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15 **Occurrence of potentially pathogenic arcobacters in shellfish.**

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35

36 **Abstract**

37 Considering that several recent cases of human gastroenteritis have been associated with species
38 from the *Arcobacter* genus, and that few data are currently available about the occurrence of this
39 genus in Italian shellfish, the aim of the present study was to evaluate the occurrence of *Arcobacter*
40 spp. and the presence of virulence-associated genes. The approach consisted of cultural and
41 biomolecular (multiplex-PCR and 16S-RFLP) methods identifying isolates, followed by PCR
42 assays aimed at the *cadF*, *ciaB*, *cjl349*, *irgA*, *hecA* putative virulence genes. *Arcobacter* spp. was
43 detected in 16/70 (22.8%) shellfish samples. Specifically, *Arcobacter* spp. was highlighted in 10/42
44 (23.8%) mussel and in 6/28 (21.4%) clam samples. Subsequently, biomolecular assays revealed *A.*
45 *butzleri* in 12/16 (75%) and *A. cryaerophilus* 1B in 4/16 (25%) isolates. PCRs aimed at the five
46 putative virulence genes demonstrated widespread distribution of these genes among *Arcobacter*
47 isolates and some differences from the results published by other authors. Our research provides
48 more information regarding the health risks associated with the consumption of raw bivalve
49 molluscs and underlines the need to implement an adequate control plan by performing intensive
50 and continuous monitoring in order to guarantee human health.

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

52

53 **1. Introduction**

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

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

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

68 *Arcobacter* is a bacterial genus of Gram-negative, slightly curved rods, positive for oxidase and
69 usually motile, found both in animal and environmental sources (González and Ferrús, 2011;
70 Tabatabaei et al., 2014). This genus currently encompasses 22 species (Levican et al.; 2015; Nieva-
71 Echevarría et al., 2013; Whiteduck-Léveillé et al., 2015; Zhang et al., 2015), some of which have
72 been associated with human and animal illnesses: *A. butzleri* and *A. cryaerophilus* have been
73 associated with diarrhea and enterocolitis and occasionally with bacteriemia, endocarditis, and
74 peritonitis (Collado and Figueras, 2011; Figueras et al., 2014; Hsueh et al. 1997; Kayman et al.,
75 2012; Lau et al., 2002; Lerner et al., 1994; McGregor et al., 2015; On et al., 1995; Woo et al.,
76 2001; Yan et al., 2000). Moreover, *A. butzleri* is the species recognised as the etiological agent of
77 traveller's diarrhea (Jiang et al., 2010). Despite the high persistence and prevalence of these two
78 species in clinical cases, food, and the environment, *A. skirrowii* and *A. thereius* have also been
79 recovered in stool samples of patients with diarrhea or enterocolitis (Van den Abeele et al., 2014;
80 Wybo et al., 2004).

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

83 rooting strategies. Bacteria from the genus *Arcobacter* have been considered natural inhabitants of
84 environmental waters because they have been found in various water sources, including surface
85 water, ground water, rivers, lakes, sea water, sewage and plankton (Collado et al., 2008; Collado et
86 al., 2009; Fera et al., 2004; Moreno et al., 2003). Indeed, *A. marinus*, *A. aquimarinus*, *A. ellisii*, *A.*
87 *molluscorum*, *A. venerupis* and *A. bivalviorum* have been detected in water and shellfish,
88 confirming that these species are natural inhabitants of aquatic environments. Moreover, the
89 presence of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* species in water and shellfish as observed
90 by Collado et al. (2008) and Fera et al. (2004), may be associated with sewage outlets and faecal
91 pollution (Ottaviani et al., 2013). This organism may persist in marine environmental waters under
92 suitable conditions for survival and rooting, adhering to zooplankton as previously suggested by
93 Collado et al. (2008) and Fera et al. (2004). Also, the ability of *A. butzleri* to persist in seawater in a
94 VBNC (viable but non-culturable) state allows it to survive in the absence of nutrients (Fera et al.,
95 2008). Furthermore, *A. butzleri* can persist on surfaces, given its ability to form biofilms (Assanta et
96 al., 2002; Kjeldgaard et al., 2009).

97 These survival strategies, together with the virulence potential of some *Arcobacter* spp. that may
98 have a whole suite of putative virulence genes (*cadF*, *cjl349*, *ciaB*, *mviN*, *pldA*, *tlyA*, *hecA*, *hecB*
99 and *irgA*), underlining their importance in its pathogenicity (Doudah et al., 2012, Ferreira et al.,
100 2015; Levican et al., 2013).

101 Considering the high prevalence of *Arcobacter* species in seawater (Collado et al., 2008; Fera et al.,
102 2008) and the ability of bivalve shellfish, as a result of their filter-feeding activity, to bio-
103 concentrate pathogens, the aim of the present study was to assess the presence of *Arcobacter*
104 species carrying virulence-associated genes in shellfish from Southern Italy in order to evaluate the
105 potential routes of *Arcobacter* spp.

106

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% O₂, 10% CO₂, 85% N₂), produced by

131 the CampyGen 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

2.3.4 Detection of virulence genes

Five putative *Arcobacter* virulence genes (*ciaB*, *cadF*, *cj1349*, *hecA* and *irgA*) were detected using the primers and conditions designed by Doudah et al., (2012).

3. Results

3.1 Cultural Analysis

The cultural analysis carried out on shellfish showed typical small, smooth, translucent, and watery colonies in 16 (22.8%) of the 70 shellfish samples. All organisms resulted Gram-negative, slightly curved rods, oxidase- and catalase-positive and were presumptively identified as *Arcobacter* spp. Specifically, *Arcobacter* spp. were highlighted in 10/42 (23.8%) mussel and in 6/28 (21.4%) clam samples.

3.2 Molecular analysis

3.2.1 m-PCR

The m-PCR carried out on the 16 isolates showed the characteristic amplicon of *A. butzleri* in 12/16 (75%) and the *A. cryaerophilus* amplicon in 4/16 (25%).

3.2.2 16S rDNA-RFLP

Digestion of the 16S rRNA gene with the endonuclease *MseI* produced the expected species-specific *A. butzleri* and *A. cryaerophilus* 1B RFLP patterns, thus confirming the m-PCR results (Table 1).

3.2.3 Detection of virulence genes

Arcobacter isolates tested by PCR for the presence of putative virulence genes showed amplicons with expected sizes for the different virulence genes (Table 2). Indeed, all (12/12) isolates of *A.*

180 *butzleri* harbored *cadF*, *hecA* and *irgA* genes but the *ciaB* gene was present only in 5/12 and *cjl349*
181 only in 2/12 of the isolates. All five investigated virulence genes (*cadF*, *ciaB*, *cjl349*, *irgA* and
182 *hecA*) were simultaneously detected in only 2/12 *A. butzleri* strains. Among the *A. cryaerophilus*
183 isolates, 4/4 strains were positive for the *cadF* gene and 3/4 and 1/4 for the *hecA* and *ciaB* genes,
184 respectively. None of the strains possessed the *cjl349* or *irgA* genes.

185

186 **4. Discussion**

187 This is the first study of the occurrence of putative virulence genes in potentially pathogenic
188 *Arcobacter* species in shellfish from Italy by cultural methods and biomolecular analysis. In
189 agreement with previous studies carried out in different geographical areas (Collado et al., 2014;
190 Collado et al., 2009; Levican et al., 2014), this study confirms that potentially pathogenic
191 *Arcobacters* are frequently found in edible lamellibranch mollusc samples. In this sense, *A. butzleri*
192 was the most prevalent species (75%) found in this and other studies (Collado et al., 2014; Nieva-
193 Echevarria et al., 2013;). Although both *A. butzleri* and *A. cryaerophilus* were associated with
194 several cases of human disease (Collado and Figueras, 2011; Figueras et al., 2014; Hsueh et al.
195 1997; Kayman et al., 2012; Lau et al., 2002; Lerner et al., 1994; McGregor et al., 2015; On et al.,
196 1995; Woo et al., 2001; Yan et al., 2000), little is known about the route of infection and
197 transmission of this species. Moreover, the mechanism of pathogenicity related to this genus still
198 needs to be fully understood. The present research evaluated the presence of five putative virulence
199 factors in isolates from shellfish. In agreement with previous studies carried out by Doudiah et al.
200 (2012), Karadas et al. (2013) and Tabatabaei et al. (2014), all analyzed strains harbored a high
201 occurrence of the *cadF* gene. However, in contrast with the same authors who reported a high
202 occurrence of the *ciaB* and *cjl349* genes and a low presence of the *irgA* gene in all *A. butzleri*
203 isolates tested, the present study showed a high occurrence of various different genes. All the
204 analyzed *A. butzleri* strains harbored the *irgA* and *hec* genes. In this study, all the strains of *A.*

205 *cryaerophilus* investigated harbored at least one gene i.e. *cadF* (4/4), *hecA* (3/4) and *ciaB* (1/4),
206 while none of the strains studied had the *cj1349* or *irgA* genes. The *cadF* gene was widely
207 distributed among *Arcobacter* isolates. Our results were similar to those reported by Levican et al.
208 (2014), who found that the *irgA* gene was absent in all *A. cryaerophilus* isolates studied. However,
209 the detection of *ciaB* was lower in our study. When comparing our results for *A. cryaerophilus*
210 recovered from mussels and clams with those obtained by Collado et al. (2014) for the same strain
211 and shellfish species, *cadF* and *hecA* were present in most of our samples but absent in Collado et
212 al.'s (2014).

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

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

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

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

234

235 **5. Conclusions**

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

240

241

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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)	<i>Mussels- Mytilus galloprovincialis</i>	January-2014	<i>A. butzleri</i>	<i>A. butzleri</i>
2	7	Bari	<i>Mussels- Mytilus galloprovincialis</i>	February-2014	<i>A. cryaerophilus</i>	<i>A. cryaerophilus 1B</i>
3	21	Noci (BA)	<i>Mussels- Mytilus galloprovincialis</i>	May-2014	<i>A. cryaerophilus</i>	<i>A. cryaerophilus 1B</i>
4	24	Noci (BA)	<i>Mussels- Mytilus galloprovincialis</i>	May-2014	<i>A. cryaerophilus</i>	<i>A. cryaerophilus 1B</i>
5	25	Noci (BA)	<i>Mussels- Mytilus galloprovincialis</i>	September-2014	<i>A. butzleri</i>	<i>A. butzleri</i>
6	28	Noci (BA)	<i>Mussels- Mytilus galloprovincialis</i>	September-2014	<i>A. butzleri</i>	<i>A. butzleri</i>
7	34	Noci (BA)	<i>Mussels- Mytilus galloprovincialis</i>	October-2014	<i>A. butzleri</i>	<i>A. butzleri</i>
8	37	Noci (BA)	<i>Mussels- Mytilus galloprovincialis</i>	October-2014	<i>A. butzleri</i>	<i>A. butzleri</i>
9	38	Noicattaro (BA)	<i>Mussels- Mytilus galloprovincialis</i>	January-2015	<i>A. butzleri</i>	<i>A. butzleri</i>
10	39	Noicattaro (BA)	<i>Mussels- Mytilus galloprovincialis</i>	January-2015	<i>A. butzleri</i>	<i>A. butzleri</i>
11	1V	Noci (BA)	<i>Clams-Tapes philippinarum</i>	September-2014	<i>A. butzleri</i>	<i>A. butzleri</i>
12	5V	Noci (BA)	<i>Clams-Tapes philippinarum</i>	October-2014	<i>A. butzleri</i>	<i>A. butzleri</i>
13	6V	Noicattaro (BA)	<i>Clams-Tapes philippinarum</i>	October-2014	<i>A. butzleri</i>	<i>A. butzleri</i>
14	11V	Noicattaro (BA)	<i>Clams-Tapes philippinarum</i>	December-2014	<i>A. butzleri</i>	<i>A. butzleri</i>
15	15V	Valenzano (BA)	<i>Clams-Tapes philippinarum</i>	January-2015	<i>A. butzleri</i>	<i>A. butzleri</i>
16	16V	Altamura (BA)	<i>Clams-Tapes philippinarum</i>	February-2015	<i>A. cryaerophilus</i>	<i>A. cryaerophilus 1B</i>

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388 **Table 2**

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

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