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Arcobacter spp. in environmental waters in Sicily: occurrence, antimicrobial resistance and relationship with bacteria indicators of fecal pollution

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Introduction

Arcobacter spp. are emerging enteropathogens with a wide geographical distribution and associated with bacteraemia, gastroenteritis and diarrhea in humans. The genus *Arcobacter* was proposed in 1991 to group aerotolerant bacteria formerly classified in the genus *Campylobacter.* The presence of *Arcobacter* spp. is reported in different types of food of animal and non-animal origin and in aquatic environments. A wide range of farmed animals, in which *Arcobacter* spp. has been frequently isolated in the intestinal tract and from faeces, are asymptomatic hosts and potential sources of contamination of water, food and environment. Many *Arcobacter* species have been isolated from multiple locations, suggesting that these organisms are metabolically flexible and can survive under an array of environmental conditions. The species *Arcobacter butzleri*, *A. cryaerophilus* and *A. skirrowii* have been more commonly associated with animal and human infections. *A. butzleri* is the most important and prevalent species, classified as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002). Contaminated water is considered as an important source of *Arcobacter* infection to human beings. These bacteria have been recovered from several aquatic sources (lakes and rivers, groundwater, wastewater and seawater and from plankton). The isolation of arcobacters from drinking water have also been reported as well as *Arcobacter* outbreaks associated with contaminated water. Some authors reported associations between occurrence of *Arcobacter* spp. and bacterial indicators of fecal pollution. Furthermore, resistance to antibiotics commonly used in medical and veterinary therapy, observed in *Arcobacter* spp., highlights the importance of research in this area. Aims of the present study were to evaluate the presence of *Arcobacter* spp. from different water sources, to determine the antimicrobial resistance of the isolates and to assessed the possible correlation with the levels of fecal indicator bacteria (*E. coli* and enterococci).

Materials and methods

A total of 100 water samples were collected from different sites in Sicily, between February and December 2017, and analyzed for the presence of *Arcobacter* by bacteriological and molecular methods (multiplex-PCR); in this study it was considered interesting to include seaweed samples, taken together with samples of seawaters. The samples examined are reported in Table 1. Bacteriological method: 200 ml of each water sample were filtered using a 0.45 µm nitrocellulose membrane filter (Sartorius). The filters were placed into sterile bags with 30 ml of *Arcobacter* enrichment broth (Oxoid, UK) supplemented with Cefoperazone, Amphotericin B and Teicoplanin (CAT) selective supplement (Oxoid, UK) and incubated a 30 °C for 48h under aerobic condition. After incubation, 200 µl of the broth were then dropped onto the surface of 0.45 µm nitrocellulose membrane filter (Sartorius), placed onto two selective agar plates: trypticase soy agar (TSA) supplemented with 5% Laked Horse blood (Oxoid) with CAT and modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) supplemented with CAT (Fig 1). Plates were incubated at room temperature for 30 min, after the filters were removed and the plates were incubated at 30°C for 48h to 72 h under aerobic conditions. Subsequently, suspected colonies grown within the filter area with a diameter between 0.5 mm and 2 mm, were picked, subcultured onto blood agar and incubated at 30 °C for 48h (Fig 2, 3). The isolates referable at *Arcobacter* genus (Gram negative, spiral- shaped, motile, oxidase and catalase positive, urease negative), were stored in 20% (v/v) nutrient broth –glycerol at -80 °C, for subsequent molecular identification. Biomolecular method: total DNA from each characterized *Arcobacter* isolate was extracted according to the protocol developed by Houf et al, 2000 and as also described in Ertas et al, 2010. The primers and PCR assay conditions previously described by Houf et al, 2000 were used for specific identification of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. The primers amplify a 401-bp fragment from *A. butzleri*, 257-bp fragment from *A. cryaerophilus*, and a 641-bp fragment from *A. skirrowii*. The m-PCR procedure is described in Di Noto et al, 2018. DNA from reference strains *A. butzleri* (NCTC 12481), *A. cryaerophilus* (NCTC 11885) and *A. skirrowii* (NCTC 12713) were used as positive controls (Fig 4). Antimicrobial susceptibility testing of the *Arcobacter* isolates against 10 antibiotics was performed on Mueller-Hinton agar (Oxoid) by disk diffusion method according to the guidelines of the Clinical and Laboratory Standard Institute (CLSI). Amoxicillin-clavulanic acid (AMC, 30µg), ampicillin (AMP, 10µg), cefalotin (KF 30µg), cefotaxim (CTX, 30µg), ciprofloxacin (CIP, 5µg), erythromycin (E, 10µg), gentamicin (CN, 10 µg), nalidixic acid (NA 30µg), streptomycin (S, 10µg), tetracycline (TE, 30 µg) disks (OXOID, UK) were used. Enumeration of fecal indicator bacteria: MPN procedures Colilert®-18/Quanti-Tray (IDEXX) for *E. coli* (ISO 9308-2: 2012) and Enterolert®-E/Quanti-Tray (IDEXX) for enterococci (AFNOR IDX 33), according to the manufacturer's instructions, were used in the water samples studied.

Results and conclusions

Arcobacter spp. were detected in 27 (27%) out of 100 samples examined (Table 1). All drinking water samples analysed were negative. However, *Arcobacter* spp. has been identified in a sample of non-chlorinated source water (spring water) used for drinking. All the isolates analyzed with multiplex PCR (m-PCR) at species level, showed the characteristic amplicon of *A. butzleri* in 27/28 (96.4%) and of *A. cryaerophilus* amplicon in 1/28 (3.8%) (Table 1). *A. butzleri* and *A. cryaerophilus* were co-isolated from one water sample of artificial pond with acquatic birds. All the stains isolated by seaweed samples, were identified as *A. butzleri*. The specie *A. skirrowii* was not detected in any of the samples. All the isolates of *Arcobacter* spp. were found to be resistant to ampicillin, cefalotin, cefotaxim, nalidixic acid and tetracycline, and susceptible to gentamicin, streptomycin and erytromycin (except two isolated from sea waters) and to ciprofloxacin (except one isolate of *A. butzleri* from river and one isolate of *A. cryaerophilus* from pond) (Table 2). Multidrug resistance, defined as resistance to three or more tested antibiotics, was observed in all isolates tested. The *E. coli* and enterococci levels obtained in all positive water samples examined for *Arcobacter* spp. are shown in Table 3. Some sample positive for *Arcobacter* had levels of *E.coli* and/or enterococci above the standards estabilished in the European Directive for the bathing waters (2006/7/EC), for example in 3/9 (33.3%) and in 1/3 (33.3%) of the river and seawater samples respectively: in a river sample levels of $1.7x10^6$ MPN/100ml of *E.coli* and $2.0x10^5$ MPN/100ml of enterococci were detected. In the same water sample of artificial pond in which *A. butzleri* and *A. cryaerophilus* were isolated, the levels of *E. coli* and enterococci were $1.8x10^3$ MPN/100ml and $1.2x10^3$ MPN/100ml respectively. However, *A. butzleri* has indeed been detected even in samples with low or completely absent levels of bacterial indicators of fecal contamination: *A. butzleri* was identified in a non-chlorinated source water sample (spring water) used to drink with 0 MPN/100 ml of *E. coli* and enterococci. This work supports previous studies of arcobacters findings in environmental water samples and their presence is recognized as a potential risk for human health. Moreover, our results show that pathogen bacteria of the genus *Arcobacter* may be present in aquatic sources, even with low or no values of *E.coli* and enterococci. It is important to deepen these investigations mainly in the water, which can be used for water supply and irrigation, to better assess the dangers associated with human health. Furthermore, it is recognized that the waters represented a reservoir of antibiotic resistance genes.

Sample (source)	No. of samples	*Arcobacter* spp. (%)	*A.butzleri*	*A. cryaerophilus*
Rivers	11	9 (81.8)	9	-
Streams	5	2 (40)	2	-
Ponds	8	5 (62.5)	5	1
Well water	20	2 (10)	2	-
Springs water	17	1 (5.8)	1	-
Drinking water	12	0	-	-
Seawater	21	3 (14.2)	3	-
Seaweed	6	5 (83.3)	5	-
Total	100	27 (27%)	27 (96.4%)	1 (3.6%)

Table 1: Prevalence of *Arcobacter* species in the water samples collected

Fig 1: cultural method with membrane filtration; Fig 2-3: suspected colonies of *Arcobacter* in mCCDA and in blood agar

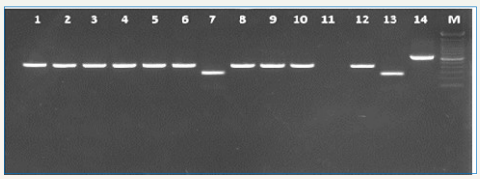

Fig 4: Multiplex PCR results of *Arcobacter* isolates. Lanes 1-6: *A. butzleri*; lane 7: *A. cryaerophilus*; lanes 8-10: *A. butzleri*; lane 11: negative control; lane 12: positive control (*A. butzleri*, NCTC 12481); lane 13: positive control (*A. cryaerophilus*, NCTC 11885); lane 14: positive control (*A. skirrowii*, NCTC 12713); lane M: 100 bp DNA Ladder

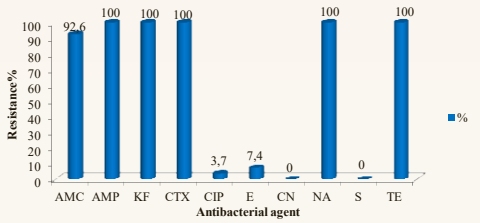

Table 2: Antibiotic resistance (%) of *A. butzleri* isolates

No. of sample	Locality (province)	Type of sample	*Arcobacter* species isolated	E. coli MPN/100 ml	enterococci MPN/100 ml
1	Palermo	River	*A.butzleri*	$1.7x10^6$	$2.0x10^5$
2	Trapani	River	*A.butzleri*	$1.9x10^3$	$7.7x10^2$
3	Trapani	River	*A.butzleri*	$2.3x10^2$	$2.0x10^1$
4	Agrigento	River	*A.butzleri*	$2.1x10^2$	$8.4x10^1$
5	Agrigento	River	*A.butzleri*	$1.5x10^2$	$3.5x10^1$
6	Messina	River	*A.butzleri*	$1.3x10^2$	$7.0x10^1$
7	Messina	River	*A.butzleri*	$2.0x10^1$	8 0
8	Messina	River	*A.butzleri*	$1.1x10^1$	$1.0x10^1$
9	Palermo	River	*A.butzleri*	$1.6x10^3$	$6.3x10^1$
10	Palermo	Stream	*A.butzleri*	$7.7x10^2$	$5.8x10^2$
11	Palermo	Stream	*A.butzleri*	$4.1x10^2$	$5.0x10^1$
12	Agrigento	Artificial Pond	*A.cryaerophilus* *A.butzleri*	$1.8x10^3$	$1.2x10^3$
13	Agrigento	Artificial Pond	*A.butzleri*	$1.2x10^3$	$5.0x10^2$
14	Palermo	Artificial Pond	*A.butzleri*	$3.0x10^2$	$1.3x10^2$
15	Palermo	Artificial Pond	*A.butzleri*	$1.2x10^1$	$1.1x10^1$
16	Agrigento	Artificial Pond	*A.butzleri*	8.0	$1.1x10^1$
17	Agrigento	Well water	*A.butzleri*	0	0
18	Agrigento	Well water	*A.butzleri*	$8.0x10^1$	0
19	Palermo	Spring water	*A.butzleri*	0	0
20	Palermo	Seawater	*A.butzleri*	$7.2x10^2$	$4.9x10^2$
21	Palermo	Seawater	*A.butzleri*	$4.0x10^1$	$3.0x10^1$
22	Messina	Seawater	*A.butzleri*	$2.8x10^1$	$1.2x10^1$

Table 3: *E.coli* and enterococci levels in water samples

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