ISOLATION OF *Arcobacter* SPECIES FROM DIFFERENT WATER SOURCES AND CHARACTERIZATION OF ISOLATED SPECIES BY MOLECULAR TECHNIQUES

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by
Funda AKINCIOĞLU

July 2011
İZMİR

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ACKNOWLEDGEMENTS

Firstly, I would like to thank to my advisor Prof. Dr. Halil İbrahim ATABAY for his support, kind and helpful approach.

I also want to thank to my co-advisor Assoc. Prof. Dr. Yusuf BARAN for his academic support and comments.

I thank to Assist. Prof. Dr. Çağatay CEYLAN for his help and comments.

I am especially grateful to my colleague Ph.D. student Çelenk MOLVA for her endless technical input, emotional support, sharing with me her valuable time and all knowledge.

I am also grateful to my colleague, Ph.D. student Hatice YAVUZDURMAZ, for her helpful and friendly approach, specialist Evrim BALCI and Dane RUSÇUKLU from İzmir Institute of Technology Biotechnology and Bioengineering Research Centre.

I would like to give thanks to veterinarian Bülent KAFA from Bornova Veterinary Control and Research Institute for giving samples.

I wish to express my thanks to my friends from the Department of Food Engineering sharing lovely time and their intimate behaviours.

Special thanks to my family for enduring all the difficulties during my life and my thesis.

ABSTRACT

ISOLATION OF *Arcobacter* SPECIES FROM DIFFERENT WATER SOURCES AND CHARACTERIZATION OF ISOLATED SPECIES BY MOLECULAR TECHNIQUES

Arcobacter is a Gram-negative, spiral-shaped bacterium and belongs to the family *Campylobacteraceae*. They are known as a potential foodborne and waterborne pathogen. The aim of this study was to determine the prevalence of *Arcobacter* spp. in different water sources in addition to their phenotypic and genetic characterization.

One hundred and fifteen samples collected from various water sources in İzmir and surrounding area were used to isolate *Arcobacter*. The isolated strains were identified at the genus level by PCR showing that 42 samples (37%) were found to contain *Arcobacter*. Then, a multiplex PCR (m-PCR) was used to differentiate the isolates at the species level, revealing that 21 samples (sewage *n*=13, river *n*=7 and drinking water *n*=1) were positive for *A. butzleri*. The remaining undifferentiated isolates (*n*=21) were further analysed by 16S rRNA gene sequencing, displaying that 19 were identified as *A. butzleri* with a similarity level of between 96-99%. In addition, the antimicrobial susceptibility of the *Arcobacter* isolates obtained was tested using a disc diffusion method. All the isolates tested (*n*=39) were found susceptible to tetracycline and ciprofloxacin, but resistant to vancomycin.

This is the first study carried out in İzmir to determine the prevalence and distribution of *Arcobacter* spp. from various water sources. The study showed that water sources including drinking water are common reservoirs and potential transmission vehicles for this emerging pathogen, suggesting that appropriate intervention measures should be taken to protect human health.

ÖZET

ÇEŞİTLİ SU KAYNAKLARINDAN *Arcobacter* TÜRLERİNİN İZOLASYONU VE İZOLE EDİLEN TÜRLERİN MOLEKÜLER YÖNTEMLERLE TANIMLANMASI

Arcobacter gram negatif, spiral şekilli bir bakteri olup, *Campylobacteraceae* ailesine aittir. Potansiyel bir gıda kaynaklı ve su-kaynaklı patojen olarak bilinir. Bu çalışmanın amacı, farklı su örneklerinden *Arcobacter* türlerini izole etmek ve izolatların fenotipik ve genotipik karakterlerini belirlemektir.

İzmir ve çevresindeki çeşitli su kaynaklarından *Arcobacter* türlerini izole etmek için 115 örnek toplandı. İzole edilen örnekler PCR ile cins seviyesinde tanımlandı. Sonuç olarak, 42 örneğin (% 37) *Arcobacter* içerdiği saptandı. Daha sonra, *Arcobacter* izolatlarını tür seviyesinde tanımlamak için multiplex-PCR (m-PCR) tekniği kullanıldı. m-PCR, 21 pozitif örneğin *A. butzleri* (kirli su birikintisi= 13 tane, dere ve akarsu= 7 tane, kaynak suyu= 1 tane) olduğunu gösterdi. İzolatların geri kalanı (n=21), 16S rRNA gen sekanslama yöntemi ile analiz edildi. Sekans sonuçları, tanımlanamayan 21 suşun 19'unun % 96-99 oranlarında *A.butzleri* olduğunu gösterdi. Daha sonra, izolatların (n=42) karakterizasyonu için bir çok biyokimyasal test uygulandı. Ayrıca, izolatlar antimikrobiyal duyarlılıklarının belirlenmesi amacıyla disk difüzyon yöntemi ile test edildi. Antibiyotik test sonuçları, test edilen tüm *Arcobacter* izolatlarının (n=39) tetrasiklin ve siprofloksasine duyarlı olduğunu fakat vankomisine dirençli olduğunu gösterdi.

Bu çalışma, çevresel su örneklerinden *Arcobacter* türlerinin prevalansının belirlenmesi amacıyla İzmir'de gerçekleştirilmiş ilk çalışmadır. Sonuç olarak, bu çalışma, su kaynaklarının bu patojen için yaygın bir rezervuar ve bulaşma aracı olduğunu göstermiştir. Dolayısıyla, insan sağlığının korunması için gerekli tedbirlerin alınması zaruridir.

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AEB : Arcobacter Enrichment Broth
bp : Base pair
DNA : Deoxyribonucleic Acid
dNTP : Deoxynucleotide triphosphate
EDTA : Ethylene Diamine Tetra Acetic acid
ERIC : Enterobacterial Repetitive Intergenic Consensus
FISH : Fluorescent In Situ Hybridization
GC-MS : Gas Chromatography – Mass Spectrometry
HPLC : High Performance Liquid Chromatography
min : Minutes
µl : Microliter
µM : Micromolar
mM : Milimolar
PCR : Polymerase Chain Reaction
m-PCR : Multiplex Polymerase Chain Reaction
PFGE : Pulsed-Field Gel Electrophoresis
RAPD : Random Amplified Polymorphic DNA
RNA : Ribonucleic Acid
rRNA : Ribosomal RNA
SDS : Sodium Dodecyl Sulfate
sec : Seconds
spp. : Species
TAE : Tris Acetate EDTA
wt/vol : Weight/volume

CHAPTER 1

INTRODUCTION

The genus *Arcobacter* (means "arc-shaped" in Latin) is included in the family *Campylobacteraceae*. The other members of the family *Campylobacteraceae* are *Campylobacter* and *Helicobacter*. They are motile, Gram-negative, non-spore forming, curved, occasionally straight rods, which may also appear as spiral (Figure 1.1) (Vandamme and De Ley, 1991).

Arcobacter was first isolated in Belfast, UK from aborted bovine fetuses by Ellis et al. (1977). The genus name *Arcobacter* was proposed by Vandamme et al. (1991). *Arcobacter* spp. have been considered as potential zoonotic foodborne and waterborne agents (Assanta et al., 2002; Houf et al., 2004). The isolation of increasing number of *Arcobacter* from foods of animal origin and from cases of human enteritis has enhanced the significance of arcobacters as a potential food safety concern (Wesley, 1997).

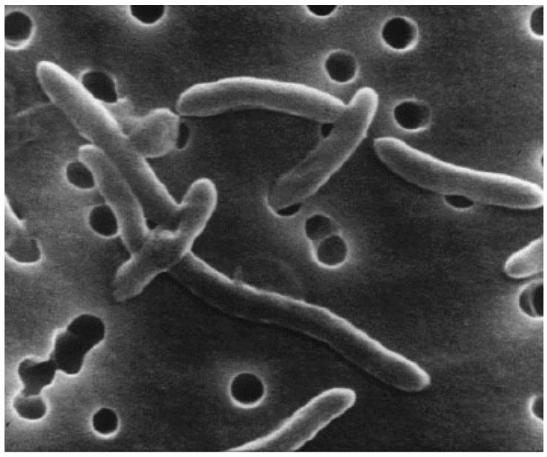


Figure 1.1. Scanning electron microscopy image of *A. butzleri* in filter with 0.45 μm pores (Source: Manke and Dickinson 1996)

1.1. General Characteristics of *Arcobacter* spp.

Arcobacters are Gram-negative, non-spore forming rods, belonging to the family *Campylobacteraceae* (Figure 1.2a). The genus *Arcobacter* is closely related to the genus

Campylobacter; however, the members of the genus *Arcobacter* are aerotolerant and are able to grow at temperatures below 30 °C. They are microaerophilic and grow at temperature ranges of 15-37 °C and a pH range of 6.8-8.0. Optimal growth occurs under microaerobic conditions (3–10 $\%O_2$) and the bacteria do not require hydrogen (Vandamme et al. 1991). Arcobacters are helical rods of 1-3 µm by 0.2-0.4 µm and sometimes may produce long cells of up to 20 µm (Wesley, 1994). They have single polar flagellum and display typical corkscrew-like motility (Figure 1.2b).

Colonies seem off-white, white or greyish color on blood agar plates. After 48 h incubation, they seem 2-4 mm in diameter and convex with entire edges (Collins et al., 1996).

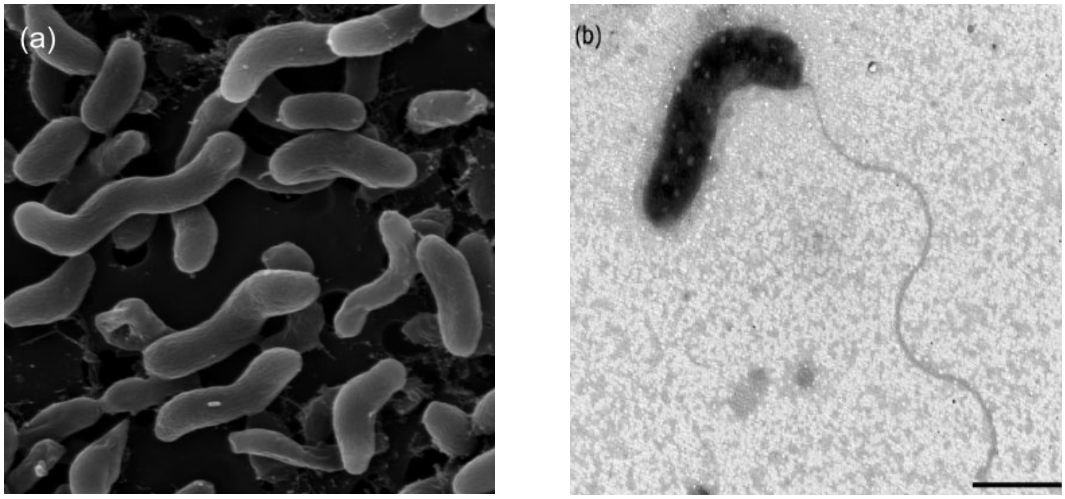


Figure 1.2. (a) Scanning electron microscopy image of cells of *A.halophilus* sp. nov. Bar, 1µm. (b) Negatively stained cells of *A. halophilus* showing single polar flagellum Bar, 0.5 µm. (Source: Donachie et al., 2005; Collado et al., 2009)

They are chemoorganotrophs that can use organic acids and amino acids as carbon sources via citric acid/ tricarboxylic acid cycle, but carbohydrates are neither oxidized nor fermented (Table 1.1).

Table 1.1. General characteristics of the genus *Arcobacter*
(Source: Vandamme et al., 1991b)

- Optimal growth under microaerobic conditions, with a respiratory type of metabolism
- Able to grow in aerobic conditions
- Hydrogen is not required for microaerobic growth
- Grows anaerobically
- Growth at 15 and 30 ^{o}C, not at 4^{o}C; growth is variable at 37 ^{o}C
- Chemoorganotrophs
- Carbohydrates are neither fermented nor oxidized
- No acidic or neutral end products produced
- Serum or blood enhances, but is not essential for growth
- Energy is obtained from amino acids or tricarboxylic acid cycle intermediates, not from carbohydrates
- Oxidase activity is present
- Most strains produce catalase
- Hippurate hydrolysis negative
- Most strains grow in the presence of 0.032% methyl orange
- Indoxyl acetate is hydrolyzed by most species except *Arcobacter mytili* (Collado et al., 2009a)
- No hydrolysis of gelatin, casein, starch and tyrosine
- Methyl red and Voges-Proskauer tests are negative
- No arylsulfatase or lecithinase activity
- Most reduce nitrate to nitrite
- Generally susceptible to nalidixic acid and resistant to cephalothin
- The mol% G–C of the DNA= 27-31

1.2. Taxonomy

The genus *Arcobacter* was proposed by Vandamme et al. (1991) to accommodate two aerotolerant *Campylobacter* species: *C. cryaerophila* (*A. cryaerophilus*) and *C. nitrofigilis* (*A. nitrofigilis*). *A. cryaerophilus* was isolated from different sources originated from farm animals and from the milk of cows with mastitis (Neill et al., 1985). *A. nitrofigilis* is a nitrogen-fixing bacterium isolated from roots of salt marsh plant called Spartina alterniflora (McClung et al., 1983). The genus was

enlarged with the reclassification of *C. butzleri*, described by Kiehlbauch et al., (1991b) as *A. butzleri* (Kiehlbauch et al., 1991a) and with the description of *A. skirrowii* (Vandamme et al., 1992a).

Two groups (named 1A and 1B or 1 and 2) were differentiated within *A. cryaerophilus* by their different restriction fragment length polymorphisms (RFLP) of the ribosomal RNA (rRNA) genes (Kiehlbauch et al., 1991b), their whole-cell protein and fatty acid contents (Vandamme et al., 1992a) and by their amplified fragment length polymorphism (AFLP) profiles (On et al., 2003). The new researchs suggest that *A. cryaerophilus* 1A and 1B belong to two separate taxa (On et al., 2001a; Vandamme et al., 2005; Debruyne et al., 2010). Also, livestock and broiler carcass studies showed that group 1B is more prevalent than 1A (Kabeya et al., 2003a; Son et al., 2007).

Two additional species were described in 2005; *A.cibarius*, isolated from broiler carcasses in Belgium (Houf et al., 2005), and *A. halophilus* isolated from a hypersaline lagoon in Hawaii (Donachii et al., 2005). The latter is the first obligate halophilic *Arcobacter* spp.

Recently, seven new species have been added to the genus; *A. mytili* isolated from mussels is the first species of the genus unable to hydrolyze indoxyl-acetate (Collado et al., 2009a). *A. thereius* (Houf et al., 2009) isolated from hypersaline lagoon and *A. marinus* isolated from seawater (Kim et al., 2010). *Arcobacter defluvii* was isolated from sewage samples (Collado et al., 2010) and *Arcobacter molluscorum* was recovered from shellfish (Figueras et al., 2010). Two new species related with animals defined as *Arcobacter trophiarum* was recovered from fattening pigs (Sarah De Smet et al., 2011) and *Arcobacter valdiviensis* was isolated from a chicken cloacal swab samples (Collado et al., submitted). All accepted species were shown at Table 1.2. with their sources and their references.

The discovery of new species has enlarged the genus to nine validly published species and the taxonomy of these species has been based on the analysis of the 16S rRNA gene. They show a similarity of interspecies levels of the 16S rRNA genes ranging from 92.0 to 98.8% (Collado and Figueras, 2011).

Table 1.2. Currently accepted species of the genus *Arcobacter*

SPECIES	TYPE STRAIN	SOURCE	REFERENCES
A. nitrofigilis	LMG 7604	Roots from *Spartina alterniflora* (Canada)	McClung et al., 1983 Vandamme et al., 1991
A. cryaerophilus	LMG 9904	Brain, aborted bovine fetus (Ireland)	Neill et al., 1985 Vandamme et al., 1991
A. butzleri	LMG 10828	Faeces, human with diarrhea (USA)	Kiehlbauch et al., 1991a Vandamme et al., 1992b
A. skirrowii	LMG 6621	Faeces, lamb with diarrhoea (Belgium)	Vandamme et al., 1992b
A. cibarius	LMG 21996	Broiler carcasses (Belgium)	Houf et al., 2005
A. halophilus	ATCC BAA 1022	Hypersaline lagoon (USA)	Donachie et al., 2005
A. mytili	CECT 7386	Mussels (Spain)	Collado et al., 2009a
A. thereius	LMG 24486	Pig abortion and duck cloacal samples (Denmark)	Houf et al., 2009
A. marinus	JCM 15502	Seawater associated with starfish (Korea)	Kim et al., 2010
A. defluvii	LMG 25694T	Sewage samples (Spain)	Collado et al., 2010
A. molluscorum	LMG 25693T	Shellfish (Spain)	Figueras et al., 2010
A. trophiarum	LMG 25534T	Fattening pigs (Belgium)	Sarah De Smet et al., 2010

ATCC, American Type Culture Collection, Rockville, Md; CECT, Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; LMG, Culture Collection of the Laboratorium voor Microbiologie Gent, Universiteit Gent, Gent, Belgium; JCM, Japan Collection of Microorganisms;

1.3. Prevalence of *Arcobacter* spp. in Different Sources

1.3.1. Foods of Animal Origin

Arcobacter spp. are frequently isolated from foods of animal origin. The highest prevalence is found in chickens, followed by pork, beef and lamb meat (Table 1.3) (Patyal et al., 2011).

Among *Arcobacter* spp. isolated from meat samples, *A. butzleri* is found most, followed by *A. cryaerophilus* (de Boer et al., 1996; Houf et al., 2003; Rivas et al., 2004; Kabeya et al., 2004; Ongor et al., 2004; Morita et al., 2004). *A.skirrowii* is often not

detected at all or only at low rates. These can be due to a low prevalence in meat or by the fact that it is more difficult to isolate than *A. butzleri* and *A. cryaerophilus*.

Like *Campylobacter*, a high prevalence of *A. butzleri* and *A. cryaerophilus* is observed on chicken carcasses from markets and abattoirs (Houf et al., 2002a; Atabay et al., 2003; Kabeya et al., 2004).

Although a high prevalence of *Arcobacter* species on chicken meat is reported worldwide, the bacteria are rarely detected in the intestinal contents of these animals. It was therefore assumed that contamination of meat probably arises from other sources and occurs as cross-contamination during slaughtering and processing (Atabay and Corry, 1997).

Table 1.3. Incidence of *Arcobacter* in foods of animal origin

Food Product	**Number of samples**	**Percentage of positivity**	**Country**	**References**
Chicken carcass	201	97	France	Manke et al., 1998
	170	81	Germany	Harrass et al., 1998
	480	83	Belgium	Houf et al., 2002a
	75	85	Turkey	Atabay et al., 2003
	61	65.3	Czech Republic	Vytrasova et al., 2003
	41	48	Japan	Morita et al., 2004
	50	52.3	USA	Johnson and Murano, 1999
	41	48	Japan	Morita et al., 2004
	10	100	Thailand	Morita et al., 2004
Chicken meat	80	65	France	Festy et al., 1993
	220	24.1	The Netherlands	De Boer et al., 1996
	52	65.4	Belgium	Houf et al., 2000
	100	48	Japan	Kabeya et al.2004
	15	20	USA	Villarruel-Lopez et al., 2003
	94	62	Northern Ireland	Scullion et al., 2006
Turkey meat	395	77	USA	Manke et al., 1998
	17	24	Denmark	Atabay et al., 2006
Duck carcass	10	80	UK	Ridsdale et al., 1998
	10	70	Denmark	Atabay et al., 2006
Ground beef	45	28.9	USA	Villarruel-Lopez et al., 2003
	108	34	Northern Ireland	Scullion et al., 2006
	32	22	Australia	Rivas et al., 2004
	90	2.2	Japan	Kabeya et al., 2004
Minced beef	97	5.1	Turkey	Ongor et al., 2004
	68	1.5	The Netherlands	De Boer et al., 1996
Ground pork	299	55.8	USA	Collins et al., 1996
	200	32	USA	Ohlendorf and Murano, 2002
	27	3.7	Italy	Zanetti et al., 1996
	101	35	Northern Ireland	Scullion et al., 2006
	21	23.8	Belgium	Van Driessche and Houf, 2007
Minced pork	26	19.2	Belgium	Van Driessche and Houf, 2007
Sheep meat	13	15	Australia	Rivas et al., 2004

1.3.2. Raw Milk

A study showed that *A. butzleri* was the only species isolated from raw milk samples (%46) in Northern Ireland (Scullion et al., 2006). Also, *A. cryaerophilus* (5 samples) and *A. butzleri* (1 sample) were isolated from sows in absence of clinical signs of mastitis (Pianta et al., 2007). Also in a study from Turkey, *A. butzleri* and *A.skirrowii* were detected from raw milk (Ertas et al., 2010). The reasons of *Arcobacter* contamination in milk probably arises from bulk tanks and farm conditions such as water trough and faeces.

1.3.3. Water

The water may play an important role in the transmission of these organisms and drinking water has been shown as a major risk factor causing diarrhea associated with *Arcobacter* spp. *Arcobacter* spp. (mostly *A. butzleri*) were isolated from a drinking water reservoir (Jacob et al., 1993), river or surface water (Moreno et al., 2003; Morita et al., 2004; Diergaardt et al., 2004), ground water (Rice et al., 1999), sewage (Stampi et al., 1999; Moreno et al., 2003) and seawater from coastal environment (Fera et al., 2004).

A novel coastal marine sulfide-oxidizing autotrophic bacterium called as "*Candidatus Arcobacter* sulfidicus" produces hydrophilic filamentous sulfur. This organism was detected without culturing and 16S rRNA gene sequences of the isolates were analysed (Wirsen et al., 2002). Also, the deep sea sediment samples taken from Suruga Bay (1159 m) were shown 93.9% homology with *A. nitrofigilis* in 16S rRNA gene sequence analysis (Li et al., 1999). Most of the marinal related species were obtained uncultivated. These uncultured isolates were also detected in salt marsh sediments (McClung et al., 1983), North Sea sediments (Llobet-Brossa et al., 1998), North Sea bacterioplankton (Eilers et al., 2000), in association with worms (Naganuma et al., 1997), a hypersaline cyanobacterial mats (Teske et al., 1996). And, several new habitats are also defined such as sewage (Heylen et al., 2006), oysters (Romero et al., 2002), oil field environments (Sette et al., 2007) and associated with cod larviculture (Mclntosh et al., 2008).

So far, five outbreaks have been reported from different counties. The first case was the detection of *A. butzleri* from diarrhoeic children in Thailand (Taylor et al., 1991). Then, the students of an Italian nursery school showed recurrent abdominal cramps with no diarrhoea from *A. butzleri* (Vandamme et al., 1992b). The first waterborne outbreak occurred at Girls Scout Camp in Idaho with nausea, vomiting, abdominal cramps and diarrhoea (Rice et al., 1999). Another outbreak happened in Ohio from drinking water due to *A. butzleri* (Fong et al., 2007). Also a recent investigation showed that *A. cryaerophilus* and other pathogens were isolated from stool samples of patients in Slovenia during an outbreak due to contamination of drinking water system (Kopilovic et al., 2008).

There is little information about the effects of drinking-water treatment on *Arcobacter* spp., but *A. butzleri* was found to be sensitive to chlorine disinfection (Rice et al., 1999). Consumption of contaminated and/or untreated water is an important source of arcobacters. *A. butzleri* can easily attach to water distribution pipe surfaces (stainless steel, copper and plastic), which causes the regrowth of these bacteria in the water distribution system. This is a significant problem in drinking water and food processing plants with respect to public health (Assanta et al., 2002).

1.4. Pathogenicity

Pathogenic mechanism of *Arcobacter* is not exactly known; but, some species may produce cytolethal distending toxin (CDT) that causes rounding (cytotoxicity) of cultured cell lines. In addition, presence of other cytotoxic factor that causes cell elongation and vacuole formation has been reported (Carbone et al., 2003). Hemagglutin, a glycoprotein of 20 kDa has been found in *Arcobacter*, which possibly interacts with a glycan receptor containing D-galactose for bacterial adhesion (Tsang et al., 1996). Arcobacters cause abortion and stillbirth in cows, sheep, and pigs (Skirrow, 1994; On et al., 2002). The organism has been isolated from uterus, oviduct, and placental tissues (Elllis et al., 1978; de Oliveira et al., 1998; Schroeder-Tucker et al., 1996).

Three species, *A. cryaerophilus*, *A. butzleri*, and *A. skirrowii*, have been isolated from faeces of humans and various animals with diarrhea, from cattle with mastitis, and from meat products of animal origin. These species have been also associated with

enteritis, recurrent cramps, bacteremia, endocarditis, peritonitis, appendicitis and abortion (Wesley, 1996).

The most commonly isolated species from human cases is *A. butzleri* as a human entero-pathogen but there are some cases for *A. cryaerophilus* and *A. skirrowii*. For instance, isolation of *A. skirrowii* from an elderly patient with chronic diarhoea was reported (Wybo et al., 2004). Also, *A. butzleri* and *A. cryaerophilus* were reported in an infection with bacteremia together and separately (On et al., 1995; Hseuh et al., 1997; Yan et al., 2000; Lau et al., 2002).

The common symptoms of *Arcobacter* infection are persistent diarrhoea accompanied by abdominal pain and stomach cramps (Lerner et al., 1994; Vandamme et al., 1992b). So, it is hard to differentiate an arcobacter infection due to the similarities of these symptoms with those of campylobacteriosis.

The first isolation of *Arcobacter* from a human was shown an Italian adult with intermittent diarhea and abdominal pain, known by the name *Campylobacter cryaerophila* (Tee et al., 1988). The prevalence of *Arcobacter* from human stools of patients with diarhoea, *A. butzleri* was the fourth common *Campylobacter*-like organism isolated (Vanderberg et al., 2004; Prouzet-Mauleon et al., 2006).

The isolation of *Arcobacter* species from faeces of healthy people were also reported in some studies (Vandenberg et al. 2004; Houf and Stephan, 2007; Samie et al., 2007). Furthermore, *A. cryaerophilus* was found in 1.4% of stool specimens from asymptomatic people (Houf and Stephan, 2007). In South Africa, 3% of the population was reported to harbor *Arcobacter* spp. without showing any symptom Samie et al., 2007). Increasing data about human infection related with *Arcobacter* spp. is not enough due to the lack of standard isolation and identification methods (Vandenberg et al., 2004; Snelling et al., 2006; Figueras et al., 2008).

To find out the virulence mechanism of *Arcobacter* species, virulence factors were investigated. The enteritis induced from *A. butzleri* showed an epithelial barrier dysfunction causing diarrhea (Bücker et al., 2009). The induction of cytokine interleukin-8 in human Caco-2 and porcine IPI-2I cell lines for *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius* (Ho et al., 2007) were investigated and the results indicated that *A. cibarius* showed the highest adhesion ability, but no correlation was detected between invasiveness and adhesion for tested strains.

The pathogenic activity of *Arcobacter* species was based on knowledge available about *Campylobacter* and *Helicobacter* species. After the genome of *A.*

butzleri RM4018 is completely identified, it is understood that *A.butzleri* is most similar to *Wollinella succinogenes* (Miller et al., 2007). Also, the genes encoding the CDT (*cdtABC*) were absent on *A. butzleri* RM4018.

Another recent and virulence-related study investigated *Arcobacter* flagellin genes (*flaA* and *flaB*) and mutational studies revealed that only *flaA* founded important for motility (Ho et al., 2008).

After all, the recent studies give new perspectives and opportunites to find information about *Arcobacter* species. But still there are lots of missing parts to understand the mechanism of infections of this potential foodborne bacterial pathogen.

1.5. Isolation

At present, no optimal isolation method for all *Arcobacter* species is available (Houf et al., 2001). Direct isolation leads to lower recovery rates than isolation after enrichment (Van Driessche et al., 2003, 2004 and 2005). Many of the isolation protocols were time-consuming and lacked specificity. Although aerotolerance is a distinctive characteristic which distinguishes *Arcobacter* spp. from *Campylobacter* spp., this is often not observed on initial isolation on media used for the isolation of campylobacters (de Boer et al., 1996).

Arcobacters may be isolated using selective media or membrane filtration methods described for the isolation of *Campylobacter* species. However, these methods are suboptimal for growth of arcobacters, which may be overgrown by campylobacters present in the same specimens. Samples should be incubated at 24-30 ^{o}C to enhance the selectivity of the procedure, and an enrichment step has been recommended (Atabay et al., 1998).

The difficulty in isolation of *A. cryaerophilus* and *A. skirrowii* is probably due to their susceptibility to antimicrobials and other components used in the isolation media (Atabay et al., 1998; Houf et al., 2001; Kabeya et al., 2003b and 2004).

Furthermore, colonies of *A. skirrowii* isolated from animal faeces are only visible after 72 h (Van Driessche et al., 2003) or after 4 days of incubation in a human case study (Wybo et al., 2004). Tiny colonies of *A. skirrowii* from swine faeces were only recovered after filtering the enrichment culture through 0.45 µm membrane filters

and incubation for 4 days (Ho et al., 2006). Poor growth of *A. skirrowii* may allow overgrowth by enteric bacteria in samples that are incubated for 1 or 2 days.

Atabay and Corry (1997) used sensitive isolation methods that allowed them to detect a wide range of *Campylobacter* and *Arcobacter* species from chicken carcasses. These methods involved the use of different selective agents and/or lower concentrations of antibiotics in the isolation media. Hydrogen was also included in the microaerobic atmosphere. Samples were plated onto nonselective blood agar, and incubation was carried out at different temperatures (most often 30 ^{o}C and 37 ^{o}C). Arcobacters were isolated only at 30 ^{o}C, under aerobic conditions after enrichment.

1.5.1. Isolation Media

Commercially available isolation media include cefoperazone, amphotericin B and teicoplanin agar (CAT) for *Arcobacter* spp. and charcoal cefoperazone, deoxycholate agar (CCDA) that is more specific for *A. butzleri*. CAT agar was reported to support growth of a wider range of arcobacters than mCCDA (Corry and Atabay, 1997).

A new commercial enrichment broth, called Arcobacter Broth (AEB) has been developed which may be used with CAT or mCCDA selective agents for isolation of *Arcobacter* spp. or *A. butzleri* (Table 1.4). This medium supports the good growth of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* although *A. nitrofigilis* grows poorly. The growth characteristics in Arcobacter Broth compare favourably with those on media designed for the isolation of campylobacters (Atabay and Corry, 1998). Use of this medium was reported that it allows high population densities and eliminates the necessity for biphasic growth methods which may be technically more difficult (Dickson et al., 1996).

When eight strains of *Campylobacter* spp. were tested, none grew in the AEB, probably because this medium contains no oxygen-quenching system, such as blood, which neutralizes the effect of atmospheric oxygen (Atabay and Corry, 1998; Corry et al., 1995). Another advantage of AEB is that *Arcobacter* spp. can reach higher population densities than using biphasic growth methods (Dickson et al., 1996).

A pre-enrichment in Arcobacter Selective Broth (ASB), followed by plating onto semisolid Arcobacter selective medium using cefoperazone, trimethoprim and

cycloheximide, has been suggested for isolation from raw poultry meats. The addition of piperacillin into the medium prevents the outgrowth of *Pseudomonas* spp. from raw meats at the isolation temperature of 24 °C (de Boer et al., 1996).

Table 1.4. Media and procedures used for isolation of *Arcobacter* species (Sources: Scullion et al., 2004; Hamill et al., 2008)

	Enrichment		**Isolation**	
Method	**Formulation antibiotics (mg/liter)**	**Incubation conditions**	**Plating medium antibiotics (mg/l)**	**Incubation conditions**
Ellis et al., 1977	EMJH 5-Fluorouracil (100)	30°C, 48-72 h, mO_2	Blood agar, no antibiotics	30°C, 48□72 h, mO_2 and O_2
de Boer et al., 1996	ASB Cefoperazone (32), Piperacillin (75) Trimethoprim (20) Cycloheximide (100)	24°C, 48 h, O_2	ASM Cefoperazone(32), Piperacillin(75) Trimethoprim(20) Cycloheximide(100)	24°C, 48-72 h, O_2
Collins et al., 1996	EMJH 5-Fluorouracil (200)	30°C, 9 days, O_2	CVA agar Cephalothin (20) Vancomycin (10) Amphotericin B (5)	30°C, up to 7 days, O_2
Atabay and Corry, 1997	CAT broth[a] Cefoperazone (8) Amphotericin B (10) Teicoplanin (5)	30°C, 48 h, mO_2	Blood agar No antibiotics Membrane filtration	30°C, up to 7 days, O_2
Johnson and Murano, 1999	JMB Cefoperazone (16), 5-Fluorouracil (200)	30°C, 48 h, O_2	JM agar Cefoperazone (32)	30°C, 48 h, O_2
Houf et al., 2001	Arcobacter broth Cefoperazone (16), Amphotericin B (10) 5-Fluorouracil (100) Novobiocin (32) Trimethoprim (64)	28°C, 48 h, mO_2	Arcobacter plating medium Cefoperazone (16), Amphotericin B (10) 5-Fluorouracil (100) Novobiocin (32) Trimethoprim (64)	30°C, 24-72 h, mO_2

[a] CAT broth is composed from AEB supplemented with cefoperazone, amphotericin and teicoplanin.
EMJH: Ellinghausen-McCullough-Johnson-Harris semisolid medium; ASB, Arcobacter selective broth; ASM, Arcobacter selective medium; CVA, cephalotin, vancomycin and amphotericin B agar; AEB: Arcobacter enrichment broth; JM, Johnson-Murano broth; O2, aerobic conditions; mO2, microaerobic conditions

Enrichment microaerobically at 30 ^{o}C in an enrichment broth for arcobacters (Lab M BROTH, LAB 135) containing cefoperazone as the selective agent together with a filter method onto mCCDA agar, incubated aerobically at 30 ^{o}C, is effective for recovery of the *Arcobacter* from raw poultry, with no growth of competing microflora (Lammerding et al., 1996). Although both mCCDA and CAT agars support growth of *Arcobacter* spp., the latter tends to support a wider range of arcobacters and campylobacters than the former (Corry and Atabay, 1997). Although *Arcobacter* spp. are resistant to the level of cefoperazone present in mCCDA (32 mg/mL compared with 8 mg/mL in CAT), they generally grow better on CAT, suggesting that there might be a synergistic inhibitory effect of deoxycholate and cefoperazone at 32 mg/mL present in mCCDA (Corry and Atabay, 1997).

1.6. Characterization Methods

1.6.1. Phenotypic Characterization

Identification of different species within the genus *Arcobacter* using standard biochemical tests is difficult because of the variability and atypical reactions of some strains (On, 1996; Atabay et al., 1998; Phillips, 2001). The main phenotypic tests used for species identification are presented in Table 1.5.

Campylobacter, *Helicobacter* and *Arcobacter* may also be distinguished by whole cell protein profiling (Vandamme et al., 1991). SDS-PAGE of whole cell proteins has been shown to be successful in species level identification of *Arcobacter* spp. isolated from poultry at the abattoir (Ridsdale et al., 1998). Whole-cell fatty acid analysis was not able to distinguish *A. butzleri* from *A. cryaerophilus* subgroup 2, but differentiated all other arcobacters (Vandamme et al., 1992b). Furthermore, Atabay et al., (1998 and 2003) used SDS-PAGE method of whole-cell proteins successfully for species level identification of *A. butzleri* in several studies.

Table 1.5. Phenotypic characteristics of *Arcobacter* species
(Source: On, 1996; Atabay et al., 1997; Donachie et al., 2005; Houf et al., 2005, 2009; Collado et al., 2009a ; Kim et al., 2010; Figueras et al., 2010)

Biochemical Tests	***A. butzleri***	***A. cryaerophilus***	***A. skirrowii***	***A. cibarius***	***A. nitrofigilis***	***A. thereius***	***A. mytili***	***A. halophilus***	***A. marinus***	***A. molloscorum***	***A. defluvii***	***A. trophiarum***
Catalase activity	V	-	+	V	+	+	+	-	-	+	+	+
Urease activity	-	-	-	-	+	-	-	-	-	-	+	-
Indoxyl acetate hydrolysis	+	+	+	+	+	+	-	+	+	-	+	+
Nitrate reduction	+	+	+	-	+	+	+	+	+	+	+	-
Growth in/on												
O_2 37°C	+	V	+	-	V	-	+	+	+	+	+	-
mO_2 37°C	+	V	+	+	-	-	+	+	+	+	+	-
Mac Conkey agar	+	V	-	+	-	V	+	-	-	+	+	V
Minimal medium	+	-	-	+	-	+	-	-	-	-	+	-
% 4 NaCl	+	V	+	-	+	-	+	+	+	+	-	+
% 1 glycine	+	+	+	-	-	+	+	+	+	-	-	V
Resistance to Cefoperazone (64mg/l)	+	+	+	+	-	+	-	-	-	+	V	+

+, ≥95% Strains positive; -, ≤11% strains positive; V, 12–94% strains positive. O_2, aerobic conditions; mO_2, microaerobic conditions.

1.6.2. Genotyping and Molecular Identification of Arcobacters

Phenotyping methods such as biotyping or antibiograms are of limited use to discriminate the strains within the species and/or subspecies level. The development and application of molecular techniques have been useful for typing of strains. DNA-based methods were also established for rapid and specific identification of *Arcobacter* spp. (Harmon and Wesley, 1996; Hurtado and Owen, 1997; Marshall et al., 1999; Al Rashid et al., 2000; Gonzalez et al., 2000; Winters and Slavik, 2000; Moreno et al., 2003).

Genotypic methods such as ribotyping (Kiehlbauch et al., 1991b; Taylor et al., 1991, pulsed field gel electrophoresis (Lior and Wang, 1993), and random and repetitive motif based polymorphic DNA analyses (Lior and Wang, 1993; Vandamme et al., 1993) were performed on limited numbers of strains.

DNA-based methods used for the identification of *Arcobacter* spp. are; RFLP (Carderelli-Leite et al., 1996; Hurtado and Owen 1997; Kiehlbauch et al., 1991; Marshall et al., 1999); sequencing of 16S rRNA gene (Lau et al., 2002); PCR-DGGE (Petersen et al., 2007); *rpoB-rpoC* (Morita et al. 2004) and *gyrA* genes (Abdelbaqi et al., 2007a); several multiplex PCR techniques (Brightwell et al., 2007; Harmon and Wesley, 1997; Houf et al., 2000; Kabeya et al., 2003); real-time PCR (Brightwell et al., 2007); microarray technique (Quiñones et al., 2007); real-time fluorescence resonance energy transfer PCR (Abdelbaqi et al., 2007b) and 16S rDNA-RFLP (Figueras et al., 2008) methods.

Besides PCR-based methods, there are other DNA-based useful techniques as well. Fluorescent in situ hybridization (FISH) and PCR methods were compared for the differentiation of *Arcobacter* and *Campylobacter* spp. isolated from sewage and sludge samples. The results showed methods were efficient (Moreno et al., 2003). In a recent study, the FISH method gave better results than PCR in terms of rapidity and sensitivity on *Arcobacter* spp. in estuarine water in Southern Italy (Fera et al., 2010). Moreover, high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) were used to analyse four *Arcobacter* species for their phospholipid and fatty acid patterns (Jelinek et al., 2006). The whole cell proteins of *Arcobacter* species were examined using SDS-PAGE for the identification differentiation of *Arcobacter* isolates (Atabay et al., 2003). The most recently employed method is matrix-associated laser desorption/ionization–time of flight (MALDI-TOF) MS which is based on composing complex fingerprints of biomarker molecules by measuring the exact mass/charge ratio of peptides and proteins in bacterial identification (Alispahic et al., 2010). *A. butzleri*, *A. skirrowii* and *A. cryaerophilus* were investigated at this study. The results showed that mass signal pattern of *A. cryaerophilus* and *A. skirrowii* as well as *A. butzleri* and *A. skirrowii* shared a number of common mass peaks thatwere 100% frequent (in 135 spectra). But, mass signal patterns of *A. cryaerophilus* were completely different to *A. butzleri*.

Multi-locus Sequence Typing (MLST) method has also been used in 374 *Arcobacter* strains including 275 *A. butzleri*, 72 *A. cryaerophilus*, 15 *A. skirrowii*, 8 *A. cibarius* isolated from different geographical locations and food sources. Seven loci, which were *aspA*, *atpA* (*uncA)*, *glnA*, *gltA*, *glyA*, *pgm* and *tkt*, were used. As a result, this method could be used successful in discrimination of *Arcobacter* strains and in *Arcobacter* related diseases (Miller et al., 2009).

1.6.2.1. Molecular Methods for Identification and/or Differentiation of *Arcobacter* spp.

1.6.2.1.1. Multiplex PCR (m-PCR)

A multiplex PCR is first described by Chamberlain et al. (1988) to diagnoge Duchenne muscular dystrophy. Basically, a m-PCR is that amplifies two or more genes in a single PCR reaction. Each primer set is designed to its target gene amplifying a PCR product of a size unique to the target gene. This technique saves time and labor since more than one target DNA sequence is detected for each reaction (Elnifro et al., 2000), but might not be useful if the PCR products are close in their size. Detection is achieved by agarose gel electrophoresis using ethidium bromide as staining dye.

m-PCR targeting the 16S and 23S rRNA genes have been described for the routine detection and identification of different *Arcobacter* spp. by many researchers (Harmon and Wesley, 1997; Houf et al., 2000; Fera et al., 2004).

A genus-specific PCR and three species-specific PCR assays, based on a target sequence comprising the most variable region of 23S rDNA, have been shown to be reliable in identifying reference strains and field isolates. This specific PCR may be used either for classification at the genus level using the primer combination ARCO1 and ARCO2 or together with the species-specific primers ARCO1-BUTZ, ARCO1-CRYAE and ARCO1-SKIR for identification at the species level. This protocol used two primer sets: one targeting a section of the 16S rRNA genes (ARCO1 and ARCO2) (Harmon and Wesley 1996) and the other amplifying a portion of the 23S rRNA genes unique to *A. butzleri* (ARCO-BUTZ) (Bastyns et al., 1995). PCR amplification resulted in all of the *Arcobacter* isolates tested yielding a 1223 bp product, whereas *A. butzleri* yielded both a 1223 bp and a 686 bp products. The 1223 bp multiplex PCR product identified all of the isolates as *Arcobacter* spp. while the presence of both the 1223 bp and 686 bp amplicons identified 66 strains as *A. butzleri* agreeing with results obtained by other methods (Harmon and Wesley, 1997).

Houf et al. (2000) designed an m-PCR assay amplifying a band per species with primers targeting the 16S and 23S rRNA genes for the simultaneous identification of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. The selected primers amplify a 257 bp fragment from *A. cryaerophilus*, a 401 bp fragment from *A. butzleri* and a 641 bp fragment from *A. skirrowii*. Though this assay was valuable for identifying the three

important *Arcobacter* species, it did not provide the means for identifying and distinguishing *A. cryaerophilus* 1A from 1B group. Thus, Kabeya et al. (2003) developed an m-PCR using primers that specify the 23S rRNA to identify the above three species, and to differentiate *A. cryaerophilus* 1A from 1B.

A PCR oligo hybridization strategy using PCR amplification of the partial *glyA* gene with three degenerate primers based on conserved *glyA* region, followed by species-specific oligo probe hybridizations to identify *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *A. butzleri* and *A. butzleri*-like strains, may provide a useful diagnostic tool for identification. Although oligo probes have a high specificity, they are not as sensitive as probes based on DNA fragment hybridizations (Al Rashid et al., 2000).

1.6.2.1.2. 16S and 23S Ribosomal RNA-Restriction Fragment Length Polymorphism (RFLP)

This technique involves amplification of the 16S or 23S ribosomal gene followed by enzymatic restriction and gel electrophoresis. The DNA fingerprints can be produced using PCR with universal primers whereas fingerprints for subgroups can be generated with species-specific primers.

Species of *Arcobacter* are reliably identified using RFLP or ribotyping involving the hybridization of *Pvu*II-digested chromosomal DNA with probes for the 16S rRNA gene (Kiehlbauch et al., 1991; Wesley et al., 1996).

The 23S rRNA gene is larger with more variable regions than the 16S rRNA gene and so provides the basis for an identification scheme that is potentially more discriminating and specific than 16S rRNA gene (Hurtado and Owen, 1997). A molecular identification scheme based on restriction profiles targeting the 23S rRNA gene, using three restriction endonucleases *Hpa*II, *Cfo*I and *Hinf*I allows differentiation of *A. butzleri* and *A. nitrofigilis* but not between *A. cryaerophilus* and *A. skirrowii* which have identical patterns with all restriction enzymes.

1.6.2.2. Genotyping of the Isolated *Arcobacter* Strains

1.6.2.2.1. Random Amplification of Polymorphic DNA (RAPD)-PCR and Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

PCR-based methods that have been used for the genotyping of *Arcobacter* spp. include the random amplification of polymorphic DNA (RAPD) and an enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) (Atabay et al., 2002; Houf et al., 2002b).

RAPD-PCR technique was found to be a useful technique to reveal epidemiologic association among the isolated strains. Houf et al. (2002b) optimised an ERIC-PCR and a RAPD-PCR for the characterization of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. Both methods gave efficient typeability and discriminatory power, but the fingerprints generated with the ERIC-PCR were more reproducible and complex than those obtained with the RAPD-PCR.

1.6.2.2.2. Pulsed Field Gel Electrophoresis (PFGE)

While PCR-based methods seem to be advantageous in saving time, PFGE is generally considered to be the gold standard for most of the relevant pathogens in food hygiene. Hume et al. (2001) used PFGE for genotyping *Arcobacter* strains isolated from nursing sows and growing pigs on three farms. Furthermore, *Arcobacter* strains isolated from ground chicken, pork, beef and lamb meats were genotyped by PFGE (Rivas et al., 2004). Although, a number of isolates with indistinguishable fingerprints were obtained, results from these two studies show that PFGE can also be used as a genotyping tool for the discrimination *Arcobacter* strains to investigate outbreaks.

1.6.2.2.3. Amplified Fragment Length Polymorphism (AFLP)

Whole-genomic fingerprinting by AFLP is a high resolution genotyping method. The digestion of target DNA with two restriction enzymes of differing cutting

frequencies, ligation of half-site with specific adaptor nucleotides, selective amplification of adapted genomic fragments and detection of subsequent PCR products has been used for genotyping for a range of microorganisms (Savelkoul et al., 1999), including *C. jejuni* and *C. coli* (Duim et al., 1999; Kokotovic and On, 1999).

On and Harrington (2001b) have established that AFLP may be used for species identification of the family *Campylobacteraceae*, including four species of *Arcobacter* and that the method is effective in identifying genetic diversity among different clonal types from distinct geographical areas. A comparison of m-PCR (Houf et al., 2000) and AFLP- profiling for speciation of *Arcobacter* spp. (Scullion et al., 2001) resulted in a good correlation between the two methods although three *A. skirrowii* isolates tested gave 'atypical' AFLP profiles (i.e. not similar to the type strain) whereas a PCR method (Harmon and Wesley, 1996) confirmed them as *Arcobacter* spp. AFLP is a demanding technique and requires a large reference database, although once adopted in a routine laboratory it is very useful and highly discriminatory technique.

1.7. Antimicrobial Activity

A. butzleri, *A. cryaerophilus* and *A. skirrowii* strains isolated from humans, chicken carcasses, meat and pork were generally found to be resistant to antimicrobials (Kabeya et al., 2004; Son et al., 2007; Abdelbaqi et al., 2007b). Also, multidrug resistance observed in different studies (Kabeya et al., 2004; Son et al., 2007). *Arcobacter* species were found resistant to trimethoprim-sulphamethoxazole and members of the broad-spectrum beta-lactams including cephalosporins (Atabay and Aydın, 2001; Fera et al., 2003; Kabeya et al., 2004). However, there are a high number of strains of *A. butzleri* resistant to other antibiotics such as clindamycin, azithromycin and/or nalidixic acid (Son et al., 2007). Furthermore, erythromycin and ciprofloxacin resistance were observed in a study (Houf et al., 2004). Fluroquinolones and tetracycline have been suggested for the treatment of infections produced by *A. butzleri* in humans and animals (Vandenberg et al., 2006; Son et al., 2007). The antibacterial activity of some antibiotics within coumarins and quinolones are known associated with DNA gyrase (Maxwell, 1997). This gene mutation generates resistance to these antibiotics. DNA gyrase have two subunits, *gyrA* and *gyrB*. Moreover, the *gyrA* gene was identified in *Arcobacter* species and contains quinolone-resistance determining

region (QRDR). This region mutation was associated with ciprofloxacin resistance in two clinical isolates of *A. butzleri* and one *A. cryaerophilus* ciprofloxacin-resistant strains (Abdelbaqi et al., 2007b). In another study showed that *A. butzleri, A. cryaerophilus* and *A. skirrowii* were found to be highly resistant to novobiocin (Houf et al., 2001). Besides commercial antimicrobial disc, a study tested the antimicrobial activity of seventeen spices and medicinal plants extracts against *A. butzleri*, *A. cryaerophilus* and *A. skirrowi*. The results showed that cinnamon, barberry, chamomile, sage and rosemary extracts had strong antimicrobial activity toward the arcobacter strains tested (Cervenka et al., 2006).

Four methods have been used determine the antimicrobial susceptibility of arcobacters: agar and broth (micro) dilution (Fera et al., 2003; Houf et al., 2001; Kiehlbauch et al., 1992), disc diffusion (Kabeya et al., 2004; Atabay and Aydın, 2001; Harrass et al., 1998) and E-test (Yan et al., 2000; Vanderberg et al., 2006).

The whole genome of *A. butzleri* (RM4018) revealed high antibiotic resistant genes for regions associated with the presence or absence of specific genes (Miller et al., 2007). For instance, *cat* gene (encoding a chloramphenicol O-acetyltransferase) was related to chloramphenicol resistance, three putative β-lactamase genes or *lrgAB* operon were associated with the β-lactam resistance and the absence of the *upp* gene (encoding for uracil phosphoribosyltransferase) with the 5-fluorouracil resistance (Miller et al., 2007).

1.8. Thesis Objectives

The purpose of this study was to isolate *Arcobacter* species from different environmental sources such as sewage, river, drinking and tap water and to determine their distribution in these habitats. Isolated strains will also be characterized by number of phenotypic tests and molecular techniques involving different PCR assays and 16SrRNA gene sequencing, in order to differentiate *Arcobacter* isolates at the genus and species level. Antibiotic susceptibility patterns of the isolated strains using a disc diffusion method will also be carried out.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used in this study are shown in Appendix A.

2.1.2. Media

The media used in this study are listed in Appendix B.

2.1.3. Reagents and Solutions

Reagents and solutions used are presented in Appendix C and Appendix D.

2.2. Methods

2.2.1. Sample Collection

One hundred-fifteen samples were collected from İzmir and surrounding area including 66 sewage, 25 river, 16 drinking and 8 tap water (Table 2.1). The samples were collected in autumn from the end of February to the middle of April. They were placed in sterile plastic bottles aseptically and immediately transferred to the laboratory within three hours for the analysis.

Table 2.1. Sampling Sites, Sources and Numbers of Samples

Sampling Site	**Origin of Sample**	**No. of Samples Collected**
Kuşçular-Yağcılar	Sewage	7
	Drinking water	3
Güzelbahçe	River	5
	Sewage	5
	Drinkig water	1
Çeşmealtı	River	5
Zeytinalanı	Sewage	6
	River	3
	Drinking water	1
Balıklıova	Sewage	1
	Drinking water	2
Gülbahçe village	Sewage	7
	River	3
	Drinking water	3
Gümüşkoy	Sewage	1
Karapınar	River	2
Ildırı	Sewage	2
Barbaros	Sewage	6
Birgi	River	2
Kadıovacık	Sewage	4
	Drinking water	2
Zeytinler	Sewage	3
Uzunkuyu	Sewage	3
	Drinking water	1
Old Çeşme Road	Sewage	4
	Drinking water	1
	River	3
Torasan	Sewage	8
İçmeler	Sewage	6
	Drinking water	2
	River	2
Özbek	Sewage	3
Buca	Tap water	1
Güzelyalı	Tap water	1
Gaziemir	Tap water	1
Karşıyaka	Tap water	2
Bornova	Tap water	2
Narlıdere	Tap water	1
		Total : 115

2.2.2. Optimization of Isolation Procedure for Enrichment of Water Samples

Due to the lack of standard method for isolation of *Arcobacter* spp., a general isolation method was tried to be optimized for the isolation of arcobacters from environmental samples. For this purpose, three available methods were applied for the enrichment of water samples. In the first method, a membrane filtration method was used with 0.45 µm pore size and 47 mm diameter nitrocellulose membrane filter (Millipore, Billerica, MA, USA) that had been placed into the vacuum filter system (Sartorius AG, Goettingen, Germany). Then, 200 ml of water sample were injected into the system. After filtration, the membrane was put into 20 ml of AEB broth supplemented with CAT supplement (Oxoid, SR174E) and incubated under microaerobic conditions at 26-30 °C for 2 to 4 days. The microaerobic atmosphere was achieved by evacuating two-third of the anaerobic jar and replacing it with a gas mixture to give final concentration of 6% O_2 using an automated anaerobic system (Anoxomat, Mart Microbiology, The Netherlands). The filters were then placed onto mCCDA and the sample homogenate were allowed to filter passively for 45 min at 30°C in air. After that, the filters were carefully removed with sterile forceps and discarded and the culture plates were incubated microaerobically at 30 °C up to 6 days by examining daily after 2 days.

In the second method, 200 ml of sample were filtered using a syringe fitted with 0.45 µm pore size membrane filters (Minisart, Sartorius Stedim Biotech S.A.) and, then, centrifuged at 3.500 *g* for 15 min. Thereafter, the supernatant was discarded and the pellet was resuspended in 20 ml AEB broth with CAT supplement and incubated under the same conditions as given above.

In the last method, 200 ml of sample were centrifuged directly at 3.500 *g* for 15 min. The supernatant was discarded again and the pellet was resuspended in 20 ml AEB broth with CAT supplement and the broths were incubated under the same conditions as above.

After each enrichment procedure mentioned above, 100 µl of broth suspension were filtered onto blood agar plates containing 5% sheep blood as described by Atabay and Corry (1997) using 0.45 µm pore size 47 mm diameter nitrocellulose membrane filter (Millipore, Billerica, MA, USA). Briefly, the filters were placed onto blood agar plates and then, the suspension was inoculated onto the membrane filters. After leaving

the plates at room temperature about 30 min the filters were removed and the inocula were evenly spread across the agar plates. All plates were incubated for 48-72 h at 30 °C microaerobically until visible colonies were observed. Then, *Arcobacter*-like colonies were picked and subcultured onto mCCDA and the plates were incubated at 30 °C aerobically for 48 h until pure cultures of the isolates were obtained. Two-three colonies were picked from each plate for further examination.

2.2.3. Phenotypic Characterization

The isolates were grown on mCCDA agar microaerobically at 30 °C for 24-48 h unless stated otherwise for phenoytypic characterization.

2.2.3.1. Gram Staining

A few drops of sterile 0.9% NaCl suspension were placed onto the microscope slides. A loopful of culture was suspended in NaCl and heat fixation was achieved. The cells were Gram-stained as follows: immersion in crystal violet for 1 min, rinse under the tap water, iodine for 1 min, rinse again, 95% alcohol for 10-15 sec, rinse again, safranine or carbol fuchsin for 30 sec (Appendix C). After rinsing, the slides were air-dried and examined under light microscopy.

2.2.3.2. Motility

The cells were suspended in 10-12 µl of sterile 0.9% NaCl on the microscope slides and covered with lamel. The slide was examined under phase-contrast microscope for characteristic darting, corkscrew-like motility of *Arcobacter* spp.

2.2.3.3. Oxidase Test

A loopful of culture was smeared onto the commercial oxidase test strips (Merck). After 2-3 min, the appearance of deep purple color showed the presence of oxidase activity in the isolates.

2.2.3.4. Catalase Test

Catalase activity was detected by adding a drop of 3% hydrogen peroxide solution onto an isolated colony. Immediate and vigorous bubbling indicated a strong catalase reaction whereas no bubble formation indicated a negative test. A weak reaction occurs slowly with lower bubble formation.

2.2.3.5. Indoxyl-acetate Hydrolysis

The isolates grown on mCCDA agar were smeared onto Indoxyl-acetate impregnated test strips (Fluka) and then, a drop of sterile distilled water was added onto the strips. Then the strips were incubated for 5-10 min. The appearance of green-blue color was indicative of indoxyl-acetate hydrolysis.

2.2.3.6. H_2S Reduction

The H_2S reduction was tested using triple sugar iron agar slants (Appendix B). H_2S reduction resulted in the formation of a black precipitate due to the metabolism of ferrous sulfate.

2.2.3.7. DNase Activity

Extracellular nuclease activity was determined on DNase test agar (Oxoid) as described by the manufacturer. Isolates were spotted onto the DNase agar. Following 18 h incubation at 30 ^{o}C, the plates were carefully flooded with 1 N HCl that precipitated DNA and allowed to stand for a few min. DNase positive cultures showed a distinct clear visible zone around the growth.

2.2.3.8. Growth in the Presence of 0.04 % TTC

The isolates were grown on BHI agar (Appendix B) containing 0.04% 2,3,5-triphenylamone-N-oxide (TTC) microaerobically at 30 °C for 24-48 h. When the bacteria used TTC during their growth, the colony color changes into pink or red.

2.2.3.9. Growth at Different Temperatures

Isolates were grown on mCCDA agar under microaerobically at 30 °C, 37 °C and 42 °C for 24-48 h.

2.2.3.10. Aerotolerance

Growth at 25 °C, 30 °C and 37 °C aerobically were also tested on mCCDA.

2.2.3.11. Growth at Different NaCl Concentrations

Isolates were grown on BHI agar containing 2% and 3.5% NaCl microaerobically at 30 °C for 24-48 h.

2.2.3.12. Growth on MacConkey Agar

Isolates were grown on MacConkey agar (Appendix B) at 30 °C aerobically for 24-48 h.

2.2.4. Molecular Characterization

2.2.4.1. Extraction of Bacterial Genomic DNA

2.2.4.1.1. Commercial Genomic DNA Isolation Kit

Bacterial genomic DNA were extracted by a commercial DNA isolation kit (In vitrogen) as described by the manufacturer. The isolates were grown on mCCDA agar for 48 h at 30 °C at microaerobically. The cells were collected and resuspended in 1 ml of sterile water. The suspension was centrifuged at 13.500 *g* for 5 min to pellet the bacteria. The supernatant was discarded and the pellet was used for genomic DNA isolation based on the manufacturers' instructions. The concentrations of DNA were measured with Nanodrop (8000-Thermo Scientific) and adjusted to 25-60 ng/µl using elution buffer provided by the kit. The DNA was stored at -20 ^{o}C.

2.2.4.1.2. Boiling Method

A modified alkali lysis protocol (Debruyne et al., 2008) was used to extract the DNA from the *Arcobacter* isolates. A loopful of culture grown on mCCDA agar for 48 h at microaerobically was suspended in 40 µl of lysis buffer containing 0.25% (w/v) SDS (Appendix D) and 0.5 M NaOH. Then, the suspension was heated at 95 °C for 15 min in a water bath and immediately cooled on ice. After cooling, 150 µl distilled water were added and the tubes were centrifuged at 13.000 *g* for 5 min. The supernatant containing genomic DNA was transferred into another sterile tube. The concentrations of DNA were measured with Nanodrop (8000-Thermo Scientific) and adjusted to 25-60 ng/µl. The DNA was stored at -20 ^{o}C.

2.2.4.2. Oligonucleotide Primers for Genus-specific PCR for *Arcobacter* Isolates

Universal primers reported by Harmon and Wesley (1996) were used in the genus PCR. These primers amplified a 1223 bp region of 16S rRNA gene. The sequences of primers were shown in Table 2.2.

Table 2.2. Sequences and the positions of genus PCR primers
(Source: Harmon and Wesley, 1996)

Primers	Position	Nucleotide sequence (5'-3')
Arco I	224 - 244	AGAGATTAGCCTGTATTGTAT
Arco II	1426 - 1446	TAGCATCCCCGCTTCGAATGA

2.2.4.3. Identification by PCR at the Genus Level

Selected sequences were amplified by PCR using a reaction mixture containing 2.5 µl *Taq* 10X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µl (10 pmol) of each of the DNA primers, 1.5U of *Taq* DNA polymerase (Fermentas) and 2 µl bacterial DNA. The PCR mixture was adjusted to 30 µl with sterile dH_2O. All the steps were performed on ice. The PCR amplification was performed in a thermal cycler (Bio-rad, C-1000) with the following amplification conditions: a denaturation step for 4 min at 94 °C; 29 amplification cycles: denaturation 1 min at 94 °C, annealing 1 min at 56 °C, and 1 min extension at 72 °C; the final extension step is 7 min at 72 °C.

2.2.4.4. Agarose Gel Electrophoresis of Amplified PCR Products

After the completion of PCR reaction, 1.0% agarose-ethidium bromide gel was prepared by adding 1.0 gr agarose to 100 ml 1xTAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA [pH 8.0]) and boiling (Appendix D). The solution was cooled to 40-50 ^oC before adding 7,5 µl of ethidium bromide (10 mg/ml) into 50 ml 1xTAE solution. The solution was poured into a gel tray and allowed to solidify. 10 µl of each PCR product were mixed with 1/5 volume of gel-loading dye before loading and electrophoresed at

90V for 40 min in 1xTAE buffer. The bands were visualized and recorded in a gel documentation system (Vilber Lourmat, France).

2.2.4.5. Oligonucleotide Primers of Multiplex PCR (m-PCR)

The sequences for m-PCR primers were targeted to three species of the *Arcobacter* 16S rRNA and 23S rRNA genes (Houf et al., 2000). The selected primers amplify a 257 bp fragment from *A.cryaerophilus*, a 401 bp fragment from *A. butzleri* and a 641 bp fragment from *A.skirrowi* (Table 2.3).

Table 2.3. Sequences and the positions of m-PCR primers
(Source: Houf et al., 2000)

Strains	Primers	Position	Nucleotide sequence (5'-3')
A. butzleri	BUTZ	959-983	CCTGGACTTGACATAGTAAGAATGA
16S rDNA	ARCO	1357-1338	CGTATTCACCGTAGCATAGC
A. skirrowii	SKIR	705-723	GGCGATTTACTGGAACACA
A. cryaerophilus	CRY1	105-124	TGCTGGAGCGGATAGAAGTA
23S rDNA	CRY2	359-340	AACAACCTACGTCCTTCGAC

2.2.4.6. Multiplex PCR (m-PCR)

Selected sequences were amplified by PCR using a reaction mixture containing 2.5 µl *Taq* 10X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µl (10 pmol) of each of the above five primers, 1.5U of *Taq* DNA polymerase (Fermentas) and 2 µl bacterial DNA.

The reactions were performed in a thermal cycler (Bio-rad C-1000) with the following amplification conditions: a denaturation step for 3 min at 94 °C; 34 amplification cycles: denaturation 30 sec at 94 °C, annealing 30 sec at 60 °C, and 1 min extension at 72 °C; the final extension step is 7 min at 72 °C.

2.2.4.7. Agarose Gel Electrophoresis of Amplified m-PCR Products

The gel was made by adding 1.25 gr agarose to 100 ml 1xTAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA [pH 8.0]) by boiling. The solution was cooled to 40-50 ^{o}C before adding 7,5 µl of ethidium bromide (10 mg/ml) into 50 ml 1xTAE solution (Appendix D). The solution was poured into a gel tray including combs. After the gel was solidified, the combs were removed. The casting tray was placed into the tank containing 1xTAE buffer. 10 µl of each PCR product mixed with 2 µl of 6xGel loading dye (Fermentas) and 4 µl of DNA molecular weight marker were loaded into the wells. The electrophoresis was performed for approximately at 90V for 40 min. The bands were visualized on an UV illuminator and recorded in a gel documentation system (Vilber Lourmat, France).

2.2.5. 16S Ribosomal RNA (rRNA) Gene Sequencing of Unidentified *Arcobacter* Isolates for Classification at the Species Level

The 16S ribosomal RNA (rRNA) gene of the 21 *Arcobacter* isolates, which were unidentified by m-PCR, was amplified by PCR using primers ArcoI and ArcoII; their sequences were given on section 2.2.4.2 (Harmon and Wesley 1996). These amplified isolates were prepared to apply 16S rRNA sequencing analysis. Firstly, Sephadex and spin columns used to purify amplified PCR products. Then, The DNA nanogram levels were measured with Nanodrop spectrophotometry (Nanodrop, 8000-Thermo Scientific). Because, the amplified PCR products range should be 3-10 ng/µl for 200-500 bp. After that, the isolates were sequenced by using ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit with forward primer (Arco I) as one-sided sequencing. The sequence were performed in PCR machine with proper conditions: a denaturation step for 1 min at 96 °C; 29 amplification cycles: denaturation 10 sec at 96 °C, annealing 30 sec at 56 °C, and 4 min extension at 60 °C; hold at 4 °C. The PCR product applied cycle sequences were passed through Sephadex and spin column. The amplified and sequenced isolates were put into wells inside machine (Applied Biosystem, ABI 3130XL). The idea of sequencing is based on that each fluorescence colour represents each nucleotide. Therefore, the sequence of the PCR product was compared with known

16S rRNA gene sequences available in the GenBank (NCBI) database by the basic local alignment search tool (BLAST) and/or MEGA 5.05 program.

2.2.6. Antimicrobial Activity

40 *Arcobacter* strains isolated in this study were tested for their antibiotic susceptibility by disc diffusion method. Firstly, the isolates were grown on mCCDA agar plates for 48 h under microaerobic conditions at 30 °C. Then, the isolates were suspended using a sterile swab to give a density of McFarland 0.5. Each isolate were then spread on Muller-Hinton agar plates with 5% sheep blood using a sterile swab. After that, antibiotic discs were placed onto the blood agar media carefully with a sterile forceps. Finally, all plates were incubated at 30 °C for 24-36 h microaerobically.

The isolates were tested for antimicrobial their activity with thirteen antibiotic discs which are nalidixic acid (5 µg), ampicillin (10 µg), tetracycline (30 µg), iminepem (10 µg), gentamicin (10 µg), vancomycin (5 µg), ciprofloxacin (5 µg), rifampicin (5 µg), azythromycin (15 µg), metronizadole (50 µg), carbenicillin (100 µg), erythromycin (15 µg), and polymyxin B (300 µg). All antibiotics supplied from Oxoid.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Optimization of Enrichment Step

Optimization was done using a reference strain of *Arcobacter butzleri* LMG 10828. AEB were used for comparisons and understanding which method could yield more colonies. Firstly, the reference strain was grown on blood agar with 5% sheep blood microaerobically for 48 h at 30 °C. Thereafter, a suspension of the reference strain was made corresponding to McFarland 0.5 (Approx. $1.5x10^8$ cfu/ml) in 10 ml 0.9% NaCl. Then, 100 µl of this suspension were taken into 200 ml of AEB. As a final concentration, there were 50-100 colonies in AEB and this suspension was used as a reference point for each method. These procedures were performed for three methods used for the isolation purposes during this study, and third method was found to give higher number of colony counts, although other two methods were also productive. The environmental water samples were initially examined using the three methods for comparison.

The first method was found efficient for drinking and tap water but not for sewage and river samples. These samples were passively filtered before vacuum filtration but the vacuum system did not allow them to pass through due to their viscosity.

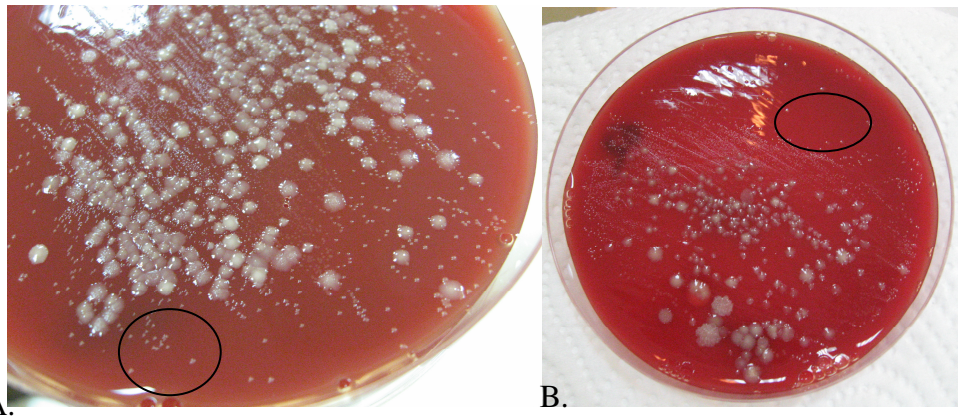
When the filters were placed into the AEB broth supplemented with CAT, the undesired bacteria were also grown during incubation. These colonies could inhibit and/or mask the growth of *Arcobacter* species (Fera et al., 2004). Therefore, injection syringe fitted with 0.45 µm pore size membrane filters were used for filtration of 200 ml water sample instead of normal membrane filters to eliminate the growth of undesired bacteria. However, this method was not effective since the syringes were plugged due to the viscosity of the samples. Also, it was expensive, time consuming and hard to apply for each sample.

Due to the limitations of these two methods, the third method was preferred. In this method, there was no filtering procedure. Size range is variable for arcobacters, so

any different size of *Arcobacter* spp. can be caught with this method. Moreover, CAT supplement were used to inhibit different other bacterial species.

3.2. Isolation

After incubation of the water samples using third method, 100 µl of broth suspension were filtered onto blood agar plates containing 5% sheep blood using 0.45 µm pore size 47 mm diameter nitrocellulose membrane filter (Atabay and Corry, 1997), the membrane filtration technique resulted in the growth of different colonies (Figure 3.1). It was found that 72 samples out of 115 showed *Arcobacter*-like colonies on the blood agar plates after the incubation. Then, these colonies were subcultured onto mCCDA until pure cultures were obtained for further characterization.



A. B.

Figure 3.1. *Arcobacter* colony morphology on blood agar plates A. and B. Black circles indicate the *Arcobacter*-like colonies.

3.3. Phenotypic Characterization

Seventy-two isolates representing single sample were chosen for phenotypic characterization (Table 3.1).

3.3.1. Gram Staining

Seventy-two isolates were Gram-stained and examined under light microscope. Based on the microscopy, these 65 isolates were Gram-negative and seemed slender

curved rod, S-shaped, small in size resembling *Arcobacter* cell morphology (Figure 3.2).

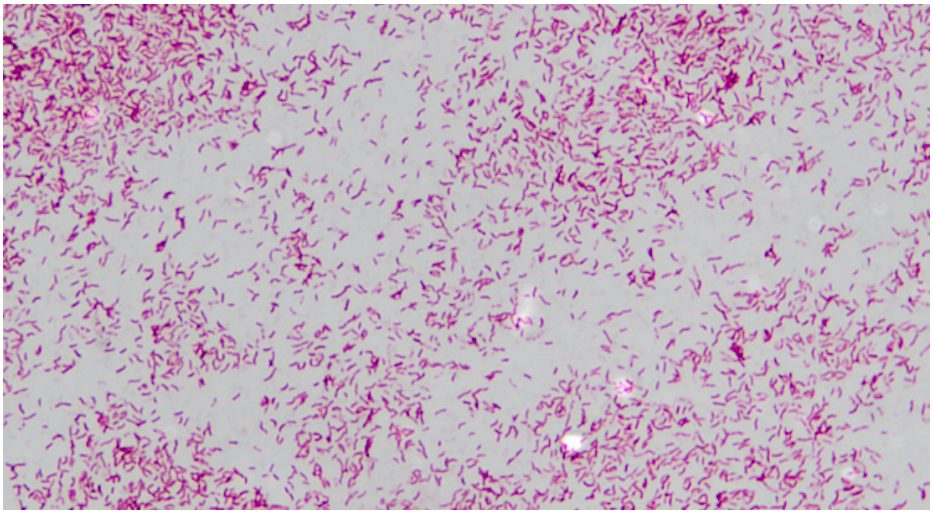


Figure 3.2. Gram straining image of the isolate 11.

3.3.2. Motility

Seventy-two isolates were examined under phase contrast microscopy for motility and it was found that 61 of them gave typical darting, corkscrew-like motility.

3.3.3. Oxidase Test

Sixty-one isolates that showed characteristic cell morphology and motility typical of arcobacters were tested for oxidase activity and 61 isolates were found oxidase positive (Figure 3.3).

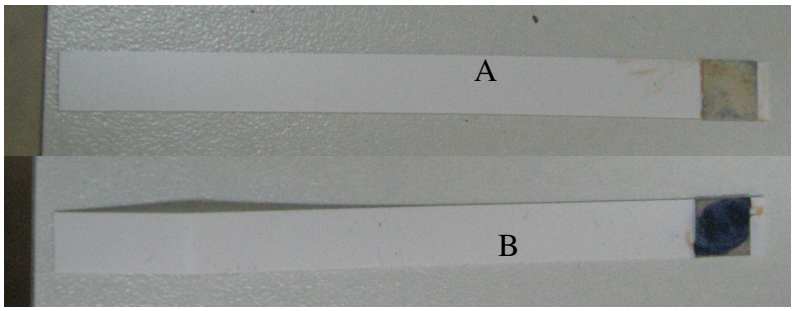


Figure 3.3. Oxidase test of *Arcobacter* isolates. A) Oxidase-negative isolate 59; B) Oxidase positive isolate 30-2.

3.3.4. Other Phenotypic Characterization Tests

Presumptively characterized *Arcobacter* isolates by the phenotypic tests using gram staining, motility and oxidase test were further identified as *Arcobacter* using several molecular techniques. As a result, 42 of these 61 isolates were classified as *Arcobacter* at the genus level. The following tests were, therefore, only applied to these 42 isolates.

3.3.4.1.Catalase Test

Forty-two isolates showed catalase activity (Figure 3.4). However, six of the isolates tested gave strong reaction, nine of isolates were moderately positive, and 27 of isolates were poorly positive. All isolates were identified as *A. butzleri* by molecular techniques. These results were consistent with general characteristics of *A. butzleri* which were given in section 1, Table1.5.

Figure 3.4. Catalase test result of the isolate 57-2. Gas bubbles indicate the presence of catalase activity.

3.3.4.2. Indoxyl-acetate Hydrolysis

The catalase test positive forty-two isolates were also found to be able to hydrolyze indoxyl-acetate (Figure 3.5). Green-blue color shows the positive result for *Arcobacter*.

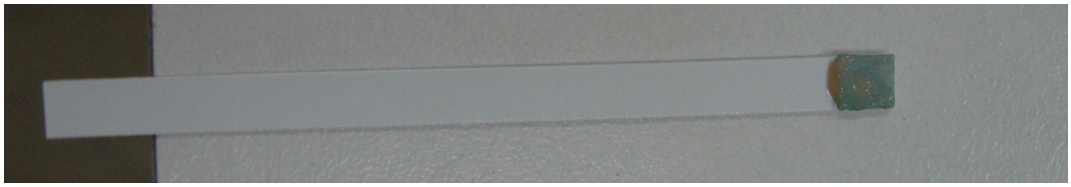


Figure 3.5. Indoxyl-acetate hydrolysis of the isolate 31.

3.3.4.3. H_2S Reduction

TSI agar was used to determine the ability of bacteria to ferment glucose, lactose and/or sucrose and their ability to reduce sulfur to hydrogen sulfide. There was no black precipitation indicating non-reduction of H_2S, which shows negative result (Figure 3.6), for forty-two isolates examined. However, there were three isolates (4-2, 38-2,57-2) that gave yellow color in slant and butt which means glucose and lactose (or sucrose) fermentation. This may be due to possibility of the presence of potential new strains or species of *Arcobacter* that may ferment carbohydrates. However, it may be also possible that the sequence analysis used might have given a false result.

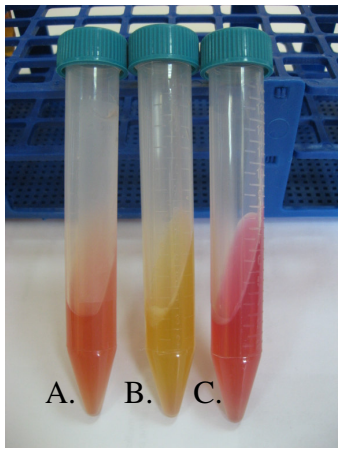


Figure 3.6. H_2S reduction test on TSI agar. A. Negative control. B.The isolate 4-2 which fermented the glucose and lactose (or sucrose). C. The isolate 11 which only shows the growth on TSI agar.

3.3.4.4. DNase Activity

None of the forty-two isolates were found positive for DNase activity.

3.3.4.5. Growth in the Presence of 0.04% TTC

41 out of 42 isolates could grow in the presence of 0.04% TTC (Figure 3.7). The results were shown in Table 3.1.

Figure 3.7. *Arcobacter* species on BHI agar supplemented with 0.04% TTC.

3.3.4.6. Growth at Different Temperatures

The growth of the forty-two isolates under microaerobic atmosphere at 30 °C, 37 °C and 42 °C and aerobic growth at 25 °C, 30 °C and 37 °C were determined on mCCDA agar (Figure 3.8). All isolates grew well at 30 °C under both aerobic and microaerobic conditions. Also, all isolates grew well at 25 °C under aerobic condition. Moreover, 35 of the 42 isolates grew in microaerobic and aerobic conditions, but 5 isolates (30-2, 65-1, 104-2, 105, 107) grew poorly in microaerobic conditions. Also, the isolate 30-2 grew poorly in aerobic conditions. The 35 isolates tested grew at 42 °C microaerobically although 14 of them grew poorly.

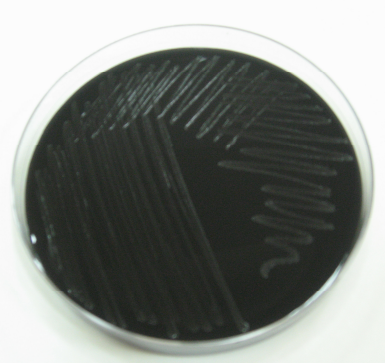


Figure 3.8. The micraerobic growth of the isolate 19 at 37 °C on mCCDA agar.

3.3.4.7. Growth at Different NaCl Concentrations

The forty-two isolates were inoculated onto BHI agar containing 2.0% and 3.5% NaCI (w/v) concentrations (Table 3.1). 42 isolates gave positive results for 2.0% NaCl while 9 isolates did not grow in the presence of 3.5% NaCI (Figure 3.9).

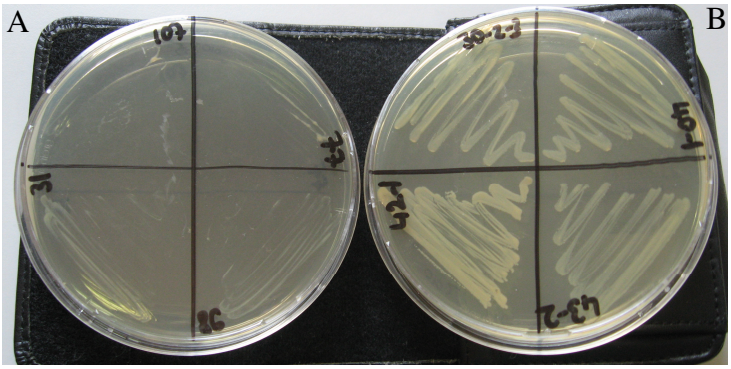


Figure 3.9. *Arcobacter* species on BHI agar with different NaCl concentrations. A: The slow growth in the presence of 3.5% NaCl concentration; B: The growth in the presence of 2.0% NaCl concentrations.

3.3.4.8. Growth on MacConkey Agar

The forty-two isolates tested for growth on MacConkey agar (Figure 3.10). The results showed that the isolate 6, 88 and 104-1 were negative which means no growth on MacConkey agar (Table 3.1). Also, the isolates 4-2, 38-2 and 57-2 showing positive result in TSI agar grew on MacConkey agar and the colony colors changed to pink due to lactose fermentation which reduced the pH of the agar below 6.8. These lactose-

fermented colonies might be a potential new strain of *Arcobacter* or there is a false result in the sequencing analysis used for the clarification of the identity of the isolates.

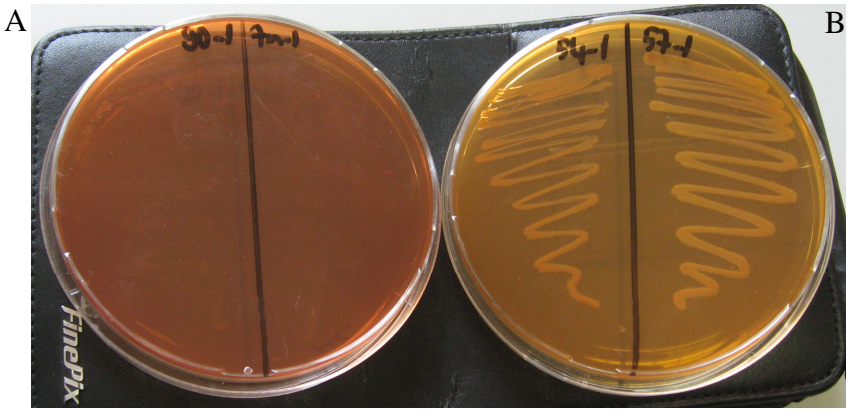


Figure 3.10. The image of *Arcobacter* spp. on MacConkey agar. A: shows the isolates 90-1 and 7-1 before incubation at 30 °C aerobically. B: shows the isolates 54-1 and 57-1 after incubation at 30 °C aerobically.

Total phenotypic characteristics of *Arcobacter* isolates from environmental samples in this study showed almost the same results with those of previous studies given in Table 1.5 for *A. butzleri*. However, three isolates (4-2, 38-3 and 57-2) showed lactose fermentation in TSI agar and MacConkey agar. General knowledge about arcobacters is that they neither ferment nor oxidize the carbohydrates (Vandamme et al., 1991b).

Table 3.1. Some phenotypic tests results of *Arcobacter* isolates from this study (No:42)

Strains	Catalase	30 °C mO_2	37 °C mO_2	42 °C mO_2	25 °C O_2	30 °C O_2	37 °C O_2	2.0 % NaCl	3.5 % NaCl	0.04 % TTC	MacConkey Agar	Molecular Ident. of the isolates
4-2	2	+	+	+*	+	+	+	+	+	+	+[a]	Seq.A.b.
6	1	+	-	+*	+	+	-	+	+*	+	-	A.b.
7-1	1	+	+	+	+	+	+	+	+	+	+*	A.b.
7-2	3	+	-	-	+	+	-	-	-	+	+	Seq.A.b.
11	1	+	+	+	+	+	+	+	+	+	+	A.b.
17-3	1	+	+	+*	+	+	+	+	+	+	+	A.b.
19	1	+	++	++	+	+	++	+	+	+	+	Seq.A.b.
30-2	1	+	+*	-	+	+	+*	+	+	+	+	A. spp.
31	1	+	+	+	+	+	+	+	+	+	+*	A.b.
36	2	+	++	+*	+	+	+	+	+	+	+	Seq.A.b.
37-1	1	+	-	+*	+	+	-	+	+	+	+	Seq.A.b
38-2	3	+	+	+*	+	+	+	+	+	+	+[a]	Seq.A.b
40-1	2	+	+	++	+	+	+	+	+	+	+	Seq.A.b
40-3	3	+	-	-	+	+	-	+	+	+	+	A.b
42-1	2	+	++	-	+	+	+	+	+	+	+	Seq.A.b
43-2	1	+	-	-	+	+	-	+	+	+	+	Seq.A.b
46	2	+	+	+*	+	+	+	+	+	+	+	Seq.A.b
47	1	+	+	+	+	+	+	+	+	+	+	Seq.A.b
50-1	2	+	+	+*	+	+	+	+	+	+	+	Seq.A.b
50-2	1	+	+	+*	+	+	+	+	+	+	+	Seq.A.b
52	2	+	-	-	+	+	-	+	+	+	+	Seq.A.b.
53	2	+	+	+*	+	+	+	+	+	+	+	A. spp.
54-1	3	+	++	++	+	+	+	+	+*	+	+	Seq.A.b.
54-2	1	+	+	+	+	+	+	+	-	+	+	Seq.A.b.
56-2	2	+	-	+*	+	+	-	+	+	+	+	Seq.A.b.
57-1	3	+	+	+	+	+	+	+	+	+	+	Seq.A.b.
57-2	3	+	+	+	+	+	+	+	+	+	+[a]	Seq.A.b.
58	1	+	+	+	+	+	+	+	-	-	+	A.b.
62-2	1	+	+	-	+	+	+	+	-	+	+	A.b.
65-1	1	+	+*	+*	+	+	+	-	-	+	+*	A.b.
77	1	+	+	+*	+	+	+	+	-	+	+	A.b.
88	1	+	+	+*	+	+	+	+	+	+	-	A.b.
89-1	1	+	+	+	+	+	+	+	+	+	+*	A.b.
90-1	1	+	+	+*	+	+	+	+	-	+	+	A.b.
96-1	1	+	+	+	+	+	+	+	+*	+	+*	A.b.
98	1	+	+	+	+	+	+	+	-	+	+	A.b.
100	1	+	+	+	+	+	+	+	+	-	+	A.b.
102	1	+	+	+	+	+	+	+	+	+	+	A.b.
104-1	1	+	+	+	+	+	+	+	+	+	-	A.b.
104-2	1	+	+*	+	+	+	+	+	+	+	+	A.b.
105	1	+	+*	+	+	+	+	+	+*	+	+	A.b.
107	1	+	+*	+	+	+	+	+	-	+	+	A.b.

+ growth, - no growth. +* poorly positive. +[a] gave pink color colonies on MacConkey agar.
++ strong positive at respective temperature on mCCDA agar plate.
1: poor positive, 2: moderately positive, 3: strong positive for catalase test.
A.b.; *A. butzleri,* Seq.A.b.; 16S rRNA gene sequence result identifies as *A. butzleri.*
A. spp.; defined as *Arcobacter* spp. at genus level

3.4. Molecular Characterization

3.4.1. Extraction of Bacterial Genomic DNA

A commercial bacterial genomic DNA isolation kit (In vitrogen) was used to extract DNA from the 61 isolates obtained in this study. The DNA quality and purity were determined based on the A_{260}/A_{280} ratios (the absorbance of nucleic acids to absorbance of amino acids) measured by Nanodrop. 1.80-2.00 levels was chosen to use in molecular studies.

In addition, the boiling method with alkaline lysis buffer was used to compare the results of the DNA isolations. It was concluded that although purities of DNA were almost the same, the concentrations of DNA were higher in the boiling method than the commercial isolation kit (Table 3.2). Both DNA extraction methods were applied to the first 30 isolates, but the remaining isolates were processed with the boiling method.

Table 3.2. Comparison of commercial genomic DNA isolation kit and boiling method for *Arcobacter* isolates

	Commercial genomic DNA isolation kit		**Boiling method**	
Isolate	**ng/µl**	**A 260/280**	**ng/µl**	**A 260/280**
4-2	29,66	2,04	243,43	1,92
7-2	27,84	1,70	390,69	1,92
19	24,69	1,90	597,44	2,08
30-2	37,56	1,74	596,05	1,98
38-2	55,97	1,94	193,84	1,98
43-2	20,72	1,87	469,40	1,97
50-1	17,78	1,85	347,44	1,94
54-2	80,12	1,78	302,16	2,01

3.4.2. Identification of the *Arcobacter* Isolates by PCR at the Genus Level

Upon PCR amplification, 42 out of the 61 isolates analysed were *Arcobacter* positive by PCR at the genus level as *Arcobacter*, yielding a 1223 bp product (Harmon and Wesley, 1996) (Figure 3.11). To overcome the problems encountered during the assay related with the reproducibility, the concentration of $MgCl_2$ was increased to 3.0 mM (Figure 3.12). Increasing the $MgCl_2$ level resulted in strong bands in genus specific

PCR. This results is important to get strong (clear) bands, and also important to apply 16S rRNA gene sequencing analysis for unidentified isolates.

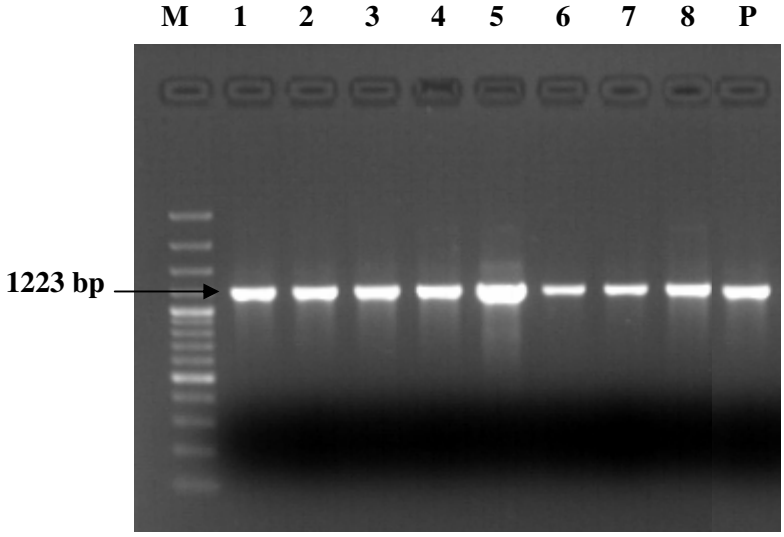


Figure 3.11. Gel photograph of 16S rRNA genus specific PCR for *Arcobacter* isolates. M: 100 bp DNA Ladder, Lanes 1-8: isolates 4-2, 11, 19, 58, 65-1, 89-1, 100, 104-1. P: *A. butzleri* LMG 10828 as positive control.

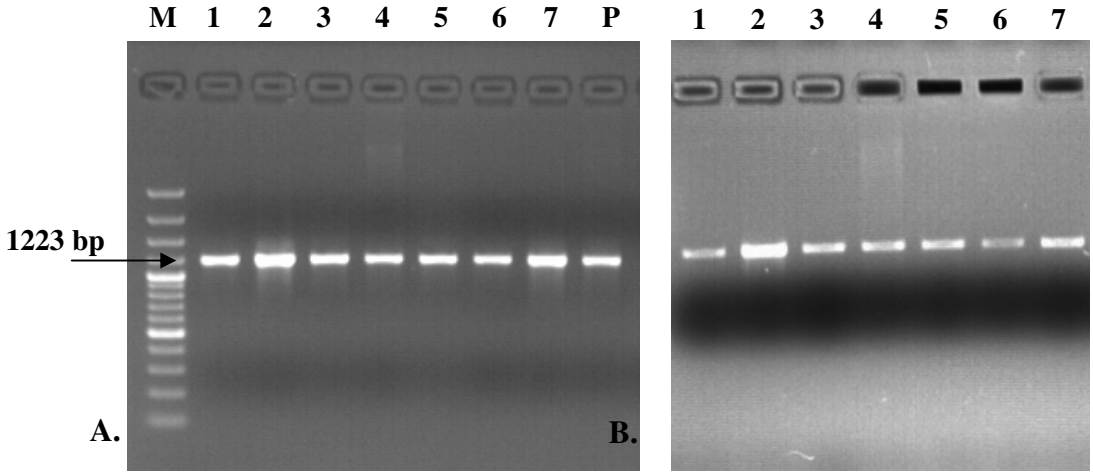


Figure 3.12. Gel photograph of 16S rRNA genus specific PCR for *Arcobacter* isolates with different $MgCl_2$ concentrations. A: 3.0 mM $MgCl_2$ concentration M: 100 bp Marker. Lanes 1-7: isolates 4-2, 6, 36, 42-1, 57-1, 43-2 and 7-2; B: 1.5 mM $MgCl_2$ concentration. Lanes 1-7: isolates 4-2, 6, 36, 42-1, 57-1, 43-2 and 7-2. P: *A.butzleri* LMG 10828 as positive control.

3.4.3. Differentiation of the *Arcobacter* Isolates by m-PCR at the Species Level

To differentiate 42 *Arcobacter* strains isolated out of this study at species level, the primers and PCR conditions specific for *A. butzleri*, *A. skirrowii* and *A. cryaerophilus* were used as described earlier (Houf et al., 2000). The results showed that 21 of the isolates eaxmined (Table 3.3) were *A. butzleri* (Figure 3.13). Twenty-one Gram-negative, rod-shaped, slightly curved, non-spore-forming bacteria that gave a negative result in *Arcobacter* species-specific PCR assay but yielded an amplicon in the *Arcobacter* genus-specific PCR test. These *Arcobacter* isolates, which were designated as *Arcobacter* spp. at the genus level (Table 3.4) were subjected to 16S rRNA gene sequencing for further characterization and classification in Section 3.4.4.

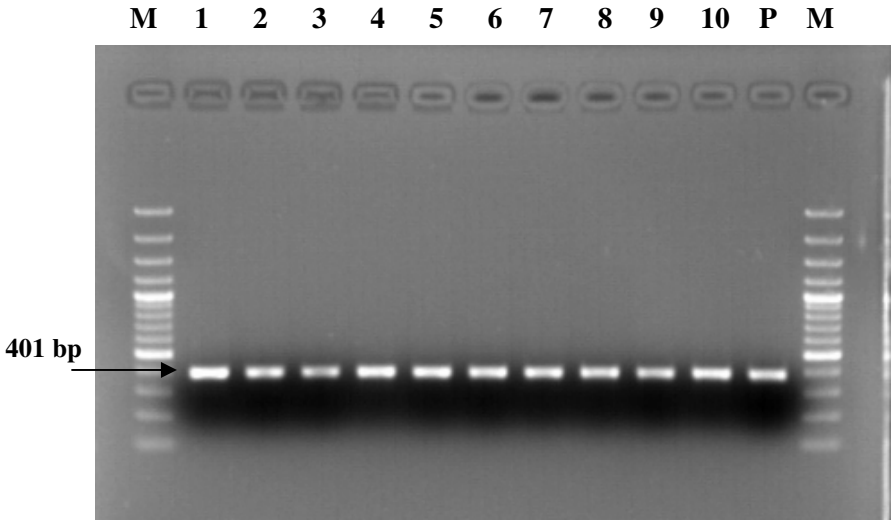


Figure 3.13. Agarose gel electrophoresis of m-PCR products. M: 100 bp Marker. Lanes 1-10 isolates: 7-1, 17-3, 31, 40-3, 58, 62-2, 77, 89-1, 96 and 98. P: *A.butzleri* LMG 10828 as positive control.

The results of the identity of *Arcobacter* isolates from environmental samples via molecular techniques such as PCR at the genus level and m-PCR at the species level were in accordance with previous studies. For example, Rice et al., (1999) used PCR and m-PCR to idenfity the isolates obtained from ground water and the results showed the presence of *A. butzleri* in non-chlorinated water. Although, the most common strain of *Arcobacter* seems as *A. butzleri*, Collado et al., (2010) reported that *A. cryaerophilus* and *A.skirrowii* in sewage effluents and a drinking water treatment plant were also found.

We employed enrichment procedure without direct culture to recover *Arcobacter* strains from the water samples throughout this study based on our preliminary laboratory trial results (data not shown). Fera et al. (2004) indicated that after enrichment period (24h), only *A. butzleri* was detected, even though *A. cryaerophilus* and *A. butzleri* were exist in pre-enriched samples. Similarly, Gonzalez et al. (2010) also indicated that the enrichment step suppresses some co-existing *Arcobacter* strains in wastewater samples. In parallel with these studies, we were also able to recover mostly *A. butzleri* strains from the environmental samples examined. This also explains why the majority of the isolates detected were *A. butzleri* in this study. It may probably be possible to recover more species of *Arcobacter* if a direct plating and/or molecular detection procedure, such as real-timre PCR, would be included.

Table 3.3. The distribution of identified and classified *Arcobacter* species according to their origin and sampling sites in this study.

Isolate No	**Species**	**Source**	**Sampling site**
6	*A. butzleri*	Sewage	Gülbahçe
7-1	*A.butzleri*	Sewage	Gülbahçe
11	*A.butzleri*	River	Güzelbahçe
17-3	*A.butzleri*	River	Old Çeşme Road
31	*A.butzleri*	River	Zeytinalanı
40-3	*A.butzleri*	River	Birgi
58	*A.butzleri*	Sewage	Gülbahçe
62-2	*A.butzleri*	River	İçmeler
65-1	*A.butzleri*	River	İçmeler
77	*A.butzleri*	Sewage	Torasan
88	*A.butzleri*	Sewage	Gülbahçe
89-1	*A.butzleri*	River	Gülbahçe
90-1	*A.butzleri*	Sewage	Gümüşkoy
96-1	*A.butzleri*	Sewage	Ildırı
98	*A.butzleri*	Sewage	Yağcılar
100	*A.butzleri*	Sewage	Kuşçular
102	*A.butzleri*	Sewage	Kuşçular
104-1	*A.butzleri*	Sewage	Kuşçular
104-2	*A.butzleri*	Sewage	Kuşçular
105	*A.butzleri*	Sewage	Kuşçular
107	*A.butzleri*	Drinking water	Kuşçular

Table 3.4. The distribution of unidentified *Arcobacte*r isolates according to their origin and sampling sites in this study.

Isolate No	Species	Source	Sampling site
4-2	*Arcobacter* spp.	River	Gülbahçe
7-2	*Arcobacter* spp.	Sewage	Gülbahçe
19	*Arcobacter* spp.	Sewage	Old Çeşme Road
30-2	*Arcobacter* spp.	Drinking water	Zeytinalanı
36	*Arcobacter* spp.	Sewage	Zeytinalanı
37-1	*Arcobacter* spp.	River	Zeytinalanı
38-2	*Arcobacter* spp.	Sewage	Barbaros
40-1	*Arcobacter* spp.	River	Birgi
42-1	*Arcobacter* spp.	Sewage	Uzunkuyu
43-2	*Arcobacter* spp.	Sewage	Uzunkuyu
46	*Arcobacter* spp.	Sewage	Birgi
47	*Arcobacter* spp.	Sewage	Birgi
50-1	*Arcobacter* spp.	Sewage	Uzunkuyu
50-2	*Arcobacter* spp.	Sewage	Uzunkuyu
52	*Arcobacter* spp.	Drinking water	Kadıovacık
53	*Arcobacter* spp.	Drinking water	Kadıovacık
54-1	*Arcobacter* spp.	River	Uzunkuyu
54-2	*Arcobacter* spp.	River	Uzunkuyu
56-2	*Arcobacter* spp.	Sewage	Barbaros
57-1	*Arcobacter* spp.	Sewage	Kadıovacık
57-2-2	*Arcobacter* spp.	Sewage	Barbaros

3.4.4. 16S rRNA Gene Sequencing of *Arcobacter* spp.

The 16S rRNA gene sequencing showed that most of the isolates were assigned to *A.butzleri* (Appendix E) within 96-99% ranges, but two isolates (30-2 and 53) could not be identified. The reason for failure to identify these isolates could be lack of appropriate sequence of isolates and/or they could be a possible new strain/species (Table 3.6). The 16S rRNA gene sequencing was shown as a useful technique for clarifying the identification of *Arcobacter* strains when other PCR-based methods were not effective.

Table 3.5. The results showing the identification of *Arcobacter* isolates after 16S rRNA gene sequence analysis

Isolate No	DNA ng/µl	Genus	Species	Identity (%)	Source
4-2	29,66	W	*A.butzleri*	99	River
7-2	27,84	W-M	*A.butzleri*	99	River
19	24,69	M	*A.butzleri*	99	Sewage
30-2	37,56	W	ND	ND	drinking water
36	16,62	M	*A.butzleri*	99	Sewage
37-1	33,76	W	*A.butzleri*	99	River
38-2	55,97	M	*A.butzleri*	99	Sewage
40-1	33,96	M	*A.butzleri*	96	River
42-1	48,11	W	*A.butzleri*	99	Sewage
43-2	20,72	W	*A.butzleri*	99	Sewage
46	23,35	M	*A.butzleri*	99	Sewage
47	52,12	M	*A.butzleri*	99	Sewage
50-1	17,78	M	*A.butzleri*	99	Sewage
50-2	39,38	W	*A.butzleri*	99	Sewage
52	81,32	M	*A.butzleri*	95	drinking water
53	12,36	W	ND	ND	drinking water
54-1	15,65	M	*A.butzleri*	99	River
54-2	80,12	S	*A.butzleri*	99	River
56-2	27,8	W	*A.butzleri*	91	Sewage
57-1	33,93	M	*A.butzleri*	98	Sewage
57-2	19,02	W	*A.butzleri*	99	Sewage

W: weak band at genus specific PCR for *Arcobacter* isolates. M: Moderate band at at genus specific PCR for *Arcobacter* isolates. S: strong band at genus specific PCR for *Arcobacter* isolates

Table 3.6. Characteristics of the *Arcobacter* isolates which were not identified by both m-PCR and 16S rRNA gene sequencing

Strain	Catalase	30 °C mO_2	37 °C mO_2	42 °C mO_2	25 °C O_2	30 °C O_2	37 °C O_2	2.0 % NaCl	3.5 % NaCl	0.04 % TTC	MacConkey agar	Molecular ident. with genus PCR
30-2	1	+	+*	-	+	+	+*	+	+	+	+	A. spp.
53	2	+	+	+*	+	+	+	+	+	+	+	A. spp.

+ growth, - no growth. +* poorly positive.
++ strong positive at respective temperature on mCCDA agar plate.
1: poor positive, 2: moderately positive, 3: strong positive for catalase test.
A. spp.; defined as *Arcobacter* spp. at genus level.

3.5. Antimicrobial Susceptibility Test

Because, at present, there is no standardized reference for comparison of the antibiotic resistance level of *Arcobacter* spp., the results were interpreted with *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Campylobacter* spp. standards of National Committee for Clinical Laboratory Standards (NCCLS, 2009). The results are shown in Table 3.7.

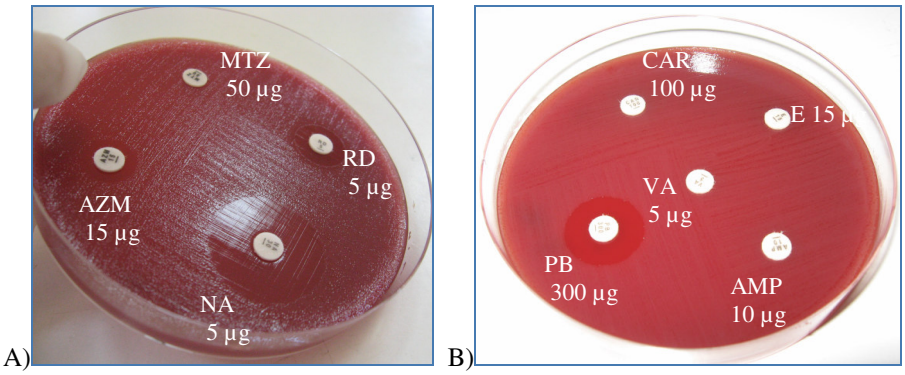


Figure 3.14. A) The image shows the antibiotic test of isolate 36 for AZM (15µg), MTZ (50 µg), RD (5 µg) and NA(5 µg). B) The image shows the antibiotic resistance of isolate 4-2 for AMP (10 µg), VA (5 µg), E (15 µg), CAR (100 µg) and PB (300 µg).

In this study, *Arcobacter* isolates, which were obtained from the environmental samples and identified by molecular techniques, were examined for their resistance to antibiotics.

The disc diffusion antibiotic test results indicated that all *Arcobacter* spp.(38/38) are resistant to vancomycin, most of them resistant to ampicillin (30/33), carbenicillin (18/19), metronizadole (17/18), rifampicin (31/39), erythromycin (16/19) and nalidixic acid (24/34). Also, the isolates tested were mostly susceptible to tetracycline (3/16), iminepem (3/16) and ciprofloxacin (4/31). Resistance to azythromycin, gentamicin and polymyxin B were intermediate (see also Table 3.7 for details). All isolates showed multiple resistance to the antibiotics tested even if they belonged to different classes.

Tetracycline and ciprofloxacin susceptibility were also determined by Son et al. (2007) for *A. butzleri*. The isolates from broiler carcasses in the study of Son et al. (2007) and those from this study showed higher level of resistancy to azythromycin. The isolates from this study displayed higher resistance to erythromycin (79%) and nalidixic acid (70,58%) than the isolates obtained from broiler carcasses in the study of

Son et al. (2007) for *A. butzleri*. Increased erythromycin resistance of *Arcobacter* isolates in human and broiler isolates were also reported by Houf et al. (2004).

In another study, Kabeya et al. (2004) reported that *Arcobacter* species isolated from meats showed susceptibility to tetracycline, and resistance to nalidixic acid. Also, all the isolates from this study and the ones from the study of Kabeya et al. (2004) showed high resistance to vancomycin.

Moreover, this study showed that *Arcobacter* isolates from the environmental samples presented resistance to gentamicin, but the studies conducted by Atabay and Aydın (2001) using broiler chicken isolates, and Vanderberg et al. (2006) using clinical isolates showed susceptibility of the *Arcobacter* isolates to gentamicin. This could be due to the differences in their sources and/or their location of isolation.

Table 3.7. Disc diffusion test results with class of antibiotics for *Arcobacter* spp.

Antibiotics	**Class**	**Conc. (µg)**	**Results**
Ampicillin (AMP)	P	10	30/33 (90% R)
Azithromycin (AZM)	M	15	12/18 (66% R and I)
Carbenicillin (CAR)	P	100	18/19 (94,73% R)
Ciprofloxacin (CIP)	Q	5	4/31 (12% R)
Erythromycin (E)	M	15	16/19 (79% R, 21% I)
Gentamicin (CN)	AG	10	5/31 (16% R, 84 I)
Iminepem (IPM)	CP	10	3/16 (18,75% R)
Metronidazole (MTZ)	N	50	17/18 (94,44% R)
Nalidixic acid (NA)	Q	30	24/34 (70,58% R)
Polymyxin B (PB)	LP	300	2/18 (11% R, 27,7% S, 61% I)
Rifampicin (RD)	R	5	31/39 (80% R, 20% I)
Tetracycline (TET)	T	30	3/16 (18,7% R)
Vancomycin (VA)	GP	5	38/38 (100 % R)

AG: aminoglycoside; **CP:** carbapenem; **GP:** glycopeptide; **M:** macrolide; **LP:** Lipopeptide; **P:** Beta-lactam penicillin; **Q:** quinolone; **R:** rifampin; **T:** tetracycline; **N:** not assigned.

CHAPTER 4

CONCLUSIONS

This study shows the presence of *Arcobacter* spp. and their distribution at the species level in the environmental samples such as sewage, river and drinking water. Therefore, the results obtained in this study highlight the importance of prevention and control of *Arcobacter* contamination in water.

One hundred-fifteen samples collected from İzmir and surroundings were examined for the prevalence of *Arcobacter* spp. After enrichment, a membrane filtration technique has been shown as an effective method for the isolation of various *Arcobacter* spp. Use of membrane filters with 0.65 µm pore size might increase the isolation rate but a problem might arise due to contaminating flora.

Extraction of bacterial genomic DNA were also done by means of a commercial kit and a boiling method. Only three of the sequenced isolates were detected with PCR after the boiling method whereas all gave positive results with the commercial extraction kit. Although the commercial kit is expensive, it is sensitive and time-saving.

In this study, 42 of the 115 (36,5%) environmental samples examined were found to contain *Arcobacter* spp. (drinking water= 6, river=11, sewage= 25). Twenty-one of the 42 isolated *Arcobacter* strains were identified as *A. butzleri* by m-PCR. 16S rDNA sequencing was shown as an effective technique for the identification of the strains that could not be identified by m-PCR.

Use of some phenotypic tests gave a useful presumptive identification of *Arcobacter* isolates at the genus level but not at the species level. Therefore, phenotypic identity of the isolates should be confirmed by molecular techniques. Molecular techniques such as ERIC-PCR, AFLP, and PFGE (Shah et al., 2011) could be used to discriminate the isolated strains within species level. 16S rDNA-RFLP (Figueras et al., 2008) can also be used to differentiate a wider variety of *Arcobacter* spp. besides m-PCR method.

Incubation time of the enrichment step could be prolonged to find a possible new species but background flora could dominate the plates. Therefore, antibiotic test results can be useful to determine a better environment to recover susceptible *Arcobacter*

strains. The antibiotic susceptibility test results could also be used for the treatment of diseases caused by these foodborne pathogens.

Consumption of contaminated water is an important source of arcobacters. *A. butzleri* can easily attach to water distribution pipe surfaces (stainless steel, copper and plastic), which causes the re-growth of these bacteria in the water distribution system (Assanta et al., 2002). Therefore, biofilm formation of the strains could be elucidated to understand the adhesion mechanism on water-distribution systems. Also, there is little information about the effects of drinking-water treatment on *Arcobacter* spp., but *A. butzleri* was found to be sensitive to chlorine desinfection (Rice et al., 1999).

This study showed that proper water treatments should be applied to prevent *Arcobacter* contamination in water sources for human health.

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APPENDIX A

CHEMICALS USED IN EXPERIMENTS

CHEMICAL	**CODE**
Campylobacter Blood-Free Selective Agar Base (modified CCDA)	Oxoid CM0739
Glycerol	Merck 1.04092
Sodium chloride	Merck 1.06404
Immersion oil	Merck 1.04699
Nutrient broth No:2	Oxoid CM0067
Tris Base	Amresco 0826
EDTA	Applichem A2937
Ethidium bromide	Sigma-Aldrich E7637
Ethanol absolute (puriss)	Sigma-Aldrich 32221
Taq DNA polymerase MBI	Fermentas EP0401
dNTP set	Fermentas R0181
Agarose (Standard)	Sigma A9539
Bromophenol blue	Merck 1.08122
Sodium dodecyl sulphate	Meerck 8.17034
Sodium hydroxide	Sigma-Aldrich 06203
Hydrochloric acid (HCl)	Merck 1.00317
6x loading dye solution Gene RulerTM	Fermentas R0611
1 kb DNA Ladder Gene RulerTM	Fermentas SM0313
100 bp DNA Ladder Gene RulerTM	Fermentas SM0323 ready to use
DNase agar	Oxoid CM0321
Iodine	Sigma I-3380
Safranine	Fluka 84120
Carbol fuchsin (ready to use)	Difco 3321-75
Crystal violet	Merck 1.15940
Hydrogen peroxide (% 30)	Sigma-Aldrich H1009

Arcobacter broth	Oxoid CM0965
Brain Heart Infusion Broth	Oxoid CM1135
Triple Sugar Iron Agar	Oxoid CM0277
Mac Conkey Agar	Oxoid CM0005
Agar	Applichem A0949
C.A.T selective supplement	Oxoid SR0174E
Indoxyl strips	Fluka 04739
Bactident Oxidase	Merck 1.13300
Campy Gen	Oxoid CN0025A
Mueller–Hinton Broth	Oxoid CM0405

APPENDIX B

CULTURE MEDIA

B.1. *Arcobacter* Broth

	g/l
Arcobacter Broth	24
Agar	15

Ingredients are dissolved in distilled water by stirring with gentle heating. Medium is sterilised by autoclaving at 121°C for 15 min.

B.2. Brain Heart Infusion (BHI) Agar

	g/l
Brain Heart Infusion Broth	37
Agar	15

Components are added into distilled water and mixed thoroughly. Medium is sterilised by autoclaving at 121°C for 15 min.

B.3. MacConkey Agar

	g/l
Peptone	20
Lactose	10
Bile salts	5
Neutral red	0.075
Agar	12

47 g of powder is suspended in distilled water and brought to the boil to dissolve completely. Medium is sterilised at 121°C for 15 min.

B.4. DNase Agar (Oxoid CM321)

	g/l
Tryptose	20
DANN	2
NaCl	5
Agar	12

39 g of powder is suspended in distilled water and brought to the boil to dissolve completely. Medium is sterilized at 121°C for 15 min.

B.5. Triple Sugar Iron (TSI) Agar

65 g/l of powder is suspended in distilled water and brought to the boil to dissolve completely. Medium is sterilised at 121°C for 15 min. After sterilization, medium is distributed in 5 ml amounts into sterile tubes. Tubes are allowed to set in the sloped position.

B.6. Nutrient Broth No:2

	g/l
Yeast extract	3
Peptone	5
NaCl	8

Ingredients are dissolved in distilled water by stirring. The pH is adjusted to 6.8. Medium is sterilized in the autoclave for 15 min at 121°C.

APPENDIX C

REAGENTS AND SOLUTIONS

C.1. Gram's Iodine Solution

	g/l
Iodine	1 g
Potassium iodide	2 g
Distilled water	300 ml

Ingredients are grinded in a mortar and dissolved by adding water slowly. The prepared solution is mixed well by stirring.

C.2. Crystal Violet Solution

5 g/l crystal violet is dissolved in sufficient water and the solution is mixed well by stirring.

C.3. Safranine Solution

Safranine	0.25 g
Alcohol	10 ml
Distilled water	100 ml

Safranine is dissolved in the alcohol. Water is added and the solution is filtered through paper.

APPENDIX D

BUFFERS AND STOCK SOLUTIONS

D.1. 50 X TAE

242 g of Tris base is dissolved in deionised water. 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) are added. Volume is adjusted to 1000 ml with deionised water.

D.2. 1 X TAE

20 ml of 50X TAE buffer is taken and 980 ml of deionised water is added to obtain 1X TAE buffer.

D.3. 10 X TBE

108 g of Tris base and 55 g boric acid are mixed and dissolved in 800 ml of deionised water. 40 ml of 0.5 M EDTA (pH 8.0) is added. The volume is brought to 1000 ml with deionised water.

D.4. 1M Tris-HCl (pH 7.2/ pH 8.0)

121.1 g of Tris base is dissolved in 800 ml of deionised water. The desired pH is obtained by adding concentrated HCl. The volume of the solution is brought to 1000 ml with deionised water.

D.5. 1 x TE (pH 8.0)

10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0) is mixed.

D.6. 0.5 M EDTA pH 8.0

186.1g of EDTA are added to 800 ml of deionised water. Solution is stirred vigorously and the pH is adjusted to 8.0 with NaOH pellets (~ 20 g). Volume is completed to 1000 ml with deionised water. Solution is dispensed into aliquots and sterilised by autoclaving.

D.7. Ethidium Bromide Stock Solution (10 mg/ml)

1 g of ethidium bromide is dissolved in 100 ml of deionised water by stirring on a magnetic stirrer to dissolve the dye completely. Solution is transferred to a dark bottle and stored at room temperature.

D.8. 10% Sodium Dodecyl Sulfate (SDS)

100 g of SDS is dissolved in 900 ml of deionised water. Solution is heat to 68˚C to dissolve. The pH is adjusted to 7.2 by adding a few drops of concentrated HCl. The volume is brought to 1000 ml with water.

D.9. Gel-loading Dye (6X)

2 ml of 10xTBE, 6 ml of glycerol is mixed in a falcon and the volume is adjusted to 20 ml with sterile deionised water. Bromophenol blue is added until the adequate colour is obtained.

D.10. dNTP (10X)

20 µl of each 100mM dATP, dCTP, dGTP and dTTP are taken and mixed in an eppendorf tube. 920 µl of sterile deionised water is added to dilute the solution to a final concentration of 2 mM. Solution is mixed gently and stored at –20 °C.

D.11. 20 % Glycerol Stock

20 ml glycerol and 80 ml Nutrient Broth No:2 are autoclaved separately at 121 °C for 15 min and then mixed aseptically.

APPENDIX E

16S rRNA GENE SEQUENCES OF ISOLATES

Isolate 7-2 (Identified by sequencing analysis)

gb|FJ968634.1| *Arcobacter butzleri* strain ED-1 16S ribosomal RNA gene, partial sequence

Length=1509
Score = 1795 bits (1990), Expect = 0.0
Identities = 1016/1022 (99%), Gaps = 6/1022 (1%)
Strand=Plus/Plus

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Query  2     TGGCCTACCA-GACGATGACGCATAACTGGTTTGAGAGGATGATCAGTCACACTGGAACT  60
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Sbjct  260   TGGCCTACCAAGACGATGACGCATAACTGGTTTGAGAGGATGATCAGTCACACTGGAACT  319

Query  61    GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGACGAAA  120
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Sbjct  320   GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGACGAAA  379

Query  121   GTCTGATGCAGCAACGCCGCGTGGAGGATGACACATTTCGGTGCGTAAACTCCTTTTATA  180
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Sbjct  380   GTCTGATGCAGCAACGCCGCGTGGAGGATGACACATTTCGGTGCGTAAACTCCTTTTATA  439

Query  181   TAAGAAGATAATGACGGTATTATATGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGC  240
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Sbjct  500   GGTAATACGGAGGGTGCAAGCGTTACTCGGAATCACTGGGCGTAAAGAGCGTGTAGGCGG  559

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Query  361   ATCTAGAATGTGGGAGAGGTAGATGGAATTTCTGGTGTAGGGGTAAAATCCGTAGAGATC  420
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Sbjct  620   ATCTAGAATGTGGGAGAGGTAGATGGAATTTCTGGTGTAGGGGTAAAATCCGTAGAGATC  679

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Sbjct  680   AGAAGGAATACCGATTGCGAAGGCGATCTACTGGAACAATATTGACGCTGAGACGCGAAA  739

Query  481   GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTACACTA  540
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Sbjct  740   GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTACACTA  799

Query  541   GTTGTTGTGAGGCTCGACCTTGCAGTAATGCAGTTAACACATTAAGTGTACCGCCTGGGG  600
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Sbjct  800   GTTGTTGTGAGGCTCGACCTTGCAGTAATGCAGTTAACACATTAAGTGTACCGCCTGGGG  859

Query  601   AGTACGGTCGCAAGATTAAAACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGC  660
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Sbjct  920   ATGTGGTTTAATTCGACGATACACGAAGAACCTTACCTGGACTTGACATAGTAAGAATGA  979

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Sbjct  1040  TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCTTAG 1099

Query  841   TTGCTAACAGTTCGGCTGAGAACTCTAAGGAGACTGCCTACGCAAGTAGGAGGAAGGTGA  900
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Sbjct  1100  TTGCTAACAGTTCGGCTGAGAACTCTAAGGAGACTGCCTACGCAAGTAGGAGGAAGGTGA 1159

Query  901   GGATGACGTCAAGTCATCATGGCCCCTTACGTCCAGGGGCTACACACGTGCTACAATGGG  960
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Query  961   GGTATACAAAGAGCAGCAATACGGTGACGTGGAGCAAAATCTCAAAAATGCCTCCCCAGT 1020
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Sbjct  1217  GGTATACAAAGAGCAGCAATACGGTGACGTGGAGC-AAATCTCAAAAATGCCT-CCCAGT 1274

Query  1021  TC  1022
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Sbjct  1275  TC  1276
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Isolate 11 (Identified by m-PCR)

gb|FJ968634.1| *Arcobacter butzleri* strain ED-1 16S ribosomal RNA gene, partial sequence

Length=1509
Score = 1669 bits (1850), Expect = 0.0
Identities = 929/930 (99%), Gaps = 1/930 (0%)
Strand=Plus/Plus

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Sbjct  260   TGGCCTACCAAGACGATGACGCATAACTGGTTTGAGAGGATGATCAGTCACACTGGAACT  319

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Sbjct  320   GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGACGAAA  379

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Sbjct  380   GTCTGATGCAGCAACGCCGCGTGGAGGATGACACATTTCGGTGCGTAAACTCCTTTTATA  439

Query  192   TAAGAAGATAATGACGGTATTATATGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGC  251
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Sbjct  440   TAAGAAGATAATGACGGTATTATATGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGC  499

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Query  372   ATCTAGAATGTGGGAGAGGTAGATGGAATTTCTGGTGTAGGGGTAAAATCCGTAGAGATC  431
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