1 **Occurrence of emerging food-borne pathogenic *Arcobacter* spp. isolated from pre-cut (ready-**

2 **to-eat) vegetables.**

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Abstract

Given that changes in consumer food behaviours have led to an increase in the demand for pre-cut ready-to-eat (RTE) vegetables, and that few data are currently available about the occurrence of *Arcobacter* spp. in such foods, the aim of the present study was to assess the occurrence of *Arcobacter* spp. that carry virulence-associated genes on pre-cut RTE vegetables, using cultural and biomolecular assays. *Arcobacter* was detected in 44/160 (27.5%) of the samples. Specifically, biomolecular identification methods revealed that 40/44 (90.9%) isolates corresponded to *A. butzleri* in and 4/44 (9.1%) to *A. cryaerophilus.* Studying the incidence of 9 virulence-associated genes revealed the widespread distribution of these genes among the *Arcobacter* isolates tested. The results obtained in our research provided plenty of information on the health risks associated with the direct consumption of raw vegetables, and highlight the need to implement further studies at each level of the production chain, in order to obtain further information to help protect human health.

*Keywords***:** pre-cut vegetables (ready-to-eat); emerging food-borne pathogens; *Arcobacter butzleri; Arcobacter cryaerophilus*

1. Introduction

In recent years, the trend towards healthier eating has led to an increase in the consumption of vegetables (Losio et al., 2015), an important source of vitamins, minerals, antioxidants, fibre, and phytonutrients for human health (Tango et al., 2014). However, changes in consumer food choices have contributed to an increase in demand for pre-cut ready-to-eat (RTE) vegetables. These products are increasingly being chosen by consumers because they are generally eaten without further processing. Pre-prepared, minimally-processed vegetables have previously been washed,

47   selected, cut and are packed in modified atmospheres in order to extend shelf life, thus improving

48   both the potential acceptable quality and the safety of the product.

49   Generally, vegetables carry a natural non-pathogenic epiphytic microbiota. Still, raw vegetables

50   may represent an important source of risk for human health because they are also carriers of

51   pathogenic micro-organisms (Harris et al., 2003; Park et al., 2012). Indeed, their complex surface

52   and porosity unfortunately facilitate pathogen attachment and survival (Report of the Scientific

53   Committee on Food, 2002; Said, 2012).

54   Documented cases of human infections are often associated with the consumption of food of

55   animal origin (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease

56   Prevention and Control), 2015). Moreover, the last decade has witnessed an increase in documented

57   outbreaks in Europe, United Kingdom and United States, associated with the consumption of raw

58   fruits, vegetables, and unpasteurized fruit juice (Buck et al., 2003; EFSA and ECDC, 2015;

59   Hausdorf et al., 2011; Report of the Scientific Committee on Food, 2002; Sagoo et al., 2003).

60   Although the European Union's food safety policy aims to ensure a high level of protection of

61   human health, the epidemiological data of ready-to-eat foodstuffs is not exclusively linked to

62   bacteria such as *Salmonella* spp.*, VTEC E. coli* and *Listeria monocytogenes* (Erickson, 2010).

63   Indeed, viruses (hepatitis A and Noroviruses), parasites (*Cyclospora cayetanensis* and

64   *Cryptosporidium*) and other bacteria (*Campylobacter jejuni*) are also involved in outbreaks

65   (Abadias et al., 2008; Harris et al., 2003; Kärenlampi and Hänninen, 2004; Verhoeff-Bakkenes,

66   2011). Furthermore, many outbreaks are underestimated where the causative agent is unknown

67   (Buck et al., 2003; EFSA and ECDC, 2015) or due to their small size or long incubation period

68   which means consumers find it hard to recall the infection or because of the long period between

69   the food exposure and illness (EFSA and ECDC, 2015; Erickson, 2010; Sagoo et al., 2003). Thus,

70   the incidence of infections caused by consuming pre-cut RTE vegetables remains greatly

71   underestimated or misunderstood.

72   Most of the agents involved in outbreaks are zoonotic, as is the case for *Arcobacter*, currently

73   regarded as an emerging and food-borne pathogen, of growing importance for public health (Calvo

74   et al., 2013; Collado and Figueras, 2011). Still, a number of actual research projects and reports of

75   human diseases (e.g., diarrhoea; enterocolitis; bacteraemia) (Arguello et al., 2015; Collado and

76   Figueras, 2011; Figueras et al., 2014; Van den Abeele et al., 2014; Webb et al., 2016) and animal

77   illnesses (e.g., gastrointestinal disorders, mastitis, reproductive problems) (Levican and Figueras,

78   2013; Van Driessche and Houf, 2003; Woo et al., 2001) have been associated with poorly known

79   bacteria belonging to the *Arcobacter* genus (Ramees et al., 2014; Van den Abeele et al.*,* 2014). The

80   genus *Arcobacter* has morphological and genetic characteristics related to *Campylobacter* spp.

81   However, arcobacters differentiate from *Campylobacter* species because they are able to grow

82   under aerobic and microaerobic conditions at 30 °C (Collado and Figueras, 2011). Given both the

83   homologies with the genus *Campylobacter* and the lack of a standardized isolation protocol for

84   identification, it is conjectured that *Arcobacter* spp. can often be mistaken for *Campylobacter* spp.,

85   or that *Campylobacter* isolates cover *Arcobacter* spp. (Collado and Figueras, 2011; Figueras et al.,

86   2014).

87   Data on the occurrence of *Arcobacter* spp. in vegetables is scarce (González and Ferrús, 2011;

88   Hausdorf et al., 2013). Arcobacters are a group of Gram-negative, slightly curved, rod-shaped

89   bacteria, isolated from environmental sources (e.g., water, surfaces, sewage) (Collado et al., 2011;

90   Giacometti et al., 2015; Hsu and Lee, 2015; Serraino and Giacometti, 2014; Šilha et al., 2015),

91   different foods of animal origin (e.g., chicken meat, pork, beef, lamb, milk, shellfish) (Collado and

92   Figueras, 2011; Giacometti et al., 2015; Gonzáles et al., 2014; Mottola et al., 2016; Van Driessche

93   and Houf, 2007; Zacharow et al., 2015), vegetables (González and Ferrús; 2011) and clinical

94   samples such as animal and human stools (Figueras et al., 2014; Van den Abeele et al., 2014; Webb

95   et al., 2016).

96   Given reports from all over the world documenting outbreaks associated with the direct

97   consumption of raw vegetables (Harris et al., 2003) and considering the lack of data regarding both

98   the occurrence and public health risks from contamination of *Arcobacter* spp. for these food types,

99   the aim of the present study was to assess the occurrence of *Arcobacter* spp. on pre-cut RTE

100   vegetables using a biomolecular assay. A further objective was to examine the distribution of

101   putative virulence genes associated with *Arcobacter* isolates.

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103   **2.   Materials and methods**

104   *2.1 Sampling*

105   A total of 160 pre-cut RTE vegetables including lettuce (*Lactuca sativa L*), spinach

106   (*Spinacia oleracea*), rocket (*Eruca vesicaria*) and valerian (*Valeriana officinalis*) were obtained

107   from supermarkets in the Apulia region (SE Italy) between February and September 2015 as

108   reported in **Table 1**. The samples were taken to the laboratory in cooled containers (+4 °C) and

109   processed within 24 h of purchase.

110

111   *2.2 Cultural analysis*

112   For *Arcobacter* isolation, 20 g of sample were added to 180 mL (1:10 wt/vol) of *Arcobacter*

113   Broth (Oxoid, Basingstoke, UK), in sterile bags, and homogenized using a stomacher (PBI

114   International, Milan, Italy) at 11 000 rev min $^{-1}$ for 2 min. Then, 20 mL of broth culture were added

115   to 20 mL of double-strength *Arcobacter* broth (Oxoid, Basingstoke, UK) supplemented with

116   Cefoperazone, Amphotericin B and Teicoplanin (CAT selective supplement SR0174E; Oxoid,

117   Basingstoke, UK), as previously reported by González & Ferrús (2011). The samples were then

118   incubated at 30 °C under aerobic conditions for 48 h. After enrichment, 80 μL of the broth was

119   dropped onto the surface of a 0.45 μm membrane filter (Sartorius Stedim Biotech GmbH,

5

120 Germany), placed onto selective agar plates prepared by suspending 24 g of *Arcobacter* broth

121 (Oxoid, Basingstoke, United Kingdom) and 12 g of agar technical no. 3 (Oxoid, Basingstoke,

122 United Kingdom) and supplemented with a selective antibiotic mix supplement (cefoperazone [16

123 mg/litre], amphotericin B [10 mg/litre], 5-fluorouracil [100 mg/litre], novobiocin [32 mg/litre], and

124 trimethoprim [64 mg/litre]) as described by Houf et al., (2001). Plates were incubated at room

125 temperature for 30 min. After filtration, the filters were removed and the dishes were incubated at

126 30 °C under both aerobic and microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2), produced by

127 the Campy*Gen* gas generating system (Oxoid, Basingstoke, UK). After incubation, one *Arcobacter*

128 colony per sample (i.e. small colourless, translucent, and convex with an intact edge) was picked,

129 subcultured onto Blood Agar (BA) and incubated for 48 h at 30 °C. The colonies were confirmed

130 morphologically by Gram staining and by oxidase determination (Oxidase strips, Oxoid Microbact,

131 Basingstoke, UK) and catalase activity.

132

133 *2.3 Biomolecular analysis*

134 *2.3.1 DNA extraction and purification*

135 DNA extraction and purification was performed as described by Mottola et al. (2016).

136 Briefly, colonies identified as *Arcobacter* spp. were transferred onto *Arcobacter* broth (Oxoid,

137 Basingstoke, UK) and incubated at 30 °C for 48 h. One millilitre of pure AB culture of presumptive

138 *Arcobacter* spp. was centrifuged at 7500 rpm for 10 min at room temperature. DNA extraction and

139 purification was performed with the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and

140 eluted with 80 μL AE Elution Buffer (QIAGEN, Hilden, Germany). The DNA concentration and

141 purity were established by evaluating the ratio A_{260nm}/A_{280nm} using a NanoDrop 2000/2000c

142 Spectrophotometer (Thermo Scientific, MA, USA).

143

144 *2.3.2 Genus-specific PCR*

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The presence of *Arcobacter* spp. was examined using genus-specific PCR oligonucleotide primers (Eurofins, Milan, Italy) (**Table 2**) reported by Harmon and Wesley (1996). The PCR reactions were performed in a final volume of 50 μL, using 25 μL of HotStarTaq Master Mix 2X (Qiagen), containing 2.5 units of HotStarTaq DNA polymerase, 1.5 mM of $MgCl_2$ and 200 μL of each dNTP. Then, 50 pmol of each oligonucleotide primer and 2 μL (50 ng) of DNA were added. The amplification profile involved an initial denaturation step at 95 °C for 15 min, followed by 25 cycles at 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min. A primer extension step (72 °C for 7 min) followed the final amplification cycle. The positive (*A. butzleri* ATCC 49616[T]) and negative (no added template) controls for the extraction were included. The PCR reactions were processed in a Mastercycler Personal (Eppendorf, Hamburg, Germany). All reactions were performed in duplicate.

The PCR reaction products were separated by gel electrophoresis on 1.5% (w/v) agarose NA (Pharmacia, Uppsala, Sweden) gel in 1X Tris-borate-EDTA (TBE) buffer containing 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0 (USB, Cleveland, OH, USA), and stained with Green Gel Safe 10000X Nucleic Acid Stain (5 μl/100 ml) (Fisher Molecular Biology, USA). A Gene Ruler[TM] 100 bp DNA Ladder Plus (MBI Fermentas,Vilnius, Lithuania) was used as the molecular weight marker. Image acquisition was performed using UVITEC (Eppendorf).

2.3.2 m-PCR

The presence and the identity of the *Arcobacter* isolates was determined using a multiplex-PCR assay as described by Houf et al. (2000). Briefly, 2 μL (50 ng) of DNA template were added to 48 μL of the reaction mixture containing 5 μL 10X PCR Rxn Buffer (Invitrogen, Carlsbad, CA, USA), 1.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.2 mmol each of deoxyribonucleotide triphosphate (Applied Biosystems), 1.3 μL of 50 mmol $MgCl_2$ (Invitrogen, Carlsbad, CA, USA). Then 50 pmol of the primers ARCO, BUTZ, CRY1, CRY2 and 25 pmol of

primer SKIR were added (**Table 2**). The amplification followed conditions described by Houf et al.

(2000) and involved an initial denaturation step at 94 °C for 5 min, followed by 32 cycles of

denaturation at 94 °C for 45 sec, primer annealing at 61 °C for 45 sec, and chain extension at 72 °C

for 30 sec. The final extension was carried out at 72 °C for 1 min. The amplifications were carried

out in a Mastercycler personal (Eppendorf, Milan, Italy). The positive (*A. butzleri* ATCC 49616[T], *A.*

cryaerophilus ATCC 43158[T], and *A. skirrowii* ATCC 51132[T]) and negative controls (no added

template) were included to verify the purity of the extraction reagents. All reactions were performed

in duplicate. The generated m-PCR products were separated by gel electrophoresis on 1.5% (w/v)

agarose NA (Pharmacia, Uppsala, Sweden) gel in 1X Tris-borate-EDTA (TBE) buffer containing

0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0 (USB, Cleveland, OH, USA), and stained

with Green Gel Safe 10000X Nucleic Acid Stain (5 μl/100 ml) (Fisher Molecular Biology, USA). A

Gene Ruler™ 50 bp DNA Ladder Plus (Invitrogen, Life Technologies, Italy) was used as the

molecular weight marker. Image acquisition was performed using UVITEC (Eppendorf).

2.3.3 16S rDNA-RFLP

In order to confirm the m-PCR results (Levican and Figueras, 2013), the 16S rDNA-RFLP

assay described by Figueras et al. (2008) was performed. Briefly, the amplification of 1026 bp of

the 16S rRNA gene was carried out with a 50 μL PCR mixture containing 5 μL (100 ng) of DNA

template, 0.5 μM each of primers CAH16S1am and CAH16S1b (**Table 2**), 5μL 10X PCR Buffer

(Invitrogen, Carlsbad, CA, USA), 1.5 μL of 50 mmol $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 200

μM each of deoxyribonucleotide triphosphate (Applied Biosystems) and 2.5 U of Taq DNA

polymerase (Invitrogen, Carlsbad, CA, USA). The amplification profile involved an initial

denaturation step at 95 °C for 2 min, followed by 31 cycles of denaturation at 94 °C, for 30 sec,

primer annealing at 55 °C, for 30 sec, and chain extension at 72 °C for 1.30 min. The final

194 extension was carried out at 72 °C for 10 min. The PCR reactions were processed in a 2720

195 Thermal Cycler (Applied Biosystems). PCR-amplified products were analyzed by electrophoresis

196 on 2% (w/v) agarose NA (Pharmacia, Uppsala, Sweden) gel in 1X Tris-borate-EDTA (TBE) buffer

197 containing 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0 (USB, Cleveland, OH, USA),

198 and stained Green Gel Safe 10000X Nucleic Acid Stain (5 μl/100 ml) (Fisher Molecular Biology,

199 USA). A Gene Ruler^TM 100 bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania) was used as

200 the molecular weight marker. Image acquisition was performed using UVITEC (Eppendorf). The

201 1026 bp amplicon was, then, digested using *MseI* endonuclease (Fermentas, Schwerte, Germany).

202 Restriction fragments were separated in 3.5% (w/v) agarose gel electrophoresis in 1X Tris-borate-

203 EDTA (TBE) buffer containing 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0 (USB,

204 Cleveland, OH, USA), and stained with Green Gel Safe 10000X Nucleic Acid Stain (5 μl/100 ml)

205 (Fisher Molecular Biology, USA). A Gene Ruler^TM 100 bp DNA Ladder Plus (MBI Fermentas,

206 Vilnius, Lithuania) was used as the molecular weight marker. Image acquisition was performed

207 using UVITEC (Eppendorf). Identification was performed comparing each obtained 16S rDNA-

208 RFLP pattern with those defined as characteristic for the different *Arcobacter* species (Figueras et

209 al., 2008).

210

211   *2.3.4   Detection of virulence genes*

212   Nine putative *Arcobacter* virulence genes (*ciaB, cadF, cj1349*, *hecA* and *irgA, mviN, tlyA,*

213 *hecB* and *pldA*) were detected using the primers and conditions designed by Douidah et al. (2012).

214 *A. butzleri* LMG10828^T and *A. thereius* LMG24486^T were used as positive and negative control,

215 respectively. Briefly, the PCR reactions were performed in a final volume of 50 μL, containing 2 μL

216 of DNA extract (50 ng), 1.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.2 μmol

217 each of deoxyribonucleotide triphosphate (Applied Biosystems), 0.2 mmol $MgCl_2$ for the primer

218 sets for *ciaB, cj1349, hecA* and *irgA* (**Table 2**). For the primer sets for *cadF* (**Table 2**), 5 μL 10X

9

219   PCR buffer and 1.5 mmol MgCl₂ were used. 2 μL of DNA template were added in the PCR

220   reaction. PCR consisted in an initial denaturation step at 94 °C for 30 sec, followed by 32 cycles of

221   denaturation at 94 °C for 45 sec, primer annealing at 56 °C for 45 sec for primers designed for *ciaB,*

222   *cj1349, hecA* and *irgA* and at 55 °C for the primer sets for *cadF*, while an extension step was

223   performed at 72 °C for 45 sec. Final extension was carried out at 72 °C for 3 min. PCR-amplified

224   products were detected by electrophoresis on 1.5 % (w/v) agarose gel in a 1X Tris-borate-EDTA

225   (TBE) buffer containing 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0 (USB,

226   Cleveland, OH, USA), and stained with Green Gel Safe 10000X Nucleic Acid Stain (5 ll/100 ml)

227   (Fisher Molecular Biology, USA). A Gene RulerTM100 bp DNA Ladder Plus (MBI Fermentas,

228   Vilnius, Lithuania) was used as the molecular weight marker. Image acquisition was performed

229   using UVITEC (Eppendorf).

230

231   *2.3.5   Statistical analysis*

232   In order to analyse the association between the presence of *Arcobacter* strains and types of

233   vegetables analyzed, a Pearson's chi-square test was performed. A *P* value of <0.05 was considered

234   as statistically significant.

235   In addition, the nonparametric Friedman rank sum test was applied to evaluate the seasonal

236   variation in the presence of *Arcobacter* spp. in pre-cut RTE vegetables.

237

238   **3. Results**

239   *3.1 Cultural analysis*

240   Small, smooth, translucent, and watery presumptive *Arcobacter* spp. colonies were detected

241   in 52 of the 160 vegetable samples analyzed (32.5 %) (**Table 3**).

242

243   *3.2 Biomolecular analysis*

244   *3.2.1 Genus-specific PCR*

245   Genus-specific PCR performed on isolates gave positive results in 44/52 cases (84.6%)

246 (**Tables 3 and 4**).

247

248   *3.2.1 m-PCR*

249   The m-PCR carried out on the 52 isolates confirmed the same 44 isolates identified by

250 genus-specific PCR as *Arcobacter* spp. Specifically, with the m-PCR found the characteristic

251 amplicon of *A. butzleri* was found in 40/44 (90.9%) and the one of *A. cryaerophilus* amplicon in

252 4/44 (9.1%) cases (**Table 4**).

253

254   *3.2.2 16S rDNA-RFLP*

255   Digestion of the 16S rRNA gene with the *MseI* endonuclease produced the expected species-

256 specific *A. butzleri* and *A. cryaerophilus* 1B RFLP patterns for the same 40 and 4 isolates,

257 respectively, thus confirming the m-PCR results (**Table 4**).

258

259   *3.2.3 Detection of virulence genes*

260   The occurrence of the presence and distribution of nine putative virulence genes in

261 *Arcobacter* spp. strains recovered from pre-cut RTE vegetables are shown in **Table 5**. *Arcobacter*

262 isolates tested by PCR for the presence of putative virulence genes showed amplicons with expected

263 sizes for the different virulence genes. Indeed, all (40/40) *A. butzleri* harboured the genes *ciaB,*

264 *cj1349, mviN, tlyA* and *pldA*. Lower detection rates were observed for the genes *cadF* in 34/40

265 (90%), *hecA* in 12/40 (30%) and *hecB* in 28/40 (70%) *A. butzleri* strains. *CadF* and *mviN* were

266 simultaneously detected in all (4/4) *A. cryaerophilus* strains. The *ciaB, cj1349, hecA, hecB, irgA,*

267 *pldA* and *tlyA* genes were not detected. None of the *A. butzleri* and *A. cryaerophilus* strains

268 possessed the *irgA* genes.

269

270   *3.2.4   Statistical analysis*

271   Statistical analyses of the association between *Arcobacter* strains and different types of

272 vegetables showed no significant differences (P >0.05). In addition, the nonparametric Friedman

273 rank sum test used to evaluate the seasonal variation in the presence of *Arcobacter* spp. of RTE

274 fresh-cut vegetables showed highly significant results (P <0.001).

275

276   **4. Discussion**

277   According to European Regulation pre-cut vegetables (ready-to-eat) are "food intended by

278 the producer or the manufacturer for direct human consumption without the need for cooking or

279 other processing effective to eliminate or reduce to an acceptable level micro-organisms of

280 concern" (Commission Regulation (EC) No. 2073/2005). Indeed, they can be obtained from fresh

281 products through selection, washing, peeling, cutting, sanitization, rinsing, drying and packaging, in

282 order to extend their shelf life and preserve their nutritive and sensorial properties (de Oliveira et

283 al., 2011; Francis et al., 1999). Nevertheless, these steps may not be fully efficient in reducing RTE

284 fresh-cut vegetable contamination.

285   The increase in widespread outbreaks associated with these food types, such as the one in

286 2011 in Germany (EFSA Panel on Biological Hazards (BIOHAZ), 2013), highlights the need to

287 address aspects related to the control of specific hazards of concern in fresh vegetable products.

288 Given that the microbial contamination of RTE prepared vegetables is underestimated, and given

289 the association of several outbreaks linked with the consumption of RTE vegetables (Ackers et al.,

290 1998; Herman et., 2015), more information about microbial contamination by emerging pathogens

291 of pre-cut RTE vegetables is required. The results of the present study highlight the occurrence of

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292 *Arcobacter* spp. in pre-cut RTE vegetables by cultural methods and biomolecular analysis.

293 *Arcobacter*-positive RTE fresh-cut vegetable samples confirm the results obtained in a previous

294 study carried out in Spain (González and Ferrús, 2011) and Germany (Hausdorf et al., 2011, 2013)

295 evaluating the presence of arcobacters in fresh lettuces for human consumption, in carrot wash

296 water and in a spinach-processing plant, respectively.

297   The data from the present research confirm that *Arcobacter* spp. are found in different

298 RTE vegetables. In particular, the presence of *A. butzleri* and *A. cryaerophilus* in 27.5 % of RTE

299 fresh-cut vegetable samples was similar to the prevalence observed by González and Ferrús (2011)

300 and Hausdorf et al. (2013), who found an incidence of *Arcobacter* spp. of 20% and 35%,

301 respectively. Moreover, the results from the present study suggest that the potential risk factors for

302 the contamination and cross-contamination of vegetables is probably due to the supply chain, from

303 production through processing to point-of-sale. This is in agreement with the results obtained by

304 Hausdorf et al. (2013) who detected *Arcobacter* spp. in samples collected at several steps of the

305 production chain in a spinach-processing plant.

306   Various researchers have highlighted the fact that bacteria of the genus *Arcobacter* have

307 been found in several sources, such as food, water, surfaces, sewage (Collado et al., 2011;

308 Giacometti et al., 2015; Hsu and Lee, 2015; Mottola et al., 2016; Serraino and Giacometti, 2014;

309 Šilha et al., 2015). Indeed, the contamination of food may occur during the production steps, where

310 either contaminated irrigation water or organic fertilizers obtained from different sources (e.g., peat,

311 animal waste, plant waste from agriculture, sewage sludge) are employed (Jung et al., 2014).

312 Moreover, surfaces in contact or not in contact with food or water used during irrigation or washing

313 vegetables may be involved in post-harvest contamination. Thus, it is recommended that monitoring

314 of water used during minimal processing be performed from farm to fork (EFSA BIOHAZ Panel,

315 2014). Food safety measures such as Good Agricultural Practices (GAP), Good Manufacturing

316 Practices (GMPs), Good Hygienic Practices (GHPs) and Hazard Analysis and Critical Control Point

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317 (HACCP) are also required (Report of the Scientific Committee on Food, 2002, EFSA BIOHAZ

318 Panel, 2014).

319   This study highlights the need to assess both the prevalence of arcobacters among the

320 vegetable types analyzed and *Arcobacter*'s seasonality. Although the distribution of arcobacters

321 among the various vegetable types shows no statistical correlation (P>0.05), the statistical analysis

322 performed in the present study showed a high prevalence of arcobacters during the summer months

323 (P <0.001).

324   The authors believe that the differences in prevalence of arcobacters in the period analyzed

325 may well be due to environmental parameters such as seasonal temperature variability, as observed

326 by Levican et al. (2014). In addition, the tendency for arcobacters to be more common over the

327 summer is probably due to inadequate management of the cold chain temperature, leading to better

328 survival of these bacteria species. In any event, further analysis over a longer sampling period is

329 required.

330   Considering the lack of a clear pathogenic role played by *Arcobacter* spp., the present study

331 investigated the presence and distribution of nine putative virulence genes in arcobacters recovered

332 from vegetables. PCR analysis data highlighted a complex virulence profile, suggesting that many

333 isolates are potential illness producers. In this study, the *tlyA*, *cj1349*, *mviN*, *pldA*, and *ciaB* genes

334 were present in 100% of the *A. butzleri* strains. Our results were similar to those reported in

335 previous studies by Collado et al. (2014); Douidah et al. (2012); Girbau et al. (2015); Karadas et al.

336 (2013) and Zacharow at al. (2015). Overall, in accordance with Girbau et al. (2015) and Karadas at

337 al. (2013), the prevalence of 100% of the *ciaB*, *cj1349*, *mviN*, *pldA* and *tlyA* genes confirms the

338 hypothesis that these genes are most prevalent in food sources. The presence of *ciaB* and *mviN*, two

339 virulence genes in common with those of *C. jejuni* that contribute to host-cell invasion suggests that

340 arcobacters may be homologous with *Campylobacter* in their pathogenic role. The detection of only

341 *cadF* and *mviN* genes in all *A. cryaerophilus* strains suggests that these strains may be less virulent

14

than *A. butzleri* isolates. Moreover, in agreement with other studies (Douidah et al., 2012; Girbau et al., 2015), no *A. cryaerophilus* strains were found to harbour multiple virulence genes. This difference could be explained by an inappropriate use of primers because they were designed from the *A. butzleri* RM4018 genome (Douidah et al., 2012).

From a hygiene point of view, detection of the potential emerging pathogens *A. butzleri* and *A. cryaerophilus* provided much information regarding the health risks associated with the direct consumption of raw vegetables. The European Union's food safety policy on microbiological criteria for foodstuffs includes vegetables as RTE foods. This definition presupposes that a producer using the label "Ready-to-eat foods" guarantees its safety. Today, in order to protect consumer health, European legislation on vegetable safety requires the assessment of only *E. coli*, *Salmonella* spp. and *Listeria monocytogenes* contamination (Commission Regulation (EC) No. 1441/2007). In response to the outbreak of Shiga toxin-producing *E. coli* (STEC) in May 2011 in the Union and given that the consumption of sprouts was identified as the most likely origin of the outbreaks, the European Union published Commission Regulation (EU) No. 209/2013 amending Regulation (EC) No. 2073/2005 as regards microbiological criteria for sprouts, introducing Shiga toxin-producing *E. coli* (STEC) O157, O26, O111, O103, O145 and O104:H4. However, there is no requirement to search for other potential pathogenic bacteria such as *Campylobacter* and *Arcobacter*.

5. Conclusions

Given the increase in food-borne disease related to the consumption of RTE pre-cut vegetables and the difficult epidemiological traceability for fruits and vegetables as carriers of food-borne pathogens, further epidemiological studies should be performed to acquire further data regarding the link between the consumption of RTE vegetables and human illness, in order to revise and update the legislation.

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521

522 **Table 1**
523 Type and number of fresh-cut RTE vegetables analyzed during the sampling period
524

Samples	No of samples	No. of samples analyzed during the sampling period							
		February	March	April	May	June	July	August	September
Lettuce (*Lactuca sativa L)*	65	8	10	10	10	8	7	8	4
Spinach (*Spinacia oleracea*)	37	5	7	4	4	5	3	5	4
Rocket (*Eruca vesicaria*),	33	4	2	4	3	4	4	7	5
Valerian (*Valeriana officinalis*)	25	2	2	2	1	4	5	5	4
Total	160	19	21	20	18	21	19	25	17

525
526

Table 2

528 Oligonucleotide primers

Method	Primers	Nucleotide sequence (5'- 3')	Genes	Amplicon Size (bp)	References
Genus-specific PCR	ARCOI	AGAGATTAGCCTGTATTGTATC	16S rRNA	1223	Harmon & Wesley (1996)
	ARCOII	TAGCATCCCCGTtaTTCGAATGA			
m-PCR	ARCO (R)	CGTATTCACCGTAGCATAGC	16S rRNA		Houf et al. (2000)
	BUTZ (F)	CCTGGACTTGACATAGTAAGAATGA	16S rRNA	401	Houf et al. (2000)
	SKIRR (F)	GGCGATTTACTGGAACACA	16S rRNA	641	Houf et al. (2000)
	CRY 1 (F)	TGCTGGAGCGGATAGAAGTA	23S rRNA	257	Houf et al. (2000)
	CRY 2 (R)	AACAACCTACGTCCTTCGAC	23S rRNA		
16S rRNA	CAH16S1am (F)	AACACATGCAAGTCGAACGA	16S rRNA	1026	Figueras et al. (2008)
	CAH16S1b (R)	TTAACCCAACATCTCACGAC	16S rRNA		Marshall et al. (1999)
Virulence genes:					
cadF	cadF (F)	TTACTCCTACACCGTAGT	*cadF*	283	Douidah et al. (2012)
	cadF (R)	AAACTATGCTAACGCTGGTT			
ciaB	ciaB (F)	TGGGCAGATGTGGATAGAGCTTGGA	*ciaB*	284	Douidah et al. (2012)
	ciaB (R)	TAGTGCTGGTCGTCCCACATAAAG			
cj1349	cj1349 (F)	CCAGAAATCACTGGCTTTTGAG	*cj1349*	659	Douidah et al. (2012)
	cj1349 (R)	GGGCATAAGTTAGATGAGGTTCC			
irgA	irgA (F)	TGCAGAGGATACTTGGAGCGTAACT	*irgA*	437	Douidah et al. (2012)
	irgA (R)	GTATAACCCCATTGATGAGGAGCA			
hecA	hecA (F)	GTGGAAGTACAACGATAGCAGGCTC	*hecA*	537	Douidah et al. (2012)
	hecA (R)	GTCTGTTTTAGTTGCTCTGCACTC			
hecB	*hecB*-F	CTAAACTCTACAAATCGTGC	*hecB*	528	Douidah et al. (2012)
	hecB-R	CTTTTGAGTGTTGACCTC			
mviN	*mviN*-F	TGCACTTGTTGCAAAACGGTG	*mviN*	294	Douidah et al. (2012)
pldA	*pldA*-F	TTGACGAGACAATAAGTGCAGC	*pldA*	293	Douidah et al. (2012)
	pldA-R	CGTCTTTATCTTTGCTTTCAGGGA			
tlyA	*tlyA*-F	CAAAGTCGAAACAAAGCGACTG	*tlyA*	230	Douidah et al. (2012)
	tlyA-R	TCCACCAGTGCTACTTCCTATA			

529

530

531 **Table 3**
532 Confirmed presence of *Arcobacter* spp. in the different fresh-cut vegetables during the sampling
533 period

		Sampling period and No. of detected isolates (%)							
Samples	**No of positive samples (%)**	**February**	**March**	**April**	**May**	**June**	**July**	**August**	**September**
Lettuce	13/65(20)	0 /8 (0)	0 [a]/10 (0)	0/10 (0)	1/10 (10)	2/8 (25)	2/7 (28.6)	4/8 (50)	4/4 (100)
Spinach	9/37 (24,3)	0 [a]/5 (0)	0/7 (0)	0/4 (0)	1/4 (25)	1/5 (20)	1/3 (33)	3/5 (60)	3/4 (75)
Rocket	9/33 (27,3)	0/4 (0)	0/2 (0)	0/4 (0)	0/3 (0)	1 [a]/4 (25)	1/4 (25)	3/7 (42.9)	4[a]/5 (80)
Valerian	13/25(52)	0/2 (0)	0 [a]/2 (0)	0/2 (0)	0/1 (0)	2/4 (50)	4/5 (80)	4/5 (80)	3/4 (75)
Total	44/160 (27,5)	0/19 (0)	0/21 (0)	0/20 (0)	2/18 (11)	6/21 (28.6)	8/19 (42)	14/25 (56)	14/17 (82.3)

534 [a] Eight presumptive isolates were not confirmed as belonging to *Arcobacter* spp.
535

536 **Table 4**
537 Molecular identifications of *Arcobacter* spp. of the 52 presumptive isolates (*A= absent;*
538 *P=present*)

Sample No.	Reference of the isolate	Isolate source	Collection date	Genus-specific PCR	m-PCR	16S rDNA-RFLP
1	12/O	Lettuce	March '15	*A*	*A*	-
2	20/O	Lettuce	April '15	*A*	*A*	-
3	26/O	Lettuce	April '15	*A*	*A*	-
4	31/O	Lettuce	May '15	*P*	*A. butzleri*	*A. butzleri*
5	40/O	Lettuce	June '15	*P*	*A. butzleri*	*A. butzleri*
6	43/O	Lettuce	June '15	*P*	*A. butzleri*	*A. butzleri*
7	44/O	Lettuce	June '15	*A*	*A*	-
8	47/O	Lettuce	July '15	*P*	*A. butzleri*	*A. butzleri*
9	51/O	Lettuce	July '15	*P*	*A. butzleri*	*A. butzleri*
10	54/O	Lettuce	August '15	*P*	*A. butzleri*	*A. butzleri*
11	55/O	Lettuce	August '15	*P*	*A. butzleri*	*A. butzleri*
12	57/O	Lettuce	August '15	*P*	*A. butzleri*	*A. butzleri*
13	60/O	Lettuce	August '15	*P*	*A. butzleri*	*A. butzleri*
14	62/O	Lettuce	September '15	*P*	*A. butzleri*	*A. butzleri*
15	63/O	Lettuce	September '15	*P*	*A. butzleri*	*A. butzleri*
16	64/O	Lettuce	September '15	*P*	*A. butzleri*	*A. butzleri*
17	65/O	Lettuce	September '15	*P*	*A. butzleri*	*A. butzleri*
18	66/O	Spinach	February '15	*A*	*A*	-
19	83/O	Spinach	May '15	*P*	*A. butzleri*	*A. butzleri*
20	87/O	Spinach	June '15	*P*	*A. butzleri*	*A. butzleri*
21	88/O	Spinach	July '15	*P*	*A. cryaerophilus*	*A. cryaerophilus 1B*
22	91/O	Spinach	August '15	*P*	*A. butzleri*	*A. butzleri*
23	93/O	Spinach	August '15	*P*	*A. cryaerophilus*	*A. cryaerophilus 1B*
24	94/O	Spinach	August '15	*P*	*A. butzleri*	*A. butzleri*
25	96/O	Spinach	September '15	*P*	*A. cryaerophilus*	*A. cryaerophilus* 1B
26	98/O	Spinach	September '15	*P*	*A. cryaerophilus*	*A. cryaerophilus* 1B
27	99/O	Spinach	September '15	*P*	*A. butzleri*	*A. butzleri*
28	115/O	Rocket	June '15	*P*	*A. butzleri*	*A. butzleri*
29	116/O	Rocket	June '15	*A*	*A*	-
30	118/O	Rocket	July '15	*P*	*A. butzleri*	*A. butzleri*
31	121/O	Rocket	August '15	*P*	*A. butzleri*	*A. butzleri*
32	123/O	Rocket	August '15	*P*	*A. butzleri*	*A. butzleri*
33	126/O	Rocket	August '15	*P*	*A. butzleri*	*A. butzleri*
34	131/O	Rocket	September '15	*P*	*A. butzleri*	*A. butzleri*
35	132/O	Rocket	September '15	*A*	*A*	-
36	133/O	Rocket	September '15	*P*	*A. butzleri*	*A. butzleri*
37	134/O	Rocket	September '15	*P*	*A. butzleri*	*A. butzleri*
38	135/O	Rocket	September '15	*P*	*A. butzleri*	*A. butzleri*
39	139/O	Valerian	March '15	*A*	*A*	-
40	143/O	Valerian	June '15	*P*	*A. butzleri*	*A. butzleri*

41	145/O	Valerian	June '15	*P*	*A. butzleri*	*A. butzleri*
42	147/O	Valerian	July '15	*P*	*A. butzleri*	*A. butzleri*
43	148/O	Valerian	July '15	*P*	*A. butzleri*	*A. butzleri*
44	149/O	Valerian	July '15	*P*	*A. butzleri*	*A. butzleri*
45	151/O	Valerian	July '15	*P*	*A. butzleri*	*A. butzleri*
46	152/O	Valerian	August '15	*P*	*A. butzleri*	*A. butzleri*
47	153/O	Valerian	August '15	*P*	*A. butzleri*	*A. butzleri*
48	154/O	Valerian	August '15	*P*	*A. butzleri*	*A. butzleri*
49	155/O	Valerian	August '15	*P*	*A. butzleri*	*A. butzleri*
50	157/O	Valerian	September '15	*P*	*A. butzleri*	*A. butzleri*
51	159/O	Valerian	September '15	*P*	*A. butzleri*	*A. butzleri*
52	160/O	Valerian	September '15	*P*	*A. butzleri*	*A. butzleri*

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550 **Table 5**
551 Presence of putative virulence genes in *Arcobacter* strains used in this study

Species	Source	No. Of Strain	No. (%) of strains generating specific gene amplicon								
			cadF	*ciaB*	*cj1349*	*irgA*	*hecA*	*mviN*	*tlyA*	*hecB*	*pldA*
A. butzleri	Valerian	8	4 (50)	8 (100)	8 (100)	0 (0)	0 (0)	8 (100)	8 (100)	4 (50)	8 (100)
	Lettuce	12	12 (100)	12 (100)	12 (100)	0 (0)	8 (66.6)	12 (100)	12 (100)	8 (66.6)	12 (100)
	Rocket	12	12 (100)	12 (100)	12 (100)	0 (0)	0 (0)	12 (100)	12 (100)	8 (66.6)	12 (100)
	Spinach	8	8 (100)	8 (100)	8 (100)	0 (0)	4 (50)	8 (100)	8 (100)	8 (100)	8 (100)
A. cryaerophilus	Spinach	4	4 (100)	0 (0)	0 (0)	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	0 (0)

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