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Abstract

Considering that several recent cases of human gastroenteritis have been associated with species from the *Arcobacter* genus, and that few data are currently available about the occurrence of this genus in Italian shellfish, the aim of the present study was to evaluate the occurrence of *Arcobacter* spp. and the presence of virulence-associated genes. The approach consisted of cultural and biomolecular (multiplex-PCR and 16S-RFLP) methods identifying isolates, followed by PCR assays aimed at the *cadF, ciaB, cjl349, irgA, hecA* putative virulence genes. *Arcobacter* spp. was detected in 16/70 (22.8%) shellfish samples. Specifically, *Arcobacter* spp. was highlighted in 10/42 (23.8%) mussel and in 6/28 (21.4%) clam samples. Subsequently, biomolecular assays revealed *A. butzleri* in 12/16 (75%) and *A. cryaerophilus* 1B in 4/16 (25%) isolates. PCRs aimed at the five putative virulence genes demonstrated widespread distribution of these genes among *Arcobacter* isolates and some differences from the results published by other authors. Our research provides more information regarding the health risks associated with the consumption of raw bivalve molluscs and underlines the need to implement an adequate control plan by performing intensive and continuous monitoring in order to guarantee human health.

Keywords: *Arcobacter,* food-borne pathogen, bivalve molluscs, putative virulence factors

1. Introduction

Each year, food-borne and water-borne zoonotic diseases affect tens of millions of people (Painter et al., 2013). Most reports of food and water-borne illnesses are associated with the consumption of food of animal origin (eggs, mixed foods, fish, molluscs, chicken, dairy) or of contaminated water (EFSA and ECDC, 2015). The prevalent etiological agents involved in the outbreaks are bacteria (*Camplylobacter jejuni, Salmonella enteritidis* and *S. typhimurium*, verocytotoxigenic *E. coli,*

Listeria monocytogenes), viruses (caliciviruses) and parasites (*Cryptosporidium parvum, Cryptosporidium hominis)* (EFSA and ECDC, 2015). Despite advances in food safety, in some cases, the causative agent associated with disease may be underestimated or unknown due to the lack of a specific protocol for their detection and identification in clinical laboratories (EFSA and ECDC, 2015; Figueras et al., 2014; Levican et al., 2013; Ramees et al., 2014).

Recently, several cases of human gastroenteritis have been associated with some species belonging to the *Arcobacter* genus, a *Campylobacter*-like organism, able to grow and survive both in aerobic and microaerophilic conditions, at temperatures below 30 °C, which differentiates them from the *Campylobacter* species (Figueras et al., 2014; Van den Abeele et al., 2014).

Arcobacter is a bacterial genus of Gram-negative, slightly curved rods, positive for oxidase and usually motile, found both in animal and environmental sources (González and Ferrús, 2011; Tabatabaei et al., 2014). This genus currently encompasses 22 species (Levican et al.; 2015; Nieva-Echevarría et al., 2013; Whiteduck-Léveillée et al., 2015; Zhang et al., 2015), some of which have been associated with human and animal illnesses: *A. butzleri* and *A. cryaerophilus* have been associated with diarrhea and enterocholitis and occasionally with bacteriemia, endocarditis, and peritonitis (Collado and Figueras, 2011; Figueras et al., 2014; Hsueh et al. 1997; Kayman et al., 2012; Lau et al., 2002; Lerner at al., 1994; McGregor et al., 2015; On et al., 1995;Woo et al., 2001;Yan et al., 2000). Moreover, *A. butzleri* is the species recognised as the etiological agent of traveller's diarrhea (Jiang et al., 2010). Despite the high persistence and prevalence of these two species in clinical cases, food, and the environment, *A. skirrowii* and *A. thereius* have also been recovered in stool samples of patients with diarrhea or enterocolitis (Van den Abeele et al., 2014; Wybo et al., 2004).

The probable human pathogenic potential of *Arcobacter* may be associated with some species' ability to survive in common food-storage conditions and with their environmental survival and

rooting strategies. Bacteria from the genus *Arcobacter* have been considered natural inhabitants of environmental waters because they have been found in various water sources, including surface water, ground water, rivers, lakes, sea water, sewage and plankton (Collado et al., 2008; Collado et al., 2009; Fera et al., 2004; Moreno et al., 2003). Indeed, *A. marinus*, *A. aquimarinus*, *A. ellisii*, *A. molluscorum*, *A. venerupis* and *A. bivalviorum* have been detected in water and shellfish, confirming that these species are natural inhabitants of aquatic environments. Moreover, the presence of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* species in water and shellfish as observed by Collado et al. (2008) and Fera et al. (2004), may be associated with sewage outlets and faecal pollution (Ottaviani et al., 2013). This organism may persist in marine environmental waters under suitable conditions for survival and rooting, adhering to zooplankton as previously suggested by Collado et al. (2008) and Fera et al. (2004). Also, the ability of *A. butzleri* to persist in seawater in a VBNC (viable but non-culturable) state allows it to survive in the absence of nutrients (Fera et al., 2008). Furthermore, *A. butzleri* can persist on surfaces, given its ability to form biofilms (Assanta et al., 2002; Kjeldgaard et al., 2009).

These survival strategies, together with the virulence potential of some *Arcobacter* spp. that may have a whole suite of putative virulence genes (*cadF, cj1349, ciaB, mviN, pldA, tlyA, hecA, hecB* and *irgA*), underlining their importance in its pathogenicity (Douidah et al., 2012, Ferreira et al., 2015; Levican et al., 2013).

Considering the high prevalence of *Arcobacter* species in seawater (Collado et al., 2008; Fera et al., 2008) and the ability of bivalve shellfish, as a result of their filter-feeding activity, to bio-concentrate pathogens, the aim of the present study was to assess the presence of *Arcobacter* species carrying virulence-associated genes in shellfish from Southern Italy in order to evaluate the potential routes of *Arcobacter* spp.

2. Materials and methods

2.1 Sampling

A total of 70 shellfish samples were obtained from a local fish market in the Apulia region (SE Italy) between January 2014 and February 2015. The samples were made up of 42 mussel (*Mytilus galloprovincialis*) and 28 clam (*Tapes philippinarum*) samples, which were taken to the laboratory in cooled containers (4 °C) and processed within 24 h of purchase.

2.2 Cultural Analysis

Shellfish were aseptically prepared for analysis according to the UNI EN ISO 6887-3/2003 standard procedure (UNI EN ISO 6887-3:2003, 2003). For *Arcobacter* isolation, 10 g of flesh and intra-valvular liquid were added to 90 mL (1:10 wt/vol) of *Arcobacter* broth (Oxoid, Basingstoke, UK) supplemented with Cefoperazone, Amphotericin B and Teicoplanin (CAT selective supplement SR0174E; Oxoid, Basingstoke, UK), in sterile bags and homogenized using a stomacher (PBI International, Milan, Italy) at 11 000 rev min^{-1} for 1 min, as previously reported by other authors (Bonerba et al., 2015; Collado et al., 2009). Then the bags were closed and incubated at 30 °C under aerobic conditions for 48 h. After enrichment, 200 µL of the broth was inoculated by passive filtration onto the surface of a 0.45µm membrane filter (Sartorius Stedim Biotech GmbH, Germany), placed onto selective agar plates prepared by suspending 24 g of *Arcobacter* broth (Oxoid, Basingstoke, United Kingdom) and 12 g of agar technical no. 3 (Oxoid, Basingstoke, United Kingdom) and supplemented with a selective antibiotic mix supplement (cefoperazone [16 mg/liter], amphotericin B [10 mg/liter], 5-fluorouracil [100 mg/liter], novobiocin [32 mg/liter], and trimethoprim [64 mg/liter]) as described by Houf et al. (2001). Plates were incubated at room temperature for 30 min. After filtration, the filters were removed and the dishes were incubated at 30 °C under both aerobic and microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2), produced by

131 the Campy*Gen* gas generating system (Oxoid, Basingstoke, UK). After incubation, typical
132 *Arcobacter* colonies (i.e. small colorless, translucent, convex with an intact edge) were picked,
133 subcultured onto Blood Agar and incubated for 48h at 30 °C. The colonies were confirmed
134 morphologically by Gram staining and by determination of oxidase (Oxidase strips, Oxoid
135 Microbact, Basingstoke, UK) and catalase activity.
136

137 *2.3 Biomolecular analysis*

138 *2.3.1 DNA extraction and purification*

139 The colonies identified as *Arcobacter* spp. were transferred onto *Arcobacter* broth (BA) (Oxoid,
140 Basingstoke, UK) and incubated at 30 °C for 48h. One milliliter BA pure culture of presumptive
141 *Arcobacter* spp. was centrifuged at 7500 rpm for 10 min at room temperature. DNA extraction and
142 purification was performed with the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and
143 eluted with 80 µL AE Elution Buffer (QIAGEN, Hilden, Germany). The DNA concentration and
144 purity were established by evaluating the ratio A_{260nm}/A_{280nm} using a NanoDrop 2000/2000c
145 Spectrophotometer (Thermo Scientific, MA, USA).
146

147 *2.3.2 m-PCR*

148 In order to determine the identity of the *Arcobacter* isolates, a multiplex-PCR assay was performed
149 using primers and conditions described by Houf et al. (2000).

150

151 *2.3.3 16S rDNA-RFLP*

152 The 16S rDNA-RFLP assay described by Figueras et al. (2008) was performed to confirm the m-
153 PCR results (Levican and Figueras, 2013).
154

155 *2.3.4 Detection of virulence genes*

156 Five putative *Arcobacter* virulence genes (*ciaB, cadF, cj1349, hecA* and *irgA*) were detected using
157 the primers and conditions designed by Douidah et al., (2012).
158
159 **3. Results**
160 *3.1 Cultural Analysis*
161 The cultural analysis carried out on shellfish showed typical small, smooth, translucent, and watery
162 colonies in 16 (22.8%) of the 70 shellfish samples. All organisms resulted Gram-negative, slightly
163 curved rods, oxidase- and catalase-positive and were presumptively identified as *Arcobacter* spp.
164 Specifically, *Arcobacter* spp. were highlighted in 10/42 (23.8%) mussel and in 6/28 (21.4%) clam
165 samples.
166
167 *3.2 Molecular analysis*
168 *3.2.1 m-PCR*
169 The m-PCR carried out on the 16 isolates showed the characteristic amplicon of *A. butzleri* in 12/16
170 (75%) and the *A. cryaerophilus* amplicon in 4/16 (25%).
171
172 *3.2.2 16S rDNA-RFLP*
173 Digestion of the 16S rRNA gene with the endonuclease *MseI* produced the expected species-
174 specific *A. butzleri* and *A. cryaerophilus* 1B RFLP patterns, thus confirming the m-PCR results
175 (**Table 1**).
176
177 3.2.3 *Detection of virulence genes*
178 *Arcobacter* isolates tested by PCR for the presence of putative virulence genes showed amplicons
179 with expected sizes for the different virulence genes (**Table 2**). Indeed, all (12/12) isolates of *A.*

butzleri harbored *cadF*, *hecA* and *irgA* genes but the *ciaB* gene was present only in 5/12 and *cj1349* only in 2/12 of the isolates. All five investigated virulence genes (*cadF*, *ciaB*, *cj1349*, *irgA* and *hecA*) were simultaneously detected in only 2/12 *A. butzleri* strains. Among the *A. cryaerophilus* isolates, 4/4 strains were positive for the *cadF* gene and 3/4 and 1/4 for the *hecA* and *ciaB* genes, respectively. None of the strains possessed the *cj1349* or *irgA* genes.

4. Discussion

This is the first study of the occurrence of putative virulence genes in potentially pathogenic *Arcobacter* species in shellfish from Italy by cultural methods and biomolecular analysis. In agreement with previous studies carried out in different geographical areas (Collado et al., 2014; Collado et al., 2009; Levican et al., 2014), this study confirms that potentially pathogenic *Arcobacters* are frequently found in edible lamellibranch mollusc samples. In this sense, *A. butzleri* was the most prevalent species (75%) found in this and other studies (Collado et al., 2014; Nieva-Echevarria et al., 2013;). Although both *A. butzleri* and *A. cryaerophilus* were associated with several cases of human disease (Collado and Figueras, 2011; Figueras et al., 2014; Hsueh et al. 1997; Kayman et al., 2012; Lau et al., 2002; Lerner et al., 1994; McGregor et al., 2015; On et al., 1995; Woo et al., 2001; Yan et al., 2000), little is known about the route of infection and transmission of this species. Moreover, the mechanism of pathogenicity related to this genus still needs to be fully understood. The present research evaluated the presence of five putative virulence factors in isolates from shellfish. In agreement with previous studies carried out by Douidah et al. (2012), Karadas et al. (2013) and Tabatabaei et al. (2014), all analyzed strains harbored a high occurrence of the *cadF* gene. However, in contrast with the same authors who reported a high occurrence of the *ciaB* and *cj1349* genes and a low presence of the *irgA* gene in all *A. butzleri* isolates tested, the present study showed a high occurrence of various different genes. All the analyzed *A. butzleri* strains harbored the *irgA* and *hec* genes. In this study, all the strains of *A.*

cryaerophilus investigated harbored at least one gene i.e. *cadF (4/4), hecA (3/4)* and *ciaB* (1/4), while none of the strains studied had the *cj1349* or *irgA* genes. The *cadF* gene was widely distributed among *Arcobacter* isolates. Our results were similar to those reported by Levican et al. (2014), who found that the *irgA* gene was absent in all *A. cryaerophilus* isolates studied. However, the detection of *ciaB* was lower in our study. When comparing our results for *A. cryaerophilus* recovered from mussels and clams with those obtained by Collado et al. (2014) for the same strain and shellfish species, *cadF* and *hecA* were present in most of our samples but absent in Collado et al.'s (2014).

Similarly, in *A. cryaerophilus* recovered from a gastroenteritis case, Figueras et al. (2014) detected only the *ciaB* gene, again in contrast with our results.

The different results observed in the present study in relation to those found by other authors could be linked to several factors such as the different identification and cultural methodology used or the climatic and geographical areas where samples were collected. Moreover, further investigations into the presence and distribution of virulence factors are required. Especially, further comparison of the data between *Arcobacters* isolated from food and strains detected in clinical cases will be required if we are to understand whether there is a correlation between contaminated food consumption and disease. However, International Standard procedures are required to facilitate the comparison of data observed by other researchers.

The results of the present study provided much information regarding the health risks associated with the consumption of raw bivalve molluscs. As previously observed for *Vibrio,* this study confirms that standard shellfish purification technologies are inefficient at removing all pathogenic and potentially pathogenic bacteria (Carraro et al., 2015; Di Pinto et al., 2005; Di Pinto et al., 2006; Tantillo et al., 2004). Moreover, even though the legislation regulating food safety requires assessment of *E. coli* and *Salmonella* contamination (EC Regulation no. 1441/2007), the increase in food-borne disease related to the consumption of raw shellfish suggests that further epidemiological

data are required to establish more specific microbiological criteria in seafood and to apply new depuration technologies in order to guarantee food safety. Therefore, effective national and European food control systems are essential to protect consumer health by implementing routine research into the emerging pathogens in this food chain.

5. Conclusions

In summary, the results in the present paper demonstrate that bivalve molluscs are potential pathogenic *Arcobacter* hosts. Furthermore, additional studies are needed to provide data that will help extend knowledge and confirm the role played by contaminated shellfish consumption in human disease.

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383 **Table 1**

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

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388 **Table 2**

389 Occurrence of virulence-associated genes in *Arcobacter* isolates
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Species	Isolate	Strain source	*cadF*	*ciaB*	*cj1349*	*irgA*	*hecA*
A. butzleri	4	*Mussels- Mytilus galloprovincialis*	+	-	-	+	+
	25	*Mussels- Mytilus galloprovincialis*	+	-	-	+	+
	28	*Mussels- Mytilus galloprovincialis*	+	-	-	+	+
	34	*Mussels- Mytilus galloprovincialis*	+	-	-	+	+
	37	*Mussels- Mytilus galloprovincialis*	+	+	+	+	+
	38	*Mussels- Mytilus galloprovincialis*	+	+	+	+	+
	39	*Mussels- Mytilus galloprovincialis*	+	+	-	+	+
	1V	*Clams-Tapes philippinarum*	+	-	-	+	+
	5V	*Clams-Tapes philippinarum*	+	-	-	+	+
	6V	*Clams-Tapes philippinarum*	+	+	-	+	+
	11V	*Clams-Tapes philippinarum*	+	-	-	+	+
	15V	*Clams-Tapes philippinarum*	+	+	-	+	+
A. cryaerophilus 1B	21	*Mussels- Mytilus galloprovincialis*	+	-	-	-	+
	24	*Mussels- Mytilus galloprovincialis*	+	+	-	-	+
	7	*Mussels- Mytilus galloprovincialis*	+	-	-	-	+
	16V	*Clams-Tapes philippinarum*	+	-	-	-	-

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