PREVALENCE AND DIVERSITY OF *ARCOBACTER* SPP. IN POULTRY MEAT IN NEW ZEALAND

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Errata sheet

- Both the terms "isolate" and "culture" are used to mean the same thing.
- Sections 3.4.2.1/3.4.2.2- Numbers of poultry sampled was 150, each were cultured by seven different methods. A total of 210 isolates were obtained as presumptive arcobacters out of which 189 were confirmed as *Arcobacter spp.* by PCR.
- Section 3.4.2- The number of isolates is the number out of 189 (i.e. PCR identified isolates).
- Page 59- The PFGE patterns are of the cultures isolated simultaneously from the same poultry sample by more than one method.
- Discussion- There are a number of *Arcobacter* genus specific PCR. When the study was designed there was no information on the species *A. cibarius,* so it was not thought necessary to include genus-specific PCR.
- Section 4.1.2.1- Poultry rearing shed surroundings like effluent or stagnant water, are a good source of arcobacters (Gude et al., 2006), from whence the crates and transportation vehicles may be contaminated. Once introduced in a processing plant, arcobacters may remain viable in processing equipments and water (Houf et al., 2002b; Houf et al., 2003). Thus, in a slaughterhouse with poor hygiene, these sources (processing equipments and water) may contribute to heavy contamination (also cross contamination). Similarly, improper packaging practices may result in cross-contamination contributing ultimately to high contamination rates.
- Section 4.1.3.1- A reviewer commented "The statement that the source of contamination for producers B and C appeared to be lower than A is not strictly true." This statement was made based on the diversity index as arcobacters from Producer B and Producer C were less diverse compared to those from Producer A. However, less diversity does not necessarily mean a common (or few) sources

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ABSTRACT

The microaerophilic bacterium *Arcobacter* has received increased attention in recent years as an emerging foodborne human pathogen. Although phenotypically related, arcobacters differ from campylobacters in their ability to grow aerobically and at lower temperatures. Poultry are considered a significant reservoir of this organism, with an isolation rate of up to 72% in faecal samples, and up to 100% in meat samples. To date, four species; *A. butzleri*, *A. skirrowii*, *A. cryaerophilus*, and *A. cibarius* have been isolated from poultry. The first three species have also been found to be associated with human and animal illnesses such as diarrhoea, bacteraemia, mastitis and abortions. The organisms are also found in raw meat products as well as in surface and ground water. Since most laboratories still do not use appropriate isolation techniques, the occurrence of this organism in food sources and their role in human illnesses is greatly underestimated.

This is the first investigation of the prevalence of arcobacters in poultry meat in New Zealand. The aim of this study was to compare the most commonly used *Arcobacter* isolation methods. In addition, this study aimed to estimate the prevalence of *Arcobacter* spp. in retail poultry in New Zealand. Other aims include comparison of genetic diversity of *Arcobacter* spp. isolated from three different poultry producers, and by different methods, and estimation of overall genetic diversity of arcobacters present in New Zealand.

During the period of May to October 2005, a total of 150 fresh, whole, retail poultry carcass produced by three different producers were purchased through two supermarket outlets in Palmerston North, New Zealand. Isolation of *Arcobacter* was done by seven different techniques. *Arcobacter*-like organisms were identified presumptively by phenotypic tests; temperature tolerance, aerotolerance, motility, and oxidase production. These presumptive arcobacters were confirmed by a species-specific multiplex PCR (m-PCR) either as *A. butzleri*, *A. cryaerophilus* or *A. skirrowii*. DNA sequencing was done for selected isolates from both species to further confirm the PCR results. The PCR positive isolates were subjected to Pulsed-Field Gel Electrophoresis (PFGE) following restriction digestion with *Eag*I.

It was found that 55.3 % of 150 retail poultry sold in New Zealand were harbouring *Arcobacter* species. Two species; *A. butzleri* and *A. cryaerophilus* were detected by m-PCR which was later confirmed by sequencing. A total of 189 isolates were detected by six methods from 83 retail poultry samples. *A. butzleri* was the predominant species and was detected in 51.3% of the samples, whereas *A. cryaerophilus* was detected only in 8% of the samples. *A. butzleri* and *A. cryaerophilus* accounted for 92.6% (n=175) and 7.4% (n=14) of the isolates, respectively. *A. butzleri* was the only *Arcobacter* species present in 46.6% samples, and *A. cryaerophilus* only in 3.3% of the samples. Both species were detected simultaneously in 4.6% of the samples. There was a wide variation among the prevalence rate of *Arcobacter* spp. in retail poultry from different producers varying from 30 to 98%. There was also a wide variation among the isolation rates of different methods varying from 3.3 to 39.3%. The best isolation method was found to be *Arcobacter*-broth enrichment followed by passive filtration through a sterile filter of 0.45µm, onto blood-agar plates. No single isolation method detected all arcobacters. PFGE of *Arcobacter* isolates demonstrated the occurrence of multiple genotypes of both *A. butzleri* and *A. cryaerophilus* in the retail poultry from the same producers, and even in a single poultry. The possible explanations for the large amount of heterogeneity include multiple sources of contamination, the occurrence of multiple parent genotypes for both species in a single poultry carcass, and a high degree of genomic recombination among the progeny of historical parent genotypes.

This study highlights the high prevalence of *Arcobacter spp.* in poultry meat in New Zealand. It also indicates prevalence of arcobacters in poultry carcass varies greatly with the choice of isolation method and none of the currently available methods are appropriate for the detection of all species of arcobacters in New Zealand. Therefore, two or more methods should be used in parallel. The level of contamination of poultry carcass may vary with the processing practices of a slaughterhouse. To eliminate or reduce arcobacters in retail poultry, maintenance of slaughter hygiene is of utmost importance. This may be achieved by regular microbiological monitoring of carcasses according to the HACCP principles. Further studies comparing the fingerprinting pattern of *Arcobacter* spp. isolates obtained from retails poultry with human isolates are necessary to test the hypothesis that poultry meat is an important source for *Arcobacter* infection in human.

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LIST OF ABBREVIATIONS

AA	*Arcobacter* agar
AB	*Arcobacter* broth
AFLP	Amplified fragment length polymorphism
ASB	*Arcobacter* selective broth
ASM	*Arcobacter* selective medium
ATCC	American type culture collection
BPW	Buffered peptone water
CAT	Cefoperazone-amphotericin-trimethoprim
CCDA	Charcoal-cefoperazone-deoxycholate agar
CHEF	Contour clamped homogenous electric field
CIN	Cefsulodin-irgasan-novobiocin
CLO	*Campylobacter* like organism
CVA	*Campylobacter*-cefoperazone-vancomycin-amphotericin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
EMJH	Ellinghausen-McCullough-Johnson-Harris
ERIC	Enterobacterial repetitive intergenic consensus
ESR	Environmental Science and Research
FA	Fatty acid
FAME	Fatty acid methyl ester
FISH	Fluorescent *in situ* hybridization
HACCP	Hazard Analysis Critical Control Point
G+C	Guanine plus cytosine
ISH	*In situ* hybridization
Kb	Kilobase
MQ	Milli-Q
NCBI	National Centre for Biotechnology Information
OD	Optical density
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAPD	Random amplification of polymorphic DNA
REP	Repetitive extragenic palindromic
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TSI	Triple sugar iron
UPGMA	Unweighted pair group method using arithmetic averages

CHAPTER 1: LITERATURE REVIEW

1.1 General introduction

The family *Campylobacteraceae* includes the bacterial species belonging to the genera *Campylobacter* and *Arcobacter* (Vandamme and Ley, 1991). The genus *Arcobacter* (Latin for 'arc-shaped organism') includes bacteria that were formerly known as "aerotolerant campylobacters" because of their similarity with campylobacters, and ability to grow in atmospheric oxygen. The microbiological and clinical features of arcobacters are similar to campylobacters (Vandenberg *et al.*, 2004). However, members of the genus *Arcobacter* are differentiated from *Campylobacter* by their ability to grow at lower temperatures and in air (Neill *et al.*, 1985; Tee *et al.*, 1988; Kiehlbauch *et al.*, 1991a).

Arcobacters have received attention in recent years because of their association with food production, and animal and human illnesses. To date, seven species have been differentiated within the genus *Arcobacter*: *A. butzleri, A. skirrowii, A. cryaerophilus, A. cibarius, A. nitrofigilis, "Candidatus Arcobacter* sulfidicus*"* and *A. halophilus* sp. nov. Among these, the first three species have been isolated from various food-items as well as from animal and human illnesses. *A. cibarius* has recently been isolated from poultry meat (Houf *et al.*, 2005).

The remaining three species: *A. nitrofigilis, "Candidatus Arcobacter* sulfidicus*"* and *A. halophilus* are free-living and are not considered animal pathogens. *A. nitrofigilis* is a nitrogen-fixing bacterium occurring on the roots of *Spartina alterniflora,* a salt-marsh plant (McClung *et al.*, 1983). *"Candidatus Arcobacter* sulfidicus*"* is an autotrophic, sulphur oxidizing species found to be occurring in marine environments (Wirsen *et al.*, 2002). *A. halophilus* has recently been found to be occurring in hypersaline lagoon water (Donachie *et al.*, 2005).

In humans, arcobacters are mainly isolated from cases of gastroenteritis and septicaemia (Lehner *et al.,* 2005). These organisms have been associated with animal diseases

including abortion (Ellis *et al.*, 1977; Ellis *et al.*, 1978; Neill *et al.*, 1985; Fernandez *et al.*, 1995; On *et al.*, 2002), mastitis (Logan *et al.*, 1982) and diarrhoea (Wesley *et al.*, 2000). Poultry is considered to be the most significant reservoir as up to 72% of the cloacal content samples (Atabay *et al.*, 2006) and up to 100% of poultry meat samples (Houf *et al.*, 2001a; Morita *et al.*, 2004) has been found to be harbouring arcobacters. Besides poultry meat, other food items like beef, pork, and lamb may also be contaminated with arcobacters (Golla *et al.*, 2002; Vytrasova *et al.*, 2003; Rivas *et al.*, 2004). Furthermore, surface and ground waters have also been found to be contaminated with different species of *Arcobacter* (Dhamabutra *et al.*, 1992; Jacob *et al.*, 1993; Musmanno *et al.*, 1997; Jacob *et al.*, 1998; Rice *et al.*, 1999; Frias-Lopez *et al.*, 2002; Amisu *et al.*, 2003; Moreno *et al.*, 2003; Diergaardt *et al.*, 2004; Fera *et al.*, 2004; Maugeri *et al.*, 2004; Morita *et al.*, 2004).

Among the arcobacters, *A. butzleri* is the most common species associated with human and animal illnesses, as well as food items (Ho *et al.*, 2006). In humans, *A. butzleri* has been associated with enteritis, abdominal cramps (Vandamme *et al.*, 1992a), appendicitis, septicaemia and bacteraemia (Taylor *et al.*, 1991; Lerner *et al.*, 1994; On *et al.*, 1995; Hsueh *et al.*, 1997; Vandamme, 2000; Yan *et al.*, 2000). *A. butzleri* has also been isolated from various animals including primates, pigs, horses, and cattle; and from various food products including poultry, pork, beef, and lamb (Lehner *et al.*, 2005).

A. cryaerophilus has been isolated from humans with abdominal illness, septicaemia, and pneumonia (Tee *et al.*, 1988; Hsueh *et al.*, 1997; Engberg *et al.*, 2000). This species has also been isolated from aborted foetuses of cattle, pigs and sheep (Fernandez *et al.*, 1995; Neill *et al.*, 1980); from pig faeces and from cattle with mastitis (Vandamme, 2000). Preputial fluid of boars has also been found to be harbouring this species of *Arcobacter* (De Oliveria *et al.*, 1999).

Recently, *A. skirrowii* has been isolated from a case of chronic diarrhoea in an elderly patient (Wybo *et al.*, 2004). Among animals, this species has been recovered from sheep and cattle with diarrhoea; from aborted porcine, ovine and bovine foetuses, and from preputial fluids of bulls (Vandamme, 2000).

The role of *Arcobacter* spp. has not been clearly defined in terms of human foodborne illness (Hsueh *et al.*, 1997; Yan *et al.*, 2000; Houf *et al.*, 2001a; Wybo *et al.*, 2004), and the infection rate in humans has not been clearly established (Vandenberg *et al.*, 2004). Although the pathogenicity of the organism is not clearly understood, the cytotoxic effects of the enterotoxin produced have been reported (Musmanno *et al.*, 1997).

Little is known about the risk factors associated with *Arcobacter* infection in humans. Transmission is believed to be by the oral route, through consumption of contaminated food or water (Marinescu *et al.*, 1996a; Jacob *et al.*, 1998; Rice *et al.*, 1999). Human-to-human transmission may also occur (Vandamme *et al.*, 1992a). It has been suggested that, because of the phylogenetic proximity, transmission mechanisms that have been described for *C. jejuni* may be applicable to *Arcobacter* spp. (Wesley, 1997).

Despite wide-occurrence and high isolation rate in different foods and water, data on the incidence and clinical importance of *Arcobacter* in humans are scare. This may be because most laboratories do not use appropriate culture conditions to detect all *Campylobacter* spp. and related organisms (Vandenberg *et al.*, 2004). Also, difficulty in assessing the infection rate may be due to the transient nature of the infection and similarity of symptoms with campylobacteriosis coupled with failure of *Campylobacter* isolation techniques to detect this organism. It has been suggested that, when the detection is based entirely on culturing on selective media, approximately 95% of *Campylobacter* infections are found to be caused by *Campylobacter jejuni* or *C. coli*. However, with modifications in isolation and identification techniques, other related species, including *Arcobacter* spp., may also be detected (Lastovica *et al.*, cited in Vandenberg *et al.*, 2004). Thus, it appears that lack of use of a suitable isolation technique is hindering the estimation of the true prevalence of different species arcobacters and their public health significance.

1.2 Taxonomy and historical review

The genus *Arcobacter* is one of the four genera of the family *Campylobacteraceae* (Vandamme and Ley, 1991). To date seven species have been differentiated within the genus *Arcobacter*: *A. butzleri, A. skirrowii, A. cryaerophilus, A. cibarius, A. nitrofigilis, "Candidatus Arcobacter* sulfidicus" and *A. halophilus* sp. nov. Among these species, the first three have been isolated from various food-items as well as from animal and human illnesses. These bacteria were called 'aerotolerant campylobacters' until the present name '*Arcobacter*' was given by Vandamme *et al.* in 1991.

Aerotolerant *Campylobacter*-like organisms were first isolated in the UK from aborted bovine and porcine foetuses in the 1970s (Ellis *et al.*, 1977; Ellis *et al.*, 1978). The workers were unable to further classify these organisms at that time.

In 1983, the species *Campylobacter nitrofigilis* was proposed for a group of *Campylobacter*-like organisms isolated from the rhizosphere of *Spartina alterniflora*, a salt marsh plant (McClung *et al.*, 1983).

In 1985, Neill *et al.* performed an extensive phenotypic characterization of aerotolerant *Campylobacter* strains isolated from various animal sources. These organisms were designated a single species, *Campylobacter cryaerophila*, on the basis of aerotolerance and ability to grow at 25 °C (Neill *et al.*, 1985). They found that the aerotolerant strains were only distantly related to strains of the other *Campylobacter* species examined and concluded that these strains formed a novel group.

In 1991, following extensive DNA homology studies, the species *Campylobacter butzleri* was proposed for aerotolerant *Campylobacter*-like organisms isolated from human enteritis (Kiehlbauch *et al.*, 1991a). The genus name '*Arcobacter*' was described as a second genus within the family *Campylobacteraceae* to encompass the bacteria *Campylobacter nitrofigilis*, *Campylobacter cryaerophila*, and an unnamed *Campylobacter* sp. strain, formerly known as aerotolerant campylobacters (Vandamme and Ley, 1991). Later, these two species were named as *Arcobacter nitrofigilis* comb. nov. (type species) and

Arcobacter cryaerophilus comb. nov., respectively (Vandamme *et al.*, 1991). In 1992, based on the results of DNA-DNA hybridization, *Campylobacter butzleri* was transferred to the genus *Arcobacter* as *A. butzleri* comb. nov., and a new species *A. skirrowii* was proposed (Vandamme *et al.*, 1992b).

Within the genus *A. cryaerophilus*, two subgroups referred to as subgroup 1 or group 1A and subgroup 2 or group 1B have been differentiated (Kiehlbauch *et al.*, 1991a; Vandamme *et al.*, 1992b). Strains of these subgroups vary in their whole-cell protein and fatty acid profiles, restriction fragment length polymorphism (RFLP) patterns, and DNA-DNA hybridizations (Vandamme, 2000). However, because these subgroups are phenotypically indistinguishable, they are regarded as a single species (Vandamme, 2000).

The publication of new species within the genus *Arcobacter* is ongoing. A novel group of bacteria occurring in sea water oxidizing sulphur derivatives were found to be phylogenetically related to *Arcobacter* and have been placed in the category *Candidatus* as "*Candidatus Arcobacter* sulfidicus" (Wirsen *et al.*, 2002). The occurrence of a "*Arcobacter skirrowii*-like" species in pig abortions and turkey faeces has been mentioned (On *et al.*, 2003). The existence of *Arcobacter cibarius* as a fourth species occurring in retail poultry carcasses has recently been published (Houf *et al.*, 2005). The nomenclature of a single bacterial isolate obtained from saline lagoon water has been published as *Arcobacter halophilus* (Donachie *et al.*, 2005).

1.3 Microbiology of arcobacters

1.3.1 Morphology

The members of the genus *Arcobacter* are Gram-negative, non-spore forming bacilli, curved, helicoid or S-shaped, 0.2 to 0.9 μm wide and 0.5 to 3 μm long (Vandamme, 2000). Cells in old cultures may form spherical or coccoid bodies and loose spiral filaments up to 20 μm long. The organisms display a corkscrew-like or darting motility by means of a single polar unsheathed flagellum at one or both ends of the cell (Ellis *et al.*, 1977; Vandamme, 2000).

A. butzleri has a diameter of 0.2 to 0.4μm and is 1 to 3 μm in length. After 3 days of incubation on blood agar, the colonies have a diameter of 2 to 4 mm, generally round shaped, and are whitish in colour (Euzeby, 2005).

A. cibarius is a slightly curved bacillus, having a diameter of 0.5μm and length of 1.5μm. The species is slightly motile although some cells have a very clear motility. After 3 days of incubation at 28°C in microaerobic atmosphere, the colonies obtained on blood-agar are whitish, slightly convex, round, smooth, nonhaemolytic, and about 2 mm in diameter (Euzeby, 2005).

A. cryaerophilus has an average size of 0.4×1.8 μm, with some forms longer than 20 μm. After 2-3 days of incubation, the colonies are smooth, convex and 1 mm in diameter, and have a regular contour (Euzeby, 2005).

A. skirrowii has a diameter of 0.2 to 0.4 μm and length of 1 to 3 μm. After 3 days of incubation, the colonies obtained on blood agar plates have a diameter of 2 to 3 mm and are often alpha-haemolytic. They are greyish and tend to spread out over the wet medium (Euzeby, 2005).

1.3.2 Growth and survival

In general, biochemical and physiological characteristics are similar in the members of the family *Campylobacteraceae* (Vandamme, 2000). Energy is obtained from amino acids or tricarboxylic acid cycle intermediates, not from the carbohydrates since the latter are neither fermented nor oxidized (Ellis *et al.*, 1977). Cells have a respiratory and chemoorganotrophic type of metabolism. Microaerophilic conditions are needed for primary isolation, but upon subsequent subculture, the organisms become more tolerant to atmospheric oxygen (Tee *et al.*, 1988).

All the species of *Arcobacter* grow at 15, 25 or 30°C and growth is optimal in a microaerobic atmosphere (3 to 10% oxygen), but they can grow in atmospheric oxygen or in anaerobic conditions. Under aerobic conditions, these bacteria grow well at 15 and 30°C, and under anaerobic condition at 35 to 37°C (Euzeby, 2005). Optimum pH requirement ranges from 6.0 to 7.0 for *A. butzleri*, and 7.0 to 7.5 for *A. cryaerophilus* (D'Sa and Harrison, 2005).

Arcobacters can survive freezing for up to 6 months at -20°C and for up to 24 months at -70°C, but are rapidly inactivated by heating to 55°C and above (D'Sa and Harrison, 2005). They are susceptible to normal chlorination procedures used for water treatment plants (Rice *et al.*, 1999) and to γ irradiation (Collins *et al.*, 1996b).

1.3.3 Isolation

Because of their fastidious growth requirements, isolation of arcobacters from meat or environmental samples requires an enrichment step. Also, to suppress the accompanying contaminants in samples, a variety of antibiotic supplements are often needed to be incorporated in the media to make them 'selective'. As the cultural characteristics of campylobacters and arcobacters are similar, methods used for isolation of arcobacters have been derived from those developed for campylobacters. Most commonly used ingredients for *Arcobacter* media are shown in Table 1.

The first isolation of arcobacters was done by Ellis *et al.* (1977) from aborted bovine foetuses. They had used Ellinghausen–McCullough–Johnson–Harris (EMJH) isolation medium containing rabbit serum (2%), agar (0.15%), with and without 5-fluorouracil (100 mg/L). The incubation was done at 30°C. They were able to obtain arcobacters from the internal organs of 15 of the 34 aborted foetus samples, and nine of the 17 control foetuses.

An enrichment broth and selective plating medium for the isolation of arcobacters from food samples has been described (Lammerding *et al.* 1996). The enrichment broth contained peptone, Lab Lemco powder, yeast extract, NaCl, resazurin, and cefoperazone. The plating medium was a modification of CCDA (Charcoal-cefoperazone-deoxycholate

agar), supplemented with cefoperazone (32 mg/L). The isolation protocol involved incubation in enrichment broth, filtration of the broth through 0.45μm pore size membrane, and plating onto modified CCDA plates. It was found that the enrichment broth and the modified CCDA plates (in combination with filtration) inhibited the growth of *P. aeruginosa*, *E. coli*, *S. aureus*, *Salmonella* sp., *C. jejuni*, and *L. monocytogenes*, but not that of arcobacters. Using this protocol, 97% of 125 poultry carcasses in Canada were found to be harbouring arcobacters.

Table 1. Commonly used ingredients and antibiotic supplements in *Arcobacter* media, and their specific properties

Ingredients		Composition of *Arcobacter* media (mg/L)					
Name	Properties	AB	CAT broth	AA	CAT agar	J&M broth	J&M agar
Bile salts	Makes media selective for Gram-negative enteric bacteria					250	
Charcoal	Quench toxic oxygen compounds					3%	
Lysed blood	Quench toxic oxygen compounds	5%	5%				5%
Sodium pyruvate	Source of carbon	500				500	500
Sodium thioglycolate	Maintains reducing conditions in media	500				500	500
5-fluorouracil	Inhibits campylobacters and promotes arcobacters	100		100			
Amphotericin B	Antifungal antibiotic	10	10	10	10		
Cefoperazone	Inhibit Gram-negative bacteria, mainly enteric flora	16	8	16	8	32	32
Novobiocin	Inhibit Gram-positive bacteria	32		32			
Teicoplanin	Inhibit Gram-positive bacteria		4		4		
Trimethoprim	Inhibit Gram-negative bacteria	64		64			

(AB=*Arcobacter* broth; AA=*Arcobacter* agar; J&M= Johnson and Murano medium)

The development of an isolation protocol for arcobacters based on their swarming ability on semisolid medium has been reported (De Boer *et al.* 1996). In this study, an *Arcobacter* selective enrichment broth (ASB) and an *Arcobacter* selective semisolid medium (ASM) was formulated for the recovery of *Arcobacter* from retail meat products. Basal media used for ASB and ASM were Brucella broth and Muller-Hinton agar, respectively. Both of these media contained cefoperazone (32 mg/L), piperacillin (75 mg/L), trimethoprim (20 mg/L), and cycloheximide (100 mg/L) as selective substances. The protocol involved enrichment of samples in ASB followed by transfer of 40μl of ASB onto ASM, and examination for the

presence of motility zones. This method isolated arcobacters from 24% of 220 poultry meat samples.

A modified cefsulodin-irgasan-novobiocin (CIN) medium for the recovery of *Arcobacter* spp. from pork has been developed (Collins *et al.* 1996a). Enrichment was done using EMJH with 5-fluorouracil (200 mg/L), which was followed by plating on to three different media. Modified CIN was compared with CVA agar (brain heart infusion agar supplemented with 10% bovine blood and cephalothin (20 mg/L), vancomycin (10 mg/L), and amphotericin B (5mg/L)) as well as brain heart infusion agar supplemented with 10% bovine blood but without antibiotics. $MgCl_2$ was used at the rate of 2 g/L in the modified selective medium. Using this media, it was shown that 89% of the 149 pork samples were positive for *Arcobacter* spp.

A study comparing the growth performance of campylobacters and arcobacters on a variety of enrichment and direct isolation media has been published (Atabay and Corry, 1997). It was found that, enrichment, either in CAT broth or in ASB (Lammerding *et al.*, 1996) inhibited campylobacters, and allowed the growth of all of arcobacters from all 15 poultry carcasses tested, all of which were negative for arcobacters without enrichment. Plating onto CAT agar following enrichment was found to yield overgrowth of competitive organisms. Incubation at lower temperature (30 vs. 37°C) yielded wider variety of arcobacters. It was recommended that, when examining poultry for campylobacters and arcobacters, both direct plating and enrichment protocol should be included.

The CAT enrichment-filtration method developed by Atabay and Corry (Atabay and Corry, 1997) was modified by On *et al.* (2002) for use with biopsy samples taken from aborted porcine foetuses. The modifications included the use of two incubation temperatures (25 and 37°C), which improved the taxonomic diversity of isolates obtained compared with incubation at 37°C alone (On *et al.*, 2002). Here, tissue samples from liver and kidneys of aborted foetuses were enriched in CAT enrichment broth followed by spotting of broth onto blood agar plates upon which a cellulose acetate filter (pore size 0.65μm) had been placed. Arcobacters were detected in approximately 40% of the aborted foetuses.

The efficacy of Oxoid *Arcobacter* broth, supplemented with CAT was evaluated and its productivity was compared with two campylobacter enrichment media, Preston broth and LabM *Campylobacter* enrichment broth (Atabay and Corry, 1998). *Arcobacter* broth supported good growth of A. *butzleri*, *A. cryaerophilus*, and *A. skirrowii* although *A. nitrofigilis* grew poorly. It was revealed that Preston broth and to a lesser extent LabM *Campylobacter* enrichment broth, were not effective for detecting *Arcobacter* strains.

A range of solid (plating) media and enrichment broth were tested by Johnson and Murano (Johnson and Murano, 1999a; Johnson and Murano, 1999b). The solid medium containing cefoperazone (32 mg/L), thioglycolic acid (0.05%), sodium pyruvate (0.05%) and sheep's blood (5%; pH 6.9) added to a basal nutrient mix (J&M agar) was found to support the optimum growth of arcobacters at 30°C (Johnson and Murano, 1999b). The enrichment broth contained cefoperazone (32 mg/L), 5-fluorouracil (200 mg/L), activated charcoal (3%), thioglycolic acid (0.05%), sodium pyruvate (0.05%), and bile salts (0.25%). This broth, called 'JM enrichment broth', together with plating on this JM agar resulted in *Arcobacter* spp. being detected in 42 out of 50 poultry samples compared with 15 with method of De Boer *et al.* (1996), and 24 with method of Collins *et al.* (1996a). Johnson and Murano concluded that their method allowed the best recovery of *Arcobacter* and the greatest inhibition of other bacteria, and had the further advantage of using aerobic incubations, thereby eliminating the need for a modified atmosphere for incubations (Johnson and Murano, 1999a).

In 2001, Houf *et al.* (2001a) developed a selective supplement comprising amphotericin B (10 mg/L), cefoperazone (16 mg/L), 5-fluorouracil (100 mg/L), novobiocin (32 mg/L), and trimethoprim (64 mg/L). Using this supplement in enrichment and plating media, arcobacters were isolated from up to 100% of the poultry meat samples. The growth performance of *A. skirrowii* was however found to be poor with this supplement. Early studies by the same workers (Houf *et al.*, 2001b) had revealed that *A. skirrowii* is the species most susceptible to antimicrobial agents used in selective media. This may explain the low recovery rates reported to date for this organism.

Recently, Scullion *et al.*, (2004) compared protocol of Johnson and Murano (Johnson and Murano, 1999a) with other two protocols: Houf *et al.* (2001a) and On *et al.* (2002). It was found that Houf *et al.* method resulted in the highest recovery (68%) of arcobacters followed by Johnson and Murano (50%) and On *et al.* method (28%). Use of Houf *et al.* and Johnson and Murano method together increased the number of positive samples detected by approximately 25% compared with use of either method alone. Johnson and Murano method detected *A. cryaerophilus* in more samples than did the other two methods, and *A. skirrowii* was detected by only Johnson and Murano method.

While comparing the media used for isolation of *Arcobacter* spp. Houf *et al.* (2001a) technique appears to be the best in terms of high detection rates (up to 100%), and ease of preparation. In spite of detection of a range of species, the media used in the Johnson and Murano method is cumbersome and time consuming to prepare, and thus has not been used widely.

1.4 Identification and subtyping of arcobacters

Several phenotypic and molecular methods have been employed for the identification and/or subtyping of arcobacters. The most commonly employed methods are reviewed in the following sections.

1.4.1 Phenotypic identification methods

Observation of morphology, temperature tolerance, and biochemical tests are the most commonly used tests that have been used for the phenotypic characterization of arcobacters. The phenotypic tests that have been employed for the identification of arcobacters are shown in Table 2.

Identification of isolates of arcobacters to the species level, differentiating among species as well as between arcobacters and campylobacters, and subtyping by using classical

phenotypic tests is difficult and may give erroneous results because of a lack of clear-cut differentiating tests (Vandamme *et al.*, 1991; Vandamme *et al.*, 1992b; Yan *et al.*, 2000). Thus, relying on conventional phenotypic methods may lead to considerable underestimation of the true incidence of arcobacters in food commodities, and in animal and human illness (Manke *et al.*, 1998).

Table 2. Differential phenotypic characteristics between *Arcobacter* and *Campylobacter* species*

Characteristics	*A. butzleri*	*A. cryaerophilus*	*A. skirrowii*	*A. nitrofigilis*	*A. halophilus*	*A. cibarius*	*C. jejuni*
Alpha-haemolysis	-	-	+	-	-	-	+
Catalase activity	V	V	+	+	-	V	+
Oxidase activity	+	+	+	+	+	+	+
Hippurate hydrolysis	-	-	-	-	-	-	+
Urease	-	-	-	+	-	-	-
Nitrate reduction	+	+	+	+	+	-	+
Selenite reduction	-	-	V	V	?	-	V
H_2S(TSI)	-	-	-	-	-	-	-
Indoxyl acetate hydrolysis	+	+	+	+	+	+	+
Growth at 15°C (air)	+	+	+	+	+	?	-
Growth at 25°C (air)	+	+	+	+	+	V	-
Growth at 37°C (microaerobic)	+	V	+	V	+	+	+
Growth at 42°C(microaerobic)	V	-	V	-	-	-	+
Growth on minimal medium	+	-	-	-	-	+	-
Growth on MacConkey agar	V	V	-	-	-	V	-
Growth in glycine (1%)	-	V	-	-	-	-	+
Growth in NaCl (4%)	-	-	+	+	+	-	-
Resistance to nalidixic acid	V	V	S	S	S	V	S
Resistance to cephalothin (32 mg/L)	R	R	R	S	S	R	R
Resistance to cefoperazone (64 mg/L)	R	R	R	S	S	R	R

+, Characteristic present in 90% of the strains examined; -, characteristic present in less than 11% of the strains examined; V, variable reaction; ?, not known; S, susceptible; R, resistant

(*Source: Vandamme *et al.*, 1991; Vandamme, 2000; Yan *et al.*, 2000; On *et al.*, 2003; Wybo *et al.*, 2004; Donachie *et al.*, 2005; Houf *et al.*, 2005).

1.4.1.1 Dark-field Microscopy

Rapid identification of arcobacters has been done by dark-field microscopy (Lammerding *et al.*, 1996; Schroeder-Tucker *et al.*, 1996; De Oliveria *et al.*, 1999; Atabay *et al.*, 2003; Fernandez *et al.*, 2004; Houf *et al.*, 2005). The technique involves direct examination of the presumptive colonies suspended in saline under a dark field microscope. The organisms are seen as small comma-shaped or spiral rods exhibiting characteristic darting or corkscrew motility. Dark-field microscopy is simple, rapid, and inexpensive, and is used for the presumptive diagnosis of *Campylobacter* enteritis in humans (Paisley *et al.*, 1982).

1.4.1.2 Temperature and aerotolerance test

The spiral or curved cellular morphology of arcobacters may not be a useful criterion to facilitate identification to genus level, as this characteristic is similar to campylobacters. Some of the phenotypic tests that differentiate arcobacters from campylobacters are aerotolerance, growth on MacConkey agar, growth at 15°C, 25°C, and 37°C, and no growth at 42°C (Vandamme *et al.*, 1992a; Marinescu *et al.*, 1996a; Schroeder-Tucker *et al.*, 1996; Hsueh *et al.*, 1997; Yan *et al.*, 2000; Atabay *et al.*, 2003).

1.4.1.3 Biochemical tests

Basic biochemical tests that are routinely used for the identification of campylobacters are also used for the identification of arcobacters to the species level. Commonly, *Arcobacter* isolates are tested for the presence of catalase and oxidase, tolerance to sodium chloride (3.5%), growth on MacConkey agar, and hydrolysis of indoxyl acetate (Schroeder-Tucker *et al.*, 1996).

Arcobacter spp. produce positive results for oxidase test, nitrate reduction test, and hydrolysis of indoxyl acetate (Marinescu *et al.*, 1996b; Euzeby, 2005). They give negative results for oxidation or fermentation of sugars, production of indole, production of lecithinase, Voges-Proskauer reaction, reduction of nitrites, production of hydrogen sulphide in TSI (Triple Sugar Iron) medium, hydrolysis of urea, hippurate, esculin, casein, tyrosine, and starch, and liquefaction of gelatine (Marinescu *et al.*, 1996b; Schroeder-Tucker *et al.*, 1996; Vandamme, 2000; Euzeby, 2005). A variable result is observed, according to the species, for the catalase test, reduction of nitrates, hydrolysis of DNA, growth in the presence of 1% glycine, 2% and 4% NaCl, and 1% bile, growth on MacConkey agar, and sensitivity to cadmium chloride (Marinescu *et al.*, 1996a; Euzeby, 2005).

Some biochemical tests are also useful for speciation of arcobacters. 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 (Kiehlbauch *et al.*, 1991b; Vandamme *et al.*, 1992b; Schroeder-Tucker *et al.*, 1996). It has been suggested that *A. butzleri* (weak-to-negative catalase reaction) can be distinguished from other species of *Arcobacter* (strong catalase reaction) by the catalase test (De Oliveria *et al.*, 1997; 1999; Yan *et al.*, 2000).

The API CAMPY® system has been tested for the identification of arcobacters. Harrass *et al.* (1998) employed this system for the identification of *Arcobacter* isolates obtained from poultry carcasses. The authors argued that since the genus *Arcobacter* has not been included in the analytical profile index of the API CAMPY®, *Arcobacter* isolates cannot be identified suitably using this scheme. Yan *et al.* (2000) mentioned that this scheme had misidentified *A. butzleri* as *Campylobacter coli*.

The usefulness of biochemical tests is however hampered by the fastidious growth requirements of arcobacters and their relatively inert biochemical character (Vandamme, 2000).

1.4.1.4 Antibiotic sensitivity test

Antibiotic sensitivity tests may be used in combination with other phenotypic tests for the presumptive identification of arcobacters. As with campylobacters, the three most commonly used antibiotics for sensitivity testing are nalidixic acid, cephalothin and cefoperazone (Table 2). Disk diffusion test (On *et al.*, 1995; Hsueh *et al.*, 1997; Yan *et al.*, 2000) and agar dilution test (Houf *et al.*, 2001b; Houf *et al.*, 2004) have been used for testing antibiotic sensitivity of arcobacters. Although the agar dilution method is considered the reference method, the disk diffusion method could also be a reliable and convenient method (Gaudreau and Gilbert, 1997).

A variable sensitivity is observed with nalidixic acid (30 µg per disc) for *A. butzleri*, *A. cryaerophilus*, and *A. cibarius*; whereas *A. skirrowii*, and *A. nitrofigilis* are susceptible to it (Euzeby, 2005). With regards to cephalothin (30 µg per disc) and cefoperazone (30 µg per disc), *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, and *A. cibarius* are resistant, whereas *A.*

nitrofigilis and *A. halophilus* are susceptible (Euzeby, 2005). On *et al.* (1995) observed that *A. butzleri, A. cryaerophilus* and *A. skirrowii* were resistant to nalidixic acid (32 mg/L), metronidazole (4 mg/L), carbenicillin (32 mg/L) and cefoperazone (64 mg/L). Yan *et al.* (2000) observed that an isolate of *A. butzleri* from a human patient was susceptible to nalidixic acid and resistant to cefazolin in the disk test. Hsueh *et al.* (1997) found an isolate of *A. cryaerophilus* 1B obtained from a human patient was susceptible to nalidixic acid (30-μg disk) but resistant to cephalothin (30 μg disk).

Harrass *et al.* (1998) evaluated the usefulness of antimicrobial resistance tests to differentiate 87 isolates of *Arcobacter.* They observed that resistance to sulfamethoxazole/trimethoprim, cefazolin, and ampicillin were predominant, while resistance to nalidixic acid, chloramphenicol, and clindamycin were less frequent, and all 87 isolates were susceptible to aminoglycosides and minocycline. It was concluded that, antimicrobial resistance testing, in combination with growth and tolerance tests and plasmid analysis gave a highly specific and detailed characterization and differentiation of *A. butzleri* isolates obtained from poultry carcasses.

It should be noted in antimicrobial susceptibility testing that numerous factors may affect the result. Examples of such factors include the size of the inoculum and the composition of the basal medium (On and Holmes, 1991).

1.4.2 Biotyping

Phenotypic tests that evaluate the capability of a microorganism to generate or use biochemical substrates, for differentiating within a species, is referred to as biotyping.

A biotyping scheme has been developed for *A. butzleri* and *A. butzleri*-like isolates recognizing 16 biotypes numbered 1A, 1B to 8A, 8B, based on their ability to produce urease, rapid H_2S, DNase and the utilization of sodium acetate (Lior and Woodward, 1993). Using this scheme, Marinescu *et al.* (1996b) identified 3A, 4A, 6A, 7A and 8A biotypes among 162 *A. butzleri* and one *A. butzleri*-like isolate obtained from poultry samples. Out

of these, biotype 8A was the most common followed by 7A and 4A. Lior's scheme differentiated the 44 strains of *A. butzleri* obtained from meat samples into the biotypes 2A, 3A, 3B, 4A, 4B, 5A, 6A, 7A, 7B, 8A, and 8B; 8A being the most common followed by 8B and 4A (De Boer *et al.* 1996). Similarly, this scheme was useful in subtyping 18 strains of *A. butzleri* obtained from river samples (Musmanno *et al.* 1997).

As with biochemical tests, the usefulness of biotyping is hampered by the fastidious growth requirements of arcobacters and their relatively inert biochemical character, so is not employed commonly.

1.4.3 Serotyping

Serotyping involves the use of specific antibodies to detect homologous antigens, and is most widely applied for typing of Gram-negative enteric bacterial pathogens. For most foodborne pathogens, agglutination techniques are employed. For campylobacters, a serotyping scheme, based on soluble heat-stable or heat-labile antigens, has been widely used (Penner and Hennessy, 1980; Lior *et al.*, 1982; On, 1996; Frost *et al.*, 1998).

A serotyping scheme for *A. butzleri* has been described by Lior and Woodward (Lior and Woodward, 1994). In Lior's approach, antisera produced from rabbits using heat-labile antigens were used for slide agglutination tests of live bacteria. This scheme recognized 65 serotypes (in 14 serogroups) of *A. butzleri* obtained from human and nonhuman sources. The same serotypes of *A. butzleri* were found to be common among human, poultry, pig, and water. No cross-reactivity was observed with the antisera against *C. jejuni*, *C. coli*, and *C. lari*.

Using Lior's scheme, 13 strains of *A. butzleri* obtained from 10 children from an outbreak of abdominal cramp has been serotyped (Vandamme *et al.* 1992a). Serotyping by using antiserum prepared against the outbreak strains revealed that all of the strains belong to serotype 1. This scheme has also been employed for typing of arcobacters obtained from poultry samples (Marinescu *et al.* 1996a, 1996b). Twenty-two different serogroups were

recognized among 162 *A. butzleri* and one *A. butzleri*-like isolate; serotype 1 being the most predominant followed by 26 and 19 (Marinescu *et al.*, 1996b). The authors mentioned that *A. butzleri* isolated from poultry meat and from humans with diarrhoeal illness were belonging to the same serotype (serotype 1). Similar findings has been reported by Lammerding *et al.* (1996)

Serotyping is in limited use for subtyping of arcobacters. The main disadvantage of this method is lack of the availability of serotyping reagents. Production of antisera to the large number of strains would be too time consuming, costly and impractical.

1.4.4 Molecular/ Genotypic methods

These techniques involve detection and characterization of molecules (fatty acids, proteins, nucleic acids, and other chemicals) produced by bacteria. Genotyping, a commonly used molecular method, refers to the direct DNA-based analysis of chromosomal or extrachromosomal genetic material (Tyler and Farber, 2003). Molecular methods may be broadly classified into three categories on the basis of the type of macromolecules targeted for characterization (Swaminathan and Matar, 1993): fatty-acid based methods, protein based methods, and nucleic acid based methods.

1.4.4.1 Cellular fatty acid profiles

Since the fatty acid (FA) composition of bacterial cells may vary significantly between taxa, its profiling has been employed for classification and identification of several bacteria, including campylobacters (Vandamme, 2000). Briefly, the method involves saponification of the whole-cell FAs, esterification with an alcohol, extraction of FA methyl esters (FAMEs) with an organic solvent, separation by gas chromatography and identification by comparing their retention times with those of known standards (On, 1996).

Several authors used cellular fatty acid methyl ester analysis for the differentiation and identification of arcobacters. Lambert *et al.* (1987) described the use of cellular fatty acid analysis for the differentiation of *Campylobacter* and *Campylobacter*-like organisms,

including *A. cryaerophilus*. Tee *et al.* (1988) used gas chromatography analysis of fatty acid for the identification of a human isolate of *A. cryaerophilus*. Kiehlbauch *et al.* (1991a) used this technique for the characterization of 78 strains of aerotolerant campylobacters and found them to be *A. butzleri*. Vandamme *et al.* (1992b) reported that fatty acid analysis was useful in distinguishing all species of arcobacters, with the exception of being unable to differentiate *A. butzleri* from *A. cryaerophilus* subgroup 2. Hsueh *et al.* (1997) employed this technique for the identification of a bacterial isolate recovered from a person with bacteraemia. In combination with biochemical tests, the isolate was identified as *A. cryaerophilus* 1B.

1.4.4.2 Protein profiling

Examination of the protein content of a living cell gives an indication of the genetic organization of an organism. Among the different types of protein profiling, profiles obtained from whole bacterial cell by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) are most commonly used for identification of bacteria, including campylobacters (On, 1996).

The comparison of whole-cell protein patterns obtained by highly standardized SDS-PAGE has been used for screening and identifying a large number of strains of arcobacters. A good correlation has been observed between a high similarity in whole-cell protein content and level of DNA-DNA hybridization (Vandamme *et al.*, 1992b). Atabay *et al.* (2003) described the simultaneous use of a SDS-PAGE and a multiplex PCR for the detection of arcobacters from retail poultry carcass. Both the methods detected arcobacters from 42 samples out of 44, and the species were found to be *A. butzleri* by both methods. Wybo *et al.* (2004) mentioned SDS-PAGE profiling technique was useful for confirming the identification of *A. skirrowii* obtained from a patient with chronic diarrhoea. Houf *et al.* (2005) found this technique, in combination with DNA-DNA hybridization, rRNA gene sequencing, and DNA base composition analysis, to be useful in differentiating an *Arcobacter* isolate obtained from poultry carcass to be a novel strain.

In spite of being highly sensitive, protein profiling techniques are not suitable for routine identification studies since they are very laborious, time-consuming, and technically demanding to run patterns in an adequately standardized way (Vandamme, 2000).

1.4.4.3 DNA-base compositions

One of the distinctive features of DNA that has taxonomic significance is its mole percent guanine-plus-cytosine content (mol% G+C). Among the bacteria, the mol% G+C value is constant for a specific organism. All of the G+C values are determined by thermal denaturation method. Although closely related bacteria have similar mol% G+C values, two organisms that have similar mol% G+C values are not necessarily closely related.

The G+C content of the DNA of arcobacters ranges from 27 to 31 mol% (Vandamme, 2000). In one study, the G+C content the DNA of genus *Arcobacter* was found to be 28-31 mol% (Vandamme *et al.*, 1991). Kiehlbauch *et al.* (1991a) found this G+C content to be 29-32 mol% for five *A. cryaerophilus* reference strains. Tee *et al.* (1988) mentioned the G+C content of DNA of *A. cryaerophilus* from human faecal samples to be 31.1±1 mol%. Houf *et al.* (2005) found that G+C content of *A. cibarius* ranged between 26.8 and 27.3 mol%.

1.4.4.4 Hybridization techniques

Hybridization techniques depend on the detection of a signal generated after the binding of a labelled probe with the target nucleic acid. Hybridization takes place when the sequence of the probe is adequately similar to that of the target nucleic acid and that a duplex is formed and held together by hydrogen bonds from nucleotide pairing. The target nucleic acid as well as the probe may be single- or double-stranded RNA or DNA.

1.4.4.4.1 DNA-DNA hybridization

This technique involves hybridization of the entire DNA-contents of both organisms under examination. The degree of DNA-DNA binding is determined spectrophotometrically and is expressed as a percentage. DNA binding values of 70% or more indicate that there is

significant DNA homology (Vandamme *et al.*, 1991), and indicates a direct relationship at species level.

The DNA-DNA hybridization technique has been found to be useful in speciation of *Arcobacter* spp. and differentiation of the two subgroups of *A. cryaerophilus*. Kiehlbauch *et al.*(1991a) found two distinct hybridization groups among the 78 aerotolerant campylobacters of human and animal origin by DNA-DNA hybridization. *A. cryaerophilus* belonged to a DNA hybridization group which was genetically and phenotypically heterogeneous, and was further differentiated as DNA hybridization group 1A and 1B; and *A. butzleri* belonged to DNA hybridization group 2. Employing this technique, Vandamme *et al.* (1992b) identified five groups of *Arcobacter* strains as *A. cryaerophilus* (two distinct subgroups), *A. butzleri*, *A. nitrofigilis*, and *A. skirrowii*.

This technique has been regarded as a reference method and has also been used to confirm the results of other techniques. In an outbreak of abdominal cramps in humans, the causative organisms identified as *A. butzleri* by SDS-PAGE of whole-cell proteins and cellular fatty acid analysis was confirmed by DNA-DNA hybridization (Vandamme *et al.*, 1992a). *A. cryaerophilus* obtained from faecal samples of a man which was presumptively identified by biochemical tests and liquid-gas chromatography was confirmed by this test (Tee *et al.* 1987; 1988). DNA-DNA hybridization test also confirmed the existence of a novel species of *Arcobacter* as the novel strain (*A. cibarius*) had binding percent of below 47 with *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (Houf *et al.*, 2005).

Although it is generally regarded as the reference method, DNA-DNA hybridization technique has limited practical application in a routine laboratory or for examination of large numbers of strains in a reference laboratory.

1.4.4.4.2 In situ hybridization

In situ hybridization (ISH) involves hybridization of a labelled nucleic acid probe with a DNA or RNA sequence *in situ* (in the cells). The probe can be either radioactively labelled

and detected by autoradiography or fluorescently labelled (abbreviated FISH) and detected by immunocytochemistry.

Fluorescent *in situ* hybridization (FISH) with rRNA oligonucleotide probes has been used for detection and identification of different microorganisms, including arcobacters. Using this technique, Snaidr *et al.* (1997) found that 4% of the microorganism cells present in an activated sludge plant were *Arcobacter* spp.

A rapid FISH protocol to detect arcobacters in naturally and artificially contaminated samples has been developed (Moreno *et al.* 2003). The probe was targeting partial 16S rRNA gene sequence. The detection range of FISH assay was found to vary between 10^2cells/ml (after culture enrichment) to 10^4cells/ml (without enrichment). It was found that 100% of the water samples (n=10) and sludge samples (n=10) were positive for *Arcobacter* spp.

The main advantage of FISH techniques is its rapidity as DNA is not necessary to be extracted from bacteria, so can be conducted without culture, and results may be directly observed in the samples.

1.4.4.4.3 Restriction fragment length polymorphisms (RFLP) and Ribotyping

These techniques involve southern blot hybridization of genomic DNA digested with a six-cutter restriction enzyme and hybridization with a universal rRNA probe (Swaminathan and Matar, 1993; Jay, 2000; Newell *et al.*, 2000). The occurrence of several copies of the rRNA genes (coding for 16S and 23S rRNA) at different locations on the chromosome and their high degree of conservation among bacteria make these genes ideal target for probing (Newell *et al.*, 2000).

RFLP and ribotyping has expedited the identification and/or subtyping of *Arcobacter* spp. from a variety of sources. Kiehlbauch *et al.* (1991b) have mentioned that RFLP patterns were useful in differentiating the species: *A. butzleri* and *A. cryaerophilus*, from other closely related bacteria (*Campylobacter* like organisms; CLOs). De Oliveria *et al.* (1999)

have described the use of ribotyping to identify *Arcobacter* spp. obtained from preputial fluids of pigs.

Besides speciation, the technique has also found useful to discriminate between the two hybridization groups of *A. cryaerophilus*. Out of 50 porcine abortion-related isolates, ribotyping identified 16% as *A. cryaerophilus* DNA group 1A, 60% as *A. cryaerophilus* DNA group 1B, and 8% as *A. butzleri* (Schroeder-Tucker *et al.* 1996). However, remaining 16% were not able to be classified by ribotyping patterns. In another study, out of 18 isolates of *Arcobacter* spp., two were identified as *A. butzleri*, six as *A. cryaerophilus* hybridization group A, and seven as *A. cryaerophilus* hybridization group B (De Oliveria *et al.* 1999).

PCR-RFLP is a modification of conventional RFLP technique which involves an additional step of PCR amplification of a target sequence. Hurtado and Owen (1997) reported a rapid two-step identification scheme based on PCR-RFLP analysis of the 23S rRNA gene. The scheme was found to useful in differentiating the isolates belonging to the *Campylobacter*, and *Arcobacter* genera. Marshall *et al.* (1999) described a PCR-RFLP analysis of the 16S rRNA gene for differentiating isolates belonging to the *Campylobacter*, *Arcobacter*, and *Helicobacter* genera. The technique also differentiated *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* by producing unique fingerprints for all three species.

1.4.4.5 Polymerase chain reaction (PCR)

PCR is an *in-vitro* method involving enzymatic amplification of specific DNA sequence using oligonucleotide primers that hybridize to the region of interest in the target DNA. Ribosomal RNA, an essential part of prokaryotic and eukaryotic ribosomes, is genetically stable and consists of conserved and variable regions. The latter may vary considerably among different bacterial species and are therefore targets for PCR amplification. PCR uses primers to get the copying process started. The extraordinary ability of PCR to exponentially and rapidly replicate a target DNA sequence has made it a very powerful tool for the detection of infectious agents. The difficulties in routine detection, isolation and identification make arcobacters ideal candidates for PCR identification.

Several investigators have targeted the 16S or 23S rRNA gene in order to identify the species level members of the *Arcobacter*. Based on a 23S rDNA area, Bastyns *et al.* (1995) developed a PCR assay for the identification of arcobacters,. They found the amplification of this 23S rDNA area was useful for genus-specific and species-specific detection of arcobacters. The species-specific assay was able to differentiate the three species *A. cryaerophilus*, *A. butzleri* and *A. skirrowii*.

A genus-specific PCR assay for the detection of *Arcobacter* spp has been described (Harmon and Wesley 1996). The assay was able to detect the four species of arcobacters, *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, and *A. nitrofigilis*. The advantage of this protocol was it utilized either purified DNA or a crude bacterial cell lysate, and the amount of time required was reduced (8 h vs. several days). Later, a multiplex-PCR assay for the simultaneous detection of *Arcobacter* spp. and the differentiation of *A. butzleri* from other arcobacteria was developed by them (Harmon and Wesley, 1997). Two sets of primers were used in this protocol. The first set of primers targeted the 16S rRNA genes of *Arcobacter* spp., and the second set amplified the 23S rRNA genes unique to *A. butzleri*.

Surez *et al.* (1997) developed a nested PCR test for detection of arcobacters in gastric samples from swine. The primers were targeting the 16S rRNA gene of members of rRNA superfamily VI. The PCR products were differentiated and confirmed by hybridization with an internal oligonucleotide probe. The results of nested PCR were also compared with single step PCR and direct culture methods. *Arcobacter* spp. were recovered from 4 of 71 samples and the nested PCR test was found to be more sensitive than single step PCR.

Gonzalez *et al.* (2000) developed a genus-specific PCR-culture technique to detect *Arcobacter* spp. in fresh poultry meat. The primers were targeted to amplify the 16S rRNA gene from *Arcobacter* spp. PCR amplification was conducted following a short selective enrichment of poultry samples. Using this assay 53% of the 96 retail poultry samples were found to be positive for the presence of *Arcobacter* spp.

Using a variable 16S rRNA and 23S rRNA region, Houf *et al.* (2000) developed a species-specific multiplex-PCR assay for the simultaneous detection and identification of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*. Three primers sets were designed to amplify a 257 bp fragment of 23S rRNA gene from *A. cryaerophilus*, a 401 bp fragment of 16S rRNA gene from *A. butzleri*, and a 641 bp fragment of 16S rRNA gene from *A. skirrowii*. The assay was found to be specific as no PCR product was generated for closely related bacteria.

A genus-specific multiplex PCR assay for the simultaneous detection and identification of *Campylobacter* spp. and *Arcobacter* spp. has been described (Winters and Slavik 2000). The primer sets amplified a 159 bp fragment of 16S rRNA genes of *C. jejuni* and 1223 bp fragment of 16S rRNA genes of *A. butzleri*. The protocol was compatible with a variety of food products like poultry and pork, and fruits and vegetables.

Kabeya *et al.* (2003a) developed a species-specific PCR assay for the identification of the arcobacters. The one-step PCR assay was shown to be capable of providing a rapid species differentiation of *Arcobacter* strains. Moreover, by using this PCR assay, it was possible to differentiate between *A. cryaerophilus* 1A and 1B.

A PCR assay for identification of *Arcobacter* strains from environmental water and activated-sludge samples has been evaluated (Moreno *et al.* 2003). The assay was performed on naturally and artificially contaminated samples, with and without enrichment. The detection range of PCR assay varied between 1cell/ml (after enrichment) to 10^3cells/ml (without enrichment).

The use of a PCR technique combined with an enzyme-linked immunosorbent assay (PCR-ELISA) for the quantitative detection of *Arcobacter* spp. in poultry meat has been described (Antolin *et al.* 2001). The primers were targeted to amplify 181 bp DNA fragment of the 16S rRNA gene from *Arcobacter* spp. It was found that the detection threshold for the PCR-ELISA assay was 10 CFU/g.

Although highly discriminating PCR assays have been developed for species identification of *Arcobacter*, an inherent limitation often encountered with PCR assays in the inability to distinguish between bacterial strains. Among the PCR protocols mentioned here, the protocol of Houf *et al.* (2000) has been used extensively for speciation of *Arcobacter* spp. No PCR protocol has yet been published for the detection of a recently discovered species *A. cibarius.*

1.4.4.6 Repetitive element PCR (Rep-PCR)

This is a PCR-based fingerprinting method that targets the amplification of repetitive elements (rep elements) in the bacterial genome. The rep elements targeted for PCR amplification useful in subtyping of Gram-negative bacteria are enterobacterial repetitive intergenic consensus (ERIC) and the repetitive extragenic palindromic (REP) sequences (Versalovic *et al.*, 1991; Olive and Bean, 1999).

Rep-PCR has been used for assessing the genetic diversity and epidemiological relationships among *Arcobacter* spp. isolates. This technique revealed that 14 outbreak-related strains of *A. butzleri* obtained from the cases of abdominal cramps in children had an identical fingerprinting pattern (Vandamme *et al.* 1993). In another study, Rep-PCR employed for assessing the genetic diversity of 121 *A. butzleri* isolates from turkey meat revealed 86 different patterns, indicating multiple sources of contamination (Manke *et al.* 1998). Driessche *et al.* (2005) found this technique was useful in subtyping 164 isolates of *Arcobacter* spp. obtained from faecal samples of healthy cattle. A high degree of heterogeneity was observed among the isolates and it was suggested that animals could be colonized by multiple genotypes. It was further suggested that infection is transmitted by direct contact and no vertical transmission occurs in cattle.

Houf *et al.* (2002a) optimized Rep-PCR for subtyping of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* strains. Ninety-eight percent of the 228 *Arcobacter* isolates (182 *A. butzleri* and 46 *A. cryaerophilus*) from 24 poultry samples were typeable among which 131 types (91 *A. butzleri* and 40 *A. cryaerophilus*) were detected. The fingerprint profile was

compared with random amplification of polymorphic DNA (RAPD) and both methods were found to be equally discriminatory.

1.4.4.7 Random amplification of polymorphic DNA (RAPD)

RAPD involves the use of arbitrary primers for amplification of target DNA sections by normal PCR. Whole genomic DNA is used and PCR is performed at low stringency allowing primer to bind at various positions of the target DNA resulting in several amplicons of various sizes (Swaminathan and Matar, 1993; Newell *et al.*, 2000).

RAPD has been successfully employed for identification and typing of *Arcobacter* spp (Houf *et al.* 2002a; 2003). Using this technique, 95% of the 228 *Arcobacter* isolates (182 *A. butzleri* and 46 *A. cryaerophilus*) from 24 poultry samples were typeable among which 128 types (89 *A. butzleri* and 39 *A. cryaerophilus*) were detected (Houf *et al.*, 2002a). Using RAPD together with ERIC-PCR, a total of 1,079 *Arcobacter* isolates obtained from various sources including slaughter equipment, processing water and the poultry carcass were differentiated into 159 *A. butzleri* types and 139 *A. cryaerophilus* types (Houf *et al.*, 2003). The extreme heterogeneity among the isolates suggested that arcobacters were acquired from different sources.

1.4.4.8 Amplified-fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) analysis involves digestion of chromosomal DNA with a combination of two restriction endonucleases followed by PCR amplification and detection of fragments between adjoining restriction sites in the whole genetic content of the given organism (Newell *et al.*, 2000).

The potential of AFLP has been examined for identification and subtyping of *Arcobacter* species. Numerical analysis of the AFLP pattern from the 72 strains produced five phenons at 29% similarity level, four of which represented the species *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. nitrofigilis* (On *et al.* 2003). The remaining phenon suggested the existence of a new species for the isolates obtained from pig abortions and turkey faeces,

and was called '*Arcobacter skirrowii*-like'. At 91% similarity level, AFLP differentiated five subtypes among the 73 strains obtained from six different sample types and six different countries (On *et al.* 2004). So, it was suggested that distinct subtypes of *A. butzleri* may be found in a given environment. In another similar study, at 90% similarity level, AFLP differentiated 12 genotypes among 20 *A. butzleri* strains obtained from poultry plant effluent (Amisu *et al.*, 2003)

AFLP is being increasingly employed routinely for subtyping of microorganisms with increased availability of automated DNA sequencers. The major advantage of this technique is that prior sequence knowledge of the amplification target is not necessary.

1.4.4.9 DNA sequencing

This is a common technique employed for identification of unknown organisms and involves sequence analysis of 16S rRNA and its comparison with rRNA sequences available in the international database (Vandamme, 2000). The similarity or diversity of two bacterial strains can also be determined by this technique.

A number of studies have employed sequencing of the 16S rRNA gene for the identification and differentiation of arcobacters. Using this technique, Yan *et al.* (2000) found that PCR product of two *Campylobacter*-like isolates obtained from human blood culture samples were having 100% sequence similarity with the 16S rRNA gene of *A. butzleri.* Lau *et al.* (2002) mentioned that 16S rRNA gene sequencing was useful in the identification of a strain of *A. butzleri* isolated from the blood culture of a patient with acute gangrenous appendicitis. On *et al.* (2003) employed this technique for identification of a groups of arcobacters that had distinct AFLP patterns than the known species. These strains were found to be novel species within the genus *Arcobacter* and were named "*Arcobacter skirrowii*-like". Similarly, Diergaardt *et al.* (2004) employed this technique for confirmative identification of *Campylobacter*-like isolates obtained from drinking and environmental water sources. Out of 22 *Campylobacter*-like isolates, 19 were identified as *A. butzleri.*

1.4.4.10 Pulsed-field gel electrophoresis (PFGE)

The technique involves embedding bacterial cells in agarose followed by *in-situ* lysis, digestion of the chromosomal DNA with restriction endonucleases that cleave infrequently, and electrophoresis of the DNA fragments in pulsed electric fields. The infrequent cutting enzymes generate 5-20 very large molecular weight DNA fragments (Tyler and Farber, 2003), and allows clear separation of DNA fragments ranging from 10 to 800 kb (Schwartz and Cantor, 1984).

To determine the relatedness (similarity or diversity) among strains, the DNA restriction patterns of the strains are compared with one another. Usually when strains have less than 3 band differences, they are considered to be closely related (Tenover *et al.*, 1995). However there are no standardized criteria for interpreting the fragment patterns. *Arcobacter* isolates that are >90% similar on the dendograms generated by specific software programs has been considered related for *Arcobacter* spp (On *et al.* 2004). Software packages such as BioNumerics are used in generating dendograms which employs dice similarity coefficient and the PFGE patterns are clustered by the unweighted pair group method using arithmetic averages (UPGMA). The total number of PFGE patterns in a given population, along with the values for total number of strains in the sample population, and number of strains belonging to a particular subtype may then be used for diversity index (DI) calculation. Simpson's index of diversity has been used commonly for this purpose (Hunter and Gaston, 1988). A DI with an absolute value of zero indicates that the population is clonal whereas a value closer to one indicates a high genetic diversity.

PFGE was first used to study the chromosomal DNA of *Arcobacter* spp. by Hume *et al.* (2001). Three endonucleases: *Ava*I, *Eag*I, and *Sac*II, were found to be useful for strain differentiation of arcobacters, *Eag*I and *Sac*II being more suitable for differentiation among the isolates. In this study multiple genotypes for the *A. butzleri* and *A. cryaerophilus* isolates were obtained from pigs of different ages at a farrow-to-finish pig farm, suggesting considerable genotypic variation and genetic rearrangement.

Rivas *et al.* (2004) employed PFGE for examination of the similarity of *A. butzleri* isolates recovered from ground poultry, pork, beef and lamb meats from different location and time-periods. Fingerprint patterns following restriction with the endonucleases *Sac*II, *Eag*I and *Sma*I were found to be useful for strain differentiation. Among the 31 *A. butzleri* isolates recovered from different sources, 15-18 different PFGE patterns were observed across all three enzymes. Among the three enzymes used, *Sma*I was found to be less discriminatory but when used in combination with other enzymes, the discriminatory power of the combination was increased. When compared with Rep-PCR and RAPD, PFGE was found to be the most discriminatory subtyping technique.

PFGE has also been employed for investigating the mode of transmission of *Arcobacter* spp. Ho *et al.* (2005) employed this technique for studying the transmission of *Arcobacter* species from carrying sows to their piglets. The genomic DNA of *Arcobacter* spp isolated from sows and newborns were digested with *Eag*I. High similarity of PFGE profile *Arcobacter* isolates from sows and their respective offspring revealed that *Arcobacter* spp. may be transmitted both vertically and horizontally.

Among the various molecular typing methods, PFGE and AFLP have been commonly used for subtyping of *Arcobacter* spp. PFGE is considered to be the most discriminatory molecular epidemiological tools available for subtyping of arcobacters (Son *et al.*, 2006) and is regarded as 'gold-standard' of all molecular typing methods (Olive and Bean, 1999).

1.5 Epidemiology of *Arcobacter*

1.5.1 Arcobacters and humans illness

Limited information is available on the worldwide contribution of *Arcobacter* spp. towards human illness. Regardless of the fact that specific techniques are rarely employed in routine laboratories to isolate and identity *Arcobacter*, evidence for its substantive role in human illness as an emerging pathogen is increasing. Table 3 list the cases of isolation of *Arcobacter* spp. in different countries of the world.

Table 3. Isolation of arcobacters from human illness in different countries of the world

Country	Description	Reference
Australia	*A. cryaerophilus* isolated from a 35-year-old man having intermittent diarrhoea for 4-6 months	Tee *et al.*, 1988
	A. butzleri isolated from two children and four adults having enteritis, diarrhoea, abdominal cramps, vomiting and fever	Lauwers *et al.*, 1996
Belgium	*A. skirrowii* found to be associated with chronic diarrhoea in a 73-old-man with chronic diarrhoea persisting for two months	Wybo *et al.*, 2004
	Out of 40,995 patients with abdominal illness, *A. butzleri* and *A. cryaerophilus* detected respectively in 67 and 10 patients	Vandenberg *et al.*, 2004
Chile	*A. butzleri* isolated from cases of chronic diarrhoea in two children having recurrent abdominal cramps, diarrhoea, and vomiting	Fernandez *et al.*, 2004
Denmark	*A. butzleri* and *A. cryaerophilus* isolated from faecal samples; no information on patient history and symptoms	Engberg *et al.*, 2000
France	*A. butzleri* and *A. butzleri*-like organisms isolated from two children (19 month and 3-year-old) having diarrhoea, but no abdominal cramps, fever or vomiting	Marinescu *et al.*, 1996a
Germany	*A. butzleri* detected in faecal samples of an adult man and a woman; both having severe abdominal cramps and profuse diarrhoea	Lerner *et al.*, 1994
Italy	*A. butzleri* isolated from 10 children with recurrent abdominal cramps, but no fever or diarrhoea	Vandamme *et al.*, 1992a
Taiwan	*A. cryaerophilus* 1B detected in blood sample of a 72-year-old women having uraemia and haematogenous pneumonia	Hsueh *et al.*, 1997
	A. butzleri isolated from a 60-year-old man with bacteraemia and liver cirrhosis; symptoms were fever and haematemesis	Yan *et al.*, 2000
Thailand	*A. butzleri* and *A. cryaerophilus* isolated from 15 (2.37%) of 631 children with mild diarrhoea	Taylor *et al.* 1991
UK	*A. butzleri* detected in blood samples of a neonate with bacteraemia; the neonate was experiencing hypotension and hypothermia	On *et al.*, 1995

There has been only one large scale study reporting the prevalence rate of arcobacters in patients with diarrhoeal illness (Vandenberg *et al.* 2004). In this study, out of 67,599 stool samples obtained from 40,995 patients, *A. butzleri* and *A. cryaerophilus* were found in 67 (97 isolates) and 10 patients (13 isolates), respectively, accounting for a prevalence rate of 0.18%. Arcobacters accounted for 4% of the 1,906 *Campylobacter* like organisms (CLOs) isolated. The most prominent clinical symptom observed was acute or persistent watery diarrhoea. Except bloody diarrhoea, other clinical features were similar to *C. jejuni* infection. The acute diarrhoea lasted for 3-15 days while the persistent one lasted for between 2 weeks to 2 months. Other important clinical features observed were: fever (temperature >38°C); nausea or vomiting or both; and abdominal pain.

Limited information is available on the risk factors for infection and transmission of *Arcobacter spp.* in humans. No particular age groups seemed to be susceptible to *Arcobacter* infection since the illness is found in neonates to 90-year-old patients (On *et al.*,

1995; Vandenberg *et al.*, 2004). Consumption of contaminated food or water is considered to be the most important source of infection (Marinescu *et al.*, 1996a; 1996b; Hsueh *et al.*, 1997). In a few occasions, person-to-person transmission (Vandamme *et al.*, 1992a), and intrauterine transmission (On *et al.*, 1995) has been suspected. There is no information about how arcobacters cause disease, their virulence factors or their pathogenicity. The difficulty encountered in the establishment of pathogenicity for each *Arcobacter* species, the sources and routes of infection are probably at least partly due to failure of their detection in routine laboratory tests.

1.5.2 Arcobacters in foods

Poultry meat is considered to be the most important source of arcobacters. *Arcobacter* spp. has been isolated from poultry carcasses with recovery rates of up to 100% (Table 4). In addition, there are small number of reports of *Arcobacter* detection in carcasses of other birds including 77% in turkey (Manke *et al.*, 1998), and 80% in ducks (Ridsdale *et al.*, 1998). Eggs are however considered to be free of arcobacters (Zanetti *et al.*, 1996; Phillips, 2001), and thus do not present a public health risk.

Table 4. Isolation rates of arcobacters from poultry carcasses in different parts of the world

Country	Species detected	Isolation rates (%)	No. of samples	Reference
Australia	All A. *butzleri*	73	22	Rivas *et al.*, 2004
Belgium	*A. butzleri* -64%, *A. cryaerophilus* -9%, both together -11% samples	90 100	71 (broiler) 34 (layer)	Houf *et al.*, 2001a
Brazil	*A. butzleri* -41%,	46	80	De Oliveria *et al.*, 2001
Canada	*A. butzleri* 67% isolates,	97	125	Lammerding *et al.*, 1996
Denmark	*A. butzleri* in 100% samples, *A. cryaerophilus* in 16%,	100	30	Atabay *et al.*, 2006
France	*A. butzleri* -99%	81	201	Marinescu *et al.*, 1996b
Japan	*A. butzleri*-55%, *A. cryaerophilus* –30%,	20	180	Maruyama *et al.*, 2001
	A. butzleri -15%, *A. cryaerophilus* -2%, and *A. skirrowii* in 1% of samples	23	100	Kabeya *et al.*, 2004
Mexico	*A. butzleri*- in 73%, *A. skirrowii*- in 23%	40	45	Villarruel *et al.*, 2003
Spain	No speciation	53	95	Gonzalez *et al.*, 2000
Thailand	No speciation	100	10	Morita *et al.*, 2004
Netherlands	All *A. butzleri* or *butzleri*-like	24.1	224	De Boer *et al.*, 1996
Turkey	All *A. butzleri*	95	44	Atabay *et al.*, 2003
	A. butzleri -in all 25, *A. cryaerophilus*- in 13, and *A. skirrowii*- in 2 samples	100	25	Atabay *et al.*, 1998
UK	*A. butzleri* - 33, *A. cryaerophilus* -3, and *A. skirrowii* -1 sample	68	50	Scullion *et al.*, 2004
USA	No speciation	84	50	Johnson and Murano, 1999a

Besides poultry meat, arcobacters are found to be occurring in a number of foods of animal origin; beef and pork being the most common (Table 5). In Chile, arcobacters have also been reported as occurring in oysters (Romero *et al.*, 2002).

Table 5. Prevalence rate of arcobacters in beef, pork and lamb meat in different parts of the world

Origin	Description	Reference
Australia	*A. butzleri* isolated from ground meat samples of pork 29% (n=21), beef 22% (n=32) and lamb 15% (n=13); no other species detected;	Rivas *et al.*, 2004
Canada	*Arcobacter* spp. isolated from 1.5% (n=68) of minced beef samples, 0.5% (n=194) pork samples, and 4.9% (n=61) of the mixed minced pork/beef samples	De Boer *et al.*, 1996
Czech Republic	*Arcobacter* spp. detected in 50% (n=9) of retail beef samples; two pork samples tested negative	Vytrasova *et al.*, 2003
Italy	*A. butzleri* detected in 3.7% (n = 27) of pork loin samples; no other species detected;	Zanetti *et al.*, 1996
Turkey	*A. butzleri* detected in 5% (n=97) of minced beef meat samples; no other species detected	Ongor *et al.*, 2004
USA	*Arcobacter* spp. detected in 90% (n=149) ground pork samples; no speciation done	Collins *et al.*, 1996a
USA	*Arcobacter* spp. detected in 32% (n=200) of ground pork samples; the detection rate ranged from 0-68% among different plants	Ohlendorf and Murano, 2002

As seen above, the abundant presence of *Arcobacter* spp. in foods of animal origin suggests an important role of contaminated food in the transmission of these bacteria.

1.5.3 Arcobacters in water and environment

Water is considered to have a significant role in transmission of arcobacters both to animals and humans. Table 6 lists the isolation of arcobacters from water and environmental samples in different parts of the world. Worldwide, arcobacters have been detected in various proportions in different types of water including surface water, ground water, sea water, sewage and sludge.

Some researchers have described *Arcobacter* spp. as ubiquitous organisms (On *et al.*, 1995). In one study, arcobacters were found to be more abundant than campylobacters in sludge samples (Moreno *et al.*, 2003). This may be because arcobacters are capable of growing in atmospheric oxygen and at lower temperatures than campylobacters (Wesley *et al.*, 2000). These organisms have been found occurring in poultry farm surroundings like

stagnant water and sludge (Gude *et al.*, 2005), which may be a source of continuing infection in individual farms.

Table 6. Isolation of arcobacters from water and environment in different countries of the world.

Origin	Description	Reference
Belgium	*Arcobacter* spp. detected in 91% (n=24) water samples before being used in poultry processing plant	Houf *et al.*, 2003
Caribbean	*Arcobacter* spp detected in coral reefs	Frias-Lopez *et al.*, 2002
Germany	79% (n=147) of *Campylobacter*-like strains isolated from drinking water treatment plants identified as *Arcobacter* spp.; 100 strains were *A. butzleri;*	Jacob *et al.*, 1998
Germany	4% of all cells in activated sludge samples were *Arcobacter* spp.	Snaidr *et al.*, 1997
Japan	*A. butzleri* detected in 23% (n=17) of river water samples; no other species detected;	Morita *et al.*, 2004
Nigeria	26 (14%) of the poultry abattoir waste water samples positive for *A. butzleri*; no other species detected;	Amisu *et al.*, 2003
South Africa	*A. butzleri* isolated from 9% (n=11) of surface water samples and 54% (n=4) of sewage water samples; tap water samples (n=5) and ground water samples (n=4) free of arcobacters	Diergaardt *et al.*, 2004
Spain	100 % (n=10) of river water and 100% (n=10) activated sludge sample positive for *Arcobacter* spp; speciation not done;	Moreno *et al.*, 2003
Thailand	*A. cryaerophilus* and *A. cryaerophilus*-like organisms isolated from 47% and 26% (n=156) water samples, respectively; source was 36 canals of Bangkok metropolitan area; no seasonal variation on isolation rates	Dhamabutra *et al.*, 1992
Thailand	*A. butzleri* detected in 100% (n=7) canal water samples from Bangkok; no other species detected; the isolates were genetically diverse	Morita *et al.*, 2004
USA	*A. butzleri* isolated from contaminated well water; suspected to have been associated with abdominal illness in group of 117 girls	Rice *et al.*, 1999

Limited information is available on survival of *Arcobacter* spp. in environment. It has been reported that *A. butzleri* can remain viable for up to 16 days in groundwater (Rice *et al.* 1999). Houf *et al.* (2003) suggested that arcobacters survive the scalding water temperatures (52°C) in poultry processing plants, which have implications as to how cross-contamination between poultry carcass can be controlled during processing. However, these bacteria can easily attach to various water distribution pipe surfaces, like stainless steel, copper, and plastic (Assanta *et al.*, 2002) which makes them potentially difficult to control in processing plants. As these organisms are susceptible to chlorination (Rice *et al.*, 1999), chlorinated water may be considered free of arcobacters.

1.5.4 Arcobacters in animals

1.5.4.1 Poultry

Although live poultry are susceptible to infection in natural or experimental conditions (Wesley and Baetz, 1999), it has been argued that arcobacters may not be normal inhabitants of the poultry intestine (Atabay *et al.*, 1998; Eifert *et al.*, 2003; Gude *et al.*, 2005), or that their habitat in living birds in unknown (Houf *et al.*, 2000). A recent study indicated cloacal contents of poultry are naturally colonized by various species of *Arcobacter* (Atabay *et al.*, 2006). As with the retail carcasses, poultry faecal samples may have a high isolation rate of up to 72% (Table 7) indicating that contamination of carcasses occurs during processing (Gude *et al.*, 2005; Atabay *et al.*, 2006).

Table 7. Prevalence rate of arcobacters in poultry faecal/cloacal swab samples in different countries of the world.

Country	Species detected	Prevalence (%)	No of samples	Reference
Belgium	None	0%	30	Houf *et al.*, 2000
Denmark	*A. butzleri* (n=13) *A. cryaerophilus* (n=9)	72%	29	Atabay *et al.*, 2006
Japan	*A. butzleri*- 47.1% *A. cryaerophilus*- 55.9% isolates	14.5 %	234	Kabeya *et al.*, 2003b
UK	No speciation	1.6%	60	Atabay and Corry, 1997
USA	*A. butzleri* in 1% sample, others not speciated	15%	407	Wesley and Baetz, 1999

1.5.4.2 Pigs

Pigs are considered to be an important reservoir of arcobacters. Table 8 summarizes the isolation of arcobacters from pigs in different countries of the world. Since the first isolation of *Arcobacter*-like organisms from aborted porcine foetuses in the United Kingdom (Ellis *et al.*, 1978), a number of studies have been undertaken to estimate the occurrence of these organisms in pigs. Although the majority of the pigs are found to be healthy carrier of arcobacters (Driessche *et al.*, 2003; Kabeya *et al.*, 2003b; Driessche *et al.*, 2004), the organisms are also found to be associated with a variety of illnesses, such as infertility and reproductive problems (De Oliveria *et al.*, 1997), and gastric ulcers (Surez *et al.*, 1997). While their pathogenicity is not yet clearly established, arcobacters are found to

be capable of colonizing neonatal piglets (Wesley *et al.*, 1996). Transmission may occur horizontally or vertically (Ho *et al.*, 2005).

Table 8. Prevalence rate of arcobacters in pigs in different countries of the world.

Origin	Description	Reference
Belgium	*Arcobacter* spp. detected in 16-85% (n=294) faecal samples from healthy pigs; excretion ranged from 0 to 10^4 CFU/g faeces; most predominant species- *A. butzleri,*	Driessche *et al.*, 2004
Brazil	17 *Arcobacter* isolates obtained from visceral organs of aborted foetus and sows with reproductive problems; 12 (71%) were *A. cryaerophilus* 1B, four (24%) were *A. cryaerophilus* 1A, and one (6%) was *A. butzleri.*	De Oliveria *et al.*, 1997
Brazil	24% (n=74) of the preputial swab samples positive for arcobacters; 8 were *A. cryaerophilus* 1B, 7 were *A. cryaerophilus* 1A and 2 were *A. butzleri.*	De Oliveria *et al.*, 1999
Denmark	*Arcobacter* spp. detected in >40% (n=55) aborted pig foetuses; 11 isolates were *A. cryaerophilus*, 10 were *A. skirrowii,*	On *et al.*, 2002
Japan	10% (n=250) of the faecal samples positive for arcobacters; *A. butzleri* the most prevalent species (60%) followed by *A. cryaerophilus* (36%); 13.3% (n=15) of the vaginal swab samples positive for *A. butzleri* only;	Kabeya *et al.*, 2003b
The Netherlands	>42% (n=144) sow's rectal swab samples positive for arcobacters; *A. skirrowii* - the predominant species, followed by *A. cryaerophilus* or *A. butzleri*; seasonality not found; infection of the newborn piglets ranged from 38.5 to 83.3% in each litter (litter size=4-17)	Ho *et al.*, 2006
UK	82% (n=17) of the aborted foetuses 18% (n=11) of the normal foetuses were harbouring *Arcobacter*-like organisms;	Ellis *et al.*, 1978
USA	*Arcobacter* spp. detected in 40.4% (n=952) of porcine faecal samples	Harmon and Wesley, 1996
USA	Arcobacters detected in 46% faecal samples (n=1,057) of market weight pigs; no species differentiation done	Wesley *et al.*, 1999
USA	*Arcobacter* spp. recovered from 43% (n=30) of porcine abortion cases; *A. cryaerophilus* 1B was the predominant species followed by *A. cryaerophilus* 1A and *A. butzleri.*	Schroeder-Tucker *et al.*, 1996
USA	2.85% (n=350) caecal samples from slaughtered pigs were positive for arcobacters; All were *A. butzleri*; pigs were colonized by multiple *Arcobacter* genotypes;	Hume *et al.*, 2001

1.5.4.3 Cattle

A summary of isolation of arcobacters from cattle in different parts of the world is shown in Table 9. In cattle, arcobacters has been isolated from a wide range of specimens including faeces (Driessche *et al.*, 2005), vaginal swabs (Kabeya *et al.*, 2003b), mastitic milk (Logan *et al.*, 1982), preputial sheath wash (Gill, 1983), and various visceral organs (Kiehlbauch *et al.*, 1991a). The prevalence rate in faecal samples in cattle has been found to range from

3.6 to 39%, with much higher rate in dairy cows (Wesley *et al.*, 2000; Golla *et al.*, 2002; Driessche *et al.*, 2003; Kabeya *et al.*, 2003b).

With the exception of a few abortions, diarrhoea and mastitis, a number of animals (11% of 276) have also been found to serving as healthy carrier of these organisms (Driessche *et al.*, 2005). Transmission may occur horizontally but vertical transmission has not been reported (Driessche *et al.*, 2005).

Table 9. Isolation of arcobacters from cattle in different countries of the world.

Origin	Description	Reference
Belgium	-Arcobacters detected in faecal samples of 11 % (n=276) of the animals; 5.9 to 11% in dairy cattle, 18.9 % in young cattle and 27.3% in calves; *A. cryaerophilus* predominant species followed by *A. butzleri* and *A. skirrowii*. - *Arcobacter* excretion ranged from 0 to 10^4 CFU/g faeces	Driessche *et al.*, 2005
Belgium	-Arcobacters detected in faecal samples of 39%(n=50) of the animals; *A. butzleri* isolated from 13, *A. cryaerophilus* from five and *A. skirrowii* from two samples	Driessche *et al.*, 2003
Canada	-4% of 198 isolates of campylobacters obtained from bovine faeces identified as arcobacters, four were *A. butzleri*, and *A. skirrowii*, and the remaining were *Campylobacter* spp.	Inglis and Kalischuk, 2003
Japan	-Arcobacters detected in 3.6% (n=332) faecal samples; *A. butzleri* (83.3% of isolates) the most prevalent species, followed by *A. cryaerophilus* 1B (16.7%); -Detected in 5 (8.1%) of 61 vaginal swab samples; four *A. butzleri*, one *A. cryaerophilus* 1B. -The seasonal positive rate varied from 1.4% in spring to 7.6% in summer	Kabeya *et al.*, 2003b
Turkey	-9.5% (n=200) of the rectal swab samples positive; *A. butzleri* most prevalent (7%) followed by *A. cryaerophilus* (2%) and *A. skirrowii* (0.5%)	Ongor *et al.*, 2004
UK	*Arcobacter* like organisms isolated from 44% (n=34) of aborted bovine foetuses	Ellis *et al.*, 1977
USA	*Arcobacter* spp. identified in 14.3% (n=1,682) of healthy cows; No information on different species	Wesley *et al.*, 2000
USA	9% (n=200) of the cattle sampled tested positive for *A. butzleri*; highest incidence for *A. butzleri* (i.e. 18%) observed for dairy cows; no other species detected	Golla *et al.*, 2002

1.5.4.4 Other animals

Besides poultry, pigs and cattle, arcobacters have also been recovered from other animal species. Driessche *et al.* (2003) isolated arcobacters from 16.1% (n=62) of ovine and 15.4% (n=13) of equine faecal samples. *A. butzleri* was the only species detected in equines whereas both *A. butzleri* and *A. cryaerophilus* were detected in ovines, the latter species being predominant. Wesley *et al.* (1995) have also mentioned the detection

Arcobacter spp. in aborted equine foetuses. A number of studies have recovered *A. butzleri* from primates with or without diarrhoeal illness (Kiehlbauch *et al.*, 1991a; Anderson *et al.*, 1993; Higgins *et al.*, 1999). Other animals from which arcobacters have been isolated include raccoon (Hamir *et al.*, 2004) tortoise and ostrich (Kiehlbauch *et al.*, 1991a).

There are several reasons behind the variation in isolation rates of *Arcobacter*, even from similar sample types. The most important reason may be the variation in isolation medium. Besides, hygienic practices during production and/or processing, sample size and sampling methods, and identification methods may influence the number of positive samples (Madden *et al.*, 2000; Atabay *et al.*, 2003; Ho *et al.*, 2006).

While the earlier laboratory methods favoured isolation of campylobacters instead of arcobacters, the precise role of the latter in human illness is still unknown. However, with improved isolation and identification methods, there is increasing evidence that *Arcobacter* is an emerging human pathogen (Phillips, 2001; Vandenberg *et al.*, 2004; Ho *et al.*, 2006). Nevertheless, currently available isolation techniques are not standardized and need further improvement as they are not optimal for all species of arcobacters (Atabay *et al.*, 1998; Houf *et al.*, 2001a; Houf *et al.*, 2001b). The widespread occurrence of arcobacters in food and water also requires the development of effective isolation methods for accurately assessing their prevalence and significance in human diseases.

While there are no epidemiological studies on the routes of transmission of arcobacters to humans, circumstantial evidence suggests that transmission results from consumption of contaminated food (mainly poultry) and water (Marinescu *et al.*, 1996b; Rice *et al.*, 1999). As the precise role of poultry meat in human *Arcobacter* infection is still unclear, molecular fingerprinting of these organisms may contribute to our knowledge of the epidemiological behaviour including contamination sources and transmission routes.

1.6 Aims and objectives

The isolation of *Arcobacter* species requires specific conditions, and the current methods may not be optimal for all species. The failure of isolation or very low isolation rate of these organisms may be due to the lack of information about the most appropriate isolation method. This study will thus compare the most commonly followed *Arcobacter* isolation protocols and recommend the most appropriate one for isolation of arcobacters from poultry meat in New Zealand.

Nothing is known about the prevalence of *Arcobacter* spp. in poultry meat in New Zealand, and its potential clinical significance as a foodborne pathogen. The overall objective of this study is to determine the potential role of poultry meat as a source of *Arcobacter* spp. in New Zealand.

The study aims:

- To establish the prevalence rate of *Arcobacter* species in poultry meat in New Zealand,
- To compare seven different techniques of *Arcobacter* isolation from poultry meat,
- To identify the species of *Arcobacter* prevalent in poultry meat in New Zealand,
- To compare the relatedness (similarity or diversity) among *Arcobacter* strains isolated from different poultry producers and by different methods.

CHAPTER 2: MATERIALS AND METHODS

2.1 Sample collection

To determine the prevalence of *Arcobacter* spp., a total of 150 fresh, whole retail poultry carcasses were purchased from three different poultry producers, 50 from each, through two supermarket outlets in Palmerston North, New Zealand, during the period of May to October 2005. Following purchase, the poultry carcasses were transported to the microbiology laboratory at Massey University. During transportation and storage, retail poultry packs were maintained at 4 to 6°C. A period of period of approximately 1-4 h elapsed between purchase and analysis. The packaging condition was checked for its intactness, and 'use by' date of each sample was noted.

2.2 Media preparation and quality control

Most of the media used in this study were prepared at Central Sterile and Media Supply of Massey University, Palmerston North. The antibiotic supplements used for CAT broth and CAT agar were the commercially available ones (CAT supplement, Oxoid, Basingstoke, UK). Antibiotic supplements used in *Arcobacter* broth and *Arcobacter* agar were purchased separately from Sigma (St. Louis, USA), and the supplement mix was prepared in the lab as described in Appendix 6. Those media were checked periodically for the ability to grow *A. butzleri*, *A. cryaerophilus*, *C. jejuni* and *E. coli* reference strains.

2.3 Isolation

Seven different methods were evaluated for their ability to support the growth of arcobacters, and these are summarized in Figure 1. Isolation was done from each carcass by each method. In all methods, the initial processing of the poultry carcass was the same. The poultry carcass was placed in a stomacher bag (BA6042; Seward Limited, UK) and 200 ml of sterile buffered peptone water (BPW; Appendix 1) was poured over it. The carcass and BPW was massaged manually through the bag for about 5 minutes. The BPW was filtered through sterile cheese cloth into a 250 ml sterile Nalgene centrifuge bottle.

The BPW was centrifuged for 15 min at 16000x g at 5-15°C using refrigerated superspeed centrifuge (Life Technologies, USA). The supernatant was discarded and the pellet was resuspended in 10 ml of sterile BPW.

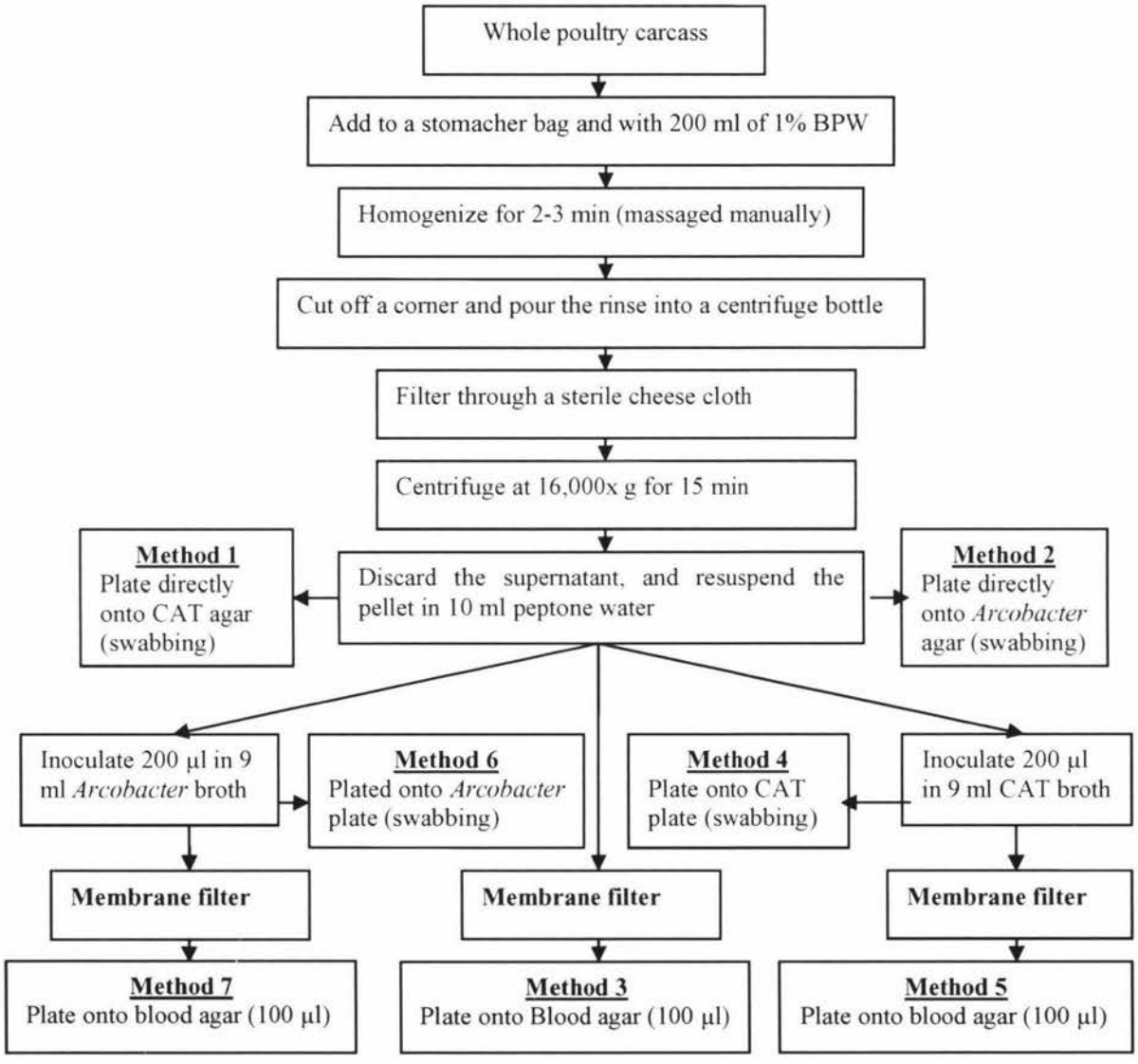

Figure 1. Flow diagram of procedures for isolation of *Arcobacter* species from poultry meat samples

In **Method 1**, direct plating was done by swabbing resuspended pellet onto *Campylobacter* selective agar (Lab M, Bury, England) containing three antibiotics: cefoperazone, amphotericin B, and teicoplanin (CAT agar, Appendix 5). The plates were incubated at 27±2°C for 48±4 hours, under microaerobic condition Microaerobic atmospheres

containing <1% O_2 and ~20% CO_2 were created in rectangular plastic jars (AnaeroPack System™, Mitsubishi Gas Chemicals, Japan) by using a gas pack (AnaeroPack System™) generating CO_2, and absorbing O_2. In **Method 2,** direct plating was done by swabbing onto *Arcobacter* agar (AA; Appendix 3) containing five antibiotics: cefoperazone, amphotericin B, 5-fluorouracil, novobiocin, and trimethoprim, as suggested by Houf *et al.,* (2001a). The plates were also incubated microaerobically at 27±2°C for 48±4 hours. **Method 3** was a modification of the protocol of Steele and McDermott (1984), and involved inoculation of 100 µl of suspension onto Columbia horse or sheep blood agar plates (Global Science & Technology Ltd., Auckland New Zealand) after being allowed to pass passively for 2 hours through a sterile membrane filter (Sartorius, Germany) of 0.45µm pore size. The incubation conditions were identical to method 1 or 2.

For the remaining four methods, 200 µl of the suspension was cultivated by enrichment in *Campylobacter* enrichment broth (Lab M, Bury, England) containing three antibiotic supplements; cefoperazone, amphotericin, and trimethoprim (CAT broth, Appendix 4; On *et al.*, (2002)) or *Arcobacter* broth (AB; Appendix 2; Houf *et al.*, (2001a)) microaerobically at 27±2°C for 48±4 hours. In **Method 4** (modified from protocol of Atabay *et al.,* 1998), CAT broth was swabbed onto CAT agar plate. In **Method 5** (protocol of On *et al.*, 2002), 100µl of this CAT broth was plated on to Columbia horse or sheep blood agar after passing it passively for 2 hours through a membrane filter of 0.45µm pore size. In **Method 6** (protocol of Houf *et al.*, 2001a), AB was swabbed onto AA. In **Method 7** (modified from protocol of Houf *et al.* 2001a), 100µl of this AB was plated on to Columbia horse or sheep blood agar after passing it passively for 2 hours through a membrane filter of 0.45µm pore size. As with all methods, incubation was done microaerobically for 48±4 hours at 27±2°C.

2.4 Identification of isolates

2.4.1 Presumptive identification

2.4.1.1 Colony morphology

A pure culture was obtained by subculturing single colonies from any of the isolation techniques onto blood agar plates. Reference strains were obtained for *A. butzleri* (ATCC 49616), *A. cryaerophilus* (ATCC 43158), and *Campylobacter jejuni* (ESR, 3785) from the Institute of Environmental Science and Research Limited (ESR), Wellington, New Zealand. Colony morphology of the isolates from pure culture was compared with these reference strains.

After 2-3 days of incubation on blood agar plates, *A. butzleri* reference strain colonies have a diameter of 2 to 4 mm, and are generally rounded in shape and whitish in colour. Similarly *A. cryaerophilus* reference strain colonies have a size of 1 mm, they are smooth, convex and have regular contour. The colonies become flattened and take an irregular form and their size is variable. Isolates having characteristic morphology were subjected to further phenotypic tests.

2.4.1.2 Biochemical tests

The ability of isolates to produce oxidase was tested using oxidase strips (Oxoid Limited, UK). Arcobacters are oxidase positive and produce purple-blue colouration within 10 seconds whereas there is no colour change in negative isolates.

2.4.1.3 Dark-field microscopy

A few presumptive *Arcobacter* colonies grown on blood agar plates were suspended in 0.9% sterile saline onto a glass slide. The suspension was inspected under 12.5×20 magnifications using an Olympus dark-field microscope. The suspension preparation was observed for characteristic morphology and motility. In dark-field microscopy, arcobacters

appear as small comma-shaped or spiral rods exhibiting typical darting or corkscrew motility

2.4.1.3 Storage

Presumptive isolates of *Arcobacter* spp. isolated from subsequent pure culture in sheep or horse blood agar were preserved in 15% glycerol broth and stored at -80°C for later molecular characterization.

2.4.2 Confirmative identification

2.4.2.1 Polymerase chain reaction (PCR)

Multiplex polymerase chain reaction (m-PCR) was performed, for all presumptive *Arcobacter* isolates, as described by Houf *et al.* (2000). For speciation of arcobacters either as *A. butzleri*, *A. cryaerophilus*, or *A. skirrowii*, three primer sets were used (Table 10).

2.4.2.1.1 DNA extraction

The presumptive arcobacters stored at -80°C were streaked onto blood agar plates and incubated microaerobically for 48 h at 27±2°C. For extraction of DNA, 2-3 colonies of pure culture presumptive arcobacters were suspended in 1 ml of saline in an Eppendorf tube. The suspension was boiled in a water bath for 10 minutes to destroy nucleases/proteases. This boiled suspension was used as template DNA for the PCR reaction.

2.4.2.1.2 PCR amplification

PCR reactions were performed in a reaction mixture (50 µl final volume) containing 2 µl of lysed bacteria (DNA template), 5 µl of 10×PCR buffer (Invitrogen, USA), 1.5 unit of *Taq* polymerase (Invitrogen), 0.2 mmol 1/L of each deoxyribonucleotide triphosphate (Geneworks, Australia), 1.5 mmol/L $MgCl_2$ (Invitrogen), 28.2 µl of distilled water and 50

pmol of the primers ARCO, BUTZ, CRY1, CRY2, and 25 pmol of the primer SKIR (Invitrogen). PCR involved 32 cycles of denaturation (94°C, 45s), primer annealing (61°C, 45s), and chain extension (72°C, 30s). For all experiments, a Perkin Elmer GeneAmp System 2400 PCR thermocycler was used. Amplified products from each sample were mixed with 5 µl of loading buffer (4mM cresol red, 60% sucrose) and were detected by electrophoresis in 1.5% agarose (certified molecular biology agarose, Bio-Rad, USA) in 0.5×Tris-borate-EDTA (TBE) buffer (Appendix 8) at 100 V for 40 min. Gels were stained with ethidium bromide. An UV transilluminator (GelDoc 2000, Bio-Rad, USA) with an analyst computer program (Quantity One 4.2 software, Bio-Rad) was used for visualization.

Table 10. Sequence and origin of the sets of primers (GenBank)

Species and target	Primers	Position	Primer sequence
A. butzleri	BUTZ	959-983	5'-CCTGGACTTGACATAGTAAGAATGA-3'
16S rDNA	ARCO	1357-1338	5'-CGTATTCACCGTAGCATAGC-3'
A. skirrowii	SKIR	705-723	5'-GGCGATTTACTGGAACACA-3'
16S rDNA	ARCO	1358-1339	5'-CGTATTCACCGTAGCATAGC-3'
A. cryaerophilus	CRY1	105-124	5'-TGCTGGAGCGGATAGAAGTA-3'
23S rDNA	CRY2	305-340	5'-AACAACCTACGTCCTTCGAC-3'

2.4.2.2 DNA sequencing

To confirm the result of PCR, the amplification products from eight isolates; three *A. butzleri* and five *A. cryaerophilus*, selected at random were sequenced at the Allan Wilson Centre in Massey University. PCR-amplified *A. butzleri* and *A. cryaerophilus* species-specific PCR products were purified using the QIAquick PCR purification kit (Qiagen 28104, MA, USA). The amount of DNA in each template was quantified by performing electrophoresis in 1.5% agarose gel against low mass DNA ladder (Invitrogen). Two sequencing reactions were prepared per isolate; one with forward primer and another with reverse primer. Each sequencing reaction of the *A. cryaerophilus* samples contained 2µl primer (CRY1 or CRY2; 25 pmol/µl), 1µl template DNA (5 ng/µl) and 12.0 µl distilled water. Similarly, sequencing reaction of *A. butzleri* contained 2µl primer (ARCO or BUTZ; 25 pmol/µl), 0.5 µl template DNA (25 ng/µl) and distilled water 12.5 µl.

Sequencing reactions were performed with the BigDye™ terminator ready reaction cycle sequencing kit (Version 3.1, Applied Biosystems Inc., CA, USA). Using BLAST

procedure, nucleotide sequence of the PCR products from both species was compared with the previously published sequence of *A. butzleri* and *A. cryaerophilus* on the GenBank database of the National Centre for Biotechnology Information (NCBI).

2.5 Pulsed-field gel electrophoresis

All isolates obtained during the study that were identified as *Arcobacter* spp. were subjected to PFGE with the restriction enzyme *EagI* (New England Biolabs, USA). PFGE was done as described by Hume *et al.* (2001), with some modifications in plug preparation step.

2.5.1 Preparation of Plugs

Arcobacter colonies that were incubated microaerophilically for 48-72 hours at 27±2°C on blood agar plates were swabbed gently without disturbing the agar surface using sterile cotton swabs and suspended in 2 ml of phosphate buffered saline (PBS) (Appendix 11) in a Falcon tube (Biosciences Discovery, USA). The optical density (OD) of the suspension was adjusted to 1.00 ± 0.20 using a turbidity meter (Microscan, USA). A 400 µl aliquot of each bacterial suspension was placed into an Eppendorf tube, and 20 µl of 20mg/ml proteinase K (Appendix 9) was added. An equal amount of Seakem Gold agarose (1%) (Appendix 10) brought to 55°C was added to the cell suspension with gentle pipetting 5-6 times to mix well. Following mixing, 400 µl of the cell-agarose suspension was immediately dispensed into the wells of reusable plug molds (Bio-Rad, USA). Two to three plugs were made from each isolate. Plugs were allowed to cool and solidify at room temperature for 10-15 min or at 4°C for 5 min. Plugs were placed in universal plastic tubes (Techno-Plus, Australia) containing 5 ml of lysis buffer (Appendix 14) and 25µl of proteinase K and incubated at 55° for 2-3 hours using a shaking waterbath (Bellco Biotechnology, USA).

After incubation, the lysis buffer was drained out and the plugs were washed with MiliQ (MQ) water. The plugs were incubated in 10-15 ml of MQ water at 55°C in the shaking

waterbath for 10-15 min. The MQ water was replaced with 10-15 ml of TE buffer (Appendix 16) and incubated for 10-15 min. This washing with TE was repeated another three times. Plugs were stored in 2 ml of TE buffer at 4°C until ready for restriction digestion.

2.5.2 Restriction digestion of PFGE plugs

Each plug was removed from the storage tube and placed on a sterile glass slide. One third (about 2 mm) of the plug was sliced off with a scalpel and placed into 100 µl of digestion mastermix (Table 11). The remainder of the plug was placed into 1 ml of TE buffer and stored at 4°C until required. Ethanol was used to sterilize the glass slide and scalpel after slicing each plug specimen. Incubation was done at 37°C for four hours.

Table 11. Incubation conditions for restriction of plugs with *Eag*I (New England Biolabs, USA)

S.N.	Components	Volume (per plug)
1	Sterile MQ water	86 µl
2	Restriction enzyme 10X buffer	10 µl
3	Restriction enzyme (10 units/µl)	4 µl
	Total	100 µl

2.5.3 Electrophoresis

Electrophoresis was done in 1% pulsed-field certified agarose (Cambrex Bioscience, USA) prepared in 0.5×TBE (Appendix 8). The electrophoresis cell of a CHEF Mapper (Bio-Rad, USA) was filled with 2.2 litre of 0.5×TBE buffer, and circulated for about one hour at 14°C. Restriction digested plugs were loaded on the gel along with a low-molecular-weight marker (New England Biolabs, USA), and two lambda-markers (New England Biolabs, USA). The gel was placed in the electrophoresis tank and ran using the following conditions: Initial switch time 0.1 seconds, final switch time 90 seconds, run time 20 hours, angle 120°, gradient 6V/cm, temperature 14°C, and ramping factor linear.

2.5.4 Staining and documentation

The gel was removed from the tank and stained in ethidium bromide solution (Appendix 17) for 10 minutes. The gel was then rinsed with sterile MQ water and photographed under

UV light using the Gel-DOC 2000 (Bio-Rad, USA). Using Bionumerics software (Version 4.0, Applied-Maths, Belgium), inverted Tiff images of the PFGE profiles were digitised, normalised and subjected to cluster analysis. Band positions of digitised images determined automatically by computer were checked manually. Bands were described by their sizes in kilobases (Kb) relative to one of the molecular weight markers (Low molecular weight marker; New England Biolab) and were identified from the highest molecular weight downwards. In generating dendograms, the dice similarity coefficient was used and patterns were clustered by the unweighted pair group method using arithmetic averages (UPGMA), at 0.5% optimization and 3.0% position tolerance.

2.6 Statistical analysis

From the sampling events and sample size, a 95% confidence interval for the overall prevalence and the prevalence in different poultry producers was calculated as described in Appendix 18.

Differences in the proportions of prevalence rates were analysed by a contingency table using Chi-square analysis. Subsequent individual comparisons were undertaken using a procedure analogous to the Tukey test (Zar, 1984) as mentioned in Appendix 19. All proportions were transformed $p' = \arcsin \sqrt{p}$ for the Tukey test.

Simpson's index of diversity was calculated to estimate the subtype diversity of *Arcobacter* spp., according to Hunter and Gaston (1988) as mentioned in Appendix 20.

CHAPTER 3: RESULTS

3.1 Presumptive identification of *Arcobacter* spp.

A total of 150 retail poultry carcasses from three producers were collected from two supermarket outlets in Palmerston North during this study period. Based on colony morphology (Figure 2), dark-field microscopy, and an oxidase test, a total of 210 isolates were identified as presumptive *Arcobacter* spp. Cells of each of the two species examined demonstrated motility under the conditions mentioned in the methodology. Most of the cells clearly exhibited a rapid, darting motility, but a few cells appeared to be poorly motile

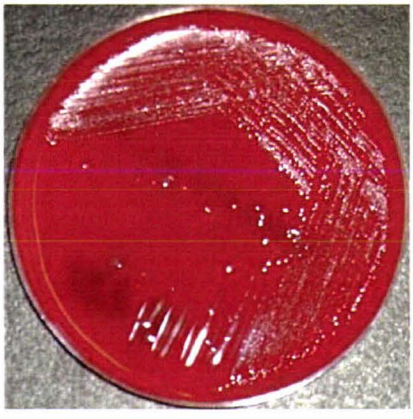

Figure 2. Typical colony morphology of *Arcobacter butzleri*

3.2 Confirmative identification of *Arcobacter* spp.

3.2.1 Polymerase chain reaction

The presumptive *Arcobacter* isolates were subjected to confirmative identification by a multiplex PCR (m-PCR). A total of 189 out of 210 presumptive isolates were confirmed to be *Arcobacter* spp. Ninety-two (92.5%) percent of these isolates were identified as *A. butzleri*, and the remaining 7.4% as *A. cryaerophilus*. The PCR primers used targeted regions of the 16S and 23S rRNA genes which amplified a 257 bp and 401 bp fragment, respectively, in *A. cryaerophilus* and *A. butzleri*, as seen in the agarose gel after electrophoresis (Figure 3).

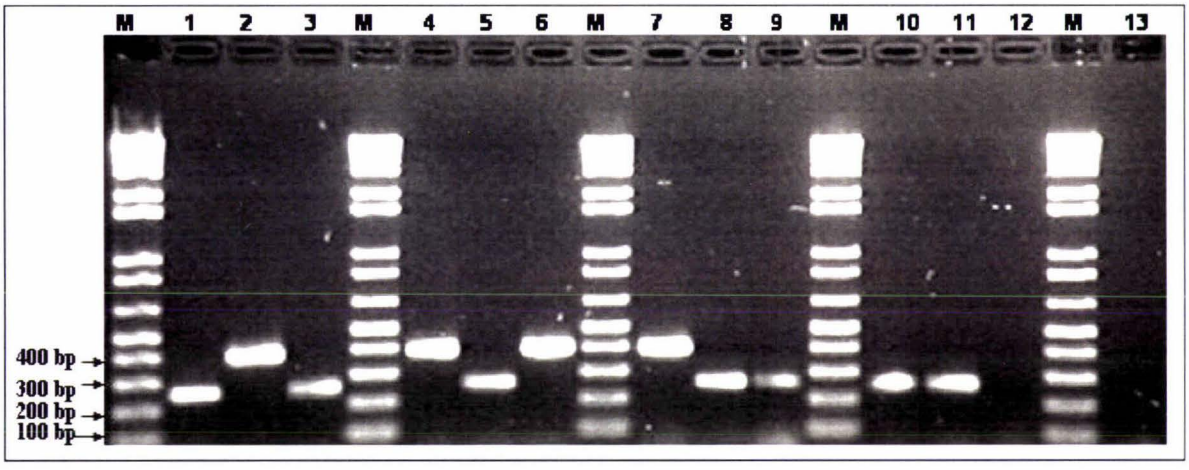

Figure 3. PCR of *Arcobacter* spp. isolated from poultry carcass samples

Lane M: 100 kb molecular size markers; lanes 1, 3, 8, 9, 10 & 11: *A. cryaerophilus* showing a 257 bp product; lanes 2, 4 & 7: *A. butzleri* showing a 401 bp product; lane 5: *A. cryaerophilus* reference strain (ATCC 43158); lane 6: *A. butzleri* reference strain (ATCC 49616); lane 12: presumptive *Arcobacter* spp.; lane 13: Negative control

3.2.2 DNA sequencing

To further confirm the results of PCR identification of the *Arcobacter* isolates, PCR-mediated direct nucleotide sequence analysis of the rRNA genes was performed. Results of sequencing confirmed the PCR identification results (Table 12).

Table 12. Sequence similarity of *A. butzleri* and *A. cryaerophilus* to the published database

Sample no	PCR result	Primer sequence	Sequence similarity (%)
130.2	*A. cryaerophilus*	5'-TGCTGGAGCGGATAGAAGTA-3'	98
		5'-AACAACCTACGTCCTTCGAC-3'	100
162.5	*A. cryaerophilus*	5'-TGCTGGAGCGGATAGAAGTA-3'	99
		5'-AACAACCTACGTCCTTCGAC-3'	100
163.7	*A. cryaerophilus*	5'-TGCTGGAGCGGATAGAAGTA-3'	99
		5'-AACAACCTACGTCCTTCGAC-3'	100
160.2	*A. cryaerophilus*	5'-TGCTGGAGCGGATAGAAGTA-3'	98
		5'-AACAACCTACGTCCTTCGAC-3'	100
157.5	*A. cryaerophilus*	5'-TGCTGGAGCGGATAGAAGTA-3'	99
		5'-AACAACCTACGTCCTTCGAC-3'	100
162.2	*A. butzleri*	5'-CGTATTCACCGTAGCATAGC-3'	100
		5'-CCTGGACTTGACATAGTAAGAATGA-3'	100
162.6	*A. butzleri*	5'-CGTATTCACCGTAGCATAGC-3'	100
		5'-CCTGGACTTGACATAGTAAGAATGA-3'	100
163.6	*A. butzleri*	5'-CGTATTCACCGTAGCATAGC-3'	99
		5'-CCTGGACTTGACATAGTAAGAATGA-3'	100

Comparison of the nucleotide sequence of the entire amplicon showed that the 16 rRNA gene of *A. butzleri* isolates shared 99 to 100% sequence identity, while that of 23S rRNA

gene of *A. cryaerophilus* isolates shared 98 to 100% similarity, with the sequence of the respective strains published in GenBank.

3.3 Prevalence of *Arcobacter* spp. in retail poultry

The PCR test confirmed the isolation of *Arcobacter* spp. in 83 (55.3%) of 150 poultry meat samples. Two species; *A. butzleri* and *A. cryaerophilus* were detected in 78 (52.0%) and 12 (8.0%) poultry carcasses, respectively. *A. butzleri* was the only *Arcobacter* spp. detected in 71 (47.3%) poultry carcasses whereas five (3.3%) carcasses contained *A. cryaerophilus* alone. Both the species were detected simultaneously in seven (4.6%) carcasses.

3.3.1 Comparison among producers

The prevalence of *Arcobacter* spp. in retail poultry carcasses from various producers is shown in Figure 4. A high variation was observed in the prevalence rate. Among the three producers, Producer B had the highest number of poultry carcasses positive for *Arcobacter* spp. followed by poultry carcasses from Producer C and A. Forty-nine (98%) of the 50 poultry carcasses from Producer B harboured *Arcobacter* spp. A total of 132 isolates were obtained from these 49 poultry carcasses out of which eight isolates were *A. cryaerophilus* and the remaining 124 isolates were *A. butzleri*. Nineteen (38%) of the 50 poultry carcasses from Producer C harboured *Arcobacter* spp. A total of 27 isolates were obtained from these 19 poultry carcasses, of which four isolates were *A. cryaerophilus* and the remaining 23 isolates were *A. butzleri*. Fifteen (30%) of the 50 poultry carcasses from Producer A harboured *Arcobacter* spp. A total of 30 isolates were obtained from these 15 poultry carcasses, of which two isolates were *A. cryaerophilus* and the remaining 28 isolates were *A. butzleri*.

Overall, the Chi-squared analysis showed there was a significant difference ($P<0.05$) in prevalence rates between producers. Subsequent comparisons using the Tukey test showed the difference in prevalence rates between Producer 'A and B', and 'B and C' were highly significant ($P<0.05$), whereas the difference in prevalence rates between Producer 'A and C' was not statistically significant ($P>0.05$) (see Appendix 19.1).

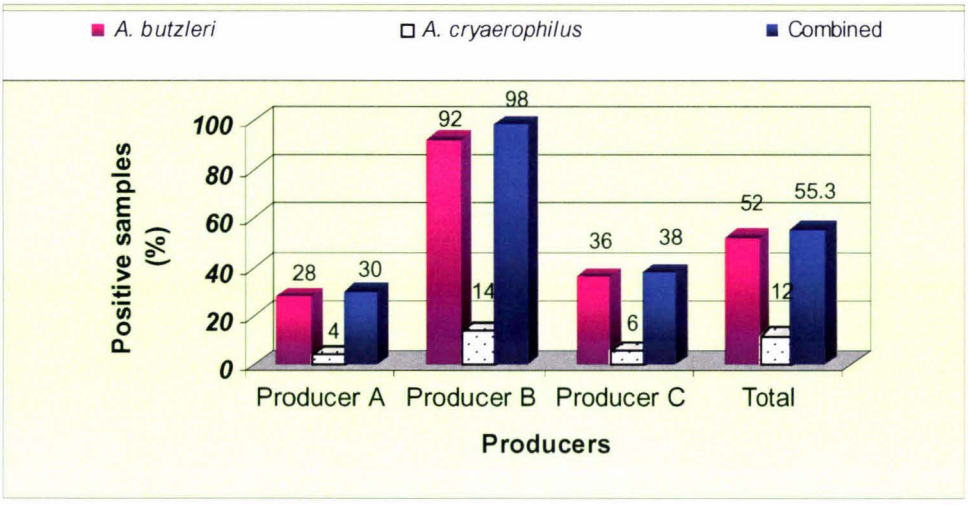

Figure 4. Percentage of poultry carcasses from different producers positive to *Arcobacter* spp.

Table 13 demonstrates that, prevalence of *Arcobacter* spp. in the population of retail poultry from Producer A ranges between 17.3% and 42.7%, that from Producer B between 94.1% and 100%, and Producer C between 24.5% and 51.4%. Thus, at 95% confidence interval, the prevalence of *Arcobacter* spp in poultry meat samples in New Zealand ranges between 47.3% and 63.2%.

Table 13. Calculation of 95% confidence intervals for the prevalence of *Arcobacter* spp. in different poultry producers

Source type	Producer A	Producer B	Producer C	Overall
Sample size	50	50	50	150
Total number of positive samples	15	49	19	83
Total measured prevalence for *Arcobacter* spp.	30%	98%	38%	55.3%
Variance	0.0042	0.00039	0.00471	0.00164
Standard error of prevalence	0.06480	0.01979	0.06864	0.04049
95% confidence interval for prevalence (p±1.96 SEp)	0.30±0.127	0.98±0.0387	0.38±0.1345	0.55±0.079
Upper confidence limit	42.7%	100%	51.4%	63.2%
Lower confidence limit	17.3%	94.1%	24.5%	47.3%

The packaging condition of poultry carcasses from Producer A was excellent and that of Producer B were the worst. Out of 50 poultry carcasses from Producer B, 38 were leaking, while 27 from C and only two from A were leaking.

3.3.2 Comparison of the isolation methods

Arcobacter spp. was detected in 55.5% of 150 poultry meat samples tested by a combination of the six methods (Figure 5). Method 7 was the best with an isolation rate of 39.3%, followed by Method 6 (38.6%), Method 5 (31.3%), Method 2 (8%), Method 1 (5.3%), and Method 4 (3.3%). Method 3 did not detect any isolates of *Arcobacter*, and was not included in the statistical analysis. The difference in isolation rates of Methods '6 and 7', '5 and 6', '1 and 2' and '1 and 4' was not statistically significant (P>0.05). The remaining comparisons between methods were significantly different to each other (see Appendix 19.2).

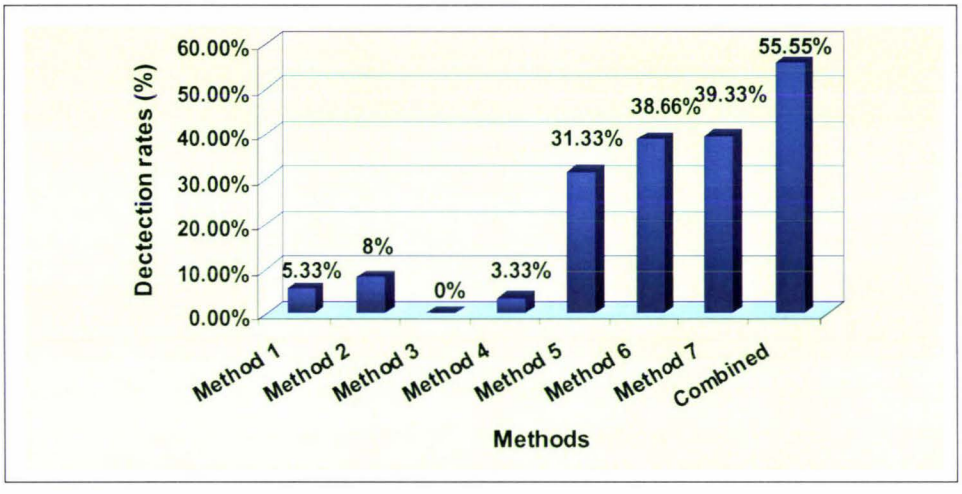

Figure 5. *Arcobacter* spp detection rates of seven different methods

Method 1. Direct inoculation on CAT agar; Method 2. Direct inoculation on *Arcobacter* agar; Method 3. Direct inoculation on 'filter-blood agar'; Method 4. CAT broth enrichment to CAT agar; Method 5. CAT broth enrichment to 'filter-blood agar'; Method 6. *Arcobacter* broth enrichment to *Arcobacter* agar; Method 7. *Arcobacter* broth enrichment to 'filter-blood agar'

A total of 175 *A. butzleri* and 14 *A. cryaerophilus* isolates were obtained from 83 retail poultry samples by combination of the six different methods. From 26 carcass samples, a positive culture of *A. butzleri* was obtained by one method only, from 20 carcasses by two methods, from 25 carcasses by three methods, and from 12 carcasses by four methods. Similarly, from 10 carcass samples, a positive culture of *A. cryaerophilus* was obtained by one method only, and from two carcasses by two methods.

There was a wide variation among the number of *Arcobacter* isolates of both species detected by different methods (Figure 6). Method 7 detected 59 isolates of *Arcobacter* out

of which 58 isolates were *A. butzleri* and only one isolate was *A. cryaerophilus*. Method 6 detected 58 isolates of *Arcobacter* out of which 56 isolates were *A. butzleri* and two isolates were *A. cryaerophilus*. Method 5 detected 47 isolates of *Arcobacter* out of which 42 isolates were *A. butzleri* and five isolates were *A. cryaerophilus*. Method 4 detected five isolates of *Arcobacter* all of which were *A. butzleri*. Method 2 detected 12 isolates of *Arcobacter* out of which 10 isolates were *A. butzleri* and two isolates were *A. cryaerophilus*. Method 1 detected eight isolates of *Arcobacter* out of which four isolates were *A. butzleri* and four isolates were *A. cryaerophilus*. It appears that Method 5 is most appropriate for isolation of *A. cryaerophilus* followed by Method 1. None of the methods detected all arcobacters of both species.

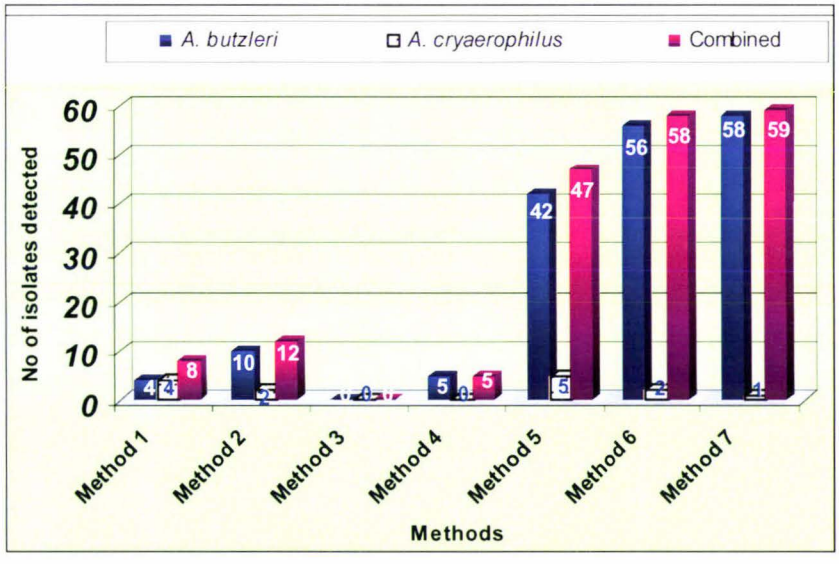

Figure 6. Number of *Arcobacter* spp. isolates detected by seven different methods

Method 1. Direct inoculation on CAT agar; Method 2. Direct inoculation on *Arcobacter* agar; Method 3. Direct inoculation on 'filter-blood agar'; Method 4. CAT broth enrichment to CAT agar; Method 5. CAT broth enrichment to 'filter-blood agar'; Method 6. *Arcobacter* broth enrichment to *Arcobacter* agar; Method 7. *Arcobacter* broth enrichment to 'filter-blood agar'

3.4 Diversity among *Arcobacter* spp. isolates

3.4.1 PFGE of *Arcobacter* spp. isolates

Genomic DNA from 168 *Arcobacter* spp. isolates were characterized by PFGE using the restriction enzyme *Eag*I. Digestion with *Eag*I endonuclease yielded PFGE fragments patterns useful for genotypic strain differentiation (Figure 7). Although the reference

strains showed only five to seven bands. the enzyme digestions for the genomic DNA from *Arcobacter* isolates generated between four and fourteen fragments ranging from approximately 20 kb to greater than 1000 kb in length. A PFGE profile was not obtained for 12 *A. butzleri* and two *A. cryaerophilus* isolates digested with *Eag*I endonuclease. Numerous attempts resulted in an undigested single band.

3.4.1.1 PFGE of *A. butzleri* isolates from poultry meat samples

Out of 175 *A. butzleri* isolates characterized by PFGE. 56 isolates (one isolate per poultry carcass sample) were selected for comparison among the three producers. On the basis of >90% (90 to 100%) similarity of the PFGE patterns at 0.5% optimization and 3.0% position tolerance (Figure 7). 21 band patterns of *A. butzleri* isolates were obtained (Table 14).

Table 14. PFGE restriction patterns and the subtype diversity index of 56 *A. butzleri* isolates from three different producers.

Patterns	Producer A	Producer B	Producer C	Total
A		2		2
B		1		1
C	1		1	2
D		7		7
E		1		1
F	2			2
G		2		2
H		1		1
I		1	2	3
J	1	1		2
K			4	4
L	1			1
M		1		1
N		1		1
O		6	7	13
P	1	2		3
Q		1		1
R		3		3
S	1	3		4
T		1		1
U	1			1
No of isolates	**8**	**34**	**14**	**56**
No of subtypes	**7**	**16**	**4**	**21**
Diversity Index	**0.964**	**0.919**	**0.692**	**0.922**

Six out of these 21 patterns were common to more than one producer. however none of the patterns was common to all three sources. Table 14 shows that highest subtype diversity index was found for *A. butzleri* isolates obtained from poultry carcasses from Producer A

(0.964), followed by Producer B (0.919), and Producer C (0.692). The overall diversity index for *A. butzleri* was 0.922.

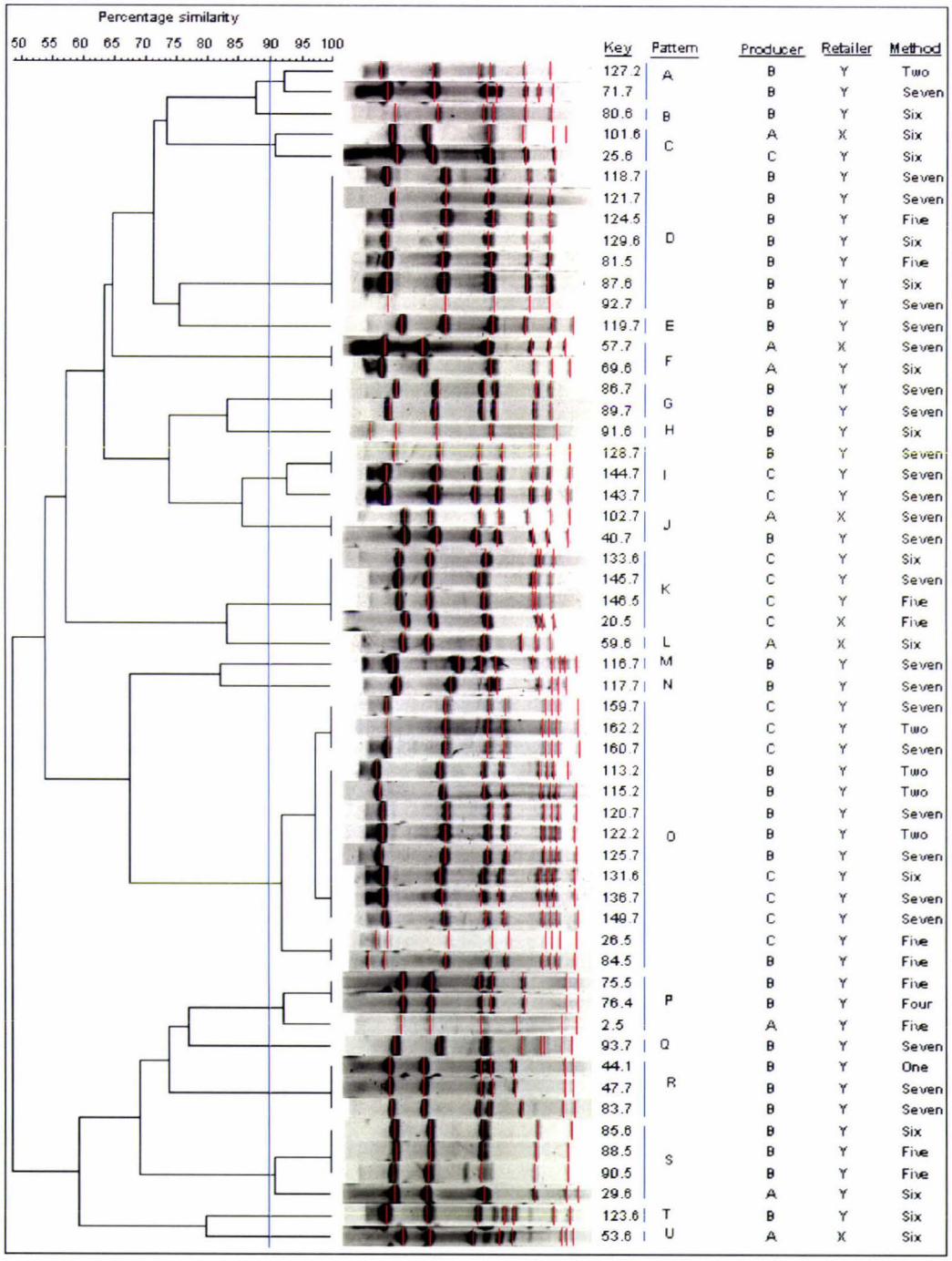

Figure 7. PFGE profile of 56 isolates of *Arcobacter butzleri* selected at random for diversity index calculation

3.4.1.2 PFGE of *A. cryaerophilus* isolates from poultry meat samples

Twelve isolates of *A. cryaerophilus*, each obtained from different poultry meat samples were selected for comparison among the three producers. On the basis of >90% (90 to 100%) similarity of the PFGE patterns at 0.5% optimization and 3.0% position tolerance (Figure 8), seven band patterns of nine *A. cryaerophilus* isolates were obtained (Table 15). The remaining three isolates were not suitable for comparison as the chromosomal DNA in plugs of two of them were undigested, and one smeared on repeated attempts.

Table 15. PFGE restriction patterns and the subtype diversity index of 12 *A. cryaerophilus* isolates from three different producers .

Patterns	Producer A	Producer B	Producer C	Total
A	-	-	1	1
B	-	1	-	1
C	-	1	-	1
D	1	-		1
E	-	2	-	2
F	-	-	2	2
G	-	1		1
No of isolates	**1**	**5**	**3**	**9**
No of subtypes	**1**	**4**	**2**	**7**
Diversity Index	**Undefined**	**0.900**	**0.667**	**0.944**

None of the seven patterns of *A. cryaerophilus* were common to more than one producer. Table 15 shows that highest subtype diversity was found among the isolates obtained from poultry meat from Producer B (0.900) followed by Producer C (0.667), while that from Producer A could not be defined. Diversity index of *A. cryaerophilus* from Producer B and C were inconsistent with that of *A. butzleri* from the respective producers. The overall diversity index indicated that *A. cryaerophilus* were more diverse than *A. butzleri* (0.944 vs 0.922) across poultry carcasses from three producers. The lowest similarity among *A. butzleri* isolates was 50% while that among *A. cryaerophilus* isolates was 55%.

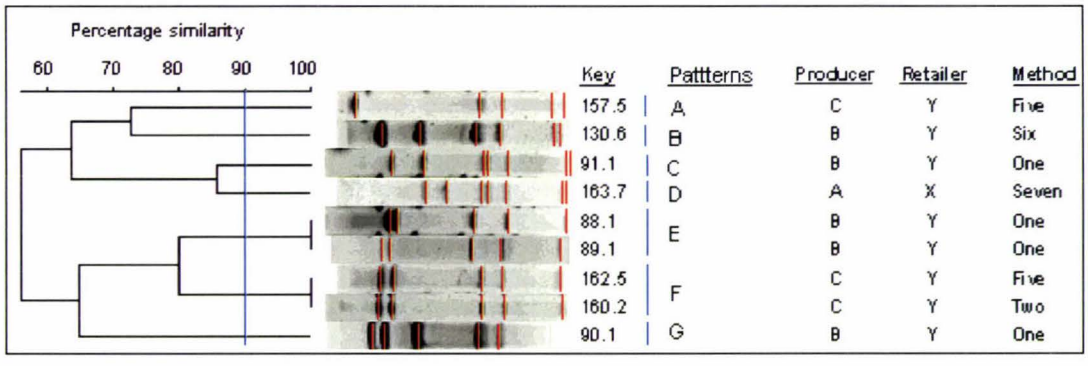

Figure 8. PFGE profile of nine *A. cryaerophilus* isolates for diversity index calculation

3.4.2 Comparison of *Arcobacter* isolates from different producers

3.4.2.1 Comparison of isolates from Producer A

Genomic DNA of 13 isolates of *A. butzleri* and one isolate of *A. cryaerophilus* obtained from 14 poultry meat samples from Producer A characterized by PFGE were compared. Restriction digestion with *Eag*I yielded six to eight bands in PFGE ranging in size from <40 kb to 1000 kb. Seven band patterns of eight *A. butzleri* isolates were obtained (Table 14). Of the seven, four band patterns (C, J, P, and S) were shared with other producers. Patterns J, P and S were shared with Producer B whereas pattern C was indistinguishable from that of Producer C. The remaining patterns F, L, and U were unique to *A. butzleri* isolates from poultry meat from Producer A. The diversity index for *A. butzleri* isolates from Producer A was calculated to be 0.964 and lowest similarity among isolates obtained from this producer was 56.41%.

3.4.2.2 Comparison of isolates from Producer B

Genomic DNA of 55 isolates of *A. butzleri* and six isolates of *A. cryaerophilus* obtained from poultry meat from Producer B were characterized by PFGE. Restriction digestion with *Eag*I yielded five to nine bands in PFGE ranging in size from <40 kb to 1000 kb. Sixteen band patterns of 34 *A. butzleri* isolates were obtained (Table 14). Of the 16, five band patterns (I, J, O, P and S) were shared with other producers. Pattern I and O were shared with Producer C whereas pattern J, P, and S were indistinguishable from that of Producer A. The remaining 11 patterns (A, B, D, E, G, H, M, N, Q, R and T) were unique to *A. butzleri* isolated from poultry meat from Producer B. The diversity index for *A. butzleri* isolates from Producer B was calculated to be 0.919 and lowest similarity among isolates obtained from this producer was 47.77%.

Four PFGE patterns were detected among the five isolates of *A. cryaerophilus* from Producer B (Table 15). None of these four patterns were common to other producers. The diversity index was calculated to be 0.900 and the lowest similarity among these isolates was 52.89%.

3.4.2.3 Comparison of isolates from Producer C

Genomic DNA of 14 isolates of *A. butzleri* and three isolates of *A. cryaerophilus* obtained from poultry meat from Producer C were characterized by PFGE. Restriction digestion with *Eag*I yielded five to nine bands in PFGE ranging in size from <40 kb to 1000 kb. Four band patterns of 14 *A. butzleri* isolates were obtained (Table 14), out of which three band patterns (C, I, and O) were shared with other producers. Pattern C was shared with Producer A whereas pattern I and O was indistinguishable from that of Producer B. Pattern K was unique to *A. butzleri* isolates from poultry meat from Producer C. The diversity index for *A. butzleri* isolates from Producer C was calculated to be 0.692, and lowest similarity among isolates obtained from this Producer was 44.29%.

Two PFGE patterns were detected among the three isolates of *A. cryaerophilus* from Producer C (Table 15). None of these two patterns were common to other producers. The diversity index was calculated to be 0.667 and the lowest similarity among these isolates was 60.01%.

While comparing PFGE pattern of *A. butzleri* from three producers (Table 14), it appears that isolates from Producer A and Producer B are most common (three patterns common), followed by that between Producer B and C (two patterns common), and Producer C and A (one pattern common).

3.4.3 Comparison of *Arcobacter* isolates from different isolation methods

Out of the 83 *Arcobacter* spp. positive poultry carcasses, positive culture from 57 samples were detected by more than one method, 37 samples by more than two methods, and 12 samples by more than three methods. The PFGE patterns of the *Arcobacter* isolates obtained by different methods from the same carcass were not consistent. The number of occasions the positive culture isolates from the same poultry meat sample that were similar or dissimilar to each other, at different similarity levels, is shown in Table 16.

Methods '1 and 5', '2 and 5', '4 and 6', and '4 and 7' simultaneously detected *Arcobacter* positive cultures from the same poultry meat samples once only, and thus a comparison can not be made.

Table 16. Similarity of PFGE patterns of *Arcobacter* spp. isolated simultaneously from the same poultry sample by more than one method.

Methods	Total number of occasions occurring together	90 to 100 % similar (% occasions)	80 to 89% similar (% occasions)	70 to 79% similar (% occasions)	< 70% similar (% occasions)
1 and 5	1	-	-	-	1 (100%)
2 and 5	1	1* (100%)	-	-	-
2 and 6	6	1* (16%)	-	-	5 (84%)
2 and 7	4	-	-	-	4 (100%)
4 and 5	2	1 (50%)	1 (50%)	-	-
4 and 6	1	1 (100%)	-	-	-
4 and 7	1	-	1 (100%)	-	-
5 and 6	13	5 (38%)	-	2 (15%)	6 (46%)
5 and 7	10	3 (30%)	-	1 (10%)	6 (60%)
6 and 7	28	21 (75%)	2 (7%)	1 (3.5%)	4 (14%)
Total	**67**	**34**	**3**	**4**	**27**

* *A. cryaerophilus*

Two isolates of *A. butzleri* detected by Method 1 and 5 were related by about 40% only (Figure 9).

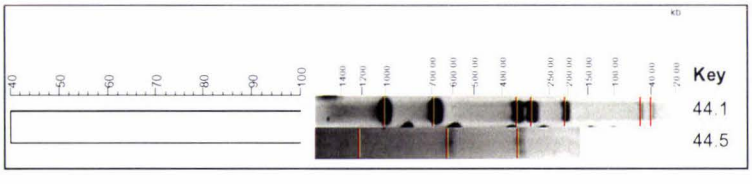

Figure 9 Two isolates of *A. butzleri* detected by method 1 and 5.

Two isolates of *A. cryaerophilus* detected by Method 2 and 5 were 99.99% similar (Figure 10).

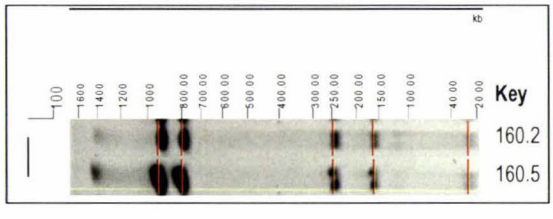

Figure 10. Two isolates of *A. cryaerophilus* detected my methods 2 and 5

Two isolates of *A. butzleri* detected by Methods 4 and 6 were 99.99% similar (Figure 11).

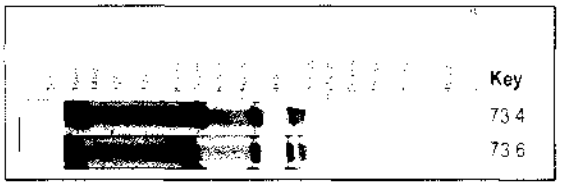

Figure 11. Two isolates of *A. butzleri* detected by methods 4 and 6

Two isolates of *A. butzleri* detected by method 4 and 7 were 80% similar (Figure 12).

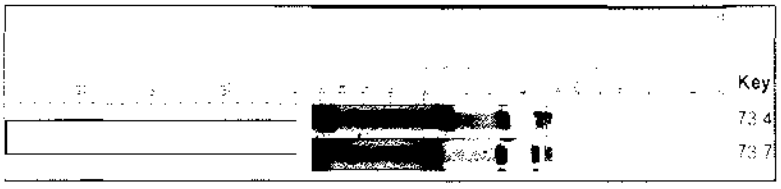

Figure 12. Two isolates of *A. butzleri* detected by method 4 and 7

3.4.3.1 Comparison of isolates from Method 2 and 6

Method 2 and 6 simultaneously detected *Arcobacter* positive cultures from six poultry meat samples (Table 16). Out of this 16% (n=1) of the time, the isolates were 100% similar (Figure 13), and 80% (n=5) of the occasions the isolates were <70% similar (Figure 14).

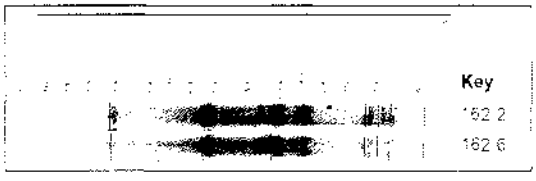

Figure 13 Two isolates of *A. butzleri* detected by method 2 and 6

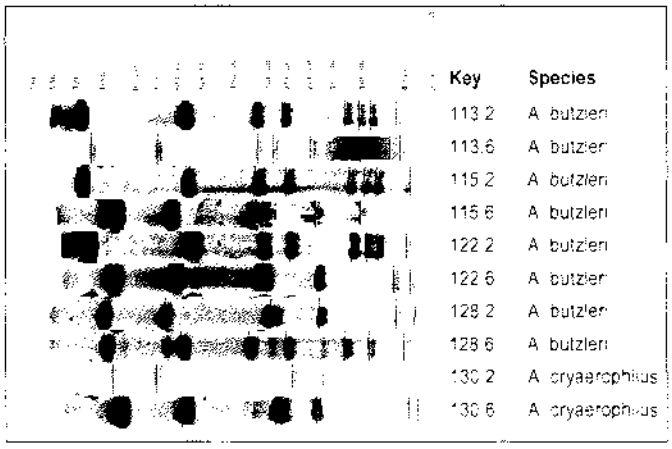

Figure 14. Ten isolates of *A. butzleri* and two isolates of *A. cryaerophilus* detected by method 2 and 6

3.4.3.2 Comparison of isolates from Method 2 and 7

Method 2 and 7 simultaneously detected *Arcobacter* positive cultures from four poultry meat samples (Table 16). Out of this, 100% of the occasions the isolates were <70% similar (Figure 15).

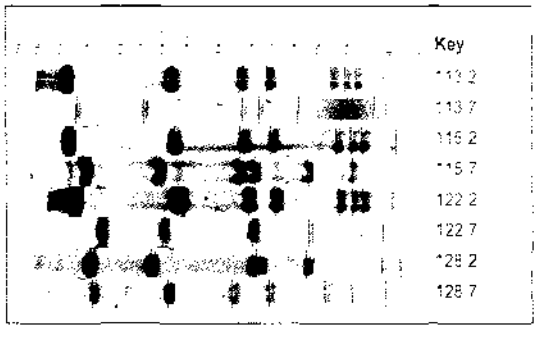

Figure 15 Eight isolates of *A. butzleri* detected by method 2 and 7

3.4.3.3 Comparison isolates from Method 4 and 5

Method 4 and 5 simultaneously detected *Arcobacter* positive culture from two poultry meat samples (Table 16). Out of this 50% (n=1) of the occasions, the isolates were 99.99% similar (Figure 16), and 50% (n=1) of the occasions the isolates were 80% similar (Figure 17).

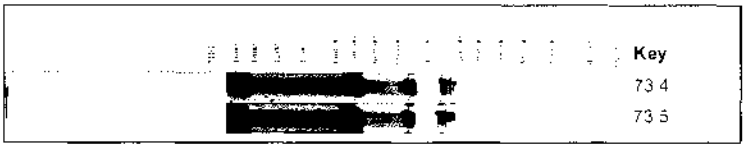

Figure 16. Two isolates of *A. butzleri* detected by method 4 and 5

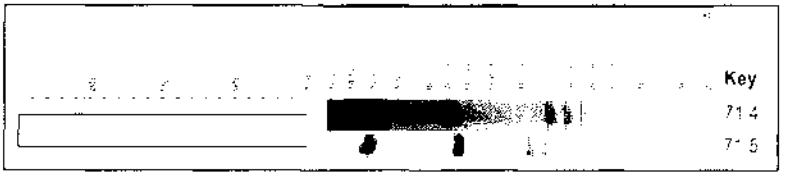

Figure 17. Two isolates of *A. butzleri* detected by method 4 and 5

3.4.3.4 Comparison of isolates from Method 5 and 6

Method 5 and 6 simultaneously detected *Arcobacter* positive cultures from 13 poultry meat samples (Table 16). Out of this 15% (n=2) of the occasions, the isolates were 70-79% similar (Figure 18). 38% (n=5) of the occasions the isolates were 100% similar (Figure 19), and 46% (n=6) of the occasions the isolates were < 70% similar (Figure 20).

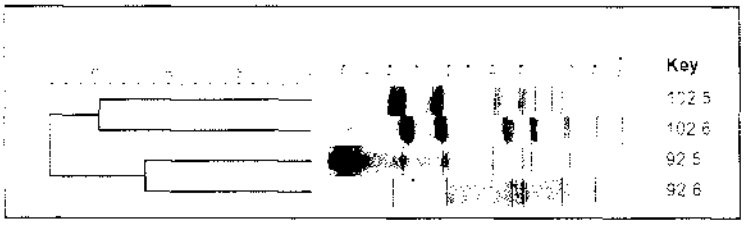

Figure 18 Four isolates of *A. butzleri* detected by method 5 and 6

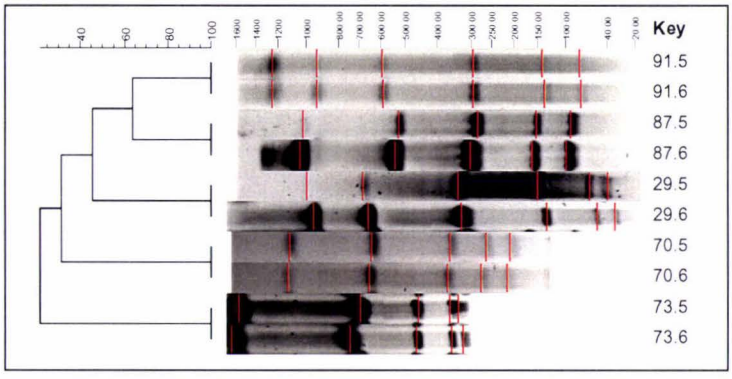

Figure 19 Ten isolates of *A. butzleri* detected by method 5 and 6

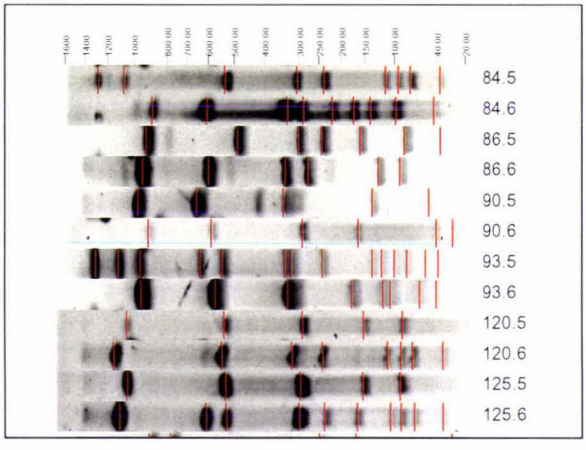

Figure 20 Twelve isolates of *A. butzleri* detected by method 5 and 6

3.4.3.5 Comparison of isolates from Method 5 and 7

Method 5 and 7 simultaneously detected *Arcobacter* positive cultures from 10 poultry meat samples (Table 16). Out of this, 30% (n=3) of the occasions, the isolates were 100% similar (Figure 21), 10% (n=1) of the occasions the isolates were 80-89% similar (Figure 22), and 60% (n=6) of the occasions the isolates were < 70% similar (Figure 23).

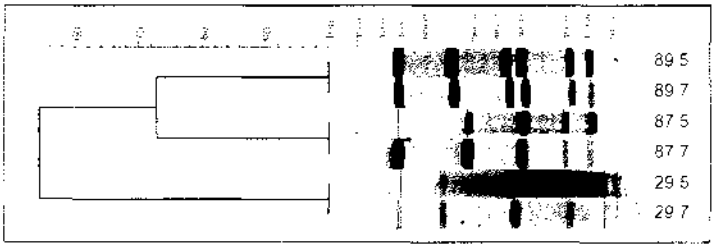

Figure 21 Six isolates of *A. butzleri* detected by method 5 and 7

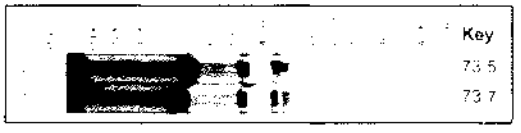

Figure 22 Two isolates of *A. butzleri* detected by method 5 and 7

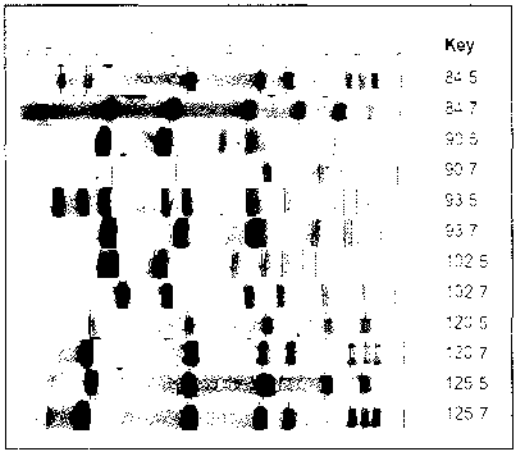

Figure 23 Twelve isolates of *A. butzleri* detected by method 5 and 7

3.4.3.6 Comparison of isolates from Method 6 and 7

Method 6 and 7 simultaneously detected *Arcobacter* positive cultures from 28 poultry meat samples (Table 16). Out of this 75% (n=21) of the occasions, the isolates were 100% similar (Figure 24). 7% (n=2) of the occasions the isolates were 80-89% similar (Figure 25). 3.5% (n=1) of the occasions the isolates were 70-79% similar (Figure 26), and 14% (n=4) of the occasions were the isolates were <70% similar (Figure 27).

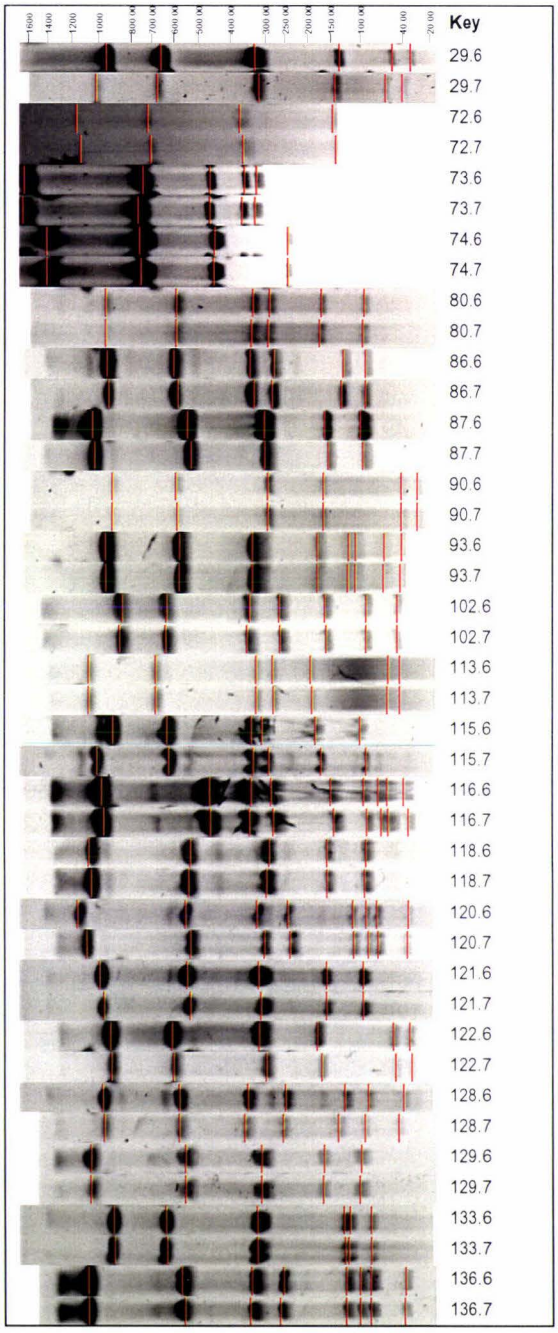

Figure 24 Forty-two isolates of *A. butzleri* detected by method 6 and 7

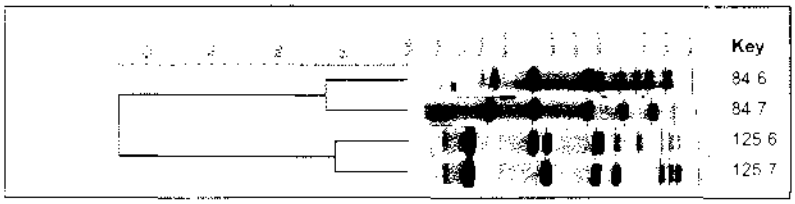

Figure 25 Four isolates of *A. butzleri* detected by method 6 and 7

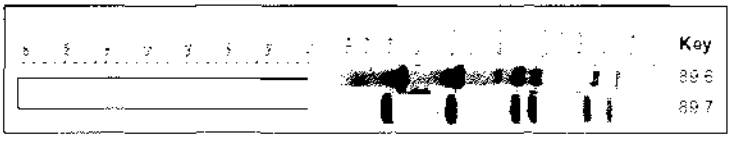

Figure 26 Two isolates of *A. butzleri* detected by method 6 and 7

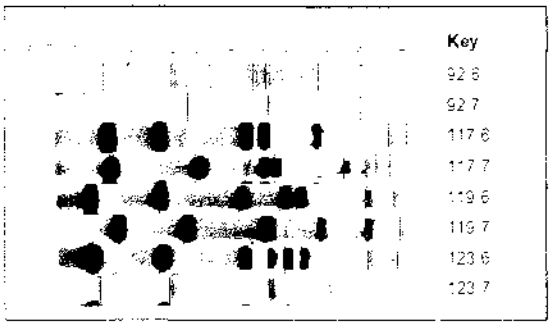

Figure 27 Eight isolates of *A. butzleri* detected by method 6 and 7

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 Discussion

This study compared the most commonly used *Arcobacter* isolation methods to isolate arcobacters from poultry meat samples and showed that use of a combination of methods significantly increases the isolation rates. Using these methods, a high prevalence of *Arcobacter spp.* in retail poultry in New Zealand was found. Two species; *A. butzleri* and *A. cryaerophilus* were detected, the former being the predominant one. In both species, a high level of diversity was found among the large number of isolates tested.

4.1.1 Identification methods

In this study, out of 210 isolates presumptively identified as *Arcobacter* spp. by growth characteristics (growth at 27°C and in air), oxidase test, and dark-field microscopy, 189 were confirmed to be *Arcobacter* spp. by the m-PCR. Multiplex PCR developed by Houf *et al.* (2000) was found to be highly sensitive as this PCR identification was later confirmed by sequencing of DNA of selected samples. Earlier studies have also mentioned that this m-PCR technique is highly sensitive for the identification of three species of *Arcobacter*; *A. butzleri, A. cryaerophilus* and *A. skirrowii* (Houf *et al.*, 2001a; Scullion *et al.*, 2004; Atabay *et al.*, 2006) It may be possible the remaining 21 isolates (out of 210) belonged to recently discovered species *A. cibarius*. However, as no PCR protocol has been published for detection of *A. cibarius*, we could not rule out their presence.

4.1.2 Prevalence of *Arcobacter* spp. in retail poultry carcass

This study detected *Arcobacter* spp. in 55.3% of retail poultry carcasses. At 95% confidence interval, this prevalence rate ranges between 47% and 63% (Table 13). These results are consistent with previous studies in Spain (Gonzalez *et al.*, 2000) and Japan (Morita *et al.*, 2004). This is however, lower than the prevalence rate of 73% in Australia (Rivas *et al.*, 2004), 81% in France (Lammerding *et al.*, 1996), 84% in the USA (Johnson and Murano, 1999a), 90-100% in Belgium (Houf *et al.*, 2001a), 95% in Turkey (Atabay *et*

al., 2003), 97% in Canada (Lammerding *et al.*, 1996), and 100% in the UK (Atabay *et al.*, 1998) and Denmark (Atabay *et al.*, 2006). However, the present study prevalence rate was higher than that of 20% in Japan (Maruyama *et al.*, 2001), 24% in The Netherlands (De Boer *et al.*, 1996), and 40% in Mexico (Villarruel-Lopez *et al.*, 2003). This direct comparison of prevalence rate is of little significance as sampling techniques, sensitivity of isolation medium and culture conditions might have influenced the number of positive samples (Madden *et al.*, 2000; Atabay *et al.*, 2003; Driessche *et al.*, 2003). The present study employed one of the most sensitive isolation methods (Method 6) having detection rates of up to 100% (Houf *et al.*, 2001a). This suggests that none of the retail poultry samples in the present study has been identified false negative.

In this study, only two species- *A. butzleri* and *A. cryaerophilus* were detected which is consistent with the previous studies of Houf *et al.* (2001a; 2002b), Maruyama *et al.* (2001) and Atabay *et al.* (2006). However, a number of studies have also reported *A. skirrowii* together with *A. butzleri* and *A. cryaerophilus* (Atabay *et al.*, 1998; Villarruel-Lopez *et al.*, 2003; Kabeya *et al.*, 2004; Scullion *et al.*, 2004). But, no *A. skirrowii* were detected in the present study.

Several studies have indicated that *A. butzleri* is the most common species isolated from retail poultry carcass followed by *A. cryaerophilus* and *A. skirrowii* (Atabay *et al.*, 1998; Kabeya *et al.*, 2004; Scullion *et al.*, 2004; Atabay *et al.*, 2006). In this study, the species *A. butzleri* was the only *Arcobacter* spp. detected in 47.3%, and *A. cryaerophilus* alone in 3.3%, and both species together in 4.6% of the retail poultry carcasses. This is similar to Houf *et al.* (2001a) who reported the isolation of *A. butzleri* alone in 64% of samples (n=100), *A. cryaerophilus* alone in 9%, and both species together in 11% samples.

Usually the isolation rate of *A. skirrowii* is very low, 1-2% (Kabeya *et al.*, 2004; Scullion *et al.*, 2004). So, its absence in the present study is not that surprising Study of earlier workers has revealed that only the use of CAT supplement or 5-fluorouracil fully supports the growth of all three *Arcobacter* species, and *A. skirrowii* is the species most susceptible to antimicrobial agents used in selective media (Houf *et al.*, 2001b). This may explain the

low recovery rates reported to date for this species. As we have used a range of selective supplements and culture conditions, the failure of isolation of *A. skirrowii* in the present study may be because of its non occurrence in New Zealand retail poultry.

4.1.2.1 Comparison among producers

A high variation was observed in the isolation rates of arcobacters in retail poultry carcasses from the three producers. The prevalence was highly variable as 15% to 98% of poultry carcasses were found to be positive for *Arcobacter* spp. in three producers. Retail poultry carcasses from Producer B appeared to be the most contaminated as 98% of the 50 poultry carcasses sampled were harbouring arcobacters.

As arcobacters may be commonly present in slaughtering equipment (Houf *et al.*, 2002b) and water (Houf *et al.*, 2003), and contamination of carcasses occurs mainly at the processing plants (Gude *et al.*, 2005), it appears that the slaughtering, processing and packaging practices in the processing plants of different producers is variable and is the most likely source of contamination. Processing facilities of Producer A may be the most hygienic followed by that of Producer C, while that of Producer B appears to be the most contaminated. Further study is necessary to test the hypothesis that *Arcobacter* contamination of poultry carcass depends on the slaughtering practices in the processing plants.

The variation in prevalence rate was also found to be consistent with the packaging condition of the retail poultry from different producers. Bad packaging may be a confounding factor associated with other unhygienic practices that are associated with *Arcobacter* contamination. It is possible that bad packaging conditions of Producer B and Producer C might have contributed to cross-contamination of retail poultry from these producers.

A. butzleri is the most commonly detected *Arcobacter* species in human and animal illness, as well as in food, and the environment. Consistent with the findings of earlier studies, *A. butzleri* was found to be the predominant species detected in all three producers accounting

for 85-93% of all isolates detected. This rate is slightly lower than that reported for *A. butzleri* in poultry meat in France where it was 99% of all *Arcobacter* isolates detected (Marinescu *et al.*, 1996b). However, it is higher than the rate of 67% of all *Arcobacter* isolates detected in poultry meat in Canada (Lammerding *et al.*, 1996).

In this study, *A. cryaerophilus* comprised 15-17% of all *Arcobacter* isolates detected in poultry meat from different producers. In poultry faecal samples, *A. cryaerophilus* has been found to be more abundant than *A. butzleri* (Kabeya *et al.*, 2003b; Atabay *et al.*, 2006). The comparatively high prevalence rate of *A. cryaerophilus* in retail poultry carcasses from Producer B (14%) might have been contributed by the faecal-carcass contamination at their processing plants.

4.1.2.2 Comparison of the isolation methods

There was a high variation among the isolation rates of different methods. Method 7 isolated arcobacters from highest number of retail poultry (39.6%) followed by Method 6 (38.6%), and Method 5 (31.3%). Method 7 involved incubation of 200 µl of poultry wash water in *Arcobacter* broth, as developed by Houf *et al.* (2001a), followed by passive passage of the broth onto blood agar plate through a membrane filter. *Arcobacter* broth contained a selective supplement comprising amphotericin B (10 mg/L), cefoperazone (16 mg/L), 5-fluorouracil (100 mg/L), novobiocin (32 mg/L), and trimethoprim (64 mg/L). Using this supplement in enrichment and plating media, Houf *et al.* (2001a) isolated arcobacters from up to 100% of the poultry meat samples. Scullion *et al.* (2004) also mentioned this Houf *et al.* method resulted in highest recovery (68%) compared to Johnson and Murano (Johnson and Murano, 1999a) and On *et al.* method (On *et al.*, 2002), which yielded a isolation rate of 50% and 28%, respectively.

It has been mentioned that *A. skirrowii* is the species most susceptible to antimicrobial agents used in selective media (Houf *et al.*, 2001b). The growth performance of *A. skirrowii* has also been found to be poor with the supplement used in *Arcobacter* broth (Houf *et al.* 2001a). This may explain the failure to detect *A. skirrowii* by Method 7 in the present study. Scullion *et al.* (2004) also mentioned that *A. skirrowii* was not detected by

Houf *et al.* (2001a). method, and *A. cryaerophilus* was detected in fewer samples than did the Johnson and Murano (1999a) and On *et al.* (2002) method. So it appears that, although Method 7 in the present study detected the maximum number of *A. butzleri*, it did not detect a number of *A. cryaerophilus* isolates possibly present in the retail poultry which were detected by other methods. This method also did not detect any *A. skirrowii* which may have been present.

The slightly lower detection rate of Method 6 compared to Method 7 may be because in Method 6, the supplement used in *Arcobacter* broth was also used in the plating media. Persistent exposure to antibiotic supplements might have inhibited the growth of some *Arcobacter* spp present in the retail poultry.

Method 5 isolated the highest number of *A. cryaerophilus* isolates (n=5). This method also isolated a significantly greater number of isolates of arcobacters than Method 4 (5 vs. 47 isolates). The first step in these techniques (Method 4 and Method 5) was same and involved enrichment of 200µl of poultry wash suspension in CAT broth containing the antibiotic supplements cefoperazone (8 mg/L), amphotericin B (10 mg/L), and teicoplanin (4 mg/L). Later on, in Method 4, enriched CAT broth was streaked on to CAT plates, while in Method 5 enrichment in CAT broth was followed by passive passage of the broth onto blood agar plate through a membrane filter. As with Method 6, persistent exposure to antibiotic supplements might have inhibited the growth of a significant number of *Arcobacter* isolates in Method 4. Atabay and Corry (1997) has also mentioned that CAT broth enrichment is most productive when used in parallel with membrane filtration on non-selective agar. It has also been mentioned that plating onto CAT agar might yield overgrowth of competitive organism (Atabay and Corry, 1997; Houf *et al.*, 2000; Rivas *et al.*, 2004). In Method 4 in the present study (CAT broth enrichment followed by CAT agar plating), a similar overgrowth of accompanying microflora was observed.

Method 5 employed media developed by On *et al.* (2002). In the present study, the detection rate of this method was found to be lower than that of Method 6 or Method 7, but the difference was only significantly less than Method 7 and not significantly less than

Method 6. This finding is inconsistent with the observation of Scullion *et al.* (2004) who found that the method developed by On *et al.* (2002: Method 5) has a significantly lower isolation rate than the method developed by Houf *et al.* (2001a; Method 6) (28% vs. 68%). This is interesting because Scullion *et al.*, (2004) had compared 50 samples only whereas the present study compared 150 samples. The larger sample size in the present study should have been more likely to detect a difference.

CAT broth enrichment has been mentioned to support the growth of different *Arcobacter* strains (Atabay and Corry, 1997), namely; *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (Atabay and Corry, 1998). However, CAT broth enrichment in the present study (Method 5) did not detect any *A. skirrowii*, although the highest number of *A. cryaerophilus* was detected by this method. Using the same media, Atabay *et al.* (2006) were also unable to detect *A. skirrowii* from retail poultry carcasses, while that species was detected in retail ducks and turkeys in the same study. This suggests *A. skirrowii* was indeed absent in retail poultry carcasses examined in this study.

Two methods in the present study; Method 1, and Method 2, involved direct plating, respectively onto CAT agar, and *Arcobacter* agar plates. As in Method 5, CAT medium was found to yield greater numbers (50% of the isolates detected by this technique) of *A. cryaerophilus* isolates (n=4), however, the overall prevalence detected by this technique was very low (5.3%). Plating directly onto *Arcobacter* agar plates (Method 2) gave a slightly higher prevalence rate compared to Method 1 (8% vs. 5.3%). This is consistent with the higher prevalence rate detected by Method 7 or Method 6 compared to Method 5. The detection rate of Method 2 in the present is however very low compared to direct isolation rate of 71% (Houf *et al.*, 2001a). This may be because New Zealand strains of arcobacters are more sensitive to the antibiotic supplements. Further work is necessary to confirm this. It is also possible that most of the retail poultry samples in New Zealand contain a very low number of arcobacters and direct plating methods detected these bacteria only from those samples containing a very high number.

Consistent with the findings of Atabay and Corry (1997), Method 3 did not detect even a single isolate of *Arcobacter* spp. No preenrichment was done in this technique, and it involved a passive passage of the poultry wash onto blood agar plate through a membrane filter. A lot of overgrowth of the competing microorganisms was detected on the blood agar plates. Probably accompanying organisms inhibited the arcobacters present in the sample while it was passing through the filter. This method was included in the present study as it has been found to be 100% sensitive for the isolation of *Campylobacter* spp. (Atabay and Corry, 1997). Thus, present study finding further confirms that *Campylobacter* isolation techniques may not be suitable for isolation of arcobacters.

The present study indicated that none of the currently available methods are suitable for isolation of all species of *Arcobacter*. Method 6 or 7; (*Arcobacter* supplement enrichment) is most suitable for isolation of *A. butzleri*; however, a number of *A. cryaerophilus* isolates were not detected by this technique. Method 5 (CAT supplement enrichment) isolated the maximum number of *A. cryaerophilus* isolates; however a number of *A. butzleri* isolates were not detected by this method. So, it appears that both CAT supplement and *Arcobacter* supplement enrichment should be used in parallel to detect the maximum possible number of arcobacters of different species.

In this study, two of the retail poultry carcasses detected positive for *Arcobacter* spp. by direct isolation method were found to be negative by enrichment methods. In one study, enrichment isolation method was found to inhibit the recovery of *A. cryaerophilus* from poultry samples which was earlier detected together with *A. butzleri* on direct isolation (Houf *et al.*, 2002b). It has also been mentioned that enrichment favours the overgrowth of *A. butzleri* (Houf *et al.*, 2003). So, it appears that enrichment media should be used together with direct isolation methods.

This study also indicated that the use of two or more than two methods together significantly increases the isolation rates of arcobacters. The highest recovery was found when isolation rates of Methods 5 and 7 were combined. The 39.3% isolation rate of Method 7 was increased to 48.6% when used in combination with Method 5. The number

of *A. cryaerophilus* detected increased from one with Method 7 to six by the combination. When three Methods 5, 6, and 7 were used together, the isolation rate was increased to 53.3% and the number of *A. cryaerophilus* isolates increased to eight. Given sufficient resources, we recommend that, a variety of isolation methods should be used in parallel, to maximize the recovery rates. This approach parallels the recommended methodology for isolating arcobacters from poultry meat samples, where two enrichment media are utilized (Scullion *et al.*, 2004). Given the fact that all three *Arcobacter* species are potential foodborne pathogens, development of a selective supplement suitable for isolation of all three species is of utmost importance.

4.1.3 Diversity among *Arcobacter* spp. isolates

Relatively few molecular fingerprinting studies have been undertaken to assess the genetic diversity of *Arcobacter* spp. We employed PFGE for assessing the diversity among the isolates of *Arcobacter* spp. obtained from different poultry producers and those detected by different isolation methods. In PFGE, the percentage similarity of the fingerprinting patterns gives an indication of genetic relatedness (i.e. being clonal) and being derived from a common source (Tenover *et al.*, 1995)

The restriction enzyme *Eag*I has been suggested to be most discriminatory when compared to *Ava*I, *Sma*I and *Sac*II, and is being extensively used in the PFGE of *Arcobacter* isolates (Hume *et al.*, 2001; Rivas *et al.*, 2004; Ho *et al.*, 2005). In the present study, digestion of genomic DNA with the restriction enzyme *Eag*I produced between four to 14 fragments, most frequently five to seven, as detected by Ho *et al.* (2005), and Hume *et al.*(2001), and were useful for strain differentiation. Consistent with the observation of these studies, genomic DNA from 12 *A. butzleri* and two *A. cryaerophilus* in the present study was unable to be digested by *Eag*I.

High diversity was observed among the isolates of *A. butzleri* and *A. cryaerophilus*. A total of 28 types (21 *A. butzleri* types and seven *A. cryaerophilus* types) from 65 retail poultry carcasses were discerned. The diversity of patterns was especially interesting because a

particular pattern never occurred in retail poultry from all three producers. A number of earlier studies have indicated that a high level of genetic diversity exists in *Arcobacter* spp.(Son *et al.,* 2006), and multiple types may occur in a single location (Houf *et al.*, 2002a; On *et al.*, 2002), and even in a single animal (Hume *et al.*, 2001; Atabay *et al.*, 2002; Houf *et al.*, 2002a; On *et al.*, 2002; On *et al.*, 2004; Driessche *et al.* 2005). Results of the present study largely agree with the aforementioned studies and confirm that *A. butzleri* and *A. cryaerophilus* are highly diverse species, with multiple genotypes often in circulation in a given processing plant and even in single retail poultry.

The large amount of genotypic variation in the isolates from the present study suggests that there were multiple parent genotypes for the *A. butzleri* and *A. cryaerophilus* isolates. The plausible explanation for high genetic diversity among arcobacters is that a high degree of genomic recombination may have occurred among the progeny of a limited number of parent genotypes and that the genotypes had been present at the facility for an extended period. Given the ability of members of the family *Campylobacteraceae* to exhibit genomic rearrangement (Hume *et al.*, 2001), the latter case may be more plausible.

The presence of multiple genotypes of arcobacters in retail poultry from single producer may indicate there are numerous sources of contamination. Although from one producer, the birds are likely to have come from different farms carrying arcobacters with them. However, it has been suggested that arcobacters may not be normal inhabitants of the poultry intestine (Atabay *et al.*, 1998; Eifert *et al.*, 2003; Gude *et al.*, 2005), and there is no strong evidence suggesting live poultry are susceptible to *Arcobacter* infection (Wesley and Baetz, 1999). As arcobacters are commonly isolated from poultry transportation crates (Houf *et al.*, 2002; Eifert *et al.*, 2003; Gude *et al.*,2005) the high prevalence of arcobacters in poultry cloacal contents is believed to be a result of direct contamination during transportation (Atabay *et al.*, 2006). This might have increased the diversity of *Arcobacter* spp. even in a single producer in the present study. But most of the diversity may be the result of contamination by arcobacters during slaughtering. This may be supported by the fact that most slaughtering equipment in poultry processing plants are contaminated with

arcobacters (Houf *et al.*, 2002a), and carcass contamination may occur during processing (Gude *et al.*, 2005; Atabay *et al.*, 2006).

In spite of the high genetic diversity observed among *Arcobacter* isolates, some common restriction patterns were detected among retail poultry carcasses from different producers. Six out of 21 PFGE patterns of *A. butzleri* were common to more than one producer which provides evidence of some identical clones contaminating retail poultry from different producers.

4.1.3.1 Comparison of *Arcobacter* isolates from different producers

It has been mentioned that the poultry are rarely colonized by arcobacters during rearing, and most contamination is believed to occur during transportation of the birds and processing of the carcasses (Gude *et al.*, 2005; Houf *et al.*, 2003). In this study, isolates of *A. butzleri* from Producer A were most diverse (DI =0.964) which indicates number of sources of contamination was highest in this particular producer. It may be possible that this producer (Producer A) receives live birds reared on a wide range of farms, and also the transportation crates being used might have been harbouring a wide variety of *A. butzleri* strains. The cross contamination among carcasses while processing appears to be minimal as the prevalence was the lowest for this producer. After processing and packaging too, little cross contamination might have occurred as the packaging conditions for retail poultry were very good and only two out of 50 packs were leaking. So, it appears that, although retail poultry from this producer had the lowest prevalence rate, multiple contamination sources during processing might have contributed to the highest diversity among the isolates obtained.

The source of contamination of *A butzleri* for Producer B and C appear to be lower than that for Producer A. One possible reason for this increased similarity of PFGE patterns in *A. butzleri* from Producer B is most of the poultry carcasses (98%) from this producer were harbouring arcobacters which might be an evidence of cross-contamination or poor hygiene. Also a significant number of the retail poultry packs (38 out of 50) from this producer were leaking, and cross contamination might have occurred among the samples.

The least diversity (DI=0.692) of *A. butzleri* isolates from Producer C suggests there were only a few contamination sources for poultry carcasses from this producer. Cross-contamination might have occurred among the retail poultry carcasses following processing, as a significant number of the retail poultry packs (27 out of 50) from this producer were leaking. Thus, the present study shows no correlation between the rate of contamination of poultry carcasses and diversity of *Arcobacter* isolates detected.

Consistent with the DI of *A. butzleri* isolates, the DI of *A. cryaerophilus* was found to be higher for isolates from Produce B than Producer C, which again suggests the source of contamination was higher for Produce B than for Producer C. Because of the limited number of samples, the diversity index of *A. cryaerophilus* from Producer A could not be determined. However, the PFGE pattern of the *A. cryaerophilus* isolate from Producer A was found to be <90% similar to that from Producer B or C which may indicate the source of contamination was not common to other producers.

4.1.3.2 Comparison of *Arcobacter* isolates from different isolation methods

It appears different isolation techniques detect different strains of arcobacters. We observed that a number of isolation methods detected positive cultures of *Arcobacter* from single poultry sample. In total, a positive culture of *Arcobacter* was detected by more than one method in 57 out of 83 positive poultry carcasses. The isolates detected from the same poultry carcass were not always 100% related to each other.

Method 2 and 6 together detected 12 isolates of arcobacters. Out of the six occasions isolates of *Arcobacter* were detected together by these methods, 84% of the occasions the isolates were <70% similar. As Method 2 involves direct plating onto *Arcobacter* agar whereas Method 6 uses *Arcobacter* broth enrichment followed by plating onto *Arcobacter* agar, the difference in culture conditions might have allowed the detection of different *Arcobacter* strains from the same poultry samples. Method 2 might have detected isolates resistant to the inhibitory effects of competing flora whereas Method 6 might have detected even the isolates 'viable but non culturable' on direct isolation.

Method 2 and 7 detected together eight isolates of arcobacters. Out of the four occasions isolates of arcobacters were detected together by these methods, 100% of the occasions the isolates were unrelated. Again, the reason may be difference in the culture conditions of the two methods. Direct plating onto *Arcobacter* agar in Method 2 might have detected only the isolates present in high number or those resistant to competitive flora while in Method 7 the antibiotic supplements might have inhibited some isolates but favoured the growth of some others.

Method 4 and 5 detected together four isolates of arcobacters. Out of the two occasions isolates of arcobacters detected together by these methods, 100% of the occasions the isolates were >80% similar. This may be because both of these methods used the similar culture conditions; CAT broth enrichment followed by plating onto selective agar (CAT Agar; Method 4) or non-selective agar (Blood agar; Method 5).

Method 5 and 6 detected together 26 isolates of arcobacters. Out of the 13 occasions isolates of arcobacters detected together by these methods, 38% of the occasions the isolates were 90-100% similar, 15% of occasions were 70-79% similar, and 46% of occasions were <70% similar. The diversity among the isolates detected together by these two methods may be because of the composition of the selective supplements used in the culture media, as Method 5 involved CAT broth enrichment (containing cefoperazone (8 mg/L), amphotericin B (10 mg/L), and teicoplanin (4 mg/L)) where as Method 6 involved *Arcobacter* broth enrichment (containing amphotericin B (10 mg/L), cefoperazone (16 mg/L), 5-fluorouracil (100 mg/L), novobiocin (32 mg/L), and trimethoprim (64 mg/L)). Further, in Method 5 enrichment was followed by plating onto non-selective media, whereas in Methods 6 subculture was done on *Arcobacter* agar.

Method 5 and 7 detected together 20 isolates of arcobacters. Out of the 10 occasions isolates of arcobacters detected together by these methods, 60% of the occasions the isolates were <70% related to each other. Again, as with Method 5 and 6, the detection of highly diverse isolates might have been possible by the use of isolation media. Method 5 had employed CAT broth enrichment whereas Method 7 had employed *Arcobacter* broth enrichment, both of which contain different antibiotic supplements (Table 1).

Method 6 and 7 together detected highest number (n=56) of *Arcobacter* isolates. Out of the 28 occasions isolates of arcobacters detected together, on 75% of the occasions the isolates were 90-100% related to each other. This might have occurred because the media used in both methods is similar. Both methods use *Arcobacter* broth enrichment, however in Method 6 subculture in done onto *Arcobacter* agar plates, whereas in Method 7 onto blood agar plates containing membrane filters. The remaining 25% of the occasions, the isolates were highly diverse which indicates that a single poultry carcass may indeed harbour multiple genotypes of *Arcobacter*.

The results suggest that different isolation methods detected different strains of arcobacters. Consequently, to detect the diverse range of arcobacters present in the samples, a combination of direct and enrichment methods should be used in parallel. This finding also further supports the fact that arcobacters are highly diverse and multiple genotypes may be present in a single animal. However, as no single isolation method was repeated on the same retail poultry sample, a comparison could not be made as to whether different isolation methods detected different *Arcobacter* strains or a single poultry was indeed harbouring multiple genotypes. Further research repeating the same isolation methods on the same poultry sample is necessary to ascertain this.

4.2 Conclusion

Poultry meat is considered one of the most important sources for the foodborne pathogens including *Arcobacter spp*. Because of the lack of use of suitable isolation technique, the exact burden of *Arcobacter*-related infections in humans is still clearly not known.

Arcobacter spp. has been isolated from retail poultry in different countries at different rates. However, no studies have been undertaken to estimate the prevalence of this organism in New Zealand. This study indicated that 55.3% of retail poultry sold in New Zealand are harbouring *Arcobacter* spp. *A. butzleri* is the predominant species with a prevalence rate of 51.3% and *A. cryaerophilus* is present in 8% poultry carcasses. Poultry from different producers are harbouring arcobacters at different level. Probably the slaughtering practices in the processing plants are the main factors for the differences in prevalence rates.

This study indicates that prevalence of arcobacters in poultry carcasses varies greatly with the choice of isolation method, and none of the currently available methods are suitable for isolation of all species of *Arcobacter*. Method 7 is the best single isolation method followed by Method 6 and Method 5. Method 6 and 7 are particularly useful for isolating *A. butzleri* whereas Method 5 is appropriate for the isolation of *A. cryaerophilus*. The remaining methods are not optimal for isolation of arcobacters. However, direct isolation methods like Method 1 seem appropriate for isolation of *A. cryaerophilus*. The results from the present study indicate that a number of isolation methods should be used in parallel to increase the recovery rate and range of species and strains of arcobacters. When Method 5 and 7 are used together, the recovery rate and range of diversity of *Arcobacter* increases dramatically.

PFGE analysis of genomic DNA of *A. butzleri* and *A. cryaerophilus* shows a high degree of diversity of the organisms obtained from the poultry carcasses from a single producer, or even from a single poultry carcass. The explanations for the large amount of heterogeneity include multiple sources of contamination, the presence of multiple parent genotypes for both species in a single poultry carcass, and a high degree of genomic recombination among the progeny of parent genotypes. A few PFGE fingerprinting patterns common between poultry carcasses from different producers have provided evidence of presence of some identical clones, probably originating from a common source.

Contamination of poultry carcasses appears to occur from multiple sources, most probably at the processing plants, and the level of contamination may vary with the processing conditions. To eliminate or reduce arcobacters in retail poultry, maintenance of slaughter hygiene is of utmost importance. This may be achieved by regular microbiological monitoring of carcasses according to the HACCP principles. This study highlighted high prevalence of *Arcobacter* spp. in poultry meat in New Zealand which is considered as the most significant source of *Arcobacter* infection in humans. Further studies comparing the molecular fingerprinting pattern of *Arcobacter* spp. isolates obtained from retail poultry with human isolates, are necessary to test the hypothesis that poultry meat is an important source of *Arcobacter* infection in humans.

APPENDIX

1. Buffered peptone water

Ingredients	Per liter
Difco buffered peptone water (Difco, USA)	20 g
Distilled water	1000 ml

Directions:

Dissolve 20 gram of peptone powder in 1 litre of distilled water. Sterilize by autoclaving at 121°C for 15 minutes. Allow it cool and store at 4°C.

2. *Arcobacter* broth

Ingredients	Per liter
Arcobacter broth (Oxoid, Basingstoke, UK)	28 g
Lysed horse blood (Venous Supplies Limited, Auckland, New Zealand)	50 ml
Cefoperazone (Sigma, St. Louis, USA)	16 mg
Amphotericin B (Sigma, St. Louis, USA)	10 mg
5-fluorouracil (Sigma, St. Louis, USA)	100 mg
Novobiocin (Sigma, St. Louis, USA)	32 mg
Trimethoprim (Sigma, St. Louis, USA)	64 mg
Sodium pyruvate (Sigma, St. Louis, USA)	500 mg
Sodium thioglycolate (Sigma, St. Louis, USA)	500 mg

Directions:

Dissolve 28.0 grams of *Arcobacter* broth powder in 1 litre of distilled water. Add thioglycolate and pyruvate, 0.05% of each, and dissolve. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 50°C. Aseptically add the antibiotics after reconstituting them as mentioned in Appendix 6. Aseptically add 50 ml of lysed horse blood. Mix well and aseptically distribute into sterile containers.

3. *Arcobacter* agar

Ingredients	Per liter
Arcobacter broth (Oxoid, Basingstoke, UK)	28 g
Plain Agar (Bacto, NJ, USA)	15 g
Cefoperazone (Sigma, St. Louis, USA)	16 mg
Amphotericin B (Sigma, St. Louis, USA)	10 mg
5-fluorouracil (Sigma, St. Louis, USA)	100 mg
Novobiocin (Sigma, St. Louis, USA)	32 mg
Trimethoprim (Sigma, St. Louis, USA)	64 mg

Directions:

Dissolve 28.0 grams of *Arcobacter* broth powder in 1 litre of distilled water. Add 12 grams of agar and dissolve. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 50°C. Add the antibiotics after reconstituting them as mentioned in Appendix 6. Mix well and aseptically distribute into sterile containers.

4. CAT broth

Ingredients	Per liter
Campylobacter enrichment broth (Lab M, Bury, England)	27.6 g
CAT supplement (Oxoid, Basingstoke, UK)	2 vials
Lysed horse blood (Venous Supplies Limited, Auckland, New Zealand)	50 ml

Directions:

Weigh 27.6 grams of *Campylobacter* enrichment broth powder, disperse in 1 liter of distilled water. Allow to soak for 10 minutes, swirl to mix and sterilize by autoclaving at 121°C for 15 minutes. Cool to 47°C, and supplement with 2 vials of CAT supplement (Oxoid SR 0174E), and 50 ml lysed horse blood. Mix thoroughly and aseptically dispense into sterile containers.

5. CAT agar

Ingredients	Per liter
Campylobacter blood free selective medium (Lab M, Bury, England)	45.5 g
CAT supplement (Oxoid, Basingstoke, UK)	2 vials

Directions:

Weigh 45.5 grams of *Campylobacter* enrichment agar powder; disperse in 1 liter of distilled water. Allow to soak for 10 minutes, swirl to mix and sterilize by autoclaving at 121°C for 15 minutes. Cool to 47°C, and add 2 vials of CAT supplement. Pour into sterile Petri dishes ensuring continuous mixing during pouring to prevent charcoal setting.

6. Reconstitution of antibiotics

S.N.	Ingredients	Solvents	Amount per unit	Exact amount
1	Cefoperazone	Distilled water (dw)	0.5 gm in 100 ml, sterilize using .22 µm filter	3.2 ml dw for 16 mg cefoperazone
2	5-fluorouracil	2 M NaOH	1 gm in 50 ml dw and 2 ml NaOH	5 ml dw and 0.2 ml (2 M) NaOH for 100 mg 5-fluorouracil
3	Trimethoprim	0.05 N HCl at 50°C	0.5 gm in 30 ml HCl	3.84 ml HCl for 64 mg trimethoprim
4	Novobiocin	Distilled water (dw)	100 mg in 1 ml	0.32 ml dw for 32 mg novobiocin
5	Amphotericin-B	Distilled water (dw)	0.05 g in 100 ml, filter sterilize	20 ml distilled water for 10 mg amphotericin B
6.	CAT supplement	Distilled water (dw)	4 ml per vial	4 ml per vial

- Dissolve every antibiotic separately in their respective solvents at higher concentration (once dissolved, distilled water can be used to dilute all the antibiotics(Anon, 2002))
- Make a final volume of 50 ml by adding 17.44 ml distilled water to the antibiotic solution. This solution will contain 16 mg cefoperazone, 10 mg amphotericin, 100 mg 5-flurururacil, 32 mg novobiocin and 64 mg trimethoprim.
- Store the solution for 5 days at 4°C, 14 days at -20°C, and 5 months at -70°C. Freeze in sterile plastic tubes or bottles.

7. 15% Glycerol Broth

Ingredients	Per liter
Nutrient broth (Difco, USA)	80 g
Glycerol	150 ml

Directions: Dissolve the nutrient broth in 1L distilled water and add glycerol. Autoclave at 121°C for 15 minutes and store at 4°C.

8. 5×TBE buffer

Ingredients	Per liter
Tris-base	54 g
Boric-acid	27.5 g
0.5M EDTA (pH8)	20 ml
Distilled water	800 ml

Directions:

Mix and adjust the volume to 1 L with distilled water. For making 0.5×TBE working-solution, dilute 1 L of stock-solution to 10 L with distilled water.

9. Proteinase K (20 mg/ml)

Ingredients	Per 20 ml
Proteinase K (Amresco, USA)	0.40 g
Sterile MQ water	20 ml

Directions:

Weigh the Proteinase K into a sterile 30 ml vial and add the measured water. Mix to dissolve and then filter through 0.2 µm filter into a second sterile 30 ml vial. Aseptically dispense 400 µl volumes into sterile 1.5µl tubes and store at -20°C.

10. Seakem gold agarose (1% in 0.5% TBE)

Ingredients	Per 100 ml
Agarose (Cambrex Bioscience, USA)	1 g
0.5X TBE Buffer	99 ml

Directions:

Weigh the agarose in 250 ml bottle. Add the TBE and swirl gently to disperse the agarose. Allow to stand for 15 min to aid rehydration of the agarose. Loosen the cap and microwave for 60 sec, mix gently and repeat heating for 15 sec intervals until the agarose in completely dissolved. Place in waterbath at 55-60°C until ready to pour.

11. Phosphate buffered saline

Add 1 tablet of Phosphate buffered saline tablet (BR0014G, Oxoid) to 100 ml distilled water. Sterilize by autoclaving at 121°C for 15 minutes, and store at 4°C.

12. 0.5 M EDTA, pH (8.0)

Ingredients	Per litre
$Na_2EDTA.2H_2O$	186.1 g
10 N NaOH	~50 ml
MQ water	~1000 ml

Directions:

Mix EDTA with 800 ml of water. Add 10 N NaOH slowly, checking pH until the pH is 8.0. Make the volume up to 1 L and then dispense 500 ml volumes in 1 L bottles. Autoclave at 121°C for 15 min and store at room temperature.

13. Sarcosyl 10%

Ingredients	Per 100 ml
Sarcosyl	10 g

Sterile MQ water	90 ml

Directions:

Carefully add the sarcosyl to the water in a sterile container. Dissolve by mixing gently and warming to 50-60°C. Store at room temperature.

14. Lysis buffer

Ingredients	**Per 200 ml**
50 mM Tris	10 ml of 1M Tris, pH 8.0
50 mM EDTA	20 ml of 0.5 M EDTA, pH 8.0
1% Sarcosyl	20 ml of 10% Sarcosyl
Sterile MQ water	150 ml

Directions:

Measure the Sarcosyl, Tris, EDTA, and sterile water into a 500 ml bottle and mix gently. Store at 4°C.

15. 1 M Tris-HCl (pH 8.0)

Ingredients	**Per litre**
Tris-HCl	157.6 g
MQ water	~900 ml

Directions:

Dissolve Tris-HCl in 800 ml of water. Adjust the pH to 8.0 and then make the volume up to 1 L. Dispense 500 ml volumes in 1 L bottles and autoclave at 121°C for 15 min, and store at room temperature.

16. TE-buffer

Ingredients	**Per liter**

10 mM Tris	10 ml of 1 M Tris, pH 8.0
1 mM EDTA	2 ml of 0.5 M EDTA, pH 8.0
Sterile MQ water	~1000 ml

Directions:

Mix the solutions, make the volume up to 1 litre and store at room temperature.

17. Ethidium bromide stock solution

Ingredients	**Per 200 ml**
Ethidium bromide	0.8 g
Distilled water	200 ml

Directions:

Mix in brown-walled plastic Nalgene bottle and label 'toxic'. Store at room temperature. To make working-solution, add 1 ml of stock in 1 L of distilled water.

18. Calculation of confidence interval for prevalence rate

Binomial distribution can be made from sampling events. If repeated samples of the same number of individuals 'n' were selected, the calculated prevalence rate 'p' would vary from sample to sample. This inconsistency is defined by the standard error (SE) of the mean, and is estimated from the sample.

$SE(p) = [p(1-p)/n]^{1/2}$

[Here 'p' denotes the proportion of test positive samples].

$Variance = p(1-p)/n$

Confidence interval for a proportion $= p \pm z [p(1-p)/n]^{1/2}$

[Value of z for 90%, 95% and 99% confidence interval is 1.65, 1.96, and 2.567, respectively].

A 95% confidence interval would allow for a 2.5% chance that the population proportion is lower than the lower confidence limit and 2.5% chance that the population proportion is higher than the upper confidence limit.

An example of calculation of 95% confidence interval for the overall prevalence rate is shown below. Table 13 shows the 95% confidence interval for prevalence of *Arcobacter* spp. in poultry from different producers.

For overall prevalence:

Sample size=150

Arcobacter spp. positive samples=83

Total measured prevalence for *Arcobacter* spp. (p) = 83/150=0.5533

Variance = [0.5533(1-0.5533)/150] =0.00164

SE (p) = $0.00164^{1/2}$ =0.04049

95% confidence interval = 0.5533± 1.96 x 0.04049 =0.5533 ± 0.07936

19. Comparison of proportions

19.1 Comparison of proportions of prevalence rates between producers

Using Chi-square ($\chi 2$), testing the null hypothesis (H_0) that proportion of prevalence rate is the same in all three producers:

Here,

Number of samples for Producer A (n_A) = 50, positive samples (X_A) = 15, proportion (p_A) = 0.30; Number of samples for Producer B (n_B) = 50, positive samples (X_B) = 49, proportion (p_B) =0.988; Number of samples for Producer C (n_C) = 50, positive samples (X_C) = 19, proportion (p_C) =0.38

$$\bar{p} = \frac{\sum Xi}{\sum ni} = \frac{15+49+19}{50+50+50} = 88/150 = 0.5533$$

$$\bar{q} = 1 - \bar{p} = 1 - 0.5533 = 0.4466$$

$$\chi 2 = \sum \frac{(Xi - ni\bar{p})}{ni\bar{p}\bar{q}} = \frac{[15-(50)(0.5533)]2+[49-(50)(0.5533)]2+[19-(50)(0.5533)]2}{50(0.5533)(0.4466)}$$

$$= \frac{(160.40+455.39+74.995)}{12.355} = 55.90$$

Tabulated value of $\chi 2$ at 0.001 level of significance and 2 DF =13.816

So, $P < 0.001$

Therefore, reject the null hypothesis.

Now, we have to determine which proportions are different from which other. For this purpose, the Tukey test is used as follows:

Here,

Total DF=150-1=149

Groups DF=3-1=2

Error DF (v) =149-2=147

Level of significance (α) =0.05

Total number of proportions being tested=3

Standard error (SE) = $\sqrt{205.18/n+0.5} = \sqrt{205.18/50.5} = 2.01$

Calculated value of q = Difference in proportion/ SE

Tabulated value of q (at 0.05 level of significance, 147 error DF, and 3 numbers of means being tested) = 3.314

Null hypothesis (H_0) = Proportion of prevalence rate is the same in all three producers

Alternative hypothesis (H_A) = Proportion of prevalence rate is not the same in all three producers

Producers ranked by proportion	Producer B	Producer C	Producer A
Ranked Producer proportions	49/50=0.98	19/50=0.38	15/50=0.30
Ranked transformed proportions	82.29	38.17	33.34

Table 17. Comparison of proportions of prevalence rates among producers

Comparisons	Difference in proportions	SE	q (calculated value)	q (tabulated value)	Conclusion
B vs. A	48.95	2.01	24.35323383	3.314	Reject H_0
B vs. C	44.12	2.01	21.95024876	3.314	Reject H_0
C vs. A	4.83	2.01	2.402985075	3.314	Accept H_0

Overall conclusion: There is significant difference ($P<0.05$) between the prevalence rates of Producers B and A, and B and C, but not between the Producers C and A.

19.2 Comparison of proportions of isolation rates between methods

First, testing the null hypothesis (H_0) that proportion of prevalence rate is the same in all three producers.

Here,

Number of samples for Method 1 (n_1) = 150, positive samples (X_1) = 8, proportion (p_1) = 0.053; Number of samples for Method 2 (n_2) = 150, positive samples (X_2) = 12, proportion (p_2) =0.08; Number of samples for Method 4 (n_4) = 150, positive samples (X_4) = 5, proportion (p_4) =0.033; Number of samples for Method 5 (n_5) = 150, positive samples (X_5) = 47, proportion (p_5) = 0.313; Number of samples for Method 6 (n_6) = 150, positive samples (X_6) = 58, proportion (p_6) =0.386; Number of samples for Method 7 (n_7) = 150, positive samples (X_7) = 59, proportion (p_7) =0.393

$$\bar{p} = \frac{\sum Xi}{\sum ni} = \frac{8+12+5+47+58+59}{150+150+150}$$

=189/900

= 0.210

$\bar{q} = 1 - \bar{p} = 1 - 0.210 = 0.790$

$$\chi 2 = \sum \frac{(Xi - ni\bar{p})}{ni\bar{p}\bar{q}} =$$

$$\frac{[8-(150\times 0.210)]2+[12-(150\times 0.210)]2+[5-(150\times 0.210)]2+[47-(150\times 0.210)]2+[58-(150\times 0.210)]2+[59-(150\times 0.210)]2}{(150\times 0.210\times 0.790)}$$

=3333.50/24.88

= 133.98

Tabulated value of $\chi 2$ at 0.001 level of significance and 5 DF =20.515

So, $P < 0.001$

Therefore, reject the null hypothesis.

Now, we have to determine which proportions are different from which other. For this purpose, the Tukey test is used as follows:

Here,

Total DF=150-1=149

Groups DF=6-1=5

Error DF (v) =149-5=144

Level of significance (α) =0.05

Total number of proportions being tested=6

Standard error (SE) = $\sqrt{205.18/n+0.5} = \sqrt{205.18/150.5} = 1.16$

Calculated value of q = Difference in proportion/ SE

Tabulated value of q (at 0.05 level of significance, 147 error DF, and 3 numbers of means being tested) = 4.03

Null hypothesis (H_0) = Proportion of isolation rate is the same in all six methods

Alternative hypothesis (H_A) = Proportions of isolation rate is not the same in all six methods

Methods ranked by proportion	Method 7	Method 6	Method 5	Method 2	Method 1	Method 4
	59/150	58/150	47/150	12/150	8/150	5/150
Ranked Method proportions	=0.393	=0.386	=0.313	=0.08	=0.053	=0.033
Ranked transformed proportions	38.76	38.17	33.96	16.64	13.18	10.3

Table 18. Comparison of the proportions of isolation rates between different methods

Comparisons	Difference in proportions	SE	q (calculated value)	q (tabulated value)	Conclusion
7 vs. 4	28.46	1.167	24.38731791	4.03	Reject H_0
7 vs. 1	25.58	1.167	21.91945159	4.03	Reject H_0
7 vs. 2	22.12	1.167	18.9545844	4.03	Reject H_0
7 vs. 5	4.8	1.167	4.11311054	4.03	Reject H_0
7 vs. 6	0.59	1.167	0.505569837	4.03	Accept H_0
6 vs. 4	27.87	1.167	23.88174807	4.03	Reject H_0
6 vs. 1	24.99	1.167	21.41388175	4.03	Reject H_0
6 vs. 2	21.53	1.167	18.44901457	4.03	Reject H_0
6 vs. 5	4.21	1.167	3.607540703	4.03	Accept H_0
5 vs. 4	23.66	1.167	20.27420737	4.03	Reject H_0
5 vs. 1	20.78	1.167	17.80634105	4.03	Reject H_0
5 vs. 2	17.32	1.167	14.84147386	4.03	Reject H_0
2 vs. 4	6.34	1.167	5.432733505	4.03	Reject H_0
2 vs. 1	3.46	1.167	2.964867181	4.03	Accept H_0
1 vs. 4	2.88	1.167	2.467866324	4.03	Accept H_0

Overall conclusion: The difference between methods '6 and 7', '5 and 6', '1 and 2' and '1 and 4' are not statistically significant ($P>0.05$). The remaining methods are all significantly different from each other ($P<0.05$).

20. Calculation of diversity index

Simpson's diversity index, which was developed for the description of species diversity within an ecological habitat can be derived from the following equation (Hunter and Gaston, 1988):

$$DI = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

Where N is total number of strains in the sample population, S is the total number of types described, and n_j is the number of strains belonging to the jth type. An example of calculation of diversity index for *A. butzleri* from Producer A (as listed in Table 14) is given below. Here,

No. of isolates (N) =8; No of subtypes (S) =7

No. of strains belonging to pattern C (n1) =1; No. of strains belonging to pattern F (n1) =2

No. of strains belonging to pattern J (n1) =1; No. of strains belonging to pattern L (n1) =1

No. of strains belonging to pattern P (n1) =1; No. of strains belonging to pattern S (n1) =1

No. of strains belonging to pattern U (n1) =1

So, diversity index=1-[(1×0+2×1+1×0+1×0+1×0+1×0+1×0)]/8×7=1-2/56 =0.964

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