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 considered 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. PRCs 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. thereus 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 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) 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, Ampicillin and Tetracyclines (CAT) (CAT selective supplement; Oxoid, Milan, Italy). The samples were then incubated at 30°C under aerobic conditions for 48 h.
After enrichment, 200 μL of the broth was dropped onto the surface of a 0.45 μm 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 mL of Elution Buffer (QIAGEN, Hilden, Germany) and purity were established by evaluating the DNA concentration with 80 mL of Elution Buffer (QIAGEN, Hilden, Germany) and eluted with the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and eluted with 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’-TCTCAATTATGGAYCAAACAC-3’) and rpoB-Arc-R (5’-AGTTATACCATCCATGGCATGC-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, tlyA, hecB and pldA) were detected using the primers and conditions described by Douidah 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, glmA, gltA, gycA, 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

| Samples  | No. (%) of Arcobacter spp. positive samples | No. (%) of A. butzleri positive samples | No. (%) of A. cryaerophilus 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)                                  |

Table 1. Cultural and molecular identification results.
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 irog. 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 ST338 and 652 were found in 2 (13%) isolates.

**Discussion**

*Arcobacter* spp. are emerging foodborne pathogens causing public health concern worldwide (Ramees et al., 2017). Given the documented cases of arcobacterioses, 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 (Figueras 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 | cadF | ciaB | cj1346 | irogA | hecA | mviN | tlyA | hecB | pldA |
|-------------|-----------------|------|------|--------|-------|------|------|------|------|------|
| A. butzleri | 15              | 15   | 2    | 5      | 0     | 2    | 13   | 15   | 15   | 15   |
| A. cryaerophilus | 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 | aspA | atpA | glaA | glnA | gltA | glyA | pgm | tkt | ST |
|---------|----|---------|-------------|------------|------|------|------|------|------|------|-----|-----|----|
| 1       | 20/O | A. butzleri | 721         | 13 | 4 | 40 | 15 | 521 | 244 | 58 | 538* |
| 2       | 21/O | A. butzleri | 721         | 13 | 4 | 40 | 15 | 521 | 244 | 58 | 538* |
| 3       | 34/O | A. butzleri | 837         | 268 | 186 | 153 | 123 | 635* | 306 | 210 | 651* |
| 4       | 37/O | A. butzleri | 837         | 268 | 186 | 153 | 123 | 635* | 306 | 210 | 651* |
| 5       | 54/O | A. butzleri | 837         | 268 | 186 | 153 | 123 | 635* | 306 | 210 | 651* |
| 6       | 35/O | A. butzleri | 799         | 19 | 17 | 17 | 12 | 551 | 68  | 65 | 526  |
| 7       | 83/O | A. butzleri | 799         | 19 | 17 | 17 | 12 | 551 | 68  | 65 | 526  |
| 8       | 38/O | A. butzleri | 799         | 19 | 17 | 17 | 12 | 551 | 68  | 65 | 526  |
| 9       | 71/O | A. butzleri | 799         | 19 | 17 | 17 | 12 | 551 | 68  | 65 | 526  |
| 10      | 63/O | A. butzleri | 799         | 19 | 17 | 17 | 12 | 551 | 68  | 65 | 526  |
| 11      | 26/O | A. butzleri | 836         | 81 | 62 | 128 | 148 | 465 | 244 | 183 | 652* |
| 12      | 27/O | A. butzleri | 836         | 81 | 62 | 128 | 148 | 465 | 244 | 183 | 652* |
| 13      | 39/O | A. butzleri | 838         | 3 | 42 | 2 | 15 | 684* | 21  | 4  | 653* |
| 14      | 42/O | A. butzleri | 838         | 3 | 42 | 2 | 15 | 684* | 21  | 4  | 653* |
| 15      | 48/O | A. butzleri | 838         | 3 | 42 | 2 | 15 | 684* | 21  | 4  | 653* |
| 16      | 1/O  | A. cryaerophilus | 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 (Douidah 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, improperly 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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