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Abstract

Some species of the Arcobacter genus are considered emerging foodborne and waterborne enteropathogens. However, the presence of Arcobacter spp. in vegetables very little is known, because most studies have focused on foods of animal origin. On the other hand, quinolones are considered as first-line drugs for the treatment of infection by campylobacteria in human patients, but few data are currently available about the resistance levels to these antibiotics among Arcobacter species. Therefore, the aim of this study was to investigate the presence and diversity of arcobacters isolated from fresh vegetables such as lettuces, spinaches, chards and cabbages. Resistance to quinolones of the isolates was also investigated. One hundred fresh vegetables samples purchased from seven local retail markets in Valencia (Spain) during eight months were analysed. The study included 41 lettuces, 21 spinaches, 34 chards and 4 cabbages. Samples were analysed by culture and by molecular methods before and after enrichment. By culture, 17 out of 100 analysed samples were Arcobacter positive and twenty-five isolates were obtained from them. Direct detection by PCR was low, with only 4 % Arcobacter spp. positive samples. This percentage increased considerably, up 20 %, after 48 h enrichment. By polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), 17 out of the 25 isolates were identified as A. butzleri and 8 as A. cryaerophilus. Only two A. butzleri isolates showed resistance to levofloxacin and ciprofloxacin. The sequencing of a fragment of the QRDR region of the gyrA gene from the quinolones-resistant isolates revealed the presence of a mutation in position 254 of this gene (C-T transition). This study is the first report about the presence of pathogenic species of Arcobacter spp. in chards and cabbages and confirms that fresh vegetables can act as transmission vehicle to humans. Moreover, the presence of A. butzleri quinolone resistant in vegetables could pose a potential public health risk.

Keywords	Arcobacter spp. detection; vegetables; quinolone resistance; gyrA

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Universitat Politècnica de València
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Dr. Tortorello
Editor-in-Chief
Food Microbiology

January 23rd, 2016

Dear Dr;

We deeply appreciate that you have found acceptable, after minor changes and transforming it to a short communication, our submitted manuscript entitled **"Isolation, molecular identification and quinolone-susceptibility testing of *Arcobacter* spp. isolated from fresh vegetables in Spain"** for publication in "Food Microbiology". We have follow your instructions to response the reviewer's comments.

Yours sincerely,

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RESPONSE TO THE EDITORS AND REVIEWERS

Thanks for the reviewers comments that have improved the quality of our manuscript. We have highlighted in green the changes done in the manuscript. Additionally the text has been slightly shorted to fit in the requirements for short communications, as requested by the editor. We have also added five new references in the text that have been also highlighted.

A short overview on different foods that was reported to be contaminated by Arcobacters could be useful in the introduction. It has been done accordingly

Why did the Authors choose an incubation temperature of 37°C; usually other Authors chose lower temperatures for Arcobacter detection; and how this choice may have influenced the results?

We have chosen an incubation at 37ºC under microaerophilic atmosphere because some authors have stated that these organisms grow optimally for primary isolation under these conditions, and sometimes aerotolerance at 30ºC is not observed on initial isolation (Mansfield and Forsythe, 2000, Rev. Medical Microbiol., 11:161-170). In addition, our research group published an article in 2007 comparing the isolation rates obtained when the samples were enriched under microaerophilic conditions at 37ºC or in aerobic conditions at 30ºC, and our results indicated that a slight improvement in isolation rate when the enrichment was under microaerophilic conditions at 37ºC was observed (González *et al*., 2007, J. Food Prot., 70: 341-347).

Can the Authors give some details on the sampled vegetable if appropriate (for example were they prewashed and packed vegetables?)

All vegetables were fresh, unpacked, not washed and without a previous step of disinfection.

Please consider in the discussion, were appropriate, the following article: Anna Mottola et al. Occurrence of emerging food-borne pathogenic Arcobacter spp. isolated from pre-cut (ready-to-eat) vegetables. International Journal of Food Microbiology 236 (2016) 33–37. It has been done accordingly

Table 2 - Use decimal points instead of commas. It has been done accordingly

Line numbering throughout the manuscript should be used to facilitate review and comment. It has been done accordingly

Isolation, molecular identification and quinolone-susceptibility testing of *Arcobacter* spp. isolated from fresh vegetables in Spain

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ABSTRACT

Some species of the *Arcobacter* genus are considered emerging foodborne and waterborne enteropathogens. However, the presence of *Arcobacter* spp. in vegetables very little is known, because most studies have focused on foods of animal origin. On the other hand, quinolones are considered as first-line drugs for the treatment of infection by campylobacteria in human patients, but few data are currently available about the resistance levels to these antibiotics among *Arcobacter* species. Therefore, the aim of this study was to investigate the presence and diversity of arcobacters isolated from fresh vegetables such as lettuces, spinaches, chards and cabbages. Resistance to quinolones of the isolates was also investigated.

One hundred fresh vegetables samples purchased from seven local retail markets in Valencia (Spain) during eight months were analysed. The study included 41 lettuces, 21 spinaches, 34 chards and 4 cabbages. Samples were analysed by culture and by molecular methods before and after enrichment. By culture, 17 out of 100 analysed samples were *Arcobacter* positive and twenty-five isolates were obtained from them. Direct detection by PCR was low, with only 4 % *Arcobacter* spp. positive samples. This percentage increased considerably, up 20 %, after 48 h enrichment. By polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), 17 out of the 25 isolates were identified as *A. butzleri* and 8 as *A. cryaerophilus*. Only two *A. butzleri* isolates showed resistance to levofloxacin and ciprofloxacin. The sequencing of a fragment of the QRDR region of the *gyrA* gene from the quinolones-resistant isolates revealed the presence of a mutation in position 254 of this gene (C-T transition).

This study is the first report about the presence of pathogenic species of *Arcobacter* spp. in chards and cabbages and confirms that fresh vegetables can act as transmission vehicle to humans. Moreover, the presence of *A. butzleri* quinolone resistant in vegetables could pose a potential public health risk.

Key words: *Arcobacter* spp. detection, vegetables, quinolone resistance, *gyrA*.

1. Introduction

Arcobacters are Gram-negative, slender, motile, spiral-shaped rods, members of the ε-Proteobacteria subdivision, belonging to the family *Campylobacteraceae*. *Arcobacter* spp. are fastidious organisms that can be differentiated from *Campylobacter* species by its ability to grow in aerobic conditions and at lower temperatures, between 15 and 30 °C (**Ünver *et al*., 2013**). This genus currently consists of 23 species that have been isolated from humans and animals, as well as water and food sources (**Keyman *et al*., 2012; Levican *et al*., 2013; Levican *et al*., 2015; Sasi *et al*., 2013; Whiteduck-Leveillee *et al*., 2015a; Whiteduck-Leveillee *et al*., 2015b; Zhang *et al*., 2015**). The species *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. cibarius* and *A. thereius* have been associated with animal and human infections **(Collado and Figueras 2011; Houf *et al*., 2005; Van den Abeele *et al*., 2014)**. The number of foodborne and waterborne diseases caused by these organisms has increased in recent years **(Hsu and Lee, 2015)**, which represents a challenge for Food Safety.

Although *Arcobacter* epidemiology is not well known, it has been suggested that water plays a significant role in the transmission of the pathogen (**Collado *et al*., 2008; Collado *et al.*, 2010; González *et al*., 2007)**. Different studies reported the presence of *Arcobacter* in various water environment: wastewater, lakes and river, recreational beach, ground water, seawater and drinking water **(Hsu and Lee, 2015)**. Food products of animal origin have also been suggested as an important potential transmission route of *Arcobacter* **(Collado**

and Figueras, 2011). Shellfish are another potential pathogenic *Arcobacter* hosts **(Mottola *et al.*, 2016a).** Arcobacters have been also detected in fresh lettuces in Spain (**González and Ferrús 2011**), in a spinach-processing plant **(Hausdorf *et al*., 2013)** and in a carrot-processing plant in Germany **(Hausdorf *et al*., 2011)**. Moreover, *Arcobacter* spp. has recently been isolated from pre-cut ready-to-eat vegetables **(Mottola *et al*., 2016b).** Vegetables can be contaminated through irrigation water, or directly from faecal discharges of infected animals (**Hausdorf *et al*., 2013**). Based on published data, the weighted average prevalence of *Arcobacter* in food, from highest to lowest, is as follows: dairy products, pork, seafood, beef, poultry, lamb, vegetable and rabbit **(Hsu and Lee, 2015)**.

In general, *A. butzleri* is the most prevalent species in food, followed by *A. cryaerophilus* and *A. skirrowii* **(Collado *et al.*, 2009)**. In fact, *A. butzleri* was in the list of microbes considered a serious hazard to human healthy by the International Commission on Microbiological Specifications for Foods **(ICMFS, 2002).**

The identification of *Arcobacter* species is still problematic, due to irregular biochemical reactions of some isolates, as well as the low metabolic activity of microorganism. (**Atabay *et al*., 2006**). Several DNA-based techniques have been described for identifying some *Arcobacter* species, such as: m-PCR (**Houf *et al*., 2000**), real-time PCR (**Brightwell *et al*., 2007**), sequencing of 16S rRNA (**Lau *et al*., 2002)**, *rpoB, rpoC* (**Morita *et al*., 2004**) and *gyrA* genes (**Abdelbaqi *et al*., 2007),** and microarray techniques (**Quiñones *et al*., 2007**). These methods are not able to identify all the species included in the genus. However, the 16S rRNA-RFLP method developed by **Figueras *et al.* (2012**) allows the identification of up to 17 of the 23 species currently recognized.

Fluoroquinolones are one of the most commonly prescribed antimicrobial agents in the world and are used to treat a variety of bacterial infections in humans (**Ünver *et al*., 2013**). Due to the wide use (and overuse) of these drugs, the number of quinolone-resistant bacterial strains has been growing steadily since the 1990s. This fact threatens their clinical utility (**Aldred *et al*., 2014**). There are limited researches about of *Arcobacter* antimicrobial susceptibility to fluoroquinolones of clinical use. However, it has been reported that the resistance to these antimicrobial agents is related to the presence of a mutation in the QRDR region of the *gyrA* gene (C to T transition), which leads to a substitution in the position 85 (Thr to Ile) in the GyrA protein (**Abdelbaqi *et al*., 2007; Van den Abeel *et al*., 2016**).

Therefore, the aim of this work was to investigate the presence, diversity and quinolone susceptibility of *Arcobacter* spp. isolated from fresh vegetables, as well as to analyze the mutations associated with quinolone resistance.

2. Materials and methods

2.1. Sample preparation

A total of 100 vegetable samples collected from November 2014 to June 2015 in the city of Valencia, Spain were examined. Lettuces (41), chards (34), spinaches (21) and cabbages (4) were purchased from different local retail establishments. All vegetables were fresh, unpacked, not washed and without a previous step of disinfection. Samples were transported to the laboratory under refrigeration and analyzed within 3 h of sampling.

All samples were weighed and homogenized individually: 20 g of each sample were mixed with 180 mL (1:10, wt/vol) of *Arcobacter* broth (Oxoid, UK) inside of a stomacher bag for 2 min. Twenty mL of the homogenized samples were inoculated into 20 mL of *Arcobacter* broth (AB) with double concentration of Cefoperazone – Amphotericin B -Teicoplanin (2CAT) selective supplement (Oxoid, UK), and the mix was incubated for enrichment at 37± 1 °C under microaerophilic conditions (CampyGen sachets, Oxoid, UK) for 48±2 h. After enrichment, 100 µL of the enrichment broth was transferred onto the surface of *Arcobacter* agar plates with 5% defibrinated sheep blood, using a 0.45 µm cellulose membrane filter, according to the technique described by **Atabay and Corry (1997**). After an hour of incubation at 37 °C, the filters were removed and the plates were incubated at 37±1 °C for 48±2 h. Grey-white and round small clear colonies with 2-3 mm

diameter were checked by Gram stain. The Gram-negative suspicious *Arcobacter* colonies were streaked on plates and confirmed by conventional PCR.

2.2. DNA extraction and PCR detection

For direct PCR detection from samples, DNA extraction was performed from culture broths (before and after 48 h enrichment period). To identify the presumptive *Arcobacter* isolates, colonies were harvested and re-suspended in 300 µL of Tris-EDTA (TE), centrifuging for 2 min at 12000–16000 xg and subsequently submitted to DNA extraction. The bacterial DNA was extracted using a commercial kit (GenElute Bacterial Genomic DNA Kit, Sigma- Aldrich, USA), following the manufacturer's instructions.

Specific *Arcobacter* spp. PCR was performed as described by **Bastyns *et al.*** (1995) using ARCO1 (5'-GTCGTGCCAAGAAAAGCCA-3') and ARCO2 (5'-TTCGCTTGCGCTGACAT-3') primers that amplified a 331-bp fragment of 23S rDNA gene. *A. butzleri* DSM 8739 was included as a positive control throughout the study. PCR products (5 µL) were detected by electrophoresis on 1.2% (wt/vol) agarose gel prepared in 1 X Tris–Acetate–EDTA (TAE) buffer and stained with Red Safe (Ecogen, Spain) at 100 V for 45 min. The amplicons were visualized in a UV transilluminator. A 100-bp DNA ladder (Thermo Fisher Scientific, Germany) was used as a molecular weight marker.

2.3. Species identification of *Arcobacter* isolates

For species identification, we used the 16S rRNA-RFLP technique described by **Figueras *et al.* (2012**). A 1026-bp fragment of the 16S rRNA gene from all the isolates was amplified using CAH1am (5′-AACACATGCAAGTCGAACGA-3′) and CAH1b (5′-TTAACCCAACATCTCACGAC-3′) primers. The amplification was developed in a total volume of 50 µL including 1X NH_4 buffer, 1.5 mM $MgCl_2$, deoxynucleoside triphosphates at a final concentration of 0.2 mM each, 0.5 µM of each primer, 5 U of *Taq* polymerase (Ecogen, Spain) and 1 µL of DNA template, following the conditions described by **Figueras *et al.* (2008)**. The PCR product was purified using a commercial Kit (GenElute PCR Clean-Up Kit, Sigma-Aldrich, USA) according to the manufacturer's instructions. After purification, PCR products (10 µL) were digested with 10 U of the endonuclease *Mse*I (Thermo Fisher Scientific, Germany) and 2 µL of 10 X buffer R, in a total volume of 30 µL. The reaction mixture was incubated at 65 °C for 5 h. For discriminating between *A. butzleri*, *A. thereius* and *A. trophiarum*, an enzymatic digestion with *Mnl*I endonuclease (Thermo Fisher Scientific, Germany) was performed: 10 µL of the PCR product were digested with 10 U of the enzyme and 2 µL of 10 X buffer G, and the mix was incubated at 37 °C for 5 h.

Restriction fragments were separated by electrophoresis on 3.5% of agarose gels prepared in 1 X TAE buffer at 90 V for 2.5 h. A 50-bp ladder (Thermo Fisher Scientific, Germany) was used as molecular weight marker. The gels were stained with Gel Red Nucleotid Acid (Biotium, USA) according to the manufacturer´s instructions. Finally, the gels were photographed on a UV transilluminator.

In all assays, DNA templates from reference strains *A. butzleri* DSM 8739, *A. cryaerophilus* 1A CECT 8222, *A. defluvii* CECT 7697, *A. mytili* CECT 7386, *A. molluscorum* CECT 7696 and *A. ellisii* CECT 7837 were used to compare the restriction patterns obtained.

In addition to the PCR-RFLP experimental analysis, a computational simulation was performed by downloading from the GenBank database the 16S rRNA gene of the reference strains of the species enumerated formerly. The 1026-bp fragment from each reference strain was analysed by REBASE (http://rebase.neb.com/rebase/rebtools.html), using the restriction enzymes *Mse*I and *Mnl*I. The results were compared with experimental assays.

2.4. Antimicrobial susceptibility

The antimicrobial susceptibility test was carried out using the disc diffusion (BD, USA) and E-test strips (BioMérieux, France) methods. The antimicrobial agents used in this study were ciprofloxacin and levofloxacin. In brief, the 25 isolates obtained were grown on *Arcobacter* agar with 5% defibrinated sheep

blood at 37 °C under microaerobic conditions for 48 h. After incubation, a suspension of each organism was prepared in *Arcobacter* broth and adjusted to McFarland 0.5. The suspensions were spread onto *Arcobacter* agar using a sterile cotton-tipped swab, and then the antibiotic discs (5 µg/disc) and strips (0.002 to 32 µg/mL) were placed onto the agar plates. Finally, the plates were incubated in microaerobic atmosphere at 37±1 °C for 48±2 h. After incubation period, the diameter of the inhibition zones surrounding discs and the Minimum Inhibitory Concentration (MIC) of the E-test were measured and the results were interpreted according to the National Committee for Clinical Laboratory Standards to *Campylobacter* (**CLSI, M45-A2, 2010**). A disc diffusion zone of ≤ 6 mm and a MIC value ≥ 4 µg/ml indicates resistance while a MIC value ≤ 1 and a disc diffusion zone of > 6 mm indicates susceptibility.

2.5. Sequencing of the *gyrA* gene of resistant *Arcobacter* isolates

The resistant isolates as well as two susceptible isolates and the reference strain *A. butzleri* DSM 8739 were analyzed by sequencing of the QRDR region of the *gyrA* gene. Primers used were F–QRDR (5'-TGGATTAAAGCCAGTTCATAGAAG´-3) and R2–QRDR (5'-TCATMGWATCATCATAATTTGGWAC´-3), which generate a 344-bp PCR fragment of the *gyrA* gene (**Abdelbaqi *et al*., 2007**).

PCR products were purified by using a commercial Kit (GenElute Bacterial Genomic DNA Kit, Sigma-Aldrich, USA) following the manufacturer's instructions. Finally, the purified PCR products were sequenced by Instituto de Biología Molecular y Celular de Plantas, Universitat Politècnica de València (IBMCP-UPV, Valencia, Spain).

3. Results

3.1. *Arcobacter* spp. detection by conventional PCR

Arcobacter spp. was detected in 20 out of the 100 analysed samples (20%) after 48 h enrichment in AB supplemented with 2CAT at 37 °C under microaerophilic conditions. The highest detection levels were obtained in spinaches (42.90%, 9/21) and cabbages (25%, 1/4), while lettuces and chards showed percentages of 14.63 (6/41) and 11.76 (4/34), respectively. Only in four of the 20 positives samples, the detection was possible without enrichment (**Table 1**).

3.2. *Arcobacter* spp. detection by culture

Seventeen out of 100 vegetables samples (17%) were contaminated with *Arcobacter*. The highest presence was found in spinaches (38.10%, 8/21), followed by cabbages (25.0%, 1/4), chards (11.76%, 4/34) and lettuces (9.76 %, 4/41). (**Table 2**). Twenty-five isolates were obtained from the 17 positive samples, 13 from spinaches, 6 from chards, 5 from lettuces and 1 from cabbages.

3.3. Identification of *Arcobacter* spp. isolates by PCR-PFLP

The method used in this work allows the identification of up to 17 of the 23 species currently recognized. After *Mse*I restriction, two different patters were obtained: pattern I (365, 216 and 138-bp) specific for *A. cryaerophilus*, and pattern II (548, 216 and 138-bp) shared by three species, *A. butzleri*, *A. thereius* and *A. trophiarum* **(Fig. 1)**. The digestion with *Mnl*I enzyme of the isolates sharing the same profile after *Mse*I restriction, yielded only one pattern (267, 173, 147 and 106-bp), specific for *A. butzleri*.

Finally, two different species were identified by 16S rRNA-RFLP assay with *Mse*I and *Mnl*I. Seventeen isolates (68%) were identified as *A. butzleri* and eight (32%) as *A. cryaerophilus* (**Table 2**). All experimental RFLP patterns were similar to the obtained by computational *in silico* assay. To our knowledge, this is the first report of *A. cryaerophilus* isolation from lettuces, chards and cabbages.

3.4. Antibiotic susceptibility test and analysis of the QRDR of the *gyrA* gene

All isolates of *A. cryaerophilus* isolates were found to be susceptible to levofloxacin and ciprofloxacin. Only two isolates of *A. butzleri* were resistant to both antibiotics (11.76%, 2/17).

A 344-bp fragment of *gyrA* gene was amplified from all the isolates. The sequencing of the PCR product revealed the presence of a mutation in position 254 of *gyrA* gene (C-T transition) in the two resistant *A. butzleri* isolates, while the susceptible isolates and the reference strain *A. butzleri* DSM 8739 showed no mutation.

4. Discussion

The genus *Arcobacter* includes species considered emerging food and waterborne pathogens. Most of the analysis are focused on foods of animal origin and wastewater samples; however few studies have investigated its presence in vegetables. Nowadays, vegetable consumption is growing, because they are considered healthy and safe. However, vegetables could act as transmission vehicles for *Arcobacter* to humans. The bacterial load in vegetable is influenced by a multitude of factors, e.g., the poor hygienic practice of field workers, the handling during processing (**Beuchat and Ryu, 1997**) or the quality of water (**Collado *et al*., 2008 and 2010; Moreno *et al*., 2003**). Vegetables do not seem to be a reservoir for *Arcobacter* spp. However, this type of food can be contaminated through water used for irrigation as well as postharvest washing (**Hausdorf *et al*., 2011**). In this case, the reuse of wash water or insufficient disinfection procedures may lead to cross-contamination of other batches of vegetables by residual microorganisms (**Hausdorf *et al*., 2011**). Another possible reason for the *Arcobacter* detection in vegetables is the presence of soil particles, contaminated with animal faecal discharges adhering to them (**Hausdorf *et al*., 2013**).

Arcobacter spp. were detected in 20% of the vegetable samples using conventional PCR after selective enrichment. The highest number of contaminated samples was found in spinaches (42.9%), followed by lettuces (14.63%), cabbages (25%) and chards (11.76%). Although not many studies are available about the occurrence of arcobacters on vegetables, the contamination levels obtained in lettuces are similar to those reported by **González and Ferrús (2011**), who found incidence levels of *Arcobacter* spp. of 14% and 20%, by conventional and real-time PCR, respectively. In another study developed in a spinach-processing plant in Germany, *Arcobacter* spp. were detected in 35% of the analysed samples by using genus-specific quantitative PCR.

Among the 20 PCR positive samples detected after 48 h enrichment, only 4 samples were also *Arcobacter* positive before enrichment, suggesting high contamination levels in these samples. It has been reported that a previous enrichment step of the samples increases the level of viable cells, providing better sensitivity of PCR detection (**Collado *et al*., 2008; Denis *et al*., 2001**).

Seventeen samples (17%) were positive by culture, and 25 isolates were obtained. The most contaminated samples were the spinaches (38.10%), followed by cabbages (25%), chards (11.76%) and lettuces (9.76%). This is the first report about isolation of *Arcobacter* from chards and cabbages. Regarding isolation rates, **González and Ferrús (2011**) isolated the bacteria from 7 out of 50 fresh lettuces (14%). More recently, the presence of *Arcobacter* spp. was also found in pre-cut vegetables in Italy, who confirm the presence of *Arcobacter* in 27.5% (44/160) ready-to eat vegetables, including lettuce, spinach, rocket and valerian **(Mottola *et al*., 2016b).**

In our study, the detection rates by conventional PCR (20%) and culture (17%) were almost similar. Only in one sample was not possible to detect the organism by PCR, although we could obtain an isolate by culture. These results could be due to the possible presence of inhibitor substances in the sample or in the enrichment broth (**Rahini *et al*., 2014**).

By 16S rRNA-RFLP method, seventeen isolates (68%) were identified as *A. butzleri* and eight (32%) as *A. cryaerophilus*. *A. butzleri* has been described as the most frequently isolated species from food and water samples (**Collado and Figueras, 2011).** In vegetables samples, the few studies published indicate that *A. butzleri* is also the most prevalent. In fact, until 2016, *A. butzleri* was the unique specie isolated from this type of samples (**González and Ferrús, 2001; Hausdorf *et al*., 2013).** In 2016, four *A. cryaerophilus* isolates were obtained by **Mottola *et al.* (2016b)** from 37 spinach pre-cut (ready to eat) samples, but it was not possible to isolate this specie from the other samples tested in the study, including lettuce, rocket and valerian. However,

it is remarkable that in our study *A. cryaerophilus* has been also isolated from lettuces, chards and cabbages, and not only from spinaches. In addition, this is the first report about the presence of pathogenic species of *Arcobacter* spp. in chards and cabbages.

Fluoroquinolones are potential drugs to treat infections due to campylobacteria in human patients (**Vandenberg *et al*., 2006**). In our study, all *A. cryaerophilus* isolates and 15 *A. butzleri* isolates were susceptible to quinolones. However, two *A. butzleri* isolates showed resistance by disc diffusion method and E-test strips. Different levels of quinolone susceptibility from clinical isolates have been published. **Vandenberg *et al*., (2006)** found that most *A. butzleri* isolates from diarrheal stool specimens in Belgium were susceptible to ciprofloxacin (96.7%). **Van den Abeele *et al*. (2016)** obtained similar results from human *Arcobacter* isolates, most *A. butzleri* strains were susceptible to ciprofloxacin (87%), whereas half of the *A. cryaerophilus* isolates (51%) showed high-level resistance (MIC >32 mg/L). However, **Mandisodza *et al*. (2012)** found that all arcobacters isolated from fecal samples from humans with diarrhea in New Zealand were susceptible to ciprofloxacin. In another study carried out by **Fera *et al*. (2003)** on 30 *Arcobacter* spp. strains isolated from environmental samples, fluoroquinolones had a good activity against *A. butzleri* and *A. cryaerophilus*, although of the two species tested, *A. butzleri* was found to be less susceptible . As there is no recommendation by the CLSI, susceptibility tests for *Arcobacter* species are not standardized and there is currently no available data that can be used for the interpretation of the test results. This fact could be one of the reasons of the variability in the susceptibility reported among different studies **(Atabay and Aydin, 2001).**

In both *A. butzleri* resistant isolates, the sequencing of QRDR fragment revealed the presence of a mutation in position 254 of *gyrA* gene (C-T transition), which was absent in the susceptible isolates and the reference strain *A. butzleri* DSM 8739. We compared the results obtained by the disc diffusion method and E-test with those obtained by the sequencing of the 344-bp fragment of *gyrA* and the C-254 to T mutation could be the cause of quinolone resistance as this change was not present in the susceptible isolates tested. The presence of the mutations causes the substitution of Thr to Ile in position 85 in the GyrA protein (**Abdelbaqi *et al*., 2007**; **Van den Abeele *et al*., 2016**), and has been related to the acquisition of clinical resistance to quinolone agents.

In conclusion, the results of this study show important levels of *Arcobacter* contamination in fresh vegetables that are generally consumed raw. To our knowledge, this is the first report of *A. cryaerophilus* isolation from lettuces, chards and cabbages, and is the first study to prove the presence of pathogenic species of *Arcobacter* spp. in chards and cabbages. In addition, the presence of quinolone resistant isolates of *A. butzleri* in vegetables could be a major public health concern, as ciprofloxacin is one of the most commonly used and best performing fluoroquinolones.

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Fig. 1. 16S rRNA-RFLP patterns obtained for *Arcobacter* isolates using the restriction enzyme *Mse*I. Lanes M: 50-bp ladder; lanes 1 and 5: lettuce isolates; lane 2: cabbage isolate; lanes 3, 6 and 7: chards isolates; lane 4: *A. cryaerophilus* CECT 8222; lane 8: spinach isolate; lane 9: *A. butzleri* DSM8739.

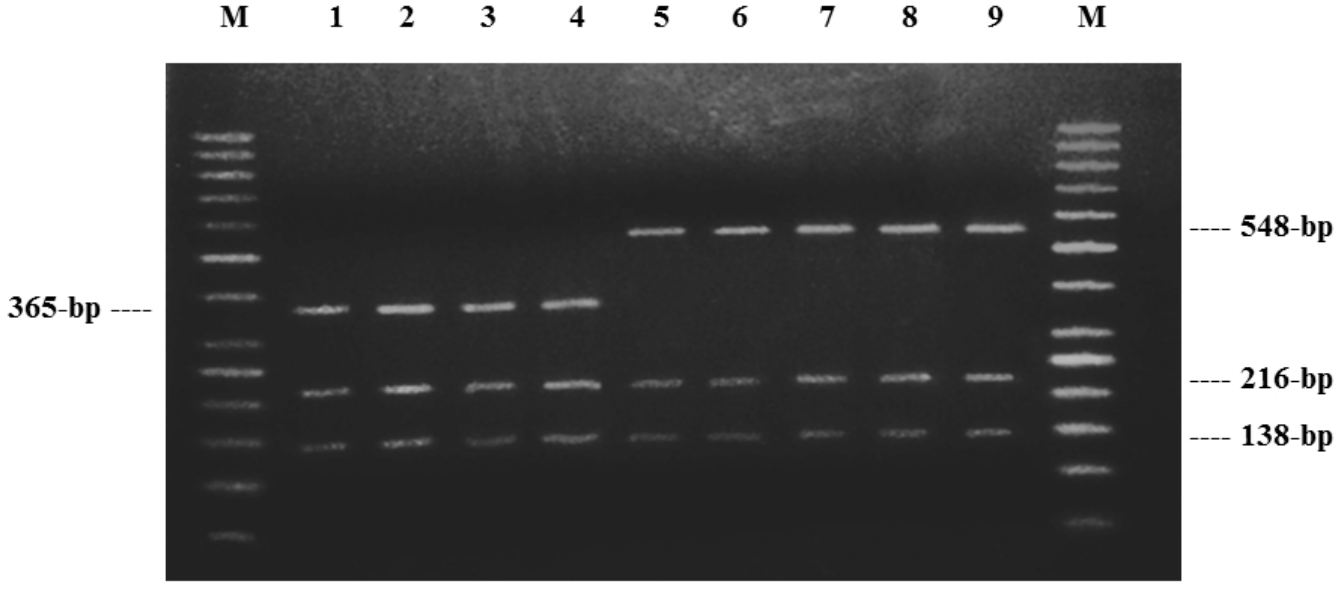
M
1
2
3
4
5
6
7
8
9
M
365-bp ----
---- 548-bp
---- 216-bp
---- 138-bp

Table 1. *Arcobacter* spp. detection by PCR before and after enrichment

		N° (%) of positive samples	
Type of sample	N° of samples	PCR before enrichment	PCR after enrichment
Lettuces	41	1 (2.44)	6 (14.63)
Spinaches	21	3 (14.29)	9 (42.90)
Chards	34	-	4 (11.76)
Cabbages	4	-	1 (25)
Total	100	4	20

Table 2. *Arcobacter* spp. detection by culture

Sample (*n*)	N° positive samples (%)	N° isolates	*A. butzleri*		*A. cryaerophilus*	
			N° sample	N° isolates (%)	N° sample	N° isolates (%)
Lettuce (41)	4 (9.76)	5	2	3(12)	2	2(8)
Spinach (21)	8 (38.10)	13	6	11(44)	2	2(8)
Chard (34)	4 (11.76)	6	1	3(12)	3	3(12)
Cabbage (4)	1 (25.00)	1	-	-	1	1(4)
Total (100)	17	25	9	17(68)	8	8(32)

Highlights

- *Arcobacter* detection in lettuces, spinaches, chards and cabbages by PCR and cultural methods.
- First *Arcobacter cryaerophilus* isolation from lettuces, chards and cabbages.
- First report about the presence of pathogenic species of *Arcobacter* spp. in chards and cabbages.
- Characterization of *Arcobacter* quinolone-resistance of isolates obtained from vegetables samples.