GENETIC DIVERSITY OF *CAMPYLOBACTER* ON BROILER CARCASSES

by

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(Under the Direction of Mark E. Berrang)

ABSTRACT

*Campylobacter jejuni* and *C. coli* are the most important human enteropathogens among the campylobacters. The objective of this study was to determine how diversity in *Campylobacter* found on chicken carcasses collected from re-hang and post chill sites at 17 poultry processing plants in the United States is impacted during processing by sequencing the Short Variable Region of the flaA locus. Seventy percent of carcasses had one *flaA*-SVR type detected. *Campylobacter* genetic diversity decreased as carcasses proceeded through processing; carcasses sampled at re-hang had significantly more genetic diversity in *Campylobacter* populations than carcasses sampled at post chill. There was more diversity in *Campylobacter* on carcasses collected during winter than spring, summer, or fall. There were certain types that were present at re-hang that were not present at post chill, and vice versa, suggesting that there are certain types that are prone to perish during processing while others may survive or persist in the stressful processing environments.

INDEX WORDS: *Campylobacter*, genetic diversity, *fla* typing, *flaA*-SVR
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CHAPTER 1

INTRODUCTION

_Campylobacter jejuni_ and _C. coli_ are spiral, curved, or s-shaped rods that are 0.2 to 0.8 μm in width and 0.5 to 5 μm in length and the most important human enteropathogens among the campylobacters, affecting an estimated 2.4 million cases each year in the U.S. alone (2, 10). In the U.S., there were approximately 5,712 laboratory-confirmed cases of campylobacteriosis in 2006, according to the Centers for Disease Control and Prevention; second only to _Salmonella_ infections (2). Although _C. jejuni, C. coli, C. lari_, and _C. upsaliensis_ are all capable of causing sickness in humans, most cases of campylobacteriosis are caused by _C. jejuni_ (85%) with the majority of the remaining caused by _C. coli_ (2, 9, 10). Risk factors for contracting campylobacteriosis include consumption and handling of raw and undercooked poultry, most commonly chicken and turkey products, cross contamination with other foods, and contaminated milk and water (1, 4, 11, 15, 8).

Both _C. coli_ and _C. jejuni_ colonize the intestines of food-producing animals and humans (16). The favored environment appears to be the intestines of avians, which may be due the body temperature of 42° C, which is the optimal growth temperature for _Campylobacter_. There have been many investigations into the possible sources of contamination of poultry flocks with _Campylobacter_, but no definitive factor has been determined to explain its incidence (4, 8). Since _Campylobacter_ is ubiquitous, it is easy for the flock to be exposed to many potential sources of contamination; current sampling
and culture methods may not be adequate to recover all bacterial cells present. In avians, *Campylobacter* is a commensal that colonizes the gut. After one member of a flock has become contaminated, *Campylobacter* is transmitted from that animal to other animals vertically and horizontally (5, 7).

*C. jejuni* and *C. coli* can be found on 90% of poultry in the U.S. (17), and from 18% to >90% of poultry in Europe, varying from country to country (12). Carry over contamination from a positive flock to a negative one is a source for contamination in processing (3, 12). Cross contamination in the slaughter plant due to contaminated equipment and processing water, is very difficult to control. Finished products that are heavily contaminated with high numbers of microbes are considered undesirable from a food safety and quality point of view. There are various steps in processing designed to eliminate or control these microbes, such as multiple washes and chilling. In some instances, processing aids are used to further reduce the microbial load on chicken carcasses by removing surface contamination.

Measures taken to reduce or eliminate the prevalence of *Campylobacter* are being researched and applied in the food industry. Prevalence in foodstuffs is likely to have a huge impact on the health care industry, as *Campylobacter*-related illness and hospitalization costs are $8 billion in the United States alone (6). The underlying principle for reduction of *Campylobacter* infection in humans is to prevent colonization of poultry, or to reduce the prevalence on carcasses during processing.

Information relative to the diversity of *Campylobacter* and nature of infection helps in the investigations of adaptation. Molecular subtyping is an important tool for epidemiological studies; it helps in tracing sources and routes of transmission of human
infection, identifying and monitoring specific strains over time and different regions with important characteristics. Molecular subtyping also contributes to the development of strategies to control transmission, elucidate sources, and determine possible routes of contamination in the food chain (13, 14, 19).

The objective of this study were

1. Determine the genetic diversity of *Campylobacter* collected from rehang and post-chill sites by sequencing the Short Variable Region (SVR) of the flagellin locus in *Campylobacter*

2. Determine how diversity in *Campylobacter* found on chicken carcasses is impacted by processing
References


CHAPTER 2
LITERATURE REVIEW

History

_Campylobacter_ was first observed in 1886 by Theodore Escherich (34), who described it as a non culturable _Vibrio_ organism found in the stool of infants with diarrhea. Before the second half of the 20th century, it was more recognized as a veterinary disease than a human pathogen. John Macfayden and Stewart Stockman (125) were pioneers in veterinary _Campylobacter_ research. They were responsible for determining its role in animal husbandry as the etiologic agent responsible for veterinary deaths and stillbirths. Until 1987, campylobacters were classified as _Vibrio_ species. Vibrionic abortion was a major cause of abortion in sheep and cattle in the entire world. “V. fetus,” now known as _C. fetus_, was found to be the cause (126). It was also first found to cause diarrhea and enteric disease in animals.

Bacteria that currently belong to the _Campylobacter_ species were originally difficult to classify and were assigned to a _Vibrio_ species due to cell morphology. _V. jejuni_ was classified in 1927, while _V. coli_ was classified in 1944. _V. jejuni_ was associated with bovine dysentery, human gastroenteritis, and aborted sheep fetuses and _V. coli_ was associated with pigs with diarrhea (30). However, there were differences between these vibrionic organisms and those in the _Vibrio_ family, including microaerophilic growth requirements, and non-fermentative metabolism (139). In 1973, Vèron and Chatelain (140) performed a comprehensive study on the taxonomy of these
microaerophilic, vibrionic organisms, and added *C. coli*, *C. jejuni*, and *C. sputorum* to the genus *Campylobacter* that was created in 1963 by Sebald and Vèron. During the 1980’s there was an explosion of *Campylobacter* research, where *C. conscious* (131), *C. sputorum* subsp. *mucosalis* (67), *C. nitrofigilis* (74), *C. hyointestinalis* (44), *C. lari* (9), *C. pylori* (73), *C. cryaerophila* (85), *C. cinaedi* (136), *C. fennelliae* (136), and *C. mustelae* (39) were isolated and classified. In rRNA homology studies of a portion of the 16S rRNA sequence, results showed that *Campylobacter* species belongs to three major phylogenetic clusters: the first one includes *C. fetus*, *C. hyointestinalis*, *C. sputorum*, *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. conscious* and *C. mucosalis*. The second includes *C. pylori*, *C. fennelliae*, and *Wolinella succinogenes*. And the third includes *C. nitrofigilis*, and *C. cryaerophila* (134). Revision of *Campylobacter* taxonomy was initiated in 1989 by Goodwin. The genus *Helicobacter* was created to include *C. pylori* and *C. mustelae*. *C. cinaedi* and *C. fennelliae* was changed to *H. cinaedi* and *H. fennelia* (45). Organisms in the second cluster of campylobacters were given the name *Arcobacter* (139). The family *Campylobacteriaceae* is now comprised of *Campylobacter*, *Arcobacter*, *Helicobacter*, *Sulfurospirillum* and *Bacteroides ureorlyticus* (139).

**General Characteristics**

Bacterial cells in the genus *Campylobacter* are spiral, curved, or s-shaped rods that are 0.2 to 0.8 μm in width and 0.5 to 5 μm in length (139). Old or abused cultures form coccoid bodies, that may not grow by standard microbiological techniques, which is termed the viable but not culturable (VBNC) state (53). *Campylobacter* are non-sporeforming, microaerophilic rods with atmospheric requirements of an O₂
concentration of 3 to 15%, a CO₂ concentration of 3 to 5%, and the balance nitrogen. Some campylobacters require H₂ in its microaerophilic environment and growth may be enhanced due to its addition. Mobility and chemotaxis are due to an unsheathed flagella at one or both ends of the cell. Metabolism is respiratory and chemoorganic (139). C. gracilis is non motile and C. showae has multiple flagella (139). Typical biochemical reactions that help to identify this organism are reduction of fumarate to succinate, negative methyl red reaction, and acetoin and indole production (139). C. jejuni and C. coli are commonly referred to as thermophilic campylobacters, which grow best at 37 to 42 C with a genome of about 1600 to 1700 kb (34). C. jejuni can be differentiated from C. coli by its ability to hydrolyze hippurate. (52)

Isolation

In early Campylobacter research, culture and isolation were problematic. Originally, selective filtration, using the small cell size and motility, was the method used to separate C. jejuni from fecal samples (18, 27). It is difficult to isolate Campylobacter spp. from food samples because of their low numbers and the presence of damaged cells. Isolation from food may require an additional pre-enrichment step, where damaged cells are not inhibited, with a reduced temperature of 37°C to promote growth and survival. After a short enrichment step, usually 12 hours, the sample is then plated onto selective media. Campylobacter is resistant to bacitracin, novobiocin, polymixin B, cephalothin, vancomycin, vitampicin (17, 103), and these antibiotics are commonly added to media to aid in selection for campylobacters. However, with the addition of some antibiotics, such as cephalothin, some strains of C. jejuni, C. coli, and C. lari are missed due to sensitivity (14). Media generally includes antibiotics, as well as ingredients to neutralize possible...
toxic effects from oxygen and light, such as lysed blood, charcoal, a combination of ferrous sulphate, sodium metabisulphite and sodium pyruvate; also haemin or haematin.

**Campylobacter and Human Infection**

*C. jejuni* and *C. coli* are the most important human enteropathogens among the campylobacters, causing an estimated 2.4 million cases each year in the U.S. alone (5,41). *C. jejuni* is the predominant species in poultry and *C. coli* is commonly found in swine. In the U.S., there were approximately 5,712 laboratory-confirmed cases of campylobacteriosis in 2006, according to the CDC; second only to *Salmonella* infections (5). The incidence of infection has decreased by 30% from baseline studies, conducted from 1996 to 1998; most of the decline occurred by 2001, due to implementation of HACCP programs in 1996 (4).

Risks for contracting campylobacteriosis are consumption and handling of raw and undercooked poultry, most commonly chicken and turkey products, cross contamination with other foods, and contaminated milk and water (3, 8, 79, 121, 132). *C. jejuni* and *C. coli* can be found on 90% of poultry in the U.S. (129), and from 18% to >90% of poultry in Europe, varying from country to country (87). Nordic countries typically have lower prevalence than southern countries (87). Although *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* are all capable of causing sickness in humans, most cases of campylobacteriosis are caused by *C. jejuni* (85%) and the majority of the remaining caused by *C. coli* (5, 40, 41). Most cases occur as isolated, sporadic events, not as part of large outbreaks (23). Active surveillance through FoodNet indicates about 12.71 cases are diagnosed each year for every 100,000 persons on the U.S. population.
Symptoms of the disease are fever, abdominal cramps, and diarrhea (23). Illness lasts about a week, and the innate immune response is responsible for termination of colonization and disease. There are an estimated 124 fatal cases a year, in the U.S., usually in the immunocompromised (23). About one in every 1000 diagnosed cases can result in chronic sequelae, which includes reactive arthritis and Gillian-Barrè Syndrome (GBS).

Worldwide, there are estimated to be about 400 million cases of campylobacteriosis per year (41). In the U.S., common source outbreaks are due to the consumption of raw milk, contaminated food sources, and contaminated drinking water. Between 1978 and 1987, consumption of raw milk accounted for over half of all outbreak-associated cases of campylobacteriosis. Between 1988 and 1996, consumption of contaminated food sources accounted for 83% of outbreaks (41). Asymptomatic human carriers in susceptible populations are uncommon, but not impossible.

*Campylobacter* is known to behave differently depending on the host population. In susceptible populations, exposure to *Campylobacter* is infrequent. The host’s immune response is responsible for ending disease and colonization. The immune response may also provide short-term protection against future infection. Endemic populations encounter *Campylobacter* from different sources which is evidenced by different strains and species present (120). The organism is present, probably transiently, since no colonization is evident.

**Guillian-Barrè Syndrome (GBS)**

GBS is an acute post infectious autoimmune-mediated disorder affecting the peripheral nervous system, clinically defined by flaccid paralysis, areflexia and
albuminocytological dissociation in the spinal fluid (48). Ascending paralysis evolves rapidly and weakness of limbs and respiratory muscles is common (20). There are no specific tests to provide a confirmatory diagnosis of GBS. Known antecedents associated with the development of GBS are infections with *C. jejuni*, *Mycoplasma pneumoniae*, cytomegalovirus, Epstein-Barr virus, varicella-zoster virus and some vaccines (rabies, swine influenza) (57). GBS incidence is from 0.4 to 4.0 cases/100,000 (13, 57). The first confirmed link between infection with *C. jejuni* and GBS was reported by Rhodes and Tattersfield in 1982. *C. jejuni* has been isolated from approximately 15% of patients with GBS (84). The risk of developing GBS after *Campylobacter* infection is 1/1000 and for the serotype, about 1 per 200 (19). *C. jejuni* may trigger GBS through molecular mimicry between lipooligosachharides (LOS) in the bacterial cell wall and gangliosides in peripheral nerve tissue (124). Prior *Campylobacter* infection is associated with GBS, however, proof of infection is not needed to diagnose the condition (31).

Pathogenesis

Both *C. coli* and *C. jejuni* colonize the intestines of food-producing animals and humans (122). The favored environment appears to be the intestines of birds, which may be due the body temperature of 42°C, which is the optimal growth temperature for *Campylobacter*. In some birds, particularly poultry, *Campylobacter* is a commensal. In humans, *Campylobacter* infection causes sickness. Pathogenesis of *C. jejuni* is characterized by motility, chemotaxis, translocation across host cell membranes, adherence, invasion and toxin production. Motility, conferred to *C. jejuni* and *C. coli* by its polar flagella, is important in colonization of the host (82, 97). The flagellar filament protein is comprised of flaA and flaB, which are coded by the adjacent *flaA* and *flaB* genes.
genes. Mutants with reduced expression of \textit{flaA} of \textit{flaB} can have truncated flagella and reduced invasiveness \cite{47, 143}. Both proteins are needed for full motility and virulence. Chemotaxis is important for bacterial cells to respond to stimulus. Non chemotactic cells showed a threefold increase in adherence and invasion of INT 407 cells, compared to wild-type isolate, but was unable to colonize mice or cause symptoms \cite{151}.

Translocation may permit bacteria access to underlying tissues and could promote their dissemination throughout the host. In 1992, Everest et al. noted that 86\% of \textit{Campylobacter} isolates from individuals with colitis were able to translocate across polarized Caco-2 cells versus 48\% of strains isolated from individual with non-inflammatory disease. The consensus among investigators is that after \textit{C. jejuni} is ingested and passed through the stomach, it initially colonizes the jejunum and ileum, and then the colon \cite{1, 124}. After the bacterial cell adheres to the receptor site on the host cell, a subpopulation is then able to invade the host cell and cause mucosal damage and inflammation \cite{35}. A number of \textit{Campylobacter} species, including strains of \textit{C. jejuni}, \textit{C. coli}, \textit{C. lari}, \textit{C. fetus}, and \textit{C. upsaliensis}, produce cytolethal distending toxin (CDT) \cite{61, 81}. The production of CDT by \textit{Campylobacter} isolates was first reported by Johnson and Lior in 1988. CDT causes cell distension evident through elongation, swelling, and enlargement of the nuclei of host cells. CDT ultimately disintegrates and kills cells. CDT is encoded by three genes, \textit{cdtA}, \textit{cdtB}, and \textit{cdtC} \cite{102}. CDT is heat labile and trypsin sensitive \cite{61}. All three components of the toxin are necessary for full activity \cite{66}. 
Campylobacter and Poultry Rearing

There have been many investigations into the possible sources of contamination of poultry flocks with Campylobacter, but no definitive factor has been determined to explain its incidence (8, 26). Since Campylobacter is ubiquitous, it is possible for the flock to be exposed to many potential sources of contamination; current sampling and culture methods may not be adequate to recover all bacterial cells present. Studies on the prevalence of Campylobacter in poultry have indicated that C. jejuni is more common than C. coli (32, 62). Colonization usually occurs in chicks around 2-3 weeks of age, and the rapid spread from one broiler to the entire grow out house of 10,000 to 30,000 birds may be due to a low infective dose, which may be as low as 35-40 cells (23, 128).

Vertical and Horizontal Transmission

After one member of a flock has become contaminated, Campylobacter is transmitted from that animal to other animals vertically and horizontally (15, 26, 106). Vertical transmission, a controversial topic among scientists, is passage of bacteria from hen to chick through contaminated eggs. There has been much research done to determine the role vertical transmission plays in Campylobacter transmission, with contrasting results (21, 26) Campylobacter and other pathogens can colonize the reproductive tract, as the egg passes through the tract it can become contaminated with pathogens that were a part of the flora of the hen (15). Breeder hens are usually colonized by multiple strains of C. jejuni (58). C. jejuni has also been isolated from rooster semen (87, showing that Campylobacter may also be transmitted sexually in poultry. Intact eggshells are permeable to C. jejuni. When immersed in an infectious organism, 4% of eggshells were experimentally infected (26). Eggs infected by
immersion can be colonized inside the shell or on the shell. An egg can become contaminated through the feces from the infected hen in the nest. Although Cox (26) have shown vertical transmission can occur, others believe it is rare (58, 62, 99, 109).

Horizontal transmission is from bird to bird, generally within a flock. Risk factors for horizontal transmission include bird age, flock size, lack of biosecurity, carry over from a previous flock, contaminated air, soil, and water (87). Most scientists agree that feed, feed additives, and fresh litter are not sources of contamination (10). C. jejuni has been isolated from drinking water lines and reservoirs in chicken houses. The same phenotype and genotype in water reservoirs has been found in chicken feces (87, 100). C. jejuni is known to survive in water under favorable conditions, however, because of antimicrobial usage and water flow, water lines and reservoirs may not be a Campylobacter-friendly environment. Overall, Newell (87) suggested that water contaminated with C. jejuni constitutes a low risk. In countries where litter is not reused and the grow-out house is cleaned between flocks, contamination is sporadic and unpredictable (118). In the U.K., 15% of sequential flocks in 100 houses showed evidence of genotypically identical strains, which suggests strains were being carried over between flocks (117). However, Nordic studies (87) show that there is variance of strains from one flock to another, suggesting disinfection can be successful in eliminating C. jejuni.

Poultry are coprophagic; consumption of contaminated feces and cecal droppings is an important characteristic of Campylobacter spread in a grow-out house. Line and Hiett (70) estimated the colonization dose 50% (CD50) to be 316 CFU when coprophagia was eliminated. Chickens exposed to the outside environment are likely to encounter
more than one strain or species of *Campylobacter* capable of establishing a persistent commensal relationship during the lifetime of the bird (16). Barn-reared broiler chicken flocks are likely to be exposed to a somewhat more limited set of *Campylobacter* types (90) but these flocks have also been reported to exhibit the dominance of one *Campylobacter* type only to be replaced with another type later in rearing (16). Currently the factors that enable one species or strain to become the dominant type in the chicken gut are largely unknown but it appears to be more complicated than being the first strain to establish colonization. Shreeve (118) also noticed in some cases a flock may have several genotypes present, yet subsequent flock may have only one of the genotypes found in the first flock, suggesting some types are more successful at surviving transmission. It is also common to isolate more than one species or subtype from the same bird (16, 114, 118).

*Campylobacters* can be carried into broiler houses on clothing, boots, hands, and equipment (87). Biosecurity measures are taken but may not be that effective; the level needed prevent colonization is not known. When birds are restricted in their movement, those who are close to doors without hygiene barriers are the first ones to become colonized (87).

*Campylobacter* prevalence in poultry flocks is higher in summer than in winter, and it varies with latitude; number of cecal organisms per bird and strain types present varies between seasons (56, 142). There is a peak in human *Campylobacter*-related sickness and prevalence in warmer months (8, 60). This may be due to migratory birds or insects (8, 59). In a 2006 study (8), 95% of *Campylobacter*-positive flocks were detected in the April-September period, and there was a peak in July to September. However, it
was found that after controlling for the season and farm, the seasonal prevalence did not change during the study period.

**Campylobacter and Poultry Processing**

Carry-over contamination from a positive flock to a negative one can be a source for contamination in processing (7, 87). Cross contamination in the slaughter plant due to contaminated equipment and processing water, is very difficult to control. The presence of *C. jejuni* and *C. coli* in the intestine and liver may be important for the transmission of this bacterium to poultry meat (33, 129). Barrios (8) and Miwa (80) showed that efforts to improve sanitation at slaughterhouses have been difficult since *C. jejuni* are spread from intestinal contents.

Poultry processing is comprised of several steps—slaughter, scalding, defeathering, evisceration, and chilling. Damage to the intestines during the slaughter and evisceration processes can lead to direct contamination of carcasses and other organs. Berrang and Dickens (11) studied the presence and level of *Campylobacter* on broiler carcasses throughout the processing plant. In that study, samples were collected immediately before scald, post-scald, post-pick before transfer to the evisceration line, immediately after the removal of the viscera, after the final washer, and post-chill.

**Scald**

The scalding process is meant to loosen feathers in the follicles; *Campylobacter* has been recovered from scald water (126). Miwa (80) found that at the beginning of a processing day, scald and evisceration water is *Campylobacter*-negative, and the same *flaA*-RAPD profile was recovered from carcasses after defeathering and evisceration when negative flocks were processed after positive ones. Berrang and Dickens (11)
found prevalence was highest pre-scald and counts dropped significantly, after carcasses were scalded.

**Defeathering**

The defeathering process involves automated pickers to remove feathers (11, 80). These pickers may cause inter-flock cross-contamination, due to finger-like projections applying pressure to carcasses that may cause leakage of intestinal contents onto the surface of the pickers. *Campylobacter*-negative flocks are indirectly contaminated by *Campylobacter*-positive flocks during defeathering (146). Berrang and Dickens (11) concluded that *Campylobacter* populations and the percentage of positive carcasses increase significantly during the picking process.

**Evisceration**

During evisceration, there is a high chance for bacterial contamination, if the removal of the viscera leads to rupture of the intestines and leakage of fecal material. *Campylobacter* can be spread from the intestines to the outside of the carcass.

**Chilling**

Chilling is an important critical control point for microbiological contamination. Despite chilling water being a source for cross contamination in processing (111), this step also washes surface *Campylobacter* off and post-chill carcasses have lower bacterial loads than anywhere else on the line (11, 107). There are certain parameters that must be met in order for this critical control point to be effective—the temperature must be correct, organic matter should be kept at a minimum, pH must be the optimum range for chlorine to be effective, and concentration of antimicrobials must be monitored (108).
The post-chill area of the processing line has been found to have the least prevalence of *Campylobacter* (11).

Overall, *Campylobacter* prevalence drops as flocks move through the plant. However, *Campylobacter* counts detected on positive carcasses leaving the chill tank were not significantly different from those detected on positive carcasses leaving the scald tank (11). Defeathering and evisceration are the two points in processing with the highest microbial load.

**Campylobacter genetic diversity in poultry processing**

There is a direct correlation between high *Campylobacter* prevalence and high genetic diversity on a carcass (68). Changes in fla type distribution occur during processing (89). These changes in fla type suggests that some strains are more resistant to the stressors encountered during processing. The substantial genetic diversity of *Campylobacter* is evidenced by strain-to-strain variability in virulence and tolerance to certain environmental stressors (95). Subtypes that are more robust may contaminate the abattoir environment, surviving through carcass chilling, and carry over into subsequent flocks (89).

**Processing Aids**

Finished poultry products that are heavily contaminated with high numbers of microbes are considered undesirable from a food safety and quality point of view. There are various steps in processing designed to eliminate or control these microbes, such as chilling and multiple washes. In some instances, processing aids are used to further reduce the microbial load on chicken carcasses by removing surface contamination. Common chemical processing aids include trisodium phosphate, acidified sodium
chlorate, food-grade acids, peroxycarids, and chlorine. These aids not only are effective at washing and killing unattached bacterial cells from the surface of poultry meat, they are also effective in removing and killing attached cells.

*Trisodium phosphate (TSP)*

TSP is a white, free flowing crystalline material with a pH of 11.8 at a concentration of 12% (115). When residual TSP on carcasses enters the chill tank, a rise in pH results, rendering chlorinated chill water ineffective in reducing microbial loads (108). TSP is more effective against gram-negative bacteria. Mechanisms of action include destruction effect due to the high pH (11), removal of non-attached bacteria, removal of surface fat, which by default removes bacteria, and an effect on the bacterial cell wall (63). It is effective in reducing the bacterial load up to 2 log CFU/ml when the concentration is between 10 and 12% (28, 36, 127). Del Rio (28) found that after dipping for 15 mins and storage at 3°C, 12% TSP reduced natural microflora and spoilage organisms 0.5 to 2 log CFU/ml. Arritt (6) found that there was a more than one log reduction in bacterial counts when 10% TSP was applied before or after immersion in bacterial suspension. TSP has been shown to not alter the sensory characteristics detrimentally (125).

*Acidified sodium chlorite (ASC)*

ASC is a solution of sodium chloride acidified with an organic acid to form chlorus acid. ASC operates at a pH of 2.3-3.2 (63). It is a broad-spectrum disinfectant, which oxidizes the microbial cell wall, and attacks sulfide and disulfide linkages in proteins (63). It attaches to the amino acid component of the cell membrane. It can be administered in a spray or dip, usually at concentrations of 1000 ppm. It is effective
against bacteria, viruses, fungi, yeasts, molds and some protozoa (63). It was found to reduce *Campylobacter* prevalence on broiler carcasses from 73.2% positive to 49.1% (63). Application of 0.1% ASC resulted in a greater than 1 log reduction in *Campylobacter* when applied before and after inoculation (6). Sinhamahapatra (119) found treatment with 1200 ppm ASC spray, was equivalent to a 70°C hot water spray in the ability to lower coliform counts. Del Rios (28) showed that 1200 ppm ASC lowered mold and yeasts counts by 1.45 log CFU/g and did not detrimentally effect sensory characteristics.

*Food-grade acids (FGAs)*

Food-grade acids include citric acid, lactic acid, succinic acid, and propionic acid. They are soluble in water and GRAS substances approved for use in the food industry. They work by disrupting the cellular membrane and acidify cellular contents (63). They are stable in the presence of organic material, but they can corrode processing instruments and can cause off flavors, odors and colors in end products (63). 0.1% acetic acid added to scald water reduced *Salmonella* and *Campylobacter* counts by 0.5 to 1.5 CFU/ml (93). Sinhamahapatra (119) found that a lactic acid carcass dip was effective in reducing total plate counts by 1.36 log CFU/ml. Berrang (12) added organic acids to the cloaca prior to scalding and recorded a statistically significant reduction in *Campylobacter* numbers when compared to control groups. Zhao (152) found that carcass dips in 2% acetic acid at 4°C reduced populations of *Campylobacter* by 1.4 log CFU.
Peroxyacids

Peroxyacids are a combination of acetic acid, octanoic acid, and hydrogen peroxide, they are commonly clear colorless liquid; oxidation/reduction reactions take place in water they generate water, acetic acid, octanoic acid. When solubilized in water the acids act by lowering pH, disrupting the cellular membrane and acidifying cellular contents. Spraying poultry carcasses for 15 sec with a 200mg/l peroxyacid solution reduces *C. jejuni* by 2 log, which was a greater effect than water alone (116).

**Reduction/Elimination of Campylobacter in poultry**

*Campylobacter* is a worldwide threat to human health and measures taken to reduce or eliminate its prevalence are being researched and applied in the food industry. Reduction of prevalence in foodstuffs is likely to have a huge impact on the health care industry, as *Campylobacter*-related illness and hospitalization costs were $8 billion in the United States alone (19). The underlying principle for reduction of *Campylobacter* infection in humans is to prevent colonization of poultry, or to reduce the prevalence on carcasses during processing. Methods that are currently employed to prevent or treat colonization by *Campylobacter* include vaccination, competitive exclusion, phage therapy, antimicrobial therapy, and alternative feed formulation.

**Vaccination**

The goal of vaccination is to prevent infection and colonization of poultry by pathogens. There are some main characteristics a perfect vaccine should have. Since *Campylobacter* is quickly spread to thousands of chicks within a matter of days, a quick protective response is beneficial. The vaccine should be cost-effective and easy to administer as there are upwards of 30,000 birds in one flock. The vaccine should not be
harmful or deleterious to animals or humans. The vaccine should either prevent colonization or provide a 2-3 log reduction in bacterial loads in colonized animals. A risk assessment by Rosenquist (107) showed that a 2-log reduction in chickens at the time of slaughter is sufficient to have an impact on human health. A vaccine of choice should also provide cross-protection between C. coli and C. jejuni, since both species colonize in poultry. Currently, there is no vaccine that possesses all these characteristics, but there are some that have potential to become very useful in the poultry industry. Some vaccines that are currently employed that are effective against Campylobacter are experimental colonization with wild-type C. jejuni administered orally, which produces about a 1-log reduction upon challenge (23). Formalin inactivated C. jejuni with and without E. coli heat labile toxin administered orally produced a 1.5 log reduction in bacterial numbers upon challenge (105). Native-flagellin vaccine administered with heat killed C. jejuni offers a 1-2 log reduction upon challenge (147, 148) and attenuated Salmonella expressing CjaA offers greater than a 6-log reduction upon challenge (150).

The main difficulty of formulating a vaccine for Campylobacter is due to the genotypic and phenotypic instability of the bacterium. A conserved protective antigen is difficult to pinpoint, and until it is found, one vaccine may not be suitable for use in poultry for Campylobacter. Another obstacle to the development of an effective vaccine is the apparent change in a dominant colonizing type observed by Bull (16) which affects the usefulness of a particular vaccine.

Competitive exclusion

Competitive exclusion (CE) in chickens is the introduction of bacteria from the gut of mature birds to newly hatched chicks in order to rapidly develop the microflora of
chicks to protect against colonization by pathogens, particularly *Salmonella*. It is solely meant to be prophylactic, not treatment after colonization. CE is independent of breed, strain, sex of birds, but there is a difference in protective capability. It is usually administered in the drinking water or as a spray in the hatchery. Problems include uneven distribution of the protective effect across the flock and reduced viability of anaerobic organisms over time (113). CE is also used to reestablish intestinal microflora after antibiotic therapy. BroilAct®, a CE product established by the Orion Company in Finland, consisting of mixed culture derived from the cecal contents of a single hen from 1987. It included 32 different types of bacteria, including 22 strictly anaerobic rods and cocci, 10 facultatively anaerobic rods and cocci, and no spore-formers (113). BroilAct® has a protective effect not only for *Salmonella*, but also for *Campylobacter jejuni* and *E. coli* (49, 50).

**Campylobacter phage**

*Campylobacter*-specific bacteriophages are ubiquitous in the environment and have been isolated from sewage, broiler chickens, and porcine manure (7,46, 64, 110). Bacteriophages are self-replicating, and self-limiting, and have host specificity that is not seen in broad antimicrobials and they do not disrupt the gut bacterial flora (140). *Campylobacter* phage have been used with limited success as a treatment to reduce the numbers of *Campylobacter* in poultry microflora (7, 141). Since they are commonly isolated from poultry excrement, their use will not introduce any new biological entity into the food chain. Wagenaar (141) found that there is a 3-log reduction in *C. jejuni* when phage is administered after colonization. When phage was administered before colonization, it was delayed, but not prevented. Atterbury (7) found *Campylobacter*
phage on retail meat, but only when *Campylobacter* was present. Phage also has the ability to overcome the genetic and phenotypic instability of *Campylobacter*. They are constantly adapting to mutating host defenses, thus maintaining an evolutionary balance. Successful administration of phage therapy must be carefully timed to have maximum effect. Phage therapy may be used as a biocontrol agent, but is best used in combination with other biocontrol measures.

*Antimicrobials*

There are many concerns with the use of antimicrobials in food production including their effect on human health, and the rise of antibiotic resistant bacteria; this has led to the ban or restriction in uses in food production. The EU has banned the use of many antibiotics, especially for use as growth promoters (7, 113, 138). In the U.S., the use of antibiotics has also come under examination. The use of bacteriophages may help fill the void left by the ultimate discontinuation of antibiotics.

*Feed formulation*

Feed formulations can alter the microenvironment of the chicken gut, making it unsuitable for certain bacteria to flourish. Mucin composition, pH, free volatile fatty acid content, non-soluble polysaccharide content, and gas production are dependent on feed formulation and can have an effect on the ability of certain pathogens to colonize the gut (137). However, since *Campylobacter* is not considered a poultry pathogen, but commensal, feed formulations have had a limited effect on its colonizing capabilities. A study by Line (69) showed that supplementing poultry feed with *Saccharomyces boulardii* reduced *Salmonella* colonization from 70% to 20 and 5% in the 1x and 100x yeast treated birds, respectively. However, the yeast supplemented feed had no effect on
colonization of *Campylobacter*. Udayamputoor (137) found that protein source had an effect on colonization by *Campylobacter*. Experimental groups fed a diet with plant protein had significantly lower cecal colonization, when compared to experimental groups fed a diet containing animal protein or a mixture of plant and animal proteins.

**Molecular Subtyping**

Clarification of the diversity and nature of *Campylobacter* infections helps in the investigation of disease. Molecular subtyping is an important tool for epidemiological studies; it helps in tracing sources and routes of transmission of human infection, identifying and monitoring specific strains over time and different regions with important characteristics. Molecular subtyping also lends itself to the development of strategies to control transmission, sources and possible contamination in the food chain (88, 91, 144). Methods for subtyping must be evaluated in the context in which they are used and must be able to give reliable, reproducible data (91). Discriminatory index is important for distinguishing results and choosing the correct typing method for the sample (78). In addition, discriminatory index is used to compare discriminatory power (DP) of typing method. A method with high DP may be better suited for a particular goal; to attain a greater DP two methods can be combined. Reproducibility is important between and within labs to be able to compare results and draw meaningful conclusions. Molecular typing methods must be standardized so that more information can be ascertained from results. It is possible for results to differ due to primers used, temperatures for PCR, culture methods and conditions, restriction enzymes used, number of restriction enzymes, etc. Efforts for standardization include databases with electrophoretic profiles and sequences, and standard primers.
Typing methods

There are three major types of typing schemes—phenotypic, genotypic and nucleic acid analysis. In phenotypic methods, observable physical or biochemical characteristics of an organism, which are influenced by genetic make up and environmental conditions, are used as the basis of separation. Major phenotypic methods include serotyping, phage typing, and biotyping. In genotypic methods, the genetic makeup of the organism, apart from the physical attributes is used as the basis of separation. Common genotypic methods include random amplified polymorphic (RAPD) DNA-PCR, restriction fragment length polymorphism (RFLP)-PCR, pulse-field gel electrophoresis (PFGE), \textit{fla} typing, and ribotyping. With heterogeneous groups of strains, PFGE, and \textit{fla} typing have greater discriminatory power than RAPD analysis or ribotyping (151). In nucleic acid analysis, the genome or a particular gene sequence is used for classification. Among the three major methods, nucleic acid analysis is the most reproducible. Those methods are fast, eliminate experimental variation, and facilitate interlaboratory comparisons (78).

Phenotypic methods

Serotyping

Serotyping separates bacteria based on antibody-antigen reactions. \textit{Campylobacter} serotyping was developed in Canada in the 1980’s by Penner and Lior (149). Penner schemes are based on the heat stable antigens using passive hemagglutinations (101) and Lior is based on the heat labile antigens using bacterial agglutination (71). The Penner scheme is used as the basis for the typing scheme in the Laboratory of Enteric Pathogens (LEP), Public Health Laboratory Service in the United
Kingdom (42). Advantages of serotyping include the wide acceptance as a valid typing method (144). Disadvantages of serotyping include a high level of untypeable strains, in human and veterinary samples (up to 20% in some instances), culturing conditions may render an isolate untypeable, ambiguous results, transient antigen expression, and cross-reactivity between certain antigens (38). The method requires a panel of antisera that is costly to maintain, is laborious and requires at least 5 to 7 days to complete (38, 96, 112), considering the need to repeatedly subculture isolates before testing. Using the LEP scheme, up to 40% of poultry isolates are untypeable.

*Phagotyping*

Phagotyping uses viruses as markers to identify microorganisms. Since bacteriophages are host-specific, identification to subspecies is possible. *Campylobacter* phage typing has many different schemes. The primary typing scheme was developed in the U.S. and uses common poultry phage. The scheme was adapted by other countries, with the basic or main phages remaining the same, but other phages added that were isolated from *Campylobacter* positive birds in that particular country. Disadvantages of phagotyping include the appearance of a unique phage pattern of a strain that Reacts with phage but Does Not Conform to a designated type (RDNC) (65). An advantage of phagotyping is the host specificity of phages. Host specificity reduces the chance of phages interacting with the wrong bacteria. Another advantage of phagotyping is its application as a complement to serotyping. Serotyping classifies bacteria into 20 broad groupings and phagotyping can further classify 6 to 29 subgroups from each of those 20 serogroups, thereby enhancing the discriminatory power of this epidemiological tool (42). There are 66 known serotypes and 76 known phage types for a total of 5016 possible
combinations. In Frost’s study, 336 phage-serotype combinations were identified among a sample of 2407 isolates, which may be indicative of the relationship between phage and serotypes.

**Biotyping**

Biotyping is used to distinguish isolates according to biochemical reactions and metabolic activities. Differentiation is based upon the results of the biochemical tests. The discriminatory index depends on the number of tests administered. There are many types of tests or combinations of tests that can be used in order to discriminate, which is an advantage of biotyping. The discriminatory power may be increased as the number of tests administered. Biotyping is very common and widely accepted as a separatory method. For example, Skirrow developed a typing scheme based on hippurate hydrolysis, a rapid H₂S test in iron-containing media, and resistance to nalidixic acid for the differentiation of campylobacters into *C. jejuni*, *C. coli*, and a third group, the nalidixic acid-resistant thermophilic campylobacters. Disadvantages of biotyping include cost in supplies and labor. Cost and labor increases as discriminatory power increases, due to the increased number of tests performed. However, recent developments have made it possible to automate and perform many tests at one time. Biotypes may be dependent upon growth conditions and therefore reproducibility may be difficult, inter and intralaboratory.

**Genotypic methods**

**Polymerase Chain Reaction (PCR)**

PCR is used as the basis of many genotypic typing schemes. PCR is an automated, rapid, sensitive way to amplify DNA. PCR is comprised of three basic
steps—denaturation, annealing, and extension. The original double stranded DNA is denatured to form a single strand to which a primer can anneal. After annealing, a complimentary strand is formed. In theory, a single piece of DNA is enough to begin a reaction that multiplies exponentially to contain over one million pieces of DNA, depending on the number of cycles performed by the thermal cycler. PCR may amplify the whole genome or a specific gene; it is dependent upon the primers used in the reaction. Primer selection is depends on the target gene and reaction conditions. Specificity of amplification reaction depends on selectivity of primers, enzymes used, condition of primers and enzymes, and reaction conditions. PCR has become increasingly automated and more complex. Examples of this include real time PCR, reverse transcriptase PCR, and hot start-cold end PCR. PCR is also used in combination with other methods to increase discriminatory power. Limitations in PCR include inhibitors present in the sample matrix, condition of the primers and enzymes, and product assumption. Some samples are taken from a food matrix, especially in the case of *Campylobacter*, without enrichment. Some matrix constituents may have an inhibitory effect on the PCR efficiency. Advantages of PCR include automation of procedure, quick turnaround time, usefulness in detection. Disadvantages include high capital input for equipment, and cell viability is not known, as DNA can be amplified from dead or living cells.

*Random Amplified Polymorphic DNA (RAPD)*

RAPD is a whole genome typing method in which a single oligonucleotide is used to amplify a gene. The primer has to have the ability to bind in both forward and reverse directions. When performing RAPD, annealing temperatures must be taken into account.
Primer selection is the greatest determinant of the number of bands, since the primer acts on DNA in random places, random bands are produced. RAPD can have high discriminatory potential due to its ability to determine polymorphism in the entire genome (144). However, RAPD can have low reproducibility due to random digestion. Low reproducibility is also due to the ratio of DNA to primer concentration, model of thermal cycler, magnesium concentration or brand of taq DNA polymerase, which all effect banding patterns.

A combination of fla and RAPD typing have successfully been applied to Campylobacter (2, 82, 83, 98). However, disadvantages such as minor differences in band patterns and weak band patterns make RAPD’s discriminatory capacity somewhat poor, which may lead to subjective interpretation of results (72, 92, 144).

PCR-Restriction fragment length polymorphism (RFLP)

PCR-RFLP is a method in which a particular sequence is amplified and enzymes are used to digest genomic DNA at certain sites. The results are precise band lengths, which produce identifiable electrophoretic patterns that can theoretically be reproduced because of the specificity of restriction enzymes used. Advantages of RFLP are simple setup, low maintenance costs, and rapid processing of samples. The level of discrimination can be improved with multiple restriction enzymes. Disadvantages of RFLP are difficulty in choosing an appropriate target for amplification; prior knowledge of genome is needed, and a small section of genome is examined. RAPD analysis has been compared to fla typing in a study, and it was found that fla typing is more discriminatory when analyzing broiler liver and intestine samples for C. jejuni and C. coli (33).
**Pulsed-field gel electrophoresis (PFGE)**

PFGE begins with whole cells that are lysed in agarose blocks to reduce contamination and shearing of DNA. The genomic DNA is washed to remove contaminants and extracellular materials. The plug containing DNA is digested with restriction enzymes, that are chosen for optimum typing of each pathogen, which cleave the DNA into large fragments. The fragments are separated by multi-directional pulsed electric fields. PFGE is the method that is currently employed by epidemiologists at the U.S. Centers for Disease Control and Prevention as the standard for PulseNet (130). PulseNet is a national network of public health and safety agencies that exchange standardized PFGE patterns on food-borne pathogenic bacteria. Regulation and standardization of methods, especially through CDC’s PulseNet has led to high reproducibility, rapid exchange of information between various laboratories nationally and internationally. Currently, PFGE is regarded as the ‘gold standard’ for subtyping methods, being the most discriminatory among commonly applied subtyping methods (78). Yet, without standardization of enzymes and other experimental conditions, reproducibility is questionable, and results are sometimes open to personal interpretation.

**fla typing**

Flagella are responsible for motility, chemotaxis and virulence in bacteria. Flagella of gram-negative cells are composed of a filament, basal body, and hook. Filament is comprised of protein subunits encoded by a gene called *fla* (38, 51, 76). The genes that code for these protein subunits are what are analyzed in *fla* typing. Campylobacters have two encoding genes, *flaA* and *flaB*. In *C. jejuni* the two genes are roughly 1.73 kb, arranged in tandem, and are separated by a segment of about 0.2 kb in
length (37, 51). Expression of \textit{flaA} and \textit{flaB} are differentially regulated, with each gene containing its own distinct promoter, $\sigma^{28}$ for \textit{flaA} and $\sigma^{54}$ for \textit{flaB} (47, 54). There are conserved regions and variable regions, which makes this locus suitable for typing. The flagellin B gene (\textit{flaB}) is, in contrast to \textit{flaA}, not essential for motility, or pathogenicity, and is thought to be a genetic reservoir for \textit{flaA}. However, both gene products are required for fully functional flagella (47). \textit{flaB} may serve as a gene donor, in which parts can be introduced into \textit{flaA} through homologous recombination to compensate for deletions, mutations or to increase the immunogenic repertoire of a gene (78). Because both highly conserved and variable regions are present (75), this locus is suitable for RFLP, and RAPD analysis of PCR products. The conserved regions in the \textit{flaA} locus are partially conserved in other species; \textit{fla} typing is useful for the majority of \textit{C. coli} strains, some strains of \textit{C. lari}, and \textit{C. helveticus} (94). Despite its simple and quick applications, disadvantages for the \textit{fla} typing scheme include difficulty in interlaboratory comparisons, and genetic instability. There are many techniques used in \textit{fla} typing and there is no standard for what methods should be used for comparison. Difficulty encountered in comparing results may be overcome by standardization of restriction enzymes and electrophoretic conditions.

\textit{Ribotyping}

Ribotyping is based on the presence of conserved copies of the rRNA coding for the 23S and 16S genes. \textit{Campylobacter} has three ribosomal gene copies, which make this method less discriminatory than others. Ribotyping cannot discriminate between \textit{C. fetus} subsp. \textit{fetus} and \textit{C. fetus} subsp. \textit{venerealis} (29). The choice of restriction enzyme is very important. The combination of enzymes used in ribotyping increases the
discriminatory power. This method samples the entire genome, and is not largely affected by small polymorphisms unless at the restriction site. Ribotyping can be fully automated and is reproducible. However, it is costly and only a limited number of samples can be analyzed at one time.

**Nucleotide analysis**

Direct nucleotide sequencing (with or without PCR amplification) is becoming increasingly automated and is an alternative method for genotyping bacterial isolates. The advantage of sequence analysis is that it is highly reproducible and the results are easy to interpret. However, interpretation is highly dependent on computerized comparison programs and the parameters set by the software packages used (144). Sequence analysis has been applied to the flagellin locus in several studies (51, 75).

*Sequencing SVR of flaA locus*

Sequence analysis of the flagellin locus has been used successfully as an epidemiologic typing method by Meinersmann (75, 77), Mellman (78), Fitzgerald (38), and Harrington (51). There is up to 30% variability among *C. jejuni* isolates, over two regions in the *flaA* gene, the first, a shorter one, from sequence positions 450 to 600 and a longer one, from sequence position 700 to 1450. The shorter variable region, termed the SVR is commonly used as a locus for *fla* typing. Meinersmann (75) found analysis using the SVR has similar discriminatory power as analysis of the entire *fla* gene. Fitzgerald (38) found that SVR analysis was able to differentiate between outbreak strains of *Campylobacter jejuni* and sporadic strains. Mellmann (78) found that analysis of the SVR provides adequate discrimination in short term epidemiology. An advantage of analysis of the SVR is that it can be sequenced with a twofold redundancy over the entire
region with a single pair of forward and reverse primers that bind to conserved flanking sequences, while analysis of the whole gene or a long variable region requires many more primers (75). Fitzgerald (38) compared various methods of subtyping outbreak isolates and found analysis of the SVR to be more discriminatory than serotyping and PCR-RFLP of the entire flaA gene, but less discriminatory than PFGE. When using the SVR as an epidemiological tool, care must be taken since the entire genome is not represented.

**Genomic recombination of C. jejuni**

*Campylobacter* is a genus of organisms that is known to participate in genetic recombination. Genetic instability can be defined as an event leading to a change in the genetic organization of a bacterial strain. Mechanisms leading to instability can be summarized as point mutations as a result the activity of mobile elements, i.e., *Campylobacter* phage; random or programmed recombinations of DNA segments; high frequency mutations; incorporation of foreign DNA after natural transformation (145).

Some genotypic and phenotypic subtyping methods are susceptible to genetic instability. Genetic recombination commonly occurs at the flagellin locus, and this can affect the applicability of many typing schemes, PFGE and *fla* typing, in particular. Harrington showed direct evidence for intragenomic flagellin recombination in a non mutant (*C. jejuni* TGH9011) strain and evidenced intergenomic recombination within natural populations of *C. jejuni* (51).

Typing can be affected by recombination through difficulty in identifying clones, or methods may differentiate strains that are of the same origin that may have been subject to recombination (88) Recombination may occur in *Campylobacter fla* genes to give this bacterium a biological advantage over other pathogenic species. *flaB* can serve
as a genetic reservoir for flaA and during recombination introduction of flaB allows for
Campylobacter to retain full motility (2). Recombination makes fla typing unsuitable for
long-term surveillance of bacteria.

**Phenotypic vs. genotypic methods**

It is very difficult to compare genotypic and phenotypic methods for subtyping
due to a lack of standard strains for comparison. Some serogroups have a variation in
their phenotypic and genetic heterogeneity. Some groups are very closely related while
others are not. Having a standard set of strains will eliminate variation and difficulty
comparing genotypic and phenotypic methods. It is generally accepted that phenotypic
characteristics can be unstable. For example, loss of toxin production by a
Campylobacter does not necessarily alter the strain genetic identity. Generally,
genotypic methods have greater discriminatory power than phenotypic methods.
However, combining a genotypic method with serotyping or combining two independent
genetic methods usually results in greater discriminatory power and more complete strain
identification than a single genotyping method alone. The decision of which method to
use is dependent upon the objectives of research and the desired discriminatory power.
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CHAPTER 3

GENETIC DIVERSITY OF CAMPYLOBACTER ON BROILER CARCASSES COLLECTED AT RE-HANG AND POST-CHILL IN 17 U.S. POULTRY PROCESSING PLANTS*

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Abstract

*Campylobacter jejuni* and *C. coli* are the most important human enteropathogens among the campylobacters. The objective of this study was to determine how diversity in *Campylobacter* found on chicken carcasses collected from re-hang and post-chill sites at 17 poultry processing plants in the United States is impacted during processing by sequencing the Short Variable Region of the *flaA* locus. Seventy percent of carcasses had one *flaA*-SVR type detected. *Campylobacter* genetic diversity decreased as carcasses proceeded through processing; carcasses sampled at re-hang had significantly more genetic diversity in *Campylobacter* populations than carcasses sampled at post-chill. There was more diversity in *Campylobacter* on carcasses collected during winter than spring, summer, or fall. There were certain types that were present at re-hang that were not present at post-chill, and vice versa, suggesting that some subtypes may be prone to perish during processing while others may survive or persist despite stressors encountered with the processing environments.
Introduction

Campylobacter jejuni and C. coli are the most important human enteropathogens among the campylobacters, affecting an estimated 2.4 million cases each year in the U.S. alone (4, 12). Risks for contracting campylobacteriosis are consumption and handling of raw and undercooked poultry, most commonly chicken and turkey products, cross contamination with other foods, and consumption to contaminated milk and water (2, 6, 19, 28, 33).

Both C. coli and C. jejuni colonize the intestines of food-producing animals and humans (29). The favored environment appears to be the intestines of avians, which may be due the body temperature of 42° C, which is the optimal growth temperature for Campylobacter. There have been many investigations into the possible sources of contamination of poultry flocks with Campylobacter, but no definitive factor has been determined to explain its incidence (6, 10). Since Campylobacter is ubiquitous, it is easy for the flock to be exposed to many potential sources of contamination; current sampling and culture methods may not be adequate to recover all bacterial cells present. In some birds, particularly poultry, Campylobacter is a commensal. After one member of a flock has become contaminated, Campylobacter is transmitted from that animal to other animals.

C. jejuni and C. coli can be found on 90% of poultry in the U.S. (32) and from 18% to >90% of poultry in Europe, varying from country to country (22). Carryover contamination from a positive flock to a negative one is also a source for contamination in processing (5, 22). Cross-contamination in the slaughter plant due to contaminated equipment and processing water, is very difficult to control. Finished products that are
heavily contaminated with high numbers of microbes are considered undesirable from a food safety and quality point of view. There are various steps in processing designed to eliminate or control these microbes, such as chilling and multiple washes. In some instances, processing aids are used to further reduce the microbial load on chicken carcasses by removing surface contamination.

Measures taken to reduce or eliminate the prevalence of *Campylobacter* are being researched and applied in the food industry. Prevalence in foodstuffs is likely to have a huge impact on the health care industry, as *Campylobacter*-related illness and hospitalization costs are $8 billion per year in the United States alone (9). The underlying principle for reduction of *Campylobacter* infection in humans is to prevent colonization of poultry, or to reduce the prevalence on carcasses during processing. Clarification of the diversity and nature of *Campylobacter* infections helps in the investigations of disease. Molecular subtyping is an important tool for epidemiological studies; it helps in tracing sources and routes of transmission for human infection, identifying and monitoring specific strains over time and in different regions. Molecular subtyping also lends itself to the development of strategies to control transmission, sources and possible contamination of the food chain (23, 25, 36).

In this study, the Short Variable Region (SVR) of the flagellin locus was analyzed to determine the genetic diversity of populations of *Campylobacter* collected from rehang and post-chill sites at 17 broiler processing plants around the United States. We hypothesized that the diversity in *Campylobacter* populations would decline as carcasses move towards the end of processing, and the population diversity would not affected by the choice of chemical processing aid.
Materials and Methods

Origin of Isolates

*Campylobacter* isolates were from a previous USDA-FSIS study conducted in 2005 (7).

Isolation of Colony for DNA extraction

Isolates were removed from freezer and streaked for isolation on tryptic soy agar (TSA) (Remel, Inc; Lenexa, KS; USA) with 5% sheep blood, incubated at 42°C for 24 to 36 hours. An isolated colony chosen for subculture was streaked for a lawn on TSA and grown for 24 hours to be used in DNA isolation.

DNA Isolation

DNA extraction was performed using an isolation kit from (Puregene; Gentra Systems; Minneapolis, MN) following the manufacturer's instructions.

PCR for speciation

An automated BAX PCR (DuPont Qualicon, Wilmington, DE) system was used to identify the species of the isolates as *C. jejuni* or *C. coli*. Reference strains for campylobacter included *C. coli* ATCC 33559 and *C. jejuni* ATCC 33560, as described (12). For the DNA samples that did not produce identifiable product during electrophoresis was then subject to a multiplex PCR as described by Wang (36).

PCR for flaA gene

*FlaA*-SVR PCR was performed as described by Meinersmann (19) with the following primers: FLA4F: 5'GGATTTCGTATTAACAAATGGTGC 3' and FLA625RU: 5'CAAGWCCTGTCCWACTGAAG 3’, and the PCR cycles included: 94 °C for 2 min and then were cycled 30 times at 94 °C for 15 s, 50 °C for 30s, 72 °C for 1 min. Samples were incubated at 72 °C for 2 min and held at 4 °C until processed.
Amplification products were analyzed by electrophoresis at 130V for 60 mins using 1X TBE (0.89 Tris borate, 0.02M EDTA) running buffer on 2% agarose gels (Seakem LE agarose, Cambrex Bio Science Rockland, Inc., Rockland, ME). Gels were stained with 10mg/ml of an ethidium bromide solution (Sigma) and visualized on a UV gel documentation system. The PCR products were cleaned using the Qiagen BioRobot 3000 (Qiagen, Inc., Valencia, CA)

**Sequencing**

Cleaned PCR product was sent to the Integrated Biomolecular Resources laboratory within the Core Technologies at the Eastern Regional Research Center of the USDA ARS (600 East Mermaid Lane, Wyndmoor, PA) for dye-terminator DNA sequencing using Applied Biosoysystem 3730 DNA sequencer and the Applied Biosystem 3130 Genetic Analyzer (Applied Biosystems, Forest City, CA). Sequence data were assembled with Sequencher 4.7™ (Gene Codes Corporation, Inc; Ann Arbor, MI; USA). PAUP version 4.0b10 (Sinauer Associates, Inc., Sunderland, MA) was used to generate distance matrixes.

**Statistical analysis**

Statistical analysis was carried out using the Community Analysis Package 3.0, Species Diversity and Richness (2004, Pisces Conservation, Ltd. Lymington, UK) to find Simpson’s Index and statistical differences through a randomization test by Solow (30).

**Method for naming types**

Nucleotide and peptide sequences for alleles of *Campylobacter* FlaA SVR have been collected on a curated database on the Internet (http://hercules.medawar.ox.ac.uk/flaA/). Each detected type was entered into the database to determine if its flaA-SVR sequence
was already published. Those types that were found in the database were identified by the published number. If a type was not found in the database, its closest relative was the basis of the name. For example, in this study there were 4 sequences most closely related to the published sequence of \textit{flaA}-SVR type 54. These four were given the names 54.1, 54.2, 54.3, and 54.4. The current study made use of the \textit{Campylobacter jejuni} Multi Locus Sequence Typing website (http://pubmlst.org/campylobacter/) developed by Keith Jolley and Man-Suen Chan and sited at the University of Oxford.

\textbf{Results and Discussion}

Understanding how \textit{Campylobacter} is transmitted and contaminates carcasses during processing will help in designing interventions to reduce or eliminate it in broiler production. Studying diversity of bacterial populations also may help scientists to identify bacteria that may have adapted for survival in the processing environment or may survive stresses and more likely to cause human sickness. In this study, we studied two distinct areas of a poultry processing line. We found that diversity declined as carcasses moved towards the end of processing.

Out of 35 flocks, \textit{C. jejuni} was the only species detected in 21 flocks, \textit{C. jejuni} and \textit{C. coli} were detected in 13 flocks (Table 1). There were three flocks primarily comprised of \textit{C. coli}, and all samples from these flocks were taken during the spring sampling.

In total, 1249 isolates were sequenced; they were collected from 17 plants, representing 35 flocks. Seventy-three \textit{flaA}-SVR types were identified (Figure 1). There was greater diversity in rehang samples than there was in post-chill samples. \textit{Campylobacter} from plant 8 was the most diverse, with eighteen types present, while
plant 3 had the least diversity, with one type present (Data not shown). Plant 3 was used as the basis for comparison to find significant differences in all diversity indices. The five plants with the lowest diversity were significantly less diverse than the other 12 plants. Plant 3 had no diversity in rehang and post-chill samples, as seen in Table 2. Only flaA-SVR type 57 was found in this processing facility, which may suggest proliferation of one type on the farm and at arrival for slaughter, and possibly type 57 has flourished in this processing environment.

In this study, for the purposes of statistical analysis, diversity was analyzed in two ways: independent and dependent observations. Independent observations refer to type richness, or the number of types per sample. It does not account for the abundance of each type at each sampling occasion. A sampling occasion is defined as each instance a sample was taken. For example, there are four seasons and two sites (re-hang and post-chill) which makes eight possible sampling occasions for each detected type at each plant. By analyzing independent observations, equal weight was given to each type, minimizing the effect of abundance on diversity. For dependent observations, relative abundance of each independent observation was analyzed. It was important in this study to differentiate between the two types of observations to detect diversity changes because some variables, such as season, chemical treatment may affect dependent observations (abundance of a type) but not necessarily independent observations (number of types at each sampling occasion). When performing statistical analysis on independent observations, we did not detect a statistically significant difference in diversity for any variable (season, site, chemical). All tables and figures shown reflect the analysis of dependent observations, unless noted.
Campylobacter prevalence in poultry flocks varies with latitude; number of cecal organisms per bird but is generally higher in summer than in winter (14, 34). In the current data, fall was the least diverse season with a diversity index 0.71, as shown in Table 3. Winter was the most diverse season. When considering independent observations, summer was the most diverse season, with a diversity index of 0.98; however, there was no significant difference in diversities in independent observations. There is a peak in human Campylobacter-related sickness and prevalence in warmer months (6, 16). Migratory birds and insects are suggested sources (6, 15). Over all seasons, there was a significant difference in diversity among fall, winter, and spring and summer. There was no significant difference observed between spring and summer. Over the four samplings that roughly represented the four seasons, there was a significant difference in diversities between the rehang and post-chill sites in the fall and winter seasons and no significant difference in diversity between rehang and post-chill sites in the spring and summer, as shown in Table 4. When comparing the diversity of all rehang samplings over all seasons, there was no significant difference, the same was noted for as post-chill samples. When comparing the diversity for each site, not differentiating for plants, it was found that rehang is significantly more diverse than post-chill, with diversity indices of 0.9466 and 0.9243, respectively.

Chicken carcasses produced in the United States and in the United Kingdom are frequently contaminated with multiple subtypes of Campylobacter (11, 18, 25). Two carcasses, (0.5%) had four genotypes detected, as seen in Table 5. By far, the majority of carcasses, 70%, had one flaA-SVR type detected n them.
In accordance with the results presented by Lindmark (2006) there may be a correlation between high flock prevalence and presence of several genotypes on carcasses. In the current study, results from plant three were in agreement with Lindmark’s observation. Plant 3 had the lowest numbers, $0.78 \pm 0.23 \log \text{CFU/ml}$ at re-hang, $0.00 \pm 0.00 \log \text{CFU/ml}$ post-chill and low prevalence (Table 7), and the lowest diversity indices of 0.00. Plant 8 had the highest diversity, 0.83 and one of the highest prevalence and numbers among the studied plants, $4.33 \pm 0.10 \log \text{CFU/ml}$ and $1.06 \pm 0.11 \log \text{CFU/ml}$ for re-hang and post-chill respectively. However, counter to Lindmarks’ assertion, in Plant 4 had the highest numbers post-chill and almost the highest in re-hang samples, $1.19 \pm 0.11 \log \text{CFU/ml}$ and $4.38 \pm 0.10 \log \text{CFU/ml}$, respectively, with a diversity index of 0.54.

Chemical processing aids are used in poultry processing facilities to reduce the bacterial loads on carcasses; they may change the microtopography of chicken skin kill, damage or remove bacteria (13). Some chemical processing aids have an effect on the pH of the rinse water in the processing facility. Trisodium phosphate (TSP) for example, raises the pH and reduces the efficiency of chlorine as an antimicrobial. It may be expected to see a higher prevalence and genetic diversity in facilities that used TSP as a chemical processing aid. This was observed for the TSP utilizing plants, 4, 8, 10, 17, and 20. Plants that utilize TSP had the highest genetic diversity in post-chill sites as well as the highest prevalence of *Campylobacter*, as shown in Table 6 and Table 8 while plants utilizing hypochlorous acid system (HAS) had the lowest genetic diversity in the post-chill sample, with one *flaA*-SVR type surviving chemical treatment detected. Plants
using a blend of food grade acids (FGA) and chlorine dioxide (CD) as processing aids did not have significantly more diversity in the rehang site than at the post-chill site.

Alter (3) found that AFLP type distribution changes during processing, beginning with a broad diversity at the beginning of slaughter, with only a few highly genetically related Campylobacter subtypes present after a 24 h chilling period. In the current study there was less diversity later in processing, but the types found may not be very closely related. A lower diversity was observed in the post-chill site when compared to the rehang site, the overall index of diversity for rehang samples was 0.9466 and for post-chill samples the overall index of diversity was 0.9243. A high diversity in C. jejuni genotypes was isolated at the beginning of the slaughter line, with a significantly lower diversity found at post-chill sites. In many plants, prevalence was very low in post-chill sites, which will affect diversity.

In Campylobacter, genetic recombination is known to occur. In some studies, isolates with similar PFGE profiles were concluded to be of clonal origin, a product of genomic rearrangements (36). In addition, Rivoal (27) suggests that genotypes with similar but not identical fla and rib rRNA gene profiles are derivatives of a more commonly isolated subtype. In the current study, there were some flaA-SVR types that were closely related, but more study is needed to prove if there is a single origin for these types.

It has been reported that carcasses sampled towards the end stage of processing have been contaminated with campylobacters even when the bacteria was not isolated from the chickens at the arrival at the abattoir (1, 8, 21, 24). In addition, it has been shown that the subtypes of campylobacters found on carcasses from colonized chicks are
not always those that were most prevalent in the guts of birds (1, 24). In the current study, there were several subtypes detected post-chill that were not detected at re-hang, which may be indicative of contamination occurring during processing (Figures 1 and 2). The scalding process and the chilling period put selective pressure on the \( C. \textit{jejuni} \) population. Certain stress-resistant subpopulations may survive and ultimately remain in the food chain leading to the conclusion that different genotypes not only exhibit a different potential for colonization but also a different potential to survive environmental stressors (3, 26, 31). There were many types found at the re-hang site that were not found at post-chill (Figures 1 and 2). This may show that some types are prone to perish during processing while others survive to retail or some types may flourish in stressful environments.

A change in diversity for independent samples may be an indication of whether some types of \textit{Campylobacter} are more resistant to treatment. However, because of the propensity of \( C. \textit{jejuni} \) to engage in lateral gene transfer, the flaA-SVR cannot be expected to be linked to the trait that confers the resistance; we can only conclude that there are resistance traits.

For this study, diversity of \textit{Campylobacter} on broiler carcasses was examined. It was found that diversity decreased as a carcass proceeded through processing, and the season during which the carcass was sampled may affect on the diversity of types detected. We also found that the chemicals used in reprocessing or added to chill tanks may impact the diversity of \textit{Campylobacter}. It must be kept in mind that in this study, we considered independent observations, yet, the sample size was inadequate to determine statistical significance. For future research, it may be possible to include a
larger sample size to determine the effects of processing on independent observations. It may also be promising to study those types that are able to survive processing or proliferate in the processing environment to determine any special characteristics possessed, and their possible impact on human health.
References


of Campylobacter on broiler carcasses collected at rehang and post-chill in 20 U.S. processing plants. *J Food Prot* 70:1556-1560.


free-range broiler farms and comparison with isolates of various origins. 


Table 1: Percentage of *C. jejuni* and *C. coli* detected on carcasses from each of 17 commercial processing plants sampled across seasons in 2005.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Season</th>
<th>% <em>C. jejuni</em></th>
<th>% <em>C. coli</em></th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Spring</td>
<td>100</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>100</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>Spring</td>
<td>100</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>Fall</td>
<td>100</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>4</td>
<td>Fall</td>
<td>100</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>100</td>
<td>0</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>96.55</td>
<td>3.45</td>
<td>58</td>
</tr>
<tr>
<td>5</td>
<td>Fall</td>
<td>84.38</td>
<td>15.62</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>100</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>2.78</td>
<td>97.22</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>Summer</td>
<td>100</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>7</td>
<td>Winter</td>
<td>100</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>100</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>100</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>8</td>
<td>Winter</td>
<td>78.38</td>
<td>21.62</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>100</td>
<td>0</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>83.33</td>
<td>16.67</td>
<td>48</td>
</tr>
<tr>
<td>9</td>
<td>Winter</td>
<td>100</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>Winter</td>
<td>100</td>
<td>0</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>100</td>
<td>0</td>
<td>53</td>
</tr>
<tr>
<td>Week</td>
<td>Season</td>
<td>Value1</td>
<td>Value2</td>
<td>Value3</td>
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<td>----------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>11</td>
<td>Spring</td>
<td>3.28</td>
<td>96.72</td>
<td>61</td>
</tr>
<tr>
<td>14</td>
<td>Winter</td>
<td>100</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>0</td>
<td>100</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>100</td>
<td>0</td>
<td>55</td>
</tr>
<tr>
<td>15</td>
<td>Winter</td>
<td>89.74</td>
<td>10.26</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>46.3</td>
<td>53.7</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>90.32</td>
<td>9.68</td>
<td>31</td>
</tr>
<tr>
<td>16</td>
<td>Winter</td>
<td>100</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>26.67</td>
<td>73.33</td>
<td>30</td>
</tr>
<tr>
<td>17</td>
<td>Winter</td>
<td>100</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>100</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>22.73</td>
<td>77.27</td>
<td>66</td>
</tr>
<tr>
<td>18</td>
<td>Winter</td>
<td>94.74</td>
<td>5.26</td>
<td>38</td>
</tr>
<tr>
<td>20</td>
<td>Summer</td>
<td>89.19</td>
<td>10.81</td>
<td>37</td>
</tr>
</tbody>
</table>
Table 2: Simpson’s Index of Diversity for *Campylobacter* detected on broiler carcasses from 17 commercial processing plants at re-hang, post chill or combined, over all seasons

<table>
<thead>
<tr>
<th>Plant</th>
<th>Simpson's Index of Diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>0.0282&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>18</td>
<td>0.0715&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>0.2361&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>0.2390&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>0.4518&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>0.4629&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16</td>
<td>0.5229&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>0.5442&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>0.5538&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>0.6796&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>17</td>
<td>0.7672&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>0.7746&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>0.7748&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>0.7882&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>0.7904&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>0.8313&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Different superscripts represent significant difference in diversity in randomization test 

(p≤0.05)
Table 3: Simpson’s Index of Diversity for *Campylobacter* detected on broiler carcasses during each season, all processing plants combined

<table>
<thead>
<tr>
<th>Season</th>
<th>Simpson's Index of Diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA</td>
<td>0.7170&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>W</td>
<td>0.9195&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SP</td>
<td>0.8802&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SU</td>
<td>0.8960&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Different superscripts represent significant difference by randomization test (p≤0.05).
Table 4: Simpson’s Index of Diversity for *Campylobacter* detected on broiler carcasses during each season at re-hang and post-chill sites, all processing plants combined

<table>
<thead>
<tr>
<th>Season</th>
<th>Site</th>
<th>Simpson's Index of Diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fall</td>
<td>Re-hang</td>
<td>0.7684&lt;sup&gt;aZ&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>0.4805&lt;sup&gt;bY&lt;/sup&gt;</td>
</tr>
<tr>
<td>Winter</td>
<td>Re-hang</td>
<td>0.9136&lt;sup&gt;aZ&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>0.8667&lt;sup&gt;bY&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spring</td>
<td>Re-hang</td>
<td>0.8772&lt;sup&gt;cZ&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>0.8196&lt;sup&gt;cY&lt;/sup&gt;</td>
</tr>
<tr>
<td>Summer</td>
<td>Re-hang</td>
<td>0.9127&lt;sup&gt;cZ&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>0.8065&lt;sup&gt;cY&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Diversity indices within the same plant at different sites with different superscripts are significantly different by randomization test (p≤0.05).

<sup>z</sup> Diversity index at the same Site in different plants with different superscripts are significantly different by randomization test (p≤0.05).
Table 5: Number of unique *flaA*-SVR types detected per broiler carcass \(^a\)

<table>
<thead>
<tr>
<th>No</th>
<th>Types/ Carcass</th>
<th>Carcass Site</th>
<th>Total Carcasses</th>
<th>No Carcasses</th>
<th>% Total Carcasses</th>
<th>% Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Re-hang</td>
<td>212</td>
<td>50.84%</td>
<td>68.17%</td>
<td>19.42%</td>
<td>76.42%</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>81</td>
<td>19.42%</td>
<td>76.42%</td>
<td>5.52%</td>
<td>21.70%</td>
</tr>
<tr>
<td>2</td>
<td>Re-hang</td>
<td>86</td>
<td>20.62%</td>
<td>27.65%</td>
<td>5.52%</td>
<td>21.70%</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>23</td>
<td>5.52%</td>
<td>21.70%</td>
<td>0.48%</td>
<td>1.89%</td>
</tr>
<tr>
<td>3</td>
<td>Re-hang</td>
<td>11</td>
<td>2.64%</td>
<td>3.54%</td>
<td>0.48%</td>
<td>1.89%</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>2</td>
<td>0.48%</td>
<td>1.89%</td>
<td>0.48%</td>
<td>0.64%</td>
</tr>
<tr>
<td>4</td>
<td>Re-hang</td>
<td>2</td>
<td>0.48%</td>
<td>0.64%</td>
<td>0.48%</td>
<td>0.64%</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>0</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
</tbody>
</table>

\(^a\) ≤ four isolates tested per carcass

n = 417
Table 6: Simpson’s Index of Diversity for *Campylobacter* detected on broiler carcasses for carcass treatments at re-hang and post-chill, all processing plants combined

<table>
<thead>
<tr>
<th>Carcass Treatment</th>
<th>Site</th>
<th>Simpson’s Index of Diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASC(^1)</td>
<td>Re-hang</td>
<td>0.8455(^a^)</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>0.5410(^b^)</td>
</tr>
<tr>
<td>CD(^2)</td>
<td>Re-hang</td>
<td>0.5933(^a^)</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>0.3857(^a^)</td>
</tr>
<tr>
<td>FGA(^3)</td>
<td>Re-hang</td>
<td>0.4882(^a^)</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>0.5779(^a^)</td>
</tr>
<tr>
<td>HAS(^4)</td>
<td>Re-hang</td>
<td>0.5631(^a^)</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>0.0000(^b^)</td>
</tr>
<tr>
<td>NO TREATMENT</td>
<td>Re-hang</td>
<td>0.6222(^a^)</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>0.4962(^b^)</td>
</tr>
<tr>
<td>PA(^5)</td>
<td>Re-hang</td>
<td>0.8563(^a^)</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>0.6445(^b^)</td>
</tr>
<tr>
<td>TSP(^6)</td>
<td>Re-hang</td>
<td>0.9306(^a^)</td>
</tr>
<tr>
<td></td>
<td>Post-chill</td>
<td>0.8281(^b^)</td>
</tr>
</tbody>
</table>

\(^a,b^\) Values within carcass treatments with different superscripts are significantly different by randomization test (p \(\leq 0.05\))

\(^1\) ASC, Acidified sodium chlorite (Sanova), Ecolab Inc. St. Paul, MN

\(^2\) CD, Chlorine dioxide, Ashland Specialty Chemical, Boonton, NJ

\(^3\) FGA, Blend of food grade acids (FreshFX), SteriFX Inc., Shreveport, LA
4 HAS, Hypochlorous acid system (Tomco₂), Tomco Equipment Co., Loganville, GA

6 PA, Peroxyacetic acid based antimicrobial (Inspexx 100), Ecolab Inc. St. Paul, MN

7 TSP, Trisodium phosphate, Danisco USA Inc., New Century, KS
Table 7: *Campylobacter* prevalence and numbers (mean log CFU/ml carcass rinse ± standard error) detected on broiler carcasses at re-hang and post-chill for 17 different commercial processing plants sampled four times, 2005 (7)

<table>
<thead>
<tr>
<th>Plant</th>
<th>No.+</th>
<th>log CFU/ml</th>
<th>No.+</th>
<th>log CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22$^Y$</td>
<td>1.62$^A$ ± .26</td>
<td>5$^Z$</td>
<td>0.15$^B$ ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>30$^Y$</td>
<td>2.21$^A$ ± 0.24</td>
<td>3$^Z$</td>
<td>0.28$^B$ ± 0.16</td>
</tr>
<tr>
<td>3</td>
<td>10$^Y$</td>
<td>0.78$^A$ ± 0.23</td>
<td>0$^Z$</td>
<td>0.00$^B$ ± 0.00</td>
</tr>
<tr>
<td>4</td>
<td>40$^Y$</td>
<td>4.38$^A$ ± 0.10</td>
<td>36$^Y$</td>
<td>1.19$^B$ ± 0.11</td>
</tr>
<tr>
<td>5</td>
<td>39$^Y$</td>
<td>3.35$^A$ ± 0.17</td>
<td>18$^Z$</td>
<td>0.45$^B$ ± 0.11</td>
</tr>
<tr>
<td>6</td>
<td>20$^Y$</td>
<td>2.35$^A$ ± 0.38</td>
<td>20$^Y$</td>
<td>0.93$^B$ ± 0.16</td>
</tr>
<tr>
<td>7</td>
<td>35$^Y$</td>
<td>2.52$^A$ ± 0.18</td>
<td>14$^Z$</td>
<td>0.38$^B$ ± 0.08</td>
</tr>
<tr>
<td>8</td>
<td>40$^Y$</td>
<td>4.33$^A$ ± 0.10</td>
<td>34$^Z$</td>
<td>1.06$^B$ ± 0.11</td>
</tr>
<tr>
<td>9</td>
<td>16$^Y$</td>
<td>1.36$^A$ ± 0.30</td>
<td>1$^Z$</td>
<td>0.05$^B$ ± 0.05</td>
</tr>
<tr>
<td>10</td>
<td>40$^Y$</td>
<td>3.55$^A$ ± 0.16</td>
<td>20$^Z$</td>
<td>0.47$^B$ ± 0.11</td>
</tr>
<tr>
<td>11</td>
<td>29$^Y$</td>
<td>1.64$^A$ ± 0.20</td>
<td>8$^Z$</td>
<td>0.27$^B$ ± 0.10</td>
</tr>
<tr>
<td>14</td>
<td>40$^Y$</td>
<td>3.50$^A$ ± 0.16</td>
<td>31$^Z$</td>
<td>0.89$^B$ ± 0.10</td>
</tr>
<tr>
<td>15</td>
<td>39$^Y$</td>
<td>4.49$^A$ ± 0.19</td>
<td>22$^Z$</td>
<td>0.41$^B$ ± 0.07</td>
</tr>
<tr>
<td>16</td>
<td>39$^Y$</td>
<td>3.05$^A$ ± 0.18</td>
<td>3$^Z$</td>
<td>0.08$^B$ ± 0.06</td>
</tr>
<tr>
<td>17</td>
<td>40$^Y$</td>
<td>4.19$^A$ ± 0.14</td>
<td>31$^Z$</td>
<td>0.96$^B$ ± 0.11</td>
</tr>
<tr>
<td>18</td>
<td>28$^Y$</td>
<td>2.67$^A$ ± 0.32</td>
<td>11$^Z$</td>
<td>0.34$^B$ ± 0.10</td>
</tr>
<tr>
<td>20</td>
<td>30$^Y$</td>
<td>2.98$^A$ ± 0.29</td>
<td>13$^Z$</td>
<td>0.34$^B$ ± 0.09</td>
</tr>
<tr>
<td>mean</td>
<td>30$^Y$</td>
<td>2.66$^A$ ± 0.06</td>
<td>14$^Z$</td>
<td>0.43$^B$ ± 0.02</td>
</tr>
</tbody>
</table>
number positive out of 40 tested, 10 per replication

A,B Mean *Campylobacter* numbers from different sites within the same plant with different superscripts are significantly different (P<0.01) by students T test.

Y, Z *Campylobacter* prevalence values from different sites within the same plant with different superscripts are significantly different (P<0.01) by Chi Square test for independence
Table 8. *Campylobacter* counts (mean log CFU/ml carcass rinse ± standard error) from whole broiler carcass rinse samples collected at re-hang and post-chill as affected by reprocessing chemical treatment (7)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>n</th>
<th>Re-hang</th>
<th>Post-chill</th>
<th>Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAS(^1)</td>
<td>40</td>
<td>1.62(^{cd}) ± 0.26</td>
<td>0.15(^{cd}) ± 0.07</td>
<td>1.47(^{cd}) ± 0.26</td>
</tr>
<tr>
<td>PA</td>
<td>100</td>
<td>1.94(^{cd}) ± 0.16</td>
<td>0.15(^{cd}) ± 0.06</td>
<td>1.79(^{bc}) ± 0.17</td>
</tr>
<tr>
<td>ASC</td>
<td>140</td>
<td>2.48(^{bc}) ± 0.14</td>
<td>0.04(^d) ± 0.02</td>
<td>2.33(^b) ± 0.14</td>
</tr>
<tr>
<td>FGA</td>
<td>120</td>
<td>1.63(^d) ± 0.17</td>
<td>0.31(^c) ± 0.06</td>
<td>1.32(^{cd}) ± 0.15</td>
</tr>
<tr>
<td>TSP</td>
<td>250</td>
<td>3.73(^a) ± 0.08</td>
<td>0.76(^a) ± 0.04</td>
<td>2.97(^a) ± 0.07</td>
</tr>
<tr>
<td>CD</td>
<td>70</td>
<td>2.82(^b) ± 0.26</td>
<td>0.41(^{bc}) ± 0.07</td>
<td>2.41(^{ab}) ± 0.24</td>
</tr>
<tr>
<td>none</td>
<td>80</td>
<td>2.43(^{bc}) ± 0.21</td>
<td>0.65(^{ab}) ± 0.10</td>
<td>1.78(^{bd}) ± 0.15</td>
</tr>
<tr>
<td>mean</td>
<td>800</td>
<td>2.66 ± 0.06</td>
<td>0.43 ± 0.02</td>
<td>2.23 ± 0.06</td>
</tr>
</tbody>
</table>

\(^{a,b,c,d}\) Values within columns with no like superscripts are significantly different (\(p< 0.05\)) by Tukey’s honest significant difference test.
Figure 1: Dendrogram showing relatedness of *Campylobacter flaA*-SVR types detected on broiler carcasses from 17 different commercial processing plants sampled four times, 2005
Figure 2: Frequency of *Campylobacter flaA*-SVR types detected across all plants and seasons, for re-hang and post-chill sites (Dependent observations)
Figure 3: Unique *Campylobacter flaA*-SVR types detected across all plants and seasons, for re-hang and post-chill sites (Independent observations)
CHAPTER 4

CONCLUSION

_Campylobacter jejuni_ and _C. coli_ are the most important human enteropathogens among the campylobacters, affecting an estimated 2.4 million cases each year in the U.S. alone. Risks for contracting campylobacteriosis are consumption and handling of raw and undercooked poultry, most commonly chicken and turkey products, cross contamination with other foods, and contaminated milk and water. _C. jejuni_ and _C. coli_ can be found on 90% of poultry in the U.S., and from 18% to >90% of poultry in Europe, varying from country to country. Carry over contamination from a positive flock to a negative one is a source for contamination in processing. In some instances, processing aids are used to further reduce the microbial load on chicken carcasses by removing surface contamination. However, use of these processing aids may have an additional effect on the microbial loads in addition to lowering counts. Clarification of the diversity and nature of _Campylobacter_ helps in the investigations of disease.

In this study, the Short Variable Region (SVR) of the flagellin locus was analyzed to determine the genetic diversity of populations of _Campylobacter_ collected from rehang (post-defeather) and post chill sites at 17 plants around the United States. Each population was analyzed for changes in diversity due to different variables. Diversity was affected by season, chemical processing aid, and site from which the sample was taken. It is also noted that several types may have been able to resist certain environmental stressors and adapt to the processing environment. Future study is needed
to determine any traits or characteristics that are prevalent in these types and enable survival and possible impacts on human health.