Short Term Mission Report

Anna Mottola

Identification and genetic diversity of *Arcobacter* spp. Italian and Spanish sources.

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Introduction

Arcobacter is a genus of Gram-negative, slightly curved bacteria found in both animal and environmental sources (González and Ferrús, 2011; Tabatabaei *et al.*, 2014). This genus currently consists of 21 species, some of them are potential emerging food-borne enteropathogens (Levican *et al*., 2014; Nieva-Echevarria *et al*., 2013; Levican and Figueras, 2013; Levican *et al*., 2015; Whiteduck-Léveillée *et al*., 2015). These bacteria are considered members of seawater microbiota, wastewater and drinking water reservoirs (Collado *et al.,* 2008). Bivalve molluscs, due to their ability to concentrate microorganisms from contaminated water during their filter-feeding activities, can bio-concentrate *Arcobacter* spp. and can be considered an important health risk for human health because in southern Italy they are often eaten poorly cooked and/or raw (Collado *et al.,* 2009; Levican *et al.,* 2014; Ottaviani *et al*., 2013).

Objectives

Considering that the Research and Development objective of Unit of Environmental Mycology and Microbiology of the University Rovira I Virgili of Reus, Tarragona, Spain was studying the taxonomy and epidemiology of *Arcobacter* in clinical and environmental sources, the fellowship with the Food Safety of the Department of Veterinary Medicine of Bari aimed at identifying and genotyping the genus *Arcobacter* in shellfish using DNA-based methods. The further aim of the project was estimating the incidence of the species present in samples in order to understand their pathogenicity and to obtain epidemiological data to demonstrate that bivalve molluscs are a possible source for human contamination**.**

The project involved the use of different DNA-based approaches like the application and the comparison of m-PCR described by Houf et al., in 2000, the sequencing of the *rpoB* gene and the 16S rRNA-RFLP analysis, to evaluate the assay in a more reliable, simple, fast, and inexpensive way.

More the project evaluated ERIC-PCR and MLST genotyping methods for phylogenetic analysis. MLST typing system based on the seven housekeeping loci (*aspA, atpA, glnA, gltA,glyA, pgm* and

tkt) was developed to obtain further data on the genetic diversity of genus *Arcobacter* and provide and clarify genotypic information for strain characterization (Collado and Figueras, 2011).
Given the association of *Arcobacters* to human infection and that little is known about their mechanism of virulence, the final objective of the study was to apply PCR-based assays to detect putative virulence genes (*ciaB, cj1349, cadF, irgA* and *hecA*) and to obtain information about their role in human infections.

Methodology

Previous isolation and identification of Arcobacter spp. in Italian laboratories.

In this study, a total of 70 shellfish samples (42 mussels - *Mytilus galloprovincialis*- and 28 clams - *Tapes philippinarum*) harvested between January 2014 and February 2015 from local fish market of Apulia region (South Italy) were previously analyzed in the laboratory of Food Microbiology of the department of Veterinary Medicine of Bari to detect *Arcobacter* spp.

In 16 on 70 samples (10 of mussels and 6 of clams) colonies that proved to be Gram negative, slightly curved rods, oxidase and catalase positive and able to grow under both aerobic and microaerobic conditions at 30 °C were observed and identified as *Arcobacter* spp.

In order to confirm the isolates identified as *Arcobacter* spp. by phenotypic tests, the bacterial DNA was extracted and a genus specific PCR was performed, using primers and conditions reported by Harmon and Wesley (1996). PCR results were then sequenced.

DNA preparation

Prior to species identification, spectrophotometry via NanoDrop 2000/2000c (Thermo Scientific, MA, USA) at 260 and 280 nm was used to take initial readings of both purity and concentration. Due to limitations of spectrophotometry, concentration was further verified on agarose gel using GeneRuler 1kb Plus (Thermo Scientific, MA, USA) quantitative marker.

Molecular identification of Arcobacters.

The characterization at specie level of the strains was attempted using specific *Arcobacter* identification methods i.e. Multiplex-PCR (m-PCR), the sequencing of the *rpoB* gene and the restriction fragment length polymorphism of the 16S rRNA gene (16S rDNA-RFLP).

Multiplex-PCR

Multiplex-PCR was performed using the primers described by Houf et al. (2000). Primers amplify a 401-bp fragment of 16S rRNA gene for *A. butzleri*, 641-bp of 16S rRNA gene for *A. skirrowii* and a 257-bp fragment of 23S rRNA gene for *A. cryaerophilus* species. PCR reaction was carried out with a 50µL PCR mixure containing 2 µL (50 ng) of DNA template, 5µL 10X PCR Rxn Buffer (Invitrogen, Carlsbad, CA, USA), 1.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.2 mmol of each deoxyribonucleotide triphosphate (appl. Biosystems), 1,3 µL of 50 mmol $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), and 50 pmol of the primers ARCO, BUTZ, CRY1, CRY2 (Tab.1), and 25 pmol of primer SKIR (Tab.1). The amplification profile involved an initial denaturation step at 94 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 45 sec, primer annealing at 61 °C for 45 sec, and chain extension at 72 °C for 30 sec. The final extension was carried out at 72 °C for 1 min. The PCR reactions were processed in a 2720 Thermal Cycler (appl. Biosystem).

The m-PCR products thus generated were separated by gel electrophoresis on 1.5% (w/v) agarose gel in 1X Tris-borate-EDTA (TBE) buffer, and were visualized after staining with the RedSafe™ gel (INtRON Biotechnology) using UV transilluminator Gel Doc™ XR+(BioRad) with an analyst computer software (Image Lab 5.1).
A Gene Ruler™ 50 bp DNA Ladder Plus (Invitrogen) was used as the molecular weight marker.

Rpo-B PCR

PCR amplification of the rpoB-gene was performed using primers rpoB15-F and rpoB2-R (Tab. 1).

The reaction mixture for the rpoB-gene were performed in a final volume of 50 µL containing primers at a final concentration of 25 mol, 5 µL (100 ng) of DNA template, 5µL 10X PCR Buffer (Invitrogen, Carlsbad, CA, USA), 2,5 µL of 50 mmol MgCl2 (Invitrogen, Carlsbad, CA, USA), 250 µmol of each deoxyribonucleotide triphosphate (appl. Biosystems) and 1U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA).

The amplification profile involved an initial denaturation step at 94 °C for 30 sec, followed by 36 cycles at 94 °C for 30 sec, 54 °C for 30 sec and 72 °C for 30 sec. A final extension was carried out at 72 °C for 7 min. The PCR reactions were processed in a 2720 Thermal Cycler (appl. Biosystem). PCR amplified products were analyzed by electrophoresis on 1 % (w/v) agarose gel in 1X Tris-borate-

EDTA (TBE) buffer, and were visualized after staining with the RedSafe™ gel (INtRON Biotechnology) using UV transilluminator Gel Doc™ XR+(BioRad) with an analyst computer software (Image Lab 5.1). A Gene Ruler™ 100 bp DNA Ladder Plus (Invitrogen) was used as the molecular weight marker.

RpoB products were sequenced by EUROFINS (Milan, Italy). The sequence analysis was carried out using Lasergene SeqMan version 7.0.0. MEGA software version 6.0 (Tamura *et al*., 2011) was used for alignments, for calculating genetic distances and for clustering using the neighbour-joining, maximum parsimony and maximum likelihood algorithms.

16S rDNA-RFLP

The amplification of 1026 bp amplicon was carried out with a 50µL PCR mixure containing 5 µL (100 ng) of DNA template, 0,5 µM each primer primers CAH16S1am and CAH16S1b (Tab. 1), 5µL 10X PCR Buffer (Invitrogen, Carlsbad, CA, USA), 1,5 µL of 50 mmol $MgCl_2$(Invitrogen, Carlsbad, CA, USA), 200 µM each deoxyribonucleotide triphosphate (appl. Biosystems) and 2,5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The amplification profile involved an initial denaturation step at 95 °C for 2 min, followed by 31 cycles of denaturation at 94 °C, 30s, primer annealing at 55 °C, 30s, and chain extension at 72 °C for 1.30 min. The final extension was carried out at 72 °C for 10 min. The PCR reactions were processed in a 2720 Thermal Cycler (appl. Biosystem).

PCR amplified products were analyzed by electrophoresis on 2 % (w/v) agarose gel in in 1X Tris-borate-EDTA (TBE) buffer using a Gene Ruler™ 100 bp DNA Ladder (Invitrogen) and were visualized after staining with the RedSafe™ gel (INtRON Biotechnology) using UV transilluminator Gel Doc™ XR+(BioRad) with an analyst computer software (Image Lab 5.1).

The amplicon 1026 bp was then digested using the *Mse*I endonuclease (Fermentas, Schwerte, Germany).

Restriction fragments were separated in 3.5% (w/v) agarose gel electrophoresis in in 1X Tris-borate-EDTA (TBE) buffer at 100 V for 100 min.

A Gene Ruler™ 50 bp DNA Ladder Plus (Invitrogen) was used as the molecular weight marker.

Genotyping of Arcobacters.

Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR)

To avoid repeated analysis of the same strains, ERIC-PCR was preliminary performed.

Then, *Arcobacter* strains isolated respectively in the laboratory of Food Microbiology of the department of Veterinary Medicine of Bari (16 samples) and in the Unit of Environmental Mycology and Microbiology of the University Rovira I Virgili (31 strains) were compared to perform the genetic discrimination of *Arcobacter* strains isolated in different sources.

In particular, samples isolated in Spanish laboratories included 25 *A. butzleri* strain, isolated respectively from human stool (15), water (5) and shellfish (5), 3 strains of *A. cryaerophilus* 1A isolated in water and 3 samples of *A. cryaerophilus* 1B detected respectively from water (2) and human stool (1).

ERIC-PCR assay was performed using oligonucleotide primers described by Varsalovic *et al.* 1991 (Tab. 1).

The PCR reaction was carried out with a 50 µL PCR reaction mixture containing 2 µL (50ng) of DNA template, 1.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 2 mmol of each deoxyribonucleotide triphosphate (appl. Biosystems),4 µL of 50mmol $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), and 25 pmol of the primers foward ERIC 1R and reverse ERIC 2 (Tab. 1).

PCR involved an initial denaturation step at 95 °C for 5 min, followed by 41 cycles of denaturation at 94 °C for 1 min, primer annealing at 25 °C for 1 min, and chain extension at 72 °C for 10 min. The PCR reactions were processed in a 2720 Thermal Cycler (appl. Biosystem).

Amplified products were detected by electrophoresis in 2% agarose gels stained with the RedSafe™ gel (INtRON Biotechnology) at 80V for 120 min. Band pattern were visualized with UV transilluminatior Gel Doc™ XR+(BioRad) with an analyst computer software (Image Lab 5.1)..
A Gene Ruler™ 50 bp DNA Ladder Plus (Thermo Scientific) was used as the molecular weight marker.
Results obtained were analyzed using BioNumerics version 6.6 software (Applied Maths, Ghent, Belgium).

Multi-locus Sequence Typing (MLST)

MLST primers used were described by Miller *et al*., 2009. Each MLST reaction mixture was performed in a final volume of 50 µL containing 5 µL (100 ng) of DNA template, 5µL 10X PCR Buffer (Invitrogen, Carlsbad, CA, USA), 2,5 µL of 50 mmol $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 250 µmol of each deoxyribonucleotide triphosphate (appl. Biosystems) and 1U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR for MLST were performed on a 2720 Thermal Cycler (appl. Biosystem).

The reaction mixture was subjected to denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 3 min,53 °C for 30 sec and 72 °C for 2 min. Afterwards, a final extension step at 72 °C was achieved for 5 min.

PCR amplified products were analyzed by electrophoresis on 1 % (w/v) agarose gel gel in 1X Tris-borate-EDTA (TBE) buffer, and were visualized after staining with the RedSafe™ gel (INtRON Biotechnology) using UV transilluminator Gel Doc™ XR+ (BioRad) with an analyst computer software (Image Lab 5.1). A Gene Ruler™ 100 bp DNA Ladder Plus (Invitrogen) was used as the molecular weight marker.

The MLST gene products were sequenced by Eurofins (Milan, Italy). The sequence analysis was carried out using Lasergene SeqMan version 7.0.0.

The isolates arcobacters MLST profiles were compared to the *Arcobacter* specific MLST scheme (http://pubmlst.org/arcobacter/).

Detection of Putative Virulence Genes

The Putative Virulence Factors *ciaB, cj1349, cadF, irgA* and *hecA* virulence factors, of *Arcobacter* strain isolated in Italian shellfish, were detected by PCR-based analysis. Positive (LMG10828) and negative (LMG24486) control were included in the reaction. The oligonucleotide primers are listed in Tab 1.

The PCR reactions were performed in a final volume of 50 µL, containing 50 ng/ µL, 1.5U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA Invitrogen, Carlsbad, CA, USA), 0,2 µmol of each deoxyribonucleotide triphosphate (appl. Biosystems) , 0.2 mmol $MgCl_2$ for the primers sets for *ciaB, cj1349, hecA* and *irgA*. For the primers sets for *cadF* 5 µL 10X PCR buffer and 1.5 mmol $MgCl_2$ were used. PCR involved in an initial denaturation step at 94 °C for 30 sec, followed by 32 cycles of denaturation at 94 °C for 45 sec, primer annealing at 56 °C for 45 sec for primers designed for *ciaB,*

cj1349, hecA and *irgA* and 55 °C for the primer sets for *cadF* and an extension step was performed at 72 °C for 45 sec. Final extension was carried out at 72 °C for 3 min.

PCR amplified products were detected by electrophoresis on 1,5 % (w/v) agarose gel gel in 1X Tris-borate-EDTA (TBE) buffer at 100 V for 60 min. Gels were visualized after staining with the RedSafe™ gel (INtRON Biotechnology) using UV transilluminator Gel Doc™ XR+ (BioRad) with an analyst computer software (Image Lab 5.1). A Gene Ruler™ 100 bp DNA Ladder Plus (Invitrogen) was used as the molecular weight marker.

Results and discussions

The results of the molecular investigations reveals the presence of *Arcobacter butzleri* (12 on 16 strains) and *Arcobacter cryaerophilus* 1B (4 on16 strains) in Italian *Arcobacter* isolates analyzed by simultaneous methods previously described (Tab. 2). The results obtained using different assay for molecular identification methods (m-PCR, *rpoB* gene sequencing, 16S-RFLP) are concordant, but show that some methods have better performance than others.

For example, *rpoB* gene sequencing is a good method to estimate microbial diversity for its universal presence in all prokaryotes and for its capacity to contain phylogenetic information (Case *et al.,* 2007). Nonetheless, as Levican and Figueras previously observed in 2013, results obtained confirms that the performance of the m-PCR used in the present study was 100% reliable for the identification of *A. butzleri* only. However, as Figueras and colleagues observed in 2012, 16S rRNA-RFLP is a rapid, reliable, reasonably inexpensive and reproducible method for the investigation of the prevalence of *Arcobacters* in food products.

The diversity of species was also confirmed using genotyping methods. The application of both ERIC-PCR and MLST showed high variability in *Arcobacter* species characterized.

In particular, the comparison of *Arcobacter* strains analyzed by ERIC-PCR showed a high variability in *Arcobacter* species characterized.

ERIC-PCR sequences were found to be present in all 16 *Arcobacters* strains isolated in Italian laboratories and reveals that there were high variability in isolates. In fact, the analysis of the results demonstrate the presence of 12/16 different polymorphism.

BioNumerics software analysis detected three pairs of clones: two pairs of clones of *A. butzleri* were observed in mussel samples, while the same strain of *A. butzleri* isolated from mussels was detected also in an *A. butzleri* strain isolated from clam.

This data suggest that the same clone is circulating in the environment and that probably the presence of the same clone is associated with the water used during the shellfish depuration system.

Important results were also observed in the dendogram obtained by comparison of *Arcobacter* samples isolated in Italian shellfish and *Arcobacter* strains detected in different Spanish sources.

The comparison of Italian and Spanish strains reveals some close similarities. Specifically, results obtained reveal some similarities between an *Arcobacter butzleri* strain detected in Italian clam and a strain isolated in Spanish mussel. However, these results indicated possible homogeneity and their phylogenic relationship. (Fig. 1)

Comparing MLST genotype method and ERIC-PCR applied to Italian *Arcobacter* samples, similar results were observed.

In fact, the presence of clones identified by ERIC-PCR were confirmed by MLST assay.

Several new MLST alleles and sequence types were previously defined in this study, but still need to be submitted in Miller database. The analysis of MLST genes, shows that a large amount of diversity exists within the *A. butzleri* and *A. cryaerophilus* species in shellfish.

According with literature data (Lehmann et al., 2015; Figueras at al., 2014; Kayman et al., 2012; Nieva-Echevarria et al., 2013), results obtained demonstrate that to discriminate bacteria strain it is essential to combine both molecular identification and genotyping techniques.

In agreement with Miller at al., 2009, among PCR assays, the use of MLST genotyping is the best accurate and useful method to perform strain discrimination in an accurate epidemiological study but it is expensive, and requires particular levels of competence and much time. It also appears that, the application of ERIC-PCR methods, demonstrates that this system is cheaper and faster than MLST. Moreover ERIC-PCR is a useful method to perform a discrimination for the major *Arcobacter* species associated with several cases of human infection. However, the reliability of ERIC-PCR as a specific typing method is questionable in the light of the fact that results can't be

compared with other authors data because a reference database that give the possibility perform a large epidemiological study is not available.

Considering that *A. butzleri*¸ *A. cryaerophilus* and *A. skirrowii* are considered *potential human pathogen* (Collado and Figueras, 2011), and given that the risk for human health associated to these species is not fully understood at present, the evaluation of Putative Virulence Factors (*ciaB* that encodes for the invasin*, cj1349* and *cadF* that encode for the fibronectin binding protein, *irgA* encoding for the iron-regulated outer membrane protein and hecA virulence factors that encode filamentous hemagglutinin) performed in this study gives data that may be correlated with the role played by *Arcobacter* in human infection.

All *A. butzleri* and *A. cryaerophilus* isolates investigated carried *cadF* gene, while the other genes studied were mainly present only in *A. butzleri* analyzed (Tab 3).

The presence of the high prevalence of three particular virulence genes (*cadF, irgA* and *hecA*) in all *Arcobacter butzleri* suggest that this species may be more virulent than the *A. cryaerophilus* studied.

The detection of *A. butzleri* and *A. cryaerophilus*, two of the species considered *potential human pathogens* (Collado et al., 2011), and the presence of some putative virulence factor support the thesis that shellfish may represent a risk for human health especially when these products are traditionally eaten poorly cooked and/or raw.

In conclusion, the application of identification and genotyping methods, and the detection of Putative Virulence Genes, allows the possibility to investigate the impact of *Arcobacter* spp. on public health, including the exposure source and clinical gastroenteritis cases, especially when these food matrix are eaten poorly cooked or raw. Despite the low number of *Arcobacters* analyzed, the study performed suggests that the analysis of *Arcobacter* prevalence and genotype correlation between Italian and Spanish strains in Italian shellfish may be useful to perform a future epidemiological study. The fellowship between the Spanish and Italian Universities will be useful to perform of a risk analysis in shellfish and to implement new routine research also of the emerging pathogen.

Acknowledgements

This work was supported by Società Italiana di Diagnostica di Laboratorio Veterinaria (SIDiLV), which I thank for selecting my project and giving me the possibility to move to Spain and to improve this research. This experience gave me the possibility to learn the implementation of additional techniques to perform the identification and genotype bacteria. Collaborations in such international environment increased my knowledge and expertise In addition, new scientific connections focused on the creation and transfer of knowledge and technology will be established between Italian and Spanish research groups.

I wish to thank Prof. Maria José Figueras, for the professionalism, enthusiastic support, motivation and guidance through every step of this short-term mission. I also acknowledge fundamental assistance provided by my fellow Alba Pérez-Cataluña for her patience, assistance and unconditional friendship.

I would also like to thank Nuria, Fadua, Carolina, and all the members of staff at Unit of Micology and Microbiology Ambiental of the University Rovira I Virgili because all them give me full hospitality and friendship.

I'm very grateful to my phD supervisor Prof. Marilia Giupeppina Tantillo and to my phD tutor, Prof. Elisabetta Bonerba for their constant guidance, encouragement and advices. A special thanks goes to Prof Angela Di Pinto for her precious scientific support, and to all researchers and colleagues of the section of food safety of the Department of Veterinary Medicine of Bari for their *virtual* constant presence.

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Tab 1: Primers used for the 16S genus specific PCR, m-PCR, 16S rRNA-RFLP, ERIC-PCR, rpoB gene and virulence genes

Method	Primer Sequence 5' to 3'	Sequence 5' to 3'	Target	Size (bp)	Reference
16S Genus-specific PCR	ARCO I	AGAGATTAGCCTGTATTGTATC	16S rRNA	1223	Harmon and Wesley (1996)
	ARCO II	TAGCATCCCCGCTTCGAATGA			
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	---------	Versalovic *et al*. (1991)
	ERIC 2 (R)	ATGTAAGCTCCTGGGGATTCAC	Genome		Houf *et al.* (2002)
rpoB	rpoBArc15F	TCTCAATTTATGGAYCAAAC	*rpoB-gene*	900	Collado *et al.* (unpublished)
	rpoBArc24R	AGTTATATCCATTCCATGGCAT	*rpoB-gene*		
Virulence genes:					
cadF	cadF-F	TTACTCCTACACCGTAGT	*cadF*	283	Duidhal *et al.* (2012)
	cadF-R	AAACTATGCTAACGCTGGTT			
ciaB	ciaB-F	TGGGCAGATGTGGATAGAGCTTGGA	*ciaB*	284	
	ciaB-R	TAGTGCTGGTCGTCCCACATAAAG			
cj1349	cj1349-F	CCAGAAATCACTGGCTTTTGAG	*cj1349*	659	
	cj1349-R	GGGCATAAGTTAGATGAGGTTCC			
irgA	irgA-F	TGCAGAGGATACTTGGAGCGTAACT	*irgA*	437	
	irgA-R	GTATAACCCCATTGATGAGGAGCA			
hecA	hecA-F	GTGGAAGTACAACGATAGCAGGCTC	*hecA*	537	
	hecA-R	GTCTGTTTTAGTTGCTCTGCACTC			

Tab 2: Molecular identification results

N. sample	Isolate name	Country	Strain source	Collection date	16-S rRNA	m-PCR Houf 2000	*rpo-B*	RLFP
1	4	Torre a mare (BA)	*Mussles- Muytilus galloprovincialis*	January-2014	*A. butzleri*	*A. butzleri*	*A. butzleri*	*A. butzleri*
2	7*	Bari	*Mussles- Muytilus galloprovincialis*	February-2014	*A. cryaerophilus 1B*	*A. cryaerophilus*	*A. cryaerophilus 1B*	*A. cryaerophilus 1B*
3	21*	Noci (BA)	*Mussles- Muytilus galloprovincialis*	May-2014	*A. cryaerophilus 1B*	*A. cryaerophilus*	*A. cryaerophilus 1B*	*A. cryaerophilus 1B*
4	24*	Noci (BA)	*Mussles- Muytilus galloprovincialis*	May-2014	*A. cryaerophilus 1B*	*A. cryaerophilus*	*A. cryaerophilus 1B*	*A. cryaerophilus 1B*
5	25*	Noci (BA)	*Mussles- Muytilus galloprovincialis*	September-2014	*A. butzleri*	*A. butzleri*	*A. butzleri*	*A. butzleri*
6	28*	Noci (BA)	*Mussles- Muytilus galloprovincialis*	September-2014	*A. butzleri*	*A. butzleri*	*A. butzleri*	*A. butzleri*
7	34	Noci (BA)	*Mussles- Muytilus galloprovincialis*	October-2014	*A. butzleri*	*A. butzleri*	*A. butzleri*	*A. butzleri*
8	37*	Noci (BA)	*Mussles- Muytilus galloprovincialis*	October-2014	*A. butzleri*	*A. butzleri*	*A. butzleri*	*A. butzleri*
9	38	Noicattaro (BA)	*Mussles- Muytilus galloprovincialis*	January-2015	*A. butzleri*	*A. butzleri*	*A. butzleri*	*A. butzleri*
10	39	Noicattaro (BA)	*Mussles- Muytilus galloprovincialis*	January-2015	*A. butzleri*	*A. butzleri*	*A. butzleri*	*A. butzleri*
11	1V	Noci (BA)	*Clams-Tapes philippinarum*	September-2014	*A. butzleri*	*A. butzleri*	*A. butzleri*	*A. butzleri*
12	5V	Noci (BA)	*Clams-Tapes philippinarum*	October-2014	*A. butzleri*	*A. butzleri*	*A. butzleri*	*A. butzleri*
13	6V*	Noicattaro (BA)	*Clams-Tapes philippinarum*	October-2014	*A. butzleri*	*A. butzleri*	*A. butzleri*	*A. butzleri*
14	11V	Noicattaro (BA)	*Clams-Tapes philippinarum*	December-2014	*A. butzleri*	*A. butzleri*	*A. butzleri*	*A. butzleri*
15	15V	Valenzano (BA)	*Clams-Tapes philippinarum*	January-2015	*A. butzleri*	*A. butzleri*	*A. butzleri*	*A. butzleri*
16	16V	Altamura (BA)	*Clams-Tapes philippinarum*	February-2015	*A. cryaerophilus 1B*	*A. cryaerophilus*	*A. cryaerophilus 1B*	*A. cryaerophilus 1B*

*A different color is used to show clones identified by ERIC-PCR

Tab 3: Putative Virulence Factors Results

N. samples	Isolate name	Country	Strain source	Collection date	Species detected	*cadF*	*ciaB*	*cj1349*	*irgA*	*hecA*
1	4	Torre a mare (BA)	Mussles- *Muytilus galloprovincialis*	January-2014	*A. butzleri*	+	-	-	+	+
2	7	Bari	Mussles- *Muytilus galloprovincialis*	February-2014	*A. cryaerophilus 1B*	+	-	-	-	+
3	28	Noci (BA)	Mussles- *Muytilus galloprovincialis*	September-2014	*A. butzleri*	+	-	-	+	+
4	34	Noci (BA)	Mussles- *Muytilus galloprovincialis*	October-2014	*A. butzleri*	+	+	-	+	+
5	38	Noicattaro (BA)	Mussles- *Muytilus galloprovincialis*	January-2015	*A. butzleri*	+	-	-	+	+
6	39	Noicattaro (BA)	Mussles- *Muytilus galloprovincialis*	January-2015	*A. butzleri*	+	-	-	+	+
7	1V	Noci (BA)	Clams-*Tapes philippinarum*	September-2014	*A. butzleri*	+	-	-	+	+
8	5V	Noci (BA)	Clams-*Tapes philippinarum*	October-2014	*A. butzleri*	+	+	+	+	+
9	6V	Noicattaro (BA)	Clams-*Tapes philippinarum*	October-2014	*A. butzleri*	+	+	+	+	+
10	11V	Noicattaro (BA)	Clams-*Tapes philippinarum*	December-2014	*A. butzleri*	+	+	-	+	+
11	15V	Valenzano (BA)	Clams-*Tapes philippinarum*	January-2015	*A. butzleri*	+	-	-	+	+
12	1/O	Altamura (BA)	Clams-*Tapes philippinarum*	February-2015	*A. cryaerophilus 1B*	+	-	-	-	-

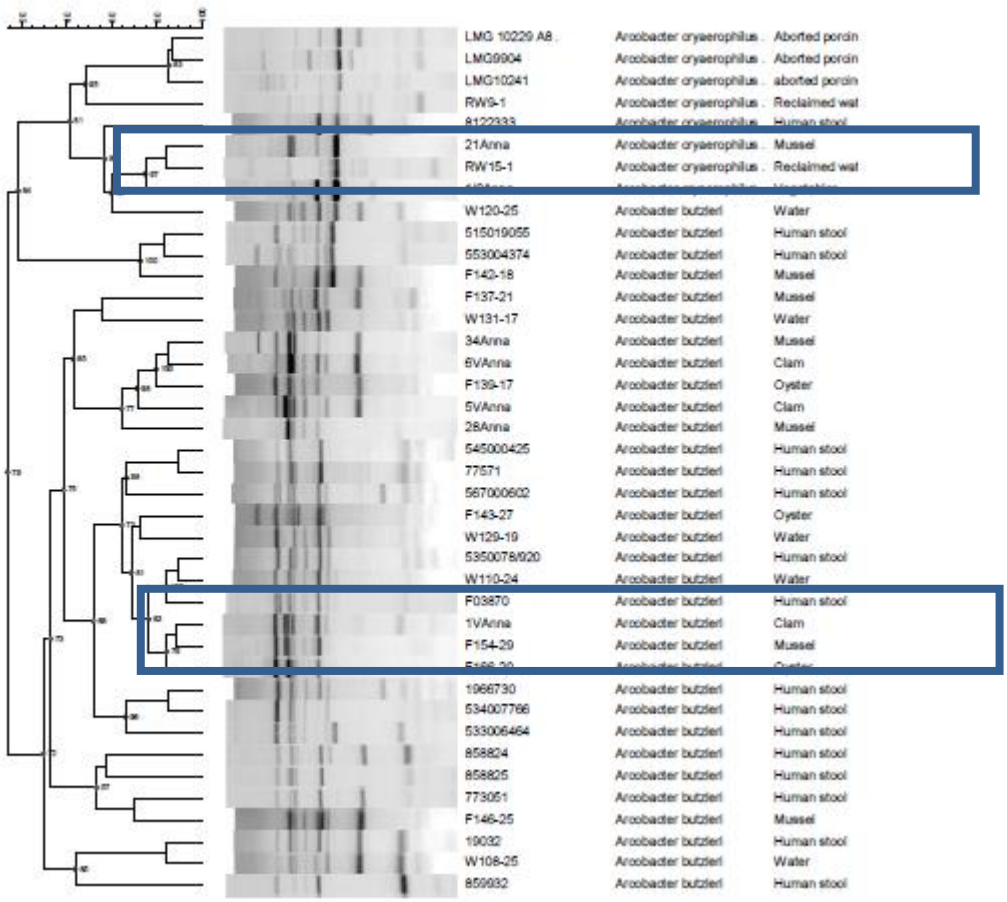

Fig. 1: ERIC-PCR dendogram showing the clustering of the Italian *Arcobacter* strain recovered in shellfish and Spanish strain recovered in different sources.