Detection of *Arcobacter* spp. in food products collected from Sicilia region: A preliminary study

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Abstract

The aim of the study was to evaluate the occurrence of *Arcobacter* spp. in food samples collected from Sicilia region. A total of 91 food products of animal origin (41 meat, 17 fresh milk, 18 shellfish) and 15 samples of fresh vegetables, were examined by cultural method and confirmed by biochemical analysis and PCR methods. The detection of *Arcobacter* spp. was performed, after selective enrichment, on two selective agar plates: Arcobacter agar and mCCD (modified charcoal cefoperazone deoxycholate) agar supplemented with CAT (Cefoperazone, Amphotericin B and Teicoplanin). *Arcobacter* species were isolated using the membrane filtration technique. In 13 (14.3%) out of the 91 tested samples, the presence of *Arcobacter* spp. was found: the isolates were confirmed by multiplex PCR and identified as belonging to the species *A. butzleri* and *A. cryaerophilus.* The highest prevalence rate was observed in chicken meat (8.8%) followed by shellfish (3.3%). Negative results have been obtained for raw milks and vegetables samples. The preliminary study highlights the importance of this emerging pathogen and the need for further studies on its prevalence and distribution in different types of food for human consumption.

Introduction

During recent years, *Arcobacter* spp. has been identified as an emerging foodborne zoonotic pathogen worldwide (Ho *et al*., 2006) and associated with enteritis and abortion in animals and bacteraemia, gastroenteritis and diarrhea in humans (Jiang *et al.*, 2010; Figueras *et al.*, 2014; Van den Abeele, 2014). Three species of *Arcobacter* namely *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* are more commonly associated with clinical conditions (Collado and Figueras 2011; Ramees *et al.*, 2017). In particular, *A. butzleri* has been classified as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002).

The genus *Arcobacter* was proposed in 1991 to group aerotolerant bacteria formerly classified in the genus *Campylobacter* (Vandamme *et al.*, 1991). To date, 27 species of *Arcobacter* genus have been reported with a significant genetic diversity that have been isolated from different sources, including domestic and wild animals, birds, foods of animal origin, vegetables (Ramees *et al.*, 2017) and, recently, from marine gasteropod mollusks (Tanaka *et al.*, 2017). Arcobacters have also been recovered from a variety of foods of animal origin, namely meat (chicken, beef, pork), raw milk and seafood (bivalve mollusks) and also from water and vegetables (Rivas *et al.*, 2004; Collado *et al.*, 2008; Collado and Figueras 2011; Shah *et al.*, 2011; Levican *et al.*, 2014; Molva and Atabay, 2016; Mottola *et al.*, 2014a, 2016b).

Poultry species particularly act as an important reservoir of *Arcobacter* spp. and as a major source of infection spread (Houf *et al.*, 2002; Hassan, 2017; Ramees *et al.*, 2017).

Arcobacter spp. infection in human may be associated to the consumption and/or manipulation of contaminated raw or poorly cooked food of animal origin and/or contaminated water (Collado and Figueras 2011). Some studies also show the presence of *Arcobacter* spp. in dairy farm as sources of milk and milk product contamination (Serraino *et al.*, 2013; Giacometti *et al.*, 2015), in pre-cut RTE vegetables (Mottola *et al.*, 2016a) and in shellfish (Mottola *et al.*, 2016b; Leoni *et al.*, 2017).

In the last years, DNA- based assays used for the identification of *Arcobacter* species have been developed, more rapid and with higher specificity than conventional identification methods, among which Multiplex PCR (Houf *et al.*, 2000; Levican and Figueras, 2013).

The present study was designed with an aim to know the occurrence of *Arcobacter* spp. in various food samples of animal origin and in fresh vegetables collected in Sicilia region by utilizing both bacteriological and molecular methods.

Materials and Methods

Sample collection

A total of 91 food products of animal origin (41 meat products, 17 raw milk, 18 live bivalve mollusks) and 15 samples of fresh vegetables were collected from Sicilia region and analysed between January and June 2017. Particularly were examined n=15 poultry samples (chicken quarters, wings and carcasses), n=10 beef meat (rolled and minced meat), n=16 pork meat (minced and sausage), n=17 of bovine raw milk, n=13 mussels (*Mytilus galloprovincialis*) and n=5 clams (*Tapes philippinarum*) and n=15 of variety fresh vegetables (6 lettuce, 4 celery, 2 parsley, 3 artichokes). Vegetables and meat samples were collected from local retails markets while raw milk from dairy farms. Some carcasses of poultry have been withdrawn to the production. Bivalve mollusks came from classified relaying and production areas of the Sicilia region. All samples were transported to the laboratory, kept cool and analyzed within 24 h.

Isolation

For isolation of *Arcobacter,* 25 g of samples (meat, shellfish, vegetables) were aseptically inoculated in a 1:10 ratio in *Arcobacter* enrichment broth (Oxoid, UK) supplemented with Cefoperazone, Amphotericin B and Teicoplanin (CAT) selective supplement (SR0174, Oxoid, UK), homogenized with stomacher and incubated at 30°C under microaerophilic condition for 48h (Bonerba *et al*., 2015).

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The skin of the neck, when it present, has been withdrawn by carcasses of poultry. Each raw milk sample was previously centrifuged at 3500 × g for 10 min at 22°C, the upper phase removed and the sediment (20 mL) was added to 20 mL of enrichment broth (Ertas *et al.*, 2010) and incubated as before.

Subsequently, 200 µL of the broth was filtered using 0.45 µm pore size nitrocellulose membrane filters (Sartorius), placed onto two selective agar plates: trypticase soy agar (TSA) supplemented with 5% defibrinated sheep blood and with CAT (Oxoid, UK) and modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) supplemented with CAT antibiotics (Mottola *et al.*, 2016a; Ramees *et al.*, 2017). After a incubation for 60 min at 30°C, the filters were removed and the plates were incubated under aerobic conditions at 30°C for 48h. Subsequently, presumptive *Arcobacter* colonies (small colourless, translucent, convex with an entire edge) were picked, subcultured onto blood agar and incubated at 30°C for 48h. Purified isolates were further confirmed morphologically by Gram staining and biochemical analysis (catalase, oxidase, urease tests and motility, indoxyl acetate hydrolysis, salt tolerance and growth on McConkey agar). 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, after molecular identification.

Molecular analysis

DNA Extraction

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). Five colonies of each strain grown on blood agar were suspended in 1 mL of sterile distilled water and centrifuged at 16,000 g for 10 min at 10°C. Bacterial cell pellets were washed with 500 µL of sterile distilled water and then 100 µL of the suspension boiled in thermomixer for 10 min to lyse the cells. After another centrifugation (16,000 g for 10 min), 2 mL of each supernatant was used as the DNA template for the multiplex PCR assay.

Multiplex PCR

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* (Table 1). PCR reactions were performed in a 50 µL reaction mixture (2X PCR master mix Promega) contained: 2 µL template DNA, 5 µL of 10× PCR buffer, 1.25 mM MgCl2, 0.2 mM deoxynucleoside triphosphate mixture, 50 pmol of each of the primers, ARCO, BUTZ, CRY1, CRY2, 25 pmol of the primer SKIR and 1.5 U Taq DNA polymerase (Ertas *et al.*, 2010).

PCR amplification was performed in an automatic thermocycler (2720 Applied Biosystems) with an initial denaturation at 94°C for 2 min, 32 cycles of denaturation (94°C, 45 s), primer annealing (61°C, 45 s) and final extension (72°C, 30 s). The amplification products were then separated in 1.5% agarose gels with SYBR Safe DNA gel stain, at 100 V for 40 min and the bands were visualized with a UV transilluminator (GelDoc, Euroclone).

DNA from reference strains *A. butzleri* (NCTC 12481), *A. cryaerophilus* (NCTC 11885) and *A. skirrowii* (NCTC 12713) were used as positive controls and sterile distilled water was used as negative control.

Results

Microbiological analysis

The cultural characteristics, the mor-

Table 1. Oligonucleotide primers for amplification of *Arcobacter* spp.

A. butzleri,	BUTZ ARCO	5'-CCTGGACTTGACATAGTAAGAATGA-3' 5'-CGTATTCACCGTAGCATAGC-3'	401bp
A. skirrowii	SKIR ARCO	5'-GGCGATTTACTGGAACACA-3' 5'CGTATTCACCGTAGCATAGC-3'	641bp
A. cryaerophilus	CRY1 CRY2	5'-TGCTGGAGCGGATAGAAGTA-3' 5'-AACAACCTACGTCCTTCGAC-3'	257bp

Table 2. Prevalence of *Arcobacter* spp in the samples examined.

Type of sample	N. examined	Positive samples (%)	*Arcobacter* spp. (PCR multiplex)
Chicken meat	15	8 (8.8)	*A. butzleri*
Meat products	26	2 (2.2)	*A. butzleri*
Bivalve mollusks	18	3 (3.3)	*A. butzleri* (2) *A. cryaerophilus* (1)
Bovine raw milk	17	0	
Fresh vegetables	15	0	
Total	91	13 (14.3)	*A. butzleri* (92.3) *A. cryaerophilus* (7.7)

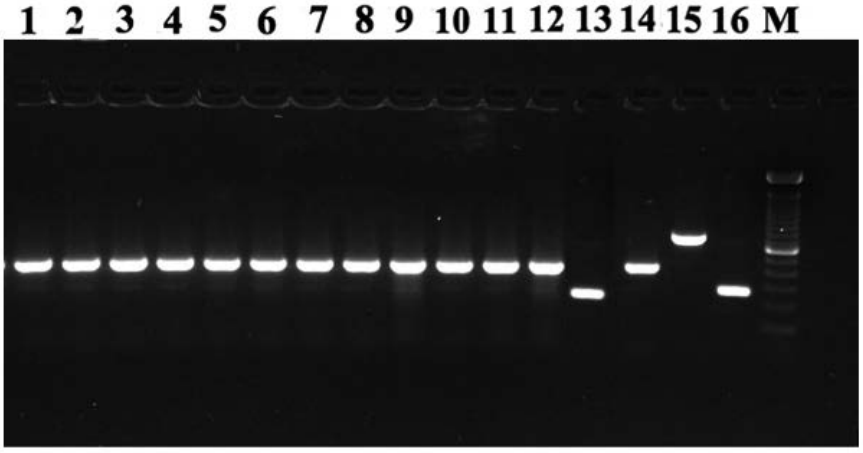

Figure 1. Identification of *Arcobacter* isolates by multiplex PCR. Lanes 1-12 *A. butzleri* isolates; lane 13 *A. cryaerophilus* isolate; lanes 14-15-16 positive controls (*A. butzleri, A. skirrowii, A. cryaerophilus*), lane M: 100 bp ladder.

phological examination and the biochemical analysis performed on samples analysed, have allowed the isolation of presumptive *Arcobacter* species in 13 (14.3%) of the 91 samples examined (Table 2). The suspect colonies showed typical white to whitish-gray color, small (2-4 mm) diameter, convex and translucent aspect; Gram's negative staining, oxidase and catalase positive and urease negative test revealed presumptive *Arcobacter* spp. isolates. Specifically *Arcobacter* spp. was highlighted in 8/15 (8.8%) chicken meat, in 2/26 (2.2%) minced meat (beef and pork) and in 3/18 (3.3%) bivalve mollusks (1 sample of mussel and 2 of clams). In the samples of chicken, *Arcobacter* were identified in the carcasses, especially by the skin of the neck. The samples of bovine raw milk and of fresh vegetables have tested negative (Table 2).

Biomolecular analysis

All the presumptive isolates were confirmed as *Arcobacter* spp. by molecular analysis with multiplex PCR (m-PCR). The molecular analysis carried out on the 13 isolates, showed the characteristic amplicon of *A. butzleri* in 12/13 (92.3%) and the *A. cryaerophilus* amplicon in 1/13 (7.7%) (Figure 1). Therefore, based on m-PCR, the most prevalent species in the food samples analysed, were *A. butzleri* (n=12) followed by *A. cryaerophilus* (n=1).

Discussions

Arcobacter spp. has been reported to be as an emerging hazard for the public health (Collado and Figueras, 2011; Ramees *et al.*, 2017). The current state on the transmission of arcobacters to human, suggests that the potential routes are represented by food and water contaminated: several reports showed that *A. butzleri* is the most common species and has been associated with human disease, such as enteritis, severe diarrhea, bacteremia and septicemia (Ferreira *et al.*, 2014).

Arcobacter spp. have been isolated from a variety of food products for human consumption (chicken, pork, beef meat, raw milk and dairy products, seafood, vegetables) (Ramees *et al.*, 2017). Chicken meat particularly has been reported with highest prevalence for *Arcobacter* spp. followed by pork and beef (Shah *et al.*, 2011). In general, the presence of these microrganisms in food processing environments, indicates possible persistence or cross contamination (Houf *et al.*, 2002). Lehmann *et al.* (2015) reported the prevalence of *Arcobacter* spp. as 27% from poultry meat and 2% from minced meat (beef and pork). Similarly, De Smet *et al.* (2010) reported the presence of *Arcobacter* from pre- and post-chilled bovine carcasses indicating the need for hygienic practices to interrupt the transmission cycle. In all these works, *A. butzleri* was the species most frequently isolated. A recent study on chicken meat in Turkey, report the prevalence of the species *A. butzleri*, followed by *A. cryaerophilus* and *A. skirrowii*: based on the type of samples, the carcasses resulted the most contaminated, followed by drumsticks (Molva *et al.*, 2016). Several studies have shown the occurrence of *Arcobacte*r spp. in shellfish (Collado *et al.*, 2009; Laishram *et al.*, 2016; Levican *et al.*, 2014; Mottola *et al.*, 2016a; Leoni *et al.*, 2017). In Italy, Mottola *et al.* (2016a), report a prevalence of *Arcobacter* spp. in the 23,8% of mussel and in the 21,4% of clam samples, collected from local fish market in the Apulia region: the isolates were identified as *A. butzleri* (75%) and *A. cryaerophilus* (25%). A survey of the occurrence in mussels and in clams from the Central Adriatic Sea, has been detected *Arcobacter* spp. in 30% of samples (33% and 22% respectively) and *A. butzleri* shall be reported as the most common species (20%) followed by *A. cryaerophilus* (9%) and *A. skirrowii* (1%) (Leoni et al., 2017). Arcobacters have been also detected in fresh vegetables such as lettuces in Spain (Gonzales and Ferrus, 2011), in a spinach-processing plant (Hausdorf *et al.*, 2013) and from pre-cut ready-to-eat vegetables (lettuces, spinach, rocket, valerian) (Mottola *et al.*, 2016b).

In our study, thirteen samples (14.3%) were positive for *Arcobacter* spp. by culture method: the most contaminated samples were the chicken meat (8.8%) followed by shellfish (3.3%) and meat products (2.2%). As reported in the literature, the chicken meat, especially poultry carcasses, proved to be the most contaminated samples followed by pork and beef, especially minced meat (De Smet *et al.*, 2010; Lehmann *et al.*, 2015; Shah *et al.*, 2011). In this study, *Arcobacter* spp. were isolated from chicken carcasses and the samples of the most contaminated meat products were minced meat. Few samples were examined in this study, but the results obtained were similar to those reported by other authors.

Regarding bivalve mollusks, we instead found the clams more contaminated than mussels. *Arcobacter* spp. were not isolated from the samples of bovine raw milk and of fresh vegetables analyzed: probably a majority numbers of samples must be collected and among these latter, particularly the broad-leaved vegetables should be more examined. In fact, according to some authors, the vegetables do not seem to be a reservoir for *Arcobacter* spp. but this type of food, broad-leaved vegetables particularly, can be contaminated through irrigation water as well as postharvest washing (Gonzales *et al.,* 2017; Hausdorf *et al.*, 2013).

At the moment, preliminary data obtained show a lower prevalence in the samples examined than bibliographic data and need further analysis.

However, the results of the work also show that the application of the implemented cultural method and the use of membrane filtration, as indicated by other authors, result to be more effective for the isolation of *Arcobacter* spp. The molecular analysis with multiplex PCR (m-PCR) allowed to confirm as *Arcobacter* spp. all the presumptive isolates.

In our study, the m-PCR method identified the species *A. butzleri* and *A. cryaerophilus*, which were recovered from 92.3% and 7.7% of the samples, respectively. *A. butzleri* was the predominant species present in samples analyzed.

However, the m-PCR technique described by Houf *et al.* (2000) is a sensitive assay targeting the 16S and 23S rRNA genes for the simultaneous identification of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. This PCR method has the advantage of 100% reliable identification of *A. butzleri*, but false positive reaction occurs for *A. cryaerophilus* and *A. skirrowii* (Levican and Figueras, 2013).

Therefore, other techniques for the correct identification of these species and of other potential pathogenic *Arcobacter* spp., should be used (Levican and Figueras, 2013; Ramees *et al.*, 2017).

Conclusions

In summary, the preliminary results obtained in the present work, demonstrate that food products of animal origin can be vehicle of potential pathogenic *Arcobacter*. These results of the occurrence of *Arcobacter* spp. also can add new data available for this important zoonotic pathogen. Furthermore, additional studies are needed to provide information on its prevalence and distribution in different types of food for human consumption.

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