

Occurrence and characterization of *Arcobacter* spp. from ready-to-eat vegetables produced in Southern Italy

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Abstract

Given that the number of foodborne illness outbreaks linked to the consumption of ready-to-eat vegetables has been widely documented and considering that data on the occurrence of *Arcobacter* spp. in such foodstuffs are lacking, the aim of the present study was to evaluate the presence of *Arcobacter* spp. and the occurrence of virulence factors as well as to genotype *Arcobacter* spp. in ready-to-eat (RTE) vegetable samples, using cultural and biomolecular assays. *Arcobacter* spp. was detected in 16/110 (14.5%) samples, with *A. butzleri* being detected in 15/16 and *A. cryaerophilus* in 1/16 isolates. PCRs aimed at the nine putative virulence genes demonstrated widespread distribution of such genes among *A. butzleri* and *A. cryaerophilus* isolates. In addition, multilocus sequence type (MLST) analysis revealed a low genetic diversity within the arcobacters isolates. The results underline the need to develop an appropriate surveillance system based on biomolecular characterization for an integrated microbiological risk assessment of ready-to-eat vegetables, and consequently of composite foods.

Introduction

A great number of recent foodborne illness outbreaks reveal a strong link with leafy green vegetables, mostly lettuce and spinach and ready-to-eat (RTE) salads made with them (EFSA and ECDC, 2018).

A wide range of well-known pathogens (e.g. *Listeria monocytogenes*, *Salmonella* spp., *Bacillus cereus*, *Thermophilic Campylobacter*, *Yersinia enterocolitica*)

have been shown to be responsible for most vegetable-related illness. However, based on surveillance and monitoring reports for food and waterborne disease outbreaks in the European Union, a large number of foodborne illnesses are associated with unknown aetiological agents (EFSA and ECDC, 2018). Among these, emerging pathogens from the genus *Arcobacter* might be involved (Mottola *et al.*, 2016a).

The genus *Arcobacter* includes 29 species, some of which have gained growing importance for public health (Figueras *et al.*, 2014; Barboza *et al.*, 2017). Specifically, *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. thereius* are recognized as emerging zoonotic agents, most likely to be causative agents of human diseases and animal illnesses (Barboza *et al.*, 2017; Ferreira *et al.*, 2017). Also, a recent case of bacteremia in Maryland associated with *A. mytili* was described by Vasiljevic *et al.* (2019). The infectious dose has not yet been established and the incidence of arcobacteriosis seems to be low compared with other foodborne microorganisms, probably both because these bacteria are not routinely investigated and because there is no standardized accurate analytical method for their detection and characterization (Figueras *et al.*, 2014). However, point-source *Arcobacter* spp. outbreaks have been associated with exposure to faecally-contaminated drinking water wells, as well as with the manipulation or consumption of contaminated raw or poorly-cooked food or direct contact with animals (Ramees *et al.*, 2017). Arcobacters have been isolated from chicken meat, milk, pork and seafood (Mottola *et al.*, 2016a; Ramees *et al.*, 2017). Recently, close contact with pets has been suggested as another potential infection source for humans (Fera *et al.*, 2009). The presence of *Arcobacter* spp. in vegetables is known, but few data are currently available about its occurrence in RTE vegetables (Mottola *et al.*, 2016b; González *et al.*, 2017).

Given the limited information about the occurrence of arcobacters in vegetables and the wide use of vegetables in the preparation of composite foods (Mottola *et al.*, 2020), the aim of the present study was to assess the occurrence, the presence of virulence-associated genes and genotyping isolates of *Arcobacter* spp. isolated from RTE vegetables, in order to elucidate and achieve a specific risk assessment for the sector.

Materials and Methods

Sampling

The occurrence of *Arcobacter* spp. was investigated in 110 RTE vegetables collected

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from a RTE vegetables processing plant from Apulia region (SE, Italy), during a self-control routine survey. Specifically, the samples were made up of a final product, including 56 lettuces, and 54 rocket salads. All samples were transferred to the laboratory in cooled containers at 4±2°C and processed within 4 h of sampling.

Cultural analysis

The isolation of *Arcobacter* spp. from vegetables was performed in accordance with González and Ferrús (2011) and with Mottola *et al.* (2016b, 2020). Briefly, 20 g of sample were added to 180 mL (1:10 wt/vol) of Arcobacter Broth (AB) (Oxoid, Milan, Italy), in sterile bags, and homogenized using a stomacher (PBI International, Milan, Italy) at 11 000 rev min⁻¹ for 2 min. Then, 20 mL of the stomacher-processed samples was added to 20 mL of double-strength AB supplemented with Cefoperazone, Amphotericin B and Teicoplanin (CAT) (CAT selective supplement; Oxoid, Milan, Italy). The samples were then incubated at 30°C under aerobic conditions for 48 h.

After enrichment, 200 μ L of the broth was dropped onto the surface of a 0.45 μ m pore-size nitrocellulose membrane filter (Sartorius Stedim Biotech GmbH, Germany), placed onto plates of Trypticase Soy Agar (TSA) (Oxoid, Milan, Italy) supplemented with 5% of sheep blood.

Plates were incubated at room temperature for 30 min. After filtration, the filters were removed and the plates were incubated at 30°C both under aerobic and microaerobic conditions.

After incubation, one *Arcobacter*-like colony per sample (i.e. small colourless, translucent, and convex with an intact edge) was picked, subcultured onto TSA supplemented with 5% sheep blood and incubated for 48 h at 30°C. Purified isolates were further confirmed morphologically by Gram staining and biochemical analysis (catalase, oxidase, indoxyl acetate-hydrolysis, and urease tests). Isolates were then stored at -80°C in Trypticase Soy broth (TSI) supplemented with glycerol (30% vol/vol).

Biomolecular analysis

DNA extraction and purification

The colonies identified as *Arcobacter* spp. were transferred onto AB and incubated at 30°C for 48 h. One milliliter AB pure culture of presumptive *Arcobacter* spp. was centrifuged at 7500 rpm for 10 min at room temperature. DNA extraction was performed with the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and eluted with 80 μ L of Elution Buffer (QIAGEN, Hilden, Germany). The DNA concentration and purity were established by evaluating the ratio A260nm/A280nm using a filter BioPhotometer D30 (Eppendorf, Milan, Italy).

Identification of *Arcobacter* spp. isolates by multiplex-PCR

In order to determine the identity of the *Arcobacter* isolates, a m-PCR assay was performed using primers and conditions described by Houf *et al.* (2000). The amplification products were confirmed by electrophoresis in 1.5% agarose NA (Pharmacia, Uppsala, Sweden) gel in 1X Tris Acetate-EDTA buffer (TAE) (Sigma-Aldrich, Milan, Italy) containing 40 mM

Tris-acetate and 1 mM EDTA, pH 8.3 (Sigma Aldrich, Milan, Italy), and stained with Green Gel Safe 10000X Nucleic Acid Stain (Fisher Molecular Biology, USA) at 100V for 45 min. A Gene Ruler™ 100 bp DNA Ladder (Invitrogen, Life Technologies, Italy) was used as the molecular weight marker. Positive (*A. butzleri* ATCC 49616, *A. cryaerophilus* ATCC 43158 and *A. skirrowii* ATCC 51132) and negative (pure distilled water) controls for the extraction and PCR were included. Image acquisition was performed using UVITEC (Eppendorf).

Arcobacter rpoB gene sequencing

All *Arcobacter* isolates identified by mPCR were further confirmed using the *rpoB* gene sequencing using primers rpoB-Arc-F (5'-TCTCAATTTATGGAYCAAAC-3') and rpoB-Arc-R (5'-AGTTATATCCATTCCATGGCAT-3') and conditions described by Levican, 2013. The amplification products were confirmed by electrophoresis as above described. A Gene Ruler™ 100 bp DNA Ladder (Invitrogen, Life Technologies, Italy) was used as the molecular weight marker. Image acquisition was performed using UVITEC (Eppendorf). PCR product purification and sequencing analysis were performed by EUROFINs GENOMICS S.r.l. (Milan, Italy). The sequence analysis was carried out using Lasergene SeqMan version 7.0.0.

Detection of virulence genes

Nine putative *Arcobacter* virulence genes (*ciaB*, *cadF*, *cj1349*, *hecA*, *irgA*, *mviN*, *thyA*, *hecB* and *pldA*) were detected using the primers and conditions described by Doudah *et al.* (2012). In order to confirm the specificity of the amplicons, PCR product purification and sequencing analysis were performed by EUROFINs GENOMICS S.r.l. (Milan, Italy). The sequence analysis was carried out using Lasergene SeqMan version 7.0.0.

Multilocus sequence typing

Strain characterization was performed by MLST carried out using primers and conditions previously described by Miller *et al.* (2009). Specifically, the seven housekeeping genes (*aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm* and *tkt*) included in the *Arcobacter* scheme of the PubMLST

database (<http://pubmlst.org/arcobacter/>) were amplified and the sequenced. MLST housekeeping gene products were purified and sequenced by EUROFINs GENOMICS S.r.l. (Milan, Italy). The sequence analysis was carried out using Lasergene SeqMan version 7.0.0. Alleles and Sequence Type (ST) were compared with the *Arcobacter* pubMLST database (<http://pubmlst.org/Arcobacter/>), which allowed immediate identification of existing alleles or ST. Any new alleles or ST (i.e. those not recognized by the pubMLST system) were submitted to the database curator to be assigned new allele or ST numbers.

Results

Cultural analysis

The cultural analysis showed typical small, smooth, translucent, and watery colonies in 16/110 (14.5%) of the analyzed samples. Specifically, *Arcobacter* spp. were highlighted in 11/56 (20%) lettuce and 5/54 (9%) rocket salad samples (Table 1). All organisms resulting Gram-negative, with slightly curved rods, indoxyl acetate-hydrolysis positive, oxidase- and catalase-positive, and urease-negative were presumptively identified as *Arcobacter* spp.

Biomolecular analysis

m-PCR

The m-PCR carried out on the 16 isolates showed the characteristic 401-bp amplicon, specific for *A. butzleri*, in 15/16 (94%) isolates and 257-bp fragment, specific for *A. cryaerophilus*, in 1/16 (6%) isolates (Table 1).

rpoB gene sequencing

The sequencing of the *rpoB* gene confirmed the results obtained by m-PCR (Table 1).

Detection of virulence genes

PCR results for the nine putative virulence genes of *A. butzleri* and *A. cryaerophilus* isolates are shown in Table 2. *Arcobacter* isolates tested by PCR for the presence of putative virulence genes showed amplicons with expected sizes for the

Table 1. Cultural and molecular identification results.

Samples	No. (%) of <i>Arcobacter</i> spp. positive samples	No. (%) of <i>A. butzleri</i> positive samples	No. (%) of <i>A. cryaerophilus</i> positive samples
Lettuce	11/56 (20)	11/16 (69)	0/16 (0)
Rocket	5/54 (9)	4/16 (25)	1/16 (6)
Total	16/110 (14.5)	15/16 (94)	1/16 (6)

different virulence genes and the specificity of the results was confirmed by subsequent sequence analysis. All the 15 *A. butzleri* isolates harbored the genes *cadF*, *tlyA*, *hecB*, and *pldA*. Thirteen out of fifteen *A. butzleri* isolates were positive for the gene *mviN*. The genes *ciaB* and *hecA* were simultaneously detected in 2/15 *A. butzleri* isolates. The gene *cj1346* was observed in 5 *A. butzleri* isolates, while no isolate was positive for the gene *irgA*. The *A. cryaerophilus* isolate was positive only for the genes *cadF*, *ciaB* and *mviN* genes.

MLST

All the isolates were successfully typed by MLST and new alleles and sequence types (STs) were identified (Table 3). Specifically, 42 alleles were identified across all seven loci and 2 (5%) were previously unreported. A total of 6/16 (38%) STs were identified among the analysed *Arcobacter* spp. (Table 3). Overall, 5/6 (83%) STs were previously unreported and resulted from new allele sequences. All STs were identified more than once. Specifically, ST 526 was shared by 5 (31%) isolates, STs 651 and 653 were identified in 3 (19%) isolates, and ST538 and 652 were found in 2 (13%) isolates.

Discussion

Arcobacter spp. are emerging food-borne pathogens causing public health concern worldwide (Ramees *et al.*, 2017). Given the documented cases of arcobacteriosis, such as acute gastrointestinal infections associated with the exposure to or the consumption of contaminated water or poorly cooked meat/shellfish or rare cases of bacteremia, the emergence of arcobacters has gained growing importance during recent years (Figuera *et al.*, 2014; Barboza *et al.*, 2017; Vasiljevic *et al.*, 2019). The consumption of *Arcobacter*-contaminated food or water is considered the route of transmission to human and animals, although this has not yet been proven. Such a hypothesis relies on the high prevalence of arcobacters in the intestinal tract and fecal samples from healthy farm animals and in many food products (Ramees *et al.*, 2017).

Given that *Arcobacter* spp. contamination in fresh vegetables has also been reported (González and Ferrús, 2011; Mottola *et al.*, 2016b; González *et al.*, 2017), even though available data are scarce, the prevalence of *Arcobacter* found in RTE

vegetables in this study assumes key sanitary importance. Specifically, the results obtained in this study agree with previous studies, although with variable prevalence rates (González and Ferrús, 2011; Mottola *et al.*, 2016b; González *et al.*, 2017; Ramees *et al.*, 2018; Vicente-Martins *et al.*, 2018; Kim *et al.*, 2019). However, the results of this study are in contrast with Di Noto *et al.* (2018), who found vegetable samples negative for *Arcobacter* spp. The presence of *Arcobacter* spp. in RTE vegetables indicate that key risk factors for contamination of vegetable products could include environmental, animal and human sources. In fact, we suppose that the occurrence of arcobacters probably occurs at farm level through the direct use of untreated wastewater for irrigation and inappropriate handling of animal manure for vegetable cultivation, or during poorly hygienic or improper post-harvest, handling, processing and packaging procedures, in agreement with Antwi-Agyei *et al.* (2015). Even though the pathogenic role of arcobacters still needs to be clarified, the occurrence rate observed in the current study indicates that RTE vegetables have a probable role as a source of *Arcobacter* spp. infections to human beings, and

Table 2. Occurrence of putative virulence genes in *Arcobacter* isolates.

Species	No. of isolates	No. of isolates generating a virulence-associated gene amplicon								
		<i>cadF</i>	<i>ciaB</i>	<i>cj1349</i>	<i>irgA</i>	<i>hecA</i>	<i>mviN</i>	<i>tlyA</i>	<i>hecB</i>	<i>pldA</i>
<i>A. butzleri</i>	15	15	2	5	0	2	13	15	15	15
<i>A. cryaerophilus</i>	1	1	1	0	0	0	1	0	0	0

Table 3. Multilocus sequence type (MLST) results of the 16 isolates considered, according to the *Arcobacter* MLST database.

Isolate	ID Isolate	Species	PubMLST ID	<i>aspA</i>	<i>atpA</i>	<i>glnA</i>	<i>gltA</i>	<i>glyA</i>	<i>pgm</i>	<i>tkt</i>	ST
1	20/O	<i>A. butzleri</i>	721	13	4	40	15	521	244	58	538*
2	21/O	<i>A. butzleri</i>	721	13	4	40	15	521	244	58	538*
3	34/O	<i>A. butzleri</i>	837	268	186	153	123	635*	306	210	651*
4	37/O	<i>A. butzleri</i>	837	268	186	153	123	635*	306	210	651*
5	54/O	<i>A. butzleri</i>	837	268	186	153	123	635*	306	210	651*
6	35/O	<i>A. butzleri</i>	799	19	17	17	12	551	68	65	526
7	83/O	<i>A. butzleri</i>	799	19	17	17	12	551	68	65	526
8	38/O	<i>A. butzleri</i>	799	19	17	17	12	551	68	65	526
9	71/O	<i>A. butzleri</i>	799	19	17	17	12	551	68	65	526
10	63/O	<i>A. butzleri</i>	799	19	17	17	12	551	68	65	526
11	26/O	<i>A. butzleri</i>	836	81	62	128	148	465	244	183	652*
12	27/O	<i>A. butzleri</i>	836	81	62	128	148	465	244	183	652*
13	39/O	<i>A. butzleri</i>	838	3	42	2	15	684*	21	4	653*
14	42/O	<i>A. butzleri</i>	838	3	42	2	15	684*	21	4	653*
15	48/O	<i>A. butzleri</i>	838	3	42	2	15	684*	21	4	653*
16	1/O	<i>A. cryaerophilus</i>	720	221	79	61	71	265	154	180	539*

*Novel STs or alleles.

consequently could pose public health concerns.

Also, in this study, both conventional cultural methods as well as molecular techniques report the emerging pathogen *A. butzleri* as the most prevalent species in RTE vegetables, followed by *A. cryaerophilus*, in accordance with previous studies (González and Ferrús, 2011; Mottola *et al.*, 2016b; González *et al.*, 2017; Ramees *et al.*, 2018; Vicente-Martins *et al.*, 2018; Kim *et al.*, 2019). However, according to Levican *et al.* (2016) the high occurrence of *A. butzleri* is probably due to the use of enrichment which can cover, and thus underestimate, detection of other species.

Molecular analysis of the occurrence of putative virulence genes indicates a various virulence profile among isolated arcobacters, supporting their potential pathogenic role and indicating a relevant veterinary and public health significance. In particular, in agreement with previous studies investigating the occurrence of virulence factors in *A. butzleri* isolated from various food matrices and environments, this study highlighted a high occurrence of genes *cadF*, *tlyA*, *hecB*, *pldA*, followed by the genes *cj1349* and *ciaB*, encoding for fibronectin-binding proteins, hemolysin, hemolysin activation protein, phospholipase A and the *Campylobacter jejuni* invasion antigen B, respectively (Doudah *et al.*, 2012; Mottola *et al.*, 2016a,b; Sekhar *et al.*, 2017; Kim *et al.*, 2019; Šilha *et al.*, 2019). These results might be relevant from a food-safety angle, given that the same virulence factors have been observed in *A. butzleri* isolated from clinical stool samples in Spain and Czech Republic (Pérez-Cataluña *et al.*, 2017; Šilha *et al.*, 2019).

Regarding the isolate of *A. cryaerophilus*, in agreement with previous studies, we found that our isolate was positive for the genes *ciaB*, *cadF* and *mviN* (Figueras *et al.*, 2014; Barboza *et al.*, 2017). Even though we considered only one isolate of *A. cryaerophilus*, the pathogenicity studies of *A. cryaerophilus* need to be improved given that Figueras *et al.* (2014) and Barboza *et al.* (2017) observed similar results in *A. cryaerophilus* isolates recovered from episodes of human diarrhea, probably due to the consumption of contaminated meat or fish.

Therefore, the presence of virulence genes in *Arcobacter* spp. increases the chances of fresh and RTE vegetables acting as transmission vehicles to humans, posing a potential public health risk. Considering that the mechanisms of pathogenicity of *Arcobacter* species are still poorly understood, more studies are needed in order to better understand the potential

relationship between the prevalence of the putative virulence genes, the source of the isolates and the potentially pathogenic properties of arcobacters isolates.

Given the wide occurrence of *Arcobacter butzleri* in water, soil, feces, RTE foods and in processing plants (Giacometti *et al.*, 2013; Mottola *et al.*, 2016a,b, Ramees *et al.*, 2017), we suppose that the occurrence of *A. butzleri* might have several origins. Firstly, improper agricultural and processing of vegetables might play a crucial role. In fact, raw material, manure used as fertilizer, fecal matter of wild animals or fecal contaminated soil or water used in production are potential contamination sources in vegetables (Mottola *et al.*, 2016b). Secondly, *A. butzleri* survival and resistance to stress created during food storage and processing might justify its presence in RTE vegetables (Ferreira *et al.*, 2016). Indeed, Kjeldgaard *et al.* (2009) reported that it seems that chilled temperatures do not act as a barrier to preserving food against *Arcobacter butzleri*, which can survive and even form biofilms. In addition, Ferreira *et al.* (2016) demonstrated that *A. butzleri* is able to survive within several sanitizing food-processing environments (e.g. chlorine of 0.2% – 0.5%; 5% ethanol or 0.2% sodium chloride). However, such a hypothesis needs to be better clarified in order to better understand the presence and resistance of *Arcobacter* spp. in foods.

Further, in this study MLST analysis revealed a low genetic diversity within the *A. butzleri* isolates, largely contrasting with previous studies performed by Miller *et al.*, (2009); Merga *et al.* (2013); Pérez-Cataluña *et al.* (2017); that observed a diversity of arcobacters in the analyzed samples. In this study, among the 16 isolates considered we found only 6 STs (38 %), proof of the persistence of specific isolates in the samples, which in turn probably indicates that they had colonized the processing plant environment. In addition, we suppose that the low variability in the genotypes is probably due to limited sources of contamination or to unrepresentative sampling. In fact, all RTE vegetables considered in this study came from a small vegetable processing plant in the Apulia region, supplied by a handful of local vegetable growers, which meant that no association between the sequence types and the host or geographical sources could be found. Even though we analyzed a low number of isolates and observed only 2 new alleles and 5 previously unreported STs, the presence of new alleles and new STs provides new information for the *Arcobacter*-pubmlst database. Surprisingly, ST526 was previously isolated from carrot

in 2015, suggesting that some isolates are circulating in food. However, our results highlight a drawback in the *Arcobacter*-pubmlst database (<https://pubmlst.org/arcobacter/>) given that it does not consider “vegetables” in its “source” records, but such information is only voluntarily provided by researchers. Considering this, genotypic information about arcobacters in vegetables could be underestimated. Consequently, the database needs to be updated and more studies are required about the genotyping of arcobacters from vegetables in order to acquire more information from the MLST database.

Conclusions

The study confirms the presence of *Arcobacter* spp. in RTE vegetables, mainly due to contamination of the raw material. Our results, therefore, require produce risk factors along the food chain to be handled by focusing on the implementation of strict food safety measures and enhancing producers, suppliers and handling training and controls, in order to apply a farm-to-fork approach to controls and reduce the sanitary risks related to the RTE vegetable production process (Report of the Scientific Committee on Food, 2002; Mottola *et al.*, 2016b). In addition, in order to clarify the role of RTE vegetables in the transmission of arcobacters to humans, the notification of gastrointestinal illness related with the consumption of RTE vegetables and strict intervention strategies in the event of outbreaks are required. Furthermore, the identification of specific virulence factors and the molecular genotyping of emerging pathogens *A. butzleri* and *A. cryaerophilus* highlight the need to start an innovative surveillance system based on biomolecular characterization for an integrated microbiological risk assessment of RTE vegetables, aimed at implementing an innovative control of the supply chain, and consequently to guarantee consumers' health (Di Pinto *et al.*, 2012) Considering this, molecular techniques that allow the rapid and accurate quantification, identification and characterization of *Arcobacter* spp. in foods are needed to perform microbial risk-assessment of *Arcobacter* spp. in the RTE vegetable chain and an innovative food-management system.

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