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Study of *Arcobacter* spp. contamination in fresh lettuces detected by different cultural and molecular methods

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Abstract

Arcobacters are considered potential emerging food and waterborne pathogens. However, there is no data on the presence of *Arcobacter* spp. in fresh vegetables. Therefore the objective of this research was to study the presence of *Arcobacter* spp. in fresh lettuces.

Fifty fresh lettuces purchased from different local shops in Valencia (Spain) were analyzed. The assay was performed simultaneously by cultural and molecular methods. Isolates were identified by real-time, multiplex PCR and restriction fragment length polymorphism analysis of PCR-amplified DNA fragment (PCR-RFLP). Finally, all the isolates were genotyped using the randomly amplified polymorphic DNA (RAPD-PCR) method.

Arcobacter sp. was detected in 10 of the 50 samples (20%) by real-time PCR, being *A. butzleri* the unique detected species by mPCR. The detection levels obtained by conventional PCR (7 samples/50, 14%) were slightly lower. These seven samples were found to be positive also by culture isolation. All 19 obtained isolates were identified as *A. butzleri* by multiplex PCR and PCR-RFLP. Great genetic heterogeneity among the isolates was observed by RAPD-PCR profiling.

To our knowledge, this is the first study in which *Arcobacter* spp. is detected in fresh vegetables such as lettuces. Although these foods are generally considered safe, given the large quantities consumed and the fact that further cooking is absent, lettuces could be a source of arcobacters of public health concern.

1. Introduction

The genus *Arcobacter* is a member of the Gram-negative, ε-Proteobacterial subdivision and belongs to the family *Campylobacteraceae*. Arcobacters are fastidious, microaerophilic, non-sporing, motile, spiral-shaped organisms that can grow between 15 and 39 °C. These organisms also have the ability to grow aerobically at 30ºC, which is a distinctive feature that differentiates *Arcobacter* species from *Campylobacter* species.

Arcobacter presently contains six species: *Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter nitrofigilis*, *Arcobacter skirrowii*, *Arcobacter cibarius* and *Arcobacter halophilus* (Donachie et al., 2005; Houf et al., 2005, Vandamme et al., 1992). Recently a number of potentially novel species have been described: *A. thereius* sp. nov., isolated from pigs and ducks (Houf et al., 2009), *A. marinus* sp. nov. (Kim et al., in press), and *A. mytili* sp. nov., isolated from mussels (Collado et al., 2009). Among them, only *A. butzleri, A. skirrowii*, *A. cryaerophilus* and *A. cibarius* have been associated with animal and human infections (Houf et al., 2005; Van Driessche et al., 2005). Furthermore, the majority of isolated arcobacters belong to one of three species *Arcobacter butzleri*, *A. cryaerophilus* or *A. skirrowii* (Miller et al., 2009).

The direct connection between consumption of *Arcobacter* contaminated food or water and human illness has not been established yet, although it is likely that transmission of arcobacters takes place via these routes. It has been suggested that water may play an important role in transmission (Fera et al., 2004; González et al.,

2007; Moreno et al., 2003; Rice et al., 1999). Raw meat is also considered as another source of *Arcobacter* infection in humans.

Different studies reported the detection of *Arcobacter* spp. in various types of water including ground water, surface water, raw sewage and sea water (Diergaardt et al., 2004; Lehner et al., 2005). They are also commonly present on food of animal origin with the highest prevalence for poultry, followed by pork and beef (Rivas et al., 2004). However, to date no information is available about the presence of *Arcobacter* spp. in fresh vegetables and given that in recent years the consumption of salads has increased, driven by the trend towards healthier eating, it could be interesting to monitoring its microbiological contamination.

Standardized *Arcobacter* detection methods have yet to be established. Several studies comparing different culture based protocols have been published (Ohlendorf et al., 2002; Scullion et al., 2004). However, it takes on average 4 to 5 days from receipt of a sample to the confirmation of an isolate as *Arcobacter*. Over the last few years, molecular assays, such as PCR based methods, have already proved to be valuable tools for rapid *Arcobacter* detection and identification (González et al., 2007; Houf et al., 2000). Generally, these methods are more rapid, sensitive and specific than culture, and nowadays they are evolving to automated procedures, which allow for a real-time monitoring of the process of DNA amplification. Therefore the objective of this research was to study the presence of *Arcobacter* spp. in fresh lettuces for human consumption using different cultural and molecular methods.

2. Materials and methods

2.1. Sample processing.

Fifty fresh lettuces purchased from seven different local retail shops in the city of Valencia (Spain) between January and July of 2009 were analyzed. Samples were transported to the laboratory, stored at 5ºC, and examined within 1 h of sampling. SYBR Green real-time PCR, conventional and multiplex PCR, and cultural methods were performed simultaneously. To confirm the results each food sample was tested twice in different experiments.

The samples (20 g) were individually homogenized for 2 min in a homogenizer (Stomacher Lab-Blender 400, Seward Medical, London, England) with 180 ml (1:10 dilution) of Arcobacter Enrichment Basal Medium (Oxoid CM965, Basingstoke, England). Subsequently, 20 ml of double-strength Arcobacter Broth (AB) with Cephoperazone-AmphotericinB-Teicoplanin (CAT) selective supplement (Oxoid SR174E) were inoculated with 20 ml of the homogenized samples and mixed thoroughly and incubated for enrichment at 30°C under microaerophilic conditions (Oxoid Campy*Gen* sachets, Oxoid CN0035) for 48 h. Although *Arcobacter* spp. are capable of aerobic growth, the optimal growth condition for primary isolation is microaerobic (Mansfield and Forsythe, 2000).

For direct PCR detection of *Arcobacter* spp. in the lettuce samples, 1 ml aliquots of the homogenized samples were processed before and after the 48 h enrichment period. The samples were centrifuged for 10 min at 12.000 rpm to pellet the bacteria and DNA was subsequently extracted using a commercial food DNA extraction Kit (Speedtools Food DNA, Biotools B&M Labs., S.A., Madrid, Spain).

For isolation of bacteria, 80 µl of each broth was dropped on a 0.45 µm cellulose membrane filter laid on the surface of sheep blood agar plates with CAT, taking care to avoid spilling the inoculum over the edge of the filter. After one hour incubation at 30ºC in aerobic atmosphere, the filters were removed and the plates were incubated for 48 h at 30ºC under microaerophilic conditions. This technique was previously used to isolate *Arcobacter* spp. from chickens (Atabay and Corry, 1997), and it depends on the ability of arcobacters, but not the competitive biota, to pass through a membrane filter. One to four presumptive *Arcobacter* colonies (small, white or grey, round colonies) were selected from each plate, checked by Gram stain microscopic appearance and for their ability to grow on blood agar aerobically at 30ºC (to differentiate from *Campylobacter* spp.). Identification was confirmed by real-time and conventional PCR as described below.

2.2. Molecular methods.

Cells from an exponential growth of the purified cultures were harvested and resuspended in 500 µl of Tris-EDTA (TE) buffer. After that, DNA extraction and purification was performed using a genomic DNA extraction Kit (GeneElute Bacterial Genomic DNA Kit, Sigma-Aldrich, USA). Presumptive arcobacters were identified by real-time and conventional PCR. Species identification was performed using a recently developed 16S rDNA-RFLP technique and a multiplex PCR assay. The discrimination among all the isolates recovered from the same sample and belonging to the same species was carried out by RAPD-PCR. For ensuring reproducibility of results, all the isolates were analysed twice in different experiments.

140 *Arcobacter* sp. detection was carried out by real-time PCR using ARCO1 (5'-
141 GTCGTGCCAAGAAAAGCCA-3') and ARCO2 (5'-TTCGCTTGCGCTGACAT-
142 3') primers (Bastyns et al., 1995). The mixture consisted of 2 µl of DNA, 0.5 µM of
143 each primer, 2 mM $MgCl_2$ and 2 µl of LightCycler Fast-Start DNA Master SYBR
144 Green I Mix (Roche Diagnostics GmbH, Mannheim, Germany) in a total reaction
145 volume of 20 µl. The reactions were performed in a LightCycler 2.0 real-time PCR
146 system (Roche Diagnostics Ltd, Rotkreuz, Switzerland) according to González et al.
147 (2010).

148 Detection by conventional PCR was done using the same primers (ARCO 1
149 and ARCO2) that amplified a 331-bp fragment of 23S rRNA gene. Then, for
150 simultaneous detection of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*, a species-
151 specific multiplex PCR assay, using the primers described by Houf et al. (2000), was
152 performed. Primers amplify a 401-bp fragment of 16S rRNA gene for *A. butzleri*, a
153 641-bp of 16S rRNA gene for *A. skirrowii* and a 257-bp fragment of 23S rRNA gene
154 for *A. cryaerophilus* species. Both PCR assays were developed as described by
155 González et al. (2007).

156 PCR products (15 µl) were detected by electrophoresis on 1.5% (w/v) agarose
157 gel in 1× Tris-Acetate-EDTA (TAE) buffer at 90V for about 90 min, and visualized
158 by UV transillumination after staining with ethidium bromide (0.5 µg/ml). A 100-bp
159 DNA ladder (Fermentas, Burlington, Canada) was used as a molecular weight
160 marker.

161 Species identification of the isolates by PCR-RFLP analysis was performed
162 using the 16S rDNA-RFLP method designed by Figueras et al. (2008) that is able to

discriminate the 6 currently accepted species. Firstly, a 1026-bp fragment of the 16S rDNA from all the isolates was amplified using CAH1a mod (5'-AACACATGCAAGTCGAACGA-3') and CAH1b (5'-TTAACCCAACATCTCACGAC-3') primers. Then, PCR products (10 µl) were digested with 10 U of the enzyme *Mse*I (Fermentas) in a final volume of 30 µl at 65°C for 5 h. Restriction fragments were separated by electrophoresis on 3.5% (w/v) agarose gels in TAE 1× buffer with ethidium bromide at 85V for 3 h. GeneRuler 100-bp DNA Ladder Plus (Fermentas) was used as a standard for molecular size determination.

For all the assays, DNA templates from reference strains *A. butzleri* DSM 8739, *A. cibarius* DSM 17680, *A. cryaerophilus* DSM 7289, *A. halophilus* DSM 18005, *A. nitrofigilis* CECT 7204, and *A. skirrowii* CIP 103588 were used as positive controls. Negative controls in which DNA was replaced with sterile distilled water were also included in every assay.

The characterization of the isolates was carried out by RAPD-PCR analysis using the 1254 primer 5′-CCGCAGCCAA-3′ (Akopyanz et al., 1992) according to González et al. (2010). Amplified PCR products (15 µl each) were separated by electrophoresis in 2.5% (w/v) agarose gels run in 1× TAE buffer with ethidium bromide at a constant voltage of 90V for 3.5 h. Finally, DNA fragments were viewed under UV transillumination. Patterns with at least one different band were considered as different types. Isolates which presented the same pattern and had been recovered from the same sample were considered to be the same strain.

185 The PCR reactions were performed with an automatic gradient thermocycler
186 (Eppendorf AG, Hamburg, Germany). All the reagents (*Taq* polymerase, dNTP and
187 $MgCl_2$) were provided by Ecogen (Spain) and the primers were prepared by TIB
188 MOLBIOL (Germany).

189

190 **3. Results and discussion**

191 All the *Arcobacter*-positive lettuces had been purchased from the same retail
192 shop. *Arcobacter* sp. was detected in 10 of the 50 samples (20%) by real-time PCR,
193 but just in one of them the detection was possible without enrichment (sample L22).
194 The detection rate using conventional PCR was slightly lower. Seven out of the 10
195 real-time PCR positive samples also gave a positive result after 48 h enrichment in
196 AB supplemented with CAT at 30°C under microaerophilic conditions. Arcobacters
197 were not found on the initial suspensions by conventional PCR, except for one of the
198 samples (sample L22), as with the real-time PCR (Table 1).

199 To confirm the results each food sample was tested twice and, for all samples,
200 repeated PCR analysis yielded consistent results. All the other lettuce samples
201 analyzed were negative and remained negative when tested by both PCR assays even
202 after the enrichment period.

203 When multiplex PCR was applied to enrichment broths, *A. butzleri* was the
204 only detected species in all of the 10 PCR-positive samples (Table 1), although this
205 PCR is able to detect simultaneously *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*.
206 Therefore, it can be assumed that they were not present in the samples.

As expected, some real-time PCR-positive samples were negative by culture, but negative samples by real-time PCR were always *Arcobacter*-negative by means of selective plating or conventional PCR. The detection rates by real-time PCR were higher than isolation, showing that arcobacters were present in the samples although they were not able to be recovered, probably because the numbers were very low. Alternatively, DNA but not viable bacteria could have been present in the samples. However, it is unlikely because an enrichment step was included to avoid false positive results. In fact, it has been reported that the combination of PCR with an enrichment step increases the level of viable cells, while dead cells and inhibitors are diluted (Denis et al., 2001). In addition, bacterial contamination levels in food products are often lower than those in clinical samples. Therefore, although real-time PCR is especially useful for quick detection without enrichment, we included an enrichment step as that is often required for food analyses. It seems that differences in recovery rates of *Arcobacter* spp between the two PCR assays may be due to a hundredfold difference in their detection limits (González et al., 2010). Moreover, the time for isolation by culture methods required at least 5 days and further biochemical identification while the total analysis time by real-time PCR, even after previous 48 h enrichment, was reduced to 2 days. The application of molecular methods to rapidly and unequivocally detect and identify foodborne pathogens in foodstuffs is offering a valid alternative to traditional microbiological testing (Rantsiou et al., 2010).

Seven samples were found to be positive by culture. They were the same seven samples *Arcobacter*-positive with the conventional PCR assay. A total of 19 isolates were obtained from these samples. All positive samples, other than sample 22, required 48 h of enrichment and then plating before presumptive *Arcobacter* was

231 detected (Table 1). However, sample L22 was found to be also positive by direct
232 plating and by PCR on the initial suspensions, suggesting higher contamination levels
233 than the others.

234 The application of the multiplex PCR assay generated the 401-bp fragment of
235 16S rRNA gene typical for *A. butzleri* for all isolates examined (Table 1). However,
236 as the multiplex PCR technique only enables the identification of *A. butzleri*, *A.*
237 *cryaerophilus* and *A. skirrowii*, the 16S rDNA-RFLP assay was used for confirmation
238 of *A. butzleri*. Digestion with restriction enzyme *Mse*I yielded the six expected
239 specific patterns for the *Arcobacter* reference strains (Figueras et al., 2008). The 19
240 isolates produced fingerprints that were identical to that of *A. butzleri* DSM 8739
241 reference strain (Table 1).

242 *A. cryaerophilus* and *A. skirrowii* were not isolated in this study. The most
243 probable reason for this may be that they were not present in the lettuces, as they
244 were not detected either by direct PCR of the samples, and the isolation method used
245 in the current study is also able to detect those other two species of *Arcobacter*
246 (Atabay et al., 2003). Among *Arcobacter* spp. isolated from food of animal origin and
247 water, *A. butzleri* is found most, followed by *A. cryaerophilus. A. skirrowii* is rarely
248 detected due to its low prevalence or by the fact that it is more difficult to isolate than
249 *A. butzleri* and *A. cryaerophilus* (Lehner et al., 2005). *A. butzleri* seems to be highly
250 prevalent in animal and chicken meat, as well as various types of water samples
251 (Diergaardt et al., 2004; Ho et al., 2006; Lehner et al., 2005), though its prevalence in
252 raw vegetables has been very rarely studied (Winters and Slavik, 2000). Therefore,

253 the lack of published data about *Arcobacter* spp. contamination in fresh lettuces limits
254 the ability to compare our results with other studies.

255 A total of 9 different RAPD-PCR profiles, with 4-10 amplified DNA
256 fragments ranging from 260 to 2800-bp, could be distinguished among the 19 *A.*
257 *butzleri* isolates obtained from the lettuce samples. DNA patterns of the isolates
258 showed a substantial intra-species genetic heterogeneity. This great genetic variation
259 has been reported previously by other authors (Atabay et al., 2002; Houf et al., 2002;
260 Houf et al., 2003). The same profile was never detected in the isolates belonging to
261 different samples, except for the isolates from samples L41 and L42, which presented
262 identical patterns (Figure 1; Table 1). What is more, in some isolates from the same
263 sample more than one genetic profile was detected. The four *A. butzleri* isolates of the
264 sample L18 showed 3 different patterns and the isolates of sample L22 obtained by
265 direct plating presented a different genetic profile from those isolates obtained from
266 the same sample after enrichment (Table 1). As this method is limited by its
267 reproducibility, because it uses a single nonspecific primer and low annealing
268 temperatures, all the isolates were analysed twice and no variation in the RAPD-PCR
269 patterns was observed.

270 Our results have proved that RAPD-PCR analysis is a valuable and simple
271 technique able to discern among *Arcobacter* isolates. In the present study all the
272 *Arcobacter*-positive samples were purchased from only one of the seven shops. This
273 may indicate a contamination during manipulation at retail instead of a contamination
274 of the vegetables in the field; however, it is unlikely because different RAPD-PCR
275 profiles among the isolates obtained from different lettuces were observed.

To our knowledge, this is the first study in which *Arcobacter* spp. is detected in fresh vegetables such as lettuces. These foods are generally considered safe and *Arcobacter* contamination levels seem to be rather lower than in animal food products and waters. However, given the large quantities of vegetables that are consumed and the fact that further cooking is absent, these foods could be considered as a potential public health risk. As there are no previous published data on the incidence of *Arcobacter* spp. in raw vegetables, and no standard detection method is available, further studies including more samples, and more kind of fresh vegetables would be needed before any definitive conclusions can be drawn.

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449 Table 1. Detection and identification of *Arcobacter* spp. in fresh lettuces

Sample	Incubation[a]	Real-time PCR[b]	PCR[b]	Multiplex PCR[b,c]	Number of isolates[b,c,d]	RAPD-PCR profiles
L18	0_h	-	-	-	-	
	48_h	+	+	*A b*	4 (*A b*)	I, II, III
L22	0_h	+	+	*A b*	3 (*A b*)	IV
	48_h	+	+	*A b*	4 (*A b*)	V
L40	0_h	-	-	-	-	
	48_h	+	+	*A b*	1 (*A b*)	VI
L41	0_h	-	-	-	-	
	48_h	+	+	*A b*	2 (*A b*)	VII
L42	0_h	-	-	-	-	
	48_h	+	+	*A b*	1 (*A b*)	VII
L46	0_h	-	-	-	-	
	48_h	+	-	*A b*	-	
L47	0_h	-	-	-	-	
	48_h	+	+	*A b*	3 (*A b*)	VIII
L48	0_h	-	-	-	-	
	48_h	+	-	*A b*	-	
L49	0_h	-	-	-	-	
	48_h	+	-	*A b*	-	
L50	0_h	-	-	-	-	
	48_h	+	+	*A b*	1 (*A b*)	IX

450 [a] 0_h, sample diluted in AB broth before enrichment; 48_h, sample after enrichment
451 [b] +, *Arcobacter* spp. detected; -, *Arcobacter* spp. non detected
452 [c] *A b*, *Arcobacter butzleri*
453 [d] Identification of the isolates by multiplex PCR and PCR-RFLP analysis in brackets
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459 **Figure legends**

460 **Figure 1.** RAPD-PCR profiles of representative *A. butzleri* isolates obtained from
461 different lettuce samples. Lanes M, 100-bp DNA Ladder Plus with band sizes
462 indicated on right (bp); lanes 1-4: isolates sample L18; lane 5: isolate sample L42;
463 lanes 6-9, 13: isolates sample L47; lane 10: isolate sample L40; lane 11: isolate
464 sample L41; lane 12: isolate sample L22.

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