Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.
PREVALENCE AND DIVERSITY OF *ARCOBACTER* SPP. IN POULTRY MEAT IN NEW ZEALAND

A thesis presented in the partial fulfillment of the requirements for the degree of Master of Science in Veterinary Microbiology and Public Health at Massey University, Palmerston North, New Zealand.

SHRAWAN BHANDARI
2006
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 EagI.
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 cloaca 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 bacteremia (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 cryaerophilus*, 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 cryaerophilus*, 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. *butsleri* has a diameter of 0.2 to 0.4 µm 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 0.5 µm and length of 1.5 µm. 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

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Composition of <em>Arcobacter</em> media (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>AB</strong></td>
</tr>
<tr>
<td>Bile salts</td>
<td></td>
</tr>
<tr>
<td>Charcoal</td>
<td></td>
</tr>
<tr>
<td>Lysed blood</td>
<td></td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td></td>
</tr>
<tr>
<td>Sodium thioglycolate</td>
<td></td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td></td>
</tr>
<tr>
<td>Cefoperazone</td>
<td></td>
</tr>
<tr>
<td>Novobiocin</td>
<td></td>
</tr>
<tr>
<td>Teicoplanin</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim</td>
<td></td>
</tr>
</tbody>
</table>

(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. MgCl₂ 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*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>A. butzleri</th>
<th>A. cryaerophilus</th>
<th>A. skirrowii</th>
<th>A. nitrofigilis</th>
<th>A. halophilus</th>
<th>A. cibarius</th>
<th>C. jejuni</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-haemolysis</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase activity</td>
<td>V</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>V</td>
</tr>
<tr>
<td>Oxidase activity</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hippurate hydrolysis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Selenite reduction</td>
<td>-</td>
<td>-</td>
<td>V</td>
<td>V</td>
<td>?</td>
<td>-</td>
<td>V</td>
</tr>
<tr>
<td>H2S(TSI)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indoxyl acetate hydrolysis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 15°C (air)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 25°C (air)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>V</td>
</tr>
<tr>
<td>Growth at 37°C (microaerobic)</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 42°C (microaerobic)</td>
<td>V</td>
<td>-</td>
<td>V</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth on minimal medium</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth on MacConkey agar</td>
<td>V</td>
<td>V</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>V</td>
</tr>
<tr>
<td>Growth in glycine (1%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Growth in NaCl (4%)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Resistance to nalidixic acid</td>
<td>V</td>
<td>V</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>V</td>
<td>S</td>
</tr>
<tr>
<td>Resistance to cephalothin (32 mg/L)</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Resistance to cefoperazone (64 mg/L)</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

*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 nitrates, 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_{2}S, 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 \(10^2\) cells/ml (after culture enrichment) to \(10^4\) cells/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 be 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* IA and IB.

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 1 cell/ml (after enrichment) to $10^3$ cells/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 is their 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: *Aval*, *Eagl*, and *SacII*, were found to be useful for strain differentiation of arcobacters, *Eagl* and *SacII* 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 *SacI*, *EagI* and *Smal* 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, *Smal* 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 *EagI*. 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

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

There has been only one large scale study reporting the prevalence rate of arcobacters in patients with diarrhoeal illness (Vandenberge 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

<table>
<thead>
<tr>
<th>Country</th>
<th>Species detected</th>
<th>Isolation rates (%)</th>
<th>No. of samples</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>All <em>A. butzleri</em></td>
<td>73</td>
<td>22</td>
<td>Rivas et al., 2004</td>
</tr>
<tr>
<td>Belgium</td>
<td><em>A. butzleri</em> - 64%, <em>A. cryaerophilus</em> - 9%, both together - 11% samples</td>
<td>90</td>
<td>71 (broiler)</td>
<td>Houf et al., 2001a</td>
</tr>
<tr>
<td>Japan</td>
<td><em>A. butzleri</em> - 67% isolates,</td>
<td>97</td>
<td>125</td>
<td>Lammerding et al., 1996</td>
</tr>
<tr>
<td>Denmark</td>
<td><em>A. butzleri</em> in 100% samples, <em>A. cryaerophilus</em> in 16%,</td>
<td>100</td>
<td>30</td>
<td>Atabay et al., 2006</td>
</tr>
<tr>
<td>France</td>
<td><em>A. butzleri</em> - 99%</td>
<td>81</td>
<td>201</td>
<td>Marinescu et al., 1996b</td>
</tr>
<tr>
<td>Mexico</td>
<td><em>A. butzleri</em>- in 73%, <em>A. skirrowii</em>- in 23%</td>
<td>40</td>
<td>45</td>
<td>Villarruel et al., 2003</td>
</tr>
<tr>
<td>Spain</td>
<td>No speciation</td>
<td>53</td>
<td>95</td>
<td>Gonzalez et al., 2000</td>
</tr>
<tr>
<td>Thailand</td>
<td>No speciation</td>
<td>100</td>
<td>10</td>
<td>Morita et al., 2004</td>
</tr>
<tr>
<td>Netherlands</td>
<td>All <em>A. butzleri</em> or butzleri-like</td>
<td>24.1</td>
<td>224</td>
<td>De Boer et al., 1996</td>
</tr>
<tr>
<td>Turkey</td>
<td>All <em>A. butzleri</em></td>
<td>95</td>
<td>44</td>
<td>Atabay et al., 2003</td>
</tr>
<tr>
<td>UK</td>
<td><em>A. butzleri</em>- in all 25, <em>A. cryaerophilus</em>- in 13, and <em>A. skirrowii</em>- in 2 samples</td>
<td>100</td>
<td>25</td>
<td>Atabay et al., 1998</td>
</tr>
<tr>
<td>USA</td>
<td>No speciation</td>
<td>84</td>
<td>50</td>
<td>Johnson and Murano, 1999a</td>
</tr>
</tbody>
</table>
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

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

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.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td><em>Arcobacter</em> spp. detected in 91% (n=24) water samples before being used in poultry processing plant</td>
<td>Houf et al., 2003</td>
</tr>
<tr>
<td>Caribbean</td>
<td><em>Arcobacter</em> spp detected in coral reefs</td>
<td>Frias-Lopez et al., 2002</td>
</tr>
<tr>
<td>Germany</td>
<td>79% (n=147) of <em>Campylobacter</em>-like strains isolated from drinking water treatment plants identified as <em>Arcobacter</em> spp.; 100 strains were <em>A. butzleri</em>;</td>
<td>Jacob et al., 1998</td>
</tr>
<tr>
<td>Germany</td>
<td>4% of all cells in activated sludge samples were <em>Arcobacter</em> spp.</td>
<td>Snaidr et al., 1997</td>
</tr>
<tr>
<td>Japan</td>
<td><em>A. butzleri</em> detected in 23% (n=17) of river water samples; no other species detected;</td>
<td>Morita et al., 2004</td>
</tr>
<tr>
<td>Nigeria</td>
<td>26 (14%) of the poultry abattoir waste water samples positive for <em>A. butzleri</em>; no other species detected;</td>
<td>Amisu et al., 2003</td>
</tr>
<tr>
<td>South Africa</td>
<td><em>A. butzleri</em> 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</td>
<td>Diergaardt et al., 2004</td>
</tr>
<tr>
<td>Spain</td>
<td>100% (n=10) of river water and 100% (n=10) activated sludge sample positive for <em>Arcobacter</em> spp. speciation not done;</td>
<td>Moreno et al., 2003</td>
</tr>
<tr>
<td>Thailand</td>
<td><em>A. cryaerophilus</em> and <em>A. cryaerophilus</em>-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</td>
<td>Dhamabutra et al., 1992</td>
</tr>
<tr>
<td>Thailand</td>
<td><em>A. butzleri</em> detected in 100% (n=7) canal water samples from Bangkok; no other species detected; the isolates were genetically diverse</td>
<td>Morita et al., 2004</td>
</tr>
<tr>
<td>USA</td>
<td><em>A. butzleri</em> isolated from contaminated well water; suspected to have been associated with abdominal illness in group of 117 girls</td>
<td>Rice et al., 1999</td>
</tr>
</tbody>
</table>

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 is 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.

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

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.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td><em>Arcobacter</em> spp. detected in 16-85% (n=294) faecal samples from healthy pigs; excretion ranged from 0 to 10⁴ CFU/g faeces; most predominant species - <em>A. butzleri</em>,</td>
<td>Driessche et al., 2004</td>
</tr>
<tr>
<td>Brazil</td>
<td>17 <em>Arcobacter</em> isolates obtained from visceral organs of aborted foetuses and sows with reproductive problems; 12 (71%) were <em>A. cryaerophilus</em> 1B, four (24%) were <em>A. cryaerophilus</em> 1A, and one (6%) was <em>A. butzleri</em>.</td>
<td>De Oliveria et al., 1997</td>
</tr>
<tr>
<td>Brazil</td>
<td>24% (n=74) of the preputial swab samples positive for arcobacters; 8 were <em>A. cryaerophilus</em> 1B, 7 were <em>A. cryaerophilus</em> 1A and 2 were <em>A. butzleri</em>.</td>
<td>De Oliveria et al., 1999</td>
</tr>
<tr>
<td>Denmark</td>
<td><em>Arcobacter</em> spp. detected in &gt;40% (n=55) aborted pig foetuses; 11 isolates were <em>A. cryaerophilus</em>, 10 were <em>A. skirrowii</em>,</td>
<td>On et al., 2002</td>
</tr>
<tr>
<td>Japan</td>
<td>10% (n=250) of the faecal samples positive for arcobacters; <em>A. butzleri</em> the most prevalent species (60%) followed by <em>A. cryaerophilus</em> (36%), 13.3% (n=15) of the vaginal swab samples positive for <em>A. butzleri</em> only;</td>
<td>Kabeya et al., 2003b</td>
</tr>
<tr>
<td>The Netherlands</td>
<td><em>A. skirrowii</em> - the predominant species, followed by <em>A. cryaerophilus</em> or <em>A. butzleri</em>; seasonality not found; infection of the newborn piglets ranged from 38.5 to 83.3% in each litter (litter size=4-17)</td>
<td>Ho et al., 2006</td>
</tr>
<tr>
<td>UK</td>
<td>82% (n=17) of the aborted foetuses 18% (n=11) of the normal foetuses were harbouring <em>Arcobacter</em>-like organisms;</td>
<td>Ellis et al., 1978</td>
</tr>
<tr>
<td>USA</td>
<td><em>Arcobacter</em> spp. detected in 40.4% (n=952) of porcine faecal samples</td>
<td>Harmon and Wesley, 1996</td>
</tr>
<tr>
<td>USA</td>
<td>Arcobacters detected in 46% faecal samples (n=1,057) of market weight pigs; no species differentiation done</td>
<td>Wesley et al., 1999</td>
</tr>
<tr>
<td>USA</td>
<td><em>Arcobacter</em> spp. recovered from 43% (n=30) of porcine abortion cases; <em>A. cryaerophilus</em> 1B was the predominant species followed by <em>A. cryaerophilus</em> 1A and <em>A. butzleri</em>.</td>
<td>Schroeder-Tucker et al., 1996</td>
</tr>
<tr>
<td>USA</td>
<td>2.85% (n=350) caecal samples from slaughtered pigs were positive for arcobacters; All were <em>A. butzleri</em>; pigs were colonized by multiple <em>Arcobacter</em> genotypes;</td>
<td>Hume et al., 2001</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>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; <em>A. cryaerophilus</em> predominant species followed by <em>A. butzleri</em> and <em>A. skirrowii</em>. -Arcobacter excretion ranged from 0 to 10⁴ CFU/g faeces</td>
<td>Driessche et al., 2003</td>
</tr>
<tr>
<td>Belgium</td>
<td>Arcobacters detected in faecal samples of 39% (n=50) of the animals; <em>A. butzleri</em> isolated from 13, <em>A. cryaerophilus</em> from five and <em>A. skirrowii</em> from two samples</td>
<td>Driessche et al., 2005</td>
</tr>
<tr>
<td>Canada</td>
<td>4% of 198 isolates of campylobacters obtained from bovine faeces identified as arcobacters, four were <em>A. butzleri</em>, and <em>A. skirrowii</em>, and the remaining were <em>Campylobacter</em> spp.</td>
<td>Inglis and Kalischuk, 2003</td>
</tr>
<tr>
<td>Japan</td>
<td>Arcobacters detected in 3.6% (n=332) faecal samples; <em>A. butzleri</em> (83.3% of isolates) the most prevalent species, followed by <em>A. cryaerophilus</em> 1B (16.7%); -Detected in 5 (8.1%) of 61 vaginal swab samples; four <em>A. butzleri</em>, one <em>A. cryaerophilus</em> 1B. -The seasonal positive rate varied from 1.4% in spring to 7.6% in summer -9.5% (n=200) of the rectal swab samples positive; <em>A. butzleri</em> most prevalent (7%) followed by <em>A. cryaerophilus</em> (2%) and <em>A. skirrowii</em> (0.5%)</td>
<td>Kabeya et al., 2003b</td>
</tr>
<tr>
<td>Turkey</td>
<td><em>Arcobacter</em> like organisms isolated from 44% (n=34) of aborted bovine foetuses</td>
<td>Ongor et al., 2004</td>
</tr>
<tr>
<td>UK</td>
<td><em>Arcobacter</em> spp. identified in 14.3% (n=1,682) of healthy cows; No information on different species</td>
<td>Ellis et al., 1977</td>
</tr>
<tr>
<td>USA</td>
<td><em>Arcobacter</em> spp. identified in 14.3% (n=1,682) of healthy cows; No information on different species</td>
<td>Wesley et al., 2000</td>
</tr>
<tr>
<td>USA</td>
<td>9% (n=200) of the cattle sampled tested positive for <em>A. butzleri</em>; highest incidence for <em>A. butzleri</em> (i.e. 18%) observed for dairy cows; no other species detected</td>
<td>Golla et al., 2002</td>
</tr>
</tbody>
</table>

### 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
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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.