Molecular Epidemiology of Arcobacter in Cattle

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By

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Abstract

The primary objective of this study was to investigate the molecular epidemiology of the emerging human foodborne pathogen *Arcobacter* spp. in cattle, in order to gain a better understanding of its diversity, epidemiology and transmission. The study formed part of a larger collaborative study investigating the transmission, evolution and control of foodborne diseases based at the University of Liverpool, as part of the DEFRA and HEFCE-funded Veterinary Training and Research Initiative (VTRI).

A series of five previously published methods were compared and evaluated for the isolation of *Arcobacter* spp. from animals faeces. The aim of this was to determine a single sensitive, specific and effective method for the isolation of *Arcobacter* spp., as no standardised method was published. A method comprising a five antibiotic enrichment broth (Houf *et al.*, 2000) followed by direct plating onto mCCDA agar with an added three-antibiotic supplement was found to be the most effective method and was adopted for use throughout the remainder of the project. It was also determined that *Arcobacter* spp. do not survive well in frozen faecal samples and that isolation should be carried out on fresh samples where possible.

The prevalence of *Arcobacter* and *Campylobacter* spp. on four cattle farms in Cheshire, UK, was investigated using a series of five cross-sectional studies over a 12-month period. It was found that the prevalence of *Arcobacter* spp. appears to peak in summer, a probable effect of temperature. Analysis also showed that the prevalence of *Arcobacter* spp. from cattle faeces is related to sampling environment (a higher recovery occurred when cattle grazing outdoors were sampled), age (recovery higher in younger animals) and the individual farm, suggesting that further investigation into the effect of farm management practices on *Arcobacter* spp. prevalence is required.

The molecular typing methods macro-restriction PFGE, ERIC-PCR and MLST were applied to eight hundred isolates from farms in Cheshire and Lancashire in order to investigate the diversity of *Arcobacter* spp. in cattle in the North West the UK. PFGE and ERIC-PCR were found to be of limited use in the typing of *Arcobacter* spp., however MLST demonstrated a high level of diversity amongst the isolates and was found to be a useful tool in the molecular epidemiology of *Arcobacter* spp.

The whole genome sequence of an *A. butzleri* isolate from a clinically healthy dairy cow was obtained using 454 high-throughput sequencing technology. Upon comparison with a previously published human-isolated *A. butzleri* whole genome sequence a surprisingly high level of variation was discovered. The two genomes differed in a total of 502 regions, with an overall difference in sequence of approximately 20%. Regions showing variation included genes encoding regulatory, sensing and survival systems, which suggests some level of host-adaptation in *A. butzleri.*

Further study is recommended in order to fully investigate the effect of farm management practices on *Arcobacter* spp. prevalence in cattle, and to investigate potential links between *A. butzleri* sensing and survival systems and the host environment of the isolate.

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Chapter One

Introduction and Literature Review: The Molecular Epidemiology of *Arcobacter*.

Arcobacter spp. are Gram negative, spiral-shaped bacteria with a single polar flagellum, which belong to the family *Campylobacteraceae*. They differ from the closely related genus *Campylobacter* in that they are able to grow at temperatures as low as 15°C and in aerobic conditions, while most *Campylobacter* spp. do not grow below 30°C, and are unable to grow well at the optimal growth temperature of most *Campylobacter* species, 42°C. The genus *Arcobacter* currently contains eight species, of which five are considered to be emerging human foodborne pathogens. *A. nitrofigilis*, *A. halophilus* and *Candidatus A. sulfidicus* are not currently associated with human or animal sources. *A. butzleri*, *A. skirrowii*, *A. cryaerophilus*, *A. cibarius* and *A. mytili* have all been isolated from potential sources of human infection such as meat and water. *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have also been isolated from human faecal samples (Vandenberg *et al.*, 2004; On *et al.*, 1995; Hsueh *et al.*, 1997; Vandamme *et al.*, 1992a; Wybo *et al.*, 2004; Woo *et al.*, 2001; Samie *et al.*, 2007; Houf and Stephan, 2007; Kielbauch *et al.*, 1991; Prouzet-Mauleon *et al.*, 2006; Tee *et al.*, 1988).

The first report of an *Arcobacter* species was by Ellis *et al.* (1977), who isolated a Spirillium/Vibrio-like organism from bovine foetuses, naming it *Campylobacter cryaerophilus*. The separate genus, *Arcobacter*, was proposed in 1991 (Vandamme *et al.*, 1992b) when hybridisation experiments showed *Campylobacter nitrofigilis*, *C. cryaerophilus* and an unnamed *Campylobacter* formed a distinct genus, which was named *Arcobacter*. This genus is widespread and is found in a variety of sources, such as meat, water and animal faeces. Many habitats of *Arcobacter* spp. are human foodstuffs and are therefore

potential sources of human infection. The numerous sources of *Arcobacter* spp. will be described here.

1.1. *Arcobacter* spp. in Poultry

Arcobacter spp. appears to be a common contaminant of poultry products, therefore posing a potential threat to human health due to its suggested nature as a human foodborne pathogen. As a result, a large number of studies have been carried out which focus on the prevalence and diversity of *Arcobacter* spp. in poultry and poultry products.

The prevalence of *Arcobacter* spp. tends to be low in studies using cloacal swabs from poultry, leading to the conclusion that *Arcobacter* spp. are not natural inhabitants of the poultry gut. Kabeya *et al.* (2003a) found 14.5% (n=234) *Arcobacter* prevalence in chicken cloacal samples in Japan; Wesley and Baetz (1999) found 15% prevalence (n=407) from chicken cloacal swabs in the USA. From poultry faeces, Amisu *et al.* (2003) found 14% *Arcobacter* prevalence in poultry abattoir effluent in Nigeria (n=150). Andersen *et al.* (2007) found 2% (n=298) and 2.1% (n=145) *Arcobacter* prevalence in turkey cloacal swabs and caecal contents, respectively, and up to 67% prevalence in turkey drinking water on the same farms (n=46), supporting the belief that *Arcobacter* does not frequently colonise the poultry gut, and that carcass contamination must occur at or after slaughter (Andersen *et al.*, 2007).

Studies of poultry meat, however, suggest a much higher incidence of *Arcobacter* contamination compared to cloacal swabs. Atabay *et al.* (1997, 1998) found 100% *Arcobacter* prevalence (n=35) on supermarket and abattoir chickens in Turkey, and similarly Morita *et al.* (2004) found 100% *Arcobacter*

prevalence (n=10) on chicken meat in Thailand and 48% on chicken meat in Japan (n=41). A second Japanese study (Kabeya *et al.*, 2004), however, found much lower prevalence on shop-bought chicken carcasses, with only 23% of chickens being contaminated (n=100). Houf *et al.* (2001a) reported a prevalence of 65% on broiler meat in Belgium (n=52), and a similar study in Australia revealed a prevalence of 73% (n=22) on chicken meat (Rivas *et al.* 2004). Andersen *et al.* (2007) reported 93% prevalence on turkey carcasses at slaughter in the USA (n=150). A study in Belgium and Turkey which compared the presence of *Arcobacter* on fresh and frozen shop-bought chickens gave prevalences of 95% (n=44) and 23% (n=31), respectively (Atabay *et al.*, 2003), suggesting the inability of *Arcobacter* spp. to survive after freezing. Son *et al.* (2007) isolated *Arcobacter* from broiler carcasses during different stages of processing and showed that prior to scalding, the *Arcobacter* prevalence of the carcasses was 96.8% (n=125). The prevalence was lower at other stages of processing, (61.3% pre-chill; n=75 and 9.6% post chill; n=125), with the overall prevalence being 55.1% (n=325). A study of chickens and turkeys in Turkey found prevalences of 68% (n=100) in chicken meat but only 4% (n=100) in turkey meat (Aydin *et al.* 2007), suggesting different prevalence of *Arcobacter* spp. colonisation in different poultry species, although this has yet to be investigated further. However, studies by Atabay *et al.* (2008) found 18% (n=90) *Arcobacter* prevalence in cloacal swabs of domestic geese, and 70% prevalence (n=10) in duck cloacal samples, 11% in turkey flocks (n=37) and 100% (n=30) in chicken meat swabs (Atabay *et al.*, 2006). In another study of ducks, Risdale *et al.* (1999) found five of ten oven-ready duck carcasses to be

contaminated with *A. cryaerophilus*. These prevalence data are summarised in Table 1.1.

In chicken processing plants, *Arcobacter* spp. have been isolated from carcasses and slaughter equipment (Houf *et al.* 2002b), although the precise source of contamination is unclear. Van Driesche and Houf (2007b) attempted to determine the source of chicken meat contamination by investigating whether *Arcobacter* spp. can colonise any part of the chicken as part of the natural flora. They did not find any *Arcobacter* on the carcass skin, feathers or intestinal tract of the birds, although *Arcobacter* spp. were isolated from all neck skin samples (n=45). It was suggested that water used in the processing of the carcasses may be a possible source and this was further investigated by Ho *et al.* (2008b), who attempted to determine whether water used in poultry slaughterhouses was a likely source of *Arcobacter* contamination of poultry meat. Supply water, water draining off carcasses during processing, and the carcasses and intestinal tracts of processing poultry were examined. No *Arcobacter* spp. were found in any of the supply water samples, in contrast with the study of Houf *et al.* (2007b), but *Arcobacter* spp. were found in almost all draining-off water, carcass and intestinal samples, suggesting that water supplies are unlikely to be a source of *Arcobacter* contamination in this case, but that the bacteria were present and colonising the gut of the birds (Ho *et al.*, 2008b). It is possible that *Arcobacter* spp. may be introduced to slaughterhouses via the poultry intestinal tracts, and may then go on to contaminate carcasses and machinery. High prevalence in chicken meat and on chicken carcasses, however, suggests that the source may not necessarily be intestinal. It is not thought that *Arcobacter* spp. cause any clinical signs of disease in poultry (Wesley and Baetz, 1999).

In 2008, Lipman *et al.* sampled the intestinal tract, oviduct magnum mucosa, ovarian follicles and eggs of two breeding hen flocks. *A. butzleri, A. skirrowii* and *A. cryaerophilus* were all isolated from the intestinal tracts with a prevalence of 85% in one flock (n=40), and 20% in the other (n=30), and from 15% of oviduct magnum mucosa samples (n=40). However, no *Arcobacter* spp. were isolated from any of the eggs or ovarian follicles of either flock, suggesting that the bacteria may colonise the intestinal tract and oviduct of hens, but might not be transmitted vertically from hens to their eggs (Lipman *et al.*, 2008). In contrast, studies have shown that vertical transmission of the closely related genus, *Campylobacte*r, from breeders to broilers does occur in chickens (Hiett *et al.*, 2002; Cox *et al.*, 2005; Byrd *et al.*, 2007), suggesting a difference in the abilities of *Arcobacter* spp. and *Campylobacter* spp. to colonise chickens.

Overall, the literature provides conflicting reports concerning the sources of *Arcobacter* spp. that are detected in chickens, but studies agree that the prevalence of *Arcobacter* spp. in chickens is high, and that this poses a potential threat to human health.

Table 1.1. Prevalences of *Arcobacter* spp. in poultry-related sources worldwide.

Reference	Location	Source	Sample size	Prevalence (%)
Kabeya *et al.* (2003a)	Japan	Chicken cloacal swabs	234	14.5
Wesley and Baetz (1999)	USA	Chicken cloacal swabs	407	15
Andersen *et al.* (2007)	USA	Turkey cloacal swabs Turkey caecal contents Turkey carcass swabs	298 145 46	2 2.1 93
Amisu *et al.*(2003)	Nigeria	Poultry abbatoir effluent	150	14
Houf *et al.* (2002)	Belgium	Chicken carcasses Chicken slaughter equipment	na	na
Van Driessche and Houf (2007a)	Belgium	Chicken carcass - Skin - Feathers - Intestinal tract - Neck skin	 10 10 10 45	 0 0 0 100
Ho *et al.* (2008b)		Slaughterhouse supply water Draining off water Chicken carcasses Intestinal tracts	na 4 140 40	0 100 68 85
Atabay *et al.* (1997)	UK	Supermarket chickens	20	100
Atabay *et al.* (1998)	Turkey	Abbatoir chickens	15	100
Morita *et al.* (2004)	Thailand Japan	Chicken meat	10 41	100 48
Kabeya *et al.* (2004)	Japan	Chicken carcasses	100	23
Houf *et al.* (2001)	Belgium	Broiler meat	52	65
Rivas *et al.* (2004)	Australia	Chicken meat	22	73
Atabay *et al.* (2003)	Belgium and Turkey	Fresh chicken meat Frozen chicken meat	44 31	95 23
Son *et al.* (2007)		Broiler prior to scalding Broiler pre-chill Broiler post-chill	125 75 125	96.8 61.3 9.6
Aydin *et al.* (2007)	Turkey	Chicken meat Turkey meat	100 100	68 4
Atabay *et al.* (2008)	Turkey	Cloacal swab of Geese	90	18
Atabay *et al.* (2006)	Turkey	Duck cloacal samples Turkey flocks Chicken meat	10 37 30	70 11 100
Risdale *et al.* (1999)		Duck carcasses	10	50

In 2008 Houf *et al.* identified a novel *Arcobacter* species, *A. cibarius*, in broiler flocks, a vehicle for transmission in the human food chain. It has since been identified in piggery effluent irrigated soil, a further link to the human food chain.

1.2. *Arcobacter* spp. in Pigs

Arcobacter spp. appear also to be common in pigs and have been reported in the porcine reproductive tract, being thought to cause abortion in some cases. De Oliveira *et al.* (1997) isolated *A. cryaerophilus* and *A. butzleri* from aborted porcine foetuses, porcine uterine and oviductal tissues and placenta, and later found a prevalence of 26.7% (n=60) in preputial swabs taken at slaughter (de Oliveria *et al.* 1999). Similarly, On *et al.* (2002) reported 42% *Arcobacter* spp. prevalence in porcine abortions (n=55), whilst Suarez *et al.* (1997) isolated *Arcobacter* spp. from porcine gastric samples. *Arcobacter* spp. have also been reported in pork meat, as presented in Table 1.2.

Table 1.2. Prevalences of *Arcobacter* spp. in pork products worldwide.

Reference	Location	Source	Sample size	Prevalence (%)
Rivas *et al.* (2004)	Australia	Retail pork	21	29
Villaruel-lopez *et al.* (2003)	Mexico	Retail pork	45	51.1
Kabeya *et al.* (2004)	Japan	Retail pork	100	7
Van Driessche and Houf (2007a)	Denmark	Retail pork products Pork carcasses	47 169	21 96.4

Further studies have focused on porcine faeces; Van Driessche *et al.* (2003) found a prevalence of 43.9% *Arcobacter* spp. in pig faecal samples, and later prevalences ranging from 16% to 85% in faecal samples on four different pig farms (Van Driessche *et al.* 2004). Kabeya *et al.* (2003) reported *Arcobacter* spp. in 10% of pig faeces tested and 13.3% in pig vaginal swabs, and

Chinivasagam *et al.* (2007) reported *A. butzleri, A. cryaerophilus* and *A. cibarius* in piggery effluent irrigated soil.

These studies show that *Arcobacter* spp. are very common in pigs and their products, causing disease in some cases. Pigs and their products might function as a vehicle of *Arcobacter* transmission to humans, due to the popularity of consuming pork in many countries.

1.3. *Arcobacter* spp. in Cattle

Arcobacter spp. have been detected in raw beef samples at prevalences ranging from 2% (Kabeya *et al.*, 2004) to 37% (Aydin *et al.*, 2007), proving that *Arcobacter* spp. are present in beef, which may act as a vehicle for transfer to humans.

Table 1.3. Prevalences of *Arcobacter* spp. in beef products worldwide.

Reference	Location	Source	Sample size	Prevalence (%)
Villarruel-lopes *et al.* (2003)	Mexico	Raw ground beef	45	28.8
Kabeya *et al.* (2004)	Japan	Retail beef	90	2
Rivas *et al.* (2004)	Australia	Retail ground beef	32	22
Scullion *et al.* (2006)	Northern Ireland	Retail raw beef	108	34
Aydin *et al.* (2007)	Turkey	Retail raw minced beef	27	37
Ongor *et al.* (2004)	Turkey	Minced beef	97	5

Arcobacter spp. have also been isolated frequently from cattle faeces. In the US, Wesley *et al.* (2000) reported 14.3% *Arcobacter* prevalence in dairy cattle (n=2085), while Golla *et al.* (2002) found an overall prevalence of 9% in

dairy and beef cattle (n=200). Ongor *et al.* (2004) studied the faeces and meat of clinically healthy cattle in Turkey and found *A. butzleri* prevalences of 10% (n=200) and 5% (n=97) respectively. Kabeya *et al.* (2003a) found *Arcobacter* spp. in 4% of cattle faeces (n=332) and 8% of cattle vaginal swabs (n=61) in Japan. Van Driessche *et al.* (2003) found a prevalence of 39% in cattle faeces in Belgium, the highest prevalence reported to date. Aydin *et al.* (2007) reported *Arcobacter* in cattle gall bladders (8%; n=50) and cattle rectal swabs (6.9%; n=173). The differences in prevalence found in these studies may be partly explained by the variety of isolation techniques used for *Arcobacter* spp. The use of different isolation techniques can give very different results, as will be discussed later (Chapter 1, section 1.7.). All of these studies reported the isolation of *Arcobacter* spp. from clinically healthy cattle, highlighting the fact that *Arcobacter* spp. are typically carried by cattle without causing disease. While disease can occur, it is uncommon. This will be discussed later.

Very few studies have been conducted concerning *Arcobacter spp.* in milk or dairy products, however, *Arcobacter* spp. were isolated at a prevalence of 46% in raw milk (n=101) by Scullion *et al.* (2007). Since *A. cryaerophilus* and *A. butzleri* were isolated from clinically healthy dairy cows in Brazil by Pianta *et al.* (2007) it is probable that *Arcobacter* spp. are present in the milk of clinically healthy cattle.

1.4. *Arcobacter* as a cause of disease in animals

Arcobacter spp. have been associated with septicaemia, abortion, enteritis and mastitis in various animals (Ellis *et al.*, 1977; Higgins and Degre, 1979; De Oliveira *et al.*, 1997; On *et al.*, 2002; Wesley, 1997; Higgins *et al.*, 1999), whilst

at the same time frequently, and more commonly, being isolated from clinically healthy animals (Atabay *et al.*, 2008; Van Driessche *et al.*, 2005; Van Driessche *et al.*, 2004; Ongor *et al.*, 2004; Wesley *et al.*, 2000), leading to some discussion as to whether *Arcobacter* spp. are normal gut flora of the animals in which they have been found.

A. butzleri, A. skirrowii and *A. cryaerophilus* have been associated with illness in animals, having been isolated from milk from a cow with mastitis and associated with livestock diarrhoea and aborted livestock foetuses (Rivas *et al.*, 2004). In the case of livestock abortions, On *et al.* (2002) suggested that *Arcobacter* spp. may be responsible for the majority of livestock abortions in developed countries, after reporting *Arcobacter* in over 90% of abortions in Danish pigs, with no other apparent cause found.

1.5. *Arcobacter* spp. in water and the environment.

Almost certainly the most common habitat of *Arcobacter* spp. is the environment. *Arcobacter* spp. have been frequently isolated from various water sources and while it is possible that its presence in water is due to faecal contamination, a number of studies have led to the conclusion that *Arcobacter* spp. are naturally water-borne pathogens. The sequencing of an *A. butzleri* whole genome (Miller *et al.*, 2007), and its comparison with the whole genomes of other organisms suggested that *Arcobacter* spp. are free-living water-borne pathogens. This is because the genome contains an unexpectedly large number of genes relating to environmental survival and sensing, and is unexpectedly similar to water-dwelling organisms such as *Sulfurimonas denitrificans* (Miller *et al.*,

2007). This is supported also by the existence of *A. nitrifigilis, A. halophilus* and *Candidatus A. sulfidicus*, environment-associated *Arcobacter* species.

The first report of *Arcobacter* spp. in water was by Jacob *et al.* (1993) who isolated *A. butzleri* from a drinking water reservoir in Germany. The same group subsequently isolated *Arcobacter* spp. from drinking and surface water (Jacob *et al.*, 1996), and from drinking water and sewage treatment plants (Jacob *et al.*, 1998). *Arcobacter* spp. have also been found in activated sludge in Germany (Snaidir *et al.*, 1997), ground water in the USA, (Rice *et al.*, 1999), coral surfaces in the Netherlands Antilles (Frias-Lopez *et al.*, 2002), water and mussels in two brackish lakes in Italy (Maugeri *et al.*, 2000), wastewater in Spain (Gonzalez *et al.*, 2007), ground water, drinking water and surface water in South Africa (Diergaardt *et al.*, 2004), and 100% of canal water samples tested in Thailand (Morita *et al.*, 2004). As well as being isolated from freshwater, *Arcobacter* spp. have been isolated from seawater; Maugeri *et al.* (2004 and 2005) isolated *A. butzleri* from seawater and plankton in Italy and found it to be more commonly associated with plankton than free-living in water. Fera *et al.* (2004) found only one *A. butzleri* isolate from seawater and plankton samples, although PCR testing of the samples suggested that a higher prevalence of *Arcobacters* was present, leading to the suggestion that culturable and non-culturable forms may have been present.

A new species, *Arcobacter mytili*, was recently isolated from saltwater mussels (*Mytilus* spp.) in Catalonia, Spain (Collado *et al.* 2009a). Hence mussels may act as a vehicle for transmission of *Arcobacter* to humans.

Fong *et al.* (2007) found *Arcobacter* spp. in seven out of sixteen wells tested after an outbreak of gastrointestinal illness in the USA, suggesting that

Arcobacter was the cause of the illness, although this was not verified by association with human illness. Assanta *et al.* (2002) observed the ability of *A. butzleri* to attach to water pipe surfaces, finding that the organism was able to form extracellular fibrils that allowed it to attach readily to a number of surface types. Moreno *et al.* (2004) assessed the effect of chlorination on *A. butzleri* and found that the organism remained viable after treatment. The assumption is that *Arcobacter* spp. are naturally present in water as an environmental pathogen, although Collado *et al.* (2008) found that *Arcobacter* spp. in numerous environmental water sources was significantly associated with the presence of faecal pollution, leading to the suggestion that *Arcobacter* contamination of water has, in fact, a faecal origin.

In addition, several environmental-associated *Arcobacter* species exist. *A. nitrofigilis*, for example, has never been isolated from humans or animals. It was originally isolated from the roots of the salt marsh plant *Spartina alterniflora* (McLung and Patriquin, 1980). A recently identified environmental species, *Arcobacter halophilus*, which has never been isolated from humans or animals, was first recovered from a hypersaline lagoon (Donachie *et al.* 2005). A third environmental species, C*andidatus Arcobacter sulfidicus* was initially isolated from deep-sea vents and identified as a member of the *Arcobacter* genus by phylogenetic analysis of 16s rRNA sequence (Wirsen *et al.*, 2002).

1.6. *Arcobacter* spp. in humans

The knowledge of *Arcobacter* infection in humans is limited mostly to the findings of two large scale studies in France and Belgium, and a number of smaller studies and individually reported cases. Vandenberg *et al.* (2004)

reported *A. butzleri* to be the fourth most commonly found *Campylobacter*-like organism in an eight year study of 67,599 hospital-submitted diarrhoeic stool samples in Belgium. Prouzet-Mauleon *et al.* (2006) also found that *A. butzleri* was the fourth most common *Campylobacter*-like organism isolated in their study of 2,855 human diarrhoeic stool samples submitted as part of a *Campylobacter* surveillance network. This study found an overall prevalence of 1%. Houf and Stephan (2007) studied the faeces of 500 asymptomatic, healthy individuals in Switzerland and found *A. cryaerophilus* to be present in 1.4% of the samples. In Limpopo, South Africa, Samie *et al.* (2007) sampled the stools of 322 individuals. The study found infection of 6.2% with *A. butzleri*, 2.8% with *A. cryaerophilus* and 1.9% with *A. skirrowii*. In a similar study, Kownhar *et al.* (2007) found 1.5% of diarrhoeic HIV patients to be infected with *Arcobacter* spp. in southern India. A study of both healthy and diarrhoeic patients by Engberg *et al.* (2000) recovered *A. butzleri* and *A. cryaerophilus* from the diarrhoeic samples. These studies show that the isolation of *Arcobacter* spp. from humans is fairly common, and is not necessarily associated with clinical disease, since arcobacters can also be isolated from healthy individuals. Detection of *Arcobacter* spp. is not included in routine diagnostic screening of faecal samples in the UK (www.hpa-standardmethods.org.uk/documents/bsop/pdf/bsop30.pdf - last accessed 15.2.2010) and, as a result, *Arcobacter* in humans in the UK is rarely reported. The only report to date of *Arcobacter* detection in a human in the UK is of *A. cryaerophilus* in one diarrhoeal sample, which was isolated after screening 2893 diarrhoeic faecal samples for enteric pathogens as part of an investigation into the occurrence of enteric pathogens in the UK (Tompkins *et al.* 1999).

Symptoms of *Arcobacter* infection reported in humans include persistent, watery diarrhoea (Vandenberg *et al.,* 2004), abdominal pain and cramps (Vandamme *et al.*, 1992a) and nausea, vomiting and fever (Dediste *et al.,* 1998). However, a number of *Arcobacter* isolates have been from healthy individuals with no clinical symptoms (Houf and Stephan, 2007; Woo *et al.*, 2001). The relatively small number of human cases reported, along with the fact that in many cases the *Arcobacter* spp. were isolated alongside other pathogens, means that *Arcobacter* is currently regarded only as a potential emerging human foodborne pathogen (Miller *et al.*, 2007). Data so far are inconclusive regarding the nature of *Arcobacter* spp. as human pathogens.

Four confirmed human outbreaks of *Arcobacter* infection have occurred. The first was in an Italian school in 1992 (Vandamme *et al.* 1992a); ten children were diagnosed with *A. butzleri* infection after suffering severe abdominal cramps. Another occurred at a girls' school camp in the USA, when faecal contamination of a well used for drinking water occurred (Rice *et al.*, 1999). A third occurred after groundwater contamination in Ohio, USA (Fong *et al.*, 2007) and a fourth occurred in Slovenia, where *A. cryaerophilus* was isolated from a stool sample of a patient involved in a water-borne multi-microbial outbreak (Kopilovic *et al.*, 2008). No other outbreaks of *Arcobacter* infection have since been reported, although numerous reports of individual, sporadic cases have been published. In the first of these, Lerner *et al.* (1994) reported *A. butzleri*-associated persistent diarrhoea and abdominal cramps in two patients. Wybo *et al.* (2004) reported finding *A. skirrowii* in a patient with chronic diarrhoea. *Arcobacter* has also been associated with other clinical symptoms; On *et al.* (1995) reported *A. butzleri* in a neonate with bacteraemia, Hsueh *et al* (1997)

reported *A. cryaerophilus* in a pneumonia patient with bacteraemia, and Yan *et al.* (2000) reported *A. butzleri* in a patient with liver cirrhosis, a high fever and oesophageal bleeding. Woo *et al.* (2001) isolated *A. cryaerophilus* from an otherwise healthy road traffic accident victim, whilst Lau *et al.* (2002) isolated *A. butzleri* from a patient with acute gangrenous appendicitis. *A. butzleri, A. skirrowii* and *A. cryaerophilus* are the only *Arcobacter* species to have been reported as being isolated from humans thus far, with, of the three, *A. butzleri* being the most commonly associated with disease.

1.7. *Arcobacter* isolation methods

A variety of methods have been employed for the isolation of *Arcobacter* spp. from samples, ranging from modified techniques for the isolation of *Campylobacter* and *Leptospira*, to those involving specially designed, *Arcobacter*-specific, media. This may explain the difference in prevalence reported in different studies. Atabay and Corry (1998) evaluated the use of *Arcobacter* broth (Oxoid, UK) with added CAT (cefoperazone, amphotericin, teicoplanin) selective antibiotic supplement. Johnson and Murano (1999) developed JM broth and plates after evaluating separate components of media selective for Gram-negative organisms. They found that JM medium, comprising a basal nutrient mix with 0.05% thioglycolic acid, 0.05% sodium pyruvate, and 5% sheep's blood, was the most effective for the growth of *Arcobacter* spp. More recently, Houf *et al.* (2001a) developed an *Arcobacter*-specific isolation method involving the use of commercially available *Arcobacter* media (Oxoid, UK) with a supplement consisting of five antibiotics. Houf *et al.* (2001b) found that all *Arcobacter* isolates tested were highly resistant to the

antifungal agents amphotericin B and cyclohexamide, and also 5-fluorouracil. Many of the strains were also resistant to novobiocin. These compounds, along with trimethoprim, were used in combination in the isolation media developed by Houf *et al.* (2001a) and now widely used for the isolation of *Arcobacter* spp. In addition, Hamill *et al.* (2008) evaluated the use of Hugh and Leifson's medium during *Arcobacter* isolation and found that it could be used to detect non-*Arcobacter* spp. and hence eliminate them. Scullion *et al.* (2003) compared several published methods and found in two separate studies that Johnson and Murano broth, modified to include streaking onto enrichment media at 24 hours and 48 hours, was significantly more effective than other methods tested, and that microaerobic incubation improved the selectivity of the method (Hamill *et al.*, 2007; Scullion *et al.*, 2003).

The fact that several different isolation methods for *Arcobacter* spp. can be used is problematic, meaning that the results of studies carried out using different methods are not comparable. Naturally, certain factors such as antibiotics included in selective media are likely to affect the strains isolated as some strains are likely to be more susceptible than others, leading to a possible bias in results. The use of a standard selective isolation media would negate this problem.

1.8. Identification of *Arcobacter* spp.

It is widely accepted that phenotypic methods of identification are difficult to use in this genus, due to its limited biochemical activities (Atabay *et al.* 2006; On., 1996), thus promoting the need for more reliable, molecular identification techniques. A genus-specific PCR assay was developed by

Harmon and Wesley (1997). This targeted the 16S rRNA genes and gave a product of 1223 base pairs (bp), enabling identification of an *Arcobacter* isolate to genus level. Similarly, Gonzalez *et al.* (2000) developed a genus-specific PCR assay which targeted the 16S rRNA, producing a 200bp product. In 2000, Houf *et al.* developed a multiplex PCR assay, again based on the 16S and 23S rRNA genes, which enabled the identification of *A. butzleri, A. skirrowii* and *A. cryaerophilus*, with each giving a different sized product (401bp, 641bp and 257bp respectively).

While the PCR assay of Houf *et al.* (2000) has for several years been accepted as the gold standard for *Arcobacter* identification and has been used in many studies, Figueras *et al.* (2008) found discrepancies in the method, with some strains identified as *A. skirrowii* using the Houf multiplex PCR assay, being identified as *A. nitrofigilis* after 16S rRNA sequence analysis and RFLP, including *A. nitrofigilis* type and reference strains. At the time of the design of the Houf multiplex PCR assay, only four *Arcobacter* species had been discovered, and so the PCR assay was designed based on very limited data. As of December 2009 eight *Arcobacter* species have been identified, with further novel species suggested, making this PCR assay somewhat redundant. Hence, Figueras *et al.* (2008) devised a 16S rDNA-RFLP method to identify all *Arcobacter* spp. known at that time. The development of PCR assays which, it is hoped, will be able to discriminate between more *Arcobacter* spp. is planned for the near future (M. Figueras, personal communication).

1.9. Molecular epidemiology and typing of *Arcobacter.*

A number of different molecular typing methods have been used to study the genetic diversity of *Arcobacter* spp. to date, the most recently developed methods being *Arcobacter*-specific multi-locus sequence typing (MLST) and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR).

MLST is already widely used for the typing of *Campylobacter* and other bacterial species; as of July 2009 almost thirty bacterial MLST schemes were hosted on the Oxford University PubMLST website (www.pubmlst.org). The *Arcobacter* MLST scheme was devised by William Miller of the US department of Agriculture, and features the same seven housekeeping genes utilised in *Campylobacter jejuni* and *C. coli* schemes (Dingle *et al.,* 2001; Dingle *et al.*, 2005), providing the additional benefit of possible cross-comparison of alleles between the *Arcobacter* and *C. jejuni/coli* databases (Miller *et al.*, 2009). This technique involves sequencing short lengths of seven housekeeping genes. These are genes which are highly conserved within the genus and yet show a certain amount of variation between isolates of the same species. Comparison of these genes makes for a robust, portable and very reliable, sensitive and specific typing method. MLST has proved a popular and successful method for typing *Campylobacter* isolates (Dingle *et al.*, 2001; Dingle *et al.*, 2005; Maiden, 2006; Miller *et al.*, 2006), and a recently developed *Arcobacter* MLST technique is now being used in a small number of studies worldwide. Similar to the popular *C. jejuni* MLST method, the *Arcobacter* method makes use of seven housekeeping genes, *aspA* (477bp), *atpA* (489bp), *glnA* (474bp), *gltA* (429bp), *glyA* (507bp), *pgm* (503bp) and *tkt* (462bp) (Miller *et al.* 2009). Each housekeeping gene is sequenced and submitted to a curated online database.

Each allele is assigned a number based on existing sequence data, and new alleles are assigned numbers by the curator. These form a seven-number allelic profile, which is in turn given a number, referred to as the sequence type of the isolate. Groups of isolates with similar sequence types can indicate clonal complexes, which are also assigned numbers. MLST is the most portable of typing methods to date, with data being stored on a central database accessible by anyone, and a standardised methodology with little room for discrepancy. As it is a sequence-based method, it is objective and very accurate, unlike band-based typing methods which can be subject to inaccuracy and variations between laboratories (Faria *et al.*, 2008).

ERIC PCR is a PCR-based method which uses forward and reverse primers to amplify sections of DNA that occur between repetitive elements found throughout the bacterial genome. This technique was optimised by Houf *et al.* (2002) and found to be more sensitive and specific when compared with random amplified polymorphic DNA-PCR (RAPD; a PCR-based technique that amplifies random sections of DNA. Houf *et al.*, 2002), leading to its use in a number of recent studies (Van Driessche *et al.*, 2004; Van Driessche *et al.*, 2005; Van Driessche and Houf, 2007a; Aydin *et al.*, 2007). ERIC-PCR uses specific primers which amplify areas of sequence between repeated motifs that occur throughout the genome, producing a pattern of different sized bands after agarose gel electrophoresis. However, some doubts exist concerning the reliability of ERIC-PCR. The PCR reaction features an extremely low annealing temperature, just 25°C (Houf *et al.* 2002), which creates a very high risk of non-specific binding and therefore reduces the reliability of the bands, making the technique equivalent to a RAPD technique.

RAPD uses short primers of nine or ten nucleotides and low annealing temperatures in order to amplify random fragments of DNA. The primers anneal to any sites in the genome that are complementary and thus produce random fragments of varying lengths which are then visualised by agarose gel electrophoresis.

Another commonly used molecular typing technique is macrorestriction pulsed field gel electrophoresis (PFGE) which uses a rare-cutting restriction enzyme to cut whole genomic DNA, and the resulting banding patterns are then visualised following pulsed-field gel electrophoresis. Bacterial cells are suspended in agarose blocks, lysed using a proteinase enzyme, and a restriction enzyme is used to cut the DNA into fragments of varying sizes, which are then visualised following agarose gel electrophoresis run at a low voltage over a number of hours. This uses a pulsed field, where the current is applied in pulses from different directions in order to maintain clarity of the bands on the gel.

A number of studies have used PFGE to type *Arcobacter* strains, using the enzymes *KpnI, KspI, EagI, SmaI* and *SacII.* Hume *et al.* (2001) first adapted an existing PFGE protocol for use with *Arcobacter* species in order to investigate the genotypic variation of *Arcobacter* in pigs. They concluded that a large amount of genotypic variation exists amongst *Arcobacter* isolates in swine, suggesting colonisation by multiple parent genotypes which have undergone genomic rearrangement during passage through animals, a common feature of the *Campylobacteraceae* (Hume *et al.* 2001). Later, Rivas *et al.* (2004) further developed the scheme, in order to characterise *Arcobacter* species from meat.

Ho *et al.* (2006) used PFGE to demonstrate vertical transmission of *Arcobacter* species from sows to piglets, while Son *et al.* (2006) used PFGE to

demonstrate high levels of diversity in *Arcobacter* species from broiler carcasses, obsreving a much higher genetic diversity in *Arcobacter* strains than *Campylobacter* strains in the same study. Rivas *et al.* (2004) used PFGE, based on the method of Hume *et al.* (2001), to study the diversity of *A. butzleri* isolates from meat, noting that PFGE appeared more discriminatory than other genotyping methods, including ERIC-PCR, rep-PCR and RAPD. These studies demonstrated the value of PFGE as a genotyping technique capable of showing diversity within the *Arcobacter* genus.

Amplified fragment length polymorphism (AFLP) is a further molecular typing method used in *Arcobacter* research. Genomic DNA is cut by two restriction enzymes, and then adaptors are annealed to the fragment, to which oligonucleotide primers are bound. Fragments are then amplified by PCR and visualised on a gel. Amisu *et al.* (2003) used AFLP to study the genetic diversity of *A. butzleri* strains from poultry effluent, concluding that AFLP provided a useful tool for the molecular epidemiological study of *A. butzleri*. On *et al.* (2003, 2004) and Gonzales *et al.* (2007) have also used AFLP to study the genetic diversity of *Arcobacter* spp. and found it a useful epidemiological tool. All, in agreement with other studies, found a large degree of heterogeneity, showing that numerous different *Arcobacter* genotypes may be present at any one time in a given environment.

Gonzalez *et al.* (2007) typed *Arcobacter* and *Campylobacter* isolates from chicken and water using both PFGE and AFLP and found a large amount of heterogeneity among all isolates. This study determined that PFGE is a useful tool for studying epidemiological relationships in closely related isolates, although not particularly sensitive, therefore AFLP was capable of detecting the

smaller genomic variations and would be more useful for the differentiation of isolates.

Restriction fragment length polymorphism (RFLP) is a molecular typing technique that uses a restriction enzyme to detect polymorphisms in rRNA, and hybridisation to specific probes to produce fragments that are separated and visualised by agarose gel electrophoresis. A rare-cutting restriction enzyme is used to cut whole genomic DNA into large fragments and the resulting fragments are separated by agarose gel electrophoresis. This is followed by Southern blotting, in which the fragments in the gel are transferred to a nitrocellulose or nylon membrane and then hybridised to labelled probes (usually labelled with colorimetric or chemiluminescent substrates). These probes are designed to be homologous to the gene being studied, which produces bands of varying sizes on the gel due to the varying locations of the restriction sites within the gene in different isolates. A derivative of this method, ribotyping, involves the use of probes designed to amplify sections of the 23S or 16S ribosomal RNA of bacteria and results in a smaller number of bands, simplifying analysis but making the technique less sensitive (Olive and Bean, 1999). The Southern blotting RFLP method is, however, rather outdated, being replaced by simpler, more direct PCR-RFLP methods. In PCR-RFLP, specific primers are used to first amplify a target locus, e.g. a specific gene, which is then subjected to restriction digestion. The bands are typically visualised on an agarose or polyacrylamide gel with ethidium bromide stain, which negates the need for Southern blotting. Different PCR-RFLP schemes exist, often focussing on a specific locus or gene. One example of this is the *fla* typing scheme, which is commonly used for typing *Campylobacter* species. *fla* typing schemes target the

flaA, or both *flaA* and *flaB* flagellin gene loci. These genes comprise conserved terminal regions which surround variable central regions. The desired region is PCR-amplified and the resulting product is subjected to RFLP, producing banding patterns which differ between isolates, due to the variable regions between the *fla* genes (Ayling *et al.*, 1996; Nachamkin *et al.*, 1996).

RFLP and PCR-RFLP have been used in a number of typing and differentiation studies of *Arcobacter* spp. (Wesley *et al.* 1996; Hurtado and Owen, 1997; de Oliveira *et al.* 1997; Marshall *et al.* 1999; Moreno *et al.* 2004; Figueras *et al.* 2008) differentiate *Arcobacter* and *Campylobacter* spp. and to study the diversity of different *Arcobacter* strains.

Ho *et al.* (2008a) showed *Arcobacter* spp. to possess much shorter versions of the two flagellin genes, *flaA* and *flab,* possessed by the closely related *Campylobacter* spp.. *Arcobacter* spp. appear to lack a large section of the variable central region, making the development of a *fla*-sequence typing method with a suitable discriminatory ability difficult.

1.10. *Arcobacter* Whole Genome Sequencing.

The first bacterial genome to be sequenced was that of *Haemophilus influenzae* (Fleischmann *et al.*, 1995), using a shotgun sequencing method designed by Sanger *et al.* (Sanger *et al.*, 1977; Sanger *et al.*, 1982, Fleischmann *et al.*, 1995). Sanger's method, known as the chain-termination method, involves in vitro amplification of the target DNA in *E. coli* plasmids, and the use of ddNTPs (dideoxynucleotides) to create random, short chains of DNA starting and ending with specific bases depending on the dNTP used, which are then separated and visualised on a polyacrylamide gel. The breaking up of DNA into

smaller fragments is repeated, resulting eventually in overlapping fragments that allow the re-assembly of the whole genome with the fragments in the correct order. This method, however, is limited by its requirement for a cloning step, which means bias may occur against areas of DNA that do not replicate well in *E. coli* (used as a cloning vector) or that encode toxic compounds that may limit its replication in these circumstances (Hall, 2007). This means directed sequencing of non-clonable regions is needed (Medini *et al.*, 2008). Newer sequencing technologies such as 454 and polony sequencing, both of which involve parallel sequencing by synthesis on a solid support, and single molecule sequencing methods have been developed that avoid the inherent biases of previous methods (Hall, 2007).

454 whole genome sequencing (Roche, USA, www.454.com) is a recently developed high-throughput, next-generation sequencing method which involves a two-step approach. Firstly, oligonucleotide adaptors are attached to sheared DNA, which is attached to beads. The beads are used in a PCR amplification so that multiple copies of the DNA fragment are present on each bead (Margulies *et al.*, 2005). Secondly, pyrosequencing is performed in parallel on each bead in a 96-well plate. Pyrosequencing is a pyrophosphate-based sequencing method; wells contain luciferase and sulfurylase enzymes and nucleotides are added sequentially across the plate during the sequencing run. Inorganic pyrophosphate is released upon the addition of each new nucleotide to the sequence, leading to the generation of light when the pyrophosphate is converted to ATP using the sulfurylase and luciferase enzymes (Ronaghi *et al.*, 1996). The 454 technology has a read length of 400 base pairs and a run length of 500 mega bases, meaning a whole genome can be sequenced in a single run,

taking a fraction of the time when compared to Sanger sequencing methods (Roche, USA).

With the availability of fast, reliable and affordable whole genome sequencing methods such as these, whole genome sequence data for virtually any organism can now be obtained, and whole genome sequencing is beginning to become standard practice for the investigation of microbial organisms. The increasing accessibility of whole genome sequencing is demonstrated by the fact that in 2007, 279 complete bacterial genomes were present on the public databases (Hall, 2007). As of July 2009, there are more than 1900, and this number is continually growing, especially with the imminent completion of the University of Liverpool's "Fifty Genomes for Fifty Years" (www.liv.ac.uk/vetseq/) and other similar large-scale genome sequencing projects.

To date, the genomes of forty nine epsilonproteobacteria have been sequenced, including various *C. jejuni* subspecies (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006; Pearson *et al.*, 2007), strains of *C. coli* (Fouts *et al.*, 2005), *C. fetus* (CP000487.1, unpublished), *C. upsaliensis* (Fouts *et al.*, 2005), *C. lari* (Fouts *et al.*, 2005), *Sulfuromonas denitrificans*; a sulphur-oxidising chemolithautotroph (Sievert *et al.*, 2008), *Wolinella succogenes* (Baar *et al.*, 2003), *Helicobacter hepaticus* (Suerbaum *et al.*, 2003), *H. pylori* (Tomb *et al.*, 1997; Oh *et al.*, 2006), and other members of the phylum. In 2007, the first whole genome sequence of an *Arcobacter* was completed and published. An *A. butzleri* isolate from a human clinical case was sequenced in the USA and yielded some surprising results.

Miller *et al.* (2007) determined a fully annotated whole genome sequence of *A. butzleri* isolate RM4018, a human clinical isolate. Shotgun high-throughput sequencing was carried out, and reads were assembled using a computerised genome assembly program before annotation. The genome was found to contain 2,341,251 bp making it the second largest epsilonproteobacterial genome at that time, being smaller than *Sulfurovum* strain NBC37-1 and larger than all other epsilonproteobacterial genomes including *Campylobacter* spp., *S. denitrificans* and *W. succogines* (Miller *et al.*, 2007).

The study found that the majority of the *A. butzleri* proteome, predicted based on the genome, was most similar to those of the *Helicobacteraceae* members *S. denitrificans* and *W. succinogenes*, and that many of the genes present, such as those involved in sulphur metabolism and signal transduction, had been described only in these species, being unique to this subdivision. A proportion of the genes identified were most similar to those epsilonproteobacteria associated with deep-sea vents, *Nitratiruptor* and *Sulfurovum*. Another observation of note was that a major proportion of the *A. butzleri* genome was dedicated to mechanisms involved in growth and survival in different environmental conditions, such as proteins associated with respiration, signal transduction, chemotaxis, and DNA repair and adaptation, and sensing systems such as two-component systems, which feature a surface component to detect extracellular changes, and an intracellular component to affect responses to these changes, and ECF family σ-factor pairs (Miller *et al.*, 2007).

1.11. Background and Aims of This Study.

While the genus *Arcobacter* is currently considered to be very closely related to the genus *Campylobacter*, and may exhibit similar features in its clinical symptoms of disease and sporadic infection in humans, and high prevalence in food animals, many important aspects of *Campylobacter* research have not yet been applied to its relative, *Arcobacter*. One important aspect of *Campylobacter* infection in man is its seasonal prevalence. It is thought that the seasonal peaks in *Campylobacter* prevalence and the number of clinical cases reported in humans are related, and that the defining cause of this seasonality may be the key to preventing or reducing some human infections.

During a study of *Campylobacter jejuni* prevalence in the north west UK cattle in 2006 it was observed that *Arcobacter* was isolated commonly from the cattle faeces using *Campylobacter*-specific methods, and that *Arcobacter* recovery appeared to increase when *C. jejuni* prevalence was lower (unpublished data). This project aimed to isolate both *Arcobacter* and *Campylobacter* from various groups of cattle in the north west UK, and to observe the prevalence of each over a period of one year. This data was then used to investigate whether the *Arcobacter* and *Campylobacter* prevalences may be related, and whether factors such as management group (i.e. lactating adults, non-lactating adults, calves, fattening adults and young stock), season and farm type (beef vs dairy) affect *Arcobacter* prevalence.

Aim 1. In view of the lack of a widely accepted, standardised *Arcobacter* isolation method, this study aimed to compare five published methods to determine which method was best suited for use in the project. A pilot study was carried out using cattle, sheep and badger faecal samples from the North West

UK and Gloucester. The optimum isolation method was then used for subsequent studies.

Aim 2. The chosen isolation method was used, along with a *Campylobacter*-specific method, to isolate *Arcobacter* spp. and *Campylobacter* spp. from four farms in Cheshire, UK, in order to determine whether any seasonal change in prevalence occurred. Farm management practices were noted, in order to determine factors that may affect *Arcobacter* prevalence.

Aim 3. This project aimed to genotype a number of *Arcobacter* isolates in order to determine the diversity of *Arcobacter* isolates in UK cattle. High levels of diversity have been shown in *Arcobacter* isolates from various sources worldwide, but this study aimed to be the first report of the diversity of *Arcobacter* spp. from cattle in the UK.

Aim 4. Finally, this project aimed to determine an *A. butzleri* whole genome sequence for comparison with an existing *A. butzleri* genome. Annotation and bioinformatic analysis of this genome sequence may provide insight into disease and survival mechanisms, and may provide us with information regarding the transmission and survival of *A. butzleri* in cattle.

Chapter Two

General Materials and Methods.

2.1. Sample collection.

2.1.1. Cattle and Sheep

Faecal samples were observed being voided, then 4-5 grams were immediately collected in a sterile plastic universal tube with built in scoop, to avoid any contamination of the samples during collection. Samples were then stored at ambient temperature in an insulated box, to avoid any major changes in temperature before processing. Samples were processed within three hours of collection.

On each farm visit the housing status (indoors/outdoors) of the animals and management groups/age were noted.

2.1.2. Badgers

Sixteen faecal samples were collected in sterile containers from badger latrines at a national park in Gloucester, where routine surveillance of badger habitats was carried out daily. Containers were placed in a secure container and transferred to the University of Liverpool by post at ambient temperature. All samples were processed immediately upon receipt, no more than seven days after collection.

2.2. Isolation methods.

For all isolation methods used, 1g faeces was inoculated into 9ml enrichment broth and incubated in specific conditions before being streaked onto solid media after twenty four hours. Plates were incubated and colonies showing typical morphology of *Campylobacter* or *Arcobacter* spp. were selected and sub-cultured to purity twice on Columbia agar plates (LabM, Bury, UK) with 5% defibrinated horse blood before being processed for molecular analysis.

2.2.1 *Arcobacter* isolation methods

H broth - An *Arcobacter*-specific broth, here called *Arcobacter* broth, made up of 18g peptone (Oxoid, Basingstoke, UK), 1g yeast extract (Lab M, Bury, UK) and 5g sodium chloride (Sigma, UK) per litre, with the addition of a five antibiotic supplement made up of 5-fluorouracil (100mg/ml), amphotericin B (10mg/ml), cefoperazone (16mg/ml), novobiocin (32mg/ml) and trimethoprim (64mg/ml), (all Sigma-Aldrich, UK) as described by Houf *et al.* (2001).

AC broth - A second *Arcobacter*-specific broth that comprised *Arcobacter* broth as described above, but with the addition of supplements cefoperazone (8mg/l), amphotericin B (10mg/l) and teicoplanin (4mg/l) (CAT) supplement (Oxoid, UK), as described by Atabay and Corry (1998).

H solid medium - *Arcobacter* broth plus 12g per litre of agar no. 1 (Lab M, Bury, UK) and the same five-antibiotic supplement as described above (Houf *et al.* 2001).

CC solid Medium – modified CCDA. A charcoal agar base (Lab M, Bury, UK) with added cefoperazone (8mg.l^{-1}), amphotericin B (10mg.l^{-1}) and teicoplanin (4mg.l^{-1}) supplement (Oxoid, Cambridge, UK).

All *Arcobacter*-specific methods involved an 18-24 hour incubation in an enrichment broth followed by a 72 hour incubation with interim examinations to visually confirm growth on a solid medium, all under aerobic conditions at 30°C.

2.2.2. Specific *Campylobacter* isolation method

C broth - A *Campylobacter*-specific enrichment broth, CEB (Lab M, Bury, UK) with the addition of 5% defibrinated horse blood and cefoperazone (20mg/l), vancomycin (20mg/l), trimethoprim (20mg/l) and cyclohexamide (50mg/l)

(CVTC) supplement (Lab M, Bury, UK) as described by Kemp *et al.* (2005). The corresponding solid medium, C medium, was a specific *Campylobacter* isolation medium containing CCDA (Lab M, Bury, UK.) with added cefoperazone (32mg/l) and amphotericin B (10mg/l) antibiotic supplement (Lab M, Bury, UK) as described by Kemp *et al.* (2005). Pure cultures were inoculated into C broth and incubated for 18-24 hours under microaerobic conditions (3% hydrogen, 11% oxygen, 12% carbon dioxide and 73% nitrogen) at 37°C, in a VAIN (DWS, Shipley, UK) then streaked onto C solid medium and incubated for 72 hours under the same conditions, with interim visual examinations to confirm growth.

For the *Arcobacter* isolation pilot study (Described in Chapter Three), five different combinations of the above methods were used in order to determine the best method for the isolation of *Arcobacter* spp. from animals in the UK. Figure 2.1. illustrates the media combinations used.

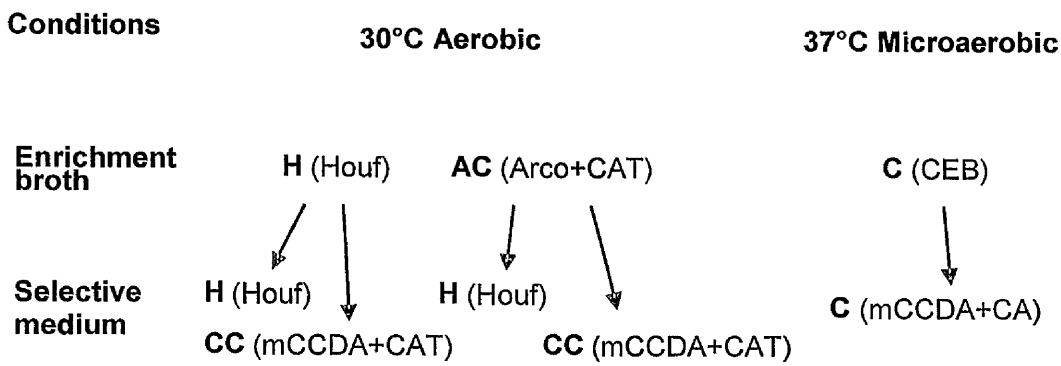

Figure 2.1. The five media combinations used in the *Arcobacter* isolation pilot study.

2.2.3. Sub-culturing on blood agar plates.

After isolation using specific media, colonies were streaked to purity on Columbia agar plates (Columbia agar base, Lab M, Bury, UK) with 5% defibrinated horse blood in order to obtain only pure cultures. In the *Arcobacter* isolation pilot study (Chapter Three) ten colonies were selected from each solid medium plate in order to ensure a diversity of isolates was represented.

After isolation, all isolates were stored at -80°C on cryogenic beads in Microbank vials (Pro-Lab, Neston, UK) or processed immediately for further analysis.

2.3. Extraction of samples from frozen storage.

Samples were recovered from storage by inoculation of one or two cryogenic beads (Pro-Lab, Neston, UK) onto Columbia agar (Lab M, Bury, UK) with 5% defibrinated horse blood and incubation either in aerobic conditions at 30°C (*Arcobacter* only) or in microaerobic conditions (see section 2.2.2.) at 37°C (*Campylobacter* only) for up to 72 hours.

2.4. Preparation of DNA.

For each isolate, pure colonies were grown on Columbia agar with 5% defibrinated horse blood, and approximately 5µl of cells were harvested and suspended by mixing in 150µl distilled water in a 1.7ml eppendorf tube (Eppendorf, Cambridge, UK). The suspension was heated at 100°C for fifteen minutes and centrifuged at 12000g for ten minutes. Each suspension was stored at 4°C short term or -80°C long term, for use in PCR analysis.

2.5. Chelex DNA extraction method.

For higher quality DNA from isolates, to be used for MLST analysis, approximately 5µl of cells were harvested as described in section 2.4. and a cell suspension was created by mixing in 300µl chelex solution (Walsh *et al.*, 1991). Stock solution comprised 10g chelex powder (Bio-Rad, Hemel Hempstead, UK) in 50ml sterile distilled water (in-house). Suspensions were mixed well by vortexing, and then heated at 95°C for ten minutes before centrifuging at 12000g for two minutes. Supernatant (50µl) was then diluted in 450µl sterile distilled water. The extractions were stored at 4°C short term or -80°C long term for use in molecular analysis. At the time of each batch of extractions, an extraction from an *A. butzleri* control isolate (NC12481) was carried out. One micro litre of this extract was then used as a positive extract control in subsequent PCR assays.

2.6. Polymerase Chain Reaction.

All PCR reactions were carried out using ABI 2720 thermal cyclers (Applied Biosystems, Warrington, UK). Primer sequences and the sizes of predicted amplicons are shown in Appendix I. Reactions were carried out in batches of 96 using 96-well plates. All PCR reagents (PCR buffer, dNTP mix, $MgCl_2$, Taq polymerase, oligonucleotide primers and ReddyMix PCR mix) were supplied by Thermo Scientific, Hemel Hempstead, UK, and all PCR products were visualised by running on a 1.5% w/v or 2% w/v agarose gel, as described in section 2.6.1.

2.6.1. Visualisation of PCR products by agarose gel electrophoresis.

PCR products were visualised by running on a 1.5% agarose gel made using 1g Hi-pure low EEO agarose (Biogene, Cambridge, UK) in 50ml 1xTris-borate ethanoate (TBE) buffer (Sigma-aldrich, UK) unless stated otherwise. For all standard PCR reactions, 25µl PCR product was run on a gel with the addition of 2µl 6x gel loading solution (Sigma-aldrich, Dorset, UK). Reactions using ReddyMix did not require this, as ReddyMix already contains a loading buffer. Eighteen µl of 100-bp Superladder (100µg/ml in TE buffer; Thermo Scientific, UK) was loaded at the start of each gel, and after every 25 wells if running a large gel. Large gels (200 wells, 30cm x 30cm) and medium gels (60 wells, 20cm x 30cm) were run at 120v for 75 minutes. Small gels (30 wells, 7cm x 10cm) were run at 120v for 20 minutes. Large 2% w/v gels used for products of the Wang *Campylobacter* PCR were run at 120v for 120 minutes. Bands were visualised and photographed using either Gel-Doc 2000 (Bio-Rad, USA) or UVitec UVPro MW transilluminators.

2.6.2. *Arcobacter* genus-specific 16S rRNA PCR (Gonzalez *et al.* 2000).

An *Arcobacter* genus-specific PCR which targets the 16S rRNA gene was applied to all isolates. Reactions were carried out in 25µl volumes, using 24µl ReddyMix PCR master mix (Thermo Scientific, Hemel Hempstead, UK), which contains 1.5mmol $MgCl_2$, and 1µl template DNA. Primers used were forward primer Arc1 and reverse primer Arc2 (Gonzalez *et al.*, 2000) at a concentration of 1µmol per litre each. Reaction conditions were as follows:

Table 2.6.2. Cycling conditions for the *Arcobacter* genus-specific 16S rRNA PCR.

Initial denaturation	94°C	3 minutes
30 cycles	94°C	1 minute
	59°C	1 minute
	72°C	1 minute
Final elongation	72°C	7 minutes

The 181-bp products were visualised by agarose gel electrophoresis as described later.

2.6.3. *Arcobacter* speciation multiplex 16S and 23S PCR (Houf *et al.* 2000).

Any isolates positive using the *Arcobacter* genus-specific PCR were then identified to species level using the *Arcobacter* multiplex PCR of Houf *et al.* (2000). Reactions (25µl) were carried out using ReddyMix PCR master mix as described above (section 2.6.1.), with 1µl template DNA. The primers used were forward primer ARCO with reverse primers BUTZ and SKIR, all of which were used at a concentration of 1µmol per litre. A second, separate reaction was carried out using the forward primer CRY1 and reverse primer CRY2 at the same concentration. In a variation on the described method, in which all five primers are used in a single multiplex reaction, this second reaction was carried out separately as this was found to give clearer, more reliable results. All reaction conditions were as follows:

Table 2.6.3. Cycling conditions for the *Arcobacter* speciation multiplex 16S and 23S PCR.

Initial denaturation	94°C	2 minutes
30 cycles	94°C	30 seconds
	59°C	30 seconds
	72°C	1 minute
Final elongation	72°C	7 minutes

For the *Arcobacter* PCR assays, a positive control template was included in every 96-well batch of reactions. Positive controls used were *A. skirrowii* (NC12713), *A. butzleri* (NC12481) and *A. cryaerophilus* (NC11885). Molecular grade water was used as a negative control in every batch of reactions.

Isolates that were negative in the *Arcobacter* genus-specific PCR were considered to be putative *Campylobacter* species and were tested using *Campylobacter* species-specific PCR assays (sections 2.6.4. to 2.6.8.) capable of detecting *C. jejuni, C. coli, C. lari, C. lanienae, C. hyointestinalis* and *C. fetus*, all of which are likely to be found in cattle in the UK.

2.6.4. *Campylobacter* colony multiplex PCR for detection of *C. jejuni, C. coli* and *C. lari* (Wang *et al.* 2002).

The first *Campylobacter*-specific PCR to be carried out on the isolates was adapted from the multiplex PCR assay developed by Wang *et al.* (2002), which targets the *hipO* gene in *C. jejuni* and *glyA* in *C. coli* and *C. lari*. Each 25µl reaction contained 0.5µl 20mM dNTP mix, 2.5µl 10x PCR buffer, 20mM $MgCl_2$, 1U Taq polymerase, 0.5µM *C. jejuni* primers CJF and CJR , 1µM *C. coli* primers

CCF and CCR and 0.5μM *C. lari* primers CLF and CLR, with 2.5μl template DNA. Reaction conditions were as follows:

Table 2.6.4. Cycling conditions for the *Campylobacter* colony multiplex PCR for detection of *C. jejuni, C. coli* and *C. lari.*

Initial denaturation	95°C	6 minutes
30 cycles	96°C	30 seconds
	59°C	30 seconds
	72°C	30 seconds
Final elongation	72°C	7 minutes

Products of this PCR were visualised on a 2% w/v agarose gel run at 120V for 75 minutes and stained with ethidium bromide (see section 2.7.) in order to allow detection of the smaller *C. coli* fragments.

2.6.5. *Campylobacter* CeuE gene PCR for detection of *C. jejuni* and *C. coli* (Gonzalez *et al.* 1997).

A second *Campylobacter*-specific PCR to detect *C. jejuni* and *C. coli* designed by Gonzalez *et al.* (1997) was carried out in order to confirm the results of the Wang PCR. This PCR targets the *ceuE* siderophore transport protein of *C. jejuni* and *C. coli*. Each 25μl reaction contained 2.5μl 10x PCR buffer, 0.5μl 20mM dNTP mix, 3.5mM $MgCl_2$, 1μM each of COL1, COL2, JEJ1 and JEJ2 primers, 1U Taq polymerase and 1μl template DNA. Reaction conditions were as follows:

Table 2.6.5. Cycling conditions for the *Campylobacter* CeuE gene PCR for the detection of *C. jejuni* and *C. coli.*

Initial denaturation	94°C	5 minutes
30 cycles	94°C	30 seconds
	57°C	30 seconds
	72°C	1 minute
Final elongation	72°C	7 minutes

2.6.6. *Campylobacter* 16S rRNA Duplex PCR for the detection of *C. hyointestinalis* and *C. fetus* (Linton *et al.* 1996).

A Duplex PCR based upon that designed by Linton *et al.* (1996), which targets a region of the 16S rRNA genes that is similar in these two species, was used for the detection of *C. hyointestinalis* and *C. fetus*. A common forward primer, here named ChyoF, and two reverse primers, ChyoR and CfetR were used at a concentration of 1mM each, with a reaction mixture made up of 0.5µl 20mM dNTP mix, 2.5µl 10x PCR buffer, 2.5mM $MgCl_2$, 1U Taq polymerase and 1µl template DNA. Reaction conditions were as follows:

Table 2.6.6. Cycling conditions for the *Campylobacter* 16S rRNA duplex PCR for the detection of *C. hyointestinalis* and *C. fetus*.

Initial denaturation	94°C	4 minutes
	94°C	1 minute
25 cycles	64°C	1 minute
	72°C	1 minute
Final elongation	72°C	7 minutes

2.6.7. *C. lanienae* PCR Logan *et al.* (2000).

This PCR was carried out using ReddyMix PCR mix as described earlier. The primers CLAN76F and CLAN1021R at a concentration of 1mM each were used, giving a product of 920 bp. The cycling conditions were as follows:

Table 2.6.7. Cycling conditions for the *C. lanienae* PCR.

Initial denaturation	94°C	5 minutes
	94°C	1 minute
30 cycles	58°C	1 minute
	72°C	1 minute
Final elongation	72°C	7 minutes

2.6.8. *Campylobacter* 16S rRNA PCR for the detection of *C. lari* (Linton *et al.* 1996.

On occasion, some isolates produced faint bands at around 250 bp on the *Campylobacter* colony multiplex PCR (Wang *et al.*, 2002) that were not reliable enough to be able to assign to species. These suspected *C. lari* isolates were tested using the 16S rRNA PCR of Linton *et al.* (1996) for the detection of *C. lari*, using the primers LL1 and LL2 at a concentration of 1mM each. Reactions were carried out in 25µl volumes, using ReddyMix PCR mix as described earlier. The cycling conditions were as follows:

Table 2.6.8. Cycling conditions for the *Campylobacter* 16S rRNA PCR for the detection of *C. lari.*

Initial denaturation	94°C	4 minutes
25 cycles	94°C	1 minute
	64°C	1 minute
	72°C	1 minute
Final elongation	72°C	7 minutes

As in the *Arcobacter* PCRs, a positive control template was used in every batch of reactions (one per 96-well plate). Control templates used were isolates *C. jejuni* (NC11168), *C. coli* (012), *C. lari* (017), *C. lanienae* (NC13004), *C. hyointestinalis* (NC11608) and *C. fetus subsp. fetus* (NC10842).

2.6.9. Further identification of *Arcobacter* species.

Any isolates identified as *Arcobacter* spp. by the *Arcobacter* genus-specific PCR, but unable to be assigned to species, were subject to *groEL* gene sequencing (Karenlampi *et al.*, 2004). The primers M13H60F and T7H60R were used at a concentration of 0.67mM each and identified using the online BLAST tool (www.ncbi.nlm.nih.gov/BLAST). For any isolates that could not be successfully identified using the *groEL* sequencing, sequencing of the 16S region was carried out using the universal 16S primers, 16sUF and 16sUR (N.J. Williams, personal communication). *groEL* sequencing was carried out in-house while 16S sequencing was carried out by GeneService, London, UK. Sequencing results were checked for sequencing artefacts using CHROMAS (Technylesium, Queensland, Australia) uploaded onto the BLAST website (www.ncbi.nlm.nih.gov/BLAST) and an online BLAST database search was performed. The closest match to each isolate (>99% identity) was noted. Isolates with 100% identity were assigned to species.

Chapter Three

An Evaluation of *Arcobacter* Isolation Methods.

3.1. Introduction.

Arcobacter spp. are Gram negative bacteria that differ from the closely related *Campylobacter* spp. in that they are able to grow below 30°C, the normal minimum growth temperature of *Campylobacter* spp., and down to temperatures as low as 15°C and in aerobic conditions. The genus *Arcobacter* currently contains seven species, of which four are considered to be emerging human foodborne pathogens. While *A. nitrofigilis*, *A. halophilus* and *Candidatus A. sulfidicus* are not associated with human or animal sources, *A. butzleri*, *A. skirrowii*, *A. cryaerophilus*, *A. cibarius* and *A. mytili* have all been isolated from potential sources for human infection, including meat and water, while *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have all been isolated from human faecal samples and directly associated with disease in humans (Vandenberg *et al.*, 2004; On *et al.*, 1995; Hsueh *et al.*, 1997; Vandamme *et al.*, 1992a; Wybo *et al.*, 2004; Woo *et al.*, 2001; Samie *et al.*, 2007; Houf and Stephan, 2007; Kielbauch *et al.*, 1991; Prouzet-Mauleon *et al.*, 2006; Tee *et al.*, 1988).

Arcobacter spp. have been reported to cause disease in cattle and were originally isolated from aborted bovine foetuses (Ellis *et al.*, 1977). However, it was not until 2000 that Wesley *et al.* (2000) reported the first isolation of *Arcobacter* from clinically healthy cattle. Since then, *Arcobacter* spp. have been isolated from healthy cattle in Belgium (Van Driessche *et al.*, 2005), Japan (Kabeya *et al.*, 2003), Turkey (Ongor *et al.*, 2004; Aydin *et al.*, 2007), the USA (Golla *et al.*, 2002; Wesley *et al.*, 2000), New Zealand (McFadden *et al.*, 2005) and from beef or beef products from Thailand (Vindigni *et al.*, 2007), Northern Ireland (Scullion *et al.*, 2006), Turkey (Ongor *et al.*, 2004; Aydin *et al.*, 2007), Australia (Rivas *et al.*, 2004), Japan (Kabeya *et al.*, 2003), Mexico (Villarruel-

Lopez *et al.*, 2003), Czech republic (Vytrasova *et al.*, 2003), USA (Golla *et al.*, 2002) and the Netherlands (De Boer *et al.*, 1996), showing that the organism can be present not only in diseased cattle, but also in healthy animals and their products, illustrating the importance of cattle and beef as potential sources of *Arcobacter* infection of humans.

A variety of methods have been employed for the isolation of *Arcobacter* spp. from faecal samples, ranging from modified techniques for the isolation of *Campylobacter* and *Leptospira*, to those involving media specially designed for the isolation of *Arcobacter*. The first reported isolation of an *Arcobacter* used the medium specially designed for the isolation of *Leptospira;* Ellinghausen-McCulloch-Johnson-Harris (EMJH) medium supplemented with 5-fluorouracil (Ellis *et al.* 1977). Atabay and Corry (1998) evaluated the use of *Arcobacter* broth (Oxoid, UK) with added CAT (cefoperazone, amphotericin, teicoplanin) supplement, and found it to be useful for the isolation of *Arcobacter* from frozen storage. Johnson and Murano (1999) developed JM broth and solid medium, which were determined to be the most sensitive, specific and efficient in a comparison of three *Arcobacter* isolation methods based on isolation from raw poultry (Scullion *et al.*, 2004) and Houf *et al.* (2001) developed an *Arcobacter*-specific isolation method involving the use of *Arcobacter* media with a supplement consisting of five antibiotics (cefoperazone, trimethoprim, amphotericin, novobiocin and 5-fluorouracil). This method has been used in a number of studies on *Arcobacter* prevalence, and appears to be the most commonly used method in the literature. Other isolation methods used have been based on the use of EMJH p-80 and brucella broth (Ongor *et al.*, 2004).

Despite the range of isolation methods used previously, no single standard method for the isolation of *Arcobacter* spp. from faecal samples has yet been established, leaving a need for one to be developed. The lack of a standard method means that comparing and validating data from different studies is difficult. Some methods may be biased towards the isolation of strains which are resistant to the antibiotic supplements used in the media, for example, making results between studies using different techniques non-comparable. It has been suggested that a lack of a standard *Arcobacter* isolation method means that many human cases go undetected, and that the application of such a method could lead to the potential discovery of more human infections than are currently observed (Prouzet-Mauleon *et al.,* 2006), providing a more accurate representation of *Arcobacter* prevalence around the world and thus leading to improved efforts to control infection.

The aim of this study was to determine the most sensitive and specific method for the isolation of *Arcobacter* spp. from animal faecal samples. Secondly, the effect of freezing on *Arcobacter* in faecal material was investigated to determine whether it would be feasible to use faecal samples from past studies that had been kept in frozen storage.

3.2. Materials and Methods

3.2.1. Comparison of *Arcobacter* Isolation Methods

Sample Collection

Seventy seven faecal samples were collected from cattle, sheep and badgers on six farms in Cheshire and Lancashire and a wildlife park in Gloucestershire, UK. Four dairy cattle farms and two sheep farms were sampled,

along with the wildlife park which had a large population of badgers. Each location was sampled once with between six and twelve faecal samples being collected from each. Cattle samples were classified by management group (unweaned calves, weaned calves, dry adults and lactating adults) and three samples from each management group were obtained from each farm. On one occasion, weaned calves were not available and samples from very young stock (<3 months of age) were collected instead.

Table 3.1. Sample numbers and the corresponding type, farm and location of each.

Sample numbers	Sample type	Sampling location	Location
1 – 11	cattle	Farm1	Wirral
12 – 23	cattle	Farm 2	Cheshire
24 – 29	sheep	Sheep farm 1	Lancashire
30 – 41	cattle	Farm 3	Lancashire
42 – 53	cattle	Farm 4	Lancashire
54 - 65	sheep	Sheep farm 2	Wirral
66 - 77	badger	Wildlife park	Gloucester

Samples were processed within three hours of collection on all occasions, except the badger samples, which were processed immediately after being received by post.

Sample Processing

A total of five isolation methods were compared in this study, using three separate enrichment broths and three different solid media in combination, all based on methods from published studies. In short, method HH was based on the method of Houf *et al.* (2001) and used *Arcobacter* broth and solid medium (based on the *Arcobacter* media of Oxoid, UK) with an added five-antibiotic supplement. Method HCC used the same broth but with cefoperazone charcoal desoxycholate (CCDA) agar plates plus CA supplement. Method ACCC used *Arcobacter* broth with added CA supplement and the aforementioned CCDA+CA plates. Method ACH used the *Arcobacter* and CA broth with Houf solid medium, and Method CC was a *Campylobacter*-specific method using *Campylobacter* enrichment broth and CCDA with CA solid medium. A more detailed description of each method and the incubation and growth conditions can be found in Chapter Two, General Materials and Methods, section 2.2.1. Ten separate colonies were taken from the each of the five solid specific media in order to ensure all present bacterial strains were represented, giving a potential maximum of fifty isolates per sample collected.

The sensitivity and specificity of each of the five methods was calculated and the results compared in order to identify the best isolation method.

3.2.2. The effect of freezing on the viability of *Arcobacter.*

Immediately after inoculation of 1g of each faecal sample into enrichment broth, the remainder of each faecal sample was frozen at -80°C. The samples were then removed one week later, defrosted and subjected again to the same five isolation methods after thawing. The resulting isolates were then tested

using the *Arcobacter* genus-specific PCR (Gonzalez *et al.* 2000) to determine the overall numbers of *Arcobacter* isolated after freezing.

3.3. Results

3.3.1. Comparison of *Arcobacter* isolation methods

In total, 1266 isolates were recovered from the 77 animal faecal samples using all five isolation methods. Of these, 478 (38%) were identified as belonging to the genus *Arcobacter* and then assigned to species. Of the remainder, 231 were identified as the genus *Campylobacter* and assigned to species, and the remainder (557) were unidentified. Table 3.2. shows the number of animals positive for each species on each of the farms sampled, using each of the five isolation methods.

Table 3.2. The number of animals on each farm carrying *Arcobacter* or *Campylobacter spp.*, detected using five isolation methods. Only three species of *Arcobacter* were detected.

	HH[1] n=30			
Location	*A. butzleri n=12*	*A. skirrowii n=7*	*A. cryaerophilus n=4*	*Campylobacter spp. n=7*
farm 1	3	3	1	1
farm 2	1	0	0	0
farm 3	4	1	1	0
farm 4	2	3	1	0
sheep	2	0	1	0
badger	0	0	0	6
	HCC[2] n=50			
	A. butzleri n=17	*A. skirrowii n=17*	*A. cryaerophilus n=8*	*Campylobacter spp. n=8*
farm 1	3	3	1	2
farm 2	3	0	1	2
farm 3	5	6	5	1
farm 4	4	8	1	0
sheep	2	0	0	2
badger	0	0	0	1
	ACH[3] n=33			
	A. butzleri n=8	*A. skirrowii n=11*	*A. cryaerophilus n=1*	*Campylobacter spp. n=13*
farm 1	2	5	0	0
farm 2	1	0	0	0
farm 3	3	2	0	0
farm 4	1	2	0	0
sheep	1	2	1	5
badger	0	0	0	8
	ACCC[4] n=43			
	A. butzleri n=10	*A. skirrowii n=12*	*A. cryaerophilus n=2*	*Campylobacter spp. n=19*
farm 1	3	4	0	2
farm 2	1	0	0	0
farm 3	5	5	2	3
farm 4	1	1	0	4
sheep	0	2	0	5
badger	0	0	0	5
	CC[5] n=48			
	A. butzleri n=6	*A. skirrowii n=13*	*A. cryaerophilus n=9*	*Campylobacter spp. n=19*
farm 1	0	2	5	0
farm 2	0	1	2	2
farm 3	3	7	1	8
farm 4	2	2	0	0
sheep	1	1	1	9
badger	0	0	0	0

[1] **HH based on the method of Houf *et al.* (2001).**

[2] **HCC based on the methods of Houf *et al.* (2001) and Kemp *et al.* (2005)**

[3] **ACH based on the methods of Atabay and Corry (1998) and Houf *et al* (2001)**

[4] **ACCC based on the methods of Atabay and Corry (1998) and Kemp *et al.* (2005)**

[5] **CC based on the method of Kemp *et al.* (2005).**

The five isolation methods HH, HCC, ACH, ACCC and CC were compared for sensitivity and specificity using all samples where *Arcobacter* was isolated, irrespective of sample type or management group. Table 3.3. shows the sensitivity and specificity of each method. Sensitivity was calculated as the number of samples shown to be positive for *Arcobacter* using a specific method, as a percentage of the number of samples shown to be positive overall, by any method. HCC was shown to have the greatest sensitivity (70.7%) of the five methods tested. Specificity was calculated as the percentage of isolates obtained using a single method that was identified as *Arcobacter* spp. HCC was found also to be the most specific of the five methods tested, with a specificity of 64.1%.

Table 3.3. The sensitivity and specificity of each isolation method tested.

Method	*Arcobacter* positive samples (of a total of 77)	*Arcobacter* sp. Isolates	*Campylobacter* sp. and non-campylobacteraceae isolates	Sensitivity (%)	Specificity (%)
HH	17	94	63	41.5	59.9
ACH	18	63	199	43.9	24.0
ACCC	18	59	290	43.9	16.9
HCC	29	177	99	70.7	64.1
CC	18	96	111	43.9	46.4

As shown in Table 3.3., the sensitivities of the other four methods were much lower. In terms of specificity, HH was of a similar level to HCC, with a specificity of 59.9%. The other three methods were much less specific, at 46.4% (CC), 24.0% (ACH) and 16.9% (ACCC). The most frequently isolated *Arcobacter* species was *A. skirrowii*, which constituted 47% of all *Arcobacter*s recovered. This was followed by *A. butzleri* at 41%, then *A. cryaerophilus* at

12%. However, this varied according to each method, as illustrated in Figure 3.1.

The largest proportion of *Arcobacter*s was retrieved using HCC. Of all the isolates obtained using this method, 62% were *Arcobacter* spp. Of these, almost equal proportions were obtained of *A. skirrowii* (29%) and *A. butzleri* (26%), along with 6% *A. cryaerophilus*. It would be recommended, therefore, that for any study requiring isolation of *A. butzleri, A. skirrowii* and *A. cryaerophilus* at levels representative of the true population, the method HCC should be used.

Over all methods, the most frequently isolated *Arcobacter* spp. was *A. skirrowii*, forming 17.8% of the total isolates obtained. *A. butzleri* formed 15.5% of all isolates and *A. cryaerophilus* formed 4.5%. *Campylobacter* spp. accounted for 18.2% of all isolates, while 44.0% were unidentified or 'other'. After *groEL* gene sequencing these were found to include *Pseudomonas, Psychrobacter, Acinetobacter, Saccharophagus* species and *Escherichia coli*.

The most common *Campylobacter* spp. isolated in the study was *C. jejuni* (126 isolates) but *C. coli, C. lari, C. hyointestinalis, C. fetus* and *C. lanienae* were also isolated. Figure 3.1. shows the proportions of species isolated using each method.

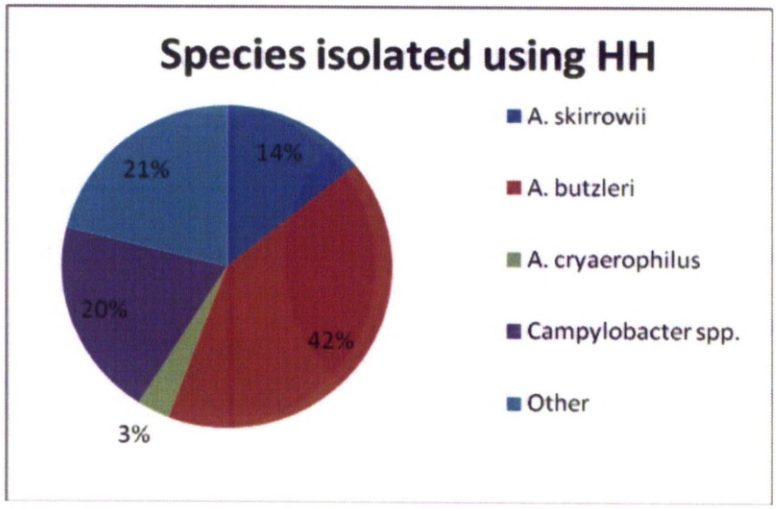

Figure 3.1a. Species proportions isolated using the HH method.

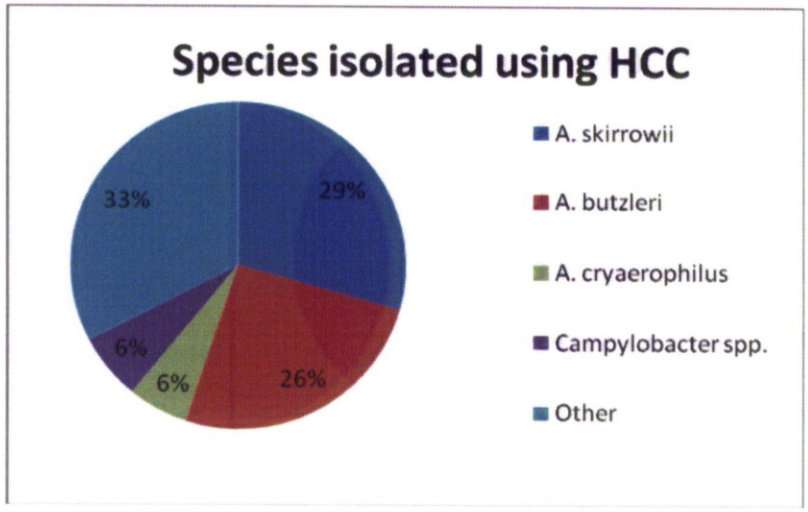

Figure 3.1b. Species proportions isolated using the HCC method.

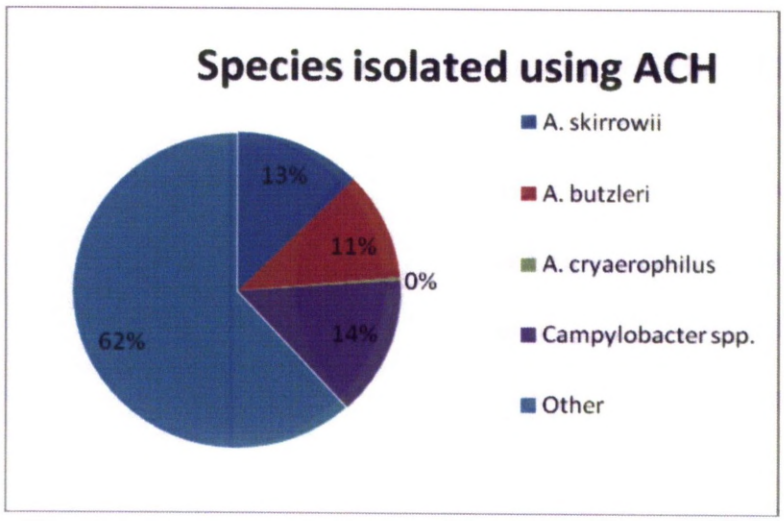

Figure 3.1c. Species proportions isolated using the ACH method.

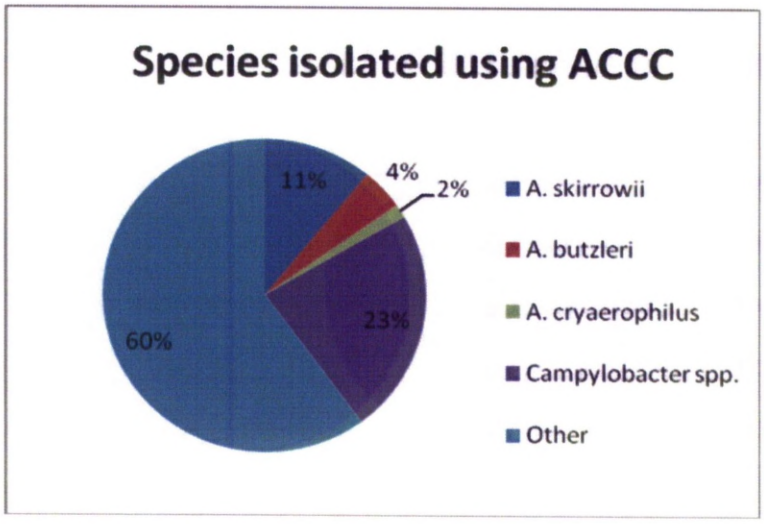

Figure 3.1d. Species proportions isolated using the ACCC method.

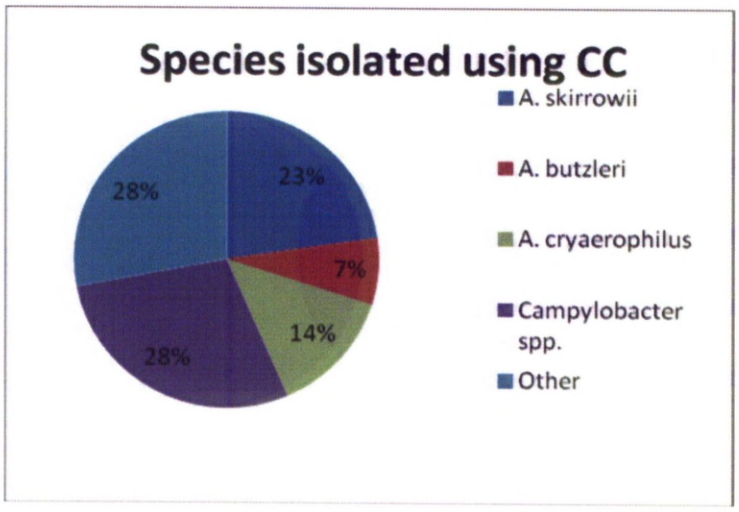

Figure 3.1e. Species proportions isolated using the CC method.

Of the four dairy cattle management groups sampled during this study, the younger groups of cattle were found to have a higher prevalence of *Arcobacter* species using these methods. Weaned calves had an overall prevalence of 84.5%, while unweaned calves followed with a prevalence of 83.4%. Of the adults, lactating cows had a prevalence of 68.7%, while dry adults had the lowest overall prevalence, with a value of 48.9%.

3.3.2. Investigation into the effect of freezing.

750 isolates were recovered from the same faecal samples using the same five isolation methods, after the samples had been frozen at -80°C for one week. Of these, 149 (19%) were identified as belonging to the *Arcobacter* genus using PCR (Gonzales *et al.* 2000). The isolates were not assigned to species. There was a 61% reduction in the number of isolates recovered from the frozen samples, when compared to fresh samples.

Of the 41 *Arcobacter*-positive samples identified using fresh faecal samples, 22 remained positive for at least one *Arobacter* isolate after freezing,

while *Arcobacter* spp. were not recovered from the remaining 19 previously positive faecal samples. *Arcobacter* spp. were recovered from three additional faecal samples after freezing, where no *Arcobacters* had been recovered when fresh.

3.4. Discussion.

The main aim of this study was to compare five methods for the isolation of *Arcobacter* from animal faeces in order to select a method for subsequent use as a standard *Arcobacter* isolation method. Such a method would have to be highly sensitive and specific, whilst effectively isolating *Arcobacter* species from faecal samples. This study compared five *Arcobacter* isolation methods based on published methods (from Houf *et al.,* 2001, Kemp *et al.,* 2005, Atabay and Corry, 1998) and found that enrichment H broth, developed by Houf *et al.* (2001), and plating onto CCDA with CAT plate medium (CC plates) incubated aerobically at 30°C was the most sensitive and specific method of the five tested (Table 3.3.).

Using all methods, it was possible to isolate *A. butzleri, A. skirrowii* and *A. cryaerophilus*, although in different proportions, as illustrated in Figures 3.1a-e. Differences in the frequency of isolation of particular species with different methods is most likely due to the varying sensitivities of the species to the antibiotic supplements used in the media, or contaminants. *A. skirrowii* was the most frequently isolated *Arcobacter* overall, followed by *A. butzleri* then *A. cryaerophilus*, and Method HCC appears to be more representative of these overall results than the next most specific method, HH. This supports the

conclusion that HCC is the best method of those tested for specific *Arcobacter* isolation from faecal samples.

A. butzleri, *A. skirrowii* and *A. cryaerophilus* were all isolated from cattle in this study, whereas *A. butzleri* was the only *Arcobacter* species isolated from sheep (n = 4). No *Arcobacter* spp. were isolated from badger faeces. This is the first study to report the three species from cattle in the UK. A small number of isolates were identified as *Arcobacter* using the genus-specific PCR of Gonzalez et al. (2000), but could not be assigned to species using the multiplex PCR (Houf *et al.* 2000). It was considered that these isolates were *A. cibarius* (Houf *et al.*, 2005), for which there are currently no PCR primers available. However, after *groEL* gene sequencing and subsequent BLAST analysis it was determined that none of the isolates were *A. cibarius*. The isolates identified this way included further *A. butzleri* and *A. skirrowii* isolates, as well as *Pseudomonas* sp., *Acinetobacter* sp., *Escherichia coli* and a *Saccharophagus* sp.; likely to have been mistakenly isolated from the original faecal samples.

Studies outside the UK generally report a low *Arcobacter* prevalence in sheep. Aydin *et al.* (2007) found no *Arcobacter* spp. in 68 sheep faecal samples, although a small number of isolates from sheep faeces and meat have been reported (On *et al.*, 2004; Vandamme *et al.*, 1992b; Rivas *et al.*, 2004). To the author's knowledge, this is the first report of *Arcobacter* in sheep in the UK. *Arcobacter* have been found in wildlife samples in the UK, including wood mice, rabbits, badgers and birds (Jones *et al.*, poster presentation, 14th International Workshop for Campylobacter, Helicobacter and Related Organisms, 2007) although none were recovered from badger samples in this study. The badger samples used in this study were dry in consistency and had spent up to seven

days in the postal system. These factors may have caused the lack of *Arcobacter* recovered from the samples.

A number of the isolates obtained in the study remained unidentified. One hundred of these were Gram stained and examined microscopically for the morphological characteristics of the *Campylobacteraceae*, which are Gram-negative, curved or spiral rods. All of the 100 isolates examined lacked the typical morphology associated with *Campylobacter*, and so no further analysis of these unidentified isolates was carried out. It is assumed that non-*Campylobacter*-like colonies had been selected during the isolation process. Ten single colonies were selected from each sample on solid media. As *Arcobacter* and *Campylobacter* colonies are quite small and often difficult to identify by eye, it is probable that some of the colonies picked were neither *Arcobacter* nor *Campylobacter* species, leading to the presence of the unidentified isolates.

Finally, all 77 faecal samples were frozen at -80°C or one week, then thawed and the isolation processes repeated. A total of 149 *Arcobacter*s were obtained from a total of 750 isolates. This is considerably lower than the 469 *Arcobacter*s from a total of 1266 isolates obtained from the samples before freezing. Hence for optimal isolation of *Arcobacter* spp. from faecal samples, the samples used must be fresh and not frozen, and the use of stock frozen samples is not recommended.

While this study has determined the best method of *Arcobacter* isolation from cattle faecal samples out of a total of five methods, a great many more remain in use throughout the *Arcobacter* research community. Ideally a comparison of a larger number of methods should be carried out in order to truly determine the most sensitive and specific method for future use.

Of all the known sources of *Arcobacter*, cattle are a group in which the presence of this genus is less commonly studied. A comparison of methods that takes into account *Arcobacter* recovery from different animals and different sources (e.g. water), which are more commonly studied may be advantageous.

Chapter Four

An investigation into factors affecting *Arcobacter* prevalence in cattle using a series of cross-sectional studies.

4.1. Introduction.

Prevalences of *Arcobacter* spp. in cattle of up to 37% have been found in Turkey (Aydin *et al.*, 2007), up to 39% in Belgium (Van Driessche *et al.* 2003), and higher prevalences found in the UK (Chapter Three, section 3.3.), with prevalences of *Campylobacter* spp. (up to 51%) being reported from dairy cattle in the UK by Kwan *et al.* (2008).

A number of studies have reported an apparent seasonal prevalence of *Campylobacter* spp. in dairy cattle in the UK (Skirrow, 1991; Stanley *et al.*, 1998a; Kwan *et al.*, 2008), with peaks occurring in spring and autumn. Similarly, Meanger and Marshall (1989) found a peak in *C. jejuni* and *C. coli* in autumn, compared to summer and winter, while Kwan *et al.* (2008) and Grove-White *et al.* (2009) found summer peaks of *C. jejuni* prevalence in dairy cattle in the north west UK. The prevalence of *Arcobacter* spp. was found to increase as levels of *Campylobacter* spp. decreased (D. Grove-white, personal communication) in a similar study, a pattern that has not, to the author's knowledge, been investigated to date.

Cattle in the UK are kept both indoors and outdoors, with many herds being housed indoors for the colder winter months (October to April, approximately), and being moved outside to pasture once the weather improves (April/May). Cattle as carriers of *Arcobacter* spp. are well documented worldwide (Villaruel-lopes *et al.* 2003; Kabeya *et al.* 2004; Rivas *et al.* 2004; Scullion *et al.* 2006; Aydin *et al.* 2007), however, very little published data exists on *Arcobacter* prevalence in cattle in the UK.

The aim of this study was to investigate the prevalence of *Arcobacter* and *Campylobacter* species in faecal pats from dairy and beef cattle on four farms.

In order to investigate whether any peaks in seasonal prevalence occur in *Arcobacter* spp. and whether the prevalence of *Arcobacter* is affected by cattle management, serial cross-sectional studies were undertaken.

4.2. Materials and Methods.

4.2.1.Cross-Sectional Studies

A series of cross-sectional studies were carried out on five occasions (named A to E) between December 2007 and November 2008. Four farms were selected in Cheshire, comprising two dairy and two beef farms. Farms 1 and 4 were beef farms that supply beef to a national supermarket and a local butcher, respectively. Farms 2 and 3 were dairy farms, both of which supply their raw milk to larger companies for processing and distribution. Cattle on Farms 3 and 4 were housed indoors all year round. Farms 1 and 2 housed cattle indoors during the winter and outdoors at pasture during the summer. Both beef farms purchased young animals on a regular basis. Each farm was sampled once every three months, with up to fifty cattle faecal pats being sampled on each occasion. Samples were collected from different management groups, in Table 4.1.

Table 4.1. The target numbers of samples collected from each management group on each farm.

Dairy farms	dry cattle	lactating cattle	unweaned calves.	weaned calves
Beef farms	fattening cattle	heifers/young stock	calves	
Number sampled	20	20	5	5

Samples of freshly voided faecal pats (approximately 5g) were collected from up to forty adult cattle and ten calves on each farm. The housing status (indoors or outdoors) of each animal was recorded. *Arcobacter* spp. and *Campylobacter* spp. were isolated using the HCC and CC isolation methods respectively (Chapter Two, Sections 2.1.1. and 2.2.2.) and identified using PCR (Chapter Two, Section 2.6.). Four colonies were selected from each plate based on typical morphology in order to obtain diverse strains from each faecal pat. Any isolates subsequently identified as *Arcobacter* spp., isolated using the CC method were discarded, as were any *Campylobacter* spp. isolated using HCC, in order to avoid possible bias created by either method isolating the wrong species.

The sampling sessions took place at three-month intervals, over a period of one year, as follows:

Session A: November and December 2007 (Winter)

Session B: February 2008 (Winter)

Session C: May 2008 (Summer)

Session D: July and August 2008 (Summer)

Session E: October 2008 (Winter).

Winter was defined as 1st October – 31st March, and Summer was defined at 1st April – 30th September.

4.2.2.Statistical Analyses.

Univariate analysis

Robust univariate analysis was carried out using STATA version 9 (StataCorp., Texas, USA). The effects of season, sampling environment (indoors or outdoors), farm type (beef vs. dairy), and age group (young vs. adult) were

tested, as well as the effects of sampling environment and age group on each farm. The six management groups recorded during sampling were recoded into two variables of age group. On dairy farms, weaned and unweaned calves were recoded as "young", and dry and lactating adults were recoded as "adult". On beef farms, calves and young stock (up to five months of age) were recoded as "young" and fattening animals and heifers were recoded as "adult". Robust standard errors (Huber, 1981) were calculated to allow for farm-level clustering. A probability value of less than 0.05 was taken to indicate significance.

Multivariate Logistic Regression Modelling

Multivariable logistic regression models, with farm specified as a fixed effect, were fitted, with the binary outcome variable being the presence or absence of *Arcobacter* spp. in a faecal sample. A backward stepwise model building strategy (Kirkwood and Sterne, 2003) was employed to determine which variables should be included in the final model. Interactions between variables in the final model were observed, and variables were retained only if they improved the model fit. Variables considered to improve model fit were defined as those producing a probability (P) value of less than 0.2. Time was included in the model as four sine and cosine functions (i.e. harmonic regression) in order to allow modelling of possible seasonality (Stolwijk *et al.,* 1999). Four time covariates, x_1, x_2, x_3 and x_4 were generated as follows: x_1 $\cos(2\pi t/52)$, x_2 $\sin(2\pi t/52)$, x_3 $\cos(4\pi t/52)$, x_4 $\sin(4\pi t/52)$, where t = sampling date. Similarly, models were also fitted for each individual *Arcobacter* species, namely *A. butzleri, A. skirrowii* and *A. cryaerophilus.*

4.3. Results.

4.3.1. General results.

In total, 792 faecal samples were collected, and 1628 isolates were obtained, of which 1218 were identified as *Arcobacter* spp. and 406 were identified as *Campylobacter* spp. A total of 337 (42.5%) faecal samples were positive for at least one species of *Arcobacter,* and 120 (15.1%) animals tested positive for *Campylobacter* spp. Figure 4.1. shows the proportion of each *Arcobacter* species isolated. Total numbers of *Arcobacter*- and *Campylobacter*-positive pats and prevalences during the five cross sectional studies (covering a period of eleven months) are shown in Table 4.2.

Table 4.2. The numbers of *Arcobacter* and *Campylobacter* positive faecal samples in each session.

Date	Session	Total faecal samples	*Arcobacter* positive n (%)	*Campylobacter* positive n (%)
November/ December 2007	A	160	62 (39)	20 (13)
February 2008	B	158	17 (11)	40 (25)
May 2008	C	155	70 (45)	31 (20)
July/August 2008	D	180	113 (63)	23 (13)
October 2008	E	139	75 (54)	6 (4)
Mean			**67 (42)**	**24 (15)**

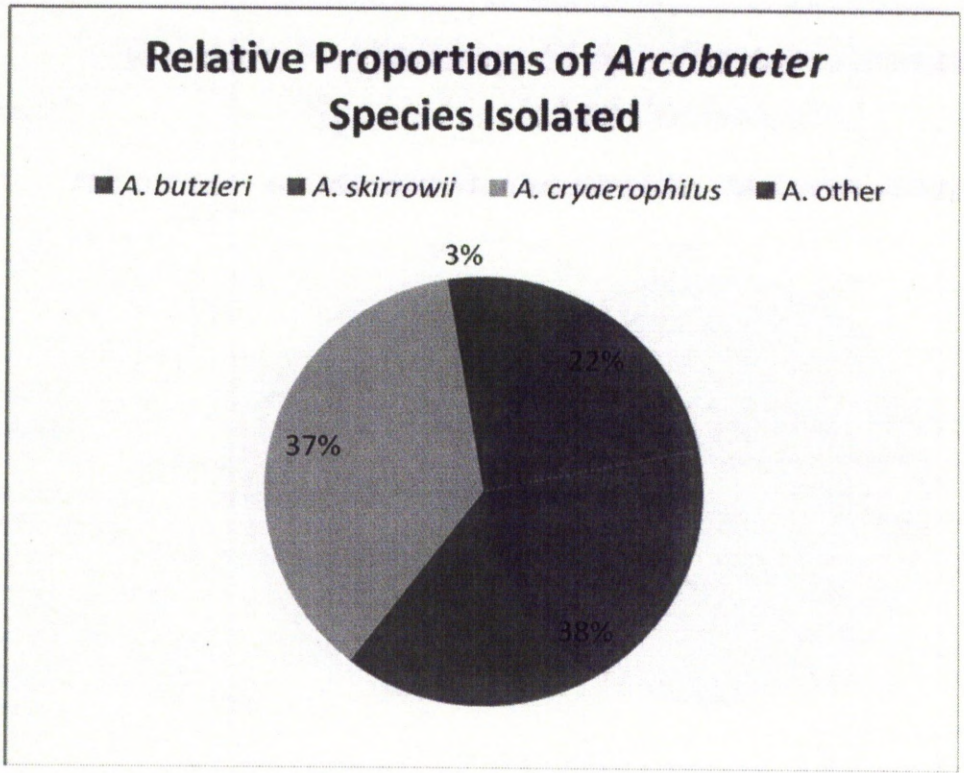

Figure 4.1. Proportion of each *Arcobacter* species recovered from *Arcobacter*-positive faecal samples.

Nine faecal samples (1.1%) contained all three *Arcobacter* species, a further fifteen (1.9%) with *A. butzleri* and *A. skirrowii*, twenty-three (2.9%) with *A. butzleri* and *A. cryaerophilus*, and sixty-nine (8.7%) with *A. cryaerophilus* and *A. skirrowii*. Table 3 shows the total numbers of each species isolated from the 792 faecal samples.

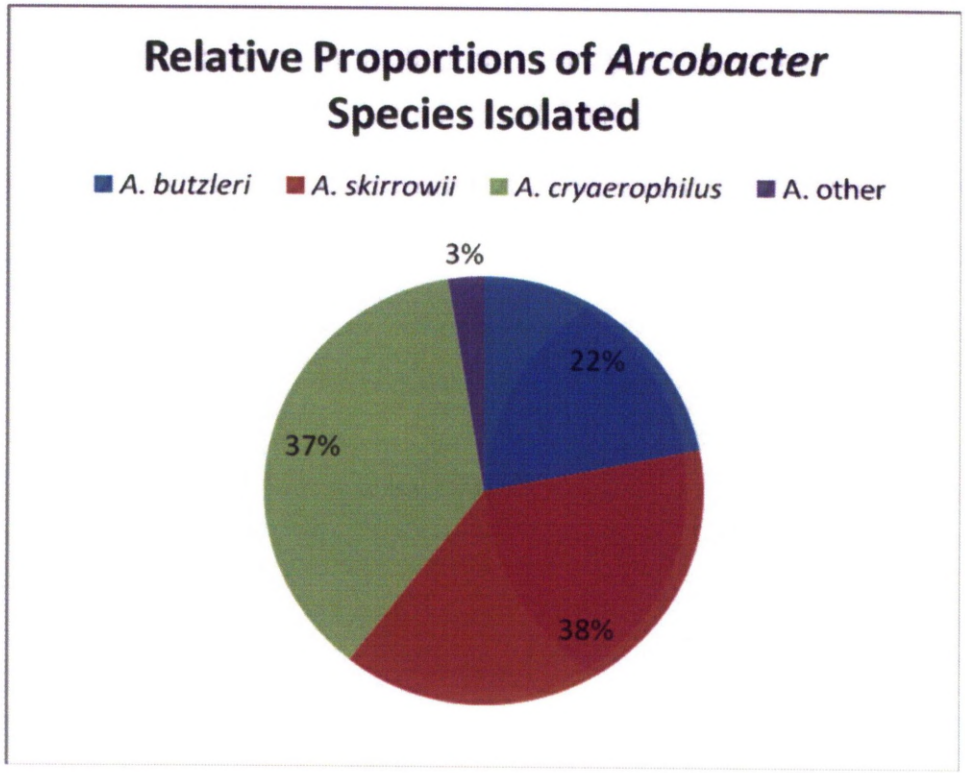

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Table 4.3. Numbers of *Arcobacter* and *Campylobacter* species isolated.

Species	Number of Isolates
A. butzleri	265
A. skirrowii	471
A. cryaerophilus	449
Unidentified *Arcobacter* species	34
C. jejuni	217
C. coli	24
Campylobacter lari	2
Campylobacter hyointestinalis	15
Campylobacter fetus	52
Campylobacter lanienae	9
Unidentified *Campylobacter species*	87

The 34 isolates referred to here as "Unidentified *Arcobacter* species" were *Arcobacter* species which had tested positive in the genus-specific PCR of Gonzalez *et al.* (2000), but were unidentifiable using the species-specific PCR of Houf *et al.* (2001). For identification, isolates were subjected to PCR amplification and sequencing of a fragment of the *groEL* gene (Karenlampi *et al,* 2004), but amplicons could not be obtained for any of the isolates. The isolates were then subjected to 16S rRNA sequencing (N.J. Williams, personal communication: Appendix I) and analysed using an online BLAST search. The isolates were all confirmed as belonging to the genus *Arcobacter*, but species could not be assigned.

4.3.2. Statistical Analyses

Univariate Analysis

The effect of season on *Arcobacter* and *Campylobacter* prevalence was investigated using STATA version 10, Tables 4.4. to 4.7.

Table 4.4. Results of univariate analysis using robust standard errors on four beef and dairy farms in Cheshire, UK, using the presence or absence of *Arcobacter* spp as the binary outcome. * indicates a statistically significant result as p = <0.05.

Variable	Proportion (95% CI)	Probability
Season		
Winter	54.6% (13.9 – 95.3)	0.1466
Summer	66.3% (46.5 – 86.0)	
Sampling Environment		
Inside	43.2% (15.2 – 71.2)	0.5591
Outside	61.0% (17.6 – 104.3)	
Farm Type		
Beef	47.3% (137.2 – 231.9)	0.1783
Dairy	32.6% (10.8 – 54.3)	
Age Group		
Adult	46.5% (26.1 – 66.9)	0.5293
Young	64.2% (12.9 – 115.5)	
Age Group on Dairy Farms		
Adult	38.3% (32.8 – 43.9)	<0.001*
Young	85.4% (78.2 – 92.6)	
Age Group on Beef Farms		
Adult	58.6% (51.8 – 65.5)	0.015*
Young	53.6% (46.5 – 60.8)	

Table 4.5. Results of univariate analysis using robust standard errors on four beef and dairy farms in Cheshire, UK, using the presence or absence of *A. butzleri* as the binary outcome. No significance was observed.

Variable	Proportion (95% CI)	P value
Season		
Winter	88.5% (0.82 – 0.95)	0.4526
Summer	18.2% (-0.86 – 0.45)	
Sampling Environment		
Inside	13.2% (0.08 – 0.26)	0.0939
Outside	79.7% (18.4 – 1.41)	
Farm Type		
Beef	81.0% (0.13 – 1.49)	0.1303
Dairy	9.5% (-0.26 – 0.45)	
Age Group		
Adult	16.5% (0.06 – 0.27)	0.4663
Young	89.6% (0.69 – 1.10)	
Age Group on Dairy Farms		
Adult	12.0 % (0.48 – 1.28)	0.0639
Young	97.9% (0.96 – 1.00)	
Age Group on Beef Farms		
Adult	23.2% (0.10 – 0.37)	0.6580
Young	85.4% (-0.57 – 2.28)	

Table 4.6. Results of univariate analysis using robust standard errors on four beef and dairy farms in Cheshire, UK, using the presence or absence of *A. skirrowii* as the binary outcome. * The only significant variable was sampling environment.

Variable	Proportion (95% CI)	P value
Season		
Winter	80.4% (0.62 – 0.99)	0.3621
Summer	28.7% (0.49 – 0.52)	
Sampling Environment		
Inside	26.9% (0.15 – 0.39)	0.0041*
Outside	95.9% (87.5 – 1.04)	
Farm Type		
Beef	71.3% (-0.14 – 1.56)	0.2918
Dairy	18.2% (-0.70 – 1.06)	
Age Group		
Adult	23.7% (0.03 – 0.44)	0.9449
Young	77.1% (0.53 – 1.01)	
Age Group on Dairy Farms		
Adult	20.0% (-1.30 – 1.71)	0.7571
Young	87.5% (-0.51 – 1.80)	
Age Group on Beef Farms		
Adult	29.0% (0.07 – 0.51)	0.9416
Young	71.9% (-0.78 – 2.22)	

Table 4.7. Results of univariate analysis using robust standard errors on four beef and dairy farms in Cheshire, UK, using the presence or absence of *A. cryaerophilus* as the binary outcome. No significance was observed.

Variable	Proportion (95% CI)	P value
Season		
Winter	83.3% (0.62 – 1.04)	0.1346
Summer	26.6% (0.12 – 0.41)	
Sampling Environment		
Inside	19.8% (-0.29 – 0.42)	0.5161
Outside	73.2% (0.38 – 1.08)	
Farm Type		
Beef	75.7% (-0.92 – 2.43)	0.5269
Dairy	17.4% (-0.01 – 0.36)	
Age Group		
Adult	23.5% (0.65 – 0.88)	0.4479
Young	83.7% (0.53 – 1.14)	
Age Group on Dairy Farms		
Adult	20.0% (0.10 – 0.30)	0.1145
Young	90.6% (0.68 – 1.14)	
Age Group on Beef Farms		
Adult	28.6% (-0.75 – 1.32)	0.6140
Young	80.2% (-1.42 – 3.02)	

Multivariate Logistic Regression Modelling

The final models are presented in Tables 4.8. to 4.12. Farm identity was included as a fixed effect. Farm type was not included in the models due to co-linearity (a high level of correlation) with farm identity.

Table 4.8. Results of logistic regression modelling including covariates associated with the probability of isolating *Arcobacter* spp. from cattle faeces on farms in Cheshire. All variables were found to have a significant effect.

Covariate	Odds Ratio	P Value (95% CI)
Farm 2	0.23	<0.001 (0.13 – 0.39)
Farm 3	0.16	<0.001 (0.09 – 0.27)
Farm 4	0.26	<0.001 (0.16 – 0.42)
Age group	2.46	<0.001 (1.68 – 3.61)
Sampling Environment	2.21	0.012 (1.19 – 4.11)
Tcos2	0.40	<0.001 (0.31 – 0.52)
Tsin2	0.32	<0.001 (0.24 – 0.43)
Tsin4	0.74	0.003 (0.60 – 0.90)
Tcos4	1.21	0.321 (0.83 – 1.78)

Farm identity was a fixed effect with Farm 1 as baseline. There remains considerable variation associated with farm identity. Age group was a fixed effect with "adult" as baseline. There remains an effect of age group. Sampling environment was a fixed effect with "indoors" as baseline. Harmonic regression analysis showed a significant effect of time, Figure 4.2.

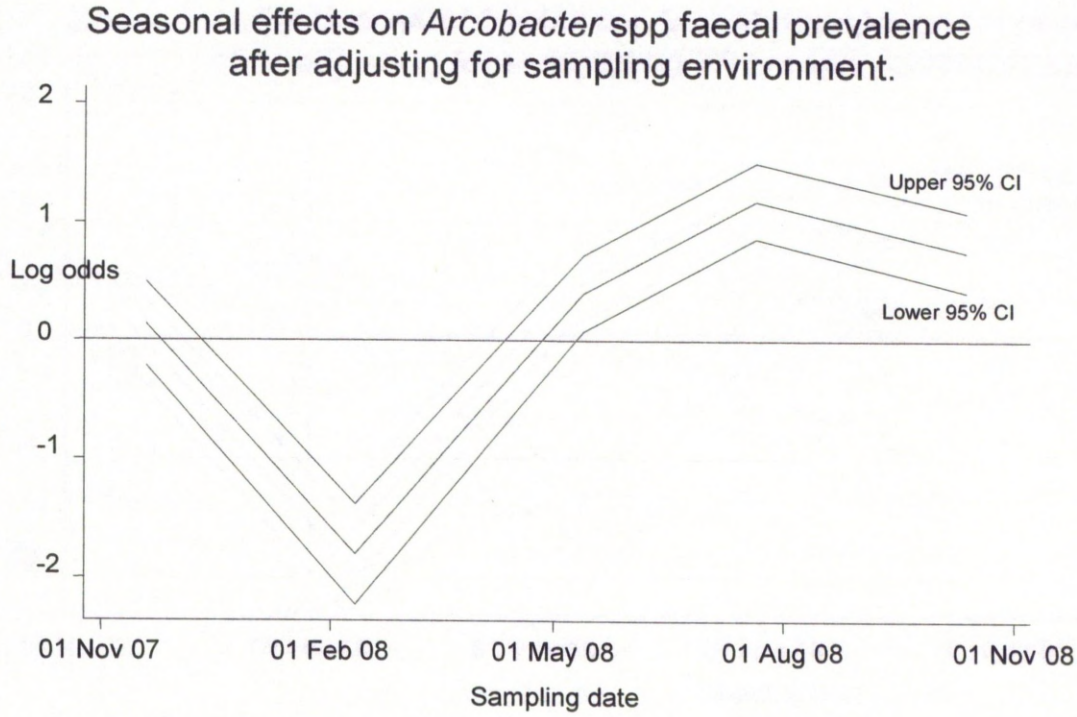

Figure 4.2. Seasonal effects on *Arcobacter* spp. recovery from faecal samples after adjusting for sampling environment, age group and farm.

The outcome of the logistic regression model using the presence or absence of *A. butzleri* as the binary outcome is shown in Table 4.9.

Table 4.9. Results of Logistic regression modelling including covariates associated with the probability of isolating *Arcobacter butzleri* from cattle faeces on farms in Cheshire. All variables were found to have a significant effect except Tcos2 and Tsin4.

Covariate	Odds Ratio	P Value (95% CI)
Farm 2	0.25	<0.001 (0.12 – 0.55)
Farm 3	0.19	<0.001 (0.10 – 0.40)
Farm 4	0.57	0.043 (0.34 – 0.98)
Sampling Environment	0.55	0.150 (0.24 – 1.24)
Age Group	1.92	0.010 (1.17 – 3.18)
Tcos2	0.83	0.272 (0.60 – 1.16)
Tsin2	0.54	0.001 (0.37 – 0.78)
Tsin4	1.04	0.806 (0.79 – 1.36)
Tcos4	2.14	0.005 (1.26 – 3.63)

Farm identity was a fixed effect with Farm 1 as baseline. There remains considerable variation associated with farm identity. Sampling environment was a fixed effect with "indoors" as baseline. There remains an effect of sampling environment. Age group was a fixed effect with "adult" as baseline. There remains an effect of age group. Harmonic regression analysis of time showed a significant effect of time, represented in Figure 4.3.

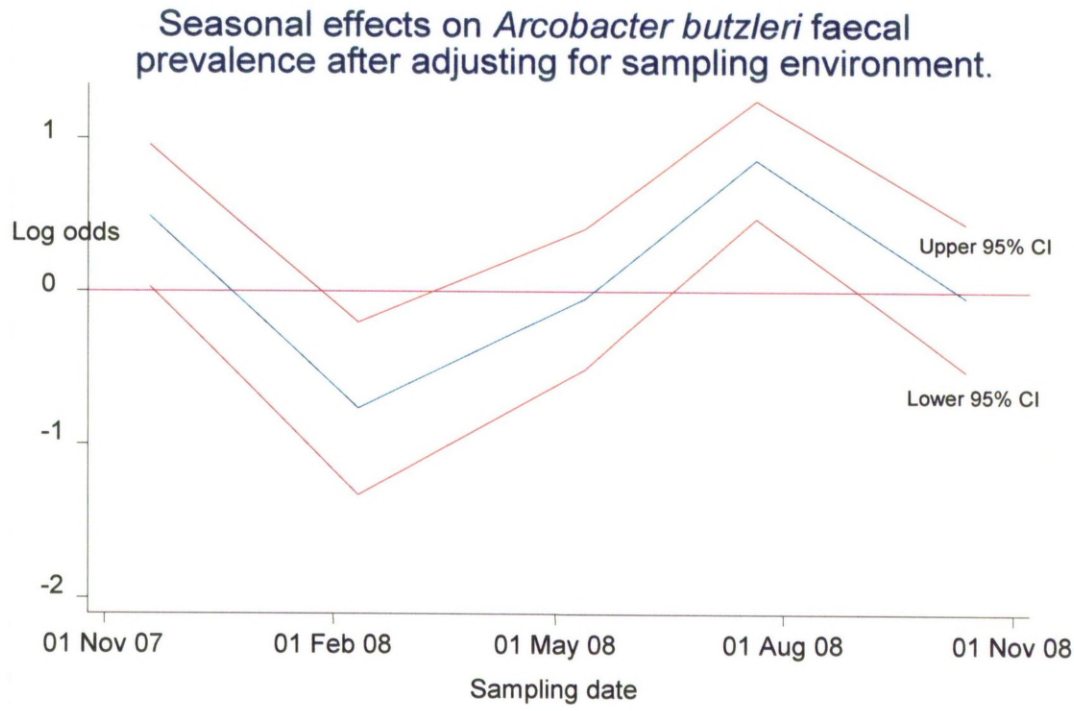

Figure 4.3. Seasonal effects on *A. butzleri* recovery from faecal samples after adjusting for sampling environment, age group and farm.

The outcome of the logistic regression model using presence or absence of *A. skirrowii* as the binary outcome is shown in Table 4.10.

Table 4.10. Results of Logistic regression modelling including covariates associated with the probability of isolating *Arcobacter skirrowii* from cattle faeces on farms in Cheshire.

Covariate	Odds Ratio	P Value (95% CI)
Farm 2	0.86	0.638 (0.46 – 1.62)
Farm 3	0.53	0.011 (0.32 – 0.86)
Farm 4	0.49	0.003 (0.31 – 0.79)
Sampling environment	19.9	<0.001 (6.84 – 5.76)
Age Group	1.63	0.014 (1.10 – 2.39)
Tcos2	0.39	<0.001 (0.28 – 0.53)
Tsin2	0.35	<0.001 (0.25 – 0.49)
Tsin4	0.66	0.001 (0.51 – 0.85)
Tcos4	0.75	0.200 (0.49 – 1.16)

Farm identity was a fixed effect with Farm 1 as baseline. There remains considerable variation associated with farm identity. Sampling environment was a fixed effect with "indoors" as baseline. There remains a significant effect of sampling environment. Age group was a fixed effect with "adult" as baseline. There remains a significant effect of age group.

Harmonic regression analysis of time showed a significant effect of time, represented in Figure 4.4.

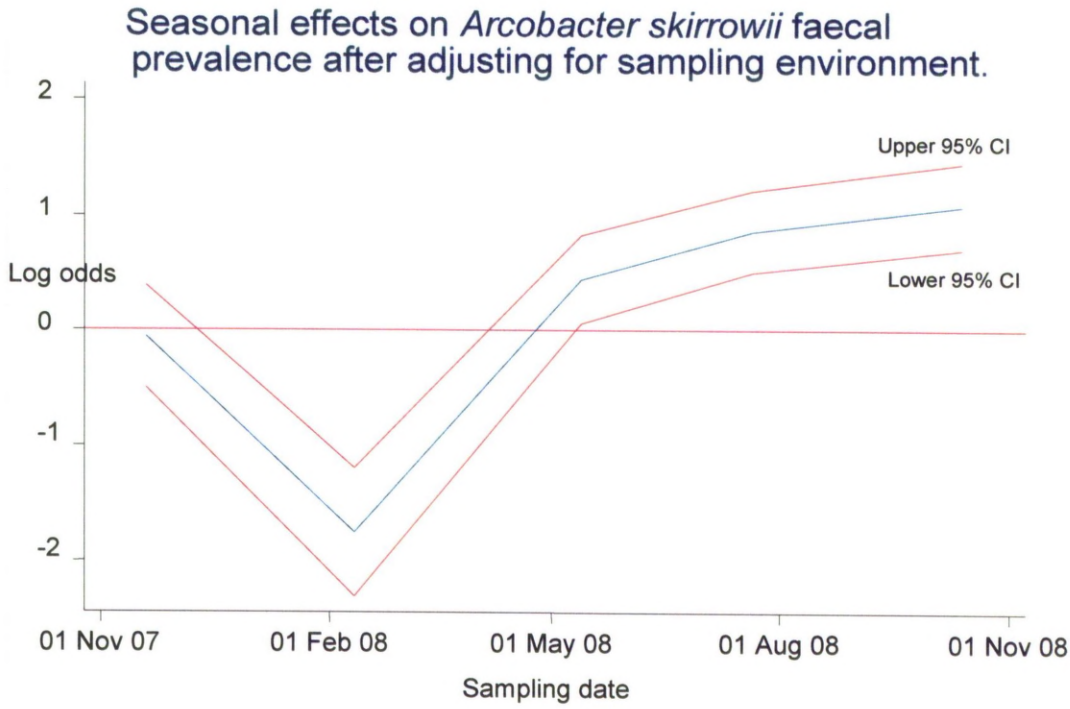

Figure 4.4. Seasonal effects on *A. skirrowii* recovery from faecal samples after adjusting for sampling environment, age group and farm.

The outcome of the logistic regression model using presence or absence of *A. cryaerophilus* as the binary outcome is shown in Table 4.11.

Table 4.11. Results of Logistic regression modelling including covariates associated with the probability of isolating *Arcobacter cryaerophilus* from cattle faeces on farms in Cheshire.

Covariate	Odds Ratio	P Value (95% CI)
Farm 2	0.34	<0.001 (0.21 – 0.55)
Farm 3	0.27	<0.001 (0.16 – 0.45)
Farm 4	0.22	<0.001 (0.13 – 0.37)
Age Group	0.36	0.011 (1.13 – 2.59)
Tcos2	0.08	<0.001 (0.38 – 0.70)
Tsin2	0.08	<0.001 (0.33 – 0.65)
Tsin4	0.09	0.011 (0.57 – 0.93)
Tcos4	0.24	0.872 (0.67 – 1.62)

Farm identity was a fixed effect with Farm 1 as baseline. There was considerable variation in the effect of farm, with odds ratios ranging from 0.22 (95% CI 0.13 – 0.37) to 0.34 (95% CI 0.21 – 0.55). This suggests that even after adjusting for the other covariates, there remains considerable variation associated with farm identity.

Age group was a fixed effect with "adult" as baseline. After adjusting for the other covariates, there remained an effect of age group, with the odds ratio of recovering *A. cryaerophilus* from young cattle being 0.36 (95% CI 1.13 – 2.59).

Harmonic regression analysis of time showed an effect of time, represented in Figure 4.5.

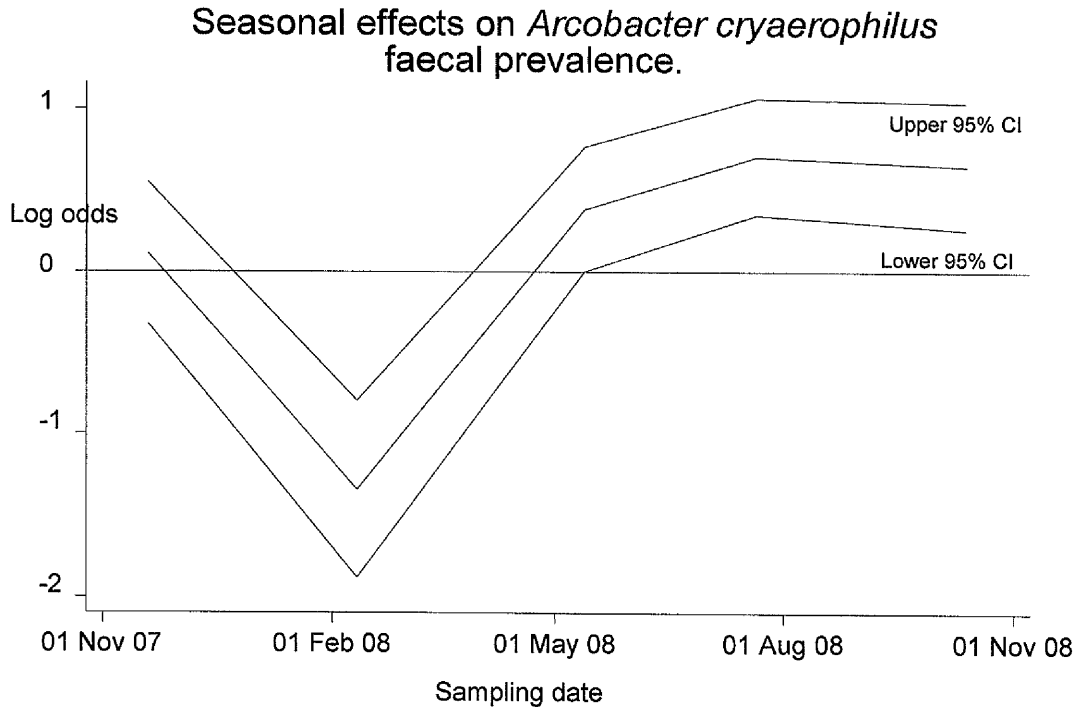

Figure 4.5. Seasonal effects on *A. cryaerophilus* recovery from faecal samples after adjusting for sampling environment, age group and farm.

Table 4.12. A summary of the significance of effects of management factors on prevalence of *Arcobacter* spp. in faecal pats on four farms in Cheshire, as determined by both robust univariate analysis and multivariate logistic regression.

Covariate	Effect on *Arcobacter* spp. recovery	Effect on *A. butzleri* recovery	Effect on *A. skirrowii* recovery	Effect on *A. cryaerophilus* recovery
Univariate analysis (with Robust Standard Errors)				
Sampling Environment	no	no	yes	no
Farm Type	no	no	no	no
Age Group	no	no	no	no
Season	no	no	no	no
Multivariate Regression Analysis				
Sampling Environment	yes	yes	yes	no
Age Group	yes	yes	yes	yes
Farm	yes	yes	yes	yes
Time	yes	yes	yes	yes

4.4. Discussion

The aim of this study was to investigate farm management factors that may affect the prevalence of *Arcobacter* in cattle, and to investigate any possible seasonality. Univariate analysis of the data showed a significant effect of season (summer v winter) on *Arcobacter* spp. prevalence when not allowing for clustering (P = <0.001), however this lost significance when clustering was taken into account (P = 0.1466). Multivariate analysis of the data collected, which adjusts for any confounders in the data, showed a significant drop in *Arcobacter* spp. prevalence during winter (February), followed by a significant peak in the summer (August), showing a statistically significant seasonality.

This is the first report of a statistically significant seasonal prevalence in *Arcobacter* spp., although previous studies have identified possible seasonal variation. Andersen *et al.* (2004) identified a possible seasonal trend in the prevalence of *Arcobacter* in commercial turkeys, and Wesley *et al.* (2000) found a possible seasonal trend in the prevalence of *Arcobacter* in cattle in the USA, isolating *Arcobacter* spp. at a higher prevalence after May than before May ($P = 0.02$). Manke *et al.* (1998) reported a higher prevalence of *Arcobacter* spp. from poultry meat in spring and summer, and Stampi *et al.* (1999) reported a higher recovery from water in spring and summer. In contrast, however, Kabeya *et al.* (2003b) found no statistically significant seasonal prevalence of *Arcobacter* spp. on cattle, poultry and swine farms in Japan (Kabeya *et al.*, 2003b).

Given the close phenotypic relatedness of the two, the higher prevalence of *Arcobacter* spp. during the summer might be similar to the seasonal patterns of the closely related genus, *Campylobacter.* Peaks in human *Campylobacter* infection have been repeatedly reported worldwide, with many studies finding a main peak during the summer months of May to July, in a number of countries (Kovats *et al.,* 2004; Louis *et al.*, 1995). Investigations into possible causes of this summer peak in human infections have revealed a statistically significant link with climate, and more specifically temperature (Kovats *et al.*, 2004; Louis *et al.,* 1995). In a study of human *Campylobacter* infections in Europe, Canada, the USA and New Zealand, an annual peak in human infections was weakly associated with increased temperatures approximately three months prior to the occurrence of the peak (Kovats *et al.,* 2004). Similarly, a study of human *Campylobacter* infections reported over a ten year period showed a peak in May, June and July every year, which was found to be significantly associated with

daily average temperature and sunshine (Louis *et al.*, 1995). After correction for autocorrelation was applied, temperature was found to have the single biggest effect (P = <0.001) and rainfall was found to have no significant effect (Louis *et al.*, 1995).

Similarly, a peak in the recovery of *Campylobacter* from animals during the summer months has also been reported; Willis and Murray (1997) reported a peak from May to October in *C. jejuni* recovered from broiler carcasses, and Jones *et al.* (1991) reported seasonal peaks in *Campylobacter* spp. in farm animals and in environmental sewage, which appeared to correspond with seasonal peaks in human infection. Kwan *et al.* (2008) reported a peak in *C. jejuni* recovery from dairy cattle in Cheshire, UK, during June, followed by a second peak in November, and Grove-White *et al.* (2009) reported a peak in *C. jejuni* in dairy cattle in the UK during summer. This study found a peak in *Campylobacter* prevalence from February to May.

It appears, then, that *Campylobacter* infection in humans is related to temperature, and that as related seasonal peaks have been found to occur in animals (Jones *et al.* 1991; Willis *et al.*, 1997; Kwan *et al.*, 2008), it appears likely that transmission of *Campylobacter* spp. is somehow affected positively by warmer temperatures. Wesley *et al.* (2000) reported that *Arcobacter* was more frequently detected in dairy cows in the southern USA than those in the north, again suggesting higher numbers in warmer climates. Studies into the effect of very low temperatures on *Arcobacter spp.* have shown an inability to persist in faecal matter (See Chapter Three, section 3.2.2.) and a reduction in recovery from poultry carcasses after freezing (Atabay *et al.*, 2003). This may provide some explanation for the drop in the prevalence of *Arcobacter* during the winter,

since very low temperatures are likely to prevent the survival of *Arcobacter* in the environment. A more thorough investigation into the effect of temperature and climate on the prevalence of *Arcobacter* in cattle and the environment would enable a better understanding of the ability of *Arcobacter* to survive freezing temperatures during winter. A longitudinal study of cattle, cattle housing and fresh water on farms might provide more insight into the seasonal prevalence shown in this study.

Univariate analysis of sampling environment suggested that the effect of sampling environment (i.e. whether cattle are kept indoors or outside) on the likelihood of recovering *Arcobacter* spp., when allowing for clustering, is not significant, and that sampling environment had no significant effect on the recovery of *A. butzleri* or *A. cryaerophilus*. However, recovery of *A. skirrowii* was shown to be significantly higher when sampled outside. Multivariate analysis, taking confounders into account, showed that the recovery of *Arcobacter* spp. tended to be higher when samples were taken outside, and the same was found with *A. skirrowii*. However, cattle being kept outdoors appeared to have no significant effect on the recovery of *A. butzleri* or *A. cryaerophilus*.

One possible explanation for the higher overall prevalence of *Arcobacter* spp. when cattle faeces sampled outside may be that cattle grazing at pasture are naturally more likely to be exposed to environmental sources of *Arcobacter*, such as water, soil and agricultural run-off (Jacob *et al.*, 1996; Rice *et al.*, 1999; Diergaardt *et al.*, 2004; Morita *et al.*, 2004; Collado *et al.*, 2008). Analysis of an *A. butzleri* whole genome sequence (Miller *et al.*, 2007), suggested that *A. butzleri* is a free-living, water-borne opportunistic pathogen, a fact supported by the many reports of *Arcobacter* in water (Jacob *et al.*, 1993; Jacob *et al.*, 1996;

Jacob *et al.*, 1998; Rice *et al.*, 1999; Maugeri *et al.*, 2000; Morita *et al.*, 2004). A study conducted in June and July 2007 of ground water, cattle trough water, ponds and canal water on and around Farm 1 revealed a high prevalence of *Arcobacter* spp. in the samples (unpublished data). That study, coupled with the numerous reports of *Arcobacter* spp. in water and the environment suggest that cattle at pasture are likely to come into frequent contact with *Arcobacter* spp., and the presence of *Arcobacter* in cattle drinking troughs on farm 1 (unpublished data) suggests that ingestion of *Arcobacter* by the cattle is likely.

Another factor that may affect the prevalence of *Arcobacter* in cattle housed outdoors is the common practice of slurry spreading. Slurry, including animal manure and treated human sewage (also known as biosolids; http://www.defra.gov.uk/foodfarm/landmanage/waste/sludge/index.htm), is spread over agricultural land in order to fertilise soil. *Arcobacter* has been widely reported in cattle slurry as well as agricultural run-off (Wesley *et al.*, 2000; Golla *et al.*, 2002; Ongor *et al.*, 2004; Van Driessche *et al.*, 2003). The spreading of slurry onto the pastures on which cattle are subsequently grazed may increase the risk of exposure of cattle to *Arcobacter* whilst grazing.

Another factor to take into account is the possibility that the increased prevalence of *Arcobacter* spp. in cattle sampled outdoors is actually a reflection of increased faecal shedding of *Arcobacter*, rather than a genuine increase in prevalence. Generally, large differences in the diets of cattle housed indoors compared to the diet of cattle at grass exist. It is possible that the diet of the outdoor-grazing cattle affects the gut flora in some way, which leads to increased faecal shedding of *Arcobacter* spp.

Multivariate regression analysis of different age groups of cattle (young vs. adult) revealed a significantly higher likelihood of recovering *Arcobacter* spp. in young cattle. Hume *et al.* (2001) found the opposite in pigs, with a higher prevalence in older animals, which may be explained by the fact that pigs are monogastric while cattle are ruminants. One possible explanation for the higher prevalence in younger animals may be that younger cattle possess certain characteristics of gut function that affect the ability of the gut to be colonised by *Arcobacter* spp. Robinson *et al.* (2005) found that the presence of whole grain in the faeces of young cattle was significantly associated with the recovery of *Campylobacter* from the faeces, and referred to the fact that young cattle appear to show incomplete development of the rumen, or rumen flora, characterised by the presence of whole grain in the faeces. Possibly this lack of development of the gut of young cattle allows *Arcobacter* to colonise the gut more easily, explaining the higher prevalence in young cattle.

Univariate analysis of farm type (beef vs. dairy) revealed no significant effects, while multivariate analysis suggested a higher likelihood of recovering *Arcobacter* spp. from beef farms. After multivariate analysis comparing farm identity, it became apparent that the result was due mainly to a much higher prevalence on Farm 1 compared to the other farms, leading to the variable, "farm type" being excluded from the model due to co-linearity (a high level of correlation between the two variables). Farm 1 had a significantly higher prevalence of all three *Arcobacter* spp..

Farm 1 was a beef farm in Cheshire, UK, with a herd size of approximately 500, where cattle were kept outdoors in summer (May to September) and indoors in winter. Farm 4 had a herd size of approximately 300

and cattle were housed in sheds all year round. Herd size has been found to have a significant effect on the prevalence of *Arcobacter,* with suggestions being made that larger herds of animals increase the likelihood of mixing larger numbers of susceptible hosts with carrier animals (Wesley *et al.*, 2000). It is possible, therefore, that the larger herd size on Farm 1 provides one explanation for the higher prevalence found there.

Another explanation may be the possibility of increased exposure of animals on Farm 1 to environmental or other sources of *Arcobacter.* Farm 1 was located in a rural area with streams, water troughs and ponds onsite. A study of the water sources on this farm in 2007 revealed a very high prevalence of *Arcobacter* spp. in all the samples, including the cattle drinking troughs (unpublished data). As water is a known source of *Arcobacter* (Miller *et al.*, 2007; Jacob *et al.*, 1993; Jacob *et al.*, 1996; Jacob *et al.*, 1998; Rice *et al.*, 1999; Morita *et al.*, 2004), and as the cattle on Farm 1 were kept outdoors during the summer, it is possible that Farm 1 has the highest prevalence due to an increased risk of exposure of the cattle to *Arcobacter*s in the water sources on the farm. In contrast, the other beef farm, Farm 4, was located in a semi-rural area with no streams of ponds onsite. In addition, cattle on Farm 4 were housed indoors all year round, and thus may be less likely to be exposed to any *Arcobacter* in the environment. Additionally, the presence of *Arcobacter* spp. in wildlife animals (badgers, rabbits, wood mice, bank voles) on Farm 1 has been confirmed (Unpublished data). It is possible that such animals act as reservoirs of *Arcobacter* spp., and that the presence of cattle outdoors on Farm 1 creates a greater risk of exposure to these reservoirs.

Farm 2 was a dairy farm located within one mile of Farm 1, with its dairy herd kept outdoors during the summer, but its *Arcobacter* prevalence was found to be much lower than on the nearby Farm 1. One explanation for this may be that Farm 2 had a smaller herd size than Farm 1. Farm 3 was a dairy farm on which the cattle were housed indoors all year round, and which also had a much lower prevalence than Farm 1. Further studies into farm management practices, as well as an investigation into potential wildlife reservoirs of *Arcobacter* may provide further insight into the large variations in the prevalence of *Arcobacter* on these farms.

In conclusion, statistical analysis of prevalence data has shown that there is seasonality of *Arcobacter* spp. in cattle on the four farms studied, and that some management practices, i.e. housing status of the cattle and age group, appear to have an effect on the prevalence of *Arcobacter* in cattle. Further investigation of these factors may lead to a better understanding of the mechanisms of transmission and colonisation of *Arcobacter* spp., which may have potential implications for human health. Peaks in *Arcobacter* prevalence do not appear to be related to the prevalence of *Campylobacter*, but rather to environmental factors such as temperature and exposure to potential reservoirs.

Chapter Five

Molecular Typing of *Arcobacter* spp.

5.1. Introduction

Molecular typing techniques play a vital role in the surveillance of bacteria that cause infectious disease and the study of bacterial populations in virtually any environment. Molecular typing techniques have gradually replaced phenotypic and serological typing methods in recent years, and are recognised as being more sensitive, specific, stable and reliable. Numerous studies have utilised molecular typing techniques to study pathogens of interest, for example those which exhibit antibiotic resistance or virulence, and those involved in pandemic and epidemic infections of humans.

An important field in which molecular typing is also important is the study of population dynamics and epidemiology. Molecular typing can be used to study the evolution of a pathogen, its spread and modes of transmission, to classify bacteria based on genetic diversity and to determine how bacterial strains evolved from a common ancestor or independent sources (Foley *et al.*, 2009). Typing can also be used to investigate bacterial diversity below species level, allowing discrimination between strains and subtypes (Li *et al.*, 2009).

Molecular typing methods can generally be separated into three main categories; DNA banding pattern-based methods, DNA sequencing-based methods and DNA hybridisation-based methods (Li *et al.*, 2009).

5.1.1. Band-based Typing Methods

The majority of molecular typing methods are banding pattern-based techniques. In these, fragments of DNA are obtained by PCR assay and/or restriction enzyme digestion and separated by agarose gel electrophoresis, resulting in a banding pattern or "DNA fingerprint", which can then be analysed in a number of ways. One example of a band-based typing method commonly used to type

Arcobacter spp. is macro-restriction pulsed-field gel electrophoresis (PFGE; Hume *et al.*, 2001), which uses a rare-cutting DNA restriction enzyme to cut chromosomal DNA, fragments of which are then separated on an agarose gel using alternating or pulsed fields. Other methods include amplified fragment length polymorphism (AFLP; On *et al.*, 2003), random amplified polymorphic DNA (RAPD; Atabay *et al.*, 2002) and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR; Houf *et al.*, 2002), which use oligonucleotide primers to measure the relative positions of the intergenic consensus DNA sequence, producing differently sized bands which are visualised on an agarose gel.

5.1.2. Sequence-based Typing Methods

Probably the most widely used sequence-based method is multi-locus sequence typing (MLST; Maiden *et al.*, 2006), in which specific regions of seven or more housekeeping genes are amplified by PCR and sequenced, and the data used to form an allelic profile. The data can be stored in an online database which can be used to compare alleles and isolates (www.pubmlst.org and www.mlst.net). Sequence-based typing methods are more subjective and repeatable than band-based methods, as the same sequence data should be obtained regardless of the laboratory where the sequencing is carried out, and hence these methods are less prone to variability due to human error or the use of different equipment and protocols. These methods are also more portable, with data usually in electronic format and therefore easily transferred between different locations or individuals.

A number of molecular typing techniques have been used to study the epidemiology of *Arcobacter* spp. and overall, studies seem to concur that the genus *Arcobacter* contains many different strains with high levels of heterogeneity

(Gonzalez *et al.*, 2007; Aydin *et al.*, 2007; Son *et al.*, 2006; Atabay *et al.*, 2006; On *et al.*, 2004).

In this study ERIC-PCR, macro-restriction PFGE and MLST were used to type *Arcobacter butzleri*, *A. skirrowii* and *A. cryaerophilus* isolates from cattle in order to observe and compare the diversity of *Arcobacter* isolates obtained in the north west UK during 2007 and 2008 (Chapter Three, Four).

5.2. Materials and Methods

5.2.1. ERIC-PCR

Three hundred isolates of *A. butzleri, A. skirrowii* and *A. cryaerophilus* obtained during the farm survey described above were selected for typing using ERIC PCR. The isolates were selected at random, by hand, from a list of isolates obtained during the survey (Chapter Three). All had been previously identified to species level using an *Arcobacter* genus-specific PCR (Gonzalez *et al.* 2000) and an *Arcobacter* species-specific PCR (Houf *et al.*, 2000).

The selected isolates were typed using ERIC-PCR based upon the technique described by Houf *et al.* (2002), using 1µl aliquots of boiled cell lysate. The oligonucleotide primers ERIC1R ($^{5'}$ATGTAAGCTCCTGGGGATTCAC$^{3'}$) and ERIC2 ($^{5'}$AAGTAAGTGACTGGGGTGAGCG$^{3'}$) (Versalovic *et al.*, 1991) were used at a concentration of 0.5µM each, along with 5U *Taq* DNA polymerase (Abgene, UK), 4mM $MgCl_2$, 2.5µl 10x PCR buffer, 0.25µl dNTP mix and 1µl template DNA. Reactions were carried out in 25µl volumes and cycling conditions were as follows:

Table 5.1. Cycling conditions for ERIC-PCR (Houf et al., 2002).

Initial denaturation	94°C	4 minutes
40 cycles	94°C	1 minute
	25°C	1 minute
	72°C	1 minute
Final elongation	72°C	7 minutes

ERIC-PCR products were separated on a 2% (w/v) agarose gel (Chapter Two, Section 2.6.1.) for 150 minutes at 100V. Gels were visualised using the Gel-Doc 2000 (Bio-Rad, USA). The results were analysed using BioNumerics software (Version 4.0, Applied-Maths, St. Martens-Latem, Belgium) using a position tolerance of 2%.

5.2.2. Macro-restriction PFGE.

PulseNet PFGE method

Macro-restriction PFGE was carried out using a method based upon a one-day standardised *Campylobacter* PFGE protocol from PulseNet, USA (http://www.cdc.gov/pulsenet/protocols.htm; see Appendix II) with the modifications described below. The stored isolates were grown under aerobic conditions at 30°C on Columbia agar containing 5% lysed horse blood for 72 hours. Some isolates with scant growth were cultured in *Arcobacter* broth (see section 2.2.1.) before incubation on blood agar. Approximately 20-40µl of cells from each plate was suspended in 1ml sterile phosphate buffered saline (PBS, Sigma-Aldrich, Dorset, UK) and the optical density adjusted to 0.4 with the addition of PBS. Four

hundred microlitres of the suspension were mixed with 25µl proteinase K at a concentration of 20mg/ml (Sigma-Aldrich, UK) and 400µl 1% PFGE agarose (BioRad, USA) prepared in 0.5x tris-borate EDTA (TBE) (Sigma-Aldrich, UK). The mixture was transferred to disposable plug moulds and allowed to set at 4°C before being incubated, with shaking at 120rpm, in 3ml cell lysis buffer containing 25µl proteinase K (20mg/ml) at 54°C for 15 minutes. Plugs were washed once in sterile distilled water for 20 minutes at 54°C, and then washed three times in 1x Tris EDTA (TE) (Sigma-Aldrich, UK) at 54°C for 20 minutes. Plugs were washed once more in 0.1xTE for 20 minutes at 37°C and half a block (approximately 5mm length) was then digested with forty units of restriction enzyme; either *Eag*I, *Sma*I or *Kpn*I, in the recommended buffer L (Thermo scientific) for 2 hours at 37°C with shaking at 120rpm. Blocks were placed in 1% PFGE certified agarose (Bio-Rad, USA) in 0.5x TBE on a CHEF DRIII machine (Bio-Rad) for 16 hours using an initial switch time of 6.7s, final switch time of 38.3s and a temperature of 14°C. Gels were stained for 20 minutes in 0.1% ethidium bromide solution in distilled water and visualised using a Gel-Doc 2000 (Bio-Rad, USA). Banding patterns on gel images were analysed using the BioNumerics software package (version 5.0) with a position tolerance of 2%.

5.2.3. MLST.

A multi-locus sequence typing (MLST) scheme was used, following a protocol based on that of Miller *et al.* (2009).

A total of 800 isolates of *A. butzleri, A. skirrowii* and *A. cryaerophilus* were subjected to MLST. These comprised 300 isolates obtained during a farm survey (Chapter Three, Section 3.2.1.) and 250 *A. butzleri* isolates obtained during a second farm survey (Chapte Four, Section 4.2.1.), along with 125 *A. skirrowii* and 125 *A.*

cryaerophilus isolates from the same study, which had been selected at random using the Survey Toolbox programme from www.ausvet.com (http://www.ausvet.com.au/content.php?page=res_software#st).

Larger numbers of *A. butzleri* isolates than *A. skirrowii* or *A. cryaerophilus* were selected for typing because *A. butzleri* was considered to be the species most commonly associated with foodborne illness in humans and thus is potentially most relevant in a typing study. MLST allowed the comparison of isolates with those already on the online database, including isolates from humans.

Using DNA extracted using the chelex method (Chapter Two, Section 2.5.), seven PCR reactions per isolate were carried out using the primer pairs of Miller *et al.* (2009), in Table 5.2. The *glyA*1 primer set, based on the *lysS* gene, was selected to represent the locus *glyA* described in the original methodology (Miller *et al.*, 2009). PCR reactions were carried out in batches of up to 46 isolates using 96-well plates (Starlab, Milton Keynes, UK). The PCR reaction mix was as follows: 10xPCR buffer (Thermo Scientific, UK), 2.5mM $MgCl_2$ (Thermo Scientific, UK), 250µM (each) dNTPs (Thermo Scientific, UK), 50pmol each primer (forward and reverse, VH Bio, UK), 1U Taq polymerase (Thermo Scientific, UK) and 2µl template. Reactions were carried out in 50µl volumes. PCR reactions were carried out using ABI 2700 and 2720 thermal cyclers using the following conditions: an initial denaturation step of 94°C for seven minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 30 seconds, 72°C for two minutes, and a final extension step of 72°C for seven minutes. Product (10µl) from six randomly selected wells were run on a 2% agarose gel for twenty minutes at 120V along with 10µl each of the positive and negative controls, and separated and visualised by agarose gel electrophoresis (Chapter Two, Section 2.6.1.) in order to confirm the presence of

amplicons before attempting to sequence them. The brightness of the amplicons on the gel was used to determine the amount of water used for dilution after PEG-precipitation (below).

A PEG-precipitation reaction was then carried out in order to clean up the PCR products: Sixty microlitres of 20% (w/v) PEG_{8000} (Appendix IV) in 2.5M NaCl was added to each well containing PCR product in the 96-well plate. Plates were then sealed, vortexed briefly and centrifuged at 500rpm (Eppendorf Centrifuge 5810R, Hamburg, Germany) for one minute to ensure the mix was in the bottom of each well, then incubated at 37°C for 15 minutes, 20°C for 30 minutes or at 4°C overnight. Plates were then centrifuged at 2750rcf (Eppendorf Centrifuge 5810R, Hamburg, Germany) for one hour to enable the formation of a pellet on the bottom of each well. The PEG mixture was then removed by inverting the plate onto folded absorbent paper and centrifuging upside-down at 500rpm (Eppendorf Centrifuge 5810R, Hamburg, Germany) for one minute. Pellets were washed twice with 150µl 70%w/v ice cold ethanol by centrifuging at 2750rcf for twenty minutes, and the ethanol removed each time by inverted centrifuging at 2750rcf for one minute. Plates were air-dried for ten minutes, and the pellets resuspended in sterile molecular grade water (Sigma, UK) according to the brightness of the PCR product. Products giving very bright bands were resuspended in 50µl, whereas products with very faint bands were resuspended in 5µ1. The plates were then sealed with adhesive film, vortexed briefly to ensure pellets were resuspended, centrifuged at 500rpm (Eppendorf Centrifuge 5810R, Hamburg, Germany) for one minute and stored at -20°C until the sequencing reaction could be conducted.

Separate PCR sequencing reaction mixes were made up for each forward and reverse primer using 2.38µl molecular grade water (Sigma, UK), 1.87µl 5x buffer,

0.25µl BigDye Terminator (Applied Biosystems) and 4µl 0.67µM primer (forward or reverse) per reaction. Eight point five microlitres of the mixture containing the forward primer was added to the wells of odd-numbered columns of a sterile 96-well plate and the same amount of the mixture containing the reverse primer was added to even-numbered columns. Of each product for sequencing, 1.5µl was added to the relevant wells and the locations of products on the plate were recorded in a spreadsheet. The plates were loaded onto an Applied Biosystems ABI 2720 thermal cycler and run for thirty cycles of 96°C for ten seconds, 50°C for five seconds and 60°C for two minutes, followed by a final holding step at 4°C.

Ethanol precipitation was then carried out. The contents of the wells were each washed with 52µl of a solution of 100% ethanol and 3M sodium acetate, then vortexed and centrifuged briefly at 500rpm (Eppendorf Centrifuge 5810R, Hamburg, Germany) before being incubated at room temperature for forty five minutes. The mixture was then removed by placing the plate upside down on folded absorbent paper and centrifuging at 500rpm for 1 minute. The resulting pellet was washed once by addition of 150µl ice-cold 70% w/v ethanol and centrifuged at 2750rcf (Eppendorf Centrifuge 5810R, Hamburg, Germany) for ten minutes. The ethanol was then removed by placing the plate upside down onto absorbent paper and centrifuging at 500rpm for one minute. The plate was allowed to dry in air at room temperature for ten minutes before the addition of 10µl HiDi formamide (Applied Biosystems) per well. Plates were briefly vortexed, then spun at 500rpm for one minute followed by heat denaturation on a hot block (Grant Instruments, Cambridge, UK) at 94°C for two minutes.

Plates were then loaded onto an ABIPRISM 3130xl Sequencer with ABI 3130xl genetic analyser and ABI 3130 data collection software v3.0 using ABI 3130 POP-7 polymer and run on the FastSeq50 program.

MLST allele sequences were edited using the STARS software (University of Oxford, 2001) after it had been set up to connect to the *Arcobacter* PubMLST database (http://pubmlst.org/*Arcobacter*/) sited at the university of Oxford and hosted by Keith Jolley (Jolley *et al.*, 2004). New alleles were quality checked using CHROMAS (Technylesium, Australia) and submitted to the database curator for allele assignment. Alleles that were not novel were assigned the relevant number using the online database query tool. Once new allele numbers had been assigned, the seven-number allelic profiles were submitted to the curator who then assigned a sequence type (ST) and entered the data onto the database. Isolates that did not feature novel allele sequences were compared to existing alleles on the database and automatically assigned the correct ST.

Table 5.2. ***Arcobacter*** **MLST amplification and sequencing primers. Courtesy of William Miller, taken from Miller *et al.* (2009). The final column, labelled "amplification", shows which species were successfully amplified using each primer set. The column labelled B represents *A. butzleri*, S represents *A. skirrowii*, C represents *A. cryaerophilus*, N represents *A. nitrofigilis*, Sl represents an *A. skirrowii*-like species and Cb represents *A. cryaerophilus* group b (Oliveria *et al.*, 1999). Y = successful amplification, V = weak amplification of some strains.**

		Oligonucleotide primer set											
		Forward (5' → 3')		Reverse (5 → 3')		Amplification[a]						
Locus	Allele size	Primer	Sequence	Primer	Sequence	B	C	S	Sl	Cb	N	Amplicon size (bp)
aspA	477	*aspA* BF	ATTTTRAGAGATTCT TTTCRCRATAAA	*aspA* BR	AACATTATTCATACA AATTTCAGSATT	Y	V	V	V	V	V	711
		aspA CF2	AAATATTRMGAGAT GCTTTTTATGGAA	*aspA* CR2	TACAAACTTCAGGAT TWGCWGTAAT		Y					702
		aspA SF	GCTTATCCAACTGC WATTAAAMTTACA	*aspA* SR	CTTCAGGATTTGCTG YAATTCC			Y	Y			760
		aspA CibF	AGCCTTAAGATTTTA AGAGATGCTTTC	*aspA* CibR	AGGATTTGCTGTAAT TCCTTTTATACA					Y		699
		aspA NF	CTTTGAGATTTTTAA GGGATTGTTTTG	*aspA* NR	ATTCATACAAACATC TTCATTTGCAGT						Y	713
atpA	489	*atpA* BF	CWGTTGCKATTGAT ACAATTCTTAA	*atpA* BR	CAATTTGTTTTTCAAT AACTAATGGTTT	Y						774
		atpA CF	GATACAATTCTTAA YCAAAAAGGTGA	*atpA* CR	AAAACTTCWACCATT CTTTGWCCAA		Y	Y	Y	Y	Y	719
glnA	474	*glnA* BF	TGCAGTTAGTGCWT CTMCTTTAGATAA	*glnA* BR	ATAGRTTTTTCCCATC TTTCCAA	Y						734
		glnA CF2	AAATGGAATGCCTT TTGATGGTT	*glnA* CR1	TTRTCWCCATAAAGW GGTTTTGGCA		Y	Y	Y	Y	Y	657
gltA	429	*gltA*B F	TTGATGGAGARAAT TCTGAGTTAAG	*gltA* BR	GGAACATTTTTAACA TCACCAATTA	Y						706

		*gltA*C F	TGATATWGCTGATT TRGCTGGTAAA	*gltA* CR	CCAATCATTCTTARYT GATCCATAAC		Y	Y	Y	Y	Y	651
glyA [b]	50 7	ABIy sS	AAATGGAYGARGAY TTTGTWAATGC	*glyA* BR	CATCTTTYCCTGAAA ATGGTTTATT	Y						1189
		ABIy sS	AAATGGAYGARGAY TTTGTWAATGC	*glyA* CR	GCATCTTTTCCWSWR AATGGTTTAT		Y	Y	Y	Y	V	~120 0[c]
		glyA BNF	ATGCAGAAGGTTAT CCATATAAAAG	ABa da2	GTAACACCWACATAC TCTTTRAATACTCT	Y	V	V	Y	Y	N	~130 0[c]
		glyA CF	TGCAAATGTTCAAC CWCATAGTGGA	ABa da3	AWAAGWGCYTTCCA WACATTTATTT		Y	Y				~130 0[c]
		glyA CF	TGCAAATGTTCAAC CWCATAGTGGA	ABa da8	TTTTCTTTCWATKCCC CATCT		Y	Y				~130 0[c]
		glyA BF	TGGWTGTAAATWTG CAAATGTTCAA	*glyA* BR	CATCTTTYCCTGAAA ATGGTTTATT	Y						735
		glyA CF	TGCAAATGTTCAAC CWCATAGTGGA	*glyA* CR	GCATCTTTTCCWSWR AATGGTTTAT		Y	Y	Y	Y	V	723
pgm	50 3	*pgm* ABF I	TCCRAAAAATYTRA CWYTAAAAGGT	*pgm* ABR	AAAGTCTRATTTTATT YTCTGTKCC	Y	N	N	N	N	N	758
		pgm ACF I	GGATTAAGAATTGT YCTTGAYTGTGC	*pgm* ACR	TCAACATCTTTTYTAT TTTTACCTTCA		Y				N	767
		pgm ASF1	AAAAGGKCTTAGAA TTGTWCTTGATTG	*pgm* ACR	TCAACATCTTTTYTAT TTTTACCTTCA			Y	Y		N	771
		pgm ACib F	TTCCCAAAAGATTTA ACGCTAAAAG	*pgm* ACR	TCAACATCTTTTYTAT TTTTACCTTCA					Y	N	791
t *k* *t*	46 2	*tkt*AB F	GCTGATATTGCAAC AGTWTTAAGTA	*tkt*A BR	TAAATCCWGCTTTTT CTTTWGATTT	Y	V	V	V	V	N	721
		*tkt*AC F	CTCCTATGGGAMTK GCTGATATTG	*tkt*A CR2	ATTAAAYCCAGCTTT TATTTTTGCTTG		Y	Y	Y		N	737
		*tkt*AC F	CTCCTATGGGAMTK GCTGATATTG	*tkt*A CibR	GGATTAAATCCTGCT TTTTCTTTAGATT					V	N	739

Resulting sequence data were edited and analysed using STARS MLST analysis software (NERC, Oxford, UK) and CHROMAS.

5.2.3a. Phylogenetic Analysis

Molecular evolutionary analysis was carried out using MEGA version 4 (Tamura, Dudley, Nei and Kumer, 2007). Neighbour-joining trees were constructed for each locus, using pairwise and multiple alignment using the ClustalW algorithm within MEGA: with the parameters; gap opening penalty, 15; gap extension penalty, 6.66; delay divergent cutoff 30%.

eBURST analysis was carried out on the 53 sequence types identified in this study. Analysis was carried out using eBURST version 3 (Imperial College, London). Group definition was set at 6 out of 7 loci.

The classical Maynard-Smith index association test for linkage disequilibrium was carried out on the 53 sequence types using START version 2.0.

5.3. Results.

5.3.1. ERIC-PCR

Typing of 300 isolates was carried out using ERIC-PCR and the results were analysed using BioNumerics (Version 4.0., Applied Maths, St Martens-Latem, Belgium). Figure 5.1. shows a dendrogram produced using BioNumerics based on the ERIC-PCR results.

The ERIC-PCR produced a total of 134 different banding patterns from the 300 isolates showing, in agreement with previous *Arcobacter* typing studies, that a large degree of genetic diversity exists between the *Arcobacter* isolates studied. Figure 5.1. shows that separate clusters of individual species occurred, but with no overall grouping of species.

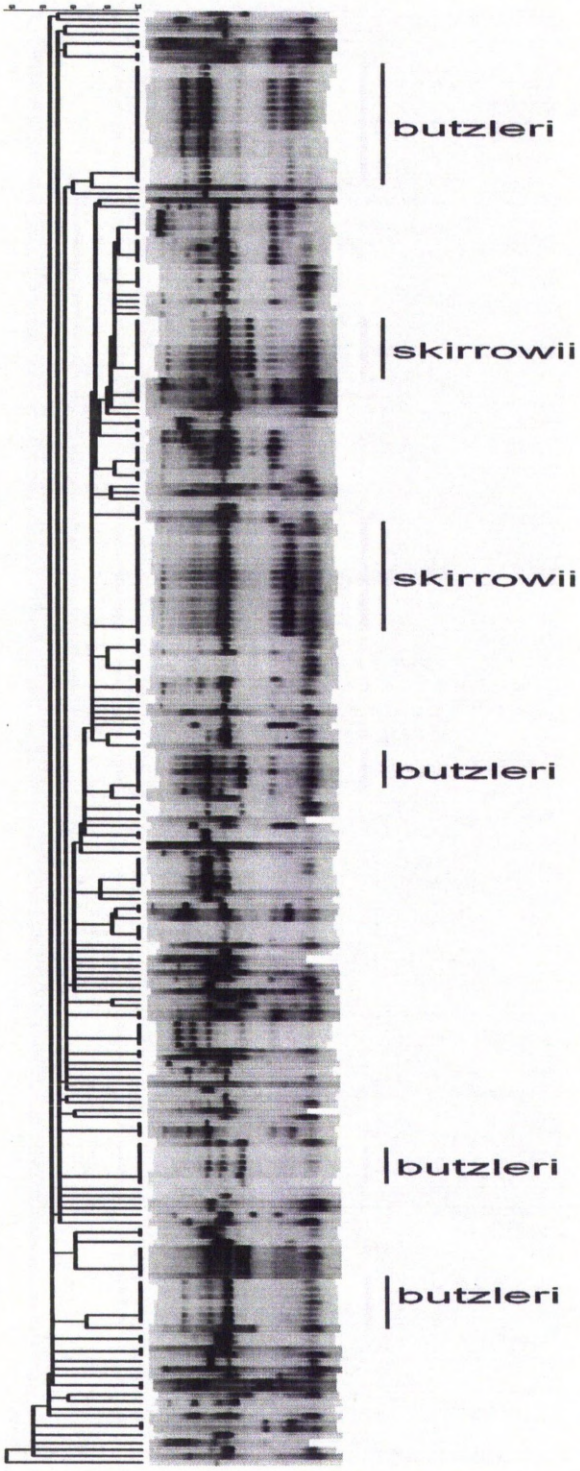

Figure 5.1. Similarity dendrogram produced using BioNumerics v4.0, using the banding patterns produced by ERIC-PCR for 300 *Arcobacter* isolates with groups of isolates of the same species labelled. Note that isolates of the same species form separate clusters.

5.3.2. PFGE.

Macro-restriction PFGE was undertaken using the same set of 300 isolates that were used in ERIC-PCR above. Isolates were analysed using the restriction enzymes *kpn*I, *Sma*I, *ksp*I, *sag*I and *eag*I using the method described in Section 5.2.2, although no successful results were obtained. With most isolates no discrete bands were produced, and when bands were produced there were too few to allow any meaningful analysis. Attempts were made to optimise the protocol through the introduction of additional water and TE washes, longer digestion times (increased from two hours to six hours) with higher concentrations of enzyme (concentration doubled from forty units per sample to eighty units per sample) and higher concentrations of bacterial cells, but without success. Problems arose with reviving bacterial cells from frozen culture. When suitable bacterial cell concentrations were obtained and the method was completed, gel electrophoresis produced poor quality banding patterns that were unsuitable for analysis, probably due to ineffective restriction enzyme digestion.

Figure 5.2. shows examples of the results of PFGE experiments, demonstrating the incomplete digestion of the fragments.

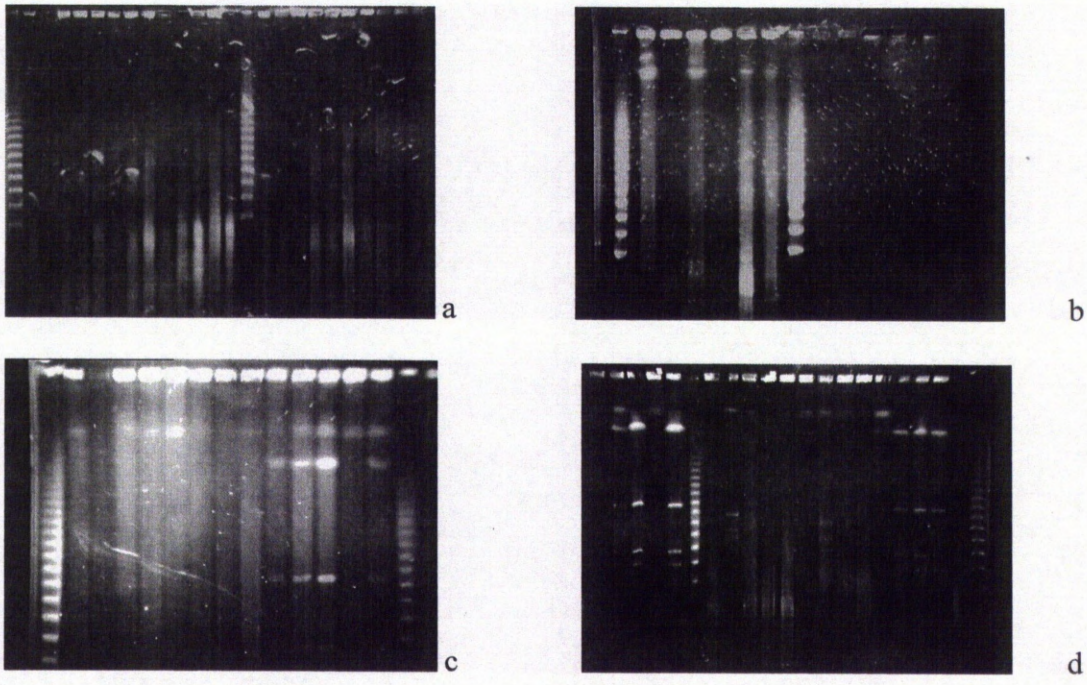

Figure 5.2. Gel images of PFGE analysis. Images a and b were obtained after completing the original protocol, using the *Sma*I enzyme, and both show a lack of discrete bands. Images c and d were obtained after increasing the restriction enzyme incubation time to four hours, with eighty units of the enzyme *Kpn*I, and these show some bands produced after digestion, but of insufficient quality or number for valid analysis.

5.3.3. MLST.

MLST (Miller *et al.* 2009) was applied to a total of 652 isolates of *A. butzleri, A. skirrowii* and *A. cryaerophilus* from cattle and sheep on six farms. A total of 800 isolates were originally selected for MLST, made up of the same 300 isolates used in the ERIC-PCR and PFGE studies, plus five hundred isolates obtained during the 2007-2008 cross sectional studies (Chapter Four). However, only 652 of these remained viable after frozen storage. Of the 652 isolates to which the scheme was applied, a total of 249 (38%) complete allelic profiles were obtained,

and 143 (22%) of these, all belonging to the species *A. butzleri*, were assigned sequence types (STs) by the database curator; the remaining 106 were of insufficient quality to be assigned STs. The 143 isolates given STs were made up of 53 different STs, two of which (18 and 138) were existing STs and 51 of which were novel.

A number of alleles obtained for *A. skirrowii* and *A. cryaerophilus* isolates could not be assigned sequence types due to the presence of imperfections in the sequence traces. All 53 of the sequences assigned STs belonged to the species *A. butzleri*. A list of the isolates and alleles can be found in Appendix V.

As the *Arcobacter* MLST scheme was only developed in 2009, the majority of alleles produced using this technique were novel, previously unidentified alleles, which had to be submitted to the MLST site curator for assignment. Any sequence traces submitted as new alleles need to be of very high quality with no ambiguous peaks or weak signals. This meant that a large number of alleles in this study were not assigned an allele number as time constraints prevented the multiple repeats of the sequencing process that would be required to obtain traces of acceptable quality.

The amount of variation at each locus differed. The minimum number of different alleles identified at each locus is shown in Table 5.3. Some alleles occurred which could not be assigned numbers, meaning each locus potentially features more alleles than are shown here.

Table 5.3. The minimum number of discrete alleles identified at each locus sequenced.

Locus	Minimum number of different alleles identified
aspA	72
atpA	41
glnA	26
gltA	41
glyA	102
pgm	55
tkt	40

Of the seven loci used in the MLST scheme, *glyA* was found to feature the most variation, with at least 102 alleles identified and *gltA* had the least variation, with at least 26 alleles identified amongst all three species.

5.3.3a. Phylogenetic analysis of alleles at each locus

Phylogenetic analysis of each of the seven loci was completed using the MEGA evolutionary analysis software, using sequence data from all alleles. Figures 5.3a. to 5.3g. show the neighbour-joining trees produced using the alleles obtained at each individual locus.

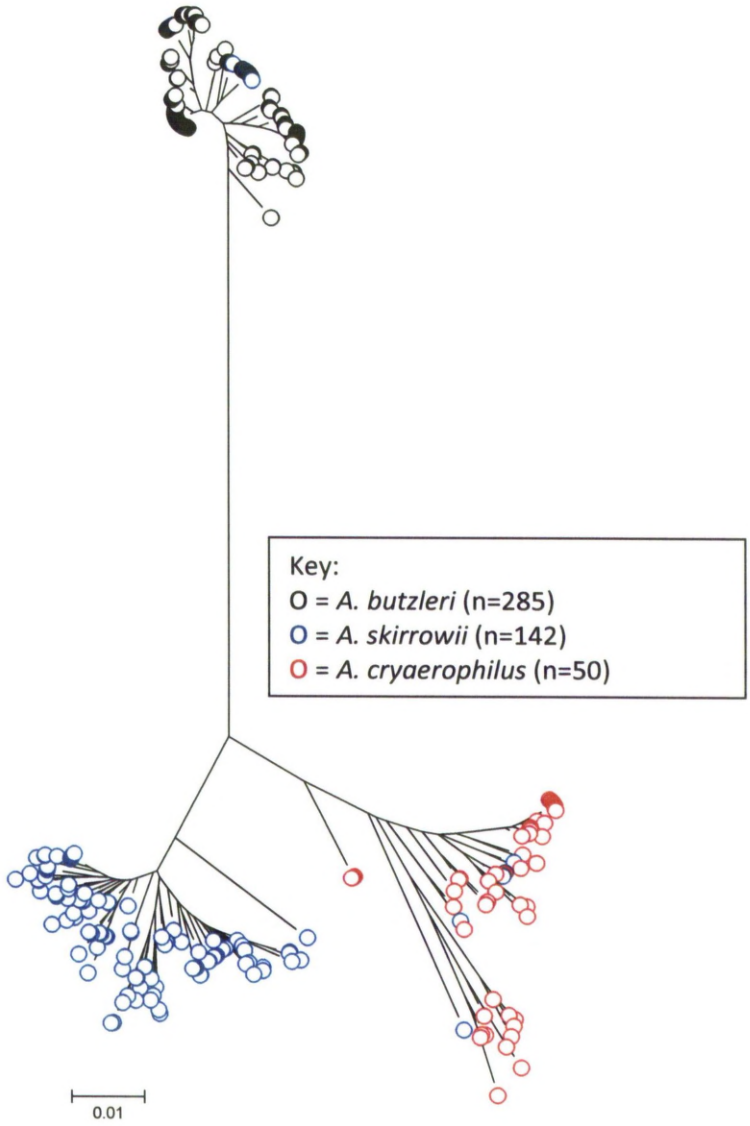

Figure 5.3a. Neighbour-joining tree of all *aspA* alleles obtained (n= 477).

aspA

When *asp*A alleles were analysed, the three *Arcobacter* species formed three distinct clusters with *A. skirrowii* and *A. cryaerophilus* being the more closely related, with *A. butzleri* more distant. Several *A. skirrowii* isolates were located in both the *A. butzleri* and *A. cryaerophilus* clusters (Figure 5.3a).

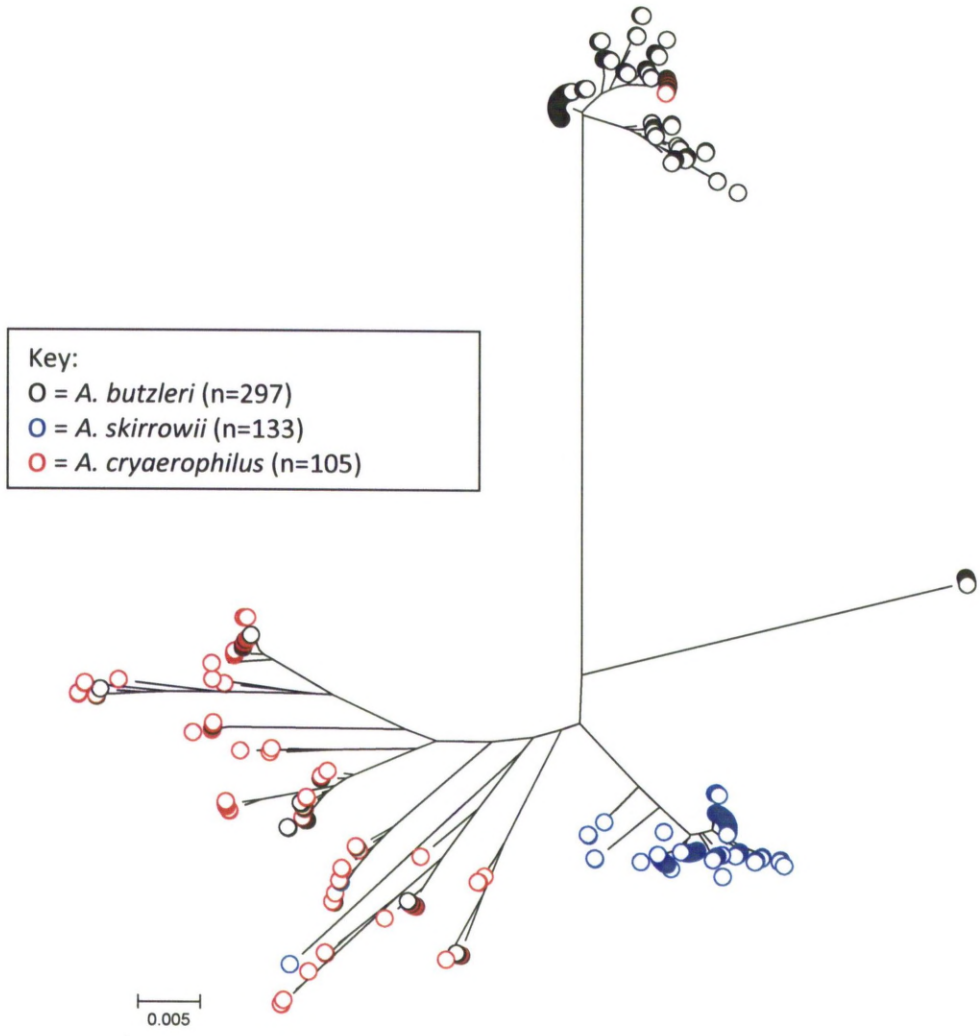

Figure 5.3b. Neigbour-joining tree of all *atpA* alleles obtained (n= 535).

atpA

When *atp*A alleles were compared, *A. butzleri* and *A. skirrowii* formed distinct clusters, while the *A. cryaerophilus* isolates were more widely distributed along a branch. A second small *A. butzleri* cluster occurred much further down the tree, nearer to the *A. skirrowii* and *A. cryaerophilus* clusters than the main *A. butzleri* cluster. Isolates of both *A. skirrowii* and *A. cryaerophilus* could be seen within the main *A. butzleri* cluster, while *A. butzleri* and *A. skirrowii* isolates occurred within the *A. cryaerophilus* cluster, Figure 5.3b.

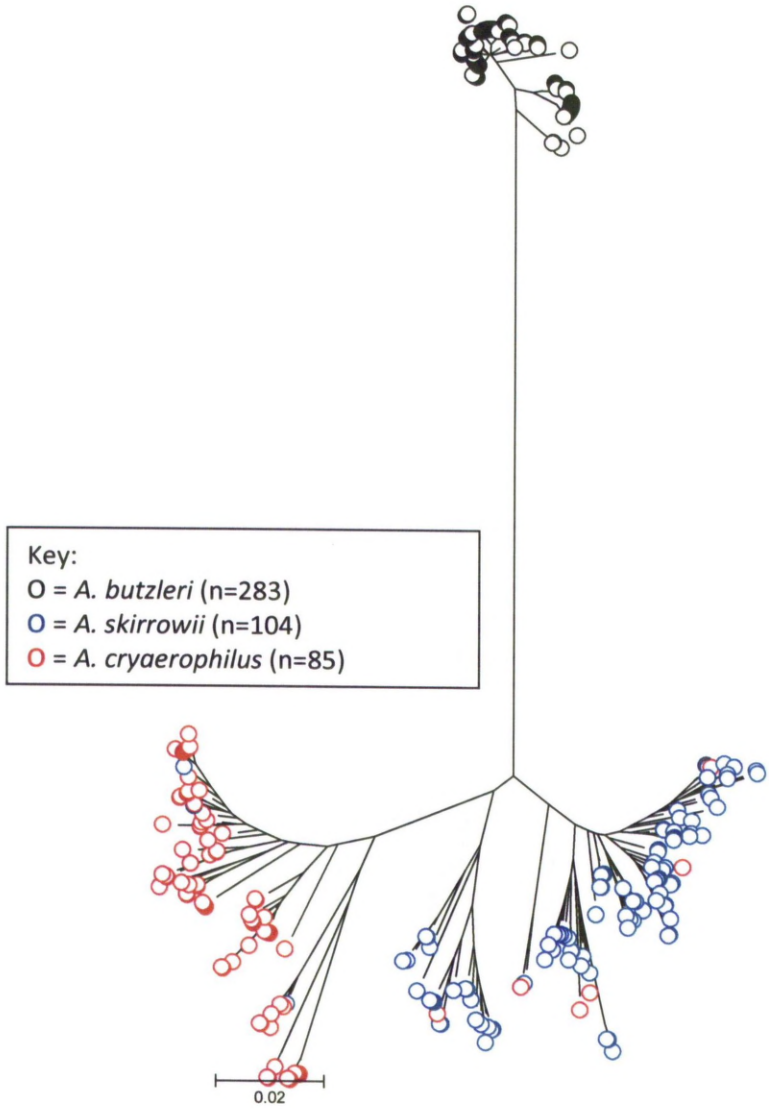

Figure 5.3c. Neighbour-joining tree of all *glnA* alleles obtained (n= 472).

glnA

Using the *glnA* locus, *A. butzleri* isolates formed a distinct and homogeneous cluster, while *A. skirrowii* and *A. cryaerophilus* isolates formed two larger clusters spread across one end of the tree. One *A. skirrowii* subgroup occurred along the *A. cryaerophilus* branch. Isolates of *A. skirrowii* were present within the *A. cryaerophilus* cluster, and vice versa, Figure 5.3c.

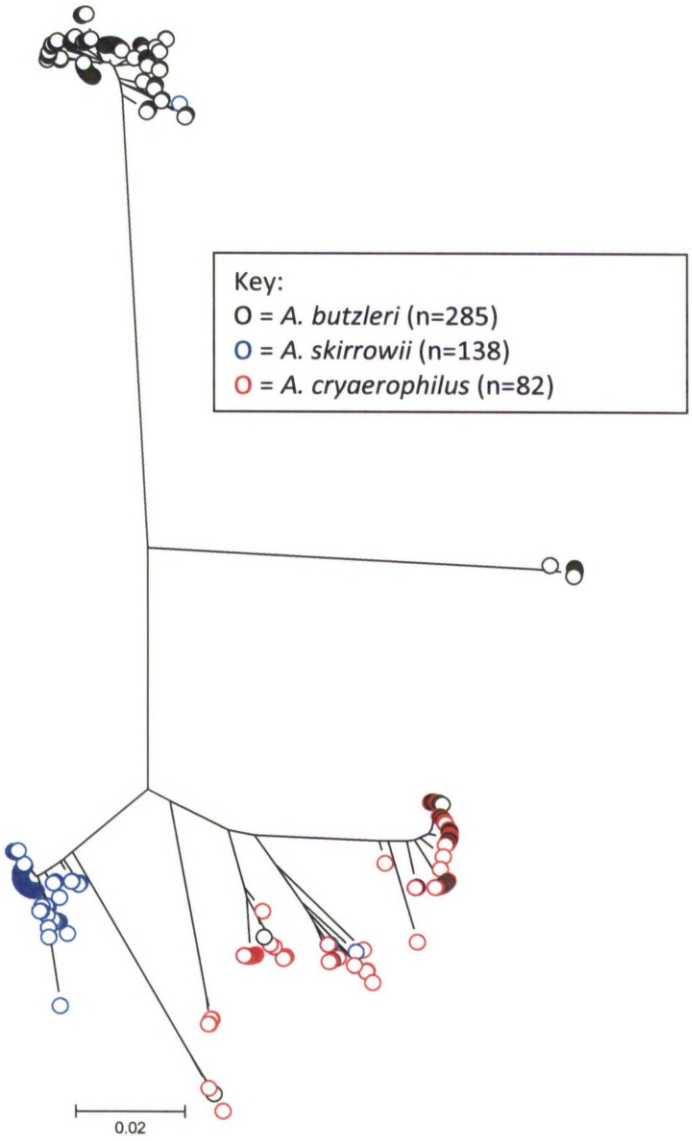

Figure 5.3d. Neighbour-joining tree of all *gltA* alleles obtained (n= 505).

gltA

On the basis of the *gltA* locus, *A. butzleri* isolates again largely formed a single homogeneous cluster. *A. skirrowii* isolates formed a small, distinct cluster near to the *A. cryaerophilus* isolates, which showed diversity. As in the *atpA* tree, a second, smaller *A. butzleri* cluster appeared further down the tree, nearer to the *A. skirrowii* and *A. cryaerophilus* clusters. A third cluster consisting of one *A. butzleri* isolate, along with two *A. cryaerophilus* isolates occurred along the *A. skirrowii*

branch. *A. butzleri* and *A. skirrowii* isolates occurred within the *A. cryaerophilus* cluster, while a small number of *A. skirrowii* isolates appeared within the main *A. butzleri* cluster, Figure 5.3d.

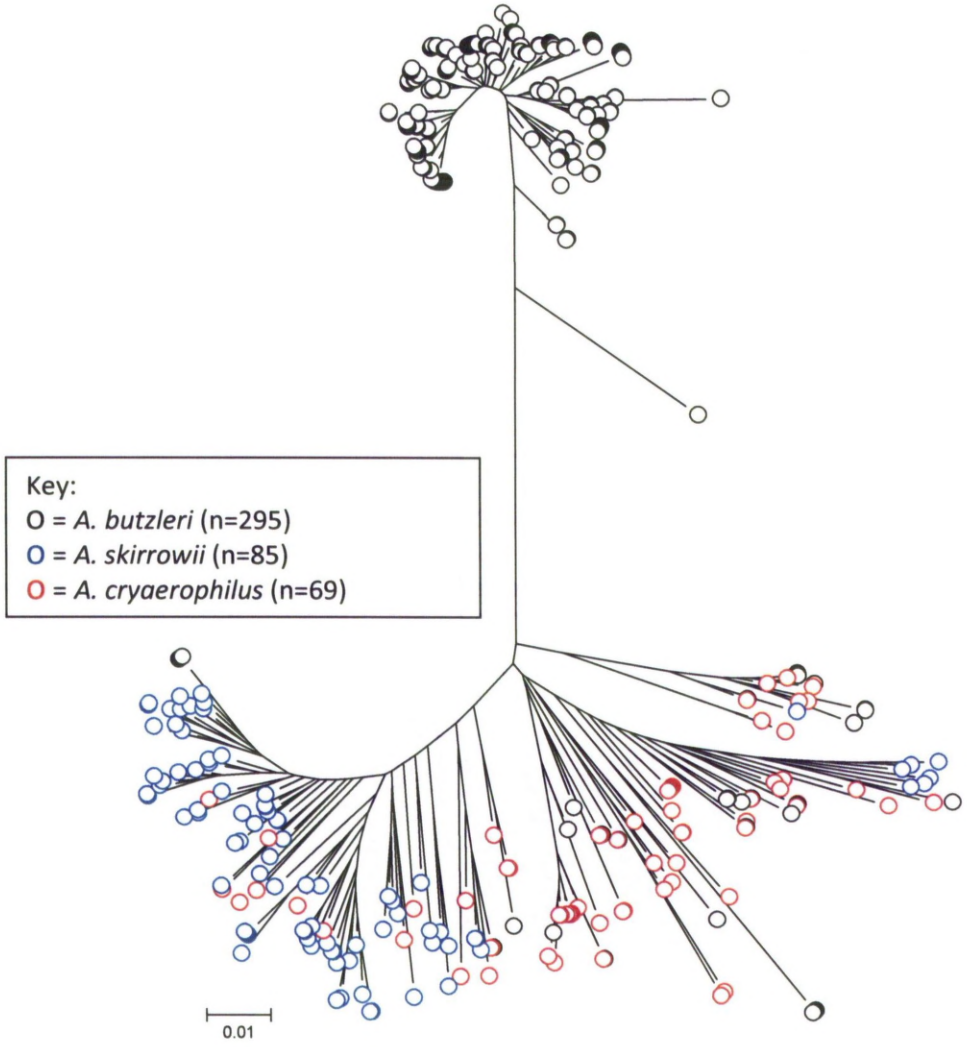

Figure 5.3e. Neighbour-joining tree of all *glyA* alleles obtained (n= 449).

glyA

Clustering on the basis of the *glyA* locus showed a great deal more diversity than the other loci. *A. butzleri* isolates formed a distinct cluster, while *A. skirrowii* and *A. cryaerophilus* isolates formed a second, mixed cluster. However, a number of *A. butzleri* isolates occurred within the *A. skirrowii/A. cryaerophilus* cluster, and a

single *A. butzleri* isolate formed an outlier halfway up the main tree, separate from the main *A. butzleri* cluster, Figure 5.3e.

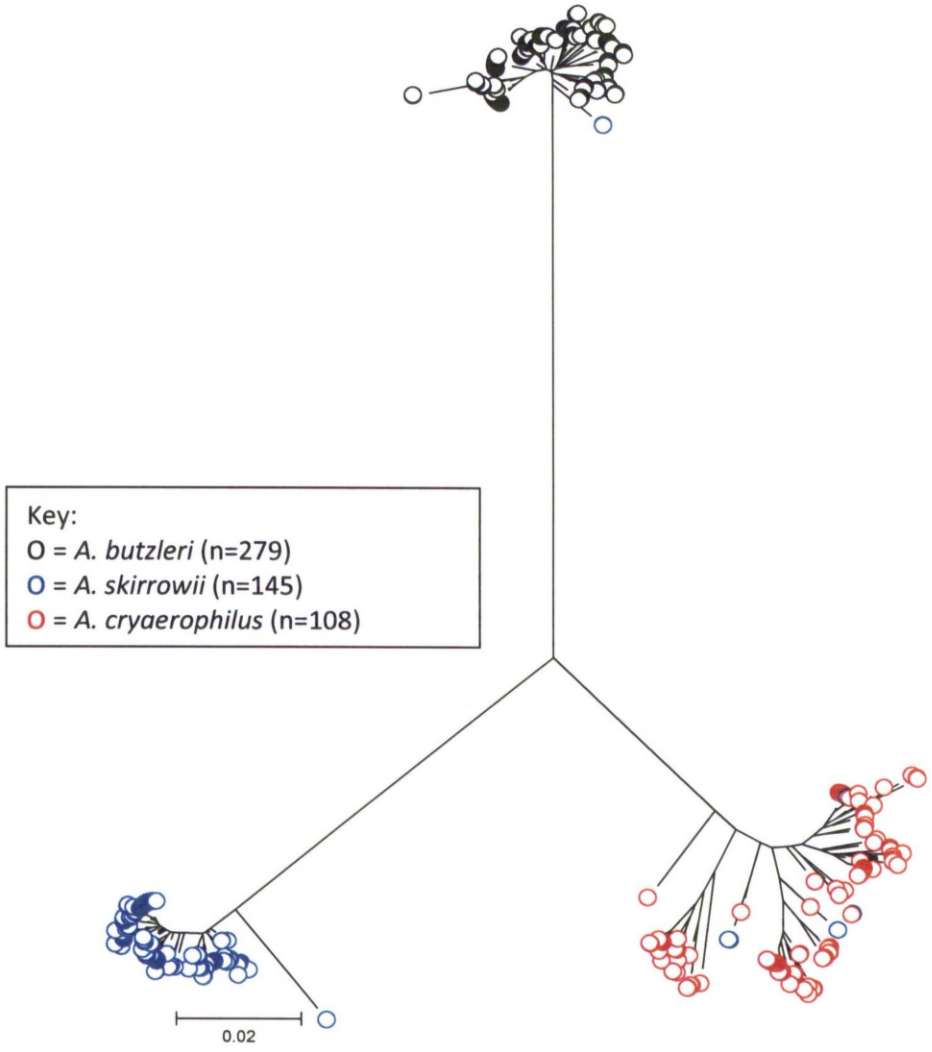

Figure 5.3f. Neighbour-joining tree of all *pgm* alleles obtained (n= 532).

pgm

On the basis of the *pgm* locus, three distinct clusters were formed, with approximately equal distances between them. Small numbers of *A. skirrowii* isolates occurred within both the *A. butzleri* and *A. cryaerophilus* clusters, Figure 5.3f.

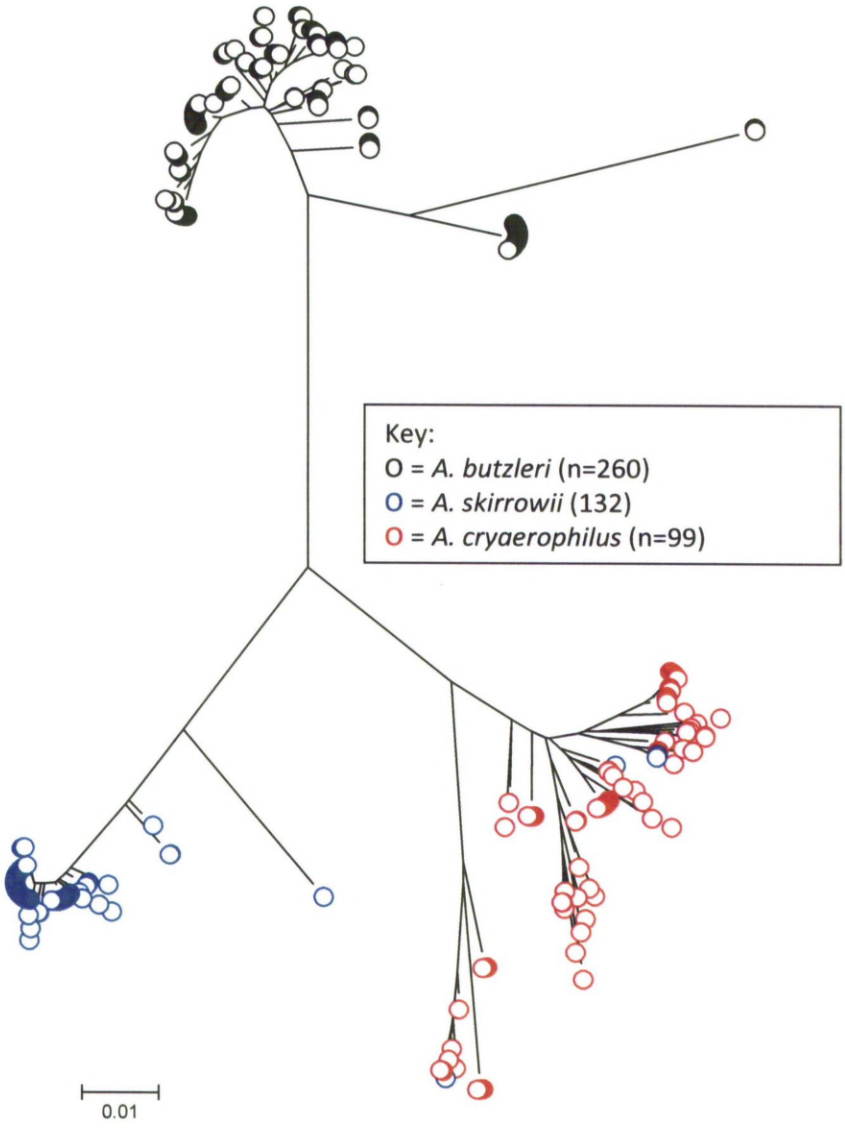

Figure 5.3g. Neighbour-joining tree of all *tkt* alleles obtained (n= 491).

tkt

On the basis of the *tkt* locus, isolates formed three distinct clusters, with some isolates forming outliers. A small number of *A. skirrowii* isolates were found within the *A. cryaerophilus* cluster, Figure 5.3g.

5.3.3b. eBURST analysis of sequence types

eBURST analysis was carried out using the 53 different sequence types identified in this study, all of which belonged to the species *A. butzleri.* The STs formed five groups, with 42 singletons. No founding strain was identified. The eBURST output is summarised in Table 5.4.

Table 5.4. eBURST analysis of the 53 sequence types. No founding strain was identified.

Group 1	3 isolates, 3 STs, predicted founder: multiple candidates				
Isolate	**Frequency**	**Single locus variants**	**Distance**	**Group**	**Subgroup**
31153	1	2	1.0	8%	0%
34063	1	2	1.0	9%	0%
34709	1	2	1.0	16%	0%
Group 2	2 isolates, 2 STs, Predicted founder: none				
Isolate	**Frequency**	**Single locus variants**	**Distance**		
36078	1	1	1.0		
36080	1	1	1.0		
Group 3	2 isolates, 2 STs, Predicted founder: none				
Isolate	**Frequency**	**Single locus variants**	**Distance**		
34439	1	1	1.0		
34427	1	1	1.0		
Group 4	2 isolates, 2 STs, Predicted founder: none				
Isolate	**Frequency**	**Single locus variants**	**Distance**		
30618	1	1	1.0		
35222	1	1	1.0		
Group 5	2 isolates, 2 STs, Predicted founder: none				
Isolate	**Frequency**	**Single locus variants**	**Distance**		
35758	1	1	1.0		
30878	1	1	1.0		
Singletons	42 isolates				
35678 31171 36110 34597 35898 36076 34724 34436 35286 34720 34688 34304 31711 36107 31166 36106 35184 36104 34042 35601 35982 31387 35660 36069 30852 36067 34423 31894 36091 35659 34031 35687 35681 35265 35296 36082 35717 35716 34029 35160 31141 30642					

Allelic sequence data for each of the 53 *A. butzleri* sequence types was concatenated in the order *aspA, atpA, glnA, gltA, glyA, pgm, tkt* and a neighbour-joining tree produced using MEGA version 4.0., Figure 5.4.

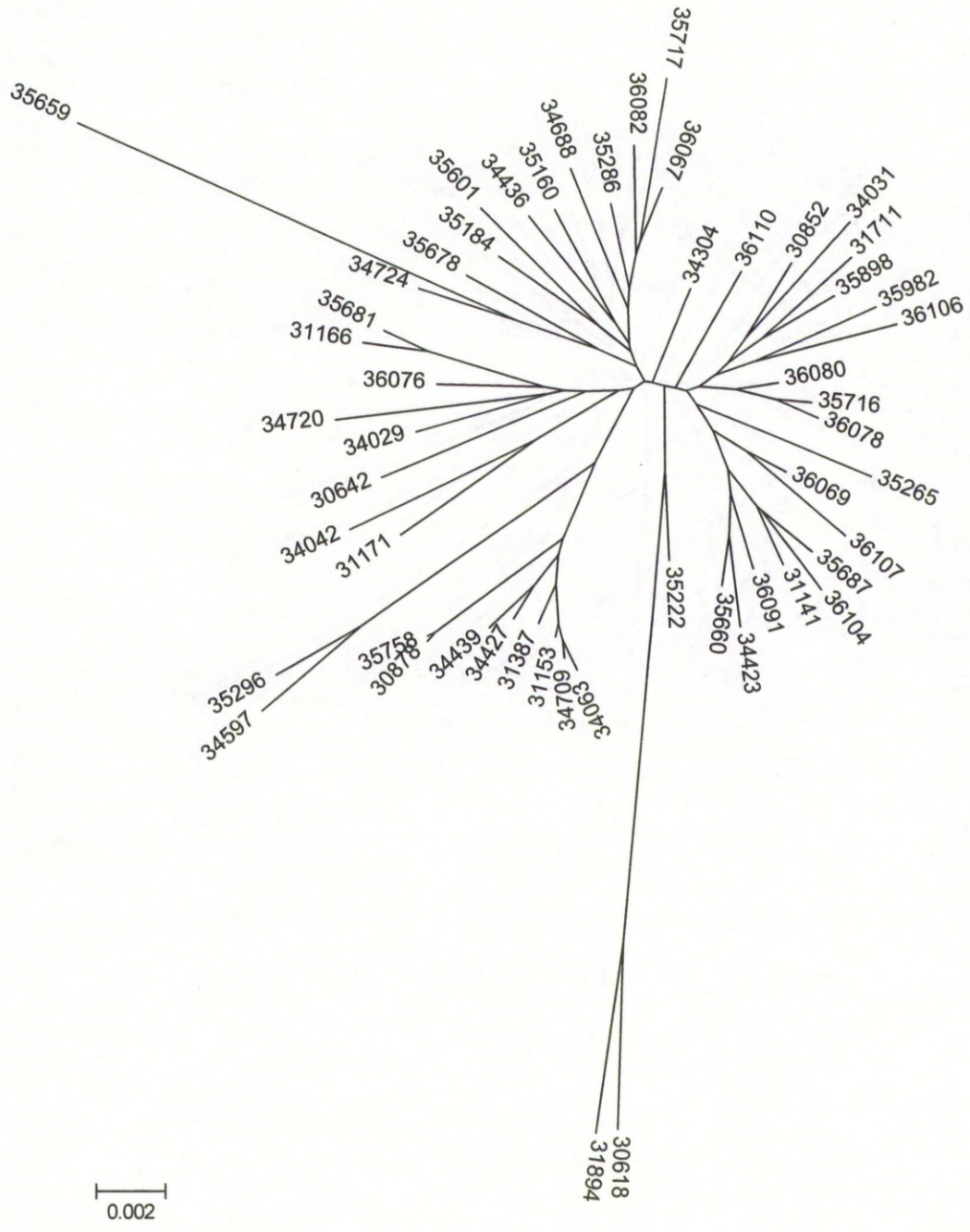

Figure 5.4. Neighbour-joining tree constructed using the concatenated sequence data of the fifty three unique *Arcobacter butzleri* sequence types identified.

5.3.3c. Linkage Disequilibrium

The Maynard-Smith index of association method was used to test linkage disequilibrium, which demonstrates the clonality of a population. The following results were obtained:

Observed variance (Vo)	1.0303
Expected variance (Ve)	0.5493
Index of association (IA)	0.8757
Mean trial variance	0.5506
Max trial variance	0.6977
5% critical value (LMC)	0.6004

The observed variance was greater than the maximum variance obtained in 1000 trials ($p = 0.00$). Significant linkage disequilibrium was detected, showing that the *Arcobacter* population tested is weakly clonal.

5.3.3d. Distribution of Sequence types

Table 5.5. shows the *A. butzleri* sequence types present on each farm. STs found on multiple farms are highlighted in red.

Table 5.5. The distribution of sequence types amongst the six farms sampled. STs found on multiple farms are highlighted in red.

Farm (details)	Number of isolates assigned STs	Number of STs present	STs present
1 (beef, Cheshire)	37	13	18, 294, 301, 302, 303, 311, 333, 340, 343, 345, 346, 352, 353
2 (dairy, Cheshire)	13	6	297, 299, 304, 309, 310, 354
3 (dairy, Wirral)	11	5	18, 298, 300, 341, 347
4 (beef, Wirral)	50	23	18, 138, 293, 296, 306, 308, 327, 328, 329, 330, 331, 332, 335, 336, 337, 339, 342, 344, 348, 349, 351, 356, 357
5 (dairy and sheep, Lancashire)	17	4	292, 295, 305, 307
6 (dairy, Lancashire)	15	3	308, 334, 355

Sequence type 18 was found on three farms in Cheshire, while 308 was found on one farm in Cheshire and one in Lancashire, approximately 100km away. On Farm five, sequence type 292 was found in both sheep and dairy cattle.

In the case of those faecal samples that featured two STs, both STs found in the pat were isolated from other pats on the same farm. From pats 47 and 48 (from which ten isolates each were originally obtained), seven and eight isolates respectively were analysed, yielding STs. Pat 47 was found to contain one ST, and 48 was found to contain two, suggesting that the diversity of isolates per pat is low

In the pilot study (Chapter Three), up to ten isolates were selected from each faecal pat sampled. Table 5.6. shows the number of isolates from each pat analysed using MLST, and the STs present in each.

Table 5.5. The distribution of sequence types amongst the six farms sampled. STs found on multiple farms are highlighted in red.

Farm (details)	Number of isolates assigned STs	Number of STs present	STs present
1 (beef, Cheshire)	37	13	18, 294, 301, 302, 303, 311, 333, 340, 343, 345, 346, 352, 353
2 (dairy, Cheshire)	13	6	297, 299, 304, 309, 310, 354
3 (dairy, Wirral)	11	5	18, 298, 300, 341, 347
4 (beef, Wirral)	50	23	18, 138, 293, 296, 306, 308, 327, 328, 329, 330, 331, 332, 335, 336, 337, 339, 342, 344, 348, 349, 351, 356, 357
5 (dairy and sheep, Lancashire)	17	4	292, 295, 305, 307
6 (dairy, Lancashire)	15	3	308, 334, 355

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In the pilot study (Chapter Three), up to ten isolates were selected from each faecal pat sampled. Table 5.6. shows the number of isolates from each pat analysed using MLST, and the STs present in each.

Table 5.6. Distribution of sequence types in faecal samples.

Faecal sample number	Number of isolates analysed	STs present
4	2	300
7	2	347
12	2	299
20	1	297
25	3	292
30	2	292
31	2	307
32	2	295, 302
33	2	305, 295
34	3	292
35	2	292
47	7	308
48	8	355, 308
50	1	334

Table 5.7. shows the relative abundance of the sequence types identified. ST 308 occurred the most frequently, in 14 isolates.

Table 5.7. The relative frequency of STs amongst the 143 *A. butzleri* isolates assigned a sequence type.

ST	Frequency
297	1
303	1
304	1
305	1
311	1
327	1
328	1
329	1
333	1
334	1
336	1
337	1
338	1
340	1
342	1
344	1
345	1
348	1
349	1
350	1
353	1
356	1
138	2
295	2
299	2
300	2
310	2
330	2
332	2
341	2
347	2
351	2
294	3
298	3
307	3
309	3
335	3
339	3
352	3
355	3
357	3
293	4
306	4
331	4
343	4
354	4
296	5
302	5
346	5
301	6
18	8
292	11
308	14

5.4. Discussion.

The aim of this study was to utilise and compare the results of three methods for the typing of *Arcobacter* isolates from cattle. ERIC-PCR, PFGE and MLST were used. Of these, MLST, a sequence-based typing method, gave the most useful results.

ERIC-PCR is a band-based typing technique which uses PCR to amplify specific areas of enteropathogen DNA, known as repetitive intergenic sequences. In this study, banding patterns were successfully produced using the ERIC-PCR technique of Houf *et al.* (2002). The repeatability of the ERIC-PCR results was tested by carrying out the method twice, using the same isolates each time and comparing the results. The results obtained (Figure 5.1.) were almost identical each time, with minor differences in the brightness of a few bands, suggesting that the technique has good repeatability.

While analysis of the banding patterns revealed a large amount of diversity amongst the 300 isolates, in accordance with a previous study of arcobacters using the same method (Miller *et al.* 2009), the technique appeared to show no grouping of related strains, such as those belonging to the same species. This was unexpected, as grouping together of strains of the same species would usually be expected, even in organisms known to be unusually diverse. Therefore, two *A. butzleri* whole genome sequences were searched for the presence of the ERIC-PCR primer sites. These were not found. The banding patterns were likely to have been produced by random amplification of nonspecific sites, making the technique equivalent to a RAPD method. This is supported by the fact that the annealing temperature for the primers in this technique is very low, 25°C (Houf *et al.*, 2002). Studies using ERIC-PCR and RAPD to type *Arcobacter* isolates have found similarly high levels of heterogeneity

(Houf *et al.*, 2002; Atabay *et al.*, 2002; Aydin *et al.*, 2006; Van Driessche *et al.*, 2007; Ho *et al.*, 2007), however, a significant finding of this study is that, based on whole genome sequence data, it is clear that ERIC-PCR, as developed for *Arcobacter*, is not a true ERIC technique, and is therefore of limited value.

The same 300 isolates were subject to macro-restriction PFGE, and despite using several different restriction enzymes and attempting to optimise the method, no acceptable results were obtained. This is believed to be due to a combination of an inability to retrieve sufficient quantities of cells of some isolates from frozen storage on cryogenic beads, and the inability of the isolates to be suitably digested by the restriction enzymes. The inability to retrieve isolates from frozen storage may be an effect of freeze-thawing or the presence of phenotypic characteristics which prevent the survival of genetic material during freezing, as has been shown to be the case in *Campylobacter* spp. (Murinda *et al.*, 2004). Digestion produced either too few bands to make analysis possible or failed completely, producing smearing. Over 100 isolates were tested in total, without success. It was concluded that the enzymes available were unable to suitably digest the *Arcobacter* DNA, possibly due to nuclease activity, and that the method was unsuitable for the typing of *Arcobacter* isolates from cattle in this instance. PFGE has been successfully used previously to type *Arcobacter* isolates using the same enzymes as were applied in this study (Hume *et al.*, 2001; Rivas *et al.*, 2003; Ho *et al.*, 2006).

Rivas *et al.* (2003) obtained between four and eight fragments for the majority of *A. butzleri* isolates (n=31) from ground meat digested using *Sac*II, *Eag*I and *Sma*I enzymes. However, they reported that four of the isolates failed to produce bands, even after formaldehyde treatment to prevent nuclease activity (Rivas *et al.*, 2003). Ho *et al.* (2006b) also reported the failure of a large number of

isolates (n=10) to be digested with *Eag*I, and acceptable results were obtained for only 50% of the isolates. Formaldehyde treatment was not used in the study (Ho *et al.*, 2006b). Formaldehyde treatment of isolates was not carried out in the present study, due to health and safety considerations due to the hazardous nature of the method. However, the isolates have been archived and remain available for subsequent study.

MLST was carried out on 652 isolates consisting of *A. butzleri* (n=356) , *A. skirrowii* (n=174) and *A. cryaerophilus* (n=122), all isolated from the faeces of cattle (Chapters Three and Four). Overall, 249 complete allelic profiles were obtained, comprising 53 separate sequence types. A total of 3347 alleles were sequenced, out of a possible 4494 (74%). Neighbour-joining trees were calculated using the alleles at each locus (although these alleles were not all assigned STs), and e-BURST analysis of 53 *A. butzleri* STs was completed, Figure 5.4.

The neighbour-joining trees produced using the alleles at each of the seven loci are similar to those of Miller *et al.* (2009) using *Arcobacter* isolates from different sources, including humans with diarrhoea. *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* generally form three distinct clusters, with the *A. butzleri* cluster being more distant from the other two, Figures 5.3a. to 5.3g. For the *gly*A locus, Figure 5.3e., *A. skirrowii* and *A. cryaerophilus* did not form distinct clusters, instead forming one mixed cluster that is separate from the *A. butzleri* cluster, a result similar to that found by Miller *et al.* (2009), and indicating lateral transfer of genes between these species.

In each of the seven trees produced for the different loci, there appear to be isolates that cluster within unexpected species groups. For example, for the *asp*A locus, several *A. skirrowii* isolates were present within both the *A. butzleri* and *A.*

cryaerophilus clusters, Figure 5.3a., and in the *atp*A tree, isolates of all three species appear in all three clusters. A similar unexpected clustering of a small number of isolates occurs in all of the trees calculated in this study.

One possible explanation for these apparently anomalous alleles may be lateral transfer, where genetic material from one species is directly transferred to isolates of another, resulting in the presence of genes from a different species within an isolate. Lateral transfer, particularly between *A. skirrowii* and *A. cryaerophilus* has been found to occur in *Arcobacter* in MLST studies (W. Miller, personal communication). Lateral transfer between *Campylobacter jejuni* and *C. coli*, closely related to *Arcobacter* spp., has also been reported (Dingle *et al.*, 2005; Miller *et al.*, 2005; Wilson *et al.*, 2009). Lateral transfer is unlikely to be the best explanation in all cases in this study, as lateral transfer tends to occur very infrequently (W. Miller, personal communication). Some isolates may have been incorrectly identified at the species level, particularly as some doubts have been raised about the reliability of the standard *Arcobacter* speciation PCR assay (Figueras *et al.*, 2008).

Due to the failure of all isolates to be assigned a sequence type, it is not possible to determine whether the anomalous alleles in each tree belong to the same isolates. The presence of alleles from the same isolates being present in the 'wrong' species cluster at multiple loci might suggest that the isolate had apparently been wrongly identified.

All isolates in this study were identified to species level using the multiplex PCR assay of Houf *et al.* (2000), but this method has recently been shown to be inaccurate, for example, it incorrectly identifies *A. halophilus* isolates as *A. skirrowii* and *A. cryaerophilus*, and fails to distinguish *A. skirrowii* and *A. nitrofigilis* (Figueras *et al.*, 2008). At the time of the PCR assay's design, only three species of

Arcobacter had been identified, and little was known about the genus. The PCR primers were designed based on limited information and as such are now outdated. Newer, more reliable identification techniques are now being developed (Figueras *et al.,* 2008). As a result, it is possible that in some of the cases in this study, isolates were incorrectly assigned to species. As the Houf PCR assay was apparently the best method of identification to species level in this study, it may not be possible to rely completely on its outcome. The isolates have been archived and are available for use in subsequent studies.

Due to the lack of biochemical activity in *Arcobacter* spp., phenotypic tests for the presence of *Arcobacter* are generally unreliable (Oporto *et al.*, 2007; Hamill *et al.*, 2008). In addition, phenotypic methods can be cumbersome and time consuming, with large amounts of time and effort often being required to obtain a small number of isolates (Aydin *et al.*, 2006). Phenotypic methods are therefore usually discounted in favour of faster, more accurate molecular methods and specific isolation techniques for the detection and characterisation of *Arcobacter* isolates (Hamill *et al.*, 2007).

The *Arcobacter* MLST scheme features a PCR amplification stage, with oligonucleotide primers that were designed to work specifically in the genes of individual *Arcobacter* species. This suggests that any isolates that had been incorrectly assigned to species using the PCR of Houf *et al.* (2000) would fail at the MLST PCR stage, and thus would not be included in the MLST results. However, anomalous alleles were obtained. Of the seven loci used in the *Arcobacter* MLST scheme, oligonucleotide primers used for the *pgm* locus are the most species-specific (W. Miller, personal communication), and primers used for the *atp*A locus are the least. This means *atp*A is the locus most likely to feature incorrectly identified

alleles, and *pgm* the least likely. In this study, both loci feature a similar number of anomalous alleles, Figures 5.3b., 5.3f., suggesting that many of the anomalous alleles are most likely to have arisen due to misidentified isolates.

One way to further investigate anomalous alleles would be to carry out an orthologous identification method, such as a 16S rRNA-related PCR identification method, e.g. AFLP, to define the true species of the isolates.

e-BURST analysis of the 53 sequence types revealed that no single predicted founding strain was present in the dataset. The STs formed five groups, with 42 singletons, demonstrating the large amount of genetic diversity within the isolates. A neighbour-joining tree was constructed using the concatenated allele data of isolates representing the 53 sequence types, Figure 5.4. The tree shows a similar level of relatedness between most of the sequence types, with three isolates; 35659, 30618 and 31894 occurring at greater distances from the rest of the group, indicating greater diversity. Of these three outliers, isolates 30618 and 31894 (STs 347 and 334 respectively) appear closely related, since they diverge at a greater distance from the remainder of the group. These two isolates originated in cattle on very geographically distant farms, 30618 was isolated from a dairy farm in Cheshire, while 31894 came from a dairy farm in North Lancashire. ST 334 occurs only once, in isolate 31894, and ST 347 occurs twice, with both isolates obtained from the same animal. The third outlier, isolate 35659 (ST 336) was obtained from a beef cattle farm in Cheshire and occurs only once.

Index of association testing by the Maynard-Smith method revealed significant linkage disequilibrium within the dataset, indicating that the population is weakly clonal, a feature widely reported in *C. jejuni* (Dingle *et al.*, 2001; Manning *et al.*, 2003; Suerbaum *et al.*, 2001). A weakly clonal population is thought to consist

of complexes or lineages, made up of isolates that are linked by a common ancestor (Holmes *et al.*, 1999), hence clonal complexes and lineages may be identified in *Arcobacter* once more isolates have been typed.

The *Arcobacter* MLST database was created in 2009, and contains 357 sequence types and 593 isolates (as of February 2010); small numbers compared to the *Campylobacter jejuni* and *coli* database (as of February 2010). Clonal complexes have not yet been identified in *Arcobacter*, and associations of STs with sources or locations have not been established.

For the isolates investigated in this study no apparent associations of STs with location were identified. Of the 53 STs present, only two were found on more than one farm, with the remaining 51 STs being found only on one farm each, possibly as a result of the number of isolates being relatively small. ST 18 was found in one animal on farm 3, three animals on farm 1 and one animal on farm 4, all of which are farms in Cheshire. ST 308 was found in two animals on farm 6, in Lancashire, and two animals on farm 4, in Cheshire. A full list of the alleles and STs for each isolate can be found in Appendix V.

Of the 300 isolates from the pilot study (Chapter Three), 49 isolates from fourteen faecal pats were successfully analysed using MLST. Three of the pats contained two sequence types, while the rest featured one. The same STs were found in several animals on each farm. On average, each ST was present in two to five isolates from different animals, showing a very high level of genotypic diversity. This appears to be characteristic of *Arcobacter* spp., as demonstrated in previous typing studies (Miller *et al.*, 2009; Gonzalez *et al.*, 2007; Aydin *et al.*, 2007; Son *et al.*, 2006; Atabay *et al.*, 2006; On *et al.*, 2004).

The presence of a large amount of diversity in *Arcobacter*'s housekeeping genes may be due to horizontal gene transfer or point mutations (Miller *et al.*, 2009). No clustering of STs associated with geographical location or animal host was observed. MLST analysis of a much larger collection of isolates from a larger variety of sources might lead to the identification of clonal complexes, as in *C. jejuni* and *C. coli* (Dingle *et al.*, 2005).

In conclusion, this study has shown that considerable genetic diversity occurs in the *Arcobacter* isolates obtained from cattle in this study. Of the three methods studied, the MLST scheme of Miller *et al.* (2009) appears to be of the greatest value, providing a level of sensitivity, repeatability and portability not achieved by PFGE or ERIC-PCR. With modifications, particularly formaldehyde treatment for nuclease activity and thiourea treatment to prevent DNA damage, PFGE may prove to be a useful tool in *Arcobacter* typing, although lacking the repeatability, portability and sensitivity of MLST. Based on the results from this study, ERIC-PCR should not be relied upon as an effective typing method for *Arcobacter* spp. Future work should include typing, preferably by MLST, of a much larger dataset, including isolates from different sources, so that possible associations of particular strains with foodborne infection can be investigated.

Chapter Six

An *Arcobacter butzleri* Whole Genome Sequence.

6.1. Introduction.

Arcobacter spp. are emerging human foodborne pathogens that have been isolated from numerous types of food and water, animals and environmental sources. Of the *Arcobacter* spp. identified to date, *A. butzleri* is most commonly associated with human disease, and as a result, it has been the species most frequently studied. A number of molecular typing methods have been used to study the genotypic diversity of *A. butzleri*, with sequencing-based methods such as multi-locus sequence typing (MLST, Miller *et al.*, 2009) becoming increasingly popular. An important issue in the study of any potential human foodborne pathogen is that of the disease-causing mechanisms involved in its pathogenicity, which has become more feasible with the advent of whole genome sequencing, especially with the emergence of high throughput sequencing methods (Van Putten *et al.*, 2009).

Whole genome sequencing allows the detailed analysis of genetic information, enabling identification of features throughout the genome. With the advent of faster, improved sequencing methods in recent years, whole genome sequencing of microorganisms has become a fast, reliable way to investigate bacterial strains in detail as well as allowing detailed comparison with other isolates (Yang *et al.*, 2009; Hall, 2007).

In 2007 Miller *et al.* published the first *A. butzleri* genome, obtained using high throughput sequencing. The isolate used, RM4018, originated from a human in the USA showing clinical signs of disease. The genome was found to have greater similarity to the *Helicobacteraceae*, *Shewanella denitrificans* and *Wolinella succinogenes,* than to the *Campylobacteraceae*. A large proportion of the genome was found to be dedicated to genes coding for mechanisms involved in environmental sensing and survival, such as two-component sensing systems, and

the genome featured many genes associated with organisms that are non-host-associated, supporting previous suggestions that *Arcobacter* is predominantly a free-living, water-borne organism. The identification of virulence-associated genes supports the classification of *A. butzleri* as an emerging human pathogen (Miller *et al.* 2007).

As well as the human isolate RM4018, a set of twelve additional *A. butzleri* isolates from a variety of sources, Table 6.1., were subjected to a DNA hybridisation microarray in the same study. Probes for 2238 genes were featured on the microarray which was developed based on the RM4018 whole genome sequence. The RM4018 DNA was used as a reference and was competitively hybridised with the other twelve strains. Comparative genomic indexing of the twelve strains tested resulted in the compilation of a set of 1676 core genes (i.e. those which were present in all 13 strains tested) and revealed high level of divergence between the 13 isolates, none of which were of bovine origin. A total of 42 coding sequences (CDS; areas of sequence which encode a functional protein) were present only in strain RM4018.

Table 6.1. The sources and geographical locations of 13 *Arcobacter butzleri* isolates compared by Miller *et al.* (2009). The entire genome of strain RM4018 was sequenced.

Isolate name	Source	Location
RM4018	Human diarrhoeal stool	USA
RM1588	Chicken	USA
RM1591	Turkey carcass	USA
RM4128	Human stool	South Africa
RM4462	Human stool	USA
RM4467	Primate rectal swab	USA
RM4596	Turkey	USA
RM4843	Chicken carcass	USA
RM4850	Horse	USA
RM5516	Pig	USA
RM5538	Human stool	USA
RM5541	Human stool	USA
RM5544	Human stool	Thailand

Numerous studies have shown that while a single reference genome for a species is useful, a single genome may give only a limited "snapshot" of the real make up of the species (Hall, 2007; Tettelin *et al.*, 2008). With a genus that is particularly heterogeneous, as *Arcobacter* has been shown to be through genotyping studies, multiple genomes will be required to give a more representative view of the species. This study aimed to determine an *A. butzleri* whole genome sequence from a UK cattle isolate and subsequently compare it with that obtained by Miller *et al.* (2007) by using bioinformatics methods to identify genes present in the genome and through laboratory testing using phenotypic and genotypic methods.

6.2. Materials and Methods.

A. butzleri strain 7h1h was obtained from a clinically healthy dairy cow in Cheshire, UK during 2007. The isolate was from a clearly isolated individual colony from which DNA was originally extracted using the boiled lysate method (see Chapter Two, Materials and Methods, section 2.4.). The isolate was subjected to both an *Arcobacter* genus-specific 16S rRNA PCR assay (Gonzalez *et al.*, 2000) and a species-specific PCR assay (Houf *et al.*, 2000) and was found to be of the species *A. butzleri*. DNA was extracted again from a frozen culture using the chelex method (Chepter Two, Section 2.5.) and tested again using PCR. The presence of clear, bright and well-defined single bands after PCR amplification, and an absorbance ratio of 260/280nm, confirmed the DNA extract of this isolate to be of sufficient quality for whole genome sequencing.

6.2.1. 454 Sequencing and annotation

High quality DNA was extracted using a QIAamp mini DNA extraction kit (Qiagen, UK) and sent to the Liverpool Centre for Genomics Research where 454 sequencing was performed using the 454 genome sequencer FLX, following the manufacturer's instructions (Roche 454 Life Sciences, Basel, Switzerland). Briefly, the technique consisted of creating fragment libraries by fragmentation, attachment of adapter sequences, refinement of the ends and selection of adapted molecules. Paired-end libraries were then produced using hydroshear shearing, circularisation, addition of adapters and selection as for the fragment library. Libraries were amplified by emPCR and beads containing the fragments were recovered and enriched. Sequencing primers were added and libraries were deposited onto a PicoTiterPlate plate and sequenced. The resulting reads were assembled to form a

pseudogenome using 454s Newbler assembler (Version version 2.0.01.12), using the published RM4018 genome as a guide. GLIMMER version 3.02 (http://www.cbcb.umd.edu/software/glimmer/) was used to identify putative ORFs and an in-house PERL script was used to identify and merge ORFs that were likely to have been split during the sequencing process. The resulting pseudogenome was then annotated using bioinformatics tools, a process in which each individual ORF, gene or coding region (n=2420) is separately investigated using online searches and computer programmes, and ultimately assigned a name and function. Putative functions were assigned to each gene using ARTEMIS (Rutherford *et al.*, 2000) and ACT: ARTEMIS Comparison Software (Carver *et al.*, 2005) and the BLAST online tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The pseudogenome, along with the genome of RM4018 was also uploaded onto the curated online SEED database (www.rast.mnpdr.org), which features annotation and comparison facilities using Rapid Annotation using Subsystems Technology (RAST). Using this software, the 7h1h pseudogenome was automatically annotated and compared with that of RM4018.

6.2.2. PCR Screening for specific genes

After annotation of the genome sequence ten predicted coding sequences, including five that were absent from 7h1h when compared to RM4018, and five of the genes previously identified as "core genes" by Miller *et al.* (2007), were selected for the PCR screening of a panel of ninety-two additional *A. butzleri* isolates, selected from four previous studies in the UK. The screening panel included isolates obtained from humans, cattle, sheep, badgers, birds, rabbits, wood mice and water, all of which were archived isolates at the University of Liverpool, with the exception of the human-derived isolates, which were provided by W. Miller, USDA, Albany,

California, USA. The details of the areas selected and their functions are shown in Table 6.2. All primer sets were designed to test for the presence of a given gene by amplifying a part of it.

Table 6.2. Details of the regions of genomic plasticity (RGPs) selected for PCR screening, including the location of each on the original genome, and the function of each gene.

Gene Locus Tag	RGP type (Insertion, deletion or divergent)	Location on Genome	Putative Function
Abu 987	Deleted from 7h1h	990377 – 991738 (RM4018)	Transcriptional regulator, GntR family
Abu 1814	Deleted from 7h1h	1815547 – 1816881 (RM4018)	O-antigen polymerase
Orf 2356	Inserted into 7h1h	2146785 – 2148164 (7h1h)	Glycerol phosphotransferase
Orf 1258	Inserted into 7h1h	1122911 – 1125052 (7h1h)	Toxin secretion ABC transporter (ATP-binding and membrane protein); hlyB-like protein
Orf 739	Inserted into 7h1h	686093 – 687121 (7h1h)	Glycosyl transferase, group 1
GlsA (Abu 2331)	Deleted from 7h1h	2337847 – 2338761 (RM4018)	Glutaminase A
Abu 991	Deleted from 7h1h	994469 – 996028 (RM4018)	AcrB/AcrD/AcrF family protein
Abu 1030	Deleted from 7h1h	1031657 – 1039207 (RM4018)	Hypothetical membrane protein
Orf 1448	Inserted into 7h1h	1305851 – 1308048 (7h1h)	Histidine kinase
Orf 1254	Inserted into 7h1h	117348 – 1119690 (7h1h)	Exonuclease

All isolates had been previously identified as *A. butzleri*, *A. skirrowii* or *A. cryaerophilus* using the PCR assay of Houf *et al.* (2001), apart from three isolates, which gave an *Arcobacter* genus amplicon using the PCR assay of Gonzalez *et al.* (2002), and were identified as *Arcobacter* spp. based on universal 16S sequencing (N.J. Williams, personal communication), however these isolates did not yield specific amplicons using the PCR assay of Houf *et al.*, (2001). Isolates stored on cryogenic beads (ProLab, Neston) were recovered on Columbia agar with 5% defibrinated horse blood and DNA was extracted using the Chelex 100 method (Chapter Two, Section 2.5.). All extracted DNA was stored at 4°C prior to use.

Ten sets of oligonucleotide primers were designed using BatchPrimer3 (http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi), based on the genome sequences of *A. butzleri* isolates 7h1h and RM4018. The primer sequences and product sizes are shown in Table 6.4. 25µl PCR reactions contained 0.5µl 20mM dNTP mix, 2.5µl 10x PCR buffer, 1.5mM $MgCl_2$, 1U *Taq* polymerase and 1µl template DNA. Reactions for Abu987, ORF2356, ORF1258 and Abu991 were carried out with an annealing temperature of 59°C, while reactions for Abu1814, *gls*A and Abu1030 had annealing temperatures of 65°C. Cycle conditions were as follows:

Table 6.3. Cycling conditions of the *Arcobacter* specific gene screening PCR assay.

Initial denaturation	94°C	5 minutes
30 cycles	94°C	30 seconds
	59°C/65°C	30 seconds
	72°C	1 minute
Final elongation	72°C	7 minutes

Table 6.4. Oligonucleotide primers designed to detect ten selected regions of genomic plasticity, and their resulting product sizes.

Primer Names	Sequence (5' – 3')	Product size (bp)
Abu987F Abu987R	GCAGGAACAAAACTGCCTTC CATCATTTTCTTTTGCCCAAT	703
Abu1814F Abu1814R	TGGATAGTGCATATGCTTTTATGA CATCACCAGTTCCAACACCA	678
Orf2356F Orf2356R	TTAGCCCCTCATTCGCCTAT AACTCCATGCCACAATTGAA	600
Orf1258F Orf1258R	TGGTGTTGCAAATCCAATCT GCCAATTTGGATCTATTGTCG	704
Orf739F Orf739R	CAAGGGGTGGAGAACTAGCA CTTCCATAACGCTTCGTGGT	708
GlsAF GlsAR	TTCCAGCTCTTGCAAATGTAAA ACCGCTTTTTCCAGGAAGTC	695
Abu1030F Abu1030R	GGGCACCAAACAATGCTTAT AGCAAGTGTTGCTGTTGCAC	692
Orf1448F Orf1448R	GGCTCAAAAGGATACAATCCA AAACCAATTCCTATCCCATCTTC	683
Orf1254F Orf1254R	ACGGGTGCTGGGAAAAGTA TCTTTTTCAAAACTGATTTGCTCA	695
Abu991F Abu991R	TGTGGATTTAAAAGGCGAAAA CCAAGTGAAACAATCAAAGCAA	702

Agarose gel electrophoresis was used to visualise the PCR products (Chapter Two, Section 2.7.).

6.2.3. Omnilog analysis

As well as the annotation of the sequence by assigning gene names and functions using bioinformatics methods, both strain 7h1h and the USA strain RM4018 (courtesy of Bill Miller, USDA, USA) were subjected to analysis using the BIOLOG Omnilog system. This was carried out in collaboration with the Veterinary Laboratories Agency in Weybridge, Surrey (M. Woodward and M. Abuoun). Selected phenotypic characteristics of the two strains were determined and compared.

The Omnilog system is a fully automated microbial identification and characterisation system developed by Biolog, CA, USA. Using 96-well plates, microbial samples are incubated under differing conditions and the growth curve monitored using phenotype microarray technology and recorded in real-time. The system is made up of numerous plates, each of which is used to test a particular phenotypic feature such as optimum pH, salt tolerance and the ability to utilise a specific carbon source. Each well contains a different growth medium into which the sample is inoculated at a set turbidity. The plates are incubated over a set period and the microbial growth in each well monitored, producing growth curves for each well. Table 6.5. shows the range of plates available, and the features tested by each (See also Appendix VI).

Table 6.5. Phenotypic features tested by Biolog Omnilog plates 1 – 10.

Plate Name	**Tests**
PM01	Carbon sources
PM02A	Carbon sources
PM03B	Nitrogen sources
PM04A	Phosphorus and sulphur sources
PM05	Nutrient supplements
PM06	Peptide nitrogen sources
PM07	Peptide nitrogen sources
PM08	Peptide nitrogen sources
PM09	Osmolytes
PM10	pH

In this study, the phenotypic characteristics shown in Table 6.5 of isolates 7h1h and RM4018 were compared using this system. Plate PM05 was first used to assess the growth of the two *Arcobacter* strains, using sodium succinate/ferric citrate as a carbon source. After both strains were grown successfully, plates PM02 (to test

usage of carbon sources), PM03 (to test usage of nitrogen sources) and PM05 were then used for tests over a period of 96 hours, in order to monitor the growth in different conditions over a longer period. Lastly, phenotypic testing using plates PM01-PM10 was carried out, using sodium succinate/ferric citrate as a carbon source, for the standard time of 72 hours.

6.3. Results.

6.3.1. General Results.

A 2,223,498 bp pseudogenome was obtained for strain 7h1h. It was found to feature 2420 predicted open reading frames (ORFs). No plasmids were identified. A complete list of the ORFs identified along with their predicted functions and levels of similarity to published ORFs can be found in Appendix IX). Table 6.6. shows a summary of the subsystem features identified in the 7h1h genome using the RAST genome annotation tool (Aziz *et al.*, 2008).

Table 6.6. A summary of the subsystem features identified by the RAST annotation program.

Subsystem Feature	Number of Predicted ORFs of Each Subsystem Feature Present in 7h1h Genome	Number of Predicted ORFs of Each Subsystem Feature Present in RM4018 Genome
Phages, prophages and transposable elements	5	0
Cofactors, vitamins, prosthetic groups, pigments	67	110
Cell wall and capsule	54	61
Potassium metabolism	9	8
Photosynthesis	0	0
Plasmids	0	0
Miscellaneous	2	2
Membrane transport	12	6
RNA metabolism	21	40
Nucleosides and nucleotides	26	36
Protein metabolism	67	130
Cell division and cell cycle	18	32
Motility and chemotaxis	12	12
Regulation and cell signalling	8	8
Secondary metabolism	0	0
DNA metabolism	18	14
Virulence	29	39
Fatty acids, lipids and isoprenoids	39	46
Nitrogen metabolism	16	16
Dormancy and sporulation	0	0
Respiration	49	51
Stress response	39	41
Metabolism of aromatic compounds	1	5
Amino acids and derivatives	104	179
Sulfur metabolism	11	11
Phosphorus metabolism	3	20
Carbohydrates	48	59
Total	**658**	**926**

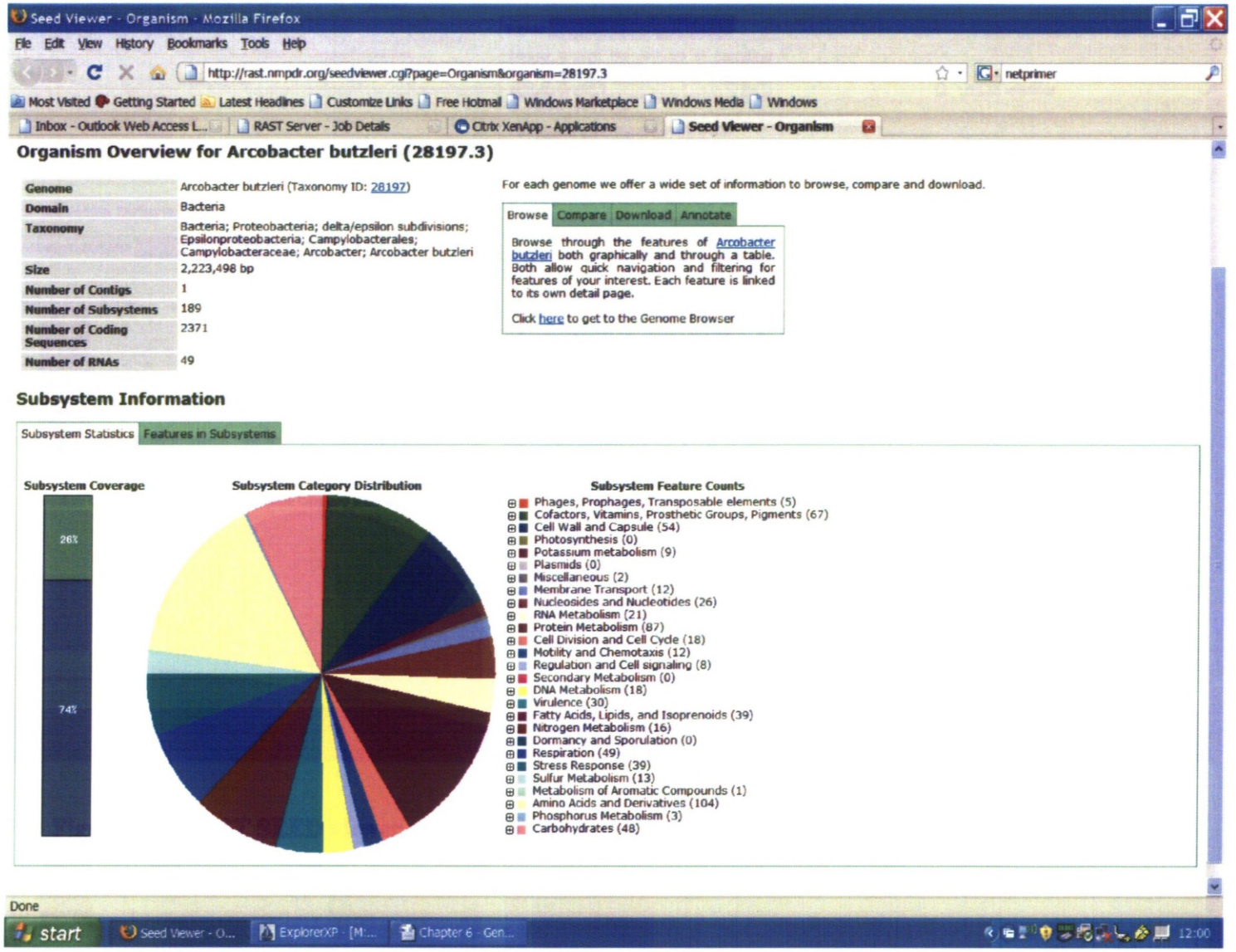

Figure 6.1. RAST SEED viewer screenshot, showing an overview of the predicted subsystems of 7h1h.

After comparison with the USA strain, RM4018, 502 regions of genomic plasticity (RGP) were identified. These regions were either insertions (open reading frames present in 7h1h but absent from RM4018), deletions (ORFs absent from 7h1h but present in RM4018) or divergent when compared to RM4018. The RGPs consisted of 326 insertions, 108 deletions and 68 divergent ORFs, giving an overall difference between the two genomes of approximately 20%. Of the 1676 "core" genes identified by Miller *et al.* (2007), 154 (9.2%) were absent from strain 7h1h, Table 6.7.

Table 6.7. "Core" genes present in RM4018 but absent from the 7h1h genome, n=154.

Name of Gene Absent from 7h1h (where applicable)	Name of Region on RM4018 Genome	Putative Function
rpsI	AB0098	50S ribosomal protein L13
appA	AB0099	Oligopeptide ABC transporter, periplasmic substrate-binding protein
	AB0100	Conderved hypothetical protein, HAD-superfamily hydrolase
	AB0109	Hypothetical protein
	AB0110	ABC transporter, periplasmic substrate-binding protein, putative
	AB0129	Conserved hypothetical protein
lrgA	AB0179	LrgA family protein
aas	AB0190	2-acylglycerophosphoethanolamine acyltransferase/acyl-acyl carrier protein synthetase
glnD	AB0191	Protein-P-II Uridylyltransferase
	AB0192	Conserved hypothetical protein
	AB0225	Conserved hypothetical protein
gatC	AB0226	Glutamyl-tRNA (Gln) amidotransferase, subunit C
tgt	AB0227	Queuine tRNA-ribosyltransferase
phnA	AB0263	Phosphonoacetatehydrolase
	AB0264	Conserved hypothetical protein, predicted metal-dependent hydrolase
dnaX	AB0283	DNA polymerase III, gamma and tau subunits
	AB0491	Amidohydrolase family protein
	AB0562	Probable sodium/hydrogen antiporter
	AB0595	Conserved hypothetical protein
	AB0596	Hypothetical TPR repeat protein

	AB0630	Two-component response regulator
	AB0631	Two-component sensor histidine kinase
	AB0653	Conserved hypothetical protein
	AB0723	Hypothetical protein
	AB0724	Two-component sensor histidine kinase
	AB0725	Two-component response regulator
	AB0739	Conserved hypothetical protein
	AB0749	2-hydroxy-6-oxohepta-2,4-dienoate hydrolase
alr	AB0789	Alanine racemase
uvrC	AB0790	Excinuclease ABC, subunit C
	AB0820	Hypothetical protein
	AB0887	Hypothetical protein
	AB0898	Oxidoreductase, short-chain dehydrogenase/reductase family
	AB0901	Cytochrome c family protein
	AB0937	Cobalt ABC transporter, permease protein, putative
	AB0951	Conserved hypothetical protein
	AB0961	Hypothetical protein
	AB0985	Acetyltransferase, GNAT family
	AB0987	Sigma factor regulatory protein, FecR/PupR family
	AB0990	Transcriptional regulator, GntR family
guaB	AB1026	Inosine-5-monophosphate dehydrogenase
	AB1027	Conserved hypothetical protein
	AB1028	Hypothetical protein
	AB1029	Two-component sensor histidine kinase
	AB1030	Two-component response regulator
	AB1031	Outer membrane efflux protein, putative
	AB1032	Conserved hypothetical protein
	AB1036	DnaJ domain protein
	AB1037	DnaJ domain protein
	AB1038	Glycosyl hydrolase
	AB1050	Conserved hypothetical protein (DUF24 domain protein)
	AB1051	Conserved hypothetical protein
lig	AB1071	DNA ligase
	AB1072	Glutathionylspermidine synthase family protein
	AB1114	Hypothetical protein
	AB1115	Two-component response regulator
	AB1133	Conserved hypothetical integral membrane protein
	AB1134	MiaB-like tRNA modifying enzyme
	AB1136	Conserved hypothetical protein
	AB1139	Conserved hypothetical protein
	AB1169	Two-component sensor histidine kinase
prlC	AB1203	Oligopeptidase A
hemK	AB1204	Modification methylase
hemN1	AB1205	Oxygen-independent coproporphyrinogen III oxidase
nudH	AB1206	(Di)nucleoside polyphosphate hydrolase
lysC	AB1207	Aspartokinase
	AB1208	Conserved hypothetical protein
holB	AB1209	Putative DNA polymerase III delta prime subunit HolB

lpxK	AB1254	Lipid A biosynthesis protein LpxK
	AB1259	DnaJ domain protein
	AB1271	Conserved hypothetical protein
dltA	AB1286	D-alanine activating enzyme
dltB	AB1287	D-alanyl transfer protein
	AB1324	Hypothetical protein
	AB1325	Conserved hypothetical protein
	AB1326	Hypothetical protein
mraW	AB1327	S-adenosyl-methyltransferase
	AB1377	Conserved hypothetical protein
ftsZ	AB1385	Cell division protein FtsZ
ftsA	AB1386	Cell division protein FtsA
	AB1387	Conserved hypothetical membrane protein
	AB1404	Hypothetical protein
	AB1405	Hypothetical protein
hypC	AB1415	Hydrogenase expression/formation protein HypA
	AB1429	Hypothetical protein
	AB1430	Conserved hypothetical protein (DUF1504 domain protein)
	AB1464	Conserved hypothetical protein (DUF125 domain protein)
	AB1467	Conserved hypothetical protein
aceE	AB1480	Pyruvate dehydrogenase E1 component
aceF	AB1481	Dihydrolipoamide acetyltransferase
lpdA	AB1482	Dihydrolipoamide dehydrogenase
	AB1514	Two-component response regulator
	AB1515	Mn2+ and Fe2+ transporter, NRAMP family
	AB1516	Heavy-metal transporting ATPase
	AB1517	Heavy-metal transport protein, MerT homolog
	AB1518	Transglutaminase family protein
	AB1519	Putative FdhC protein
fdhB2	AB1520	Formate dehydrogenase, iron-sulfur subunit FdhB
	AB1565	Conserved hypothetical membrane protein (DUF6)
	AB1566	Conserved hypothetical protein
	AB1572	Conserved hypothetical protein
	AB1646	Two-component sensor histidine kinase
purH	AB1647	Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
purL	AB1648	Phosphoribosylformylglycinamidine synthase II
	AB1649	Conserved hypothetical protein
	AB1650	Peptidase, M23/M37 family
folE	AB1651	GTP cyclohydrolase I
corA	AB1652	Magnesium and cobalt transport protein
ctsF	AB1653	Campylobacter transformation system protein CtsF
ctsE	AB1654	Campylobacter transformation system protein CtsE
	AB1707	Hypothetical protein
	AB1708	Hypothetical protein
era	AB1709	GTP-binding protein Era homolog
	AB1711	Mg chelatase-related protein

	AB1712	Hypothetical protein
	AB1713	Polypeptide deformylase
clpP	AB1714	ATP-dependent Clp protease, proteolytic subunit
tig	AB1715	Trigger factor
	AB1716	Conserved hypothetical protein
	AB1718	Conserved hypothetical protein
nspC	AB1719	Carboxynorspermidine decarboxylase
	AB1749	Conserved hypothetical protein, putative cytochrome
	AB1750	Two-component response regulator
	AB1751	Acetyltransferase, GNAT family
	AB1752	Putative nickel transporter
	AB1774	Ferredoxin-like protein
waaE	AB1807	ADP-heptose synthase
gmhA	AB1808	D-sedoheptulose 7-phosphate isomerase
waaF	AB1810	Lipopolysaccharide heptosyltransferase II
	AB1811	Conserved hypothetical protein
	AB1812	Putative heptosyltransferase
	AB1813	Conserved hypothetical protein
	AB1814	Conserved hypothetical membrane protein
	AB1817	Putative acetyltransferase
cspA	AB1875	Cold-shock protein, DNA-binding
	AB2104	tRNA pseudouridine synthase
	AB2105	Conserved hypothetical protein
	AB2106	Conserved hypothetical protein
ctpA	AB2107	Carboxyl-terminal protease family protein
purC	AB2108	Phosphoribosylaminoimidazole-succinocarboxamide synthase
purS	AB2109	Phosphoribosylformylglycinamidine synthetase
purQ	AB2110	Phosphoribosylformylglycinamidine synthase I
	AB2111	Conserved hypothetical protein
	AB2155	Conserved hypothetical protein
metY	AB2156	O-acetylhomoserine sulfhydrylase
recJ	AB2247	Single-stranded DNA-specific exonuclease
	AB2248	Hypothetical protein
	AB2249	Conserved hypothetical protein
thiJ	AB2250	4-methyl-5(beta-hydroxyethyl)-thiazole monophosphate synthesis protein ThiJ
dnaE	AB2251	DNA polymerase III, alpha subunit
surE	AB2252	Stationary-phase survival protein, SurE
	AB2253	Hypothetical protein
	AB2312	Methyltransferase
thyX	AB2315	Thimidylate synthase ThyX

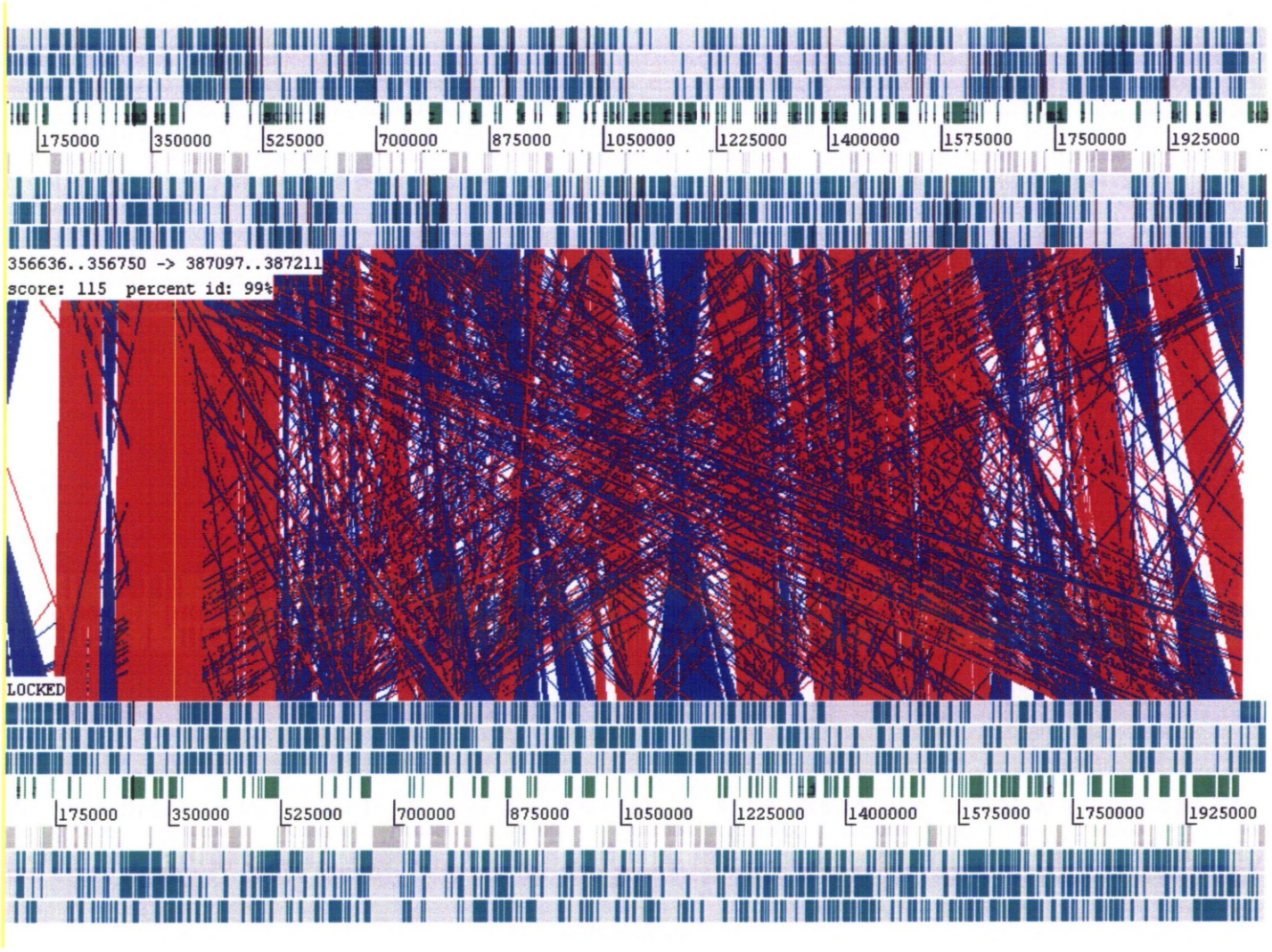

Figure 6.2. A screenshot from ACT, showing an overall comparison of the two genomes, with 7h1h at the top, and RM4018 at the bottom. Red areas signify 97-100% identity between the two genomes, while blue areas represent regions occurring in reverse orientation.

Figure 6.2. shows a screenshot of the ACT program, which was used to compare the two genomes using the BLAST algorithm.

Table 6.8. Differences in the numbers of features of interest between 7h1h and RM4018. Deletions are those ORFs that are deleted from 7h1h in comparison to RM4018, and insertions are ORFs that are present in 7h1h and not RM4018.

Subsystem Feature	Number in 7h1h	Number in RM4018	Details of RGPs
Stress Response	39	41	2 deletions
Sulphur Metabolism	11	11	none
Two-component system	32	37	1 divergent, 5 deletions
TonB receptor	18	19	4 divergent, 1 insertion, 2 deletions
ECF sigma family	4	7	3 deletions

Comparison of the two genomes using the RAST SEED viewer (http://rast.nmpdr.org/seedviewer.cgi) resulted in a list of 362 predicted differences (differences were defined as ORFs with a similarity less than 80%). The full list can be seen in Appendix VIII. Features with 0% identity are those which do not occur in both genomes, and are therefore either insertions or deletions.

6.3.2. PCR screening results

A panel of 95 isolates were selected for PCR screening to detect the presence of seven putative genes that had been suggested to be lying within regions of genomic plasticity when comparing the two *A. butzleri* genomes. The genes comprised Abu987, a transcriptional regulator deleted from 7hh; Abu1814, an O-antigen polymerase deleted from 7h1h; Orf2356, a glycerol transferase inserted in

7h1h; Orf1258, an ABC transporter inserted in 7h1h; *gls*A, glutaminase A deleted from 7hh; Abu991; an AcrB/AcrD/AcrF family protein deleted from 7h1h, and Abu1030, a membrane protein deleted from 7h1h. Isolates screened included *A. butzleri, A. cryaerophilus, A. skirrowii* and unidentified *Arcobacter* isolates retrieved from cattle, water, sheep and wildlife animals in the UK, and isolates from humans in the USA, Denmark, South Africa and Thailand (Miller *et al*, 2009). Table 6.9. shows the results of the screening PCR assays, showing the presence or absence of each gene. The percentage carriage of each gene amongst isolates from each source is presented in Table 6.10.

Multiple amplicons were obtained for ORF739, ORF1254 and ORF1448 using combinations of 2.5mM $MgCl_2$, 1.5mM $MgCl_2$ and annealing temperatures of 59°C, 62°C and 65°C. Time constraints meant that further optimisation of these PCR assays was not possible, so no acceptable results were obtained.

Table 6.9. Results of the gene screening PCRs. 1 indicates PCR-positive, blank indicates PCR-negative.

Isolate	Species	Source	Abu987	Abu1814	Orf2356	Orf1258	GlsA	Abu991	Abu1030
C93	*A. butzleri*	Rabbit	1	1					
C95	*A. butzleri*	Rabbit							
C100	*A. butzleri*	Badger	1	1		1			
C326	*A. skirrowii*	Cattle							
C340	*A. butzleri*	Cattle	1						
C341	*A. butzleri*	Cattle	1						
C425	*A. skirrowii*	Cattle							
C495	*A. skirrowii*	Cattle							
C496	*A. skirrowii*	Cattle							
C503	*A. butzleri*	Cattle						1	
C505	*A. butzleri*	Cattle	1					1	
C8	*A. cryaerophilus*	Cattle							
C325	*A. skirrowii*	Cattle							
C30	*A. cryaerophilus*	Cattle							
W30385	*A. butzleri*	water	1			1		1	1
W30386	*A. butzleri*	water	1				1	1	
W30391	*A. butzleri*	water	1	1		1			
W30397	*A. butzleri*	water	1						
W30400	*A. butzleri*	water	1						
W30411	*A. butzleri*	water	1						

W30423	*A. butzleri*	water	1	1				1	1
W30429	*A. butzleri*	water	1	1			1	1	1
W32903	*A. butzleri*	water	1			1		1	
W30469	*A. butzleri*	water	1			1		1	
W32908	*A. butzleri*	water	1						
W32888	*A. butzleri*	water	1	1				1	
W32892	*A. butzleri*	water	1			1		1	
W32885	*A. butzleri*	water	1						
W32867	*A. butzleri*	water	1	1			1		1
W32862	*A. butzleri*	water	1	1			1	1	1
W32875	*A. butzleri*	water	1	1		1		1	
W33195	*A. butzleri*	water		1		1			
W33204	*A. butzleri*	water	1						
W33225	*A. butzleri*	water	1						
W33229	*A. butzleri*	water	1			1	1	1	
W33232	*A. butzleri*	water	1	1				1	
W33104	*A. butzleri*	water	1			1	1	1	
W32994	*A. butzleri*	water	1						
P30670	*A. skirrowii*	Cattle							
P30675	*A. skirrowii*	Cattle							
P30684	*A. skirrowii*	Cattle							
P31166	*A. butzleri*	Sheep	1				1	1	
P31853	*A. butzleri*	Cattle		1					
P32200	*A. skirrowii*	Sheep							
P32209	*A. butzleri*	sheep							
A8d	*A. cryaerophilus*	Cattle					1		
A9c	*A. skirrowii*	Cattle							
A25a	*A. skirrowii*	Cattle							
A29c	*A. skirrowii*	Cattle							
A32c	*A. skirrowii*	Cattle			1				
A58a	*A. cryaerophilus*	Cattle							
A78c	*A. cryaerophilus*	Cattle							
A785a	*A. skirrowii*	Cattle							
A110b	*A. skirrowii*	Cattle							
A110d	*A. cryaerophilus*	Cattle							
A485c	*A. cryaerophilus*	Cattle							
A327c	Unknown *Arcobacter*	Cattle			1		1		
A306b	*A. skirrowii*	Cattle					1		
A306c	*A. cryaerophilus*	Cattle							
A336b	*A. skirrowii*	Cattle							
A339b	*A. skirrowii*	Cattle							
A343c	*A. cryaerophilus*	Cattle							
A506c	*A. cryaerophilus*	Cattle							
A518b	*A. cryaerophilus*	Cattle							
A708a	*A. cryaerophilus*	Cattle							
A622c	*A. skirrowii*	Cattle							
A602c	*A. skirrowii*	Cattle							
A340a	*A. skirrowii*	Cattle							
A723a	*A. cryaerophilus*	Cattle							
A671c	*A. skirrowii*	Cattle							
A400c	*A. cryaerophilus*	Cattle							
A441a	Unknown *Arcobacter*	Cattle							
A436a	Unknown *Arcobacter*	Cattle							
A34a	*A. skirrowii*	Cattle							
A496b	*A. cryaerophilus*	Cattle							
A475a	*A.*	Cattle							

	cryaerophilus								
A18b	*A. cryaerophilus*	Cattle							
RM5556	*A. butzleri*	Human	1	1				1	1
RM3790	*A. butzleri*	Human	1						
RM4129	*A. butzleri*	Human	1	1					
RM4463	*A. butzleri*	Human		1					
RM5230	*A. butzleri*	Human	1				1	1	
RM5519	*A. butzleri*	Human	1	1			1	1	
RM5529	*A. butzleri*	Human	1	1			1	1	
RM5534	*A. butzleri*	Human	1					1	
RM5542	*A. butzleri*	Human	1	1			1	1	1
RM5543	*A. butzleri*	Human		1			1	1	
RM5549	*A. butzleri*	Human					1	1	
RM5530	*A. butzleri*	Human					1	1	
RM5533	*A. butzleri*	Human		1			1	1	
30005	*A. skirrowii*								
30006	*A. butzleri*			1			1	1	
30007	*A. cryaerophilus*								
7h1h	*A. butzleri*	Cattle			1	1			
RM4018	*A. butzleri*	Human	1	1			1	1	1
Total			**38**	**22**	**3**	**11**	**20**	**28**	**8**

Table 6.10. Percentage carriage of the genes in each source, according to PCR assays.

		Presence (%)						
Source	**No. of Isolates**	**Abu987**	**Abu1814**	**Orf2356**	**Orf1258**	**GlsA**	**Abu991**	**Abu1030**
Rabbit	2	50	50	0	0	0	0	0
Badger	1	100	100	0	100	0	0	0
Cattle	48	6	2	6	2	6	4	0
Water	24	96	38	0	38	25	54	21
Sheep	3	33	0	0	0	33	33	0
Human	14	64	64	0	0	64	71	21
All	95	40	23	3	12	21	31	8

6.3.3. Omnilog Results

Omnilog analysis of the two *A. butzleri* strains, 7h1h and RM4018 revealed a number of phenotypic differences. In terms of metabolic differences, RM4018 appeared to utilise certain carbon and nitrogen sources more effectively than 7h1h, while 7h1h appeared to utilise some sulphur and phosphorus sources that RM4018 did not. The main results of note are presented in Table 6.11. Figure 6.3. shows an

example of a growth curve produced using the Omnilog system, showing mor abundant growth on L-asparagine by RM4018. The full results are presented in Appendix VII.

Table 6.11. The main phenotypic differences as determined by Omnilog analysis of 7h1h and RM4018.

Omnilog plate name	Type of test	Growth on substance	Result RM4018	Result 7h1h
PM01	Carbon sources	L-asparagine	+	-
PM01	Carbon sources	L-glutamic acid	+	-
PM03	Nitrogen sources	L-glutamine	+	-
PM03	Nitrogen sources	L-tryptophan	+	-
PM03	Nitrogen sources	L-tyrosine	+	-
PM04	Phosphorus and sulphur sources	Taurine	-	+
PM04	Phosphorus and sulphur sources	Butane sulfonic acid	-	+
PM04	Phosphorus and sulphur sources	Methane sulfonic acid	-	+
PM04	Phosphorus and sulphur sources	L-cysteic acid	-	+

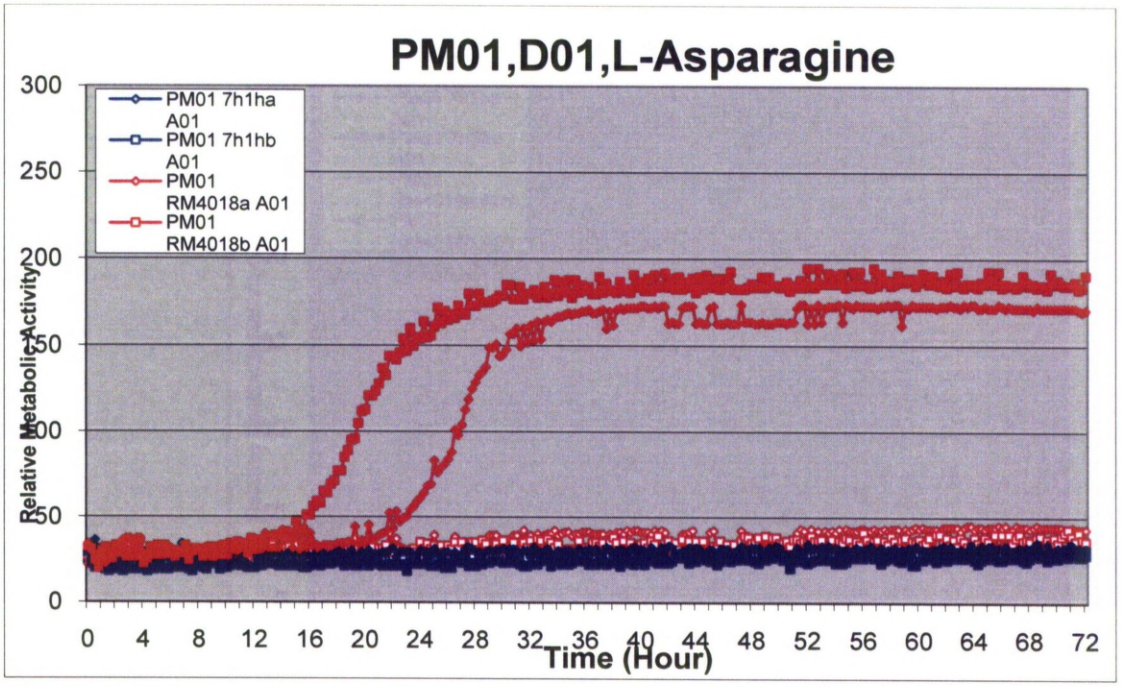

Figure 6.3. Omnilog output chart showing the growth curve of 7h1h and RM4018 using L-asparagine as a carbon source, over a time period of 72 hours. Each isolate was tested in duplicate, hence 7h1ha, 7h1hb, RM4018a and RM4018b.

6.4. Discussion.

Miller *et al.* (2007) found that the genome of *A. butzleri* strain RM4018 showed greater similarity to *S. denitrificans* and *W. succinogenes* than to other members of the *Campylobacteraceae*. The presence of a larger than expected number of genes associated with environmental sensing and survival, and which are more commonly found in free-living, non-host-associated organisms, suggests that *A. butzleri* is a free-living, water-borne organism. The presence of virulence-related genes, including the virulence factor MviN, fibronectin binding proteins and phospholipases, supported *A. butzleri*'s classification as an emerging human pathogen (Miller *et al.* 2007).

The main aim of this study was to obtain a second *A. butzleri* whole genome sequence, that of an isolate from cattle, in order to compare it with that of the human isolate RM4018, in order to gain a better understanding of *A. butzleri* and its mechanisms. Upon comparing the whole genome sequences of these two isolates, a surprising amount of variation became apparent. At the gene level, i.e. comparing the nucleotide sequences of the two genomes, the two isolates differed overall by approximately 20%, a level of difference not usually expected in two isolates of the same species (Falush, 2009). The isolates differed at 562 ORFs, which were therefore classified as RGPs (regions of genomic plasticity), and 9.0% of the genes identified by Miller *et al.*, (2007) as being "core" were absent from 7h1h.

The RGPs identified in this study included a variety of genes. Of interest when considering the survival of *A. butzleri* in a variety of environmental conditions was the presence of six two-component sensing systems within the RGP group. Five of these were deleted from 7h1h and one was divergent. Two-component sensing systems are mechanisms that allow an organism to sense and respond to its environment (Stock *et al.* 2000). These mechanisms feature a membrane-bound histidine kinase molécule, which senses specific environmental stimuli and ilicits a response via the associated response regulator, usually by controlling the expression of specific genes (Mascher *et al.* 2006). These systems are widely found in prokaryotes and are usually involved in the sensing of environmental changes such as in pH, osmolarity and temperature (Mascher *et al.* 2006). Seven of these receptors were shown to differ between the two genomes, comprising two deletions from 7h1h, one insertion into 7h1h and four divergent genes, having possible implications for the survival of the organism in harsh environments such as in soil and water.

Similarly, RM4018 posesses three ECF (extracytoplasmic function) sigma family proteins that are deleted from 7h1h. ECF sigma family proteins are a subfamily of sigma factors; proteins which regulate promoter selectivity. The ECF family of sigma factors are small regulatory proteins which, upon recieving a stimulus from the environment, can bind to RNA polymerase and stimulate transcription (Helmann, 2002).

The presence of large numbers of these systems in the *A. butzleri* genome (Miller *et al.* 2007) supports the notion that *A. butzleri* is a free-living environmental species (Miller *et al.* 2007). The fact that the RM4018 genome posesses more of these systems than 7h1h suggests that the human-isolated RM4018 may be better able to adapt to a wider range of environmental conditions, while 7h1h may be better suited to a single (cattle) host.

The two genomes also appear to differ greatly in the number of virulence-related genes. The RAST SEED viewer shows 56 virulence-related subsystem features in the RM4018 genome, and 30 putative virulence-related subsystem features in 7h1h. This substantial difference in numbers may be significant in relation to the pathogenicity of *A. butzleri*, particularly as RM4018, the human-isolated strain, posesses more virulence factors. This suggests that the human-isolated strain posesses a greater ability to cause disease than the cattle-derived isolate.

The first gene to be screened, Abu987, was predicted to encode a transcriptional regulator of the GntR family, that was present in the genome of RM4018 and absent from 7h1h. It was identified as one of the "core" genes by Miller *et al.* (2007) after hybridisation of 13 *A. butzleri* isolates. In this study it was

present in 40% of the screened samples overall, and was particularly prevalent in water isolates, with 96% carriage. Abu987 was found in all sources.

The *gntR* family of proteins are a family of transcriptional regulators that are responsible for transcription of a variety of different proteins and are widely distributed throughout the bacterial kingdom. The widespread nature of this family is probably reflected in the presence of the *gntR* gene in all source types in this study. Abu987 was present at much lower levels in cattle (6%, n=48) than any of the other groups, including wildlife (Rabbit: 1 isolate, Badger: 1 isolate) and water (96%, n=24), which form potential reservoirs for *Arcobacter* infection of cattle; and the idea of Abu987 being less common in cattle is supported by the fact that the gene was deleted from 7h1h, a strain isolated from cattle. This suggests that wildlife and *Arcobacter* spp. may not be the source of infection of cattle.

Abu1814 encodes a putative O-antigen polymerase and was deleted from 7h1h, but present in RM4018 as one of the "core" genes. O-antigen is a polysaccharide found on the outer surface of bacterial cells, and is linked to virulence and host immune response (Miller *et al.*, 2007). The gene Abu1814 was found in only 2% of the cattle isolates, but 64% of human isolates, 38% of water samples and 23% overall. The low carriage of this gene amongst isolates from cattle may indicate adaptation by the bacteria to the host. The lipopolysaccharide (LPS) on the cell surface is known to affect virulence (host antibodies recognise the surface antigens) and survival (the condition of the membrane is important for structural integrity and protection of the cell).

Orf2356 was a putative glycerol phosphotransferase gene that was present in 7h1h and deleted from RM4018. Glycerol phosphotransferase is an enzyme that

catalyses the reaction whereby diphosphate and glycerol are converted to phosphate and glycerol 1-phosphate, and vice-versa. The fact that it was absent from all groups, except in a small number of cattle, suggests that this enzyme is not essential for the survival of *A. butzleri*. If this enzyme was found in cattle isolates specifically, for example due to some form of adaptation for survival in a bovine host, it would be expected to be seen in larger numbers of cattle. As it is, the percentage carriage in cattle was 6%, suggesting that the enzyme may be absent from the majority of *A. butzleri* strains, regardless of source.

Orf1258 encodes a putative toxin secretion ABC transporter (ATP-binding and membrane protein), deleted from RM4018 but present in 7h1h. ABC transporters are transmembrane proteins that utilise energy from ATP to carry out transport and other processes. Toxin secretion ABC transporters specifically transport toxins through the outer membrane, secreting them outside the cell (Binet *et al.*, 1997). Orf1258 was common in water isolates and was found in the one badger isolate tested. It was absent from human, sheep and rabbit isolates, and present only in 2% of the cattle isolates. The fact that *A. butzleri* isolates possess a toxin secretion system, as well as a toxin activating acetyltransferase, suggests *A. butzleri* is capable of causing disease.

glsA, a gene encoding glutaminase A, was deleted from 7h1h and present in RM4018. Glutaminase A is required to convert glutamine to glutamate, an important stage in bacterial metabolism. This gene was absent from the badger and rabbit isolates, present in 6% of cattle isolates, and present in 25%, 33% and 64% of water, sheep and human isolates, respectively. The absence of *glsA* in 7h1h suggests this isolate is unable to metabolise glutamine unlike other isolates, as described later in this chapter, in the discussion of Omnilog results (Page 166 onwards). It is

possible that the ability to metabolise glutamine is related to the availability of glutamine in the host environment, and that isolates in cattle and wildlife animals have less need to metabolise glutamine than isolates in cattle and humans. With such a small dataset, it is possible only to make assumptions from these results. A larger study involving more isolates from more sources would be required to show any real difference in the carriage of these genes.

Abu991 was an Acr family protein that was deleted from 7h1h and present in RM4018, as one of the "core" genes. Acr proteins are a family of regulatory proteins that are often involved in major metabolic pathways. Abu991 was absent from the three wildlife (rabbit and badger) isolates, present at low levels (4%) in cattle isolates and present in 54%, 33% and 71% of water, sheep and human isolates, respectively. The higher level of carriage amongst the human isolates may indicate an increased need for metabolic regulatory processes in these isolates, compared to those from other sources.

Abu1030 was a hypothetical membrane protein deleted from 7h1h, present in RM4018 and included as one of the "core" genes (Miller *et al.*, 2007). This gene was absent from all sources except water (21%) and human (21%). As a membrane protein, this may have implications for virulence or survival, and further investigation into the nature and function of this gene could elucidate this.

Results of the Biolog Omnilog analysis highlighted some major differences in the metabolic processes of the two *A. butzleri* isolates. Isolate RM4018 showed significantly more growth than 7h1h when L-asparagine was used as a carbon source. *Campylobacteraceae* utilise amino acids for energy, not carbohydrates (www.bergeys.org/multitaxachapter). L-asparagine is an amino acid which is

converted to aspartic acid during anaerobic respiration. L-asparaginases in Gram-negative bacteria have been linked to anaerobic fumarate respiration by providing aspartate, which is converted to fumarate. After hydrolytic cleavage by asparaginase, aspartate is catabolised to produce fumarate and ammonia (Kroger *et al.*, 2002). The inability of 7h1h to utilise L-asparagine suggests one or more genes relating to L-asparagine use may be absent, suggesting that this amino acid is not required for respiration in 7h1h. Both 7h1h and RM4018 feature a single predicted L-asparaginase gene (AB1589 in RM4018 and ORF1740 in 7h1h), which are highly similar (98%). 7h1h posesses one glutamine-hydrolyzing asparagine synthetase, *asnB1*. RM4018 posesses three; *asnB1* (glutamine-hydrolysing, 97% similar to that of 7h1h), *asnB2* and *asnB*, providing a possible explanation for their difference in L-asparagine use.

Many Gram-negative bacteria are known to posess two L-asparaginases, one of which is thought to have a role in anaerobic fumarate respiration (Jennings *et al.*, 1990). Fumarate respiration is thought to be the most widespread form of bacterial anaerobic respiration (Kroger *et al.*, 2002), and fumarate is reduced by the enzyme, methylmenaquinol:fumarate reductase (mfr) in the epsilonproteobacteria, which is encoded by the gene, *frd* in *C.jejuni* (Guccione *et al.*, 2009). Both 7h1h and RM4018 contain the three *frd* genes, *frdA*, *frdB* and *frdC*, with a high level of similarity (99-100%), suggesting that these genes are not responsible for the phenotypic difference in L-asparagine use.

RM4018 was found to utilise L-glutamic acid as a carbon source, while 7h1h did not. Organisms can synthesise glutamine from L-glutamic acid, which is required for the synthesis of essential cell proteins, and glutamine is essential for ammonia transfer. Organisms can use glutamine and glutamic acid for the same

purpose without having to convert the glutamic acid. 7h1h posesses 2 glutamine synthases and several glutamine amidotransferases, while RM4018 posesses the same genes with a high level of similarity, plus one additional gene, *glsA*, which encodes glutaminase A, the enzyme required to convert glutamine into glutamate, possibly explaining the phenotypic difference in glutamic acid metabolism shown in this study.

A similar result was found when using L-glutamine as a nitrogen source. RM4018 appeared to utilise L-glutamine while 7h1h did not, which may also be related to 7h1h's lack of a *glsA* gene.

RM4018 was shown to utilise L-tryptophan as a nitrogen source, while 7h1h did not. L-tryptophan is an amino acid that is essential for protein structure. RM4018 has 3 tryptophan synthases (*trpA*, *trpB2*, *trpB1*) and 7h1h has the same 3, suggesting that the phenotypic differences in tryptophan use are not related to these genes.

For nitrogen metabolism, many epsilonproteobacteria employ two enyme transport systems, *Nap* (periplasmic nitrate reductase) and *Nrf* (periplasmic cytochrome c nitrite reductase). It has been reported that all epsilonproteobacteria lack a *napC* gene in their *nap* gene cluster (Kern and Simon, 2009), and this is the case in strains 7h1h and RM4018, which feature highly similar *nap* gene clusters. *Arcobacter* has previously been shown to be capable of nitrogen fixation and nitrate respiration (Kaeberlein *et al.*, 2002), which may be related to *Arcobacter*'s natural environmental habitat. 7h1h features two *nrfH* genes, two *nrfI* genes and one *nrfA* gene, while RM4018 features two *nrfH* genes, two *nrfA* genes and one *nrfI* gene.

This difference in the *nrf* gene cluster may explain some of the phenotypic differences in the nitrogen-reducing ability of the two strains.

On testing L-tyrosine as a nitrogen source 7h1h showed no growth, whereas RM4018 did. Tyrosine is not normally hydrolysed by the *Campylobacteraceae* (Vandamme *et al.*, Bergey's manual of systematic bacteriology, www.bergeys.org/multitaxachapter). In other bacteria, tyrosine is catabolized along with phenylalanine to form fumarate and acetoacetate, and tyrosine kinases have been found to phosphorylate a number of substrates, forming an important regulatory feature (Grangeasse *et al.*, 2001). Both 7h1h and RM4018 feature a single protein tyrosine phosphatase gene, with the two genes being 95% similar, however neither strain features a tyrosine kinase. It is notable that protein tyrosine phosphatases have been linked with virulence in bacteria (Guan and Dixon, 1993).

7h1h was found to utilise taurine (2-aminoethanesulfonate) as a sulphur source, while RM4018 did not. Bacterial degradation of taurine releases sulphates and ammonia (Ikeda *et al.*, 1963), and taurine can be used by some bacteria as a sole source of sulphur for growth (Chien *et al.*, 1997). Despite its apparent ability to utilise taurine as a sole sulphur source, none of the genes related to this process (*tauR*, *tpa*, *ald* and *xsc*; Bruggeman *et al.*, 2004; Denger *et al.*, 2009) were found in the 7h1h genome, suggesting that its use of taurine as a sulphur source is regulated in 7h1h by some unknown gene(s).

Butane sulfonic acid and methane sulfonic acid could be utilised as sulphur sources by 7h1h, and not by RM4018. Both are naturally occurring acids in the atmosphere that form part of the sulphur cycle, and are deposited on the earth, often through precipitation. For sulphur metabolism, RM4018 was found to posess a set

of *sox* genes for sulphur oxidation, as well as genes for the sulphate ABC transporter *cysATW*, the sulphate binding protein Sbp, the ATP sulfhydrylase *cysDN*, the adenosine phosphphosulfate reductase *cysH*, the sulphite reductase proteins *cysI* and *cysJ* and the siroheme synthase *cysG* (Miller *et al.*, 2007). 7h1h on the other hand, does not posess any of the *sox* genes required for sulphur oxidation. Of the other sulphur-related genes found in RM4018, 7h1h posesses *cysATW*, *sbp*, *cysH*, *cysI* and *cysJ*, and does not posess *cysDN*. These differences in the sulphur metabolism-related genes of the two strains may go some way towards explaining the ability of 7h1h to utilise sulphur for growth.

A number of microorganisms have been reported to be able to utilise L-cysteic acid as a sole sulphur source (Stapley *et al.*, 1970). Cysteine is oxidised as cysteic acid, which is decarboxylated into taurine by the enzyme, sulfinoalanine decarboxylase (CSAD; Sekowska *et al.*, 2000); genes encoding this enzyme do not appear to be present in either the 7h1h or RM4018 genomes. An alternative pathway is that the cysteic acid is transformed into cysteine and sulphite by a cysteine lyase (Sekowska *et al.*, 2000). Both 7h1h and RM4018 appear to posess one selenocysteine lyase-encoding gene, with 99% similarity between the two, which suggests that an alternative gene or pathway is responsible for the phenotypic difference in cysteic acid use.

After comparison of the two *A. butzleri* genomes, it is clear that the isolates differ in many ways. Differences in survival and sensing systems may well be related to the source of the isolate, and studies of additional isolates from other sources would provide interesting future work. These results suggest a degree of host or niche adaptation by *A. butzleri*, based on the results of PCR assays. A more detailed study would be required to properly investigate this, as the results of a single

PCR assay for each gene may not be adequately reliable, due to sequence divergence at the primer binding sites, or similar.

Chapter Seven

Concluding Discussion.

Arcobacter spp. are emerging human foodborne pathogens that have been isolated from various animals, foodstuffs, humans and the environment. Despite their abundance, relatively little is known about the mechanisms of transmission and infection of the genus. This project aimed to further our understanding of the diversity, transmission and epidemiology of *Arcobacter* spp., with a particular focus on *Arcobacter* in cattle. This study formed part of a larger collaborative study, the DEFRA and HEFCE-funded Veterinary Training Research Initiative (VTRI), which aimed to use veterinary research to improve understanding of infectious diseases and to better prepare the UK for future outbreaks of infectious disease. The University of Liverpool hosted a branch of this scheme which focussed on the transmission, evolution and control of foodborne diseases, of which this project formed a small part.

Arcobacter spp. were originally classified as "aerotolerant *Campylobacter*s" and share many features with their fellow *Campylobacteraceae.* In a University of Liverpool study into the molecular epidemiology of *C. jejuni* on dairy farms in the north west UK it was noted that on many sampling occasions *Arcobacter* spp. were isolated from the cattle faeces more often than the intended *C. jejuni.* The recovery of *Arcobacter* spp. appeared to increase as *C. jejuni* recovery decreased (unpublished data), leading to the design of this study.

The aims of this study were to:

1) Evaluate five methods for the isolation of *Arcobacter* spp. from animal faeces and choose the most sensitive and specific for use in subsequent studies.

2) Determine the prevalence of *Arcobacter* in cattle on a number of beef and dairy farms in Cheshire, and investigate factors affecting the prevalence.

3) Determine the genotypic diversity of *Arcobacter* spp. from cattle in Cheshire, using molecular typing techniques.

4) Obtain an *A. butzleri* whole genome sequence and compare it to an existing *A. butzleri* whole genome.

Despite many studies into the prevalence of *Arcobacter* spp. in numerous sources worldwide, a standard *Arcobacter* isolation method has not emerged. The methods used differ significantly, making comparisons between studies difficult. The first aim of this project was to compare five published methods for the isolation of *Arcobacter* spp. and determine which method was the most appropriate in this study, in terms of sensitivity and specificity. It is referred to as HCC subsequently. A combination of an *Arcobacter* selective broth containing five antibiotics (Houf *et al.*, 2001), with modified charcoal agar with added CAT antibiotic supplement, was the most sensitive and specific method, with the least bias toward one particular species.

Different estimates of the prevalence of *Arcobacter* have been published worldwide, and some of this variation is probably due to the differing sensitivities and specificities of the different isolation methods used. Isolation of *Arcobacter* spp. from chicken carcasses resulted in prevalences of 23% (Kabeya *et al.*, 2004, Japan), 65% (Houf *et al.*, 2001, Belgium), 100% (Atabay *et al.*, 1997 and 1998, UK), 73% (Rivas *et al.*, 2004, Australia), and 90% (Gonzalez *et al.*, 20007, Spain). Most of these studies used different isolation techniques. Atabay and Corry (1997) reported very different prevalences when comparing four methods for the isolation of *Arcobacter* spp., and Scullion *et al.* (2004) found prevalences ranging from 28% to 68% when comparing three different isolation methods to analyse raw chicken samples. Both studies point out that a standard sensitive and specific method was

required in order to allow the true prevalence of *Arcobacter* spp. worldwide to be determined. The method HCC, demonstrated to be the most sensitive and specific in this study, is an affordable, easy method that does not require any specialist filtration or plating equipment, and based on the results reported here it is recommended for use as a standard technique for the isolation of *Arcobacter* spp. from faeces.

The prevalence of *Arcobacter* spp. on four farms over a period of one year was investigated via a series of five cross-sectional studies. Each study sampled up to fifty individual faecal pats from cattle from various age and management groups (e.g. lactating adults, dry adults, weaned calves, unweaned calves, heifers, fattening adults) on two dairy farms and two beef farms, all in Cheshire. Robust Univariate and multivariate logistic regression analysis of the resulting data suggested *Arcobacter* spp. were more likely to be recovered from cattle grazing at pasture, and from young cattle. Prevalence was also highest during the summer, an effect similar to that shown in *Campylobacter* spp. (Kwan *et al.*, 2008; Grove-White *et al.*, 2009). Farm type (i.e. beef vs. dairy) was shown to have an effect after univariate analysis, with prevalence appearing higher on beef farms, but after multivariate analysis this was shown to be due to a very high prevalence of *Arcobacter* spp. on one particular beef farm.

A number of publications have associated increases in temperature with increases in the prevalence of *C. jejuni*, explaining the summer peak in human and animal hosts commonly found in this species (Kovats *et al.*, 2004; Louis *et al.*, 2005). Due to the level of similarity between *Arcobacter* spp. and *Campylobacter* spp., it is possible that the same explanation may be applied here, particularly as the apparent peak in the prevalence of *Arcobacter* spp. in this study also occurred during

the summer. A study of *Arcobacter* spp. in cattle in the USA also associated an increase in prevalence with an increase in temperature (Wesley *et al.*, 2000), which is supported by this study. This study tested the effect of freezing on *Arcobacter* recovery, as part of a pilot study into *Arcobacter* isolation (Chapter Three), and found that recovery of *Arcobacter* spp. from animal faecal samples was reduced after freezing the samples for one week. This apparent sensitivity of *Arcobacter* spp. to very low temperatures may go some way to explaining the lower recovery of *Arcobacter* spp. during the winter, as is similar with *Campylobacter* spp.

One factor not investigated in this study was the effect of diet on the prevalence of *Arcobacter* spp. in cattle. The prevalence of *Arcobacter* spp. was found to be higher when cattle were sampled outdoors, and also during the summer. The diet of dairy cattle when kept outdoors at pasture (usually in the summer) is very different to that of cattle housed indoors. It is possible that the higher prevalence in cattle sampled outdoors is related to the diet of grass being consumed by the cattle at that time. Further investigation into the effect of diet, and the link between diet and environment on the prevalence of *Arcobacter* spp. would provide an interesting opportunity for further work on this subject.

Other future work would be to carry out a longitudinal study over a longer time period. The conclusions of this study are based on serial cross sectional data taken over a period of one year. Although the data suggest a summer peak in *Arcobacter* prevalence, the true seasonal prevalence can only be shown using longitudinal data, over multiple years. With the apparent peak in *Arcobacter* prevalence occurring in summer in this study, it would be useful to determine whether a summer peak in human *Arcobacter* infections occurs, similar to that found in *C. jejuni*. *Arcobacter* was shown to be the fourth most common cause of bacterial

enteritis in two clinical studies in Belgium and France (Vandenberg *et al.*, 2004; Prouzet-Mauleon *et al.*, 2006), suggesting that *Arcobacter* spp. may be a significant cause of human gastrointestinal infection. Despite this knowledge, *Arcobacter* spp. are not included in routine laboratory testing of clinical samples in the UK, thus allowing no estimation of the prevalence of *Arcobacter* spp. amongst the UK population.

Molecular typing of *Arcobacter* isolates in this study revealed a high level of diversity amongst the three species tested, which is in agreement with previous studies (Gonzalez *et al.*, 2007; Aydin *et al.*, 2007; Son *et al.*, 2006; Atabay *et al.*, 2006; On *et al.*, 2004). The ERIC-PCR typing technique was found to be of little use, due to a lack of sites for the binding of the primers on the *A. butzleri* genome and its low annealing temperature (encouraging nonspecific binding of the primers), a significant finding as this technique is currently widely used in *Arcobacter* typing studies.

Macro-restriction PFGE failed in this study due to the lack of bands preventing any meaningful analysis, however MLST was found to be a very useful tool in the study of the molecular epidemiology and diversity of *Arcobacter* spp.

An MLST scheme for the typing of *Arcobacter* spp. was used in this study, using the same seven loci as applied to *C. jejuni* and *C. coli*, potentially allowing a direct comparison of *Arcobacter* and *Campylobacter* isolates (Miller *et al.*, 2009). As of 21st March, 2010, the *Arcobacter* MLST database (www.pubmlst.org/arcobacter/) contained three hundred and fifty seven different sequence types (STs), sixty six (18.5%) of which were submitted from this study. No clonal complex data has yet been calculated using the database, but phylogenetic analysis of the alleles identified in this study showed the formation of clusters

according to species at each locus, with a few outliers. One finding of note was that several isolates at each allele appeared in the 'wrong' cluster, suggesting the occurrence of lateral transfer in *A. skirrowii, A. butzleri* and *A. cryaerophilus*. This warrants further investigation.

Given an increase in the number of isolates submitted to the *Arcobacter* database, it will be possible to assign clonal complexes (CCs), allowing researchers to determine whether any association between CCs and sources exists, as has been demonstrated in *C. jejuni*, where specific CCs are often associated with particular sources, e.g. poultry, and even specifically with human infection (Dingle *et al.*, 2005). Similar associations in *Arcobacter* spp. may allow a better understanding of the status of *Arcobacter* as a foodborne pathogen in humans, as well as providing an insight into the transmission of *Arcobacter* spp., although this may be limited by the fact that *Arcobacter* is not routinely tested for in the UK.

An *A. butzleri* whole genome sequence was determined using the high-throughput 454 sequencing technology (Roche 454 Life Sciences, USA) based at the Advanced Genomics Facility, at the University of Liverpool. The sequenced isolate, 7h1h, was recovered from the faeces of a clinically healthy dairy cow on a farm in Cheshire during 2007. This was the second *A. butzleri* whole genome to be sequenced, the first being an isolate from a human clinical case of diarrhoea in the USA, published by Miller *et al* (2007). The 7h1h pseudogenome was found to be approximately 80% similar to the Miller genome, with 502 regions that differed between the two, some of which were related to environmental sensing and survival, having significance for host adaptation. Like the Miller isolate, a large proportion of the 7h1h genome was devoted to environmental sensing and survival, supporting suggestions that *A. butzleri* is a water-borne opportunistic pathogen (Miller *et al.*,

2007). A number of phenotypic differences were identified after comparing the two genomes using the Biolog Omnilog system (Biolog, CA, USA), including the ability of the isolates to utilise specific amino acids for energy, and their ability to use different nitrogen and carbon sources.

The considerable amount of genotypic and phenotypic variation between the two genomes, coupled with the fact that many of the differences (n=18, Chapter six, Table 6.7.) related to environmental sensing and survival, suggests that the survival and sensing abilities of *A. butzleri* may differ between hosts. Work is underway to determine whether particular genes are associated with particular host animals or environmental sources (W. Miller, personal communication), which will provide a better insight into the ability of *A. butzleri* to adapt to an environment. Further molecular analysis of the 7h1h pseudogenome will be necessary to ensure the genes are arranged in the correct order, and to verify the findings of this study.

Sequencing of further *Arcobacter* genomes will undoubtedly provide important information on the nature of this organism, its transmission and its disease-causing ability.

This study has shown that *A. butzleri, A. skirrowii* and *A. cryaerophilus* are present at high prevalences in cattle in Cheshire, and that considerable genetic diversity exists within the genus, as assessed by MLST and whole genome sequencing. Further study is required to determine whether particular genotypes are related to source or pathogenicity. This study also showed that ERIC-PCR, a typing technique widely used in studies on *Arcobacter* spp., is of limited use in this genus due to a lack of primer binding sites in the *Arcobacter* genome. Cattle appear to be a common reservoir host of *Arcobacter*, without suffering clinical illness as a result. The prevalence of *Arcobacter* spp. in cattle appears to peak during the summer, with

a drop in winter, which appears to be affected by the age and housing status of the cattle, and possibly by herd size and exposure to environmental water. Further study is recommended to determine whether this summer peak is a true seasonal effect.

Appendix

Appendix I. Details of the primers used for each PCR assay, and the resulting band sizes.

PCR Name	Reference	Forward Primer	Reverse Primer	Fragment Size (bp)
Arcobacter Genus-specific 16s rRNA PCR	Gonzalez et al. 2000	Arc1 5' AGAACGGGTTATAGCTTGCTAT 3'	Arc2 5' GATACAATACAGGCTAATCTCT 3'	181
Arcobacter multiplex 16s and 23s PCR	Houf et al., 2000	Arco 5' CGTATTCACCGTAGCATAGC 3' Cry1 5' TGCTGGAGCGGATAGAAGTA 3'	Butz 5' CGTGGACTTGACATAGTAAGAATGA 3' Skir 5' GGCGATTTACTGGAACACA 3' Cry2 5' AACAACCTACGTCCTTCGAC 3'	401 641 257
Campylobacter colony multiplex PCR for detection of *C. jejuni, C. coli* and *C. lari*	Wang et al., 2002	CJF 5' ACTTCTTTATTGCTTGCTGC 3' CCF 5' GTAAAACCAAAGCTTATCGTG 3' CLF 5' TAGAGAGATAGCAAAAGAGA 3'	CJR 5' GCCACAACAAGTAAAGAAGC 3' CCR 5' TCCAGCAATGTGTGCAATG 3' CLR 5' TACACATAATAATCCCACCC 3'	323 126 251
Campylobacter CeuE gene PCR for detection of *C. jejuni* and *C. coli*	Gonzalez et al., 1997	JEJ1 5' CCTGCTACGGTGAAAGTTTTGC 3' COL1 5' ATGAAAAAATATTTAGTTTTTGCA 3'	JEJ2 5' GATCTTTTTGTTTTGTGCTGC 3' COL2 5' ATTTTATTATTTGTAGCAGCG 3'	793 894
Campylobacter 16S rRNA PCR for the detection of *C. lari*	Linton et al., 1996	LLF (CL594F) 5' CAAGTCTCTTGTGAAATCCAAC 3'	LLR (CL1155R) 5' ATTTAGAGTGCTCACCCGAAG 3'	561
Campylobacter 16S rRNA Duplex PCR for the detection of *C. hyointestinalis* and *C. fetus*	Linton et al., 1996	CHyoF (CFCH57F) 5' GCAAGTCGAACGGAGTATTA 3'	CHyoR (CH1344R) 5' GCGATTCCGGCTTCATGCTC 3' CFetR (CF1054R) 5' GCAGCACCTGTCTCAACT 3'	1287 997
C. lanienae PCR	Logan et al., 2000	CLAN76F 5' GTAAGAGCTTGCTCTTATGAG 3'	CLAN1021R 5' TCTTATCTCTAAGAGGTTCTT 3'	920
groEL PCR	Karenlampi et al., 2004	H60F 5' GGNGAYGGNACNACNACNGCNACNGT 3'	H60R 5' TCNCCRAANCCNGGNGCYTTNACNGC 3'	593
16s universal identification PCR	N. J. Williams, Personal Communication	16sUF 5' GGTGGAGCATGTGGTTTAATTCG 3'	16SUR 5' CTGATCTACGATTACTAGCGATTCCG 3'	424

Appendix II

PulseNet PFGE Protocol

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One-Day (24-26 h) Standardized Laboratory Protocol for Molecular Subtyping of *Campylobacter jejuni* by Pulsed Field Gel Electrophoresis (PFGE)

BIOSAFETY WARNING: Please read all instructions carefully before starting protocol. Treat all plasticware, glassware, pipets, spatulas, etc. that come in contact with the cell suspensions or plugs as contaminated materials and dispose of, or disinfect according to the guidelines of your institution. Disinfect plug molds before they are washed. Contaminated items should be disinfected with 10% bleach for at least 30 minutes if they will be washed and reused.

Day 0

Streak an isolated colony from test cultures onto Trypticase Soy Agar with 5% defibrinated sheep blood (TSA-SB) plates (or comparable media) for confluent growth. It is recommended that a storage vial of each culture be created. To do this stab small screw cap tubes of TSA, HIA, or similar medium.with the same incolating loop used to streak the plate. This will ensure that the same colony can be retested if necessary. Incubate cultures at microaerobically 37°C for 14-18 h.

Day 1

1.

Turn on shaker water bath or incubator (54-55°C), stationary water baths (55- 60°C) and spectrophotometer (or equivalent instrument such as the Dade Microscan Turbidity meter or bioMérieux Vitek colorimeter).

2.

Prepare **TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0)** as follows:

10 ml of 1 M Tris, pH 8.0
2 ml of 0.5 M EDTA, pH 8.0
Dilute to 1000 ml with sterile Ultrapure water (Clinical Laboratory Reagent Water (CLRW))

3. Prepare 1% SeaKem Gold agarose in **TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0)** as follows:
   a. Weigh 0.50 g SeaKem Gold (SKG) into 250 ml screw-cap flask.
   b. Add 49.5 ml TE Buffer; swirl gently to disperse agarose.
   c. Remove cap, cover loosely with clear film, and microwave for 30-sec; mix gently and repeat for 10-sec intervals until agarose is completely dissolved.

d. Place flask in a 55-60°C water for 15 minutes or until ready to use.

Note: The TE Buffer used to make the plug agarose is also used to wash lysed PFGE plugs.

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4. Label small tubes (Falcon 2054 tubes or equivalent) with culture numbers.

5. Transfer 2 ml of phosphate-buffered saline (PBS: 0.01 M, pH 7.2-7.4) or 0.85% NaCl to small labeled tubes (Falcon 2054 tubes). Use sterile polyester-fiber or cotton swab that has been moistened with sterile PBS to remove some of the growth from agar plate; suspend cells in PBS.
6. Adjust concentration of cell suspensions to:
    a. Dade Microscan Turbidity Meter: 0.35 - 0.45 (measured in Falcon 2054 tubes). For Falcon tubes 2057 adjust to 0.52 - 0.64.
    b. Spectrophotometer: 610 nm wavelength, absorbance (Optical Density) of 0.680 (range of 0.570 to 0.820).

c.
bioMérieux Vitek colorimeter: ≈ 20% transmittance (measured in Falcon 2054 tubes)

Note: The values in Steps 7a, 7b and 7c give satisfactory results at CDC; each laboratory may need to establish the optimal concentration needed for satisfactory results

CASTING PLUGS

Label wells of PFGE plug molds with culture number. When reusable plug molds are used, put strip of tape on lower part of reusable plug mold before labeling wells.
Note 1: Unused plug agarose can be kept at room temperature and reused 1-2 times. Microwave on low-medium power for 10 -15 sec and mix; repeat for 5 -10 sec intervals until agarose is completely melted. This agarose melts rapidly!
Note 2: Proteinase K solutions (20 mg/ml) are available commercially. Alternatively, a stock solution of Proteinase K can be prepared from the powder in sterile Ultrapure water (CLRW). For best results, aliquot in 300-500 µl into small tubes and store in a freezer (-20 °C) until ready to use. Just before use, thaw appropriate number of vials needed for the samples; keep Proteinase K solutions on ice. If the Proteinase K stock solution was prepared from powder, discard any thawed solution at the end of work day. Store commercially prepared Proteinase K solutions according to directions provided by the supplier.

1. Add 20 µl of Proteinase K (20 mg/ml stock) to labeled 1.5-ml microcentrifuge tubes (200 µl is needed for 10 cell suspensions.).
2. Transfer 400 µl (0.4 ml) of adjusted cell suspensions to each labeled tube and mix gently with pipet tip. The use of a 1000 µl pipet (P-1000) and tip is recommended for this step; the use of a smaller pipet and tip might cause DNA shearing.
3. Add 400 µl (0.4 ml) melted 1% SeaKem Gold agarose to the 400 µl cell suspension and mix gently by pipeting up and down two to three times. Over-pipeting could cause DNA shearing. Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60°C).

4.
Immediately, dispense part of mixture into appropriate well(s) of disposable plug mold. Do not allow bubbles to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose and are useful if repeat testing is required. Allow plugs to solidify at room temperature for 10-15 min. They can also be placed in the refrigerator (4°C) for 5 minutes.

Note: The generation of cell suspension and the subsequent casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis. If large numbers of samples are being prepared, it is recommended that they be processed in batches of ~10 samples at a time. Once the first batch of isolates are in the cell lysis incubation, then start preparing the cells suspensions the next group samples, and so on. All batches can be lysed and washed together, since additional lysis time will not affect the initial batches.

LYSIS OF CELLS IN AGAROSE PLUGS

Note: When 50-ml tubes are used for lysis, two plugs (reusable plug molds) or three plugs (disposable plug molds) of the same strain can be lysed in the same tube.

1.

Label 50-ml polypropylene screw-cap or 50-ml Oak Ridge tubes with culture numbers.

2. Prepare Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) as follows:

25 ml of 1 M Tris, pH 8.0
50 ml of 0.5 M EDTA, pH 8.0
50 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)1
Dilute to 500 ml with sterile Ultrapure water (CLRW)

3.

Calculate the total volume of Cell Lysis/Proteinase K Buffer needed as follows:

a. 5 ml Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) is needed per tube (e. g., 5 ml x 10 tubes = 50 ml).

b.

25 µl Proteinase K stock solution (20 mg/ml) is needed per tube of the cell lysis buffer (e. g., 25 µl x 10 tubes = 250 µl). The final concentration of Proteinase K in lysis buffer is 0.1 mg/ml.

c.

Prepare the master mix by measuring the correct volume of Cell Lysis Buffer and Proteinase K into appropriate size test tube or flask and mix well.

Note: Discard any thawed Proteinase K stock solution that was prepared from powder by the user at end of work day. Store commercially prepared Proteinase K solutions according to directions of the supplier.

4. Add 5 ml of Proteinase K/Cell Lysis Buffer to each labeled 50 ml tube.

5. If a flat edge is wanted on the plugs, trim excess agarose from top of plugs with scalpel or

1The N-Lauroylsarcosine, Sodium salt can be added directly to the other ingredients and allowed to dissolve. See page 13 of this document or Section 5a of the PulseNet PFGE Manual.

razor blade. Open mold and transfer plugs from mold with a 6-mm wide spatula to appropriately labeled tube. Be sure plugs are under buffer and not on side of tube.

Note: The excess agarose, scalpel, spatula, tape, etc. are contaminated. Dispose of or disinfect them appropriately.

6. Remove tape from reusable mold. Place both sections of plug mold, spatulas, and scalpel in 70% isopropanol (IPA) or other suitable disinfectant. Soak them for 15 minutes before washing them. Discard disposable plug molds or disinfect them in 10% bleach for 30-60 minutes if they will be washed and reused.
7. Place tubes in rack and incubate in a 54-55°C shaker water bath for 15-30 min with constant and vigorous agitation (175-200 rpm). Be sure water level in water bath is above level of lysis buffer in tubes.
8. Pre-heat enough sterile Ultrapure water (CLRW) to 54-55°C so that plugs can be washed two times with 10-15 ml water (200-250 ml for 10 tubes).

WASHING OF AGAROSE PLUGS AFTER CELL LYSIS

Note: Most laboratories will find that their plugs are sufficiently stable to perform the following washing steps at 54-55°C. However, if you notice that your plugs are nicked along the edges or breaking it will be necessary for your laboratory to lower the water bath or incubator to 50°C for the following washing steps.

1. Remove tubes from water bath, and carefully pour off lysis buffer. Plugs can be held in tubes with a screened cap (Bio-Rad) or spatula.

Note: Be sure to remove all of the liquid during this and subsequent wash steps by touching lip of tube onto an absorbent paper towel.

2. Add at least 10-15 ml sterile Ultrapure water (CLRW) to each tube. Discard water. This acts as a quick wash to remove lysis buffer left behind from the previous step. Add 10-15 ml of sterile Ultrapure water (CLRW) that has been pre-heated to 54-55°C to each tube and shake the tubes vigorously in a 54-55°C water bath for 10-15 min.
   a. Pre-heat enough sterile TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0) in a 54-55°C water bath so that plugs can be washed three times with 10-15 ml TE (300-350 ml for 10 tubes) after beginning last water wash.
3. Pour off water, add at least 10 -15 ml pre-heated (54-55°C) sterile TE Buffer, and shake the tubes vigorously in 55°C water bath for 10-15 min.
4. Pour off TE and repeat wash step with pre-heated TE two more times.
5. Decant last wash and add 5 ml sterile TE (room temperature). Store plugs in 5 ml sterile TE buffer at 4°C until ready to do the restriction digestion. Plugs can be transferred to smaller tubes (17-mm x 100-mm, 12-mm x 75-mm, etc.) for storage. Plugs made with disposable plug molds can be stored in 2 ml round bottom tubes containing 1.5 to 1.7 ml of TE.

RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS WITH SmaI

Note: A small slice of the plug or the entire plug can be digested with the primary restriction enzyme; SmaI. Restriction digestion of a small slice of the plug is recommended because less enzyme is required and other slices of the plug can be subjected to restriction analysis with other enzymes. KpnI is recommended as the secondary enzyme for analysis of Campylobacter jejuni isolates. The use of a secondary enzyme is useful in situations where the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable

Note: All PulseNet PFGE protocols use a strain of Salmonella choleraesuis ss. choleraesuis serotype Braenderup H9812 (ATCC BAA-664) as the molecular size standard. PFGE plugs (or blocks) of the Salmonella strain H9812 are made according the "PulseNet One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of E. coli O157:H7, Salmonella serotypes, and Shigella sonnei by PFGE" as described in the training manual (www.cdc.gov/pulsenet). This strain is used as a size standard for the normalization and analysis of PFGE patterns for all the organisms tracked by PulseNet, including E. coli O157:H7, Salmonella, Shigella, Listeria monocytogenes, Vibrio cholerae and Campylobacter jejuni. After plugs of the size standard are made, approximately 2-mm slices are cut and restricted with 40-50 Units of XbaI enzyme for 2 hours at 37°C. The plug slices are loaded on the electrophoresis gel in lanes 1, 5, 10 (10-well gel), 1, 5, 10, 15 (15-well gel), or 1, 5, 10, 15, 20 (20-well gel. New lots of S. Braenderup H9812 PFGE plugs should be tested with "old" lots to confirm that the pattern and band intensity is the same and that no observable genetic changes have occurred.

SmaI Restriction Reactions (KpnI conditions appear in parenthesis)

1.

Label 1.5-ml microcentrifuge tubes with culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for the size standard plug slice.

Note: The appropriate restriction buffer will vary between vendors and may differ between enzymes from the same vendor. Always use the restriction buffer recommended by the vendor for the particular restriction enzyme.

a. Optional Pre-Restriction Incubation Step: Prepare a master mix by diluting the appropriate 10X restriction buffer (Roche Applied Science or equivalent) 1:10 with sterile Ultrapure water (CLRW) according to the following table:

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water	180 µl	1800 µl	2700 µl
Restriction Buffer	20 µl	200 µl	300 µl
Total Volume	200 µl	2000 µl	3000 µl

c. Add 200 µl diluted 1X restriction buffer to labeled 1.5-ml microcentrifuge tubes.

d. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.

e. Cut a ~2.0 mm-wide slice from each test samples and the appropriate number of S. ser. Braenderup H9812 standards with a scalpel (or single edge razor blade, cover slip, etc.) and transfer to tube containing restriction buffer. Be sure plug slice is under buffer. Replace rest of plug in original tube that contains 5 ml TE buffer and store at 4°C.

Note: The shape and size of the plug slice that is cut will depend on the size of the comb teeth that are used for casting the gel. Gel wells that are cast with combs that have 10-mm-wide teeth will require a different size plug slice than those cast with combs that have smaller teeth (5.5-mm) teeth. The number of slices that can be cut from the plugs will also depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

f. Incubate plug slices in a 25°C water bath for 5-10 min or at room temperature for 10-15 min.

g. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to cut plug slice with pipet tip and that plug slice is not discarded with pipet tip.

2.

Prepare the restriction enzyme master mix by diluting 10X restriction buffer 1:10 with sterile Ultrapure water (CLRW) and adding SmaI restriction enzyme (40 U/sample) according to the following table. Mix in the same tube that was used for the diluted restriction buffer:

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20°C) at all times.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water	179 µl	1790 µl	2685 µl
Restriction Buffer	20 µl	200 µl	300 µl
Enzyme (40 U/µl)	1 µl	10 µl	15 µl
Total Volume	200 µl	2000 µl	3000 µl

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20°C) at all times.

a.

Optional Addition of Bovine Serum Albumulin (BSA): Several restriction enzyme vendors specifically recommend the addition of 1X BSA to enzyme restriction mixtures. However, BSA can be added to all enzyme restriction mixtures and may assist in reducing the incidence of incomplete restriction. If BSA is added to the enzyme reaction mixture, the volume of BSA added should be deducted from the volume of water to maintain the total volume of 200 µl per slice. For example, if (NEB BSA; 2µl of 100X per plug slice) the amount of water is 173 µl instead of 175 µl for each plug slice.

3. Add 200 µl restriction enzyme mixture to each tube. Close tube and mix by tapping gently; be sure plug slices are under enzyme mixture.

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2 De-ionized water (does not need to be sterilized).

4. Incubate sample and control plug slices at 25°C (room temp or water bath/chiller or incubator) for 4 h. For KpnI, incubate for 4-6 hours at 37°C.
5. If plug slices will be loaded into the wells (Option B, page 9), continue with Steps 1-4 of the next section (CASTING AGAROSE GEL) approximately 1 h before restriction digest reaction is finished so the gel can solidify for at least 30 minutes before loading the restricted PFGE plugs.

CASTING AGAROSE GEL

A. Loading Restricted Plug Slices on the Comb:

1. Confirm that water bath is equilibrated to 55-60°C.
2. Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables.

5X TBE:

Reagent	Volume in milliliters (ml)					
5X TBE	200	210	220	230	240	250
Reagent Grade Water2	1800	1890	1980	2070	2160	2250
Total Volume of 0.5X TBE	2000	2100	2200	2300	2400	2500

10X TBE:

Reagent	Volume in milliliters (ml)					
10X	10	10	11	11	12	12

TBE	0	5	0	5	0	5
sterile Ultra pure water (CLR W)	1900	1995	2090	2185	2280	2375
Total Volume of 0.5X TBE	2000	2100	2200	2300	2400	2500

3. Make 1% SeaKem Gold (SKG) Agarose in 0.5X TBE as follows:
    a. Weigh appropriate amount of SKG into 500 ml screw-cap flask. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.
    b. Remove cap, cover loosely with clear film, and microwave for 60-sec; mix gently and repeat for 15-sec intervals until agarose is completely dissolved.
    c. Recap flask and place in 55-60°C water bath.

    Mix 1.0 g agarose with 100 ml 0.5X TBE for 14-cm-wide gel form (10 or 15 wells)

    Mix 1.5 g agarose with 150 ml 0.5X TBE for 21-cm-wide gel form (15 wells)

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

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4. A small volume (2-5 ml) of melted 1% SKG agarose may be needed to fill wells after plugs are loaded. Prepare 50 ml by melting 0.5 g agarose with 50 ml 0.5X TBE in 250 ml screw-cap flask as described above. Unused SKG agarose can be kept at room temperature, melted, and reused several times. Microwave for 15-20 sec and mix; repeat for 10-sec intervals until agarose is completely melted. Place in 55-60°C water bath until ready to use. Alternatively, save approximately 5 ml of the melted agarose used to cast the gel in a pre-heated (55-60°C) 50 ml flask and place in 55-60°C water bath until used.

Note: Confirm that gel form is level on leveling table, that front of comb holder and teeth face the bottom of gel, and that the comb teeth touch the gel platform.

5. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.
6. Remove plug slices from tubes; put comb on bench top and load plug slices as close to the bottom edge of the teeth as possible. Load the plug slices in the following order:
    a. Load Salmonella serotype Branderup H9812 standards in lanes (teeth) 1, 5, 10 (10-well gel) or in lanes 1, 5, 10, 15 (15-well gel).
    b. Load samples on remaining teeth of the comb.

7. Remove excess buffer with tissue. Allow plug slices to air dry on the comb for 5-10 minutes or seal them to the comb with 1% SKG agarose (55-60°C).
8. Position comb in gel form and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth, that the lower edge of the plug slice is flush against the black platform, and there are no bubbles.
9. Carefully pour the agarose (cooled to 55-60°C) into the gel form.
10. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run.)
11. Turn on cooling module (14°C), power supply, and pump (setting of 70 for a flow of 1 liter/minute).
12. Remove comb after gel solidifies for 30-45 minutes.
13. Fill in wells of gel with melted and cooled (55- 60°C) 1% SKG Agarose (optional). Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

B. Loading Restricted Plug Slices into the Wells:

1. Follow steps 1-4 in "Option A" above (Loading Restricted Plug Slices on the Comb).

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2. Cool melted SKG agarose in 55-60°C water bath for 15-20 min; carefully pour agarose into gel form (casting stand) fitted with comb. Be sure there are no bubbles.

Note: Confirm that gel form is level on gel-leveling table before pouring gel, that front of comb holder and teeth face bottom of gel, and the bottom of the comb is 2-mm above the surface of the gel platform.

3. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer depends on whether residual buffer was left in tubing, or if unit was flushed with water after the last gel was run.)
4. Turn on cooling module (14°C), power supply, and pump (setting of 70 for a flow of 1 liter/minute) approximately 30 min before gel is to be run.
5. Remove enzyme/buffer mixture; add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.
6. Remove comb after gel solidifies for at least 30 minutes.
7. Remove restricted plug slices from tubes with tapered end of spatula and load into appropriate wells. Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure that are no bubbles.
    a. Load Salmonella serotype Branderup H9812 standards in lanes 1, 5, 10 (10-well gel) or in lanes 1, 5, 10, 15 (15-well gel).
    b. Load samples in remaining wells.

Note: Loading the plug slices can be tedious; each person has to develop his/her own technique for consistently placing the plug slices in the wells so the lanes will be straight and the bands sharp.

8. Fill in wells of gel with melted 1% SKG Agarose (equilibrated to 55- 60°C). Allow to harden for 3-5 min. Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

ELECTROPHORESIS CONDITIONS

A. SmaI: Select following conditions on Chef Mapper for Campylobacter jejuni.
Auto Algorithm
50 kb - low MW
400 kb - high MW
Select default values except where noted by pressing "enter".
Change run time to 18 hours
(Default values: Initial switch time = 6.76 s; Final switch time = 35.38 s).
B. KpnI: Select following conditions on Chef Mapper for Campylobacter jejuni.
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Two State
Gradient – 6.0V
Change run time to 18 hours
Included Angle – 120
Initial switch time =5.2 s
Final Switch time =42.3 s
Select default values except where noted by pressing "enter".
Note: The electrophoresis running times recommended above are based on the equipment and reagents used at the CDC. Running times in your laboratory may vary (faster or slower) and should be determined empirically.
Note: Make note of the initial milliamp (mAmp) reading on the instrument. The initial mAmps should be between 110-170 mAmps. A reading outside of this range may indicate that the 0.5X TBE buffer was prepared improperly and the buffer should be remade.

Day 2

STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL

1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide by diluting 40 µl of ethidium bromide stock solution (10 mg/ml) with 400 ml of reagent grade water (this volume is for a staining box that is approximately 14-cm x 24-cm; a larger container may require a larger amount of staining solution). Stain gel for 20 - 30 min in covered container.

Note: Ethidium bromide is toxic and a mutagen; the solution can be kept in dark bottle and reused 5 - 6 times before discarding according to your institution's guidelines for hazardous waste or use the destaining bags recommended for disposal of ethidium bromide (Section 10).

2. Destain gel in approximately 500 ml reagent grade water for 60 - 90 min; change water every 20 minutes. Capture image on Gel Doc 1000, Gel Doc 2000, or

equivalent documentation system. If background interferes with resolution, destain for an additional 30-60 min.

Note: If both a digital image and conventional photograph are wanted, photograph gel first before capturing digital image.

3. Follow directions given with the imaging equipment to save gel image as an *.img or *.1sc file; convert this file to *.tif file for analysis with BioNumerics software program The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Ensure that the image is in focus and that there is little to no staturation (over-exposure) in the bands. Additional instructions are provided in PNL07 of the PulseNet QA/QC manual.

4.

Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2 L reagent grade water or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 min before draining water from chamber.

5.

If the lowest band in the H9812 standard does not migrate within 1 -1.5 cm of the bottom of the gel, the run time will need to be determined empirically for the conditions in each laboratory.

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Please note the following if PFGE results do not have to be available within 24 hours:

1. Plugs can be lysed for longer periods of time (up to 2 hours).
2. The washing steps with TE to remove the lysis buffer from the PFGE plugs can be done for longer periods of time (15-30 min) and at lower temperatures (37°C or room temperature). They can be started on Day 1 and finished the morning of Day 2 after overnight refrigeration of the plugs in TE.
3. The restriction digestion can be done for longer periods of time (4-16 hours). Restrict for at least 4 hours when using 20 units of SmaI.

Use of trade names and commercial sources is for identification purposes only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

NOTE: CLIA LABORATORY PROCEDURE MANUAL REQUIREMENTS

Efforts have been made to assure that the procedures described in this protocol have been written in accordance with the 1988 Clinical Laboratory Improvement Amendments (CLIA) requirements for a procedure manual (42 CFR 493.1211). However, due to the format required for training, the procedures will require some modifications and additions to customize them for your particular laboratory operation.

Any questions regarding the CLIA requirements for a procedure manual, quality control, quality assurance, etc., should be directed to the agency or accreditation organization responsible for performing your laboratory's CLIA inspection. In addition, some states and accreditation organizations may have more stringent requirements that will need to be addressed.

Appendix III

Defra/VTRI Sequencing Unit

Campylobacter MLST Protocol (manual method)

Version 3

September 2007

CONTENTS

Extract Preparation

Reagents/Equipment required -

sterile phosphate buffered saline (PBS)
Sterile 1.5ml microcentrifuge tubes
Sterile plastic loops
Dry block heater (Grant UBD4)
Microcentrifuge
Storage racks
Freshly grown *Campylobacter* (or beads)

1. Switch block heater on and set temperature to 100°C.
2. Dispense 150µl sterile PBS into sufficient 1.5ml tubes; label tubes with unique identifier (usually culture collection number).
3. Add *either* a light inoculum of bacteria *or* 1 or 2 beads to each labelled tube (SINGLE BEAD IS THE PREFERRED OPTION).
4. Mix by vortexing.
5. Incubate in block heater at 100°C for 15 minutes.
6. Centrifuge at 13000rpm for 10 minutes.
7. Freeze at -20°C until required. Store at 4°C when thawed.

DO NOT VORTEX BEFORE USE. IF PELLET IS DISTURBED, RE-CENTRIFUGE BEFORE USE.

PCR - reactions

Reagents/Equipment required -

PCR reagents (dNTPs [10mM], 10x buffer, $MgCl_2$ [25mM], *Taq* DNA polymerase [5Uμl^{-1}], primers [10μM], molecular grade H_2O)
96-well non-skirted microtitre plates (AbGene)
Adhesive PCR film (AbGene)
DNA extracts
Thermal cycler
Centrifuge with microtitre plate rotor
Plate vortexer

1. In Excel create a spreadsheet to indicate which DNA isolate will be in each well of the half-microtitre plate. Use the blank form "Blank batch form.xls", and fill in the blank fields as appropriate. This sheet will act as a sample tracking sheet throughout the MLST process. Remember to include a negative control. Assign unique identifier to the plate in the format : MLST PCR XXX *A & B*, where *XXX* is the unique PCR batch number, A is the locus on the left hand side of the plate, and B is the locus on the right hand side of the. Record in the comments box if primers used differed from the standard set (Appendix I). Save a copy as the batch name i.e. MLST PCR XXX.

2. Mix PCR reagents together (Master mix) in the following quantities;

	1x Master Mix	52x Master Mix
Sigma molecular grade H_2O	36.75μl	1911μl
10x buffer	5.0μl	260μl
$MgCl_2$ (25mM)	3.0μl	156μl
dNTPs (10mM stock)	1.0μl	52μl
Forward primer (10μM stock)	1.0μl	52μl
Reverse primer (10μM stock)	1.0μl	52μl
Taq polymerase (5 units/μl)	0.25μl	13μl

3. Aliquot 48 μl master mix per microtitre well and tap plate gently to ensure liquid is in the bottom of the well. Pipette 2 μl DNA (boiled lysate) onto the side of each well as per plate layout created in Excel.

4. Gently tap plate to move DNA to well bottom and carefully seal the plate with adhesive film, paying particular attention at the edges. Vortex and spin plate briefly at 500 rpm.

5. Place plate in thermal cycler and load program with the following conditions;

C. jejuni isolates / primers;

95 °C for 3 min
94 °C for 20 sec
50 °C for 20 sec } X 35
72 °C for 1 min
72 °C for 5 min
4 °C forever.

C. coli isolates / primers;

95 °C for 3 min
94 °C for 30 sec
50 °C for 30 sec } X 35
72 °C for 1 min
72 °C for 5 min
4 °C forever.

6. Mix 5 µl of each sample with 1 µl 6x loading buffer and load into wells of a 2% agarose gel containing ethidium bromide 0.5 µg / ml. Electrophorese at about 120 V for 20 min and visualise DNA on a U.V. transilluminator.

The method can be halted here indefinitely, with products being stored at 4°C for up to 2 weeks, or at -20°C for indefinite storage.

PCR product clean-up (PEG precipitation)

1. Aliquot 60 µl 20% (w/v) PEG_{8000}, 2.5M NaCl per well, using a multichannel pipette, seal wells with adhesive film, vortex and briefly spin the plate at 500 rcf to ensure mix is at the bottom of the wells. Incubate the plates for either 15 min at 37 °C, 30 min at 20 °C or overnight at 4 °C. (Longer incubations do not have a detrimental effect on the clean up procedure).

2. Spin at 2750 rcf at 4 °C for 60 min.

3. To remove PEG, place folded blue tissue into the bottom of the centrifuge plate holders and gently invert the plate onto blue tissue. Spin at 500 rpm for 60 sec.

4. Wash pellet twice with 150 µl 70% ice-cold ethanol. i.e. add 150 µl per well and spin at 2750 rcf for 10 min. Remove ethanol by inversion of plate onto blue tissues, and then spin inverted plate on folded clean blue at 500 rpm for 60 sec. Repeat.

5. Air dry plate on bench for 10 min.

6. Re-suspend pellet in STERILE milliQ water. Re-suspension volume is dependant on intensity of PCR product observed following PCR e.g. Barely visible products are re-suspended in 5 µl with more intense products re-suspended in volumes up to 50 µl. Volumes for each locus batch are determined with reference to intensity of product band on gel image. Seal lid carefully, vortex and spin briefly.

7. Resuspended products can be stored long-term at -20°C, or short-term at 4°C.

Sequencing reactions

1. Create a speadsheet in Excel to indicate which isolate/primers will be in which wells, such that the PCR product from well A1 will be in A1 and A2, the forward primer will be A1 and the reverse in A2. PCR product from A2 in A3 and A4 etc, according to the sequence plate pipetting guide sheet in Appendix VII. The name of the isolate is unimportant but the extension must be in the format indicated. (*pgm* and *tkt* are expressed as eg. pgm_1 and tkt_1). Use template "Sequencing plate template (aspA).xls". Assign a unique identifier in the format : VTRI_XXX*loc*_dd.mm.yy, where XXX is the unique batch number, *loc* is the locus to be sequenced, and *dd.mm.yy* is the date on which the sequencing reaction was run. Save a copy as the run name. Remember to fill in all the appropriate fields. For subsequent loci, use the "replace" function in Excel to change the locus name, and save a copy under the name of that locus.

	1	2	3	4	5	6	7	8	9	10	11	12
A	806.aspA1	806.aspA2	869.aspA1	869.aspA2	1030.aspA1	1030.aspA2	1200.aspA1	1200.aspA2	1267.aspA1	1267.aspA2	1431.aspA1	1431.aspA2
B	808.aspA1	808.aspA2	875.aspA1	875.aspA2	1062.aspA1	1062.aspA2	1202.aspA1	1202.aspA2	1280.aspA1	1280.aspA2	1434.aspA1	1434.aspA2
C	809.aspA1	809.aspA2	882.aspA1	882.aspA2	1075.aspA1	1075.aspA2	1209.aspA1	1209.aspA2	1291.aspA1	1291.aspA2	1491.aspA1	1491.aspA2
D	815.aspA1	815.aspA2	892.aspA1	892.aspA2	1079.aspA1	1079.aspA2	1210.aspA1	1210.aspA2	1293.aspA1	1293.aspA2	1495.aspA1	1495.aspA2
E	818.aspA1	818.aspA2	912.aspA1	912.aspA2	1094.aspA1	1094.aspA2	1212.aspA1	1212.aspA2	1310.aspA1	1310.aspA2	1506.aspA1	1506.aspA2
F	825.aspA1	825.aspA2	920.aspA1	920.aspA2	1190.aspA1	1190.aspA2	1218.aspA1	1218.aspA2	1417.aspA1	1417.aspA2	1540.aspA1	1540.aspA2
G	834.aspA1	834.aspA2	923.aspA1	923.aspA2	1192.aspA1	1192.aspA2	1219.aspA1	1219.aspA2	1418.aspA1	1418.aspA2	1558.aspA1	1558.aspA2
H	850.aspA1	850.aspA2	935.aspA1	935.aspA2	1196.aspA1	1196.aspA2	1221.aspA1	1221.aspA2	1423.aspA1	1423.aspA1	blank	blank

2. Open the file **plate3700.xls** and enable macros. Copy and paste the data from the above spreadsheet (excluding cell letter/number) into it and select the Save cell. Save as a .txt file. This is required as a list file for STARS to rename the data prior to analysis. Do NOT save changes to plate3700.xls.

3. Make up master mix in required volume. Make two batches of 50 aliquots per sequencing plate :

	1x Master Mix	50x Master Mix
Molecular grade H_2O 119 µl	2.38 µl	
5x buffer 93.5 µl	1.87 µl	

Big Dye	0.25 µl
12.5 µl	
Primer (forward OR reverse) [0.67µM]	4 µl
200 µl	

N.B. Sequencing primers are at 0.67µM i.e. 1:15 dilution of PCR primer concentration (see Appendix I). Sequencing primers are not necessarily the same as the PCR primers.

4. Add 8.5µl of master mix containing forward primer to wells of columns 1,3,5,7,9 and 11; 8.5µl of master mix containing reverse primer to wells of columns 2,4,6,8,10 and 12

5. Pipette 1.5 µl of the first PCR product onto the side of wells A1 and A2. Repeat for remainder of wells as per plate layout. Spin briefly to move DNA template to bottom of wells.

6. Place plate in thermal cycler and load program with the following conditions;

96 °C for 10 sec
50 °C for 5 sec
60 °C for 2 min
} X 30
4 °C forever.

7. Do not stop at this point. Proceed immediately to precipitation unless sequencing reaction runs overnight.

Sequencing reaction clean-up (ethanol precipitation)

a. Per plate mix 7000 µl 100% ethanol and 280 µl 3M sodium acetate and aliquot 52 µl per well.

b. Replace adhesive film, vortex and briefly spin (500 rpm). Incubate at room temp for 45 min and spin at 2750 rcf (4 °C) for 1 h.

c. Remove adhesive film and gently invert plate onto absorbent tissue. Spin inverted plate on fresh tissue (500 rpm) for < 1min.

d. Wash pellet once by addition of 150 µl ice-cold 70% ethanol per well, cover plate with film and spin at 2750 rcf for 10 min.

e. Remove adhesive film, invert plate onto absorbent tissue and give a final short inverted spin at 500 rpm.

f. Air dry at room temp for 10 minutes. Recover plate with adhesive film and store at –20 °C prior to sequencing (MAXIMUM 72 hours).

g. Aliquot 10µl HiDi (formamide) per well, vortex and briefly spin.

h. Heat denature 2mins at 94°C. Allow to cool, remove film, and load plate onto sequencer.

Appendix IV

MLST Routine Reagent Recipes

20% PEG_{8000}/2.5M $NaCl_2$ - 200g PEG_{8000}
146.1g $NaCl_2$

Make up to 1l in Duran bottle with distilled H_2O.

Put in magnetic stirrer and leave to stir overnight.

3M sodium acetate - 24.6g sodium acetate in 100ml of distilled H_2O.

BIOLOG

Phenotype MicroArrays™

PM1 MicroPlate™ Carbon Sources

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 M-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 M-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

PM2A MicroPlate™ Carbon Sources

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin	A4 β-Cyclodextrin	A5 γ-Cyclodextrin	A6 Dextrin	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin	A11 Mannan	A12 Pectin
B1 N-Acetyl-D-Galactosamine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose	B4 Amygdalin	B5 D-Arabinose	B6 D-Arabitol	B7 L-Arabitol	B8 Arbutin	B9 2-Deoxy-D-Ribose	B10 I-Erythritol	B11 D-Fucose	B12 3-O-β-D-Galacto-pyranosyl-D-Arabinose
C1 Gentiobiose	C2 L-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 α-Methyl-D-Glucoside	C7 β-Methyl-D-Galactoside	C8 3-Methyl Glucose	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside	C11 β-Methyl-D-Xyloside	C12 Palatinose
D1 D-Raffinose	D2 Salicin	D3 Sedoheptulosan	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose	D7 Turanose	D8 Xylitol	D9 N-Acetyl-D-Glucosaminitol	D10 γ-Amino Butyric Acid	D11 δ-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 α-Keto Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionic Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-L-Proline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenylalanine	H3 L-Pyroglutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 Sec-Butylamine	H7 D,L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone	H10 2,3-Butanediol	H11 2,3-Butanone	H12 3-Hydroxy 2-Butanone

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Phenotype MicroArrays™

PM3B MicroPlate™ Nitrogen Sources

A1 Negative Control	A2 Ammonia	A3 Nitrite	A4 Nitrate	A5 Urea	A6 Biuret	A7 L-Alanine	A8 L-Arginine	A9 L-Asparagine	A10 L-Aspartic Acid	A11 L-Cysteine	A12 L-Glutamic Acid
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-Isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-Methionine	B8 L-Phenylalanine	B9 L-Proline	B10 L-Serine	B11 L-Threonine	B12 L-Tryptophan
C1 L-Tyrosine	C2 L-Valine	C3 D-Alanine	C4 D-Asparagine	C5 D-Aspartic Acid	C6 D-Glutamic Acid	C7 D-Lysine	C8 D-Serine	C9 D-Valine	C10 L-Citrulline	C11 L-Homoserine	C12 L-Ornithine
D1 N-Acetyl-D,L-Glutamic Acid	D2 N-Phthaloyl-L-Glutamic Acid	D3 L-Pyroglutamic Acid	D4 Hydroxylamine	D5 Methylamine	D6 N-Amylamine	D7 N-Butylamine	D8 Ethylamine	D9 Ethanolamine	D10 Ethylenediamine	D11 Putrescine	D12 Agmatine
E1 Histamine	E2 β-Phenylethyl-amine	E3 Tyramine	E4 Acetamide	E5 Formamide	E6 Glucuronamide	E7 D,L-Lactamide	E8 D-Glucosamine	E9 D-Galactosamine	E10 D-Mannosamine	E11 N-Acetyl-D-Glucosamine	E12 N-Acetyl-D-Galactosamine
F1 N-Acetyl-D-Mannosamine	F2 Adenine	F3 Adenosine	F4 Cytidine	F5 Cytosine	F6 Guanine	F7 Guanosine	F8 Thymine	F9 Thymidine	F10 Uracil	F11 Uridine	F12 Inosine
G1 Xanthine	G2 Xanthosine	G3 Uric Acid	G4 Alloxan	G5 Allantoin	G6 Parabanic Acid	G7 D,L-α-Amino-N-Butyric Acid	G8 γ-Amino-N-Butyric Acid	G9 ε-Amino-N-Caproic Acid	G10 D,L-α-Amino-Caprylic Acid	G11 δ-Amino-N-Valeric Acid	G12 α-Amino-N-Valeric Acid
H1 Ala-Asp	H2 Ala-Gln	H3 Ala-Glu	H4 Ala-Gly	H5 Ala-His	H6 Ala-Leu	H7 Ala-Thr	H8 Gly-Asn	H9 Gly-Gln	H10 Gly-Glu	H11 Gly-Met	H12 Met-Ala

PM4A MicroPlate™ Phosphorus and Sulfur Sources

A1 Negative Control	A2 Phosphate	A3 Pyrophosphate	A4 Trimeta-phosphate	A5 Tripoly-phosphate	A6 Triethyl Phosphate	A7 Hypophosphite	A8 Adenosine- 2'-monophosphate	A9 Adenosine- 3'-monophosphate	A10 Adenosine- 5'-monophosphate	A11 Adenosine- 2',3'-cyclic monophosphate	A12 Adenosine- 3',5'-cyclic monophosphate
B1 Thiophosphate	B2 Dithiophosphate	B3 D,L-α-Glycerol Phosphate	B4 β-Glycerol Phosphate	B5 Carbamyl Phosphate	B6 D-2-Phospho-Glyceric Acid	B7 D-3-Phospho-Glyceric Acid	B8 Guanosine- 2'-monophosphate	B9 Guanosine- 3'-monophosphate	B10 Guanosine- 5'-monophosphate	B11 Guanosine- 2',3'-cyclic monophosphate	B12 Guanosine- 3',5'-cyclic monophosphate
C1 Phosphoenol Pyruvate	C2 Phospho-Glycolic Acid	C3 D-Glucose-1-Phosphate	C4 D-Glucose-6-Phosphate	C5 2-Deoxy-D-Glucose 6-Phosphate	C6 D-Glucosamine-6-Phosphate	C7 6-Phospho-Gluconic Acid	C8 Cytidine- 2'-monophosphate	C9 Cytidine- 3'-monophosphate	C10 Cytidine- 5'-monophosphate	C11 Cytidine- 2',3'-cyclic monophosphate	C12 Cytidine- 3',5'-cyclic monophosphate
D1 D-Mannose-1-Phosphate	D2 D-Mannose-6-Phosphate	D3 Cysteamine-S-Phosphate	D4 Phospho-L-Arginine	D5 O-Phospho-D-Serine	D6 O-Phospho-L-Serine	D7 O-Phospho-L-Threonine	D8 Uridine- 2'-monophosphate	D9 Uridine- 3'-monophosphate	D10 Uridine- 5'-monophosphate	D11 Uridine- 2',3'-cyclic monophosphate	D12 Uridine- 3',5'-cyclic monophosphate
E1 O-Phospho-D-Tyrosine	E2 O-Phospho-L-Tyrosine	E3 Phosphocreatine	E4 Phosphoryl Choline	E5 O-Phosphoryl-Ethanolamine	E6 Phosphono Acetic Acid	E7 2-Aminoethyl Phosphonic Acid	E8 Methylene Diphosphonic Acid	E9 Thymidine- 3'-monophosphate	E10 Thymidine- 5'-monophosphate	E11 Inositol Hexaphosphate	E12 Thymidine 3',5'- cyclic monophosphate
F1 Negative Control	F2 Sulfate	F3 Thiosulfate	F4 Tetrathionate	F5 Thiophosphate	F6 Dithiophosphate	F7 L-Cysteine	F8 D-Cysteine	F9 L-Cysteinyl-Glycine	F10 L-Cystoic Acid	F11 Cysteamine	F12 L-Cysteine Sulfinic Acid
G1 N-Acetyl-L-Cysteine	G2 S-Methyl-L-Cysteine	G3 Cystathionine	G4 Lanthionine	G5 Glutathione	G6 D,L-Ethionine	G7 L-Methionine	G8 D-Methionine	G9 Glycyl-L-Methionine	G10 N-Acetyl-D,L-Methionine	G11 L- Methionine Sulfoxide	G12 L-Methionine Sulfone
H1 L-Djenkolic Acid	H2 Thiourea	H3 1-Thio-β-D-Glucose	H4 D,L-Lipoamide	H5 Taurocholic Acid	H6 Taurine	H7 Hypotaurine	H8 p-Amino Benzene Sulfonic Acid	H9 Butane Sulfonic Acid	H10 2-Hydroxyethane Sulfonic Acid	H11 Methane Sulfonic Acid	H12 Tetramethylene Sulfone

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PM5 MicroPlate™ Nutrient Supplements

A1 Negative Control	A2 Positive Control	A3 L-Alanine	A4 L-Arginine	A5 L-Asparagine	A6 L-Aspartic Acid	A7 L-Cysteine	A8 L-Glutamic Acid	A9 Adenosine-3',5'-cyclic monophosphate	A10 Adenine	A11 Adenosine	A12 2'-Deoxy Adenosine
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-Isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-Methionine	B8 L-Phenylalanine	B9 Guanosine-3',5'-cyclic monophosphate	B10 Guanine	B11 Guanosine	B12 2'-Deoxy Guanosine
C1 L-Proline	C2 L-Serine	C3 L-Threonine	C4 L-Tryptophan	C5 L-Tyrosine	C6 L-Valine	C7 L-Isoleucine + L-Valine	C8 trans-4-Hydroxy L-Proline	C9 (5) 4-Amino-imidazole-4(5)-Carboxamide	C10 Hypoxanthine	C11 Inosine	C12 2'-Deoxy Inosine
D1 L-Ornithine	D2 L-Citrulline	D3 Chorismic Acid	D4 (-)Shikimic Acid	D5 L-Homoserine Lactone	D6 D-Alanine	D7 D-Aspartic Acid	D8 D-Glutamic Acid	D9 D,L-α,ε-Diamino-pimelic Acid	D10 Cytosine	D11 Cytidine	D12 2'-Deoxy Cytidine
E1 Putrescine	E2 Spermidine	E3 Spermine	E4 Pyridoxine	E5 Pyridoxal	E6 Pyridoxamine	E7 β-Alanine	E8 D-Pantothenic Acid	E9 Orotic Acid	E10 Uracil	E11 Uridine	E12 2'-Deoxy Uridine
F1 Quinolinic Acid	F2 Nicotinic Acid	F3 Nicotinamide	F4 β-Nicotinamide Adenine Dinucleotide	F5 δ-Amino-Levulinic Acid	F6 Hematin	F7 Deferoxamine Mesylate	F8 D-(+)-Glucose	F9 N-Acetyl D-Glucosamine	F10 Thymine	F11 Glutathione (reduced form)	F12 Thymidine
G1 Oxaloacetic Acid	G2 D-Biotin	G3 Cyano-Cobalamine	G4 p-Amino-Benzoic Acid	G5 Folic Acid	G6 Inosine + Thiamine	G7 Thiamine	G8 Thiamine Pyrophosphate	G9 Riboflavin	G10 Pyrrolo-Quinoline Quinone	G11 Menadione	G12 Myo-Inositol
H1 Butyric Acid	H2 D,L-α-Hydroxy-Butyric Acid	H3 α-Ketobutyric Acid	H4 Capryllic Acid	H5 D,L-α-Lipoic Acid (oxidized form)	H6 D,L-Mevalonic Acid	H7 D,L-Carnitine	H8 Choline	H9 Tween 20	H10 Tween 40	H11 Tween 60	H12 Tween 80

PM6 MicroPlate™ Peptide Nitrogen Sources

A1 Negative Control	A2 Positive Control: L-Glutamine	A3 Ala-Ala	A4 Ala-Arg	A5 Ala-Asn	A6 Ala-Glu	A7 Ala-Gly	A8 Ala-His	A9 Ala-Leu	A10 Ala-Lys	A11 Ala-Phe	A12 Ala-Pro
B1 Ala-Ser	B2 Ala-Thr	B3 Ala-Trp	B4 Ala-Tyr	B5 Arg-Ala	B6 Arg-Arg	B7 Arg-Asp	B8 Arg-Gln	B9 Arg-Glu	B10 Arg-Ile	B11 Arg-Leu	B12 Arg-Lys
C1 Arg-Met	C2 Arg-Phe	C3 Arg-Ser	C4 Arg-Trp	C5 Arg-Tyr	C6 Arg-Val	C7 Asn-Glu	C8 Asn-Val	C9 Asp-Asp	C10 Asp-Glu	C11 Asp-Leu	C12 Asp-Lys
D1 Asp-Phe	D2 Asp-Trp	D3 Asp-Val	D4 Cys-Gly	D5 Gln-Gln	D6 Gln-Gly	D7 Glu-Asp	D8 Glu-Glu	D9 Glu-Gly	D10 Glu-Ser	D11 Glu-Trp	D12 Glu-Tyr
E1 Glu-Val	E2 Gly-Ala	E3 Gly-Arg	E4 Gly-Cys	E5 Gly-Gly	E6 Gly-His	E7 Gly-Leu	E8 Gly-Lys	E9 Gly-Met	E10 Gly-Phe	E11 Gly-Pro	E12 Gly-Ser
F1 Gly-Thr	F2 Gly-Trp	F3 Gly-Tyr	F4 Gly-Val	F5 His-Asp	F6 His-Gly	F7 His-Leu	F8 His-Lys	F9 His-Met	F10 His-Pro	F11 His-Ser	F12 His-Trp
G1 His-Tyr	G2 His-Val	G3 Ile-Ala	G4 Ile-Arg	G5 Ile-Gln	G6 Ile-Gly	G7 Ile-His	G8 Ile-Ile	G9 Ile-Met	G10 Ile-Phe	G11 Ile-Pro	G12 Ile-Ser
H1 Ile-Trp	H2 Ile-Tyr	H3 Ile-Val	H4 Leu-Ala	H5 Leu-Arg	H6 Leu-Asp	H7 Leu-Glu	H8 Leu-Gly	H9 Leu-Ile	H10 Leu-Leu	H11 Leu-Met	H12 Leu-Phe

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PM7 MicroPlate™ Peptide Nitrogen Sources

A1 Negative Control	A2 Positive Control: L-Glutamine	A3 Leu-Ser	A4 Leu-Trp	A5 Leu-Val	A6 Lys-Ala	A7 Lys-Arg	A8 Lys-Glu	A9 Lys-Ile	A10 Lys-Leu	A11 Lys-Lys	A12 Lys-Phe
B1 Lys-Pro	B2 Lys-Ser	B3 Lys-Thr	B4 Lys-Trp	B5 Lys-Tyr	B6 Lys-Val	B7 Met-Arg	B8 Met-Asp	B9 Met-Gln	B10 Met-Glu	B11 Met-Gly	B12 Met-His
C1 Met-Ile	C2 Met-Leu	C3 Met-Lys	C4 Met-Met	C5 Met-Phe	C6 Met-Pro	C7 Met-Trp	C8 Met-Val	C9 Phe-Ala	C10 Phe-Gly	C11 Phe-Ile	C12 Phe-Phe
D1 Phe-Pro	D2 Phe-Ser	D3 Phe-Trp	D4 Pro-Ala	D5 Pro-Asp	D6 Pro-Gln	D7 Pro-Gly	D8 Pro-Hyp	D9 Pro-Leu	D10 Pro-Phe	D11 Pro-Pro	D12 Pro-Tyr
E1 Ser-Ala	E2 Ser-Gly	E3 Ser-His	E4 Ser-Leu	E5 Ser-Met	E6 Ser-Phe	E7 Ser-Pro	E8 Ser-Ser	E9 Ser-Tyr	E10 Ser-Val	E11 Thr-Ala	E12 Thr-Arg
F1 Thr-Glu	F2 Thr-Gly	F3 Thr-Leu	F4 Thr-Met	F5 Thr-Pro	F6 Trp-Ala	F7 Trp-Arg	F8 Trp-Asp	F9 Trp-Glu	F10 Trp-Gly	F11 Trp-Leu	F12 Trp-Lys
G1 Trp-Phe	G2 Trp-Ser	G3 Trp-Trp	G4 Trp-Tyr	G5 Tyr-Ala	G6 Tyr-Gln	G7 Tyr-Glu	G8 Tyr-Gly	G9 Tyr-His	G10 Tyr-Leu	G11 Tyr-Lys	G12 Tyr-Phe
H1 Tyr-Trp	H2 Tyr-Tyr	H3 Val-Arg	H4 Val-Asn	H5 Val-Asp	H6 Val-Gly	H7 Val-His	H8 Val-Ile	H9 Val-Leu	H10 Val-Tyr	H11 Val-Val	H12 Y-Glu-Gly

PM8 MicroPlate™ Peptide Nitrogen Sources

A1 Negative Control	A2 Positive Control: L-Glutamine	A3 Ala-Asp	A4 Ala-Gln	A5 Ala-Ile	A6 Ala-Met	A7 Ala-Val	A8 Asp-Ala	A9 Asp-Gln	A10 Asp-Gly	A11 Glu-Ala	A12 Gly-Asn
B1 Gly-Asp	B2 Gly-Ile	B3 His-Ala	B4 His-Glu	B5 His-His	B6 Ile-Asn	B7 Ile-Leu	B8 Leu-Asn	B9 Leu-His	B10 Leu-Pro	B11 Leu-Tyr	B12 Lys-Asp
C1 Lys-Gly	C2 Lys-Met	C3 Met-Thr	C4 Met-Tyr	C5 Phe-Asp	C6 Phe-Glu	C7 Gln-Glu	C8 Phe-Met	C9 Phe-Tyr	C10 Phe-Val	C11 Pro-Arg	C12 Pro-Asn
D1 Pro-Glu	D2 Pro-Ile	D3 Pro-Lys	D4 Pro-Ser	D5 Pro-Trp	D6 Pro-Val	D7 Ser-Asn	D8 Ser-Asp	D9 Ser-Gln	D10 Ser-Glu	D11 Thr-Asp	D12 Thr-Gln
E1 Thr-Phe	E2 Thr-Ser	E3 Trp-Val	E4 Tyr-Ile	E5 Tyr-Val	E6 Val-Ala	E7 Val-Gln	E8 Val-Glu	E9 Val-Lys	E10 Val-Met	E11 Val-Phe	E12 Val-Pro
F1 Val-Ser	F2 β-Ala-Ala	F3 β-Ala-Gly	F4 β-Ala-His	F5 Met-β-Ala	F6 β-Ala-Phe	F7 D-Ala-D-Ala	F8 D-Ala-Gly	F9 D-Ala-Leu	F10 D-Leu-D-Leu	F11 D-Leu-Gly	F12 D-Leu-Tyr
G1 Y-Glu-Gly	G2 Y-D-Glu-Gly	G3 Gly-D-Ala	G4 Gly-D-Asp	G5 Gly-D-Ser	G6 Gly-D-Thr	G7 Gly-D-Val	G8 Leu-β-Ala	G9 Leu-D-Leu	G10 Phe-β-Ala	G11 Ala-Ala-Ala	G12 D-Ala-Gly-Gly
H1 Gly-Gly-Ala	H2 Gly-Gly-D-Leu	H3 Gly-Gly-Gly	H4 Gly-Gly-Ile	H5 Gly-Gly-Leu	H6 Gly-Gly-Phe	H7 Val-Tyr-Val	H8 Gly-Phe-Phe	H9 Leu-Gly-Gly	H10 Leu-Leu-Leu	H11 Phe-Gly-Gly	H12 Tyr-Gly-Gly

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PM9 MicroPlate™ Osmolytes

A1 NaCl 1%	A2 NaCl 2%	A3 NaCl 3%	A4 NaCl 4%	A5 NaCl 5%	A6 NaCl 5.5%	A7 NaCl 6%	A8 NaCl 6.5%	A9 NaCl 7%	A10 NaCl 8%	A11 NaCl 9%	A12 NaCl 10%
B1 NaCl 6%	B2 NaCl 6% + Betaine	B3 NaCl 6% + N-N Dimethyl glycine	B4 NaCl 6% + Sarcosine	B5 NaCl 6% + Dimethyl sulphonyl propionate	B6 NaCl 6% + MOPS	B7 NaCl 6% + Ectoine	B8 NaCl 6% + Choline	B9 NaCl 6% + Phosphoryl choline	B10 NaCl 6% + Creatine	B11 NaCl 6% + Creatinine	B12 NaCl 6% + L- Carnitine
C1 NaCl 6% + KCl	C2 NaCl 6% + L-proline	C3 NaCl 6% + N-Acetlyl L-glutamine	C4 NaCl 6% + β-Glutamic acid	C5 NaCl 6% + γ–Amino -n- butyric acid	C6 NaCl 6% + Glutathione	C7 NaCl 6% + Glycerol	C8 NaCl 6% + Trehalose	C9 NaCl 6% + Trimethylamine -N-oxide	C10 NaCl 6% + Trimethylamine	C11 NaCl 6% + Octopine	C12 NaCl 6% + Trigonellino
D-1 Potassium chloride 3%	D2 Potassium chloride 4%	D3 Potassium chloride 5%	D4 Potassium chloride 6%	D5 Sodium sulfate 2%	D6 Sodium sulfate 3%	D7 Sodium sulfate 4%	D8 Sodium sulfate 5%	D9 Ethylene glycol 5%	D10 Ethylene glycol 10%	D11 Ethylene glycol 15%	D12 Ethylene glycol 20%
E1 Sodium formate 1%	E2 Sodium formate 2%	E3 Sodium formate 3%	E4 Sodium formate 4%	E5 Sodium formate 5%	E6 Sodium formate 6%	E7 Urea 2%	E8 Urea 3%	E9 Urea 4%	E10 Urea 5%	E11 Urea 6%	E12 Urea 7%
F1 Sodium Lactate 1%	F2 Sodium Lactate 2%	F3 Sodium Lactate 3%	F4 Sodium Lactate 4%	F5 Sodium Lactate 5%	F6 Sodium Lactate 6%	F7 Sodium Lactate 7%	F8 Sodium Lactate 8%	F9 Sodium Lactate 9%	F10 Sodium Lactate 10%	F11 Sodium Lactate 11%	F12 Sodium Lactate 12%
G1 Sodium Phosphate pH 7 20mM	G2 Sodium Phosphate pH 7 50mM	G3 Sodium Phosphate pH 7 100mM	G4 Sodium Phosphate pH 7 200mM	G5 Sodium Benzoate pH 5.2 20mM	G6 Sodium Benzoate pH 5.2 50mM	G7 Sodium Benzoate pH5.2 100mM	G8 Sodium Benzoate pH 5.2 200mM	G9 Ammonium sulfate pH8 10mM	G10 Ammonium sulfate pH 8 20mM	G11 Ammonium sulfate pH 8 50mM	G12 Ammonium sulfate pH8 100mM
H1 Sodium Nitrate 10mM	H2 Sodium Nitrate 20mM	H3 Sodium Nitrate 40mM	H4 Sodium Nitrate 60mM	H5 Sodium Nitrate 80mM	H6 Sodium Nitrate 100mM	H7 Sodium Nitrite 10mM	H8 Sodium Nitrite 20mM	H9 Sodium Nitrite 40mM	H10 Sodium Nitrite 60mM	H11 Sodium Nitrite 80mM	H12 Sodium Nitrite 100mM

PM10 MicroPlate™ pH

A1 pH 3.5	A2 pH 4	A3 pH 4.5	A4 pH 5	A5 pH 5.5	A6 pH 6	A7 pH 7	A8 pH 8	A9 pH 8.5	A10 pH 9	A11 pH 9.5	A12 pH 10
B1 pH 4.5	B2 pH 4.5 + L-Alanine	B3 pH 4.5 + L-Arginine	B4 pH 4.5 + L-Asparagine	B5 pH 4.5 + L-Aspartic Acid	B6 pH 4.5 + L-Glutamic Acid	B7 pH 4.5 + L-Glutamine	B8 pH 4.5 + Glycine	B9 pH 4.5 + L-Histidine	B10 pH 4.5 + L-Isoleucine	B11 pH 4.5 + L-Leucine	B12 pH 4.5 + L-Lysine
C1 pH 4.5 + L-Methionine	C2 pH 4.5 + L- Phenylalanine	C3 pH 4.5 + L-Proline	C4 pH 4.5 + L-Serine	C5 pH 4.5 + L-Threonine	C6 pH 4.5 + L-Tryptophan	C7 pH 4.5 + L-Tyrosine	C8 pH 4.5 + L-Valine	C9 pH 4.5 + Hydroxy- L-Proline	C10 pH 4.5 + L-Ornithine	C11 pH 4.5 + L-Homoarginine	C12 pH 4.5 + L-Homoserine
D-1 pH 4.5 + Anthranilic acid	D2 pH 4.5 + L-Norleucine	D3 pH 4.5 + L-Norvaline	D4 pH 4.5 + α- Amino-N- butyric acid	D5 pH 4.5 + p- Aminobenzoate	D6 pH 4.5 + L-Cysteic acid	D7 pH 4.5 + D-Lysine	D8 pH 4.5 + 5-Hydroxy Lysine	D9 pH 4.5 + 6-Hydroxy Tryptophan	D10 pH 4.5 + D,L-Diamino pimelic acid	D11 pH 4.5 + Trimethyl amine-N-oxide	D12 pH 4.5 + Urea
E1 pH 9.5	E2 pH 9.5 + L-Alanine	E3 pH 9.5 + L-Arginine	E4 pH 9.5 + L-Asparagine	E5 pH 9.5 + L-Aspartic Acid	E6 pH 9.5 + L-Glutamic Acid	E7 pH 9.5 + L-Glutamine	E8 pH 9.5 + Glycine	E9 pH 9.5 + L-Histidine	E10 pH 9.5 + L-Isoleucine	E11 pH 9.5 + L-Leucine	E12 pH 9.5 + L-Lysine
F1 pH 9.5 + L-Methionine	F2 pH 9.5 + L- Phenylalanine	F3 pH 9.5 + L-Proline	F4 pH 9.5 + L-Serine	F5 pH 9.5 + L-Threonine	F6 pH 9.5 + L-Tryptophan	F7 pH 9.5 + L-Tyrosine	F8 pH 9.5 + L-Valine	F9 pH 9.5 + Hydroxy- L-Proline	F10 pH 9.5 + L-Ornithine	F11 pH 9.5 + L-Homoarginine	F12 pH 9.5 + L-Homoserine
G1 pH 9.5 + Anthranilic acid	G2 pH 9.5 + L-Norleucine	G3 pH 9.5 + L-Norvaline	G4 pH 9.5 + Agmatine	G5 pH 9.5 + Cadaverine	G6 pH 9.5 + Putrescine	G7 pH 9.5 + Histamine	G8 pH 9.5 + Phenylethylamin e	G9 pH 9.5 + Tyramine	G10 pH 9.5 + Creatine	G11 pH 9.5 + Trimethyl amine-N-oxide	G12 pH 9.5 + Urea
H1 X-Caprylate	H2 X-α-D- Glucoside	H3 X-β-D- Glucoside	H4 X-α-D- Galactoside	H5 X-β-D- Galactoside	H6 X-α- D- Glucuronide	H7 X-β- D- Glucuronide	H8 X-β-D- Glucosaminide	H9 X-β-D- Galactosaminid e	H10 X-α-D- Mannoside	H11 X-PO4	H12 X-SO4

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