) antigens and the Lior scheme which is based on bacterial agglutination of of heat-labile antigens (Lior *et al.*, 1982). High numbers of untypeable strains, time consuming protocol and the cost has prevented the use of the above serological methods.

Molecular identification methods exploit the DNA sequences which are conserved and specific to that particular species. These methods are quick, very discriminative and reliable, hence these days they have taken over most of the traditional methods of detection and identification. There are plenty of molecular methods available,

but some of them are advantageous over others, (Table 2.3) sometimes some methods are cheaper than the other. Hence, depending on a lab's resources and requirements some methods are preferred over the others.

Multiplex Polymerase Chain Reaction (mPCR)

Multiplex PCR is similar to PCR except that there are more than one pair of primers in the reaction mixture. The very first use of PCR was to amplify the number of copies of a specific region of DNA, in order to produce enough DNA to be adequately tested. A PCR reaction mix is made up of one set primers (forward and reverse), a thermostable DNA polymerase the DNA template to be amplified and synthetic oligonucleotides. Usually, at least a million-fold increase of a specific section of a DNA molecule can be achieved in a single PCR reaction. The basic PCR reaction involves a denaturation step, annealing step and an elongation step, these three steps are repeated 30-40 times to get the desired amplification.

An example of multiplex PCR primers (Table 2.4): primers specific to the *cadF* gene region, for *Campylobacter* genus identification; primers specific to an unidentified gene for *C. jejuni* identification; and primers specific to *ceuE* gene for *C.coli* identification (Cloak & Fratamico, 2002) . A second multiplex reaction (Table 2.3) consists primers for 16S rDNA gene for *Campylobacter* genus, hippuricase gene primers for *C. jejuni* and aspartokinase gene primers for *C. coli* identification respectively (Oyarzabal *et al.*, 2005) . Both the above multiplex reactions have been successfully used to identify *Campylobacter* genus, and *C. jejuni*, *C. coli* species.

In recent years, PCR has been successfully applied to detect *Campylobacter* spp. in water, dairy products, and diarrheic samples (Linton *et al.*, 1997). PCR can be used to not only detect but also to differentiate various *Campylobacter* species (Al Rashid *et al.*, 2000; Oyarzabal *et al.*, 1997). It has been proven to be one of the quick and discrete methods for species and genus identification (Oyarzabal *et al.*, 2007). This technique allows the detection not only of viable bacteria but also of the non-culturable forms of *Campylobacter*. In the case of *Campylobacter* specific sequences like fragments of 16S or 23S rRNA genes and their intervening sequences, or parts of regions of the flagellin genes (*fla*) or part of gene encoding binding-protein-dependent transport system for sidephore enterochelin (*ceuE*) (Gonzalez *et al.*, 1997) can be targets for species identification. For example, PCR primers specific to16S rRNA gene or 23S rRNA gene (Eyers *et al.*, 1993) are used to detect *Campylobacter*, primers specific to hippuricase (*HipO*) gene are be used to detect *C. jejuni*, primers specific to the enterochelin uptake system (*ceuE*) gene are used for detection of *C. coli*.

Pulsed-Field Gel Electrophoresis (PFGE)

One of the significant advances in technology related to mapping of genomes of organisms over the past 25 years has been Pulsed-Field Gel Electrophoresis (PFGE). The new way to separate large fragments of DNA, PFGE was 1st introduced by Schwartz and Cantor in 1984 (Schwartz & Cantor, 1984). Conventional electrophoresis, a well established technique by the early 1970's permits the separation of DNA fragments up to 30kbp, thus fragments greater than 40-50kbp which are important for whole genome analysis or genome mapping cannot be separated. The ability of this technique to resolve

DNA fragments ranging from 6000kb (Gardiner, 1991) to very large fragments with size limit to over 10,000kb (10Mb). That's why it is often considered the "gold standard" of molecular typing methods.

PFGE is one of the most discriminating methods for sub-typing *Campylobacter* spp. (Michaud *et al.*, 2001; On *et al.*, 1998; Yan *et al.*, 1991). It can be used to establish the degree of relatedness among different strains of the same species based on the DNA band patterns at the end of the elecrophoresis gel run.

PFGE works by periodically altering the electric field orientation. The large extended coiled DNA fragments are forced to change orientation and size dependent separation is re-established because the time taken for the DNA to reorient is size dependent (Figure 2.4). Two factors that influence DNA migration rates through conventional gels are charge differences between DNA fragments & 'molecular sieve' effect of DNA pores.

For PFGE, bacterial isolates grown, either in broth or on solid media are combined with molten agarose and poured into small molds. The results are agarose plugs containing the whole bacteria. The embedded bacteria are then subjected to in situ detergent-enzyme lysis and digestion with an infrequently cutting restriction enzyme. The digested bacterial plugs are then inserted into an agarose gel and subjected to electrophoresis in an apparatus in which the polarity of the current is changed at predetermined intervals. The pulsed field allows clear separation of DNAs from a few kilobases (kb) to over 10 megabase pairs (Mb).

The electrophoretic patterns are visualized following staining of the gels with a fluorescent dye such as ethidium bromide. Gel results can be photographed, and the data can be stored by using one of the commercially available digital systems.

For subtyping of *Campylobacter* spp. PFGE is one of the most discriminatory method currently available (On *et al.*, 1998). The PulseNet, the National Molecular SUbtyping Network for Foodborne Diseases, Center for Disease Control and Prevention (CDC), in 1996 introduced a system where standardized PFGE protocols by PulseNet were used to compare DNA fingerprints of various pathogens between different laboratories. A protocol has also been developed for molecular sub-typing *Campylobacter jejuni* and other *Campylobacter* species (Ribot *et al.*, 2001).

One of the factors that have limited the use of PFGE is the time involved in completing the analysis. While the procedural steps are straightforward, the time needed to complete the procedure can be 2 to 3 days. This can reduce the laboratory's ability to analyze large numbers of samples.

Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) is a molecular technique in which organisms may be differentiated by analysis of patterns derived from restriction of their DNA. If two organisms differ in the distance between sites of restriction of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another. The regions amplified are usually between 150-3,000 base pairs in length. The slight differences

between organisms of different species sometimes can cause the addition or removal of a restriction endonuclease recognition site. DNA can be processed by RFLP either directly or by using PCR product.

In the case of *Campylobacter* the restriction fragment length polymorphism of an amplified segment of the *fla*A gene (PCR-RFLP) is a common approach (Harrington *et al.*, 2003; Nachamkin *et al.*, 1993), when one is looking for polymorphisms within the same PFGE patterns, or the strains that fail to give a PFGE pattern. Methodology includes PCR amplification, restriction of the amplicons with *Dde*I and separation of different fragments on the agarose gel and then analysis.

Some advantages of RFLP are, results are consistent and can be used for future comparison work and it is relative easy to perform. Limitations are its less sensitive in phylogenetic resolution (in comparison to sequencing).

Denaturing Gradient Gel Eectrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis (DGGE) is a powerful genetic analysis technique that can be used for detecting single base changes and polymorphisms in genomic, cloned, and PCR amplified DNA. It is an important genetic fingerprinting technique used when there is a need to separate individual sequences from a complex mixture. It is one of the fast molecular sub-typing methods which facilitates separation of equal lengths of DNA fragments that have differences in base sequences (Myers *et al.*, 1985; Orita *et al.*, 1989). The DGGE technique was originally formulated for detecting DNA polymorphisms and single base changes. In the present day DGGE has evolved to become a very important molecular tool in the area of microbial ecology. It is useful for

analyzing and characterize a bacterial community (Dabert *et al.*, 2001; Muyzer & Smalla, 1998; Simpson *et al.*, 1999). It is also useful to subtype bacterial species within a large population (Bennasar *et al.*, 1998; Farnleitner *et al.*, 2000a; Farnleitner *et al.*, 2000b; Manzano *et al.*, 1997). DGGE is particularly advantageous when a metagenomic approach is used to determine the population of microorganisms that are difficult to culture.

The basic principle of DGGE is the electrophoretic separation of amplified PCR products of equal length in a sequence specific way. The sequence-specific separation of DNA molecule of similar size is based on melting temperature Tm of the double stranded (ds) DNA (Figure 2.5).

The DGGE gel is made up of a denaturing gradient consisting of varying concentrations of urea and formamide. Thus when the double stranded DNA is exposed to the increasing denaturant environment it melts in distinct segments, called the melting domains. The Tm of individual domains is sequence specific and is based on the G-C content of that DNA molecule. Thus in the presence of a denaturing environment a DNA molecule may have different melting domains determined by the nucleotide sequence. The mobility of the DNA molecules is slowed down at the point where DNA strands dissociate forming a partially single stranded molecule with no further movement in the gel. The GC clamp that is artificially incorporated during the amplification step of the sample of interest before the DGGE gel run prevents the complete denaturation of the DNA strands thus creating a ‘Y’ structured molecule (Myers *et al.*, 1985). This branched molecule sharply reduces the mobility in a polyacrylamide gel.

Universal primers 338 F, containing the G + C clamp at its 5' end, and 518R are commonly used for the amplification of the 16S rRNA gene (Muyzer *et al.*, 1993; Sheffield *et al.*, 1989). The amplicon size is around 180 bases long. Subtyping of *C. jejuni* and *C. coli* without culturing the sample has been done using DGGE. A variable region of approximately 170-210 base pairs from the 3V end of *flaA* to the 3V end of the intergenic region that separates the tandemely oriented *flaA* and *flaB* genes was used (Studer E., 1998).

Applications of DGGE include a rapid and effective method for screening samples for genetic mutations and variantions. Also, DNA fragment melting points can be determined using perpendicular DGGE. Denaturing gradient gels are used to detect non-RFLP polymorphisms. Limitations of this technique are it's not easy to work with and works well only for short (<500 bp) fragments of DNA.

2.8. Marination of Meat for Control of *Campylobacter* spp.

Marination is an age old method used to preserve foods. Various organic acid based marinades have been in use to prevent the growth of microorganisms in meat products. Applying marinade to meat or poultry enhances its flavor (Chen, 1982), increases its moisture retention and tenderness (Ang, 1987), preserves its color (Cassidy, 1977), and inhibits the warmed over flavor (Mahon, 1963). The spices and extracts that are added to the marinades also have preservative and antioxidant properties which are antifungal, antibacterial and antiviral (Farag, 1989; Larrondo, 1995). The antibacterial activity is based on low pH, high NaCl concentration, and various spices added to the marinade (Perko-Makela, 2000). If the marination of meat is done for a long time there

may be a possibility in reduction in its antimicrobial activity. Some researchers have concluded that the buffering capability of meat may neutralize the acidic marinade and thus the marinade may become less acidic and less antimicrobial (Bjorkroth *et al.*, 2005). In one of the studies (Perko-Makela, 2000) of effects of marination on *Campylobacter jejuni*, the survival of *C. jejuni* on marinated chicken drumsticks and sliced breasts strips stored at 4^{o}C was studied. There was no significant difference in *C. jejuni* numbers in between the marinated and non-marinated chicken meat. The marinade that was used was an emulsion of vegetable oil, water, spices and salt.

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Table 2.1. Phenotypic and Biochemical tests for detection of *Campylobacter* species

Organism	Catalase	Nitrate reduction	Hippurate hydrolysis	Indoxyl acetate hydrolysis	Growth at		Susceptibility to	
					25°C	42°C	Nalidixic acid	Cephalothin
C.jejuni subsp.*jejuni*	+	+	+	+	-	+	Sensitive	Resistant
C.jejuni subsp. *doylei*	Variable	-	Variable	+	-	-	Sensitive	Sensitive
C.coli	+	+	-	+	-	+	Sensitive	Resistant
C.fetus subsp.*fetus*	+	+	-	-	+	-	Variable	Sensitive
C.lari	+	+	-	-	-	+	Resistant	Resistant
C.upsaliensis	Weak	+	-	+	-	+	Sensitive	Sensitive

Table 2.2. Composition of different plate media

Adaptation from (Oyarzabal *et al.*, 2005)

Medium	Base	Supplements
Campy-Cefex	Brucella agar[a] (43 g/l); ferrous sulfate[b] (0.5 g/l); sodium bisulfite[b] (0.2 g/l); sodium pyruvate[b] (0.5 g/l); deionized water (1 l).	Laked horse blood[c] (50 ml); cefoperazone[b] (33 mg); cycloheximide[b] (0.2 g).
Modified Campy-Cefex	Brucella agar (43 g/l); ferrous sulfate (0.5 g/l); sodium bisulfite (0.2 g/l); sodium pyruvate (0.5 g/l); deionized water (1 l).	Lysed horse blood[d] (50 ml); cefoperazone (33 mg); amphotericin B[b] (2 mg).
CAMPY	Brucella agar (43 g/l); deionized water (1 l).	Lysed horse blood (70 ml); polymyxin B sulfate[e] (1 mg); trimethoprim[b] (0.01 g); vancomycin[b] (0.01 g); amphotericine B (2 mg); novobiocin[b] (0.05 g); sodium pyruvate (0.25 g); sodium metabisulfite (0.25 g); ferrous sulfate (0.25 g).
Modified CCDA[f]	Nutrient broth No.2 (25 g/l); bacteriological charcoal (4 g/l); casein hydrolysate (3 g/l); sodium desoxycholate (1 g/l); ferrous sulphate (0.25 g/l); sodium pyruvate (0.25 g/l); agar (12 g/l); deionized water (1 l).	Cefoperazone (32 mg); amphotericin B (10 mg).
Karmali[g]	Columbia agar base (39 g/l); activated charcoal (4 g/l); hemin (0.032 g/l); deionized water (1 l).	Sodium pyruvate (100 mg); cefoperazone (32 mg); vancomycin (20 mg); cycloheximide (100 mg).
Campy-Line	Brucella agar (43 g/l); ferrous sulfate (0.5 g/l); sodium bisulfite (0.2 g/l); sodium pyruvate (0.5 g/l); alpha ketoglutaric acidb (1 g/l); sodium carbonateb (0.6 g/l); deionized water (1 l).	Heminb (10 mg); polymyxin B sulfate (0.35 mg); trimethoprim (5 mg); vancomycin (10 mg); cycloheximide (100 mg); cefoperazone (33 mg); triphenyltetrazolium chlorideb (200 mg).

[a] Acumedia, Lansing, MI
[b] Sigma-Aldrich, St. Louis, MO
[c] Oxoid Inc., New York, NY
[d] College of Veterinary Medicine, Auburn University.
[e] Alexis Corporation, Lausen, Switzerland.
[f] Campylobacter Selective Blood Free Agar (CM0739), and CCDA selective supplement (SR0155, Oxoid)
[g] Campylobacter agar base (CM0935), and Campylobacter selective supplement (SR0167, Oxoid)

Table 2.3. Advantages and disadvantages of various genotyping methods and serotyping for sub-typing of campyobacters

Adapted from (Wassenaar & Newell, 2000)

Method	Discriminatory Power[a]	Typeability (%)[b]	Reproducibility[c]	Sensitivity to Genetic Instability	Time Required	Cost	Availability
PCR	Good	100	Good	Most of the times	4 - 5 h	Low	Good
fla typing	Reasonable	100	Good	Yes	< 1 day	Low	Good
DGGE	Very Good	100	Good	Yes	3 - 4 days	Average	Limited
PFGE	Good	100	Good	Yes	3 - 4 days	Average	Limited
Sequencing	Very Good	100	Good	NA[d]	2 - 3 days	Average	Limited
Serotyping	Average	~80	Good[e]	NA	< 1 day	Low	Limited

[a]The discriminatory power of a method is its ability to differentiated between genetically unrelated strains.
[b]Typeability is 100% if all strains tested are typed by a given method.
[c]The reproducibility of a method is determined by its ability to identify duplicate samples. This is irrespective of external factors, such as genetic instability.
[d]NA, not applicable.
[e]Weak serological cross-reactions can complicate interpretation of data. Multiple passages may be required to fully express antigens.

Table 2.4. New and old set of multiplex PCR primers used for genus and species identification of *Campylobacter*

	Primers	**Sequences (5'-3')**	**Tm (^{o}C)**	**References**
500	CC18F	GGTATGATTTCTACAAAGCGAG	51	Linton et al. 1997
	CC519R	ATAAAAGACTATCGTCGCGTG	50	
344	HipO-F	GACTTCGTGCAGATATGGATGCTT	56	Persson and Olsen 2005
	HipO-R	GCTATAACTATCCGAAGAAGCCATCA	56	
1062	16S-F	GGAGGCAGCAGTAGGGAATA	55	Persson and Olsen 2005
	16S-R	TGACGGGCGGTGAGTACAAG	56	
400	F2B	TTGAAGGTAATTTAGATATG	44	Konkel et al. 1999
	RIB	CTAATACCTAAAGTTGAAAC	43	
894	COLI	ATGAAAAAATATTTAGTTTTTGCA	54	Gonzalez et al. 1997
	COL2	ATTTTATTATTTGTAGCAGCG	52	
160	C-I	CAAATAAAGTTAGAGGTAGAATGT	51	Winters & Slavik 1995
	C-2	GGATAAGCACTAGCTAGCTGAT	55	

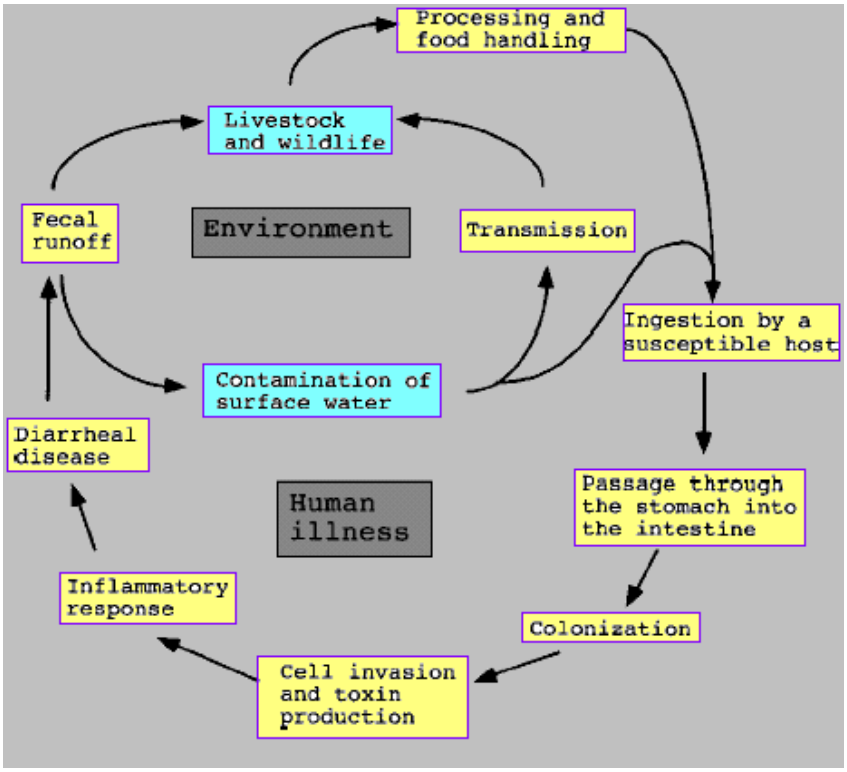

Figure 2.1. The movement of *C.jejuni* in the environment involves animals, water and food.

Adapted from (Konkel *et al.*, 2001)

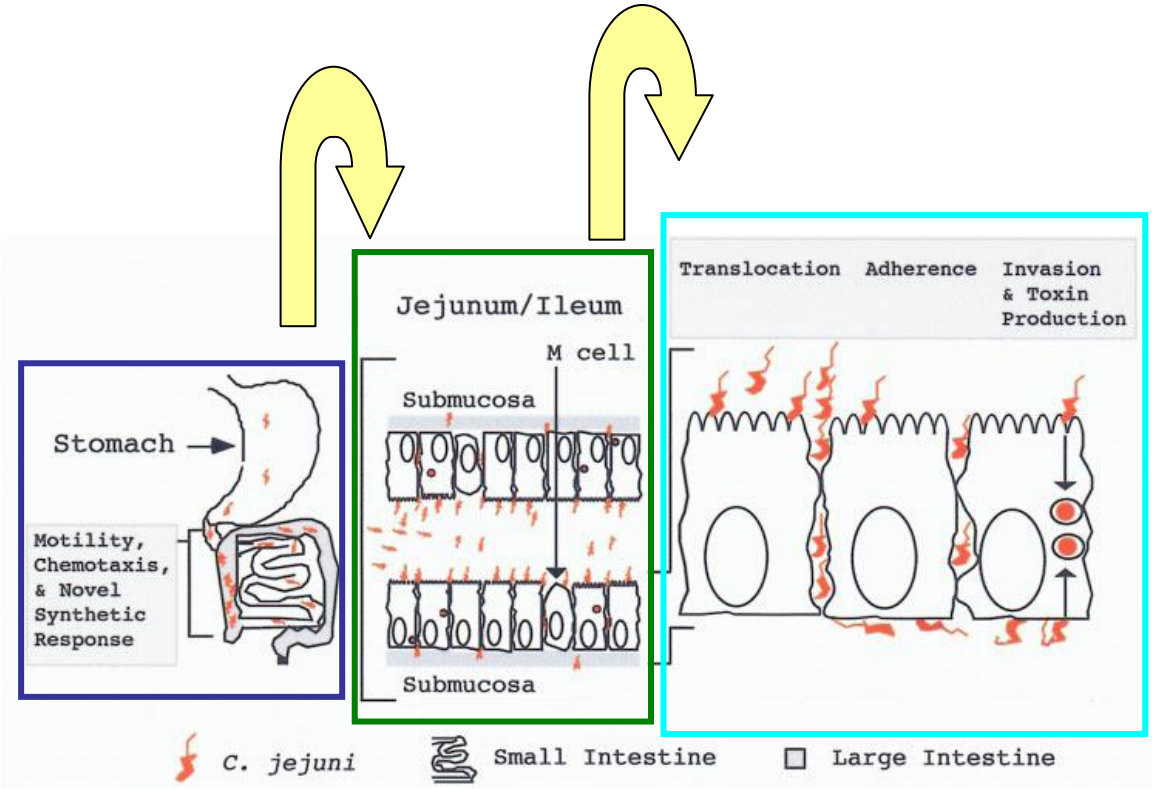

Figure 2.2. Depiction *C.jejuni* virulence determinants and their role in development of the disease. Adaptation from (Konkel *et al.*, 2001)

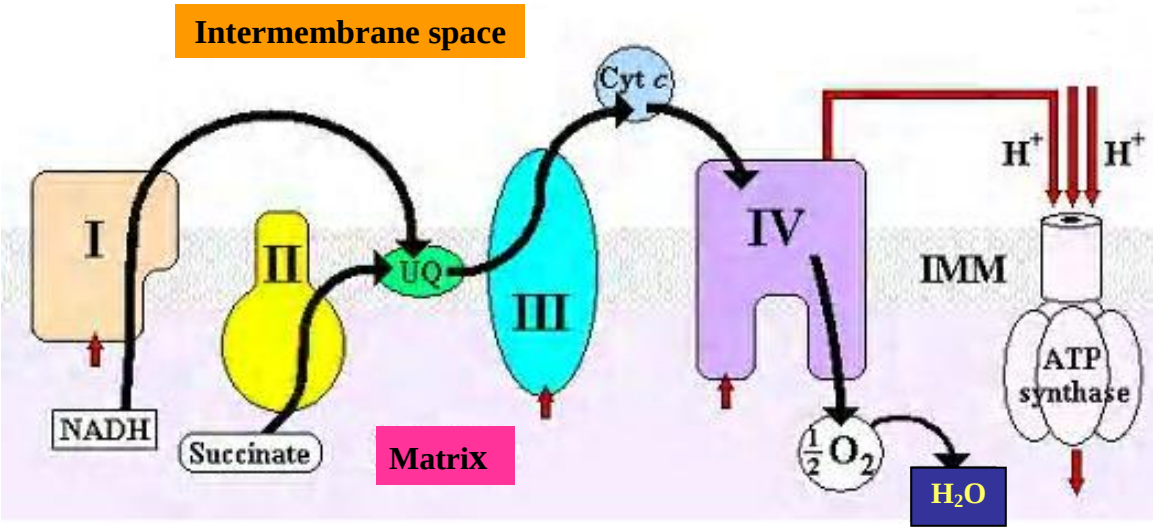

Figure 2.3. Cytochrome electron transport system (ETC) located in cytoplasmic membrane (Adaptation of figure from wikimedia.org)

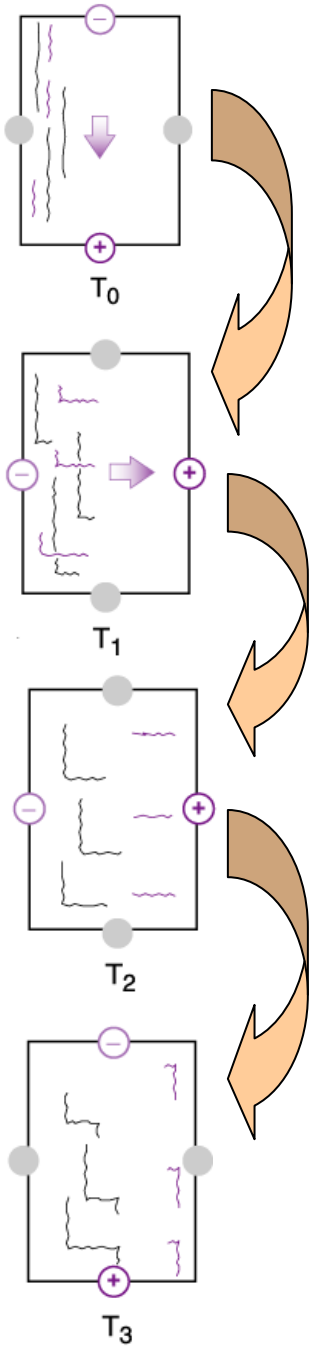

Figure 2.4. ***Principle of Pulsed-Field Gel Electrophoresis:*** Alternating electric current is applied to the field to move DNA fragments of 100 -1,000 kb. DNA molecules "squirm" through agarose and migrate parallel to the electric field. When the current is reversed; it takes more time (T) for large molecules to re-orient than smaller molecules.

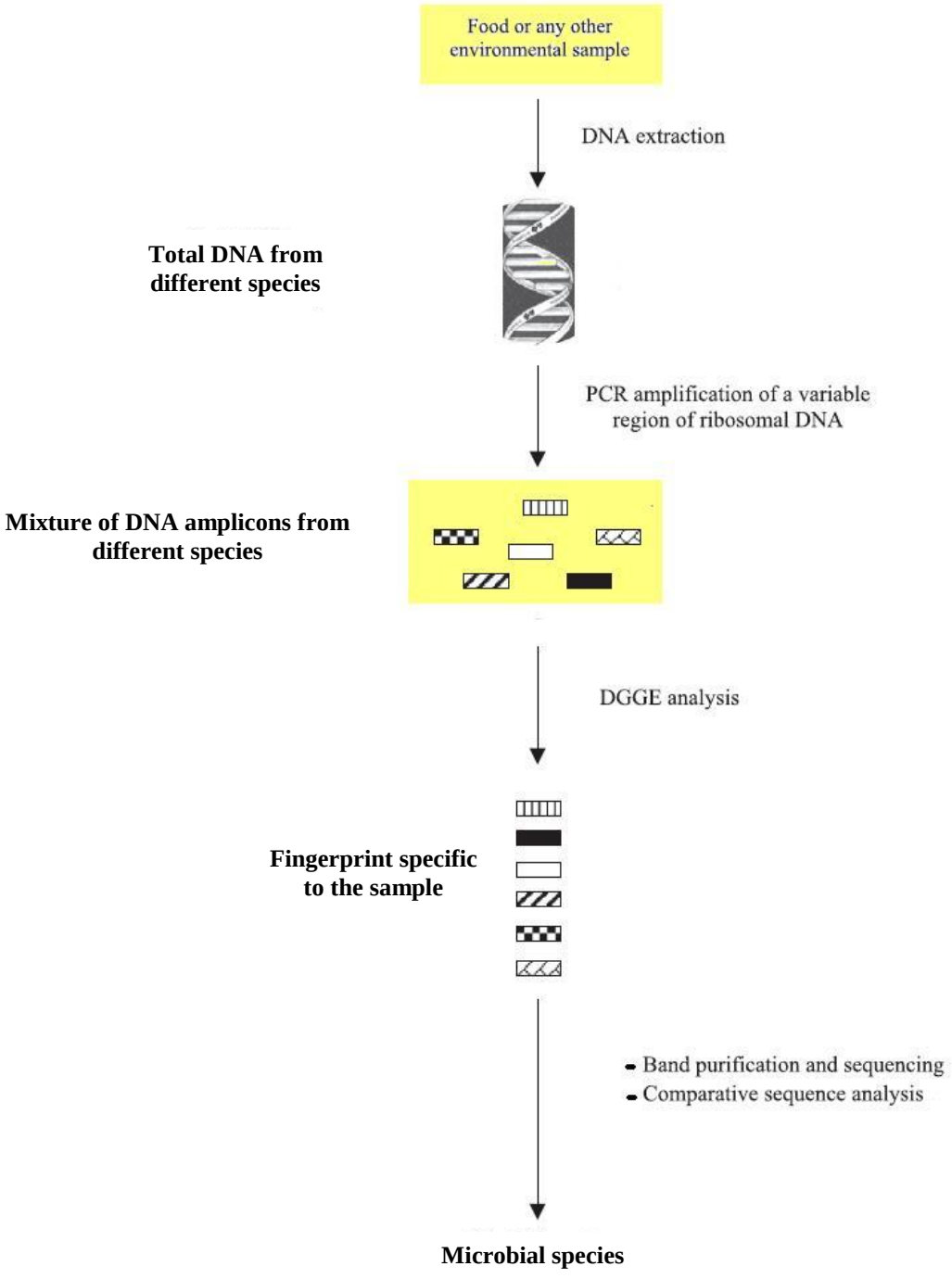

Figure 2.5. Flow diagram – Denaturing Gradient Gel Electrophoresis procedure. Adapted from (Ercolini, 2004)

CHAPTER III: EVALUATION OF OXYRASE® FOR THE ISOLATION OF *CAMPYLOBACTER* SPP. FROM RETAIL BROILER MEAT

3.1. Introduction

Campylobacter is a slow growing, Gram negative bacterium that requires microaerobic conditions, usually 5% oxygen, for growth. A standard gas mix containing 5% oxygen, 10% carbon dioxide and 85% nitrogen mix is commonly used for isolation of *C. jejuni* and *C. coli*. However, the makeup of the gas mix varies because some *Campylobacter* spp. appear to need hydrogen for growth and some mixes contain seven to 10% hydrogen in their compositions (Skirrow, 1991).

Different methods for the generating of microaerobic environments have been successfully used to grow *Campylobacter*. Some of these methods include the use of sachets (Oxoid BR56, Oxoid, UK) that generate carbon dioxide (Sails, 1998). Other systems include the evacuation-replacement system (Microbiology International, Frederick, MD), and microaerobic workstations (Don Whitley Scientific Ltd., West Yorkshire, UK). Gas-generating sachets and the evacuation-replacement systems have also been used with success with plastic bags used to freeze food products. In these cases, the bags have to have a strong closing system, usually a "ziplock" type, to prevent air from leaking during incubation (Szalanski *et al.*, 2004).

Several oxygen-quenching agents are traditionally added to all selective media to improve the isolation of *Campylobacter* spp. These agents neutralize the toxic effects of oxygen radicals and include lysed horse blood, defibrinated blood from various animals alkaline hematin (Corry *et al.*, 1995; Border *et al.*, 1974; Solomon, 1999). Several agar media use a combination of ferrous sulfate, sodium metabisulfite and sodium pyruvate as oxygen-quenching agents (George *et al.*, 1978). Charcoal and hematin are used in some charcoal based agar media (Hutchinson and Bolton, 1984), and iron salts and norepinephrine are added to Brucella agar to remove oxygen from the medium (Bowdre *et al.*, 1976).

Oxyrase®, an enzyme system that selectively removes oxygen from its surrounding environment, has also been tested for the isolation of *Campylobacter* spp. from inoculated food samples (Wonglumsom *et al.*, 2001). This enzyme is a sterile suspension of *Escherichia coli* membrane fragments and functions as an oxygen scavenger in liquid and solid media (Adler *et al.*, 1981). The cytochrome electron transport system located within the cytoplasmic membrane appears to be responsible for the oxygen-quenching activities of the membranes fragment (Adler *et al.*, 1981; Schnaitman, 1970).

Although this enzyme was tested for the growth of *Campylobacter* spp. in inoculated food samples, there are no publication evaluating the efficacy of Oxyrase addition to enrichment and plate media for the isolation of naturally occurring *Campylobacter* spp. in retail broiler meat. In this study we added Oxyrase to Bolton broth and to modified Campy-Cefex agar that were used to isolate naturally occurring *Campylobacter* spp. from commercial, retail broiler meat. Presumptive *Campylobacter*

spp. were identified with multiplex polymerase chain reaction assays and were analyzed using *fla*A-restriction fragment length polymorphism. In addition, denaturing gradient gel electrophoresis analysis was done to determine the microbial composition of Bolton broth with and without the addition of Oxyrase. The percentage of similar group of organisms growing in both Bolton and Bolton-Oxyrase broths was 85%.

3.2. Materials and Methods

Sample Preparation

Retail broiler meat was purchased from local stores. Five to six samples of boneless breasts, breast tenders and boneless thighs were purchased per week, from January 2008 to October 2008, for a total of 131 samples.

From each individual meat package representing a sample, one inch pieces were cut aseptically and thoroughly mixing. Then, 25 g of meat was weighed two times (two subsamples) in individual, sterile Whirl-Pak® bags (Nasco, Fort Atkinson, WI). One of the sub-samples was enriched by the addition of 100 ml of Bolton's broth supplemented with 5% (v/v) lysed horse blood (Oyarzabal *et al.*, 2007). The other sub-sample was enriched with 100 ml Bolton's broth supplemented with 5% (v/v) lysed horse blood and 2% of Oxyrase for Broth™ (Oxyrase, Inc. Mansfield, Ohio). This enzyme was added after the medium was autoclaved and cooled. Samples were stomached for 1 min and the sub-samples enriched in Bolton's broth were incubated under microaerobic conditions (85% nitrogen, 10% carbon oxidize, 5% oxygen; Airgas, Radnor, PA) using anaerobic jars gassed with a MACSmics Jar Gassing System (Microbiology International, Frederick, MD). The sub-samples containing Bolton's broth with Oxyrase were not

gassed with the microerobic mix. These samples were just tightly closed leaving a reduce head space in the bags.

All samples were incubated at 42^{o}C for 48 h. At 24 and 48 h, samples were transferred with a loop (~100 µl) to modified Campy-Cefex (mCC) (Oyarzabal *et al.*, 2005) agar plates. To assess the efficacy of the addition of Oxyrase to agar plates for the isolation of *Campylobacter* spp., samples were also transferred to mCC supplemented with 10% of Oxyrase for Agar™ (Oxyrase, Inc.) The enzyme was mixed with the medium and poured into normal petri dishes (Fisher) and into OxyDish™ (Oxyrase, Inc.) plates as suggested by the manufacturer. All the plates, except the mCC plates supplemented with Oxyrase and OxyDish with Oxyrase, were incubated under microaerobic conditions. All plates were incubated at 42^{o}C for up to 48 h.

Presumptive *Campylobacter* colonies were observed under phase contrast microscopy (Optiphot-2, Nikon, Tokyo, Japan) for morphology and motility of *Campylobacter* cells. Presumptive isolates were stored at -80^{o}C in tryptic soy broth (Difco, Detroit, MI) supplemented with 30% glycerol (v/v) and 5% (v/v) lysed horse blood for further analysis.

Identification of Campylobacter Isolates

Campylobacter isolates were recovered by depositing the stock cultures on a 0.65 µm cellulose filters (Millipore, Billerica, MA, USA) that was placed on mCC agar plates. Plates were incubated for 24 h at 42^{o}C under microaerobic conditions. Bacterial DNA was extracted using PrepMan™ Ultra sample preparation reagent (Applied Biosystems, Foster City, CA).

All *Campylobacter* isolates were identified to the species level using two multiplex PCR (mPCR) assays described elsewhere (Oyarzabal *et al.*, 2005; Oyarzabal *et al.*, 2007).

Subtyping of Campylobacter *Isolates*

The restriction fragment length polymorphism of an amplified segment of the *fla*A gene (PCR-RFLP) was carried out as described elsewhere (Harrington *et al.*, 2003; Nachamkin *et al.*, 1993). Briefly, PCR amplification was done in 25 μl aliquots in a PTC-100 Programmable Thermal Controller (Bio-Rad Laboratories, Hercules, CA). Amplicons were restricted with *Dde*I and DNA bands were detected in 2.5% SeaKem® Gold Agarose (Lonza, Basel, Switzerland) that were stained with ethidium bromide. Gels were visualized using a UV transilluminator (Gel-Doc System, Syngene, Frederick, MD) and a TIFF file was created using a computer program (GeneSnap) for molecular analysis (Syngene, Frederick, MD).

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

Universal primers 338 F, containing the G + C clamp at its 5' end, and 518R were used for the amplification of the 16S region gene (Muyzer *et al.*, 1993; Sheffield *et al.*, 1989). The amplification consisted of 30 cycles of 5 min of denaturation at 94^{o}C, 1 min of annealing at 55^{o}C, and 1 min of extension at 72^{o}C. The presence of PCR amplicons was confirmed by running the amplions on a 1.5% agarose gel. The denaturing gradient for the DGGE run in vertical polyacrylamide gels ranged from 45%-65%. Ingeny electrophoresis system (Ingeny phorU, Netherlands) at a voltage of 100V for 16-18 h was

used for separation of DGGE bands. Gels were stained in ethidium bromide and visualized under the UV imager. A marker strain was used in every DGGE gel run to have a standard for further analysis in Bionumerics version 4.50 (Applied Maths, Austin, TX) software program to analyze various strain and their genotypic profiles.

Sequencing

The primers used for sequencing were 338F, without the G + C clamp, and the 518R (Muyzer *et al.*, 1993; Sheffield *et al.*, 1989). The amplification consisted of 30 cycles of 5 min of denaturation at 94^{o}C, 1 min of annealing at 55^{o}C, and 1 min of extension at 72^{o}C. The presence of amplions was confirmed by running the PCR-amplified products on a 1.5% agarose gel. Amplicons were purified using the columns from Promega Wizard SV Gel (Madison, WI) and PCR clean-up system (Promega, Madison, WI). The purified amplicons along with the forward and reverse primers were sent out for sequencing to Lucigen (Middleton, WI), where the amplified products were sequenced using an ABI 3730 sequencer (Applied Biosystems). The assembly of the consensus sequence from the forward and reverse run was done with MultAlin (http://bioinfo.genotoul.fr/multalin/multalin.html) and ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The percentage similarity of the consensus sequences was calculated with the nucleotide Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical Analysis

Positive/negative data between treatments (Bolton versus Bolton with Oxyrase) were analyzed with the McNemar's Test (McNemar, 1947) and the Kappa coefficient (Cohen, 1960) using Proc Freq in SAS (SAS version 9.1; SAS Institute Incorporated, Cary, NC). A McNemar's test value ≤ 3.84 assumes the null hypothesis; that means that the test method (Oxyrase) and reference method (Bolton broth) are equivalent and cannot be rejected at the 5% level of confidence ($P < 0.05$). The Kappa coefficient is used to quantify the agreement between two raters in placing the sample of subjects into two categories. Therefore, Kappa is a chance-corrected measure of agreement between two raters. Each rater classifies each of a sample of subjects into one of two mutually exclusive categories (King, 2004). Kappa values are between 0 and 1. A Kappa value of 1.0 implies a perfect agreement. In general, Kappa values of 0.20 and 0.40 are considered a “fair agreement”, while values between 0.40 and 0.60 are considered “moderate agreement” (McNemar, 1947).

3.3. Results

The isolation rate from Bolton (microaerobic) and Bolton Oxyrase (regular atmosphere) broths did not result in significant differences (chi-square = 0.46) (Table 3.1). mCC + Oxyrase in OxyDish performed similarly to control plate media (chi-square value 1.39) (Table 3.2). However, mCC + Oxyrase in petri dishes (bagged) resulted in less number of isolates than the control plate media ($P < 0.05$) (Table 3.3).

The DGGE analysis and sequencing results from the enrichment broths, with and without the addition of Oxyrase showed that the majority of the bands (85%) were similar among the treatment (Figure 3.1), indicating the growth of similar kinds of microbial populations in both the control and test enrichment medium.

DGGE analysis showed the presence of different bands in the same samples but different liquid enrichment media (Figure 3.1). The various groups of organisms in each enrichment broth that differed are listed in the table (Table 3.4).

The multiplex PCR results (Figure 3.4) showed that in some cases different species were colonizing different sub-samples. In addition, some subsamples showed the presence of both *C. jejuni* and *C. coli* after enrichment (Figure 3.4, lanes 7 and 9).

RFLP band patterns (Figure 3.5) of sub-samples showed that in most of the cases the same strain was present in both sub-samples. However, in some cases different strains from the same *Campylobacter* spp. were colonizying the subsamples (Figure 3.5, lanes 3 and 4).

3.4. Discussion

As Bolton's broth and Bolton's broth with Oxyrase did not yielded significantly different numbers of positive samples, it may be concluded that Oxyrase works well in creating microaerobic environments in liquid enrichment media. However, Oxyrase in plate medium yielded significantly different results from the regular plating medium mCC, and it can be concluded that the use of Oxyrase in agar plates is not as efficient for the isolation of *Campylobacter* spp. from retail boiler meat.

Even though Oxyrase in liquid enrichment medium and Oxyrase in OxyDish

perform as good as the control plating medium, the cost may be a limiting factor for the incorporation of these systems in food microbiology laboratories.

One of the advantages of DGGE is that it is a culture-independent method and can be used to characterize and supervise the total microbial population in different niches. The basic principle of DGGE is the electrophoretic separation of amplified PCR products of equal length in a sequence specific way. The gel is made up of a denaturing gradient comprised of urea and formamide. The sequence-specific separation of DNA molecule of similar size is based on melting temperature Tm of the double stranded (ds) DNA. Thus when the dsDNA is exposed to the increasing denaturant environment it will melt in distinct segments, called the melting domains. The double stranded DNA will start becoming single stranded, thus DNA becomes partially melted at the Tm of lowest melting domain creating branched molecules. This branched molecule sharply reduces the mobility in a polyacrylamide gel thus allowing separation of amplicons which show even a single nucleotide substitution.

We found that five bands were almost consistently appearing throughout the samples. These bands were named A through E and were used as a marker at the end of each DGGE gel to standardize the gels and create a fingerprinting profile in BioNumerics. This fingerprinting profile allowed us to make comparison on the electrophorectic mobility of a partially melted double-strand DNA to evaluate the number of similar bands among the different treatments. The first run of sequencing for these bands gave us identity values that varied from 96% to 82%, and the top band was similar to *Acinetobacter* sp. (accession number FJ216156.1) the lowest band similar to Lactobacillus sp. (accession number AF159000.1) (Table 4.2).

The calculation of the GC content of these sequences (http://www.genomicsplace.com/gc_calc.html) showed that *Acinetobacter* had a GC content of 54.2%, and *Lactobacillus* sp. had a GC content of 47.1%. Therefore the spread of the electrophoretic runs spanned approximately 7% in the GC content from the highest to the lowest band.

DGGE analysis showed differences in bands patterns in the same samples but different liquid enrichment media indicating different set of organisms being selected in these two types of enrichment methods. The bands that were not common to both the media were processed and then sequenced. The percentage of similar group of organisms growing both in Bolton and Bolton oxyrase enrichment media was 85% and of the different group of organisms which were either present or absent in either Bolton or in Bolton Oxyrase enrichment media was 15%.

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Table 3.1. Plate media, mCC (Bolton) vs mCC (Bolton Oxyrase)

		mCC (C)		Total
		+	-	
Bolton Oxy to	+	20	15	35
mCC (CB)	-	18	78	96
Total		38	93	131

Chi square, as defined by McNemar's Test = 0.46

Kappa Coefficient is between 0 and 1

Table 3.2. Plate media, mCC vs mCC + Bolton Oxyrase in an OxyDish

		mCC (C)		
		+	-	Total
Oxydish + mCC oxy (COX)	+	6	12	18
	-	6	17	23
Total		12	29	41

Chi square, as defined by McNemar's Test = 1.39

Kappa Coefficient is between 0 and 1

Table 3.3. Plate media, mCC vs mCC + Bolton Oxyrase in a Petri Plate

		mCC (C)		Total
		+	-	
Petri plates + mCC oxy (COB)	+	7	5	12
	-	18	46	64
Total		25	51	76

Chi square, as defined by McNemar's Test = 8.52

Kappa Coefficient is between 0 and 1

Table 3.4. Results after BLAST analysis of the sequences

Marker		Number	Bacterial strain	% similarity	Accession Number
A		K 1	Uncultured *Acinetobacter* sp.	96%	FJ216156.1
B		K 2	Uncultured Nitrospirae bacterium	85%	AF540054.1
C		K 3	*Lactobacillus* sp. L21	87%	AF159000.1
D		K 4	*Lactobacillus saerimneri*	95%	AY255802.1
E		K 5	*Lactobacillus* sp. L21	82%	AF159000.1
Bolton	**Bolton oxyrase**				
	Lane 6	K 6	*Pseudomonas syringae*	98%	DQ512737.1
	Lane 6	K 7	Uncultured bacterium	79%	EU873625.1
Lane 7		K 8	*Campylobacter subantarcticus*	97%	AM933374.1
Lane 9		K 9	Uncultured bacterium	87%	FJ035391.1
	Lane 10	K 10	Uncultured bacterium	94%	EF089410.1
	Lane 10	K 11	*Campylobacter* sp. 87/06	88%	EU623475.1
Lane 11		K 12	Uncultured *Pantoea* sp.	98%	AM711573.1
Lane 13		K 13	*Lactobacillus saerimneri*	98%	AY255802.1
	Lane 16	K 14	Uncultured *Acinetobacter* sp. clone SS1B08	90%	FJ216156.1
Lane 17		K 15	*Lactobacillus* sp. L21	96%	AF159000.1
	Lane 18	K 16	*Campylobacter subantarcticus*	96%	AM933374.1

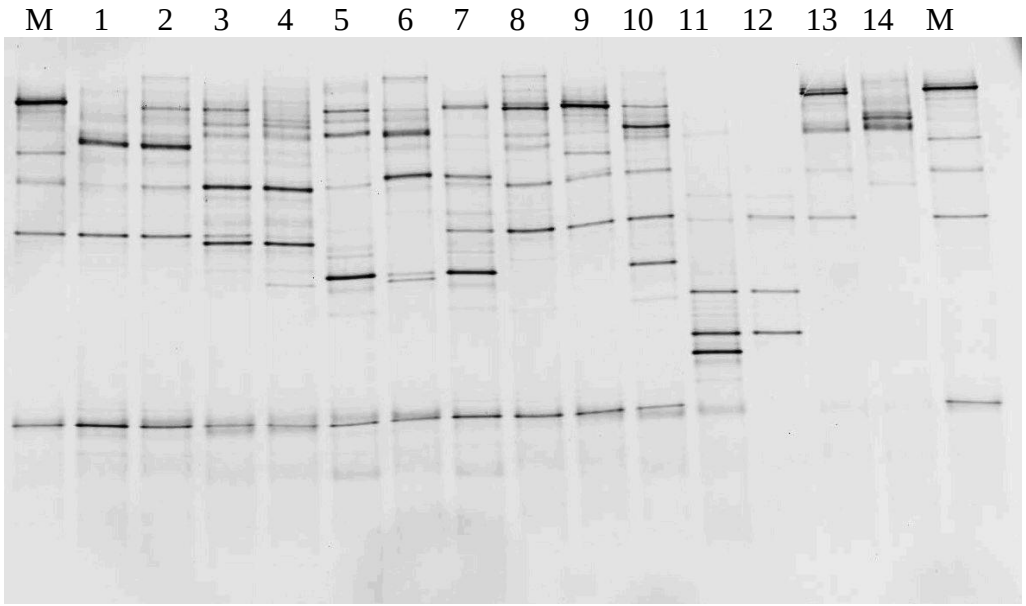

Figure 3.1. DGGE band patterns, Lane M-marker; Lanes 1, 3, 5, 7, 9, 11, 13, = Bolton samples 13, 14, 15, 16, 17, 19, 20;

Lanes 2, 4, 6, 8, 10, 12, 14 = Bolton + Oxyrase samples 13, 14, 15, 16, 17, 19, 20

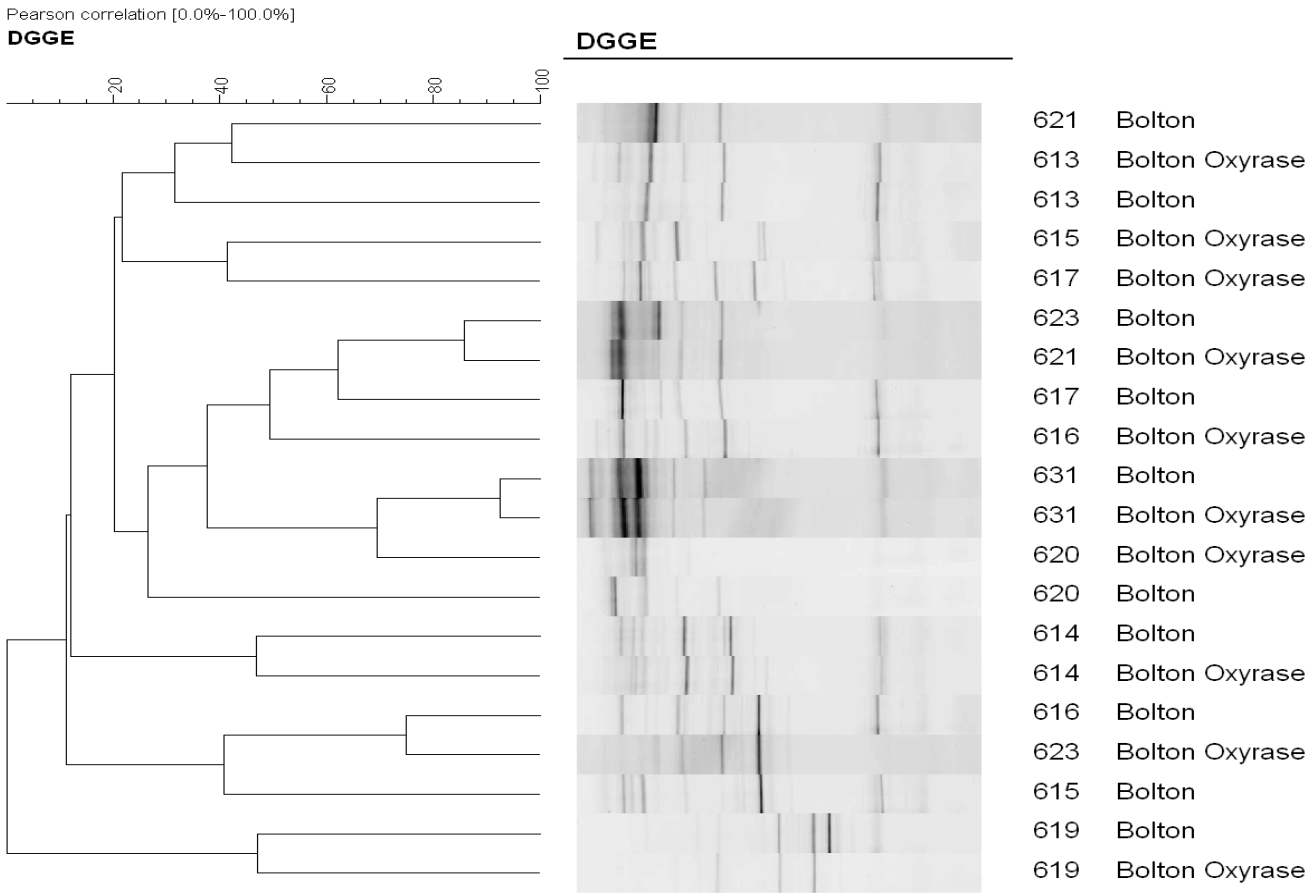

Figure 3.2. Dendrogram, Bolton and Bolton Oxyrase – Pearson's correlation

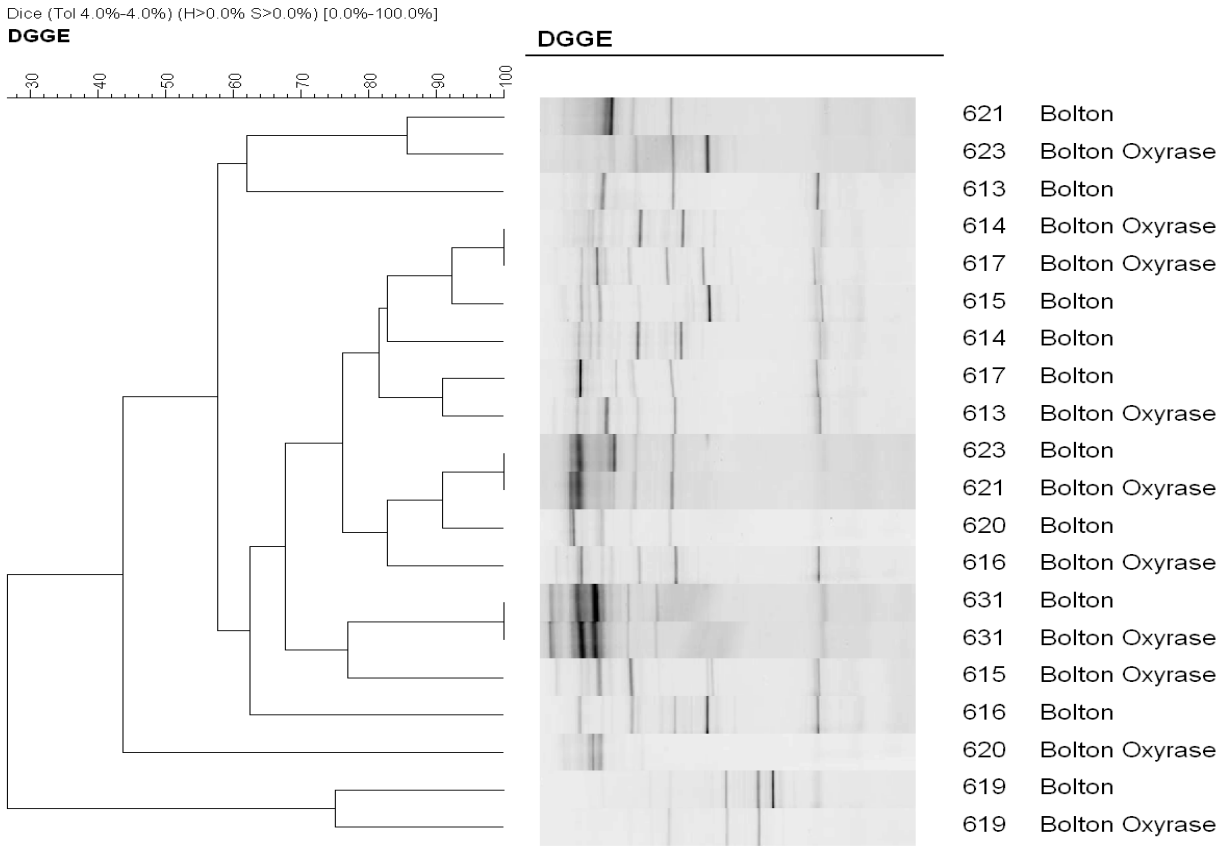

Figure 3.3. Dendrogram, Bolton and Bolton Oxyrase – Dice's correlation

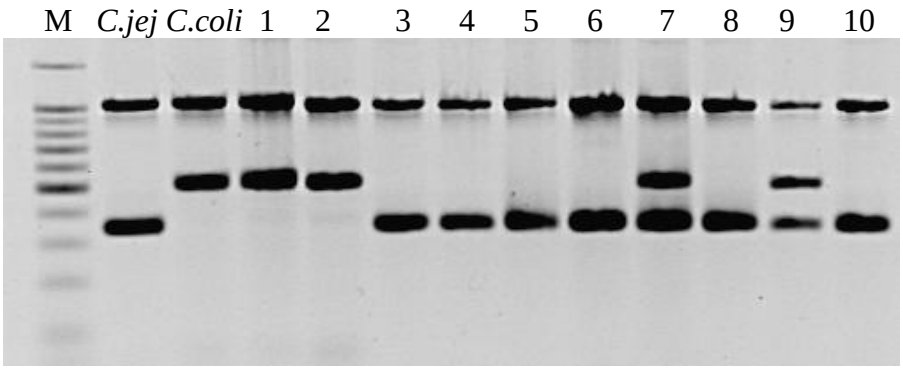

Figure 3.4. Multiplex PCR results, Lane M - marker, Lanes 1, 3, 5, 7, 9 Bolton samples ; Lanes 2, 4, 6, 8, 10 corresponding Bolton oxyrase samples

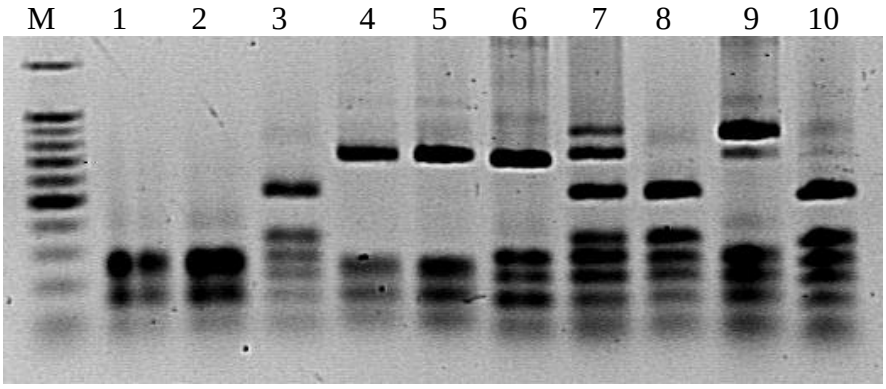

Figure 3.5. RFLP results, Lane M – marker, Lanes 1, 3, 5, 7, 9 Bolton samples ; Lanes 2, 4, 6, 8, 10 corresponding Bolton oxyrase samples

CHAPTER IV: MICROBIOLOGICAL AND MOLECUAR METHODS

EVALUATION OF ENRICHMENT MEDIA WITHOUT THE MICROAEROBIC GAS MIXTURE FOR GROWTH OF *CAMPYLOBACTER* SPP.

4.1. Introduction

Campylobacter is a Gram negative bacterium that can grow at a concentration of 5% or less of oxygen. Hence the standard atmospheric mixture containing 5% oxygen, 10% carbon dioxide and 85% nitrogen is commonly used in the isolation of *C. coli* and *C. jejuni*. However, the composition of the gas mix varies. Some *Campylobacter* spp. appear to need hydrogen for growth and some mixes contain 7 to 10% of hydrogen in their compositions (Skirrow, 1991).

Originally, a candle placed in a jar was used for the generation microaerobic environment for the growth of *Campylobacter* spp. However, over the years different methods for the generating of microaerobic environments have been successfully used to grow *Campylobacter* from clinical or food samples. Some of these methods include the use of sachets (for instance Oxoid BR56, Oxoid, UK) that generate carbon dioxide from sodium bicarbonate and citric acid, and hydrogen from sodium borohydride, with a catalyst (palladium) that converts water from hydrogen and oxygen (Sails, 1998). Some newer sachets quench the oxygen in the jar and generate carbon dioxide without the production of hydrogen (for instance CampyGen™, Oxoid). Other systems include the

evacuation-replacement system (Microbiology International, Frederick, MD), which uses a standard 5% O_2, 10% CO_2 and 85% N_2 gas mix, and microaerobic workstations, which usually include incubators, for the processing of large number of samples (Don Whitley Scientific Ltd., West Yorkshire, UK). Workstations have a modular atmosphere controlled system (MACS) that allows for a better control of the different concentrations of oxygen, carbon dioxide, nitrogen and hydrogen in the mix throughout incubation (Annable, 1998). Gas-generating sachets or envelops in jars do not usually maintain a constant atmosphere throughout incubation. Therefore the MACS technology is an important improvement; yet, these workstations are expensive and are not within the budget of many microbiology laboratories.

Gas-generating sachets and the evacuation-replacement systems have been also used with success with plastic bags used to freeze food products. In these cases, the bags have to have a strong closing system, usually a "ziplock" type, to prevent air from leaking during incubation.

The objective of the present study is to examine if *Campylobacter* enrichment in Bolton's broth from retail broiler samples really needs the microaerobic atmospheric mix. The microbial changes in the different media will be evaluated with Denaturing Gradient Gel Electrophoresis.

4.2. Materials and Methods

Sample Preparation

Five to six samples of retail broiler retail meat consisting of breast tenders, boneless breasts and boneless thighs were purchased from local stores.

This was done every week for 20 weeks, from January 2008 to November 2008. A total of 96 samples were analyzed.

From each individual meat package representing a sample, one inch pieces were cut aseptically and thoroughly mixed (Figure 4.1). Then 25 g of meat was weighed two times (two subsamples) in individual, sterile Whirl-Pak® bags (Nasco, Fort Atkinson, WI). Both the sub-samples was enriched with the addition of 100 ml of Bolton's broth supplemented with 5% (v/v) lysed horse blood (Oyarzabal *et al.*, 2007) into each bag. Samples were stomached for 1 min and one of the sub-samples enriched in Bolton's broth was incubated under microaerobic condition (85% nitrogen, 10% carbon oxide, 5% oxygen; Airgas, Radnor, PA) using anaerobic jars gassed with a MACSmics Jar Gassing System (Microbiology International, Frederick, MD), while the second sub-sample containing Bolton's broth was not gassed with the microerobic mix and was just tightly closed leaving a reduce head space in the bags. All samples were incubated at 42^{o}C for 48 h. At 24 and 48 h, samples were transferred (full loop, approximately 100 µl) to modified Campy-Cefex (mCC) (Oyarzabal *et al.*, 2005) agar plates. Modified Campy-Cefex plates were gassed after plating in jars to create microaerobic conditions as previously described. All plates were incubated at 42^{o}C for up to 48 h. Presumptive *Campylobacter* colonies were observed under phase contrast microscopy (Optiphot-2, Nikon, Tokyo, Japan) for morphology and motility of *Campylobacter* cells. Presumptive isolates were stored at -80^{o}C in tryptic soy broth (Difco, Detroit, MI) supplemented with 30% glycerol (v/v) and 5% (v/v) lysed horse blood.

Identification of Campylobacter Isolates

Campylobacter isolates were recovered by transferring the stock cultures on a 0.65 µm cellulose filters (Millipore, Billerica, MA, USA) that was placed on mCC agar plates. After 10-15 m the filter was removed and the plates were incubated for 24 h to 48 h at 42^{o}C under microaerobic conditions. *Campylobacter* colonies were picked after 24 h – 48 h and bacterial DNA was extracted using PrepMan™ Ultra sample preparation reagent (Applied Biosystems, Foster City, CA). All *Campylobacter* isolates were identified to the species level using two multiplex PCR (mPCR) assays described elsewhere (Oyarzabal *et al.*, 2005; Oyarzabal *et al.*, 2007).

Subtyping of Campylobacter Isolates

The restriction fragment length polymorphism of an amplified segment of the *fla*A gene (PCR-RFLP) was carried out as described elsewhere (Harrington *et al.*, 2003; Nachamkin *et al.*, 1993). Briefly, PCR amplification was done in 25 µl aliquots in a PTC-100 Programmable Thermal Controller (Bio-Rad Laboratories, Hercules, CA). Amplicons were restricted with *Dde*I and DNA bands were detected in 2.5% SeaKem® Gold Agarose (Lonza, Basel, Switzerland) that was stained with ethidium bromide. Gels were visualized using a UV transilluminator (Gel-Doc System, Syngene, Frederick, MD) and a TIFF file was created using a computer program (GeneSnap) for molecular analysis (Syngene, Frederick, MD).

Denaturing Gradient Gel Electrophoresis (DGGE) of Microbial Flora of Enriched Samples

Universal primers 338 F, containing the G + C clamp at its 5' end, and 518R were used for the amplification of the 16S gene (Muyzer *et al.*, 1993); (Sheffield *et al.*, 1989). The amplification consisted of 30 cycles of 5 min of denaturation at 94^{o}C, 1 min of annealing at 55^{o}C, and 1 min of extension at 72^{o}C. The presence of PCR amplicons was confirmed by running the amplions on a 1.5% agarose gel. The denaturing gradient for the DGGE run in vertical polyacrylamide gels ranged from 45%-65%. Ingeny electrophoresis system (Ingeny phorU, Netherlands) at a voltage of 100V for 16-18 h was used for separation of DGGE bands. Gels were stained in ethidium bromide and visualized under the UV imager. Bionumerics version 4.50 (Applied Maths, Austin, TX) software was used to analyze the band patterns. A marker strain was used in every DGGE gel that was run as a standard for Bionumerics analysis during the comparative analysis of genotype profiles of the strains.

Sequencing

The primers used for sequencing were 338F, without the G + C clamp, and the 518R (Muyzer *et al.*, 1993; Sheffield *et al.*, 1989). The amplification cycle consisted of 5 min of denaturation at 94^{o}C and 1 min of annealing at 55^{o}C and 1 min of extension at 72^{o}C for 30 cycles and the presence of PCR amplicons was confirmed by running the amplions on a 1.5% agarose gel. Amplicon was purified using the columns from Wizard SV Gel and PCR clean-up system by Promega (Madison, WI). The purified amplicons along with the forward and reverse primers were sent out for sequencing.

The sequencing of the amplified bands was done as described in Chapter III.

Statistical Analysis

Positive/negative data between treatments (Bolton with microaerobic gas mix versus Bolton without the gas mix) were analyzed with the McNemar's Test (McNemar, 1947); and the Kappa coefficient (Cohen, 1960) using Proc Freq in SAS (SAS version 9.1; SAS Institute Incorporated, Cary, NC). A McNemar's test value ≤ 3.84 assumes the null hypothesis; that means that the test method (Bolton without the gas mix) and reference method (Bolton with microaerobic gas mix) are equivalent and cannot be rejected at the 5% level of confidence ($P < 0.05$). The Kappa coefficient is used to quantify the agreement between two raters in placing the sample of subjects into two categories. Therefore, Kappa is a chance-corrected measure of agreement between two raters. Each rater classifies each of a sample of subjects into one of two mutually exclusive categories (King, 2004). Kappa values are between 0 and 1. A Kappa value of 1.0 implies a perfect agreement. In general, Kappa values of 0.20 and 0.40 are considered a “fair agreement”, while values between 0.40 and 0.60 are considered “moderate agreement” (McNemar, 1947).

4.3. Results and Discussion

The isolation rate from Bolton (microaerobic) and Bolton (without microaerobic gas mix) broth did not result in significant differences (chi-square = 0.21) (Table 4.1).

The DGGE analysis of the two enrichment broths showed that the majority (85%) of the bands were similar among the treatment (Figure 4.1), indicating the growth of similar kinds of microbial populations in both the control and test enrichment medium.

There was some amount of difference (20%) in presence or absence of certain bands in certain meat samples. A BLAST analysis gave us an insight into the type of microbial population that's inhabiting with Campylobacter during its growth. Some of those microorganisms were unculturable organisms.

DGGE analysis showed the presence of different bands in the same samples but different liquid enrichment media (Figure 4.2). After the assembly of the consensus sequence from the forward and reverse run was done with MultAlin and ClustalW the sequencing results of these bands was alignment in the Basic Local Alignment Search Tool (BLAST http://blast.ncbi.nlm.nih.gov/Blast.cgi) which gave the following results. The percentage of similar group of organisms growing both in Bolton under the microaerobic environment and Bolton without gas enrichment media was 80% and of the different group of organisms which were either present or absent in either Bolton with gas and in Bolton without enrichment media was 20% (Figure 4.2).

In most of the comparisons of the multiplex PCR results (Figure 4.5), the presence of same species of *Campylobacter* was seen, but some comparisons showed the presence of different strains. In a sub-sample, Bolton broth showed the presence of both *C. jejuni* and *C. coli*, whereas the equivalent strains (lanes 14) isolated from the Bolton without gas showed the presence of only *C.coli*.

The RFLP band pattern (Figure 4.6) was same for most of the strains. However, few exceptions (Figure 4.6, lanes 3 and 4, 7 and 8) showed the presence of different band patterns indicating presence of different strains.

Our results showed the presence of growth of *Campylobacter* without the presence of microaerobic gas mix. This suggests that the environment inside the enrichment bag may have oxygen concentration less than 5% which will enable the growth of *Campylobacter.*

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Table 4.1. Liquid enrichment media, Bolton Broth with microaerobic gas mix vs Bolton Broth without gas mix

		Bolton Broth		
		+	-	Total
Bolton w/o gas	+	31	13	44
	-	14	38	52
Total		45	51	96

$\chi^2 = 0.21$

Table 4.2. Results after BLAST analysis of the sequences

Marker		Number	Bacterial strain	% similarity	Accession Number
Marker A		K 1	Uncultured *Acinetobacter* sp.	96%	FJ216156.1
Marker B		K 2	Uncultured Nitrospirae bacterium	85%	AF540054.1
Marker C		K 3	*Lactobacillus* sp. L21	87%	AF159000.1
Marker D		K 4	*Lactobacillus saerimneri*	95%	AY255802.1
Marker E		K 5	*Lactobacillus* sp. L21	82%	AF159000.1
Bolton	**Bolton w/o gas**				
Lane 15		K 17	*Pseudomonas extremorientalis*	99%	EU982883.1
Lane 17		K 18	*Pseudomonas* sp. tDp10	96%	AJ971379.1
Lane 21		K 19	*Lactobacillus salivarius*	85%	FJ581418.1
	Lane 22	K 20	Uncultured bacterium	88%	EU469596.1
Lane 23		K 21	Uncultured bacterium	80%	DQ264456.1
Lane 25		K 22	*Pseudomonas* sp. R-35702	97%	AM886093.1
	Lane 26	K 23	*Lactobacillus* sp. JN4	83%	AF157041.1

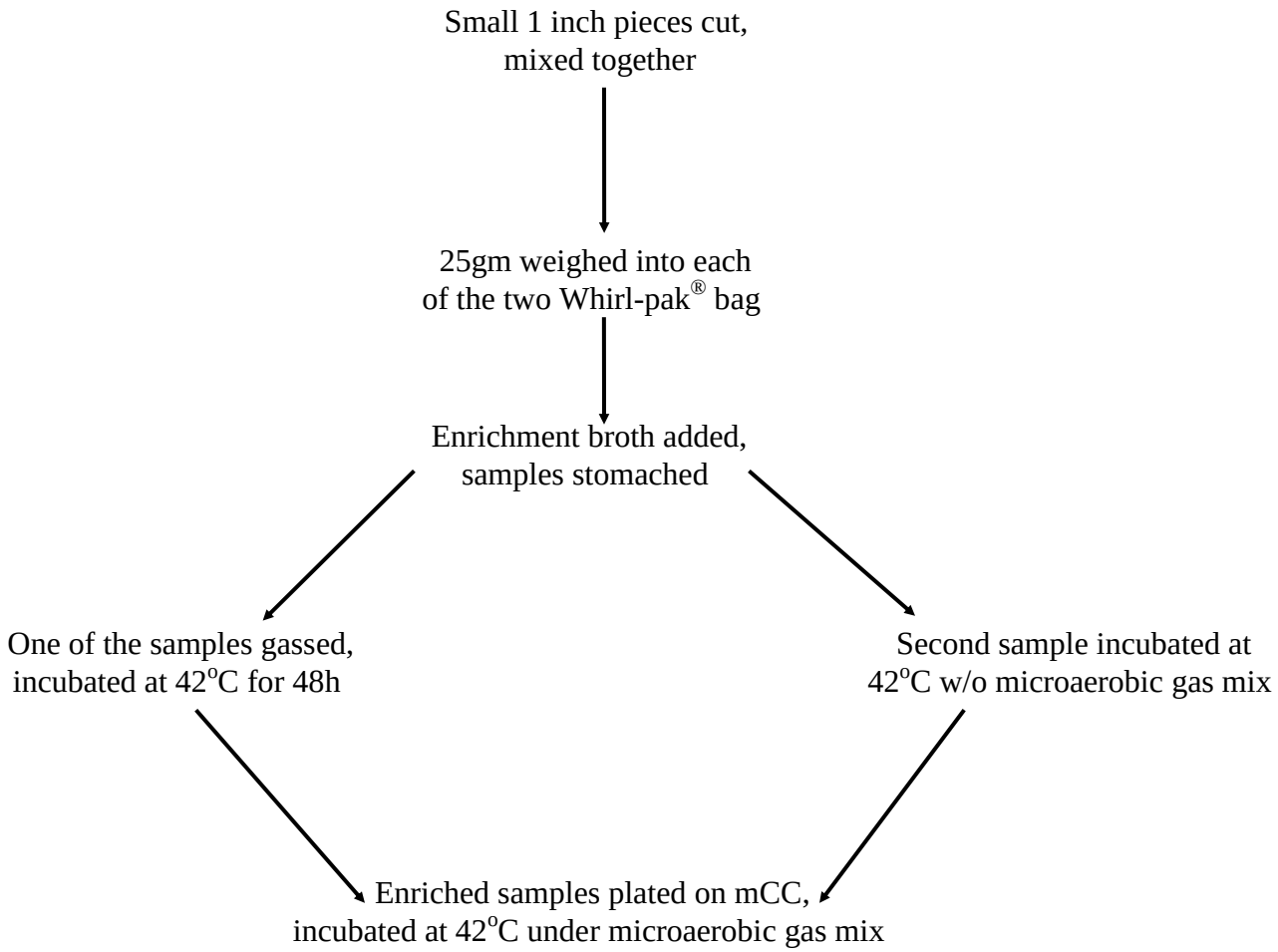

Figure 4.1. Flow Chart – Experimental procedure

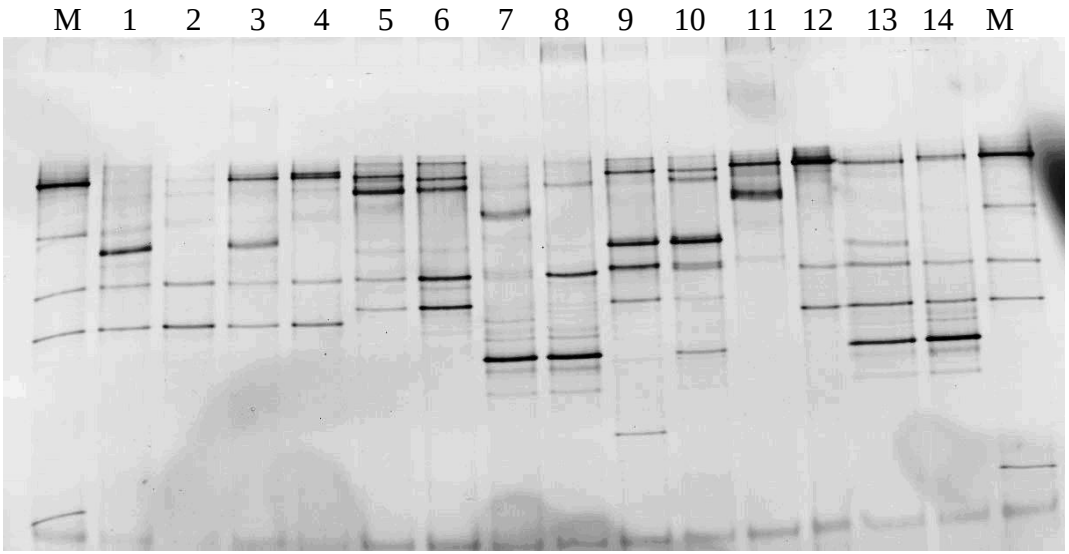

Figure 4.2. DGGE band patterns, Lane M-marker; Lanes 1, 3, 5, 7, 9, 11, 13 - Bolton with microaerobic gas mix; samples 21, 23, 31, 32, 33, 34, 35; Lanes 2, 4, 6, 8, 10, 12, 14 - Bolton without microaerobic gas mix; samples 21, 23, 31, 32, 33, 34, 35

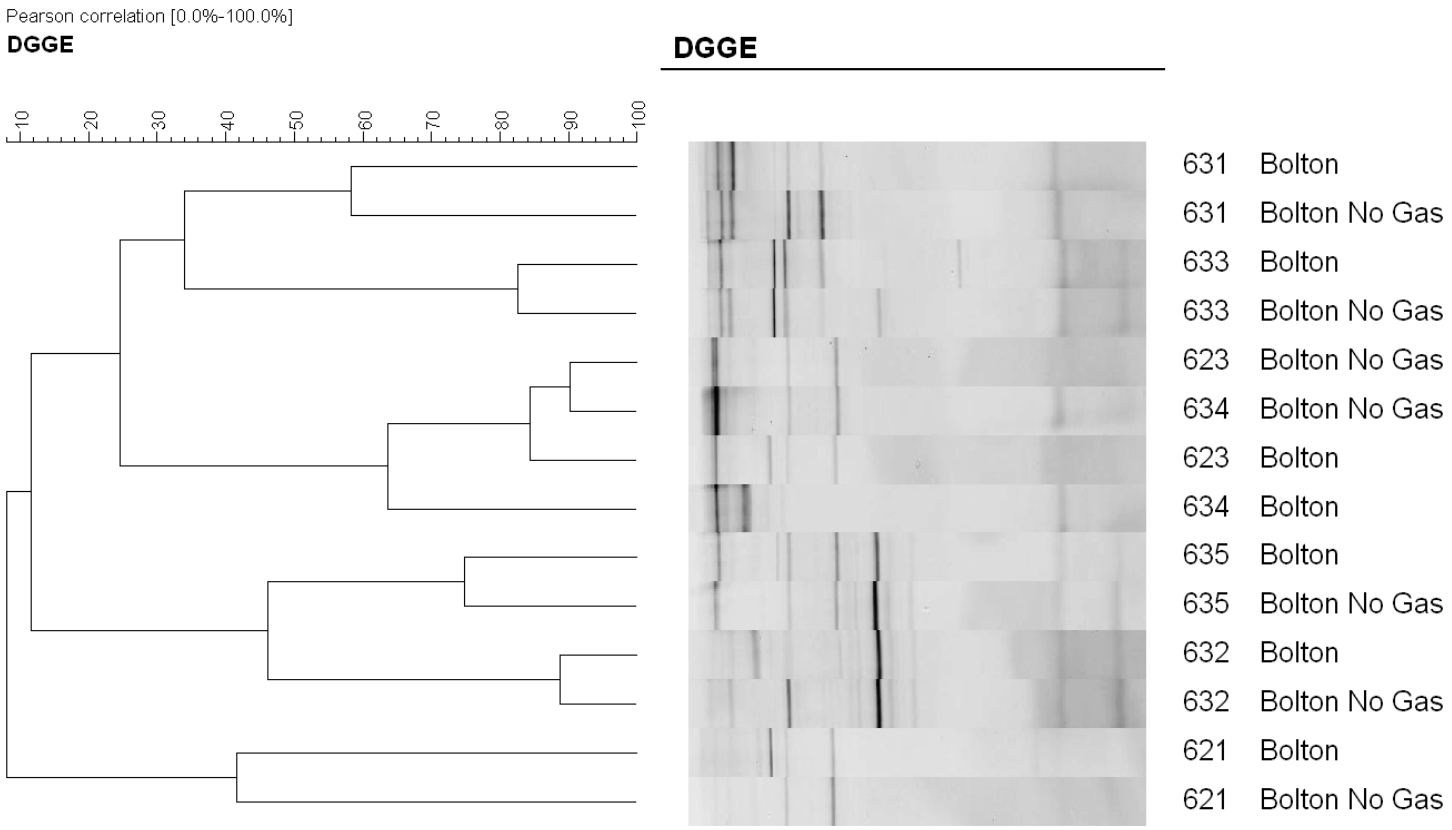

Figure 4.3. Dendrogram, Bolton and Bolton without gas – Pearson's correlation

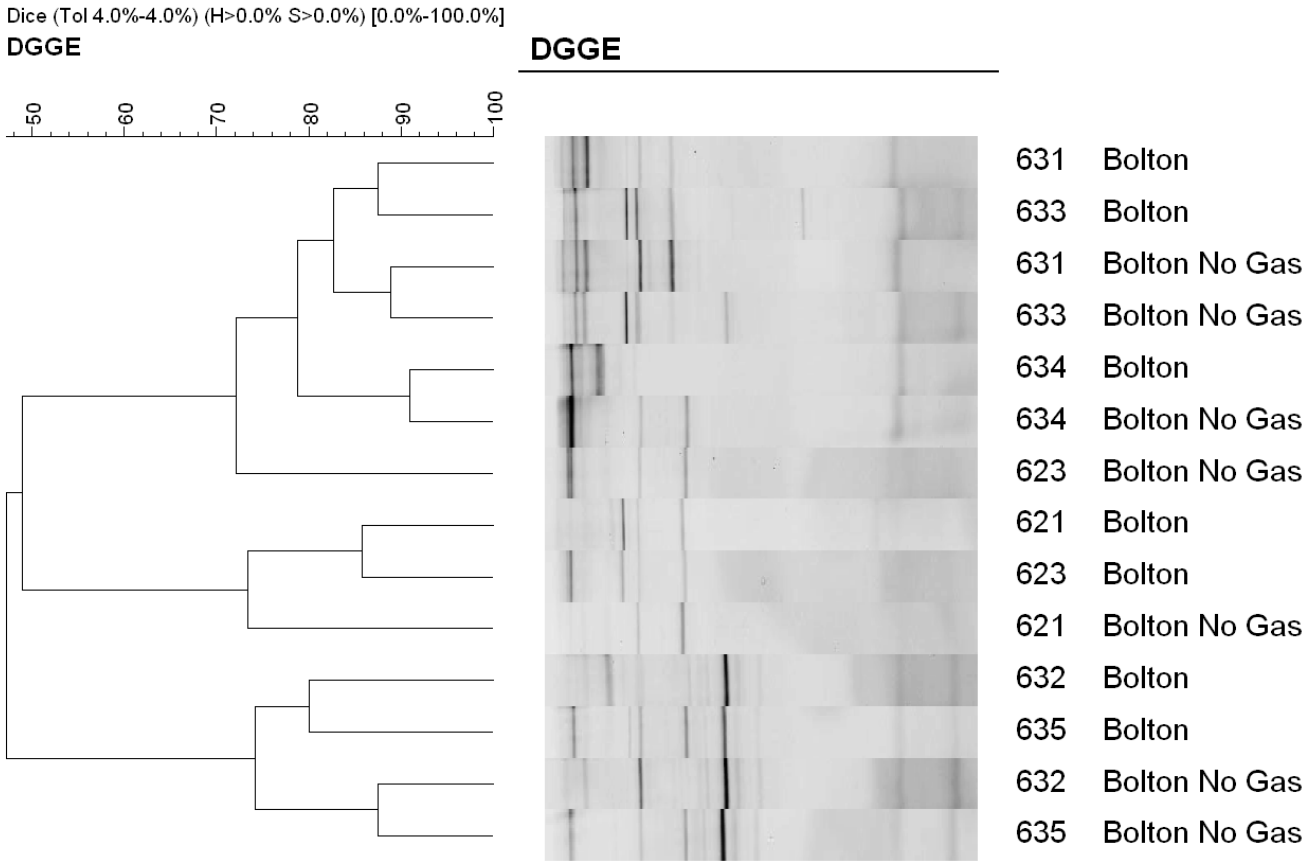

Figure 4.4. Dendrogram, Bolton and Bolton without gas – Dice's correlation

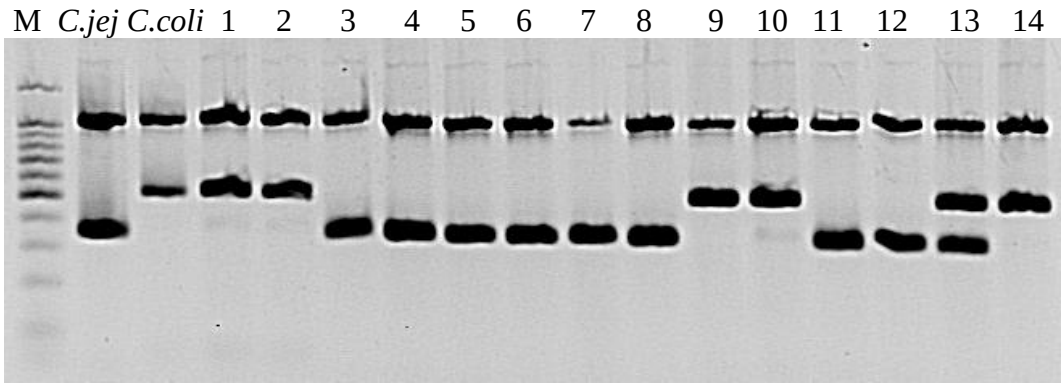

Figure 4.5. Multiplex PCR results, Lane M - marker, Lanes 1, 3, 5, 7, 9,11, 13 Bolton samples ; Lanes 2, 4, 6, 8, 10, 12, 14 corresponding Bolton without gas samples

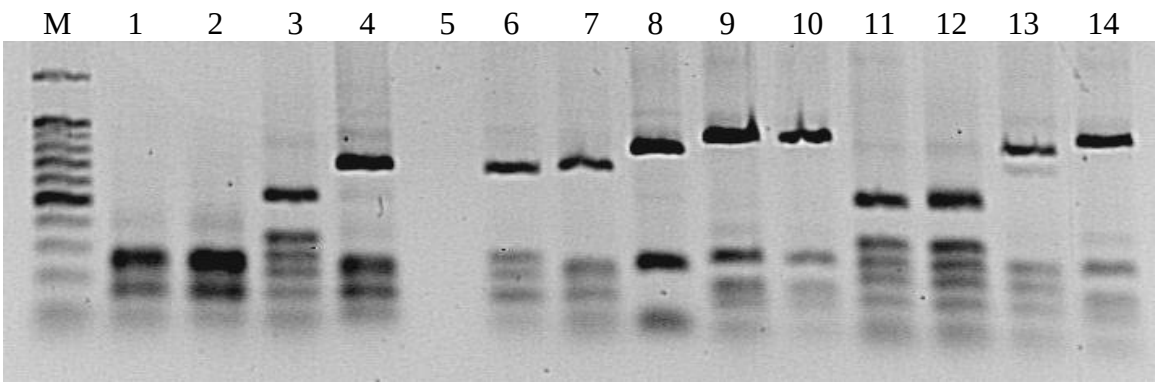

Figure 4.6. RFLP results, Lane M – marker, Lanes 1, 3, 5, 7, 9, 11, 13 = Bolton samples; Lanes 2, 4, 6, 8, 10, 12, 14 = corresponding Bolton without gas samples

CHAPTER V: ENUMERATION, PREVALENCE AND PULSED-FIELD GEL ELECTROPHORESIS ANALYSIS OF *CAMPYLOBACTER* ISOLATES OBTAINED FROM COMMERCIAL BROILER CARCASSES

5.1. Introduction

Campylobacter jejuni and *Campylobacter coli* are two important thermotolerant bacteria which are pathogenic to humans. Most of the campylobacteriosis cases are sporadic and the exact modes of infections are not well understood (Rodrigues *et al.*, 2001).

The number of *Campylobacter* spp. in poultry carcasses have been reduced over the last 10 years due to the implementation of the hazard analysis and critical control Points (HACCP) systems (Anonymous, 1996). From the changes brought about by HACCP, the increase in the water usage (Sellers, 2001), the incorporation of carcass washers (Bashor *et al.*, 2004) and the implementation of antimicrobial intervention strategies (Oyarzabal *et al.*, 2005) can be indirectly correlated to the reduction in the number of foodborne pathogens in general. Recent publications reporting prevalence of *Campylobacter* spp. in broiler carcasses have used direct plating media. However, the number of *Campylobacter* spp. per carcass is low enough that it requires an enrichment step

To better understand the epidemiology of *Campylobacter* spp. molecular methods for sub-typing are used to track the sources of *Campylobacter* pulsed-field gel electrophoresis (PFGE) is one of the most discriminating methods for subtyping of *Campylobacter* spp (On *et al.*, 1998). This method allows for the separation of large fragments of DNA (30 kbp or above), and was first introduced by Schwartz and Cantor in 1984 (Schwartz & Cantor, 1984). The ability of this technique to resolve very large fragments was initially used to map the genomes of microorganisms, after that this technique was used to compare genomes from different microorganism or different strains of the same organism.

In a previous study, six different plate media were evaluated for direct enumeration of *Campylobacter* spp. from broiler carcasses collected from four commercial broiler processing facilities (Oyarzabal *et al.*, 2005). In this article, we analyzed the data by plant to determine variations in the prevalence of *Campylobacter* spp. among plants. We also compare the efficacy of a combination of plate media versus individual plates for enumeration, and analyzed the isolates from different plants and from the same sample, same plates but different samples, same plates but different plants, and same plates and same plants with PFGE to determine the DNA variability among plants and within isolates from the same sample.

5.2. Materials and Methods

Sample Collection and Identification of the Isolates

Four commercial processing plants (A through D) were sampled three times. Twenty post-chill carcasses were collected, rinsed with 400ml of buffered peptone water and analyzed for the presence of *Campylobacter* spp. Carcass rinses were plated on six different plate media (Campy-Cefex, modified Campy-Cefex, mCCDA, Karmali, CAMPY, Campy-Line) for enumeration of *Campylobacter* spp.

Presumptive *Campylobacter* colonies were observed under a phase contrast microscope for morphology and motility. A multiplex polymerase chain reaction (PCR) assay that targets the *cadF* gene (*C. jejuni* and *C. coli*), the *ceuE* gene (*C. coli* only) and an unknown DNA fragment (*C. jejuni* only) was used to confirm the identity of *Campylobacter* isolates (Table 5.1) (Cloak & Fratamico, 2002).

Pulsed-Field Gel Electrophoresis (PFGE) Analysis

Fifty eight isolates, 20 from plant A, 23 isolates from plant B, seven from plant C and eight from plant D, were analyzed following a standardized PFGE protocol for subtyping *Campylobacter* isolates. This protocol is a slight modification of the protocol by the Centers for Disease Control and Prevention (Atlanta, GA) and has been published elsewhere (Ribot *et al.*, 2001). DNA was restricted with *Sma*I and analyzed with a contour-clamped homogeneous electric field (CHEF, Bio-Rad, Hercules, CA) in 1% agarose gel stained with ethidium bromide. Gels were visualized with a UV transilluminator (Gel-Doc System, Bio-Rad) and a digital pictured was recorded using

GeneSnap (Syngene, Frederick, MD). A lambda marker (50-kb-1,000kb; Promega, Madison, WI) was included as the reference lanes in the PFGE gels.

PFGE types were analyzed as follows: different plates but same carcass, same plates but different carcass, same plates but different plants, and same plates and same plants. Cluster comparison was done with the Dice correlation coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm from Bionumerics version 4.50 (Applied Maths, Austin, TX). The optimization and position tolerance for band analysis were set at 4%, and a cutoff of 90% was used to determine PFGE types and subtypes.

Statistical Analysis of Plate Results

Colony counts were converted to $\log_{10}$ CFU/ml and analyzed using the general lineal model procedure of SAS (SAS Release 9.1, SAS Institute Incorporated, Cary, NC). Means were analyzed for differences using Duncan test (SAS), and percentage values analyzed by a qualitative, McNemar chi-square ($\chi 2$) analyses using one degree of freedom. Chi-square values less than 3.84 indicated that percentages were equivalent and therefore the null hypothesis (similar percentages) could not be rejected at the 5% level of confidence.

5.3. Results

Plant Prevalence

Variations in the number of *Campylobacter* positive carcasses were found during the different visits to the plant. However, the average prevalence of *Campylobacter* spp.

calculated by direct plating showed that three plants had similar percentages of *Campylobacter* positive carcasses while the fourth one exhibited lowered *Campylobacter* counts (Table 5.2). The mean log of CFU/ml or carcass rinse was similar for all the four plants, and was less than 1CFU/ml.

When the results from one plate, mCC, were compared with the results of the combination of all plates; plants A and B exhibited and significant increase ($P < 0.05$) in the number of positive samples. However, plants C and D did not showed any significant differences between mCC and the results from the combination of all plates (Table 5.2). The analysis of all positive samples, from all plants, resulted in 63% positive samples, which was significantly different ($P < 0.05$) from the results of only mCC plates (50%). Therefore, the increase in the number of plate media used for the analysis of the results resulted in an increase in the frequency of positive samples, but generated a slight, non-significant decrease in the average number of *Campylobacter* spp. (Figure 5.1).

PCR Testing

From 152 positive samples, the multiplex PCR (Cloak & Fratamico, 2002) identified 143 isolates as *C. jejuni* (98 %), three as *C. coli* (2 %) and six containing both *C. jejuni* and *C. coli* (Table 5.1) (Figure 5.2). These mixed cultures were re-isolated using a 0.65 µm cellulose filter (Millipore Corp., Billerica, MA) on mCC plates. One isolated colony was then tested with the PCR protocol, and all isolates resulted as *C. jejuni*.

PFGE Analysis

Twenty isolates from plant A, 23 isolates from plant B, seven isolates from plant C and eight isolates from plant D resulted in nearly twenty different sets of PFGE band patterns when a 90% cutoff and a position tolerance of 4% were used to determine relatedness in BioNumerics (Figure 5.7).

Both *C. jejuni* and *C. coli* isolates were tested with PFGE. These results suggest an occurrence of different fingerprinting patterns from among *Campylobacter* strains collected from the rinse of the same carcass (Figure 5.6) confirming the presence of more than one strain subtype in a single carcass. The *C. coli* strains interestingly showed similar PFGE patterns (Figure 5.10) even though they were isolated from different carcasses and different plants (Figure 5.4).

5.4. Discussion

The prevalence of *Campylobacter* spp. varied with plants. In three plants, we found an incidence of more than 50% of *Campylobacter* spp. Plant C exemplifies the extreme variations that can be found in the prevalence of *Campylobacter* spp. in broiler carcasses when using only direct plating for identification. In this case, prevalence data should be reported with caution because no enrichment was used and samples that contained a low number of cells could have been misidentified as negatives.

The mean log CFU per ml of *Campylobacter* was less than 1 log CFU/ml and correlates with previous reports summarizing the current number of *Campylobacter* spp. in commercial broiler carcass in the US (Oyarzabal *et al.*, 2005).

Direct plating is useful to enumerate *Campylobacter* in carcasses that have more than 4000 CFU per carcass rinse. Yet, our prevalence results are in agreement with the findings reported by (Bashor *et al.*, 2004), in a study performed in four commercial processing plants. The authors in this report spread plated the carcass rinse onto ten agar plates, two plates were spread with 0.1 ml and eight plates were spread each with 0.25 ml. There was an overall prevalence of 68% of carcasses positive for *Campylobacter* spp. and a higher number of log CFU per ml of carcass rinse (3.1 up to 4.1). These differences may be due to the fact that samples were collected from post-evisceration, post-carcass wash, before the chiller, and because only 100 ml of buffered peptone water was used for rinsing of the carcasses.

The amount of rinse solution, in the form of buffered peptone water or other rinse solutions (Stern & Robach, 2003), and different transportation and storage times and temperatures make it difficult for the current published results to be comparable. In addition, we do not have reliable information on the survival of *Campylobacter* spp. in buffered peptone water for extended times at refrigeration temperature and under regular atmospheric conditions. Therefore, the influence of these variables should be minimized when developing experimental protocols.

Variables such as the amount of ml of rinse used the time of shaking of the carcasses and the transportation time and temperature before samples are processed may influence the final result when using direct plating to isolate *Campylobacter* spp. from processed carcasses. The chances for detection and enumeration increases when more rinse is spread-plated and the samples have large number of *Campylobacter* spp. per ml of rinse.

Table 5.3 shows the results obtained by each plating medium organized by plant. All plates except one exhibited a similar performance. In our laboratories, we have found that colonies are easier to collect from a blood-based medium than from a charcoal-based medium. Yet, the differentiation of colonies in any given plate is still subjective and the use of a phase-contrast or dark-field microscope for the identification of presumptive *Campylobacter*-like organisms can not be replaced by personal experience.

Statistical differences were found between the prevalence on m Campy-Cefex and the prevalence based on the results from all plates (Table 5. 2). An increase in the number of plates slightly decreased the average counts of *Campylobacter* spp. but significantly increased the overall number of positive (Figure 5.1). These findings highlight the fact that the number of *Campylobacter* spp. found in carcass is usually less than 1 log CFU per ml of rinse, or ~4000 CFU per carcass, and therefore direct plating may not be reliable to consistently detect thee number, of *Campylobacter* spp. present in carcasses (Oyarzabal *et al.*, 2005), Enrichment of the carcass rinses should be used when pursuing the collection of prevalence data in broiler carcasses.

In 2001, a study was published that compared the recovery of three enumeration methods in 24 post-chill broiler carcasses: a 3-tube MPN (most probable number) in which the enrichment was Hunt (Hunt, 1992), a 3-tube MPN in which the enrichment was Rosef's (Rosef, 1981) and direct plating on Campy-Cefex (0.1 ml in duplicate) with a 1:1 dilution of 25 ml the samples in 2X Bolton enrichment broth (Line *et al.*, 2001). The same study compared the efficacy of three agar plates, Campy-Cefex, mCCDA and Muller-Hinton agar supplemented with blood and antibiotics (0.1 ml induplicate), for the enumeration of *Campylobacter* from 36 freshly processed carcasses. Similarly to our

results, the authors in this study found that Campy-Cefex and mCCDA recovered a similar number of positive samples, 66.7% and 63.9%, respectively, but reported that the sum of all positive using three plates was 94.4%. These results underline, again, the fact that direct plating is a good method when several plates are used and a high number of *Campylobacter* spp. are found in the rinse (more than 1 log CFU per ml). The limitations of drawing conclusions on prevalence data from analyses performed using only 0.1 ml of the sample, in duplicate, for direct plating should not be underestimated.

The more we reduce the number of *Campylobacter* spp. on broiler carcasses, the more we will need to incorporate enrichment procedures to determine the real prevalence on the samples. Presently, the number of *Campylobacter* spp. in broiler carcass rinses is at the lowest limit of detection by direct plating. A semi-quantitative determination, where result is given in intervals according to pre-established tables and where a detection limit of 0.1 CFU/g requires analysis of 10 g (or ml) of sample, has been established by The Nordic Committee on Food analysis (Anonymous, April 2005).

Until a solid, validated methodology is developed to enumerate these low number of *Campylobacter* spp. in samples, we may have to rely on the enrichment of the samples. The results from the PCR protocol used in this study were quite robust to identified mixed cultures, and were easy to interpret. The prevalence of *C. coli* was lower than the prevalence of *C. jejuni*. This difference in the prevalence of these two species is commonly found in process broiler carcasses, and it is similar to the rate of *C. jejuni*/*C. coli* found live, commercial broilers, (Genigeorgis, 1986; Johannessen *et al.*, 2007; Potturi-Venkata *et al.*, 2007). The analysis of selected *C. jejuni* isolates with PFGE suggested a high variability in the DNA patters of *Campylobacter* strains among the four

plants, and during different visits (Figure 5.5). Considerable amount of PFGE patterns were also seen in the profiles of different isolates within the same production plant, (Figure 5.9) and even with isolates collected from the same sample (Figure 5.8). PFGE patterns for *C. coli* strains were same even though the samples were from different carcasses and sometimes from different plants (Figure 5.10). This DNA diversity within *C. jejuni* strains has been found for strains isolated from retail meat samples (Dickins *et al.*, 2002; Oyarzabal *et al.*, 2007), from live broilers (Potturi-Venkata *et al.*, 2007) and for other *Campylobacter* spp. (Bourke *et al.*, 1996).

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Table 5.1. Primer sets used in the multiplex PCR for confirmation of *Campylobacter* spp.

Product (bp)	Primers	Sequences (5'-3')	Tm (^{o}C)	References
400	F2B	TTGAAGGTAATTTAGATATG	44	Konkel et al.
	RIB	CTAATACCTAAAGTTGAAAC	43	1999
894	COLI	ATGAAAAAATATTTAGTTTTTGCA	54	Gonzalez et al.
	COL2	ATTTTATTATTTGTAGCAGCG	52	1997
160	C-I	CAAATAAAGTTAGAGGTAGAATGT	51	Winters and
	C-2	GGATAAGCACTAGCTAGCTGAT	55	Slavik 1995

Table 5.2. Counts and prevalence (%) of *Campylobacter* spp. per plant and comparison of counts and prevalence data between mCampy-Cefex and all six plating media used in the study.

			Positive per Sampling Time		LOG CFU/ml			Positive (%)		
Plant	Mean log CFU/ml[1]	Total Positive (%)	Min. (%)	Max. (%)	mCampy-Cefex	Total Plating Media	SEM[2]	mCampy-Cefex	Total Plating Media	χ^3
A	0.57^{C}	47 $(78.3)^{B}$	9 (45)	19 (95)	0.5^{A}	0.5^{A}	0.05	34 (57)	47 (78)	6.41
B	0.74^{B}	47 $(78.3)^{B}$	12 (60)	20 (100)	0.8^{A}	0.7^{A}	0.06	37 (62)	47 (78)	3.96
C	0.74^{B}	37 $(61.7)^{B}$	0	20 (100)	0.8^{A}	0.6^{B}	0.04	33 (55)	37 (62)	0.54
D	0.10^{A}	20 $(33.3)^{A}$	0	19 (95)	0.2^{A}	0.1^{B}	0.03	14 (23)	20 (33)	1.47

[1] Different letters within column indicate significant difference ($P < 0.05$).

[2] SEM = standard error of the mean.

[3] Chi-square values ≥ 3.84 indicate significance at $P < 0.05$.

Table 5.3. *Campylobacter* positive samples organized by plant and plating medium.

	Mean (log CFU/ml) per Plant [1]			
Plate	**A**	**B**	**C**	**D**
Campy-Cefex	0.64^{A}	0.99^{A}	0.92^{A}	$0.10^{A,B}$
mCampy-Cefex	0.62^{A}	0.91^{A}	$0.88^{A,B}$	0.18^{A}
mCCDA	$0.59^{A,B}$	0.86^{A}	0.92^{A}	$0.15^{A,B}$
Karmali	0.63^{A}	0.81^{A}	0.60^{C}	$0.08^{A,B}$
CAMPY	0.40^{B}	0.86^{A}	$0.73^{B,C}$	$0.07^{A,B}$
Campy-Line	$0.52^{A,B}$	0.02^{B}	0.38^{D}	0.03^{B}

[1] Different letters within column indicate significant difference ($P < 0.05$).

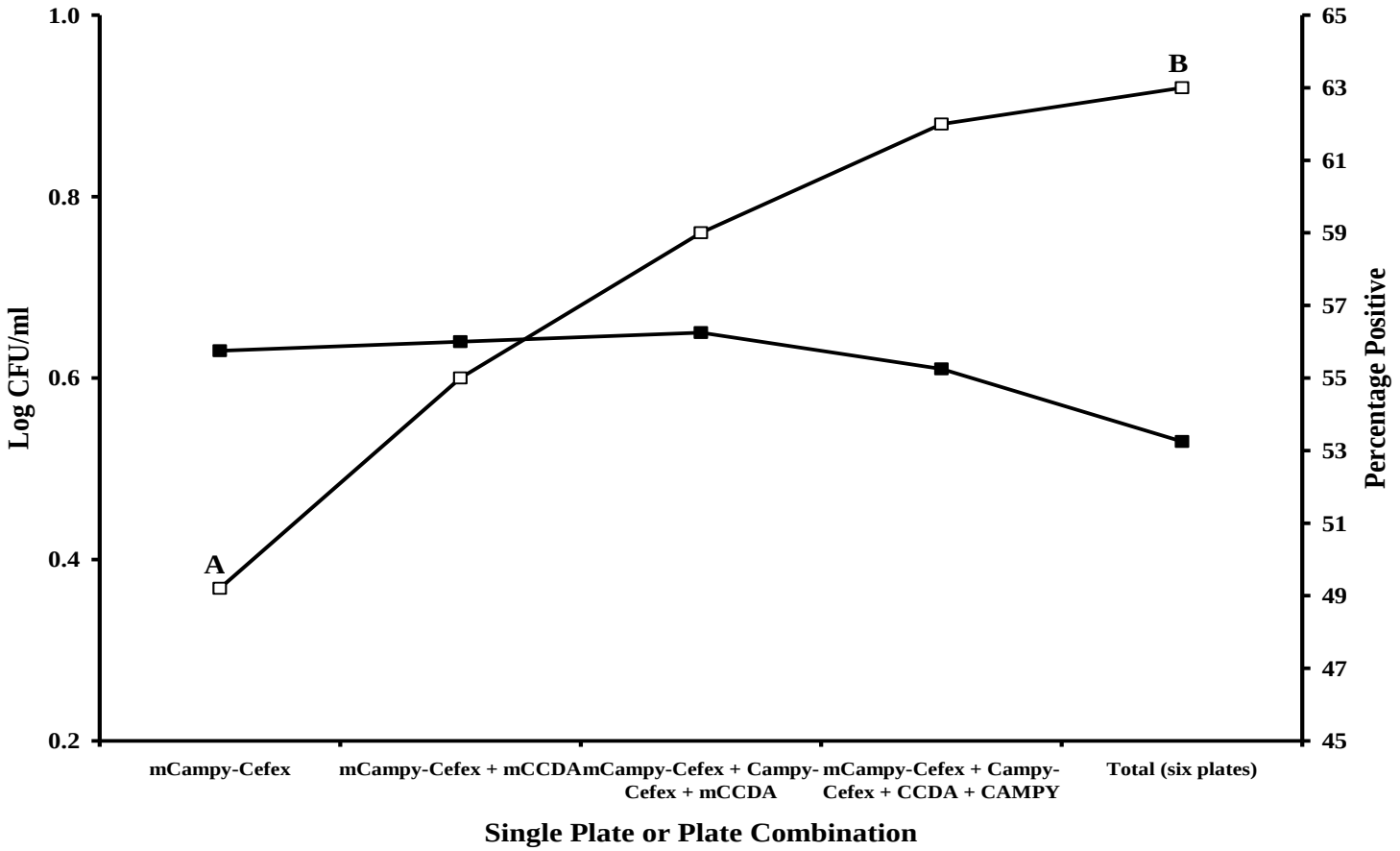

Figure 5.1. Average count of *Campylobacter* spp. (■) and percentage positive (□) for single plate or for a combination of plating media. A and B are statistically significant ($\chi^2 = 9.20$)

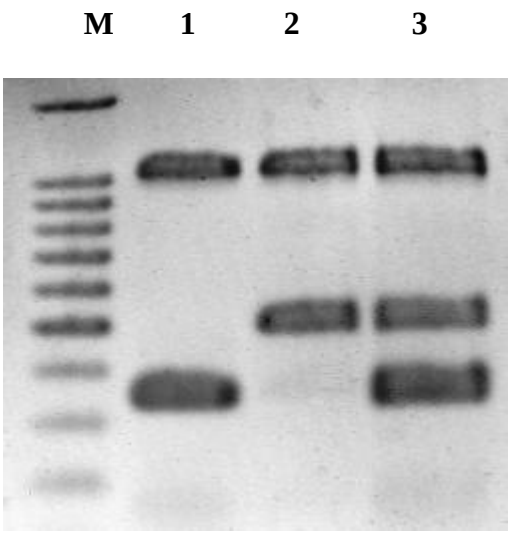

Figure 5.2. Multiplex PCR for confirmation of *Campylobacter* spp.: Lane M, 100bp ladder; Lane 1, *C.jejuni*; Lane 2, *C.coli*; Lane 3, Mixed (*C.jejuni* + *C.coli*)

1 2 3 M

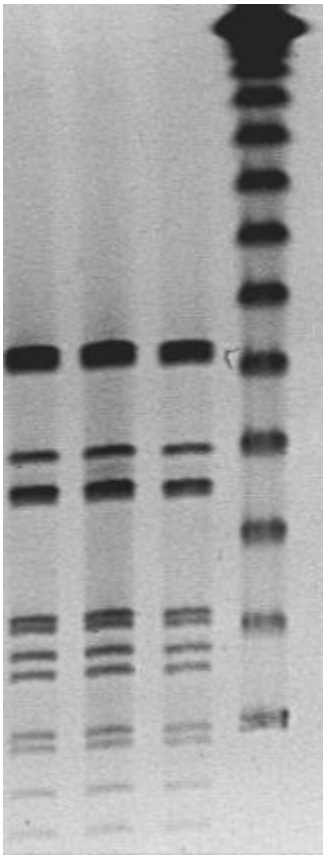

Figure 5.3. *C. coli* strains from different poultry production plants: Lane 1, 136 FDA; Lane 2, 137 FDA; Lane 3, 137 FDA; Lane M, Lambda marker

M 1 2 3 4 5 6 7

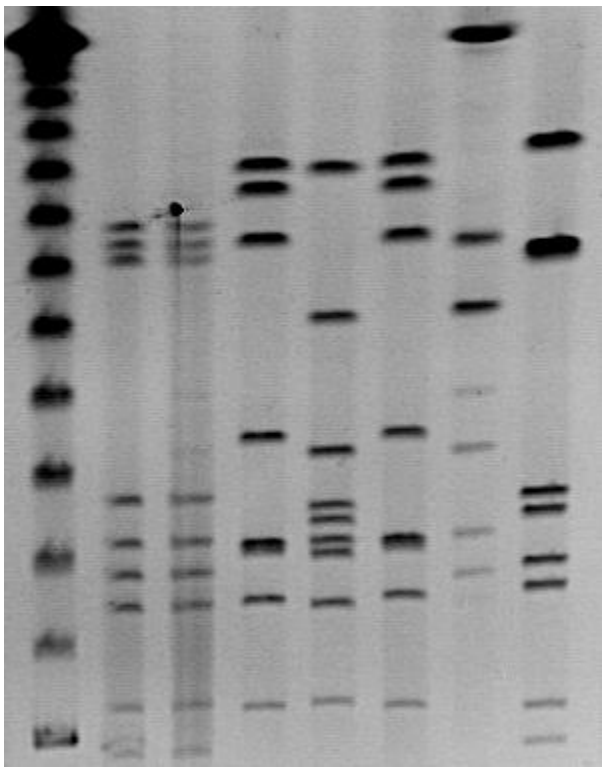

Figure 5.4. Same poultry production plant but different plate comparison: Lane M, Lambda marker; Lane 1, 17 Line (A); Lane 2, 17 CC (A); Lane 3, 119 Kar (A); Lane 4, 237 Line (D); Lane 5, 113 Kar (A); Lane 6, 24 CC (B); Lane 7, 137 mCC (B)

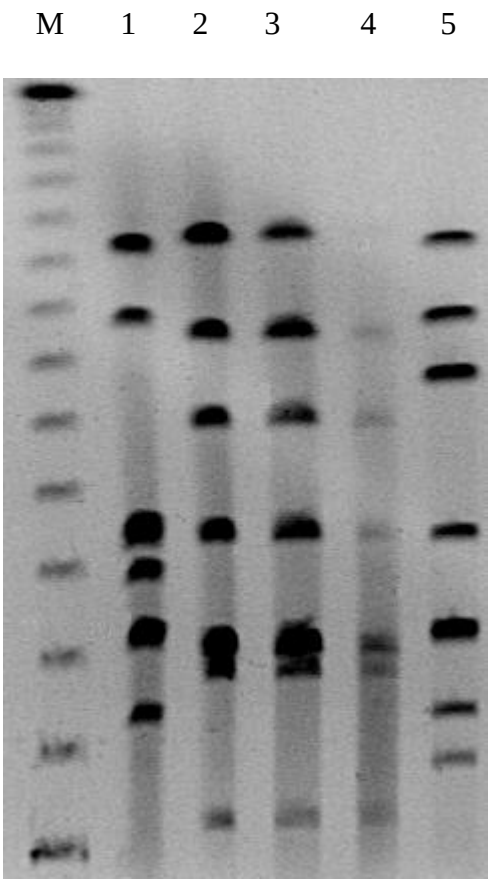

Figure 5.5. Same carcass different plate comparison: Lane M, Lambda marker; Lane 1, 216 Kar; Lane 2, 216 FDA; Lane 3, 215 Kar; Lane 4, 212 FDA; Lane 5, 215 CC

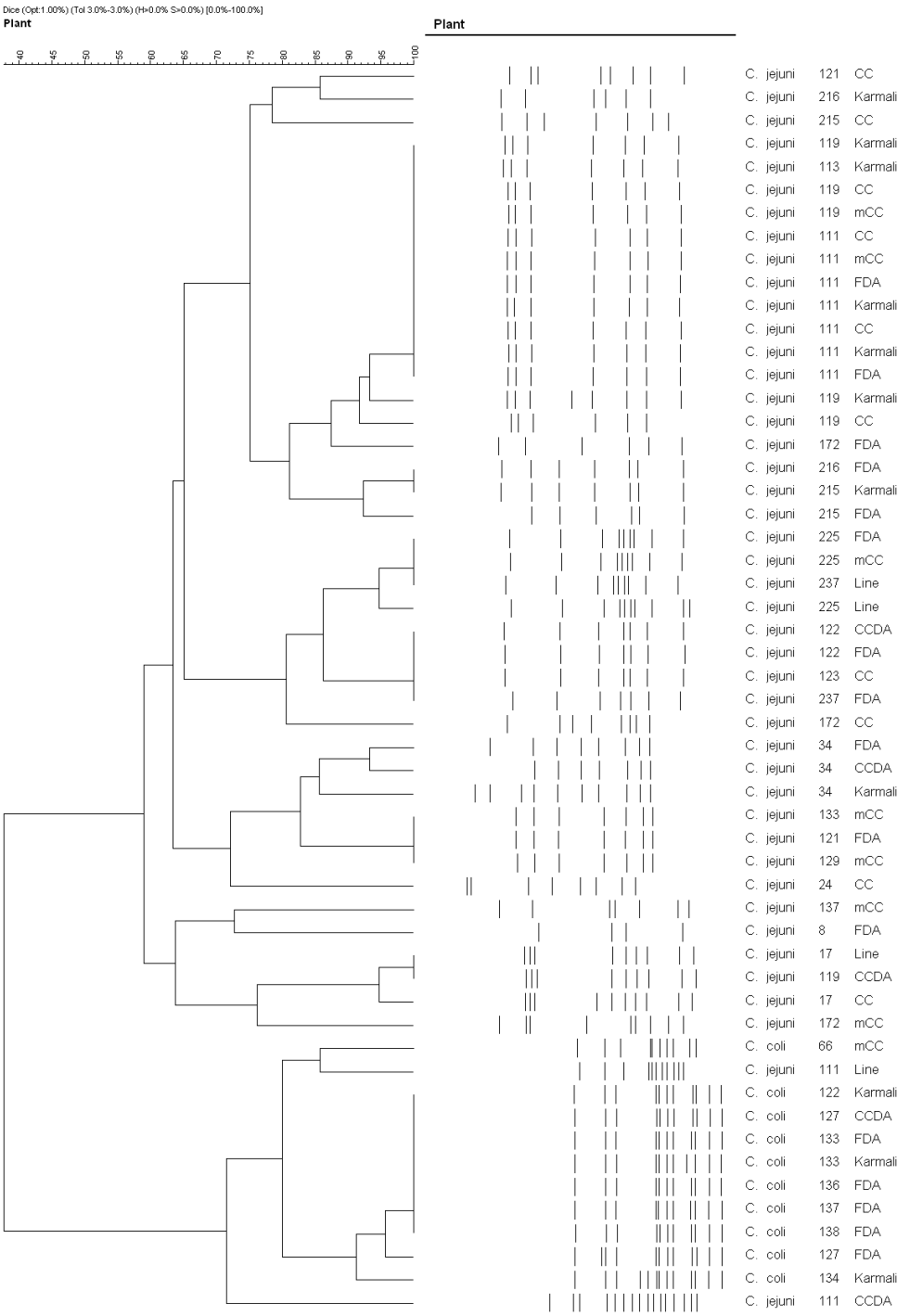

Figure 5.6. PFGE data analysis using the BioNumerics software, dendrogram of all the strains analyzed

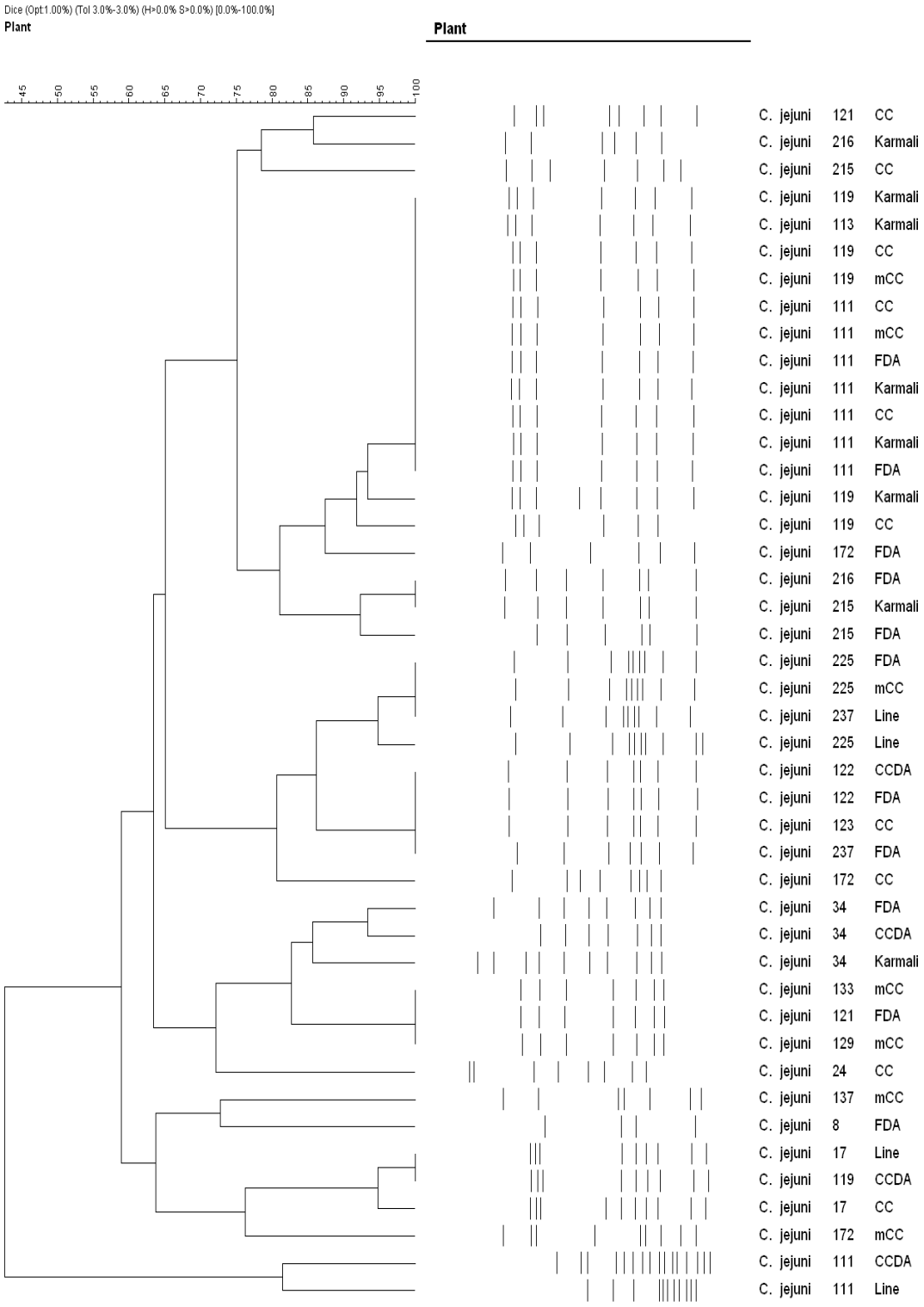

Figure 5.7. Dendrogram showing the relatedness of a subset of *Campylobacter jejuni*

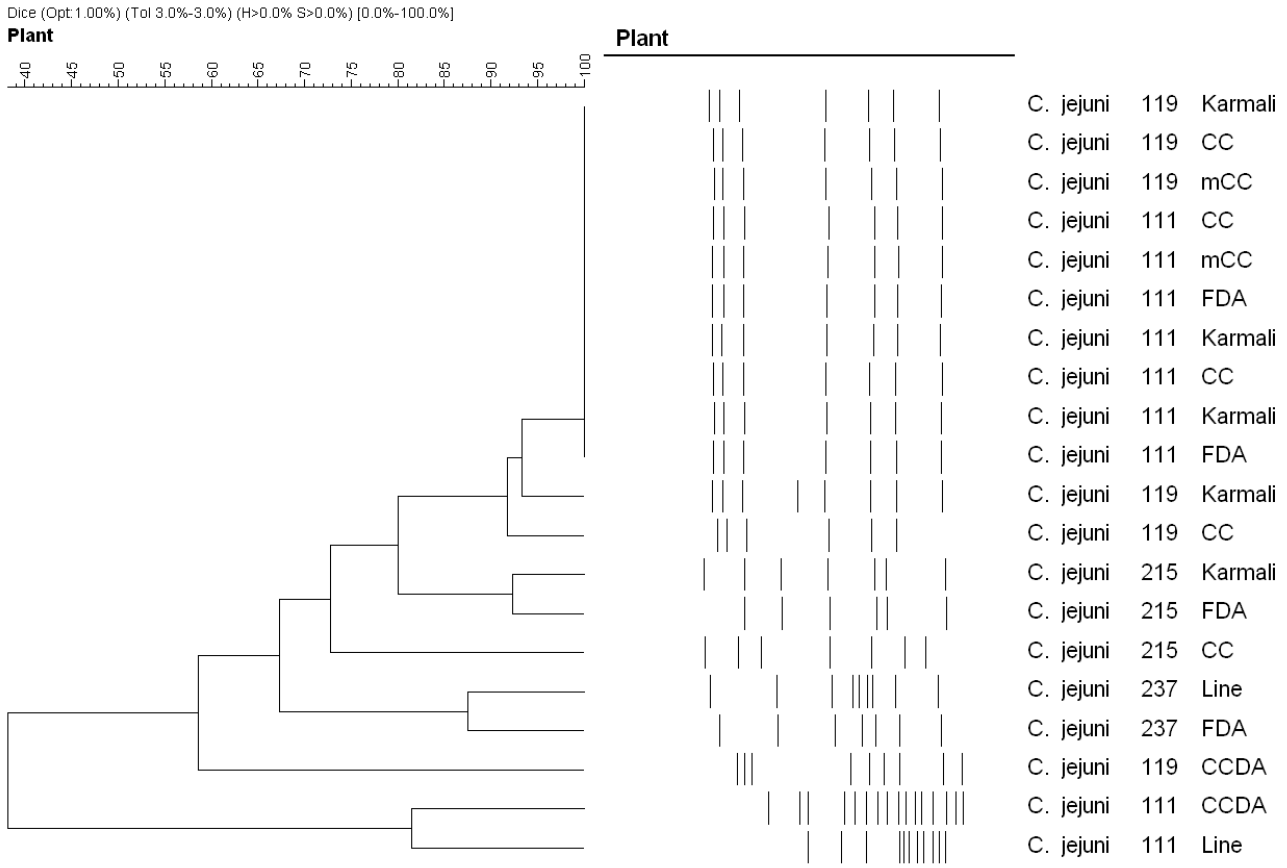

Figure 5.8. Dendrogram showing the relatedness of a subset of *Campylobacter jejuni* isolated on various plate media

Figure. 5.9. Dendrogram showing the relatedness of a subset of *Campylobacter coli* isolated on various plate media

CHAPTER VI: MULTIPLEX POLYMERASE CHAIN REACTION ASSAYS FOR SCREENING VIRULENCE GENES OF *CAMPYLOBACTER JEJUNI* ISOLATED FROM PROCESSED BROILERS

6.1. Introduction

The complete understanding of mechanism of campylobacteriosis, human diarrheal illness caused by *Campylobacter* infection is still an ongoing work and has not been completely determined. Various determinants of virulence, which are the result of the expression of various virulent genes, have been demonstrated for *Campylobacter* spp., including colonization, adhesion, invasion, and cytotoxin/ enterotoxin production (Bang *et al.,* 2003); (Ketley, 1997). Grouping *Campylobacter* spp. based on their genetic profiles in combination with their virulence abilities can be an important step towards understanding their epidemiology; such a study can also contribute to more understanding of the pathogenicity pattern of this bacterium.

Many protocols for isolation of *Campylobacter* from broiler carcasses have used direct plating media, but these are not comparable to each other, nor have they undergone full validation to warrant consistency with traditional techniques that include an enrichment step (Bashor *et al.,* 2004); (Line *et al.*, 2001; Stern & Robach, 2003); (Stern *et al.*, 2001).

A recent study conducted in our laboratories evaluated different plate media for direct enumeration of *Campylobacter* spp. on broiler carcasses (Oyarzabal *et al.*, 2005). Four commercial broiler processing facilities were sampled three times each. Twenty post chill carcasses were collected, rinsed and analyzed for the presence of *Campylobacter* spp. per visit (Oyarzabal *et al.*, 2005).

Among the virulence genes flagellin gene is the most studied and well characterized one (Nuijten *et al.*, 1990). There is evidence of involvement of the *flaA* in the colonization of *Campylobacter* (Nachamkin *et al.*, 1993); (Wassenaar *et al.*, 1993). *CadF*, has been identified as an adhesion which is a 37-kD outer membrane protein (Konkel *et al.*, 1997), and its participation in *Campylobacter* infection has been proven using animal models (Ziprin *et al.*, 1999). Cytolethal Distending Toxin (CDT) is one of the cytotoxin that has been well characterized (Pickett *et al.*, 1996); (Pickett & Whitehouse, 1999). CDT toxin is shown to be responsible for the cellular detention, and then death in cell lines (Johnson & Lior, 1988); (Pickett *et al.*, 1996); (Bang *et al.*, 2001). It has also been found that CDT blocks cell cycle in different phases (Whitehouse *et al.*, 1998). Another gene that has been associated with virulence of *Campylobacter* is *wlaN* gene. It is a β-1, 3-galactosyltransfarase responsible for synthesis of Lipooligosaccharide (LOS) (Linton *et al.*, 2000). Mutations in *docA*, encoding a periplasmic cytochrome C peroxidase reduced the colonization power of *C. jejuni* indicating its role in virulence (Hendrixson & DiRita, 2004). Studies have also shown mutations in the *fliM*, flagellar motor switch protein resulted in reduced in reduced motility (Bleumink-Pluym *et al.*, 1999); (Golden & Acheson, 2002).

The main objectives of the present work are as follow, to develop multiplex PCR assays to detect the following virulence genes: *cdtA* (blocking of eukaryotic genes in G2/early M phase), *cadF* (adhesion related), *docA* (colonization and infectivity), and several genes related to the flagella: *flgB* (basal body protein synthesis), *flgE2* (hook protein), *flhB* (protein biosynthesis and export), *fliM* (flagellar switch protein), wlaN (involved in LOS biosythensis) (Bang *et al.*, 2003); (On & Jordan, 2003) in the same samples obtained on the different plate medias which were used for PFGE analysis. Test the validity of these multiplex reactions to detect virulence genes from other samples like retail samples and human samples that are positive for the presence of *Campylobacter.*

6.2. Materials and Methods

Sources of Samples

Samples isolated from carcasses from poultry processing plants from US and Puerto Rico were analyzed for the presence of virulence genes. Nearly 90 samples from the poultry plants in the US and thirty samples from processing plants in Puerto Rico were screened.

Sample Collection

Twenty post-chill carcasses were collected, rinsed with 400ml of buffered peptone water and analyzed for the presence of Campylobacter spp. Carcass rinses were plated on six different plate media (Campy-Cefex, mCampy-Cefex, mCCDA, Karmali, CAMPY, Campy-Line) for enumeration. PFGE patterns of the isolates from different plates, same carcass; same kind of plate, different plants; and same kind of plate, same plant were compared.

Identification of Isolates

Colonies obtained on Campy-Cefex, mCampy-Cefex, mCCDA, Karmali, CAMPY, Campy-Line were observed under a phase contrast microscope (1000x) for morphology and motility.

DNA Extraction and PCR Confirmation

Strains were grown from frozen stock cultures stored at -80^{o}C in cryovials (65% TSB, 30% glycerol and 5% lysed horse blood). Cultures were streaked onto modified Campy-Cefex plates and were incubated at 42^{o}C for 36-48 h. Colonies growing on plate medium were used for the DNA extraction. DNA was extracted using Prepman Ultra^{TM} (Applied Biosystems).

A multiplex PCR targeting the cadF gene (*C. jejuni* and *C. coli*), the *ceuE* gene (*C. coli* only) and an unknown DNA fragment (*C. jejuni* only) was used to confirm the *campylobacter* spp. and genus (Cloak & Fratamico, 2002).

Statistical Analysis

Colony counts were converted to log10CFU/ml and analyzed using the general lineal procedure of SAS (SAS Institute Incorporated, Cary, NC). Means were analyzed for differences using Duncan test (SAS), and percentage values analyzed by a qualitative, McNemar chi-square ($\chi 2$) analyses (one degree of freedom). Chi-square values less than 3.84 indicated that percentages were equivalent and could not be rejected at the 5% level of confidence.

Multiplex PCR for Virulence Gene Identification

Primers: (Table 6.1) shows the published primers that were used in this study (Bang *et al.*, 2003; Konkel *et al.*, 1999; Muller, 2006) A PTC-100 Programmable thermal control (MJ Research, Inc.) was used for all the PCRs. The steps of a single amplification cycle (Table 6.2) were: 95^{o}C for 2 min (Denaturation), 50^{o}C for 1 min (Annealing)*, 72^{o}C for 2 min (Extension).

*Annealing temperatures varied according to the primer sets being used (Table 6.3). Gels were stained in 0.1% etidium bromide solution, and the image captured in a Syngene^{TM} image cabinet. The Gene Snap^{TM} program was used for further analysis of the gel.

Sequencing of the Virulence Genes

The PCR amplicons from the PCR reactions of eight virulence genes were purified and sent for amplification to the Auburn University Genomics & Sequencing Lab using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City,CA). The sequences were BLASTed to obtain nucleotide-nucleotide sequence similarities (http://ncbi.nih.gov/BLAST/).

6.3. Results

Multiplex PCR & Virulence Gene Analysis

The pre-made mix from PromegaTM was used for all the mPCRs. The use of OmniMix® (Cepheid, Sunnyvale, CA) did not give similar results. Many extra unspecific bands were seen at the end of a PCR when Omnimix was used. The four multiplex assays

developed have primers for genes *fliM* and *docA* (53^{o}C); *flgE$_2$* and *iamA* (52^{o}C); *cdtA* and *flhB* (50^{o}C); and *cadF* and *flgB* (48^{o}C). It was difficult to standardize a multiplex PCR for more than two pairs of primer set. Figure 6.1 shows how the addition of the third primer set started giving errors in the amplification reaction.

Figure 6.2 shows the respective amplified product on a gel after it has been stained with ethidium bromide. The *iamA* gene did not appear in the multiplex PCR. Howerver, when used in individually in a PCR reaction with just the primers specific to the *iamA* gene it gave a positive result for most of the samples tested.

Fifty-eight *C. jejuni* isolates obtained from one poultry processing plant were positive for *flhB, flgB, flgE2, cdtA* and *docA.* Thirty-six *C. jejuni* isolates from another processing plant were positive for the presence of these mentioned genes, except for *docA* gene.

Another set of isolates obtained from these poultry processing plants, which are being used for PFGE studies, were also screened for virulence genes. Twenty samples of this set of seventy have been analyzed for presence of virulence genes whose results are shown in (Table 6.4). The only genes that were found in all isolates were *flgB, flgE$_2$,* and *flhB.*

Out of the thirty samples from Puerto Rico that were analyzed (Table 6.4) all were negative for the presence of *iamA* genes, 2 were negative for *docA* and 1 was negative for *flhB* gene. All the thirty samples showed the presence of *flgB, cdtA* and *flgE$_2$.*

Sequencing Results

Selected sequneces from the amplicons obtained by PCR were sequenced. A Multalin analsysis of these sequences with the specific genes sequences downloaded from the NCBI database showed that the amplified products for *cdtA* gene, and *docA* gene aligned well but the sequences for *flhB*, *flgE*$_2$, *flgB* didn't align well.

6.4. Discussion

Our results showed that some primer pairs worked better at annealing temperatures different from the ones mentioned by the previous researchers. This was checked because some of the products of the multiplex reactions upon confirmation of sequence alignment didn't align well with the BLAST sequences and hence the primers that were used by previous researchers were used aligned with the corresponding genes from the BLAST sequences and the alignment was not very good. New primers will be designed and be used instead of these primers in future multiplex reactions.

It has also been observed that the amplification works with Promega^{TM} master mix alone. The pre-made mix from PromegaTM was used for all the mPCRs as the use of OmniMix® (Cepheid, Sunnyvale, CA) did not give good results. One reason might be varying levels of stringencies of the two mixes because of presence of different concentration of enzymes, MgCl2, and dNTPs, which resulted in extra, unspecific bands when using Omnimix. Use of other mix (Omnimix^{TM}) gave many extra unspecific bands; the reason may be varying levels of sensitivities between the two mixes.

Even though primers with close melting temperatures (Tms) were used, more than four or six primers in a single multiplex reaction gave unspecific products; this may be

because of binding of primers at sites other than the targeted genes, bases pairing of different primers even before they go and bind the DNA for amplification. The use of these multiplex reactions can be extrapolated for rapid screening of *Campylobacter* strains with virulent genes.

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Table 6.1. Primer sets used in the multiplex PCR for detection of virulence genes of *Campylobacter* spp. (Bang *et al.*, 2003; Konkel *et al.*, 1999; Muller, 2006)

Target gene	Primers	Sequence(5'-3')	PCR-product (bp)
fliM	CjfliM1	TCATCCTCCTCTTCAGGCTC	1011
	CjfliM2	CACCGACACACCCATAGCCTC	
docA	docA1	ATAAGGTGCGGTTTTGGC	725
	docA2	GTCTTTGCAGTAGATATG	
$flgE_2$	$flgE_2$-q-F	CATCTCACCACGACCTCCTGTTC	
	$flgE_2$-q-R	GCAAAAATCGCAATGGCTTCA	132
cdtA	GNW	GGAAATTGGATTTGGGGCTATACT	
	IVH	ATCACAAGGATAATGGACAAT	165
flhB	flhB-q-F	CAGGTGCGGATGTGGTGATC	
	flhB-q-R	CACTCCTTTGGCAACAACCCT	101
cadF	cadF2B	TTGAAGGTAATTTAGATATG	
	cadR1B	CTAATACCTAAAGTTGAAAC	400
flgB	flgB-q-F	GCACGATTTACCAAAGCTGTTTCAA	
	flgB-q-R	CACTGGTGCTTTAGCGGGTAGA	123

Table 6.2. Thermocycler conditions for 30 cycles

Steps	Temperature	Time
Denaturation	95°C	2 min
	95°C	30 sec
Annealing *	50°C	1 min
	72°C	2 min
Extension	72°C	5 min
	4°C	unlimited

*Annealing temperatures may vary depending upon the primer set being used

Table 6.3. Primer pairs that can be used in a various multiplex Polymerase chain reactions

Genes	Primers	Annealing Temperatures
fliM + *docA*	CjfliM1, CjfliM2, docA1, docA2	53^{o}C
flgE$_2$	flgE$_2$-q-F, flgE$_2$-q-R	52^{o}C
cdtA + *flhB*	GNW, IVH, flgB-q-F, flgB-q-R	50^{o}C
cadF + *flgB*	cadF2B, cadR1B, flgB-q-F, flgB-q-R	48^{o}C

Table 6.4. *Campylobacter* strains isolated from poultry processing plants screened for the presence and absence of virulence genes

Isolate ID	*fliM*	*docA*	*cadF*	*flgB*	*flgE*$_2$	*cdtA*	*flhB*
34 mCC	+	+	-	+	+	+	+
119 CCDA	+	+	+	+	+	+	+
237 line	+	+	+	+	+	+	+
34 CCDA	+	+	+	+	+	+	+
119 Kar	+	+	+	+	+	+	+
122 CCDA	-	-	+	+	+	-	+
137 mCC	+	+	+	+	+	+	+
24 Kar	+	+	+	+	+	+	+
24 FDA	+	+	+	+	+	+	+
17 CC	+	+	+	+	+	+	+
34 FDA	+	+	+	+	+	+	+
113 Kar	+	+	+	+	+	+	+
34 Kar	+	+	+	+	+	+	+
24 CCDA	+	+	+	+	+	+	+
17 mCC	+	+	+	+	+	+	+
24 CCDA	+	+	+	+	+	+	+
215 FDA	+	+	-	+	+	+	+
225 mCC	+	+	+	+	+	+	+
172 mCC	+	+	+	+	+	+	+
89 CC	-	+	+	+	+	+	+

Table 6.5. *Campylobacter* strains isolated from poultry processing plants in Puerto Rico screened for the presence and absence of virulence genes

P.rico samples	*flgB*	*docA*	*iamA*	*flhB*	*cdtA*	*flgE2*
PR11-4	+	+	-	+	+	+
PR8	+	+	-	+	+	+
PR(10)8	+	+	-	+	+	+
PR7(6)	+	+	-	+	+	+
PR5(8)	+	+	-	+	+	+
PR4(7)	+	+	-	+	+	+
PR3(6)	+	+	-	+	+	+
PR2(5)	+	+	-	+	+	+
PR17-1	+	+	-	+	+	+
PR14-5	+	+	-	+	+	+
PR14-4	+	+	-	+	+	+
PR14-2	+	+	-	+	+	+
PR14-1	+	+	-	+	+	+
PR13-4	+	-	-	+	+	+
PR13-3	+	+	-	+	+	+
PR13-2	+	+	-	+	+	+
PR13-1	+	+	-	+	+	+
PR12-4	+	+	-	+	+	+
PR11-3 (21)	+	+	-	+	+	+
PR 11-3	+	+	-	+	+	+
PR 10-5	+	+	-	+	+	+
PR10-4	+	+	-	+	+	+
PR10-3	+	+	-	+	+	+
PR10-2	+	-	-	+	+	+
PR1-4	+	+	-	+	+	+
PR1	+	+	-	+	+	+
PR	+	+	-	-	+	+

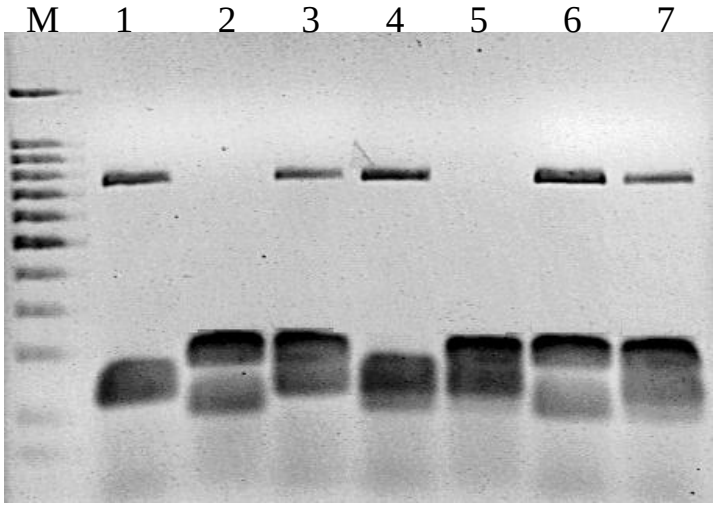

Figure 6.1. Multiplex PCR showing unspecific amplification on use of more than 2 primers for strain 34 Kar at 50^{o}C : Lanes 1 = 100bp marker, 2 = *flgB* + *docA*, 3 = *cdtA* + *flhB*, 4 = *flgB* + *docA* + *cdtA*, 5 = *flgB* + *docA* + *flhB*, 6 = *cdtA* + *flhB* + *flgB*, 7 = *cdtA* + *flhB* + *docA*, 8 = *flgB* + *docA* + *cdtA* + *flhB*.

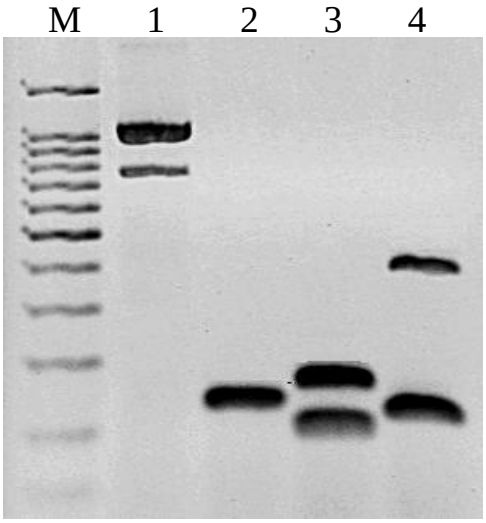

Figure 6.2. Multiplex PCR for the screening of virulence genes of Sample 237 Line: Lanes 1 = 100 bp marker, 2 = *fliM* + *docA* (53^{o}C), 3 = *flgE$_2$* + *iamA* (52^{o}C), 4 = *cdtA* + *flhB* (50^{o}C), 5 = *cadF* + *flgB* (48^{o}C).

CHAPTER VII: REDUCTION OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* ON RETAIL BROILER MEAT BY COMMERCIAL, LEMON-BASED MARINADES

7.1. Introduction

The prevalence of broiler retails samples contaminated with *Campylobacter* spp. is still high in the US (Oyarzabal *et al.*, 2007); (Wong *et al.*, 2007). Although several antimicrobial interventions strategies are currently used to reduce the number of *Campylobacter* spp. during the processing of broiler carcasses, the prevalence of contaminated carcasses and the number of *Campylobacter* cells in contaminated carcasses is still high (Oyarzabal *et al.*, 2005) Few interventions are available before cooking to further increase the number of *Campylobacter* in retail broiler meat. A process that may have an impact on bacterial foodborne pathogens in meat is the marinade process done to tenderizers and/or increase flavors in meats.

Marination is an age old method used to preserve foods. Various organic acid based marinades have been in use to prevent the growth of microorganisms in meat products. Applying marinade to meat or poultry enhances its flavor (Chen, 1982), increases its moisture retention and tenderness (Ang, 1987), preserves its color (Cassidy, 1977), and inhibits the warmed over flavor (Mahon, 1963). The spices and extracts that are added to the marinades also have preservative and antioxidant properties which are

antifungal, antibacterial and antiviral (Farag et al., 1989; Larrondo et al., 1995). The antibacterial activity is based on low pH, high NaCl concentration, and various spices added to the marinade (Perko-Makela et al., 2000). A commercial marinade usually contains oil, salt, sugar, acids, antimicrobial substances, such as sorbic and benzoic acids, and additives to reduce the hardness and change the rheological properties (elasticity and viscosity) of the meat. The acid used in commercial marinades is usually acidic or citric acid, provided usually by the addition of vinegar or lemon juice, respectively. Marinades are used to soak food products (meat or vegetables) before cooking. The consumption of marinated meat has been increasing in the last decade. In Finland, for instance, it has been estimated that around 80% of the retail poultry are sold marinated (Björkroth, 2005). Additionally, marinades have been suggested to increase product safety and shelf life, although the limited research dealing with the impact of marinades on the microbial profile of meats suggests otherwise (Björkroth, 2005).

The objectives of this work were to evaluate the reduction of *C. jejuni* and *C. coli* in retail broiler meat by the addiction of commercial marinades. In the first series of experiments, meat was artificially inoculated with *C. jejuni* or *C. coli* strains and was marinated for 0.5, 6 or 24 h. Direct plating was used to count the surviving *Campylobacter* cells and a multiplex polymerase chain reaction assays (mPCR) and pulsed-field gel electrophoresis (PFGE) were used to identify and type the strains recovered after the experiments. In the second series of experiments, we tested the efficacy of three commercial marinades in 28 meat samples that were found to be naturally contaminated with *Campylobacter* spp. For these experiment, we used a marination time of 4 h.

7.2. Materials and Methods

Marinades

The marinades used in these studies were Baja Chipotle, which contains lime juice, vinegar, salt, and spices like chipotle, chili pepper, onion, and oregano; Herb and Garlic, which contains lime juice, vinegar, dried garlic and spices; and Mesquite, which contains lime juice, garlic powder, paprika, onion powder, spices and citric acid. These marinades are manufactured by Lawry's Foods, Inc. (Fremont, CA). We also tested a lemon juice marinade containing 100% lemon juice made from concentrate (ReaLemon; Dr Pepper Snapple Group, Inc., Plano, TX). All marinades were purchased from a local food store. We also tested sterile water adjusted to pH 3 with 1N hydrochlorous acid. The composition of each of the marinade is as follow:

- Real Lemon – Contains 100% lemon juice made from concentrate.
- Baja Chipotle – Contains lime juice, vinegar, salt, and spices like chipotle, chilli pepper, onion, and oregano.
- Herb and Garlic – Contains lime juice, vinegar, dried garlic and spices.
- Mesquite – Contains lime juice, garlic powder, paprika, onion powder, spices and citric acid.

Artificial inoculation of meat samples

The *Campylobacter* strains used in these studies were *C. jejuni* 1065 and PR-13-4, and *C. coli* 947 and 956. Strains 947, 946 and 1065 have been isolated from retails,

broiler meat (Oyarzabal *et al.*, 2007), while strain PR-13-4 has been isolated from fecal material of live broilers (Oyarzabal et al., 2008). Strains were started from freezer stocks and were cultured twice in modified Campy-Cefex (mCC) agar plates (Oyarzabal et al., 2005) at 42^{o}C for 24 h. Growth from three agar plates was dissolved into 4.5 ml phosphate buffer solution (PBS) and were adjusted to an $OD_{600\ nm}$ value of 1.6 to 1.8. This inoculum was then diluted into 20 ml of PBS in a plastic, spray bottle for a final concentration of approximately 7 log CFU/ml. This inoculum was used to spray skinless, boneless breast meat that was purchased from local stores. The meat was cut in 16 pieces of square-shape (approximately 50 g each) breast meat that were spray (all sides) in a BLS II flow laminar cabinet. The pieces of meat were left to dry in the laminar flow cabinet for 1 h. The inoculated pieces of meat were then placed in plastic zip-lock bags (Glad) which were stored at ~4^{o}C for 24 h.

Marination of broiler meat

After 24 h, two inoculated pieces of breast fillet were placed in disinfected, plastic containers (The Glad Products Company, Oakland, CA) and were covered with 300 ml of the marinade. The volume of marinade allowed for the complete coverage of the two pieces of breast fillets. Each marination experiment with artificially inoculate meat included the following treatments: two samples that were not marinated (control without marination), two samples marinated in sterile distilled water adjusted to pH 3, and two samples marinated in each of the following marinades: Baja Chipotle, Herb & Garlic, Mesquite and ReaLemon. All samples were marinated in 300 ml of the marinade and in plastic containers that were closed and placed at 4^{o}C for the duration of the

marination. Three independent replicates were done for each inoculated strain. These replicates included the preparation of a new inoculation and the marination experiments. Samples inoculated with *C. jejuni* were marinated for 0.5, 6 or 24 h, while samples inoculated with *C. coli* were marinated for 6 h.

Enumeration of Campylobacter spp. after marination

At the end of the marination, meat portions were removed from the marinades, the excess of marinade was cleaned and each piece was weighed and place in sterile bags (Whirl-Pak®, Nasco, Fort Atkinson, WI). Bolton broth supplemented with 10% lysed horse blood was added at a volume of twice the weight amount of the meat. Samples were stomached for 1 minute and a 10-fold serial dilution was made in PBS tubes. Duplicate plates were made on mCC plates for enumeration of surviving *Campylobacter* cells. Agar plates were incubated under a microaerobic gas mix (10% CO_2, 5% O_2, and 85% N_2; Airgas, Radnor, PA) that was added into anaerobic jars by the evacuation/replacement methods using a MACSmics Jar Gassing System (Microbiology International, Frederick, MD). Presumptive *Campylobacter* colonies were counted on plates after incubation at 42°C for 42 h. Average counts from each marination were subtracted from the average count from the meat that were not marinated (control without marination).

The meat samples were also enriched in Bolton broth under microaerobic conditions at 42°C for 24 h. A loopful of broth (~ 100 ml) was transferred to mCC plates, which were incubated under similar conditions for 48 h. Positive samples were determined by phase contrast analysis of presumptive colonies. In selected experiments,

presumptive colonies were stored at -80^{o}C for further analysis as described under Identification and Typing of isolates. If no colonies were found on enumeration plates but the enriched sample was positive, a value of 10 CFU per g of meat was assigned for that sample. Samples were considered negative if no growth was found on enumeration plate or after the enrichment of the samples.

Marination of meat naturally contaminated with Campylobacter spp.

Forty-six breast (skinless, boneless) meat samples were screened for the presence of *Campylobacter* spp. from April 2008 to October 2008. Fourteen positive samples were used for these marination trails. Two independents marination runs were performed with each sample for a total of 28 experiments. Samples were screened for *Campylobacter* spp. by cutting 25 g of meat and enriching it in sterile plastic bags (Whirl-Pak®, Nasco, Fort Atkinson, WI) with 100 ml of Bolton broth (Oyarzabal et al., 2007). Samples were incubated at 42^{o}C under microaerobic conditions for 48 h. Enriched samples were transferred to mCC plates that were incubated at at 42^{o}C under microaerobic conditions for 48 h. Phase contrast microscopy was used to determine presumptive *Campylobacter* spp.

Fifty g pieces were cut from samples that were found to be positive for *Campylobacter* spp. for the marination experiment with naturally occurring campylobacters. Meat samples were marinated with Real Lemon, Baja Chipotle, and Herb and Garlic at 4^{o}C for 4 h, using 300 ml of each marinade per container. Two 50-g pieces were marinated per container. The pH of the different marinades was recorded before marination and at the end of the marination. After marination, the meat was

transferred into a sterile whirl pack bag using sterile forceps. Samples were enriched with 200 ml of Bolton at 42^{o}C for 48 h. Analysis of the enriched samples under phase contrast microscopy was used to determine presumptive positives. Enriched samples were also streaked onto mCC plates to confirm the presence of *Campylobacter*. Plates were incubated at 42^{o}C for 48 h. Presumptive colonies under phase contrast microscopy were stored at -80^{o}C in Brucella broth supplemented with 30% glycerol for further analysis.

Identification and characterization of isolates

Stock cultures were started on mCC paltes and bacterial DNA was extracted using Prepman Ultra^{TM} (Applied Biosystems, Foster City, CA). For selected inoculation experiments and for all naturally-occurring isolates, bacterial DNA was tested with a multiplex polymerase chain reaction (mPCR) assay that specifically reacts with *C. jejun*i and *C. coli* (Oyarzabal et al., 2007). To validate that the inoculated strain was the one recovered from the experiments when artificial inoculation was employed, we analyzed the isolates with pulsed-field gel electrophoresis (PFGE). This technique was selected because it provides a fingerprinting pattern for each isolate. These isolates can then be compared for relatedness to assure that the inoculated and the recovered strains are the same. PFGE was performed as described elsewhere (Oyarzabal et al., 2008). Briefly, A digestion of *Salmonella choleraesuis* ss. *Choleraesuis* serotype Braenderup H9812 (ATCC BAA-664) was used as the DNA marker. *Campylobacter* DNA was digested with *Sma*I and restricted DNA fragments were separated with a contour-clamped homogeneous electric field (CHEF Mapper, Bio-Rad, Hercules, CA) in 1% agarose gel stained with ethidium bromide. The gels were visualized with a UV transilluminator

(Gel-Doc System, Bio-Rad), and a picture recorded using GeneSnap (Syngene, Frederick, MD). PFGE profiles were visually compared.

Statistical analysis

The reduction by each marinade treatment was calculated by subtracting the counts after the treatment with marinades from the inoculated samples that were not treated (control without marination). In the case of samples tested for reduction in naturally occurring *Campylobacter* also the Chi square analysis (χ^2) or the McNemar's Test (McNemar, 1947) was used to calculate if there was any significant difference or not . A McNemar's test value ≤ 3.84 assumes the null hypothesis; that means that the test method (marination) and reference method (control) are equivalent and cannot be rejected at the 5% level of confidence ($P < 0.05$).

7.3. Results

Artificial inoculation of meat samples

The average weight of the pieces that were artificially inoculated with *Campylobacter* strains was 56.7 g (± 2.5 SEM). Table 2 show the counts of C. jejuni after marination times of 0.5 and 24 h. A 0.5 h marination time showed a minimal reduction, of 0.07 to 0.23 $\log_{10}$ CFU per g for Baja Chipotle; Herb & Garlic, Mesquite and Water at pH 3. ReaLemon showed a higher decreased compared to tcontrols, but the reduction was not more than 1 $\log_{10}$ CFU per g of meat.

A 24 h marination showed a higher reduction compared to non-marinated controls (Table 7.1). Water at pH 3 and Mesquite yielded a reduction of 0.24 and 0.79 $\log_{10}$ CFU

per g of meat, respectively. Baja Chipotle and Herb & Garlic resulted in a higher reduction, with 1.14 and 1.15 $\log_{10}$ CFU per g of meat, respectively. ReaLemon again showed the highest reduction with 3.59 $\log_{10}$ CFU per g of meat less than the non-marinated control.

A marination time of 6 h resulted in reductions of *C. jejuni* that were similar for Baja Chipotle, Herb & Garlic, Mesquite (0.45, 0.60 and 0.41 $\log_{10}$ CFU per g, respectively). Water did not result in any reduction. ReaLemon yielded the highest reduction (1.66 $\log_{10}$ CFU per g). This reduction was significant ($P < 0.05$) when compared to the reductions produced by the other marinades.

The pattern was similar for the reductions of *C. coli* by marindes, with the difference that the reductions were higher than the values recorded for *C. jejuni* (Table 7.2). The reduction produced by Baja Chipotle, Herb & Garlic and Mesquite were similar and in the range of 0.92 to 1.10 $\log_{10}$ CFU per g. Again, water at pH 3 resulted in a modest reduction of 0.29 $\log_{10}$ CFU per g, and ReaLemon showed the largest reduction, with 2.02 $\log_{10}$ CFU per g. The reduction by ReaLemon was significant ($P < 0.05$) when compared to the reductions produced by the other marinades.

Naturally occurring Campylobacter spp

Fourteen positive samples were used for these marination trails, with two independent experiments run with each sample. Yet, from 28 positive samples, only 15 samples yielded positive controls. The rest of the samples (13) yielded negative controls. When determining the effects of marinades in naturally-occurring *Campylobacter* spp., there was a significant ($P < 0.05$) reduction by the marinades in comparison to the control

(non-marinated). In the case of Herb and Garlic there were two *Campylobacter* positive samples after marination, while only one sample was *Campylobacter* positive after marination with for Baja Chipotle and one after marination with ReaLemon (Table 7.3).

Marinades pH

Table 7.4 shows the average pH value for each of the marinades and the broiler meat surface at the beginning and at the end of 4 h marination. These values were recorded from the experiments with naturally contaminated meat. The lowest pH values were recorded with ReaLemon. For all marinations, the pH increase by 0.2 units and was significant. The pH values for the surface of the meat before marination was 6.27 and after marination reduced to almost 3.53 (real lemon).

Pulse Field Gel Electrophoresis

PFGE comparisons between the inoculated strains and the strains collected after direct plating showed that only the inoculated strains were recovered in the experiments with the artificially inoculated meat (Figure 7.1).

7.4. Discussion

The high prevalence of *Campylobacter* spp. in broiler retails meat requires a continuous search for alternatives to reduce the incidence of these pathogens in retail meat products. Our work was aimed at determining the impact of marinations in the control of *Campylobacter* spp., mainly *C. jejuni* and *C. coli*, in retail broiler meat. We chose breast meat because this product is the most common broiler meat product

available in retail stores across the US. We also wanted to test commercial marinades and the choice of the tested marinades was done based on marinades that are commonly sold in the US (Baja Chipotle, Herb and Garlic and Mesquite). We also tested lemon juice-based marinade (ReaLemon) and sterile water at pH 3 because of their lower pH.

There are few studies evaluating the effect of marinades on the reduction of *Campylobacter* in poultry meat. Because direct contact is required for marinades to induce chemical reaction for the tenderization of meat, and to provide the antimicrobial effects, we soaked the meat in plastic containers to assure that all the surfaces of a meat were in contact with the marinade. In our studies, the marination of meat for 0.5 and 6 h resulted in a minimal reduction of *Campylobacter* spp. (less than 1 Log_{10} CFU per g of meat). The limited effect of marinades on the reduction of *C. jejuni* has been linked to the buffering capability of meat, which can quickly neutralize the pH of acidic marinades (Björkroth, 2005). The change in the acidic pH towards neutrality results in dissociation of the lipophilic acids and limits the antimicrobial effect of marinades. Interestingly, at 6 h marination, we observed that the reduction of *C. coli* was much higher than the reduction recorded for *C. jejuni* strains. We believe this is the first report on the impact of marinades on *C. coli*. This *Campylobacter* species has been found to be more susceptible to the effect of low pH than *C. jejuni*, and culture broth adjusted to pH 5 had a 5 Log_{10} CFU/ml reduction of *C. coli* versus only 2.5 Log_{10} CFU/ml reduction of *C. jejuni* during a 12-h contact time (Shaheen et al., 2007). It is apparent that more research should be done to elucidate the physiological properties of *C. coli* that make this *Campylobacter* species more susceptible to lower pH.

The antimicrobial effects of Baja Chipotle, Herb and Garlic and Mesquite applied for 6 h were similar to the effect of sterile water adjusted to pH 3. These findings suggest that the actual pH of the marinades may have been responsible for the antimicrobial effect against *Campylobacter*. However, when comparing the effects of these marinades with ReaLemon, which has a pH similar to Baja Chipotle (Table 7.4), it is clear that the pH by itself does not account for the antimicrobial effect. Also when water at pH 3 was used to marinate, the pH at the end of 4 h of marination dramatically increased to 5.98. Even on the surface of the meat the pH after marination increased the most in the case of water at pH 3. Thus pH alone doesn't seem to play a role in the reduction in numbers of campylobacters. It has been suggested that the antibacterial activity of marinades is based on low pH, organic acids, high NaCl concentration, and various spices added to the marinade (Perko-Makela et al., 2000). The most stable pH of the marinade before and after marination was seen in the case of Baja Chipotle in whose case the pH increased from 3.11 to 3.18. Yet, to overcome the buffering capacity of meat a high concentration of these substances or longer contact times are needed. In our studies, a longer contact time (24 h) of the marinade with the meat did provide a higher numerical reduction. This reduction, however, was in the range of only 1.1 Log_{10} CFU per g of meat, and only for two marinades (Baja Chipotle and Herb & Garlic). ReaLemon did yield a reduction that was higher than 3 Log_{10} CFU g of meat. However this long contact time in a lemon juice marinade (high content of citric acid) resulted in meat that lost most of its organoleptic charactersitics and was undesirable for human consumption.

The first series of experiments allowed us to decide on a more realistic marination time that could be used for the testing of naturally contamianted retal broiler meat.

A 4 h marination time was deccided and all retail meat samples that were used in these experiments were analyzed for the presence of naturally occurring *Campylobacter* spp. . We used 14 positive samples to run 28 independent experiments. From these experiments, 15 experiments had controls that were positive while 13 experiments had controls that were negative. Although the meat pieces were from the same package and were considered as one sample, we used different breast fillets and apparently *Campylobacter* was present on some fillets but absent on other fillets. These findings highlights the difficulties of testing for naturally occurring *Campylobacter* spp. in retail broiler meat. The distribution of *Campylobacter* is not uniform across the sample and tehrefore we could not garantee that the rest of the meat was positive. In addition, because *Campylobacter* spp. are usually present in low numbers in retail broiler meat (Oyarzabal et al., 2007), there was a need to enrich the samples and therefore a direct enumeration procedure was not employed.

We did find a significant decrease of *Campylobacter* spp. with Baja Chipotle, Herb & Garlic and ReLemon by chi-square analysis. However, the Kappa values and the accuracy index calculated in these comparisons suggest that the analyzed set of data is not large enough to draw any further conclusions. We believe a much larger data set should be analyzed to determine if a 4-h marination can indeed provide a consistent reduction of *Campylobacter* spp. in retail broiler meat. Some researchers have concluded that the buffering capability of meat may neutralize acidic marinades and thus the marinades may have less antimicrobial effects against bacterial pathogens (Björkroth *et al.*, 2005). However, there are several substances that may have antimicrobial effects in a marinade.

Some of these substances may be impacted by pH changes while others may just have an effect based on concentrations and contact with the bacteria.

Other studies have reported that marination did not result in significant reductions in the numbers of *C. jejuni* in boiler meat. One study (Perko-Makela et al., 2000) determined the survival of *C. jejuni* on marinated chicken drumsticks and sliced breasts strips stored at 4^{o}C. There was no significant difference in *C. jejuni* numbers between the marinated and non-marinated chicken meats. The marinade that was used was an emulsion of vegetable oil, water, spices and salt. In another study with poultry products in Finland, the low pH of marinades selected for lactic acid bacteria (LAB) bacteria and marinated meat spoiled faster. Again, no reduction of *C. jejuni* was found by the use of marinades (Lundström and Björkroth 2007).

In summary, commercial marination did not provide a consistently significant reduction of *C. jejuni* or *C. coli* in short contact times (0.5 h). A contact time of 6 h still provided inconsistent reductions that were less than 1 Log CFU/g of meat, except for ReaLemon which provided a higher reduction. However, the meat marinated in ReaLemon for 6 h lost most of its quality. *C. coli* exhibited a higher numerical reduction than *C. jejuni* by these marinades, suggesting that unique physiological properties may render *C. coli* more susceptible to low pH. Finally, the trials to assess the antimicrobial effect of marinades on retail broiler meat naturally contaminated with *Campylobacter* spp. suggested that a 4-h marination time may provide a significant reduction. Yet these findings should be corroborated with a larger number of samples, and perhaps with other commercial marinades, to withdraw any further conclusions.

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Table 7.1. Reduction of *C. jejuni* numbers under different marinades under refrigeration and two marination times (0.5 and 24 h) on inoculated *C. jejuni* in broiler breast meat.

Marinade	Mean Log CFU/g (± SEM) [a]	
	0.5 h	24 H
Baja Chipotle	0.26 (0.17) A	1.13 (0.19) B
Herb & Garlic	0.26 (0.21) A	1.15 (0.17) B
Mesquite	0.40 (0.30) A	0.76 (0.32) B
ReaLemon	0.96 (0.50) A	3.58 (0.45) A
Water (pH 3)	0.30 (0.20) A	0.26 (0.15) B

[a] Each value is the mean of there independent trials with *C. jejuni* strain B 1065 and three indent trials with *C. jejuni* strain PR-13-4. SEM = standard error of the mean. Means in a column followed by different letters are statistically different ($P < 0.05$) by Duncan's test.

Table 7.2. Reduction of *C. jejuni* and *C. coli* numbers on different marinades applied for six h under refrigeration on inoculated *C. jejuni* and *C. coli* in broiler breast meat.

Marinade	Mean Log CFU/g (± SEM) [a]	
	C. jejuni [b]	*C. coli* [b]
Baja Chipotle	0.45 (0.11) B[c]	1.08 (0.23) B[c]
Herb & Garlic	0.61 (0.20) B	1.00 (0.15) B
Mesquite	0.41 (0.09) B	0.90 (0.23) B
ReaLemon	1.66 (0.38) A	1.96 (0.25) A
Water (pH 3)	0.66 (0.03) B	0.58 (0.24) B

[a] Each value is the mean of six independent trials (three replicates per strain) for two *C. jejuni* and two *C. coli* strains. SEM = standard error of the mean. Means in a column followed by different letters are statistically different ($P < 0.05$) by Duncan's test.

[b] The reduction for *C. jejuni* and *C. coli* were not statistically similar for most marinades, except for Baja Chipotle, in which the reduction observed for *C. coli* (1.08) was statistically higher than the one recorded for *C. jejuni* (0.45).

[c] The reduction of *C. coly* was higher ($P < 0.05$ by Duncan's test) than the reduction of *C. jejuni* by Baja Chipotle.

Table 7.3. Detection of naturally occurring *Campylobacter* spp. in retail meat after the marination of the meat with Herb and Garlic, Baja Chipotle or ReaLemon for 4 h. Chi-square (McNemar) comparison between control (non-marinated) and marinated samples.

	Control Versus		
	Herb and Garlic	Baja Chipotle	Lemon Juice
Chi Square	15.1	17.1	17.1
Kappa	0.13	0.00	0.00
Sensitivity	0.13	0.00	0.00
Specificity	1.00	1.00	1.00
False-negative rate	0.87	1.00	1.00
Negative predictive value	0.50	0.46	0.46
Accuracy	0.54	0.46	0.46

Table 7.4. pH of the different marinades and the retail broiler meat used in these experiment before and after four hours of marination

Product	Time of Monitoring	pH Value (± SEM)
Meat	Before marination	6.27 (0.040) A
Water at pH 3	Before marination	3.19 (0.017) G,H
	After marination	5.98 (0.039) B
	Meat after marination	5.74 (0.134) C
Baja Chipotle	Before marination	3.11 (0.006) H,I
	After marination	3.18 (0.009) H
	Meat after marination	3.99 (0.017) E
Herb & Garlic	Before marination	3.36 (0.004) F,G
	After marination	3.53 (0.045) F
	Meat after marination	4.25 (0.021) D
ReaLemon	Before marination	2.66 (0.006) J
	After marination	2.97 (0.026) I
	Meat after marination	3.53 (0.157) F

SEM = standard error of the mean.

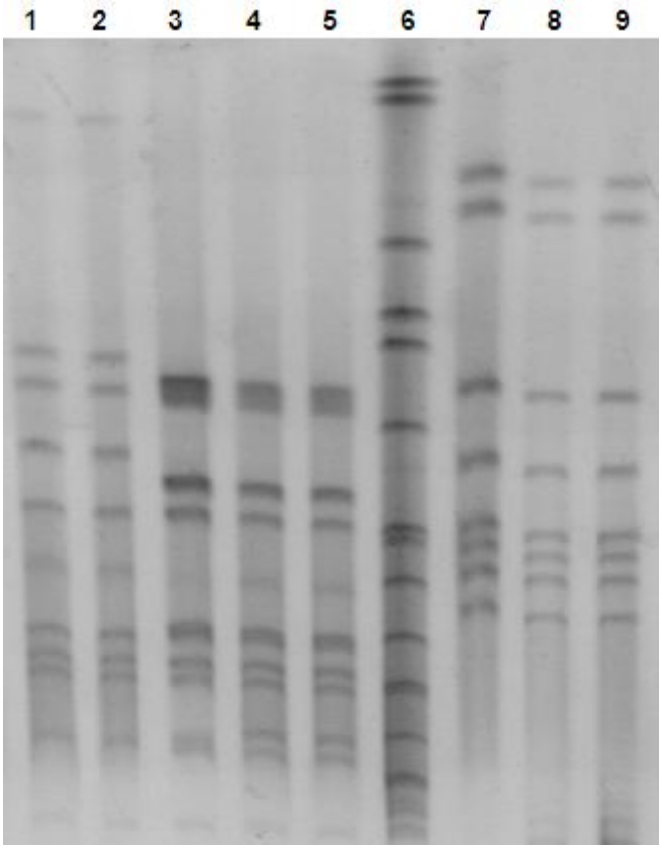

Figure 7.1. PFGE profiles of *C. jejuni* and *C. coli* isolates used in these studies. Line 1-2: *C. coli* 947 before inoculation (L1) and collected from Mesquite after inoculation (L2); Line 3-5, C. coli 956 before inoculation (L3) and collected from Herb and Garlic after inoculation (L4-5); Line 6: DNA marker, a digestion of *Salmonella choleraesuis* ss. *Choleraesuis* serotype Braenderup H9812 (ATCC BAA-664) ; Line 7-9, *C. jejuni* 1065 before inoculation (L7) and collected from Baja Chipotle after inoculation (L8-9).

DISSERTATION CONCLUSIONS

The Oxyrase study gave the following results. Oxyrase in Bolton broth performed similar to Bolton broth with microaerobic gas mix. mCC + Oxyrase in petri plate (bagged) performed poorly. mCC + Oxyrase in OxyDish performed well. DGGE analysis showed the presence of different microbiota in the two enrichment broths. RFLP analysis also showed that different strains were present.

The Bolton without microaerobic gas mix gave the following results. Microscopy and Plate results comparison showed no differences between the sub-samples for *Campylobacter* ($P > 0.05$). *Campylobacter* enriched in Bolton broth may not need a microaerobic gas mix to multiply. DGGE indicated the occurrence of diverse microbial population. RFLP analysis showed the presence of different strains.

The pulsed-field gel electrophoresis study gave the following results. PFGE data revealed a considerable amount of diversity in the genomic profile of isolates from a single carcass. More than one strain of *Campylobacter* spp. were isolated from the same carcass. High prevalence rates in the processing facilities (78%, 78% and 62%, for plants A, B and C, respectively). However, one plant D showed a lower percentage (33%).

The multiplex PCR for virulence genes study gave the following results. Three multiplex assays for six virulence genes developed. More than six primers in one assay resulted in unspecific amplicons.

Multiplex assays successfully used to screen virulence genes of *C. jejuni* strains from broiler carcasses from the US and *C. jejuni* from commercial broilers from Puerto Rico.

The marination of retail broiler meat studies gave the following results. Significant reduction in *Campylobacter* numbers in inoculated meat in the marination with real lemon for 24 h at 4^{o}C. Significant reduction in naturally occurring *Campylobacter* in retail broiler meat wen marinated with all the marinades 4 h at 4^{o}C.

Thus in combination the following studies gave the following results. Growth of *Campylobacter* from retail meat enriched in Bolton broth may not need a microaerobic gas mix. Oxyrase can be used as a supplement in the media for the growth of *Campylobacter*. Enrichment in Bolton with oxyrase and Bolton without the gas mix indicates a possibility of diverse microbial niche. Single broiler carcass inhabits more than one strain of *Campylobacter.* Multiplex PCR assays can now be used for detection of pathogenicity genes. Marinades can significantly reduce numbers of naturally occuring campylobacters.

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