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Arcobacter spp. at retail food from Portugal: Prevalence, genotyping and antibiotics resistance

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ABSTRACT

Arcobacter genus is considered a foodborne emergent pathogen able to cause disease among humans and animals. The wide distribution and high prevalence of *Arcobacter* in food is becoming a concern, since consumption of contaminated food and water is considered the most probable route of *Arcobacter* transmission to humans. In this work, ready-to-eat packaged vegetables, poultry, pork, beef and fish meat were purchased from several retail markets and supermarkets and tested for the presence of *Arcobacter* spp. Suspected colonies were isolated and identified, characterized through ERIC-PCR and resistance to nine antibiotics commonly used to treat infections by this microorganism was assessed; in parallel, *Arcobacter* spp. was directly detected on enrichment broth by multiplex PCR. The results show a high prevalence of *Arcobacter* spp. among retail food, with an overall prevalence of 60.5% obtained by both molecular and culture detection. *A. butzleri* was the most frequently isolated species (58.5%) followed by *A. cryaerophilus* (35.8%) and *A. skirrowii* (5.7%). A high genetic diversity among the isolates was observed, despite the detection of a possible cross-contamination between food samples. Also, a high rate of multidrug resistance among the isolates (85.7%) was observed. Taken together, our results suggest that the consumption of *Arcobacter*-contaminated food products is of public health concern.

1. Introduction

Arcobacter genus is a member of the *Campylobactereaceae* family, which also includes the genera *Campylobacter* and *Sulfurospirillum*. The *Arcobacter* genus was created in 1991 to accommodate the already known species: *Arcobacter nitrofigilis* and *Arcobacter cryaerophilus* (Vandamme et al., 1991). Currently, this corresponds to a vast and diverse genus comprising 26 recognized species: *A. cryaerophilus*, *A. nitrofigilis*, *A. butzleri*, *A. skirrowii*, *A. cibarius*, *A. halophilus*, *A. mytili*, *A. thereius*, *A. marinus*, *A. trophiarum*, *A. defluvii*, *A. molluscorum*, *A. ellisii*, *A. bivalviorum*, *A. venerupis*, *A. anaerophilus*, *A. cloacae*, *A. suis*, *A. ebronensis*, *A. aquimarinus*, *A. lanthieri*, *A. pacificus*, *A. faecis*, *A. acticola*, *A. porcinus* and *A. lekithochrous* (Diéguez, Balboa, Magnesen, & Romalde, 2017; Ferreira, Oleastro, & Domingues, 2017; Figueras, Pérez-Cataluña, Salas-Massó, Levican, & Collado, 2017; Park, Jung, Kim, & Yoon, 2016), with phylogenetic analysis of the 16S rRNA gene sequences found in databases indicating that these are a fraction of the total taxa (Wesley & Miller, 2010). These species have been isolated from different sources, namely humans, animals, water, foodstuff, food processing environment or others, showing that *Arcobacter* spp are widely distributed (Ferreira, Queiroz, Oleastro, & Domingues, 2016). Among *Arcobacter* species some are considered food- and waterborne pathogens, posing a risk to human health (Collado & Figueras, 2011). Indeed, the consumption of contaminated food or water is suggested as the most probable transmission route of *Arcobacter* to humans and animals, yet this must still be confirmed (Collado & Figueras, 2011). The International Commission on Microbiological Specifications for Foods has already considered *A. butzleri* and *A. cryaerophilus* serious hazard to human health, being *A. butzleri* the most prevalent and epidemiologically important species of the genus (Collado & Figueras, 2011; ICMSF, 2002). *Arcobacter* spp. are widely distributed in the food chain, often presenting resistance to several antibiotics, namely when isolated from food products available at retail stores, such as raw vegetables, meat or fish. When isolated from samples of such products,

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Arcobacter has shown high resistance to ampicillin, tetracycline, nalidixic acid and chloramphenicol (Abay, Kayman, Hizlisoy, & Aydin, 2012; Collado, Jara, Vásquez, & Telsaint, 2014; Villalobos, Jaramillo, Ulate, & Echandi, 2013; Yesilmen, Vural, Erkan, & Yildirim, 2014). Even if *Arcobacter* infections can be self-limiting, the consumption of contaminated foods with resistant *Arcobacter* species may pose a threat to human health due the lack of treatment options for severe infections, in addition to a possible transfer of resistance determinants to other pathogenic bacteria (Rahimi, 2014; Son, Englen, Berrang, Fedorka-Cray, & Harrison, 2007).

Therefore, the goals of this work were to study the prevalence of *Arcobacter* species in food samples from retail markets and supermarkets in Portugal, to evaluate the genetic diversity among isolates and to assess the resistance of strains to different antibiotics.

2. Materials and methods

2.1. Sampling and Arcobacter isolation

A total of 114 food samples were purchased from various local retail markets and supermarkets in the Centre of Portugal. The collected samples included fresh fish meat (n = 25, from aquacultures or wild-caught, in a total of 13 different fish species), fresh meat (n = 68, including poultry (n = 25), pork (n = 24), beef (n = 19)), and ready-to-eat packaged vegetables (n = 21, including several salad mixes, spinach, watercress). The samples were transported to the laboratory and processed within a maximum of three hours. Ten grams of food sample was added to 90 mL of *Arcobacter* Broth (Oxoid, Hampshire, England) supplemented CAT Selective Supplement (Oxoid, Hampshire, England) and homogenized during 60 s in a stomacher. After incubation at 30 °C during 48 h in aerobic conditions, 200 μL of enrichment broth was pipetted onto a membrane filter (with 0.45 μm pore diameter) placed onto a Blood Agar plate supplemented with 5% (v/v) defibrinated horse blood (Oxoid, Hampshire, England). Passive filtration proceeded for 30 min at room temperature, and after filter removal, the plates were incubated under aerobic conditions at 30 °C for 24 h. Afterward, four typical colonies were selected and subcultured in blood agar plates. The selected colonies tested as oxidase positive and as Gram negative rods were stored at −80 °C in brain heart infusion broth with 20% of glycerol, for subsequent molecular identification. A total of 400 μL of enrichment broth was used for molecular detection.

2.2. Molecular detection and identification for Arcobacter spp.

The molecular detection on enrichment broth and identification of isolates to species level was performed using multiplex PCR (mPCR) previously described (Douidah, De Zutter, Vandamme, & Houf, 2010; Houf, Tutenel, De Zutter, Van Hoof, & Vandamme, 2000). DNA extraction was performed by boiling method, on isolates suspension and on samples from enrichment broth. PCR products were examined by electrophoresis on 1.5% agarose gel in 1× Tris-acetate-EDTA buffer stained with Midori Green (Nippon Genetics Europe, Düren, Germany) and visualized by a UV gel documentation system. Reference strains *A. butzleri* LMG 6620, LMG 10828, *A. cryaerophilus* LMG 10829, and *A. skirrowii* LMG 6621 were used as positive controls. Multiplex PCR described by Houf et al. (2000) was used for molecular detection of *Arcobacter* spp. in enrichment broths, and for identification of suspected colonies. Isolates identified as negative or as other species rather than *A. butzleri* were further analysed through the mPCR described by Douidah et al. (2010). After this two-step algorithm, suspected isolates given a non-specific amplification were subjected to *16S rDNA* (Ménard, Dachet, Prouzet-Mauleon, Oleastro, & Mégraud, 2005) or *gyrA* (Kim, Hwang, & Cho, 2010) amplification and sequencing. Products were sequenced on both strands, using the Big Dye Terminator v1.1 Sequencing standard kit (PE Applied Biosystems Chemistry, Foster City, CA, USA) and the Automated Sequencer Genetic Analyser ABI-Prism 3130 xl (PE Applied Biosystems). The sequences were compared using the NCBI BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analysis was conducted using the leBIBIQBPP software (Flandrois, Perrière, & Gouy, 2015).

2.3. Isolates genotyping

Genetic diversity of isolates was evaluated by Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR), using the primers described by Houf, De Zutter, Van Hoof, and Vandamme (2002a). The isolates were grown in blood agar plates over 48 h at 30 °C and the DNA was extracted as described by Houf et al. (2002a). The ERIC-PCR was performed in 15 μL reactional mixture with 1.5 μL of 10× Dream Taq Buffer, 0.8 U of Dream Taq DNA Polymerase, 4 mM $MgCl_2$, deoxynucleoside triphosphate mixture at a final concentration of 200 μM and 25 pmol of each primer (ERIC1R and ERIC2). The template used was 1 μL of DNA at 25 ng/μL. PCR was performed as follows: one cycle of 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 25 °C and 2 min at 72 °C and a final extension step of 5 min at 72 °C. Amplification products were analysed by electrophoresis in 2% agarose gel at 120 V for 2 h, stained and visualized under UV light. All gels were examined using the INFOQuest software to identify the distinct genetic profiles, considering fragments between 100 and 2000 bp.

2.4. Antimicrobial susceptibility testing

The determination of minimum inhibitory concentrations (MICs) of nine antibiotics, ampicillin, cefotaxime, chloramphenicol, ciprofloxacin, levofloxacin, nalidixic acid, erythromycin, gentamicin, and tetracycline, was performed by the agar dilution method. Antibiotic dilutions ranging from 256 to 0.06 μg/mL were incorporated in Müeller-Hinton agar supplemented with 5% (v/v) of sheep defibrinated blood. The selected strains were first grown in blood agar plates at 30 °C for 48 h. Then, inocula were prepared by suspension in a saline solution (0.85% NaCl) and the turbidity adjusted to 0.5 McFarland and followed by a dilution to 10^7 cfu/mL. The plates were inoculated with 2 μL of each isolate suspension and incubated for 48 h at 30 °C, under aerobic atmosphere. A plate without antibiotic was used for growth control, and the strain *Escherichia coli* ATCC 25922 was used as control in each run.

3. Results

The overall prevalence, either by culture or by molecular detection of *Arcobacter* species in 114 retail samples distributed in five food categories was 60.5% (69/114); the highest frequency of detection was in fresh poultry meat (92.0%), followed by fresh fish (68.0%), ready-to-eat packaged vegetables (47.6%), fresh pork meat (45.8%) and fresh beef meat (42.1%) (Table 1).

When comparing detection methods, a higher number of positive samples was found by molecular detection in enrichment broths with 59 positive samples (51.8%) versus 53 positive samples (46.5%) by culture preceded by enrichment. Although this is a slight difference, only 43 (37.7%) of the samples were positive by both culture and molecular detection in enrichment broth using the mPCR described by Houf et al. (2000).

The distribution of species in positive samples showed that the most frequently isolated species was *Arcobacter butzleri* (58.5%) followed by *A. cryaerophilus* (35.8%) and *A. skirrowii* (5.7%).