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Occurrence of *Arcobacter* spp. and correlation with the bacterial indicator of faecal contamination *Escherichia coli* in bivalve molluscs from the Central Adriatic, Italy

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ABSTRACT

A total of 162 samples of bivalve molluscs (45 mussels and 117 clams) collected between December 2012 and 2014 from harvesting areas of the Central Adriatic were analysed by a culturing method for the presence of *Arcobacter* spp. Species identification was performed by PCR and sequencing analysis of a fragment of the *rpoB* gene. Overall, *Arcobacter* species were detected in 30% of samples, specifically 33% clams and 22% mussels. *A. butzleri* was the most common species (20% of the samples), followed by *A. cryaerophilus* (9%) and *A. skirrowii* (1%). A seasonal association of *A. butzleri* contamination was detected. *A. butzleri* was significantly more commonly recovered from samples collected during the winter-spring period (29%) than from those of the summer-autumn (8%). *A. cryaerophilus* was cultured from 6% to 11% of the samples collected in summer-autumn and winter-spring, respectively, but these differences were not statistically significant. *A. skirrowii* was recovered from a sample of mussels harvested in May 2014. To identify associations between the occurrence of *Arcobacter* spp. and *E. coli* levels, samples were divided into groups generating results with *E. coli* at >230 MPN/100 g and *E. coli* at ≤230 MPN/100 g, the latter corresponding to EU microbiological criteria allowed for live bivalve molluscs at retail level. *A. butzleri* was significantly more commonly detected in samples with higher *E. coli* levels (48%) than in those with lower levels of *E. coli* (10%), providing evidence for considering *E. coli* as an index organism for *A. butzleri* contamination in bivalve molluscs.

1. Introduction

Marine bivalve molluscs are filter-feeding animals capable of accumulating in their tissues microorganisms pathogenic to humans occurring in the marine environment. These are either of indigenous origin, such as *Vibrio* species (Baker-Austin et al. 2010; Leoni et al. 2016; Oliver 2015), or derived from faecal contamination, such as *Salmonella* spp., enteric viruses (hepatitis A virus, norovirus, etc.) or protozoan parasites (*Cryptosporidium* spp., *Giardia* spp., etc.) (Bellou et al. 2013; Hohweyer et al. 2013; Morrison et al. 2011). The faecally derived enteric pathogens can enter the marine ecosystem via agricultural runoffs or sewage discharges (Malham et al. 2014; Fisher et al. 2014).

Hence, traditional eating habits of consuming raw or lightly cooked bivalve molluscs may result in illness due to bivalves acting as passive carriers of food-borne pathogens (Bellou et al. 2013; Potasman et al. 2002).

The genus *Arcobacter* was proposed in 1991 to group aerotolerant bacteria formerly classified in the genus *Campylobacter* (Vandamme et al. 1991). Species within this genus have been reported as associated with cases of human gastrointestinal disease and are regarded as a group of emerging human food-borne enteropathogens (Collado and Figueras 2011). Among these, *A. butzleri* is the most commonly recognised species associated with human disease, followed by *A. cryaerophilus* (Figueras et al. 2014).

Symptoms of gastroenteritis with *Arcobacter* include abdominal pain and acute or persistent watery diarrhoea (Van den Abeele et al. 2014; Vandenberg et al. 2004). Livestock animals may also act as reservoir of *Arcobacter* species associated with human disease (Ho et al. 2006).

A. butzleri has been reported as the fourth most common *Campylobacter*-like organism detected in human stool specimens in Belgium and France (Prouzet-Mauleon et al. 2006; Vandenberg et al. 2004). In a further prevalence study performed in Belgium on 6774 stools collected from patients with enteritis between 2008 and 2013, members of the genus *Arcobacter* were the fourth most common isolated group of

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pathogens (Van den Abeele et al. 2014). Prevalence studies on *Arcobacter* spp. in stool specimens from diarrhoeal patients reported a prevalence of 1.3% for *A. butzleri* and 0.3% for *A. cryaerophilus* in Portugal (Ferreira et al. 2014), 1.4% for *A. butzleri* in Chile (Collado et al. 2013), 2.67% for *Arcobacter* spp. in India (Patyal et al. 2011) and 0.9% in New Zealand, respectively (Mandisodza et al. 2012).

After excretion, these microorganisms can contaminate food and water, which are possible routes of transmission to humans (Collado and Figueras 2011). *Arcobacter* spp. have been detected in samples from sewage (Collado et al. 2008; Diergaardt et al. 2004; Fisher et al. 2014; Merga et al. 2014), drinking water (Ertas et al. 2010; Jalava et al. 2014), lakes and river waters (Collado et al. 2008, 2010; Diergaardt et al. 2004), estuarine water (Fera et al. 2010) and seawater (Collado et al. 2008; Fera et al. 2004; Maugeri et al. 2005).

In Europe, the presence of *Arcobacter* spp. has been reported in coastal waters at sites in southern Italy and the north-east of Spain (Collado et al. 2008; Fera et al. 2004; Maugeri et al. 2005).

Few studies have assessed the occurrence of *Arcobacter* spp. in shellfish (Collado et al. 2009a; Fernandez et al. 2015; Laishram et al. 2016; Levican et al., 2014; Mottola et al. 2016; Nieva-Echevarria et al. 2013; Salas-Massó et al. 2016). *Arcobacter* spp. have been reported in samples of clams from local markets and mussels from a farm in the Ebro River Delta in Catalonia, Spain, with a prevalence of 18% (10 out of 56 samples), 40% (2 out of 5 samples) for *A. butzleri,* 14% (8 out of 56 samples), 80% (4 out of 5 samples) for *A. cryaerophilus* in mussels and clams, respectively (Collado et al. 2009a). Another study from the same area reported an overall prevalence of 30% for *Arcobacter* in bivalve molluscs collected from the Fangar and Alfacs bays in the Ebro River Delta (Levican et al. 2014). In the same study, *A. butzleri* was isolated from mussels, oysters and clams, *A. cryaerophilus* from mussels and oysters and *A. skirrowii* from mussels (Levican et al., 2014). *A. butzleri* and *A. cryaerophilus* have also been cultured from clams and mussels which were sampled at the retail level in the North of Spain, with an overall prevalence of *Arcobacter* spp. of 73% (Nieva-Echevarria et al. 2013). A study from Chile reported a 23% prevalence of *A. butzleri* in mussels (Fernandez et al. 2015). All these studies documented that bivalve molluscs could be a reservoir of *Arcobacter* spp., therefore a possible human source of infection, particularly if bivalves are consumed raw or lightly cooked.

In the European Union, sanitary safety of live bivalve molluscs is currently governed under Regulation (EC) No 854/2004 (Anon 2004), which stipulates that classified production areas must be periodically monitored to check the microbiological quality of shellfish, by monitoring the bacteriological indicator of faecal contamination *Escherichia coli.*

Although an association between the presence of *Arcobacter* spp. and higher levels of bacterial indicators of faecal pollution has been reported for environmental waters (Collado et al. 2008; Collado et al. 2010), studies performed on bivalve molluscs have not previously investigated the relationship between levels of bacterial indicators of faecal contamination and *Arcobacter* spp. in shellfish (Collado et al. 2009a; Fernandez et al. 2015; Levican et al., 2014; Nieva-Echevarria et al. 2013).

This work aimed to investigate the occurrence of *Arcobacter* spp. in bivalve molluscs collected from harvesting areas of the Central Adriatic Sea between December 2012 and 2014 and study the correlation between *Arcobacter* species and levels of the bacterial indicator of faecal contamination of bivalve molluscs *E. coli*.

2. Material and methods

2.1. Preparation of shellfish samples for microbiological analysis

A total of 162 shellfish samples, 45 of mussels (*Mytilus galloprovincialis*) and 117 of clams (*Chamelea gallina*), were collected between December 2012 and December 2014 from harvesting areas of the region Marche, in the coastal area of provinces of Ancona and Macerata. Mussels were all collected from areas not requiring purification before placing on the market. For clams, 88 and 29 samples were from bivalve mollusc harvesting areas requiring- and not-requiring- purification to meet *E. coli* health standard before placing on the market, respectively.

Samples were externally cleaned with potable drinking water and aseptically prepared for analysis in accordance with ISO 6887-3 (Anon 2003).

Seventy-five grams of flesh and liquor of the prepared shellfish were diluted 1:3, by adding 2 ml of 0.1% sterile peptone per 1 g of shellfish, homogenized in a blender and further diluted in 0.1% sterile peptone water to obtain an initial suspension of 1:10. Decimal dilutions of the homogenate were prepared in 0.1% sterile peptone and *E. coli* enumeration was performed by a 5 tubes × 3 dilution Most Probable Number (MPN) procedure according to ISO/TS 16649-3, as previously described (Ottaviani et al. 2015).

2.2. Isolation and identification of Arcobacter spp.

Arcobacter spp. isolation was performed by using an enrichment step according to the method of Collado et al. (2009b). Briefly, 10 g of flesh and liquor were homogenized with 90 ml (1:10, wt/vol) of *Arcobacter* broth supplemented with cefoperazone, amphotericin B, and teicoplanin (Arcobacter-CAT broth; Oxoid S.p.A., Rodano, Milan, Italy) in stomacher bags. After aerobic incubation at 30 °C for 48 h, 0.2 ml of the broth were inoculated by passive filtration with Millipore filters (0.45 μm, 47 mm) onto blood agar plates (Trypticase soy agar supplemented with 5% sheep blood) and incubated for 48–72 h at 30 °C under aerobic conditions.

After incubation, 4–5 presumptive *Arcobacter* colonies (small, translucent, beige to off-white,1–4 mm in diameter) were confirmed with Gram stain (Gram negative, curved or slightly curved rod) and oxidase test (positive). Species identification was performed by PCR of the *rpoB* gene with primers from Korczak et al. (2006). Briefly, bacterial growth from suspect colonies were suspended in 500 μl of sterile distilled water, heated to 95 °C for 10 min, then centrifuged at 13,000 rpm for 1 min. The supernatant was either tested by PCR immediately or stored at −20 °C. A fragment of the *rpoB* gene was amplified in a final volume of 50 μl containing 1× PCR buffer (GoTaq® Flexi DNA Polymerase, Promega, Madison, USA), 0.2 mM of each dNTP, 0.5 μM of each primer (primers CamrpoB-L and RpoB-R from Korczak et al. 2006), 2.5 mM of MgCl2 (GoTaq® Flexi DNA Polymerase, Promega, Madison, USA), 0.05 U/μl of *Taq*pol (GoTaq® Flexi DNA Polymerase, Promega, Madison, USA), and 5 μl of lysate. PCR conditions were as follows: 30 cycles at 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s and a final elongation at 72 °C for 5 min. 10 μl of PCR products were checked for presence of the *rpoB* fragment by horizontal electrophoresis in 1% agarose gel containing 0.5% of ethidium bromide. Isolates were considered positive when amplification resulted in a PCR product of the expected size by comparison to a 100-bp DNA ladder and a positive control strain (*A.butzleri* LMG 10828). Negative controls, of PCR water, were included in each batch of tests in the DNA amplification.

Before performing *Arcobacter* species identification by sequencing analysis of the *rpoB* fragments, the genetic diversity of colonies isolated from the same sample was assessed using a previously described ERIC-PCR method (Houf et al. 2002).

2.3. Sequencing analysis of rpoB gene fragments

PCR products for sequencing analysis were purified with the High Pure PCR Product Purification kit Roche Diagnostics (GmbH, Mannheim, Germany). Sequencing analysis was performed in both directions with a ABI Prism® BigDye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems™, Life Technologies, Foster City, USA), according to the manufacturer's instructions, using *rpoB* primers and an automated capillary sequencer ABI Prism® 310 Genetic Analyzer (Applied Biosystems™, Foster City, USA). Nucleotide and amino acid sequences were aligned