

PREVALENCE, GENETIC DIVERSITY, AND ANTIMICROBIAL RESISTANCE
PATTERNS OF *ARCOBACTER* AND *CAMPYLOBACTER* ON BROILER CARCASSES
DURING PROCESSING

by

INSOOK SON

(Under the Direction of Mark A. Harrison)

ABSTRACT

Broiler carcasses (n = 325) were sampled in a U.S. commercial poultry processing plant during five plant visits from August to October of 2004 at three sites along the processing line: 1) pre-scalding, 2) pre-chilling, and 3) post-chilling. *Arcobacter* species were recovered from pre-scalded carcasses more frequently (96.8%) than from pre-chilled (61.3%) and post-chilled carcasses (9.6%). For *Arcobacter* identification, a species-specific multiplex PCR assay showed that *A. butzleri* was the most prevalent species (79.1%) followed by *A. cryaerophilus* 1B (18.6%). *A. cryaerophilus* 1A was found at low levels (2.3%) and *A. skirrowii* was not isolated at all. *Campylobacter* was isolated from 92% of pre-scalded carcasses, 100% of pre-chilled carcasses, and 52% of post-chilled carcasses. For *Campylobacter* speciation, the BAX[®] PCR identified as *C. jejuni* (87.6%) as the most common species followed by *C. coli* (12.4%).

The genetic diversity of *Arcobacter* and *Campylobacter* was analyzed by pulsed field gel electrophoresis (PFGE). Genomic DNA was digested with *Kpn*I from *Arcobacter* strains and *Sma*I from *Campylobacter* strains. A total of 32.8% of *Arcobacter* isolates belonged to single-isolate groups, while only 2.3% of *Campylobacter* isolates belonged to this category. The remaining *Arcobacter* species were distributed among 25 multi-isolate PFGE groups, while *Campylobacter* species were found in just eight multi-isolate groups.

The great majority of *Arcobacter* (93.7%) and *Campylobacter* (99.5%) isolates were resistant to one or more antimicrobials. Multiple antimicrobial resistance was observed in 71.8% of the *Arcobacter* isolates and in 28.4% of the *Campylobacter* isolates. Of the *A. butzleri* isolates, 89.9% (n = 125) were resistant to clindamycin, 82% (n = 114) were resistance to azithromycin, and 23.7% (n = 33) were resistant to nalidixic acid. Resistance to tetracycline was very high in *C. jejuni* and *C. coli* at 99.5% and 96.3%, respectively.

These data suggest significant contamination of *Arcobacter* and *Campylobacter* from carcasses from different processing sites in a commercial poultry plant with a high genetic diversity of *Arcobacter*, and demonstrated resistance in *Arcobacter* and *Campylobacter* to common antimicrobial agents.

INDEX WORDS: *Arcobacter*, Broiler chickens, *Campylobacter*, Poultry processing, Genetic diversity, PFGE, Typing, Antimicrobial resistance

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DEDICATION

This dissertation is dedicated to

My parents

And

My parents-in-law

For their prayer, constant support, and unconditional love.

AND

To

My beloved husband

Kwangwook Ahn

For his unwavering encouragement, support, and patience.

Without all of you, I could never have come so far.

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CHAPTER 1

INTRODUCTION

Campylobacter is an important human pathogen and the most common cause of bacterial gastroenteritis in developed countries (6). Studies have revealed that *Campylobacter* infection is sometimes associated with serious complications including Guillain-Barré syndrome, an acute neurological disease characterized by ascending paralysis of peripheral nerves, which may lead to respiratory muscle compromise and death (5). Recently, *Arcobacter* has gained attention as an emerging foodborne pathogen following outbreaks associated with water, cattle, poultry, and ground pork and turkey products (2, 8, 10, 11). Vandamme et al. (7) proposed the genus *Arcobacter* to include the bacteria formerly known as aerotolerant *Campylobacter*. At present, this genus comprises four species; *A. butzleri*, *A. cryaerophilus* (subgroups 1A and 1B), *A. skirrowii*, and *A. nitrofigilis*. The routes of *Arcobacter* infection are unclear although they may include person-to-person contact and consumption of contaminated water and food of animal origin (9).

Contaminated poultry meat is considered an important vehicle of human infection with *Campylobacter* and *Arcobacter*. In contrast to the high contamination rate for poultry products, arcobacters have rarely been recovered from the intestinal contents or feces of chickens (1). Few reports have been published about how the processing procedures in the United States affect *Arcobacter* and *Campylobacter* species. In a U.S. poultry processing plant, there are typically six basic sections: pre-scalding, scalding, defeathering, evisceration, washing and chilling. Bacterial contamination is frequently high at the pre-scalding stage. In this study, *Arcobacter* and *Campylobacter* were isolated at pre-scalding, pre-chilling, and after chilling, where bacterial contamination is much lower. No single method for isolating *Campylobacter* has yet to be developed that is appropriate for all sample types. The choice of method depends on the expected level of *Campylobacter* in the sample material and any extraneous

bacterial flora that may be present. A combination of direct plating and sample enrichment often provides better recovery of *Campylobacter* than either technique alone and this approach was used for the isolation of *Campylobacter*. The proper identification of *Arcobacter* is essential for an understanding of its role in causing human foodborne illness. Some scientists believe that because of the similarities between *Arcobacter* and *Campylobacter*, some outbreaks attributed to *Campylobacter* may in fact be due to *Arcobacter* instead. Arcobacteriosis had been found to produce symptoms similar to those of campylobacterial illness, including persistent diarrhea, abdominal pain, vomiting, fever, chills, and malaise (4). In previous prevalence studies, the standard *Campylobacter* isolation techniques were not able to isolate *Arcobacter* because the use of a higher temperature (42°C) was not selective for *Arcobacter*. Therefore, current isolation methods have focused more on the differing temperature and oxygen requirements as a basis to select for *Arcobacter*.

Prevention of *Arcobacter* and *Campylobacter* infections in humans through consumption of contaminated poultry is dependent upon a good epidemiological understanding of these organisms. For epidemiological investigations and the subsequent development of intervention strategies to eliminate pathogens from food supply, the ability to determine the relatedness of these pathogens has gained importance. Information acquired from molecular typing and tracking of pathogens can facilitate the detection of outbreaks within flocks or herds and the detection of emerging isolates with increased virulence properties. Molecular typing and tracking also assist in the assessment of the effectiveness of current control measures, in the establishment of risk strategies, and in evaluating the effectiveness of food safety programs (3).

Research on the epidemiology of *Arcobacter* spp. dates to 1991, but few reports have been published comparing its distribution to *Campylobacter* spp. The epidemiology of *Campylobacter* infection is complex and not well understood since the organism is widely distributed in the environment and throughout the food chain. Many methods for typing and subtyping have been developed for *Campylobacter*. Phenotypic typing schemes such as Penner serotyping and several genotypic based methods including random amplification of polymorphic DNA-PCR (RAPD-PCR), *fla* typing, amplified

fragment length polymorphism fingerprinting (AFLP), and pulsed-field gel electrophoresis (PFGE) have been introduced. Genotyping methods are generally more discriminatory than phenotypic typing, but each method has its own limitations. When choosing a typing method, several factors including discriminatory power of the method for the particular organism of interest, the simplicity in performing the techniques, reproducibility, outcomes potential associated with a technique, and cost should be considered.

The human health relevance of antimicrobial resistant bacteria from food production animals has long been a point of contention between the public health and animal health communities. Most public health officials believe the primary role of antimicrobial use in food animals contributes to the increasing resistance in foodborne bacterial pathogens. As *Campylobacter* and *Arcobacter* may be transferred from animals to humans, the possible development of antimicrobial resistance in *Campylobacter* spp. and *Arcobacter* spp., due to the use of antimicrobial agents in food animals, is a matter of concern. It is therefore important to know whether antimicrobial resistant *Campylobacter* and *Arcobacter* can be isolated from animals.

It has been established that administration of antimicrobials to food animals can select for resistance among bacteria which are subsequently transmitted to humans through food or animal contact. However, the frequency of these events remains unclear. When evaluating the public health consequences of antimicrobial use in animals, it is important to consider each pathogen-antimicrobial situation individually. The susceptibility to 7 antimicrobials used by the National Antimicrobial Resistance Monitoring System (NARMS) for *Campylobacter* was examined in this study. These 7 antimicrobials are: azithromycin (AZ), ciprofloxacin (CI), erythromycin (EM), gentamicin (GM), tetracycline (TC), nalidixic acid (NA), and clindamycin (CM). This research will lead to a better understanding the contamination levels and patterns of antimicrobial resistance in *Campylobacter* spp. and *Arcobacter* spp. isolated from the poultry processing environment in the United States. This work demonstrates survival of *Arcobacter* and *Campylobacter* in the poultry processing plant, and also indicates inadequate decontamination of the processing facility resulting from either improper execution

of cleaning or disinfection procedures. An understanding of the survival and antimicrobial resistance patterns of *Arcobacter* and *Campylobacter* on broiler carcasses at different stages of processing is important for the protection of consumer health against these pathogens.

This study will provide a useful comparison of the survivability of these two organisms and also provided a snapshot view of the prevalence of *Arcobacter* and *Campylobacter* in poultry obtained from a U.S. poultry processing plant. The incidence of antimicrobial resistance in many pathogenic bacteria has continually risen over the last few decades and has become a major medical challenge. Information about the antimicrobial resistance of *Arcobacter* and *Campylobacter* species in this study will assist in choosing suitable antimicrobials for severe arcobacteriosis or campylobacteriosis.

The overall goal of this research was to examine the prevalence and contamination levels of *Arcobacter* and *Campylobacter* in poultry broiler flocks at the poultry processing plant, determine their genetic diversity, and identify antimicrobial susceptibility patterns in these organisms. This dissertation is divided into six chapters. Chapter II is a literature review, which describes the taxonomic history of *Arcobacter* and *Campylobacter*, their sources such as animal and food, human, and poultry processing plant, pathogenicity, typing method, and antimicrobial susceptibility. In Chapter III, optimization of methods for *Arcobacter* isolation and culture were investigated. Broiler carcasses were collected in a commercial poultry processing plant to determine the prevalence of *Arcobacter* and *Campylobacter* at three sites along the processing line: 1) pre-scalding, 2) pre-chilling, and 3) post-chilling. The prevalence of *Arcobacter* and *Campylobacter* from broiler carcasses was compared. Identification of *Arcobacter* species and *Campylobacter* species by PCR was also performed. In Chapter IV, the genetic diversity of *Arcobacter* and *Campylobacter* from broiler carcasses was studied using PFGE. The genetic diversity present on *Arcobacter* species was compared with the genotypes of *Campylobacter* species. In Chapter V, a broth-microdilution method was used for antimicrobial susceptibility testing of *Arcobacter* and *Campylobacter* from broiler carcasses and the patterns of antimicrobial resistance in these organisms was determined. A summary and conclusions from the three studies in Chapters III through V were included in Chapter VI.

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CHAPTER 2

LITERATURE REVIEW

Taxonomic history and general characteristics

Arcobacter

In 1977, *Arcobacter*, previously classified as an aerotolerant campylobacter (157) or *Campylobacter*-like organisms (115, 224), was first isolated by Ellis et al. (51) from aborted bovine and porcine fetuses. The genus *Arcobacter* (Latin for ‘arc-shaped bacterium’) includes bacteria that were formerly designated *Campylobacter cryaerophila* (Latin for ‘loving cold and air’) (239). *Arcobacters* are gram negative, non-spore forming, microaerophilic and generally 1 to 3 µm by 0.2 to 0.9 µm in size (223). They are curved rods, S-shaped or helical cells that are motile by means of polar flagella and exhibit a corkscrew movement (90). *Arcobacter butzleri* is only slightly curved, and *A. cryaerophilus* tends to much more helical (155). *Arcobacter* belongs to the rRNA superfamily VI of the *Proteobacteria* which also includes the genera *Campylobacter*, *Helicobacter*, *Wolinella* and *Sulfuro spirillum* (224). Unlike campylobacters, arcobacters while phenotypically similar to campylobacters, are distinguished by their ability to grow at cooler temperatures (15-25°C) and under normal atmospheric conditions (16). They also do not require hydrogen for growth. The G + C content of *Arcobacter* DNA ranges from 27 to 31 mol% (223).

At present, the genus *Arcobacter* is comprised of four species (224). *Arcobacter butzleri* and *A. cryaerophilus* are associated with human diarrheal illness and bacteremia, and with reproduction abnormalities in farm animals (115, 170, 215). *Arcobacter butzleri* is regarded as the primary human pathogen (115). Within *A. cryaerophilus*, two subgroups referred to as group 1A (115) and group 1B (227) have been identified. Their whole-cell protein, fatty acid patterns, and the restriction fragment length polymorphisms of the rRNA genes differentiate strains of these two subgroups (227). *Arcobacter*

skirrowii has been reported in farm animals and on broiler carcass, but has not been isolated from other sources (227, 243). *Arcobacter nitrofigilis* has never been associated with animal or human infection. It is a nitrogen-fixing bacterium associated with the roots of *Spartina alterniflora*, a salt marsh plant (115, 141).

Campylobacter

In 1886, Escherich observed nonculturable spiral-shaped bacteria in stool samples of children with diarrhea (57) (cited from Vandamme (223)). In 1913, *Vibrio*-like bacteria were recovered from aborted ovine fetuses by McFadyean and Stockman (143). Smith in 1918 isolated spiral bacteria in aborted bovine fetuses and noted that these strains and the vibrios of McFadyean and Stockman belonged to the same species (201). Later, he proposed the name *Vibrio fetus* for these strains (202). *Vibrio jejuni* from blood cultures of humans with gastroenteritis (119) and *Vibrio coli* from feces of pigs with diarrhea (48) were also described. Florent (69) isolated *Vibrio bubulus* from the bovine vagina (cited from Vandamme (223)) and Firehammer (64) identified *Vibrio fecalis* isolated from normal ovine feces. *V. fetus* and *V. bubulus* were transferred into the new genus *Campylobacter* in 1963 due to their low G + C DNA base composition, microaerophilic growth requirements, and nonfermentative metabolism (223). In 1989, Goodwin et al. (78) proposed a revision of *Campylobacter* taxonomy. Vandamme et al. (224), in 1991, completed another revision of the taxonomy and nomenclature of the genus *Campylobacter* and related bacteria. DNA-rRNA hybridizations provided the basis for the taxonomic structure used at present and identified *Campylobacter* spp. as a diverse yet phylogenetically distinct group, rRNA superfamily VI which consists of rRNA homology groups I (*Campylobacter* and *B. ureolyticus*), II (*Arcobacter*) and III (*Helicobacter* and *W. succinogenes*).

Campylobacters are spiral or S-shaped rods 0.2 to 0.8 µm wide and 0.5 to 5 µm long with rapid, darting, reciprocating motility (223). Energy is obtained from amino acids or tricarboxylic acid cycle intermediates, but not carbohydrates (223). The G + C content of *Campylobacter* DNA ranges from 29 to 47 mol% (223). Campylobacters are catalase and oxidase positive, and urease negative. They are microaerophilic, requiring an oxygen concentration of 3-15% and a carbon dioxide concentration of 3-5%

(222). However, some species of *Campylobacter*, such as *C. sputorum*, *C. concisus*, *C. mucosalis*, *C. curvus*, *C. rectus*, and *C. hyointestinalis* require an atmosphere containing an increased concentration of hydrogen to be isolated, but *C. coli* and *C. jejuni* do not need a hydrogen-enriched atmosphere (151). Enzymes such as superoxide dismutase (SOD), catalase, peroxidase, glutathione synthetase, and glutathione reductase in *C. jejuni* are believed to play an important role in providing protection against oxygen toxicity. *Campylobacter* are fastidious organisms that require complex growth media (222).

Campylobacter jejuni and *Campylobacter coli* are referred to as thermophilic campylobacters and grow best at 37°C to 42 °C, with an optimal temperature of 42°C, probably reflecting adaptation to the intestines of birds. *Campylobacter jejuni* and *C. coli* have a genome of approximately 1600-1700 kilobases (kb), which is relatively small compared to the genomes of other enteropathogens such as *Escherichia coli* (4500 kb) (39). *Campylobacter jejuni* and *C. coli* are very similar phenotypically, but *C. jejuni* can be differentiated from *C. coli* by its ability to hydrolyze hippurate (87).

Sources

Animals and food

Arcobacter infections in animals are associated with abortions and enteritis (237). *Arcobacter* have been isolated from dairy cattle (85), pigs (172), pork (41), and poultry (8). Contamination levels as high as several thousand organisms per gram of neck skin in poultry have been reported (8, 95). Although the exact contribution of contaminated poultry products to human infection is not known, insufficient cooking, mishandling of raw poultry, and cross-contamination are the most likely routes of transmission to humans. In cattle, *Arcobacter* spp. have been recovered in the feces of calves with diarrhea or in animals with mastitis (237). Both healthy and clinically ill pigs (237) were found to harbor *Arcobacter* spp. Boer et al. (44) reported that *Arcobacter* spp. was isolated from 1.5% of minced beef (n=68) samples tested. Harmon and Wesley (84) have identified *Arcobacter* spp. by PCR in 40% (504 of 1102) of fecal samples obtained from clinically healthy swine. In Canada (125) and The Netherlands (44), *A. butzleri* was recovered from retail-purchased whole and ground chicken, and turkey samples. A report

by Wesley et al. (240) described that *A. butzleri* colonized neonatal piglets, suggesting their invasive potential. *Arcobacter butzleri*, *A. cryaerophilus*, and *A. skirrowii* are especially common on broiler chicken carcasses and in the slaughter house although not in the intestines of the birds (14, 77, 86, 241). *Arcobacter* infection in animals has caused abortions and enteritis with clinical symptoms of mastitis (135) and diarrhea (115). Collins et al. (41) described that 90% of pork samples in a slaughter facility were positive for *Arcobacter* species. The distribution of *Arcobacter* in seafood, shell-fish and raw milk is unknown (239).

Campylobacter infection can be acquired from raw milk, contaminated water and from pets with diarrhea (114). The infectious dose can be as low as 500-800 organisms (29). Among 80 outbreaks of human campylobacteriosis reported to the Centers for Disease Control and Prevention (CDC) between 1973 and 1992, 30 outbreaks were caused by the consumption of raw milk (5). The digestive tract of clinically normal cattle has been reported to be a significant reservoir for a number of *Campylobacter* spp., with prevalence ranging from 0-80% in cattle (15). A high prevalence of *C. coli* in pigs has been reported and dressed pig carcasses have been shown to be more frequently contaminated than either beef or sheep carcasses (158). Contaminated shellfish have also been implicated as a vehicle in the dissemination of campylobacteriosis. Harvesting shellfish from *Campylobacter*-contaminated waters appears to be the most likely cause of infection (245).

Poultry processing plant

In a poultry processing plant, there are typically six basic sections: pre-scalding, scalding, defeathering, evisceration, washing, and chilling. In contrast to other food animals, poultry are eviscerated without opening the carcass, and as the skin is not normally removed, many contaminants are found on and in the skin (42).

Few reports have been published on the prevalence of *Arcobacter* from the poultry processing environment compared to those on *Campylobacter*. According to the report of Vandamme et al. (226), chickens were identified as the source of an outbreak of *A. butzleri* diarrhea. In Canada, *A. butzleri* was found from 97% (121 of 125) of the poultry carcasses obtained from five different processing plants (125).

Houf et al. (93) also examined the occurrence of *Arcobacter* and *Campylobacter* isolated from eight Belgium poultry slaughterhouses and described a higher prevalence for *Arcobacter* compared to that of *Campylobacter*.

A number of reports on *Campylobacter* isolated from each stage of the processing line in poultry plants have appeared in the literature. Slaughter and processing provide opportunities for reducing *Campylobacter* counts on food-animal carcasses (5), but the nature of the poultry processing system makes cross-contamination from *Campylobacter*-infected to *Campylobacter*-free carcasses unavoidable (145). Significant *Campylobacter* contamination can be found on the majority of chickens entering the processing plant. This contamination is easily spread from carcass to carcass during processing (113). Berrang and Dickens (26) collected poultry samples 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 to study the presence and level of *Campylobacter* on broiler carcasses and found that *Campylobacter* populations decreased due to processing. Wempe et al. (236) observed that the water used in rinsing the birds in the feather picker physically removed *Campylobacter* and thus reduced the number of organisms on the edible parts. Jeffery et al. (106) sampled 6 to 12 carcasses from 22 flocks just before evisceration for determining the prevalence of *Campylobacter*. They found skin samples 78% positive, crops 48% positive, and the intestines 94% positive. Berndtson et al. (25) recovered *Campylobacter* in 89% of neck skin samples, 93% of peritoneal cavity swab samples, and 75% of subcutaneous samples.

Broiler carcasses are washed with systems of washers using chlorinated water to remove contamination such as blood, tissue fragments, and fecal contamination (113). Limited studies have been done on evaluating the performance and effectiveness of poultry washers, and sanitizing treatments within the processing plant (6, 46). Many poultry processors use water chillers for rapid cooling of carcasses, and adding sodium chloride or trisodium phosphate to the chiller water in the presence of an electrical current was shown to reduce *C. jejuni* contamination of the chiller water (131). Sanchez et al. (188)

found that *Campylobacter* prevalence on chilled carcasses was significantly higher with immersion chilling compared to air chilling.

The report by Smith et al. (199) showed that levels of contamination on retail poultry remain high despite interventions made at the processing plant. Stern and Line (207) found *Campylobacter* spp. in 98% of retail-packaged broilers sampled from grocery stores. Another report by Willis and Murray (244) found that 69% of raw broilers from retail outlets were positive for *C. jejuni*. At present, some poultry processors are starting to monitor *Salmonella* levels on birds when they are received at the plant and incidences after processing have been greatly reduced. Many poultry processing plants do not measure *Campylobacter* levels regularly (113). Although the level of *Campylobacter* contamination is reduced during processing, it is still present on the carcass after processing at levels of 100-10,000 organisms/g. They still may not reduce the levels of contamination below a threat to public health since as few as 500 organisms can make a person ill (113).

***Arcobacter*, *Campylobacter* and human infection**

While knowledge of the clinical importance of *Arcobacter* spp. is at present limited, *A. butzleri* and *A. cryaerophilus* have been associated with human disease as causes of diarrhea (115, 215). These two species also have been isolated from human blood, including a case of neonatal bacteremia (98, 115, 170). The majority of isolates obtained from humans belongs to the species *A. butzleri*. It is highly likely that in both cases *A. butzleri* played a major causative role in acute disease although very little is known about the clinical significance of *A. butzleri* infections in humans (129). More than 50% of 22 patients with *A. butzleri*-associated diarrhea suffered from abdominal pain, nausea, fever, chills, vomiting, and malaise (117). In an outbreak at an Italian nursery and primary school, abdominal cramps caused by *A. butzleri* were reported (226). *Arcobacter skirrowii* has been isolated from the stool sample of a person with chronic diarrhea (249). Recently, during an 8-year study Vandenberg et al. (228) observed that patients infected with *A. butzleri* were more likely to have persistent diarrhea or watery diarrhea than those with *C. jejuni* infection, but they were less likely to have acute diarrhea. However, the importance

of *Arcobacter* spp. as a cause of human illness remains to be determined probably because optimal isolation techniques have not yet been established.

Campylobacter infection in humans is usually acquired through the consumption of undercooked contaminated meat or other cross-contaminated food products (222). The most common cause of sporadic cases of *Campylobacter* enteritis is by eating undercooked chicken (45). Person-to-person transmission is uncommon (189). In England and the United States, *Campylobacter* species are isolated from about 5% of patients with diarrhea and the annual incidence of *Campylobacter* enteritis is approximately 50/100,000 population for all persons and about 300/100,000 for children 1 to 4 years old (138, 197). The high isolation rate in neonates and infants is attributed in part to susceptibility on first exposure and to the low threshold for seeking medical care for infants (213). The most prominent features among patients seeking medical attention for *Campylobacter* enteritis are diarrhea, abdominal pain, and fever (30). Grossly bloody stools are common and often require medical attention. Abdominal pain is the most characteristic manifestation of illness (166) .

Pathogenesis

There is very little information on toxin production by the genus *Arcobacter*. Figura et al. (63) and Musmanno et al. (150) demonstrated the presence of cytotoxins and cytolethal distending factors in 18 strains of river water isolates. Wesley (238) reported that *A. butzleri* colonized neonate piglet intestines, suggesting an invasive potential. Mammalian cell-associated cytotoxin activity is detectable in *A. butzleri* NCTC 12481, and hemolytic activity was showed in several strains (90). A hemagglutinin, 20 kDa in size, and sensitivity to proteolytic digestion and inactivation at 80°C, has been characterized from arcobacters. It is possible that a lectin-like molecule binding to erythrocytes via a glycan receptor containing D-galactose is part of its structure (220).

The genus *Campylobacter* colonizes the gastrointestinal tract of a broad range of animals. They are commensals in most animals but notably are associated with disease in humans. The human pathogens *C. jejuni* and *C. coli* are causative agents of acute human enterocolitis and are the most

common cause of foodborne diarrhea in many industrialized countries (213). *Campylobacter* motility is accomplished by polar flagella which creates a cork-screw motion that allows the organism to penetrate the mucus layer covering the intestinal cells to colonization of the intestine (127, 160). The flagellum of *C. jejuni* consists of an unsheathed polymer of flagellin subunits, which are encoded by the adjacent *flaA* and *flaB* genes (165). The *flaA* and *flaB* genes show a very high degree of sequence identity (95%) (83, 164). The *C. jejuni* flagellum consists of FlaA protein, and the *flaA* gene is expressed at much higher levels than the *flaB* gene (222). Another factor in *C. jejuni* colonization of the intestine is chemotaxis that enables detection and movement up or down chemical gradients (222). *Campylobacter jejuni* is attracted to mucins, L-serine and L-fucose, whereas bile acids are repellants (100).

An important feature in *C. jejuni* pathogenesis is its binding and entry in host cells (248). During infection, *C. jejuni* crosses the mucus layer covering the epithelial cells and adheres to these cells. A subpopulation subsequently invades the epithelial cells resulting in the mucosal damage and inflammation (222). With *C. jejuni* a major factor involved in adherence and invasion is the flagella (81). Yao et al. (250) showed that *C. jejuni* mutants with reduced motility due to paralyzed flagella had reduced adherence and an absence of invasion, suggesting adhesion and invasion are dependent on both motility and flagellar expression. In addition, *Campylobacter* cytolethal distending toxin (CDT) activity also can undoubtedly contribute to the cytopathic effects associated with *C. jejuni* infection (233) although the role of CDT in *C. jejuni* pathogenesis has not been determined (180). While most *C. jejuni* strains have a relatively high CDT activity, *C. coli* strains show mostly low activity (181). The ability to acquire iron compounds in low concentration from the host (62), and the outer membrane constituents lipooligosaccharide (LOS) and lipopolysaccharide (LPS) of *C. jejuni* (70) can also contribute to *Campylobacter* pathogenesis.

There is increasing evidence that *C. jejuni* is an important factor in the development of Guillain-Barré syndrome (GBS). Nachamkin et al. (152) reported that the *C. jejuni* surface polysaccharide structures and flagella are sialylated, which is thought to be responsible for the ganglioside mimicry leading to GBS. This syndrome is an autoimmune-mediated disorder of the peripheral nervous system

and is one of the most common causes of acute flaccid paralysis (153). GBS is linked to infection with particular heat-stable serotypes of *C. jejuni*, and formation of autoantibodies is considered to be responsible for the demyelination leading to this disorder (27, 124). In *Campylobacter* colonization and pathogenesis, oxidative stress defense systems produced during normal metabolism to deal with toxic oxygen metabolites play an important role (211). These systems can be divided into superoxide stress defense and peroxide stress defense. The main component of the *C. jejuni* superoxide stress defense mechanism is the superoxide dismutase (SOD) protein SodB (178). The peroxide stress defense system is composed mainly of the catalase (KatA) and alkylhydroperoxide reductase (AhpC) proteins (19). The thermal stress response of *C. jejuni* and *C. coli* in the avian gut, where the normal temperature is 42°C, as well as temperatures in human hosts (37°C) and during transmission in water, milk or on meat results mainly from the induction of the expression of heat shock proteins (HSPs) (222). Konkel et al. demonstrated that among several HSPs including the GroESL, DnaJ, DnaK, and ClpB, only DnaJ has a role in *C. jejuni* pathogenesis, as a *C. jejuni dnaJ* mutant was unable to colonize chickens (123).

Isolation and identification methods

Arcobacter

The isolation and enumeration of *Arcobacter* spp. from animals has proven to be difficult. While the morphology and phenotypic characteristics of *Arcobacter* are very similar to *Campylobacter*, the selective media and growth temperature are quite different. The difference in optimum growth temperature for *Arcobacter* and *Campylobacter* may be used to help differentiate these organisms in the development of isolation protocols. A number of different isolation methods have been developed for the isolation of *Arcobacter* from humans and animals.

Originally, *Arcobacter* spp. were isolated using the *Leptospira* semisolid enrichment medium, EMJH P-80 (Ellinghausen-McCullough-Johnson-Harris Polysorbate-80) supplemented with 5-fluorouracil (100 µg/ml) (51). Since then, various enrichment and plating media have been devised for the detection of *Arcobacter* spp. by adapting different strategies (41, 44, 125). Skirrow first developed a

selective medium based on blood-agar supplemented with trimethoprim, polymyxine B and vancomycin for the isolation of *Campylobacter* (196). Modified cefsulodin-irgasan-novobiocin (CIN) and brain heart infusion agar supplemented with 10% bovine blood and cephalothin, vancomycin, and amphotericin B (CVA) following EMJH P-80 as an enrichment broth were compared (41). In 1999, Johnson and Murano (108) developed a plating media consisting of special peptone no. 2, 5% sheep's blood, 0.05% pyruvate, 0.05% thioglycolate, and 0.032 mg/liter cefoperazone for the isolation of *A. butzleri*, *A. cryaerophilus*, and *A. nitrofigilis*. They concluded this formula was superior to all other formulation in the aspect of absolute growth index, colony size, and colony differentiation values. Golla et al. (76) compared the Johnson-Murano method (108) and the method of Collins (41) to isolate *A. butzleri* from beef and dairy cattle. Houf et al. (95) described a new selective broth and media which includes amphotericin B, cefoperazone, 5-fluorouracil, novobiocin, and trimethoprim as selective supplements for the isolation of *Arcobacter* species and evaluated a new isolation procedure for poultry products. Scullion et al. (194) compared the isolation methods of Johnson and Murano (107), Houf et al. (93), and On et al. (172) to determine which was the most effective for the isolation of *Arcobacter* spp. from retail packs of raw poultry in Northern Ireland. They reported method of Johnson and Murano was the most effective *Arcobacter* isolation method.

Some *Arcobacter* are difficult to identify because of their fastidious growth requirements (35). *Arcobacters* are relatively biochemically inert, morphologically similar to campylobacters (116), and only a few phenotypic tests, including the Preston identification scheme, API Campy identification kits, and catalase test can be used to differentiate *Arcobacter* spp. (13, 168). The most reliable biochemical tests to identify *A. butzleri* include growth in 1% glycine and in 1.5% NaCl, weak catalase activity, and resistance to cadmium chloride (115, 192, 227). *Arcobacter cryaerophilus* strains show strong catalase activity and are sensitive to cadmium chloride (192, 225, 227). For the identification and differentiation of *Arcobacter* spp., several useful molecular methods have been published. These methods include DNA probes (242), PCR based methods (85, 97, 109), and analysis of whole-cell proteins using SDS-PAGE (11, 13). Oligonucleotide probes for the 16S rRNA gene of *Arcobacter* and *A. butzleri* have been described

for species identification and subtyping (242). Since probe-based methods required DNA purification, digestion, and hybridization, these methods may be too time consuming and labor intensive for diagnostic identification (85). PCR-based methods are more rapid, have a higher specificity than conventional identification, and overcome the ambiguities of biochemical identification (85). Harmon and Wesley (85) described the identification of *Arcobacter* species based on a multiplex PCR that was superior to previously described protocols. This method did not provide the means for identifying and distinguishing *A. cryaerophilus* 1A from 1B although their assay was valuable for identifying the *Arcobacter* species. A multiplex PCR reported by Winters and Slavik (246) identified *A. butzleri* and *C. jejuni* from dairy products and raw and ready-to-eat foods. Houf et al. (97) developed a multiplex PCR assay targeting the 16S and 23S rRNA genes for the simultaneous detection and identification of arcobacters. Recently, a species-specific one-step PCR assay has been described by Kabeya et al. (109) that is easy to perform and suitable for the identification of *Arcobacter* species *A. butzleri*, *A. cryaerophilus* 1A and 1B, and *A. skirrowii*.

Campylobacter

Difficulties in the isolation of *Campylobacter* had been a major obstacle in the initial development of *Campylobacter* research. In the early 1970s, Butzler et al. (38) applied a filtration method, using the small cellular size and the vigorous motility of *Campylobacter* cells to isolate them from stools of humans with diarrhea. A few years later, Skirrow (196) reported a selective supplement comprising a mixture of vancomycin, polymyxin B, trimethoprim, and a basal medium, which enabled routine diagnostic microbiology laboratories to isolate campylobacters. Bolton and Robertson (33) developed the Preston medium to isolate *Campylobacter* spp. from environmental samples. However, a possible failure in the isolation of certain strains of *C. coli* sensitive to polymyxine B, a component of the Preston medium, was reported (162). Also, a number of approaches to isolate *C. jejuni*, *C. coli*, and other species on selective media and by filtration methods were reported. Selective media include blood-free media such as charcoal cefoperazone deoxycholate agar (CCDA) (103), charcoal-based selective medium (CSM) (112), and blood-containing media such as Campy-CVA medium (184). Prominent among these

selective media are the modified CCDA-Preston blood-free medium (103) and Campy-Cefex (206). Stern et al. (209) found these two media provided reasonable selectivity and allowed for good quantitative recovery from poultry carcasses. Campy-Line agar (CLA), a recently developed medium, has been proposed for use in enumerating campylobacters from poultry carcass rinses (132). A filtration method has been used to isolate *Campylobacter* from fecal samples because of the susceptibility of some *Campylobacter* species to the various antibiotics present in the selective medias (79, 80). However, filtration using nonselective media is not as sensitive as culture with selective media. Thus, filtration methods should be used to complement selective media and not as a replacement (155).

Most *Campylobacter* species require a microaerobic atmosphere consisting of reduced oxygen and increased CO₂ (e.g., 5% O₂, 10% CO₂, and 85% N₂) for their recovery (149). Some species of *Campylobacter*, including *C. sputorum*, *C. concisus*, *C. mucosalis*, *C. curvus*, *C. rectus*, and *C. hyointestinalis* require hydrogen in the growth atmosphere. A gas mixture of 6% O₂, 6% CO₂, 3% H₂, and 85% N₂ is sufficient for isolating these hydrogen-requiring species, but a hydrogen-enriched atmosphere is not necessary for the isolation of *C. jejuni* and *C. coli* (151).

The campylobacters are generally negative in many conventional biochemical tests and identification is based on a limited number of morphological and biochemical features. For initial analysis, a Gram stain, saline wet mount phase-contrast examination of the colony, or an oxidase test should be performed (155). Several phenotypic tests for identifying *Campylobacter* spp. have been described. The most routinely useful tests for initial identification include catalase, hippurate hydrolysis, indoxyl acetate hydrolysis, nitrate reduction, production of H₂S, and antibiotic sensitivity by the disk method (21). Confirmation of *Campylobacter* isolation may be accomplished with immunologically based latex agglutination assays (88, 209). *Campylobacter* species are difficult to differentiate from *Arcobacter* species based on phenotypic tests. Thus, PCR-based assays are the most useful approach for accurately identifying *Arcobacter* species, and these have recently been described (22, 84). The range of genes reported for the identification of *Campylobacter* spp. by PCR includes 16S rRNA (75, 221), 23S rRNA (61), the cadF virulence gene (122), and the flagellin genes, *flaA* and *flaB* (176, 235). PCR has

also been adapted to allow the direct identification of *C. jejuni* from the sample material without prior enrichment steps (56). For applications involving large numbers of samples, multiplexing can be incorporated to reduce the total assay time and increase sample throughput (50). Several investigators have evaluated PCR as a method for identification of *C. jejuni* and *C. coli* from poultry (37, 55).

Typing methods for epidemiological studies

Subtyping bacterial species is useful in epidemiological studies for 1) tracing sources and routes of transmission of human infections, 2) identifying and monitoring, both temporally and geographically, specific strains with important phenotypic characteristics, and 3) developing strategies to control organisms within the food chain (159).

Serotyping methods

Two basic schemes have been adopted for serotyping *Campylobacter*. The Penner scheme (177) was based on soluble heat-stable (HS) antigens, while the Lior scheme (134) detected variation in heat-labile (HL) antigens. Jacob et al. (104) characterized a total of 147 *Campylobacter*-like strains isolated from drinking water treatment plants by serotyping. One hundred strains were typed as *A. butzleri*, 17 as *A. butzleri*-like and 6 as *C. jejuni/coli*. Vandamme et al. (226) typed *A. butzleri* causing abdominal cramps in an Italian school using the Lior method, and suggested that person-to-person transmission occurs. However, serotyping has several limitations including low reproducibility (175), non-specific reactions (32), and high levels of nontypeable strains (163). Ono et al. (173) reported that heat-stable serotyping was the least discriminatory method compared with random amplification of polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE).

Genotyping methods

Recently, molecular subtyping methods have been developed. The major advantage of genotyping methods is that they can be widely available compared to serotyping which requires a battery of specific antisera (234). The range of criteria to compare genotyping methods includes sensitivity, availability, reproducibility, rapidity, ease of use, cost, and discriminatory power. Some of these

techniques, such as PFGE, *fla* typing, RAPD, and amplified fragment length polymorphism (AFLP) are already in use in a number of laboratories. Each technique has different advantages and disadvantages that are discussed in more detail below.

a. Random amplification of polymorphic DNA (RAPD): Arbitrary PCR primers can be used to amplify random genomic DNA sequences under low-stringency PCR conditions (234). Since the entire genome of the target organism is used in RAPD to generate amplified fragments, the lengths of the PCR products, efficiency of annealing, and amplification efficiency vary with the sites primed (159). A slightly different approach for amplifying random genomic DNA fragments involves using primers specific for enterobacterial repetitive intergenic consensus (ERIC) sequences. ERIC analysis uses lower annealing temperatures to increase the number of bands that can be detected (234). RAPD and ERIC methods for *Arcobacter* isolates from poultry were optimized by Houf et al. (92, 94), who reported that variations in the quality and amount of DNA template had a major effect on the RAPD results obtained. Atabay et al. (12) examined genotypic diversity of 35 *Arcobacter* isolates from markets chickens (28 whole carcasses and seven wings) using RAPD and showed 11 distinct DNA profiles. In *Campylobacter*, RAPD fingerprinting has been applied to the typing of a range of human, animal, and environmental isolates of *C. jejuni* and *C. coli* (89, 140, 208). The combination of an ERIC primer and a randomly chosen primer have also been used for *Campylobacter*, but the reproducibility of this technique was low (52, 74). The advantage of RAPD is that prior knowledge of the target genomic DNA sequence is not needed. This method is also quick, inexpensive, and does not require a complex apparatus, while the major disadvantage is poor reproducibility, the difficulty of interpretation of minor bands and low sensitivity to genetic instability (159, 234).

b. *fla* Typing: The flagellin gene locus of *C. jejuni* contains two flagellin genes (*flaA* and *flaB*), which are arranged in tandem and are separated by approximately 170 nucleotides (179). These genes are highly conserved and a 92% identity between *flaA* and *flaB* genes has been reported for individual isolates (159). PCR primers can be synthesized based on conserved gene sequences, thus making the flagellin gene locus suitable for restriction fragment length polymorphism (RFLP) analysis of the amplification

product (234). Several *fla* typing procedures have been developed, and there is considerable variation in the PCR-RFLP procedures including the DNA preparation techniques (133, 154), primer design (146), annealing temperatures (28), and restriction enzymes used (4, 28, 154). The level of discrimination of *fla* typing is generally to be much greater than that of serotyping, but lower than that of pulsed-field gel electrophoresis. Unlike serotyping, it has a high typeability for human, veterinary, and environmental isolates (17, 139). *fla* typing has been successfully applied to campylobacters from broiler flocks (17, 105, 187, 210). Similarly, campylobacters from foodstuffs (65) and from animals and water (120) have been investigated. However, no reports using *fla* typing for *Arcobacter* isolates have to date been published. *fla* typing is relatively simple and quick and involves widely available reagents and equipment (159).

c. Amplified Fragment Length Polymorphism Fingerprinting (AFLP): AFLP analysis involves the digestion of whole-genomic DNA with two restriction enzymes, one with a 4-bp recognition site and the other with a 6-bp recognition site. PCR amplification of the digestion products is based on the restriction sites and is designed so that only those fragments flanked by both restriction sites are amplified. This method can be adapted to any bacterial species. However, the restriction enzymes and adjacent specific nucleotides used must be optimized for each species (159). On et al. (167, 171) suggested AFLP profiling was effective for the characterization of *Arcobacter* spp. and for the subsequent resolution of various taxonomic, population genetic and epidemiological problems. Recently, AFLP analysis has been also used to subtype *C. jejuni* (49, 191). The major advantage of this technique is that a random portion of the whole genome is sampled. The disadvantage is that this method is complex and requires an automated DNA sequencer and appropriate software (234). Standardized procedures also must be established in order to exchange AFLP data between laboratories (159).

d. Pulsed-Field Gel Electrophoresis (PFGE): For PFGE, bacterial cells are embedded in chromosomal grade agarose and washed to remove contaminating chemicals (212). Thin slices of the DNA-containing blocks are cut and digested with rare cutting enzymes to yield a moderate number of DNA fragments (212). To prevent chromosomal DNA shearing, the bacteria are immobilized by mixing the bacterial suspension with melted agarose before the cells are lysed (137). The DNA fragments are

then separated by a special electrophoretic method and visualized by staining in ethidium bromide (159). Variation in the presence of relevant restriction sites result in genotypic profiles (also called macrorestriction profiles) (204). PFGE used with various restriction enzymes has been regarded as the best standard typing method because it examines polymorphisms throughout the genome (137) and has a high discriminatory power (65).

Few surveys of the genetic diversity among *Arcobacter* species using PFGE have been reported. Hume et al. (102) and Rivas et al. (186) characterized *Arcobacter* isolates from a swine facility and from retail stores by PFGE using *SacII*, *EagI*, or *SmaI*. They found high levels of diversity among the *Arcobacter* isolates tested. PFGE has also been successfully used to group *C. jejuni* isolates from poultry (91, 156, 161), animals (cattle, sheep, and turkey) in farms (31, 34, 66), human outbreaks (36, 47, 111), as well as *C. coli* isolates from sows and piglet (101). Ono et al. (173) compared RAPD and PFGE for epidemiological typing of *C. jejuni* and *C. coli*. They described that the PFGE types were consistent with their RAPD types, but RAPD-DNA patterns of strains were changed depending on the concentration of template DNA. The discriminatory potential of PFGE is high, but the restriction enzymes, including *SmaI*, *Sall*, *KpnI*, *ApaI*, and *BssHII*, used to digest the chromosomal DNA vary between studies (234). Using more than one enzyme significantly increases the discriminatory power of the PFGE (73, 169). Differences in electrophoretic conditions can also lead to apparent differences in the profiles obtained for the same DNA preparation (234). Tenover et al. (218) described a set of guidelines for interpreting DNA restriction patterns generated by PFGE. They categorized indistinguishable, closely related, possibly related, and different depending on number of fragment differences. However, criteria proposed are only reliable if PFGE resolves at least 10 distinct fragments.

Until recently, one of disadvantages of PFGE method was that it is time consuming and labor-intensive, typically requiring three to four days for *Campylobacter* (65). The recent development of a rapid PFGE protocol that takes 24 to 30 h for *Campylobacter* reported by Ribot et al. (185) makes PFGE more practical compared to other genotyping methods. The PulseNet program (<http://www.cdc.gov/pulsenet>), established by Centers for Disease Control and Prevention (CDC) in

collaboration with state health departments and the U. S. Food and Drug Administration (FDA), is incorporating *Campylobacter* into their system, analyzing PFGE data by a computer network, and comparing PFGE pattern from different sources.

Antimicrobial susceptibility of *Arcobacter* and *Campylobacter*

An antimicrobial is a compound that kills or inhibits the growth of microbes, such as bacteria and fungi, but does not harm the human or animal under treatment (230). Antimicrobials in food animal production are used for three different purposes. First, antimicrobials are used to treat sick animals. Second, antimicrobials are used in the absence of disease to prevent diseases during times when animals may be susceptible to infections. This use affects a larger number of animals because it usually involves treating a whole herd or flock, which increases the likelihood of selecting for organisms that are resistant to the antimicrobials. Third, antimicrobials are commonly given in the feed at subtherapeutic doses for relatively long periods to promote the growth and feed efficiency of cattle, poultry, and swine (193).

Although antimicrobials have provided many beneficial effects in agriculture, their use has generated the problem of antimicrobials resistance. Antimicrobial resistance is the ability of bacteria or other microbes to resist the effects of an antimicrobial through various mechanisms. Antimicrobial resistance has become a food safety problem for several reasons. First, some antimicrobials resistance is increasing including fluoroquinolones and third-generation cephalosporins. These antimicrobials are commonly used to treat serious infections in humans caused by bacterial pathogens frequently found in food, such as *Salmonella* and *Campylobacter*. However, some *Salmonella* and *Campylobacter* strains have become resistant to these drugs, and therefore people may become ill from drug-resistant organisms (7). Further, healthy persons who consume a small number of *Campylobacter* may carry them for a few weeks without having any symptoms, because the growth of those few *Campylobacter* are held in check by the normal bacteria in their intestines. However, a few antimicrobial resistant *Campylobacter* ingested in food can cause illness if the person who consumed the contaminated food then takes an antimicrobial for another reason. The antimicrobial can kill normal bacteria in the gut, allowing the few *Campylobacter*

that ordinarily would be unlikely to cause illness to take over and cause illness. Third, the food supply may be a source of antimicrobial resistant genes. Harmless bacteria present in food-producing animals can become resistant, and humans could acquire these bacteria when they eat food products from these animals. Once ingested, resistant genes from these bacteria could also be transferred to pathogenic bacteria (230).

Resistant bacteria can be transferred to humans through the food supply or by direct contact with animals. For example, *Campylobacter* is a commensal in the intestines of chickens. Humans often become infected with *Campylobacter* after eating undercooked chicken. In 1989, none of the *Campylobacter* strains from ill persons that the CDC tested were resistant to fluoroquinolone antimicrobials. In 1995, the FDA approved the use of fluoroquinolones in poultry production. Soon afterwards, *Campylobacter* strains isolated from ill persons were found that were resistant to fluoroquinolones. When an ill person is treated with an antimicrobial to which the bacteria is resistant, the antimicrobial will not help and may even make the illness worse (195). The World Health Organization has recommended discontinuing use of antimicrobial growth promoters that belong to an antimicrobial class used in human medicine (7). Increasing antimicrobial resistance in the bacteria harbored by animals makes it more likely for humans who do get infected to have a resistant strain. The illness may last longer, be more serious, or more expensive to treat.

Antimicrobial resistant bacteria, including animal pathogens, human pathogens that have animal reservoirs, and commensal bacteria may be transferred to humans either through the food supply or by contact with animals (174, 247). The transfer of resistant bacteria from food-producing animals to humans is most evident in human bacterial pathogens that have food animal sources, such as *Campylobacter*, which has reservoirs in chickens and turkeys (5). It is therefore important to know whether antimicrobial resistant *Campylobacter* and *Arcobacter* can be isolated from animals. When evaluating the public health consequences of antimicrobial use in animals, it is valuable to consider each pathogen-antimicrobial situation individually. To monitor antimicrobial resistance in food-borne enteric

pathogens, the National Antimicrobial Resistance Monitoring System (NARMS) for Enteric Bacteria in the U.S. was established in 1996 (http://www.fda.gov/cvm/narms_pg.html).

History of AM susceptibility test

The study of antimicrobials began with the early pioneers of microbiology, Pasteur, Koch, and Ehrlich who made many references to antibiosis (126). In 1924, Fleming introduced the use of the ditch plate technique for evaluating antimicrobial qualities of antiseptic solutions (67), and developed a broth dilution technique using turbidity as an end-point determination (68). This has been described as a forerunner of contemporary minimum inhibitory concentration (MIC) methodology (182). In the 1940s, disc diffusion methods were developed which were widely adopted (121, 148, 229). Also, the development of methods incorporating antimicrobial agents into agar, which subsequently became known as the agar dilution technique, became available. It was recognized, however, that performing agar dilution MIC estimations for routine bacterial isolates was too time consuming and cumbersome. Another problem of diffusion or dilution techniques was that there were many variables affecting antimicrobial susceptibility testing (AST). The need for the standardization of testing, a critical issue, was recognized by several organizations and investigators. In 1966, a significant report on standardization of the disc method which became the basis of the National Committee for Clinical Laboratory Standards (NCCLS) in 1975 was published (23). The labor required in these methods as discussed earlier has led to automated AST methods. One of the automated AST methods, the Autobac disc elution system, was introduced and marketed by Pfizer Diagnostics in 1974 (144). In the same year, the McDonnell Douglas Corporation introduced the AMS System (3), which is known today as the Vitek system. In 1977, standardized microtitre trays containing antimicrobial agents were introduced. All the susceptibility techniques described above rely on phenotypic testing of the isolated bacteria. The phenotypic approach to susceptibility testing has shortcomings, such as the tests being highly dependent on experimental conditions and more than one method needed to obtain an accurate susceptibility profile (24). Furthermore, there is no international agreement on breakpoints for interpretation of antimicrobial susceptibility tests. Recently, numerous DNA-based assays have been developed for detection of

bacterial resistance genes (43, 216). The limitation of these methods is that the presence of a resistance gene may not always be indicative of resistant bacteria (24) since the expression of the gene is required for resistance. Also, if a gene coding for resistance to an antibiotic is not detected, it may not necessarily mean that the bacteria are susceptible to that particular agent particularly if the MIC is close to the breakpoint (24). If the limitations of these methods are solved and the procedures standardized, the results produced will offer a reliable guide to susceptibility.

Antimicrobial susceptibility testing and reporting are very important to facilitate and ensure appropriate treatment of foodborne disease. Interpretation of antimicrobial susceptibility results in *Arcobacter* and *Campylobacter* are difficult due to the lack of standards for testing methods and accepted breakpoints for determining resistance.

Instrumentation in AM susceptibility testing

In the past, instrumentation had little impact on disc diffusion tests, but the potential of automated zone readers has recently attracted attention due to increased interest in using routinely derived susceptibility data. The need to save labor, increased surveillance, and use of large laboratories to analyze samples has led to the use of automated systems. The manual determination of full-range MICs by agar or microbroth dilution methods is not used often in most clinical laboratories.

In order to standardize disc diffusion susceptibility testing, variables such as disc content, inoculum concentration, setting of interpretative criteria (breakpoint MICs and inhibition zone diameters), and establishment of quality control parameters must be determined (59). Among these, measurement of zone sizes is a significant variable. Automated zone readers can help reduce operator variability in reading plates and error in transcription of results, and provide automatic interpretation of zone diameters (59). The device for agar dilution methods described in 1959 (205) was mechanical, but still there were no automated analysis systems that yielded usable data. Recently, the NCCLS Sub-Committee on Veterinary Antimicrobial Susceptibility Testing approved an agar dilution protocol as a valid method for susceptibility testing of *Campylobacter* spp. (147).

In a broth microdilution system, 96-well plates contain dried antimicrobials which, when rehydrated with inoculated growth medium, provide dilution series for full MIC determination or susceptibility based on breakpoints. The broth microdilution method is preferred for testing larger number of isolates, especially when susceptibility to nalidixic acid and trimethoprim-sulfamethoxazole are being considered (136). There are two different types of reading devices currently available (59): 1) manual reading instruments for recording visually determined growth endpoint, such as the Microscan Touch SCAN-SR (Dade Behring Inc., West Sacramento, CA, USA), the Sceptor (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA), and the Sensititre Sensitouch (Trek Diagnostic Systems, East Grinstead, UK) and 2) automated reading devices, such as the Microscan autoSCAN-4 (Dade Behring Inc.), the AutoSceptor (Becon Dickinson Diagnostic Instrument Systems), and the Sensititre AutoReader (Trek Diagnostic Systems).

Antimicrobial susceptibility testing by automated systems has the advantages of labor saving and improved reproducibility. However, the instruments are expensive and the cost of consumable items is also a factor. Automated systems currently cannot be applied to all clinically important bacteria and less frequently tested agents (59).

AM resistance testing of *Arcobacter*

Severe symptoms of arcobacteriosis may require antibacterial therapy, but diarrhea caused *Arcobacter* species is usually presumed to be self-limiting. When an antibiotic is recommended for treatment, the most commonly prescribed drugs are erythromycin or a fluroquinolone such as ciprofloxacin (136). Tetracycline, doxycycline, and gentamicin are sometimes listed as alternative drugs for treatment.

Few reports on antimicrobial susceptibility testing of *Arcobacter* species have been published. Fera et al. (60) investigated AM susceptibility to 26 antimicrobial agents for *A. butzleri* and *A. cryaerophilus* by microdilution techniques. Kabeya et al. (110) found that *Arcobacter* strains isolated from beef, pork and chicken, and from retail shops showed the highest resistance to vancomycin (100%) and methicillin (97.5%) among 11 antimicrobials using a disk diffusion method. In their study, 63.5%

and 30.2% of *A. butzleri* strains were resistant to nalidixic acid and chloramphenicol, respectively. However, Atabay et al. (10) reported that all the strains of *A. butzleri* examined were susceptible to nalidixic acid and chloramphenicol, but resistant to ampicillin, amoxicillin, and amoxycillin in resistance to 23 antimicrobial testing using the disc diffusion method. *Arcobacter* species have shown susceptibility to aminoglycosides, including kanamycin and streptomycin (110). In addition, *Arcobacter* isolates have been reported to be susceptible to tetracycline (10). *A. skirrowii* is the most antimicrobial susceptible species (96), which is a possible explanation for low recoveries reported to date for this species. Currently, there is no standardized data for the interpretation of antimicrobial susceptibility testing of *Arcobacter* species.

AM resistance testing of *Campylobacter*

For the treatment of severe campylobacteriosis, the most commonly recommended drugs are erythromycin or ciprofloxacin (198). Tetracycline, doxycycline, and chloramphenicol are occasionally used as alternative drugs for treatment (72). However, resistance to several of these drugs in *Campylobacter* species has been increasing. It has been shown that *Campylobacter* in poultry can rapidly become resistance to antimicrobial agents, including fluoroquinolones (142). Resistance in *Campylobacter* to fluoroquinolones increased significantly during the 1990s (54). Resistance was reported to develop among patients after treatment with fluoroquinolones (18), and was found to coincide with the introduction of these agents in veterinary medicine (1). In the Netherlands, a report also showed a link between fluoroquinolone use in veterinary medicine and increasing fluoroquinolone resistance in human isolates of *Campylobacter* (53). This is recognized as an emerging public health problem in many European countries (54). Quinolone-resistant *Campylobacter* infection has also been associated with foreign travel or military deployment to many countries (183, 199).

Tetracycline has been used often both therapeutically and subtherapeutically as feed additives for livestock and poultry (40, 219), and resistance to this drug is also a concern with *Campylobacter* (71, 128, 130). Ge et al. (72) reported that resistance to tetracycline was very common in *Campylobacter* isolates from retail meat samples using the agar dilution method. In *C. coli* isolated from French broilers,

resistance to ampicillin, nalidixic acid, enrofloxacin, tetracycline and erythromycin was found to be significant. In an Irish poultry processing plant, *C. coli* and *C. jejuni* had high resistance to ampicillin and tetracycline (58) as determined using the disc diffusion assay.

The agar dilution method was the first method to have been standardized for antimicrobial testing of *Campylobacter* isolates, and today it is the method recommended most often for *Campylobacter* sensitivity testing (1, 155). However, as previously discussed, this method is difficult to use routinely due to the labor involved (136). The Epsilon-meter-test (E test) provides faster performance than the agar dilution method, but the E test is limited with regard to the antimicrobials available for testing (20, 99). The broth microdilution method is easy to handle, simple, can be set up more rapidly, and can be automated (136). Reports related to the broth microdilution method for antibiotic susceptibility testing of *Campylobacter* have been published (53, 128). However, there has been a lack of standardization of this method because of different broth and test parameters (136). Luber et al. (136) compared the broth microdilution method with the E test and an agar dilution test for antimicrobial susceptibility of *C. coli* and *C. jejuni* isolates. The broth microdilution method showed a more stable performance compared to the other two methods. King (118) described disc diffusion methods suitable for detecting resistance to commonly used antimicrobials. At present, a fully standardized method for testing *Campylobacter* antibiotic sensitivity testing has not been uniformly adopted. The sensitivity and specificity of new test methods assessed by comparison of performance with other methods have yet to be fully determined (136).

The functions and resistance mechanisms of antimicrobials

To monitor antimicrobial resistance, the USDA, CDC, and FDA, in partnership with state and local health departments, established the National Antimicrobial Resistance Monitoring System of Enteric Bacteria (NARMS) in 1996. For *Campylobacter*, susceptibility testing to the 8 antimicrobials using the E test method was begun in 1997. These included: ciprofloxacin (CI), clindamycin (CM), erythromycin (EM), nalidixic acid (NA), tetracycline (TC), gentamicin (GM), azithromycin (AZ), and chloramphenicol (CL). In 2005, an automated microbroth dilution assay (TREK Diagnostic System) was introduced for

Campylobacter. Chloramphenicol was eliminated from testing and florfenicol and telithromycin were added.

Ciprofloxacin is a fluoroquinolone, a subgroup of quinolones derived from nalidixic acid. The fluoroquinolones have become widely used to treat human infections with ciprofloxacin being used extensively as prophylaxis for travelers because of their activity against both gram-negative and gram-positive bacteria in urinary tract infections, osteomyelitis, and community-acquired pneumonia (82, 190). Fluoroquinolones (e.g., ciprofloxacin) are a main drug of choice for treating gastroenteritis caused by *Campylobacter* species (2). These agents block DNA replication and repair by targeting the type II topoisomerases DNA gyrase, and the type IV topoisomerases in bacterial cells (231). Fluoroquinolone resistance in *C. jejuni* appears to be due primarily to mutations in the genes encoding subunits of DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) (219). Cloning and sequencing of the *C. jejuni gyrA* gene demonstrated that mutations in *gyrA* at Thr-86, Asp-90, and Ala-70 are responsible for resistance (232).

Clindamycin is recommended as an alternative treatment for *Campylobacter* gastroenteritis (200). In Taiwan, 10% of *C. jejuni* and 50% of *C. coli* isolates from humans were resistance to clindamycin, as were 8% of *C. jejuni* and 83% of *C. coli* isolates from chicken products (130).

Erythromycin and azithromycin belong to the macrolide class of antibiotics and have similar chemical structures (231). Telithromycin, a broad-spectrum macrolide, has recently been approved for use in the United States (231). In all European Union countries the use of macrolides for growth promotion has been banned since July 1999 (54). Macrolides are bacterial protein synthesis inhibitors that binds reversibly to a site on the ribosome, causing dissociation of the peptidyl-tRNA rather than blocking peptidyltransferase activity (203, 231). Erythromycin resistance has been shown to be more frequent in *C. coli* than *C. jejuni* (198). The mechanism of resistance to erythromycin is generally through modification of the target site, the ribosome or through alteration of the antibiotic, e.g., esterification of erythromycin (9). In *C. jejuni* and *C. coli*, the resistance mechanism is not consistent with the presence of an rRNA methylase, with modification of the antibiotic, or with efflux.

Gentamicin is an aminoglycoside and is bactericidal to the cell (231). It penetrates the cell wall and binds irreversibly to the ribosome, blocking bacterial protein synthesis (219). Aminoglycoside resistance has been reported in *C. jejuni* and *C. coli*, but is less common in *C. jejuni* than in *C. coli* (219). The majority of resistance determinants are plasmid borne, but a few have been reported which seem to be chromosomally encoded (214, 217). Aminoglycoside resistance is based on modification of the antibiotic, which is then unable to interact with the ribosome. Three families of enzymes are responsible for aminoglycoside resistance in bacteria: the aminoglycoside phosphotransferases (APH), the aminoglycoside adenylyltransferases (AAD), and the acetyltransferases (219). Chromosomal mutations of ribosomal proteins and rRNA may provide aminoglycoside resistance. These antibiotics all bind to specific sites on the ribosome and modification of the binding site could greatly reduce the affinity of these compounds for the ribosome (219).

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CHAPTER 3
PREVALENCE OF ARCOBACTER AND CAMPYLOBACTER ON BROILER
CARCASSES DURING PROCESSING¹

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Abstract

Broiler carcasses (n=325) were sampled in a U.S. commercial poultry processing plant for the prevalence of *Arcobacter* and *Campylobacter* at three sites along the processing line: 1) pre-scalding, 2) pre-chilling, and 3) post-chilling. Samples (75-125 broilers per site) were collected during five plant visits from August to October of 2004. *Arcobacter* species were recovered from pre-scalded carcasses more frequently (96.8%) than from pre-chilled (61.3%) and post-chilled carcasses (9.6%). For *Arcobacter* speciation, a species-specific multiplex polymerase chain reaction assay showed that *A. butzleri* was the most prevalent species (79.1%) followed by *A. cryaerophilus* 1B (18.6%). *A. cryaerophilus* 1A was found at low levels (2.3%) and *A. skirrowii* was not isolated at all. *Campylobacter* was isolated from 92% of pre-scalded carcasses, 100% of pre-chilled carcasses, and 52% of post-chilled carcasses. For *Campylobacter* speciation, the BAX[®] PCR identified the most common species as *C. jejuni* (87.6%) followed by *C. coli* (12.4%). Overall, *Arcobacter* was isolated from 55.1% (179 of 325), while *Campylobacter* was isolated from 78.5% (255 of 325) of the carcasses from the three collection sites. Our results demonstrate significant contamination of broiler carcasses by *Arcobacter* although less than that found for *Campylobacter*.

Key words: *Arcobacter*, Broiler chickens, *Campylobacter*, Poultry processing

Introduction

Campylobacter is the most common cause of acute bacterial gastroenteritis in humans (33). The most important *Campylobacter* species related to human illness are *C. jejuni* and *C. coli* (46). *Arcobacter* species are gram-negative, aerotolerant, vibrio-like bacteria that are closely related to *Campylobacter* which can induce illness similar to *Campylobacter* and other foodborne pathogens (43). A taxonomic study by DNA/rDNA and DNA/DNA hybridization, and electrophoretic protein profiles revealed that the genus *Arcobacter* includes four species (42). *Arcobacter butzleri* and *A. cryaerophilus* (subgroups 1A and 1B), respectively, have been recognized as potential human pathogens with symptoms such as abdominal pain, nausea, vomiting or fever and can cause abortion in farm animals (21, 28, 40, 41, 43). *Arcobacter skirrowii* has also been associated with farm animals and with human illness with chronic diarrhea (25, 31, 48). *Arcobacter nitrofigilis* has rarely been reported to cause infection in animals or humans (32). While *Arcobacter* species are phenotypically similar to *Campylobacter* species, two notable features, namely marked aerotolerance and the ability to grow at cooler (15-25°C) temperatures, distinguish *Arcobacter* from *Campylobacter* (1). A recent report by Vandenberg et al. (44) suggested that *A. butzleri* was more often associated with persistent and watery diarrhea compared with *C. jejuni* during an 8-year study period.

Arcobacter and *Campylobacter* are common contaminants of broiler carcasses in poultry processing plants (2, 4, 13, 18). There have been several reports on the high levels of *Campylobacter* on broiler chickens from the farm (39) and retail chicken (50). Consequently, under-cooked and raw poultry meats are common vehicles for the transmission of human campylobacteriosis. Houf *et al.* (19) found *Arcobacter* more frequently than *Campylobacter* on neck skins of broilers before and after chilling in Belgian poultry plants. Considerable efforts have been made to reduce levels of contamination of *Arcobacter* and *Campylobacter* on processed poultry carcasses. However, few reports have been published on the prevalence of *Arcobacter* in U.S. poultry plants. Furthermore, little is known about how the processing procedures in the U.S. may affect the prevalence of *Arcobacter* and *Campylobacter*. At present, the origin of *Arcobacter* contamination and the nature of its pathogenesis are still unknown.

This is due in part to the lack of standardized isolation methods for *Arcobacter*. However, a number of studies on the development and comparison of media and enrichment broths for the recovery of *Arcobacter* from foods of animal origin such as chicken (14, 20, 22, 37), pork (8, 34), and beef (11, 17) have been reported. Similarly, no single method for successfully isolating *Campylobacter* from all sample types has yet been developed, although the method of Stern et al. (38) has been used successfully for detecting *C. jejuni* and *C. coli* in poultry. The choice of method depends on the expected level of *Campylobacter* in the sample examined and any competitive bacterial flora that may be present.

The present study was designed to compare the prevalence of *Arcobacter* and *Campylobacter* on broiler carcasses from a commercial poultry processing plant.

Materials and Methods

Sample collection

Broiler carcasses were collected during five plant visits from August to October of 2004 at a commercial poultry processing plant. Carcasses were randomly chosen by hand using new latex gloves for each carcass from three sites along the processing line: pre-scalding (n=125), pre-chilling (n=75), and post-chilling (n=125). Samples were placed into sterile plastic bags that were sealed and covered with ice in coolers for transport to the laboratory. All carcasses were subjected to a whole carcass rinse. Briefly, feathered carcasses collected at the pre-scalding site were shaken with 500 ml of sterile distilled water for 60 s. Carcasses collected at pre-chilling and post-chilling were also shaken with 100 ml of sterile distilled water for 60 s. Carcasses were then discarded. The rinses were poured into 50 ml sterile specimen cups and refrigerated at 4°C. Samples were placed in culture media within 1 h post-collection.

Isolation of *Arcobacter*

Both direct plating and enrichment methods were used for *Arcobacter* isolation. For direct plating, 100 µl aliquots of carcass rinses were spread-plated on CVA (cefoperazone, vancomycin, and amphotericin B) agar (8) containing 43 g/liter Brucella agar (Hardy Diagnostics, Santa Maria, CA), 0.5 g/liter ferrous sulfate (Sigma, St. Louis, MO), 0.2 g/liter sodium bisulfate (Sigma), 0.5 g/liter pyruvic acid (Sigma), 950 ml distilled water, 10 mg/liter vancomycin (Sigma), 10 mg/liter amphotericin B (Sigma), 33

mg/liter cefoperazone (Sigma) and 50 ml lysed horse blood (Lampire Biological Laboratories, Pipersville, PA). Serial 1:10 dilutions in PBS for pre-scald samples were also prepared and one hundred microliter aliquots of each dilution were spread-plated on CVA agar plates. All plates were incubated for 48 h at 25°C aerobically.

For enrichment, 1 ml of rinse was inoculated into 5 ml Houf broth (20) which contains 24 g/liter *Arcobacter* broth (Oxoid Ltd, Hampshire, England), 0.5 g/liter thioglycollic acid (Arcos Organics, Geel, Belgium), 0.5 g/liter pyruvic acid (Sigma), 16 mg/liter cefoperazone (Sigma), 100 mg/liter 5-fluorouracil (ICN Biomedicals, Inc., Aurora, OH), 10 mg/liter amphotericin B (Sigma), 32 mg/liter novobiocin (Sigma), 64 mg/liter trimethoprim (Sigma), and 50 ml/liter lysed horse blood (Lampire Biological Laboratories). Following aerobic incubation at 25°C for 48 h, a sterile swab was used to streak a portion of the broth onto CVA agar; plates were incubated aerobically at 25°C for 48 h. Isolates were restreaked twice on Brucella agar (BBA, Hardy Diagnostics) supplemented with 5% (vol/vol) lysed horse blood (Lampire Biological Laboratories) to ensure clonality. Presumptive identification of *Arcobacter* was performed by microscopic examination of wet mounts of colonies using phase contrast optics. Isolates were stored at -70°C in Wang's freezing medium (45) with 15% (vol/vol) glycerol and Brucella broth (Sigma).

Isolation of *Campylobacter*

Pre-scald samples were isolated by direct plating using the same procedure and medium as described for *Arcobacter*. For enrichment, 1 ml of rinse was placed in 5 ml Bolton's broth (7) which contained 27.6 g/liter *Campylobacter* enrichment broth (Acumedia Manufactures, Inc., Baltimore, MI), 5% (vol/vol) lysed horse blood (Lampire Biological Laboratories) and *Campylobacter* selective supplement (Oxoid Ltd). Enrichment cultures were incubated for 24 h at 42°C in a microaerobic atmosphere consisting of 5% O₂, 10% CO₂, and 85% N₂. Following incubation, 0.1 ml of Bolton broth was spread onto CVA agar and these plates were incubated microaerobically for 48 h at 42°C. From each positive plate, one typical *Campylobacter* colony was subcultured twice on BBA. For presumptive identification, wet mounts of suspected *Campylobacter* colonies were examined using phase contrast

microscopy. *Campylobacter* isolates were stored at -70°C in Wang's freezing medium with 15% (vol/vol) glycerol and Brucella broth (Sigma).

***Arcobacter* multiplex PCR**

Reference strains of *Arcobacter*, including *A. buzleri* (ATCC 49616), *A. cryaerophilus* 1A (ATCC 43158), *A. cryaerophilus* 1B (ATCC 49615), and *A. skirrowii* (ATCC 51132) were used as controls. Reference strains and all presumptive *Arcobacter* isolates were cultured on BBA at 25°C for 48 h under ambient atmosphere. A modified multiplex-PCR for *Arcobacter* of Kabeya et al. (24) was used for species identification. Template DNA was prepared using a commercial DNA extraction kit (Gentra Systems, Inc., Minneapolis, MN). The DNA concentration was adjusted to 25 ng/μl using $\lambda_{260\text{nm}}$ measured in a Beckman DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA). The 50 μl PCR reaction mixture contained 25 ng of DNA template, 50 pmol each N.c. 1A and ARCO-U, 10 pmol each N.c. 1B, N.butz, and N.skir, 0.5U of Jump Start™ *Taq* DNA polymerase (Sigma), 0.8 mM of dNTP's (Applied Biosystems, Warrington, UK), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM MgCl₂. Nuclease-free water was added to adjust the final reaction volume to 50 μl. Amplification was performed in a thermal cycler (PTC-200, MJ Research, Inc., Watertown, MA) using the following program: initial denaturation at 94°C for 10 min, followed by 30 amplification cycles consisting of denaturation for 30 s at 94°C, annealing for 1 min at 64°C and elongation for 1 min at 72°C. The final elongation was performed at 72°C for 7 min. The samples were held at 4°C until the PCR products were analyzed. The amplified DNA products were electrophoresed on 2% agarose gels (Seakem LE Agarose, Cambrex Bio Science Rockland, Inc., Rockland, ME) at 90V for 6 h using 1X TBE (0.89 M Tris borate, 0.02 M EDTA, pH 8.3) as the running buffer, then stained with 10 mg/ml ethidium bromide solution (Sigma). Gels were visualized using a UV gel documentation system (Fluorochem™ 8900, Alpha Innotech Corp., San Leandro, CA). A molecular weight standard (Roch, Mannheim, Germany) ranging from 100 bp to 1500 bp was included on each gel.

***Campylobacter* BAX[®] PCR**

Reference strains for *Campylobacter* included *C. coli* (ATCC 33559) and *C. jejuni* (ATCC 33560). Identification of *C. coli* and *C. jejuni* was determined using the BAX[®] PCR assay (Dupont Qualicon, Wilmington, DE) as previously described (15). Amplification products were analyzed by electrophoresis at 130V for 70 min using 1X TBE (0.89 M Tris borate, 0.02 M EDTA, pH 8.3) running buffer on 2% agarose gels (Seakem LE Agarose, Cambrex Bio Science Rockland, Inc., Rockland, ME). Gels were stained with 10 mg/ml of an ethidium bromide solution (Sigma) and visualized using a UV gel documentation system (Fluorochem[™] 8900, Alpha Innotech Corp.).

Statistical analysis

Differences in the prevalence of *Arcobacter* and *Campylobacter* between plant visits and sampling sites were determined using the chi-square test for independence. A P value of ≤ 0.05 was considered statistically significant.

Results

Arcobacter was isolated from 55.1% (179 of 325) of carcasses from three collection sites in a commercial poultry processing plant (Table 3.1). More pre-scalding samples were contaminated with *Arcobacter* (96.8%) than the other two sites (pre-chilling and post-chilling). As chickens were processed, *Arcobacter* contamination was decreased. The positive rate of *Arcobacter* from pre-chilling samples from sampling day “D” was much less than those in other sampling days, but the overall contamination rates observed for five sampling days were not significantly different ($p \leq 0.05$). The results of species-specific multiplex PCR of *Arcobacter* species were shown in Figure 3.1. The position in the gel of *Arcobacter* species was 692 b.p. for *A. butzleri*, 728 b.p. for *A. cryaerophilus* 1A, 152 b.p. for *A. cryaerophilus* 1B, and 448 b.p. for *A. skirrowii*. The speciation of *Arcobacter* positive samples is shown in Table 3.2. *Arcobacter butzleri* was found most commonly (79.1%), followed by *A. cryaerophilus* 1B (18.6%); no *A. cryaerophilus* 1A was isolated from pre-chilled and post-chilled carcasses. *Arcobacter skirrowii* was not identified in this study.

Pre-scalding and pre-chilling carcasses had the highest contamination rate of *Campylobacter*, 92% to 100% positive, respectively (Table 3.3). Compared to *Arcobacter*, *Campylobacter* was recovered 4.8% less from pre-scalding carcasses but 38.7% more from pre-chilling carcasses. Sampling day and site had a significant effect on the number of samples positive for *Campylobacter*. While all post-chilling samples were positive for *Campylobacter* on “E” sampling day, only one sample on “A” and none on “B” sampling day was *Campylobacter* positive. Processing may have less effect on the presence of *Campylobacter* compared to *Arcobacter*. The gel of *Campylobacter* speciation by BAX[®] PCR was seen in Figure 3.2. Between *Campylobacter* species, *C. jejuni* (87.6%) was far more common than *C. coli* (12.4%; Table 3.4). However, on sampling day “A”, all positive samples were identified as *C. coli* (data not shown), while for all other sampling days, *C. jejuni* was detected most commonly. Most *C. coli* were recovered from pre-scalding samples, while *C. jejuni* was distributed commonly among the three collection sites.

Overall, *Campylobacter* was isolated from 78.5% (255 of 325) of the carcasses from the three collection sites, while *Arcobacter* was isolated from 55.1% (179 of 325).

Discussion

The overall prevalence of *Arcobacter* and *Campylobacter* species on broiler carcasses at the commercial poultry plant surveyed was 55.1% (179 of 325) and 78.5% (255 of 325), respectively. The data from Belgian poultry plants surveyed by Houf et al. (19) cannot be directly compared with our data due to the different isolation method, sample type and technique. They found 96.2% and 95% (n=480) of the samples tested positive for the presence of arcobacters on broiler neck skin samples collected before and after chilling, respectively. In other studies, *A. butzleri* was recovered from 81% from poultry carcasses examined (n=201) in France (29) and 97% (n=125) of the poultry carcasses from poultry plants in Canada (27).

For *Arcobacter* identification and speciation, the concentration of PCR primers and the PCR cycling program of the multiplex PCR of Kabeya et al. (24) was changed based on preliminary experiments. This modified multiplex PCR identified *A. butzleri* as the predominant species (79.1%).

Arcobacter cryaerophilus 1B was also detected at 18.6% on total carcasses examined (n=325) compared to 8.1% from fecal samples (25). Human illness caused by *A. cryaerophilus* 1B was firstly reported in a 35-year-old man with diarrhea persisting for 4 to 6 months (40). In a previous study, 60% of *A. cryaerophilus* and 13.5% of *A. skirrowii* were isolated from chicken carcasses (n=15) from a poultry plant in the United Kingdom (1), and high levels of *A. cryaerophilus* in aborted porcine fetuses have been reported (12, 36). These authors also noted that *A. skirrowii* strains were sensitive to the deoxycholate contained in the agar plates used (1). The comparison between our isolation method and their method is not possible due in part to different sampling numbers. *Arcobacter skirrowii* was not isolated from any samples in the present study. This may be due to growth inhibition under aerobic conditions (25) or susceptibility to the antibiotics used to make the enrichment broth and selective agar. The growth of *A. butzleri* on CVA agar plates under aerobic atmosphere was faster than those of the other three species. The greater rate at which *A. butzleri* grows compared to the other *Arcobacter* spp. may account in part as to why it is encountered more frequently.

A review by Corry and Atabay (9) summarized that *C. coli* was found on 6% to 50% of broiler carcasses. Our study showed an overall rate of 12.4% for *C. coli*. *Campylobacter jejuni* has been reported to be the most frequent species recovered poultry (23, 26) and foods of animal origin (49) which is similar to our result (88%).

In the present study, as broiler carcasses were processed from pre-scalding to post-chilling, the positive percentage of *Arcobacter* was decreased sharply. Other reports (2, 9, 19) support the idea that mechanical processing decreases the presence of *Arcobacter*. Poultry may not be a natural source and *Arcobacter* (2), and contamination on the surface of broiler carcasses may occur in poultry processing plants. To our knowledge, no information has been reported on the prevalence of *Arcobacter* from carcasses from different processing sites in U.S. poultry plants.

Compared to *Arcobacter*, the contamination level of *Campylobacter* between the pre-chilled (100%) and pre-scalded carcasses (92%) was not considerably different. The reason could be the rupture of internal organs such as the ceca and colon resulting in high numbers of *Campylobacter* being leaked

onto the carcasses (3). It should be noted that the skin of poultry meat is usually not removed and high levels of *Campylobacter* may be present on and in the skin. Scalding at 50-53°C generally can reduce the numbers of *Campylobacter* significantly, but defeathering may cause recontamination with *Campylobacter* and cross-contamination between carcasses. Logue et al. (30) noted positive *Campylobacter* species (*C. jejuni* and *C. coli*) from surface swabbing of carcasses sampled at two plants (Plant A: 40.8% pre-chill vs. 37.6% post-chill; Plant B: 41.8% pre-chill vs. 19.8% post-chill). Our study found *Campylobacter* from 100% of pre-chilled carcasses and 52% in post-chilled carcasses. The different contamination rates may be due to the plants sampled, different conditions of the plant, processing procedure, and differences in the analytical methods used to process the samples.

According to decimal reduction value (*D*-values) of *Arcobacter* (10) and *Campylobacter* reported by other researchers (5), *Campylobacter* and *Arcobacter* are usually more heat sensitive than other vegetative bacteria and are inactivated easily by cooking. Human infection typically results from eating of undercooked poultry or via cross-contamination from inadequate handling of poultry products. Factors that affect the incidence of *Arcobacter* and *Campylobacter* on poultry from processing plants are dependent on the season, poultry plant examined, geographical location, and type of production (30). Seasonal variations in occurrences of *Campylobacter* in poultry products have been described in several reports (6, 16, 35, 47). No studies concerning the seasonal incidence of *Arcobacter* in poultry processing plants have been reported. However, *Arcobacter* isolated from cattle, swine and chicken farms did not show significant seasonal differences (25). When the samples in the present study collected from late August to October are compared with data (10%) from the same season from chicken farms (25), our in-plant data was higher (55.1%).

Although Atabay and Corry (2) reported that *Arcobacter* species may not colonize the poultry intestinal tract, future studies will be required to determine the levels of *Arcobacter* recovered from external and internal sites of broilers, and whether seasonal variations occur with *Arcobacter* as it does with *Campylobacter*.

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Table 3.1. *Arcobacter* on broiler carcasses from poultry processing plant

Sampling day	Processing line site			Total
	Pre-scald	Pre-chill	Post-chill	
A	100% (25/25) ^a	NS ¹	8% (2/25) ^a	54% (27/50) ^a
B	96% (24/25) ^a	NS	12% (3/25) ^a	54% (27/50) ^a
C	100% (25/25) ^a	72% (18/25) ^a	8% (2/25) ^a	60% (45/75) ^a
D	100% (25/25) ^a	28% [*] (7/25) ^b	4% (1/25) ^a	44% (33/75) ^a
E	88% (22/25) ^a	84% (21/25) ^a	16% (4/25) ^a	62.7% (47/75) ^a
Total	96.8% (121/125) ^x	61.3% (46/75) ^y	9.6% (12/125) ^z	55.1% (179/325)

¹ Not sampled

^{a, b} Values within columns with no common superscripts differ significantly ($P \leq 0.05$) by chi-square test for independence. Chi square was not done on %, but rather on # positive/ # tested.

^{x-z} Values within rows with no common superscripts differ significantly ($P \leq 0.05$) by chi-square test for independence. Chi square was not done on %, but rather on # positive/ # tested.

Table 3.2. *Arcobacter* species on broiler carcasses from poultry processing plant

Sample site	<i>Arcobacter</i> species		
	<i>A. butzleri</i>	<i>A. cryaerophilus</i> 1A	<i>A. cryaerophilus</i> 1B
Pre-scald: 67.2% (119) ^a	78.2% (93)	3.4% (4)	18.5% (22)
Pre-chill:26% (46)	78.3% (36)	0	21.7% (10)
Post-chill:6.8% (12)	91.7% (11)	0	8.3% (1)
Total:100% (177)	79.1% (140)	2.3% (4)	18.6% (33)

^a Number of samples

Table 3.3. *Campylobacter* on broiler carcasses from poultry processing plant

Sampling day	Processing line site			Total
	Pre-scalding	Pre-chilling	Post-chilling	
A	100% (25/25) ^a	NS ¹	4% (1/25) ^a	52% (26/50) ^a
B	60%* (15/25) ^a	NS	0% (0/25) ^a	30% (15/50) ^b
C	100% (25/25) ^a	100% (25/25) ^a	68% (17/25) ^b	89% (67/75) ^{cd}
D	100% (25/25) ^a	100% (25/25) ^a	88% (22/25) ^{bc}	96% (72/75) ^d
E	100% (25/25) ^a	100% (25/25) ^a	100% (25/25) ^c	100% (75/75) ^d
Total	92% (115/125) ^x	100% (75/75) ^y	52% (65/125) ^z	78.5% (255/325)

¹ Not sampled

^{a-d} Values within columns with no common superscripts differ significantly ($P \leq 0.05$) by chi-square test for independence. Chi square was not done on %, but rather on # positive/ # tested.

^{x-z} Values within rows with no common superscripts differ significantly ($P \leq 0.05$) by chi-square test for independence. Chi square was not done on %, but rather on # positive/ # tested.

Table 3.4. *Campylobacter* species on broiler carcasses from poultry processing plant

Sample site	<i>Campylobacter</i> species	
	<i>C. coli</i>	<i>C. jejuni</i>
Pre-scald: 48.9% (106) ^a	24.5% (26)	75.5% (80)
Pre-chill: 26.7% (58)	0	100% (58)
Post-chill: 24.4% (53)	1.9% (1)	98.1% (52)
Total: 100% (217)	12.4% (27)	87.6% (190)

^a Number of samples

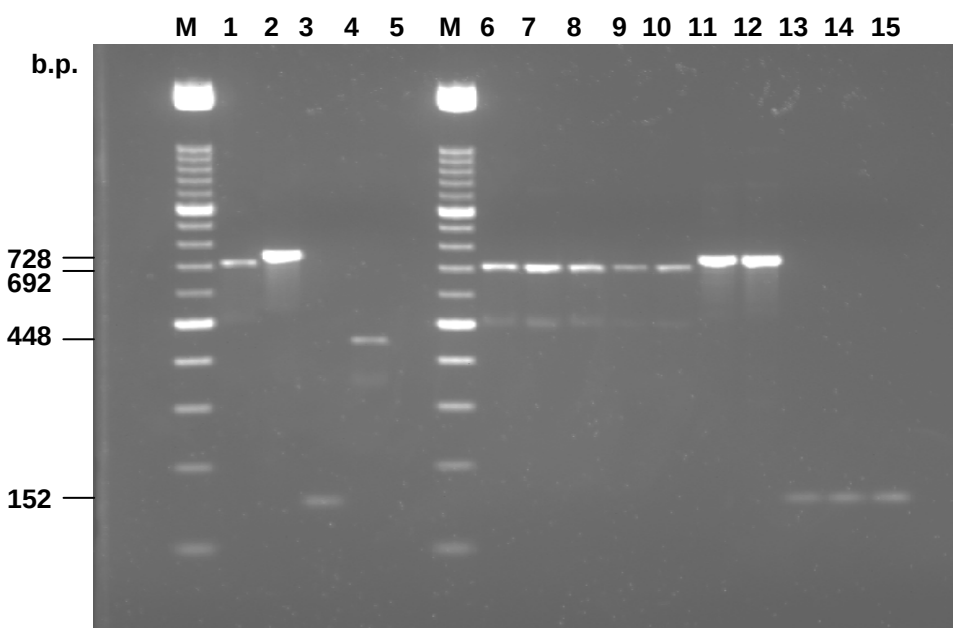


Figure 3.1. Species-specific multiplex PCR of *Arcobacter* species. Lane M, 100 base DNA ladder; 1, *A. butzleri* (ATCC 49616); 2, *A. cryaerophilus* 1A (ATCC 43158); 3, *A. cryaerophilus* 1B (ATCC 49615); 4, *A. skirrowii* (ATCC 51132); 5, negative control; 6-15, *Arcobacter* positive samples from a poultry plant.

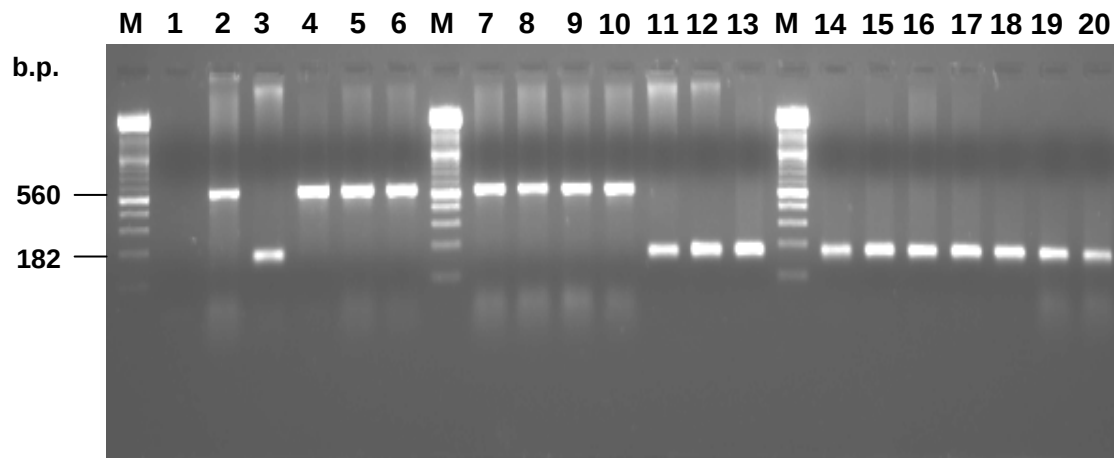


Figure 3.2. Species-specific BAX[®] PCR of *Campylobacter* species. Lane M, 100 base DNA ladder; 1, negative control; 2, *C.coli* (ATCC 33559); 3, *C. jejuni*(ATCC 33560); Lanes 4-20, *Campylobacter* positive samples from a poultry plant.

CHAPTER 4

GENETIC DIVERSITY OF *ARCOBACTER* AND *CAMPYLOBACTER* ON BROILER CARCASSES DURING PROCESSING²

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Abstract

Broiler carcasses (n=325) samples were cultured for *Arcobacter* and *Campylobacter* at three sites along the processing line (pre-scald, pre-chill, and post-chill) in a commercial poultry processing plant during five plant visits from August to October of 2004. Similarity of genomic DNA was assessed by pulsed-field gel electrophoresis (PFGE). *Campylobacter coli* (n=27) and *C. jejuni* (n=188) genomic DNA were digested with *Sma*I and *Arcobacter butzleri* (n=139), *A. cryaerophilus* 1A (n=4) and *A. cryaerophilus* 1B (n=31) was digested with *Kpn*I. All eight *Campylobacter* groups were comprised entirely of isolates from one sampling day, while more than half of the *Arcobacter* groups contained isolates from different sampling days. PFGE groups of *Arcobacter* and *Campylobacter* strains were distributed among single-isolate and multi-isolate groups. A total of 32.8% (57/174) of the *Arcobacter* isolates belonged to single-isolate groups, while only 2.3% (5/215) of the *Campylobacter* isolates belonged to this category. The remaining *Arcobacter* strains were distributed among 25 multi-isolate groups; only eight multi-isolate *Campylobacter* groups were observed. Cluster analysis showed no associations among the multi-isolate groups for either genus. Our results demonstrate far greater genetic diversity for *Arcobacter* compared to that of *Campylobacter*, and suggest the *Campylobacter* groups are specific to individual flocks of birds processed on each sampling day.

Key words: *Arcobacter*, Broiler chickens, *Campylobacter*, Genetic diversity, PFGE, Typing

Introduction

Arcobacter, which is closely related to *Campylobacter*, may be underestimated as cause of foodborne enteritis in humans. Reports of human enteritis caused by *Arcobacter* associated with foods of animal origin have drawn attention to this organism as a public health concern (23). *Arcobacter*, in particular *A. butzleri*, has been found commonly in poultry meat products (7, 40). *Campylobacter jejuni* is the most frequently isolated species of human bacterial enteritis worldwide whereas *C. coli* causes human illness less frequently (12, 28). The pathogenicity of all campylobacters isolated from poultry and whether poultry is the main source of human campylobacteriosis are debatable, but half of all sporadic cases of *Campylobacter* infection are thought to result from consumption of contaminated raw or undercooked poultry and red meats (26). *Arcobacter* and *Campylobacter* are common contaminants of broiler carcasses in poultry processing plants (2, 3, 8, 13). There have been several reports on the high levels of *Campylobacter* on broiler chickens from the farm (34) and retail chickens (41). Therefore, identification of control measures requires a good understanding of the epidemiology of *Arcobacter* and *Campylobacter* including contamination source, transmission routes, and pathogen-host interactions in poultry meat products.

A number of different typing methods have been used to investigate the genetic diversities of *Arcobacter* and *Campylobacter* species. Phenotypic methods, such as serotyping (21) and phage typing, (33) have been used for identifying the source of infection. These methods are not widely available and have limitations which include insufficient discrimination, cross-reactivity, and high levels of nontypeability (24, 25). Comparatively, genetic based methods have enhanced sensitivity, discrimination and improved availability. For *Campylobacter*, PCR-based methods, pulsed-field gel electrophoresis (PFGE), ribotyping, and DNA sequence-based typing of the *flaA* gene, have been used in epidemiological studies (39). Compared to *Campylobacter*, few reports have appeared on typing methods for *Arcobacter*. To date, PFGE, PCR-based typing methods (1, 15, 22), ribotyping (19, 20), and amplified fragment length polymorphism (AFLP) analysis (29) have been used to characterize *Arcobacter* strains.

Fitzgerald et al. (11) reported that PFGE was superior to other typing methods tested for discrimination of *C. jejuni* strains. The Centers for Disease Control and Prevention (CDC) have developed a standardized PFGE protocol for *C. jejuni* that requires 24-30 h to perform (31). Two studies have been reported using PFGE for *Arcobacter* by digestion with *EagI*, *SacII*, *AvaI*, and *SmaI* restriction endonucleases (17, 32).

Little is known about how poultry processing procedures in the United States may affect the genetic diversity of *Arcobacter* and *Campylobacter*. The purpose of the present study was to modify PFGE methods for the characterization of *A. butzleri*, *A. cryaerophilus* 1A, and *A. cryaerophilus* 1B genetic diversity on broiler carcasses from a commercial poultry processing plant. The genetic diversity of the *Arcobacter* species was compared to that of *C. coli* and *C. jejuni*.

Materials and Methods

Sample collection

Broiler carcasses were collected during five plant visits from August to October of 2004 at a commercial poultry processing plant. Carcasses were randomly chosen and collected by hand using new latex gloves for each carcass from three sites along the processing line: pre-scald (25 carcasses/visit), pre-chill (25 carcasses/visit), and post-chill (25 carcasses/visit). Samples were placed into sterile plastic bags that were sealed and covered with ice in coolers for transport to the laboratory. All carcasses were subjected to a whole carcass rinse. Feathered carcasses collected at the pre-scalding site were rinsed by shaking with 500 ml of sterile distilled water for 60 s. Carcasses collected at pre-chill and post-chill were rinsed by shaking with 100 ml of sterile distilled water for 60 s. Carcasses were then discarded. The rinses were poured into 50 ml sterile specimen cups and refrigerated at 4°C. Bacterial isolation was begun within 1 h of sample collection.

Isolation of *Arcobacter*

Both direct plating and enrichment methods were used for *Arcobacter* isolation. Serial dilutions were direct-plated on CVA (cefoperazone, vancomycin, and amphotericin B) agar (6). For enrichment, 1 ml of rinse was inoculated into 5 ml Houf broth (16). Following aerobic incubation at 25°C for 48 h, a

sterile swab was used to streak a portion of the broth onto CVA agar. All plates from direct and enriched samples were incubated aerobically at 25°C for 48 h. Isolates were restreaked twice on Brucella agar (BBA, Hardy Diagnostics, Santa Maria, CA) supplemented with 5% (vol/vol) lysed horse blood (Lampire Biological Laboratories, Pipersville, PA) to ensure clonality. Presumptive identification of *Arcobacter* was performed by microscopic examination of wet mounts of colonies using phase contrast optics. Isolates were stored at -70°C in Wang's freezing medium (37) with 15% (vol/vol) glycerol and Brucella broth (Sigma, St. Louis, MO).

Isolation of *Campylobacter*

Isolates from pre-scald samples were collected by direct plating using a similar procedure and medium as described for *Arcobacter*. For enrichment, 1 ml of rinse was placed in 5 ml Bolton's broth (4). Enrichment cultures were incubated for 24 h at 42°C in a microaerobic atmosphere consisting of 5% O₂, 10% CO₂, and 85% N₂. Following incubation, 0.1 ml of Bolton broth was spread onto CVA agar and these plates were incubated microaerobically for 48 h at 42°C. From each positive plate, one typical *Campylobacter* colony was subcultured twice on BBA. Isolates were presumptively identified as described for *Arcobacter*, and stored at -70°C in Wang's freezing medium (37).

***Arcobacter* multiplex PCR**

Reference strains of *Arcobacter*, including *A. butzleri* (ATCC 49616), *A. cryaerophilus* 1A (ATCC 43158), *A. cryaerophilus* 1B (ATCC 49615), and *A. skirrowii* (ATCC 51132) were used as controls. Reference strains and all presumptive *Arcobacter* isolates were cultured on BBA at 25°C for 48 h under ambient atmosphere. A modified multiplex-PCR for *Arcobacter* (18) was used for species identification. The 50 µl PCR reaction mixture contained 25 ng of DNA template, 50 pmol each N.c. 1A and ARCO-U, 10 pmol each N.c. 1B, N.butz, and N.skir, 0.5U of Jump Start™ *Taq* DNA polymerase (Sigma), 0.8 mM of dNTP's (Applied Biosystems, Warrington, UK), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM MgCl₂. Amplification was performed using the following thermal cycler (PTC-200, MJ Research, Watertown, MA) program: denature at 94°C for 10 min, followed by 30 amplification cycles: denature 30 s at 94°C, anneal 1 min at 64°C, elongate 1 min at 72°C; final elongation at 72°C for

7 min. Amplification products were analyzed by electrophoresis; gels were stained with ethidium bromide and visualized using a UV gel documentation system (Fluorochem™ 8900, Alpha Innotech Corp., San Leandro, CA). A molecular weight standard (Roche, Indianapolis, IN) ranging from 100 b.p. to 1500 b.p. was included on each gel.

***Campylobacter* BAX® PCR.**

Reference strains for *Campylobacter* included *C. coli* (ATCC 33559) and *C. jejuni* (ATCC 33560). Identification of *C. coli* and *C. jejuni* was determined using the BAX® PCR assay (Dupont Qualicon, Wilmington, DE) as previously described (10). Amplification products were analyzed as described for *Arcobacter*.

PFGE subtyping

Ribot et al. (31) of the Centers for Disease Control and Prevention (CDC) and PulseNet developed a 24 h standardized PFGE protocol for *C. jejuni*. The *Campylobacter* and *Arcobacter* isolates in the present study were subtyped using this protocol, although a different restriction enzyme (*KpnI*) and modified electrophoresis running conditions were used for *Arcobacter*. *Salmonella* Braenderup H9812 restricted with *XbaI* was used as the molecular size standard. *Salmonella* H9812 was grown on sheep's blood agar plates (Remel, Lenexa, KS) at 37°C for 24 h. Frozen-stored isolates of *Arcobacter* and *Campylobacter* were streaked onto BBA plates. *Arcobacter* isolates were incubated aerobically at 25°C for 48 h; *Campylobacter* isolates were incubated microaerobically at 42°C for 24-48 h. For restriction digestion of *Arcobacter*, 200 µl of a restriction enzyme mixture containing 40 U of *KpnI* (New England, Biolabs Inc., Beverly, MA) was added to microfuge tubes containing prepared gel plugs and incubated at 37°C for 4 h. For *Campylobacter*, the sliced plugs were pre-restricted in a 1X restriction buffer solution (SureCut buffer A, Roche) at 25°C for 5 min. Following pre-restriction, the buffer was removed from the plug slices, 200 µl of a restriction enzyme mixture containing 40 U of *SmaI* (Roche) was added, and the plug slices were incubated for 4 h at 25 °C.

The restriction fragments of the *Arcobacter* isolates were separated using the CHEF Mapper (BioRad, Hercules, CA) on 1% agarose gels in 0.5X TBE buffer for 18 h under a constant temperature of

14°C using the AutoAlgorithm function for the following electrophoresis conditions: an initial switch time of 6.76 s, a final switch time of 13.68 s, gradient of 6 V/cm, angle of 120°, and the range of 30-400 kbp. Electrophoresis conditions for *Campylobacter* used a final switch time of 38.35 s and a range of 50-400 kbp. The gels were stained for 30 min with ethidium bromide (10 mg/μl, Sigma), then destained three times in sterile distilled water. Gels were visualized with a UV gel documentation system (Fluorochem™ 8900).

Analysis of PFGE patterns

The PFGE patterns of *Arcobacter* and *Campylobacter* were analyzed using the BioNumerics software program (Version 3.5, Applied Maths, Austin, TX) and saved in TIFF file format. The optimization setting for *Arcobacter* and *Campylobacter* was 1.0%; band position tolerance was 0.8% for *Arcobacter* and 0.5% for *Campylobacter*. Suspected double bands were checked by examining the plotted densitometric curves of the PFGE profiles. Cluster analysis was performed using the Dice coefficient and unweighted pair group method using arithmetic averages (UPGMA). *Arcobacter* and *Campylobacter* isolates were automatically assigned to groups according to restriction pattern similarities BioNumerics. The initial groupings were manually edited by examining each PFGE pattern within a group and making appropriate changes.

Results

Preliminary PFGE studies were conducted using five isolates each of *Arcobacter* and *Campylobacter* from the same broiler carcass to determine the number of genetically distinguishable isolates from a broiler carcass. Twenty-five isolates from five broiler carcasses were analyzed and all 25 isolates displayed identical DNA profiles for both *Arcobacter* and *Campylobacter* (data not shown). Based on these results, one isolate per broiler carcass was selected and analyzed in this study.

The modified protocol for *Arcobacter*, using *KpnI* and initial switch time of 6.76 s and a final switch time of 13.68 s, produced the best separation of restriction fragment patterns. The PFGE patterns of *KpnI*-digested genomic DNA from *Arcobacter* isolates (n=174) and of *SmaI*-digested genomic DNA from *Campylobacter* isolates (n=215) were composed of 10 to 19 and 6 to 11 fragments, respectively

(Figure 4.1). Two isolates of *Arcobacter* (one *A. butzleri* and one *A. cryaerophilus* 1B) were untypeable, but all *Campylobacter* isolates examined yielded usable patterns.

The *Arcobacter* isolates were distributed among a total of 82 single-isolate and multi-isolate PFGE groups (Table 4.1). Single-isolate groups comprised 32.8% (57/174) of all *Arcobacter* strains. Among *Arcobacter* species, *A. cryaerophilus* 1B showed the highest proportion in this category (61.3%, 19/31) followed *A. cryaerophilus* 1A (50.0%, 2/4) and *A. butzleri* (25.9%, 36/139). Of the 25 same-species PFGE groups, one was *A. cryaerophilus* 1A and four were *A. cryaerophilus* 1B (Table 4.1). The majority (20/25) were comprised of *A. butzleri*, by far the most commonly isolated *Arcobacter* species in this study, and included 74.1% (103/139) of the *A. butzleri* strains. Cluster analysis demonstrated that the *Arcobacter* multi-isolate groups were highly diverse (Figure 4.2). No major clusters were observed and the isolates did not cluster by species, sampling day or collection site. Also, 52% (13/25) of the groups contained isolates collected on different sampling days (Figure 4.2).

In contrast to the diversity of *Arcobacter*, the *Campylobacter* strains were divided among just 13 single isolate and multi-isolate groups (Table 4.2). Only 2.3% (5/215) of the *Campylobacter* isolates belonged to groups with a single member. The percentage of *C. jejuni* strains in the single-isolate category was slightly higher than that of *C. coli* strains (Table 4.2). The eight same-species groups included one *C. coli*, containing 96.3% (26/27) of the *C. coli* isolates in this study. The other seven same-species *Campylobacter* groups comprised 97.9% (184/188) of the *C. jejuni* isolates. Cluster analysis showed that the *Campylobacter* multi-isolate groups were also quite diverse, although much less so than those of *Arcobacter* (Figure 4.3). As with *Arcobacter*, no major clustering was seen, nor did the isolates cluster by species, sampling day or collection site. However, in contrast to *Arcobacter*, each *Campylobacter* group was composed of isolates collected on a single sampling day (Figure 4.3).

Discussion

PFGE was used to characterize 174 *arcobacters* and 215 *campylobacters* isolated from 325 broiler carcasses from a commercial poultry processing plant. Digestion of *Arcobacter* isolates with *Kpn*I yielded PFGE patterns showing high discrimination that was useful for differentiating the genotypes.

Arcobacter butzleri, *A. cryaerophilus* 1A, and *A. cryaerophilus* 1B isolates from the three different collection sites in the processing facility were distinctly different from each other. The *Arcobacter* isolates in this study showed a much higher percentage of single-isolate groups (32.8%, 57/174 vs. 2.3%, 5/215) than the *Campylobacter* isolates (Table 4.1). More than half of the *A. cryaerophilus* 1B strains were found in single-isolate groups (61.3%, 19/31). Furthermore, a larger number of multi-isolate PFGE groups were found for *Arcobacter* (25 groups) compared to *Campylobacter* (8 groups).

Hume et al. (17) reported that there was little similarity between genotypic patterns by *Ava*I, *Eag*I, and *Sac*II–digested DNA from *Arcobacter* isolates collected from a farrow-to-finish swine facility. They also described that among these three restriction enzymes, *Eag*I and *Sac*II were better suited for differentiation of PFGE patterns than *Ava*I. Rivas et al. (32) characterized *A. buzleri* isolates from poultry, pork, beef and lamb by PFGE using *Sac*II, *Eag*I, and *Sma*I enzymes. More genotypic variation of *Arcobacter* isolates was observed in the present study than described in previous reports (17, 32).

PFGE results can be influenced by the person analyzing the band patterns, by the software program(s) used, and by the criteria used to differentiate epidemiologically related and unrelated isolates (36). In the present study, alternative typing methods were investigated before choosing PFGE (data not shown). Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and randomly amplified polymorphic DNA PCR (RAPD-PCR) have been used for genetic typing of *Arcobacter* isolated from poultry products or mechanically separated turkey (15, 22). However, when comparing PFGE, ERIC-PCR, and RAPD-PCR in a preliminary study, we found PFGE gave the best discrimination and the highest typeability and reproducibility (data not shown).

Five single-isolate and eight same-species PFGE groups were identified among 215 *Campylobacter* isolates in the present study. Among the eight *Campylobacter* same-species, the *C. coli* group (C-1) had a low similarity (29%) to the seven *C. jejuni* groups (Figure 4.3). The *Campylobacter* genotypes also varied between different sampling days. While the *Campylobacter* subgroups were genetically diverse, it was clear that some clones may have spread and persisted on the birds on the processing line. Moreover, compared to *Arcobacter*, a clear tendency for clonal dominance of one type

within each flock was observed. The PFGE patterns of the *Campylobacter* isolates in our study were less diverse than those from other reports (14). Although, the discriminatory potential of PFGE is dependent on the restriction enzyme(s) used (39), Dickins et al. (9) and Wassenaar et al. (38) also reported genetic similarity among *Campylobacter* isolates from retail chicken. However, multiple genotypes of *C. jejuni* have been isolated from a commercial broiler flock during rearing and even from gastrointestinal tracts of individual birds (14). Further, a Danish study (30) showed that *C. jejuni* can persist during successive broiler flock rotations. In our study, the one *C. coli* and seven *C. jejuni* PFGE groups were differentiated depending on sampling days, not sampling site.

Interestingly, the number of single-isolate groups of *Arcobacter* and *Campylobacter* varied by sampling site. The percentage of single isolates in *Arcobacter* strains (Table 4.1) at the three sites along the processing line were: 1) pre-scalding, 22% (26/116), 2) pre-chilling, 52% (24/46), and post-chilling, 58% (7/12). Compared to *Campylobacter*, processing appeared to have an effect on the prevalence of *Arcobacter*. Previous studies (5, 27) have also noted such trends in *Campylobacter* strains from poultry and turkeys during processing in abattoirs. In our study, *Arcobacter* strains from post-chilling showed greater genetic diversity than *Campylobacter* strains from the same processing site.

Although PFGE is somewhat labor-intensive, we found it to be very useful for comparing the genotypic diversity of *Arcobacter* and *Campylobacter*. PFGE relies on polymorphisms occurring throughout the genome and has a higher discriminatory power compared to other genetic typing methods (11). For global epidemiological studies, standardization of various procedures is necessary. Collection of a *Campylobacter* genotypic database is currently underway by PulseNet, which will allow rapid analysis and comparisons of PFGE patterns from different sources (35). Likewise, a genotypic database of *Arcobacter* by PFGE using *KpnI* as the restriction enzyme would help provide more accurate information on the sources of isolates. Future studies involving genotyping *Arcobacter* and *Campylobacter* strains from several flocks during the same sampling day are intended to address whether cross-contamination occurs between flocks during processing.

In conclusion, PFGE for *Arcobacter* can assist in tracking *Arcobacter* suspected in foodborne disease outbreaks. PFGE profiling is a valuable tool for taxonomic and epidemiological analysis of *Arcobacter* and *Campylobacter* strains.

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Table 4.1. Distribution of PFGE patterns of *Arcobacter* on broiler carcasses from poultry processing plant

Species	n	Single Isolate per group		2 or More Isolates per Group	
		Groups ^a	Isolates (%)	Groups	Isolates
<i>A. butzleri</i>	139	36	36 (25.9)	20	103 (74.1)
<i>A. cryaerophilus</i> 1A	4	2	2 (50.0)	1	2 (50.0)
<i>A. cryaerophilus</i> 1B	31	19	19 (61.3)	4	12 (38.7)
Totals	174	57	57 (32.8)	25	117 (67.2)

^aNumber of groups in each category

Table 4.2. Distribution of PFGE patterns of *Campylobacter* on broiler carcasses from poultry processing plant

Species	n	Single Isolate per group		2 or More Isolates per Group	
		Groups ^a	Isolates (%)	Groups	Isolates
<i>C. coli</i>	27	1	1 (3.7)	1	26 (96.3)
<i>C. jejuni</i>	188	4	4 (2.1)	7	184 (97.9)
Totals	215	5	5 (2.3)	8	210 (97.7)

^aNumber of groups in each category

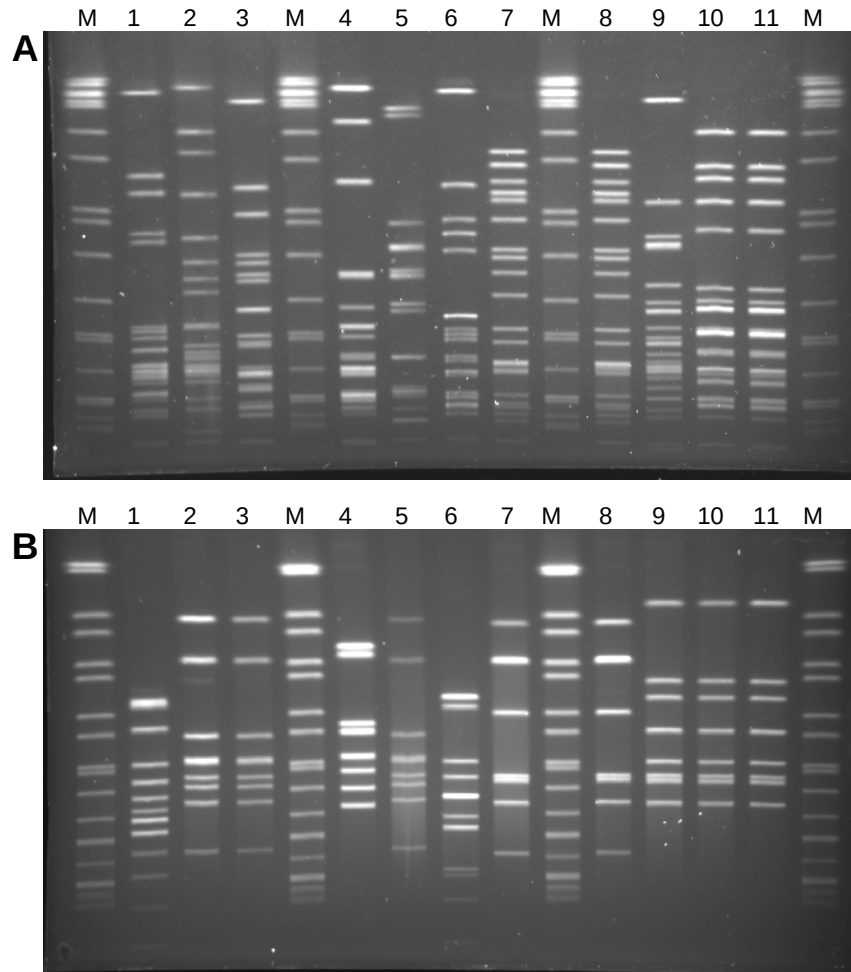


Figure 4.1. (A) PFGE *KpnI* restriction profiles of *Arcobacter* isolates: Lanes 1-6 and 10-11, pre-scalding; Lanes 7-9, post-chilling. Lane M, *Salmonella* Braenderup H9812 molecular size standard. (B) PFGE *SmaI* restriction profiles of *Campylobacter* isolates: Lanes 1-7 and 9-10, pre-scalding; Lane 8, post-chilling. Lane M, *Salmonella* Braenderup H9812 molecular size standard.

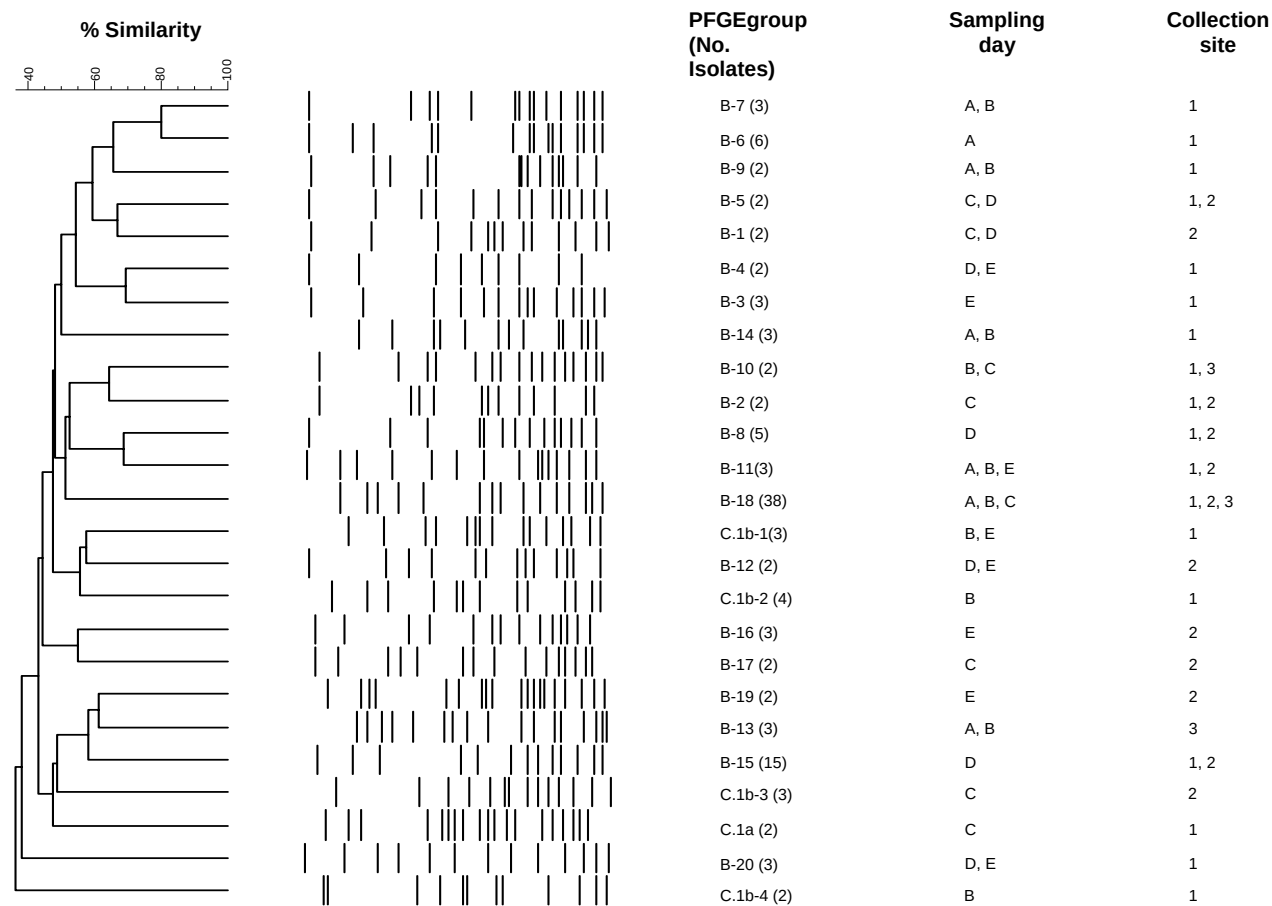


Figure 4.2. PFGE patterns of the *A. butzleri* (B-1 to B-20), *A. cryaerophilus* 1A (C.1a), and *A. cryaerophilus* 1B (C.1b-1 to C.1b-4) isolates.

Collections site: 1, pre-scalding; 2, pre-chilling; 3, post-chilling. The dendrogram was generated using UPGMA cluster analysis and Dice similarity coefficient

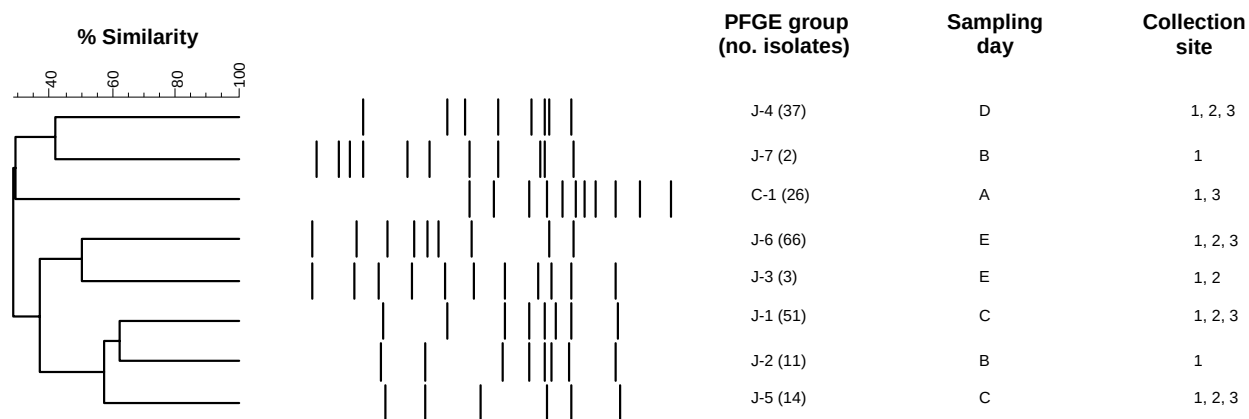


Figure 4.3. PFGE patterns of the *C. coli* (C-1) and *C. jejuni* (J-1 to J-7) isolates. Collection site: 1, pre-scalding; 2, pre-chilling; 3, post-chilling. The dendrogram was generated using UPGMA cluster analysis and Dice similarity coefficient.

CHAPTER 5

**ANTIMICROBIAL RESISTANCE PATTERNS OF *ARCOBACTER* AND
CAMPYLOBACTER ON BROILER CARCASSES DURING PROCESSING³**

³ Insook Son, Mark D. Englen, Mark E. Berrang, Paula J. Fedorka-Cray, and Mark A. Harrison. To be submitted to *International Journal of Antimicrobial Agents*, 2005

Abstract

This study examined the antimicrobial resistance of *Arcobacter* (n = 174) and *Campylobacter* (n = 215) isolated from broiler carcasses in a commercial poultry processing plant. For *Arcobacter* and *Campylobacter* species, 93.7% (n = 163) and 99.5% (n = 214) were resistant to one or more antimicrobials, and 71.8% (n = 125) and 28.4% (n = 61) were resistant to two or more antimicrobials, respectively. Of the *A. butzleri* isolates, 89.9% (n = 125) were resistant to clindamycin, 82.0% (n = 114) were resistance to azithromycin, and 23.7% (n = 33) were resistant to nalidixic acid. Resistance to tetracycline was very high in *C. jejuni* (99.5%) and *C. coli* (96.3%). Our results demonstrate substantial resistance in *Arcobacter* and *Campylobacter* to common antimicrobial agents.

Keywords: Antimicrobial resistance, *Arcobacter*, *Campylobacter*, Broiler chickens, Poultry processing

Introduction

Arcobacter, previously classified as aerotolerant *Campylobacter* (29), are gram negative, non-spore forming, microaerophilic or helical cells that are motile by means of polar flagella and exhibit a corkscrew movement (17, 44). *Arcobacter butzleri* is regarded as the primary human pathogen and *A. cryaerophilus* (subgroup 1A and 1B) is associated with human diarrheal illness and bacteremia, and with reproduction abnormalities in farm animals (23, 31, 40). *Arcobacter skirrowii* has been reported in farm animals and on broiler carcasses (45). However, the pathogenic role of *Arcobacter* in human disease is still unclear.

Infection with *Campylobacter* is recognized as a leading cause of human enteritis, and *Campylobacter jejuni* and *Campylobacter coli* are most often associated with human infections (28). Most patients with *Campylobacter* infections have a self-limited illness and do not require antimicrobial drugs except in cases with severe or prolonged symptoms, or in immunocomprised patients (36). When antimicrobial drugs are recommended for treatment, erythromycin or a fluoroquinolone such as ciprofloxacin are the drugs of choice. Tetracycline, doxycycline, chloramphenicol, and gentamicin are sometimes listed as alternative drugs (4, 36). The use of antimicrobial agents in food animals has resulted in the emergence and dissemination of antimicrobial-resistant bacteria, including antimicrobial resistant *Campylobacter* (1). Resistant *Campylobacter* strains may consequently be transmitted to humans through the food chain. Kassenborg et al. (22) found that people infected with fluoroquinolone-resistant *Campylobacter* in the U.S. were more likely to have eaten chicken or turkey outside the home than well controls. They also found that poultry is an important source of domestically acquired fluoroquinolone-resistant *Campylobacter* infection. In 1995, fluoroquinolones were first licensed for use in poultry, which is a major source of *Campylobacter* infections in humans in the United States (37). The development of quinolone resistance in *Campylobacter* spp. is primarily due to mutations in the bacterial enzyme DNA gyrase. *Arcobacter butzleri* has often been identified on poultry products, but *A. cryaerophilus* and *A. skirrowii* have also been detected on poultry (3, 27). Many reports about antimicrobial resistance in *Campylobacter* isolated from poultry production environment have been published (7, 16, 43). In foods

of animal origin, the occurrence of *Campylobacter* is much higher in poultry than in pork or beef (30). Antimicrobial-resistant *Campylobacter* from animals can infect humans through the food chain or via occupational exposure or contact (42). However, reports on antimicrobial resistance patterns in *Arcobacter* isolated from poultry are lacking. Further, there are no internationally accepted criteria of breakpoint assessment and susceptibility testing for *Arcobacter* and *Campylobacter*, and validated reference strains for quality control have not been established (6).

In this study, the antimicrobial resistance patterns of *Arcobacter* and *Campylobacter*, isolated from broiler carcasses during processing, were determined using a broth-microdilution testing. The results for the antimicrobial resistance patterns were compared between *Arcobacter* and *Campylobacter*.

Materials and Methods

Sample collection

From August to October of 2004, broiler carcasses were collected at a commercial poultry processing plant during five plant visits. Carcasses were randomly taken by hand using new latex gloves for each carcass from three sites along the processing line: pre-scalding ($n=5 \times 25=125$), pre-chilling ($n=3 \times 25=75$), and post-chilling ($n=5 \times 25=125$). Samples were placed into sterile plastic bags and covered with ice in coolers for transport to the laboratory. Feathered carcasses collected at the pre-scalding site were rinsed by shaking with 500 ml of sterile distilled water for 60 s. Carcasses collected at pre-chilling and post-chilling were rinsed by shaking with 100 ml of sterile distilled water for 60 s. Carcasses were then discarded and the rinses were poured into 50 ml sterile specimen cups and refrigerated at 4°C. Bacterial isolation was begun within 1 h of sample collection.

Isolation of *Arcobacter* and *Campylobacter*

Both direct plating and enrichment methods for *Arcobacter* and *Campylobacter* isolation were used. Serial dilutions were direct-plated on CVA (cefoperazone, vancomycin, and amphotericin B) agar (8). For enrichment, 1 ml of rinse was inoculated into 5 ml Houf broth (18) for *Arcobacter* and into 5 ml Bolton's broth (5) for *Campylobacter*, respectively. Following aerobic incubation at 25°C for 48 h for

Arcobacter enrichment, a sterile swab was used to streak a portion of the broth onto CVA agar. All *Arcobacter* plates from direct and enriched samples were incubated aerobically at 25°C for 48 h. Enrichment cultures of *Campylobacter* were incubated for 24 h at 42°C in a microaerobic atmosphere consisting of 5% O₂, 10% CO₂, and 85% N₂, and then 0.1 ml of Bolton broth was spread onto CVA agar and these plates were incubated microaerobically for 48 h at 42°C. The *Arcobacter* and *Campylobacter* Isolates were restreaked twice on Brucellar agar (BBA, Hardy Diagnostics, Santa Maria, CA) supplemented with 5% (vol/vol) lysed horse blood (Lampire Biological Laboratories, Pipersville, PA) to ensure clonality. Presumptive identification of *Arcobacter* and *Campylobacter* was performed by microscopic examination of wet mounts of colonies using phase contrast optics. For frozen storage, isolates were stored at -70°C in Wang's freezing medium (46) with 15% (vol/vol) glycerol and Brucella broth (Sigma, St. Louis, MO).

***Arcobacter* multiplex PCR**

Reference strains of *Arcobacter*, including *A. butzleri* (ATCC 49616), *A. cryaerophilus* 1A (ATCC 43158), *A. cryaerophilus* 1B (ATCC 49615), and *A. skirrowii* (ATCC 51132) were used as controls. Reference strains and all presumptive *Arcobacter* isolates were cultured on BBA at 25°C for 48 h under ambient atmosphere. A modified multiplex-PCR for *Arcobacter* (20) was used for species identification. The 50 µl PCR reaction mixture contained 25 ng of DNA template, 50 pmol each N.c. 1A and ARCO-U, 10 pmol each N.c. 1B, N.butz, and N.skir, 0.5U of Jump Start™ Taq DNA polymerase (Sigma), 0.8 mM of dNTP's (Applied Biosystems, Warrington, UK), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM MgCl₂. Amplification was performed using the following thermal cycler (PTC-200, MJ Research, Watertown, MA) program: denature at 94°C for 10 min, followed by 30 amplification cycles: denature 30 s at 94°C, anneal 1 min at 64°C, elongate 1 min at 72°C; final elongation at 72°C for 7 min. Amplification products were analyzed by electrophoresis; gels were stained with ethidium bromide and visualized using a UV gel documentation system (Fluorochem™ 8900, Alpha Innotech Corp., San Leandro, CA). A molecular weight standard (Roche, Indianapolis, IN) ranging from 100 b.p. to 1500 b.p. was included on each gel.

***Campylobacter* BAX[®] PCR**

Reference strains for *Campylobacter* included *C. coli* (ATCC 33559) and *C. jejuni* (ATCC 33560). Identification of *C. coli* and *C. jejuni* was determined using the BAX[®] PCR assay (Dupont Qualicon, Wilmington, DE) as previously described (11). Amplification products were analyzed as described for *Arcobacter*.

Antimicrobial susceptibility testing

Susceptibility testing of *Arcobacter* and *Campylobacter* isolates was conducted using the National Antimicrobial Resistance Monitoring System of Enteric Bacteria (NARMS) custom-designed *Campylobacter* panel and a Sensititre[®] semiautomated system (TREK Diagnostic Systems, Inc., Cleveland, OH, USA). The frozen bacterial strains were subcultured on BBA plates supplemented with 5% (vol/vol) lysed horse blood (Lampire Biological Laboratories). *Arcobacter* strains were incubated for 48 h at 25°C aerobically and *Campylobacter* strains were incubated for 48 h at 42°C in a microaerobic atmosphere (5% O₂, 10% CO₂, and 85% N₂). Colonies of *Arcobacter* and *Campylobacter* were suspended in Mueller Hinton broth (TREK) until the turbidity of the suspensions was adjusted to match that of a 0.5 McFarland standard. One hundred microliters of the 0.5 McFarland suspension was transferred into 11 ml of Mueller Hinton broth with laked-horse blood (TREK) which was then used to inoculate the 96-well panel to give a final concentration of 10⁵ CFU/ml. *Campylobacter* panels included a control well containing no antimicrobial drug. All panels were incubated in an anaerobic jar containing 5% O₂, 10% CO₂, and 85% N₂ at 37°C. Incubation time was 72 h for *Arcobacter* strains and 48 h for *Campylobacter* strains. Quality control ATCC strains *C. jejuni* 33560 and *A. butzleri* 49616 were tested to confirm susceptibility to all the antimicrobials at each testing. The antimicrobials tested and the resistance breakpoints (MICs) were: azithromycin, ≥2; ciprofloxacin, ≥4; clindamycin, ≥4; erythromycin, ≥32; gentamicin, ≥16; nalidixic acid, ≥32; tetracycline, ≥16. The MICs of erythromycin, ciprofloxacin, and tetracycline were classified as susceptible, intermediate, or resistant according to guidelines published by the Clinical and Laboratory Standards Institute (CLSI) for broth-microdilution susceptibility testing. The MICs for the resistance breakpoints of the other antimicrobials were those used by NARMS as

reported in the U.S. centers for Disease Control NARMS 2001 Annual Report (<http://www.cdc.gov/narms/annuals.htm>).

Data analysis

Differences in the resistance to antimicrobials by species were determined using the Wald chi-square test by logistic regression model in the SAS statistical programs (SAS Institute, NC, USA). Differences were considered statistically significant if the P-value was ≤ 0.05 .

Results

Antimicrobial resistance

Most of the 174 *Arcobacter* isolates (93.7%, n = 163) were resistant to one or more antimicrobial. The percentages by species of *Arcobacter* isolates resistant to the individual antimicrobials are shown in Table 1. The highest prevalence of resistance among all *Arcobacter* isolates was to clindamycin at 88.5% (n = 154), followed by azithromycin at 69.5% (n = 121) and nalidixic acid at 20.7% (n = 36). The percentage of *Arcobacter* isolates resistant to ciprofloxacin and erythromycin was very low, and all *Arcobacter* isolates were susceptible to gentamicin and tetracycline. Resistance in the *A. butzleri* group to azithromycin was much higher than the *A. cryaerophilus* 1B group (82.0%, n = 114 vs 12.9%, n = 4), while resistance to nalidixic acid in the *A. butzleri* group was not significantly different from the *A. cryaerophilus* 1B strains (23.7%, n = 33 vs 9.7%, n = 3). Resistance to erythromycin was observed only in *A. butzleri* at a low level (4.3%, n = 6); resistance to ciprofloxacin was only found in one *A. cryaerophilus* 1B strain (Table 1). Site of isolate collection (pre-scald, pre-chill, and post-chill) did not affect the observed antimicrobial resistance patterns (data not shown).

For *Campylobacter*, 99.1% (n = 213) of the 215 *Campylobacter* isolates were resistant to one or more antimicrobial. Percentages by species of *Campylobacter* isolates resistant to the individual antimicrobials are shown in Table 2. Tetracycline resistance was the highest at 99.1% (n = 213) for all *Campylobacter* isolates. The next most common resistance was to ciprofloxacin and nalidixic acid at 27.0 % (n = 58) for each drug. The percentages of resistance among the *C. jejuni* isolates to ciprofloxacin and nalidixic acid was more than eight times higher for the *C. coli* group (30.3%, n = 57 vs 3.7%, n = 1).

However, resistance to tetracycline for *C. jejuni* and *C. coli* was not significantly different (99.5%, n = 187 vs 96.3%, n = 26). Resistance to clindamycin was found only in *C. jejuni* at 1.6% (n = 3). All *Campylobacter* strains were susceptible to azithromycin, erythromycin, and gentamicin (Table 2).

Multiple resistance

Of the 174 *Arcobacter* isolates tested, 71.8% (n = 125) were resistant to two or more antimicrobials. A significantly higher percentage of the *A. butzleri* strains (83.5%, 116/139) and *A. cryaerophilus* 1A strains (75%, 3/4) belonged to this group compared to *A. cryaerophilus* 1B (19.4%, 6/31). The majority of these 125 *Arcobacter* strains (76%, n = 95) were resistant to two antimicrobials (Table 3). Of the 95 *Arcobacter* isolates resistant to two antimicrobials, 83 (77 *A. butzleri*, 3 *A. cryaerophilus*, and 3 *A. cryaerophilus* 1B) combined resistance to azithromycin/ clindamycin. Other resistances were found for azithromycin/ erythromycin and clindamycin/ nalidixic acid. Resistance to three antimicrobials for *Arcobacter* was found in 24% (n = 30) of the 125 multi-resistant isolates or 17.2% of all *Arcobacter* isolates (n = 174) (Table 3). Most frequently observed was the combination of azithromycin/ clindamycin/ nalidixic acid, found only in *A. butzleri* strains at 93.3% (n = 28) of these 30 strains (Table 3). The remaining two patterns of triple resistance were comprised of a single *Arcobacter* isolate each (Table 3).

For the 215 *Campylobacter* isolates, 28.4% (n = 61) were resistant to two or more antimicrobials as shown in Table 4. The highest percentage among these 61 isolates was the combination of resistance to ciprofloxacin/ nalidixic acid/ tetracycline (n = 57, *C. jejuni*). Resistance to ciprofloxacin/ nalidixic acid was found in one *C. coli* strain, and resistance to clindamycin/ tetracycline was observed in three *C. jejuni* strains.

Discussion

The use of antimicrobials in food animal production has been controversial due to antimicrobial resistance and the emergence and dissemination of resistant bacteria, an inevitable problem accompanying the use of antimicrobials. The most important reservoir for human *Campylobacter* infections are poultry and poultry products (42). The importance of *Arcobacter* as a cause of human

infection remains to be determined, in part because optimal isolation techniques have not been established. However, *Arcobacter* has been isolated from foods of animal origin, including chickens and chicken products (10, 18, 19, 35). In this study, the antimicrobial resistance patterns of *Arcobacter* and *Campylobacter* isolated from broiler carcasses in a poultry processing plant were examined and compared. To accomplish this, the NARMS criteria for *Campylobacter* were adopted to categorize the isolates as susceptible, intermediate, and resistant for *Arcobacter* because there is currently no available data that can be used for the interpretation of broth-microdilution susceptibility testing for *Arcobacter*.

The percentage of *Arcobacter* isolates (93.7%, 163/174) resistant to one or more antimicrobials was lower than that of *Campylobacter* isolates (99.5%, 214/215). However, 4.3% (6/139) of *A. butzleri* and 16.1% (5/31) of *A. cryaerophilus* 1B were susceptible to all antimicrobials. Kabeya et al. (21) reported that 63.4% of *A. cryaerophilus* isolated from meat samples were susceptible to all antimicrobials tested.

Macrolides are approved for use in broilers in the U.S. as growth promoters and therapeutics (39). Three macrolide/lincosamide agents (azithromycin, clindamycin and erythromycin) were tested in the present study. A high level of resistance in *Arcobacter* species was found to azithromycin. Among *Arcobacter* species, azithromycin resistance was much higher in *A. butzleri* than in *A. cryaerophilus* 1B. Resistance to azithromycin in *A. butzleri* strains was the second highest among the antimicrobials tested. The highest resistance for all *Arcobacter* species tested in this study was to clindamycin. This drug is recommended as an alternative treatment for *Campylobacter* gastroenteritis in humans (38). Other authors have also reported a high level (98%) of resistance to clindamycin in *A. butzleri* from human and animal isolates (24). Resistance to erythromycin was very low in *A. butzleri*, and all the *A. cryaerophilus* 1A and *A. cryaerophilus* 1B isolates were susceptible to erythromycin. Kiehlbauch et al. (24) reported that that *A. cryaerophilus* 1A isolates were generally susceptible to azithromycin and erythromycin, but *A. butzleri* and *A. cryaerophilus* 1B isolates were resistant to these macrolides in their study. Compared to the *Arcobacter* strains, all *Campylobacter* strains were susceptible to macrolides/lincosamide agents. In an Irish survey (26), the level of erythromycin resistance in *Campylobacter* isolates of poultry was much

lower than in other veterinary sources. A retrospective Canadian study by Gaudreau and Gilbert (14) reported that resistance to erythromycin occurred in 0-12.6% of human *C. jejuni* strains, most often in fewer than 5% of such strains. This indicates that macrolides/ lincosamide agents can still be considered drugs of choice for treating *Campylobacter* infections, but the decision to use azithromycin and clindamycin for treating *Arcobacter* infection requires more data from other food animal sources and human isolates.

Fluoroquinolone resistance rates in *Campylobacter* have been similar in isolates from poultry products and humans (34, 37). In contrast, data for fluoroquinolone resistance in *Arcobacter* from animal or human disease is lacking. We found all *A. butzleri* and *A. cryaerophilus* 1A were susceptible to ciprofloxacin, and only one *A. cryaerophilus* 1B was resistant to ciprofloxacin. The *Campylobacter* strains had a higher overall resistance to ciprofloxacin (27%) compared to the *Arcobacter* strains, although *C. coli* resistance was only 3.7%. Resistance to nalidixic acid was similar for both *Arcobacter* (20.7%) and *Campylobacter* strains (27%). In this regard, Fera et al. (13) reported that the fluoroquinolones, levofloxacin, marbofloxacin, enrofloxacin, and ciprofloxacin showed good activity against *A. butzleri* and *A. cryaerophilus* stains. Kabeya et al. (21) reported that 63.5% of *A. butzleri* strains were resistant to nalidixic acid using a disk diffusion test, however Atabay and Aydin (2) found that all strains of *A. butzleri* were susceptible to nalidixic acid. In an Irish poultry processing plant, the incidence of resistance to nalidixic acid was 20.5% for *C. jejuni*, but all *C. coli* strains were susceptible (12). However, an extremely high prevalence of ciprofloxacin (99%) and nalidixic acid resistance (99%) was found in *Campylobacter* strains isolated from broilers in Spain (34).

The primary mechanism of resistance to fluoroquinolones in *Campylobacter* appears to be specific point mutations in the gene encoding DNA gyrase A (*gyrA*) (9, 41). In particular, cross-resistance to both ciprofloxacin and nalidixic acid is conferred by the specific mutation Thr86-Ile in *gyrA* (15, 47). Accordingly, all of our *C. jejuni* (n=57) and *C. coli* strains (n = 1) that were resistant to ciprofloxacin were also resistant to nalidixic acid. The mechanisms of resistance to fluoroquinolones in *Arcobacter* are still not known.

All *Arcobacter* and *Campylobacter* strains included in our study were susceptible to the aminoglycoside gentamicin. Other authors (13, 21, 24) have also reported that *Arcobacter* was susceptible to aminoglycosides including kanamycin, amikacin, gentamicin, and streptomycin. *Campylobacter jejuni* and *C. coli* resistance to aminoglycosides has been reported infrequently (41). The study by Fallon et al. (12) found low streptomycin and kanamycin resistance in *C. jejuni* isolates from chicken neck flap samples (2.5% and 1.2%, respectively) and no resistance in the *C. coli* isolates.

No reports of tetracycline resistance in *Arcobacter* isolates from broiler chickens have appeared in the literature. All *Arcobacter* strains in the present study were susceptible to tetracycline. Similar results were observed in earlier reports (21, 24). These findings suggest that tetracycline may be useful for the treatment of *Arcobacter* infections in human and in veterinary medicine, along with aminoglycosides as reported previously (21). However, a high level of resistance to tetracycline in *Campylobacter* strains was observed, exceeding 99%. For *Campylobacter* species, tetracycline resistance levels were similar between *C. jejuni* (99.5%) and *C. coli* (96.3%). A similar report of high tetracycline resistance was found in Taiwan; *C. jejuni* and *C. coli* from chicken products at 83% and 90%, respectively (25). Tetracyclines have been listed as an alternative treatment in humans for *Campylobacter* gastroenteritis in the past (41). In this study, the percentages of tetracycline resistance were much higher than results reported in other studies for tetracycline resistance in *C. jejuni* (20.5%) and *C. coli* (18.2%) from poultry (12). Lucey et al. (26) also reported lower rates of tetracycline resistance at 19.4% of *C. jejuni* and *C. coli* from poultry of Ireland.

Multiple resistance for *Arcobacter* was observed in *A. butzleri* and *A. cryaerophilus* 1A; multi-resistance in *A. cryaerophilus* 1B was much lower. Resistance to three antimicrobials was found in 17.2% (30/174) of *Arcobacter* species. The majority of *A. butzleri* strains showing multidrug resistance included resistance to azithromycin/ clindamycin. Resistance to azithromycin/ clindamycin/ nalidixic acid in *A. butzleri* was the second most common pattern. The incidence of multidrug resistance in *A. butzleri* was much higher compared to *A. cryaerophilus* 1A and *A. cryaerophilus* 1B. These results are similar to the report by Kabeya et al. (21) in which *A. butzleri* exhibited more multiresistance than *A.*

cryaerophilus and *A. skirrowii*. In *Campylobacter*, resistance to three antimicrobials was found at a higher level than resistance to two antimicrobials. Among *Campylobacter* species, multiple resistance was observed more than eight times as often in the *C. jejuni* strains compared to *C. coli*. Resistance to ciprofloxacin/ nalidixic acid/ tetracycline was found in the *C. jejuni* multi-resistant isolates. A recent study by Randall et al. (33) suggested that multidrug resistance in *Campylobacter* is associated with the expression of the *cmeABC* efflux system. Another study (32) also reported that the absence in *C. jejuni* of the multidrug efflux pump (*cmeB*) resulted in a 2-4 fold increase in the susceptibility to ampicillin, ciprofloxacin, chloramphenicol, erythromycin and tetracycline.

In conclusion, *Arcobacter* and *Campylobacter* strains from a poultry processing plant showed resistance to a relatively narrow range of antimicrobials. Moreover, the percentage of multiple resistant strains was much higher in *Arcobacter* than that of *Campylobacter* strains. Long-term antimicrobial susceptibility surveillance for *Arcobacter* and *Campylobacter* isolates is needed to evaluate the effect of antimicrobial usage in food production animals.

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Table 5.1. Percentage of *Arcobacter* isolates on broiler carcasses resistant to antimicrobials by species

Antimicrobial	<i>A. butzleri</i> (n=139)	<i>A. cryaerophilus</i> 1A (n=4)	<i>A. cryaerophilus</i> 1B (n=31)	Total (n=174)
Azithromycin	82.0 (114) ^a	75 (3) ^a	12.9 (4) ^{ab}	69.5 (121)
Ciprofloxacin	0	0	3.2 (1)	0.6 (1)
Clindamycin	89.9 (125)	100.0 (4)	80.6 (25)	88.5 (154)
Erythromycin	4.3 (6)	0	0	3.4 (6)
Gentamicin	0	0	0	0
Nalidixic acid	23.7 (33)	0	9.7 (3)	20.7 (36)
Tetracycline	0	0	0	0

^{a, b} Values within columns with no common superscripts differ significantly ($P \leq 0.05$) by Wald chi-square test

Table 5.2. Percentage of *Campylobacter* isolates on broiler carcasses resistant to antimicrobials by species

Antimicrobial	<i>C. jejuni</i> (n=188)	<i>C. coli</i> (n=27)	Total (n=215)
Azithromycin	0	0	0
Ciprofloxacin	30.3 (n=57) ^a	3.7 (1) ^b	27.0 (58)
Clindamycin	1.6 (3)	0	1.4 (3)
Erythromycin	0	0	0
Gentamicin	0	0	0
Nalidixic acid	30.3 (n=57) ^a	3.7 (1) ^b	27.0 (58)
Tetracycline	99.5 (187)	96.3 (26)	99.1 (213)

^{a, b} Values within columns with no common superscripts differ significantly ($P \leq 0.05$) by Wald chi-square test

Table 5.3. Resistance patterns of *Arcobacter* isolates¹ on broiler carcasses resistant to two or more antimicrobials

No. of Resistances	Resistance pattern	No. of isolates with resistance pattern		
		<i>A. butzleri</i> (n=116)	<i>A. cryaerophilus</i> 1A (n=3)	<i>A. cryaerophilus</i> 1B (n=6)
2	AZ CM	77	3	3
2	AZ EM	5	—	—
2	CM NA	5	—	2
3	AZ CM EM	1	—	—
3	AZ CM NA	28	—	—
3	CI CM NA	—	—	1

¹ For *A. butzleri*, n=139; *A. cryaerophilus* 1A, n=4; *A. cryaerophilus* 1B, n=31; total isolates, n=174

AZ, azithromycin; CI, ciprofloxacin; CM, clindamycin; EM, erythromycin; NA, nalidixic acid; TC, tetracycline

Table 5.4. Resistance patterns of *Campylobacter* isolates¹ on broiler carcasses resistant to two or more antimicrobials

No. of Resistances	Resistance pattern	No. of isolates with resistance pattern	
		<i>C. jejuni</i> (n=60)	<i>C. coli</i> (n=1)
2	CI NA	–	1
2	CM TC	3	–
3	CI NA TC	57	–

¹ For *C. coli*, n=27; *C. jejuni*, n=188; total isolates, n=215

CI, ciprofloxacin; CM, clindamycin; NA, nalidixic acid; TC, tetracycline

CHAPTER 6

SUMMARY AND CONCLUSIONS

The studies presented were based on three primary objectives: 1) To optimize methods of *Arcobacter* isolation and culture and to compare the prevalence of *Arcobacter* and *Campylobacter* on broiler carcasses from the poultry processing plant, 2) To optimize pulsed-field gel electrophoresis (PFGE) methods for the characterization of *Arcobacter* genetic strains diversity and compare the genetic diversity of the *Arcobacter* strains with that of *Campylobacter* strains, and 3) To examine antimicrobial drug resistance in the *Arcobacter* and *Campylobacter* isolates using broth-microdilution susceptibility testing, and to compare the antimicrobial resistance patterns between both organisms.

For the first objective, samples were incubated in Houf broth followed by plating on Brucella agar supplemented with 5% lysed horse blood and cefoperazone, vancomycin, and amphotericin B (CVA) for *Arcobacter* isolation. For *Campylobacter*, sample enrichment in Bolton broth was used in conjunction with plating on CVA medium. The overall prevalence of *Arcobacter* and *Campylobacter* species on broiler carcasses was 55.1% (179 of 325) and 78.5% (255 of 325), respectively. Using a species-specific multiplex polymerase chain reaction assay, *A. butzleri* was found most commonly (79.1%), followed by *A. cryaerophilus* 1B (18.6%). *A. cryaerophilus* 1A was found at low level (2.3%) and *A. skirrowii* was not isolated at all. Between *Campylobacter* species, the BAX[®] PCR identified *C. jejuni* (87.6%) far more commonly than *C. coli* (12.4%). As broiler carcasses were processed from pre-scalding to post-chilling, the percentage of *Arcobacter* isolated was decreased sharply, while pre-scalding and pre-chilling carcasses had the highest contamination rate of *Campylobacter*, 92% to 100% positive, respectively.

For the second objective, PFGE was used characterized 174 *arcobacters* and 215 *campylobacters* isolated from 325 broiler carcasses from the commercial poultry plant. The 24 h PFGE standardized protocol for *C. jejuni* described by the Centers for Disease Control and Prevention (CDC) PulseNet was

used to examine the genetic diversity of the *Campylobacter* isolates. This protocol was modified for *Arcobacter*, using a different restriction endonuclease (*KpnI*) and electrophoresis running conditions. *KpnI* restriction digestion of *Arcobacter* genomic DNA using an initial switch time of 6.76 s and a final switch time of 13.68 s on the CHEF Mapper instrument produced both distinguishable and indistinguishable PFGE patterns. The *Arcobacter* isolates showed a much higher percentage of single-isolate groups than the *Campylobacter* isolates (32.8% vs 2.3%). *Arcobacter cryaerophilus* 1B (61.3%) and *C. coli* (3.7%) showed the highest species in each single-isolate groups. A much larger number of multi-isolate PFGE groups were found for *Arcobacter* (25 groups) compared to *Campylobacter* (8 groups). Cluster analysis demonstrated that *Arcobacter* multi-isolate groups were far more diverse than those of *Campylobacter*. No major clustering in either organism was observed, nor did the isolates cluster by species or collection site. However, each group of the *Campylobacter* isolates was composed of isolates collected on a single sampling day in contrast to the *Arcobacter* groups.

For the third objective, 93.7% of the 174 *Arcobacter* and 99.5% of the 215 *Campylobacter* isolates were resistant to one or more antimicrobials. The highest level of resistance found among all *Arcobacter* isolates was to clindamycin at 88.5%, followed by azithromycin at 69.5% and nalidixic acid at 20.7%. Resistance to gentamicin and tetracycline was not observed in the *Arcobacter* isolates, but resistance to ciprofloxacin and erythromycin was found at low levels, 0.6% and 3.4%, respectively. Among the *Campylobacter* isolates, tetracycline resistance was by far the highest at 99.1%, followed by ciprofloxacin (27%) and nalidixic acid (27%). Resistance to clindamycin was found only in *C. jejuni* at 1.6%, and all *Campylobacter* strains were susceptible to azithromycin, erythromycin, and gentamicin. For multiple antimicrobial resistance, the *Arcobacter* isolates (71.8%) had a much greater frequency of resistance to two or more antimicrobials compared to the *Campylobacter* isolates (28.4%). The combination of resistance to azithromycin and clindamycin was the most frequent (47.7%, 83/174; 77 *A. butzleri*, 3 *A. cryaerophilus* 1A, and 3 *A. cryaerophilus* 1B). Of the *Campylobacter* isolates, resistance to ciprofloxacin, nalidixic acid, and tetracycline was the most frequent combinations (26.5%, 57/215) although it was only found in *C. jejuni*.

In conclusion, significant contamination of broiler carcasses by *Arcobacter* was found although less than that found for *Campylobacter* on broiler carcasses from a commercial poultry processing plant. The genetic diversity of *Arcobacter* as determined by PFGE was far greater than that of *Campylobacter*. The restriction enzyme *KpnI* should prove valuable for establishing a genotype database for *Arcobacter* and could provide more accurate epidemiological information on the sources of isolates. The multiple antimicrobial resistance patterns of *Arcobacter* were diverse and higher than those of *Campylobacter*. This information should provide new insights on the prevalence of *Arcobacter* on carcasses from different processing sites in the poultry plant. Further, the PFGE protocol developed for *Arcobacter* can assist tracking *Arcobacter* suspected in foodborne disease outbreaks. These results will be useful in improving the prudent use of antimicrobials in agriculture, especially food animal production, and intervention strategies for reducing *Arcobacter* and *Campylobacter* contamination of food products.