A survey of mechanically separated turkey for the presence of *Arcobacter* spp. and *Arcobacter butzleri* and the genetic variability of *Arcobacter butzleri* isolates

by

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Signatures have been redacted for privacy

For the Graduate College

This manuscript is dedicated to my family and friends for their support as I pursued my goals and dreams.

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GENERAL INTRODUCTION

In 1991 the genus *Arcobacter* was proposed for the aerotolerant, gram-negative, curved shaped rods formerly identified as *Campylobacter* spp. Because arcobacters and campylobacters possess similar morphological characteristics, significant work has been done in order to properly identify and characterize arcobacters. Traditional plating methods and dark field microscopy have been superseded by genetic probes and polymerase chain reaction methods. The methods provide reliable identification in a time and cost-efficient manner.

The incidence of *Arcobacter* spp. in our food supply is still uncertain. Various surveys have been performed but there are still so many unanswered questions about *Arcobacter* spp. and its prevalence and pathogenicity.

Thesis Organization

The alternate thesis format was used and the thesis consists of two chapters. The first chapter is a literature review which contains a brief characterization of *Campylobacter* spp. followed by a descriptive account of *Arcobacter* spp. The references for the review are included in this chapter. The second chapter contains a manuscript which will be submitted to Applied and Environmental Microbiology. The references for the paper are included in this chapter. The general conclusions of the research project and recommendations for future work follow the second chapter. Attached are appendices comprising of tables of data and photographs of results used for the research project. These items are not included in the manuscript to be submitted for publication.

CHAPTER 1. REVIEW OF THE LITERATURE

Introduction

In the late 1970s, a gram negative, aerotolerant, spirillum/vibrio-like organism was isolated from aborted bovine and porcine fetuses (13, 14, 21). This organism was unable to ferment or oxidize carbohydrates, was catalase and oxidase positive, grew optimally at 30°C, was unable to grow on the primary isolation medium used for campylobacters, and lacked the heat stable antigens A, B and C of *Campylobacter fetus* (13, 14). The organism exhibited a corkscrew motility under dark field microscopy and showed various colony morphologies on solid media (13, 14, 21). Based on preliminary DNA base composition it was proposed the aerotolerant organisms be placed in the genus *Campylobacter* (13, 36). Further biochemical and physiological tests established the relationship of the aerotolerant campylobacters to the genus *Campylobacter*. The aerotolerant campylobacters comprise the fourth taxa in the genus and were named *Campylobacter cryaerophila* sp. nov. (37).

Kiehlbauch et al. performed DNA-DNA hybridizations and phenotypic tests on 78 aerotolerant *Campylobacter* isolates obtained from humans and animals with diarrheal illnesses (27). Two different DNA homology groups were identified; *C. cryaerophila* and *"C. butzleri"* sp. nov (27). It was found that the majority of the human isolates (49 out of 52) and animal isolates (15 out of 26) belonged to *"C. butzleri"* (27). Vandamme et al. also performed DNA-rRNA hybridizations on 70 animal and human strains of *Campylobacter* spp. and related taxa (49). This work showed that the *Campylobacter* spp. and the related taxa belonged to the same phylogenetic group, rRNA superfamily VI,

but a high degree of heterogeneity was seen within this group (49). It was proposed that the aerotolerant campylobacters, *C. cryaerophila*, *C. nitrofigilis* and an unnamed *Campylobacter* spp. strain be placed in a new genus, *Arcobacter* (49). Thus the rRNA superfamily VI was reclassified to contain the following genera: *Campylobacter*, *Helicobacter*, *Wolinella* and the proposed genus *Arcobacter* (49). Based on the genotypic and phenotypic differences seen in the genera *Campylobacter* and *Arcobacter* it was proposed that the two genera be assigned to a new family, *Campylobacteraceae* (50).

Further studies of the 77 aerotolerant *Arcobacter* strains (originally identified as *C. cryaerophila*) and 6 reference strains consisting of *A. nitrofigilis*, *A. cryaerophilus* and *"C. butzleri"* were analyzed using a polyphasic approach (52). The strains were analyzed by DNA-DNA and DNA-rRNA hybridizations, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins, cellular fatty acid composition and percent homology based on DNA base ratios (52). As a result of these analyses, *"C. butzleri"* was renamed *A. butzleri* comb. nov. (27, 52). Four species of *Arcobacter* were proposed as a result of these analyses: *A. butzleri*, *A. cryaerophila* (subgroup 1A and 1B), *A. nitrofigilis* and *A. skirrowii* (52).

Arcobacters and campylobacters share many of the same biochemical and morphological characteristics, thus a brief background on campylobacters will help to understand why arcobacters are important to study.

Campylobacter spp. Background

In the late 1970s campylobacters emerged as human pathogens (46). The species of campylobacters most often associated as human pathogens include the theromotolerant

(30-42°C) campylobacters, *C. jejuni* and *C. coli*, which are responsible for 80-90 % of the enteric *Campylobacter* infections (25). Other species of campylobacters, *C. upsaliensis*, *C. hyointestinalis* and *C. lari*, also cause human illness (34). In this review of the literature, campylobacters will refer to *C. jejuni* and *C. coli*.

The genus *Campylobacter* was proposed in 1963 to encompass the microaerophilic vibrios (44). Campylobacters are gram negative, curved or spiral shaped rods (0.5-8 μm by 0.2-0.5 μm) (44) which possess a darting, corkscrew motility due to the monotrichous or amphitrichous flagella (40). Differentiating between *Campylobacter* species is difficult because the organisms are relatively biochemically inert in routine laboratory tests. Campylobacters do not ferment or oxidize carbohydrates but instead use the respiratory type of metabolism which uses amino acids and the tricarboxylic acid cycle intermediates for energy (18, 40). Campylobacters are also microaerophilic, thus require oxygen concentrations of 5-10 % and carbon dioxide concentrations of 3-10 % in order to grow (6).

Campylobacters have been isolated from poultry (2, 30, 42, 60), cattle (2, 30), swine (2, 30), raw milk (2), shellfish (2) and drinking water (2). The thermotolerant campylobacters thrive in poultry, because the body temperature of the birds is between 42°C and 45°C (2). A limited number of surveys for the prevalence of thermophilic *Campylobacter*, especially *C. jejuni*, in turkey meat have been performed (30, 42, 60). The prevalence of *C. jejuni* in turkey ranges from 0.0-90 % (30, 42, 60).

Lammerding et al. isolated *Campylobacter* from 73.7 % of the turkey carcasses (n=205) using a modified Rosef's enrichment broth and Mueller-Hinton agar

supplemented with 10 % citrated sheep blood, vancomycin, trimethoprim and Polymyxin B (30). Using Lior's biotyping scheme to characterize the *Campylobacter* isolates, it was found that *C. jejuni* biotypes I and II were the most predominant in the turkey carcasses (30).

The prevalence of *C. jejuni* in fresh and frozen turkey wings purchased from a supermarket was the focus of one study (42). The method of detection for *C. jejuni* consisted of a wash fluid containing nutrient broth supplemented with polymyxin B, trimethoprim lactate and vancomycin followed by plating on brain heart infusion agar supplemented with 5 % lysed bovine blood, polymyxin B, trimethoprim lactate and vancomycin (42). The prevalence of *C. jejuni* in the fresh turkey wings (n=184) was 64.1 % and in the frozen turkey wings (n=81) was 55.6 % (42).

Another study looked at the prevalence rate of *C. jejuni* at the various slaughter stages at two turkey processing plants (60). The method of detection for *C. jejuni* consisted of an enrichment for carcass washes which contained polypeptone, yeast extract and sodium chloride supplemented with polymyxin B sulfate, trimethoprim lactate and vancomycin followed by plating on brain heart infusion agar supplemented with 5 % lysed bovine blood, polymyxin B sulfate, trimethoprim lactate and vancomycin (60). *Campylobacter jejuni* had the highest prevalence rate in the inedible portions in the turkey slaughter (60). The ceca, the feather picker drip water, the recycled water for cleaning gutters and the final carcass wash water had the highest contamination rates at 90.0, 80.6, 77.8 and 44.4 %, respectively (60). *Campylobacter jejuni* was not detected on the edible portions of the turkey, hearts, livers, wings and mechanically deboned meat.

(60). The reason for the absence of *C. jejuni* in the edible portions may be due to either the washing of all parts in chlorinated water (14-18 ppm) or undetectable levels of *C. jejuni* contamination of the parts (60).

Campylobacteriosis is a foodborne disease which causes more cases of bacterial diarrhea in the world than salmonellae (16). Outbreaks of campylobacteriosis are often due to contaminated water and milk, whereas sporadic cases are often associated with raw or undercooked poultry (2). Clinical symptoms of campylobacteriosis can range from mild to severe. In mild, non-inflammatory cases it produces a profuse watery diarrhea. In the severe cases it produces an inflammatory disease which causes slimy, bloody diarrhea, acute abdominal pain, fever and malaise (18, 25, 52). Complications due to *C. jejuni* infections can even result in Guillain-Barre syndrome in adults and children (10, 24, 43). Slaughter plants and consumers must practice safe food handling practices in order to reduce the risk of campylobacteriosis (2)

Arcobacter spp.

Characteristics

Arcobacter spp. are gram negative, non-sporeforming, curved, S-shaped or helical rods (0.2-0.9 μm by 1-3 μm) (49, 52). Motility is due to a single, polar, unsheathed flagella which causes the organism to have a darting, corkscrew motion, similar to that of *Campylobacter* (27, 49, 52). The organisms will grow at 15, 25, 30 and 37°C, but are unable to grow at 42°C, the temperature for the thermotolerant campylobacters (27, 49). *Arcobacter* spp. are capable of growing under aerobic conditions at 30°C and under

anaerobic conditions at 35-37°C, but the optimum growth occurs under microaerophilic conditions (3-10 % oxygen) (27, 52).

Arcobacter spp. are relatively biochemically inert in routine laboratory tests, thus it is often difficult to identify *Arcobacter* spp. based on the common phenotypical tests (52). Contradictions in the literature also make identification of *Arcobacter* spp. difficult. It has been reported that all species of *Arcobacter* are positive for catalase activity, but *A. butzleri* is only weakly positive (49, 52). Kiehlbauch et al. found that *A. butzleri* was either negative or weakly positive for catalase activity (27) and Schroeder-Tucker et al. found that only 50 % of the isolates tested were weakly positive for catalase activity (45). All species are positive for oxidase activity (27, 49, 52). All species are unable to ferment or oxidize carbohydrates, thus organic acids and amino acids serve as the carbon source for the organisms (27, 49, 52). All species are negative for hippurate hydrolysis, urease activity, hydrogen sulfide production and reduction of nitrite (27, 49, 52).

There are few characteristics that distinguish the various species of *Arcobacter* form one another. *Arcobacter butzleri* produces whitish, round colonies (2-4 mm in diameter) on blood agar (27, 52). They also have the ability to grow on MacConkey agar as well as reduce nitrate (52). Other characteristics of *A. butzleri* produce variable results in various strains, so they are less likely to aid in proper identification (52). *Arcobacter cryaerophilus* (formerly *A. cryaerophila*) subgroups 1A and 1B produces small, watery, beige to yellow, irregular sized colonies on blood agar (27, 36, 52). *Arcobacter skirrowii* produces grayish, flat, irregular sized colonies on blood agar (52). Most strains of *A. skirrowii* are alpha-hemolytic, and will not grow on MacConkey agar or in the presence

of 1 % oxgall (52). *Arcobacter nitrofigilis* produces whitish, round colonies on blood agar (52). Key distinguishing characteristics of *A. nitrofigilis* is its nitrogenase activity and its ability to grow in 1.5 % NaCl (52). With only a few differentiating phenotypical characteristics to identify *Arcobacter* spp. there is a great chance for the organisms to be misidentified either as other *Arcobacter* or as *Campylobacter* (20, 52).

Sources

Of the four species of *Arcobacter* only *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been isolated from animals (56). *Arcobacter butzleri* have been associated with or have been the causative agent in aborted porcine fetuses (45), animals with diarrheal illnesses (27, 52) and macaques suffering from diarrhea and colonic lesions (1). *Arcobacter cyaerophilus* have been associated with or have been the causative agent in aborted bovine and porcine fetuses (13, 14, 21, 45), reproductive tracts of farm animals (37), animal feces (37) and from dairy cows with mastitis (33). *Arcobacter skirrowii* have been isolated from preputial fluids of bulls, porcine, bovine and ovine aborted fetuses and from feces of diarrheic animals (52). The significance of *A. skirrowii* in animals is still relatively unknown (52).

Human infections caused by *Arcobacter* are primarily due to *A. butzleri* and *A. cryaerophilus* with *A. butzleri* as the primary human pathogen (56). *Arcobacter cryaerophilus* have been isolated from a human stool sample (48) and *A. cryaerophilus* 1B have been isolated from a uremic patient with hematogenous pneumonia (22). *Arcobacter butzleri* have been associated with or have been the causative agent in humans with enteritis (27). *Arcobacter butzleri* have been isolated from Thai children with

diarrhea (47), from nursery and primary school age children with abdominal cramps (41, 51), from patients with chronic diseases and diarrhea (32) and from a neonate with bacteraemia (39). The pathogenicity of *A. butzleri* will be discussed in the next section.

The prevalence of *Arcobacter* spp., particularly *A. butzleri*, in our food supply is relatively unknown. *Arcobacter butzleri* has been isolated from ground pork (8, 9), poultry (9, 15, 29) and water sources (11, 15, 23, 26).

Pathogenicity

Arcobacter butzleri, *A. cryaerophilus* and *A. skirrowii* are considered animal pathogens (56). The roles *A. butzleri* and *A. cryaerophilus* play in livestock abortions (13, 14, 21, 45), reproductive tract problems, such as, vaginal discharge from infertile sows (37) and experimental trials to induce animal abortions (21) allow for the organisms to be classified as animal pathogens. Other studies also indicate that *A. butzleri* and *A. cryaerophilus* are pathogens. In the study with macaques with diarrhea and colonic lesions, seven of the macaques had *A. butzleri* as the only identified pathogenic organism (1). Another study showed that *Arcobacter* spp., especially *A. butzleri*, were able to colonize cesarean-derived colostrum-deprived 1 day-old piglets (58). *Arcobacter butzleri* were cultured from both rectal swabs and tissues whereas *A. cryaerophilus* and *A. skirrowii* were only cultured from rectal swabs (58). Attempts to infect birds with *A. butzleri* have been unsuccessful (58). Logan et al. were successful in experimentally infecting four Friesian cows with *A. cryaerophilus* which caused mastitis 120 hours post inoculation (33).

In humans, *A. butzleri* and *A. cryaerophilus* are considered pathogens, with the majority of the isolates identified as *A. butzleri* (27, 52). Clinical symptoms of *A. butzleri* include diarrhea, nausea, vomiting, abdominal pain, fever, chills and malaise (26). Not all of these symptoms occur in each patient, but a combination of several symptoms is usually common. Some patients even have blood or mucus present in their stools (26).

Various outbreaks and isolated cases of *A. butzleri* and *A. cryaerophilus* support the claim of these two species as pathogens. As stated in the previous section, *A. butzleri* were isolated from a neonate with bacteraemia (38). In this case, *A. butzleri* were responsible for the infections which probably occurred *in utero* (38). The *A. butzleri* infection was finally controlled after numerous attempts to treat the infant with various antibiotics (38). *Arcobacter butzleri* is also responsible for an outbreak in nursery and primary school age children in Rovigo Italy (41, 51). In this outbreak, ten children (ages 3 to 7) suffered from recurrent abdominal cramps (41, 51). None of the children suffered from any other clinical symptoms previously described (41, 51). *Arcobacter butzleri* were isolated from the feces of the infected children (41, 51). This outbreak also suggested person-to-person transmission of *A. butzleri* (41, 51). In another case study, *A. butzleri* were isolated form two patients, one patient with diabetes mellitus type I and another patient with hyperuricemia and alcohol abuse (32). Both patients suffered from diarrhea and abdominal cramps (32). Again, *A. butzleri* was the only pathogen isolated from the fecal samples, and is thus believed to be the causative agent (32).

Arcobacter cryaerophilus have also been isolated from human fecal and blood samples (22, 48). In one case study, *A. cryaerophilus* were isolated from a 35 year old

homosexual male, who was not immunocompromised, but suffered from intermittent diarrhea and abdominal pain (48). *Arcobacter cryaerophilus* was the only pathogen found in the fecal sample (48). In another case study, *A. cryaerophilus* 1B was isolated from a 72 year old uremic woman with hematogenous pneumonia (22). The only bacterium isolated from this woman was *A. cryaerophilus* 1B which is thought to have caused the bacteremia (22). This case study also showed *A. cryaerophilus* 1B had an invasive nature (22).

With *Arcobacter* spp. emerging as a human pathogen it is important to be able to identify *Arcobacter* spp. from animal and food sources as well as from the person suffering from the food borne disease.

Methodology

There are several methods used to identify *Arcobacter* spp. from food and human samples. The traditional techniques which may prove to be inconclusive are giving way to the genetic based methods as a means of identification.

Enrichments

In order to have the number of arcobacters at high enough concentrations to enumerate, perform biochemical tests or to use for DNA based analyses an enrichment procedure is usually needed (8, 9, 31). Ellis et al. found that the semisolid Leptospira isolation medium, Ellinghausen-McCullough-Johnson-Harris Polysorbate-80 (EMJH P-80) consisting of phosphate buffer, salt, copper, zinc, and iron solutions, L-cystine, cobalamin (B-12), thiamine HCL (B-1), tween 80 and agar supplemented with 100 μg per

ml of 5-fluorouracil (12) worked best to isolate *Arcobacter* spp. from fetal organs and fluids (13, 14).

EMJH P-80 has been used in conjunction with selective and non-selective media to isolate *Arcobacter* spp. from ground pork (8). Collins et al. found that the use of EMJH P-80 as an enrichment and then plating onto a selective media of either cephalothin, vancomycin and amphotericin B (CVA) agar or modified cefsulodin-irgasan-novobiocin (CIN) agar, proved to have a better capabilities of isolating *Arcobacter* spp. from ground pork than the EMJH P-80 enrichment and filtering on a non-selective medium, brain heart infusion agar supplemented with 10 % defibrinated bovine blood (8). Using this method, the survey isolated *Arcobacter* spp. from 89.9 % of the pork samples (n=149) tested in the first survey and from 90.0 % of the pork samples (n=30) tested in the second survey (8). This work showed that EMJH P-80 could be used to isolate *Arcobacter* spp. from meat samples (8) which is important since EMJH P-80 had only been used to isolate *Arcobacter* spp. from animal tissue and fluid samples (13, 14, 21).

Recently, two new selective enrichment media have been described for isolating *Arcobacter* spp. from meat and poultry samples. The first new selective enrichment broth is a modification of Rosef's broth which contains peptone, Lab Lemco Powder, yeast extract, NaCl, resazurin and cefoperazone and has been used to isolate thermophilic campylobacters (31). Following incubation, the enrichment broth is filtered onto a selective medium of modified CCDA (charcoal agar) supplemented with cefoperazone, to allow the arcobacters to grow (31). Using this method, *A. butzleri* was isolated from 96.8 % of the broiler chicken carcasses (n=125) tested and from 85.7 % of the fresh

ground turkey samples (n=7) tested (31). The efficacy of this selective enrichment broth and solid medium were also examined (31). The selective enrichment broth and solid medium was successful in inhibiting the growth of competing microorganisms as long as a filtration step was used when plating from the enrichment broth (31).

The other selective enrichment broth, Arcobacter Selective Broth (ASB), consisting of Brucella broth powder, lysed horse blood, piperacillin, cefoperazone, trimethoprim and cycloheximide (9). Following incubation the ASB was plated on the isolation medium, Arcobacter Selective Medium (ASM), consisting of Mueller-Hinton broth, agar no. 3, piperacillin, cefoperazone, trimethoprim and cycloheximide (9). Using this method, *Arcobacter* spp. was isolated from 24.1 % of the poultry samples (n=220) tested and at lower rates (0.5-4.9 %) for pork and beef samples (9). The ASB and ASM did prove to be effective in isolating *Arcobacter* spp. from poultry, beef and pork samples while inhibiting the growth of competing microorganisms (9). It should be noted that neither of the new selective enrichments, the modified Rosef broth used with modified CCDA and the ASB used with ASM, were compared with any other enrichment procedure, such as EMJH P-80, for efficiency of recovery.

Genetic Probes

Genetic probes have provided microbiologists with a unique tool to detect, identify and characterize foodborne pathogens (5). Genetic probes consist of a DNA or RNA sequence that hybridizes to a highly conserved DNA or RNA sequence found only in a specific microorganism (5). This allows for probes to be either genus- or species-specific (5).

Since *Arcobacter* spp. does not react in assays routinely used in clinical laboratories and has similar morphological characteristics of *Campylobacter* spp., *Arcobacter* spp. can easily be misidentified as *Campylobacter* spp. (57). Thus an *Arcobacter* genus-specific 16S rRNA-based DNA probe (23-mer) and an *A. butzleri* species-specific 16S rRNA-based DNA probe (27-mer) were developed (57). The two probes for *Arcobacter* spp. and *A. butzleri* can be used in identification and thus eliminate the misidentification problems (57).

Ribotyping

Another method used to identify the various species of *Arcobacter* spp. is restriction fragment length polymorphisms of rDNA (ribotyping) (27, 28, 45, 57). This method relies on restriction endonucleases, such as Pvu II or Cla I, to restrict the DNA at specific sites followed by hybridization with a labelled 16S rRNA probe targeting the genes encoding 16S rRNA (27, 28). A distinct pattern for various species is obtained (27, 28). Ninety percent of the *A. butzleri* isolates exhibit a restriction fragment of 3.0-kb, whereas, all *A. cryaerophilus* (subgroup 1A and 1B) exhibit a restriction fragment of 3.2-kb (28). *Arcobacter cryaerophilus* 1B also exhibits a 2.6-kb restriction fragment (28). Kiehlbauch et al. used ribotyping to discriminate between *Arcobacter*, *Campylobacter*, *Helicobacter* and *Wolinella* as well as between the species found in each genus (28). Ribotyping is labor intensive thus it is being replaced by more rapid methods of detection.

Polymerase Chain Reaction

Traditional techniques, such as plating and biochemical and serological tests, are often very time consuming (5-10 days) and can lead to inconclusive results and

misidentification of the microorganisms (7, 57). Polymerase chain reaction (PCR) methods provide microbiologists with a way to detect and characterize microorganisms in a reliable and timely manner (1-3 days) (7). PCR can be used to identify the genus and/or species of a microorganism as well as to type or fingerprint the DNA of various strains (4, 7, 19, 20, 53).

PCR has proved to be a reliable method for detecting *Arcobacter* spp. and *A. butzleri* using purified DNA, bacterial cell lysates and directly from enrichment cultures (4, 19, 20). Bastyns et al. developed a set of genus-specific and species-specific primers based on the most variable region of the 23S rDNA gene of arcobacters (4). The genus-specific primers allowed for the identification of *Arcobacter* spp. and the species-specific primers were able to distinguish between *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (4). The variable region of the 23S rDNA gene was chosen based on the previous work on campylobacters in which species-specific primers were developed for identification of *C. fetus*, *C. hyointestinalis*, *C. mucosalis* and *C. sputorum* (3).

Harmon et al. also designed genus-specific primers based on the 16S rRNA gene of arcobacters (19, 57). These primers allowed for the identification of *Arcobacter* spp. from purified DNA, bacterial cell lysates and directly from EMJH P-80 enrichments (19). While both sets of primers identify *Arcobacter* spp. and *A. butzleri*, neither assay allowed for concurrent identification of *Arcobacter* spp. and *A. butzleri*.

Recently, Harmon et al. developed a multiplex PCR assay which incorporates the primers of the 16S rRNA gene (19) and the primers of the 23S rRNA gene (4) to allow for the concurrent identification of *Arcobacter* spp. and *A. butzleri* (20). The multiplex

PCR assay provides rapid and reliable identification of isolates and therefore could be used to determine the prevalence of *Arcobacter* spp. in food and livestock studies (20).

PCR methods have been used to type or fingerprint DNA (7). Approaches used to type or fingerprint DNA include: restriction enzyme digest of PCR amplified DNA to produce restriction fragment length polymorphisms (RFLP) patterns on agarose gels (34), amplification of the enterobacterial repetitive intergenic consensus (ERIC) motifs by PCR (53) and random amplified polymorphic DNA (RAPD) analysis which uses arbitrary primers (about 10 base nucleotides) to amplify various DNA fragments by PCR (55, 58).

Vandamme et al. used the ERIC motif primers to fingerprint *A. butzleri* isolates recovered from a human outbreak in Italy (53). The outbreak isolates were shown to be genetically similar to each other but different from the reference strains used (53).

A few studies have been done to compare the usefulness and reproducibility of the ERIC and RAPD primers (17, 39). Penner et al. found that the reproducible results among six different laboratories using the RAPD primers can be obtained as long as the reaction conditions are held constant and the temperature in the PCR tube reaches the programmed temperature during the entire PCR cycles (39). Gao et al. examined the RAPD and ERIC primers (17). This study found the RAPD primers produced variable results when subjected to different conditions (17). The $MgCl_2$ concentration, template concentration, brand of *Taq* polymerase and the brand of DNA thermal cycler all appeared to affect the reproducibility of the RAPD primers (17). Suggestions have been made that the RAPD methods must be held constant in order to give reproducible results in all laboratories (17). Gao et al. did find that the ERIC primers gave reproducible

results among the trials; however, they only work for gram negative organisms that contain the ERIC sequences (17). The ERIC and RAPD primers do show significant promise in typing or fingerprinting isolates. While these methods do require purified DNA, the results can be obtained in a timely and cost-efficient manner (7).

Of the methods available for the detection of *Arcobacter* spp. from food, an enrichment used in conjunction with the genetic based probes and PCR methods provide a means of quick and reliable identification. The implementation of these methods will increase the ability to perform large scale surveys.

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CHAPTER 2. PREVALENCE AND GENETIC VARIABILITY OF *ARCOBACTER* AND *ARCOBACTER BUTZLERI* IN MECHANICALLY SEPARATED TURKEY

A paper to be submitted to Applied and Environmental Microbiology

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Abstract

A survey for *Arcobacter* spp. and *A. butzleri* in mechanically separated turkey was conducted during the winter, summer and fall seasons. *Arcobacter* spp. and *A. butzleri* were identified by polymerase chain reaction (PCR) and species-specific oligonucleotide probes. *Arcobacter* spp. was isolated from 77 % (303 out of 395) of the total mechanically separated turkey samples collected with 74 % (223 out of 303) of the samples positive for *A. butzleri*. Of the 121 *A. butzleri* isolates tested, 86 different fingerprinting patterns were obtained indicating multiple sources of contamination.

Introduction

Aerotolerant, vibrio-like organisms were first isolated from aborted porcine and bovine fetuses (7, 8) and classified as *Campylobacter cryaerophila* (20). After morphological (19), biochemical (20), and phenotypic (13) characterizations, as well as DNA-DNA (13) and DNA-rRNA hybridizations (25), it was proposed that the heterogeneous *C. cryaerophila* be placed into the new genus *Arcobacter* (25). The four species of *Arcobacter* include *A. butzleri*, *A. cryaerophila* (subgroups 1A and 1B), *A. skirrowii*, and *A. nitrofigilis* (27).

The epidemiology of *Arcobacter* spp. is not fully understood. *Arcobacter cryaerophilus* have been isolated from aborted livestock (20) and from human stool samples (22). *Arcobacter butzleri* has been cultured from livestock (1, 13, 18) and from humans with diarrheal and/or abdominal cramps (17, 23, 26). The clinical symptoms of *A. butzleri* suggest that it is a human pathogen (5).

Arcobacter butzleri have also been isolated from water (9, 12, 14), poultry (5, 9, 16), and pork (3, 5). The overall incidence of *A. butzleri* in the food supply is unknown. An enrichment method is needed to allow the *A. butzleri* to grow to detectable levels (5, 16). A standard enrichment protocol for the isolation of *Arcobacter* spp. from meat products is not available.

Various enrichment methods have been used in studies on the prevalence of *Arcobacter* spp. in meat products (3, 5, 16). The *Leptospira* semisolid medium, Ellinghausen-McCullough-Johnson-Harris Polysorbate-80 (EMJH P-80), has been used to enrich for *Arcobacter* spp. in ground pork (3). In this study, *Arcobacter* spp. was isolated from 89.9 % of the pork samples (n=149) tested during the first survey and 90.0 % of the pork samples (n=30) tested during the second survey (3). The Arcobacter Selective Broth (ASB) and the Arcobacter Selective Medium (ASM) developed by de Boer et al. have been used to enrich for *Arcobacter* spp. in poultry, beef and pork (5). Using this enrichment method, *Arcobacter* spp. was isolated from 24.1 % of the poultry samples (n=220) tested with lower recoveries (0.5-4.9 %) for beef and pork (5). Lammerding et al. used a modified Rosef broth to enrich for *Arcobacter* spp. in poultry products (16). Using this method *A. butzleri* was isolated from 96.8 % of the broiler

chicken carcasses (n=125) tested and 85.7 % of the fresh ground turkey samples (n=7) tested (16).

Proper identification of *Arcobacter* is needed in order to fully understand its epidemiological role in causing food borne illness. The morphological similarities between *Arcobacter* spp. and *Campylobacter* spp. may lead to the misidentification of the organisms when relying on the traditional plating methods and dark field microscopy (4, 11, 31). The two organisms do show some physiological differences: *Arcobacter* spp. grows at 15°C, in the presence of oxygen and in 1.5 % NaCl whereas *Campylobacter* spp. requires growth at 37°C under microaerophilic conditions (3 to 10 % oxygen) (27).

The use of oligonucleotide DNA probes (30) and PCR-based methods (11) provide an alternative method to identify *Arcobacter* spp. These methods are based on sequences which are specific for the 16S rRNA genes of *Arcobacter* spp. and *A. butzleri* (11, 30). Another application for PCR-based methods is DNA fingerprinting (28). This method relies on the enterobacterial repetitive intergenic consensus (ERIC) sequences found in gram negative organisms which allows for the differentiation of the isolates (28).

The Nationwide Raw Ground Turkey Microbiological Survey conducted by the Food Safety and Inspection Service (FSIS) found that 25.4 % of the raw ground turkey samples (n=295) were contaminated with *C. jejuni/coli* (10). Other surveys on turkey products indicate that *C. jejuni* contamination ranges from 0-90 % (15, 21, 33). In two different studies, *Arcobacter* spp. was isolated from 24.1 % of poultry samples (n=220) (5) and 85.7 % of fresh ground turkey samples (n=7) (16). In a pilot study on turkey skin

samples conducted in our laboratory, all samples (n=12) were found to be positive for *A. butzleri* (4).

The morphological similarities between *Arcobacter* spp. and *Campylobacter* spp. as well as their presence in turkey products led to surveying mechanically separated turkey. Mechanically separated turkey is widely used in the production of both cooked and raw processed meat products. The presence of *Arcobacter* spp. in mechanically separated turkey could represent a potential food borne hazard.

The objective of this study was to determine the prevalence of *Arcobacter* spp. and *A. butzleri* in mechanically separated turkey. In addition, the summer and fall *A. butzleri* isolates were analyzed for genetic variation by DNA fingerprinting.

Materials and Methods

Sampling techniques

Three surveys were conducted. The winter (initial) survey consisted of 100 mechanically separated turkey samples which were obtained from a poultry plant (A) on four separate dates in January and February, 1996. Two additional surveys, summer and fall 1996, were conducted after the initial survey data were analyzed. The summer and fall surveys were expanded to include the initial plant (A) along with two additional plants (B and C). The summer survey consisted of 145 mechanically separated turkey samples, 25 samples from each plant (except plant B which furnished 45 samples) collected on two separate dates in July and August, 1996. The fall survey consisted of 150 mechanically separated turkey samples, 25 samples from each plant collected on two separate dates in September, 1996. The mechanically separated turkey used in the

surveys consisted of a fresh homogeneous mixture composed of skin, tissue and non-meat ingredients such as salt and sodium nitrite (6.25 %). All samples were collected using the same method; 25 samples were sent per date with five samples (75 grams each) from each of five different combos of product (~2000 lb). The surface samples were obtained from the four corners and middle of the combo. The samples were collected by plant personnel and were shipped overnight on ice to the National Animal Disease Center, Ames, IA. Figure 1 shows a general schematic flow diagram for the identification of *Arcobacter* spp. and *A. butzleri* in the winter survey samples. Figure 2 shows a general schematic flow diagram for the identification of *Arcobacter* spp. and *A. butzleri* in the summer and fall survey samples.

Arcobacter spp. enrichment techniques

Upon delivery, 10 grams of each mechanically separated turkey sample were enriched in 50 ml plastic centrifuge tubes (Blue Max, Becton Dickinson, Lincoln Park, NJ) containing 20 ml of Ellinghausen-McCullough-Johnson-Harris Polysorbate-80 (EMJH P-80) semi-solid media (6) supplemented with agar and 100 mg of 5-fluorouracil per liter (3, 21). The winter enrichments were incubated (7 days, 30°C) and subcultured (1 ml of enrichment into 9 ml of fresh EMJH P-80) and incubated for an additional 3 days at 30°C. The summer and fall enrichments were incubated (3 days, 30°C) and subcultured (1 ml of enrichment into 9 ml of fresh EMJH P-80) and incubated for an additional 3 days at 30°C. The decrease in the number of days of incubation for the summer and fall enrichments did not affect the recovery rate of *Arcobacter* spp. in the samples (data not shown).

Arcobacter spp. identification

For all surveys, a 250 µl aliquot of each subculture was used to perform the PCR reaction for the detection of *Arcobacter* spp. positive samples. The aliquots were boiled (15 minutes, 110°C) and centrifuged (1 minute, 11,000 g). A 5 µl aliquot served as the PCR template. The reagents and conditions for the PCR reaction were as described (11). The amplified DNA product was analyzed by gel electrophoresis (120 V, 1 hr) on a 1.5 % agarose gel (Seakem ME agarose, FMC Bioproducts, Rockland, ME) using a 6.5 X 10 cm horizontal gel bed (Minnie the Gel-Cicle, Hoefer Scientific Instruments, San Francisco, CA) and TBE (0.09 M Tris, 0.09 M Boric acid, 0.002 M EDTA, pH 8.5) as the running buffer. The gel was stained with ethidium bromide, visualized with UV light and photographed as described (29).

Arcobacter butzleri identification

For the winter survey, the agarose gels containing the *Arcobacter* spp. amplicons were denatured in 0.5 M NaOH, 1.5 M NaCl (30 minutes, 4°C) and neutralized in 1 M Tris-base, 1.5 M NaCl, pH 5.5 (30 minutes, 4°C). The agarose gels were then transferred onto a 0.2 µm pore size nylon membrane (Nytron, Schleicher & Schuell, Keene, NH) using the Southern blotting technique (22) with the Turboblotter Rapid Downward Transfer System (Schleicher & Schuell). After transfer, the membrane was placed on filters saturated with 0.4 N NaOH (1 minute) and then on filters saturated with 0.025 M Na_2HPO_4 (1 minute). The immobilized DNA on the membrane was crosslinked on both sides using the UV Stratalinker 1800 (Stratagene, La Jolla, CA) (30).

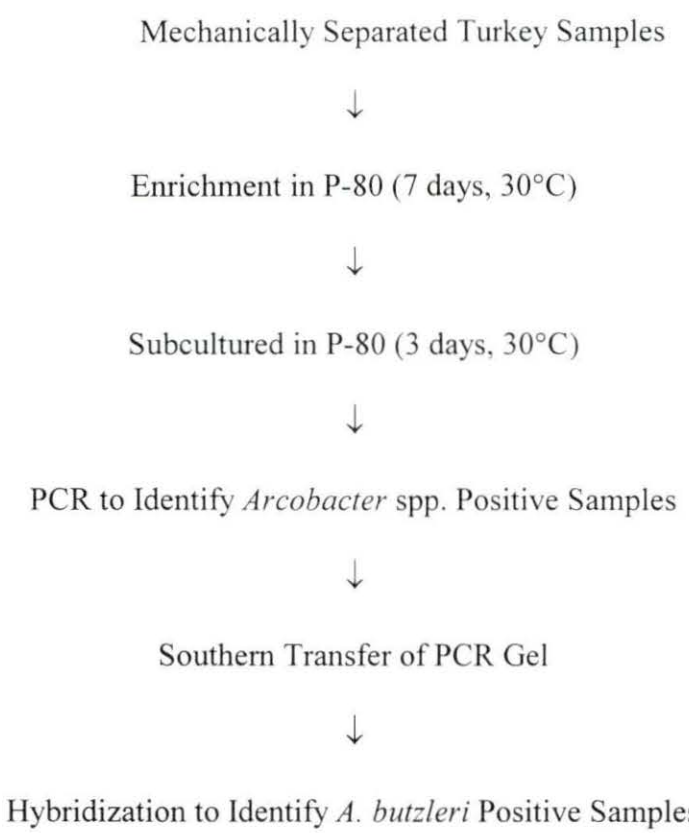

FIGURE 1. General schematic flow diagram for the identification of *Arcobacter* spp. and *A. butzleri* for the winter mechanically separated turkey samples.

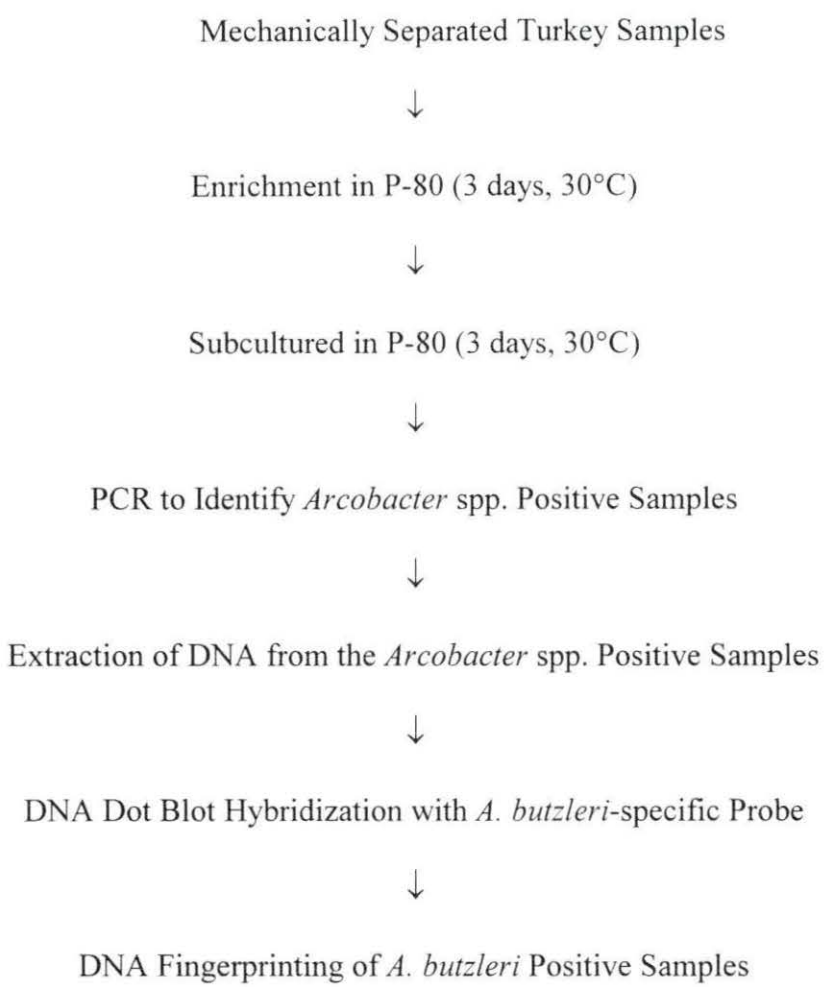

FIGURE 2. General schematic flow diagram for the identification of *Arcobacter* spp. and *A. butzleri* for the summer and fall mechanically separated turkey samples.

The membrane was prehybridized (3 hours, 37°C) using the Genius System hybridization solution (Boehringer Mannheim, Indianapolis, IN) and then hybridized (18 hours, 37°C) with the Genius System hybridization solution containing the digoxigenin (DIG)-labelled *A. butzleri* species-specific probe (30). After hybridization, the membranes were washed and incubated with the DIG detection system according to the manufacturer's directions (2). The membrane was exposed (60 minutes, room temperature) to X-ray film (X-Omat, Kodak, Rochester, NY) and then developed using the X-Omat Film Processor (Kodak) (30).

Isolates of *Arcobacter* spp. from the summer and fall surveys were identified as *A. butzleri* by dot blot hybridization with the species-specific probe (30). Purified DNA (2 μg) was immobilized on nylon membranes (Nytron, Schleicher & Schuell, Keene, NH) as described (29, 30). *Arcobacter butzleri* served as a positive control whereas *A. cryaerophilus* 1A and 1B served as negative controls for the assay. The membrane was probed with the *A. butzleri*-specific oligonucleotide probe, washed and exposed to X-ray film as described above.

DNA fingerprinting of isolates

The DNA from 121 of the summer and fall *A. butzleri* isolates were used to compare genetic variation using the PCR fingerprinting primers, ERIC 1R (5′-ATGT AAGCTCC TGGGGATTCAC-3′) and ERIC 2 (5′-AAGTAAGTGACTGGGGTGAG CG-3′) as described (28). The 50 μl PCR reaction mixture consisted of 25 pmol each of ERIC 1R and ERIC 2, 10mM Tris-HCl, 50 mM KCl, 2.0 mM $MgCl_2$, 200 mM each of the four dNTPs and 1.25 U of *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN).

PCR was performed in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) using conditions as described previously (28). The PCR product was analyzed by gel electrophoresis as described above. The photographs of the gels were scanned using the Gel Doc 1000 (Bio-Rad, Hercules, CA) and were then analyzed based on their profile analysis using the Molecular Analyst Software (Bio-Rad). This allowed for molecular weight values to be assigned to each of the isolates for comparison of banding patterns. Based on the molecular weights and visual inspection of the photographs differences between isolates were obtained.

Results

The initial (winter) survey consisted of samples from only plant A. *Arcobacter* spp. was isolated from 92 % (92 out of 100) of the samples with a total of 87 % (80 out of 92) positive for *A. butzleri*. The high prevalence rate of plant A in the winter survey led to a summer and fall survey in which the same plant plus two additional plants (B and C) were tested. Plant A had 96 % (191 out of 200) of the samples positive for *Arcobacter* spp. with 80 % (153 out of 191) positive for *A. butzleri* (Table 1). Plant B had 72 % (68 out of 95) of the samples were positive for *Arcobacter* spp. with 65 % (44 out of 68) positive for *A. butzleri* (Table 1). Plant C had 44 % (44 out of 100) of the samples positive for *Arcobacter* spp. with 59 % (26 out of 44) positive for *A. butzleri* (Table 1). A total of 77 % (303 out of 395) of the samples were positive for *Arcobacter* spp. with 74 % (223 out of 303) positive for *A. butzleri* (Table 1).

A total of 121 summer and fall *A. butzleri* isolates were analyzed for different DNA amplification patterns by PCR-based DNA fingerprinting (28). Eighty-six different

patterns were obtained from the 121 isolates. Twenty of the 86 patterns were repeated at least twice in either the same plant or in two different plants (Table 2). Overall, 71 % (86 different profiles for 121 isolates) of the isolates displayed unique DNA amplification patterns. In plant A, 64 % (38 different patterns for 59 isolates) of the isolates displayed unique patterns. In plant B, 89 % (32 different patterns for 36 isolates) of the isolates displayed unique patterns. In plant C, 81 % (21 different patterns for 26 isolates) of the isolates displayed unique patterns.

Discussion

Arcobacter spp. (77%) and *A. butzleri* (74 %) were present in 395 samples of mechanically separated turkey. Plant A had the highest recovery for both *Arcobacter* spp. (96 %) and *A. butzleri* (80 %) whereas plant C had the lowest recovery for both *Arcobacter* spp. (44 %) and *A. butzleri* (59 %). Although the cause of contamination was not examined, differences in recovery rates between plants could be due to several factors including the source of the birds, the plant environment and slaughter practices.

While plant A had the highest recovery for both *Arcobacter* spp. and *A. butzleri* it had the lowest percent (64 %) of differences in DNA amplification patterns of *A. butzleri* isolates among the three plants. The overall unique patterns (71 %) found in the summer and fall *A. butzleri* isolates indicates that contamination is probably due to multiple sources.

To aid in the identification of *Arcobacter* spp. positive samples, various modifications were tried. To detect *Arcobacter* spp. more efficiently, the number of incubation days for the enrichments was reduced from 7 days (winter survey) to 3 days

(summer and fall surveys). This modification did not affect the number of positive samples found in the survey (data not shown). PCR was also tried directly from the enrichment, but was unsuccessful due to protein denaturation of the tissue present in the enrichment. Subcultures proved to be essential in cleaning up the samples of protein and for possibly diluting potential PCR inhibitors. A direct PCR analysis was also attempted on the mechanically separated turkey samples; however, the bacterial load was not high enough to perform a direct PCR analysis. An enrichment step was needed in order to detect *Arcobacter* spp. from the samples.

In conclusion, this study shows the *Arcobacter* spp., especially *A. butzleri,* is prevalent in mechanically separated turkey. This survey employed the use of genetic-based analyses as a means of rapid and reliable identification. The high percent of DNA amplification pattern differences found among the *A. butzleri* isolates suggests that future studies should focus on seasonal and geographical variations, plant environment and sanitation practices as well as the survival of *A. butzleri* during thermal processing.

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TABLE 1. Recovery of *Arcobacter* spp. and *A. butzleri* isolated from mechanically separated turkey.

Plant	# Positive *Arcobacter* / # Samples Tested (%)	# Positive *A. butzleri* / #Samples Tested (%)
A[a]	191/200 (96%)	153/191 (80%)
B[b]	68/95 (72%)	44/68 (65%)
C[b]	44/100 (44%)	26/44 (59%)
Total[c]	303/395 (77%)	223/303 (74%)

[a] Tested in winter, summer and fall.

[b] Tested in the summer and fall.

[c] Combined data from all 3 plants.

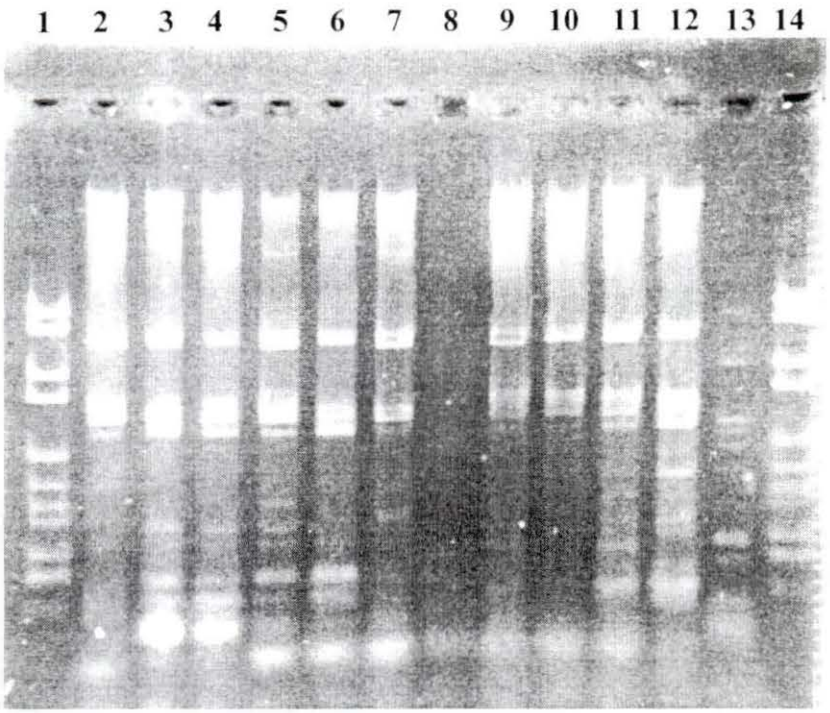

FIGURE 3. Representative PCR-based DNA fingerprints of *Arcobacter butzleri*. Lanes 1 and 14 contain the molecular weight marker VI (Boehringer Mannheim). Lanes 2-7 and 9-12 contain *A. butzleri* DNA from field samples. Lane 13 is a negative control for the PCR.

TABLE 2. DNA fingerprint patterns of *A. butzleri* isolates which repeat in either one plant or a combination of plants.

Pattern Number	Number of *Arcobacter butzleri* Isolates with the Same Pattern		
	Plant A (n=59)	Plant B (n=36)	Plant C (n=26)
2	8	1	
4	6		
5	2		
8	2		
9	2	1	
11	2		
12	1	1	
18	1	1	
25		2	
30		2	
36			3
51	1	1	
55		2	
59			3
61			2
65	2		
67	2		
68	2		
76	3		
84		2	

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GENERAL CONCLUSIONS

The goals of this study were to (1) determine the prevalence of *Arcobacter* spp. and *A. butzleri* in mechanically separated turkey and (2) to determine the genetic variations of *A. butzleri* isolates.

Arcobacter spp. and *A. butzleri* are present in mechanically separated turkey. A total of 77 % (303 out of 395) of the samples tested were positive for *Arcobacter* spp. Of the 303 samples positive, 74 % (223 out of 303) were positive for *A. butzleri.*

Several of the *Arcobacter butzleri* isolates recovered during the summer and fall (n=121) were DNA fingerprinted using a PCR-based method. This method showed that 71 % of the *A. butzleri* isolates had different fingerprinting patterns thus were genetically different. The genetic variation found among the isolates indicates multiple sources of contamination which may be due to the source of the birds or from the plant environment.

This study relied on the use of rapid detection methods, such as PCR to identify *Arcobacter* spp. and to DNA fingerprint the *A. butzleri* isolates. In a clinical or analytical laboratory time efficient and reliable methods are needed for detection and identification. This survey showed that the rapid methods can be employed to give reliable results.

Arcobacter spp. and *A. butzleri* have been shown to be present in mechanically separated turkey. Future studies as well as epidemiological surveys need to be done to further understand the role *A. butzleri* as a foodborne pathogen.

APPENDIX A

TABLES OF SEASONAL DATA

TABLE 1. Positive samples for *Arcobacter* spp. and *A. butzleri* isolated from mechanically separated turkey during the winter survey.

Plant	**# Positive** *Arcobacter* **/ # Samples Tested (%)**	**# Positive** *A. butzleri* **/ # Samples Tested (%)**
A	92/100 (92 %)	80/92 (87 %)

TABLE 2. Positive samples for *Arcobacter* spp. and *A. butzleri* isolated from mechanically separated turkey during the summer survey.

Plant	**# Positive** *Arcobacter* **/ # Samples Tested (%)**	**# Positive** *A. butzleri* **/ # Samples Tested (%)**
A	50/50 (100 %)	39/50 (78 %)
B	35/45 (78 %)	32/35 (91 %)
C	35/50 (70 %)	23/35 (66 %)
Total	120/145 (83 %)	94/120 (78 %)

TABLE 2. Positive samples for *Arcobacter* spp. and *A. butzleri* isolated from mechanically separated turkey during the fall survey.

Plant	**# Positive** *Arcobacter* **/ # Samples Tested (%)**	**# Positive** *A. butzleri* **/ # Samples Tested (%)**
A	49/50 (98 %)	34/49 (69 %)
B	33/50 (66 %)	12/33 (36 %)
C	9/50 (18 %)	3/9 (33 %)
Total	91/150 (61 %)	49/91 (54 %)

APPENDIX B

PHOTOGRAPHS OF RESULTS

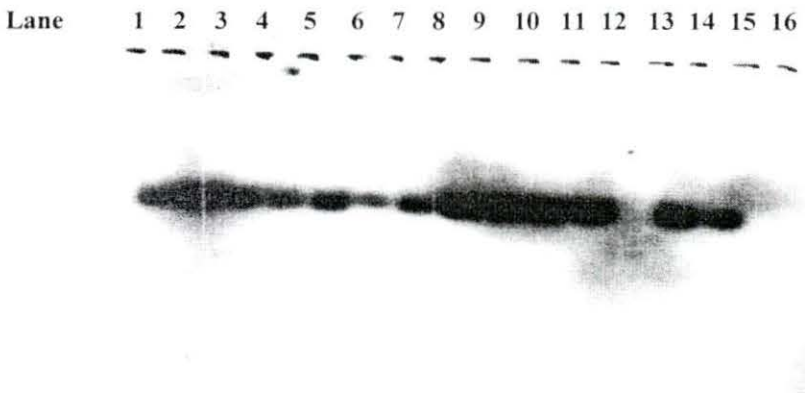

FIGURE 1. Southern blot membrane hybridized with the *Arcobacter butzleri* species-specific probe. Lanes 1 and 16 contain the molecular weight marker. Lanes 2-15 contain field samples.

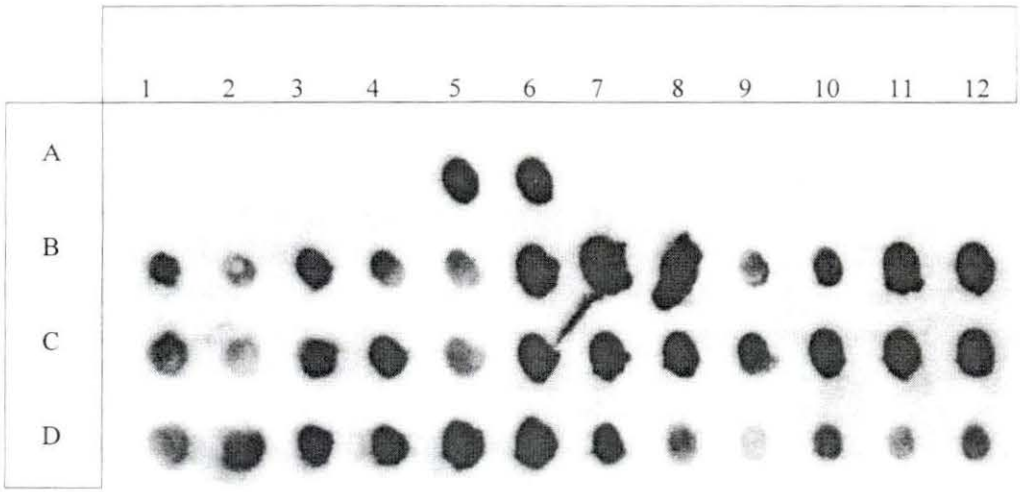

FIGURE 2. DNA dot blot of *Arcobacter butzleri* DNA of field strains isolated from mechanically separated turkey hybridized with the *A. butzleri* species-specific probe. Each sample was analyzed in duplicate. Wells A1-4 contain *A. cryaerophilus* (negative controls). Wells A5-6 contain *A. butzleri* (positive control). Wells A7-12 are empty. Wells B1-D12 contain field isolates.