SANITARY IMPORTANCE OF ARCOBACTER

Arturo Levican Asenjo

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Sanitary importance of *Arcobacter*

Doctoral Thesis

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To Karin, Fabian and Gabriela

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FREQUENTLY USED ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
ANI	Average Nucleotide Identity
ASB	Arcobacter Selective Broth
ASM	Arcobacter Selective Medium
ATCC	American Type Culture collection
BA	Blood Agar
BLAST	Basic Local Alignment Search Tool
CAT	Cefoperazone-Amphotericin B-Teicoplanin (antibiotic supplement)
CCDA	Campylobacter Cefoperazone Deoxycholate Agar
CCUG:	Culture Collection, University of Göteborg, Sweden
CDC	Center for Disease control, Culture Collection
CECT	*Colección Española de Cultivos Tipo*
CIP	Collection of Institute Pasteur
CLSI	Clinical and Laboratory Standards Institute
CSDB	*Ad hoc* committee for the re-evaluation of the species definition in bacteriology
DDH	DNA-DNA Hibridization
DGGE	Denaturing Gradient Gel Electrophoresis
DSM	*Deutsche Sammlung von Microorganisme und Zellkulturen GmbH,* German Culture Collection
ERIC	Enterobacterial Repetitive Intergenic Consensus
GAST	Global Alignment for Sequence Taxonomy
HIV	Human immunodeficiency Virus
IV	Intra Venous
LMG	*Laboratorium voor Microbiologie, Universiteit Gent,* Belgium Culture Collection
MALDI TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MLPA	Multilocus Phylogenetic Analysis
MLSA	Multilocus Sequence Analysis
MLST	Multilocus Sequence Typing
m-PCR	multiplex-PCR
NARMS	National Antimicrobial Resistance Monitoring system
NCBI	National Center for Biotechnology Information
PD	Peritoneal Dialysis
PFGE	Pulsed Field Gel Electrophoresis
RAPD	Random Amplified Polymorphic DNA
RDP	Ribosomal Database Project
RFLP	Restriction Fragment Length Polymorphism
RT-PCR	Real Time PCR
SEM	Scanning Electron Microscopy
ST	Sequence Type
TBE	Tris/Borate/EDTA buffer
TEM	Transmission Electron Microscopy
TETRA	Tetranucleotide Signature Frequency Correlation Coefficient
VBNC	Viable but Non Culturable
WWTP	Wastewater Treatment Plant

1. INTRODUCTION

1.1 The genus *Arcobacter*

Bacteria currently known as the genus *Arcobacter* were initially part of the genus *Campylobacter* because they share a similar morphology, but were considered “aerotolerant campylobacters” that were able to grow at lower temperatures. In 1983, the aerotolerant species *Campylobacter nitrofigilis* was described from roots of *Spartina alterniflora* and root associated sediments (McClung *et al.,* 1983). Two years later Neill *et al.* (1985) studied several strains of these “aerotolerant campylobacters” from animal origin, mainly from abortions, and defined the new species, *Campylobacter cryaerophila.* However, it was not until 1991, when Vandamme *et al.,* using a polyphasic approach, determined that these two species should be separated into a genus named *Arcobacter.* Therefore, the former *C. cryaerophila* became *Arcobacter cryaerophilus* and *C. nitrofigilis* became *Arcobacter nitrofigilis,* which was selected as the type species (Vandamme *et al*., 1991). The genus was amended and enlarged by Vandamme *et al.* in 1992 with the inclusion of another new species, *Arcobacter skirrowii,* recovered from diseased animals and abortions (Vandamme *et al.*, 1992), and the reclassification of *Campylobacter butzleri as Arcobacter butzleri*. This species was originally recovered from humans and animals with diarrhoea when defined by Kiehlbauch *et al.* (1991). Since then, several new species have been described from different environments, namely *Arcobacter halophilus* from a hypersaline lagoon (Donachie *et al.*, 2005); *Arcobacter cibarius* from chicken carcasses (Houf *et al.*, 2005); *Arcobacter thereius* from kidney of aborted porcine foetuses and cloacae of duck (Houf *et al.*, 2009); *Arcobacter mytili* and *Arcobacter molluscorum* from shellfish (Collado *et al.*, 2009a; Figueras *et al.*, 2011b); *Arcobacter marinus* from a mixture of seaweed, starfish and seawater (Kim *et al.*, 2010); *Arcobacter trophiarum* from faeces of pigs (De Smet *et al.*, 2011a) and *Arcobacter defluvii* from sewage (Collado *et al.*, 2011).

The species *A. cryaerophilus* includes two subgroups, named 1A and 1B, depending on their different Restriction Fragment Length Polymorphisms (RFLP) of the 16S and 23S rRNA genes (Kiehlbauch *et al.*, 1991), or named 1 and 2, depending on whole-cell protein and fatty acid content (Vandamme *et al.*, 1992). Furthermore, both subgroups clustered separately using Amplified Fragment Length Polymorphism (AFLP) analysis (On *et al.*, 2003). Regarding their prevalence, both groups have so far been isolated simultaneously only from food products and from animal and human clinical samples, subgroup 2 being much more prevalent than 1 (Collado & Figueras, 2011 and references therein). The taxonomy of *A. cryaerophilus* was reviewed by analysing several representative strains of both subgroups using the sequences of *hsp60* gene and AFLP (Debruyne *et al.*, 2010). The study concluded that the separation of the two groups should be abandoned and that the

current type strain of this species (*A. cryaerophilus* subgroup 1, LMG 24291^{T}) should be replaced by the strain of *A. cryaerophilus* subgroup 2, LMG 10829.

The 12 species that comprised the genus at the beginning of this thesis are listed in Table 1.1. An obligate microaerophilic organism that oxidises sulphides was proposed as "*Candidatus* Arcobacter sulfidicus", but it has not so far been described formally (Collado & Figueras, 2011). Furthermore, the phylogenetic analysis of the deposited 16S rRNA gene sequences (> 1.300 bp) of uncultured strains available in the Ribosomal Database Project (RDP, Cole *et al.,* 2008) suggested the existence of several potentially new *Arcobacter* spp. from different hosts and/or habitats, such as sewage, oil field environments, tidal and marine sediments, seawater, estuarine and river water, plankton, coral, tubeworms, snails, oysters, abalone, and associated with cod larviculture or with cyanobacterial mats (Wesley & Miller, 2010; Collado & Figueras, 2011).

1.1.1 Methods for the characterization of new species

1.1.1.1 16S rRNA gene

The 16S rRNA gene is still considered a good tool for separating the species of this genus and for establishing its phylogeny (Figueras *et al.*, 2011b), which is shown in Figure 1. 1. The similarity of this gene among the 12 species of this genus range from 92.1% to 98.9% (Collado & Figueras, 2011). The lowest similarity (92.1%) corresponds to *A. thereius* and *A. halophilus* and is below the 95% threshold suggested for genus differentiation (Stackebrandt & Goebel, 1994). The highest similarity is shown for *A. cryaerophilus* with *A. cibarius* (98.9%), and is far above the classical threshold (97%) usually applied to delineate species (Stackebrandt & Goebel, 1994). Other species similarities are also above this threshold, for instance, *A. molluscorum* with *A. marinus* (97.6%; Figueras *et al.*, 2011b) and *A. trophiarum* with *A. cryaerophilus* (98.2%; De Smet *et al.*, 2011a). As a result, it has been suggested that the more restrictive boundary (98.7-99%) recently proposed to separate species (Stackebrandt & Ebers, 2006) could be applied to this genus (Figueras *et al.*, 2011b).

The 16S rRNA gene has also been largely used to characterize microbial communities on the basis of its hypervariable regions (Chakravorty *et al.*, 2007). Studies have revealed that arcobacters are widely spread across several environmental communities, as reviewed by Wesley & Miller (2010). They have been detected in tidal and marine sediments, seawater, estuaries, rivers, aquifers contaminated with spills, wastewater treatment plants (WWTPs), septic tank effluent and dairy lagoon, activated sludge and cattle manure (Wesley & Miller, 2010). Associations with vertebrate and invertebrate hosts, such as coral, plankton, tubeworms, oysters, abalone, and snails have also been reported (Wesley & Miller, 2010).

Table 1.1 Species of the genus *Arcobacter*

Species	Type strain	Other designations[a]	Source	Country	References
A. nitrofigilis	LMG 7604	ATCC 33309, CCUG 15892, KCTC 2688, CCUG 15893, CECT 7204, CIP 103745, DSM 7299, LMG 6619, LMG 7604, NCTC 12251 McClung strain CI	Roots from *Spartina alterniflora*	Canada	McClung *et al*. (1983) Vandamme *et al*. (1991)
A. cryaerophilus	LMG 9904	ATCC 43158, CCM 3933, CCUG 17801, CIP 104014, DSM 7289 JCM 5361, LMG 24291, LMG 7536, NCTC 11885, R-35670 Neill A 169/B (02766)	Aborted bovine foetus (brain)	Ireland	Neill *et al*. (1985) Vandamme *et al*. (1991)
A. butzleri	LMG 10828	ATCC 49616, CCUG 30485, CDC D2686, CIP 103493 CIP 103537, DSM 8739, NCTC 12481 T , R-14508 WDCM 00065	Human with diarrhoea (faeces)	USA	Kiehlbauch *et al*. (1991) Vandamme *et al*. (1992)
A. skirrowii	LMG 6621	ATCC 51132, CCUG 10374, CIP 103538, DSM 7302, NCTC 12713, Skirrow 449/80,	Lamb with diarrhoea (faeces)	Belgium	Vandamme *et al*. (1992)
A. cibarius	LMG 21996	CCUG 48482, CECT 7203 T, CIP 108697, DSM 17680 Houf LHT-KH-2, Vandamme R-16099	Broiler carcasses	Belgium	Houf *et al*. (2005)
A. halophilus	LA31BT	ATCC BAA-1022, CCUG 53805, CIP 108450, DSM 18005	Hypersaline lagoon	USA	Donachie *et al*. (2005)
A. mytili	CECT 7386	LMG 24559, CIP 110066, Figueras F2075	Mussels	Spain	Collado *et al*. (2009a)
A. thereius	LMG 24486	CCUG 5692T, R-36847, On 16398	Pig abortion	Denmark	Houf *et al*. (2009)
A. marinus	JCM 15502	CECT 7727, DSM 21465, KCCM 90072 LMG 25634, LMG 25770, Cho CL-Sq,	Seawater associated with starfish	Korea	Kim *et al*. (2010)
A. defluvii	CECT 7397	LMG 25693, Figueras F98-3	Sewage	Spain	Collado *et al*. (2011)
A. trophiarum	LMG 25534	CCUG 59229, CIP 110286, R-39974	Pig faeces	Belgium	De Smet *et al*. (2011a)
A. molluscorum	CECT 7396	LMG 25694, Figueras SW28-11	Mussels	Spain	Figueras *et al*. (2011b)

CCUG, Culture Collection, University of Göteborg, Sweden. CECT, *Colección Española de Cultivos Tipo.* CIP, Collection of Institute Pasteur. DSM, *Deutsche Sammlung von Microorganisme und Zellkulturen GmbH.* KCTC, Korean Collection for Type Cultures. LMG, *Laboratorium voor Microbiologie, Universiteit Gent,* Belgium. NCTC, national collection of Type cultures, UK. CCM, Culture Collection of Microorganisms, Czech Republic. JCM, Japanese Collection of Microorganisms. CDC, Center for Disease Control, USA. KCCM, Korean Culture Center of Microorganisms. [a]Data obtained from StrainInfo, www.straininfo.net (Dawyndt *et al*., 2005)

The recently available massive sequencing technologies, such as pyrosequencing, provide large numbers of short sequence segments (tags), of the variable regions of the 16S rRNA gene (i.e. regions V3, ca. positions 433 – 497, V4, 576 – 682 or V6, 986 – 1043). These can be analysed using different software that will assign the sequences to particular species (Huse *et al.*, 2008), such as the RDP classifier (Cole *et al.*, 2008) or the Global Alignment for Sequence Taxonomy (GAST, Huse *et al.*, 2008). It has been stated that the pyrosequencing method is able to identify a greater number of bacterial sequences than traditional DNA approaches, providing a more in-depth comparison of bacterial diversity (Teixeira *et al.*, 2010). Despite that, no single region can differentiate among all bacteria and the targeted region should therefore be selected depending on the group of bacteria studied (Chakravorty *et al.*, 2007).

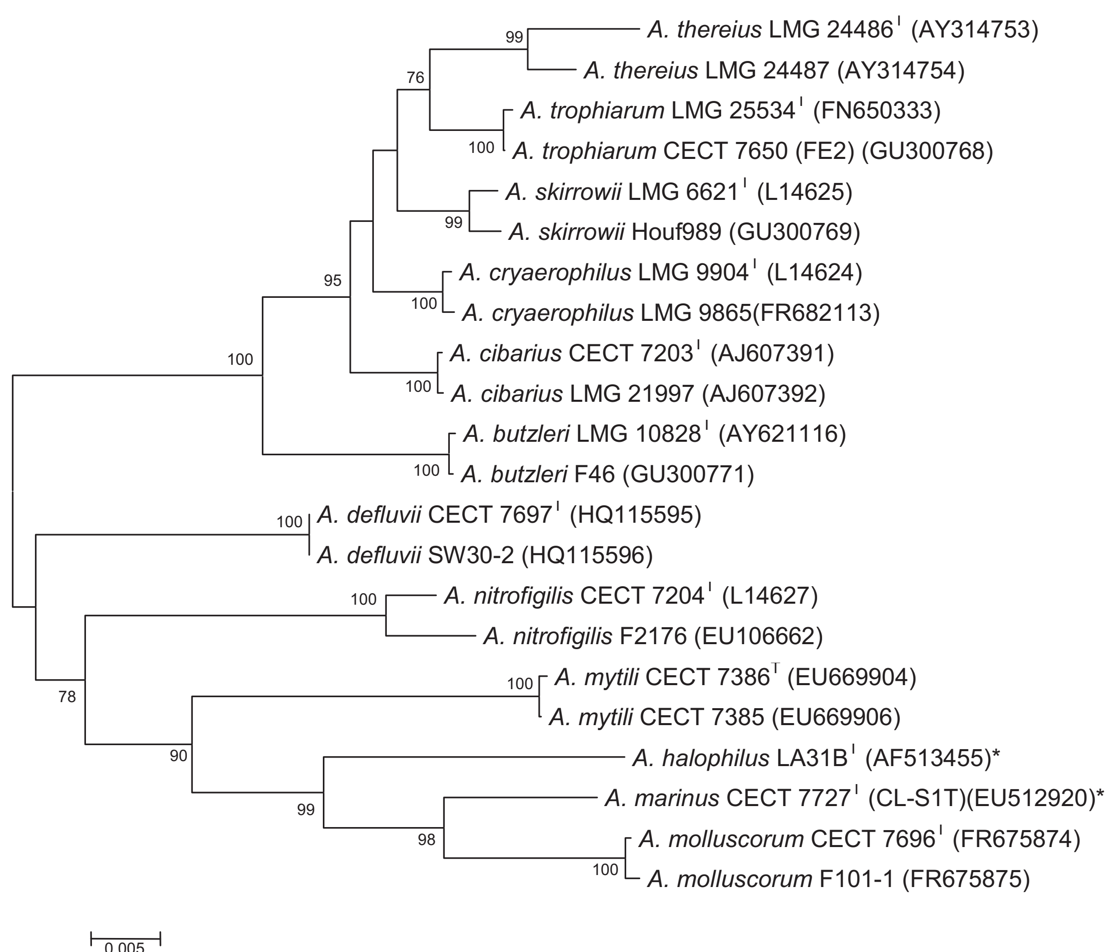

Figure 1.1 Neighbour joining tree based on 16S rRNA sequences (1401 bp) showing the phylogenetic position of the 12 *Arcobacter* species. Bootstrap values (≥70%) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 1000 nt. * Only the type strain is available so far

So far, *Arcobacter* has been detected by pyrosequencing bacterial communities present in rhizosphere soil from Antarctic vascular plants in Admiralty Bay, maritime Antarctica, using the V4 variable region (Teixeira *et al.*, 2010). In the latter study, *Arcobacter* was found in all plants analysed, i.e. *Deschampsia antarctica* and *Colobanthus quitensis*. In another study, *Arcobacter* was detected using variable region V6 in a microbial community of an experimental bioreactor model (Callbeck *et al.*, 2011). The bioreactor simulated the spoilage at low-temperature of an oil reservoir subjected to nitrate injection. A predominance of *Arcobacter* sp. was found at the bottom. Furthermore, *Arcobacter* has shown to be one of the predominant bacteria detected in two WWTPs in Milwaukee (USA) using the V6 region (McLellan *et al.*, 2010; Newton *et al.*, 2013).

1.1.1.2 DNA-DNA hybridization and other recently proposed genomic techniques

The DNA-DNA Hybridization (DDH) is currently considered the reference method for defining new bacterial species (Figueras *et al.*, 2011a and references therein). However, it is considered outdated and needs to be replaced as it does not allow a cumulative database to generate, but also because it is time consuming and prone to experimental error (Richter & Rosselló-Mora, 2009; Figueras *et al.*, 2011a). With this in mind, the “ad hoc committee for the re-evaluation of the species definition in bacteriology” has suggested that the sequences of several housekeeping genes (at least 5) could be used as an alternative to the DDH for the delineation of new bacterial species (Stackebrandt *et al.*, 2002; Figueras *et al.*, 2011a). This approach has been termed Multilocus Phylogenetic Analysis (MLPA) and has been successfully used for defining new species of the genus *Vibrio* and *Aeromonas,* in which the similarities of the 16S rRNA gene between species are very high (Figueras *et al.*, 2011a; Martinez-Murcia *et al.*, 2012). In relation to new species description in *Arcobacter*, the phylogenetic analysis of housekeeping genes like the *rpoB, gyrB* and *cpn60* genes have shown congruent results with those obtained with DDH and the 16S rRNA gene-based phylogeny (Collado & Figueras, 2011). However, they have lower intra- and interspecies similarity and therefore a higher discriminatory power than the 16S rRNA gene (Collado & Figueras, 2011). Considering this, it is important to evaluate whether a MLPA including at least 5 genes could show a good correlation with DDH results in order to validate this approach for the definition of new *Arcobacter* species.

Despite the demonstrated usefulness of MLPA in different bacteria genera (Figueras *et al.*, 2011a), it should be borne in mind that this approach could have drawbacks for certain bacterial groups, such as a putative bias in gene selection and the unavailability of amplification primers (Richter & Rosselló-Mora, 2009). Furthermore, the MLPA covers only a small proportion of the whole genome (Figueras *et al.*, 2011a). For these reasons, and considering that the current technologies make complete or partial genomes easily available,

the Average Nucleotide Identity (ANI) and Tetranucleotide Signature Frequency Correlation Coefficient (TETRA) have been proposed as other alternative methods (Richter & Rosselló-Mora, 2009). Both are based on the calculation of the degree of similarity of complete or partial (>50%) genomes. By comparing the DDH, ANI and TETRA values of several strains of different bacterial phyla, an objective boundary for species circumscription was found to correspond to a 95–96% ANI threshold reinforced by TETRA values >0.99 (Richter & Rosselló-Mora, 2009). In fact, ANI was used recently, to describe new species of *Burkholderia*, *Geobacter*, and *Vibrio*, new subspecies of *Francisella*, a new genus of *Sphaerochaeta*, and a new class of *Dehalococcoidetes* (Sentausa & Fournier, 2013 and references therein). It has also been suggested that ANI would serve not only for cultured prokaryotes but also for classifying uncultured strains if the data was combined with ecological or physiological traits (Richter & Rosselló-Mora, 2009). In fact, in their recent review, Sentausa & Fournier (2013) considered the integration of genomic data into prokaryotic taxonomic classification to be unavoidable in the near future. It is necessary to define a genomic-based method, such as ANI or another genomic comparison, to replace DDH as a standard for circumscribing prokaryotic species. They also commented that microbiologists should agree on the proposed method, and that the cut-offs established should either apply to most prokaryotes or vary according to taxonomic group.

In the genus *Arcobacter* there are only 4 complete genomes available so far, two of them are the species *A. butzleri* (strains RM4018, a derivative of ATCC 49616^T and ED-1), one is *A. nitrofigilis* DSM 7229^T and the other is *Arcobacter* sp. strain L, waiting to be formally named (Table 1.2). However, neither the ANI nor the TETRA analysis has yet been done. At present, there are several ongoing projects that are sequencing more *Arcobacter* genomes (Dr. William Miller, personal communication) so the validity of the proposed ANI and TETRA boundaries for the *Arcobacter* species should be evaluated in the near future.

1.1.1.3 Phenotypic characterization

The phenotypic characterization of *Arcobacter* species is difficult (Collado & Figueras, 2011). *Arcobacter* can be easily confused with those of *Campylobacter* because biochemical tests used to differentiate clinical bacteria, such as fermentation or oxidization of carbohydrates, often yield negative or variable results for these genera (Collado & Figueras, 2011). However, the bacteria of the genus *Arcobacter* grow at a lower temperature and in aerobic conditions (Vandamme *et al.*, 2005; Collado & Figueras, 2011).

Table 1.2 Comparison of the characteristics of the four available complete *Arcobacter* genomes

	Strains			
	A. butzleri **RM 4018**[a] Miller *et al*. (2007)	***A. butzleri*** **ED-1** Toh *et al*. (2011)	***A. nitrofigilis*** **DSM 7299**[T] Pati *et al*. (2010)	***Arcobacter sp.*** **L**[b] Toh *et al*. (2011)
Accession number	CP000361	AP012047	NC_014166.1	AP012048-49
Origin	Faeces of man with diarrhoea	Microbial fuel cell	Roots of *Spartina alterniflora*	Microbial fuel cell
Size (bp)	2,341,251	2,256,675	3,192,235	2,945,673
No. Of plasmids	0		0	1
Size plasmid (bp)	----	----	----	1,989
No. of:				
Total genes	2,259	2,158	3,224	2,845
Protein coding genes	1,011	1,454	3,154	1,812
RNA genes	ND	53	70	56
rRNA genes	5	5	4	5
Insertion elements	4	ND	0	ND
Pseudogenes	5	ND	70	ND
G+C (%)	27.05	27.10	28.36	26.6
Plasmid G+C (%)	----	----	----	46.6
Virulence				
Polar flagellum	yes	yes	yes	yes
Putative virulence genes:				
mviN	yes	ND	ND	ND
pldA	yes	ND	ND	ND
tlyA	yes	ND	ND	ND
irgA	yes	ND	ND	ND
hecAB	yes	ND	ND	ND
homolog to *iroE* (*ab0730*)	yes	ND	ND	ND
ciaB	yes	ND	ND	ND
cadF	yes	ND	ND	ND
cj1349	yes	ND	ND	ND

[a]Strain RM4018 is a derivative of the type strain of this species (ATCC 49616[T]). [b]Strain L which could belong to *A. defluvii* on the basis of the analysis of 16S rRNA gene (Collado *et al.*, 2011).

The phenotypic test that differentiates all characterized species (Table 1.3) includes only 4 enzymatic tests (catalase, urease, nitrate reduction and indoxyl acetate hydrolysis), resistance to cefoperazone and growth on several media and under different conditions. Other limitations of the phenotypic characterization of *Arcobacter* spp., and typical also for other genera, are: i) the difficulty in reproducing the results, ii) the large number of tests and

the specialized skills needed to carry them out, and iii) the variability attributable to the behaviour of the bacteria (Figueras *et al.*, 2011a). The latter is the problem for the most commonly isolated species, *A. butzleri* and *A. cryaerophilus,* which show variable results in several tests with no clear positive or negative behaviour (Table 1.3). The second edition of Bergey's Manual of Systematic Bacteriology (Vandamme *et al.*, 2005) cites growth on MacConkey agar or on minimal medium as the traits that differentiate them, despite the differences only applying to the type strains and other strains being able to yield variable results (Vandamme *et al.*, 2005; Figueras *et al.*, 2011a) (See Table 1.3).

Table 1.3 Differential characteristics among the 12 *Arcobacter* species

Characteristics	*A. nitrofigilis*	*A. cryaerophilus*	*A. butzleri*	*A. skirrowii*	*A. cibarius*	*A. halophilus*	*A. mytili*	*A. thereius*	*A. marinus*	*A. trophiarum*	*A. defluvii*	*A. molluscorum*
Growth in/on												
Air at 37 °C	V	V	+	+	-	+	+	-	+	-	+	**+**
CO_2 at 37 ºC	-	V	+	+	+	+	+	-	+	-	+	**+**
4% (w/v) NaCl	+	-	-	+	-	+	+	-	+	+[a]	-	**+**
1% (w/v) glycine	-	-	-	-	-	+[b]	+	+	+[b]	+[c]	-	**-**
MacConkey	-	V	+	-	+	-[b]	+	V	-[b]	V[a]	+	**+**
Minimal media	-	-[d]	+	-	+	-	-	+	-	-[c]	+	**-**
0.05% safranin medium	-	+	+	+	+	-[b]	-	+	+[b]	V	+	**+**
CCDA	-	+	+	+	V	-[b]	-	V	-[b]	+	+	**-**
Resistance to:												
Cefoperazone (64 mg l^{-1})	-	+	+	+	+	-	-	+	-[b]	+	V	**+**
Enzyme activity												
Catalase	+	+	V	+	V	-	+	+	-	+	+[e]	**+**
Urease	+	-	-	-	-	-	-	-	-	-	+	**-**
Nitrate reduction	+	+[f]	+	+	-	+	+[g]	+	+	-	+	**+**[h]
Indoxyl acetate hydrolysis	+	+	+	+	+	+	-	+	+	+	+	**-**

Data from Figueras *et al*. (2011b and references therein). Unless otherwise indicated: +, ≥ 95% strains positive; -, ≤11% strains positive; V, 12-94% strains positive. ND, not determined; CO_2 indicates microaerobic conditions; [a]Our strain (FE2) of this species was unable to grow in media with 4% NaCl, but grew in MacConkey agar; [b]All tested in media supplemented with 2% NaCl; [c]Test not evaluated by De Smet *et al*. (2011a); [d]Two (LMG 7537 and LMG 10241) of the four strains tested were positive; [e]Weak reaction: [f]Two (LMG 9904[T] and LMG 9065) of the four strains tested were negative; [g]Nitrate reduction was found to be positive for the 3 strains of *A. mytili* contradicting our previously published data (Collado *et al*., 2009a); [h] Nitrate is reduced after 72 h and 5 days for all strains under microaerobic and aerobic conditions, respectively.

1.1.1.4 Chemotaxonomy

It have been reported that chemotaxonomic traits can contribute to a more stable characterization of species (Tindall *et al.*, 2010); however, they have not been regularly included in the description of new *Arcobacter* species and, when carried out, results have not generally been conclusive. For instance, respiratory quinones have only been analysed for

the species *A. nitrofigilis, A. cryaerophilus, A. butzleri* and *A. skirrowii* (Vandamme *et al.*, 1992). These species have a similar profile, including menaquinone 6 (MK6) as a major respiratory quinone and a second atypical, methyl-substituted, menaquinone 6 that enables the differentiation of *Arcobacter* from *Campylobacter,* but is not useful for species differentiation (Vandamme *et al.*, 1992 and 2005). Furthermore, the whole-cell fatty acid analysis also has a questionable taxonomic value in the genus *Arcobacter,* because despite the two subgroups of *A. cryaerophilus* (1 or 2) showing different profiles, this approach cannot distinguish between *A. butzleri* and *A. cryaerophilus* subgroup 2 (Vandamme *et al.*, 1992 and 2005). A recent study only found a differential composition for the species *A. marinus* while *A. butzleri, A. cryaerophilus* and *A. skirrowii* shared the same fatty acid profile, as did *A. halophilus* and *A. nitrofigilis* (Kim *et al.*, 2010).

1.1.1.5 MALDI-TOF

The Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF), which is based on the detection of molecules, mainly proteins, has been adapted for identifying microorganisms (Welker & Moore, 2011) Software tools, such as the Biotyper (Bruker Daltoniks) or Saramis (Anagnostec), compare the obtained results with a broad database of microorganisms that include their type strains,. This has proven to be a rapid and sensitive method for identifying several bacteria (Welker & Moore, 2011). Recently, MALDI-TOF using Biotyper database, was evaluated for the differentiation of a few strains of the species *A. butzleri* (n=6), *A. cryaerophilus* (n=1) and *A. skirrowii*, (n=1) and confirmed that it was fast and reliable (Alispahic *et al.*, 2010). Therefore, it may be important to evaluate this method for the other species of the genus.

1.1.1.6 Genotyping

Genotyping methods are necessary for recognizing redundant strains of the same clone, to reveal genetic diversity among isolates, to recognize transmission routes and to trace sources of outbreaks (Collado & Figueras, 2011; Figueras *et al.*, 2011a). The most commonly used method in the genus *Arcobacter* is the Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR), and has been applied in several studies (Collado & Figueras, 2011 and references therein). However, other methods, such as the AFLP, are reported to have a better resolution (Collado & Figueras., 2011 and references therein). Moreover, the reliability of the ERIC-PCR method has been questioned by Merga *et al.* (2013) because binding sites for the primers were found to be absent in two *A. butzleri* genome sequences compared (RM4018 and 7h1h). Those authors report that the low annealing temperature used allows non-specific binding of primers to other regions and they suggest that the ERIC-PCR profile is produced randomly. Despite that, in a study on the dynamics of an *Arcobacter*

population in pigs during the fattening period, ERIC-PCR was more suitable for typing *A. thereius* strains than AFLP (De Smet *et al*., 2011b). These results were in line with those in a previous study that compared the AFLP profiles of strains representing all *Arcobacter* spp. (Debruyne *et al*., 2010), in which the profiles for *A. thereius* showed a remarkably lower number of peaks than those for other species.

An alternative method for typing is Multilocus Sequence Typing (MLST), which discriminates microbial isolates by comparing their Sequence Type (ST), which is made up of a unique combination of partial sequences of seven housekeeping loci or alleles (Joilley *et al*., 2004). The advantage of MLST over other typing methods is that sequence data is unambiguous and traceable between laboratories, allowing a global database to be created on the World Wide Web and molecular typing data for global epidemiology to be exchanged via the Internet (Urwin & Maiden, 2003). A public MLST database for the genus *Arcobacter* was created in 2009 (Miller *et al*., 2009), which includes a set of 7 genes, identical to those used for defining the MLST of *C. jejuni*, i.e. *aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm* and *tkt*; and it was designed for the 3 most commonly isolated species *A. butzleri*, *A. cryaerophilus* and *A. skirrowii.* At the time of its publication, it included 374 strains (275 *A. butzleri,* 72 *A. cryaerophilus*, 15 *A. skirrowii* and 8 *A. cibarius*) that had been isolated from Europe, Asia, Africa and the United States (Miller *et al.*, 2009). Those isolates had an allelic density (i.e. no. of alleles / no. of strains) ranging from 30% (*glnA*) to 63% (*glyA*) among the ones studied (Miller *et al.*, 2009). In addition, 61% of the strains possessed a unique ST and no more than five strains possessed the same ST (Miller *et al.*, 2009). The latter study also reports lateral gene transfer events between *A. cibarius* and *A. skirrowii* as well as between *A. cryaerophilus* and *A. thereius.* The strains included in the database did not group by host or geographical origin (Miller *et al*., 2009). This MLST scheme has so far been applied to two *Arcobacter* studies conducted by the same research group in the UK (Merga *et al.*, 2011 and 2013). In one of them, only 39 isolates, randomly selected from a total of 1260 recovered from sheep faeces and cattle faeces, were genotyped obtaining 11 different ST (Merga *et al.*, 2011). The allelic density observed ranged from between 15% (*atpA*) to 28.2% (*glyA*), and was 17.9% for the *glnA* gene. None of the obtained STs matched those available in the database (Merga *et al.*, 2011). In another study by same research group (Merga *et al.*, 2013), 104 isolates of *A. butzleri* recovered from cattle faeces were genotyped but none of the 250 *A. skirrowii* or 160 *A. cryaerophilus* could be included in the analysis due to the poor quality of sequences. The authors suggested that these results were probably due to the presence of isolates belonging to species not included in the MLST scheme, because the strains were identified using an multiplex PCR (m-PCR) method designed only to identify *A. butzleri, A. cryaerophilus* and *A. skirrowii* (Houf *et al.,* 2000). Among the 104 *A. butzleri* isolates genotyped, Merga *et al.* (2013) obtained 43 different STs; 41 of them were new at the time of

publication while the other two (ST 18 and ST 308) were already included in the *Arcobacter* MLST database. ST18 had been isolated in 2001 from turkey in Denmark and was the only ST found on different occasions and from more than one farm, while the existing ST308 corresponded to an isolate recovered from cattle faeces (Merga *et al.,* 2011). The high diversity of STs found in the studies of Merga *et al*. (2011 and 2013) corroborates the observations in Miller *et al.* (2009), who found that most alleles were infrequent and not related. This broad diversity has been reported in most studies on different kinds of samples and has been attributed to multiple sources of contamination (Aydin *et al.*, 2006) or to the putative ability of the isolates to incorporate exogenous DNA or to undergo genomic rearrangement by multiple recombination, as previously described in *Campylobacter jejuni* (Hume *et al*., 2001). Despite the advantages of MLST, such as its reproducibility and the creation of databases, it also has some limitations. For instance, it has only been possible to obtain sequences of some isolates, belonging mainly to *A. butzleri* (Merga *et al*., 2011). By contrast, the ERIC-PCR has proven useful for all species of the genus and is faster and easier to use. It is a good alternative for an initial screening of redundant clones among isolates.

1.2 Clinical and veterinary importance of *Arcobacter*

1.2.1 Incidence in humans

The true pathogenic role of *Arcobacter* in humans has not yet been clarified, although some species, mainly *A. butzleri* and *A. cryaerophilus*, have been associated with gastrointestinal diseases on several occasions, persistent diarrhoea being the main symptom (Collado & Figueras, 2011).

Since the last review (Collado & Figueras, 2011), several new studies have been conducted in different countries, i.e. New Zealand, Turkey, Chile and The Netherlands, in order to determine the presence of *Arcobacter* in faeces of patients with diarrhoea (Mandisodza *et al.*, 2012; Kayman *et al.*, 2012b; Collado *et al.*, 2013; de Boer *et al.*, 2013). In New Zealand, *A. butzleri* or *A. cryaerophilus* were isolated by culturing in 1% of 1,380 samples from faeces of patients with diarrhoea (Mandisodza *et al.*, 2012) and in Turkey, *A. butzleri* was isolated in 1.25% of 3287 samples (Kayman *et al.*, 2012b). The latter species was also recovered in Chile, with an overall detection of 0.7% from the 140 samples analysed by culturing or by a molecular detection method specific for the genus *Arcobacter* (Collado *et al.*, 2013). In The Netherlands, *A. butzleri* was detected in 0.4% of 493 samples (de Boer *et al.*, 2013) using a multiplex Real Time PCR (RT-PCR) designed to detect the latter species and several *Campylobacter* spp. from human faeces.

A recent study reports a new case of diarrhoea by *A. butzleri* in a 30-year-old male patient who was admitted to the hospital complaining of acute abdominal pain, diarrhoea and

nausea (Kayman *et al.*, 2012a). The patient had no other signs or symptoms of disease, but the stool sample collected was watery with no blood or mucus. The isolate was resistant to ampicillin, cefuroxime and clindamycin and the symptoms disappeared within 2 days of initiation of the treatment with ciprofloxacine. The source of the *Arcobacter* infection was thought to be improperly cooked chicken (on a barbeque) eaten the day before the onset of the diarrhoea (Kayman *et al.*, 2012a). In another recent study, *A. butzleri* is reported responsible for an outbreak of diarrhoea among the guests of a wedding in Wisconsin (USA). The species was detected in the analysed faeces by molecular methods, despite not being recovered by culturing (Lappi *et al.*, 2013). The 51 guests affected presented mainly diarrhoea with abdominal cramps, fatigue, nausea, chills, body/muscle aches, and headache. A case-control study of ill and healthy individuals was carried out in order to identify common factors associated with the outbreak, such as consumption of food or beverage etc., and the only significant correlation was made with the roast chicken, despite its microbiological analysis being negative (Lappi *et al.*, 2013).

It has been suggested that the significance of *Arcobacter* in human infections may be underestimated due to inappropriate detection and identification methods that have been used so far (Collado & Figueras., 2011). This seems to be supported by the results of the prospective studies mentioned above (Mandisodza *et al.*, 2012; Kayman *et al.*, 2012b; Collado *et al.*, 2013; de Boer *et al.*, 2013). In other studies, detection by molecular methods has shown an incidence of *Arcobacter* ranging between 1.4% (Collado *et al.*, 2013) and 10.9% (Samie *et al.*, 2007). Two recent studies included detection using culturing and a molecular method in parallel. One study from Chile reports 0.7% of the samples positive by culturing, and 1.4% by molecular detection (Collado *et al.*, 2013) while the other from The Netherlands reports 0% and 0.4%, respectively (de Boer *et al.*, 2013). A clear relationship between the prevalence of *Arcobacter* and the age or sex of patients had so far not been established. A higher prevalence in diabetic type 2 patients, than in non-diabetic individuals was found by Fera *et al.* (2010) in Italy. However, in two studies performed in South Africa and India that investigate HIV patients no statistical difference between the *Arcobacter* prevalence and the HIV-status was detected (Samie *et al.,* 2007; Kownhar *et al.,* 2007). Furthermore, despite *Arcobacter* spp. having a similar prevalence in countries with different levels of public health development, i.e. South Africa, Belgium, France, Turkey, New Zealand, Chile and The Netherlands (Vandenberg *et al.*, 2004; Prouzet-Mauléon *et al.*, 2006; Samie *et al.*, 2007; Mandisodza *et al.*, 2012; Kayman *et al.*, 2012b; Collado *et al.*, 2013; de Boer *et al.*, 2013), they have also been implicated in traveller's diarrhoea (Jiang *et al.*, 2010). Incidence in European and USA travellers to countries like Mexico, Guatemala and India has been established at 16% using molecular methods (Jiang *et al.,* 2010).

In relation to asymptomatic carriage of *Arcobacter*, one survey conducted in Switzerland investigated asymptomatic workers in a slaughterhouse (Houf & Stephan, 2007). The presence of these bacteria was found in 1.4% of the faeces studied. All the isolated strains were identified as *A. cryaerophilus* and showed an adhesion capacity to Caco-2 cells. The study pointed out that *A. butzleri* was not isolated from healthy humans and is therefore justified as a potential emerging pathogen (Houf & Stephan, 2007). Another survey in Italy using only a molecular detection method found 78.9% asymptomatic carriage of *Arcobacter* in diabetic type 2 (DMT2) patients versus 26.2% in non-diabetics (Fera *et al.*, 2010). The DMT2 was considered the only factor linked to the *Arcobacter* colonization, although the clinical significance of the results still needs to be determined (Fera *et al*., 2010).

Most of the reported cases of extra intestinal presentation involved bacteraemia and occurred in immunocompromised patients or those with indwelling devices (Collado & Figueras, 2011). However, a case of peritonitis, recently reported in a 63-year-old woman from Hong Kong, occurred the day after repositioning a peritoneal dialysis (PD) catheter (Yap *et al.*, 2013). The patient suffered fever, abdominal pain and turbid PD effluent from which *Arcobacter* was isolated 5 days later. The prophylactic treatment initiated before this procedure (intravenous (IV) cefazolin and oral levofloxacin) had to be switched to IV ticarcillin-clavulanate for 2 weeks. The treatment appeared to be adequate and allowed the PD catheter to be preserved.

1.2.2 Incidence in animals

In animals, *Arcobacter* spp. have been linked with abortions, mastitis and diarrhoea, and *A. butzleri, A. cryaerophilus*, *A. skirrowii* and *A. thereius* were the predominant recovered species in these cases (Collado & Figueras, 2011 and references therein). These species have also been recovered from healthy animals that could act as a reservoir of these bacteria (Collado & Figueras, 2011 and references therein). So far, *Arcobacter* spp. have been recovered from aborted foetuses and bovine, porcine and ovine placentas (Ho *et al.*, 2006a). The pathological signs observed included infertility, chronic discharge during oestrus, chronic stillborn problems and late-term abortions (Ho *et al.*, 2006a and references therein). Venereal transmission is suggested as the source of infection because *A. butzleri* and *A. cryaerophilus* were recovered from preputial fluid of bulls and *A. skirrowii* from preputial fluid of boars and fattening pigs (Ho *et al.*, 2006a and references therein). The species *A. cryaerophilus* has also been linked to an outbreak of mastitis in a dairy herd (Logan *et al.*, 1982). In fact, when four cows were infected experimentally by intramammary inoculation with the outbreak strain, all of them developed an acute clinical mastitis that resolved itself after 5 days (Logan *et al.*, 1982). On the other hand, *A. butzleri* has been associated with enteritis and diarrhoea in pigs, cattle, and horses, whereas *A.skirrowii* with

diarrhoea in sheep and cattle (Collado & Figueras, 2011 and references therein). Furthermore, *Arcobacter* spp., mainly *A. butzleri*, has been isolated from diseased exotic animals, such as rhesus macaque (*Macaca mulata*), with diarrhoea, vicuña (*Vicugna vicugna*) and equines (*Equus* sp.) with foetus abortion, rhea (*Rhea* sp.) with hepatitis, white rhinoceros (*Ceratotherium simum*) with urinary colic, and dog (*Canis lupus familiaris*) and cat (*Felis catus*) with oral disease (Wesley & Miller, 2010 and references therein). Despite most clinical cases affecting mammals, *A. cryaerophilus* was isolated on one occasion from a diseased fish, i.e. rainbow trout (*Oncorhynchus mykiss*), which suffered extensive damage of the liver, kidney, and intestine (Yildiz *et al.*, 2006). Using the analysis of 16S rRNA gene by Denaturing Gradient Gel Electrophoresis PCR (DGGE-PCR) and clone libraries methods, *Arcobacter* was recently isolated from white syndrome and brown band disease of reef-building corals (Sweet & Bythell, 2012). The relative abundance of *Arcobacter* in the latter study increased substantially in all diseased samples compared to the healthy ones.

It has been suggested that poultry, i.e., chicken, ducks, turkeys, and domestic geese might be a natural reservoir for *Arcobacter* species because there has largely been faecal shedding but no reports of any associated disease in those animals (Collado & Figueras, 2011 and references therein). Other animals considered to be important hosts and reservoirs of *Arcobacter* species are pigs, cattle, sheep, and horses, as well as pets such dogs and cats (Shah *et al.*, 2011). There are also some reports of *Arcobacter* carriage in wild and non-domesticated animals such as silvery gibbon (*Hylobates moloch*), black rhinoceros (*Diceros bicornis*), western gorilla (*Troglodytes gorilla*) and racoon (*Procyon lotor*) (Wesley & Miller, 2010).

1.3 Virulence, adhesion and invasion

The pathogenicity and virulence mechanisms of *Arcobacter* species are still not very well understood. Several studies have assessed the interaction of the species *A. butzleri, A. cryaerophilus* with different cell lines, such as Hep-2, HeLa, INT 407, CHO, Caco-2, IPI 2I and Vero (Collado & Figueras, 2011 and references therein) and among the studied *Arcobacter* strains have been observed adhesion (56%), invasion (20%) and cytotoxicity (85%) to eukaryotic cells. In a study that has assessed the cytotoxicity of the two above mentioned species and *A. skirrowii* against Vero cells (Villarruel-Lopez *et al.*, 2003), 38% of strains produced cytotoxic effects, 18% vacuolization, 39% both effects and only 6% produced no effects. Another study assessed the ability of strains belonging to *A. butzleri, A. cryaerophilus, A. skirrowii* and *A. cibarius,* to adhere, invade and induce interleukin-8 expression in human Caco-2 cells (Ho *et al.*, 2007). In that study, all strains adhered but only those of *A. cryaerophilus* invaded, whereas all strains induced IL-8 production by eukaryotic cells but there was no correlation with the adhesion or invasion behaviour of the strains (Ho

et al., 2007). However, Bücker *et al.* (2008) demonstrated that strains of *A. butzleri* are able to produce a leak flux type of diarrhoea. The differing results of the studies could be due to the different origins of strains and to the different cell lines used (Collado & Figueras, 2011 and references therein).

Several strains of the species *A. butzleri, A. cryaerophilus* and *A. skirrowii* isolated from humans and animals (chicken, pig, cattle, sheep, horse and dog) were recently evaluated using PCR for the presence of nine putative virulence genes (Douidah *et al.,* 2012). The primers were designed to target nine genes, i.e. *cadF*, *ciaB*, *cj1349*, *hecA*, *hecB*, *irgA*, *mviN*, *pldA*, *tlyA* (Table 1.4), from sequences present in the genome of *A. butzleri* strain RM 4018 (Miller *et al*., 2007). All nine genes were detected in 14% of the strains of *A. butzleri* but none of the other species possessed them all (Douidah *et al.,* 2012). Genes *cadF*, *ciaB*, *cj1349*, *mviN*, *pldA*, and *tlyA* were all detected in all *A. butzleri* strains and in 16% to 97% of the strains of *A. cryaerophilus* and *A. skirrowii* (Douidah *et al.,* 2012). Furthermore, the *irgA* gene was detected in 29.7% of strains of *A. butzleri* and only in 3% of *A. cryaerophilus*. The authors considered that the different pathogenic behaviour among species and the greater heterogeneity of their genomes were reasons that might explain the results (Douidah *et al*., 2012). The distribution of genes shows no correlation with the host they were recovered from, hampering the potential use of these genes for identifying human infectious sources (Douidah *et al*., 2012). Despite that, the study did validate a rapid and accurate PCR approach for detecting putative virulence genes in three *Arcobacter* spp., which needs further evaluation for all *Arcobacter* species. More recently, Karadas *et al*. (2013) also determined the presence of these genes by PCR in 52 strains of *A. butzleri* and studied in only six of them the capacity for adhesion and invasion to HT-29 and Caco-2 cells. However, no correlation between the virulence genes detected and the observed adhesive or invasive characteristics could be established. The authors indicated that this could be due to the few isolates compared and suggested that more strains needed to be tested (Karadas *et al*., 2013).

1.4 Antibiotic resistance

As mentioned above the main clinical presentation of *Arcobacter* infections are enteritis and bacteraemia. Both of these infections can be self-limiting and might not require antimicrobial treatment although the severity or prolongation of symptoms may justify it (Collado & Figueras, 2011 and references therein). There are no established criteria for susceptibility testing of *Arcobacter* species. Instead, the commonly used breakpoints (Mandisodza *et al.,* 2012; Kayman *et al*., 2012b; Shah *et al*., 2012b) are those defined either by the Clinical Laboratory and Standards Institute (CLSI, M45-A; CLSI, 2006) for erythromycin, tetracycline and ciprofloxacin in *C. jejuni* and *C. coli* or by the National

Antimicrobial Resistance Monitoring System for *Campylobacter* sp. (NARMS, CDC, 2006). The latter criteria are followed not only for the mentioned antimicrobials but also for nalidixic acid, gentamicin, clindamycin, chloramphenicol aminoglicosides and lincosamines. In relation to ampicillin and other antimicrobials not included in the mentioned documents, the breakpoints used are those established for *Enterobacteriaceae* by the CLSI (M100–S20; CLSI, 2010) (Mandisodza *et al.,* 2012; Kayman *et al.*, 2012b; Shah *et al.*, 2012b).

Fluoroquinolones and tetracycline have been suggested as the treatment of choice, despite being resistant to nalidixic acid and ciprofloxacin (Collado & Figueras, 2011 and references therein). A recent study that investigated the Minimal Inhibitory Concentration (MIC) of 43 *A. butzleri* strains recovered from neck skin, cecal content, carcasses of poultry and water of drainage in Portugal (Ferreira *et al*., 2013) showed resistance in 55.8% of strains for ciprofloxacine as well as in 97.7% to 100% of strains for ampicillin, amoxicillin, vancomycin, trimethoprim, piperacillin, and cefoperazone. At the same time, the only effective antibiotics were gentamicin and cloramphenicol. Different results were reported in a recent study that evaluated the resistance to antibiotics of several strains recovered from cattle, beef, milk and water using a disk diffusion method and determining the MIC by serial dilution (Shah *et al.*, 2012b). Only 6.5% of the tested strains showed resistance to tetracycline, 21.7% to ciprofloxacine and 26.1% to gentamicin. However, more strains showed resistance to erythromycin (69.6%), cefotaxime (69.6%) and ampicillin (73.9%, Shah *et al.*, 2012b). When considering the results obtained for clinical strains using different methods (Kayman *et al.*, 2012b and references therein; Mandisodza *et al.*, 2012), most isolates showed susceptiblity to ciprofloxacin, gentamicin and tetracycline and resistance to ampicillin, while erythromycin and nalidixic acid have given variable results depending on the method used. Resistance to quinolones has been linked to the use of this kind of antibiotic in livestock for preventing infections (Kayman *et al.,* 2012b). Regarding that, Sigala *et al.* (2013) isolated resistant bacteria from residential, hospital and industrial wastewater using media supplemented with antibiotics in levels above the epidemiological cut-off values, i.e. cefaclor (16 µg mL^{-1}), ciprofloxacin (8 µg mL^{-1}), doxycycline (16 µg mL^{-1}), or erythromycin (64 µg mL^{-1}). Then, they pyrosequenced the hypervariable regions (V1 through V3) of the 16S rRNA gene from the isolates and carried out a phylogenetic analysis of the sequences. They report the separation of the strains of all origins into two clusters, those resistant to doxycycline (a tetracycline) and those resistant to ciprofloxacin (a quinolone), and they also reported that the predominant bacteria in those clusters were *E. coli* and *Arcobacter* sp., respectively (Sigala *et al.*, 2013). They further indicated that it would be worthwhile to determine which resistance mechanisms are favoured in wastewater considering the different targets of the antibiotics tested (Sigala *et al.,* 2013). On that point, a mutation in the quinolones resistance-determining region of the *gyrA* gene has been shown to produce high levels of resistance in

A. butzleri and *A. cryaerophilus* (Collado & Figueras, 2011 and references therein). The latter mutation has been observed in the complete genome of *A. butzleri* RM4018, as well as other chromosomal mediated resistances due to the presence or absence of previously characterized specific genes (Miller *et al.*, 2007). For instance, it possesses the *cat* gene, encoding a chloramphenicol O-acetyltransferase, three putative β-lactamase genes associated with β-lactam resistance, and a mutation in the *upp* gene, which leads to an increased resistance to 5-fluorouracil (Table 1.4). However, these features have not been analysed for the other 3 complete genomes currently available.

1.5 Animal models

Experimental infections with *Arcobacter* have been carried out with various types of animals, such as chickens, turkeys, poults, piglets and rainbow trout (Wesley & Miller, 2010 and references therein). The recovery of animals infected experimentally varies according to the host (age of the animal, breed) and on the challenge strain (Wesley & Miller, 2010 and references therein). In one study, Beltsville white turkeys were the most suitable animal model for reproducing the diarrhoea infection by *A. butzleri in* comparison to outbreed chicken and turkeys (Collado & Figueras, 2011 and references therein). Results from experimental infections indicate that some strains of the tested species, mainly *A. butzleri*, could indeed be enteropathogens (Collado & Figueras, 2011 and references therein). Koch's classical postulates were partially fulfilled because the same challenge microbe able to cause diseases was recovered when using Beltsville white turkey and pig as animal models (Collado & Figueras, 2011 and references therein). Recently, an experimental infection with two *A. butzleri* strains recovered from the stool of healthy chicken was carried out in albino rats (Adesiji *et al.*, 2009). The studied strains produced histopathological changes in the gut of the rats (toxic ileitis) and hepatic necrosis was observed, probably produced by the diffusion of toxins from the ileum. On that point, Adesiji *et al.* (2009) suggested that these findings could be linked to persistent watery diarrhoea, which is the clinical presentation of *Arcobacter* infection in humans. The same research group recently studied the serum biochemistry and the haematological changes in rats challenged with graded doses of *Arcobacter* (Adesiji *et al.*, 2012). The challenge strains belonged to *A. butzleri* and *A. cryaerophilus* and were isolated from the caecal content of healthy chicken. They observed that the development of the diarrhoea was linked to an electrolyte imbalance and a rise in the platelets, neutrophils and lymphocyte values. The authors suggest that these haematological parameters might be good diagnostic indicators of the animal response to *Arcobacter* infections (Adesiji *et al.*, 2012).

Table 1.4 Genes present in strain *Arcobacter butzleri* RM4018[a] associated with virulence and antibiotic resistance

Gene	Homologous virulence	Assigned function of codified protein	Homologous gene in other species
ab0483	*cadF*	Fibronectin binding protein	*Campylobacter jejuni*
ab1555	*ciaB*	Invasin protein	*Campylobacter jejuni*
ab0070	*cj1349*	Fibronectin binding protein	*Campylobacter jejuni*
ab0941	*hecA*	Filamentous hemagglutinin	*Burkholderia cepacia, Acinetobacter* spp. and *E. coli*
ab0940	*hecB*	Related hemolysin activation protein	*Burkholderia cepacia, Acinetobacter* spp. and *E. coli*
ab0729	*irgA*	Iron-regulated outer membrane protein	*Vibrio cholerae* / *Eschericia coli*
ab0876	*mviN*	Siderophore	*Campylobacter jejuni*
ab0859	*pldA*	Phospholipase	*Campylobacter jejuni*
ab1846	*tlyA*	Haemolysin	*Campylobacter jejuni*
ab0730	*iroE*	Siderophore esterase	*Escherichia coli*
	Homologous resistance		**Resistant to:**
ab0785	*cat*	cloramphenicol O-acetyltranferase	Cloramphenicol
ab0578	*β-lactamase*	β-lactamase	β-lactam
ab1306	*β-lactamase*	β-lactamase	β-lactam
ab1486	*β-lactamase*	β-lactamase	β-lactam
ab1907	*upp* mutation	Uracil phosphoribosyltransferase	5-fluorouracyl
ab1799	*gyrA mutation*	DNA gyrase A	Fluorquinolones

[a]Data obtained from Miller *et al*. (2007).

1.6 Transmission routes

It has been suggested that *Arcobacter* spp. are potential food and waterborne pathogens, because these bacteria are present in different types of water and foods of animal origin (Collado & Figueras, 2011 and references therein). However, the epidemiological relationship among the isolates recovered from faeces of patients with diarrhoea and those isolated from water and food has not so far been demonstrated (Collado & Figueras, 2011).

1.6.1 Contaminated water

Up to now, 3 drinking water outbreaks have been linked to *Arcobacter.* Two of them were in USA, one on a summer camp site in Idaho (Rice *et al.,* 1999), and the other on South Bass Island, Lake Erie, Ohio (Fong *et al.*, 2007). The third case occurred in Slovenia (Kopilovic *et al.*, 2008). In all of the cases, the drinking water was faecally contaminated and although *Arcobacter* was recovered from the water and/or faeces it could not be totally proven that *Arcobacter* was the etiological agent (Collado & Figueras, 2011). It has been demonstrated in other studies that the presence of *Arcobacter* in water increases with the levels of faecal pollution (Collado *et al.*, 2008) and that experimentally these bacteria can survive for a long time in water under laboratory regulated conditions of temperature and presence of organic matter (Van Driessche & Houf, 2008a). For instance, survival has been recorded for at least 250 days at 4ºC with 1% of organic material consisting of a mixture of equal volumes of sterile horse blood and cow's milk. In addition, *Arcobacter* might have the ability to become viable but non-culturable (Collado & Figueras, 2011 and references therein). On the other hand, it has also been demonstrated that the water treatments used for producing drinking water are effective in eliminating *Arcobacter* (Collado *et al.*, 2010) but, despite that, recent studies have recovered *A. butzleri* and/or *A. skirrowii* from 3% (3/100) of drinking water samples in Turkey (Ertas *et al*., 2010) and from 11.1% (2/18) of those in Malaysia (Shah *et al*., 2012a). This is thought to be linked to the improper chlorination of water or perhaps to its ability to adhere to the pipes and to form biofilms (Assanta *et al*., 2002; Shah *et al*., 2012a). One of the problems reported from the above-mentioned summer camp outbreak was, in fact, the failure of the chlorination system (Rice *et al.,* 1999).

Arcobacter spp. have shown a high prevalence in sewage and sludge from WWTPs, being isolated in 40% to 100% of the samples at different studies (Stampi *et al.,* 1993 and 1999; Moreno *et al.*, 2003; González *et al.*, 2007 and 2010; Collado *et al.*, 2008 and 2010). In three of those studies, *Arcobacter* were detected at different sampling points in a WWTP, including the water from the post-treated outflow (Stampi *et al.,* 1993 and 1999; Moreno *et al.*, 2003). However, it is not clear if the presence of *Arcobacter* in the wastewater is due to the faecal contamination or if these microorganisms are residents growing in the sewer systems (Collado & Figueras, 2011 and references therein). In this regard, in a recent pyrosequencing study that compared the bacterial communities present at two WWTPs from Milwaukee (USA) with the ones of the human population and surface waters, they found only few *Arcobacter* sequences in the human and surface waters samples, but many sequences belonging to this taxon in the wastewater (McLellan *et al.*, 2010). A later study at the same WWTPs showed that *Arcobacter* prevalence correlated positively with the levels of ammonia, phosphorous and suspended solids

in water (VandeWalle *et al.*, 2012). Considering these results, *Arcobacter* was selected as a "sewer signature" microbe, together with *Acinetobacter* and *Trichococcus* (the most common taxa in sewage), used for detecting sewage contamination of surface waters (Newton *et al.*, 2013). More studies are needed that will determine which *Arcobacter* species the obtained 16S rRNA sequences belong to, and that will compare the dominant species recovered by conventional culturing methods at the WWTPs with those detected by pyrosequencing.

1.6.2 Contaminated food

Arcobacter spp. have been found to contaminate different types of food of animal origin, including milk, with an incidence in raw cattle milk ranging from 3.2% to 46.0% of the samples (Ertas *et al*., 2010; Shah *et al*., 2011 and 2012a). Furthermore, in a recent study of 13 cow farms and 1 dairy farm authorized to sell raw milk in Bologna, Italy (Serraino *et al*., 2013), *Arcobacter* was found at 7 cow farms and a water buffalo farm. Many studies have also investigated meat products, i.e. poultry meat, with incidence ranging from 20% to 73%, pork meat, from 0.5% to 55.8%, beef meat, from 1.5% to 55.6% and rabbit meat in 10% (Collado *et al*., 2009b; Shah *et al*., 2011; Vytrasová *et al*., 2003). It has been suggested that contamination of meat products occurs during the slaughtering process (Collado & Figueras, 2011 and references therein). For instance, De Smet *et al*. (2010) investigated the *Arcobacter* contamination on bovine carcasses in two slaughterhouses and found the same *A. butzleri* strain (as determined by ERIC-PCR) from both the chest and foreleg of two carcasses of animals coming from different farms but slaughtered on the same day in the same slaughterhouse. Similar results were observed in a study conducted in Portuguese slaughterhouses belonging to 3 different flocks (Ferreira *et al*., 2013). In that study, the same strain (determined by Pulse Field Gel electrophoresis, PFGE) was isolated from samples collected from different areas. Moreover, most of the strains (72.2%) showed the ability to form biofilms and had a high degree of resistance to several antibiotics (Ferreira *et al*., 2013). This data contributes to an understanding of the persistence mechanism of *Arcobacter* in the environment in the food chain (Ferreira *et al*., 2013).

The prevalence of *Arcobacter* in shellfish has shown to be relatively high i.e. 100% (5/5) in clams and 41.1% (23/56) in mussels (Collado *et al*., 2009b). Moreover, a wide variety of species, i.e. *A. butzleri, A. cryaerophilus, A. skirrowii, A. mytili, A. nitrofigilis* and a potential new species like *A. molluscorum* and strain F4 have all been identified (Collado *et al*., 2009b; Figueras *et al*., 2011b). As a result, it was suggested that shellfish should be considered another source of infection because they have an ability to concentrate bacterial pathogens from water and are often eaten poorly cooked or raw (Collado *et al*., 2009b). However, only a few studies have assessed the prevalence of *Arcobacter* in this kind of food (Collado & Figueras, 2011).

Recent studies have reported the presence of *Arcobacter* in vegetables. In one study, 15 lettuces from 7 retail shops were studied by RT-PCR and culturing methods (González & Ferrus, 2010). Only the lettuces obtained from one of the retail shops were positive and they probably became contaminated during manipulation during retail rather than in the field (González & Ferrus, 2010). Hausdorf *et al*. (2011) used sequences of the 16S rRNA gene to investigate the bacterial communities present in the wash-water from a carrot processing facility. *Arcobacter* was the fourth most prevalent genus, representing 9% of all clones obtained. It was suggested therefore that water or soil might be the source of contamination. Considering that vegetables are normally eaten uncooked, and the fact that the risk of contamination of vegetables is high, more studies including more samples and different vegetables are warranted.

It has been suggested that *Arcobacter* spp. can survive in food because they can tolerate high sodium chloride concentrations, desiccation, can grow at lower refrigeration temperatures and have the ability to attach to various types of surfaces (Collado & Figueras, 2011 and references therein). D'Sa & Harrison (2005), observed that a combined treatment with heat at 50°C and then a cold shock at 4º or 8°C reduced the number of *Arcobacter* cells more than using only a heat treatment at 50°C or a cold shock at 12°C or 16°C, separately. De Smet *et al*. (2010) assessed the effect of forced air-cooling on the *Arcobacter* contamination of bovine carcasses and ready to eat minced beef after cooling in two slaughterhouses. They report that when cooling reached a temperature of 7ºC and was maintained for at least 24 h, the number of arcobacters decreased significantly but it did not completely eliminate them. Isohani *et al*. (2013) investigated the ability of the type strain of *A. butzleri* (ATCC 49616^T) to tolerate what were considered sub-lethal stress temperatures, i.e. 48ºC and 10ºC, and mild and lethal acid conditions, i.e. pH 4.0 and pH 5.0, respectively. They observed that when this strain was exposed to 48ºC for 2 h it was more tolerant to acid stress (pH 4.0) than before being exposed to that high temperature. The study concluded that this ability to tolerate lethal acid conditions after being exposed to a high temperature needs to be taken into account when designing new food decontamination and processing strategies for these bacteria (Isohani *et al*., 2013).

1.7 Molecular detection and isolation of *Arcobacter*

1.7.1 Direct molecular detection

Several molecular detection methods have been developed in order to improve sensitivity and to reduce the time required in conventional culturing methods (Collado & Figueras, 2011 and references therein). There are some genus-specific PCR assays targeting the 16S rRNA (Harmon & Wesley, 1997), or the 23S rRNA genes (Bastyns *et al.*, 1995), but false negative results have been reported (Collado & Figueras, 2011). For detection at the species level, the

most commonly used method, designed by Houf *et al.* (2000), targets in a m-PCR the 16S rRNA gene of the species *A. butzleri* and *A. skirrowii* and the 23S rRNA gene of *A. cryaerophilus* (Collado & Figueras, 2011 and references therein). However, the method can confuse non-targeted species with the targeted ones, i.e. *A. nitrofigilis* and *A. mytili* with *A. skirrowii* (Collado *et al*., 2009a; Collado & Figueras, 2011) and with *A. cryaerophilus* the species *A. thereius, A. defluvii* and *A. molluscorum* (Collado *et al*., 2008; Douidah *et al*., 2010). Other methods are RT-PCRs that show better sensitivity than the m-PCR and that have provided a 2-log-unit improvement in sensitivity over conventional PCRs (Collado & Figueras, 2011 and references therein). A multiplex RT-PCR has recently been developed that can detect *A. butzleri* and campylobacters from faeces of patients with diarrhoea (de Boer *et al*., 2013). The targeted genes used in that study were the *hsp60* for *A. butzleri* and the 16S rRNA gene for *Campylobacter* sp. and for the species *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. hyointestinalis*. De Boer *et al*. (2013) determined that the sensitivity of the RT-PCR for the detection of *A. butzleri* was 10^3 CFU g^{-1} faeces, which is similar to that described for the above-mentioned m-PCR of Houf *et al*. (2000). Despite de Boer *et al*. (2013) culturing all the RT-PCR positive samples, *A. butzleri* was not recovered. The prevalence found of the latter species in the RT-PCR positive samples was 0.4%, which was identical to that for *C. coli* and *C. upsaliensis* (de Boer *et al*., 2013). On that point, Collado *et al*. (2013) have also found a higher incidence of arcobacters in faeces of patients with diarrhoea using molecular methods (1.4%) than by culturing methods (0.7%). These results demonstrate the importance of routinely screening stool samples using molecular and culturing methods in parallel in order to determine the epidemiological importance of this bacteria (Collado *et al*., 2013; de Boer *et al.*, 2013).

1.7.2 Isolation by culturing methods

Methods for isolation and detection of *Arcobacter* are still not standardized; therefore comparing results reported by different authors is difficult. Collado & Figueras (2011) indicated that the most commonly used isolation protocols are the following:

i) using an enrichment broth supplemented with Cefoperazone, Amphotericin B and Teicoplanin (CAT), followed by passive filtration of the broth through a 0.45 µm filter placed over blood agar (Atabay & Corry, 1998) and ii) incorporating amphotericin B, cefoperazone, 5-fluorouracil, novobiocin and trimethoprim in both the enrichment and the plating medium (Houf *et al.,* 2001), which was originally proposed for poultry meat after an antimicrobial *Arcobacter* susceptibility study and was also validated later for faecal specimens (Houf & Stephan, 2007). However, there is still a lack of consensus about which is the most useful (Collado & Figueras, 2011).

Recently, Merga *et al.* (2011) compared the isolation of *Arcobacter* from animal faeces using different combinations of two enrichment and 3 different plating media. The tested enrichment broths were the Arcobacter-broth (Oxoid) supplemented either with i) CAT (Arcobacter-CAT broth, Atabay & Corry, 1998) or ii) with the 5 antibiotics proposed by Houf *et al.* (2001), and the plating media were i) the one proposed by Houf *et al*. (2001), ii) the modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) supplemented with CAT or iii) the latter medium but supplemented with cefoperazone and Amphotericin B, as recommended for *Campylobacter* (Kemp *et al*., 2005). It was reported that the combination of the enrichment proposed by Houf *et al*. (2001) followed by plating in mCCDA medium supplemented with CAT performed significantly better than the other combinations (Merga *et al.*, 2011). However, the Houf *et al.* (2001) protocol was not compared to the one of Atabay & Corry (1998). In another study, Shah *et al*. (2011) compared the recovery of *Arcobacter* from milk, beef and rectal swabs of cattle using two combinations of enrichment and plating: i) enrichment in Arcobacter-CAT broth followed by passive filtration on blood agar (Atabay & Corry, 1998) and ii) enrichment in what was named Arcobacter-Selective Broth (ASB, based on Brucella broth supplemented with horse blood plus piperacillin, cefoperazone, trimethoprim and cycloheximide) followed by plating on Arcobacter- Selective Medium (ASM, with the same composition but containing agar rather than horse blood; de Boer *et al.,* 1996). Unfortunately, these authors did not include the protocol of Houf *et al*. (2001). The Atabay & Corry (1998) protocol performed the best, with 100% sensitivity although with only 34% specificity. The authors reported that this low specificity was mainly due to rapidly growing competitive bacteria such as *Pseudomonas* and *Proteus* (Shah *et al*., 2011).

There is evidence that the enrichment step enhances isolation of the *Arcobacter* species that are more resistant to the antibiotics used in the broth or those that grow faster (Collado & Figueras, 2011 and references therein). For instance, Houf *et al.* (2002) studied the genetic diversity among arcobacters isolated from poultry products, comparing the recovery after direct plating and post enrichment, and important differences were observed. .*A. cryaerophilus* was isolated in 45.8% of samples by direct plating and in only 8.3% after enrichment, while the other isolated species, *A. butzleri*, showed the inverse behaviour, i.e. 33.3% and 75%, respectively (Houf *et al*., 2002). The authors recommended isolation using both direct plating and post enrichment in parallel in order to increase the diversity of arcobacters. Similar results were found by De Smet *et al.* (2011b) from pig faeces, in which direct plating recovered more isolates than post enrichment for the species *A. thereius* (122 and 16, respectively) and *A. skirrowii* (37 and 2, respectively) despite the same number of isolates of *A. cryaerophilus* being obtained with each method. In the same study, however, the inverse results were obtained for the species *A.*

butzleri (89 and 190, respectively) and *A. trophiarum* (4 and 12, respectively). As commented, it has been hypothesized that this might be due to enrichment favouring the faster growing species (Collado & Figueras, 2011 and references therein) but this has not been demonstrated experimentally yet.

The prevalence of the *Arcobacter* species in different matrices, which have been established by direct detection from enrichment using molecular methods, might also be over-estimated. Ho *et al*. (2006b) studied the sensitivity of the m-PCR of Houf *et al.* (2000) for the simultaneous detection of the targeted species (*A. butzleri, A. cryaerophilus* and *A. skirrowii*) from mixtures containing them in different proportions. They report that when the three species were combined in equal amounts, all of them were detected but when different proportions were used, amplification favoured those species present in higher numbers.

Culturing conditions such as time of incubation or the atmosphere could also affect the isolation of *Arcobacter*. For instance, enrichment is usually incubated from 48 h to 72 h and when a shorter incubation period (< 48 h) has been used there has been less isolation (Collado & Figueras, 2011). Regarding the atmosphere of incubation, it is noteworthy that about half of the published studies incubate the samples under microaerobic conditions while the other half use aerobiosis (Collado & Figueras, 2011 and references therein). In fact, some authors indicate that, in general, arcobacters grow better under microaerobic conditions (Vandamme *et al.*, 2005; Ho *et al.*, 2006a). However, only one study has so far assessed the effect of atmosphere (aerobiosis or microaerphilia) on *Arcobacter* isolation. González *et al.* (2007) tested 20 samples from chicken carcasses and found that 7 were positive, 3 of them under aerobic and microaerobic conditions simultaneously, another 3 only in microaerophilia and the other sample only in aerobiosis. Despite there being a slightly higher recovery under microaerobic conditions, the results were not statistically significant (González *et al*., 2007). Additional studies are required that evaluate the optimal conditions of incubation for the recovery of arcobacters.

1.8 Identification of the *Arcobacter* species

Limitations in the identification methods that are currently used to recognize or identify all species correctly might be the main pitfall to establishing the true prevalence of *Arcobacter* spp. in different matrices i.e. water, food, faeces, etc. (Ho *et al*., 2006a; Collado & Figueras, 2011). As commented before, due to their metabolic characteristics, *Arcobacter* spp. are difficult to differentiate from *Campylobacter* spp. using phenotypic testing (Collado & Figueras, 2011). Nevertheless, *Arcobacter* spp. are distinguished by growing at a lower temperature and under aerobic conditions. Table 1.3 lists the most useful tests that differentiate between all species, although considering the imprecision of the phenotypic methods, different molecular

identification methods have been designed. As previously reviewed, they involve conventional PCR, m-PCR, RT-PCR, RFLP, PCR-DGGE, FISH as well as MALDI-TOF MS (Collado & Figueras, 2011 and references therein). Most of the mentioned methods were designed to detect only the species *A. butzleri* and/or *A. cryaerophilus* and/or *A. skirrowii* (Collado & Figueras, 2011). The most commonly used molecular method is the above-mentioned m-PCR developed by Houf *et al.* (2000), although several misidentifications as other species have been reported with this method (Collado & Figueras, 2011; Figueras *et al*., 2011b). In 2003, a new m-PCR method targeting the 23S rRNA gene was proposed for detecting *A. butzleri, A. cryaerophilus* and *A. skirrowii* (Kabeya *et al*., 2003) and was also able to differentiate the two subgroups of *A. cryaerophilus* (1 and 2). This method was validated using only 4 reference strains and 10 field isolates (Kabeya *et al*., 2003). Later, Pentimalli *et al.* (2008) proposed a new method that targeted the *gyrA* and 16S rRNA genes, which enable detection and identification of 4 species: *A. butzleri, A. cryaerophilus, A. skirrowii* and *A. cibarius.* However, the similarity between the sizes of the amplicons for each of the species (203, 212, 257 and 145 bp) impeded the detection of all species in the same reaction (Pentimalli *et al*., 2008). Also in 2008, Figueras *et al.* designed an RFLP method based on the digestion of the 16S rRNA gene with *Mse*I endonuclease. Apart from *A. butzleri, A. cryaerophilus* and *A. skirrowii,* three other species that had been described to that moment, i.e. *A. cibarius, A. nitrofigilis* and *A. halophilus,* were also identified. The method allowed detection of a new pattern (F2075) from shellfish (Figueras *et al*., 2008) that was later described as a new species, *A. mytili* (Collado *et al*., 2009a). Furthermore, other new RFLP patterns were encountered in some strains that corresponded to other two new species *A. molluscorum* (Figueras *et al*., 2011b) and *A. defluvii* (Collado *et al*., 2011). Other recently described species, such as *A. thereius* (Houf *et al.,* 2009) and *A. trophiarum* (De Smet *et al*., 2011a), produced the same RFLP pattern described for *A. butzleri* (Collado & Figueras, 2011). Such confusion is an important limitation that requires fixing, as well as the fact that the RFLP method was designed to be applied using polyacrylamide gel electrophoresis (Figueras *et al*., 2008), which is not routinely available in all laboratories (Douidah *et al*., 2010). Considering the limitations of the m-PCR of Houf *et al*. (2000), in 2010 the same research group proposed a new m-PCR method, targeting the 23S rRNA and *gyrA* genes able to identify 5 species linked to humans and other mammals, i.e. *A. butzleri, A. cryaerophilus, A. skirrowii, A. cibarius* and *A. thereius* (Douidah *et al.*, 2010). One year later, they designed a PCR method to complement the previous m-PCR, one that is able to detect the new species *A. trophiarum,* which they have also described from isolates recovered from animals (De Smet *et al.*, 2011a).

2. INTEREST AND OBJECTIVES

The taxonomy and epidemiology of the genus *Arcobacter* were recently reviewed by Luis Collado in his PhD thesis (Collado, 2010) and it raised several questions that became the starting point of the present thesis. Two isolates recovered by Dr. Collado, i.e. one from shellfish (F4) and one from pork meat (F41), seemed to belong to two potential new *Arcobacter* species, and a complete polyphasic characterization was required to verify if they indeed represented two new taxa. This was one of the first objectives and challenges that needed resolving. Furthermore, in the previous thesis a molecular identification method (16S rRNA-RFLP) was developed that was able to correctly characterize all *Arcobacter* species that were included in the genus until 2008. This method, contrary to most of the contemporary molecular methods, which had been designed to detect and identify only the species *A. butzleri, A. cryaerophilus* and *A. skirrowii,* was able to recognize the three remaining species that had been described at that time (*A. nitrofigilis, A. halophilus* and *A. cibarius*). The number of described new species of the genus increased exponentially from six in 2008 to 12 species at the beginning of the present thesis in 2010, creating the need to modify and update this method for characterizing all the currently accepted species. In addition, there was also necessary to evaluate whether the identification methods so far published for the species of *Arcobacter* were still useful considering the new set of species.

Over recent years, new approaches have been developed for identifying microorganisms, among which, the MALDI-TOF has become an important protagonist. This method is now routinely applied at clinical laboratories for recognizing fastidious, slow-growing bacteria or those that are difficult to identify at the species level using other methods. One study has so far used MALDI-TOF to investigate a few strains of 3 species of *Arcobacter* and it was shown to be a promising technique. However, whether MALDI-TOF is useful for identifying all the species of the genus remains to be seen.

The true prevalence of the members of this genus needs to be established not only using methods that correctly identify all the species but also employing optimal culture conditions for the recovery of these bacteria. However, so far there is a lack of consensus about which culture conditions (aerobiosis or microaerophilia, enrichment or direct plating) are the most appropriate. There is almost the same number of published studies that have used aerobiosis or microaerophilia and some authors recommend using both cultures approaches in parallel i.e. direct plating and after enrichment. Studies that systematically evaluate these factors are therefore of paramount importance.

Despite the 16S rRNA gene having been the classical tool for identifying bacteria, other housekeeping genes have proven to have a higher resolution for the differentiation of species.

Previous studies have demonstrated the usefulness of the *rpoB, gyrB* and *cpn60* genes for establishing phylogenetic relationships among the *Arcobacter* spp. However, the *ad hoc*

committee for the re-evaluation of the species definition in bacteriology has suggested that at least five housekeeping genes should be evaluated in order to circumscribe the taxon species. In fact, whether there is a sufficient degree of congruence between these MLPA techniques and the DDH for the delineation of new *Arcobacter* species has to be verified in order to be able to replace the latter technique. It is therefore important to investigate additional genes in order to propose the first MLPA scheme with at least 5 genes as an alternative tool to DDH.

It has been suggested that shellfish might be a reservoir of *Arcobacter* species because *A. mytili* and *A. molluscorum* were both discovered from these types of samples. Furthermore, contamination of shellfish with virulent *Arcobacter* strains might represent a risk to human health considering that this seafood is traditionally eaten undercooked or raw, but all this requires further investigation.

Water that has been contaminated with sewage has also been put forward as another important reservoir for new species as it has also shown a high prevalence and diversity of *Arcobacter* species. In fact, this was the origin of another recently described species (*A. defluvii*). Furthermore, a recent study that investigate the bacterial communities in sewage from two WWTPs in the USA by sequencing the 16S rRNA gene, demonstrated that *Arcobacter* was very prevalent. These results indicate that further studies are necessary to determine the diversity of *Arcobacter* species in our WWTPs, and the use of different culturing and detection approaches in parallel will enable the ideal culture conditions to be determined for those bacteria.

The clinical importance of *Arcobacter* species is not yet clarified. This is mainly due to these bacteria not being specifically searched for in clinical specimens and to the lack of standardised protocols that adequately detect and identify them. Some studies have shown that *Arcobacter* spp. can easily be confused with *Campylobacter* spp. so it would be important to find out if this occurs routinely in hospitals. On the other hand, only two studies have assessed the virulence potential of a few *Arcobacter* spp., evaluating the adhesion and invasion capacity to human Caco-2 intestinal cells and also the potential presence of some putative virulence genes. However, none of these studies has included all of the known species of the genus.

The present thesis aims to provide new data that will contribute to the improvement of the isolation and identification of all the *Arcobacter* species and that might help to understand better the epidemiology and virulence of this group of microorganisms. To achieve this, the following specific objectives have been defined:

1. To characterize one isolate from shellfish (F4) and one from pork meat (F41) using a polyphasic taxonomic approach that includes conventional methods as well as new tools (i.e. MALDI-TOF and MLPA) in order to determine if they belong to new *Arcobacter* species.

2. To actualise the 16S rRNA-RFLP method for the identification of all the species of the genus and to compare the performance of several available molecular identification methods.
3. To analyse the diversity and recovery of *Arcobacter* spp. in waste-water and shellfish, comparing the effect of different incubation and culture conditions (aerobiosis or microaerophilia, enrichment or direct plating).
4. To evaluate the adhesion and invasion capacity to Caco-2 cells and the possible presence of putative virulence genes in representative strains of all the species of the genus.
5. To re-identify, using the *rpoB* gene, *Campylobacter* strains isolated from faeces of patients with diarrhoea in order to investigate whether or not they uncover *Arcobacter* species.

3. MATERIALS AND METHODS

3.1 Investigated strains and samples

3.1.1 Type and reference strains

The type and reference strains used in the present thesis are shown in Table 3.1, while the strains received from other authors and those obtained in previous studies are shown in Table 3.2. All strains were stored frozen at -80°C in Trypticase Soy Broth (Becton Dickinson, New Jersey, USA) supplemented with 15 % glycerol (Panreac, Barcelona, Spain) and type and reference strains were also freeze-dried. The dried strains were rehydrated with brain heart infusion broth (Becton Dickinson, New Jersey, USA) and recovered on BA plates (based on Trypticase Soy Agar, TSA, supplemented with 5% sheep blood agar, Becton Dickinson, New Jersey, USA) incubated at 30°C for 48-72 h under aerobic conditions when they were needed.

Table 3.1 Type and reference strains used in this study

Species	Strain used	Source	country	References
A. nitrofigilis	CECT 7204	Roots of *Spartina alterniflora*	Canada	McClung *et al*. (1983) Vandamme *et al*. (1991)
A. cryaerophilus	LMG 9904	Aborted bovine foetus (brain)	Ireland	Neill *et al*. (1985) Vandamme *et al*. (1991)
A. butzleri	LMG 10828	Human with diarrhoea (faeces)	USA	Kiehlbauch *et al*. (1991) Vandamme *et al*. (1992)
A. skirrowii	LMG 6621	Lamb with diarrhoea (faeces)	Belgium	Vandamme *et al*. (1992)
A. cibarius	CECT 7203	Broiler carcasses	Belgium	Houf *et al*. (2005)
A. halophilus	LA31B	Hypersaline lagoon	USA	Donachie *et al*. (2005)
A. mytili	CECT 7386	Mussels	Spain	Collado *et al*. (2009a)
A. thereius	LMG 24486	Pig abortion	Denmark	Houf *et al.* (2009)
A. marinus	JCM 15502	Seawater associated with starfish	Korea	Kim *et al*. (2010)
A. trophiarum	LMG 25534	Pig faeces	Belgium	De Smet *et al*. (2011a)
A. molluscorum	F98-3	Mussels	Spain	Figueras *et al*. (2011)
A. defluvii	CECT 7397	Sewage	Spain	Collado *et al*. (2011)
A. ellisii	F79-6	Mussels	Spain	**Study 4.1**
A. bivalviorum	F4	Mussels	Spain	**Study 4.2**
A. venerupis	F67-11	Clams	Spain	**Study 4.2**
A. cloacae	SW28-13	Sewage	Spain	**Study 4.3**
A. suis	F41	Pork meat	Spain	**Study 4.3**
Species	**Reference strain**	**Source**	**country**	**Equivalences**
A. cryaerophilus	LMG 9871	Bovine abortion	UK	CCUG 17814, LMG 9905 ,Neill 02732
	LMG 9861	Bovine abortion	UK	CCUG 17802, Neill 02824
	LMG 9865	Porcine abortion	UK	CCUG 17808;Neill 02771
	LMG 10241	Porcine abortion	Canada	Higgins 88-3421R
	LMG 6622	Porcine abortion	UK	CUG 12018, Neill 02774
	LMG 10229	Porcine abortion	Canada	Higgins 87-5154R
	LMG 7537	Ovine abortion	UK	CCUG 17805, Neill 02828
	LMG 9863	Ovine abortion	UK	CCUG 17806, Neill 02799
	LMG 10829	Human blood	USA	ATCC 49615, CDC D2610
A. butzleri	LMG 11118	Human faeces	Italy	CCUG 30486, Lauwers CA4091
A. skirrowii	LMG 9911	Porcine abortion	UK	Neill 02777
A. trophiarum	CECT 7650	Duck faeces	Chile	Collado FE2

The dried or frozen strains of the species *A. nitrofigilis* needed to be initially incubated in a microaerobic atmosphere using the gas generating kit GasPakTM EZ Campy Container System (Becton Dickinson, New Jersey, USA), while subsequent incubations could be done in aerobiosis. Considering the halophilic characteristic of the species *A. marinus* and *A. halophilus,* the strains of both species were incubated in BA supplemented with 2% NaCl. The rest of the species did not have any specific requirement, despite some species showing a faster and more abundant growth than the others.

3.1.2 Sample Collection

3.1.2.1 Shellfish samples

The shellfish samples (mussels, clams and oysters) investigated in **study 4.6** were harvested from the Ebro delta farming area and were provided by the Tarragona laboratory of the *Agència de Salut Pública de Catalunya*, Spain. Ten grams of each sample was mixed with Arcobacter-CAT broth and then incubated at 30°C for 48 h under aerobic conditions. The Arcobacter-CAT broth consist in “Arcobacter broth” (peptone, 18 g L^{-1}; NaCl, 5 g L^{-1} and yeast extract, 1 g L^{-1}; Oxoid, Basingstoke, UK), supplemented with the three antibiotics Cefoperazone, Amphotericin B and Teicoplanin (CAT: Oxoid, Basingstoke, UK) according the manufacturer’s instructions (Atabay & Corry, 1998).

3.1.2.2 Water samples

The water samples included in **Study 4.7** were collected from the WWTP in the city of Reus, Spain. These samples were taken in 2 L sterile polypropylene bottles from 5 sampling points as described in the study, and were transported chilled in ice to the laboratory. Then two aliquots of 200 ml of each wastewater sample were concentrated separately by filtration using 0.45 µm membrane filters (Millipore, Molsheim, France), and then one of those filters was rolled and introduced into a tube with 9 ml of Arcobacter-CAT broth and incubated at 30°C for 48 h under aerobic conditions (enrichment step). The content of the other filter was re-suspended in 1 ml of distilled water and used as the concentrated wastewater sample for the direct molecular detection by the m-PCR method as described below in 3.2.1 and for the direct plating described in 3.2.2.

Table 3.2 Strains received from other authors

Species	Strain	Source	Country	Received from
A. cryaerophilus	FE4	Chicken cloacal swab	Chile	Dr. Luis Collado
	FE5	Chicken cloacal swab	Chile	Dr. Luis Collado
	FE6	Chicken cloacal swab	Chile	Dr. Luis Collado
	FE9	Chicken cloacal swab	Chile	Dr. Luis Collado
	FE11	Chicken cloacal swab	Chile	Dr. Luis Collado
	FE13	Chicken cloacal swab	Chile	Dr. Luis Collado
	FE14	Ovine faeces	Chile	Dr. Luis Collado
	MIC V-1	Cow faeces	Chile	Dr. Luis Collado
	MIC V3-2	Cow faeces	Chile	Dr. Luis Collado
A. skirrowii	989	Cow faeces	Belgium	Dr. Kurt Houf
	994	Cow faeces	Belgium	Dr. Kurt Houf
	E	Cow faeces	UK	Dr. Robert Madden
	F	Cow faeces	UK	Dr. Robert Madden
A. cibarius	742	Poultry carcasses	Belgium	Dr. Kurt Houf
	743	Poultry carcasses	Belgium	Dr. Kurt Houf
	745	Poultry carcasses	Belgium	Dr. Kurt Houf
	746	Poultry carcasses	Belgium	Dr. Kurt Houf
	748	Poultry carcasses	Belgium	Dr. Kurt Houf
	NC81	Piggery effluent	Australia	Dr. Nalini Chinivasagam
	NC88	Piggery effluent	Australia	Dr. Nalini Chinivasagam
A. defluvii	MIC C42	Pig faeces	Chile	Dr. Luis Collado
	CH8-2	Mussels	Chile	Dr. Luis Collado

The strains of *A. cryaerophilus* and *A. cibarius* were also used in previous studies (Collado, 2010)

3.2 Procedures for the detection, isolation and identification of *Arcobacter* species

3.2.1 DNA extraction and molecular detection by m-PCR

The DNA was extracted from 400 µl of the incubated enrichment of shellfish samples in **study 4.6** or the same volume of the concentrated wastewater samples (see 3.1.2.2) in **study 4.7**, which were transferred into an Eppendorf tube. The samples were centrifuged for 1 min at 13000 rpm and washed at least three times with distilled water, i.e. re-suspending the pellet in 1 ml of water and then centrifuging again for 1 min at 13000 rpm. Finally, the supernatant was poured out and the extraction was carried out from the pellet using the InstaGene™ DNA Purification Matrix (Bio-Rad, Hercules CA, USA) as recommended by the manufacturer.

Direct molecular detection was carried out as previously described (Collado *et al.*, 2009a) with the m-PCR designed for *A. cryaerophilus*, *A. butzleri* and *A. skirrowii* by Houf *et al.* (2000), using the primers (Table 3.3) and conditions described by the authors. The amplified products were separated by electrophoresis in 2% agarose gel and were visualised after staining with the Red Safe gel (Ecogen, Barcelona, Spain) using a trans illuminator Vilber Lourmat TFX-35C.

3.2.2 Culture procedures

Arcobacter were isolated using the passive filtration method on BA. This consists of transferring 200 µl of the sample onto the surface of a 0.45 µm membrane filter, which is placed on the BA, and then allowed to filter passively in ambient conditions for 30 min (Atabay & Corry, 1998). In **study 4.6**, the arcobacters were isolated from the enrichment (3.1.2.1) while in **study 4.7** they were isolated both directly from the concentrated wastewater samples and also from the enrichment (3.1.2.2). The plates were then incubated at 30°C for 48 h under aerobic conditions in **studies 4.6 and 4.7**, whereas in **study 4.6** they were also incubated in microaerphilia.

3.2.3 DNA extraction, genotyping and molecular identification of strains

The DNA was extracted from the isolated colonies on solid media as described above. Briefly, a single colony was picked and re-suspended in sterile MilliQ water and centrifuged 1 min at 13000 rpm. The supernatant was then poured out and the extraction was carried out from the pellet using the InstaGene™ DNA Purification Matrix (Bio-Rad, Hercules, CA, USA) following the manufacturer instructions. The concentration of each DNA sample was determined using the GenQuant pro (Amersham Biosciences) at A260.

For ERIC-PCR the primers ERIC 1R and ERIC 2 (Table 3.3) designed by Versalovic *et al*. (1991) were used following the conditions previously described by Houf *et al*. (2002). The PCR products were size-separated by electrophoresis in 2% agarose. Gels were stained using the Red Safe gel staining (Ecogen, Barcelona Spain) and visualized using a trans illuminator Vilber Lourmat TFX-35C. Gel images were saved as TIFF files, normalized with the GeneRulerTM 100bp DNA Ladder Plus (Invitrogen, Carlsbad, CA, USA), and further analysed by Bionumerics software, version 6.5 (Applied Maths, Ghent, Belgium). Patterns with at least one different band were considered different genotypes.

For identification at species level, two molecular methods were used in parallel, the mentioned m-PCR of Houf *et al*. (2000) and the 16S rRNA RFLP of Figueras *et al*. (2008). For the latter method, a 1026 bp amplicon was amplified from each strain as previously described (Figueras *et al*., 2008) using the primers CAH16S1am and CAH16S1b (Table 3.3). The size of the amplicon was verified by electrophoresis in 2% agarose gel using a 100 bp ladder (Fermentas, Schwerte, Germany) and were visualised after staining with the Red Safe gel (Ecogen, Barcelona, Spain) using a trans illuminator Vilber Lourmat TFX-35C.

The amplicon 1026 bp was then digested using the *Mse*I endonuclease (Fermentas, Schwerte, Germany). In **study 4.4** new endonucleases were searched for using the NEBcutter V

2.0 software (Vincze *et al*., 2003) (http://tools.neb.com/NEBcutter2/index.php) in order to discriminate the species that showed an equal or very similar RFLP pattern to the *Mse*I enzyme. The simulated restriction analysis of the 16S rRNA gene sequences allowed selecting the most suitable endonucleases for species discrimination. Digestions with *Mse*I and the newly selected enzymes were experimentally carried out following the manufacturer instructions under the conditions described above to confirm the expected results.

Restriction fragments were separated either in 15% polyacrylamide gel (ProtoGel, Atlanta, USA) electrophoresis in Tris-Borate-EDTA (TBE) 1X buffer at 350 V for 5 h or in 3.5% agarose gel electrophoresis in TBE 1X buffer at 100 V for 2 h. The pBR322 DNA/BsuRI (*Hae*III) (Fermentas, Schwerte, Germany) was used as a molecular weight marker for polyacrylamide gel electrophoresis or the 50 bp ladder (Fermentas, Schwerte, Germany) for agarose gel electrophoresis. The gels were stained using the Red Safe gel staining (Ecogen, Barcelona, Spain) and visualized using a trans illuminator Vilber Lourmat TFX-35C.

Table 3.3 Primers used for the m-PCR, 16S rRNA-RFLP and the ERIC-PCR (studies 4.1-4.9)

Method	Primer	Sequence 5' to 3'	Target	Size (bp)	Reference
m-PCR	ARCO (R)	CGTATTCACCGTAGCATAGC	16S rRNA		Houf *et al*. (2000)
	BUTZ (F)	CCTGGACTTGACATAGTAAGAATGA	16S rRNA	401	
	SKIRR (F)	GGCGATTTACTGGAACACA	16S rRNA	641	
	CRY 1 (F)	TGCTGGAGCGGATAGAAGTA	23S rRNA	257	
	CRY 2 (R)	AACAACCTACGTCCTTCGAC	23S rRNA		
16S rRNA	CAH16S1am (F)	AACACATGCAAGTCGAACGA	16S rRNA	1026	Figueras *et al*. (2008)
	CAH16S1b (R)	TTAACCCAACATCTCACGAC	16S rRNA		Marshall *et al*. (1999)
ERIC-PCR	ERIC 1R (F)	ATGTAAGCTCCTGGGGATTCAC	Genome	-----[a]	Versalovic *et al*. (1991)
	ERIC 2 (R)	AAGTAAGTGACTGGGGTGAGCG	Genome		Houf *et al*. (2002)

[a]Multiple bands of different sizes are expected.

3.3 Phenotypic and molecular characterization of the new species

3.3.1 Gram stain

The shape and staining behaviour of all isolates obtained from different samples were initially evaluated by Gram stain (with 0.3% carbol fuchsin), as proposed for *Campylobacter* (Ursing *et al*., 1994).

3.3.2 Motility

The motility of cells was observed in young cultures by examining wet mounts in distilled water by phase-contrast microscopy.

3.3.3 Biochemical characterization

The phenotype of the strains was characterized using the biochemical scheme of Vandamme *et al*. (2005) complemented with tests used in previous studies (On *et al*., 1996; Figueras *et al.,* 2011). Briefly, the susceptibility to different compounds was tested on nutrient agar (based on nutrient broth no. 2, Difco, supplemented with 5% sheep blood and 1.5% agar) supplemented with cefoperazone 64 mg L^{-1}, 1% glycine, 2% NaCl, 4% NaCl, 0.1% sodium deoxycholate, 1% oxgall, 0.05% safranin, 0.0005% crystal violet, 0.04% 2,3,5 triphenyl tetrazolium chloride (TTC), 0.005% basic fuchsin or 0.001% brilliant green. The indoxyl acetate hydrolysis test was carried out according to Mills & Gherna (1987) and confirmed using indoxyl acetate diagnostic tablets (IAC)-DIETABS (Rosco Diagnostica, Taastrup, Denmark). The nitrate reduction was evaluated according to Cook (1950). The capacity of growth on unsupplemented Campylobacter Blood-Free Selective Agar Base (CCDA; Oxoid, Basingstoke, UK), MacConkey agar (Oxoid, Basingstoke, UK*) and* Marine Agar (Difco, Sparks, MD, USA) was also tested. All tests were carried out at 30ºC and at least twice for the studied strains and all the type strains of *Arcobacter* species in parallel.

3.3.4 Morphological characterization by electron microscopy

The cell size, morphology and presence of flagella in *Arcobacter* strains were determined with transmission electron microscopy (JEOL 1011) after negative staining with 2% (W/V) of phosphotungstic acid solution (pH 6.9) for 1 min. For scanning electron microscopy, pieces of agar containing the growing strains were fixed in 2.5% glutaraldehyde in phosphate buffer for 24 h. The samples were then post-fixed in 1% osmium tetroxide for 2 h. After dehydration and critical point drying, specimens were mounted and coated with a thin layer of gold before examination in a JEOL JSM 6400

3.3.5 Sequencing of the 16S rRNA and other housekeeping genes

Each gene was individually amplified using the primers shown in Table 3.4. The 16S rRNA, *rpoB, gyrB* and *hsp60* genes were amplified as previously described (Collado *et al*., 2009; Debruyne *et al*., 2010; Collado *et al*., 2011). Sequencing was done either by *Macrogen Europe* (Amsterdam, The Netherlands) following the instructions of the company, or in our own laboratory. In the latter case, the amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and then sequenced using a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, USA) with an ABI PRISM 310 Genetic

Analyzer (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions. The sequence was assembled using the Seqman software (DNASTAR, USA).

The obtained DNA sequences were aligned with the CLUSTAL W software (Thompson *et al*., 1994). Genetic distances were obtained using Kimura's two-parameter model (Kimura 1980) and evolutionary trees were constructed by the neighbour joining method with the MEGA5 program (Tamura *et al*., 2011). The stability of each relationship was assessed by bootstrap analyses (1000 replicates).

Table 3.4 Primers used for amplification and sequencing of the 16S rRNA and other housekeeping genes

Method	Primer	Sequence 5' to 3'	Target	Size (bp)	Reference	Study
16S rRNA	Anti I (F)	AGAGTTTGATCATGGCTCAG	16S rRNA	1500	Martínez-Murcia *et al*. (1992)	**4.1 – 4.9**
	S (R)	GGTTACCTTGTTACGACTT	16S rRNA			
rpoB	CamrpoB-L (F)	CCAATTTATGGATCAAAC	*rpoB*	524	Korczak *et al*. (2006)	**4.1, 4.2 4.4 – 4.9**
	RpoB-R	GTTGCATGTTNGNACCCAT	*rpoB*		Collado *et al*. (2009)	
	rpoB-Arc15F[a]	TCTCAATTTATGGAYCAAAC	*rpoB*	900	Collado *et al*. (unpublished)	**4.3**
	rpoB-Arc24R[a]	AGTTATATCCATTCCATGGCAT	*rpoB*			
gyrB	gyrB-Arc-7F[a]	GTTTAYCAYTTTGAAGGTGG	*gyrB*	722	Collado *et al.* (2011)	**4.3**
	gyrB-Arc-14R[a]	CTAGATTTTTCAACATTTAAAAT	*gyrB*		Collado *et al*. (unpublished)	
	gyrB-Arc-13R[a]	ACTCTATCTCTACCTTGTTT	*gyrB*			
hsp60	H729 (F)	CGCCAGGGTTTTCCCAGTCACGAC	*hsp60*	570	Hill *et al*. (2006)	**4.1 – 4.2**
	H730 (R)	AGCGGATAACAATTTCACACAGGA	*hsp60*		Collado *et al.* (2011)	
	cpn60-Arc2F[a]	GGAGCWCAACTTGTAAAAGAAGT	*cpn*60	633	Collado *et al*. (unpublished)	**4.3**
	cpn60-Arc8R[a]	GCTTTTCTTCTATCICCAAA	*cpn*60			
gyrA	gyrA-Arc4F[a]	TAAGAGATTTAGATAAAGATAC	*gyrA*	1014	Collado *et al*. (unpublished)	**4.3**
	gyrA-Arc13R[a]	TTATCTCTTTGAAGWCCTGT	*gyrA*			
atpA	atpA-Arc5F[a]	GATACAATTCTTAACCAAAAAGG	*atpA*	751	Collado *et al*. (unpublished)	**4.3**
	atpA-Arc12R[a]	AAAACTTCWACCATTCTTTG	*atpA*			
	atpA-Arc6F[a]	TTGTATTTATGTTGCWATTGG	*atpA*			

[a]Primers used for MLPA **Study 4.3 (Levican *et al*., 2013).**

3.3.6 DNA-DNA hybridisation

3.3.6.1 DNA extraction and labelling

DNA was extracted according to Marmur (1961). The reference DNA was labelled using DIG-11-dUTP and biotin-16-dUTP using the nick-translation kit (Roche, Penzberg, Germany) and incubating for 90 min. After labelling, the DNA was precipitated with ethanol and re-suspended in 200 ml sterile MilliQ water (Urdiain *et al*., 2008).

3.3.6.2 Hybridisation

Fifteen µg of unlabelled DNA was mixed with 100–150 ng of labelled DNA, and filled to 72 µl with MilliQ water. This solution was denatured by incubating for 10 min at 100ºC and immediately chilled on ice. After a short spin of the DNA mixture, 28 µl of 1M phosphate buffer (PB) was added and mixed. The 100 µl hybridization mixtures were covered with 100 µl of light mineral oil (Sigma, Saint Louis, Missouri, USA) in order to avoid evaporation and changes in volume during incubation. Finally, all the solutions were incubated for 16 h at 30ºC below the melting point (*Tm*) of the homologous (considered non-restrictive hybridisation conditions). *Tm* was calculated with the following formula:

$$[Tm: (G+C+182.2)/2.44]$$

Separation of single and double strands

Single and double-stranded DNA was eluted on hydroxyapatite (HA) (Sigma, Saint Louis, Missouri, USA). Prior to chain separation, HA was equilibrated with 0.14M PB. Two 50 µl aliquots of each single DDH mixture were transferred to two tubes containing equilibrated HA, respectively. The DDH solution was mixed well with the HA and incubated for 15 min at Tm-35ºC. During incubation, double-stranded DNA was bound to HA and then a centrifugation was possible to separate the single strands in a new tube. The HA was washed two additional times with 450 and 500 µl of 0.14M PB respectively and incubated at TM-35ºC, then the supernatant obtained from centrifugation was collected in the tubes of single stranded DNA. The HA pellet containing bound double-stranded DNA was well mixed with 200 µl 0.4MPB, and kept at room temperature (RT) for 1–2 min. Supernatant was collected after centrifugation (2 min at 13000 rpm), and the pellet was washed again with 200 µl 0.4M PB. The final volume of double-stranded DNA was 400 µl. These final samples were denatured by boiling and they were ice-chilled prior to their detection on microtitre plates.

3.3.6.3 Detection on microtitre plates

200 µl was transferred to a well of a streptavidin coated microtitre plate (Roche, Penzberg, Germany), and incubated for 2 h at RT. Wells were then washed with 1XPBS. In each well, 200 µl of the antibody mixture (anti-digoxygenin) was added and incubated for 1h at RT. Wells were then washed again with 1X PBS. Finally, 200 µl of coating buffer 1X with 1 mg/ml p-nitrophenylphosphate (Sigma, Saint Louis, Missouri, USA) was added to each well and the plates were incubated at 37ºC. The colour development was measured at 405 nm.

3.3.7 MLPA

For the MLPA scheme **(study 4.3)** the same primers were used as described by Collado *et al*. (2009) for *gyrB* gene (Table 3.4), while new ones were designed for the *gyrA, atpA*, *rpoB* and *hsp60* genes (Annex 8.4). The PCR amplifications were performed in a reaction mixture containing 1 µl of genomic DNA, 0.2 µM each dNTP, 1 µl 10 mM of each primer (Table 3.4), 2 mM of MgCl2, 1 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and the buffer supplied with the enzyme and 40 µl of MilliQ water. PCR conditions applied were: 3 min at 94ºC, followed by 35 cycles of 15 s at 94ºC, 30 s at 55ºC and 45 s at 72ºC, followed by 5 min at 72ºC. Sequencing and sequence analysis were carried out as described (3.4.3).

3.3.8 MALDI-TOF

For the MALDI-TOF method (**studies 4.1, 4.2 and 4.3**), the *Arcobacter* colonies were grown on blood agar at 30 ºC for 72 h and then were spotted in triplicate on the MALDI-TOF MS sample plate and air-dried at room temperature. An aliquot of 1 µl matrix solution (saturated solution of cyanohydroxycinnaminic acid in 50% aqueous acetonitrile containing 2.5% trifluoroacetic acid) was added onto each sample spot and again allowed to dry. Mass spectrometric measurements were taken on a Voyager DE STR (Applied Biosystems, Carlsbad, CA, USA) using conditions described by Böhme *et al*. (2009) but the mass range was 2–20 kDa. The Bacterial Test Standard (*Escherichia coli* DH5; Bruker, Bremen, Germany) was used as an external protein calibration mixture. Mass spectra were smoothed, baselines corrected and peaks detected using the Applied Biosystems Data Explorer software. Final results were expressed for the different *Arcobacter* species as the average of 3 mass values within a deviation of ±5 Da and relative intensity ≥10% using the criteria described by Donohue *et al.* (2006), but considering peaks that were higher than 3 kDa. Furthermore, data obtained was analysed by means of BioNumerics 6.5 software (Applied Math, Ghent, Belgium). A dendrogram was constructed using the Dice similarity coefficient and the cluster analysis of similarity matrices was calculated with the unweighted pairgroup method with arithmetic averages (UPGMA).

3.4 Assays to determine the potential virulence of the isolates

3.4.1 Adhesion and invasion assays on Caco-2 cells

The bacterial suspensions for adhesion and invasion assays were prepared as follows: A colony of each strain was inoculated in Brain Heart Infusion (BHI; Difco), which was incubated under aerobic conditions for 48 h at 30ºC for *Arcobacter* strains and overnight at 37ºC for the

control strains. After the incubation period, the cultures were diluted to an optical density (600 nm) of 0.08 (ca. 10^9 cfu ml-1 of bacteria cells) for *Arcobacter* strains and of 0.05 (ca. 10^8 cfu ml^{-1}) for the control strains. Then, the cultures were centrifuged (5 minutes at 3000 rpm, 4ºC) and the resultant cell pellets were re-suspended in the same volume of warm (37ºC) Eagle's Minimum Essential Medium (EMEM, Sigma, Saint Louis, Missouri, USA) supplemented with 10% foetal bovine serum (FBS, Sigma, Saint Louis, Missouri, USA) and 1% non essential amino acids (NEAA, Sigma, Saint Louis, Missouri, USA). The bacterial viable counts were determined on BHI agar supplemented with 5% sheep blood agar following the Miles & Misra (1938) method. The number of cells (cfu ml^{-1}) of each bacterial suspension represented the mean from three enumerations.

The adhesion and invasion assays on Caco-2 cells (**study 4.8**) were carried out as described by Ho *et al*. (2008) and Townsend *et al*. (2008). In brief, 0.5 ml of a suspension of 4 x 10^4 Caco-2 cells ml^{-1} in EMEM supplemented with penicillin 10,000U and streptomycin 10,000 µg ml^{-1} (Sigma, Saint Louis, Missouri, USA) was added to each of the 24 wells of a microtitre plate, which was then incubated for 48h at 37ºC under a 5% CO_2 atmosphere (Sanyo CO_2 incubator). When the cells had formed a confluent monolayer, the medium was removed, the wells were washed twice with Phosphate Buffered Saline (PBS; Sigma, Saint Louis, Missouri, USA) and 0.5 ml of the bacterial suspension (ca 10^9 cfu ml^{-1}) was added. The plates were incubated for 2h at 37ºC to allow adhesion and invasion of the bacteria and were then washed twice with PBS to remove unbound bacteria. The cell monolayer was lysed with 1% Triton-X and the total number of bacteria associated with the Caco-2 cells was determined as described above. On the other hand, the number of adherent bacteria was calculated as the difference between the total number of bacteria associated with the Caco-2 cells and the number of intracellular bacteria. The latter was determined by inoculating another 24 well plate, which was washed twice with PBS and then supplemented with 0.5 ml of EMEM containing 125 mg ml^{-1} of gentamicin and incubated for 1h at 37ºC to kill extracellular bacteria. Despite, the sensitivity to this antibiotic was not tested for the *Arcobacter* strains, the concentration used was very high compared to the expected MICs in arcobacters for this antibiotic, i.e. 0.125 to 2 µg mL^{-1} according to the NARMS report (2006). After incubation, the cells were washed twice with PBS, lysed with 1% Triton-X and the released bacteria enumerated, as described above. All experiments were in triplicate. Results were expressed as the mean number of bacteria (log10 cfu ml^{-1}) that adhered or invaded. The limit of detection for adhesion was 1.7 x 10^4 cfu ml^{-1} and for invasion 1.7 x 10^2 cfu ml^{-1}.

3.4.2 Detection of putative virulence genes by PCR

The *Arcobacter* colonies were grown on blood agar at 30 ºC for 72 h and then the DNA was isolated using the InstaGene™ DNA Purification Matrix (Bio-Rad, Hercules CA, USA) as recommended by the manufacturer. The PCR methods were used to detect the presence of *ciaB, hecA, cj1349, cadF* and *irgA* genes using the primers (Annex 8.4) and conditions described by Douidah *et al*. (2012), using the strain *A. butzleri* LMG 10828^T as positive control. The PCR products were analysed in 2% agarose gel TBE buffer at 80 V for 90 min using the 100 bp ladder (Fermentas, Schwerte, Germany) as a molecular weight marker. The gels were stained with Red Safe DNA Gel Stain (Ecogen, Barcelona, Spain) and visualized using a trans illuminator Vilber Lourmat TFX-35C.and photographed using an UV transilluminator.

In order to confirm their identity, 27 representative amplicons of the 5 genes were sequenced using the same amplification primers as the putative virulence genes (Table 3.5). Sequencing was carried out by Macrogen Corp Europe (Amsterdam, The Netherlands). Then, a BLASTN comparison was made to confirm the presence of the studied genes in other deposited *Arcobacter* genomes.

Table 3.5 Primers used for detection of 5 putative virulence genes (study 4.8)

Method	Primer[a]	Sequence 5' to 3'	Target	Size (bp)
cadF	cadF-F	TTACTCCTACACCGTAGT	*cadF*	283
	cadF-R	AAACTATGCTAACGCTGGTT	*cadF*	
ciaB	ciaB-F	TGGGCAGATGTGGATAGAGCTTGGA	*ciaB*	284
	ciaBR	TAGTGCTGGTCGTCCCACATAAAG	*ciaB*	
Cj1349	Cj1349-F	CCAGAAATCACTGGCTTTTGAG	*Cj1349*	659
	Cj1349-R	GGGCATAAGTTAGATGAGGTTCC	*Cj1349*	
hecA	hecA-F	GTGGAAGTACAACGATAGCAGGCTC	*hecA*	537
	hecA-R	GTCTGTTTTAGTTGCTCTGCACTC	*hecA*	
irgA	irgA-F	TGCAGAGGATACTTGGAGCGTAACT	*irgA*	437
	irgA-R	GTATAACCCCATTGATGAGGAGCA	*irgA*	

[a]Douidah *et al*. (2012)

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4. RESULTS AND DISCUSSION

4.1. *Arcobacter ellisii* sp. nov., isolated from mussels

Figueras MJ, Levican A, Collado L, Inza MI, Yustes C.

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Short communication

Arcobacter ellisii sp. nov., isolated from mussels☆

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ABSTRACT

As part of a study carried out for detecting *Arcobacter* spp. in shellfish, three mussel isolates that were Gram-negative slightly curved rods, non-spore forming, showed a new 16S rDNA-RFLP pattern with a specific identification method for the species of this genus. Sequences of the 16S rRNA gene and those of the housekeeping genes *rpoB*, *gyrB* and *hsp60* provided evidence that these mussel strains belonged to an unknown genetic lineage within the genus *Arcobacter*. The similarity between the 16S rRNA gene sequence of the representative strain (F79-6^T) and type strains of the other *Arcobacter* species ranged between 94.1% with *A. halophilus* and 99.1% with the recently proposed species *A. defluvii* (CECT 7697^T). DDH results between strain F79-6^T and the type strain of the latter species were below 70% (53 ± 3.0%). Phenotypic characteristics together with MALDITOF mass spectra differentiated the new mussel strains from all other *Arcobacter* species. All the results indicate that these strains represent a new species, for which the name *Arcobacter ellisii* sp. nov. with the type strain F79-6^T (=CECT 7837^T = LMG 26155^T) is proposed.

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The genus *Arcobacter* is included in the family *Campylobacteraceae*, together with the genera *Campylobacter* and *Sulfurospirillum* and embraces a group of bacteria characterized for being aerotolerant and growing at lower temperatures than members of the genus *Campylobacter* [4,41]. The first isolation of bacteria of this genus is attributed to Ellis et al. [14] who recovered spirillum/vibrio-like microorganisms from internal organs of naturally aborted bovine foetuses. These and other isolates, were later described as *Campylobacter cryaerophila* by Neill et al. [28]. However, this species and another with similar characteristics (*Campylobacter nitrofigilis*) were allocated to the new genus *Arcobacter* in 1991 by Vandamme et al. [39] with the names *Arcobacter cryaerophilus* and *Arcobacter nitrofigilis*. The latter is the type species of the genus and is a nitrogen-fixing bacterium recovered originally from roots of the salt marsh plant *Spartina alterniflora* [27]. The amendment to the genus in 1992 by Vandamme et al. [40] included the reclassification of *Campylobacter butzleri* isolated from humans and animals with diarrhoea [23] as *Arcobacter butzleri*, and the description of the new species *Arcobacter skirrowii* isolated from the faeces of lambs with diarrhoea, aborted porcine, ovine, and bovine foetuses, and the prepuce of bulls.

Since then, the genus has expanded with the addition of several new species: *Arcobacter cibarius* from chicken meat [21], *Arcobacter halophilus* from an hypersaline lagoon [11], *Arcobacter mytili* from mussels [6], *Arcobacter thereius* from porcine abortions [22], *Arcobacter marinus* from a mixture of seawater, seaweeds and a starfish [24], *Arcobacter trophiarum* from faeces of fattening pigs [10], *Arcobacter defluvii* from sewage water [8] and finally *Arcobacter molluscorum*, from mussels and oysters [16].

The species *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*, have been associated with gastrointestinal disease and bacteraemia in humans, *A. butzleri* being the most commonly isolated species [4]. The latter was the fourth most common *Campylobacter*-like organisms isolated from the stools of patients with diarrhoea in two separate studies carried out in Belgium and France [30,42]. *Arcobacter* species have been implicated in animal diseases including abortion, septicaemia, mastitis, gastritis and enteritis [4,17,18], and are frequently isolated from meat, mainly from poultry, followed by pork and beef [4,7,18,43]. The abundant presence of the microbes in drinking water and in food of animal origin suggests that these are the transmission routes of these bacteria [4,17].

Species of this genus have been isolated from environmental waters, where it was demonstrated that their presence correlated with that of the indicators of faecal pollution [5]. In fact, it was

☆ The GenBank/EMBL/DDBJ accession numbers of the sequences of strain F79-6^T (=CECT 7837^T = LMG 26155^T) for the 16S rRNA, the *rpoB* the *gyrB* and the *hsp60* genes are FR717550, FR717542, FR717545, FR717548, respectively. The 16S rRNA, the *rpoB* the *gyrB* and the *hsp60* genes sequences of strains F79-2 (FR717551, FR717543, FR717546, FR717549, respectively) and F79-7 (FR717552, FR717544, FR717547, FR717553, respectively) have also been deposited.

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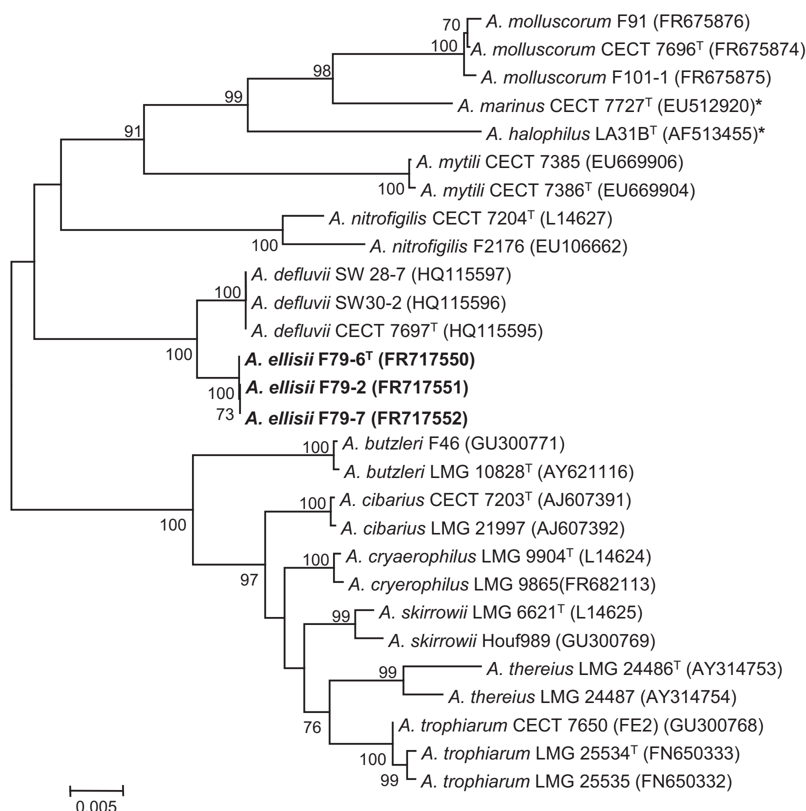

Fig. 1. Neighbour-joining tree based on 16S rRNA sequences showing the phylogenetic position of *Arcobacter ellisii* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70%) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 1000 nt. *Only the type strain is available so far.

shown that *Arcobacter* spp. entered the seawater together with faecally contaminated freshwater [5].

In a study that investigated the occurrence of arcobacters in shellfish, a high prevalence and diversity of these bacteria has been demonstrated in clams and mussels [7]. In that survey, the species *A. mytili* [6], was discovered. Very recently, another new species *A. molluscorum* has also been isolated from mussels and oysters [16].

As part of a new ongoing survey carried out for detecting *Arcobacter* in shellfish using the same isolation protocol that has been described previously [7,16], three isolates recovered from mussel samples (F79-2, F79-6 and F79-7) proved to belong to the genus on the basis of their colony morphology on blood agar (small, translucent colourless or beige to off-white), and phenotypic characteristics (Gram-negative motile slightly curved rods positive for oxidase). Molecular identification was carried out using the restriction fragment length polymorphism (16S rDNA-RFLP) designed for this genus [15] and two different multiplex PCR (m-PCR) methods [13,19]. With 16S rDNA-RFLP, the three strains showed a common pattern different from those previously described (Fig. S1), while with the two m-PCR they showed discrepant results. With the m-PCR described for the identification of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* [19], an amplicon was obtained similar to the one expected for *A. cryaerophilus* (Fig. S2). However, with the recent m-PCR designed for the identification of five *Arcobacter* species associated with humans and other mammals, the three new strains showed an amplicon similar to the one expected for *A. butzleri* [13]. However, an additional, less intense, band similar to that expected for *A. cryaerophilus* was produced by the strains F79-2 and F79-6 (Fig. S2). The new RFLP pattern observed and the contradictory results obtained by the m-PCR methods suggested that the three isolates might belong to a potential new *Arcobacter* species and required further investigation.

The three isolates were genotyped using the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), as described by Houf et al. [20], in order to find out if they were different strains. Results showed that each isolate had a different ERIC-PCR pattern, indicating that they indeed represent different strains (Fig S3) and strain F79-6^T was chosen as the type.

The 16S rRNA, *rpoB*, *gyrB* and *hsp60* genes of the three isolates were amplified and sequenced using primers and conditions previously described [6,8,9] with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The obtained sequences were assembled using SEQMAN software and the phylogenetic analyses were carried out using sequences of all type strains and other strains of all species obtained in previous studies and deposited in the GenBank. The similarity of the 16S rRNA gene sequences was determined by using EzTaxon software [3]. Independent alignments of 16S rRNA (1405 nt), *rpoB* (487 nt), *gyrB* (665 nt) and *hsp60* (555 nt) gene sequences were carried out using CLUSTAL W software [37]. Genetic distances were obtained using Kimura's two-parameter model [25] and phylogenetic trees were constructed with the neighbour-joining [32] and maximum likelihood, both using MEGA software version 4 [36], and with maximum parsimony, using PAUP software [35].

The independently obtained neigbour joining phylogenetic trees for these genes (16S rRNA, *rpoB*, *gyrB* and *hsp60*) showed that these mussel strains belonged to an unknown genetic lineage within the genus *Arcobacter* (Fig. 1, Figs. S4–S6) and this was even

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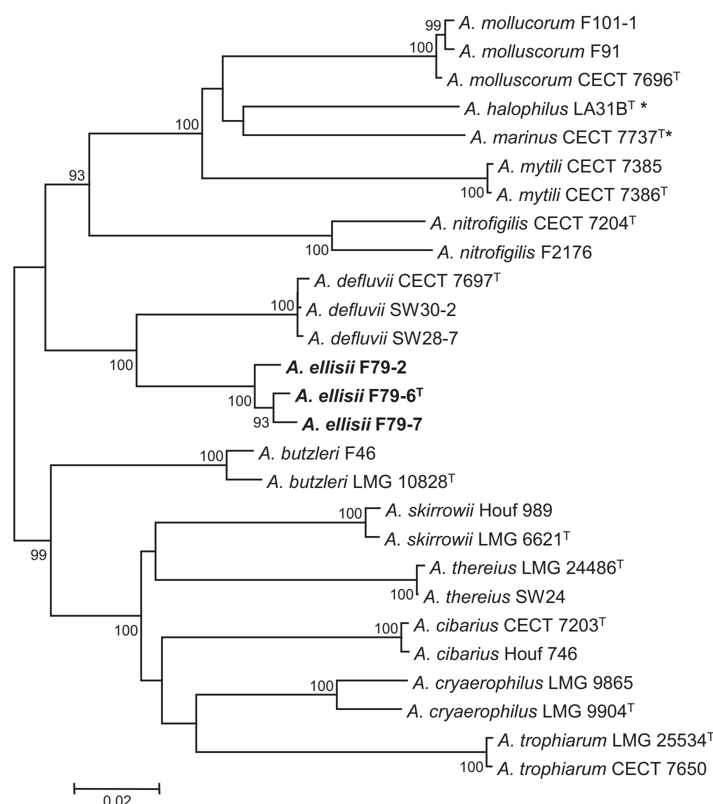

Fig. 2. Neighbour-joining tree based on the concatenated *hsp60*, *rpoB* and *gyrB* sequences showing the phylogenetic position of *Arcobacter ellisii* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70%) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt. *Only the type strain is available so far.

more evident using the concatenated sequences of the *rpoB*, *gyrB* and *hsp60* (1528 nt) genes (Fig. 2). In all the phylogenetic trees, the new species clustered with the recently described species *A. defluvii* [8]. The type strain of the latter species showed the highest 16S rRNA gene sequence similarity (99.1%) with strain F79-6^T. Clustering of the new strains and topology of the trees were similar when the analysis was done using other algorithms like the maximum parsimony and maximum likelihood (data not shown). The 16S rRNA gene sequence similarities obtained with the other species ranged between 94.1% with *A. halophilus* and 95.7% with *A. nitrofigilis*. On the basis of the 16S rRNA gene sequence similarities, the species *A. defluvii* (CECT 7697^T) with values above 97% [31,33,34] was selected for the DNA–DNA hybridization (DDH), using the methodology described in previous studies [8]. The mean and SD of direct and reciprocal DDH results obtained between strain F79-6^T and *A. defluvii* (CECT 7697^T) were 54.7% (±3.0) while between the strains F79-^{T} and F79-2 were 89.8% (±5.2). These results confirmed that these isolates belong to a new species of the genus *Arcobacter*, and ratified what we also emphasized in a previous study [16] that the new 16S rRNA gene similarity threshold >98.7% proposed for the selection of strains for DDH [34] seems to be more appropriate for this group than the original 97% threshold [33].

A complete phenotypic characterization was carried out using the recommended media and methods described previously [29,38,41]. Motility was observed by examining wet mounts in broth by phase-contrast microscopy. Cell size, morphology and presence of flagella were determined with the electron microscope as described by Collado et al. [6], confirming a typical *Arcobacter* cell size and morphology as well as a presence of a single polar flagellum (data not shown). More than 20 tests included in previous descriptions of new *Arcobacter* species [8,16] were evaluated in those strains (Table 1). All tests were conducted at 30 °C and at least twice for all the type strains of all *Arcobacter* species in parallel with the 3 new isolates, with positive and negative controls. The results of the key distinctive tests between the new strains and the other *Arcobacter* spp. are showed in Table 1. The three new strains could be differentiated from the closest neighbour *A. defluvii*, by their inability to grow on media containing 0.01% 2,3,5-triphenyl tetrazolium chloride (TTC), 1% oxgall or on media containing 0.05% safranin. Their ability to grow in microaerobic conditions at 37 °C on blood agar and at 30 °C on Campylobacter Charcoal Deoxycholate Agar (CCDA) and minimal medium and their non growth on media containing 4% of NaCl differentiated the new strains from *A. nitrofigilis*. Between 3 and 8 tests separated the new species from the rest (Table 1).

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been considered a reliable identification method for the *Arcobacter* species tested [1] and it was used therefore in the present study to characterize the potential new species. Colonies from strains F79-6^T, F79-2 and F79-7 and from all the type strains of the other *Arcobacter* spp. that had grown on blood agar at 30 °C for 72 h were spotted in triplicate (to test reproducibility) on the MALDI TOF MS sample plate and air-dried at room temperature. An aliquot of 1 μl matrix solution (saturated solution of α-cyanohydroxycinnaminic acid in 50% aqueous acetonitrile containing 2.5% trifluoroacetic acid) was added onto each sample spot and again allowed to dry [2,26]. Mass spectrometric measurements were taken on a Voyager DE STR (Applied

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Table 1
Differential characteristics of *Arcobacter ellisii* sp. nov., from other members of the genus Taxa: 1, *Arcobacter ellisii* (*n* = 3, data from this study); 2, *A. nitrofigilis* (*n* = 4); 3, *A. cryaerophilus* (*n* = 19); 4, *A. butzleri* (*n* = 12); 5, *A. skirrowii* (*n* = 9); 6, *A. cibarius* (*n* = 15); 7, *A. halophilus* (*n* = 1); 8, *A. mytili* (*n* = 3); 9, *A. thereius* (*n* = 8); 10, *A. marinus* (*n* = 1); 11, *A. trophiarum* (*n* = 11); 12, *A. defluvii* (*n* = 8); 13, *A. molluscorum* (*n* = 3). Data from On et al. [29]; Donachie et al. [11]; Houf et al. [21,22]; Collado et al. [6,8]; Kim et al. [24]; De Smet et al. [10]; Figueras et al. [16]. The specific response for type strains were coincidental or expressed in brackets; +≥95% strains positive; −, ≤11% strains positive; V, 12–94% strains positive. CO_2 indicates microaerobic conditions. All the strains grew on 0.1% sodium deoxycholate[e]. None of the strains grew on media containing 0.001% brilliant green; 0.0005% crystal violet and 0.005% basic fuchsin, or hydrolyse casein, lecithin or starch.

Characteristics	1	2	3	4	5	6	7[a]	8	9	10[a]	11	12	13
Growth in/on													
Air at 37 °C	+	V(−)	V(+)	+	+	−	+	+	−	+	−	+	+
CO_2 at 37 °C	+	−	V(+)	+	+	+	+	+	−	+	−	+	+
4% (w/v) NaCl	−	+	−	−	+	−	+	+	−	+	V(−)	−	+
1% (w/v) glycine	−	−	−	−	−	−	+	+	+	+	V(−)[b]	−	−
MacConkey	V(+)	−	V(−)	+	−	+	−	+	V(+)	−	V(+)[c]	+	+
Minimal media	+	−	−[d]	+	−	+	−	−	+	−	−[b]	+	−
0.05% safranin medium	−	−	+	+	+	+	−	−	+	+	V(+)	+	+
CCDA	+[e]	−	+	+	+	V(−)	−	−	V(−)	−	+	+	−
1% (w/v) oxgall	−	−	+	V(+)	+	+	−	+	−	−	+	+	+
0.04% TTC	−	−	+	+	V(−)	V(−)	−	−	V(−)	−	+	−	−
0.01% TTC	−	−	+	+	+	+	−	−	+	−	+	+	+
Resistance to:													
Cefoperazone (64 mg l^{-1})	−	−	+	+	+	+	−	−	+	−	+	V(+)	+
Enzyme activity													
Catalase	+	+	+	V(+)	+	V(−)	−	+[f]	+	−	+	+[f]	+
Urease	V(−)	+	−	−	−	−	−	−	−	−	−	+	−
Nitrate reduction	+	+	+(−)[g]	+	+	−	+	+[h]	+	+	−	+	+[i]
Indoxyl acetate hydrolysis	+	+	+	+	+	+	+	−	+	+	+	+	−

[a] For these strains the tests were carried out on media supplemented with 2% NaCl, with the exception of 4% (w/v) NaCl, catalase and indoxyl acetate hydrolysis [16].
[b] Test not evaluated by De Smet et al. [10] but tested by Figueras et al. [16].
[c] Strains LMG 25534[T], LMG 25535 of *A. trophiarum* and strain FE2 (CECT 7650) of this species identified in our laboratory grew on MacConkey agar contrary to the 80% positive response described for this species [10,16].
[d] Two (LMG 7537 and LMG 10241) of the four strains tested were positive [6].
[e] All strains grew, at least weakly, after 5 days of incubation.
[f] Weak reaction [6,8].
[g] Two (LMG 9904[T] and LMG 9065) of the four strains tested were negative [6].
[h] Nitrate reduction was found to be positive for the 3 strains of *A. mytili* [16] contrary to our previously published data [6].
[i] Nitrate is reduced after 72 h and 5 days for all strains under microaerobic and aerobic conditions, respectively [16].

Biosystems, Foster city, USA) using conditions described by Böhme et al. [2] but the mass range was 2–20 kDa. The Bruker Bacterial Test Standard (*Escherichia coli* DH5) was used as an external protein calibration mixture [1]. Mass spectra were smoothed, baseline corrected and peak detected using the Applied Biosystems Data Explorer software. Final results were expressed for the different *Arcobacter* species (Table S1) as the average of 3 mass values within a deviation of ±5 Da and relative intensity ≥10% using the criteria described by Donohue et al. [12] but considering peaks that were higher than 3 kDa. Furthermore, data obtained were analyzed by means of BioNumerics 6.5 software (Applied Math, Sint-Martens-Latem, Belgium). A dendrogram was constructed using the Dice similarity coefficient and the cluster analysis of similarity matrices was calculated with the unweighted pairgroup method with arithmetic averages (UPGMA).

In the obtained dendrogram the new strains cluster together and have different MALDI-TOF mass spectra than the type strains of all accepted species within the genus *Arcobacter* (Fig. S7). This is the first time that MALDI TOF MS results have been reported for all accepted species of the genus.

The polyphasic study revealed that the three isolates represent a new *Arcobacter* species for which the name *Arcobacter ellisii* (type strain F79-6[T] = CECT 7837[T] = LMG 26155[T]) is proposed.

Description of *Arcobacter ellisii* sp. nov.

Arcobacter ellisii (el.lis. N.L. gen. masc. n. ellisii of Ellis), named after W. A. Ellis, for his contribution to our knowledge of *Arcobacter*, having described the first members of *Arcobacter* as a spirillum/vibrio-like organism from naturally aborted bovine foetuses [14].

Cells are Gram-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.3–0.9 μm wide and 1–1.8 μm long. Some cells have a filamentous form up to 7 μm long. Motile by a single polar flagellum. Colonies on blood agar (BA) incubated in aerobic conditions at 30 °C for 48 h are 2–4 mm in diameter, beige to off-white, circular with entire margins, convex, and non-swarming. Pigments are not produced. All the strains grow on BA at room temperature (18–22 °C), and at 30 °C and 37 °C under both aerobic and microaerobic conditions with no significant differences. Growth is weak in anaerobic conditions at 30 °C and in aerobic conditions at 42 °C. None of the strains produce haemolysis on TSA medium supplemented with 5% sheep blood. Under aerobic conditions all the strains grow on minimal medium, CCDA, Marine Agar and media containing 2% (w/v) NaCl or 0.1% sodium deoxycholate. No growth occurs on media containing 4% (w/v) NaCl; 1% glycine; 1% oxgall, 0.01%, 0.04% or 0.1% 2,3,5-triphenyl tetrazolium chloride (TTC); 0.001% brilliant green; 0.05% safranin; 0.0005% crystal violet; 0.005% basic fuchsin and medium with 64 mg l^{-1} cefoperazone. Only two of the three strains (F79-6[T], F79-2) grow on MacConkey agar (66.6%). Strains produce oxidase and catalase activity, reduce nitrate and hydrolyse indoxyl acetate but not casein, lecithin or starch. One of the three strains (F79-2) produces urease (33.3%). Hydrogen sulphide is not produced in triple-sugar iron agar medium.

The type strain is F79-6[T] (=CECT 7837[T] = LMG 26155[T]), isolated from a sample of mussels from the Ebro Delta, Spain.

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nostic Center (MDC), Orihuela, Spain. A. L. is indebted to Universitat Rovira i Virgili for a doctoral grant. We also thank Jean Euzeby for helping to correct the epithet for the species name.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2011.04.004.

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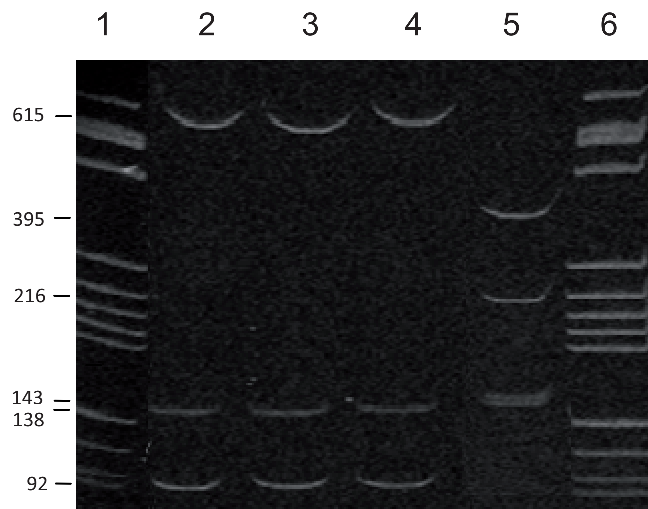


Figure S1: Polyacrylamide gel showing the common 16S DNA-RFLP pattern obtained for the strains of the new species (Lanes 2-4, F79-2; F79-6^T; F79-7 respectively), which differed from the pattern described for *A. cryaerophilus* (lane 5, LMG 9904^T) and for the other *Arcobacter* spp. [8, 15, 16]. Lanes: 1 and 6, pBR322 DNA/*Bsu*RI (*Hae*III) ladder (Fermentas).

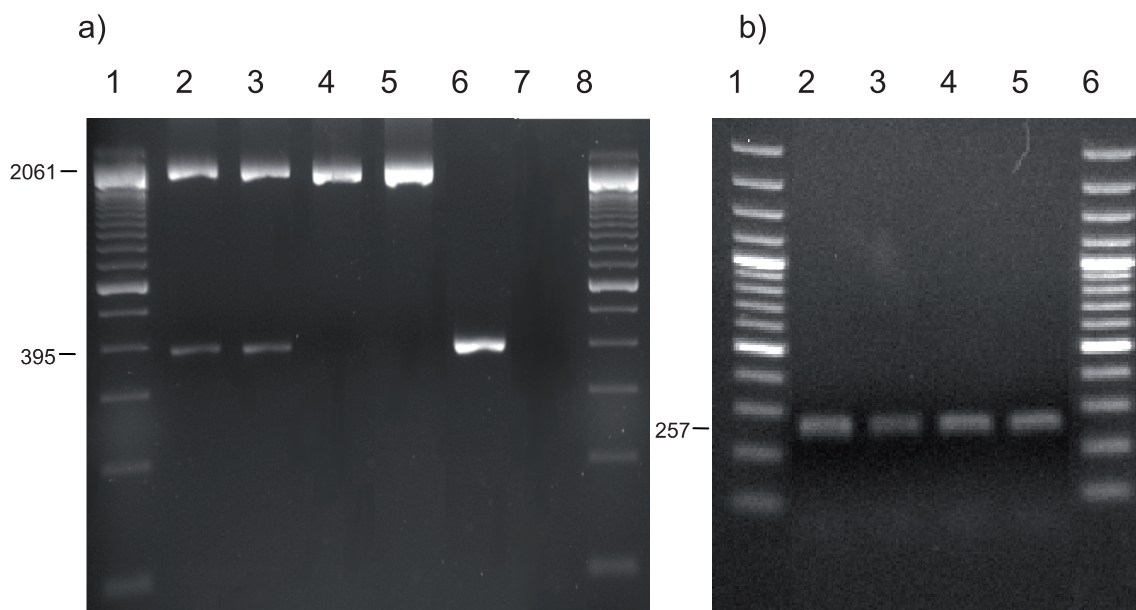


Figure S2. Agarose gels showing the amplicons obtained for the new species and for other *Arcobacter* spp. with the two m-PCR methods (a: Douidah *et al*. [13]; b: Houf *et al*. [19]).

a) The three new strains (lanes 2-4, F79-2; F79-6^{T}; F79-7 respectively) showed a band similar to that expected for *A. butzleri* (lane 5, LMG 10828^{T}) and two strains (lanes 2-3, F79-2; F79-6^{T}) also presented a band of the typical size of *A. cryaerophilus* (lane 6, LMG 9904^{T}); lane 7, negative control (MilliQ water as template DNA); lanes 1 and 8, 100nt DNA Ladder (Invitrogen).

b) The strains of the new species (lanes 2-4, F79-2; F79-6^{T}; F79-7) showed a band expected for *A. cryaerophilus* (lane 5, LMG 9904^{T}); lanes: 1 and 6, ladder 100nt (Fermentas).

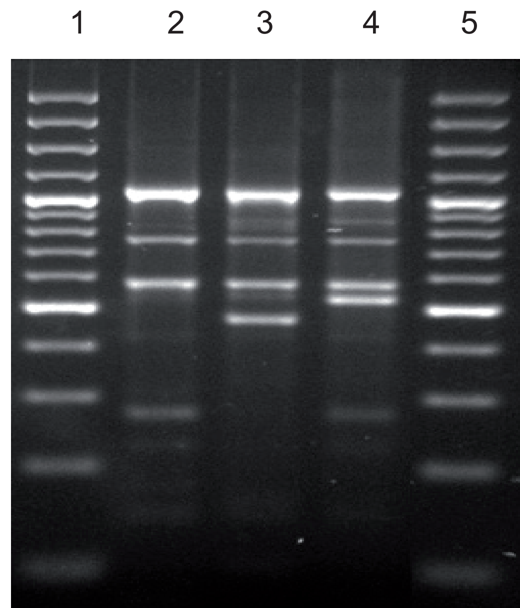
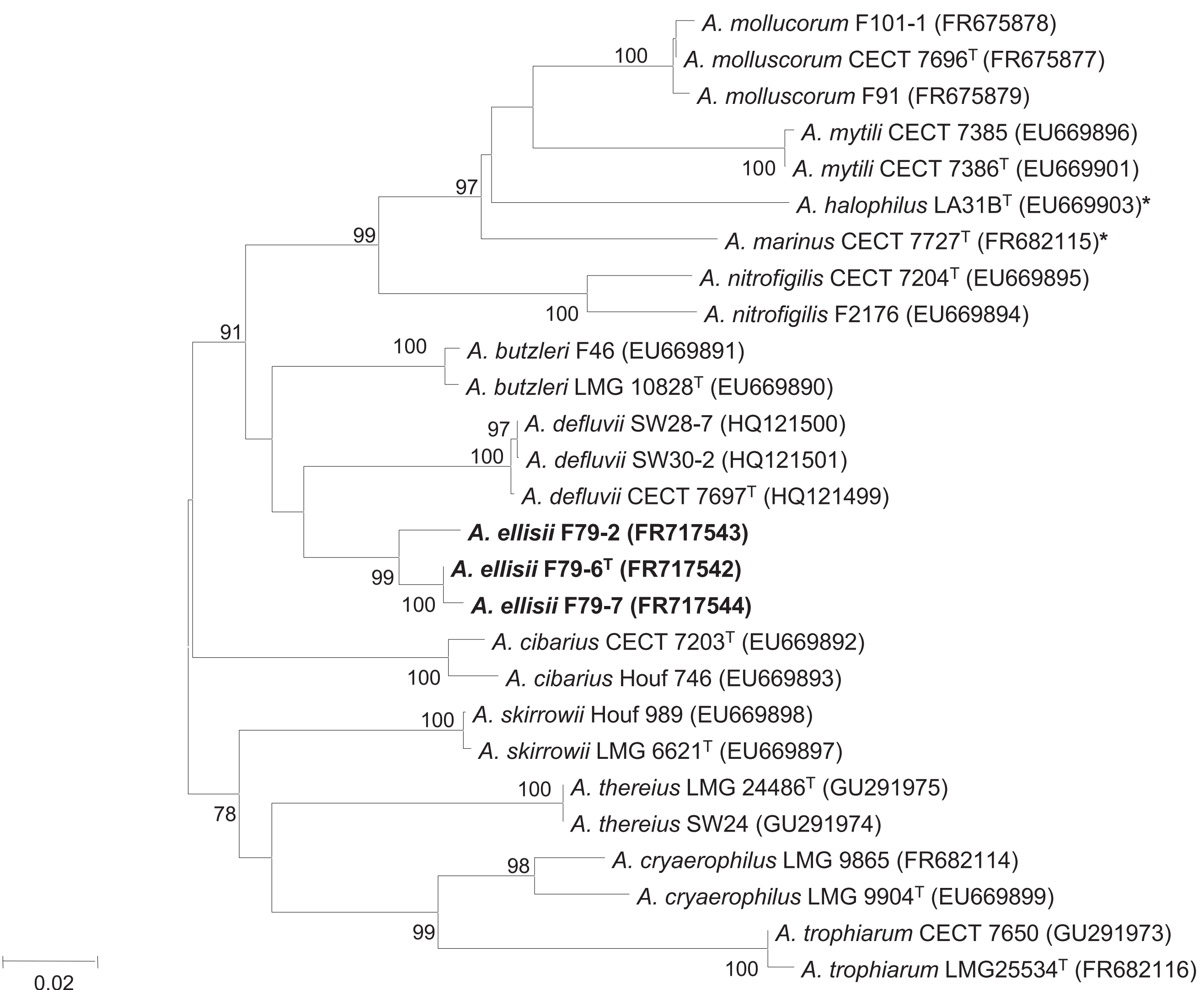


Figure S3. Agarose gel showing the three different ERIC-PCR patterns obtained for the new mussel isolates (lanes 2-4, F79-2; F79-6^{T}; F79-7 respectively). Lanes: 1 and 5, ladder 100nt (Fermentas).

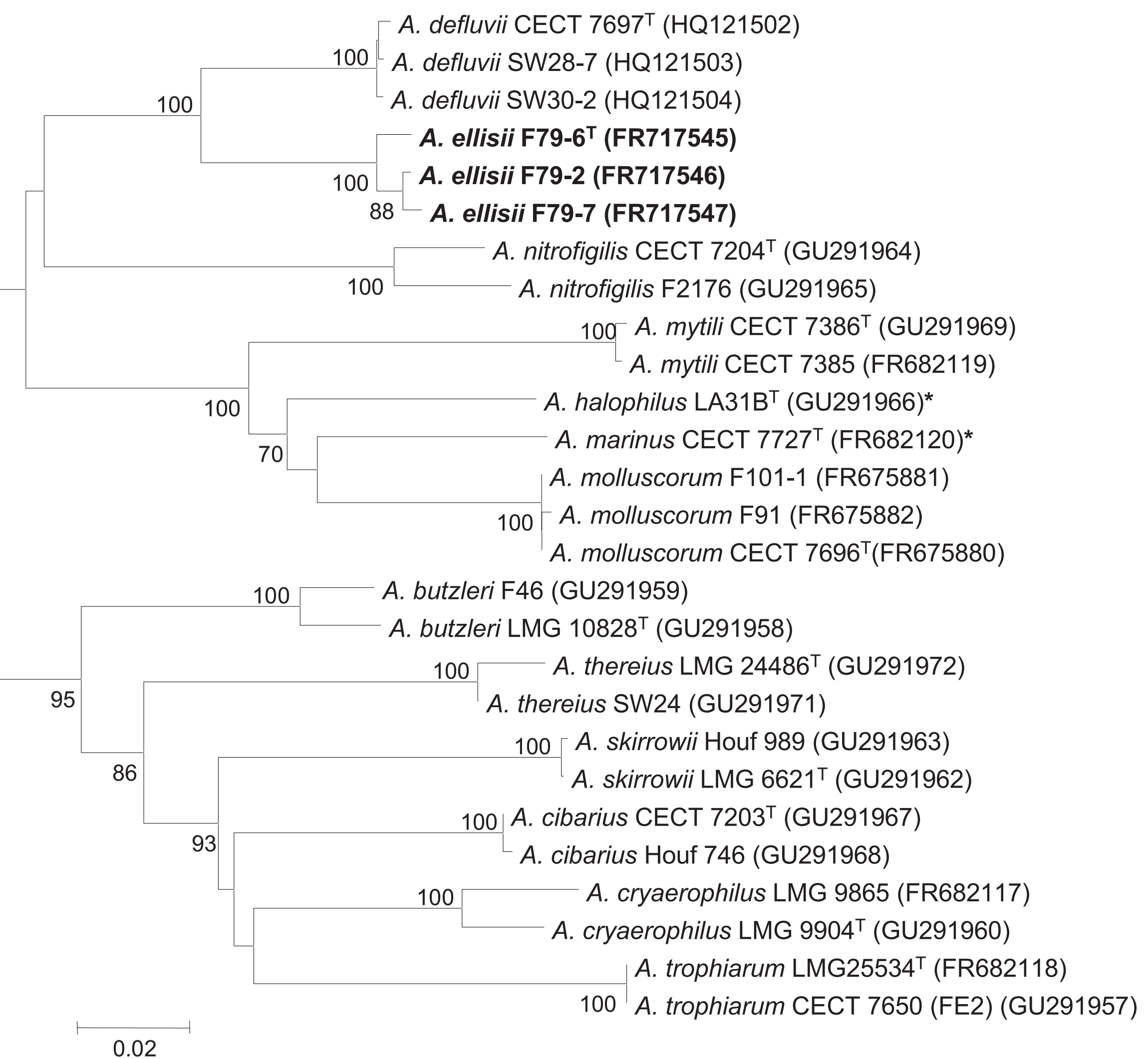
Table S1: MALDI TOF MS profiles of *Arcobacter ellisii* sp. nov and type strains of all *Arcobacter* species

A. ellisii F79-2	***A. ellisii*** F79-6T	***A. ellisii*** F79-7	***A. nitrofigilis*** CECT 7204T	***A. cryaerophilus*** LMG 9904T	***A. butzleri*** LMG 10828T	***A. skirrowii*** LMG 6621T	***A. cibarius*** CECT 7203T	***A. halophilus*** LA31BT	***A. thereius*** LMG 24486T	***A. mytili*** CECT 7386T	***A. marinus*** CECT 7727T	***A. trophiarum*** LMG 25534T	***A. defluvii*** CECT 7697T	***A. molluscorum*** CECT 7696T
4.373	**4.370**	**4.373**	4.369	4.384	3.416	3.579	3.247	4.309	4.384	3.174	3.497	3.581	3446	3.473
5.247	**5.201**	**5.248**	5.125	4.427	3.446	4.296	3.341	4.342	4.430	3.442	3.515	4.385	3.569	4.164
5.695	**5.244**	**5.734**	5.231	4.625	3.556	4.506	3.406	4.809	4.699	3.594	4.345	4.454	3.591	4.342
5.735	**5.690**	**6.119**	5.258	5.202	3.605	4.665	3.449	5.063	4.918	3.693	4.427	4.728	4.221	4.854
6.471	**5.731**	**6.467**	5.275	5.246	4.295	5.035	3.557	5.258	5.215	4.191	5.044	5.718	4.371	5.095
7.127	**6.465**	**6.829**	5.671	5.715	4.350	7.144	3.601	5.300	5.220	4.420	5.081	7.147	5.202	5.352
7.171	**6.830**	**7.168**	5.691	7.171	4.384	8.575	3.759	5.700	5.261	5.073	5.303	8.621	5.245	5.670
8.975	**7.117**	**8.972**	5.720	8.539	4.416	9.000	3.812	5.362	5.665	5.095	5.645	10.049	5.732	5.674
9.482	**7.163**	**9.478**	5.732	8.855	4.672	9.315	4.191	6.717	5.706	5.306	6.778			6.206
	10.028		7.140	9.416	5.001		4.281	6.787	6.940	5.624	7.022			6.355
					5.029		4.387	6.951	7.133	5.772	8.470			6.357
					5.229		4.444	8.437	8.345	6.341	8.690			6.838
					5.272		4.455	8.603	8.855	6.872				6.938
					5.732		4.568	8.707	9.390	7.178				7.527
					6.454		4.681	10.131		7.373				8.315
					6.826		4.703			7.532				
					6.884		4.718			8.377				
					7.204		4.804			8.636				
					8.694		5.008			8.820				
					8.832		5.016			10.144				
					9.341		5.079			12.710				
					10.048		5.175							



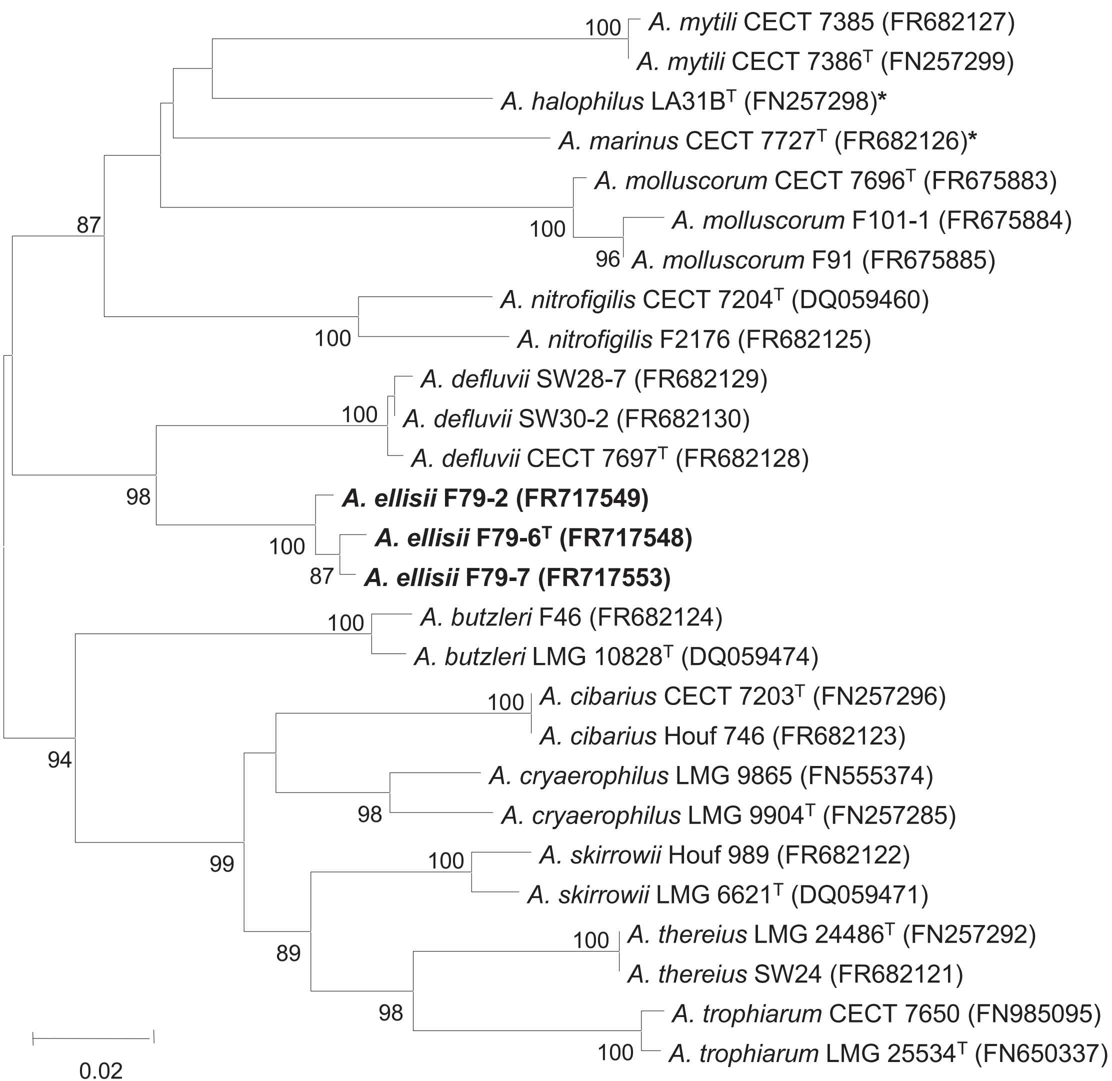
Supplementary Fig. S4. Neighbour-joining tree based on *rpoB* sequences showing the phylogenetic position of *Arcobacter ellisii* sp. nov. within the genus *Arcobacter*. Bootstrap values ($\geq$70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.

* Only type strain is available so far.



Supplementary Fig. S5. Neighbour-joining tree based on *gyrB* sequences showing the phylogenetic position of *Arcobacter ellisii* sp. nov. within the genus *Arcobacter*. Bootstrap values ($\geq$70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.

* Only type strain is available so far.



Supplementary Fig. S6. Neighbour-joining tree based on *hsp60* sequences showing the phylogenetic position of *Arcobacter ellisii* sp. nov. within the genus *Arcobacter*. Bootstrap values (≥70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.

* Only type strain is available so far.

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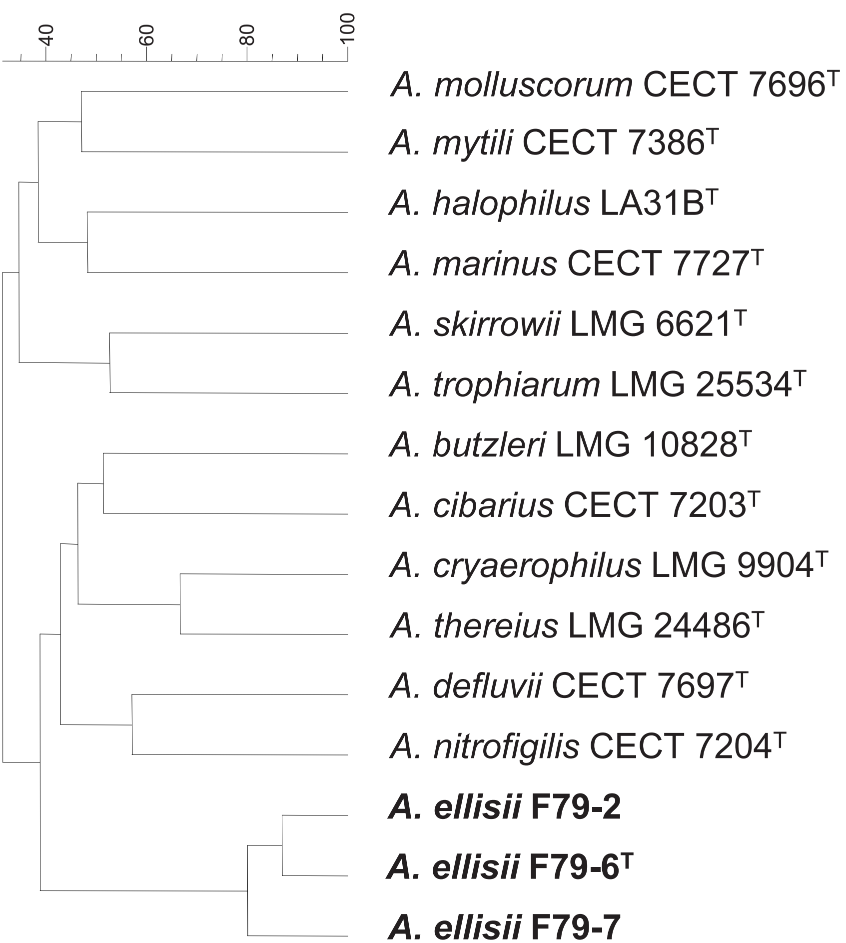

Supplementary Fig. S7. Dendrogram of MALDI-TOF comparing the mass profiles of strains of *Arcobacter ellisii* sp. nov. and those of the type strains of all *Arcobacter* species using UPGMA algorithm. The scale above the dendrogram gives percent matching mass signals between individual strains.

4.2. *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov., new species isolated from shellfish

Levican A, Collado L, Aguilar C, Yustes C, Diéguez AL, Romalde JL, Figueras MJ.

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Short communication

Arcobacter bivalviorum sp. nov. and *Arcobacter venerupis* sp. nov., new species isolated from shellfish☆

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ABSTRACT

A group of ten *Arcobacter* isolates (Gram negative, slightly curved motile rods, oxidase positive) was recovered from mussels (nine) and from clams (one). These isolates could not be assigned to any known species using the molecular identification methods specific for this genus (16S rDNA-RFLP and m-PCR). The aim of this study is to establish the taxonomic position of these isolates. The 16S rRNA gene sequence similarity of mussel strain F4^T to the type strains of all other *Arcobacter* species ranged from 91.1% to 94.8%. The species most similar to the clams' strain F67-11^T were *Arcobacter defluvii* (CECT 7697^T, 97.1%) and *Arcobacter ellisii* (CECT 7837^T, 97.0%). On the basis of phylogenetic analyses with 16S rRNA, *rpoB*, *gyrB* and *hsp60* genes, the mussel and clam strains formed two different, new lineages within the genus *Arcobacter*. These data, together with their different phenotypic characteristics and MALDI-TOF mass spectra, revealed that these strains represent two new species, for which the names *Arcobacter bivalviorum* (type strain F4^T = CECT 7835^T = LMG 26154^T) and *Arcobacter venerupis* (type strain F67-11^T = CECT 7836^T = LMG 26156^T) are proposed.

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The genus *Arcobacter* belongs to the family *Campylobacteraceae* together with the genera *Campylobacter* and *Sulfurospirillum* [3,30]. It embraces species that have been isolated from a wide diversity of habitats and hosts [3,30–32]. Vandamme et al. created the genus in 1991 [31] and expanded it in 1992 [32] to 4 species i.e. *Arcobacter nitrofigilis* (the type species of the genus) recovered for the first time from roots of *Spartina alterniflora* [23], *Arcobacter cryaerophilus* from bovine abortion foetuses [24], *Arcobacter butzleri* from human faeces [19] and *Arcobacter skirrowii* from sheep faeces [32]. Since then, it has rapidly expanded and currently includes 9 additional species, i.e. *Arcobacter cibarius* recovered from chicken meat [16], *Arcobacter halophilus* from a hypersaline lagoon [7], *Arcobacter mytili*, *Arcobacter molluscorum* and *Arcobacter ellisii* from mussels [2,11,12], *Arcobacter thereius* from porcine abortions [17], *Arcobacter marinus* from a mix of seawater, seaweed and a starfish [20], *Arcobacter trophiarum* from faeces of fattening pigs [6] and *Arcobacter defluvii* from sewage [5]. Similarity of the 16S rRNA gene within the type strains of all the species of the genus ranges from 92.0% to 99.1% [3,12]. Phylogenetic analyses based on housekeeping genes such as *gyrB*, *rpoB*, and *hsp60* have been useful for delineating closely related species and have been used in the description of the latest species, showing a good agreement with the DNA–DNA hybridization results [2,5,6,9,11,12]. The existence of several other potentially new species from diverse environments can be inferred from the16S rRNA gene sequences deposited in public databases [3,9].

The importance of the genus *Arcobacter* lies in the fact that some species are considered emerging enteropathogens and potential zoonotic agents [3,30]. The frequent isolation of species of the genus from foods of animal origin and from water suggests that these are the transmission routes of these bacteria [3,4,14]. Regarding this, in a recent study in seafood, which is often eaten uncooked, 100% of the clams and 41% of the mussel samples were positive for *Arcobacter* spp. [4]. In that study, the isolates were identified using two molecular identification methods for *Arcobacter* spp. in parallel, a multiplex PCR (m-PCR) [18] and a 16S rDNA restriction fragment length polymorphism (16S rDNA-RFLP) [10], although one strain

☆ The GenBank/EMBL/DDBJ accession numbers of the sequences of strain F4^T, F67-11^T, for the 16S rRNA gene are FJ573217 and HE565359, and for the *rpoB*, *hsp60* and *gyrB* genes are HE565362–HE565364 and HE565374–HE565376, respectively. The sequences for 16S rRNA, *rpoB*, *hsp60* and *gyrB* genes for the strains F118-2, F118-3 and F118-4 had also been deposited (HE565357, HE565358, HE565365–HE565373 and HE575529).

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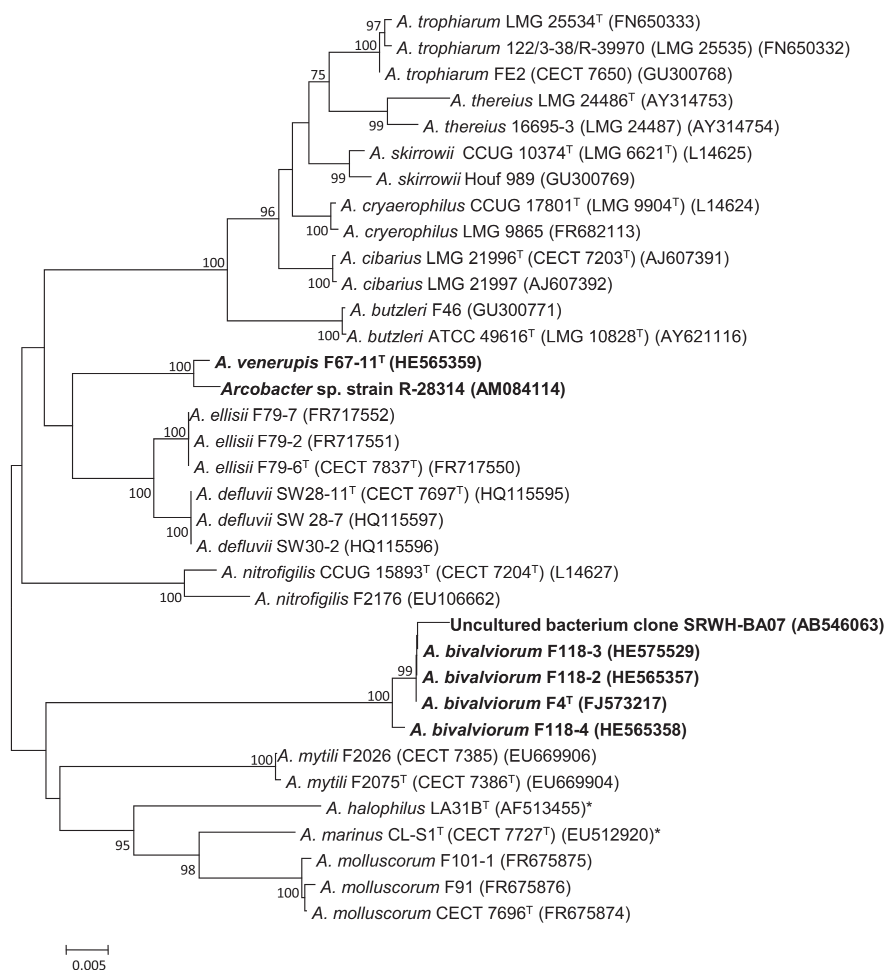

Fig. 1. Neighbour-joining tree based on 16S rRNA sequences showing the phylogenetic position of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70%) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 1000 nt. *Only type strain is available so far.

recovered from mussels (F4) could not be assigned to any known species [4]. This strain F4 appeared to be a potentially new phylogenetic line on the basis of its 16S rRNA gene sequence [4] but it remained undescribed while waiting for the isolation of other similar strains. In a more recent shellfish survey, eight *Arcobacter* isolates from mussels that were identified using the mentioned methods (m-PCR and 16S rDNA-RFLP) appeared to resemble strain F4. Furthermore, a strain recovered from a sample of clams could not be properly identified, either. The objective of the present study was to study the taxonomic position of strain F4 and the other nine isolates from mussels and clams.

Eight *Arcobacter* isolates from mussels (F118-2, F118-3, F118-4, F118-5, F118-6, F118-7, F118-8 and F118-9) were recovered from a sample collected from the Ebro delta, Catalonia (northeast Spain) in September 2010. Strain F4 was isolated from mussels in the same area in March 2007 [4], while strain F67-11^T was recovered from a sample of clams collected in January 2009 from Ferrol, Galicia (northwest Spain). All *Arcobacter* isolates were identified as such on the basis of their colony morphology on blood agar (small, translucent, beige to off-white) and their phenotypic characteristics (Gram negative, slightly curved, motile rods that produce oxidase activity) [4,5,11,12].

Considering that the 8 mussel isolates were recovered from the same sample and that isolate F4 also came from the same locality, all of them together with the clam isolate (F67-11^T) were genotyped using the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), as described previously [15]. The 8 mussel isolates showed only 3 distinctive ERIC patterns (for which isolates F118-2, F118-3 and F118-4 were chosen as the representatives) different from those shown by strain F4 (Fig. S1) and F67-11^T (data not shown).

Identification of the strains was initially attempted using two m-PCR [8,18] and the 16S rDNA-RFLP *Arcobacter* identification methods [10] in parallel. The mussel strains (F118-2, F118-3 and F118-4) behaved like strain F4 [4], i.e. they produced an amplicon of the expected size described for *A. cryaerophilus* with the m-PCR of Houf et al. [18] and the same new RFLP pattern of strain F4, which was different from the patterns shown by all other known *Arcobacter* spp. [2,5,10–12] (Figs. S2 and S3). In addition, strains F4^T (selected as the type), F118-2, F118-3 and F118-4 did not show any

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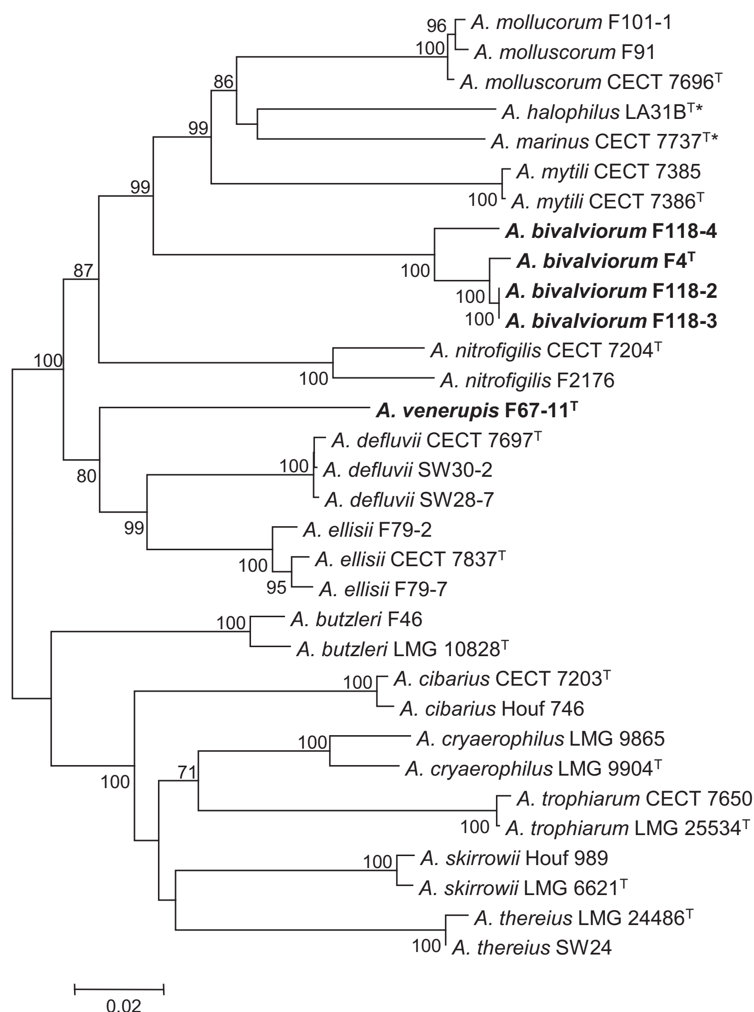

Fig. 2. Neighbour-joining tree based on the concatenated *hsp60, rpoB and gyrB* sequences showing the phylogenetic position of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. within the genus *Arcobacter*. Bootstrap values (>70%) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt. *Only type strain is available so far.

amplicon when tested with the new m-PCR designed to identify the most common *Arcobacter* species by Douidah et al. [8] (Fig. S2). The strain obtained from clams (F67-11^T) also showed different results depending on which identification method was used. With the m-PCR of Houf et al. [18] the strain showed an amplicon similar to the one described for *A. cryaerophilus* and one similar to that described for *A. butzleri* with the m-PCR of Douidah et al. [8] (Fig. S2). With the 16S rDNA-RFLP *Arcobacter* identification method [10], it showed a pattern (308, 243, 141, 138, 100, 52 nt) that could be confused with the one described for *A. marinus* [11] (308, 243, 138, 100, 52 nt) (Fig. S3).

The 16S rRNA, *rpoB*, *gyrB* and *hsp60* genes were amplified, sequenced and analysed as previously described [2,5,11]. The similarity of the 16S rRNA gene sequences was determined using EzTaxon software [1]. The 16S rRNA (1401 nt), *rpoB* (487 nt), *gyrB* (665 nt) and *hsp60* (555 nt) gene sequences were independently aligned using MEGA software version 5 [28] and CLUSTAL W [22]. Genetic distances were obtained using Kimura's two-parameter model [21] and clustered with the neighbour-joining [26], maximum parsimony and maximum likelihood methods using MEGA software version 5 [28]. The neighbour joining phylogenetic tree obtained with the 16S rRNA gene (Fig. 1), as well as those individually constructed with *rpoB*, *gyrB* and *hsp60* (Figs. S4–S6) or their concatenated sequences (*rpoB*, *gyrB* and *hsp60*, 1651 nt) (Fig. 2), showed that the group of mussel strains (F4^T, F118-2, F118-3 and F118-4) and the strain from clams (F67-11^T) belonged to two different, unknown phylogenetic lineages within the genus *Arcobacter*. The same results were obtained with the 16S rRNA gene and the concatenated sequences (*rpoB*, *gyrB* and *hsp60*) when other algorithms i.e. maximum parsimony and maximum likelihood were used (Figs. S7–S10).

The individual and concatenated trees of the *rpoB*, *gyrB* and *hsp60* genes showed that two of the mussel isolates F118-2 and F118-3 shared the same nucleotide sequences (Fig. 2 and Figs. S4–S6), despite showing different ERIC patterns (Fig. S1). In order to verify the latter result, the ERIC assay was repeated twice using different DNA extracts each time. The two assays showed different results because two different patterns were obtained on

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Table 1
Differential characteristics of *Arcobacter bivalviorum* and *Arcobacter venerupis* spp. nov. from other members of the genus. Taxa: 1, *Arcobacter bivalviorum* (n = 3); 2, *Arcobacter venerupis* (n = 1); 3, *A. nitrofigilis* (n = 4) [2,25]; 4, *A. cryaerophilus* (n = 19) [2,25]; 5, *A. butzleri* (n = 12) [25]; 6, *A. skirrowii* (n = 9) [25]; 7, *A. cibarius* (n = 15) [16]; 8, *A. halophilus* (n = 1) [7,12]; 9, *A. mytili* (n = 3) [2]; 10, *A. thereius* (n = 8) [17]; 11, *A. marinus* (n = 1) [12,20]; 12, *A. trophiarum* (n = 11) [6,12]; 13, *A. defluvii* (n = 8) [5]; 14, *A. molluscorum* (n = 3) [11]; 15, *A. ellisii* [12]. The specific responses for type strains were identical or expressed in brackets. Unless otherwise indicated: +, ≥ 95% strains positive; −, ≤11% strains positive; V, 12–94% strains positive; CO_2 indicates microaerobic conditions; CCDA: Campylobacter Charcoal Deoxycholate Agar; TTC: triphenyl tetrazolium chloride.

Characteristics	1	2	3	4	5	6	7	8[a]	9	10	11[a]	12	13	14	15
Growth in/on															
Air at 37 °C	+	−	V(−)	V(+)	+	+	−	+	+	−	+	−	+	+	+
CO_2 at 37 °C	+	+	−	V(+)	+	+	+	+	+	−	+	−	+	+	+
CO_2 at 42 °C	−	−	−	−	V(+)	−	−	−	+	−	−	−	+	+	+
0.5% (w/v) NaCl[b]	+	+	+	+	+	+	+	−	+	+	−	+	+	+	+
4% (w/v) NaCl	+	−	+	−	−	+	−	+	+	−	+	−	−	+	−
1% (w/v) glycine	−	−	−	−	−	−	−	+	+	+	+	V(−)	−	−	−
MacConkey	−	+	−	V(−)	+	−	+	−	+	V(+)	−	V(+)[c]	+	+	V(+)
Minimal medium	−	+	−	−[d]	+	−	+	−	−	+	−	−[e]	+	−	+
0.05% safranin	−	−	−	+	+	+	+	−	−	+	+	V(+)	+	+	−
CCDA	−	+	−	+	+	+	V(−)	−	−	V(−)	−	+	+	−	+[f]
0.01% sodium deoxycholate	−	−	V(−)	V(+)	+	+	+	−	+	V(−)	−	+	+	+	+[f]
1% (w/v) oxgall	−	−	−	+	V(+)	+	+	−	+	−	−	+	+	+	−
0.04% TTC	−	−	−	+	+	V(−)	V(−)	−	−	V(−)	−	+	−	−	−
0.01% TTC	−	−	−	+	+	+	+	−	−	+	−	+	+	+	−
Resistance to															
Cefoperazone (64 mg l^{-1})	−	−	−	+	+	+	+	−	−	+	−	+	V(+)	+	−
Enzyme activity															
Catalase	+	+	+	+	V(+)	+	V(−)	−	+[g]	+	−	+	+[g]	+	+
Urease	−	+	+	−	−	−	−	−	−	−	−	−	+	−	V(−)
Nitrate reduction	−	+	+	+[h]	+	+	−	+	+[i]	+	+	−	+	+[j]	+
Indoxyl acetate hydrolysis	+	+	+	+	+	+	+	+	−	+	+	+	+	−	+

[a] For these strains, the tests were carried out on media supplemented with 2% NaCl, with the exception of 0.5 and 4% (w/v) NaCl, catalase and indoxyl acetate hydrolysis [12].
[b] Growth on 0.5% (w/v) NaCl was carried out using nutrient medium supplemented with 5% sheep blood.
[c] Strains LMG 25534^T, LMG 25535 of *A. trophiarum* and strain FE2 (CECT 7650) of this species identified in our laboratory grew on MacConkey agar in contraposition with the 80% described for this species [6,12].
[d] Two (LMG 7537 and LMG 10241) of the four strains tested were positive [2].
[e] Test not evaluated by De Smet et al. [6] but tested by Figueras et al. [12].
[f] All strains grew weakly after 5 days of incubation [12].
[g] Weak reaction [2,5].
[h] Two (LMG 9904^T and LMG 9065) of the four strains tested were negative [2].
[i] Nitrate reduction was found to be positive for the 3 strains of *A. mytili* [12] in contradiction to our previously published data [2].
[j] Nitrate is reduced after 72 h and 5 days for all strains under microaerobic and aerobic conditions, respectively [12].

one occasion and the same pattern on the other (data not shown). On the basis of these new results and on the equal sequences obtained with *rpoB*, *gyrB* and *hsp60* genes, these two isolates were considered a single strain and F118-2 was used for further analyses. This is the first time that we have seen a changing ERIC PCR pattern, and this should be taken into account in future studies for strains that show very similar ERIC PCR patterns.

The 16S rRNA gene sequences of mussel strains F4^T, F118-2 and F118-4 (1401 nt) showed a similarity that ranged from 99.6% to 100%. Direct and reverse DDH experiments were carried out between two of these strains (F4^T and F118-2) as described previously [5] and the results (82.3 ± 11.4%) confirmed that they belonged to the same new species. The 16S rRNA gene sequence similarity of the 3 mussel strains (F4^T, F118-2 and F118-4) with the clam strain F67-11^T was 93.0%, while the similarity of strain F4^T with all *Arcobacter* species ranged from 91.1% with *A. cryaerophilus* (LMG 9904^T) to 94.8% with *A. defluvii* (CECT 7697^T). All these results were relatively low and far below the 97% threshold above which DNA–DNA hybridization (DDH) experiments with other known species should be carried out [9,27].

The 16S rRNA gene sequence similarities between the strain F67-11^T from clams and the type strains of all *Arcobacter* spp. ranged from 93.2% with *A. mytili* (CECT 7386^T) to 97.1% with *A. defluvii* (CECT 7697^T), followed by *A. ellisii* (CECT 7837^T) with 97.0% and *A. nitrofigilis* (CECT 7204^T) with 96.0%. The species *A. defluvii* (CECT 7697^T) and *A. ellisii* (CECT 7837^T) were selected for DDH experiments with strain F67-11^T because were also its closest neighbours in the phylogenetic trees (Figs. 1 and 2, Figs. S5, S6 and S8). The mean results obtained from direct and reverse DNA–DNA reassociation were 56.6% (±4.5) and 63.4% (±1.5), respectively, confirming that the strain F67-11^T from clams belongs to a new and different species. A BlastN analysis of the 16S rRNA sequence of strain F67-11^T, showed a 99.5% similarity with the deposited sequence of strain R-28314 (1464 nt; GenBank AM084114) from a denitrifying bacterium isolated from activated sludge at a waste water treatment plant in Ghent (Belgium) [13]. When this sequence was added to the 16S rRNA phylogenetic tree, it clustered with a 100% bootstrap with strain F67-11^T (Fig. 1). We have tried to obtain this strain in order to add it to our study, but unfortunately it was not alive anymore (de Vos, personal communication). A similar analysis was carried out with the representative of the mussel strains (F4^T) and it showed a 99.6% similarity with the sequence of an uncultured bacterium clone SRWH-BA07 (1459 nt, GenBank AB546063) from subsurface crude oil deposits in Japan. This sequence (AB546063) was included in the 16S rRNA gene phylogenetic tree and it clustered together with mussel strains (Fig. 1). These results confirm that these two new species can be found in other geographical regions and habitats.

Phenotypic characterization was carried out using the standardized test recommended for this genus in the minimal standards for describing new species of the family *Campylobacteraceae* [29] and in other *Arcobacter* publications [5,9,11,12,25,30–32]. Parallel testing was carried out with all the type strains of the species of the genus, using appropriate positive and negative controls. When examined with the transmission electron microscope [2], the cell size and morphology of the strains was determined as well as the presence of a single polar flagellum (data not shown). Phase-contrast microscopy and wet mounts were used to confirm motility. Table 1

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shows the key distinctive characteristics between the new strains and the other *Arcobacter* spp. The 3 mussel strains (F4^T, F118-2 and F118-4) showed the same phenotypic response and could be differentiated from the rest of the species of the genus, including strain F67-11^T, with at least 3 tests. The ability of the mussel strains to grow on media containing 0.5–4% (w/v) NaCl was a common characteristic they shared with *A. nitrofigilis*, *A. skirrowii*, *A. mytili* and *A. molluscorum*, but they could be differentiated from these species by their inability to reduce nitrate, among other tests (Table 1). Therefore, nitrate reduction and growth on NaCl can be two useful, initial key characteristics that differentiate this new mussel species from the other *Arcobacter* species. Strain F67-11^T from clams could be differentiated from *A. ellisii*, its closest phenotypic species, by its inability to grow in aerobic conditions at 37 °C or on media containing 0.1% sodium deoxycholate, and from the other *Arcobacter* species with at least 5 different tests.

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) profiles of the new strains F4^T, F118-2, F118-4 and F67-11^T and other representative strains (n = 42) of all *Arcobacter* spp. (Table S1) were obtained using the Voyager DE STR (Applied Biosystems, Foster city, USA) as previously described [12]. The MALDI-TOF mass spectra derived dendrogram showed that the strains F4^T, F118-2 and F118-4 clustered together and separately from all accepted species of the genus *Arcobacter* as did strain F67-11^T (Fig. 3). This represents the most complete MALDI-TOF analysis of the genus performed so far.

On the basis of the data obtained from the genetic and phenotypic characterization, this study has shown the existence of two new *Arcobacter* species, for which the names *Arcobacter bivalviorum* (type strain F4^T = CECT 7835^T = LMG 26154^T), and *Arcobacter venerupis* (type strain F67-11^T = CECT 7836^T = LMG 26156^T) are proposed. Using the identification method proposed by Figueras et al. [10], the new species *A. bivalviorum* can be clearly differentiated from the other *Arcobacter* spp. by its new specific 16S rDNA-RFLP pattern (Fig. S3). However, in the case of *A. venerupis*, attention should be paid to a thicker RFLP band of 141–138 nt that is the only difference from the pattern obtained for *A. marinus*, that shows only a thinner band (Fig. S3). As has already been commented, misidentifications will occur with the m-PCR methods [8,18] because the two new species will produce the typical *A. cryaerophilus* amplicon with the m-PCR of Houf et al. [18] and either no amplicon or the one of *A. butzleri* with the m-PCR of Douidah et al. [8] (Fig. S2).

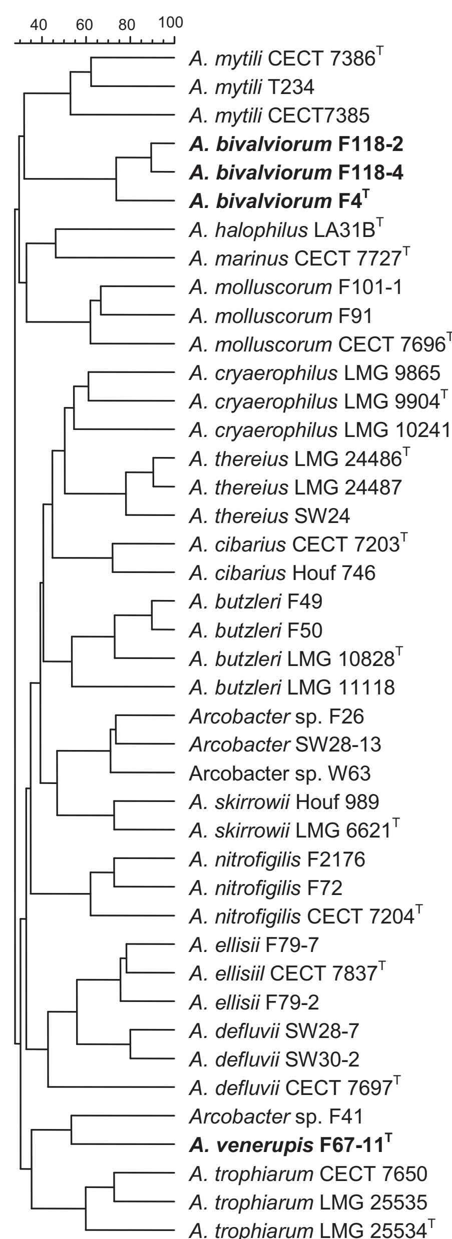

Fig. 3. Dendrogram comparing the MALDI-TOF profile similarities of strains of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. with those of representative strains of all Arcobacter species using UPGMA algorithm. The scale above the dendrogram gives percent matching mass signals between individual strains.

Description of *A. bivalviorum* sp. nov.

A. bivalviorum (bi.val.vi'o.rum. N.L. pl. neut.n. Bivalvia, scientific name of a class of molluscs; N.L. neut. gen. pl. n. bivalviorum, of bivalves of the class Bivalvia).

Cells of strains F4^T, F118-2, F118-4 are Gram-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.3–0.5 μm wide and 0.9–2.0 μm long. They are motile by a single polar flagellum. Colonies on blood agar incubated in aerobic conditions at 30 °C for 48 h are 2–4 mm in diameter, beige to off-white, circular with entire margins, convex, and non-swarming. Pigments are not produced. All the strains grow on blood agar at room temperature (18–22 °C), 30 °C and 37 °C but not at 42 °C under both aerobic and microaerobic conditions, however, growth is weak under anaerobic conditions at 30 °C. No haemolysis is observed on TSA medium supplemented with 5% sheep blood. Strains produce oxidase and catalase activity; hydrolyse indoxyl acetate but not casein, lecithin or starch; do not reduce nitrate or produce urease. Hydrogen sulphide is not produced in triple-sugar iron agar medium. Under aerobic conditions at 30 °C all the strains grow on Marine agar and on nutrient medium (0.5%, w/v NaCl) supplemented with 5% sheep blood and also on this medium containing 2% or 4% (w/v) NaCl. No growth occurs on minimal medium, Campylobacter Charcoal Deoxycholate Agar (CCDA), MacConkey agar or on nutrient media supplemented with 5% sheep blood containing 1% glycine; 0.1% sodium deoxycholate; 1% oxgall; 0.01%, 0.04% or 0.1% 2,3,5 triphenyl tetrazolium chloride (TTC); 0.001% brilliant green; 0.05% safranin; 0.0005% crystal violet; 0.005% basic fuchsin and medium with 64 mg l^{-1} cefoperazone.

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The type strain is F4^T (=CECT 7835^T = LMG 26154^T) isolated from mussels of the Ebro Delta, Spain.

Description of *A. venerupis* sp. nov.

A. venerupis (ve.ne.ru'pis. N.L. n. Venerupis, scientific generic name of Marine bivalve molluscs; N.L. gen. n. venerupis of Venerupis, isolated from the clam species *Venerupis pullastra*).

Cells of strain F67-11^T are Gram-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.3–0.6 μm wide and 0.9–2.2 μm long. It is motile by a single polar flagellum. Colonies on blood agar incubated under aerobic conditions at 30 °C for 48–72 h are 1–3 mm in diameter, beige to off-white, circular with entire margins, convex, and non-swarming. Pigments are not produced. The strain grows on blood agar at room temperature (18–22 °C), 30 °C and 37 °C but not at 42 °C under microaerobic conditions. Under aerobic conditions, it grows well at room temperature (18–22 °C) and 30 °C but not at 37 °C or 42 °C. No growth is observed under anaerobic conditions at 30 °C. No haemolysis is observed on TSA medium supplemented with 5% sheep blood. Strain produces oxidase, catalase and urease activity, reduces nitrate and hydrolyses indoxyl acetate but not casein, lecithin or starch. Hydrogen sulphide is not produced in triple-sugar iron agar medium. Under aerobic conditions at 30 °C the strain F67-11^T grows on minimal medium, MacConkey agar, Marine agar or CCDA medium and on nutrient medium (0.5%, w/v NaCl) supplemented with 5% sheep blood and also on this medium containing 2% (w/v) NaCl. No growth occurs on nutrient medium supplemented with 5% sheep blood containing 4% (w/v) NaCl; 1% glycine; 0.1% sodium deoxycholate; 1% oxgall; 0.01%, 0.04% or 0.1% 2,3,5 TTC; 0.001% brilliant green; 0.05% safranin; 0.0005% crystal violet; 0.005% basic fuchsin and medium with 64 mg l^{-1} cefoperazone.

The type strain is F67-11^T (=CECT 7836^T = LMG 26156^T) isolated from a sample of clams from the locality of Ferrol, Galicia, Spain.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2012.01.002.

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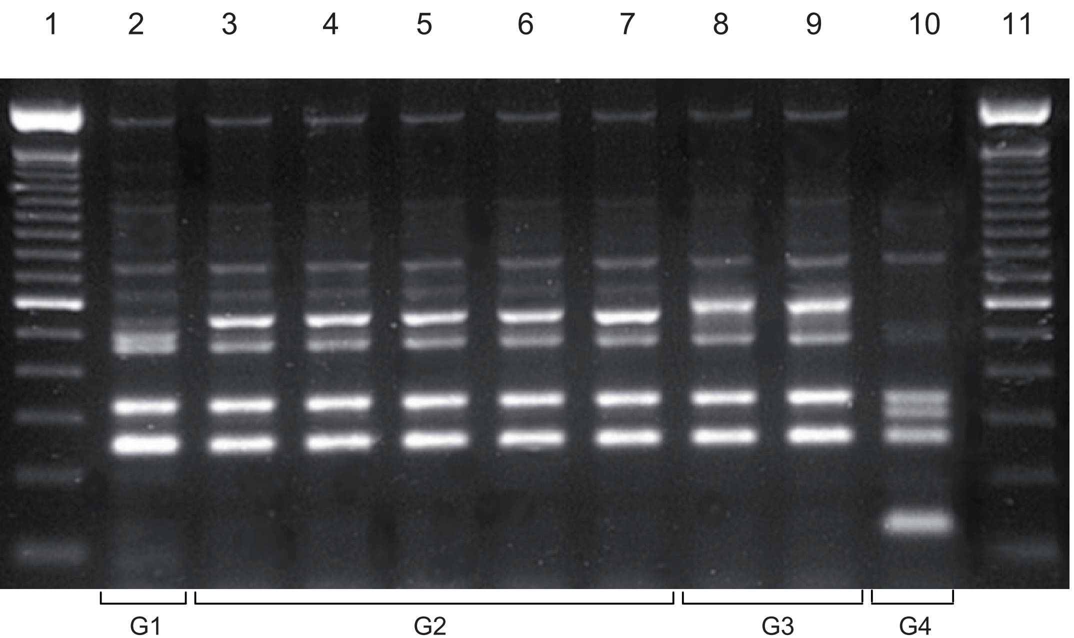
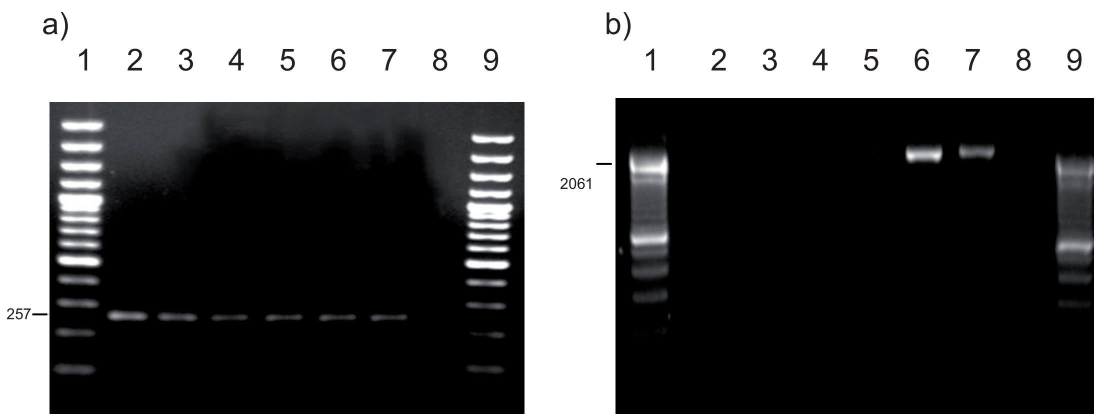
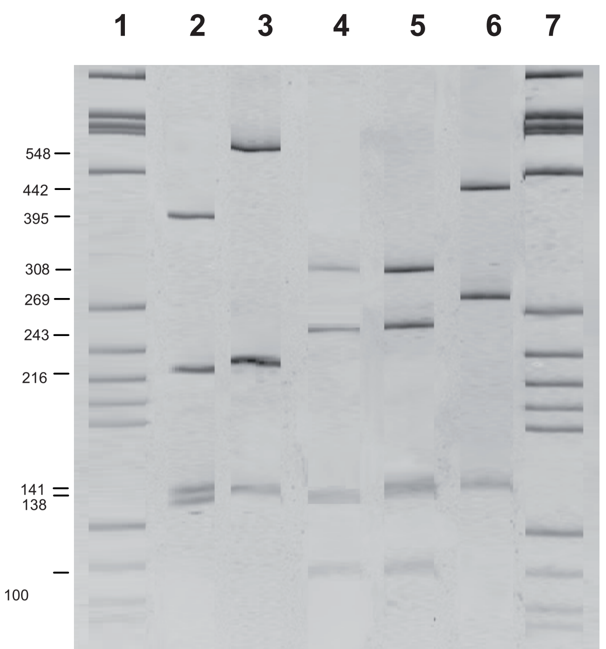


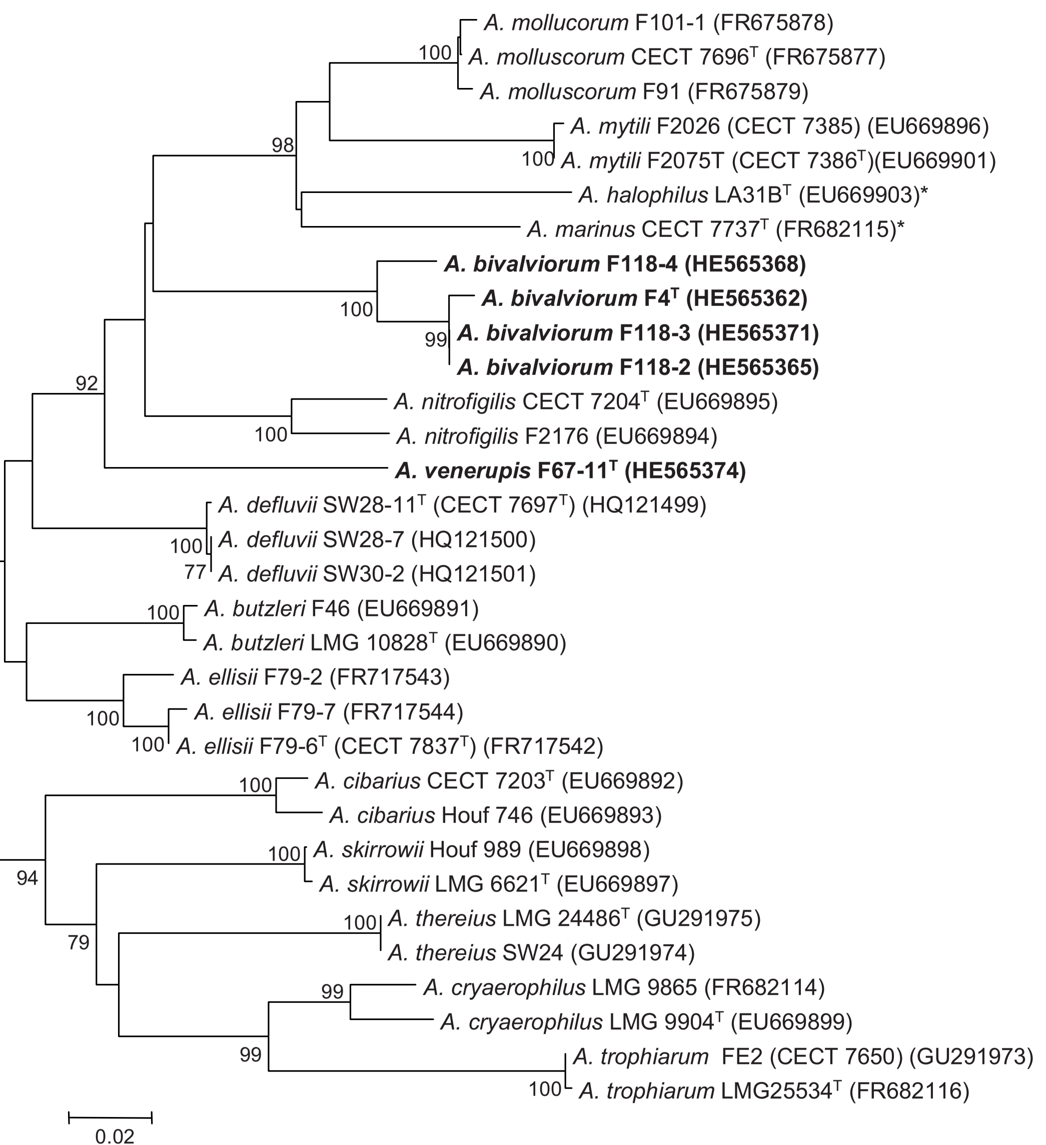
Suplementary Fig. S1 Agarose gel showing the ERIC-PCR patterns (G=Genotypes) of strain F4 and of the 8 additional new mussel isolates of *A. bivalviorum*. Lanes: 1 and 11, Ladder 100 nt (Invitrogen); Genotype 1: 2, F4; Genotype 2: 3, F118-2; 4, F118-5; 5, F118-6; 6, F118-8; 7, F118-9; Genotype 3: 8, F118-3; 9, F118-7; Genotype 4: 10, F118-4.



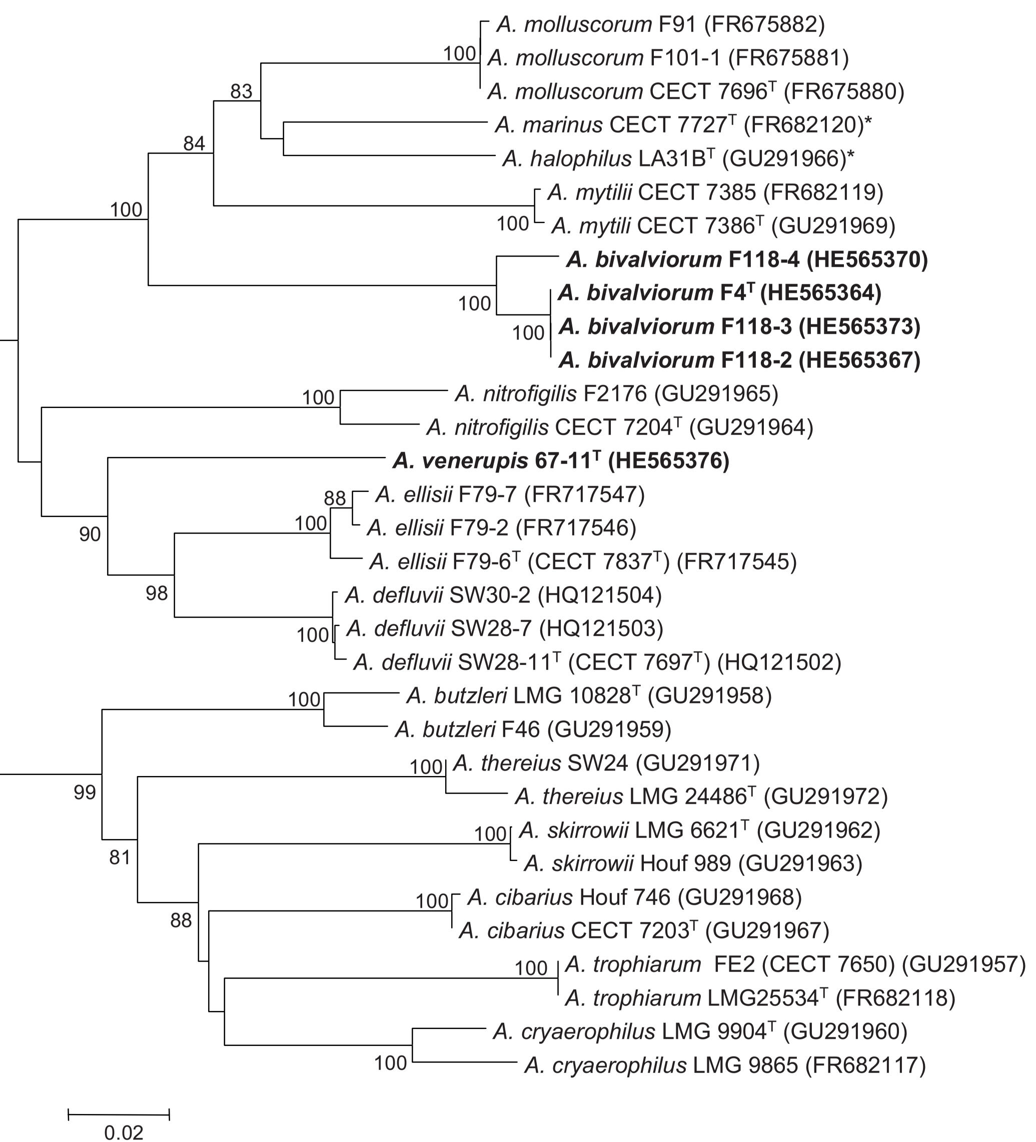
Supplementary Fig. S2. a-b: Agarose gels showing the amplicons obtained for the two new species *A. bivalviorum* and *A. venerupis* and other *Arcobacter* spp. with two m-PCR methods: a, Houf *et al*. [18]; b, Douidah *et al*. [8].
a) The four isolates of *A. bivalviorum* (lanes 3, F4^{T}; 4, F118-2; 5, F118-3; 6, F118-4) and the one of *A. venerupis* (lane 7, F67-11^{T}) showed a band similar to that expected for *A. cryaerophilus* (lane 2, LMG 9904^{T}); lanes: 1 and 9, 100 nt DNA plus Ladder (Fermentas); 8, negative control (MiliQ water as template DNA).
b) The isolates of *A. bivalviorum* (lanes 2, F4^{T}; 3, F118-2; 4, F118-3; 5, F118-4) did not produce an amplicon and the strain of *A. venerupis* (lane 7, F67-11^{T}) showed a band of the same size to that expected for *A. butzleri* (lane 6, LMG 10828^{T}); lanes: 1 and 9, Ladder 100 nt (Invitrogen); 8, negative control (MiliQ water as template DNA).



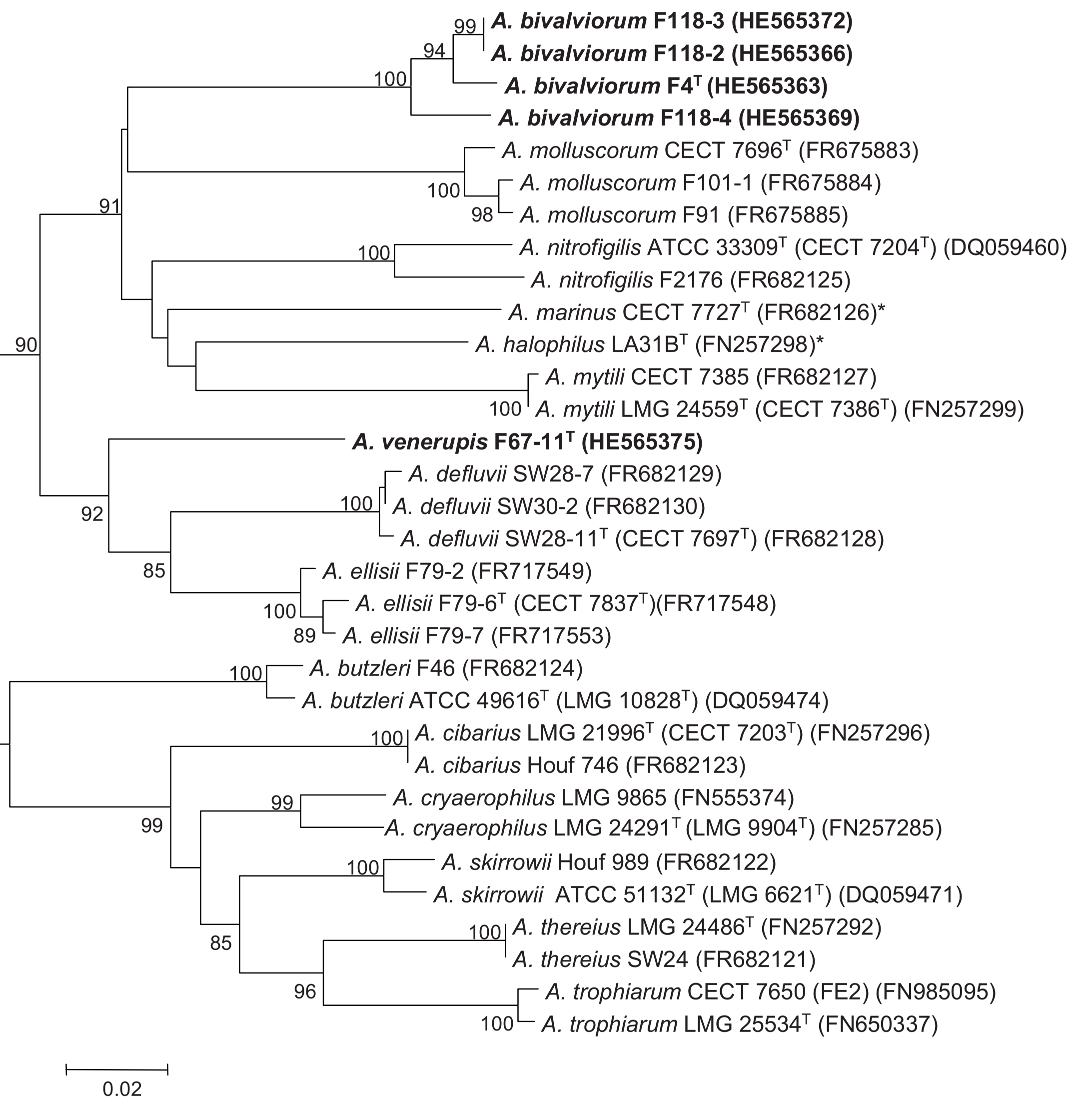
Suplementary Fig. S3: Polyacrylamide gel comparing the 16S DNA-RFLP patterns obtained for *A. venerupis* and *A. bivalviorum* with those for *A. cryaerophilus*, *A. butzleri* and *A. marinus* [10, 11]. Lanes: 1 and 7, pBR322 DNA/*Bsu*RI (*Hae*III) ladder (Fermentas); 2, *A. cryaerophilus* (LMG 9904^T); 3, *A. butzleri* (LMG 10828^T); 4, *A. marinus* (CECT 7727^T); 5, *A. venerupis* (F67-11^T); 6, *A. bivalviorum* (F4^T). The pattern of *A. bivalviorum* differs from those described for the rest of *Arcobacter* spp. [2, 5, 10-12]. Notice that the pattern of *A. venerupis* shows a double band of 141-138 nt, while the one of *A. marinus* has a single band of 138 nt. These bands agree with the result found with the computer simulation of the digestion with the *Mse*I enzyme of the 1026 nt of 16S rRNA gene sequences [10] of strains F67-11^T and CECT 7727^T.



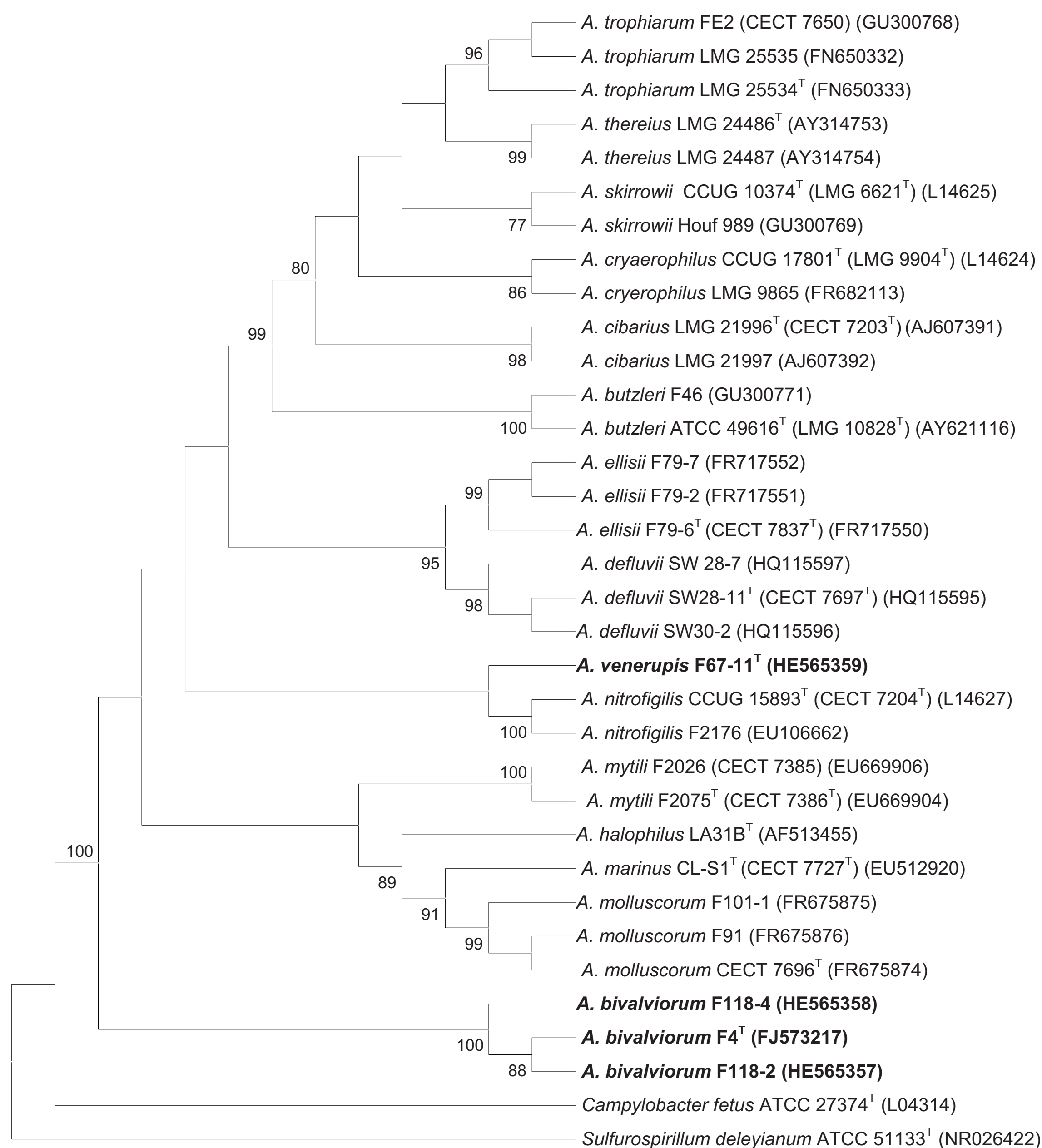
Supplementary Fig. S4. Neighbour-joining tree based on *rpoB* sequences showing the phylogenetic position of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. within the genus *Arcobacter*. Bootstrap values (≥70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.
* Only type strain is available so far.



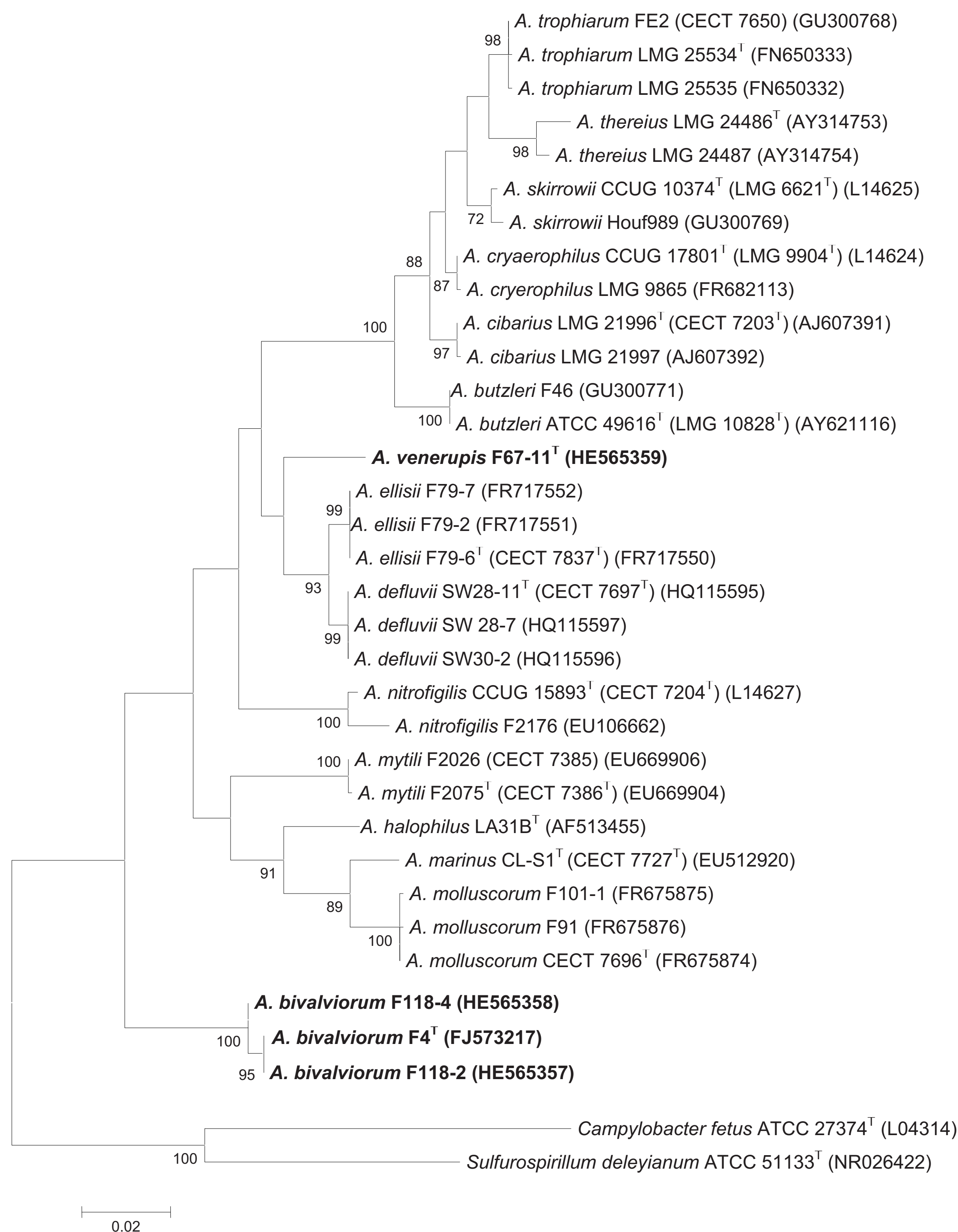
Supplementary Fig. S5. Neighbour-joining tree based on *gyrB* sequences showing the phylogenetic position of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. within the genus *Arcobacter*. Bootstrap values (≥70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.
* Only type strain is available so far.



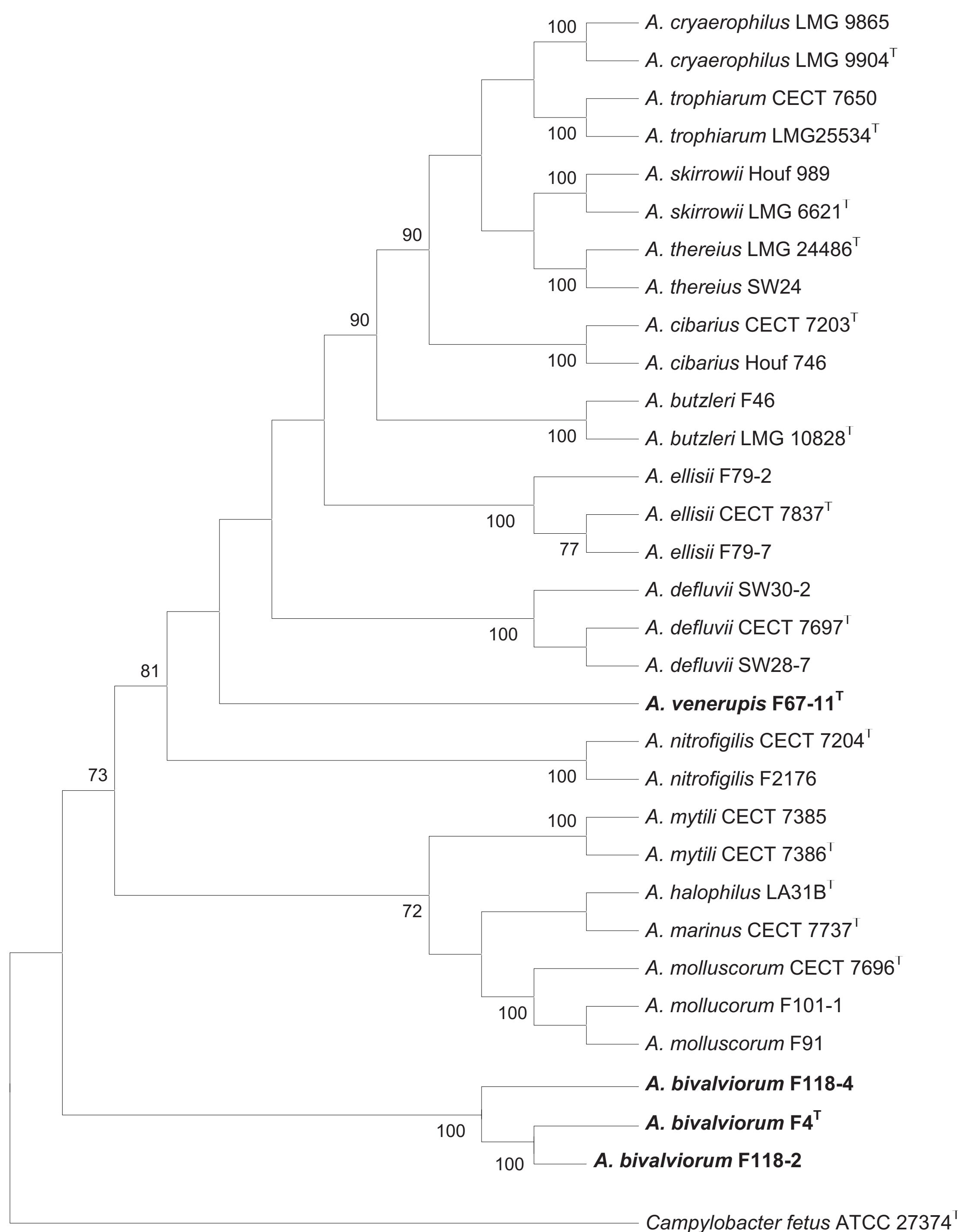
Supplementary Fig. S6. Neighbour-joining tree based on *hsp60* sequences showing the phylogenetic position of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. within the genus *Arcobacter*. Bootstrap values (≥70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.
* Only type strain is available so far.



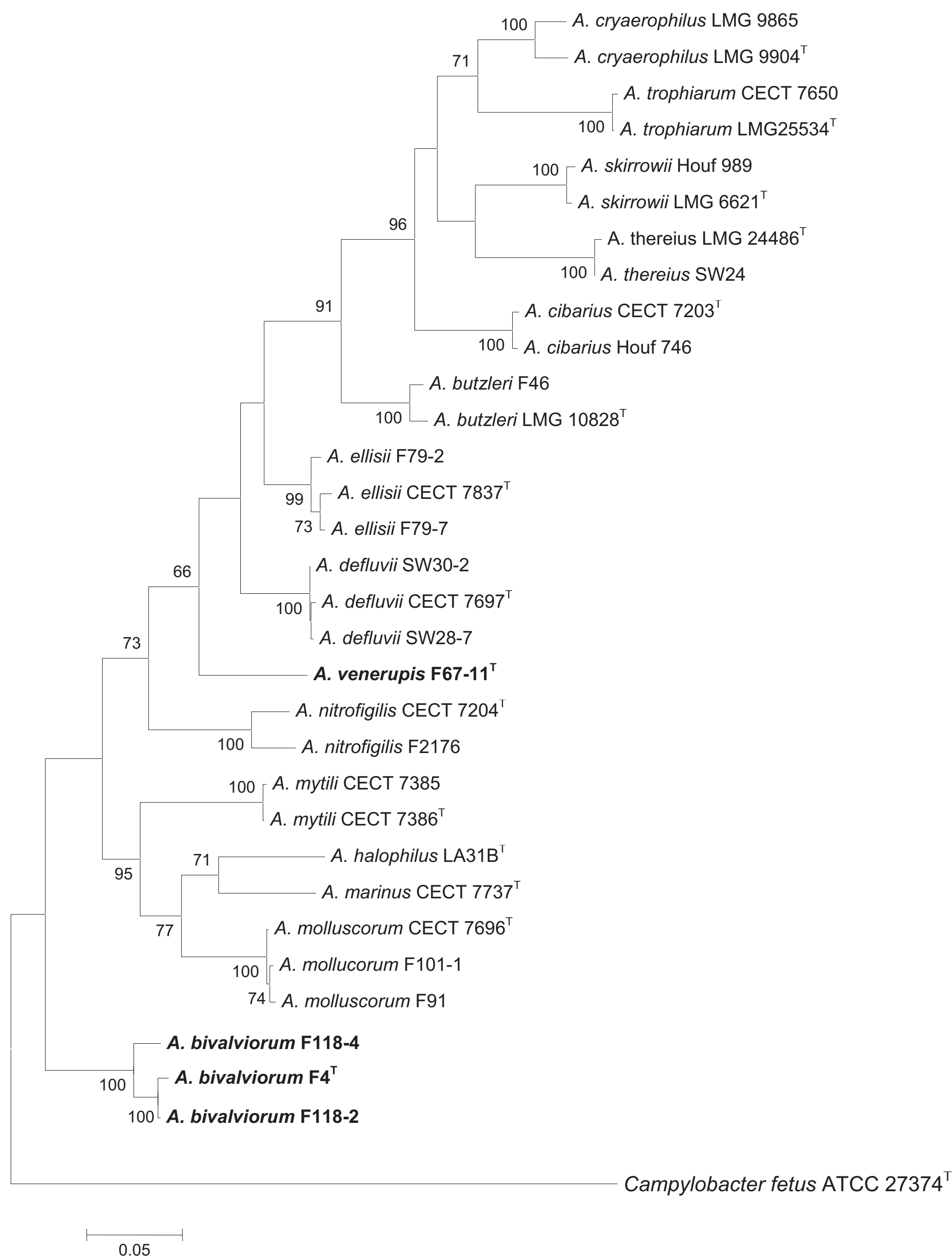
Supplementary Fig. S7. Maximum parsimony tree based on 16S rRNA sequences showing the phylogenetic position of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. within the genus *Arcobacter.* Bootstrap values ($\geq$70 %) based on 1000 replications are shown at the nodes of the tree.



Supplementary Fig. S8. Maximum likelihood tree based on 16S rRNA sequences showing the phylogenetic position of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. within the genus *Arcobacter.* Bootstrap values (≥70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.



Supplementary Fig. S9. Maximum parsimony tree based on the concatenated *hsp60*, *rpoB* and *gyrB* sequences showing the phylogenetic position of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. within the genus *Arcobacter*. Bootstrap values (≥70 %) based on 1000 replications are shown at the nodes of the tree.



Supplementary Fig. S10. Maximum likelihood tree based on the concatenated *hsp60*, *rpoB* and *gyrB* sequences showing the phylogenetic position of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. within the genus *Arcobacter*. Bootstrap values (≥70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 100 nt.

Table S1: MALDI TOF MS profiles (*m*/*z*) of *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov. and representative strains of all *Arcobacter* species

A. bivalviorum	***A. bivalviorum***	***A. bivalviorum***	***A. venerupis***	***A. butzleri***	***A. butzleri***	***A. butzleri***	***A. butzleri***	***A. cibarius***	***A. cibarius***
F4T	**F118-2**	**F118-4**	**F67-11^T**	F46	LMG 10828^T	LMG 11118	F50	CECT 7203^T	Houf 746
3,413	**3,297**	**3,299**	**3,588**	3,232	3,416	3,232	3,232	3,247	3,246
3,474	**3,409**	**3,413**	**4,235**	3,418	3,446	3,608	3,419	3,341	3,408
3,582	**3,581**	**4,185**	**4,374**	3,448	3,556	4,366	3,448	3,406	3,451
4,151	**3,603**	**4,323**	**4,708**	3,557	3,605	4,386	3,558	3,449	3,600
4,321	**4,186**	**4,356**	**5,061**	3,607	4,295	4,424	3,607	3,557	4,276
4,357	**4,323**	**4,537**	**5,697**	4,218	4,350	4,659	4,217	3,601	4,385
4,373	**4,356**	**4,760**	**5,737**	4,297	4,384	4,719	4,255	3,759	4,453
4,538	**4,538**	**4,788**	**6,424**	4,365	4,416	5,275	4,297	3,812	4,679
4,761	**4,760**	**5,080**	**6,795**	4,386	4,672	5,734	4,367	4,191	4,700
5,081	**5,082**	**5,286**	**6,822**	4,423	5,001	6,457	4,385	4,281	5,013
5,287	**5,287**	**5,329**	**7,126**	4,659	5,029	6,829	4,659	4,387	5,197
5,330	**5,329**	**5,757**	**7,166**	4,717	5,229	6,883	4,710	4,444	5,245
5,691	**5,720**	**6,288**	**7,537**	4,822	5,272	7,206	4,823	4,455	5,723
5,720	**5,758**	**6,595**	**8,457**	4,994	5,732	8,728	4,994	4,568	6,480
5,760	**6,285**	**6,817**	**9,403**	5,038	6,454	8,844	5,031	4,681	6,484
6,288	**6,593**	**7,106**	**9,916**	5,230	6,826	9,315	5,230	4,703	6,799
6,800	**6,816**	**7,161**	**10,106**	5,273	6,884		5,272	4,718	6,882
7,195	**7,156**	**8,367**		5,731	7,204		5,730	4,804	7,104
7,343	**7,193**	**9,071**		6,454	8,694		6,452	5,008	7,188
8,368	**8,365**	**9,514**		6,826	8,832		6,824	5,016	8,541
9,512	**9,066**	**10,157**		6,881	9,341		6,882	5,079	8,894
9,666	**9,513**			7,099	10,048		7,095	5,175	9,388
10,153	**10,161**			7,202			7,204	5,199	
				8,425			8,421	5,247	
				8,718			8,572	5,572	
				8,837			8,713	5,725	
				9,303			8,834	5,756	
				9,973			9,301	6,485	
							9,971	6,803	
							10,040	6,889	
								7,106	
								7,193	
								7,618	
								8,894	
								9,393	
								10,004	

Table S1-Continued

A. cryaerophilus	*A. cryaerophilus*	*A. cryaerophilus*	*A. defluvii*	*A. defluvii*	*A. defluvii*	*A. ellisii*	*A. ellisii*	*A. ellisii*	*A. halophilus*	*A. marinus*
LMG 9904^T	LMG 9865	LMG 10241	CECT 7697^T	SW28-7	SW30-2	F79-2	F79-6^T	F79-7	LA31B^T	CECT 7727^T
4,384	3,188	3,196	3446	3,228	3,227	4,373	4,370	4,373	4,309	3,497
4,427	3,404	3,419	3,569	3,590	3,590	5,247	5,201	5,248	4,342	3,515
4,625	3,425	3,426	3,591	4,369	4,369	5,695	5,244	5,734	4,809	4,345
5,202	3,587	3,565	4,221	5,198	5,024	5,735	5,690	6,119	5,063	4,427
5,246	4,174	3,576	4,371	5,200	5,200	6,471	5,731	6,467	5,258	5,044
5,715	4,279	4,174	5,202	5,204	5,244	7,127	6,465	6,829	5,300	5,081
7,171	4,386	4,256	5,245	5,244	5,732	7,171	6,830	7,168	5,700	5,303
8,539	4,411	4,386	5,732	5,732	6,450	8,975	7,117	8,972	5,362	5,645
8,855	4,465	4,413		6,451	6,826	9,482	7,163	9,478	6,717	6,778
9,416	4,637	4,597		6,825	7,079		10,028		6,787	7,022
	4,702	4,611		6,859	7,134				6,951	8,470
	4,943	4,627		7,094	7,175				8,437	8,690
	5,016	4,689		7,132	8,441				8,603	
	5,200	4,977		7,174	8,914				8,707	
	5,247	5,023		8,440					10,131	
	5,720	5,201								
	6,369	5,245								
	6,801	5,705								
	6,841	6,124								
	7,166	6,382								
	8,342	6,824								
	8,550	7,120								
	8,817	8,337								
	9,395	8,499								
	9,876	8,818								

Table S1-Continued

A. molluscorum	***A. molluscorum***	***A. molluscorum***	***A. mytili***	***A. mytili***	***A. mytili***	***A. nitrofigilis***	***A. nitrofigilis***	***A. nitrofigilis***	***A. skirrowii***
CECT 7696[T]	F91	F101-1	CECT 7386[T]	CECT 7385	T234	CECT 7204[T]	F2176	F72	LMG 6621[T]
3,473	4,164	3,474	3,174	3,009	3,439	4,369	3,188	3,186	3,579
4,164	4,259	4,164	3,442	3,174	3,593	5,125	3,422	3,293	4,296
4,342	4,313	4,313	3,594	3,358	3,692	5,231	3,573	3,420	4,506
4,854	4,345	4,331	3,693	3,443	3,694	5,258	3,585	3,439	4,665
5,095	4,856	4,346	4,191	3,693	4,321	5,275	4,135	3,572	5,035
5,352	5,076	4,855	4,420	4,191	4,330	5,671	4,226	4,219	7,144
5,670	5,098	5,074	5,073	4,259	4,343	5,691	4,297	4,295	8,575
5,674	5,351	5,095	5,095	4,262	4,420	5,720	4,372	4,370	9,000
6,206	5,635	5,276	5,306	4,420	4,734	5,732	4,405	4,479	9,315
6,355	5,674	5,578	5,624	4,790	4,790	7,140	4,569	4,756	
6,357	6,206	5,634	5,772	5,661	5,057		4,779	4,776	
6,838	6,311	5,675	6,341	5,769	5,070		5,196	5,124	
6,938	6,357	6,208	6,872	5,772	5,091		5,233	5,192	
7,527	6,838	6,358	7,178	6,338	5,188		5,277	5,229	
8,315	6,939	6,584	7,373	6,866	5,273		5,689	5,274	
	7,234	6,838	7,532	7,181	5,310		5,732	5,688	
	7,528	6,875	8,377	7,542	5,623		6,367	5,728	
	7,640	6,940	8,636	8,378	5,626		6,835	6,334	
	8,012	7,236	8,820	8,628	5,662		6,876	6,362	
	8,752	7,529	10,144	8,826	5,771		7,138	6,696	
	8,319	8,320	12,710	9,568	6,337		8,261	6,831	
	8,619	8,617			6,864		8,441	7,134	
	9,278	8,750			7,177		8,802	8,428	
	9,696	9,700			7,539			8,797	
	10,148	10,152			7,545			9,510	
	11,161				8,394			10,223	
					8,627				
					8,997				
					9,562				
					10,154				
					11,523				
					12,704				

Table S1-Continued

A. skirrowii	*A. thereius*	*A. thereius*	*A. thereius*	*A. trophiarum*	*A. trophiarum*	*A. trophiarum*	*Arcobacter* sp.	*Arcobacter* sp.	*Arcobacter* sp.	*Arcobacter* sp.
Houf 989	LMG 24486[T]	LMG 24487	SW24	LMG 25534[T]	CECT 7650	LMG 25535	SW28-13	F26	W63	F41
3,432	4,384	3,474	3,474	3,581	4,388	3,580	3,244	3,244	3,245	3,412
3,577	4,430	3,565	3,570	4,385	4,455	4,176	3,419	3,419	3,420	3,580
3,590	4,699	4,299	4,309	4,454	4,731	4,385	3,448	3,448	3,607	3,591
4,294	4,918	4,385	4,385	4,728	5,040	4,452	3,563	3,563	4,221	4,187
4,343	5,215	4,424	4,428	5,718	5,719	4,728	3,607	3,607	4,302	4,240
4,463	5,220	4,685	4,698	7,147	6,381	4,984	4,218	4,222	4,311	4,291
4,650	5,261	4,917	4,919	8,621	6,793	5,188	4,304	4,308	4,386	4,372
5,032	5,665	5,017	5,015	8,899	7,152	5,718	4,326	4,385	4,682	4,725
5,244	5,706	5,036	5,217	10,049	7,246	6,374	4,386	4,434	4,740	4,804
5,699	6,940	5,216	5,261		7,530	6,799	4,434	4,569	5,060	4,969
6,796	7,133	5,261	5,704		8,350	7,145	4,570	4,685	5,212	5,058
6,854	8,345	5,703	6,367		8,621	7,526	4,595	4,737	5,254	5,698
7,146	8,855	6,366	6,796		8,900	8,030	4,739	4,806	5,744	5,734
8,576	9,390	6,794	6,936		9,447	8,347	4,795	4,973	5,752	6,420
8,921		6,934	7,132		9,975	8,616	5,059	5,059	6,478	6,813
9,423		7,125	8,342		10,050	8,888	5,213	5,213	6,820	6,861
10,051		8,372	8,605			9,442	5,254	5,254	6,897	7,150
		8,581	8,850			9,953	5,742	5,742	7,117	7,530
		8,839	9,387			10,052	6,474	5,755	7,203	8,365
		9,356	10,017				6,825	6,830	8,587	9,438
		10,031					6,880	6,477	8,431	8,469
							7,115	6,646	8,490	8,630
							7,199	6,882	10,095	9,922
							8,378	7,051		
							8,417	7,120		
							10,090	7,202		
								8,432		
								8,491		
								8,600		
								9,461		
								9,583		
								9,928		
								10,095		

4.3. *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov., two new species isolated from food and sewage

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Short communication

Arcobacter cloacae sp. nov. and *Arcobacter suis* sp. nov., two new species isolated from food and sewage☆

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ABSTRACT

Three strains recovered from mussels (F26), sewage (SW28-13^T) and pork meat (F41^T) were characterized as *Arcobacter*. They did not appear to resemble any known species on the basis of their 16S rDNA-RFLP patterns and the *rpoB* gene analyses. However, strains F26 and SW28-13^T appeared to be the same species. The 16S rRNA gene sequence similarity of strains SW28-13^T and F41^T to the type strains of all other *Arcobacter* species ranged from 94.1% to 99.6% and 93.4% to 98.8%, respectively. Phenotypic characteristics and the DNA–DNA hybridization (DDH) results showed that they belonged to 2 new *Arcobacter* species. A multilocus phylogenetic analysis (MLPA) with the concatenated sequences of 5 housekeeping genes (*gyrA*, *atpA*, *rpoB*, *gyrB* and *hsp60*) was used for the first time in the genus, showing concordance with the 16S rRNA gene phylogenetic analysis and DDH results. The MALDI-TOF mass spectra also discriminated these strains as two new species. The names proposed for them are *Arcobacter cloacae* with the type strain SW28-13^T (=CECT 7834^T = LMG 26153^T) and *Arcobacter suis* with the type strain F41^T (=CECT 7833^T = LMG 26152^T).

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The genus *Arcobacter*, created by Vandamme et al. in 1991 [29], belongs to the family *Campylobacteraceae* and embraces Gram-negative, motile and oxidase positive, slightly curved, rod-shaped bacteria [28]. It currently includes 15 species, nine of which were isolated from environmental samples: *Arcobacter nitrofigilis*, from the roots of *Spartina alterniflora* [21]; *Arcobacter halophilus*, from a hypersaline lagoon [7]; *Arcobacter mytili*, *Arcobacter molluscorum*, *Arcobacter ellisii*, *Arcobacter bivalviorum* and *Arcobacter venerupis*, from shellfish [2,11,13,20]; *Arcobacter marinus*, isolated from both seawater and starfish [18]; and *Arcobacter defluvii*, from sewage [5]. The other six species have been described from human or animal sources: *Arcobacter butzleri* from human faeces, *Arcobacter cryaerophilus*, *Arcobacter skirrowii* and *Arcobacter trophiarum* from animal faeces [6,17,22,30]; *Arcobacter cibarius* from chicken meat [14] and *Arcobacter thereius* from porcine abortion [15]. The taxonomy of this genus has changed substantially in recent years and nine of the species have been described since 2009 [3,20]. The analysis of the 16S rRNA gene sequences deposited in GenBank indicates that there are many potentially new *Arcobacter* species that have yet to be characterized [31].

In a study that investigated the prevalence of *Arcobacter* spp. in different kinds of food [4], two strains, one from pork meat (F41^T) and the other from mussels (F26), did not resemble any *Arcobacter* species known at that time on the basis of their 16S rDNA Restriction Fragment Length Polymorphism patterns (16S rDNA-RFLP) [10]. The *rpoB* gene of both strains was sequenced and provided further evidence that they belonged to two new *Arcobacter* species but had not been described while waiting for new strains to be isolated. No other strains with the characteristics of F41^T has since been found, but another strain (SW28-13^T) isolated from sewage of a Waste Water Treatment Plant (WWTP) was found to be similar to strain F26 on the basis of its *rpoB* gene and 16S rDNA-RFLP pattern. The aim of this study was to use a polyphasic approach in order to characterize the strains F41^T, F26 and SW28-13^T as belonging to two new *Arcobacter* species.

Strain (F41^T) was recovered from pork meat purchased from a retail market, the mussel strain (F26) was collected from the Ebro river delta (both in March 2008), and the sewage strain (SW28-13^T) was isolated in March 2009 from a WWTP in the city of Reus, Spain. All strains had the expected colony morphology for *Arcobacter* species, i.e., small, translucent, beige to off-white on blood agar, and were characterized as Gram-negative, slightly curved, motile rods that produce oxidase activity [2,5,11,13,20,27]. Strains were identified by two different m-PCRs [8,16] and by the 16S rDNA-RFLP

☆ The GenBank/EMTBL/DDBJ accession numbers of the sequences of strain SW28-13^T, F26 and F41^T, for the 16S rRNA gene are HE565360, HE565361 and FJ573216, respectively, while the *gyrA, atpA, rpoB, gyrB* and *hsp60* genes of all *Arcobacter* strains included are JF802986 to JF803234 and HE997169 to HE997171.

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Table 1
Differential characteristics of *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov. from other members of the genus.

Characteristics	1	2	3	4	5	6	7	8[a]	9	10	11[a]	12	13	14	15	16	17
Growth in/on																	
Air at 37 °C	+	–	V(–)	V(+)	+	+	–	+	+	–	+	–	+	+	+	+	–
CO_2 at 37 °C	+	–	–	V(+)	+	+	+	+	+	–	+	–	+	+	+	+	+
CO_2 at 42 °C	–	–	–	–	V(+)	–	–	–	+	–	–	–	+	+	+	–	–
0.5% (w/v) NaCl	+	+	+	+	+	+	+	–	+	+	–	+	+	+	+	+	+
4% (w/v) NaCl	–	–	+	–	–	+	–	+	+	–	+	–	–	+	–	+	–
1% (w/v) glycine	–	–	–	–	–	–	–	+	+	+	+	V(–)	–	–	–	–	–
0.05% safranin	+	–	–	+	+	+	+	–	–	+	+	V(+)	+	+	–	–	–
0.1% sodium deoxycholate	+	+	V(–)	V(+)	+	+	+	–	+	V(–)	–	+	+	+	+[b]	–	–
1% (w/v) oxgall	+	–	–	+	V(+)	+	+	–	+	–	–	+	+	+	–	–	–
0.04% TTC	–	–	–	+	+	V(–)	V(–)	–	–	V(–)	–	+	–	–	–	–	–
0.01% TTC	+	+	–	+	+	+	+	–	–	+	–	+	+	+	–	–	–
Minimal medium	V(+)	+	–	–[c]	+	–	+	–	–	+	–	–[d]	+	–	+	–	+
MacConkey	+	+	–	V(–)	+	–	+	–	+	V(+)	–	V(+)[e]	+	+	V(+)	–	+
CCDA	+	–	–	+	+	+	V(–)	–	–	V(–)	–	+	+	–	+[b]	–	+
Resistance to																	
Cefoperazone (64 mg l^{-1})	–	–	–	+	+	+	+	–	–	+	–	+	V(+)	+	–	–	–
Enzyme activity																	
Catalase	+	+	+	+	V(+)	+	V(–)	–	+[f]	+	–	+	+[f]	+	+	+	+
Urease	–	–	+	–	–	–	–	–	–	–	–	–	+	–	V(–)	–	+
Nitrate reduction	+	+	+	+[g]	+	+	–	+	+[h]	+	+	–	+	+[i]	+	–	+
Indoxyl acetate hydrolysis	+	+	+	+	+	+	+	+	–	+	+	+	+	–	+	+	+

Taxa: 1, *Arcobacter cloacae* (*n* = 2); 2, *Arcobacter suis* (*n* = 1); 3, *A. nitrofigilis* (*n* = 4) [2,23]; 4, *A. cryaerophilus* (*n* = 19) [2,23]; 5, *A. butzleri* (*n* = 12) [23]; 6, *A. skirrowii* (*n* = 9) [23]; 7, *A. cibarius* (*n* = 15) [14]; 8, *A. halophilus* (*n* = 1) [7,13]; 9, *A. mytili* (*n* = 3) [2]; 10, *A. thereius* (*n* = 8) [15]; 11, *A. marinus* (*n* = 1) [13,18]; 12, *A. trophiarum* (*n* = 11) [6,13]; 13, *A. defluvii* (*n* = 8) [5]; 14, *A. molluscorum* (*n* = 3) [11]; 15, *A. ellisii* (*n* = 3) [13]; 16, *A. bivalviorum* (*n* = 3) [20]; 17, *A. venerupis* (*n* = 1) [20]. The specific responses for type strains were coincidental or otherwise expressed in brackets. Unless otherwise indicated: +, ≥95% strains positive; –, ≤11% strains positive; V, 12–94% strains positive; CO_2 indicates microaerobic conditions. TTC: 2,3,5-triphenyl tetrazolium chloride, CCDA: Campylobacter Charcoal Deoxycholate Agar.

[a] For these strains, testing was carried out on media supplemented with 2% NaCl, with the exception of 0.5 and 4% (w/v) NaCl, catalase and indoxyl acetate hydrolysis [13].
[b] All strains grew weakly after 5 days of incubation.
[c] Two (LMG 7537 and LMG 10241) of the four strains tested were positive [2].
[d] Test not evaluated by De Smet et al. [6] but by Figueras et al. [13].
[e] Strains LMG 25534^T, LMG 25535 of *A. trophiarum* and strain FE2 (CECT 7650) of this species identified in our laboratory all grew on MacConkey agar, contrary to 80% of the strains described for this species [6].
[f] Weak reaction [2,5].
[g] Two (LMG 9904^T and LMG 9065) of the four strains tested were negative [2].
[h] Nitrate reduction was positive for the 3 strains of *A. mytili* [11], contrary to our previously published data [2].
[i] Nitrate is reduced after 72 h and 5 days for all strains under microaerobic and aerobic conditions, respectively [13].

[10] specific for the genus, but discordant results were obtained with all three methods. Briefly, all strains (SW28-13^T, F26 and F41^T) produced an amplicon of the size described for *A. cryaerophilus* with the m-PCR of Houf et al. [16]; while with the m-PCR of Douidah et al. [8], strains SW28-13^T and F26 showed no amplification and strain F41T produced the expected amplicon for *A. butzleri*. Strains SW28-13^T and F26, on the other hand, produced a 16S rDNA-RFLP pattern that was different from any previously described for other *Arcobacter* spp. [10,12] (Figs. S1 and S2), and strain F41^T produced an RFLP pattern the same as the recently described species *A. defluvii* [5]. Nevertheless, with the newly proposed 16S rDNA-RFLP *Arcobacter* identification method that uses the *Bfa*I endonuclease, strain F41^T showed a species-specific RFLP pattern (580, 175, 169, and 87 bp.) different to *A. defluvii* (405, 184, 175, 93, 87 and 83 bp.) [12] (Fig. S2).

Strains SW28-13^T, F26 and F41^T were motile under the phase contrast microscope and a single polar flagellum could be seen under the transmission electron microscope (data not shown), which was also used to measure the cell size and to define the morphology of the strains, as in previous studies [2]. All strains were phenotypically characterized in parallel with the type strains of all *Arcobacter* species using the tests recommended for the family *Campylobacteraceae* and the genus *Arcobacter* [27], including those used in previous studies [2,5,11,23]. Table 1 shows the key traits that differentiate strains SW28-13^T, F26 and F41^T from other *Arcobacter* spp. The pork meat strain F41^T is unable to grow under aerobic and microaerobic conditions at 37 °C, a characteristic only previously observed for species *A. thereius* and *A. trophiarum*, despite both species having been isolated from warm blooded animals, such as pigs and ducks [6,15]. This could therefore be considered as the first discriminating trait for this species.

The 16S rRNA (1401 bp) and *gyrB* (618 bp) genes were sequenced and analysed as previously described [2,5], and the *gyrA* (686 bp), *atpA* (622 bp), *rpoB* (621 bp), *hsp60* (587 bp) as described elsewhere (L. Collado, M.J. Figueras, A. Levican & A.J. Martínez-Murcia, in preparation). EzTaxon software was used for similarities [1] and MEGA software version 5 [26] and CLUSTAL W [19] for alignments, for calculating genetic distances and for clustering using the neighbour-joining, maximum parsimony and maximum likelihood algorithms [26]. The 16S rRNA gene sequence similarity of strains SW28-13^T and F26 was 99.8% and the former was chosen as the type strain. The similarity of strain SW28-13^T to all *Arcobacter* spp., including strain F41^T, ranged from 94.1% (common to *A. halophilus* and *A. mytili*) to 99.6% (*A. ellisii*), while similarity of the strain F41^T with the other species ranged from 93.4% (*A. mytili*) to 98.9% (*A. defluvii*). In the maximum parsimony phylogenetic tree produced from the 16S rRNA gene (1401 bp) (Fig. 1), and also using other algorithms (Figs. S3 and S4), strains SW28-13^T and F26 grouped close to the species *A. ellisii* and *A. defluvii* but formed an independent phylogenetic line, as did the strain F41^T (Fig. 1).

Direct and reverse DNA–DNA hybridization (DDH) experiments were carried out for the new strains and those that showed a 16S rRNA gene sequence similarity of 97% or higher (Table 2) using the methodology described in a previous study [5], and all results were under 70% (Table 2), thus corroborating that the strains SW28-13^T and F41^T represented two new species. Furthermore, DDH experiments were carried out for strains SW28-13^T and F26 and results confirmed that they belonged to the same species (Table 2).

A multilocus phylogenetic analysis (MLPA) was carried out by concatenating 5 housekeeping genes (*gyrA*, *atpA*, *rpoB*, *gyrB* and *hsp60*, 3134 bp) (Fig. 2), as recommended by the "ad hoc committee

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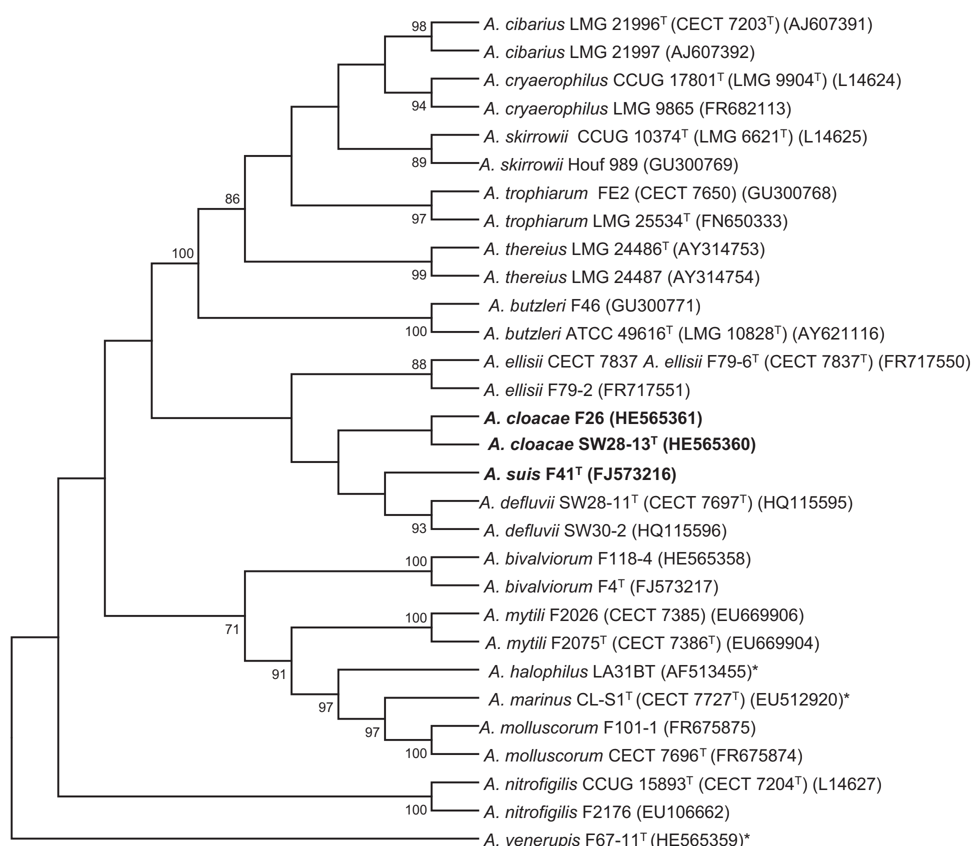

Fig. 1. Maximum parsimony tree based on 16S rRNA (1401 bp) sequences showing the phylogenetic position of *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov. within the genus *Arcobacter*. Bootstrap values (≥70%) based on 1000 replications are shown at the nodes of the tree. *Only type strain is available so far.

for the re-evaluation of the species definition in bacteriology" [24]. Both the neighbour joining phylogenetic tree (Fig. 2) and those constructed with other algorithms (Figs. S5 and S6) showed that the new strains were close to *A. venerupis*, *A. defluvii* and *A. ellisii* but formed two independent, unknown, phylogenetic lines within the genus. These results are consistent with others based on 16S rRNA phylogeny, DDH and the phenotypic characterization.

The 16S rRNA gene sequence similarities between strain SW28-13^T and its closest species *A. ellisii* is the highest (99.6%) reported between different *Arcobacter* spp., which have so far ranged from 91.1% (for *A. cryaerophilus* and *A. bivalviorum*) [20] to 99.1% (for *A. defluvii* and *A. ellisii*) [13]. However, the phylogenetic tree clearly differentiated all species, as did the DDH results. These results confirm once more that for some *Arcobacter* species the classical 97% 16S rRNA similarity suggested for the selection of strains for DDH experiments [25] is not useful [6,11,13–15,20]. Housekeeping genes have been more discriminative than the 16S rRNA gene for *Arcobacter* species in previous studies [2,5,11,13,20]. The concordance between the taxonomic delineation of *Arcobacter* spp. provided by the MLPA and the 16S rRNA gene and DDH indicates, as suggested for other genera [9], that the MLPA is a clearly alternative method to DDH and 16S rRNA gene. The resolution of the MLPA is better and the overall phylogenetic relatedness more robust (bootstrap values of 100% for all the species clusters).

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) profiles of strains SW28-13^T, F26 and F41^T were obtained as described by Figueras et al. [13]. The analysis included the type strains of all *Arcobacter* spp. (Table S1) and the resulting dendrogram showed that the strains SW28-13^T and F26 clustered together, clearly separate from all accepted species, as was strain F41^T (Fig. S7).

Table 2
16S rRNA gene similarity (%) and DNA–DNA relatedness (% ±SD) of *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov. with other species of the genus.

	16S rRNA gene similarity		DDH	
	SW28-13^T	F41^T	SW28-13^T	F41^T
A. cloacae sp. nov. SW28-13^T		98.6%		58.6% (±2.9)
A. cloacae sp. nov. F26	99.8%		88.6% (±0.6)	
A. suis sp. nov. F41^T	98.6%		58.6% (±2.9)	
A. defluvii CECT 7697^T	99.1%	98.9%	49.5% (±6.7)	55.9% (±10)
A. ellisii CECT 7837^T	99.6%	98.8%	64.4% (±2.2)	66.0% (±1.6)
A. venerupis CECT 7836^T	97.0%	97.6%	57.4% (±4.9)	59.0% (±4.2)

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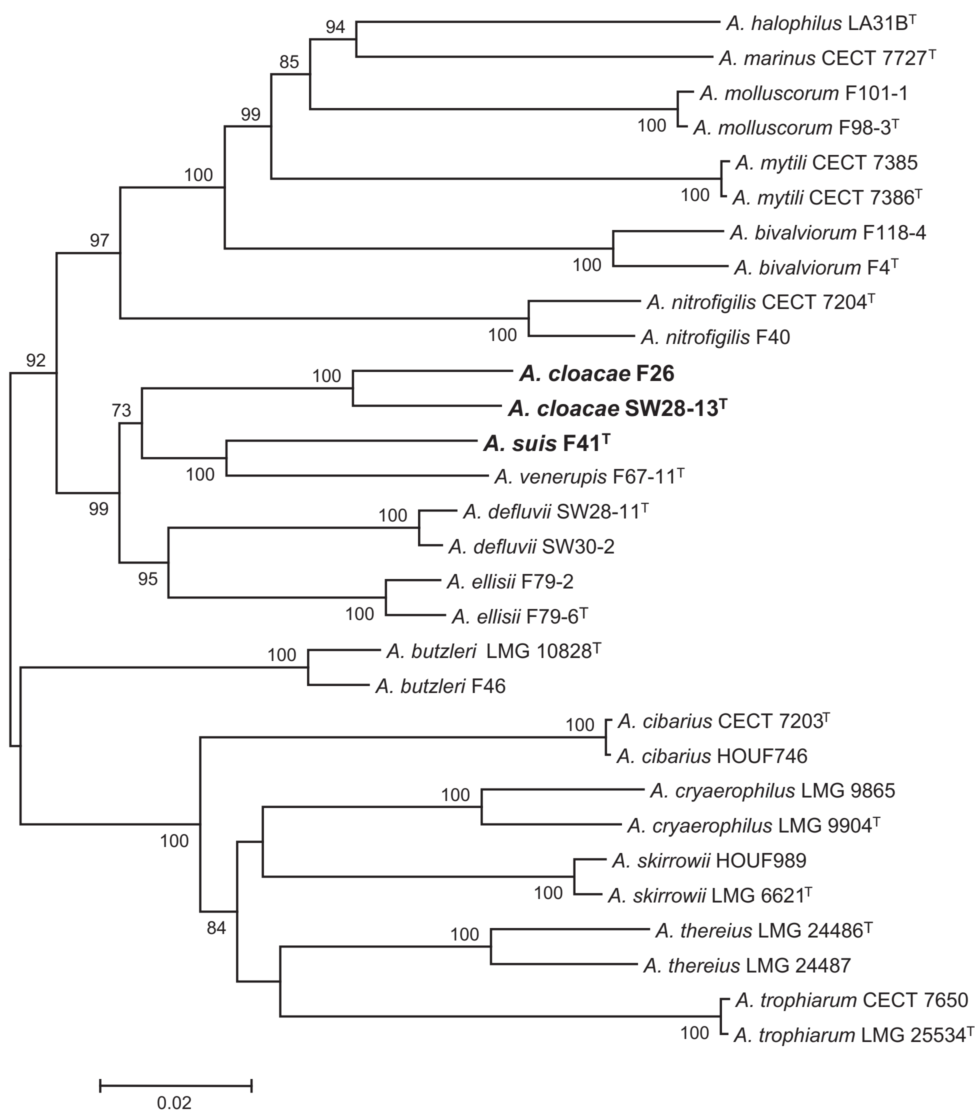

Fig. 2. Neighbour joining tree based on the concatenated sequences of *gyrA*, *atpA*, *rpoB*, *gyrB* and *hsp60* (3134 bp) sequences showing the phylogenetic position of *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov. within the genus *Arcobacter*. Bootstrap values (≥70%) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt. *Only the type strain is available so far.

Based on the polyphasic study carried out here, we have recognized two new *Arcobacter* species, for which the names *Arcobacter cloacae* (type strain SW28-13^T = CECT 7834^T = LMG 26153^T), and *Arcobacter suis* (type strain F41^T = CECT 7833^T = LMG 26152^T) are proposed.

Description of *A. cloacae* sp. nov.

A. cloacae (clo.a'ca.e. L. gen. n. cloacae. of a sewer)

Cells of strains SW28-13^T and F26 are Gram-negative slightly curved rods, non-encapsulated, non-spore forming, 0.3–0.5 μm wide and 1.0–1.5 μm long. They are motile by a single polar flagellum. Colonies on blood agar incubated in aerobic conditions at 30 °C for 48 h are 2–4 mm in diameter, beige to off-white, circular with entire margins, convex, and non-swarming. Pigments are not produced. All the strains grow on blood agar at room temperature (18–22 °C), 30 °C and 37 °C under both aerobic and microaerobic conditions with no significant differences. No growth is observed in anaerobic conditions at 30 °C or in aerobic or microaerobic conditions at 42 °C. No haemolysis is observed on TSA medium supplemented with 5% sheep blood. Strains produce oxidase and catalase activity, reduce nitrate, hydrolyse indoxyl acetate but not urea, casein, lecithin or starch. Hydrogen sulphide is not produced in triple-sugar iron agar medium. Under aerobic conditions at 30 °C all strains grow on Marine Agar, Campylobacter Charcoal Deoxycholate Agar (CCDA), MacConkey agar and on nutrient medium supplemented with 5% sheep blood also containing 0.5–2% (w/v)

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NaCl; 0.1% sodium deoxycholate; 1% oxgall; 0.01% 2,3,5-triphenyl tetrazolium chloride (TTC); 0.05% safranin or 0.0005% crystal violet. No growth occurs on nutrient medium supplemented with 5% sheep blood also containing 4% (w/v) NaCl; 1% glycine; 0.04–0.1% TTC; or 64 mg l^{-1} cefoperazone. Strain SW28-13^T, but not strain F26, is able to grow on minimal medium and on nutrient medium supplemented with 5% sheep blood containing 0.005% basic fuchsine or 0.001% brilliant green.

The type strain is SW28-13^T (=CECT 7834^T = LMG 26153^T), isolated from sewage from the Waste Water Treatment Plant of Reus, Catalonia, Spain.

This species has so far been isolated from shellfish, with an incidence of 4.3% in relation to other *Arcobacter* spp. [4], and represents 1% of the species identified in sewage obtained from a WWTP in a recent study (unpublished data). A BlastN analysis of the 16S rRNA sequence of strain SW28-13^T matched 100% with the sequences of 3 uncultured clones: MW-B27 (JQ088343, 1479 bp) and M17-10-B14 (JQ088390, 1479 bp) from water-flooded petroleum reservoirs in China and clone 42 (FJ462082, 1475 bp) from an industrial anaerobic digester in Mexico. These clones cluster with *A. cloacae* in a phylogenetic tree (data not shown).

Description of *A. suis* sp. nov.

A. suis (su'is. L. n. sus suis, a swine, pig, boar, sow; L. gen. n. suis, of a pig)

Cells of strain F41^T are Gram-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.3–0.6 μm wide and 1.2–2.1 μm long. Some cells have a filamentous form up to 7 μm long. It is motile by a single polar flagellum. Colonies on blood agar incubated under aerobic conditions at 30 °C for 48–72 h are 1–3 mm in diameter, beige to off-white, circular with entire margins, convex, and non-swarming. Pigments are not produced. The strain grows on blood agar at room temperature (18–22 °C) and 30 °C under aerobic or microaerobic conditions with no significant differences. However, it did not grow at 37 °C or 42 °C or under anaerobic conditions at 30 °C. No haemolysis is observed on TSA medium supplemented with 5% sheep blood. The strain produces oxidase and catalase activity, reduces nitrate and hydrolyses indoxyl acetate but not urea, casein, lecithin or starch. Hydrogen sulphide is not produced in triple-sugar iron agar medium. Under aerobic conditions at 30 °C, strain F41^T grows on minimal medium and MacConkey agar but not on Marine agar or CCDA. It is able to grow on nutrient medium supplemented with 5% sheep blood, also containing 0.5% (w/v) NaCl; 0.005% basic fuchsine; 0.01% TTC or 0.1% sodium deoxycholate. No growth occurs on nutrient medium supplemented with 5% sheep blood also containing 2–4% (w/v) NaCl; 1% glycine; 1% oxgall; 0.04–0.1% TTC; 0.001% brilliant green; 0.05% safranin; 0.0005% crystal violet or 64 mg l^{-1} cefoperazone.

The type strain is F41^T (=CECT 7833^T = LMG 26152^T) isolated from pork meat in Catalonia, Spain.

This species shows an incidence of 11.1% in meat in relation to other *Arcobacter* spp. [4]. A BlastN analysis of the 16S rRNA sequence of strain F41^T showed 99% similarity with 3 uncultured clones: ATB-LH-6148 (FJ535178, 1482 bp) and ATB-LH-5950 (FJ535174, 1482 bp) from carrot wash water in Germany, and clone TS1B220 (JF789595, 1499 bp) from biodegraded oil in Canada. These sequences also clustered together with *A. suis* in a phylogenetic tree (data not shown).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2012.11.003.

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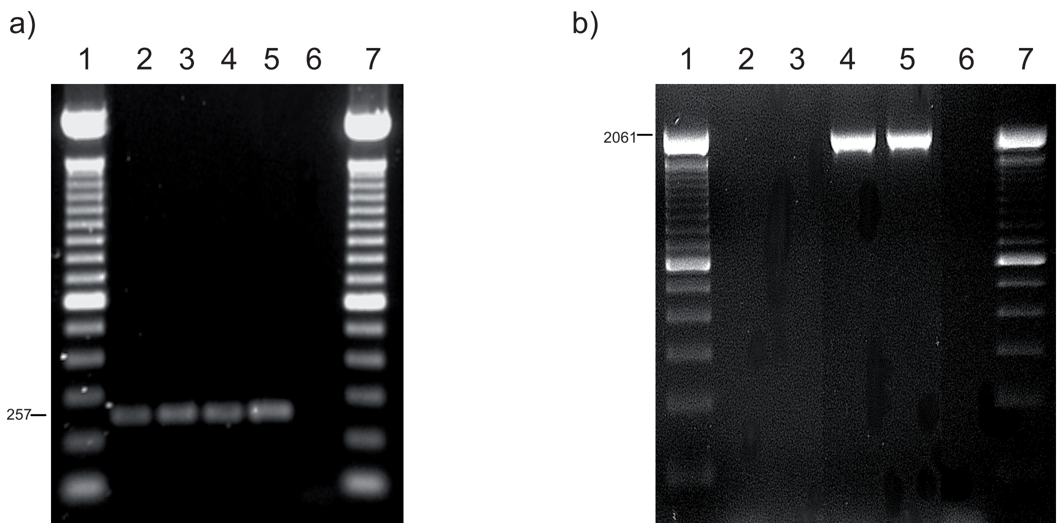

Figure S1. Agarose gels showing the amplicons obtained for *A. cloacae* sp. nov. and *A. suis* sp. nov. compared to other *Arcobacter* spp. with two m-PCR methods: a, Houf *et al*. [16]; b, Douidah *et al*. [8].
a) *A. cloacae* sp. nov. (SW28-13^T, lane 3; F26, lane 4) and *A. suis* sp. nov. (F41^T, lane 5) produced the expected amplicon for *A. cryaerophilus* (257 bp, LMG 9904^T, lane 2); lanes: 1 and 7, 100 bp Ladder (Invitrogen); 6, negative control (MiliQ water as template DNA).
b) *A. cloacae* sp. nov. (SW28-13^T, lane 2; F26, lane 3) did not produce an amplicon while *A. suis* sp. nov. (F41^T, lane 4) produced the expected amplicon for *A. butzleri* (2061 bp, LMG 10828^T, lane 5); lanes: 1 and 7, 100 bp DNA plus Ladder (Invitrogen); 6, negative control (MiliQ water as template DNA).

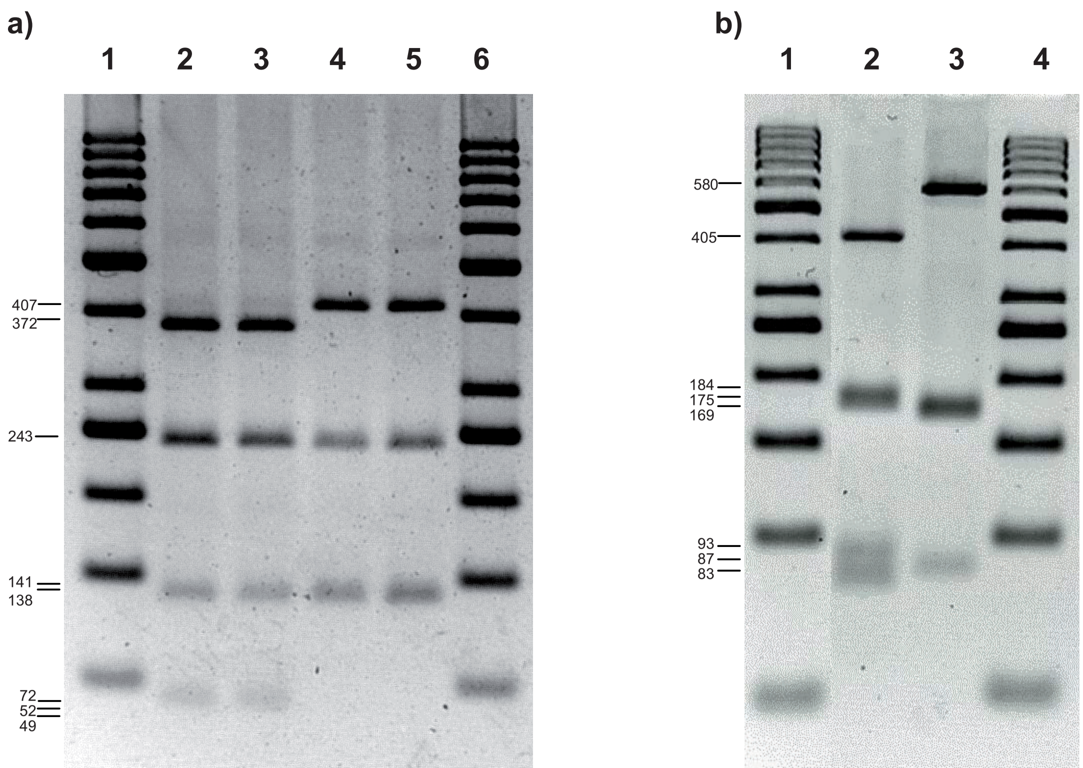

Figure S2: Agarose 3.5% gel comparing the 16S DNA-RFLP patterns [10, 12] obtained for *A. cloacae* sp. nov. and *A. suis* sp. nov. to other *Arcobacter* spp.

a) Strains of *A. cloacae* sp. nov. (SW28-13^{T}, lane 2; F26, lane 3); *A. suis* sp. nov. strain F41^{T} (lane 4) and *A. defluvii* CECT 7697^{T} (lane 5) digested with *Mse*I endonuclease [10]. Lanes: 1 and 6, 50 bp. ladder (Fermentas). The pattern obtained for *A. cloacae* is different to those described for the accepted *Arcobacter* spp. [2, 5, 10, 11, 13, 21], while the one produced by *A. suis* is similar to *A. defluvii*.

b) Strain *A. defluvii* CECT 7697^{T} (lane 3) and *A.* suis sp. nov. F41^{T} (lane 2) digested with *Bfa*I endonuclease [12]. Lanes: 1 and 4, 50 bp. ladder (Fermentas).

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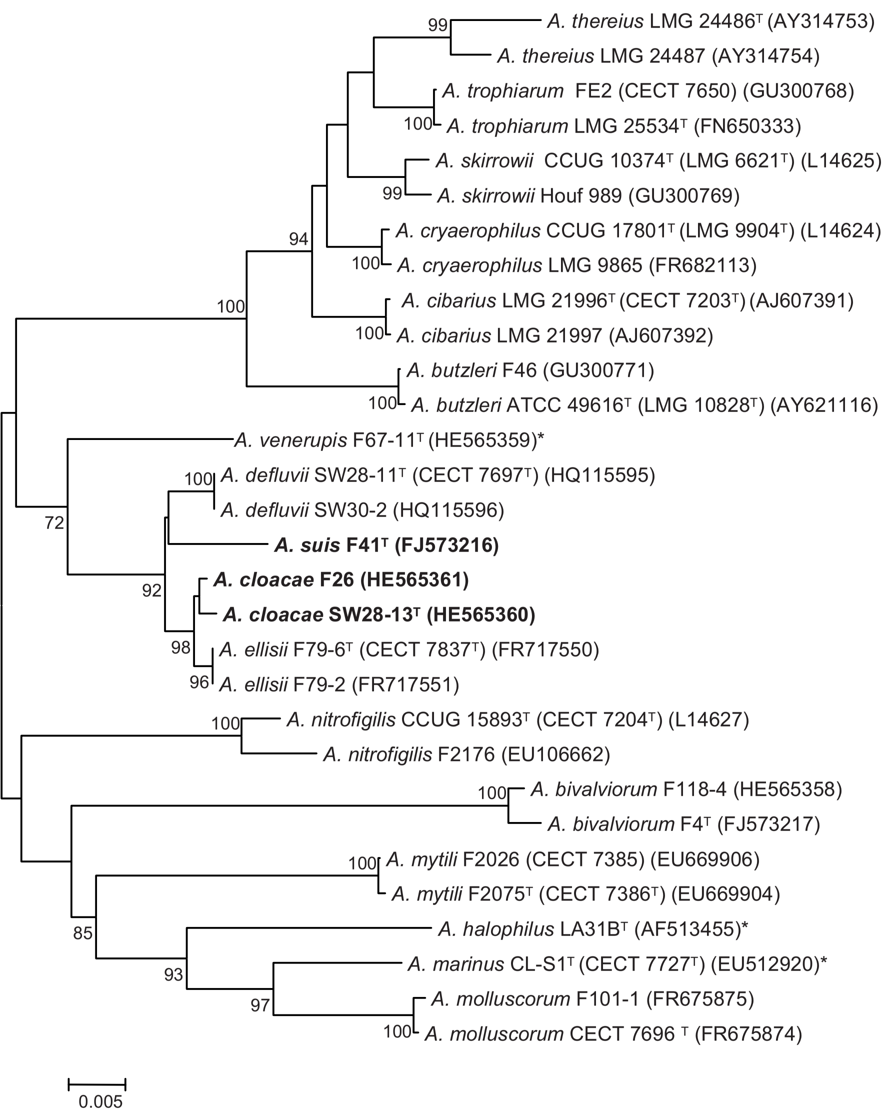

Figure S3. Neighbour joining tree based on 16S rRNA (1401 bp) sequences showing the phylogenetic position of *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov. within the genus *Arcobacter*. Bootstrap values ($\geq$70%) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 1000 nt.

* Only type strain is available so far.

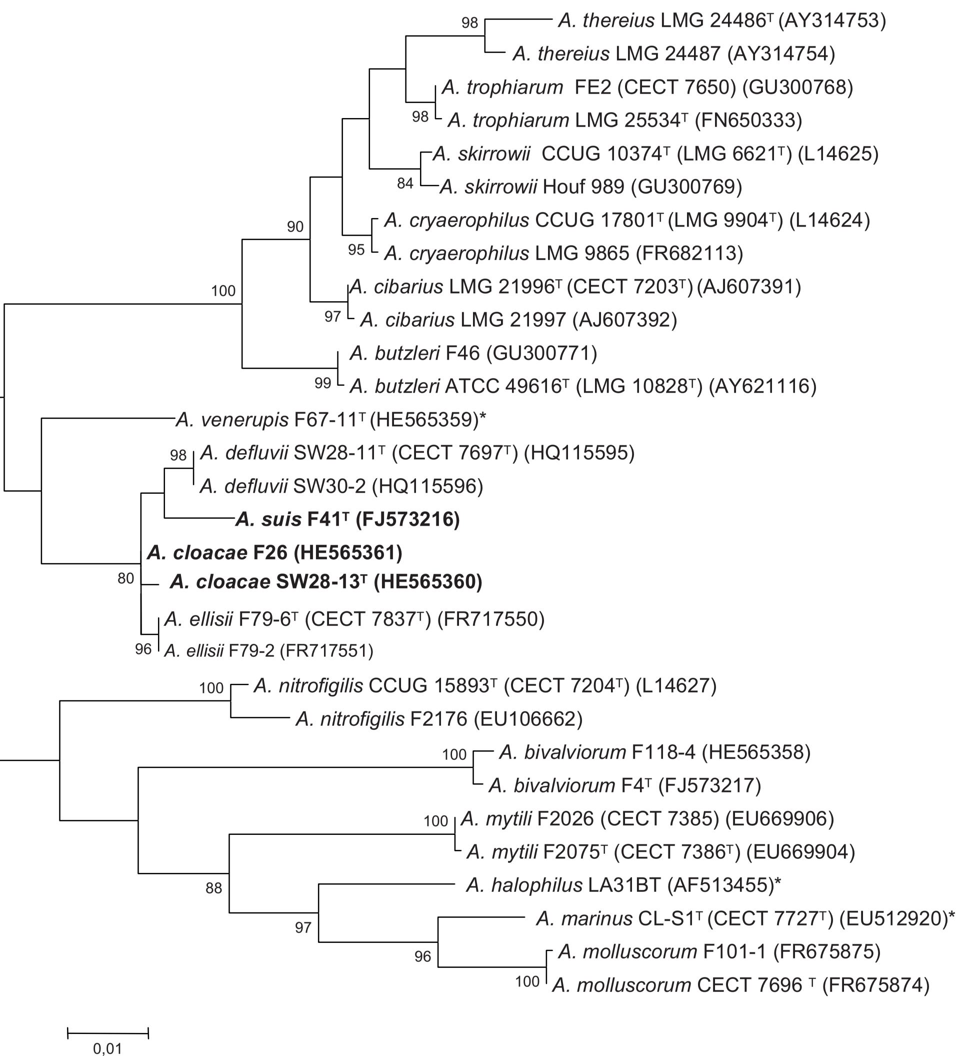

Fig. S4. Maximum likelihood tree based on 16S rRNA (1401 bp) sequences showing the phylogenetic position of *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov. within the genus *Arcobacter*. Bootstrap values ($\geq$70%) based on 1000 replications are shown at the nodes of the tree. Bar, 1 substitutions per 100 nt.
* Only type strain is available so far.

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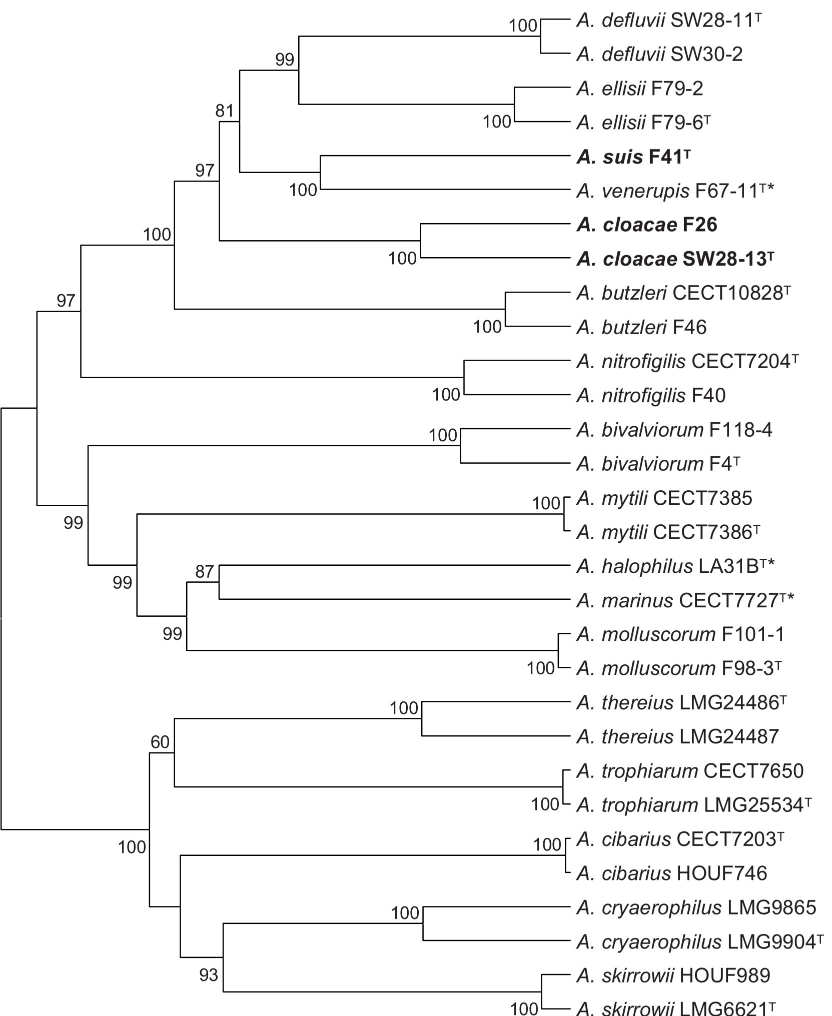

Fig. S5. Maximum parsimony tree based on the concatenated *gyrA, atpA*, *rpoB, gyrB* and *hsp60* (3134 bp) sequences showing the phylogenetic position of *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov. within the genus *Arcobacter*. Bootstrap values (≥70%) based on 1000 replications are shown at the nodes of the tree.
* Only type strain is available so far.

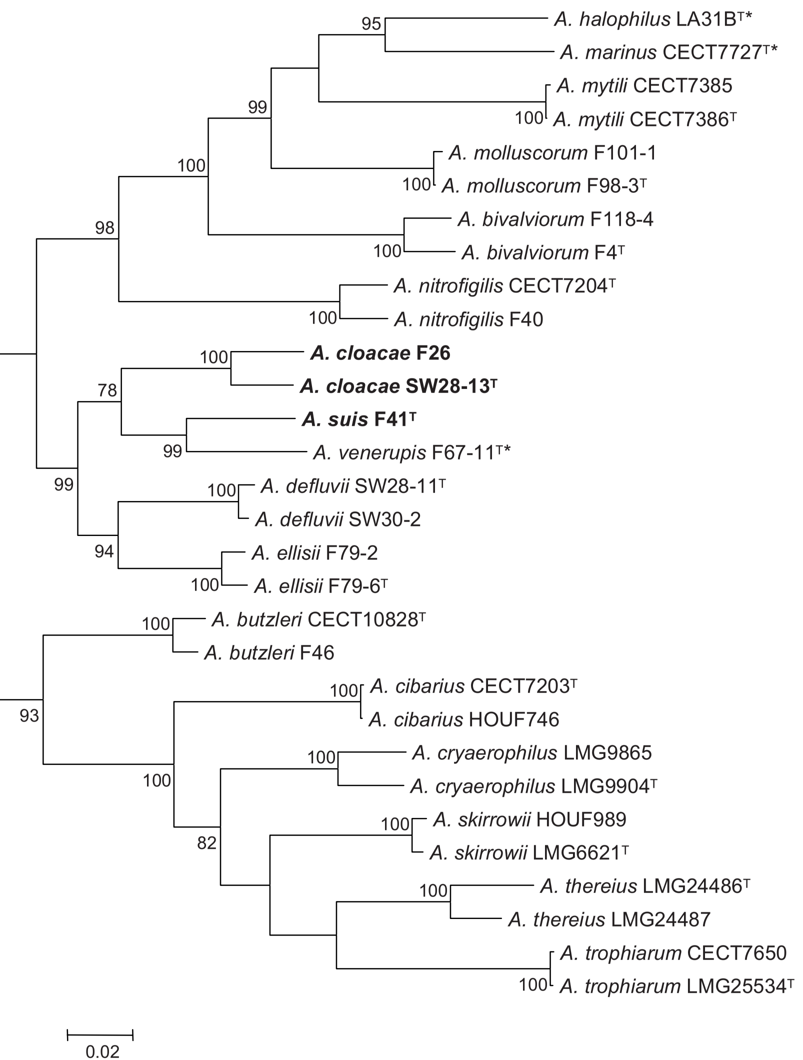

Fig. S6. Maximum likelihood tree based on the concatenated *gyrA, atpA*, *rpoB, gyrB* and *hsp60* (3134 bp) sequences showing the phylogenetic position of *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov. within the genus *Arcobacter*. Bootstrap values (≥70%) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.
* Only type strain is available so far.

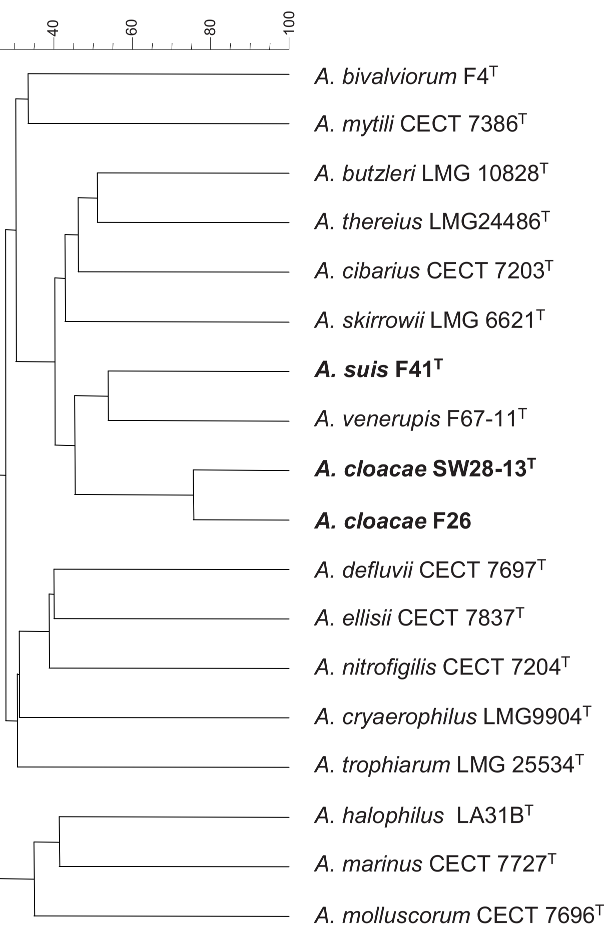

Fig. S7. Dendrogram comparing the MALDI-TOF profiles of strains of *Arcobacter cloacae* sp. nov. (SW28-13^{T} and F26) and *Arcobacter suis* sp. nov. (F41^{T}) with the type strains of all *Arcobacter* species using UPGMA algorithm. The scale above the dendrogram gives percent matching mass signals between individual strains.

Table S1: MALDI TOF MS profiles (*m*/*z*) of *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov. and the type strains of all *Arcobacter* species

A. cloacae	*A. cloacae*	*A. suis*	*A. butzleri*	*A. cibarius*	*A. cryaerophilus*	*A. defluvii*	*A. ellisii*	*A. halophilus*
SW28-13^{T}	F26	F41^{T}	LMG 10828^{T}	CECT 7203^{T}	LMG 9904^{T}	CECT 7697^{T}	F79-6^{T}	LA31B^{T}
3,244	**3,244**	**3,412**	3,416	3,247	4,384	3,446	4,370	4,309
3,419	**3,419**	**3,580**	3,446	3,341	4,427	3,569	5,201	4,342
3,448	**3,448**	**3,591**	3,556	3,406	4,625	3,591	5,244	4,809
3,563	**3,563**	**4,187**	3,605	3,449	5,202	4,221	5,690	5,063
3,607	**3,607**	**4,240**	4,295	3,557	5,246	4,371	5,731	5,258
4,218	**4,222**	**4,291**	4,350	3,601	5,715	5,202	6,465	5,300
4,304	**4,308**	**4,372**	4,384	3,759	7,171	5,245	6,830	5,362
4,326	**4,385**	**4,725**	4,416	3,812	8,539	5,732	7,117	5,700
4,386	**4,434**	**4,804**	4,672	4,191	8,855		7,163	6,717
4,434	**4,569**	**4,969**	5,001	4,281	9,416		10,028	6,787
4,570	**4,685**	**5,058**	5,029	4,387				6,951
4,595	**4,737**	**5,698**	5,229	4,444				8,437
4,739	**4,806**	**5,734**	5,272	4,455				8,603
4,795	**4,973**	**6,420**	5,732	4,568				8,707
5,059	**5,059**	**6,813**	6,454	4,681				10,131
5,213	**5,213**	**6,861**	6,826	4,703				
5,254	**5,254**	**7,150**	6,884	4,718				
5,742	**5,742**	**7,530**	7,204	4,804				
6,474	**5,755**	**8,365**	8,694	5,008				
6,825	**6,477**	**9,438**	8,832	5,016				
6,880	**6,646**	**8,469**	9,341	5,079				
7,115	**6,830**	**8,630**	10,048	5,175				
7,199	**6,882**	**9,922**		5,199				
8,378	**7,051**			5,247				
8,417	**7,120**			5,572				
10,090	**7,202**			5,725				
	8,432			5,756				
	8,491			6,485				
	8,600			6,803				
	9,461			6,889				
	9,583			7,106				
	9,928			7,193				
	10,095			7,618				
				8,894				
				9,393				
				10,004				

Table S1-Continued

A. marinus	*A. molluscorum*	*A. mytili*	*A. nitrofigilis*	*A. skirrowii*	*A. thereius*	*A. trophiarum*	*A. bivalviorum*	*A. venerupis*
CECT 7727[T]	**CECT 7696[T]**	**CECT 7386[T]**	**CECT 7204[T]**	**LMG 6621[T]**	**LMG 24486[T]**	**LMG 25534[T]**	**F4T**	**F67-11[T]**
3,497	3,473	3,174	4,369	3,579	4,384	3,581	3,413	3,588
3,515	4,164	3,442	5,125	4,296	4,430	4,385	3,474	4,235
4,345	4,342	3,594	5,231	4,506	4,699	4,454	3,582	4,374
4,427	4,854	3,693	5,258	4,665	4,918	4,728	4,151	4,708
5,044	5,095	4,191	5,275	5,035	5,215	5,718	4,321	5,061
5,081	5,352	4,420	5,671	7,144	5,220	7,147	4,357	5,697
5,303	5,672	5,073	5,691	8,575	5,261	8,621	4,373	5,737
5,645	6,206	5,095	5,720	9,000	5,665	8,899	4,538	6,424
6,778	6,357	5,306	5,732	9,315	5,706	10,049	4,761	6,795
7,022	6,838	5,624	7,140		6,940		5,081	6,822
8,470	6,938	5,772			7,133		5,287	7,126
8,690	7,527	6,341			8,345		5,330	7,166
	8,315	6,872			8,855		5,691	7,537
		7,178			9,390		5,720	8,457
		7,373					5,760	9,403
		7,532					6,288	9,916
		8,377					6,800	10,106
		8,636					7,195	
		8,820					7,343	
		10,144					8,368	
		12,710					9,512	
							9,666	
							10,153	

4.4. Updated 16S rDNA-RFLP method for the identification of all currently known *Arcobacter* spp

Figueras MJ, Levican A, Collado L.

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METHODOLOGY ARTICLE Open Access

Updated 16S rRNA-RFLP method for the identification of all currently characterised *Arcobacter* spp

María José Figueras[1*], Arturo Levican[1] and Luis Collado[2]

Abstract

Background: *Arcobacter* spp. (family *Campylobacteraceae*) are ubiquitous zoonotic bacteria that are being increasingly recognised as a threat to human health. A previously published 16S rRNA-RFLP *Arcobacter* spp. identification method produced specific RFLP patterns for the six species described at that time, using a single endonuclease (*Mse*I). The number of characterised *Arcobacter* species has since risen to 17. The aim of the present study was to update the 16S rRNA-RFLP identification method to include all currently characterised species of *Arcobacter*.

Results: Digestion of the 16S rRNA gene with the endonuclease *Mse*I produced clear, distinctive patterns for 10 of the 17 species, while the remaining species shared a common or very similar RFLP pattern. Subsequent digestion of the 16S rRNA gene from these species with the endonucleases *Mnl*I and/or *Bfa*I generated species-specific RFLP patterns.

Conclusions: 16S rRNA-RFLP analysis identified 17 *Arcobacter* spp. using either polyacrylamide or agarose gel electrophoresis. Microheterogeneities within the 16S rRNA gene, which interfered with the RFLP identification, were also documented for the first time in this genus, particularly in strains of *Arcobacter cryaerophilus* isolated from animal faeces and aborted foetuses.

Keywords: *Arcobacter*, Identification, Agarose, Polyacrylamide, 16S rRNA-RFLP, 16S rRNA gene mutations

Background

The genus *Arcobacter*, included in the family *Campylobacteraceae*, has expanded rapidly since it was first recognised in 1991 [1], and currently includes 17 species. Some of these species are considered enteropathogenic to humans and animals, as well as important zoonotic agents. *Arcobacter* species negatively impact the food industry, as many meat products are frequently contaminated with these bacteria, and multiple species have been described from shellfish [2-6]. In addition, the International Commission on Microbiological Specification for Foods classified *A. butzleri* as a serious hazard to human health [7]. However, the true incidence of *Arcobacter* species in environmental and clinical samples is thought to be underestimated because specific detection and identification methods are not normally applied and can be inaccurate [2,8].

A 16S rRNA restriction fragment length polymorphism (RFLP) method for the identification of *Arcobacter* species has previously been described [9]. The method involved a single digestion with the *Mse*I endonuclease and discriminated all *Arcobacter* species that had been described up to 2008, i.e. *A. butzleri, A. cryaerophilus, A. cibarius, A. skirrowii, A. nitrofigilis* and *A. halophilus* [9]. Further molecular methods for the identification of *Arcobacter* species have been reviewed elsewhere [2,9]. Most of the methods described target only the most common species i.e. *A. butzleri* [10,11], *A. cryaerophilus* [12] and/or *A. skirrowii* [13,14]. Even the most recently proposed identification method, m-PCR, described by Douidah *et al.* [15] in 2010, only targeted five species: *A. butzleri, A. cryaerophilus, A. skirrowii, A. cibarius* and

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A. thereius. Furthermore, using this method, the species *A. defluvii*, *A. ellisii*, *A. venerupis* and *A. butzleri* produced an identical and therefore uninformative amplicon [2,5,6].

The limitations of the current methods have arisen because of the limited testing of certain species, as well as the identification of novel species [2,4-6]. Douidah *et al.* [15] suggested that the reliance of the currently-available 16S rRNA-RFLP method on polyacrylamide gel electrophoresis was a major disadvantage for its routine use. Furthermore, the recently described species *A. thereius,* isolated from aborted pig foetuses [16], and *A. trophiarum*, which was recovered from porcine faecal matter [17], produce the same RFLP pattern as *A. butzleri* [2]. Additionally, the new species *A. venerupis*, from clams, produces a pattern that is very similar to *A. marinus* [6,18].

The aim of the present study was to update the 16S rRNA-RFLP identification method to include all the currently characterised species of *Arcobacter*, and to provide protocols for both polyacrylamide and agarose gel electrophoresis so that the method can easily be adapted.

Results

*Mse*I digestion can discriminate 10 of the 17 currently described *Arcobacter* species

Following digestion with the endonuclease *Mse*I, species-specific differential RFLP patterns were obtained for 47 of the 121 strains (38.8%), representing 12 of the 17 species that make up the *Arcobacter* genus (*A. nitrofigilis, A. cryaerophilus, A. skirrowii, A. cibarius, A. halophilus, A. mytili, A. marinus, A. molluscorum, A. ellisii, A. bivalviorum* and *A. venerupis*), including the new described species *A. cloacae* (Figure 1 and Table 1). However, *A. venerupis* produced a pattern very similar to that of *A. marinus*, with only a single 141 bp band distinguishing the two species (Figure 4 and Additional file 1: Table S1). In addition, the new species *A. suis* (F41) showed the same banding pattern as *A. defluvii*, while the characteristic *A. butzleri* pattern (Figure 4 and Additional file 1: Table S1) was also observed following *Mse*I digestion of *A. thereius* and *A. trophiarum* and 11 of the 19 (57.9%) *A. cryaerophilus* strains. Of these, nine strains (MICV1-1, MICV3-2, FE4, FE5, FE6, FE9, FE11, FE13 and FE14) were isolated from animal faeces in Valdivia, Chile, and two strains were isolated in Ireland (LMG 9863

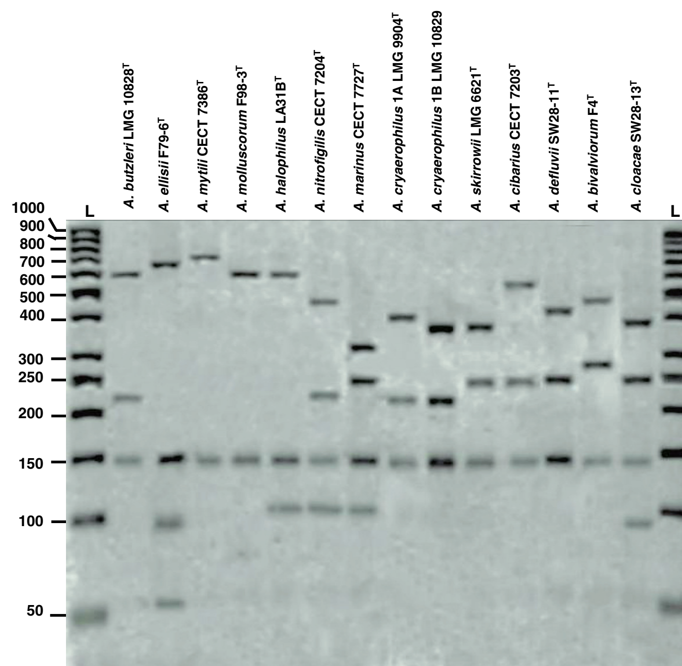

Figure 1 16S rRNA-RFLP patterns (agarose gel 3.5%) obtained for *Arcobacter* spp. using the endonuclease *Mse*I. Lanes: L, 50 bp ladder, Fermentas. The obtained patterns agree with those expected from the computer simulation (Additional file 1: Table S1). Species that share an identical or similar pattern (Additional file 1: Table S1) were: *A. butzleri*, that produced a pattern identical to those of *A. trophiarum, A. thereius* and atypical strains (n=11) of *A. cryaerophilus*; *A. marinus* CECT 7727^{T} with a pattern very similar to the one of *A. venerupis* CECT 7836^{T} and *A. defluvii* with an identical pattern to the one of *A. suis* strain F41. The identification of these species required additional digestions with other enzymes (Figures 2 – 4, Additional file 2: Table S2 and Additional file 3: Table S3).

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Table 1 *Arcobacter* spp. strains used in this study

SPECIES	STRAIN	SOURCE
A. butzleri	LMG 10828[T,¶,Ω], LMG 11118[Ω]	Human faeces
	W24-2-1, W24-05-1, W07-01-8, W03-03-6, W26-02-2, W03-02-7, W21-05-1, W2105-3, W21-05-7, W24-01-1, W10-01-1	Sea water
	SWDS1-3-2	Sewage
	F42, F46[Ω], F49, F51	Pork meat
	F15, F22, F23, F24, F25	Turkey meat
	F44, F47, F52	Chicken meat
	F43, F50[Ω], F53	Beef meat
	F1, F2, F29, F30, F38, F98-1, SAN600-1, SAN600-6, SAN512-1, SAN547-10, SAN548-8, SAN582-1, SAN582-6	Mussels
	T62	Soil
A. trophiarum	LMG 25534[T,¶,Ω], LMG 25535[¶,Ω]	Pig faeces
	CECT 7650[Ω]	Chicken cloacae
A. thereius	LMG 24486[T,¶,Ω], LMG 24487[¶,Ω]	Porcine abortion foetus
	SW24[Ω]	Sewage
	F61-1[Ω]	Pork meat
	F89-4	Mussels
	F93-4[Ω]	Clams
A. cryaerophilus	LMG 9904[T,¶,Ω], LMG 9871[¶,Ω]	Bovine abortion foetus
	LMG 9865[¶,Ω] LMG 10241[¶,Ω], LMG 6622, LMG 10229[¶,Ω]	Porcine abortion
	LMG 7537[¶], LMG 9863[¶,Ω]	Ovine abortion foetus
	LMG 10829[¶]	Human blood
	LMG 9861[¶,Ω]	Bovine abortion foetus
	FE4[Ω], FE5[¶,Ω], FE6[¶,Ω], FE9[¶,Ω], FE11[Ω], FE13[Ω]	Chicken cloacal swabs
	FE14[Ω]	Ovine faeces
	MICV1-1[¶,Ω], MICV3-2[¶,Ω]	Cow faeces
A. nitrofigilis	CECT 7204[T,¶,Ω], LMG 7547[Ω]	Roots of *Spartina alterniflora*
	F39[Ω], F40[¶], F72[Ω]	Mussels
A. skirrowii	LMG 6621[T,¶,Ω]	Lamb faeces
	LMG 9911	Porcine abortion
	Houf 989[¶,Ω], Houf 994[Ω]	Cow faeces
	S7[Ω]	Sludge
	F94-1[Ω]	Clams
	F125-1[Ω]	Mussels
	ArcoE[Ω], ArcoF[Ω]	
A. cibarius	CECT 7203[T,¶,Ω]	Chicken meat
	NC81[Ω], NC88[Ω]	Piggery effluent
	H742, H743[Ω], H745, H746[Ω], H748	Poultry carcasses
A. halophilus	LA31B[T,¶,Ω]	Hypersaline lagoon
A. mytili	CECT 7386[T,¶,Ω], CECT 7385[¶,Ω]	Mussels
	T234[Ω]	Brackish water
A. marinus	CECT 7727[T,¶,Ω]	Seawater/starfish
A. defluvii	CECT 7697[T,¶,Ω], SW28-7[¶,Ω], SW28-8, SW28-9, SW28-10, SW30-2[¶,Ω], SW30-7, SW30-8	Sewage
	MICCC4-2[Ω]	Pig faeces
	SAN599-9[Ω]	Mussels
A. molluscorum	CECT 7696[T,¶,Ω], F91[¶,Ω], F101-1[¶,Ω]	Mussels
A. ellisii	F79-6[T,¶,Ω], F79-2[¶,Ω], F79-7[¶,Ω]	Mussels
A. bivalviorum	F4[T,¶,Ω], F118-2[¶,Ω], F118-4[¶,Ω]	Mussels
A. venerupis	F67-11[T,¶,Ω]	Clams
A. suis	F41[T,¶,Ω]	Pork meat
A. cloacae	SW28-13[T,¶,Ω]	Sewage
	F26[¶,Ω]	Mussels

ATCC American Type Culture Collection, LMG Belgian Co-ordinated Collection of Micro-organisms, CECT Colección Española de Cultivos Tipo.
¶ Sequenced 16S rRNA gene.
Ω Sequenced *rpoB* gene.

and LMG 9871) from aborted ovine and bovine foetuses, respectively. The RFLP results for these 11 strains were discordant with those of m-PCR and their identity was confirmed by sequencing the 16S rRNA and *rpoB* genes.

Microhetergeneities in *A. cryaerophilus* strains interfere with RFLP identification

The chromatograms of the 16S rRNA gene sequences (1405 bp) of seven of the 11 unresolved *A. cryaerophilus* strains (MIC V1-1, MICV3-2, FE5, FE6, FE9, LMG 9863 and LMG 9871) showed mutations (i.e. microheterogeneities) at positions 192 (T→C) and 205 (A→G), which were within the target region (TTAA) of the *Mse*I endonuclease (Additional file 4: Figure S1).

Digestion with *Mnl*I and/or *Bfa*I resolves the remaining species

A second restriction digest using *Mnl*I (Fermentas) was then carried out for those strains with common or

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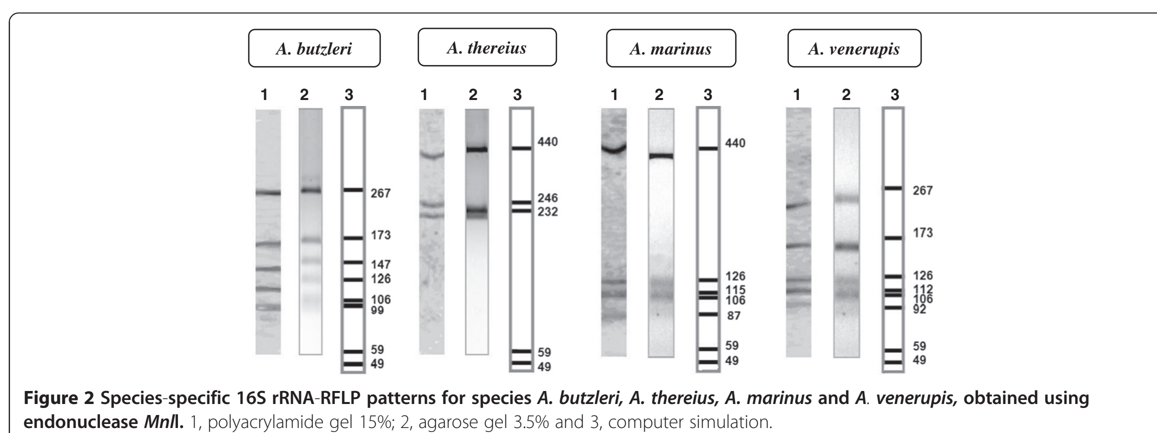

Figure 2 Species-specific 16S rRNA-RFLP patterns for species *A. butzleri, A. thereius, A. marinus* and *A. venerupis*, obtained using endonuclease *Mnl*I. 1, polyacrylamide gel 15%; 2, agarose gel 3.5% and 3, computer simulation.

similar RFLP patterns following *Mse*I digestion (Additional file 1: Table S1 and Additional file 2: Table S2). *Mnl*I generated a species-specific pattern for *A. butzleri, A. thereius, A. marinus* and *A. venerupis*, and a common pattern for *A. trophiarum* and the atypical strains of *A. cryaerophilus* (Figures 2 and 4). A further restriction digest step using *FspB*I (Fermentas), an isoschizomer of *Bfa*I, produced species-specific RFLP patterns for the separation of *A. defluvii* from *A. suis* (F41), and *A. trophiarum* from the atypical *A. cryaerophilus* strains (Figure 3 and Additional file 3: Table S3). After carrying out 16S rRNA gene restriction digests as illustrated in Figure 4, all of the 121 strains were correctly identified.

Discussion

The proposed 16S rRNA-RFLP method described here used an initial digestion with *Mse*I endonuclease, as in the original method [9], which enabled 10 of the 17 accepted species, including the recently described species *A. cloacae*, to be identified. Further digestion was necessary to resolve species that showed the *Mse*I digestion pattern of *A. butzleri* (also common to *A. thereius, A. trophiarum* and to the atypical strains of *A. cryaerophilus* with 16S rRNA gene microheterogeneities). Computer simulation revealed that two endonucleases, *Mnl*I and *Tas*I, produced discriminative patterns between the species *A. butzleri* and *A. thereius* (Figure 2 and Additional file 5: Figure S2). Furthermore, these two enzymes also produced discriminative patterns between *A. marinus* and *A. venerupis* (Figure 2), which showed distinctive but very similar patterns following *Mse*I digestion (Figure 4 and Additional file 1: Table S1). *Mnl*I was selected because it generated more distinctive banding patterns, enabling easier discrimination than *Tas*I (Additional file 5: Figure S2). Considering that *A. butzleri* is a very common species [2,8,19-21], the identification of the majority of strains will normally be obtained with this second (*Mnl*I) endonuclease reaction (Figures 1, 2, 4). In fact, 79.3% of the strains (96/121) included in the current study were correctly identified with this second digestion step.

However, a third digestion, using the enzyme *Bfa*I, was required to distinguish between *A. defluvii* and the recently described species *A. suis* and for distinguishing *A.*

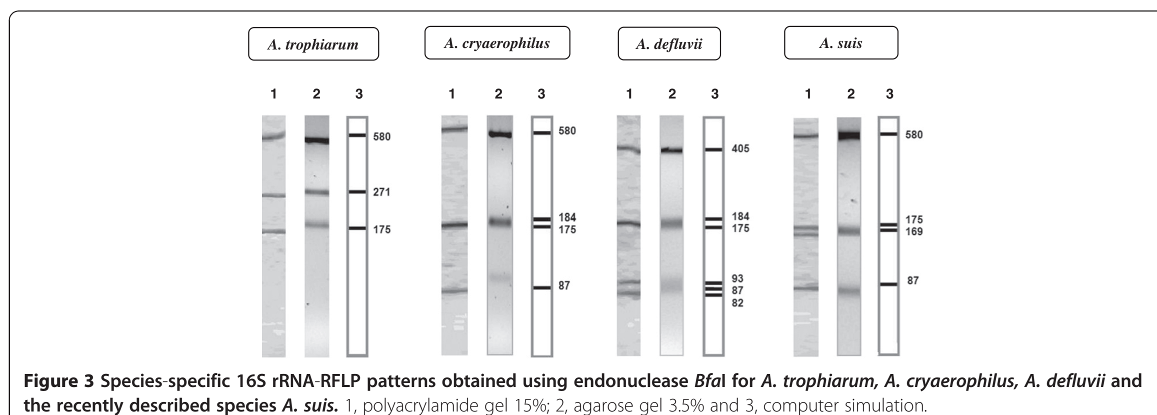

Figure 3 Species-specific 16S rRNA-RFLP patterns obtained using endonuclease *Bfa*I for *A. trophiarum, A. cryaerophilus, A. defluvii* and the recently described species *A. suis*. 1, polyacrylamide gel 15%; 2, agarose gel 3.5% and 3, computer simulation.

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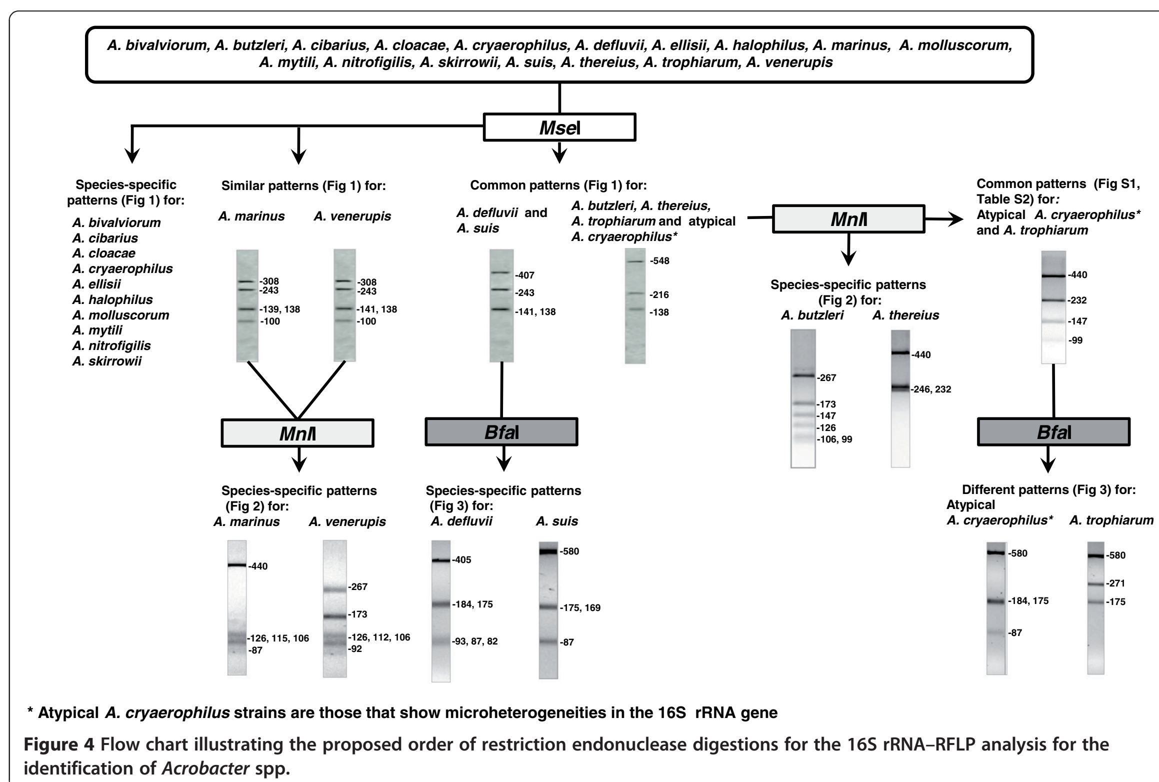

Figure 4 **Flow chart illustrating the proposed order of restriction endonuclease digestions for the 16S rRNA–RFLP analysis for the identification of *Acrobacter* spp.**

trophiarum from the atypical *A. cryaerophilus* strains following *Mnl*I digestion (Figures 3,4 and Additional file 3: Table S3). The proposed method enables reliable and fast species identification for a large collection of isolates, requiring, at most, digestion of the PCR-amplified 16S rRNA gene (1026 bp) with three restriction endonucleases (*Mse*I, *Mnl*I and/or *Bfa*I).

The original 16S rRNA-RFLP method [9] has been used to identify more than 800 *Arcobacter* strains recovered from meat products, shellfish and water in various studies [3-6,19-22]. The existing method has also helped to discover new species on the basis of novel RFLP patterns, including *A. mytili* [3], *A. molluscorum* [4], *A. ellisii* [5], *A. bivalviorum, A. venerupis* [6] and *A. cloacae* [23]. Furthermore, as well as identifying the more common *Arcobacter* species, this technique has confirmed the presence of other rare species in atypical habitats, such *A. nitrofigilis* in mussels and *A. thereius* in pork meat [20]. The updated technique described here is likely to supersede the current method in all of these areas.

The use of the 16S rRNA-RFLP method in parallel with the more commonly used molecular identification method, m-PCR [13], as well as the fact that strains with incongruent results were sequenced (*rpoB* and/or 16S rRNA gene sequencing), ensured accurate species identification, and highlighted the limitations of both identification methods [2,4-6,23]. The presence of microhete-rogeneities in the 16S rRNA gene, as in the case of the 11 atypical *A. cryaerophilus* strains, had not previously been observed. These strains produced the m-PCR amplicon expected for *A. cryaerophilus*, which targets the 23S rRNA gene [13], but showed the *A. butzleri* 16S rRNA-RFLP pattern [9]. However, *rpoB* and 16S rRNA gene sequencing results confirmed these strains as *A. cryaerophilus*. 16S rRNA-RFLP patterns that differ from those described here can be expected for any newly discovered *Arcobacter* species [3-6,9,23]. Nevertheless, intra-species nucleotide diversity (i.e. mutations or microheterogeneities in the operon copies of the 16S rRNA gene) at the endonuclease cleavage sites can also generate a novel RFLP pattern for a given isolate, or result in a pattern identical to another species [9,24,25]. In the latter situation, misidentifications may occur, as described here.

Conclusions

In conclusion, the 16S rRNA-RFLP protocols described here for the identification of *Arcobacter* spp. can be carried out using either agarose or polyacrylamide gel electrophoresis (Figures 1–3, Additional file 1: Table S1, Additional file 2: Table S2, Additional file 3: Table S3), depending on the requirements of an individual

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laboratory. It is important, however, to carry out the 16S rRNA gene digestions in the order illustrated in the flow chart (Figure 4).

The method provided in this study is reproducible, reliable, simple, fast, and reasonably inexpensive, and can be carried out efficiently in any laboratory. The technique is highly applicable for investigations of the prevalence of arcobacters in a variety of food products, water, wastewater or other environmental samples. It will enable investigators to determine the true incidence of the recently described species *A. mytili, A. marinus, A. trophiarum, A. molluscorum, A. defluvii, A. ellisii, A. bivalviorum*, *A. venerupis, A. cloacae* and *A. suis* clarifying their prevalence and epidemiology.

Methods

Bacterial strains and culture conditions

A group of 121 *Arcobacter* strains isolated from diverse origins were used in this study, including the type strains of the 17 *Arcobacter* species, as well as strains included in the original descriptions of all species (Table 1). Strains belonging to the most recently described *Arcobacter* species (*A. cloacae*, n=2, and *A. suis*, n=1) [23] were also included in the analysis.

All *Arcobacter* strains were cultured in TSA supplemented with 5% sheep blood at 30°C under aerobic conditions for 48 h in preparation for DNA extraction.

Strain identification by RFLP

All strains were identified in parallel using the 16S rRNA-RFLP method described by Figueras *et al.* [9] and the m-PCR method of Houf *et al.* [13]. Furthermore, the identities of some strains, especially those that gave either an unknown RFLP pattern, or contradictory results between the two methods (16S rRNA-RFLP and m-PCR), were confirmed by sequencing the 16S rRNA and/or the *rpoB* genes (Table 1) using primers and conditions described previously [3,26].

For the RFLP identification, total genomic DNA was extracted from all strains and used as template for the PCR amplification of a 1026 bp region of the 16S rRNA gene, as previously described [9,27]. 16S rRNA amplicons were digested with *Tru*I (Fermentas, Vilnius, Lithuania), an isoschizomer of *Mse*I, in a 30 μl final volume containing 10 μl of the amplification product, 10 U of the enzyme, 2 μl of 10× buffer, and distilled water. The reaction mixture was incubated at 65°C for 4 h. To separate the restriction fragments, the digested products were electrophoresed on 15% polyacrylamide gels (ProtoGel, Hessle, United Kingdom) at 350 V for 5 h [9], and on 3.5% agarose gels at 100 V for 90 min. In both cases, gels were prepared in 1× Tris-Borate-EDTA (TBE) buffer, and 50 bp ladder (Fermentas) was used as a molecular weight marker. Gels were stained with either SYBR Safe (Invitrogen, Carlsbad, CA, USA) or Red Safe (Ecogen, Barcelona, Spain) DNA gel stains, according to the manufacturers' instructions, and then photographed on a UV transilluminator Vilber Lourmat Model TFX-35C (Marne-la-Vallée, France).

Determination of restriction endonuclease recognition sites

Restriction endonuclease recognitions sites within the 16S rRNA sequences of all strains included in this study (Table 1 and Additional file 1: Table S1, Additional file 2: Table S2, Additional file 3: Table S3) were identified using NEBcutter V 2.0 software [28], which is available online (http://tools.neb.com/NEBcutter2/index.php). Experimental validation of the selected enzymes was carried out following the manufacturers' instructions, under the conditions described above.

Additional files

Additional file 1: Table S1. Computer simulated profiles of *Arcobacter* spp. 16S rRNA gene (1026 bp) digestion with *Mse*I endonuclease. Species with specific RFLP patterns are in bold.

Additional file 2: Table S2. Computer simulated profiles of *Arcobacter spp.16S rRNA gene* (1026 bp) digestion with *Mnl*I endonuclease. Species in bold are those that show a specific RFLP pattern that was not distinguished with *Mse*I.

Additional file 3: Table S3. Computer simulated profiles of *Arcobacter* spp. 16S rRNA gene (1026 bp) digestion with *Bfa*I endonuclease. Species in bold are those that now show a specific RFLP pattern that was not distinguished previously with *Mse*I or *Mnl*I.

Additional file 4: Figure S1. Microheterogeneities (or mutations) in the 16S rRNA gene of seven atypical *A. cryaerophilus* strains in relation to the type strain (LMG 9904[T]), strain LMG 10829 (*A. cryaerophilus* subgroup 1B) and the type strain of*A. butzleri* (LMG 10828[T]). Sequence alignment of the 16S rRNA gene (positions 190–207 in relation to *Escherichia coli*) of seven atypical *A. cryaerophilus* strains showing mutations at positions 192 (T→C) and 205 (A→G), which alter the *Mse*I restriction enzyme recognition site (TTAA). IUPAC code, Y = Pyrimidine (C or T); R = Purine (A or G).

Additional file 5: Figure S2. Agarose gel (3.5%) comparing the 16S rRNA-RFLP patterns obtained using endonucleases a\) *Tas*I and b) *Mnl*I for species *A. butzleri, A. thereius* and *A. trophiarum*. Lanes 1 and 14, 50 bp ladder (Fermentas); 2, *A. butzleri* LMG 10828[T]; 3, *A. butzleri* F42; 4, *A. butzleri* F43; 5, *A. butzleri* F44; 6, *A. butzleri* F50; 7, *A. butzleri* LMG 11118; 8, *A. thereius* LMG 24486[T]; 9, *A. thereius* SW24; 10, *A. thereius* F89-4; 11, *A. thereius* F93-4 y 12, *A.thereius* LMG 24487; 13, *A. trophiarum* CECT 7650 (identical pattern to that of the 11 atypical strains of *A. cryaerophilus*, Additional file 2: Table S2). *Mnl*I was selected because it produced more distinctive patterns among the species than *Tas*I.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MJF designed the research project, evaluated results and was principal author. LC isolated the nine strains of *A. cryaerophilus* in Chile and carried out the speciation and 16S rRNA gene mutation analyses. AL carried out the computer simulations, the experimental digestions and participated in the drafting of manuscript under the supervision of LC and MJF. All authors read and approved the final manuscript.

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Table S1. Computer simulated profiles of the digestion of the 16S rRNA gene (1026 bp) of *Arcobacter* spp. with *Mse*I endonuclease.
Species with specific RFLP pattern are in bold.

	Presence of 16S rRNA gene RFLP fragments of the following size (bp)[a]																									
	650	**615**	**551**	**548**	**519**	**442**	**434**	**407**	**395**	**372**	**366**	**365**	**308**	**269**	**243**	**216**	**167**	**143**	**141**	**139**	**138**	**100**	**99**	**92**	**72**	**52**	**49**
A. butzleri LMG 10828[T]				X												X					X					X	
A. thereius LMG 24486[T]				X												X					X					X	
A. trophiarum LMG 24486[T]				X												X					X					X	
A. cryarophilus MIC V1-1[b]				X												X					X					X	
***A. ellisii* CECT 7837[T]**		X																			X			X		X	X
***A. mytili* CECT 7386[T]**	X																		X		X					X	
***A. molluscorum* CECT 7696[T]**			X																X		X				X	X	
***A. halophilus* LA31B[T]**			X																X		X	X				X	
***A. nitrofigilis* CECT 7204[T]**							X										X				X	X				X	X
A. marinus CECT 7727[T]													X		X					X	X		X			X	
A. venerupis CECT 7836[T]													X		X				X		X		X			X	
***A. cryaerophilus* 1A LMG 9904[T]**									X							X		X			X					X	
***A. cryaerophilus* 1B LMG 10229**												X				X		X			X					X	
***A. skirrowii* LMG 6621[T]**											X				X			X			X					X	
***A. cibarius* CECT 7203[T]**					X										X						X					X	
A. defluvii CECT 7697[T]								X							X				X		X					X	
A. suis F41[T]								X							X				X		X					X	
***A. bivalviorum* CECT 7835[T]**						X								X							X					X	
***A. cloacae* SW28-13[T]**										X					X						X			X		X	X

[a]Small-size bands below 49 bp were not resolved in the electrophoresis and not included in the table.

[b]The same pattern was obtained for 10 other atypical *A. cryarophilus* strains (9 recovered from animal faeces in Chile and 2 from animal abortions in Ireland).

Table S2. Computer simulated profiles of the digestion of the 16S rRNA gene (1026 bp) of *Arcobacter* spp. with *Mnl*I endonuclease. Species in bold are those that now show a specific RFLP pattern not distinguished previously with *Mse*I enzyme.

	Presence of 16S rRNA gene RFLP fragments of the following size (bp)[a]																		
	440	**267**	**246**	**232**	**205**	**173**	**164**	**147**	**126**	**115**	**112**	**106**	**99**	**92**	**89**	**87**	**72**	**59**	**49[a]**
***A. butzleri* LMG 10828[T]**		X				X		X	X			X	X					X	X
***A. thereius* L**MG 24486[T]	X		X	X														X	X
A. trophiarum L MG 25534[T]	X			X				X					X					X	X
A. cryarophilus MIC V1-1[b]	X			X				X					X					X	X
A. cryaerophilus 1A LMG 9904[T]	X			X				X					X					X	X
A. cryaerophilus 1B LMG 10229	X			X				X					X					X	X
A. cibarius CECT 7203[T]	X			X				X					X					X	X
A. skirrowii LMG 6621[T]	X			X				X					X					X	X
***A. marinus* CECT 7727[T]**	X								X	X		X				X		X	X
***A. venerupis* CECT 7836[T]**		X				X			X		X	X		X				X	X
A. halophilus LA31B[T]	X								X			XX	X					X	X
A. molluscorum CECT 7696[T]	X								X	X		X			X			X	X
A. bivalviorum CECT 7835[T]	X								X			X	X				X		X
A. mytili CECT 7386[T]	X		X						X			X						X	X
A. nitrofigilis CECT 7204[T]		X					X		X	X		X			X			X	X
A. cloacae SW28-13[T]		X			X	X			X			X						X	X
A. ellisii CECT 7837[T]		X			X	X			X			X						X	X
A. defluvii CECT 7697[I]		X				X			X			X	X				X	X	X
A. suis F41[I]		X				X			X			X	X				X	X	X

[a]Small-size bands below 49 bp were not resolved in the electrophoresis and not included in the table.

[b]The same pattern was obtained for 10 other atypical *A. cryarophilus* strains (9 recovered from animal faeces in Chile and 2 from animal abortions in Ireland).

Table S3. **Computer simulated profiles of the digestion of the 16S rRNA gene (1026 bp) of *Arcobacter* spp. with *Bfa*I endonuclease.** Species in bold are those that now show a specific RFLP pattern not distinguished previously with *Mse*I or *Mnl*I enzymes.

	Presence of 16S rRNA gene RFLP fragments of the following size (bp)[a]												
	580	**405**	**378**	**271**	**256**	**203**	**184**	**175**	**169**	**93**	**87**	**83**	**62[a]**
***A. trophiarum* LMG 25534^{T}**	X			X				X					
***A. cryaerophilus 1A* LMG 9904^{T}**	X						X	X			X		
***A. cryaerophilus 1B* LMG 10229**	X						X	X			X		
***A. cryaerophilus* MIC V1-1[b]**	X						X	X			X		
A. skirrowii LMG 6621^{T}	X						X	X			X		
A. cibarius CECT 7203^{T}	X						X	X			X		
A. ellisii CECT 7837^{T}	X						X	X			X		
A. cloacae SW28-13^{T}	X						X	X			X		
***A. defluvii* CECT 7697^{T}**		X					X	X		X	X	X	
***A. suis* F41^{T}**	X							X	X		X		
A. butzleri LMG 10828^{T}	X			X				X					
A. thereius LMG 24486^{T}	X			X				X					
A. marinus CECT 7727^{T}	X			X				X					
A. venerupis CECT 7836^{T}			X					X	X	X	X	X	
A. halophilus LA31B^{T}			X	X		X		X					
A. molluscorum CECT 7696^{T}	X			X				X					
A. bivalviorum CECT 7835^{T}	X				X			X					
A. mytili CECT 7386^{T}	X						X	X					X
A. nitrofigilis CECT 7204^{T}			X		X			X		X	X		

[a]Small-size bands below 62 bp were not resolved in the electrophoresis and not included in the table.

[b]The same pattern was obtained for 10 other atypical *A. cryarophilus* strains (9 recovered from animal faeces in Chile and 2 from animal abortions in Ireland).

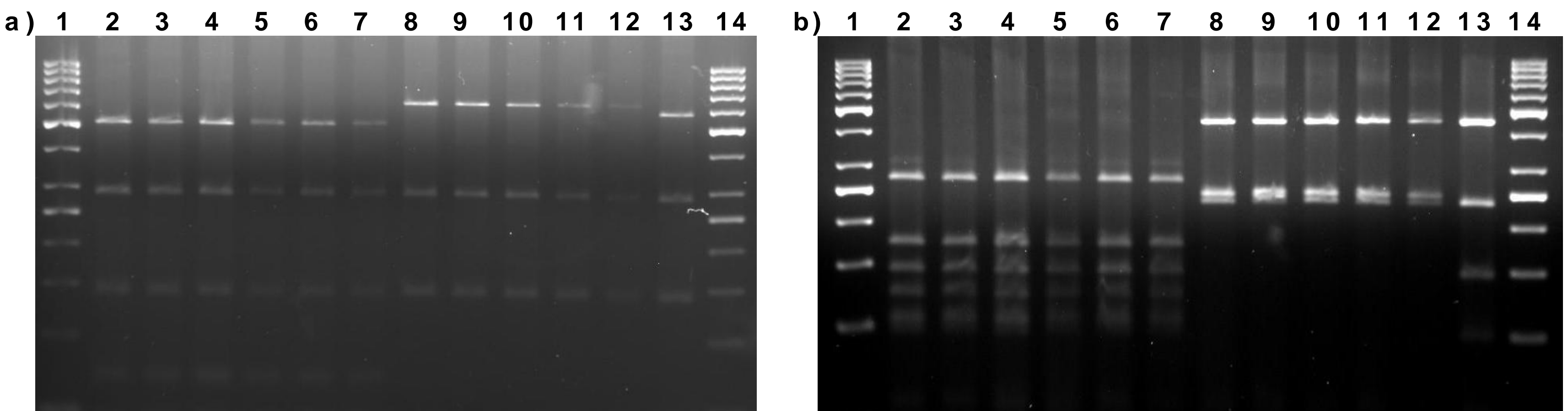

Figure S1. Agarose gel 3.5% comparing the 16S rDNA-RFLP patterns obtained using endonucleases a) *Tas*I and b) *Mnl*I. Lanes: 1 and 14, ladder 50 bp (Fermentas); 2, *A. butzleri* LMG 10828^{T}; 3, *A. butzleri* F42; 4, *A. butzleri* F43; 5, *A. butzleri* F44; 6, *A. butzleri* F50; 7, *A. butzleri* LMG 11118; 8, *A. thereius* LMG 24486^{T}; 9, *A. thereius* SW24; 10, *A. thereius* F89-4; 11, *A.thereius* F93-4 y 12, *A.thereius* LMG 24487; 13, *A. trophiarum* CECT 7650. The *Mnl*I enzyme was selected because it produced more distinctive patterns among the species than the *Tas*I.

4.5. Performance of five molecular methods for monitoring *Arcobacter* spp. and systematic literature review

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Performance of five molecular methods for monitoring *Arcobacter* spp. and systematic literature review

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Running title: Arcobacter identification

Key words: *Arcobacter*, identification, comparison, molecular methods,16S rRNA-RFLP, m-PCR, 23S rRNA, *gyrA*,

Abstract

Bacteria of the genus *Arcobacter* are considered emergent enteropathogens and potential zoonotic agents. Their taxonomy has evolved very rapidly and there are currently 17 species. The real prevalence of the species of this genus is considered underestimated because of the limitations of the available methods used for correctly detecting and/or identifying all species.

The aim of this study was to compare the ability of five PCR based methods, that mainly target regions of the 16S rRNA or 23S rRNA or the *gyrA* genes, to identify all *Arcobacter* spp., and to review systematically the results reported in the literature when using these methods.

Results. Results show that the five methods tested had misidentified as the species targeted several of the species described after their publication. Varying results were obtained depending upon the selected target regions of the mentioned genes. For instance, the worst results were obtained for certain regions of the 23S rRNA gene used for the identification of *A. cryaerophilus* because it can be confused with 8 or 11 other species depending upon the method. The results suggest that the currently known diversity of *Arcobacter* spp. in different environments may expand if reliable identification methods are applied in future studies.

Introduction

Arcobacter spp. are considered emerging enteropathogens and potential zoonotic agents that can be transmitted by food and water [1]. In fact, previous studies have demonstrated a relationship between the presence of arcobacters in water samples and bacterial indicators of faecal pollution [2,3]. This genus belongs to the *Campylobacteraceae* family and was originally proposed by Vandamme *et al*. in 1991 [4] to accommodate two aerotolerant species (A. *cryaerophilus* and *A. nitrofigilis*), which had previously been included in the genus *Campylobacter*. Since 2009, the number of newly described species has risen exponentially and it currently comprises 17 species, eight of them described in our laboratory [1,5-7].

The identification of *Arcobacter* spp. using phenotypic tests is difficult because they can be confused with *Campylobacter* spp. [1,8]. For this reason, several molecular detection and identification methods based on conventional PCR, multiplex PCR (m-PCR), Real Time PCR (RT-PCR), Restriction Fragment Length Polimorfism (RFLP), Denaturing Gradient Gel Electroforesis PCR (DGGE-PCR), Flourescence in situ Hibridization (FISH) and Matrix Assisted Laser Desorption Ionization Mass Spectra (MALDITOF MS) have been designed, as reviewed by Collado & Figueras [1]. The majority of PCR based methods [9-12] target the genus or the species *A. butzleri* and/or *A. cryaerophilus* [1], while others also included *A. skirrowii* [13,14] or *A. cibarius* [15]. In 2010, Douidah *et al*. proposed a new m-PCR method that could identify 5 species associated with in humans and other mammals, i.e. *A. butzleri, A. cryaerophilus, A. skirrowii, A. cibarius* and *A. thereius* [8]. This m-PCR was not able to detect the species *A. trophiarum,* originally isolated from pigs [16], so the same research group proposed a PCR method for this species that targets the *hsp60* gene to complement their m-PCR method [16]. In 2008, Figueras *et al*. [17] designed an RFLP based on the digestion of the 16S rRNA gene with the *Mse*I endonuclease that was able to identify the 6 species that had so far been described (*A. butzleri, A. cryaerophilus, A. cibarius, A. skirrowii, A. nitrofigilis* and *A. halophilus*). This method was recently updated with the inclusion of additional endonucleases (*Mnl*I and *Bfa*I) so that it is able to identify the 17 currently characterized *Arcobacter* spp. [18]. It has been suggested that the limitations of the identification methods in recognizing or identifying correctly all species are the main reason for inaccurate estimates of the prevalence of *Arcobacter* spp. in different matrices i.e. water, food, faeces, etc. Despite this, no study has so far evaluated comparatively the most commonly used identification methods. The aim of the present study was to compare the performance of five molecular methods for the identification of the 17 described *Arcobacter* spp. using the recently updated 16S rRNA-RFLP method as a reference [18]. Furthermore, a systematic literature review was carried out in order to analyse the results that have been obtained using these methods since their publication.

Materials and methods

The six identification methods were compared using a total of 95 strains (including type and reference strains as well as field strains) representing all currently accepted *Arcobacter* species (Table S1). The five molecular methods, selected because they were the ones that targeted the highest number of species, were the following: two m-PCRs that target *A. butzleri, A. cryaerophilus* and *A. skirrowii* [13,14], a PCR method that targets those three species and also *A. cibarius* [15], and two methods that target those 4 species and also *A. thereius,* i.e. the m-PCR of Douidah *et al*. [8], or *A. nitrofigilis* and *A. halophilus,* the 16S rRNA-RFLP of Figueras *et al,* [17]. Considering that the PCR of De Smet *et al*. [16] that targets the species *A. trophiarum* was designed by the same group to complement the m-PCR of Douidah *et al*. [8], both methods were considered as a single method for comparative purpose (Tables 1 and 2).

All isolates were grown on 5% sheep blood agar for 48 h at 30ºC under aerobic conditions. DNA was extracted using the InstaGene DNA Purification Matrix (Bio-Rad Laboratories, Hercules, CA), and quantified using the GeneQuant (Amersham Pharmacia Biotech, Cambridge, England) following the manufacturer's instructions. The PCRs were carried out in a 2720 Thermal Cycler (Applied Biosystems) using the primers and conditions described in the different studies and summarized in Table S2. The identity of all field strains was confirmed with the 16S rRNA-RFLP of Figueras *et al*. [18] and verified in some cases by sequencing the 16S rRNA and/or *rpoB* genes (Table S1).

The performance of the methods was evaluated by the percentage of strains of the targeted species that were correctly identified and considering the number of non-targeted species that gave erroneous results (Tables 1, 2 and S1).

The systematic literature review was carried out following the PRISMA guidelines [19], using the Citations Search tool in the Web of Science® V 5.8 in Thomson Reuters ISI Web of Knowledge research platform, which is available online. The platform was accessed using the Spanish national license through FECYT (*Fundación Española para la Ciencia y la Tecnología,* http://www.accesowok.fecyt.es), being last accessed on July 30th 2012. No additional source of information was used. Each of the 5 studied molecular methods was searched by author, topic (arcobacter) and year of publication to obtain the total number of citations for each method since their publication until 2012. These citations were analyzed one by one to find out the total number of strains identified at the species level. The number of strains of each species identified using any of the compared methods was the data extracted to make the calculation shown in Table S3. In those studies were no genotyping method was used, it was assumed that each isolate represents a strain.

Results and Discussion

Comparative performance of the five molecular methods

The percentage of correctly identified strains obtained with five identification methods and the number of misidentified non-targeted species revealed that the method with the lowest performance was the m-PCR of Kabeya et al. [14]. This method produced unreliable results for the 3 targeted species; only 4.8% of the *A. butzleri* were correctly identified and several non-targeted species were mistaken for it (6 species) or for *A. cryaerophilus* (8 species) or for *A. skirrowii* (3 species), despite all strains of the latter two species being correctly identified (Tables 1 and 2). Globally, the Kabeya m-PCR correctly identified only 32.6% (31/95) of the studied strains. Furthermore the method was also designed to differentiate subgroups 1A and 1B of *A. cryaerophilus*, and not all strains of these subgroups were correctly identified (Table 2). This correlates with previous *in silico* observations of Douidah *et al*. [8] in the sense that the primers designed [14], were not specific enough to provide a correct identification of *A. cryaerophilus* at the level of subgroup. Furthermore, Debruyne *et al.* [20] suggested, on the basis of results from AFLP and *hsp60* analyses, that this nomenclature (1A and 1B) should be abandoned.

The second least reliable method was the m-PCR of Houf *et al* [13], which identified correctly only 55.8% (53/95) of the strains (Table 2), including all those belonging to its targeted species, i.e. *A. butzleri, A. cryaerophilus* and *A. skirrowii* (Table 1). This method was 100% reliable for the identification of *A. butzleri* only because 9 of the 14 non-targeted species generated the typical amplicon of *A. cryaerophilus,* 2 the one of *A. skirrowii* and 2 both amplicons. In fact, only *A. cibarius* produced no amplification with this method (Table 2). These results agree with previous studies that have suggested there are possible misidentifications when using this method [1, 5-7].

A similar number of correctly identified strains (83.2%] was obtained with the other 3 methods evaluated, i.e. Pentimalli *et al.* [15]; the combined method of Douidah *et al*. [8] and De Smet *et al* [16]; and Figueras *et al.* [17]. However, the number of misidentified non-targeted species was different depending upon the method (Tables 1 and 2). For instance, with the PCR method of Pentimalli *et al.* [15], which involves 4 independent PCR reactions, the species *A. butzleri* and *A. cryaerophilus* could be confused with 4 and 1 non-targeted species, respectively, and only 60% of the strains of *A. skirrowii* were correctly identified (Table 1 and 2). Regarding the primers that these authors designed for *A. cibarius*, they were able to identify correctly all strains of this species but these strains also produced the expected amplicon for *A. butzleri* and *A. skirrowii* with their specific reactions (Table 2). Therefore, the identification of these 3 species is unreliable. In relation to the combined method of Douidah *et al.* [8] and De Smet *et al.* [16], it misidentified 4 of the non-targeted species (*A. defluvii, A. ellisii, A. venerupis* and *A. suis*) as *A. butzleri* and also 2 of the 3 strains of *A. ellisii*, as *A. cryaerophilus* (Table 2). For the remaining 4 targeted species, the method performed perfectly (Table 1). Finally, the 16S rRNA-RFLP

designed by Figueras *et al*. [17] misidentified three species (*A. trophiarum*, *A. thereius* and some strains of *A. cryaerophilus*) as *A. butzleri*, two species described since their publication (*A. suis* and *A. defluvii*) produced the same pattern, and two species (*A. venerupis* and *A. marinus*) a very similar one (Table 2). Considering these limitations, this method was recently updated with new endonucleases and produced specific results for all strains and species [18]. This updated protocol was the one used to identify all strains in this study.

Comparative evaluation of the targeted genes and designed primers

When the results were evaluated in relation to the genes used to identify the species, it was observed that the 23S rRNA gene regions targeted in the Kabeya *et al.* [14] method for *A. butzleri, A. cryaerophilus* and *A. skirrowii* were unreliable, as also was the region employed in the Houf *et al*. method [13] for *A. cryaerophilus* (Tables 1 and S2). However, the regions of this gene targeted by the m-PCR of Douidah *et al*. [8] were 100% reliable for the detection of *A. skirrowii, A. cibarius* and *A. thereius*, but not for *A. butzleri* (Tables 1, 2 and S2). Regarding the *gyrA* gene, the region used for the identification of *A. cryaerophilus* in the latter method was unreliable because *A. ellisii* was confused with this species. The same occurred with the regions used by Pentimalli *et al.* [15] for *A. cryaerophilus* and *A. skirrowii*. In fact, of the reactions that used the *gyrA* gene, the specific PCR for *A. cibarius* was the only reliable one because it did not react with any other species (Tables 1 and 2). The main reason for this poor performance of the targeted regions of 23S rRNA or *gyrA* genes (Table S2) is the limited number of sequences that had been used to derive the primers. For instance, so far the sequences of the 23S rRNA gene are only available for 8 of the 17 known *Arcobacter* species (*A. butzleri, A. cryaerophilus, A. skirrowii, A. cibarius, A. nitrofigilis, A. thereius, A. mytili* and *A. trophiarum*) and of the *gyrA* gene only for 7 species (*A. butzleri, A. cryaerophilus, A. skirrowii, A. cibarius, A. nitrofigilis, A. marinus* and *A. halophilus*). In contrast, the sequences of the 16S rRNA gene are available for all the species of the genus. The analysis of all these sequences enabled endonucleases to be selected that are able to generate RFLP species-specific patterns for all the *Arcobacter* species in the updated 16S rRNA-RFLP method recently published by Figueras *et al*. [18]. The 16S rRNA gene was previously used to design specific primers for *A. butzleri* in the methods of Houf *et al*. [13] and Pentimalli *et al*. [15] and for *A. skirrowii* with the former method. However, only the primers that targeted the 16S rRNA region chosen by Houf *et al*. [13] for the identification of *A. butzleri* (Table S2) were 100% specific, and showed no crossed-reaction with other species (Tables 1 and 2).

Literature review of the studied methods

The results of the systematic literature review that summarised the total number of strains and species identified using any the 5 methods compared (Table S3) shows that the m-PCR of Houf *et al*. [13] is the most globally referenced, with 71.9% (123/171) of the citations. This method has been used to identify 64.8% (2735/4223) of strains recorded since 2000 in the literature (Table S3). The next most-used methods were the 16S rDNA-RFLP of Figueras *et al*. [17] and the m-PCR of Douidah *et al.* [8], which have been used to identify 14.6% and 13.4% of strains, respectively (Table S3). The overall prevalent species were *A. butzleri* (63.7% of strains) followed by *A. cryaerophilus* (27.3%) and *A. skirrowii* (4.9%) (Table S3), while the other 14 species together represented only the 4.1% of studied strains (Table S3). The species diversity obtained in this revision may have some bias influenced by the different origins of the strains and/or isolation methods used in those studies.

Considering the results obtained in the present study, the strains identified as *A. butzleri* (64.5%) using the m-PCR designed by Houf *et al*. [13] could be considered as correctly identified (Table S3). However, the use of this method has probably led to an global overestimation of the species *A. cryaerophilus* and *A. skirrowii* because some of the strains identified as such could belong to several other species (Table 1 and 2). For instance, Atabay *et al.* [21] identified with the latter method [13] 6 strains as *A. skirrowii* that were not able to hydrolyze indoxyl acetate despite this being a typical phenotypic characteristic of this species. Interestingly, *A. mytili,* one of only 2 species of the genus (along with *A. molluscorum*) is indeed unable to hydrolyze indoxyl acetate and produces the typical band of *A. skirrowii* when the m-PCR of Houf *et al*. [13] is used, so the 6 mentioned strains might belong to that species. More evidence can be found in a study on the prevalence of *Arcobacter* in meat and shellfish [22], in which strains belonging to another 2 of these 13 species, i.e. *A. nitrofigilis* and *A. thereius* were recognized*.* Those strains produced the expected amplicon for *A. skirrowii* and *A. cryaerophilus,* respectively, with the Houf method [13], and the expected pattern of *A. nitrofigilis* and *A. butzleri* with the 16S rRNA-RFLP of Figueras *et al*. [17], and their identity was confirmed by sequencing the 16S rRNA and/or *rpoB* genes [22]. Furthermore, using these two methods combination, the species *A. mytili, A. molluscorum, A. defluvii, A. ellisii, A. bivalviorum, A. venerupis, A. cloacae* and *A. suis* have also been discovered [1, 5-7, 23-25].

In relation to other tested methods, the m-PCR of Douidah *et al.* [8] combined with the PCR of De Smet et al. [16] has enabled the species *A. thereius* (17.6%, 100/567), *A. trophiarum* (1.8%, 10/567) and *A. cibarius* (0.2%, 1/567) to be recognized (Table S3) in two independent studies carried out by De Smet *et al*. [26,27]. Nevertheless, the great weakness of this approach is that strains of non-targeted species might be misidentified as the more frequently isolated species, *A. butzleri* (Tables 1 and 2).

Finally, regarding the studies that used the method designed by Kabeya *et al*. [14], our results show that all the targeted species might have been overestimated because 12 of the 14 non-targeted species might be misidentified as them (Tables 1 and 2). No studies were found that used the PCR method of Pentimalli *et al*. [15]; based on our results, it is not reliable for the identification of its targeted species (Tables 1 and 2).

In this study the ability of five PCR methods to identify all *Arcobacter* spp. have been compared for the first time. None of the compared methods was completely reliable and there were different degrees of misidentification of the species described since their publication with those targeted by the method. We hope now to have highlighted that there are limitations in the compared methods and verification using reliable methods in parallel should be the way forward. Our results suggest that the currently known diversity of *Arcobacter* spp. in different environments will change in future if reliable identification methods are applied, such as the updated 16S rRNA-RFLP method [18].

Competing interests

The authors have declared that there are no competing interests.

Authors' contributions

AL carried out the experiments and literature review and drafted the manuscript, being the principal author. MJF designed the research project, evaluated results, drafted the manuscript and supervised AL. Both authors read and approved the final manuscript.

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Table 1. Comparative performance of five molecular methods for the identification of their targeted *Arcobacter* spp. (a) Targeted genes, (b) percentage of correctly identified strains of the targeted species and (c) number of non-targeted species confused as the targeted ones.

[a] The strains of the remaining nine species of the genus not listed in the table (n=28) are those of new species not targeted by the compared methods.

		Houf *et al.* [13]			**Kabeya *et al.* [14]**			**Figueras *et al.* [17]**			**Pentimalli et al. [15]**			**Douidah *et al.* [8] De Smet *et al.* [16][b]**		
Targeted species	**Strains[a]**	**a**	**b**	**c**	**a**	**b**	**c**	**a**	**b**	**c**	**a**	**b**	**c**	**a**	**b**	**c**
A. butzleri	21	16S	100	0	23S	4.8	6	16S	100	3	16S	100	4	23S	100	4
A. cryaerophilus	19	23S	100	11	23S	100[c]	8	16S	63.2	0	*gyrA*	100	1	*gyrA*	100	1
A. skirrowii	5	16S	100	4	23S	100	3	16S	100	0	*gyrA*	60	2	23S	100	0
A. cibarius	8							16S	100	0	*gyrA*	0[d]	0	23S	100	0
A. thereius	5													23S	100	0
A. trophiarum	3													*hsp60*	100	0
A. nitrofigilis	5							16S	100	0						
A. halophilus	1							16S	100	0						

[b]The method designed by De Smet *et al.* [16] only detects or identify *A. trophiarum* and it was intended to complement the m-PCR of Douidah *et al.* [8], so they are considered together as a single method
[c]The method was designed to differentiate the subgroups 1A and 1B of this species, but not all strains of these subgroups were well recognized (Table 2)
[d]Despite the 8 strains of *A. cibarius* being correctly assigned to this species, none of them was considered correctly identified because the 6 strains were also identified as *A. butzleri* and 3 strains also as *A. skirrowii* (Table 2).

Table 2. Identification results obtained for 95 strains of 17 *Arcobacter* spp. with 5 different PCR identification methods.

Species	Strains[a]	Houf *et al.* [13]	Kabeya *et al.* [14]	Figueras *et al.* [17][b]	Pentimalli *et al.* [15]	Douidah *et al.* [8] De Smet *et al.* [16]
A. butzleri **[Ab]**	21	21 Ab	1 Ab[c] 15 Ab + Acr1B[d] 5 NA[e]	21 Ab	21 Ab	21 Ab
A. cryaerophilus **(Acr)**	19	19 Acr	19 Acr	12 Acr[c] 7 Ab	19 Acr	19 Acr
Acr1A (n=6)			5 Acr1A[c] 1 Acr1B			
Acr1B (n=6)			5 Acr1B 1 Acr1A			
A. skirrowii **(Aski)**	5	5 Aski	5 Aski	5 Aski	3 Aski[cdf] 2 NA	5 Aski
A. nitrofigilis **(Anit)**	5	5 Aski	4 Acr1B[c] 1 Ab + Acr1B	5 Anit	2 Ab 2 Acr 3 NA*[c]	NA
A. halophilus **(Ahalo)**	1	1 Aski + Acr	1 Aski	1 Ahalo	NA*	NA
A. cibarius **(Acib)**	8	8 NA	3 Aski[c] 5 Aski + Acr1B	8 Acib	8 Ab 8 Acib 3 Aski	8 Acib
A. thereius **(Ather)**	5	5 Acr	1 Ab 2 Ab + Acr1B[c] 1 Acr1B 1 NA	5 Ab	5 NA*	5 Ather
A. mytili **(Amyt)**	3	3 Aski	3 Aski	3 Amyt	3 NA*	3 NA
A. marinus **(Amar)**	1	1 Acr	1 NA	1 Amar[g]	1 Ab	1 NA
A. molluscorum **(Amoll)**	3	3 Aski + Acr	3 NA	3 Amoll	3 NA*	3 NA
A. defluvii **(Adef)**	11	11 Acr	11 Ab	11 Adef	11 NA*[c]	11 Ab

A. trophiarum **(Atroph)**	3	3 Acr	2 Ab[c] 1 NA	3 Ab	3 NA*	3 Atroph
A. ellisii **(Aelli)**	3	3 Acr	3 Acr1A + Acr1B	3 Aelli	2 Aski 1 NA*[c]	1 Ab 2 Ab +Acr[c]
A. bivalviorum **(Abiv)**	3	3 Acr	3 Acr1B	3 Abiv	3 NA	3 NA
A. venerupis **(Aven)**	1	1 Acr	1 Ab	1 Aven[g]	1 Ab	1 Ab
A. cloacae **(Acloa)**	2	2 Acr	2 Ab + Acr1B	2 Acloa	2 NA*	2 NA
A. suis **(Asuis)**	1	1 Acr	1 Acr1A	1 Adef	1 NA	1 Ab
Correctly identified strains	95	53 (55.8%)	31 (32.6%)	79 (83.2%)	79 (83.2%)	79 (83.2%)

[a]All strains were identified with the RFLP method of Figueras *et al*. [18] specifically designed to recognize all species.[b]Considering that this method was designed for *A. butzleri, A. cryaerophilus, A. cibarius, A. skirrowii, A. nitrofigilis* and *A. halophilus* [17], the results for strains of other species were interpreted based on RFLP patterns described in subsequent publications [5-7, 22-24].[c]Specific result produced by the type strain when strains of this species produced more than one result. [d] "species A + species B" means that the expected amplicon for species A and B were obtained in the same reaction. [e]NA or NA*: No amplification of a band of the expected size, or (*) band/s of another size, was obtained. [f] When different results were obtained with the 4 individual PCR reactions designed by Pentimalli *et al*. [15] for *A. butzleri, A. cryaerophilus, A. skirrowii* and *A. cibarius,* they are shown in separate lines. [g]*A. venerupis* produced a pattern very similar to that of *A. marinus*, with only a single band distinguishing the two species.

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Table S1 Strains of *Arcobacter* spp. used in the study.

SPECIES	STRAIN	SOURCE
A. bivalbiorum **n=3**	F4[T,a,b], F118-2[a,b], F118-4[a,b]	Mussels
A. butzleri **n=21**	LMG 10828[T,a,b], LMG 11118[b]	Human faeces
	F42, F46[a,b], F49, F51	Pork
	F15, F22, F23, F24, F25	Turkey
	F47, F52	Chicken
	F50[b], F53	Beef
	F1, F2, F29, F30, F98-1	Mussels
	T62	Soil
A. cibarius **n=8**	CECT 7203[T,a,b]	Chicken
	NC81[b], NC88[b]	Piggery efluent
	H742, H743[b], H745, H746[b], H748	Poultry carcasses
A. cloacae **n=2**	SW28-13[T,a,b]	Sewage
	F26[a,b]	Mussels
A. cryaerophilus **n=19**	LMG 9904[T,a,b], LMG 9871[a]	Bovine abortion foetus
	LMG 9865[a,b], LMG 10241[b], LMG 6622, LMG 10229[a,b]	Porcine abortion
	LMG 9065[a], LMG 7537[a], LMG 9863[a,b]	Ovine abortion foetus
	LMG 10829[a]	Human blood
	LMG 9861[a,b]	Bovine abortion foetus
	FE4[a,b], FE5[a,b], FE6[a,b], FE9[a,b], FE11[a], FE13[a], FE17[a]	Chicken faeces
	FE14[b],	Ovine faeces
A. defluvii **n=11**	CECT 7697[T,a,b], SW28-7[a,b], SW28-8, SW28-9, SW28-10, SW30-2[a,b], SW30-7, SW30-8	Sewage
	CC42[b]	Pig faeces
	CH8-2, SAN599-9[b]	Mussels
A. ellisii **n=3**	F79-6[T,a,b], F79-2[a,b], F79-7[a,b]	Mussels
A. halophilus **n=1**	LA31B[T,a,b]	Hypersaline lagoon
A. marinus **n=1**	CECT 7727[T,a,b]	Seawater/starfish
A. molluscorum **n=3**	CECT 7696[T,a,b], F91[a,b], F101-1[a,b]	Mussels
A. mytili **n=3**	CECT 7386[T,a,b], CECT 7385[a,b]	Mussels

	T234[b]	Brackish water
A. nitrofigilis **n=5**	CECT 7204[T,a,b], LMG 7547[b]	Roots of *Spartina alterniflora*
	F39[b], F40[¶], F72[b]	Mussels
A. skirrowii **n=5**	LMG 6621[T,a,b]	Lamb faeces
	LMG 9911	Porcine abortion
	Houf 989[a,b], Houf 994[b]	Cow faeces
	S7[b]	Sludge
A. suis **n=1**	F41[T,a,b]	Pork
A. thereius **n=5**	LMG 24486[T,a,b], LMG 24487[a,b]	Porcine abortion foetus
	SW24[b]	Sewage
	F61-1[b]	Pork
	F93-4[b]	Clams
A. trophiarum **n=3**	LMG 25534[T,a,b], LMG 25535[a,b]	Pig faeces
	CECT 7650[a,b]	Chicken cloaca
A. venerupis **n=1**	F67-11[T,a,b]	Clams

ATCC: American Type Culture Collection, LMG: Belgian Co-ordinated Collection of Microorganisms, CECT: Colección Española de Cultivos Tipo. [a] Sequenced 16S rRNA gene [b]Sequenced *rpoB* gene

Table S2. Targeted gene and conditions of the PCR methods compared in this study

Author	Targeted species (expected amplicon)	Targeted gene: Position (nt)[a]	Primers (pmol)	Concentrations[b,c]	Conditions
Houf *et al*., 2000 (13)	*A. butzleri* (400) *A. cryaerophilus* (230) *A. skirrowii* (640)	16S rRNA: 959 – 1357 23S rRNA: 1720 – 1964 16S rRNA: 705 - 1357	ARCO (50) BUTZ (50) CRY1 (50) CRY2 (50) SKIR (25)	$MgCl_2$ 1.3 mmol l^{-1} *Taq* DNA polymerase 1.5 U	Initial denaturation 94ºC, 2 min and final extension 72ºC, 5 min 32 Cycles of: Denaturation 94ºC, 45 s; Annealing 61ºC, 45 s; Chain extension 72ºC, 30 s
Kabeya *et al*., 2003 (14)	*A. butzleri* (692) *A. cryaerophilus* 1A (728) *A. cryaerophilus* 1B (152) *A. skirrowii* (448)	23S rRNA: 1174 - 1865 23S rRNA: 1135 – 1865 23S rRNA: 1713 – 1865 23S rRNA: 1423 - 1865	N.c.1A (25) ARCO-U (25) N.butz (2.5) N.c.1B (2.5) N.ski (2.5)	MgCl2 1.5 mmol l-1 Taq DNA polymerase 2.5 U	Initial denaturation 94ºC, 3 min and final extension 72ºC, 5 min 30 Cycles of: Denaturation 94ºC, 30 s; Annealing 62ºC, 60 s; Chain extension 72ºC, 60 s
Figueras *et al*., 2008 (17)	Species specific patterns for species[d]	16S rRNA: 47 - 1073	CAH 1am (25) CAH 1b (25)	MgCl2 1.5 mmol l-1 Taq DNA polymerase 2.5 U	Initial denaturation 94ºC, 2 min, final extension 72ºC, 10 min 30 Cycles of: Denaturation 94ºC, 30 s; Annealing 52ºC, 30 s; Chain extension 72ºC, 90 s
Pentimalli *et al*., 2009 (15)	*A. butzleri* (203) *A. cryaerophilus* (212) *A. skirrowii* (257) *A. cibarius* (145)	16S rRNA: 803 – 1006 *gyrA*: 2337 – 2549 *gyrA*: 1366 – 1622 *gyrA*: 2364 - 2778	16S Arcobutz (30) Gyr Arcocry (30) Gyr Arcoski (30) Gyr Arcocib (50) All F and R	MgCl2 2.0 mmol l-1 Taq DNA polymerase 2.0 U	Initial denaturation 94ºC, 2 min and final extension 72ºC, 7 min 40 Cycles of: Denaturation 94ºC, 60 s; Annealing 55ºC, 60 s; Chain extension 72ºC, 60 s
Douidah *et al*., 2010 (8)	*A. butzleri* (2061) *A. cryaerophilus* (395) *A. skirrowii* (198) *A. cibarius* (1125) *A. thereius* (1590)	23S rRNA: 646 - 2707 *gyrA*: 2255 – 2640 23S rRNA: 646 – 844 23S rRNA: 646 - 1771 23S rRNA: 646 - 2236	ButR (50) SkiR (50) TheR (50) CibR (50) ArcoF (50) GyrasF (50) GyrasR (50)	MgCl2 1.5 mmol l-1 Taq DNA polymerase 1.5 U	Initial denaturation 94ºC, 2 min and final extension 72ºC, 10 min 30 Cycles of: Denaturation 94ºC, 45 s; Annealing 58ºC, 45 s; Chain extension 72ºC, 2 min
De Smet *et al*., 2011 (16)	*A. trophiarum* (382)	*hsp60*: 686 - 1068	hsp60F and R (50)	MgCl2 1.4 mmol l-1 Taq DNA polymerase 2.0 U	94ºC, 3 min, final extension 72ºC, 5 min 30 Cycles of: Denaturation 94ºC, 45 s; Annealing 58ºC, 45 s; Chain extension 72ºC, 30 s
Figueras *et al*., 2012 (18)	Species specific patterns for species[d]	16S rRNA: 47 - 1073	CAH 1am (25) CAH 1b (25)	MgCl2 1.5 mmol l-1 Taq DNA polymerase 2.5 U	Initial denaturation 94ºC, 2 min, final extension 72ºC, 10 min 30 Cycles of: Denaturation 94ºC, 30 s; Annealing 52ºC, 30 s; Chain extension 72ºC, 90 s

[a] Positions of 16S rRNA gene are based on *Escherichia coli*; 23S rRNA and *gyrA*, are based on sequences present in the *A. butzleri* RM4018 complete genome. [b] All PCR included dATP, dCTP, dGTP, and dTTP (Applied Biosystems(TM)) at a concentration of 200µM each and 5µl buffer 10X Invitrogen(TM) and miliQ water up to a final volume of 50µl; Taq DNA polymerase Invitrogen(TM). [c] For m-PCR of Kabeya *et al*. (14) 50µg of DNA was used and 100 µg for other methods. [d] The digestion of the 16S rRNA obtained amplicon (1026 bp) with the *Tru*I (17) or *Tru*I, *Mnl*I or *Bfa*I (18) generate species specific RFLP patterns for 6 and 17 species, respectively.

Table S3. Systematic literature review of 171 studies (2000-2012)[a] that identified 4223 strains of *Arcobacter* using the five PCR methods compared

[a] Of the 171 references found using the ISI Web of Knowledge (last access on July 30th 2012), Houf *et al.,* 2000 (13) was cited in 123 studies; Kabeya *et al*., 2003 (14), in 21; Douidah *et al*., 2010 (8) / De Smet *et al*., 2011 (16), in 11; Figueras *et al*., 2008 (17), in 16; and Pentimalli *et al*., 2009 (15) was cited 3 times, but only for background information.

Identified number (%) of strains of the different species in relation with the method used

Species	**n (%)**	**Houf *et al*. (13)**	**Kabeya *et al*. (14)**	**Figueras et al., (17)**	**Douidah et al., (8) De Smet et al., (16)**
***A. butzleri*:**	2690 (63.7%)	1763 (64.5%)	241 (79%)	445 (72.2%)	241 (42.5%)
***A. cryaerophilus*:**	1152 (27.3%)	850 (31.1%)	45 (14.8%)	112 (18.2%	145 (25.6%)
***A. skirrowii*:**	209 (4.9%)	87 (3.2%)	19 (6.2%)	33 (5.4%)	70 (12.3%)
Other *Arcobacter* spp.	172 (4.1%)	35 (1.3%)[b]	---	26 (4.2%)[b]	111 (19.6%)[c]
Total	4223	2735 (64.8%)	305 (7.2%)	616 (14.6%)	567 (13.4%)

[b] The remaining 14 *Arcobacter* spp. were detected using other molecular identification methods such as 16S rRNA or *rpoB* gene sequencing and/or 16S rRNA-RFLP (17) used in parallel (5-7,22-25)

[c] These strains included 100 of *A. thereius*, 10 of *A. trophiarum* and 1 of *A. cibarius* (0.2%, 1/567) identified by De Smet *et al*. (26-27) using this combination of methods.

4.6. Water temperature and incubation under aerobic and microaerobic conditions increase the recovery and diversity of *Arcobacter* spp. from shellfish

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Water temperature and incubation under aerobic and microaerobic conditions increase the recovery and diversity of *Arcobacter* spp. from shellfish.

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Running tittle: Diversity of *Arcobacter* species in shellfish

Summary

Some *Arcobacter* species are considered as emerging foodborne and waterborne pathogens and it has been suggested that shellfish could be a reservoir. However, only a few studies have investigated the presence of *Arcobacter* in this kind of food. This study assessed the prevalence and diversity of *Arcobacter* spp. in shellfish by m-PCR and culture (under different atmospheric conditions) evaluating also the possible influence of environmental parameters (temperature, salinity, etc).

Arcobacter was detected by m-PCR and/or culture in 61 (29.9%) of the 204 studied shellfish samples. Of the 476 investigated isolates, 118 belonged to different ERIC-PCR genotypes (strains) and to 11 species. This study shows the highest diversity of *Arcobacter* species ever observed in samples from any origin. The most prevalent species was *A. butzleri* (60.2%) followed by *A. molluscorum* (21.2%). The latter species together with *A. ellisii* and *A. bivalviorum* were newly discovered while conducting this study. The prevalence of *Arcobacter* was significantly higher during summer associated to an increase in water temperature. Incubation under aerobic conditions increased the number of positive samples in 41.1%, while microaerobic conditions only in 23.2%. Shellfish were confirmed as a reservoir for a remarkable diversity of *Arcobacter* spp., including new species.

Introduction

The genus *Arcobacter* currently includes 17 characterized species that belong to the class *Epsilonproteobacteria* and to the family *Campylobacteraceae* (1). Some species have been considered emerging enteropathogens to humans and animals (2, 3), in particular *A. butzleri, A. cryaerophilus* and *A. skirrowii*, which are transmitted by food and water and can also cause spontaneous abortions and mastitis in animals and bacteraemia in humans (2, 3).

The prevalence of *Arcobacter* in different type of food products, including chicken, pork, beef, and mussels ranges from 0.5% in pork meat to 73% in chicken meat (4 and references therein). It has been suggested that the intestinal tract and faecal samples of healthy farm animals (poultry, pigs, cows, etc) are a reservoir for these species (3). Arcobacters have been found to be part of the marine microbial community in studies carried out in the Wadden Sea sediments, Germany (5), brackish water near Messina in Italy (6, 7), microbial mats from Ebro delta, Spain (8) and sediments from Sweden, Norway and Korea (9) where shellfish may be present. The consumption of shellfish might be an important health risk because of their ability to concentrate bacterial pathogens from water and because they are often eaten poorly cooked and/or raw (4). Despite this important risk, only a few studies have assessed the prevalence of *Arcobacter* in shellfish. All of those studies have shown that *A. butzleri* is the most prevalent species (4, 6, 10, 11). All samples of clams and 41.1% of those from mussels were positive for *Arcobacter* (4). However, because these microbes are not routinely investigated using a standardized isolation reference method, the true incidence of the members of this genus in this food matrix is probably underestimated (3). Furthermore, despite arcobacters differing from campylobacters in their ability to grow in an aerobic atmosphere, many studies have investigated their prevalence using only microaerobic conditions (3). To date, only one study has compared the effect of different atmospheric incubation conditions i.e. aerobiosis (O_2) and microaerophilia (CO_2), on *Arcobacter* isolation, which was from chicken carcasses and did not reach any clear conclusions (12). Therefore, more studies are needed that compare the isolation of arcobacters using both culture conditions in parallel. Furthermore, current data suggests that shellfish could also be an important reservoir for species of this genus, although this hypothesis needs to be verified. This study aimed to determine whether the presence of *Arcobacter* spp. is influenced by the two atmospheric incubation conditions or by environmental parameters such as water temperature and salinity.

Materials and methods

Isolation and detection

A total of 204 shellfish, i.e. 171 samples of mussels (*Mytilus galloprovincialis*), 23 of oysters (*Crasostrea gigas*), 5 of clams (*Venerupis pullastra*) and 5 of bean clams (*Donax trunculus*), were harvested from April 2009 to December 2011 at the Fangar and Alfacs bays in the Ebro delta, Catalonia, Spain (40° 34′ 22.43″ N, 0° 39′ 12.96″ E). The average temperature and salinity during sampling days were provided by the ASPCAT laboratory in Tarragona, Spain. Isolation was carried out as described by Collado *et al*. (4). In brief, after enrichment of 10 g of the sample in 90 ml of Arcobacter-CAT broth (incubated at 30ºC in aerobiosis for 48 h), 200 µl of the broth was inoculated in parallel by passive filtration on two blood agar plates (Trypticase soy agar supplemented with 5% sheep blood; BA), one of which was incubated under aerobic (O_2) conditions and the other under microaerobic (CO_2) conditions for 48h at 30ºC. Afterwards, eight presumptive *Arcobacter* colonies (small, translucent, beige to off white; convex with an entire edge) were isolated on BA for further phenotypical and molecular identification. In parallel, a direct detection of *Arcobacter* in 400 µl of enrichment broth (4) was carried out for all samples using the m-PCR designed by Houf *et al*. (13).

Genotyping and identification

The selected colonies were identified by phenotypic testing as belonging presumptively to the genus *Arcobacter,* i.e. Gram-negative, slightly curved rods, and positive for oxidase and motility tests. The colonies that showed these characteristics were genotyped by ERIC-PCR using the primers and conditions described by Houf *et al*. (14) in order to eliminate repeated clones in the same sample and to determine the genetic diversity. The obtained ERIC-PCR patterns were analyzed using the Bionumerics software version 6.5 (Applied Maths, Ghent, Belgium). One isolate from each ERIC genotype (strain) was identified with two molecular methods in parallel, the above-mentioned m-PCR (13) and the 16S rDNA-RFLP specific for this genus (Figueras *et al*., 2008). In case of discordances between the methods or if a new RFLP pattern different from any previously described (15, 16) was observed, the identity of strain was confirmed by sequencing the *rpoB* and 16S rRNA genes as previously described (17).

Statistical analyses

In order to find any possible correlation between the prevalence of *Arcobacter,* the salinity and/or the temperature of the water, the bay from which the shellfish were harvested, or the incubation conditions, the chi-square test or the Mann-Whitney and Spearman coefficient were used. All statistical analyses were carried out using the Statistical Package for Social Sciences (v. 15.0, SPSS Inc., Chicago, IL). Statistical significance was assessed at $P < 0.05$.

Results and Discussion

Arcobacter positive samples and their relationship with environmental parameters

Overall, *Arcobacter* was found in 29.9% (61/204) of the shellfish samples studied when considering together the positive samples only by culture (13.2, 27/204), the detection obtained only by m-PCR (2.5%, 5/204), and the coincidental results between the two methods (14.2%, 29/204; Table 1). In general, fewer samples (16.7%, 34/204) were positive by m-PCR in comparison to those positive by culture (27.5%, 56/204). In a previous study that used the same culture method the overall *Arcobacter* prevalence in shellfish was slightly higher (33.3%) as was the proportion of positive samples (92.2%) that were coincidentally positive by culture and m-PCR (4). The low performance of m-PCR detection has previously been attributed to a possible presence of inhibitors in the samples (4) although it could also be due to the fact that the amount of arcobacters in the enrichment broth is below the detection limit of the m-PCR method, i.e. from 10^2 to 10^3 cfu g^{-1} (13), but no quantitative culturing was carried out in order to confirm this hypothesis. It should be remembered that this m-PCR method was originally designed to detect the species *A. butzleri, A. cryaerophilus* and *A. skirrowii* and cross reactions of those with other non-targeted species have been observed (3; Levican *et al*., unpublished results). However, the detection limits of this method for those not targeted species have not yet been determined, either. The fact that *Arcobacter* were being detected by m-PCR in only 5 samples (Table 1) could indicate the possible presence of non-viable or viable but non-culturable, arcobacters. Other authors have found a higher number of positive m-PCR samples from marine environments, 83.3% (water, small and large plankton), than those obtained by culture, 41.7% (7).

The number of positive samples for *Arcobacter* showed a seasonal variability, with a significantly higher isolation (P<0.05) in the summer when the water temperature increased to between 23ºC and 27ºC (Fig. 1). More species were isolated in July (76.9%), August (77.8%) and September (42.9%). No significant correlation was found between the prevalence of *Arcobacter* and salinity, although this parameter varied a little in the Ebro delta (mean 34.8 ± SD 1.7, Fig. 1). Finally, no significant differences were found between the prevalence of *Arcobacter* spp. in relation to the bay from which samples were harvested. This is probably related to the fact that the Alfacs and Fangar bays showed the same average temperature (20.0ºC ± 4.7º) during the same sampling period and only a slightly different mean salinity, i.e. 35.5 $^0/_{00}$ ± 2.0 and 34.2 $^0/_{00}$ ± 0.9, respectively.

Recovery under aerobic and microaerobic conditions

Regarding the incubation conditions, 23 of the 56 (41.1%) positive samples obtained by culture were only positive under aerobic conditions, while 20 (35.7%) additional samples were coincidentally positive under aerobic and microaerobic conditions and 13 (23.2%) were obtained only under microaerobic conditions. Overall, a higher number of positive samples were obtained under aerobic (43/56, 73.8%) than under microaerobic (31/56 55.3%) conditions (Table 2). There is no consensus about the optimal incubation conditions for the recovery of *Arcobacter*, but it has been stated that an optimal growth of arcobacters is obtained under microaerobic conditions (2, 18). In fact half of the studies on the prevalence of *Arcobacter* from meat samples have used aerobic incubation conditions (3 and references therein). Furthermore, no significant differences were found in the only study that has previously assessed the effect of different atmospheres (O_2 and CO_2) on *Arcobacter* isolation from chicken carcasses (12). Despite not being clear to what extent the results of the present study could be extrapolated to other types of samples, the present results indicate that 41.1% (23/56) of the shellfish samples were positive for *Arcobacter* in aerobic conditions, while only 23.2% (13/56) in microaerobic conditions. Considering that these positive samples were not coincidental, the combined use of the two methods in the present study has increased the total number of positive samples in 64.3% (36/56). Nevertheless, considering the overall better recovery, together with the lower cost and easier work under aerobic conditions, this approach seems the most convenient for routine studies.

Regarding the types of shellfish analysed, the 5 samples of bean clams (*D. trunculus*) studied were negative while the clams (*V. pullastra*) showed the highest prevalence of *Arcobacter* with 40% (2/5) positive samples, followed by mussels (32.2%; 55/171) as shown in Table S1 and 2, but the difference was not statistically significant. In a previous study (4), a higher proportion of *Arcobacter* was also isolated from clams (100%, 5/5) than from mussels (41.1%, 23/56).

Different types of molluscs, such as mussels, oysters and clams, showed a different prevalence of bacteria of other genera, such as *Vibrio* (19). This has been attributed to the bacterial characteristics, to environmental factors, as well as to host-related aspects such as filtration rate, inter-population variability and immune status (19). In this regard, clams have shown a greater risk of being contaminated with *Vibrio parahaemolyticus* than other bivalve species and it has been stated that this could in part be due to the fact that clams are buried in the sand, where microorganisms show higher concentrations than in the water column (19). In fact, in a study on the microbial communities of the Wadden sea sediments, which were analysed using *in situ* hybridisation of the 16S rRNA gene, it has been estimated that *Arcobacter* had a concentration of 10^7 cells of cm^{-3} in the upper layers of the sediments (5).

***Arcobacter* diversity in shellfish**

A total of 476 isolates were obtained from the 56 culture positive samples. These isolates were analysed using the ERIC-PCR, which showed that they belonged to 118 different genotypes, representing a genetic diversity of 24.8% (Table 3 and Figure S1). When analysed using Bionumerics software, the 118 genotypes grouped into two big clusters, one of them formed mainly by strains of the species *A. butzleri* (Figure S1, A), that also included all the A*. ellisii* strains, and the other cluster by strains of the species *A. molluscorum* (Figure S1, B). At the same time, several other minor clusters included the remaining strains of these and the other species, which seemed to group randomly, probably due to their low number (Figure S1). The genetic diversity of *Arcobacter* has so far not been determined from shellfish. However, the incidence of different genotypes ranged from 28% to 60% in other kinds of food products, such as different types of meat, as reviewed by Aydin *et al*. (21). Interestingly, despite genotypes found in shellfish being redundant, the strains recovered in different months and years were always different, indicating that specific genotypes do not remain in this environment over time. Regarding the relationship between the diversity and the incubating conditions, as observed in Table 3 the number of coincidental genotypes obtained in aerobiosis and microaerophilia was significantly lower (10.3%, $P<0.05$) than those found only under aerobiosis (31.7%) or microaerphilia (48.6%).

The 118 strains belonged to 11 species (Table 3), the most prevalent among them being *A. butzleri* (71/118; 60.2%) and, interestingly, A. *molluscorum* (25/118; 21.2%), which was in fact a species described elsewhere using some of the isolates obtained in this study (22). The third most common was *A. cryaerophilus* (6/118; 5.1%). The prevalence of *A. butzleri* did not depend on the type of shellfish and most of the new and rare species came from mussels, despite *A. molluscorum* also being isolated from oysters and *A. nitrofigilis* from clams (Table 4). In a previous study carried out in mussels from Chile, *A. butzleri* was the only species recovered (10); in another study (4) this species was the most isolated from mussels samples (43.5%) whereas *A. cryaerophilus* was the most isolated from clams (80%). In the latter study, *A. mytili* (10.7%)*, A. nitrofigilis* (7.1%)*, A. skirrowii* (3.6%) were also recovered (4). In the present study new strains of all of these species were recovered. Furthermore, we isolated for the first time from shellfish some strains of the species *A. defluvii* (0.8%), so far only known from sewage (23) and *A. thereius* (0.8%), previously known from animal faeces or abortion (24, 25). Three new *Arcobacter* spp. were discovered from isolates derived from this study, i.e. the mentioned *A. molluscorum, A. ellisii* and *A. bivalviorum* (22, 26, 27), and also another potentially new species (strain 128-2) that is waiting to be described (Table 3).

This is the first study to report the recovery of 11 different *Arcobacter* species. In fact, among the studies carried out between 2000 and 2012, about the 95% of strains were identified as *A. butzleri, A. cryaerophilus* and/or *A. skirrowii* (Levican *et al*., unpublished results). To our

knowledge, the study that has so far reported the highest diversity was the one by Collado *et al*. (4), in which 8 species were isolated from different types of meat, and 6 different species from shellfish also collected in the Ebro delta. The carriage of 5 species in faecal samples from pigs (25) and of 4 species in samples of sheep and goat (28) represented the other studies with the highest diversity of species. In this regard, the low incidence of most *Arcobacter* species observed in the different studies could be attributed to the characteristics of the analysed samples but also to the low number of isolates studied per sample, which will favour recognizing only the most prevalent species. Furthermore, several of the available detection and identification methods fail to recognize all species, confusing them with the common ones (Levican *et al*., unpublished results).

In the present study, the atmosphere incubation conditions influenced the species diversity because the two most prevalent species (*A. butzleri* and *A. molluscorum*) showed a significantly higher prevalence under aerobic conditions ($P<0.05$), as did the species *A. cryaerophilus* (Tables 2 and 3). However, other less frequent species, i.e. *A. thereius* and *A. defluvii,* were isolated under microaerobic conditions, and the low number did not allow any statistical analysis (Table 3). Therefore, the combined use of the two atmospheric conditions has not only contributed to an increase in the number of positive samples, but also to an identification of the high species diversity found in shellfish. Their use in parallel in future studies seems to be justified.

The seasonal distribution was statistically confirmed for the species *A. butzleri, A. molluscorum* ($P<0.05$), which were predominant in the samples recovered from June to October. Both species showed persistence over time because they were both isolated during the three years of sampling (Table 4). Other species, such as *A. cryaerophilus* (n=6), *A. nitrofigilis* (n=5) and *A. skirrowii* (n=2) were isolated between January and May, when the mean temperature of water was lower, ranging from 7.9ºC to 18.2ºC (Table 4). The low number of strains does not allow us to determine whether or not this is a true tendency or if the latter species were not recovered more frequently or during another period of the year due to the predominance of *A. butzleri*. It has been suggested that this species grows faster in enrichment than other species such *A. cryaerophilus, A. skirrowii* and *A. thereius,* masking their presence (14, 25).

The potential virulence of some of the strains recovered from shellfish in this study has been evaluated in another study and most of them showed adhesion and invasion capacity to the human intestinal Caco-2 cells and showed the presence by PCR of the putative virulence gene *ciaB* gene (29). The latter gene codifies for an invasion protein described in *Campylobacter jejuni* (30). It is noticeable that *A. butzleri*, the most prevalent species in the present study, together with *A. cryaerophilus* and *A. skirrowii* have been associated with cases of diarrhoea in humans (3). In fact, *A. butzleri* was the fourth most common Campylobacter-like bacteria

isolated from stool of patients with diarrhoea in two independent studies performed in Belgium and France (31, 32) and it is considered a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (33). Therefore, their presence in shellfish may have public health significance.

The results of this study confirm that shellfish from the Ebro delta, which is the second most important farming area of bivalve molluscs in Spain (34), harbour a wide diversity of arcobacters, including predominantly potentially pathogenic species, and can act as a reservoir of new *Arcobacter* species.

Conflict of Interest

All authors: No reported conflicts.

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Table 1. Prevalence of *Arcobacter* in shellfish using culturing and molecular detection by m-PCR in parallel

Shellfish type	Samples	Positive samples (%)	No. of positive samples (%) according to the method		
			Only culturing	Only m-PCR	Coincident culturing & m-PCR
Mussels	171	55 (32.2)	26 (15.2)	5 (2.9)	24 (14.0)
Oysters	23	4 (17.4)	1 (4.3)	0	3 (13.0)
Clams	5	2 (40)	0	0	2 (40)
Bean clams	5	0	0	0	0
Total	204	61 (29.9)	27 (13.2)	5 (2.5)	29 (14.2)

Table 2. Recovery by culturing of *Arcobacter* from shellfish under aerobic (O_2) and microaerobic (CO_2) incubation conditions used in parallel

Shellfish type	No. of samples	No. of positive samples (%)	No. of positive samples according to the atmosphere conditions		
			Only O_2	Only CO_2	Coincident O_2 & CO_2
Mussels	171	50 (29.2)	22 (44.0)	12 (24.0)	16 (32.0)
Oysters	23	4 (17.4)	0	1 (25.0)	3 (75.0)
Clams	5	2 (40.0)	1 (50.0)	0	1 (50.0)
Beans clams	5	0	0	0	0
Total	204	56 (27.5)	23 (41.1)	13 (23.2)	20 (35.7)

Table 3. Genetic diversity (% of different ERIC genotypes among the isolates) of *Arcobacter* species identified from the 56 positive samples obtained by culturing under different incubation conditions

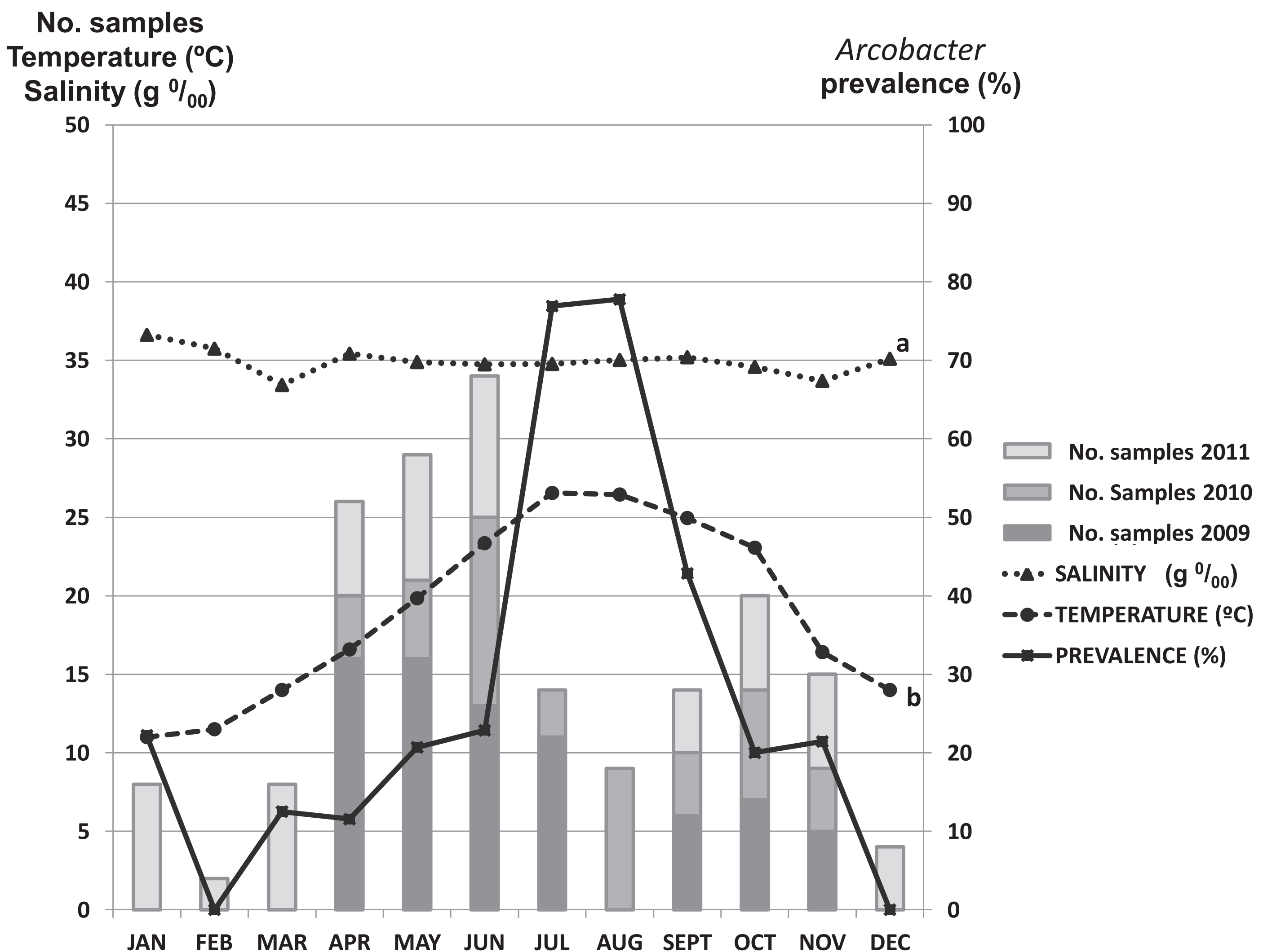
Species	No. strains / No. isolates (%)	Incidence (%) of the species	No. strains /No. isolates (%) obtained according to the atmosphere conditions		
			Only O_2	Only CO_2	Coincident O_2 & CO_2
A. butzleri	71 / 306 (23.2)	60.2	37 / 132 (28.8)	23 / 44 (52.3)	11 / 130 (8.5)
A. molluscorum	25 / 104 (24.0)	21.2	14 / 32 (43.7)	2 / 5 (40.0)	9 / 67 (13.4)
A. cryaerophilus	6 / 18 (33.0)	5.1	5 / 17 (29.4)	1 / 1 (100)	0
A. nitrofigilis	5 / 19 (26.3)	4.3	2 / 11 (18.2)	3 / 8 (37.5)	0
A. ellisii	3 / 3 (100)	2.6	2 / 2 (100)	1 / 1 (100)	0
A. bivalviorum	2 / 9 (22.2)	1.7	0	1 / 2 (50.0)	1 / 7 (14.3)
A. skirrowii	2 / 7 (28.6)	1.7	1 / 1 (100)	1 / 6 (16.7)	0
A. mytili	1 / 5 (20.0)	0.8	1 / 5 (20.0)	0	0
A. thereius	1 / 1 (100)	0.8	0	1 / 1 (100)	0
A. defluvii	1 / 2 (50.0)	0.8	0	1 / 2 (50.0)	0
***Arcobacter* sp.**	1 / 2 (50.0)	0.8	1 / 2 (50.0)	0	0
Total	118 / 476 (24.8)	100	63 / 202 (31.7)	34 / 70 (48.6)	21 / 204 (10.3)

Table 4. Relationship between the number of strains of the different *Arcobacter* spp. and the positive samples obtained in the different type of shellfish and month of sampling

			Type of shellfish				Data of sampling													
Species	**No. strains**	**No. samples**	**Mussels**	**Oysters**	**Clams**	**Beans clams**	**Year**	**No. samples/year**	**January**	**February**	**March**	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**
A. butzleri	71	31	27	2	2		2009	95	NS	NS	NS	NS	2	2	5	NS	3	2	1	NS
							2010	48	NS	NS			1	1	2	5	2	1	1	NS
							2011	61			1				NS	NS		2		
A. molluscorum	25	19	18	1			2009						1	1	4		3			
							2010							2		2	1			
							2011							2				2	1	
A. cryaerophilus	6	3	2	1			2009					1	1							
							2011				1									
A. nitrofigilis	5	4	3	1			2009					1	1							
							2011		2											
A. ellisii	3	1	1				2009						1							
A. skirrowii	2	2	2				2009					1								
							2011		1											
A. bivalviorum	2	1	1				2010										1			
A. thereius	1	1	1				2009							1						
A. mytili	1	1	1				2010										1			
A. defluvii	1	1	1				2010									1				
Arcobacter sp.	1	1	1				2011							1						

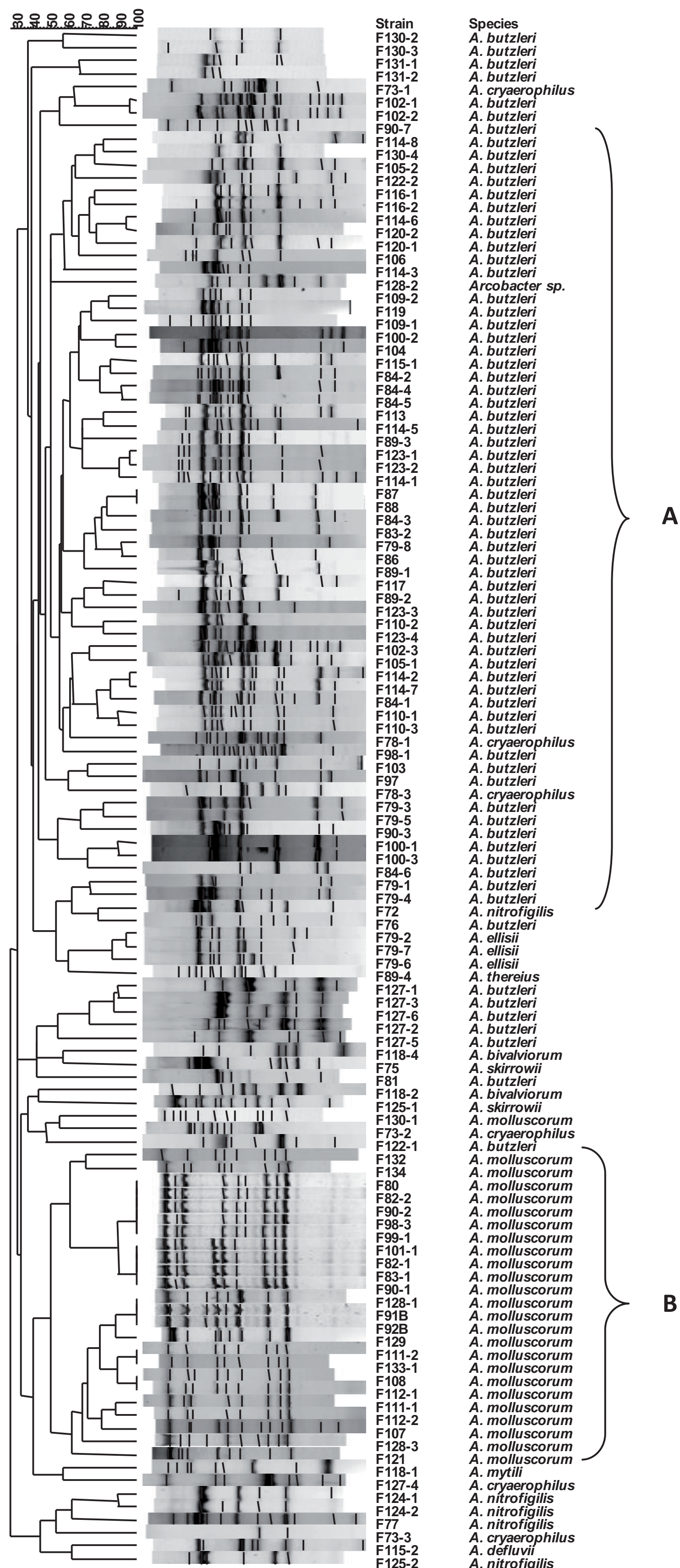
NS: No samples were collected. When no numbers are indicated it means a negative sample for any *Arcobacter* species.

Figure 1. Distribution of *Arcobacter* among shellfish samples and its relationship to the water temperature and salinity



[a] No significant correlation (*P*>0.05) between salinity and presence of *Arcobacter* spp. was found. [b] A significant positive correlation (correlation cofficient: 0.315; *P*<0.05) was found between water temperature and presence of *Arcobacter* spp.

Figure S1. ERIC-PCR dendrogram showing the genetic relationship among the 118 recovered strains



4.7. Prevalence and diversity of *Arcobacter* spp. in wastewater.

Levican A, Collado L, Figueras MJ. (in preparation)

Prevalence and diversity of *Arcobacter* in wastewater

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Abstract

The genus *Arcobacter* belongs to the family *Campylobacteraceae* and includes species considered as emerging food and water borne pathogens. *Arcobacter* are known to be present in water environments and have been linked to the presence of faecal pollution. However, only a few studies have investigated its prevalence in wastewater, the only isolated species being *A. butzleri* and *A. cryaerophilus*. However, it has been suggested that wastewater could be a reservoir for a wide range of *Arcobacter* species. This study aimed to establish the prevalence of *Arcobacter* in a WWTP using two culturing methods (direct plating and culturing after enrichment) together with parallel direct detection using an m-PCR. The genetic diversity of the isolates will be evaluated using the ERIC-PCR genotyping method.

Arcobacter spp. were present in 93.3% of the studied wastewater samples. From 178 isolates, 144 were shown to belong to different ERIC genotypes or strains (80.9%). Although some strains were recovered from different sampling points on the same sampling date, none of them persisted over the time in the WWTP. The predominant species were *A. butzleri* (53.5%) and *A. cryaerophilus* (39.6%), both of which had the widest genetic diversity, too (92.8% and 70.4%, respectively). The other species corresponded to *A. nitrofigilis* (0.7%) and to two new species *A. defluvii* (5.6%) and *A. cloacae* (0.7%). The use of both the direct plating and the culturing after enrichment enhanced the recovery of different species. *A. cryaerophilus* was the predominant species by direct plating while *A. butzleri* predominated after enrichment. The observed high prevalence and genetic diversity of *Arcobacter* spp. from wastewater confirms that this is an important reservoir for bacteria of this genus, and furthermore, new species were found.

Introduction

The genus *Arcobacter* is included together with *Campylobacter* and *Helicobacter* in the family *Campylobacteraceae*, and all of these genera include species that might be pathogenic to humans and animals (Collado & Figueras, 2011). *Arcobacter butzleri* is the fourth most common Campylobacter-like organism isolated from the stool of human patients with diarrhoea. In two independent studies carried out in France (Prouzet-Mauléon *et al*., 2006) and Belgium, the three most common are *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter upsaliensis,* (Vandenberg *et al*., 2004). It has been demonstrated that the presence of *Arcobacter* in water correlates with the presence of faecal pollution (Collado *et al*., 2008). Furthermore, *Arcobacter* has been recovered in three water outbreaks in which the drinking water was contaminated with sewage (Collado & Figueras, 2011 and references therein). Food products, especially meat, shellfish and milk have also been found contaminated with bacteria of this genus, mainly *A. butzleri*. Considering this, the *International Commission on Microbiological Specifications for Foods* has defined *A. butzleri* as a serious hazard for human health (ICMSF, 2002), and it has been identified as an important zoonotic agent to human and animals (Collado & Figueras, 2011 and references therein).

Disposal of sewage is a critical issue in modern cities that normally deliver their wastewater for treatment at Wastewater Treatment Plants (WWTPs). The objective of this treatment is to reduce degradable organic matter under controlled conditions before it is discharged into natural bodies of water (Brendecke & Pepper, 1996). However, conventionally primary and secondary treatments per se (without disinfection steps) do not eliminate the pathogens present in the water and as a result WWTP outflows contain a lot of microbes that are potentially pathogenic to humans and animals.

The presence of *Arcobacter* in water, including sewage from WWTPs has been reported in a lot of studies (Stampi *et al*., 1993 and 1999; Moreno *et al.,* 2003; González *et al*., 2007 and 2010; Collado *et al.,* 2008; McLellan *et al*., 2010). In those studies *Arcobacter* spp. were isolated in 40% to 100% of the samples studied, using different culture media and protocols, and were found in 66% to 100% of the samples when direct detection by molecular techniques were used (Moreno *et al.,* 2003; González *et al*., 2007 and 2010; Collado *et al.,* 2008). Three studies have investigated the presence of *Arcobacter* in WWTPs after the different treatments and despite differing results all of them showed the presence of *Arcobacter* at all points of the WWTP, including the water outflow (Stampi *et al.,* 1993 and 1999; Moreno *et al*., 2003). Furthermore, using pyrosequencing of the hypervariable region V6 of 16S rRNA gene, *Arcobacter* were found to be one of the predominant taxa in WWTPs in Milwaukee (USA) in contrast to their scarcity in surface waters (McLellan *et al*., 2010). In fact, considering those results, *Arcobacter* were selected as “sewer signature” microbes

together with *Acinetobacter* and *Trichococcus* (the most common taxa in sewage) in the detection of sewage contamination of surface waters (Newton *et al.*, 2013).

Studies on wastewater samples found that *A. butzleri* was more predominant than *A. cryaerophilus,* (Stampi *et al*., 1993 and 1999; Moreno *et al.,* 2003; González *et al*., 2007 and 2010; Collado *et al.,* 2008). Despite different culture protocols being used in those studies, all of them included an enrichment step in a selective broth. However, using direct and post-enrichment culturing in a study in broiler carcasses, *A. butzleri* predominated over *A. cryaerophilus* (Houf *et al*., 2002). Furthermore, De Smet *et al*. (2011) studied pig faeces using direct plating and post-enrichment. That study mostly isolated *A. skirrowii* and *A. thereius* by direct plating and *A. butzleri* and *A. trophiarum* by post-enrichment. These results were explained by the fact that some *Arcobacter* species may adapt better than others to the applied culturing conditions.

The genetic diversity in sewage has seldom been studied and methods used include Random Amplification of Polymorphic DNA (RAPD-PCR, González *et al*., 2010) and Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR, Collado *et al*., 2010). Results showed a wide range of genotypes, as happens in samples from other environments (Collado & Figueras, 2011).

The objective of this survey is to establish the prevalence and genetic diversity of *Arcobacter* spp. in a WWTP using two culturing approaches (direct plating and culturing after enrichment), using direct detection by m-PCR in parallel.

Materials and methods

Samples and water processing

The samples were collected on three occasions (April, June and October 2009) from the WWTP in Reus, Spain. There were five sampling points, at the inflow and outflow to the treatment plant, in the primary and secondary sedimentation tanks, and during the secondary biological treatment. Samples were collected into 2-litre sterile polypropylene bottles, which were then chilled in ice during transport. Microbiological assays began on the same day as sampling.

200 ml of each water sample was filtered through a 0.45 µm membrane filter (47 mm diameter) (Millipore Corp., Bedford, MA, USA), then rolled and placed into tubes containing 1ml distilled water and vigorously mixed in a vortex.

Direct molecular detection

For molecular detection, 400 µl of water from the tube was centrifuged and the pellet obtained was washed 3 times with milliQ sterile water and submitted to DNA extraction using

the InstaGene™ DNA Purification Matrix (Bio-Rad Laboratories, Hercules, CA). Direct detection was carried out in the extracts using the m-PCR designed by Houf *et al.* (2000) for the detection of A*. butzleri, A. cryaerophilus* and *A. skirrowii,* using primers and conditions described.

Culture after direct plating

For the direct detection by culturing, 200 µl of water in the tube was transferred onto the surface of a 0.45 µm membrane filter (47 mm diameter), placed on blood agar medium and allowed to filter passively under ambient conditions for 30 min (Collado *et al*., 2008). The filter was then removed and the plates aerobically incubated (30ºC, 48 to 72 h).

Culturing after the enrichment step

Post-enrichment isolation of *Arcobacter* was carried out as previously described (Collado *et al*., 2010), i.e. another aliquot of 200 ml of water was filtered through a 0.45 µm membrane filter (47 mm diameter). The filter was then introduced into tubes containing 9 ml of Arcobacter-CAT broth (Arcobacter-enrichment broth supplemented with the CAT antibiotic supplement, Oxoid, Basingstoke, UK), and incubated aerobically (30 ºC, 48 to 72 hrs). After enrichment, 200 µl of broth was transferred to blood agar medium following the same procedure as described above for direct plating.

Confirmation of the colonies

From each positive sample, eight small, colourless or beige to off-white, translucent colonies were picked, streaked to purity, and confirmed as presumptive arcobacters on the basis of their phenotypic tests (Gram negative stain, oxidase activity and motility). If both cultures, direct and post-enrichment, were positive, 16 colonies were expected from each sample, making a total of 240 isolates.

Genotyping and identification of the isolates

All isolates were genotyped using the ERIC-PCR technique, using the Houf *et al*. (2002) protocol for *Arcobacter*. DNA was extracted using the InstaGene™ DNA Purification Matrix (Bio-Rad Laboratories, Hercules, CA). The concentration of each DNA template was determined using the GenQuant pro (Amersham Biosciences, Cambridge, England) at A260 and adjusted to 25 ng ml^{-1}. Gel images were saved as TIFF files, normalized with the 100 bp DNA Ladder (Invitrogen), and further analysed by Bionumerics software, version 6.1 (Applied Maths, Belgium). Patterns with one or more different bands were considered different genotypes (Houf *et al*., 2002).

All strains (1 representative of each genotype) were finally identified using parallel techniques, the m-PCR (Houf *et al*, (2000) and the 16S rDNA-RFLP (Figueras *et al,* 2008). In strains where there were discordant results between the methods or different RFLP patterns from those described, the *rpoB* housekeeping gene was sequenced using primers and conditions described by Collado *et al*. (2009) in order to establish their identity.

Results and discussion

Prevalence and diversity of *Arcobacter* species

Arcobacter spp. were recovered from 14 of the 15 samples (93.3%). The two culture methods yielded 216 isolates, but only 178 (82.4%) showed phenotypical characteristics of *Arcobacter*. Those 178 isolates were genotyped with ERIC-PCR and the sequencing patterns indicated that they belonged to 144 different strains; the global genetic diversity was 80.9% (Table 1). In previous studies on the prevalence of *Arcobacter* spp. from wastewater samples that used different culture media and protocols, results ranged from between 40% and 100% (Moreno *et al*., 2003; González *et al*., 2007 and 2010; Collado *et al*., 2010). In the present study, *Arcobacter* spp. were isolated from all sampling points, with the exception of only one sample taken at the water outflow. In previous studies *Arcobacter* were also present at all sampling points (Stampi *et al.,* 1993 and 1999; Moreno *et al*., 2003), suggesting that conventional wastewater treatment is not able to remove the bacteria of this genus. When a genotyping method was applied, there was also a high genetic diversity. For example, Collado *et al*. (2010) reports that 90.2% of the isolates belonged to different ERIC-PCR genotypes, while González *et al*. (2010) found that all their isolates were different RAPD-PCR genotypes. In this study, despite some strains (genotypes) being recovered from different sampling points at the same time, they were never recovered again on the 3 different samplings days. This suggests that strains of *Arcobacter* do not persist over time in the WWTP. Genetic diversity might be due to multiple sources of contamination (as happens in sewage) and/or as a consequence of genomic rearrangement (González *et al*., 2010; Collado *et al*., 2010). Regarding that, De Smet *et al*. (2011) also reports a very high number of genotypes among arcobacters isolated from pig faeces, and states that such diversity hampers the identification of the possible sources of contamination.

Coincidental results were found in 134 of the 144 strains (93.1%), when a representative isolate of each genotype (or strain) was identified using two molecular methods in parallel (m-PCR and 16S rRNA-RFLP) 77 (53.4%) strains of *A. butzleri* and 57 (39.6%) strains of *A. cryaerophilus* (Table 1). Among the other 10 (6.9%) strains that produced different results, m-PCR identified one as *A. skirrowii*, one as *A. cryaerophilus* and 8 produced an amplicon similar to that expected for the latter species (257 bp), but smaller (~230 bp; Table 1).

However, by 16S rRNA-RFLP, the first strain was identified as *A. nitrofigilis* and the nine others had new RFLP patterns and could therefore not be assigned to any known species (Table 1). The *rpoB* sequences obtained from those strains confirmed that the first belonged to *A. nitrofigilis* (GeneBank HG004609) and the others to two potentially new species, defined in previous studies as *A. defluvii* (Collado *et al*., 2011) and *A. cloacae* (Levican *et al*., 2013). The present study reports the highest diversity of *Arcobacter* species so far in wastewater, as in previous studies the species isolated were, predominantly, *A. butzleri* and *A. cryaerophilus* (Stampi *et al*., 1993 and 1999; Moreno *et al.,* 2003; González *et al*., 2007 and 2010; Collado *et al.,* 2008). To our knowledge this is the first isolation of *A. nitrofigilis* from sewage, because since its description from the roots of a salt marsh plant, it has so far only been genetically identified from mussels (Collado *et al*, 2008), using the 16S rRNA-RFLP method. However, this may be due to the fact that the 16S rRNA-RFLP method is the only one available that identifies this species (Figueras *et al.*, 2008). The most prevalent species *A. butzleri* and *A. cryaerophilus* were isolated in almost equal numbers (83 and 81 isolates, respectively) from 77 (92.8%) and 57 (70.4%) strains, respectively (Table 1); therefore, *A. butzleri* showed the widest genetic diversity. In a previous study in river water that had been impacted by sewage effluents (Collado *et al*., 2010), *A. cryaerophilus* had a slightly wider diversity (95.2%) than *A. butzleri* (90.2%). Our results demonstrate that genotyping is essential for discriminating redundant strains of *Arcobacter* spp. because if no genotyping had been done we would have reported an almost equal abundance of *A. butzleri* and *A. cryaerophilus* (46.6% and 45.5%, respectively) while in fact, there were more strains of *A. butzleri* (53.5%) than *A. cryaerophilus* (39.6%). The prevalence and genetic diversity of *Arcobacter* spp. found in wastewater highlights an important reservoir for bacteria of this genus, including potentially new species.

Detection using m-PCR and with the two culturing methods

Of the 15 samples studied, 13 (86.7%) were positive by direct plating, 14 (93.3%) by post-enrichment and 6 by m-PCR (Table 2). Only one sample taken from the WWTP outflow was negative by all three methods. Compared to the culturing methods, direct detection by m-PCR (Houf *et al*., 2000) performed very badly (Table 2). However, there have been contrary results in previous studies that have investigated wastewater using the same m-PCR method (González *et al.,* 2007 and 2010; Collado *et al*., 2008). González *et al*. (2010) reports 100% of positive samples by m-PCR and only 45.5% by culturing using the same media as we have in the present study, i.e. enrichment in Arcobacter-CAT broth followed by passive filtration over a blood agar plate without antibiotics. The shorter incubation time (24 h) may have affected the recovery of *Arcobacter* by culturing, although other studies yielded the same number of positive samples by culturing and by m-PCR (Gonzalez *et al*. (2007), 66%,

and Collado *et al*. (2008) 100% of wastewater samples). The former study used a different enrichment medium, (Arcobacter-broth supplemented with 0.005% 5-fluorouracil and a different method of plating onto solid medium, which consisted of the same enrichment broth plus agar). The latter study used the same enrichment medium and incubation conditions as this study has. The different results in these three studies could be explained by the m-PCR having been carried out directly from the sample in our study, but from the enrichment broth in the other two. Under such circumstances, the possible inhibitors of the PCR reaction present in the samples might be diluted and the growth amplification might increase the level of target cells and thus the percentage of detection. It has been demonstrated that the detection of the different species by m-PCR is biased when applied after the enrichment despite seeming to be more appropriate. Ho *et al*. (2006) adjusted bacterial suspensions of *A. butzleri, A. cryaerophilus* and *A. skirrowii* to the same concentration, serially diluted 10^{--} fold and mixed in different proportions for testing by m-PCR. Those mixtures were able to detect 2 or 3 species simultaneously with the same proportion of bacterial suspensions, while in mixtures that showed different proportions, the amplification favoured the detection of only the most abundant species (Ho *et al.,* 2006). The study concludes that it is possible to detect the species that grow faster in enrichment but not those that are present simultaneously in lower numbers. Unfortunately, the authors did not provide information about the specific concentration of bacteria cells in each suspension, therefore it is not clear whether this behaviour was due to the different concentrations of the bacteria cells of each species in the mixtures or the concentration in the diluted suspensions were under the detection limit of the method (10^3 cfu ml^{-1}) previously established by Houf *et al*. (2000).

In the present study, the *Arcobacter* species detected in the positive samples also varied depending on the method. *A. butzleri* (100%) was detected in the 6 positive samples by m-PCR and together with *A. cryaerophilus* in 4 of them (66.7%) (Table 2). These results are similar to those yielded by post-enrichment culturing, i.e. 12/14 (85.7%) and 7/14 (50.0%), respectively (Table 2). On the other hand, among the 13 samples that were positive by direct plating, *A. cryaerophilus* was isolated in all of them (100%) and *A. butzleri* only in 8 (61.5%, Tables 2). Our results confirm that the enrichment step will always give the wrong idea that *A. butzleri* is the prevailing species, when in fact it is not. The m-PCR (Houf *et al*, 2000) was created only to detect *A. butzleri, A. cryaerophilus* and *A. skirrowii* but, as we have found in this study, other species can be confused with them. For example, *A. cloacae* produces the amplicon expected for *A. cryaerophilus* (257 bp) and *A. defluvii* a similar one (~230 bp) (Collado *et al*., 2011); furthermore, *A. nitrofigilis* produces the amplicon expected for *A. skirrowii* (625 bp; Collado *et al*., 2008). Despite that, Houf *et al.* (2000) claimed that no amplicon was obtained for this species when they defined the method. In 2 of the 3 samples from which the new species *A. defluvii* was isolated, the m-PCR was negative, and the other

sample was positive for *A. butzleri* and *A. cryaerophilus.* The isolated species in this sample were *A. cryaerophilus, A. nitrofigilis* and *A. cloacae* (Table 2). This might be due to an influence of different factors that were not controlled for in the present study, i.e. the presence of inhibitors, the concentration of the *Arcobacter* spp. or the sensitivity of the method for the detection of the new species.

In relation to the comparative performance of direct plating and post-enrichment, almost the same number of strains was obtained with both methods, i.e. 69 only by direct plating, 70 only by post-enrichment and 5 coincidentally by both methods (Table 3). However, the predominant species isolated by each method was different, i.e. the most abundant species recovered under direct plating conditions was *A. cryaerophilus* (46/69, 66.7%) followed by *A. butzleri* (21/69, 30.4%) (Table 3). However, the latter species was the most frequently isolated under post-enrichment culturing conditions (55/70, 78.6%) followed by *A. cryaerophilus* (10/70, 14.3%). *A. defluvii* was isolated by both methods, but more different strains of this species were obtained after enrichment than by direct plating (Table 3). The only strain of *A. nitrofigilis* was recovered by direct plating and the one of *A. cloacae* by post-enrichment (Table 3). Previous studies on these kinds of samples have included an enrichment step but not direct plating, and as commented, the only recovered species were *A. butzleri* and/or *A. cryaerophilus* (Stampi *et al*., 1993 and 1999; González *et al.,* 2007 and 2010; Collado *et al.,* 2008 and 2010). In one study where samples were cultured using the same enrichment as the present study (Collado *et al*., 2010), *A. butzleri* was 4 times more prevalent than *A. cryaerophilus* (248 vs 60 strains), this proportion being similar to that for the same species in our study by post-enrichment, i.e. 5.5 times (55 vs 10 strains) (Table 3). By direct plating the proportion *A. cryaerophilus* was 2.2 times more prevalent than *A. butzleri* (46 vs 21). A previous study on *Arcobacter* in broiler carcasses from Belgium compared the diversity of strains yielded by the two culturing methods, i.e. direct plating and by post-enrichment (Houf *et al*., 2002). In that study, 49 different strains of *A. butzleri* and 9 of *A. cryaerophilus* were recovered by post-enrichment culturing, while 31 of *A. cryaerophilus* and 42 of *A. butzleri* were recovered by direct plating. Consequently, those authors recommend the use of the two methods in parallel in order to enhance the diversity recovered. Another study (De Smet *et al*., 2011) compared the recovered isolates in faeces of pig from the same country, again using the two methods. That study recovered the same number of isolates of *A. cryaerophilus* by direct plating and post- enrichment, although more isolates were obtained by direct plating for the species *A. skirrowii* (37 vs 2) and *A. thereius* (122 vs 16). On the other hand, more isolates were obtained by post- enrichment than by direct plating for *A. butzleri* (190 vs 89) and *A. trophiarum* (12 vs 4), although the number of strains to which those isolates belonged from the two methods was not reported. It has been hypothesized that the predominance of one species over another is due to the isolation

procedure and medium used to recover the species, rather than to its higher occurrence in samples (Houf *et al.,* 2002; De Smet *et al*., 2011). However, those studies used a different medium (Houf *et al*., 2001) and protocol to the present study. For instance, Houf *et al*. (2002) selected all the colonies that grew from the direct plating but only 2-10 colonies from post-enrichment whereas De Smet *et al*. (2011) selected 10 from direct plating and only 1 from post-enrichment as a result, the reported wider diversity from direct plating had been biased by the different number of isolates studied in comparison to post-enrichment. Furthermore, in the two studies the number of positive samples for *Arcobacter* spp. ranged from only 4.6% to 37.5% by direct plating and from 11.3% to 83.3% by post- enrichment (Houf *et al.,* 2002; De Smet *et al*., 2011), so the results were probably influenced by other factors, such as the relative concentration of each species in the samples or inhibition by the antibiotics included (amphotericin B, cefoperazone, 5-fluorouracil, novobiocin, trimethoprim and cycloheximide) either in the solid or liquid agar used.

In the present study, we obtained almost equal prevalence by direct plating and by post-enrichment (86.7% and 93.3%, respectively), therefore wastewater would seem to be a good matrix, considering the number of positive results, for comparing the performance of the different isolation approaches we have chosen in the present study. In future studies, different protocols could also be evaluated in this matrix, such as that used in the present study i.e. passive filtration in blood agar after enrichment in Arcobacter-CAT broth (Atabay & Corry, 1998), and the protocol proposed by Houf *et al*. (2001).

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Table 1. Genetic diversity (No. of strains/No. of isolates) of *Arcobacter* species in wastewater and the results obtained by the two molecular identification methods (m-PCR and 16S rRNA-RFLP)

Species	No. isolates (%)	No. strains (%)	% Genetic diversity	Molecular identification m-PCR[a] / 16S rRNA-RFLP [b]
A. butzleri	83 (46.6)	77 (53.5)	92,8%	*A. butzleri* / *A. butzleri*
A. cryaerophilus	81 (45.5)	57 (39.6)	70,4%	*A. cryaerophilus* / *A. cryaerophilus*
A. defluvii[c]	12 (6.7)	8 (5.5)	66,6%	~230 bp / New pattern (407, 243, 141, 138, 52)
A. nitrofigilis[d]	1 (0.6)	1 (0.7)	100%	*A. skirrowii* / *A. nitrofigilis*
A. cloacae[e]	1 (0.6)	1 (0.7)	100%	*A. cryaerophilus* / New pattern (372, 243, 138, 92, 52, 49)
Total	178	144	80,9%	

[a] Houf *et al*. (2000); [b] Figueras *et al*. (2008). [c] New species recognized on the basis of the new RFLP pattern and described by Collado *et al*. (2011). [d] Confirmed by *rpoB* sequencing (GenBank HG004609). [e] New species recognized on the basis of the new RFLP pattern and described by Levican *et al.* (2013).

Table 2. ***Arcobacter*** **species detected according to the method at the 5 sampling points in the WWTP on the 3 different sampling occasions[a]**

Sample	Sampling point	m-PCR	Culture method: Direct	Culture method: Post-enrichment
April 2009	Inflow water	Ac + Ab	Ac + Anit	Ac + Ad+ Aclo
	Primary sedimentation tank	Ac + Ab	Ac + Ab + Ad	Ac + Ab
	Secondary biological treatment	Negative	Ac + Ad	Ac + Ad
	Secondary sedimentation tank	Negative	Negative	Ab + Ad
	Outflow water	Negative	Negative	Negative
June 2009	Inflow water	Negative	Ac + Ab	Ab
	Primary sedimentation tank	Ab	Ac + Ab	Ab
	Secondary biological treatment	Negative	Ac + Ab	Ab
	Secondary sedimentation tank	Negative	Ac	Ac + Ab
	Outflow water	Negative	Ac+ Ab	Ab
October 2009	Inflow water	Ac + Ab	Ac	Ac + Ab
	Primary sedimentation tank	Ac + Ab	Ac + Ab	Ac + Ab
	Secondary biological treatment	Negative	Ac	Ab
	Secondary sedimentation tank	Ab	Ac + Ab	Ac + Ab
	Outflow water	Negative	Ac + Ab	Ab
	Total No of positive samples	**(n=6)**	**(n=13)**	**(n=14)**
	Total No. of species:			
	A. butzleri **(Ab)**	6 (100%)	8 (61.5%)	12 (85.7%)
	A. cryaerophilus **(Ac)**	4 (66.7%)	13 (100%)	7 (50.0%)
	A. defluvii **(Ad)**	0	2 (15.4%)	3 (21.4%)
	A. nitrofigilis **(Anit)**	0	1 (7.7%)	0
	A. cloacae **(Aclo)**	0	0	1 (7.1%)

[a]The identified species are only mentioned once, independently of the number of strains obtained from each specific sample.

Table 3. ***Arcobacter*** **strains recovered from each species depending on the culturing procedure (direct plating, DP; post- enrichment, PE)**

	No. strains (%)			
Species	**Total recovered**	**Only by DP**	**Only by PE**	**Coincidentally D & PE**
A. butzleri	77 (53.5%)	21 (30.4%)	55 (78.6%)	1(20%)
A. cryaerophilus	57 (39.6%)	46 (66.6%)	10 (14.3%)	1 (20%)
A. defluvii	8 (5.5%)	1(1.4%)	4 (5.7%)	3 (60%)
A. nitrofigilis	1 (0.7%)	1(1.4%)	0	0
A. cloacae	1(0.7%)	0	1(1.4%)	0
	144	69	70	5

4.8. The adherence and invasion of human intestinal cells by *Arcobacter* species and their virulence genotypes

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1 **The adherence and invasion of human intestinal cells by *Arcobacter* species**

2 **and their virulence genotype**

3

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30 **ABSTRACT**

31 The genus *Arcobacter* is composed of 17 species which have been isolated from various 32 sources. Of particular interest are *A. butzleri, A. cryaerophilus* and *A. skirrowii* as these have 33 been associated with human cases of diarrhoea. The probable transmission routes being 34 through the ingestion of contaminated drinking water and food. To date only limited studies 35 of virulence traits in this genus have been undertaken. The present study used sixty 36 *Arcobacter* strains isolated from different sources, representing 16 of the 17 species of the 37 genus, to investigate their ability to adhere and invade the human intestinal cell line Caco-2. 38 In addition the presence of five putative virulence genes (*ciaB, cadF, cj1349, hecA* and *irgA*) 39 was screened in these strains by PCR.

40 All *Arcobacter* species except *A. bivalviorum* and *Arcobacter* sp. strain W63 adhered 41 to Caco-2 cells and most species (10/16) were invasive. The most invasive species were *A.* 42 *skirrowii*, *A. cryaerophilus*, *A. butzleri* and *A. defluvii*. All invasive strains were positive for 43 *ciaB* (encoding for a putative invasion protein). Other putative virulence genes were present 44 in other species, i.e. *A. butzleri* (*cadF, cj1349, irgA* and *hecA*), *A. trophiarum* (*cj1349*), *A.* 45 *ellisii* (*cj1349*)*,* and *A. defluvii* (*irgA*). No virulence genes were detected in strains which 46 showed low or no invasion of Caco-2 cells. These results indicate that many *Arcobacter* 47 species are potential pathogens of humans and animals.

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49 **INTRODUCTION**

50 The genus *Arcobacter* was created in 1991 (1) and is considered an atypical group within the
51 class *Epsilonproteobacteria* because its species have been isolated from many habitats and
52 hosts (2). Currently the genus is composed of 17 species (3-6). The perceived pathogenicity of
53 some species, such as *Arcobacter butzleri* and *A. cryaerophilus*, is due to their recovery from
54 stools of patients with diarrhoea and occasionally from cases of bacteraemia, endocarditis and
55 peritonitis (3). Clinical cases are probably underestimated due to the absence of specific
56 protocols for their adequate detection and identification (3).

57 In an 8 year study Vandenberg *et al*. (7) reported that the species *A. butzleri* was the
58 fourth most common *Campylobacter*-like organism isolated from 67,599 human stools. This
59 species was associated with cases of persistent and watery diarrhoea and less associated with
60 bloody diarrhoea compared to *C. jejuni*. Other *Arcobacter* species such as *A. cryaerophilus,*
61 *A. skirrowii* and *A. thereius* have also been isolated from the intestinal tracts and faeces of
62 asymptomatic farm animals, as well as being associated with diarrhoea, abortions and mastitis
63 (3, 8).

64 The pathogenicity and virulence mechanisms of *Arcobacter* spp. are still poorly
65 understood (3). Their adhesion, invasion and cytotoxicity capacity has been studied in only 4
66 *Arcobacter* species (*A. butzleri, A. cryaerophilus, A. skirrowii* and *A. cibarius*) using various
67 cell lines, i.e. Hep-2, HeLa, INT407, CHO, and Caco-2 (3 and references therein). These
68 studies showed a considerable variation in the adhesion, invasion and toxicity, depending
69 upon the origin of strains and the cell lines studied (3, 9). The publication of the *A. butzleri*
70 RM 4018 genome (10) reported the presence of several putative virulence genes in the
71 organism, such as *ciaB*, *cj1349* and *cadF.* These are homologous to genes associated with
72 pathogenicity in other closely related organisms. The *ciaB* gene in *Campylobacter* spp.
73 encodes an invasion protein injected directly into the cytoplasm of the host cells through a

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74 secretion system (11). The *cj1349*gene in *C. jejuni* encodes for proteins that enable adhesion
75 to host cells by binding specifically to fibronectin (11), and the CadF protein also induces the
76 internalization of bacterial cells by the activation of GTPases (11). In addition there are
77 homologs to the *irgA* gene encoding for an iron-regulated outer membrane protein in *Vibrio*
78 *cholerae*, and the *hecA* gene, which encodes for a filamentous hemagglutinin in
79 uropathogenic *Escherichia coli* (11). In 2012, Douidah *et al.* (12) developed primers for these
80 virulence genes and demonstrated their presence in strains of *A. butzleri, A. cryaerophilus* and
81 *A. skirrowii*. More recently, Karadas *et al.* (13) also determined the presence of these genes by
82 PCR in 52 strains of *A. butzleri*, and the adhesion and invasion capacity to HT-29 and Caco-2
83 cells in six strains. In that study, no correlation between virulence gene patterns and adhesive
84 or invasive capabilities was observed. However, the incidence of these genes and their
85 potential correlation with the adhesion or invasion capacity to human cell lines has not been
86 studied for all *Arcobacter* spp., and is the aim of the present study.
87
88 **MATERIALS AND METHODS**
89 A total of 60 *Arcobacter* strains belonging to 15 of the 17 accepted species were studied
90 (Table 1). The species *A. halophilus* and *A. marinus*, both so far only known by the type
91 strains, were not included in the study because they are halophiles and do not grow in the
92 standard media used for the cultivation of other *Arcobacter* spp. or for tissue culture studies
93 (14, 15). Strain W63 was included which represented a new *Arcobacter* species (under
94 proposal) on the basis of the 16S rRNA gene (data not shown).
95 The strains had been isolated from different sources: shellfish (n=23), meat (n=12),
96 sewage (n=11), and faeces from pigs (n=3), chickens (n=3) and sheep (n=1). Other
97 miscellaneous environmental sources were sea water (n=2), piggery effluent (n=2), roots of
98 *Spartina alterniflora* (n=1), and also porcine abortion (n=2). All strains were genetically

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99 identified using a multiplex-PCR (m-PCR; 16) and the 16S rRNA-RFLP methods specific for
100 this genus (17, 18). The identity of 40 strains (Table 1) was confirmed by sequencing the
101 *rpoB* gene as previously described (19). All strains of the same species showed unique
102 profiles when genotyped by ERIC-PCR (20) (data not shown). The control strains for the
103 adhesion and invasion assays, *Salmonella enterica* serovar Enteritidis (NCTC 3046) and
104 *Escherichia coli* K12 HB101 (Children's Hospital, Los Angeles), were obtained from the
105 Nottingham Trent University culture collection.
106
107 **Preparation of bacterial suspensions**
108 A colony of each strain was used to inoculate Brain Heart Infusion (BHI; Difco, Becton,
109 Dickinson and Company) broth which was incubated under aerobic conditions for 48 h at
110 30ºC for *Arcobacter* strains and overnight (15 ± 2 h) at 37ºC for the control strains. After the
111 incubation period, the cultures were diluted to an optical density (600 nm) of 0.08 (ca. 10^9 cfu
112 ml^{-1} of bacteria cells) for *Arcobacter* strains and of 0.05 (ca. 10^8 cfu ml^{-1}) for the control
113 strains, as per previous studies (21). The cultures were centrifuged (5 minutes at 3000 rpm,
114 4ºC) and the resultant cell pellets were resuspended in the same volume of warm (37ºC)
115 Eagle's Minimum Essential Medium (EMEM; M4655 Sigma) supplemented with 10% foetal
116 bovine serum (FBS, F7524 Sigma) and 1% non essential amino acids (NEAA, M7145
117 Sigma). The bacterial viable counts were determined on BHI agar supplemented with 5%
118 sheep blood agar following the Miles Misra (22) method. The number of cells (cfu ml^{-1}) of
119 each bacterial suspension represented the mean from three enumerations.
120 **Caco-2 adhesion and invasion assay**
121 The adhesion and invasion assays were as described previously (21, 23). Briefly, 0.5 ml of a
122 suspension of 4 x 10^4 Caco-2 cells ml^{-1} in EMEM supplemented with penicillin 10,000U and
123 streptomycin 10,000 µg ml^{-1} (P4333 Sigma) were added to each of the 24 wells of a microtitre

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124 plate which was then incubated for 48h at 37ºC under a 5% CO_2 atmosphere (Sanyo CO_2
125 incubator). When the cells had formed a confluent monolayer, the medium was removed, the
126 wells were washed twice with Phosphate Buffered Saline (PBS, D8537 Sigma) and 0.5 ml of
127 the bacterial suspension (ca 10^9 cfu ml^{-1}) was added. The plates were incubated for 2h at
128 37ºC to allow adhesion and invasion of the bacteria and were then washed twice with PBS to
129 remove unbound bacteria. The cell monolayer was lysed with 1% Triton-X and the total
130 number of bacteria associated with the Caco-2 cells was enumerated as described above.
131 The number of adherent bacteria was calculated as the difference between the total number of
132 bacteria associated with the Caco-2 cells and the number of intracellular bacteria. The latter
133 was determined by inoculating another 24 well plate which was washed twice with PBS and
134 then supplemented with 0.5 ml of EMEM containing 125 mg ml^{-1} of gentamicin and
135 incubated for 1h at 37ºC to kill extracellular bacteria. After incubation, the cells were washed
136 twice with PBS, lysed with 1% Triton-X and the released bacteria enumerated, as described
137 above. All experiments were in triplicate. Results were expressed as the mean number of
138 bacteria (log_{10} cfu ml^{-1}) that adhered or invaded. The limit of detection for adhesion was 1.7 x
139 10^4 cfu ml^{-1} (4.23 log_{10} cfu ml^{-1}) and for invasion 1.7 x 10^2 cfu ml^{-1} (2.23 log_{10} cfu ml^{-1}).
140 Values above the detection limits were defined as adherent or invasive, respectively. In order
141 to compare the adhesion and invasion results obtained for the different strains (Table 1) and
142 species (Figure 1) with those of the controls, the values per each strain or species were all
143 proportionally calculated in relation to an initial inoculum of 1.0 x 10^8 cfu ml^{-1}.
144

145 **Data analysis**

146 The range of results between the detection limit and the mean obtained for the positive control
147 (*S. enterica*) was divided into 3 categories defined as 'low', 'good' or 'high' adhesion or
148 invasion ability, as shown in Table 1.

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149 The Mann Whitney statistical test, corrected by using the Bonferroni Multiple Comparison
150 Test, was used to compare the results. For those strains where non adhesion or non invasion
151 was detected, the respective detection limit value was assigned in the data set for statistical
152 analysis. Significance was established at the p level of <0.05. The analyses were carried out
153 using the Prism version 5 (Graphpad) and the SPSS Version 20 (IBM) software.
154
155 **Detection of virulence genes**
156 Bacterial DNA was extracted using the InstaGene™ DNA Purification Matrix (Bio-Rad
157 Laboratories, Herculeus, CA). The PCR methods used to detect the presence of *ciaB*, *hecA*,
158 *cj1349*, *cadF* and *irgA* genes used the primers and conditions were as previously described
159 (12). PCR products were analysed on 2% agarose gel Tris-Borate-EDTA buffer at 80 V for 90
160 min using the 100 bp ladder (Fermentas) as a molecular weight marker. The gels were stained
161 with SYBR® Safe DNA Gel Stain (Invitrogen) and photographed using an UV
162 transilluminator. *A. butzleri* LMG 10828^T was used as the positive control strain for all PCR
163 reactions (12).
164 In order to confirm the identity of the amplicons, 28 PCR products from the 5 genes of the
165 different species were sequenced (Table 1, GenBank accession numbers HF935040-
166 HF935067). Sequences were obtained using the amplification primers by Macrogen Corp
167 Europe (The Netherlands) and then compared with the *A. butzleri* RM4018 genome
168 (GenBank: NC_009850.1) using the MEGA 5 software (24). Furthermore, a BLASTN
169 comparison was carried out to confirm the presence of the studied genes in other deposited
170 *Arcobacter* genomes.
171
172 **Microscopic observation**

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173 Strains representing those species adherent and invasive were selected for light and electron
174 microscopy examination. The experiments were performed under the described conditions
175 with the exception that Caco-2 cells were grown on coverslips placed into the 6 wells of the
176 culture plates used. For light microscopy, cells were fixed with methanol, stained for 15 min
177 with 10% Giemsa (Sigma-Aldrich) and then at least 10 fields per each slide were visualised
178 using an Olympus BX51 microscope. For transmission (TEM) and scanning (SEM) electron
179 microscopy, the cells were fixed by adding 2% glutaraldehyde solution in 0.1M phosphate
180 buffer (pH 7.4) for 2 h to the wells containing the coverslips. The cells were then rinsed with
181 0.1 M phosphate buffer (pH 7.4) and post-fixed with 1% buffered osmium tetroxide for 1 h at
182 5ºC in the dark. The fixed cells were washed in buffer and dehydrated by 15 min changes in a
183 graded series of ethanol up to 100%. The samples for TEM and SEM were then separated. For
184 TEM, loose cells were collected from the wells, transferred to eppendorf tubes and embedded
185 in Spurr resin. Ultrathin sections for TEM were stained with uranyl acetate and lead citrate
186 before examination using a Jeol 1011 at 80 kV. The coverslip preparations were used for
187 SEM, and were subjected to serial mixtures of amylacetate-ethanol in a Petri dish, in which
188 the concentration of the first substance was gradually increased through six steps to 100%.
189 The coverslips were critical-point dried with CO_2. After drying, specimens were mounted and
190 coated with a thin layer of gold before examination using a Jeol JSM 6400 at 15 kV.
191

192 **RESULTS**

193 Nearly all (14/16) *Arcobacter* species adhered to Caco-2 cells, with the exceptions of *A.*
194 *bivalviorum* and *Arcobacter* sp. strain W63, and a total of ten *Arcobacter* species invaded
195 (Figure 1). The 8 most highly invasive strains belonged to the species *A. trophiarum* (3/3), *A.*
196 *skirrowii* (1/2), *A. cryaerophilus* (1/5), *A. butzleri* (2/12) and *A. defluvii* (1/8). Most of these
197 strains had been isolated from animal faeces, and sewage (Table 1). Two strains of *A.*

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198 *trophiarum* (LMG 25535 and CECT 7650) showed similar invasion capacity as the *S.*
199 *enterica* positive control (Table 1). In fact, *A. trophiarum* was significantly ($p<0.05$) more
200 invasive than the other species (Figure 2). On the other hand, only one strain of *A. skirrowii*
201 (S7-1), showed a higher invasion capacity than *S. enterica* (Table 1). Regarding the origin of
202 strains, those recovered from faecal sources (animal faeces and sewage) were significantly
203 more invasive than those from other origins ($p<0.05$; Figure 3).

204 *CiaB* was the most prevalent virulence associated gene detected (51/60, $p<0.05$),
205 followed by *cj1349* (23/60) and *cadF* (15/60) (Table 1). Two strains of *A. butzleri* (F1 and
206 F87) isolated from mussels and one strain of *A. skirrowii* (S7-1) from sewage, were positive
207 for four or five virulence genes. *A. butzleri* F1 and *A. skirrowii* S7-1 showed significant
208 capacity to invade Caco-2 cells (Table 1). All *A. defluvii, A. trophiarum, A. butzleri, A.*
209 *skirrowii* and *A. cryaerophilus* strains possessed the *ciaB* gene, as did all strains considered as
210 highly invasive (Table 1). Some of these species possessed other genes, i.e. *A. defluvii* the
211 *irgA* gene (8/8), *A. trophiarum cj1349* (3/3), *A. butzleri cadF* (12/12), *cj1349* (11/12), *irgA*
212 (2/12) and *hecA* (1/12) and *A. skirrowii* the *cadF* (1/2), *cj1349* (1/2) and *hecA* (1/2). In
213 contrast, all strains of *A. thereius* (n=5) and *A. mytili* (n=3) and one strain of *A. cibarius* were
214 negative for all the tested genes (Table 1).

215 Bioinformatics analysis of the putative genes in sequenced *Arcobacter* strains agreed
216 with the laboratory studies. *A. butzleri* strain ED-1 (a recently released genome, GenBank:
217 NC_017187.1) possessed the 5 tested genes, as did *A. butzleri* F1 (Table 1). *Arcobacter* sp.
218 strain L (GenBank: NC_017192.1), which groups with *A. defluvii* on the basis of its 16S
219 rRNA gene sequence (24), possessed the *ciaB* and *irgA* genes which are also present in all *A.*
220 *defluvii* strains. *A. nitrofigilis* strain DSM 7299^{T} (GenBank: NC_014166.1) possessed only
221 the *ciaB* gene, as was also determined experimentally (Table 1).

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222 Both light and electron microscopy demonstrated the presence of extracellular bacteria
223 closely associated with the membrane of Caco-2 cells and intracellular bacterial cells (Figures
224 S1 and S2). In general, all *Arcobacter* species showed a homogeneous distribution of bacterial
225 cells on the Caco-2 surface without any specific pattern of adhesion. Strains of *A. trophiarum*
226 appeared to form clusters inside the Caco-2 cells (Fig S1).
227
228 **DISCUSSION AND CONCLUSIONS**
229 This is the first study of *Arcobacter* virulence potential which has included representatives of
230 all accepted *Arcobacter* species (except *A. marinus* and *A. halophilus*) and a potential new
231 *Arcobacter* species (strain W63). It has shown that most species (14/16) adhered to Caco-2
232 cells while 10/16 were invasive. All strains of *A. cryaerophilus, A. butzleri* and *A. skirrowii*
233 adhered to the human intestinal Caco-2 cells and most invaded the cell line; 5/5, 11/12 and
234 1/2, respectively. Previous studies with these 3 species, showed that overall 55/99 adhered
235 and 9/44 invaded Caco-2, CHO, HeLa, Hep-2, INT407, IPI-2I or Vero cell lines (3 and
236 references therein) but only 3 studies were performed on Caco-2 cells (13, 21, 26). Ho *et al*.
237 (21) tested 4 strains of *A. cryaerophilus*, 2 of *A. skirrowii* and 1 of *A. butzleri,* mainly isolated
238 from newborn piglets or sow amniotic fluid, and also the type strain of *A. cibarius* (LMG
239 21996^T) isolated from chicken carcasses. Although the 8 strains adhered to Caco-2 cells, only
240 two strains of *A. cryaerophilus* were able to invade. In relation to *A. cibarius,* in our study the
241 type strain (CECT 7203^T) and strain NC81 showed adhesion but no invasion capacity.
242 Although this was in agreement with previous results (21), the remaining three *A. cibarius*
243 strains showed an invasion capacity. Houf and Stephan (26) determined the ability of only 7
244 *A. cryaerophilus* strains (isolated from faeces of healthy human carriers) to attach to Caco-2
245 cells, of which only 2 adhered. The higher adhesion and invasion capacity (5/5) observed in
246 our study could be due to the different origin of strains, as previously proposed (9, 13). A

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247 recent study compared the adhesion and invasion capacity of 3 isolates of *A. butzleri* from
248 chicken meat and 3 from human origin to Caco-2 and HT-29 cells. All the isolates showed
249 adhesion and invasion of Caco-2 cells while only 4 showed adhesion to HT-29 cells and 3
250 invaded the cell line (13). Two isolates from chicken and one from human showed the
251 highest adhesion and invasion to Caco-2 and HT-29 cells, while the other two human isolates
252 were the less adhesive and less invasive to Caco-2 cells. Coincidentally the latter isolates were
253 non-invasive to HT-29 cells and therefore, it was concluded that the results were strain
254 dependent.

255 In our study, all strains of the recently described species *A. trophiarum, A. defluvii, A.*
256 *ellisii* and *A. cloacae*, were able to invade Caco-2 cells. Furthermore, the strains of *A.*
257 *trophiarum* (all from faeces of pig and chicken), were significantly more invasive than the
258 others ($p<0.05$) (Figure 2). It is notable that 100% strains of *A. trophiarum* (3/3) and *A.*
259 *thereius* (5/5) adhered, whereas 100% and 80%, respectively, invaded. This is despite both
260 species being previously described as unable to grow at 37°C under laboratory conditions (27,
261 28) yet having been isolated from warm blooded animals; pig faeces (27), porcine abortion
262 and cloacal content of ducks (28). Interestingly, the strains of these species remained viable or
263 even grew when incubated at 37ºC for 2 h in EMEM while in BHI they showed a slight
264 decrease in viable counts after incubation (data not shown). Our results could indicate that
265 EMEM and the Caco-2 cells simulate better the natural intestinal habitat than BHI.

266 The adhesion and invasion ability of mammalian cell lines by bacterial pathogens are studied
267 because these abilities are necessary for successful colonization and infection of the host (26).
268 The present study indicates that many *Arcobacter* spp., including the recently discovered
269 ones, have the potential ability to colonize and enter human cells.

270 The putative virulence genes showed a similar order of prevalence for *A. butzleri*
271 (n=12), *A. cryaerophilus* (n=5) and *A. skirrowii* (n=2), i.e. 85.0% *ciaB,* 38.3% *cj1349,* 25.0%

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272 *cadF,* 16.7% *irgA* and 3.3% *hecA,* as that previously reported (12, 13). *A. butzleri* showed the
273 highest prevalence of virulence genes; (100% *ciaB,* 91.7% *cj1349,* 91.7% *cadF,* 16.7% *irgA*
274 and 8.3% *hecA*) [n=12]. Similar results have been obtained for the latter species in previous
275 studies, because the *ciaB, cj1349* and *cadF* genes were detected in 100% of isolates while the
276 *irgA* was detected in 17.3% to 30% of the strains and the *hecA*, in 13.5% to 25.8% (12, 13).
277 Furthermore, in our study, the only strain (F1) that possessed all the 5 genes belonged to *A.*
278 *butzleri*.

279 It is plausible that there was a detection bias towards *A. butzleri* strains as the primers
280 were designed from the *A. butzleri* RM4018 genome (GenBank: NC_009850.1).
281 Nevertheless, there was correlation between the absence of virulence genes and the lack of
282 invasion of Caco-2 cells, given that the 7 strains of *A. thereius* (3 strains), *A. mytili* (3 strains)
283 and *A. cibarius* which were negative for all tested genes were either low or non-invasive
284 (Table 1). In contrast, *A. skirrowii* S7 showed the highest adhesion and invasion values and
285 possessed the four virulence related genes (*ciaB*$^+$, *hecA*$^+$, *cj1349*$^+$, *cadF*$^+$). This strain, and 2
286 strains of *A. trophiarum,* showed similar or higher invasion values ($p<0.05$) than *S. enterica*
287 (used as the positive control) and their virulence genotype included at least the *ciaB* and
288 *cj1349* genes. The occurrence of putative virulence genes in the different *Arcobacter* species
289 did correlate with those in the published whole genome sequences. Karadas *et al*. (13)
290 observed no correlation between virulence gene patterns and adhesion or invasion to Caco-2
291 and HT-29 cell lines. They also observed that the putative functional domains of *ciaB, cadF*
292 and *cj1349* genes did not change depending on the adhesion or invasion capacity. Those
293 results were in part explained by the low number of isolates compared (n=6) and it was
294 indicated that further strains needed to be tested (13).

295 With respect to possible associations between strain origin and virulence, it was
296 notable that strains from faecal sources were the most invasive ($p<0.05$) followed by those

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297 from shellfish and meat (Figure 3). Furthermore, the strains from faecal sources (n=18)
298 carried a higher proportion virulence genes (100% *ciaB*$^{+}$, 38.9% *irgA*$^{+}$, 33.3% *cj1349*$^{+}$, 11.1%
299 *cadF*$^{+}$, 5.6% *hecA*$^{+}$), as did those from food (n=35, 88.6% *ciaB*$^{+}$, 45.7% *cj1349*$^{+}$, 37.1%
300 *cadF*$^{+}$, 8.6% *irgA*$^{+}$, 2.9% *hecA*$^{+}$) when compared to the rest of strains (n=7, 42.9% *ciaB*$^{+}$,
301 14.3% *cj1349*$^{+}$ and *irgA*$^{-}$, *hecA*$^{-}$, *cadF*$^{-}$). The *irgA* gene was more prevalent in strains from
302 sewage (54.5%) compared to others (8.2%, $p<0.05$) and the *cadF* in those from food (37.1%)
303 compared to others (8.0%, $p<0.05$). It is plausible that such traits are species related, since 8
304 of the 10 strains positive for the *irgA* gene were strains of *A. defluvii.* Of these 75.0% (6/8)
305 were from sewage. Eleven of the 15 food strains positive for *cadF* gene belonged to *A.*
306 *butzleri,* of which 90.9% (10/11) were from food.
307 The *A. nitrofigilis* type strain CECT 7204^{T}, isolated from roots of *Spartina alterniflora*
308 (29) did not adhere or invade Caco-2 cells, however, it possessed the *ciaB* gene by PCR and
309 this was also confirmed when analyzing its genome. In contrast, the two other *A. nitrofigilis*
310 strains (F74 and F2176) isolated from mussels, that are considered a potential source of
311 *Arcobacter* infection (3), showed adhesion and invasion abilities and were also *ciaB*$^{+}$. In this
312 respect, the role of *ciaB* and the other genes associated with *Arcobacter* virulence, need to be
313 further studied.
314 This is the first study that demonstrates both the presence of putative virulence genes
315 associated with adhesion and invasion and complementary *in vitro* tissue culture analysis for
316 nearly all the *Arcobacter* species, using strains isolated from various sources. On the basis of
317 these results, most *Arcobacter* species were confirmed as potential human pathogens, with
318 some strains of *A. butzleri*, *A. cryaerophilus, A. skirrowii* and the recently described *A.*
319 *trophiarum* and *A. defluvii* potentially being more virulent. Further studies are warranted to
320 further characterise these virulence traits and confirm their role in infection.
321

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331

332 **Potential conflicts of interest**

333 All authors: No reported conflicts.

334

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424 **Table 1**. Virulence genotype of 60 *Arcobacter* strains and their relative adhesion and invasion

425 capacities to Caco-2 cells.

			Virulence genes					Viable count (log_{10} cfu ml^{-1})	
Species	**Strain**	**Origin**	***ciaB***	***irgA***	***hecA***	***cj1349***	***cadF***	**Adhesion**	**Invasion**
Controls									
Salmonella enterica	**NCTC 3046**		ND	ND	ND	ND	ND	6.46	4.53
Escherichia coli	**K12**		ND	ND	ND	ND	ND	6.54	NI
A. butzleri	**LMG 10828[T]**	**human blood**	+	+	+	+	+	ND	ND
Tested strains									
A. butzleri	**F1**	**mussels**	+[a]	+	+[a]	+[a]	+[a]	4.62	2.24
A. butzleri	**F15**	**turkey meat**	+	-	-	+	+	3.98	1.57
A. butzleri	**F27**	**duck meat**	+	-	-	+	+	4.69	2.73
A. butzleri	**F46[b]**	**pig meat**	+	-	-	+	+	4.73	1.96
A. butzleri	**F47**	**chicken meat**	+	-	-	+	+	4.21	2.35
A. butzleri	**F49**	**pig meat**	+	-	-	+	+	4.53	2.29
A. butzleri	**F50**	**beef meat**	+	-	-	+	+	4.69	**3.17**
A. butzleri	**F63**	**clams**	+	-	-	+	+	**5.13**	**3.10**
A. butzleri	**F71-1**	**clams**	+	-	-	+	+	**6.12**	2.72
A. butzleri	**F87**	**mussels**	+	+[a]	-	+	+	4.23	NI
A. butzleri	**SW21**	**sewage**	+	-	-	+	+	2.95	1.99
A. butzleri	**SW28-5**	**sewage**	+	-	-	-	-	**6.57**	1.70
A. cryaerophilus	**F55**	**clams**	+[a]	-	-	-	-	**5.57**	2.73
A. cryaerophilus	**F93-1**	**clams**	+	-	-	-	-	**5.52**	2.39
A. cryaerophilus	**FE4**	**chicken faeces**	+	-	-	-	-	**6.31**	**3.06**
A. cryaerophilus	**FE5**	**chicken faeces**	+	-	-	-	-	**5.37**	2.31
A. cryaerophilus	**FE14**	**sheep faeces**	+	-	-	-	-	**5.99**	2.94
A. cibarius	**NC81[b]**	**piggery effluent**	+	-	-	-	-	4.35	NI

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A. cibarius	NC88[b]	piggery effluent	-	-	-	-	-	**5.71**	2.51
A. cibarius	H743[b]	poultry meat	+	-	-	-	-	**5.64**	2.12
A. cibarius	H746[b]	poultry meat	+	-	-	-	-	**5.09**	2.12
A. cibarius	CECT7203[T,b]	poultry meat	+[a]	-	-	-	-	4.96	NI
A. skirrowii	S7-1[b]	sludge WWTP	+[a]	-	+[a]	+[a]	+[a]	**7.53**	**5.00**[c]
A. skirrowii	F28	pig meat	+	-	-	+	-	3.13	NI
A. nitrofigilis	CECT 7204[T,b]	roots *S. alterniflora*	+[a]	-	-	-	-	NA	NI
A. nitrofigilis	F72[b]	mussels	+	-	-	-	-	**5.12**	2.69
A. nitrofigilis	F2176[b]	mussels	+	-	-	-	-	**5.39**	2.91
A. thereius	LMG 24486[T,b]	porcine abortion	-	-	-	-	-	4.68	NI
A. thereius	LMG 24487[b]	porcine abortion	-	-	-	-	-	**5.08**	1.94
A. thereius	F61-1[b]	pig meat	-	-	-	-	-	4.40	1.55
A. thereius	F93-4[b]	mussels	-	-	-	-	-	**5.19**	2.18
A. thereius	SW24[b]	sewage	-	-	-	-	-	4.27	1.58
A. mytili	T234[b,d]	brackish water	-	-	-	-	-	4.15	NI
A. mytili	CECT 7385[b,d]	mussels	-	-	-	-	-	4.24	NI
A. mytili	CECT 7386[T,b,d]	mussels	-	-	-	-	-	**5.00**	NI
A. trophiarum	LMG 25535[b]	pig faeces	+	-	-	+	-	**5.08**	**4.10**[c]
A. trophiarum	LMG 25534[T,b]	pig faeces	+[a]	-	-	+[a]	-	4.08	**3.04**
A. trophiarum	CECT 7650[b]	chicken faeces	+	-	-	+	-	**5.18**	**4.21**[c]
A. defluvii	SW28-7[b]	sewage	+	+	-	-	-	**5.22**	1.99
A. defluvii	CECT 7697[T,b]	sewage	+[a]	+[a]	-	-	-	4.82	1.68
A. defluvii	SW29-1	sewage	+	+	-	-	-	**5.58**	**3.08**
A. defluvii	SW28-10	sewage	+	+	-	-	-	**5.04**	2.49
A. defluvii	SW30-8	sewage	+	+	-	-	-	4.85	1.57
A. defluvii	SW30-2[b]	sewage	+	+	-	-	-	**5.23**	2.17
A. defluvii	CH8-2[b]	mussels	+	+	-	-	-	**5.87**	1.92
A. defluvii	CC42[b]	pig faeces	+	+	-	-	-	**5.22**	2.75
A. molluscorum	F91[c,b]	mussels	+	-	-	-	-	**5.12**	NI

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A. molluscorum	**CECT 7696[T,b,d]**	**mussels**	+[a]	-	-	-	-	4.94	NI
A. molluscorum	**F101-1[b,d]**	**oysters**	+	-	-	-	-	4.26	NI
A. ellisii	**F79-2[b,d]**	**mussels**	+	-	-	-	+	**5.64**	1.87
A. ellisii	**F79-6[Tb,d]**	**mussels**	+[a]	-	-	+[a]	+[a]	**5.25**	2.80
A. ellisii	**F79-7[b,d]**	**mussels**	+	-	-	+	+	4.54	1.71
A. bivalviorum	**F4[T,b,d]**	**mussels**	+[a]	-	-	+[a]	-	NA	NI
A. bivalviorum	**F118-2[b,d]**	**mussels**	+	-	-	-	-	NA	NI
A. bivalviorum	**F118-4[b,d]**	**mussels**	+	-	-	+	-	NA	NI
A. venerupis	**F67-11[T,b,d]**	**clams**	+[a]	-	-	-	-	**5.91**	NI
A. suis	**F41[b,d]**	**pig meat**	+[a]	-	-	-	-	**5.92**	NI
A. cloacae	**F26[b,d]**	**mussels**	+	-	-	+	-	**6.11**	2.00
A. cloacae	**SW28-13[T,b,d]**	**sewage**	+[a]	-	-	+[a]	-	4.51	1.00
***Arcobacter* sp.**	**W63[b,d]**	**sea water**	+[a]	-	-	+[a]	-	NA	NI

426 NA: No adhesion detected. NI: No invasion detected. ND: Not determined. The values for adhesion and invasion were

427 proportionally calculated to an inoculum of 10^8 ufc ml-1 (8.0 log_{10} cfu ml^{-1}) for each strain. Those results classified as

428 high for adhesion (viable count > 5.0 log_{10} cfu ml^{-1}) and invasion (>3.0 log_{10} cfu ml^{-1}) are shown in bold.

429 [a]Confirmed by DNA sequencing.

430 [b]The identity of these strains was confirmed by sequencing of *rpoB* gene

431 [c]Invasion results equal or higher than the positive control *S. enterica* (NCTC 3046).

432 [d]Only available strains of these species

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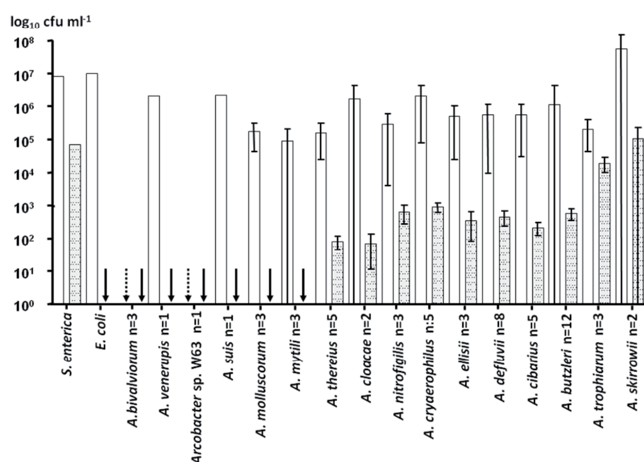

Figure 1. Adhesion (white bars) and invasion (dotted bars) to Caco-2 cells by 60 *Arcobacter* strains belonging to 15 species. Results of triplicate experiments are expressed as the mean (and standard deviation) and were proportionally calculated to an inoculum of 10^8 cfu ml^{-1}. Control strains were *Salmonella enterica,* positive for adhesion and invasion, and *Escherichia coli,* positive for adhesion and negative for invasion. Arrows indicate those cases in which adhesion (dotted) or invasion (black) was below the detection limit.

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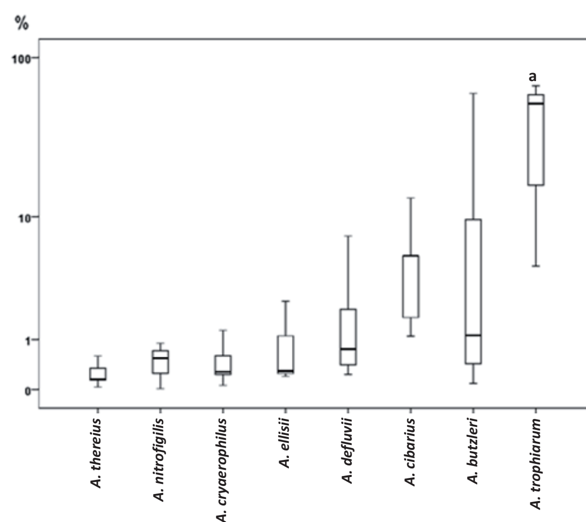

Figure 2. Boxplots showing the invasion capacity of the *Arcobacter* species. Those species in which invasion was not detected and/or that included less than 3 strains i.e. *A. skirrowii*, *A. bivalviorum, A. molluscorum, A. mytili, A. venerupis*, *A. cloacae*, *A. suis* and *Arcobacter* sp. W63, are not represented. Results are expressed as percentage of invasion in relation to positive control. The length of the box shows 50% interquartile range (25%-75%) of the variable. The line in the box indicates the median while extended lines from the box show maximum and minimum values. [a]More invasive ($p<0.05$) than the other species.

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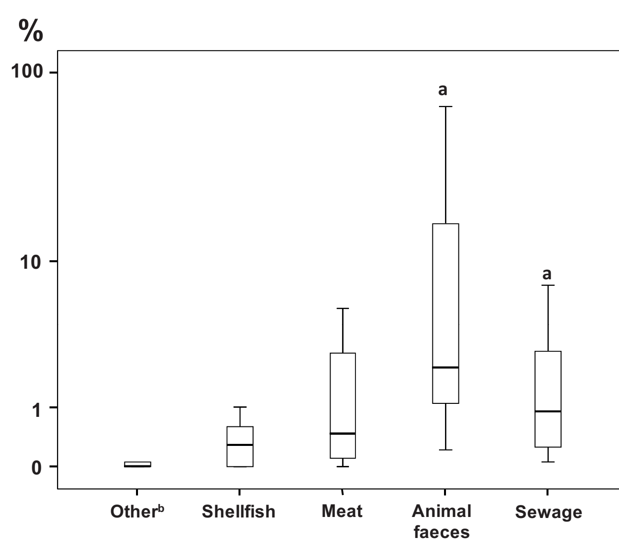

Figure 3. Boxplots showing the invasion capacity of the *Arcobacter* strains grouped by origin. The 2 strains isolated from porcine abortion were not represented. Results are expressed as percentage of invasion in relation to positive control. The length of the box shows 50% interquartile range (25%-75%) of the variable. The line in the box indicates the median while extended lines from the box show maximum and minimum values. [a]Strains from faecal sources (i.e. animal faeces and sewage together) were significantly more invasive ($p<0.05$) than strains from other origins. [b]Include strains recovered from sea water, piggery effluent, roots of *Spartina alterniflora* and porcine abortion.

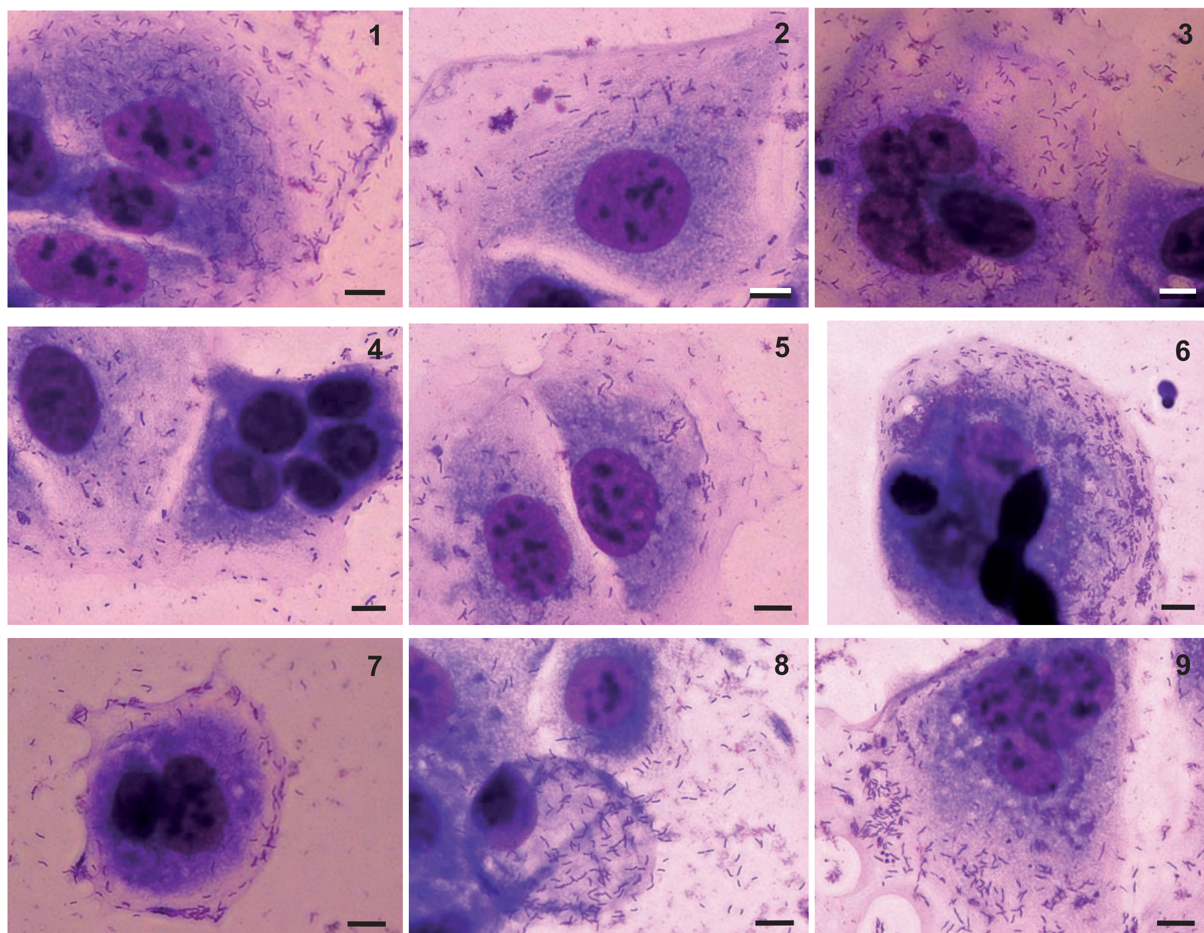

Figure S1. Giemsa stain of strains of *Arcobacter* spp. associated with caco-2 cells.1, strain F49 (*A. butzleri*); 2, strain FE4 (*A. cryaerophilus*); 3, strain S7-1 (*A. skirrowii*); 4, strain CECT 7203^{T} (*A. cibarius*); 5, strain SW29-1 (*A. defluvii*); 6, strain LMG 25534^{T} 7650 (*A. trophiarum*); 7, strain F79-6^{T} (*A. ellisii*); 8, strain F2176 (*A. nitrofigilis*); 9, strain SW28-13 ("A. cloacae"). Bar 10 μm.

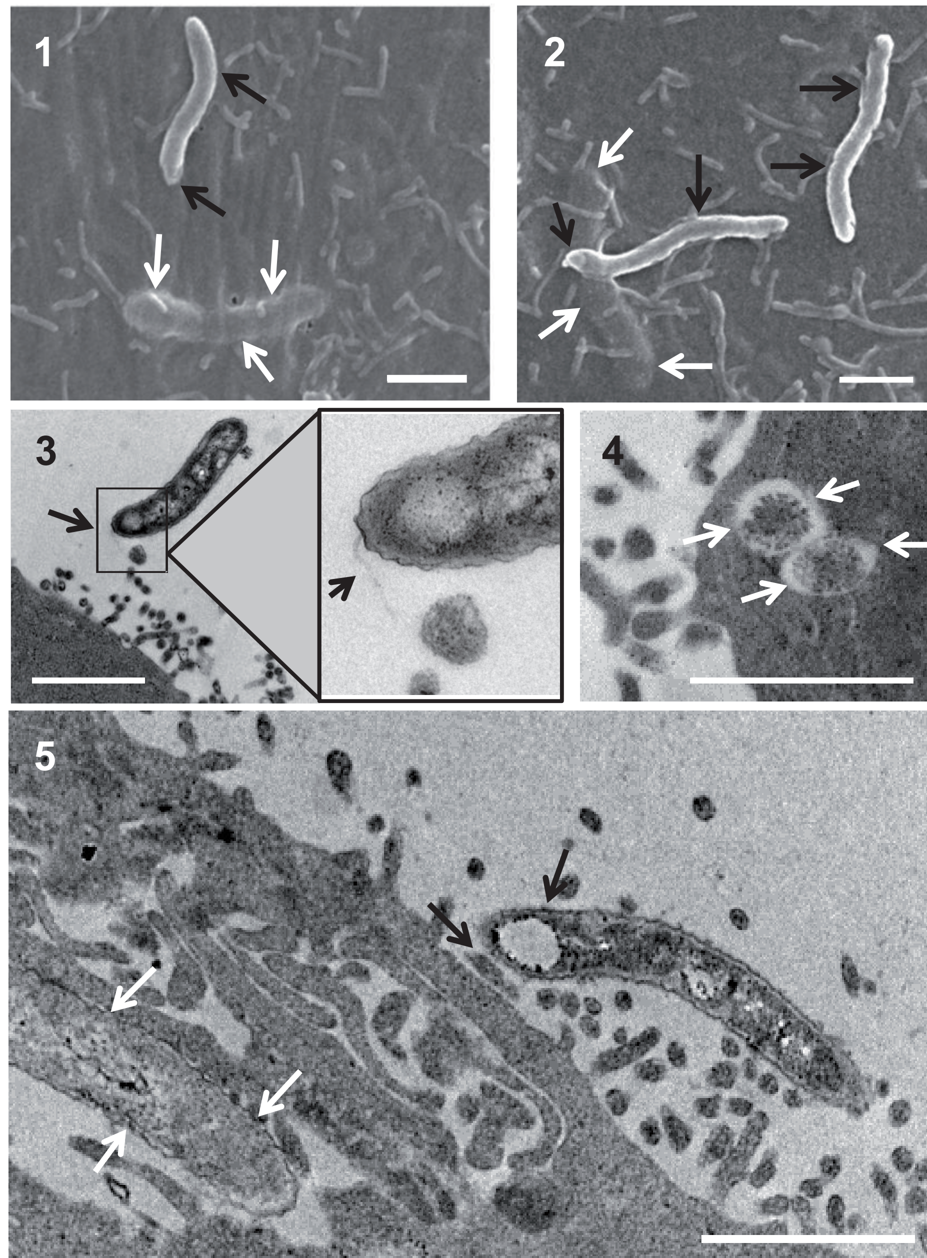

Figure S2. Scanning (SEM 1, 2) and transmission (TEM 3-5) electron micrographs showing the adhesion (black arrows) and invasion (white arrows) of *Arcobacter* strains to Caco-2 cells. Notice in image 3 and in the magnified inserted image (x3.5) a flagellum in the direction of a close by cross-sectioned microvilli, and in image 4 two cross-sectioned bactera within the cytoplasm of the Caco-2 cell. Bar 1 µm.

UNIVERSITAT ROVIRA I VIRGILI
SANITARY IMPORTANCE OF ARCOBACTE
Arturo Levican Asenjo
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4.9. A severe case of persistent diarrhoea produced by *Arcobacter cryaerophilus* but erroneously attributed to *Campylobacter* sp.

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A severe case of persistent diarrhoea caused by *Arcobacter cryaerophilus* but attributed to *Campylobacter* sp. and a review of the clinical incidence of *Arcobacter* spp.

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Keywords: *Arcobacter*; gastrointestinal disease; persistent diarrhoea; emerging or re-emerging diseases.

Abstract

Although rarely, A*rcobacter* spp. have been associated with diarrhoea and bacteraemia. We report a persistent case in a 26-year-old healthy Spanish male of bloody diarrhoea, which was attributed to *Campylobacter* but in fact, it was caused by *Arcobacter cryaerophilus,* as determined by sequencing of the *rpoB* gene. The isolate was re-identified by Matrix Assisted Laser Desorption Ionization Time of Flight MALDI-TOF and genotyped for 5 putative virulence genes and for 7 genes included in the *Arcobacter* Multilocus Sequence Typing (MLST) database. The low score obtained by MALDI-TOF indicate the need for complementing the database with more isolates. Only the *ciaB,* which encodes for an invasin, was detected. Despite the isolate belonged to a new sequence type, three of the alleles (*glnA, pgm* and *tkt*) had been found previously in isolates from faeces of patients with diarrhoea. This study together with the reviewed literature indicates that *Arcobacter* can produce bacteraemia and that the isolation from patients with diarrhoea range from 0.11% to 1.25%. This study demonstrates that *Arcobacter* species are uncover by *Campylobacter* spp., as previously suggested. This is one of the factors that lead to their underestimation together with the use of inappropriate detection and identification methods.

Introduction

Bacteria of the genus *Arcobacter,* which was created with aerotolerant species previously included in the genus *Campylobacter,* are considered emergent enteropathogens and potential zoonotic agents (1). The genus currently includes 17 species, six of them isolated from shellfish (2). Species of this genus are able to produce abortions, mastitis and gastrointestinal disorders in animals (3-5) and bacteraemia, endocarditis, peritonitis and diarrhoea in humans (6-12). So far there have been very few human diarrhoea cases reported despite it having been found that *A. butzleri* was the fourth most common *Campylobacter*-like organism isolated from human stools (13, 14). Persistent watery diarrhoea was the main symptom associated with *Arcobacter* species, in contrast to the bloody diarrhoea produced by *Campylobacter jejuni* (13). It has been suggested that *Campylobacter* isolates uncover *Arcobacter* spp. (13), which are not routinely studied with the *ad hoc* methods in clinical laboratories. However, the true impact of this confusion is unknown (1).

This study describes in detail the clinical characteristics of an acute case of diarrhoea produced by *A. cryaerophilus,* which was recognized after sequencing of the *rpoB* gene from an isolate biochemically identified as *Campylobacter* sp. The isolate was re-identified with MALDI-TOF, genotyped by Multilocus Sequence Typing (MLST) and its putative virulence genotype screened by PCR. The study intends to alert clinicians to the possible role that this poorly known bacteria genus plays in the development of human disease by showing all known clinical cases.

Case report

A 26-year-old male with no previous history of disease visited the doctor complaining of bloody watery diarrhoea of 3 weeks duration (with ca. 3 liquid depositions a day), with abdominal pain but without fever, nausea or vomiting. The patient had been attended to with similar symptoms four months before and was diagnosed with acute gastroenteritis. A stringent diet was the recommended treatment but no analyses were made at that time. Considering this previous history, a blood and stool analysis was carried out. The stool sample was examined for parasites and cultured for *E. coli*, *Salmonella*, *Shigella*, *Yersinia*, *Aeromonas*, *Plesiomonas*, *Vibrio* and *Campylobacter* species. Complete blood count (CBC) and search for Hepatitis B antigen, anti-hepatitis C virus antibody and other antibodies against HIV, CMV, adenoviruses and parvovirus B19 were all made. The patient was diagnosed with acute gastroenteritis and an empirical antibiotic treatment was initiated with amoxicillin/clavulanic acid.

The laboratory evaluation showed an almost normal CBC except for a slight relative reduction in neutrophils (37%) and an increase in the lymphocytes (51.2%). The blood culture was negative but the stool sample showed a positive culture in the *Campylobacter*

CCDA agar. Colonies grew after 3 days and were identified as *Campylobacter* sp. based on phenotypic tests (Gram stain, hippurate hydrolysis and resistance to cephalothin). The isolate was evaluated using the MicroScan WalkAway-40 automated system that revealed susceptibility to amoxicillin/clavulanic acid and to gentamicin, but resistance to ciprofloxacin and erythromycin. Considering these results and the good evolution of the patient, the empirical treatment was maintained for 8 days, time at which he recovered completely with no more diarrhoea episodes. The isolate was sent to the Unit of Microbiology at the University *Rovira i Virgili* for re-identification using the sequences of the *rpoB,* as was done routinely for all isolates identified as *Campylobacter* at the hospital. The DNA extraction, amplification and sequencing were performed using primers and conditions previously described (15). A BlastN analysis with the obtained *rpoB* sequence revealed a 99% similarity with the strain of *A. cryaerophilus* (1B, LMG 10229, Accession number EU669900), followed by a 95% similarity with the sequence of the type strain of *A. cryaerophilus* (1A, LMG 9904^{T}, Accession number EU669899), and only a 90% with a strain of the next most similar species, *A. butzleri* (ED-1, Accession number AP012047). Therefore, the isolate was identified as belonging to *A. cryaerophilus.*

Considering the rarity of the recovered microbe, the patient was contacted again in order to request him additional information. He indicated that he regularly eats raw meat and fish, and also had a dog at home and a group of laying hens fenced in the garden. Despite the patient indicating that he had not had any other episodes of diarrhoea, a new stool sample was taken to evaluate his possible carrier state. . Rectal samples from the dog and from 3 of the 6 laying hens he had at that time, as well as 2 samples of their faeces collected from the ground were taken for microbiological examination using molecular detection and culture, as described in previous studies (16). However, all samples studied yielded negative results for *Arcobacter*.

To our knowledge, only 3 cases of *A. cryaerophilus* infection have so far been reported (6, 17, 18). Those cases, together with the few available for the other species of the genus, are summarised in Table 1 and 2. The first and only case of diarrhoea due to *A. cryaerophilus* dates back to 1988 when it was still included in the genus *Campylobacter* with the name *Campylobacter cryaerophila.* The involved 35 year-old homosexual man showed intermittent diarrhoea of 4 to 6 months with abdominal pain (17). The other two are cases of bacteraemia, one in Taiwan that involved an immunocompromised 72-year-old uremic woman who showed an haematogenous pneumonia (18), and the other in a 7-year-old boy from China who had fallen into a mud pool while he was driving a mini motorcycle and suffocated (6) (Table 1). As seen in Table 1 and 2, a few other cases of diarrhoea and bacteraemia have been linked to *Arcobacter butzleri* (4, 7, 19-23) and, more rarely, to *Arcobacter skirrowii* (24). For instance, a case of acute diarrhoea caused by *A. butzleri* in a 30 year-old healthy man was reported from Turkey; it was cured with treatment with

ciprofloxacin (23). A recent case of peritonitis has been reported due to *Arcobacter* sp. in a peritoneal dialysis patient whose catheter was repositioned (25). Despite intravenous cefazolin and oral levofloxacin being given as a prophylaxis, the patient only responded after intravenous ticarcillin–clavulanate treatment for 2 weeks, with no need for the catheter to be removed.

The majority of those case studies underline the difficulty in recognising or identifying these bacteria because they grew slowly and their identification required sequencing of the 16S rRNA gene (6) or the use of specific m-PCR methods (22, 26). Considering that several hospitals use nowadays the MALDI-TOF identification technique for such fastidious, slow growing microbes, we have re-identified our *A. cryaerophilus* isolate using that method (27). The isolate was studied with the Ultraflex TOF/TOF MALDI-TOF instrument, that uses the MALDI Biotyper 2.0 software (Bruker Daltonics Bremen, Germany) after spotting directly a fresh colony in the target plate and the addition of 1 µl of the matrix (Cinaminic acid; CHCA) as described by the manufacturer. The type strain of *A. cryaerophilus* (LMG 9904^T) was used in parallel as a control. The MALDI Biotyper output for our clinical strain scored 1.493 with the strain *A. cryaerophilus* T277 CPB. A score of <1.7 normally indicates an unconfident identification, between 1.7 and 1.99 indicates a genus-level identification, and a score ≥ 2 indicates a species level identification. The second higher score was only 1.42 with a strain of the species *Pseudomonas proteolytica*. Despite the unconfident identification the first match was with an *A. cryaerophilus* and among the following bacteria listed there was no *Campylobacter* spp. The type strain of *A. cryaerophilus* (LMG 9904^T) used as control was correctly identified despite it also showing a low score (1.885). Clinicians should be aware that in the case of a strain showing this behaviour with MALDI-TOF, it is worth confirming its identity by sequencing the *rpoB* gen, so that the true incidence of these bacteria can be established. The inconclusive results obtained with MALDI-TOF could be explained by the fact that a correct identification with this method depends on the number of bacteria strains included in the database (27). The Biotyper database has only 13 *Arcobacter* strains and only 4 of them belong to the species *A. cryaerophilus.* However, it has been recently demonstrated the capacity of this method to separate strains belonging to all *Arcobacter* spp. (2, 28), therefore, it is possible that the inclusion of more strains in the Biotyper database will allow their correct identification, and this certainly will contribute to clarify the clinical importance of this genus.

The seven housekeeping genes (*aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm* and *tkt*) included in the *Arcobacter* MLST database created by Miller *et al*. (29), were sequenced from our isolate of *A. cryaerophilus* using the primers described by this authors. New alleles were being obtained for 4 genes (i.e. aspA-215, atpA-152, gltA-149 and glyA-473), while the sequences of the *glnA* (codifying for glutamine synthetase), *pgm* (phosphoglucomutase) and *tkt* (transketolase) genes corresponded to the already known alleles 59, 133 and 115,

respectively. Therefore this new clinical isolate (strain 609) belonged to a new sequence type (ST) named ST-392. Interestingly, the allele glnA-59 and the pgm-133 had been obtained from the strain 276, which was isolated from faeces of a patient with gastroenteritis in France in 2004, while the allele tkt-115 had been obtained from strain 305, which was also obtained from faeces of a patient with gastroenteritis in the USA in 2009. Apart from strains 276 and 305, the database includes only two other isolates of *A. cryaerophilus* recovered from human samples, i.e. strains 281 (from gastroenteritis) and 285 (from human blood), and both isolates share the ST-201. The few available human pathogenic strains in the database do not allow establishing a relationship between the presence of certain alleles, or STs, and virulence. Therefore, it is important that more strains isolated from human infections are included in the database as we did. In the tree constructed with the concatenated sequences of the 7 genes from each of the 75 *A. cryaerophilus* strains that are currently included in the database (Figure 1), our strain grouped with two others obtained in Europe from poultry (strain 346, ST-290 and strain 325, ST-268), being those therefore the most related strains. However, whether the *A. cryaerophilus* strain was acquired in our patient by the consumption of poultry meat, could not be demonstrated.

Five putative *Arcobacter* virulence genes (*ciaB, cadF, cj1349, hecA* and irgA) were also searched for in our strain using the primers designed by Douidah *et al*. (31). However, only the presence of the *ciaB* gene was detected, which encodes for an invasion protein in *C. jejuni.* The same result was obtained for 5 strains of *A. cryaerophilus* recovered from shellfish and from animal faeces in our laboratory (30). In the study of Douidah *et al*. (31), that included 99 *A. cryaerophilus* strains isolated from human, chicken, pig, cattle, sheep, horse and dog, the *ciaB* gene was present in the majority (92.9%) of the strains, followed by the *cj1349* (51.5%), the *cadF* (34.3%), the *hecA* (4%) and the *irgA* (3%).

Among the faecal samples at the *Hospital Sant Joan de Reus*, where the isolate of *A. cryaerophilus* was studied, *Campylobacter* was the most commonly isolated enteropathogen, representing 41.4% (65/157) of the positive stools in the last year, followed by *Samonella* (36.3%), *Aeromonas* (14.6%), *Shigella* (4.4%), *Hafnia alvei* (3.2%), and *Yersinia* (1.3%). Among the *Campylobacer*-like organisms, *C. jejuni* was the most prevalent species (82.7%), followed by *Campylobacter coli* (16.4%), while *A. cryaerophilus* was in 3rd place (0.9%), which agrees with previous studies (13, 14).

A summarized revision of studies on *Arcobacter,* including those comparing diarrheic and non diarrheic subjects, is provided in Table 3. The prevalence of *Arcobacter* species in human stools ranged from 0.1% to 1.25% in studies that derived the information from culturing, while the detection from faeces by PCR ranged from 0.4% to 13% (9, 10, 12-14, 32-37). In one study, performed in Belgium and France where the prevalence was determined by culture (13), *A. butzleri* occupied the 4th place (3.5%) among *Campylobacter*-like organisms, while *A. cryaerophilus* occupied the 7th place (0.5%). In another study

performed in South Africa using m-PCR detection, *A. butzleri* showed a higher prevalence (6.2%) after *C. jejuni* (10.2%) (10). In the same study, *A. cryaerophilus* and *A. skirrowii* showed lower incidences (2.8% and 1.9%, respectively). In other two studies that detected *Arcobacter* using the same mPCR method a higher incidence was also observed (12, 38). One of them was a case control study of faeces from diabetic patients in Italy (38). In that study it was reported a 78.9% carriage in non diarrheic faeces of type 2 diabetic patients, versus the 26.2% found for the controls (non diabetics non diarrhoea subjects). In the other one, an 8.0% incidence was reported among US/European travellers who suffered acute diarrhoea while visiting Mexico, Guatemala and India (12). Other recent studies compared the ability to detect *Arcobacter* using in parallel molecular and culture methods (36, 37). In one, Collado *et al*. (36) detected the species *A. butzleri* in 1.4% of stool samples of patients with diarrhoea, using a genus specific PCR and a species specific m-PCR method, while it was isolated from only 0.7% of samples by culture. In the other study, de Boer *et al.* (37) developed a multiplex Real Time PCR (RT-PCR) able to detect *A. butzleri* and campylobacters from faeces of patients with diarrhoea; testing this method in parallel with culture. Using this method, *A. butzleri* was detected in 0.4% of samples while it was not recovered by culture. The higher prevalence obtained using molecular methods supports the statement that *Arcobacter* spp. could be underestimated as enteropathogens because of limitations in the current culturing methods, and demonstrates the importance of routinely screening stool samples for the species of this genus using molecular methods in parallel.

The isolation in our patient of *A. cryaerophilus* in the absence of other enteropathogens and the remission of the diarrhoea symptoms after treatment with amoxicillin/clavulanic acid to which the bacteria was sensitive seems to indicate that this bacterium could be considered the etiological agent of the diarrhoea process. Despite not being able to find the contagious source of *Arcobacter* in the environment of our patient, we were able to speculate that it could have been acquired through the consumption of poorly cooked poultry meat or fish. Interestingly, the recurrent episodes and the abdominal pain seem to be a typical clinical presentation for these bacteria.

Acknowledgements

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Conflict of interest

None declared.

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SANITARY IMPORTANCE OF ARCOBACTE
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Table 1. Cases of intestinal infections associated with *Arcobacter*

Patients' sex / age	Country	Presentation	Species	Outcome	Underlying conditions	Ref.
M/35y	Australia	Chronic diarrhoea (6 months)	*A. cryaerophilus*[a]	NS	Homosexual with history of anxiety and repeated sexual exposure.	17
3y - 7y[b]	Italy	No diarrhoea, abdominal pain, occasional vomiting or fever	*A. butzleri*	Recovered 7 to 10 days after no specific treatment.	None	4
1. M/48y 2. F/52y	Germany	1. Acute watery diarrhoea (15 days) and abdominal cramps 2. Chronic diarrhoea (3 weeks) and abdominal cramps	*A. butzleri*	1. Recovered 3 days after treatment with ofloxacin 2. Recovered 2 days after treatment with doxycycline	1.T1 Diabetes mellitus 2. hyperuricemia and alcohol abuse	19
1. M/2y 2. F/1y	Chile	1. Acute mucous diarrhoea and vomiting 2. Chronic diarrhoea (4 months) with abdominal cramps and pain	*A. butzleri*	1. Recovered in 2 days with parenteral fluid therary, restricted diet but without antimicrobial treatment 2. Recovered 10 days after treatment with erythromycin	None	22
M/73y	Belgium	Chronic diarrhoea (2 months)	*A. skirrowii*	Recovered 10 days after no specific treatment.	Prosthetic aortic heart valve	24
M/30y	Turkey	Acute watery diarrhoea, abdominal pain, nausea and sweating	*A. butzleri*	Recovered 2 days after treatment with ciprofloxacin	None	23
M/26y	Spain	Persistent bloody and watery diarrhoea (3 weeks)	*A. cryaerophilus*	Recovered 8 days after treatment with amoxicillin/clavulanic acid	Acute gastroenteritis 4 months earlier	Current study

NS, not specified. [a]Originally described as *Campylobacter cryaerophila*. [b]4 males and 6 females between 3 and 7 years old.

Table 2. Extra intestinal infections associated with *Arcobacter*

Patients sex / age	Country	Presentation	Species	Outcome	Underlying disease	Ref.
Neonate	UK	Bacteraemia with hypotension, hypothermia and hypoglycaemia	*A. butzleri*	Recovered 6 days after penicillin and cefotaxime treatment	Mother had prenatal bleeding due to placenta praevia. Delivery at 26th week	20
M/72y	Taiwan	Bacteraemia and haematogenous pneumonia	*A. cryaerophilus*	Recovered 2 weeks after ceftizoxime and tobramycin treatment	Chronic renal failure, hemodialysis with arteriovenous (AV) fistula. Two months of fever and progressive cough with purulent sputum. She also had a 1-month history of anorexia and frequent loose stool 2 months before admission.	18
M/60y	Taiwan	Bacteraemia with fever and hematemesis	*A. butzleri*	Recovered 4 days after cefuroxime treatment	Chronic hepatitis B carrier, liver cirrhosis.	21
F/69y	Hong Kong	Bacteraemia with fever and lower quadrant pain	*A. butzleri*	Recovered 3 days after cefuroxime and metronidazole treatment	Gangrenous appendicitis	7
F/63y	China	Peritonitis after repositioning of catheter with fever and abdominal pain	*Arcobacter* sp.	Recovered 2 weeks after treatment with Ticarcillin-Clavulanate.	End stage renal-failure of unknown causes	25

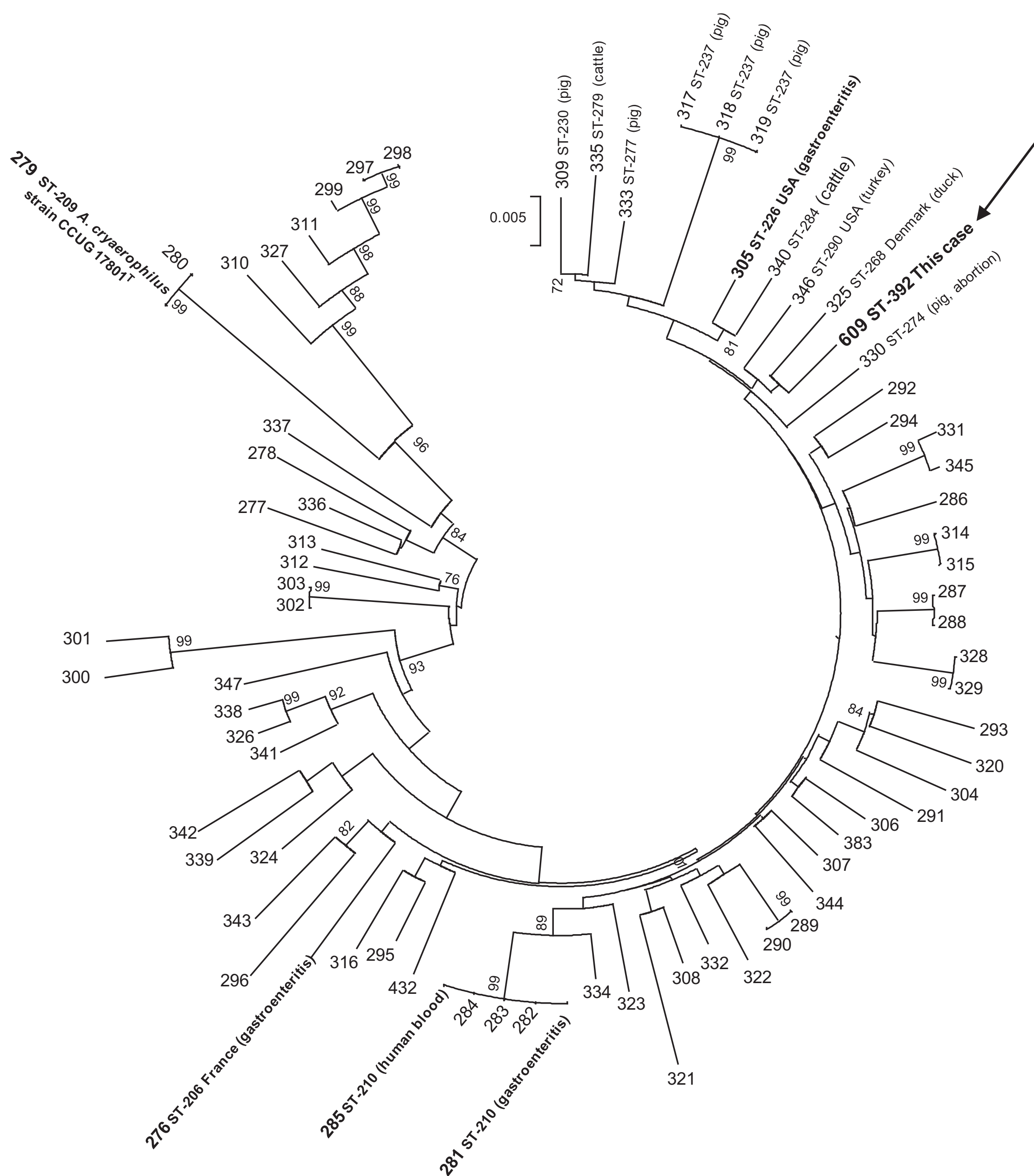
NS, not specified

Table 3. Characteristics of the patients from different diarrhoea surveys in which *Arcobacter* was detected by culture or PCR based methods

No. Patients	Country	Age (range or mean)	M/F ratio	*Arcobacter* prevalence (%) among diarrhoea patients		Type of diarrhoea and symptoms (%)							Monomicrobial infection (%)	Underlying disease (%)	Antimicrobial treatment (%)	Relapse (%)	Asymptomatic (%)	References
				Culture	PCR	Acute	Chronic	Watery	Blood	Nausea / Vomiting	Abdominal pain	Fever >38ºC						
19,535	South Africa	Paediatric (NS)	NS	0.4	ND	100	0	NS	NS	NS	NS	NS	NS	NS	NS	NS	49.7	34
67,599	Belgium and France	30d – 90y	NS	0.11	ND	50.8	16.4	50.8	6.0	27.9	29.5	32.8	82.0	16.4	26.2	6.6	19.7	13
2855[a]	France	54y	1.44	1.0	ND	59.0	3.4	NS	21.1	10.5	57.9	26.3	93.3	15.8	26.3	5.3	NS	14
322	South Africa	1m – 88y	0.77	ND	13.0	47.8	NS	NS	3.1	NS	NS	NS	83.1	13.7	NS	NS	20.8	10
400[b]	India	≥18y (NS)	1.4	1.25	ND	100	0	NS	NS	NS	NS	NS	NS	50.0	NS	NS	0	9
345	France	41.4y	0.56	0	1.2	100	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0	35
201[c]	US/Europe	NS	NS	ND	8.0	100	NS	NS	NS	NS	NS	NS	78.6	NS	NS	NS	0	12
1,380	New Zealand	49y	1.0	0.9	ND	100	0	NS	NS	8.3	NS	NS	75.0	NS	NS	NS	0	32
3,287	Turkey	26.6y	1.25	0.3	ND	100	0	33.3	0	22.2	100	11.1	NS	NS	NS	NS	0	33
140	Chile	<5 y ->50y	0.96	0.7	1.4	100	0	NS	NS	NS	NS	NS	50.0	NS	NS	NS	45.3	36
493	The Netherlands	35y	0.88	0	0.4	100	NS	NS	NS	NS	NS	NS	0.4	NS	NS	NS	NS	37

NS, not specified. ND, not done. [a]Surveillance study including only patients with diarrhoea due to *Campylobacter* like-microorganisms. [b]Case control study that included 200 HIV-1 infected patients and 200 non infected controls. [c]US or European travellers that suffered acute diarrhoea after returning from México, Guatemala or India.

Figure 1. Neighbour joining tree based on the concatenated sequences of *aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm* and *tkt* (3339 bp) showing the position of strain 609 (ST-392) among the 75 strains of *A. cryaerophilus* included in the *Arcobacter*-MLST database. Bootstrap values (≥70%) based on 1000 replications are shown at the nodes



5. GENERAL DISCUSSION

5.1. Description of new *Arcobacter* species and complementary tools for their characterization

As commented in the Interest and Objectives section, our first objective was to characterize one isolate recovered from shellfish (F4) and one from pork meat (F41), which were both recognized in a previous study as representing two potentially new species on the basis of their new and distinctive 16S rRNA-RFLP patterns and their 16S rRNA gene phylogeny (Collado *et al*., 2008). They had not been characterized up to that point because the “ad hoc committee for the re-evaluation of the species definition in bacteriology” (CSDB) recommended that a species description be based on more than a single strain (Stackebrandt *et al*., 2002; Figueras *et al*., 2011a), and no other strains had so far been isolated. Among the 594 isolates recovered from shellfish and sewage during the course of this thesis 8 other strains could also be new species on the basis of the same above mentioned criteria (see Table 5.1). However, none of them were similar to the pork meat isolate (F41), which we proposed as a new species A*. suis* in the same study as another species isolated from sewage and shellfish that we named *A. cloacae* **(Levican *et al*., 2013, study 4.3)**. Nevertheless, two strains from mussels (F118-2 and F118-4) yielded the same RFLP pattern as strain F4 and seem to belong to the same phylogenetic line (Figure 5.1). We have verified this using a polyphasic approach that included genotyping by ERIC-PCR, a phylogenetic analysis derived from the sequences of 3 housekeeping genes (*rpo*B*, gyrB* and *hsp60*) and a phenotypic characterization. In this case, the DDH experiments were not necessary because the similarity of the 16S rRNA gene sequence with its closest species was 94.8% (Table 5.1) and so below the threshold of 97% established by the CSDB to require these experiments. Using the same polyphasic approach in the same study, another new species, *A. venerupis,* was also described, but DDH was necessary in this case because the similarity of the 16S rRNA gene with the closest existing species was above 97% (Table 5.1). That latter species and another, *A. suis,* were in fact described from only one isolate but, as has been discussed in Figueras *et al.* (2011a), discovering a number of isolates of a new species can often depend on luck rather than something that can be controlled. What can be controlled, however, are the methodologies used to define accurately a new species. If a new species is well defined, it will almost certainly be recognized by other investigators in future studies. For instance, this has been the case of the species *A. ellisii, A. suis* and *A. venerupis,* which have been recognized from wash water in a spinach processing plant in Germany by constructing 16S rRNA gene clone libraries (Hausdorf *et al*., 2013). This was possible because of their description in the present thesis It is important to highlight that the 5 new species recognized in the present thesis in three studies **(4.1 - 4.3)**, were initially discovered thanks to the methodologies used to identify the isolates, i.e. the m-PCR (Houf *et al*., 2000) and the 16S

rRNA-RFLP (Figueras *et al*., 2008). The differing results between the methods and the new RFLP patterns obtained motivated the use of *rpoB* sequencing as a confirming technique, as indicated in previous studies (Collado & Figueras, 2011 and references therein), these five new species increased the number in the genus to 17. However, the most important contribution is the inclusion of two new tools for species characterization, the MALDI-TOF and the MLPA. Both methods were validated using all the species of the genus, the results from which agreed with the currently known taxonomy of this genus and made our polyphasic approach more robust.

MALDI -TOF

The analysis of the MALDI-TOF spectra obtained differentiated all known *Arcobacter* species as well as those characterized for the first time in this thesis, both, using only the type strains (**Figueras *et al*., 2011, study 4.1)** or a set 42 strains that included the type and 1 or 2 representative strains, if available, of each species. In this instance, all strains clustered by species and could be clearly differentiated **(Figueras *et al*., 2011, study 4.1; Levican *et al*., 2012, study 4.2 and Levican *et al*., 2013, study 4.3)**. One study had previously assessed the usefulness of the MALDI-TOF for *Arcobacter* spp., but there were only 8 strains of 3 species tested, i.e. *A. butzleri* (n=6), *A cryaerophilus* (n=1) and *A. skirrowii* (n=1) (Alispahic *et al.,* 2010). The method was reported as fast and reliable for differentiating *Arcobacter* from *Campylobacter,* being suitable for large scale research and clinical diagnostics (Alispahic *et al.,* 2010). Contrary to our study, Alispahic *et al.* (2010) did not construct a dendrogram but carried out the MALDI-TOF analysis using the Bruker Biotyper database (Bruker Daltonics, Bremen, Germany) designed to identify unknown isolates by comparing their MALDI-TOF profiles with those of known strains included in the database. The Bruker Biotyper and the SARAMIS databases (Anagnostec, Potsdam-Golm, Germany) are the most commonly used and have been introduced successfully in routine clinical microbiological diagnostics and in other fields of microbiology (Welker & Moore, 2011). The use of these databases has some drawbacks, however. Firstly, they are not transferable so can only be used with their own equipment, and secondly, obtaining a reliable identification depends on which bacteria strains are included in the database. For example, the Biotyper includes only 13 strains of *Arcobacter* spp. (4 strains of *A. butzleri,* 4 of *A. cryaerophilus*, 2 of *A. skirrowii* and 1 each of *A. nitrofigilis, A. halophilus* and *A. cibarius*). Therefore, many species have not yet been included in the database.

Table 5.1. Origin of the 5 new species and the results of the molecular methods used in their definition

New species	Strains	Origin	Study	Similarities of the 16S rRNA gene[a]	DDH results (% ± SD)	Other genes	MALDI-TOF[b]	Identification with 3 methods: 16S rRNA-RFLP[c]	m-PCR (2000)[d]	m-PCR (2010)[e]
A. ellisii	F79-6^T F79-2 F79-7	Mussels	**4.1**	99.1% *A. defluvii* CECT 7697^T	53.0±3.0	*rpoB* *gyrB* *hsp60*	X	New pattern 615/138/92/52/49	*A. cryaerophilus*	*A. butzleri*[f]
A. bivalviorum	F4^T F118-2 F118-4	Mussels	**4.2**	94.8% *A. defluvii* CECT 7697^T	NR	*rpoB* *gyrB* *hsp60*	XXX	New pattern 442/269/138/52	*A. cryaerophilus*	NA
A. venerupis	F67-11^T	Clams	**4.2**	97.1% *A. defluvii* CECT 7697^T 97.0% *A. ellisii* F79-6^T	56.6±4.5 63.4±1.5	*rpoB* *gyrB* *hsp60*	XXX	*A. marinus* 308/243/141/138/99/52[g]	*A. cryaerophilus*	*A. butzleri*
A. suis	F41^T	Pork meat	**4.3**	98.9% *A. defluvii* CECT 7697^T 98.8% *A. ellisii* F79-6^T 98.6% *A. cloacae* SW28-13^T 97.6% *A. venerupis* F67-11^T	55.9±1.0 66.0±1.6 59.0±4.2 58.6±2.9	*rpoB* *gyrB* *hsp60* *gyrA* *atpA*	XX	*A. defluvii*	*A. cryaerophilus*	*A. butzleri*
A. cloacae	SW28-13^T F26	Sewage Mussels	**4.3**	99.6 *A. ellisii* F79-6^T 99.1% *A. defluvii* CECT 7697^T 98.6% *A. suis* F41^T 97.0% *A. venerupis* F67-11^T	64.4±2.2 49.5±6.7 58.6±2.9 57.4±4.9	*rpoB* *gyrB* *hsp60* *gyrA* *atpA*	XX	New pattern 372/243/138/92/52/49	*A. cryaerophilus*	NA

SD, Standard deviation. NR, not required according to the criteria of CSDB criteria that establish the need for carrying out DDH experiments when the 16S rRNA gene similarity is ≥97% (Stackebrandt *et al.,* 2002). NA, no amplification. [a]Similarity with the type strain of the closest species or with the species with which DDH experiments were carried out. [b]MALDI-TOF experiments were carried out with a different number of strains depending on the study: X, means that only the type strains of each *Arcobacter* spp. were included in the analysis; XX, indicates that apart from the types another representative of each species (if available) were analysed and XXX, that apart from the types two other representative strains of each species (if available) were studied. [c]Numbers indicate the size in bp of the band obtained after digestion with the *Tru*I enzyme using the 16S rRNA-RFLP method of Figueras *et al.* (2008). [d]m-PCR method of Houf *et al.* (2000). [e]m-PCR method of Douidah *et al.* (2010). [f]Two strains (F79-2 and F79-6^T) also produced a less intense band similar to that expected for *A. cryaerophilus.* [g]This pattern is in practice indistinguishable from that of *A. marinus* (308/243/139/138/99/52).

Considering, as mentioned, that the MALDI-TOF system is used in different hospitals to characterize bacteria which are difficult to be identified with other methods (Welker & Moore, 2011) we attempted to identify with the Bruker MALDI Biotyper the clinical strain of *A. cryaerophilus* using the type strain of this species, LMG 9904^T, as control **(Figueras *et al.*, submitted, study 4.9).** The identification of the type strain showed that the two closest strains in the Biotyper database belonged indeed to the species *A. cryaerophilus* (strains T277 CPB and V441 CPB with scores 1.88 and 1.77, respectively). According to the system a score between 1.70 and 1.99 indicates a genus-level identification and <1.70 normally indicates an unconfident identification. In accordance with this, the type strain was assigned correctly to the genus. The identification of the clinical strain, however, was inconclusive, because the obtained score was only 1.49 for the closest relative *A. cryaerophilus* (strain T277 CPB). Furthermore the following closest strain belonged to another genus (*Pseudomonas proteolytica* score 1.42). The low scores are probably due to the small number of *Arcobacter* strains of this species being included in the database. In the present thesis, we have demonstrated the ability of this method to correctly separate strains belonging to all species of this genus if a complete database is used **(Levican *et al.*, 2012, study 4.2)**. Considering the availability of this technology in many hospitals, the inclusion of more strains of all species of *Arcobacter* into the Bruker MALDI Biotyper database, under the conditions described by the manufacturer, will contribute to a more accurate and successful identification of arcobacters and will enable their clinical importance to be clarified.

MLPA

This tool involves simultaneous analysis of 5 genes (Table 5.1), as recommended (Stackebrandt *et al*., 2002; Figueras *et al*., 2011a) and was applied for the first time in this genus to delineate the 2 new species *A. suis* and *A. cloacae* **(Levican *et al.*, 2013, study 4.3)**. Three of the 5 genes (*rpoB, gyrB, hsp60*) were already been used in previous descriptions of *Arcobacter* spp. (Collado *et al.,* 2009a; Figueras *et al.*, 2011b; De Smet *et al.*, 2011a), therefore, in this work we designed primers for the amplification of the other two selected genes, *gyrA* and *atpA*. We also designed new primers for *rpoB* and *hsp60* because with those available we experienced some problems in obtaining good amplification, for example the presence of other bands apart from those expected or weak amplification (data not shown). Those problems were probably due to the fact that these primers were not specifically designed for the genus *Arcobacter* (Korczack *et al*., 2006; Hill *et al*., 2006). The new primers were effective in that they were able to amplify the targeted genes in all studied strains representing all species. The

phylogenetic tree, constructed using the concatenated sequences of these 5 genes, agreed with the 16S rRNA gene and DDH results and enabled delineation of all *Arcobacter* spp. **(Levican *et al.*, 2013, study 4.3)**. Furthermore, as observed in other genera (Figueras *et al.*, 2011a), the MLPA had a better resolution and the phylogenetic relatedness was more robust (high bootstrap values) than in the 16S rRNA gene phylogenetic tree. In conclusion, the proposed MLPA proved to be an alternative method to the DDH and the 16S rRNA gene for differentiating *Arcobacter* species.

The use of both tools (MALDI-TOF and MLPA) in future studies will probably help to clarify taxonomic issues of this genus that remain, such as whether the two subgroups of *A. cryaerophilus* (1 and 2) belong to separate taxa, as suggested by Debruyne *et al*. (2010) on the basis of AFLP and *hsp60* gene analyses. Those authors observed that the two subgroups are heterogeneous, for instance, the average of DDH values in group 1 was 60% while in group 2, 73%. Moreover, the AFLP and *hsp60* analyses revealed that the majority of subgroup 2 strains grouped together according to their DDH values, while, the subgroup 1 strains formed at least 3 separate groups, with DDH values ranging from 48% to 67% (Debruyne *et al*., 2010). The authors concluded that the two *A. cryaerophilus* subgroups should be abandoned and indicated that the current type strain of the species should be substituted by strain LMG 10829 that represents the species better.

Another group formed by the species *A. bivalviorum, A. halophilus, A. marinus, A. molluscorum* and *A. mytili* also needs its taxonomic position reviewed. These species cluster together both in phylogenetic trees and in the MALDI-TOF dendrogram **(Levican *et al*., 2013, study 4.3)**. All of them have been found in marine environments and tolerate growth in 4% NaCl. They have also shown 16S rRNA similarities with the other species, ranging from 91.1% (*A. bivalviorum* with *A. cryaerophilus*) to 95.3% (*A. molluscorum* with *A. venerupis*). Therefore, their taxonomic position needs to be assessed because it has been stated that a taxa with similarity values of the 16S rRNA gene sequence below 95% taxa should be tested by other methods to establish whether separate genera are present (Tindall *et al.,* 2010).

Regarding genotyping methods, their inclusion in the description of the new species is recommended because they are useful for identification at subspecies levels and for demonstrating whether or not the isolates of a new taxon are members of a clone (Tindall *et al*., 2010). Following this recommendation, in the three **studies (4.1-4.3**) in which we described the new species we included the ERIC-PCR genotyping method that was validated for *Arcobacter* by Houf *et al.* (2002), and that it is currently the most used method for this genus (Collado & Figueras, 2011). However, in the **study 4.2** we reported for the first time a changing ERIC

pattern in repetitive experiments in one isolate of *A. bivalviorum* (F118-3), which originally showed a band of a different size to the isolate F118-2. This is in fact the established criterion for separating strains with the ERIC method (Houf *et al.*, 2002). Both isolates (F118-2 and F118-3) had identical *rpoB, gyrB* and *hsp60* sequences, therefore the ERIC-PCR experiment was repeated and then the two isolates showed the same pattern (**Levican *et al.*, 2012, study 4.2**). The reliability of ERIC-PCR method has been questioned by Merga *et al.* (2013). These authors searched for the complementary sequences to the ERIC primers in two *A. butzleri* genomes (strain RM4018 and strain 7h1h) but they were not able to find them. The authors indicate that the low annealing temperature used in the ERIC-PCR reaction (25ºC) allows non-specific binding of primers to other regions, thus producing the observed ERIC patterns randomly. However, no experimental confirmation was carried out in that study, such as the sequencing of the ERIC amplicons in order to verify their hypothesis. An alternative method for *Arcobacter* genotyping is the MLST (Miller *et al.*, 2009), which includes 7 genes for which primers are publicly available as well as a database of the so far defined sequence types (www.pubMLST.org). This scheme has some advantages over other PCR-based methods (ERIC-PCR, RAPD-PCR, PFGE, etc) such as accuracy, reproducibility and the possibility of creating worldwide databases (Urwin & Maiden, 2003). The currently available scheme (Miller *et al.*, 2009) includes only 5 of the 17 species of the genus (*A. butzleri, A. cryaerophilus, A. skirrowii, A. cibarius* and *A. thereius*) and different sets of primers should be used for amplification, depending on the species. Furthermore, Merga *et al.* (2013) investigated the diversity of *Arcobacter* by determining the MLST of 514 isolates taken from faecal samples of cattle but they were only able to obtain sequences from 20.2% (104/514) of the isolates. The sequences of the remaining 79.8% (410/514) isolates were poor quality and not useful (Merga *et al.,* 2013). The authors concluded that the results were probably due to the presence of isolates belonging to species that are not included in the MLST scheme, for which the published primers are not useful. Therefore, new primers need to be designed for sequences of all strains of all species, so that the MLST scheme could be useful for the whole genus. The MLPA scheme that we propose enables amplification of the 5 genes from strains of all species of the genus and has also proven to be able to discriminate whether or not the isolates belong to the same clone. Future studies need to determine the best genotyping method for *Arcobacter* spp. and to evaluate whether our MLPA scheme could be one of them.

Regarding 16S rRNA gene similarity, 4 of the new species we describe in this thesis (*A. ellisii, A. venerupis, A. cloacae, A. suis*) show values of between 97.0% and 99.6% with their closest species (Table 5.1). In fact, *A. cloacae* and *A. ellisii* have the highest similarity value ever

observed for this genus (99.6%). Other species have also previously shown similarities above 97%, for instance a value of 98.9% between *A. cryaerophilus* and *A. cibarius,* 97.6% between both *A. molluscorum* and *A. marinus* (Collado & Figueras, 2011). De Smet *et al*. (2011a) also observed high similarity values between *A. trophiarum* and four other species: *A. cryaerophilus* (98.2%), *A. thereius* (98.1%), *A. cibarius* (97.8%) and *A. skirrowii (*97.4%). The 16S rRNA gene similarity is one of the most important parameters for new species descriptions, because if similarity with the closest species is 97% or higher, DDH experiments are required to show if it could indeed be considered a different species (Stackebrandt & Goebel, 1992). However, Stackebrandt & Ebers (2006) proposed a more restrictive 98.7%-99% threshold, which would be more appropriate for the genus *Arcobacter,* as already suggested by Figueras *et al*. (2011b). The 16S rRNA gene is also used for identifying species by comparing them with available sequences held in different databases, using tools such as the BLASTN (NCBI) or the classifier tool of the Ribosomal Database Project (Cole *et al.*, 2008). To identify strains, their sequences need to be of a length higher than 1300 bp with <1% ambiguity with a threshold >99.5% similarity to assign them to a particular species (Janda *et al*., 2007). Our results indicate that most *Arcobacter* species comply with these guidelines. Nevertheless, constructing a 16S rRNA gene phylogenetic tree might be a useful additional tool for those species with the highest similarity values, whose identity can be determined on the basis of the grouping with type strains of known species (Figure 5.1).

Using phylogenetic trees, we have recognized that a sequence of an unnamed strain (R-28314) recovered from activated sludge in a wastewater treatment plant in Ghent (Belgium) grouped with *A. venerupis* F67-11^{T} (Heylen *et al*., 2006; **Levican *et al*., 2012, study 4.2**). Furthermore, an uncultured bacterium (SRWH-BA07) found in Japan, might belong to the new species *A. bivalviorum,* while others (MW-B27 and M17-10- B14) from water-flooded petroleum reservoirs in China and 42 from an industrial anaerobic digester in Mexico potentially belong to *A. cloacae* **(Levican *et al*., 2013, study 4.3)**. Similarly, other sequences from uncultured bacteria obtained from carrot wash water from Germany (ATB-LH-6148 and ATB-LH-5950) and from biodegraded oil in Canada (TS1B220) potentially belong to *A. suis* **(Levican *et al*., 2012, study 4.2)**. A very recent study on the diversity of bacteria communities in wash water from a spinach processing plant in Germany revealed the existence of three of the new species described in the present thesis, i.e. *A. ellisii, A. suis* and *A. venerupis* (Hausdorf *et al*., 2013). All these findings confirm that the described new species are indeed present in other geographical regions and habitats. The data also corroborates our previous assertion that irrespective of the number of strains included in a species description, it is essential that it is initially well defined,

because then other researchers will be able to recognize it, thus enhancing the diversity of the original descriptions (Figueras *et al*., 2011a).

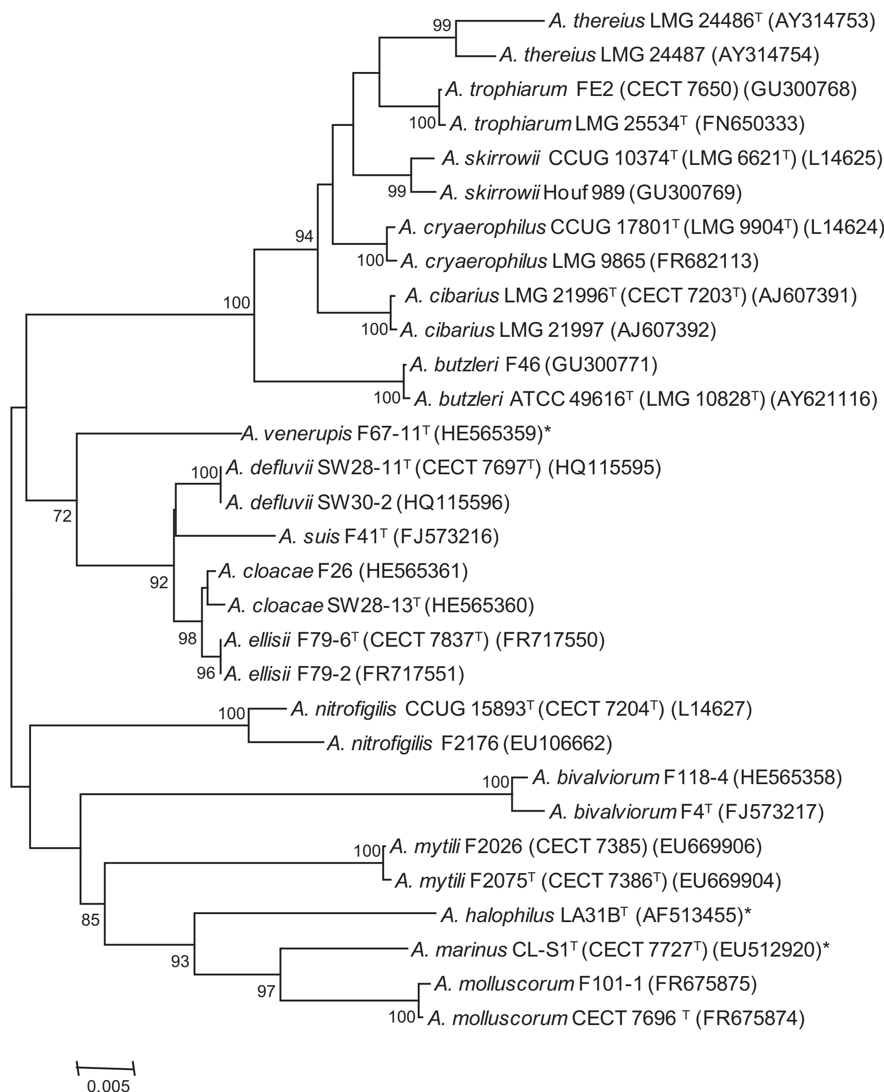


Figure 5.1. Neighbour-joining tree based on 16S rRNA (1401 bp) sequences showing the phylogenetic position of the 17 *Arcobacter* species. Bootstrap values (≥70%) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 1000 nt. * Only the type strain is available so far

In this thesis, a set of 12 atypical *A. cryaerophilus* strains, identified on the basis of *rpoB* sequencing, produced the 16S rRNA-RFLP pattern described for *A. butzleri.* The analysis of the 16S rRNA gene sequences of those strains showed that they have microheterogeneities or mutations i.e. double-sequencing signals in the chromatograms at positions 192 (T→C) and 205

(A→G). These mutations (192, **C**TAA and 205, TTA**G**) affected the cleavage site (TTAA) of the *Mse*I endonuclease generating the typical RFLP pattern of *A. butzleri* that possesses only 3 bands (548, 216 and 138) instead of *A. cryaerophilus* that possesses 4 bands (395, 216, 143 and 138) **(Figueras *et al*., 2012, study 4.4)**. When we analysed the presence of microheterogeneities among the 4 or 5 operon copies of the 16S rRNA gene in the complete four genomes available of *Arcobacter* (Table 1.2), only one of them (*A. nitrofigilis* DSM 7299^T) showed variations in 2 nucleotide positions (190, C→T and 191, T→A) in 2 of the 4 copies. Therefore, microheterogeneity seems common in *Arcobacter;* although, to our knowledge they have so far not been studied in this genus and this requires further investigation. Alperi *et al.* (2008), using a 16S rRNA-RFLP identification method previously validated for the genus *Aeromonas* (Figueras *et al*., 2000), reported the presence of microheterogeneities in 8.1% of the strains of the genus. They observed that microheterogeneity not only generates different RFLP patterns from those expected for a given species but also affects the results of identification based on the phylogenetic position in the 16S rRNA gene tree (Alperi *et al.*, 2008). Mutations in the atypical *A. cryaerophilus* strains that we have analysed did not affect the results of identification based on the phylogenetic analysis of this gene, but, as commented, they produced an identical pattern to that of *A. butzleri* and were confused with the latter species by the RFLP identification method. However, the use in parallel of the Houf *et al*. (2000) m-PCR identification method revealed that these 12 strains showed the characteristic band of *A. cryaerophilus,* and these contradictory results lead us to sequence the *rpoB* gene. These sequences, as we indicated above, confirmed that these strains all belonged to *A. cryaerophilus*.

In relation to the conventional phenotypic testing the new species were differentiated by 3 or more specific reactions from the existing ones (Table 5.2). Among the discovered species, 2 were unable to grow under aerobic conditions at 37ºC, i.e. *A. venerupis* and *A. suis*. However, *A. venerupis* was able to grow at this temperature in microaerphilia. This behaviour was previously observed in *A. cibarius*. Future studies on these two species will need to evaluate the factors that determine their growth behaviour. In relation to *A. suis,* this species was unable to grow at 37ºC either under aerobic and microaerobic conditions. This behaviour has been shown previously by only 2 species, *A. thereius* and *A. trophiarum*. Interestingly, both of those species have been recovered from samples from warm-blooded animals, i.e. aborted foetuses and/or faeces, respectively (Houf *et al.,* 2009; De Smet *et al.*, 2011a). However, we have discovered in the course of this study that strains of *A. thereius, A. trophiarum* and *A. suis* show an ability to adhere to Caco-2 cells at 37ºC **(Levican *et al*., 2013, study 4.8)**. In addition, most of the strains of *A. thereius* and all of *A. trophiarum* were also able to invade those cells at this temperature.

In fact, strains of these species remained viable, and even grew, when incubated for 2 h in Eagle's Minimal Essential Medium (EMEM) at 37ºC, while in Brain Heart Infusion (BHI) they showed a slight decrease in the viable cell counts after incubation. It seems, therefore, that EMEM plus the Caco-2 cells might simulate the natural intestinal habitat better than other culture media, such as BHI, something which should also be assessed in future studies.

Table 5.2. Phenotypic characteristics of all *Arcobacter* species

Characteristics	*A. nitrofigilis*	*A. cryaerophilus*	*A. butzleri*	*A. skirrowii*	*A. cibarius*	*A.halophilus* [a]	*A. mytili*	*A.thereius*	*A. marinus* [a]	*A. trophiarum*	*A. defluvii*	*A. molluscorum*	*A. ellisii*	*A. bivalviorum*	*A. venerupis*	*A. cloacae*	*A. suis*
Growth in/on																	
Air at 37 °C	V(-)	V(+)	+	+	-	+	+	-	+	-	+	+	+	+	-	+	-
CO_2 at 37 ºC	-	V(+)	+	+	+	+	+	-	+	-	+	+	+	+	+	+	-
CO_2 at 42ºC	-	-	V(+)	-	-	-	+	-	-	-	+	+	+	-	-	-	-
0.5% (w/v) NaCl	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+
4% (w/v) NaCl	+	-	-	+	-	+	+	-	+	-	-	+	-	+	-	-	-
1% (w/v) glycine	-	-	-	-	-	+	+	+	+	V(-)	-	-	-	-	-	-	-
0.05% safranin	-	+	+	+	+	-	-	+	+	V(+)	+	+	-	-	-	+	-
0.1% sodium deoxycholate	V(-)	V(+)	+	+	+	-	+	V(-)	-	+	+	+	+[b]	-	-	+	+
1% (w/v) oxgall	-	+	V(+)	+	+	-	+	-	-	+	+	+	-	-	-	+	-
0.04% TTC	-	+	+	V(-)	V(-)	-	-	V(-)	-	+	-	-	-	-	-	-	-
0.01% TTC	-	+	+	+	+	-	-	+	-	+	+	+	-	-	-	+	+
Minimal medium	-	-[c]	+	-	+	-	-	+	-	-[d]	+	-	+	-	+	V(+)	+
MacConkey	-	V(-)	+	-	+	-	+	V(+)	-	V(+)[e]	+	+	V(+)	-	+	+	+
CCDA	-	+	+	+	V(-)	-	-	V(-)	-	+	+	-	+[b]	-	+	+	-
Resistance to:																	
Cefoperazone (64 mg l^{-1})	-	+	+	+	+	-	-	+	-	+	V(+)	+	-	-	-	-	-
Enzyme activity																	
Catalase	+	+	V(+)	+	V(-)	-	+[f]	+	-	+	+[f]	+	+	+	+	+	+
Urease	+	-	-	-	-	-	-	-	-	-	+	-	V(-)	-	+	-	-
Nitrate reduction	+	+[g]	+	+	-	+	+[h]	+	+	-	+	+[i]	+	-	+	+	+
Indoxyl acetate hydrolysis	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+

Data from **study 4.3 (Levican *et al.*, 2013** and references therein)**.** The specific responses for type strains were coincidental or otherwise expressed in brackets. Unless otherwise indicated. +, ≥ 95% strains positive; -, ≤11% strains positive; V, 12-94% strains positive; CO_2 indicates microaerobic conditions. [a]For these strains, testing was carried out on media supplemented with 2% NaCl, with the exception of 0.5 and 4% (w/v) NaCl, catalase and indoxyl acetate hydrolysis. [b]All strains grew weakly after 5 days of incubation. [c]Two (LMG 7537 and LMG 10241) of the four strains tested were positive. [d] Test not evaluated by De Smet *et al.* (2011a) but tested by Figueras *et al.* (2011b). [e]Strains LMG 25534[T], LMG 25535 of *A. trophiarum* and strain FE2 (CECT 7650) of this species identified in our laboratory all grew on MacConkey agar, contrary to 80% of the strains described for this species. [f]Weak reaction. [g]Two (LMG 9904[T] and LMG 9065) of the four strains tested were negative. [h]Nitrate reduction was positive for the 3 strains of *A. mytili*, contrary to our previously published data (Collado *et al.*, 2009a). [i]Nitrate is reduced after 72 h and 5 days for all strains under microaerobic and aerobic conditions, respectively.

One of the limitations of phenotypic characterization is the changing behaviour of bacteria along with a lack of reproducibility of the phenotypic results (Figueras *et al.*, 2011a). For instance, when Figueras *et al.* (2011b) described the species *A. molluscorum* and they re-evaluated all the phenotypic characteristics of all the species, they reported that the species *A. mytili* was able to reduce nitrate, contrary to what it was indicated in its original description (Collado *et al*., 2009a).

During the same study, the available strains of *A. trophiarum* were unable to grow on media containing 4% NaCl, while in the description available at that time in the online version of the paper, it was indicated that all of them were able to grow (Figueras *et al*., 2011a). Those results were communicated to Prof. Houf and properly corrected in the proofs version of the paper (De Smet *et al.,* 2011a; Prof. Houf, personal communication). This lack of reproducibility has been seen before for this genus; it occurs in the most commonly isolated species, *A. butzleri* and *A. cryaerophilus,* for which the only distinctive traits reported in Bergey's Manual of Systematic Bacteriology (Vandamme *et al.*, 2005), i.e. growth on MacConkey or on minimal media, only applies to the type strains, although other strains can give variable results (**Levican *et al*., 2013, study 4.3**). Furthermore, phenotypic testing has other drawbacks, such as the large number of tests and the specialized skills needed to interpret the results (Figueras *et al.*, 2011a). These drawbacks must be considered when formulating future guidelines for new species descriptions. In fact, the existing guidelines on minimal standards for defining new *Arcobacter* species date back to 1994 and are designed for the *Campylobacteraceae* family (Ursing *et al*., 1994). It has been suggested that considering the importance of *Arcobacter*, these guidelines should be revised and updated (Figueras *et al*., 2011a). We consider that this update should include the MLPA and MALDI-TOF, which are useful tools for defining new *Arcobacter* species.

5.2. The need for a reliable molecular identification method

As commented, the five new species described in this thesis, i.e. *A. ellisii; A. bivalviorum, A. venerupis, A. cloacae* and *A. suis,* have increased the number of species of *Arcobacter* to 17. The use of the 16S rRNA-RFLP identification method (Figueras *et al*., 2008; Collado, 2010) revealed new, unknown RFLP patterns for the new species *A. ellisii, A. bivalviorum* and *A. cloacae* (Table 5.1). The RFLP patterns produced by *A. venerupis* and *A. suis* were similar to those previously described for two rarely isolated species *A. marinus* and *A. defluvii,* respectively (Table 5.1). We also found that other species, such as *A. thereius* and *A. trophiarum* and the "atypical" strains of the species *A. cryaerophilus* produced the same RFLP

pattern described for *A. butzleri* (**Figueras *et al*., 2012, study 4.4)**. Moreover, Douidah *et al.* (2010) indicates that the need for polyacrylamide gel electrophoresis in the 16S rRNA-RFLP method proposed by Figueras *et al*. (2008) means the technique is not useful in routine identification because many laboratories use only agarose gels. Therefore, there was a clear need to try to improve and update this RFLP method for all species as described in **study 4.4 (Figueras *et al*., 2012)**. The updated method includes an initial digestion with *Mse*I endonuclease, as originally described, that enabled 10 of the 17 accepted species to be differentiated. The other species that shared common or very similar RFLP patterns would be differentiated with subsequent digestion using the endonucleases *Mnl*I and/or *Bfa*I (**Figueras *et al*., 2012, study 4.4)**.

The original 16S rRNA-RFLP method has been used to identify more than 800 *Arcobacter* strains recovered from meat products, shellfish and water in various studies (Collado & Figueras, 2011; **Levican *et al*., submitted, study 4.6; Levican *et al.*, in preparation, study 4.7**). Furthermore, this method allowed other rare species from new habitats to be identified, such as *A. nitrofigilis* from mussels and *A. thereius* from pork meat and mussels, *A. defluvii* from mussels (Collado *et al*., 2008; **Figueras *et al*., 2012, study 4.4; Levican & Figueras, submitted, study 4.5**). The new 16S rRNA-RFLP protocol was developed in both polyacrylamide and agarose to make it suitable for different laboratories and so far it. is the only method able to recognize simultaneously all the 17 accepted *Arcobacter* species **(Figueras *et al*., 2012, study 4.4)**. Therefore, with this updated protocol other research groups will probably also be able to recognize a broader diversity of species as we did.

As it will be also commented latter on, the importance of *Arcobacter* in human infections is considered to be underestimated probably due to the use of inaccurate detection and identification methods (Collado & Figueras, 2011). Considering this and the increase in the number of species in recent years there seems to exist an urgent need to evaluate the performance of the available identification methods. This is evaluated in this thesis **(study 4.5).** It compares, for the first time, the accuracy of five PCR-based methods used to identify all *Arcobacter* spp. Two of those methods were m-PCRs that target *A. butzleri, A. cryaerophilus* and *A. skirrowii* (Houf *et al*., 2000; Kabeya *et al*., 2003). We have also evaluated a PCR method that targets the latter 3 species together with *A. cibarius* (Pentimalli *et al*., 2008) and another method that, apart from those 4 species, targets *A. thereius* (Douidah *et al*., 2010). The PCR of De Smet *et al*. (2011a) designed for *A. trophiarum* attempted to complement the m-PCR of Douidah *et al*. (2010), so the two methods were considered one for comparative purposes **(Levican & Figueras, submitted, study 4.5).** The results of all those method were compared

with the ones obtained with the 16S rRNA-RFLP of Figueras *et al*. (2008). In general none of the compared methods were found to be able to identify unequivocally all strains included in the study. The least reliable method was the m-PCR of Kabeya *et al*. (2003) because it identified correctly only 32.6% of studied strains. The PCR designed by Pentimalli *et al.* (2008) also performed badly because, despite identifying correctly 83.2%, the remaining strains were confused with the targeted species (*A. butzleri, A. cryaerophilus, A. skirrowii* or *A. cibarius*). The m-PCR of Houf *et al.* (2000) also performed badly, because only 55.8% of strains were correctly identified, although this method appeared to be reliable for identifying *A. butzleri,* as it identified correctly all strains of this species and none of the non-targeted species was misidentified **(Levican & Figueras, submitted, study 4.5)**. From our literature review we observed that the m-PCR of Houf *et al*. (2000) is the most commonly used method, having been used in ca. 64.8% of strains identified in different studies since 2000. Considering our results, we conclude that the widespread use of this method might have led to an overestimation of the species *A. cryaerophilus* and *A. skirrowii*, which, coincidentally, are the most reported species, together with *A. butzleri*. The m-PCR of Douidah *et al.* (2010) and the PCR of De Smet *et al*. (2011a) identified correctly 83.2% of tested strains, producing no amplicons for 10 species and were accurate for 3 (*A. skirrowii, A. thereius* and *A. cibarius*) of the 5 targeted species. However, they misidentified strains belonging to 4 species as *A. butzleri* and 1 as *A. cryaerophilus* **(Levican & Figueras, submitted, study 4.5)**. The other compared method was the 16S rRNA-RFLP method proposed by Figueras *et al*. (2008). This method also identified correctly 83.2% of tested strains, but it was only able to identify correctly 10 of 17 tested species. Our results indicate that the problem with unreliable results among the compared methods could lie with which gene is targeted and which region is used to derive the primers. The least reliable primers were those targeting the 23S rRNA gene, because using this gene the strains of *A. butzleri* were not correctly identified by Kabeya *et al*. (2003), and between 3 and 11 non-targeted species were confused as *A. butzleri, A. cryaerophilus* or *A. skirrowii* or as *A. butzleri* by the m-PCR of Douidah *et al*. (2010). This limitation is probably related to the fact that the sequences of the targeted regions of the 23S rRNA gene are only available for 8 out of the 17 *Arcobacter* spp. we observed in the GenBank so far **(Levican & Figueras, submitted, study 4.5)**.

We consider our results an important contribution, because for the first time they highlight the limitations of methods currently used for *Arcobacter* identification. Our results also support the idea that the diversity of *Arcobacter* spp. in different environments, as it is currently understood, would change if the identification methods applied in future studies were more reliable.

5.3. Exploring the prevalence and diversity of *Arcobacter* species in shellfish and wastewater

The presence of *Arcobacter* spp. in the two types of samples included in studies **4.6 (Levican *et al.*, submitted)** and **4.7 (Levican *et al.*, in preparation)**, i.e. shellfish and wastewater has been poorly studied, even though such a study clearly seems justified from the epidemiological point of view. Shellfish might possibly be a reservoir for *Arcobacter* species (Collado *et al*., 2009b) as 29% of shellfish samples investigated using molecular and culturing methods were positive for this bacteria **(Levican *et al.*, submitted, study 4.6)** and in some previous studies, this was slightly higher. For instance, 33.3% positive samples were found in different types of shellfish purchased in markets and mussels that had been collected directly from the Ebro delta (Collado *et al*., 2009) and 35.0% in mussels collected in Chile (Fernandez *et al*., 2001). Using the ERIC-PCR genotyping method (Houf *et al*., 2002), the present study determines for the first time the genetic diversity (no. of different ERIC-PCR patterns / no. of isolates) shown by 476 isolates obtained from shellfish. The results show that they belonged to 118 strains, i.e. 24.8% of diversity **(Levican *et al.*, submitted, study 4.6)**. These results were comparable to the genetic diversity found in different types of meat. For example, 30% diversity was found in beef meat (Aydin *et al*., 2007), 44.8% in chicken and broiler carcasses (Van Driessche & Houf, 2008), 65% in bovine carcasses (De Smet *et al*., 2010) and between 11.1% and 59.9% in poultry meat (Aydin *et al*., 2007 and references therein). Our most remarkable result is that we found shellfish to be a reservoir of a wide range of *Arcobacter* species. We recovered 11 different species, *A. butzleri* (60.2%) and *A. molluscorum* (21.2%) being the most prevalent. In fact, the species *A. molluscorum*, together with *A. ellisii* and *A. bivalviorum*, were discovered for the first time from these types of samples in the **study 4.6 (Levican *et al.*, submitted)**. We also isolated *A. thereius* (0.8%) and *A. defluvii* (0.8%) among others, this being the first time that they have been recovered from this environment. The large number of species identified has mainly been due to the use of the 16S rRNA-RFLP identification method (Figueras *et al*., 2008; **Figueras *et al.*, 2012, study 4.4**). The predominance of *A. butzleri* (60.2%) in shellfish is especially relevant because this species has been considered as a serious public health concern by the International Commission on Microbiological Specifications for Foods due to its abundant presence in different type of meat products (ICMSF, 2002). Taking into account that mussels and shellfish, unlike meat products, are often eaten poorly cooked or raw, the relevance of *A. butzleri* for public health could even be greater.

We found a positive correlation between the presence of *Arcobacter* in shellfish and the temperature of the water, suggesting a seasonal fluctuation. During the summer, the species mainly recovered were *A. butzleri* and *A. molluscorum* and between January and May, in different years, other species such as *A. cryaerophilus, A. nitrofigilis* and *A. skirrowii* were more abundant **(Levican *et al*., submitted, study 4.6).** Due to the low number of strains of the latter species this tendency needs to be verified in future studies as the number recovered was fairly low. Factors that can affect the prevalence of *Arcobacter* in shellfish also needs further study. For example, it is not known whether clams, which were more positive for *Arcobacter* in our study than other types of shellfish like mussels or oysters, have characteristics that favour the presence of these bacteria. Nor is it known whether the potential virulence of the strains affects the shellfish host. On that point, the **study 4.6 (Levican *et al*., submitted)** is the first to analyse the correlation between *Arcobacter* and environmental parameters and our results might contribute to the design of future studies. Another important factor derived from this study is that the incubation conditions (aerobic and microaerobic) clearly influenced the recovery of *Arcobacter.* We isolated more strains under aerobic conditions, ca. 10% more positive samples, despite the number of different species isolated in both conditions (n=9) being the same. There has only been one previous study by González *et al*. (2007) that assessed the isolation of *Arcobacter* from chicken meat using this two incubation conditions in parallel. These authors reported slightly better isolation in microaerobic conditions, however, results were not statistical different among the 7 positive samples obtained.

The presence of *Arcobacter* is associated with faecal pollution (Collado *et al*., 2008). However, few studies have investigated the presence of *Arcobacter* in wastewater (Stampi *et al*., 1993 and 1999; Moreno *et al.,* 2003; González *et al*., 2007 and 2010; Collado *et al*., 2008 and 2010). The present study has established the prevalence of *Arcobacter* spp. in a WWTP by direct culturing (86.7%) and by post-enrichment (93.3%), confirming this environment as an important reservoir for these bacteria **(Levican *et al*., in preparation, study 4.7)**. Our results show *A. butzleri* (53.4%) as the predominant species, which together with *A. cryaerophilus* (39.6%) represented 93% of the recovered strains, while *A. nitrofigilis* and two new species (*A. defluvii* and *A. cloacae*) were also isolated. However, only the species *A. butzleri* and/or *A. cryaerophilus* were isolated in previous studies using either phenotypical (Stampi *et al*., 1993 and 1999) or molecular (Moreno *et al.,* 2003; González *et al*., 2007 and 2010) methods. The high diversity of species encountered in wastewater was also observed in the study of shellfish, commented above **(Levican *et al*., submitted, study 4.6),** and in both cases we attribute this to the use of the 16S rRNA RFLP identification methods (Figueras *et al*., 2008; **Figueras *et al*.,**

2012, study 4.4) that allow recognition of all the species. The genetic diversity was very high, 80.9% **(Levican *et al*., in preparation, study 4.7),** which is similar to that previously found in these kinds of samples by other authors, i.e. 91% (Collado *et al*., 2010) and 100% (González *et al*., 2010). It has been suggested that this high genetic diversity is due to the fact that *Arcobacter* might come from many different sources and/or might be a consequence of genomic rearrangement (González *et al*., 2010; Collado *et al*., 2010). A similar number of strains was obtained by direct (n=71) and post enrichment culturing (n=73) but the predominant species recovered under the two conditions were different. Direct culturing mainly isolated *A. cryaerophilus* while post enrichment mainly isolated *A. butzleri* **(Levican *et al*., in preparation, study 4.7)**. The incidence of the species also varied depending on the culturing approach used, as occurred in previous studies (Houf *et al*., 2002; De Smet *et al*., 2011b). Houf *et al*. (2002) explained these discrepancies by the fact that the enrichment favours the faster growing species like *A. butzleri*, although this still needs to be experimentally verified. Our results **(Levican *et al*., in preparation, study 4.7)** support the recommendation of Houf *et al*. (2002) that the use of both culturing methods in parallel allows more different species and strains to be recovered than each method separately. Considering the demonstrated impact of the different employed culturing methods in the prevalence and diversity of *Arcobacter* spp., more studies in other kinds of samples are necessary to find out whether the same behaviour is observed and to determine the best growing conditions in order to define a standardised isolation method for this genus.

Detection by m-PCR performed badly in our studies on shellfish **(Levican *et al*., submitted, study 4.6)** and wastewater compared to results using culturing methods **(Levican *et al*., in preparation, study 4.7)**. In the case of shellfish, only 16.7% of samples were positive, while 24% were positive by culturing. Regarding the sewage samples, only 40% were positive by m-PCR and 93.3% by culturing. Previous studies on wastewater report a better performance of the m-PCR in comparison to culturing methods. Collado *et al*. (2008) found 100% of positive samples both by m-PCR and by culturing, while only 66% of positive samples were obtained by González *et al.* (2007) both by m-PCR and by culturing. However, in a posterior study González *et al*. (2010) found 100% using m-PCR and only 45.5% by culturing. These differences might be explained by differences in the protocols used. In all the mentioned studies, the m-PCR was not performed directly from the sample, as we did, but from the enrichment broth, which could explain the more positive samples encountered by the other authors (Collado *et al*., 2008; González *et al.,* 2007; González *et al*., 2010). However, the fewer positive samples by m-PCR in our study could also be explained by the fact that this m-PCR method (Houf *et al*., 2000) was

created only to detect *A. butzleri, A. cryaerophilus* and *A. skirrowii*. In fact other authors recovered only the species *A. butzleri* and *A. cryaerophilus* (Collado *et al*., 2008; González *et al.,* 2007; González *et al*., 2010) while we recovered those two species and also *A. nitrofigilis, A. defluvii* and *A. cloacae* **(Levican *et al*., in preparation, study 4.7)**.

5.4 Virulence and clinical importance of *Arcobacter* species

The species *A. butzleri, A. cryaerophilus* and *A. skirrowii* have been associated with human cases of diarrhoea and bacteraemia, but few studies on the virulence of these species have been carried out. We have assessed the virulence of representative *Arcobacter* spp. strains and from different sources (except *A. halophilus* and *A. marinus*), evaluating their ability to adhere and invade human intestinal Caco-2 cells and looking for the presence of five putative virulence genes (*ciaB, cadF, cj1349, hecA* and *irgA*) in those strains **(Levican *et al*., 2013, study 4.8)**.

Of the 16 studied species, 13 adhered to Caco-2 cells and 10 were invasive. The most invasive were *A. skirrowii*, *A. cryaerophilus*, *A. butzleri* and *A. defluvii,* which had been isolated predominantly from sewage and faeces. A representative set of invasive strains was selected for microscopic examination. Bacteria could be observed over the cells in all of them; however, a negative control (non-adherent, non-invasive strain), that would have differentiated adhesion and/or invasion patterns, was not included in the microscopic observation and should be considered in future studies. In a previous study on the same cell line by Ho *et al*. (2007) only the strains of *A. cryaerophilus* were found able to invade, while the other species tested (*A. butzleri, A. skirrowii* and *A. cibarius*) were only able to adhere. In another study, Houf & Stephan (2007) evaluated adhesion but not the invasion of 7 *A. cryaerophilus* strains and only 2 of them were adherent, based on microscopic observation after Giemsa staining. The differences between those studies and ours **(Levican *et al*., 2013, study 4.8)** might be explained by the different behaviour of the studied strains. In fact, the only strain we had in common with Ho *et al*. (2007) was *A. cibarius* LMG 7537^T (=CECT 7203^T), which indeed behaved similarly. A recent study that compared the adhesion and invasion capacities of 3 isolates of *A. butzleri* from chicken meat and 3 of human origin for Caco-2 and HT-29 cells, also obtained strain dependent results (Karadas *et al.,* 2013). In the latter study, despite all the isolates adhered and invaded Caco-2 cells in different degrees, only 4 adhered to HT-29 cells and only 3 invaded this cell line. Coincidentally, the least adherent and invasive isolates to Caco-2 cells were not invasive to HT-29 cells.

In relation to the presence of the investigated putative virulence genes by PCR, the strains studied in this thesis, representing 16 *Arcobacter* species, showed overall a similar prevalence (85.0% *ciaB,* 38.3% *cj1349,* 25.0% *cadF,* 16.7% *irgA* and 3.3% *hecA*) as that previously reported for *A. butzleri, A. cryaerophilus* and *A. skirrowii* (Douidah *et al*., 2012; Karadas *et al.,* 2013). Furthermore, in **study 4.8** the *A. butzleri* strains carried in general a higher proportion of these genes (100% *ciaB,* 91.7% *cj1349,* 91.7% *cadF,* 16.7% *irgA* and 8.3% *hecA*) than the other species, in accordance with a previous study (Douidah *et al*., 2012). Those authors suggested that this could be due to the different pathogenic behaviour among species or to a higher genomic heterogeneity of *A. cryaerophilus and A. skirrowii* that could make the PCR screening of the presence of the putative virulence genes less accurate. We have also considered the possibility that this is a bias due to the primers targeting these genes being derived from the complete genome of the strain *A. butzleri* RM4018 **(Levican *et al*., 2013, study 4.8)**. So far the presence of virulence genes has not been explored in the other 3 available *Arcobacter* genomes.of *A. nitrofigilis* DSM 7299^{T}, *A. butzleri* (strain ED-1) and *Arcobacter* sp. (strain L), the latter being a potential *A. defluvii* strain on the basis of the16S rRNA gene (Collado *et al*., 2011). In this regard, we carried out BLASTN analyses with the aim of detecting the presence of the five studied virulence genes in those genomes. As a result, we found that the type strain of *A. nitrofigilis* possesses only the *ciaB* gene, confirming our experimental results in that strain. Moreover, the other two genomes showed a similar presence of the virulence genes as that observed in their respective species, i.e. the *A. butzleri* strain was positive for all tested genes, whereas the *A. defluvii* strain was only positive for the *ciaB* and *irgA,* like all the studied strains of this species **(Levican *et al*., 2013, study 4.8).** Previously, Douidah *et al*. (2010) did not find any correlation between the distribution of genes and the origin of strains (human or animal origin). However, we found that the strains from faecal sources carried a higher proportion of virulence genes than strains from other origins **(Levican *et al*., 2013, study 4.8)**. In addition, the faecal strains were also the most invasive, followed by those from shellfish and meat, while the absence of virulence genes correlated with the lack of invasion to Caco-2 cells **(Levican *et al*., 2013, study 4.8)**. Our study confirmed most *Arcobacter* species as potential human pathogens, especially *A. butzleri, A. cryaerophilus, A. skirrowii, A. trophiarum* and *A. defluvii*. Despite this, further studies are warranted to characterise the studied virulence traits and to confirm their true role in *Arcobacter* infection, for instance, by using specific deletion and complemented mutants for the tested genes or experimental infections in animals.

The clinical importance of arcobacters is considered underestimated because they are not routinely searched for and there are no adequate detection and identification methods

(Collado & Figueras, 2011). Furthermore, it has been recognized that clinical arcobacters may be misidentified as *Campylobacter* spp. (Prouzet-Mauleon., 2006). Regarding this, we carried out a study in which we re-identified, by sequencing the *rpoB* gene, all isolates that had been phenotypically identified as *Campylobacter* spp. from diarrhoea faeces of patients from the *Hospital Universitari Sant Joan* (Reus, Spain). The results show that among the 116 *Campylobacter* strains studied, one strain (0.9%) in fact belongs to the species *A. cryaerophilus* **(Figueras *et al*., submitted, study 4.9).** This prevalence coincides with the results obtained in a recent study conducted in New Zealand (Mandisodza *et al*., 2012), where *Arcobacter* showed an overall prevalence of 0.9% among all patients with diarrhoea, which was even higher than that observed for *Shigella* spp. Our strain was collected from a young patient who presented bloody watery diarrhoea of 3 weeks duration (with ca. 3 liquid depositions a day), together with abdominal pain. He was submitted to antibiotic treatment and the issue cured without relapse. The need for antibiotic treatment has also been reported in other published cases because *Arcobacter* tends to produce chronic diarrhoea **(Figueras *et al*., submitted, study 4.9 and references therein).** As indicated, our findings confirm the statement that *Arcobacter* is confused for campylobacters, especially as they are not commonly searched for using specific culturing methods and also due to the lack of reliable identification methods. In this regard, we have also demonstrated that the use of other methods, such as the *rpoB* gene sequencing or the MALDI-TOF can contribute to a better identification of *Arcobacter* spp. as long as the databases are properly completed, and this may help to clarify their true clinical importance. Furthermore, our study reviews all previous clinical cases attributed to arcobacters in order to make clinicians aware of the relevance of this poorly known group of bacteria.

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6. CONCLUSIONS

1. On the basis of a polyphasic approach we have demonstrated that the strain F4 and F41 belonged to new species for which the names *A. bivalviorum* and *A. suis,* respectively, have been proposed. Moreover, during this thesis other three new species, i.e. *A. ellisii, A. venerupis* and *A. cloacae* have been described, enlarging the genus up to 17 species.

2. The new molecular tools used in the circumscription of the new species, MALDI-TOF and the proposed MLPA with 5 genes (*rpoB, gyrB, hsp60, gyrA* and *atpA*) were able to discriminate them from all existing *Arcobacter* species showing concordance with the currently known taxonomy of the genus.

3. The 16S rRNA-RFLP identification method was updated and demonstrated to be able to identify the 17 *Arcobacter* spp. using either polyacrylamide or agarose gel electrophoresis

4. None of the 5 PCR identification methods compared was able to identify unequivocally all the 17 *Arcobacter* species.

5. The parallel use of two culture approaches i.e. a direct plating and an enrichment step in *Arcobacter* CAT-broth, as well as the parallel incubation under aerobic and microaerobic conditions, increased the recovery and diversity of *Arcobacter* species obtained from wastewater and shellfish.

6. Shellfish showed the highest diversity of *Arcobacter* species ever observed in any kind of samples. They included *A. butzleri, A. molluscorum* as the most abundant followed by *A. cryaerophilus, A. nitrofigilis, A. ellisii, A. bivalviorum, A. skirrowii, A. thereius, A. defluvii* and *A. mytili*.

7. Considering the prevalence of the potential pathogenic species *A. butzleri* in shellfish, this kind of food could be considered as an important route of transmission to humans.

8. Most *Arcobacter* spp. showed to be able to adhere and invade Caco-2 cells and possessed putative virulence genes, being these characteristics common in some strains of *A. butzleri*, *A. cryaerophilus, A. skirrowii*, *A. trophiarum* and *A. defluvii.*

9. A new human case of acute diarrhea attributed to a bacteria phenotypically considered *Campylobacter* sp. but genetically identified as *A. cryaerophilus* confirmed that this confusion can produce an underestimation of the clinical relevance of *Arcobacter* spp. for humans.

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8. ANNEXES

Table 8.1. Investigated *Arcobacter* strains isolated recovered from shellfish, study 4.6

Strain	Other name	Culture collection	Data Isolation	Source	m-PCR	16S rRNA-RFLP	Identification *rpoB*	Identification 16S rRNA	Final identification
F72	322-1 (O)		06-abr-09	Mussels	*A.skirrowi*	*A.nitrofigilis*	*A.nitrofigilis*		*A.nitrofigilis*
F73-1	327 (O)		14-abr-09	Mussels	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
F73-2	327 (O)		14-abr-09	Mussels	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
F73-3	327 (O)		14-abr-09	Mussels	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
F75	397 (O)		27-abr-09	Mussels	*A.skirrowi*	*A.skirrowi*			*A.skirrowi*
F76	FM34C		12-may-09	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F77	408 (O)		04-may-09	Mussels	*A.skirrowi*	*A. nitrofigilis*	*A. nitrofigilis*	*A. nitrofigilis*	*A. nitrofigilis*
F78-1	407-7 (O)		04-may-09	Mussels	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
F78-3	407-5 (O)		04-may-09	Mussels	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
F79-1	FM79C1		04-may-09	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F79-2	FM79C2		04-may-09	Mussels	*A. butzleri*	*A. ellisii*	*A. ellisii*	*A. ellisii*	*A. ellisii*
F79-3	FM79C3		04-may-09	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F79-4	FM79C4		04-may-09	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F79-5	FM79C6		04-may-09	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F79-6	FM79O3		04-may-09	Mussels	*A. butzleri*	*A. ellisii*	*A. ellisii*	*A. ellisii*	*A. ellisii*
F79-7	FM79O41		04-may-09	Mussels	*A. butzleri*	*A. ellisii*	*A. ellisii*	*A. ellisii*	*A. ellisii*
F79-8	FM79O5		04-may-09	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F80	491-7 (O)		20-may-09	Mussels	*A.cryaerophilus*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*
F81	FM18O1		09-jun-09	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F82-1	592O2		09-jun-09	Mussels	*A.cryaerophilus*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*
F82-2	592C2		09-jun-09	Mussels	*A.cryaerophilus*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*
F83-1	676C1		07-jul-09	Mussels	*A.cryaerophilus*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*
F83-2	676C2		07-jul-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F84-1	677O1		07-jul-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F84-2	677O2		07-jul-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F84-3	677O3		07-jul-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F84-4	677C1		07-jul-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F84-5	677C3		07-jul-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F84-6	677O4		07-jul-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F86	681-O		08-jul-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F87	649-O		08-jul-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F88	650-O1		08-jul-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*

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Cont.

Strain	Other name	Culture collection	Data Isolation	Source	m-PCR	16S rRNA-RFLP	Identification *rpoB*	Identification 16S rRNA	Final identification
F89-1	652-O3		30-jun-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F89-2	652-O4		30-jun-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F89-3	652-O5		30-jun-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F89-4	652-C3		30-jun-09	Mussels	*A.cryaerophilus*	*A.butzleri*	*A. thereius*	*A. thereius*	*A. thereius*
F90-1	680-O1		08-jul-09	Mussels	*A. cry+ A.ski*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*
F90-2	680-O4		08-jul-09	Mussels	*A. cry+ A.ski*	*A. molluscorum*			*A. molluscorum*
F90-3	680-C1		08-jul-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F90-7	680-C7		08-jul-09	Mussels	*A.butzleri*	*A.butzleri*			*A. butzleri*
F91	696-O		14-jul-09	Mussels	*A.cryaerophilus*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*
F92	697-O		14-jul-09	Mussels	*A.cryaerophilus*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*
F93-4	Ameixal2C3		30-jul-09	Clams	*A. cryaerophilus*	*A. butzleri*	*A. thereius*	*A. thereius*	*A. thereius*
F97	847O1		01-sep-09	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F98-1	848C1		01-sep-09	Mussels	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
F98-3	848O3	CECT 7696	01-sep-09	Mussels	*A. cry+ A.ski*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*
F99-1	FM05C2		08-sep-09	Mussels	*A.cryaerophilus*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*	*A. molluscorum*
F100-1	18O5		29-sep-09	Oysters	*A. butzleri*	*A. butzleri*			*A. butzleri*
F100-2	18C4		29-sep-09	Oysters	*A. butzleri*	*A. butzleri*			*A. butzleri*
F100-3	18C8		29-sep-09	Oysters	*A. butzleri*	*A. butzleri*			*A. butzleri*
F101-1	58C3		29-sep-09	Oysters	*A.cryaerophilus*	*A. molluscorum*			*A. molluscorum*
F102-1	1059O3		27-oct-09	Clams	*A. butzleri*	*A. butzleri*			*A. butzleri*
F102-2	1059C1		27-oct-09	Clams	*A. butzleri*	*A. butzleri*			*A. butzleri*
F102-3	1059C4		27-oct-09	Clams	*A. butzleri*	*A. butzleri*			*A. butzleri*
F103	1060		27-oct-09	Clams	*A. butzleri*	*A. butzleri*			*A. butzleri*
F104	FM32-O1		11-nov-09	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F105-1	318-7		11-may-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F105-2	318-1		11-may-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F106	426-1		08-jun-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F107	427-1		08-jun-10	Mussels	*A. cry+ A.ski*	*A. molluscorum*	*A. molluscorum*		*A. molluscorum*
F108	514-1		29-jun-10	Mussels	*A. cry+ A.ski*	*A. molluscorum*	*A. molluscorum*		*A. molluscorum*
F109-1	547-2		22-jul-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F109-2	547-1		22-jul-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F110-1	548-8		22-jul-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F110-2	548-9		22-jul-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*

Cont.

Strain	Other name	Culture collection	Data Isolation	Source	m-PCR	16S rRNA-RFLP	Identification *rpoB*	Identification 16S rRNA	Final identification
F110-3	548-1		22-jul-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F111-1	578-2		05-ago-10	Mussels	*A. cry+ A.ski*	*A. molluscorum*	*A. molluscorum*		*A. molluscorum*
F111-2	578-1		05-ago-10	Mussels	*A. cry+ A.ski*	*A. molluscorum*	*A. molluscorum*		*A. molluscorum*
F112-1	579-4		05-ago-10	Mussels	*A. cry+ A.ski*	*A. molluscorum*	*A. molluscorum*		*A. molluscorum*
F112-2	579-1		05-ago-10	Mussels	*A. cry+ A.ski*	*A. molluscorum*	*A. molluscorum*		*A. molluscorum*
F113	580-1		05-ago-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F114-1	582-5		05-ago-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F114-2	582-9		05-ago-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F114-3	582-10		05-ago-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F114-5	582-12		05-ago-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F114-6	582-14		05-ago-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F114-7	582-8		05-ago-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F114-8	582-1		05-ago-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F115-1	599-9		12-ago-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F115-2	599-1		12-ago-10	Mussels	*A. cry debil*	*A. defluvii/A. suis*	*A. defluvii*		*A. defluvii*
F116-1	600-6		12-ago-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F116-2	600-1		12-ago-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F117	602-1		12-ago-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F118-1	611-5		16-sep-10	Mussels	*A. skirrowii*	*A. mytili*	*A. mytili*		*A. mytili*
F118-2	611-9		16-sep-10	Mussels	*A. cryaerophilus*	*A. bivalviorum*	*A. molluscorum*		*A. molluscorum*
F118-4	611-1		16-sep-10	Mussels	*A. cryaerophilus*	*A. bivalviorum*	*A. molluscorum*		*A. molluscorum*
F119	608-9		16-sep-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F120-1	609-1		16-sep-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F120-2	609-1		16-sep-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F121-1	610-9		16-sep-10	Mussels	*.A. cry + A. ski*	*A. molluscorum*	*A. molluscorum*		*A. molluscorum*
F122-1	652-7		04-oct-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F122-2	652-1		04-oct-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F123-1	735-2		12-nov-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F123-2	735-4		12-nov-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F123-3	735-8		12-nov-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F123-4	735-9		12-nov-10	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F124-1	30-12		26-ene-11	Oysters	*A. skirrowii*	*A. nitrofigilis*	*A. nitrofigilis*	*A. nitrofigilis*	*A. nitrofigilis*
F124-2	30-9		26-ene-11	Oysters	*A. skirrowii*	*A. nitrofigilis*	*A. nitrofigilis*	*A. nitrofigilis*	*A. nitrofigilis*
F125-1	32-12		26-ene-11	Mussels	*A. skirrowii*	*A. skirrowii*	*A. skirrowii*	*A. skirrowii*	*A. skirrowii*

Cont.

Strain	Other name	Culture collection	Data Isolation	Source	m-PCR	16S rRNA-RFLP	Identification *rpoB*	Identification 16S rRNA	Final identification
F125-2	32-1		26-ene-11	Mussels	*A. skirrowii*	*A. nitrofigilis*	*A. nitrofigilis*	*A. nitrofigilis*	*A. nitrofigilis*
F127-1	59-4		16-mar-11	Oysters	*A. butzleri*	*A. butzleri*			*A. butzleri*
F127-2	59-7		16-mar-11	Oysters	*A. butzleri*	*A. butzleri*			*A. butzleri*
F127-3	59-9		16-mar-11	Oysters	*A. butzleri*	*A. butzleri*			*A. butzleri*
F127-4	59-10		16-mar-11	Oysters	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
F127-5	59-11		16-mar-11	Oysters	*A. butzleri*	*A. butzleri*			*A. butzleri*
F127-6	59-1		16-mar-11	Oysters	*A. butzleri*	*A. butzleri*			*A. butzleri*
F128-1	259-5		07-jun-11	Mussels	*Acry+Aski*	*A. molluscorum*			*A. molluscorum*
F128-2	259-6		07-jun-11	Mussels	*N/A*	*Unknown*	*Arcobacter sp.*	*Arcobacter sp.*	*Arcobacter sp.*
F128-3	259-1		07-jun-11	Mussels	*Acry+Aski*	*A. molluscorum*			*A. molluscorum*
F129	260-1		07-jun-11	Mussels	*Acry+Aski*	*A. molluscorum*			*A. molluscorum*
F130-1	364-3		04-oct-11	Mussels	*A. cryaerophilus*	*A. molluscorum*			*A. molluscorum*
F130-2	364-4		04-oct-11	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F130-3	364-10		04-oct-11	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F130-4	364-1		04-oct-11	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F131-1	367-5		04-oct-11	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F131-2	367-1		04-oct-11	Mussels	*A. butzleri*	*A. butzleri*			*A. butzleri*
F132	376-1		18-oct-11	Mussels	*A. cry+ A.ski*	*A. molluscorum*			*A. molluscorum*
F133-1	377-2		18-oct-11	Mussels	*A. cry+ A.ski*	*A. molluscorum*			*A. molluscorum*
F134-1	386-1		02-nov-11	Mussels	*A. cry+ A.ski*	*A. molluscorum*			*A. molluscorum*

Table 8.2. Investigated *Arcobacter* strains recovered from wastewater, study 4.7

Strain	Other name	Culture collection	Data Isolation	Source	m-PCR	16S rRNA-RFLP	Id. *rpoB*	Id. 16S rRNA	Final identification
SW28-1	Ed1		01-April-09	Inflow	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW28-2	Ed2		01-April-09	Inflow	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW28-3	Ed3		01-April-09	Inflow	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW28-4	Ed4		01-April-09	Inflow	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW28-5	E2-1		01-April-09	Inflow	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW28-6	E2-2		01-April-09	Inflow	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW28-7	E3		01-April-09	Inflow	*230 bp*	*A. defluvii / A. suis*	*A. defluvii*	*A. defluvii*	*A. defluvii*
SW28-8	E6		01-April-09	Inflow	*230 bp*	*A. defluvii / A. suis*	*A. defluvii*	*A. defluvii*	*A. defluvii*
SW28-9	E5		01-April-09	Inflow	*230 bp*	*A. defluvii / A. suis*	*A. defluvii*	*A. defluvii*	*A. defluvii*
SW28-10	E1		01-April-09	Inflow	*230 bp*	*A. defluvii / A. suis*	*A. defluvii*	*A. defluvii*	*A. defluvii*
SW28-11	E2	CECT 7697	01-April-09	Inflow	*230 bp*	*A. defluvii / A. suis*	*A. defluvii*	*A. defluvii*	*A. defluvii*
SW28-12	Ed2v		01-April-09	Inflow	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW28-13	E8	CECT 7834	01-April-09	Inflow	*A. cryaerophilus*	*A. cloacae*	*A. cloacae*	*A. cloacae*	*A. cloacae*
SW28-14	Ed1v		01-April-09	Inflow	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW29-1	D1directo1		01-April-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW29-2	D1directo2		01-April-09	Primary treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW29-3	D1directo5		01-April-09	Primary treatment	*230 bp*	*A. defluvii / A. suis*	*A. defluvii*	*A. defluvii*	*A. defluvii*
SW29-4	D1directo6		01-April-09	Primary treatment	*230 bp*	*A. defluvii / A. suis*	*A. defluvii*	*A. defluvii*	*A. defluvii*
SW30-1	Td1		01-April-09	Biological treatment	*230 bp*	*A. defluvii / A. suis*	*A. defluvii*	*A. defluvii*	*A. defluvii*
SW30-2	Td2		01-April-09	Biological treatment	*230 bp*	*A. defluvii / A. suis*	*A. defluvii*	*A. defluvii*	*A. defluvii*
SW30-3	Td3		01-April-09	Biological treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW30-4	Td4		01-April-09	Biological treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW30-5	Td5		01-April-09	Biological treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW30-6	Td6		01-April-09	Biological treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW30-7	T8		01-April-09	Biological treatment	*230 bp*	*A. defluvii / A. suis*	*A. defluvii*	*A. defluvii*	*A. defluvii*
SW30-8	Tbiod1		01-April-09	Biological treatment	*230 bp*	*A. defluvii / A. suis*	*A. defluvii*	*A. defluvii*	*A. defluvii*
SW30-9	T1		01-April-09	Biological treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW30-10	T3		01-April-09	Biological treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW30-11	T5		01-April-09	Biological treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW30-12	T6		01-April-09	Biological treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW31-1	D2-2		01-April-09	Secondary treatment	*230 bp*	*A. cryaerophilus*	*A. defluvii*	*A. defluvii*	*A. defluvii*
SW31-2	D2-4		01-April-09	Secondary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*

Cont.

Strain	Other name	Culture collection	Data Isolation	Source	m-PCR	16S rRNA-RFLP	Id. *rpoB*	Id. 16S rRNA	Final identification
SW32-1	Ee1		26-Jun-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW32-2	Ee3		26-Jun-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW32-3	Ee4		26-Jun-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW32-4	Ee5		26-Jun-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW32-5	Ee6		26-Jun-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW32-6	Ee7		26-Jun-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW32-7	Ee8		26-Jun-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW32-8	Ed1		26-Jun-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW32-9	Ed2		26-Jun-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW32-10	Ed3		26-Jun-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW32-11	Ed4		26-Jun-09	Inflow	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW32-12	Ed5		26-Jun-09	Inflow	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW32-13	Ed6		26-Jun-09	Inflow	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW32-14	Ed7		26-Jun-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW32-15	Ed8		26-Jun-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-1	D1e1		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-2	D1e2		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-3	D1e3		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-4	D1e4		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-5	D1e5		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-6	D1e6		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-7	D1e7		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-8	D1e8		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-9	D1d1		26-Jun-09	Primary treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW33-10	D1d2		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-11	D1d3		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-12	D1d4		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-13	D1d5		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW33-14	D1d6		26-Jun-09	Primary treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW33-15	D1d7		26-Jun-09	Primary treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW33-16	D1d8		26-Jun-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW34-1	Te1		26-Jun-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*

Cont.

Strain	Other name	Culture collection	Data Isolation	Source	m-PCR	16S rRNA-RFLP	Id. *rpoB*	Id. 16S rRNA	Final identification
SW34-2	Te2		26-Jun-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW34-3	Te3		26-Jun-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW34-4	Te4		26-Jun-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW34-5	Te5		26-Jun-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW34-6	Te6		26-Jun-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW34-7	Te7		26-Jun-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW34-8	Te8		26-Jun-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW34-9	Td6		26-Jun-09	Biological treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW34-10	Td8		26-Jun-09	Biological treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW35-1	D2e4		26-Jun-09	Secondary treatment	*A. cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW35-2	D2e6		26-Jun-09	Secondary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW35-3	D2e7		26-Jun-09	Secondary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW35-4	D2e8		26-Jun-09	Secondary treatment	*A. butzleri/A.cry*	*A. butzleri*			*A. butzleri*
SW35-5	D2d1		26-Jun-09	Secondary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW35-6	D2d2		26-Jun-09	Secondary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW35-7	D2d3		26-Jun-09	Secondary treatment	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW35-8	D2d4		26-Jun-09	Secondary treatment	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW35-9	D2d5		26-Jun-09	Secondary treatment	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW35-10	D2d6		26-Jun-09	Secondary treatment	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW35-11	D2d7		26-Jun-09	Secondary treatment	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW35-12	D2d8		26-Jun-09	Secondary treatment	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW36-1	Se1		26-Jun-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW36-2	Se2		26-Jun-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW36-3	Se3		26-Jun-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW36-4	Se4		26-Jun-09	Outflow	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW36-5	Se5		26-Jun-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW36-6	Se6		26-Jun-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW36-7	Se7		26-Jun-09	Outflow	*A. Butzleri*	*A. butzleri*			*A. butzleri*
SW36-8	Se8		26-Jun-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW36-9	Sd1		26-Jun-09	Outflow	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW36-10	Sd2		26-Jun-09	Outflow	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW36-11	Sd4		26-Jun-09	Outflow	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*

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Strain	Other name	Culture collection	Data Isolation	Source	m-PCR	16S rRNA-RFLP	Id. *rpoB*	Id. 16S rRNA	Final identification
SW36-12	Sd6		26-Jun-09	Outflow	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW36-13	Sd7		26-Jun-09	Outflow	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW36-14	Sd8		26-Jun-09	Outflow	*A.cryaerophilus*	*A. cryaerophilus*			*A. cryaerophilus*
SW37-1	Ed2		09-Oct-09	Inflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW37-2	Ed3		09-Oct-09	Inflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW37-3	Ed4		09-Oct-09	Inflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW37-4	Ee1		09-Oct-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW37-5	Ee2		09-Oct-09	Inflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW37-6	Ee3		09-Oct-09	Inflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW37-7	Ee4		09-Oct-09	Inflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW37-8	Ee5		09-Oct-09	Inflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW37-9	Ee6		09-Oct-09	Inflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW37-10	Ee7		09-Oct-09	Inflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW37-11	Ed1		09-Oct-09	Inflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW38-1	D1d1		09-Oct-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW38-2	D1d2		09-Oct-09	Primary treatment	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW38-3	D1d3		09-Oct-09	Primary treatment	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW38-4	D1d4		09-Oct-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW38-5	D1d5		09-Oct-09	Primary treatment	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW38-6	D1d6		09-Oct-09	Primary treatment	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW38-7	D1d7		09-Oct-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW38-8	D1e1		09-Oct-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW38-9	D1e2		09-Oct-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW38-10	D1e3		09-Oct-09	Primary treatment	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW38-11	D1e4		09-Oct-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW38-12	D1e5		09-Oct-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW38-13	D1e6		09-Oct-09	Primary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW39-1	Td2		09-Oct-09	Biological treatment	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW39-2	Td6		09-Oct-09	Biological treatment	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW39-3	Te1		09-Oct-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW39-4	Te2		09-Oct-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW39-5	Te3		09-Oct-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*

Cont.

Strain	Other name	Culture collection	Data Isolation	Source	m-PCR	16S rRNA-RFLP	Id. *rpoB*	Id. 16S rRNA	Final identification
SW39-6	Te4		09-Oct-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW39-7	Te5		09-Oct-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW39-8	Te6		09-Oct-09	Biological treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW40-1	D2e1		09-Oct-09	Secondary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW40-2	D2e2		09-Oct-09	Secondary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW40-3	D2e3		09-Oct-09	Secondary treatment	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW40-4	D2e4		09-Oct-09	Secondary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW40-5	D2e5		09-Oct-09	Secondary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW40-6	D2e6		09-Oct-09	Secondary treatment	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW40-7	D2d4		09-Oct-09	Secondary treatment	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW40-8	D2d1		09-Oct-09	Secondary treatment	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW40-9	D2d3		09-Oct-09	Secondary treatment	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW41-1	Se1		09-Oct-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW41-2	Se2		09-Oct-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW41-3	Se4		09-Oct-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW41-4	Se5		09-Oct-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW41-5	Se6		09-Oct-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW41-6	Se8		09-Oct-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW41-7	Sd1		09-Oct-09	Outflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW41-8	Sd2		09-Oct-09	Outflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW41-9	Sd3		09-Oct-09	Outflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW41-10	Sd4		09-Oct-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW41-11	Sd5		09-Oct-09	Outflow	*A.cryaerophilus*	*A.cryaerophilus*			*A.cryaerophilus*
SW41-12	Sd6		09-Oct-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW41-13	Sd7		09-Oct-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*
SW41-14	Sd8		09-Oct-09	Outflow	*A. butzleri*	*A. butzleri*			*A. butzleri*

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Table 8.3. Strains and accession numbers of sequences used in MLPA, study 4.3

Species/strains	Other name	Source	EMBL/GenBank/DDBJ accession numbers					
			gyrB	*gyrA*	*rpoB*	*atpA*	*cpn60*	16S rRNA
A. nitrofigilis								
CECT 7204^T	MDC 1274^T	*S. alterniflora*, roots (Canada)	JF803162	JF803106	JF803189	JF802986	JF803089	L14627
F40	MDC 1634	Mussels (Spain)	GU291965	JF803108	JF803193	JF802988	JF803091	EU106662
A. cryaerophilus								
LMG 9904^T	MDC 1271^T	Aborted bovine fetus (Ireland)	JF803152	JF803130	JF803222	JF803015	JF803062	L14624
LMG 9865	MDC 1625	Aborted porcine foetus (Ireland)	FR682117	JF803131	JF803223	JF803016	JF803060	FR682113
A. butzleri								
LMG 10828^T	MDC 1272^T	Man, faeces (USA)	JF803158	JF803111	JF803200	JF802993	JF803040	AY621116
F46	MDC 1623	Pork meat (Spain)	GU291959	JF803113	JF803198	JF802994	JF803044	GU300771
A. skirrowii								
LMG 6621^T	MDC 1273^T	Lamb with diarrhoea, faeces	JF803153	JF803135	JF803217	JF803012	JF803048	L14625
HOUF 989	MDC 1631	Feces , cow (Belgium)	GU291963	JF803136	JF803221	JF803010	JF803047	GU300769
A. halophilus								
DSM 18005^T	MDC 1275^T	Water of hypersaline lagoon (USA)	JF803176	JF803101	JF803184	JF803031	JF803080	AF513455
A. cibarius								
CECT 7203^T	MDC 1270^T	Broiler, skin (Belgium)	JF803150	JF803125	JF803212	JF803006	JF803063	AJ607391
HOUF 746	MDC 1632	Poultry carcass (Belgium)	GU291968	JF803126	JF803215	JF803005	JF803064	
A. mytili								
CECT 7386^T	MDC 1636^T	Mussels (Spain)	GU291969	JF803103	JF803181	JF803033	JF803079	EU669904
CECT 7385	MDC 1637	Mussels (Spain)	FR682119	JF803104	JF803182	JF803035	JF803077	EU669906
A. thereius								
LMG 24486^T	MDC 1738	Aborted bovine foetus (Denmark)	JF803155	JF803142	JF803230	JF803022	JF803052	AY314753
LMG 24487	MDC 1740	Aborted bovine foetus (Denmark)	JF803157	JF803144	JF803234	JF803024	JF803056	AY314754
A. marinus								
CECT 7727^T	MDC 1741	Seawater (Korea)	JF803177	JF803102	JF803185	JF803032	JF803081	EU512920

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Cont.

Species/strains	Other name	Source	EMBL/GenBank/DDBJ accession numbers					
			gyrB	***gyrA***	***rpoB***	***atpA***	***cpn60***	**16S rRNA**
A. trophiarum								
LMG 25534^{T}	MDC 1749	Pig faeces (Belgium)	JF803148	JF803145	JF803227	JF803025	JF803057	FN650333
FE2 (CECT 7650)	MDC 1652	Chicken cloacal swab (Chile)	GU291957	JF803146	JF803228	JF803027	JF803058	GU300768
A. defluvii								
SW28-11^{T}	MDC 1717^{T}	Sewage (Spain)	JF803164	JF803116	JF803206	JF802999	JF803068	HQ115595
SW30-2	MDC 1718	Sewage (Spain)	JF803167	JF803119	JF803210	JF803003	JF803070	HQ115596
A. molluscorum								
F98-3^{T}	MDC 1720^{T}	Mussels (Spain)	JF803178	JF803098	JF803186	JF803030	JF803084	FR675874
F101-1	MDC 1721	Oysters (Spain)	JF803179	JF803099	JF803188	JF803029	JF803083	FR675875
A. ellisii								
F79-6^{T}	MDC 1745^{T}	Mussels (Spain)	JF803171	JF803122	JF803203	JF802998	JF803073	FR717550
F79-2	MDC 1744	Mussels (Spain)	JF803169	JF803124	JF803205	JF802996	JF803075	FR717551
A. bivalviorum								
F4^{T}	MDC 1641	Mussels (Spain)	HE565364	JF803094	JF803196	JF803038	JF803087	FJ573217
F118-4	MDC 1767	Mussels (Spain)	JF803175	JF803097	JF803197	JF803039	JF803088	HE565358
A. venerupis								
F67-11^{T}	MDC 1747	Clams (Spain)	JF803172	JF803121	JF803211	JF803004	JF803076	HE565359
A. cloacae								
SW28-13^{T}	MDC 1748^{T}	Sewage (Spain)	**HE565379**				**HE565378**	HE565360
F26	MDC 1667	Mussels (Spain)	**HE565382**	**HE997169**	**HE565380**	**HE997170**	**HE997171**	HE565361
A. suis								
F41^{T}	MDC 1642^{T}	Pig meat (Spain)						FJ573216