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Susceptibility analysis of *Arcobacter* isolated from fresh cheeses from municipal markets to fluoroquinolones in Guaranda City, Ecuador

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ABSTRACT

some species of the *Arcobacter* genus are considered emerging foodborne enteropathogens. However, the presence of this bacterium in cheese is little known. On the other hand, quinolones are considered first-line drugs for the treatment of campylobacter infection in human patients, but currently little data is available on the levels of resistance to these antibiotics among *Arcobacter* species. Thus, the objective of this study was to analyze the susceptibility of *Arcobacter* spp. isolated from 100 fresh cheeses, obtained from the municipal markets to Fluoroquinolones in Guaranda City, Ecuador. By culture, 47 out of the 100 cheese samples were positive with a total of 66 isolates. By PCR, the number of positive samples was reduced to 21 with 26 isolates. With respect to antimicrobial activity, 2 isolates showed resistance to Levofloxacin (7.69%) and 6 to Ciprofloxacin (23.08%). Nineteen strains exhibited intermediate resistance to Levofloxacin and 10 to Ciprofloxacin. This study is the first report on the presence of pathogenic species of *Arcobacter* spp. in fresh cheeses in Ecuador, which could act as a vehicle for transmission to humans and pose a potential risk to public health.

Keywords: Arcobacter, Isolation, Resistance, Fluoroquinolones.
Article type: Research Article.

INTRODUCTION

Over the years, contamination in food is due to different factors through its collection, process or transformation; among others, without proper handling, microbiological agents can proliferate (Luz & Roy 2017). In this way, the prevention of these pathogens is essential to avoid severe damage, and promote food safety before the food undergoes an alteration (FAO & OPS 2017). There are numerous bacteria with different structures, metabolism, and adaptation. In this sense, the *Arcobacter* genus is characterized by being a free-living microorganism and belongs to the order of the Epsilon proteobacteria, which are widely distributed in the environment as well as forming part of the intestinal microbiota of various animal species (Rojas 2011; Figueras *et al.* 2016). According to Rivera (2015), the symptoms that are present due to contamination with this microorganism are constant and wet diarrhea, belly pain and spasms or fever. The enteritis produced by *Arcobacter* in most of the times does not have serious consequences, however, this varies due to the severity, the time that the symptoms present and the state of the person's immune system. *Arcobacter* is an emerging pathogen that threatens food safety. Important aspects of its epidemiology are still unknown and, despite the fact that every day there are more works aimed at discovering its appearance in food, the existing risk of inoculation through the chain food is far from known. Yesilmen *et al.* (2014) points out that *Arcobacter* could be transmitted to humans through the consumption and handling raw or undercooked food. Calvo *et al.* (2013) and Mottola *et al.* (2016), on the other hand, have reported that *Arcobacter* has been isolated from cattle, pigs, sheep, goats, and birds, as well as in abortions of pigs and cattle, meat products, mollusks, milk and cheeses. In addition, other studies reported that the species belonging to the *Arcobacter* are not a natural part of the intestinal flora and humans can be infected with this pathogen in foods

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of animal origin, water, or even vegetables, among other transmission routes that are not yet well determined (González & Ferrús 2011; González *et al.* 2017; Bayas Morejòn *et al.* 2017, 2020). The identification of *Arcobacter* species is still a problem, due to the irregular biochemical reactions of some isolates, as well as the low metabolic activity of the microorganism (Atabay *et al*. 2006). These antecedents have led to the development of different studies on the application of different techniques such as PCR, which have made it possible to confirm the presence of the pathogen in different food and non-food matrices (Mansilla 2006; Rojas 2011; Rivera 2015; Bayas 2016). Fluoroquinolones are one of the most commonly prescribed antimicrobial agents in the world and are used to treat a variety of bacterial infections. However, due to the widespread excessive use of these drugs, the number of bacterial strains resistant to quinolones has increased considerably (Ünver *et al.* 2013; Aldred *et al.* 2014). In this sense, our objective of study was to help knowledge about the prevalence of this genus in foods, in this case fresh cheeses, which may pose a special risk to the consumer and their presence in the two most important markets of the Guaranda City, Ecuador.

MATERIALS AND METHODS

Study Location

This study was developed at Research Department, Faculty of Agricultural Sciences, Natural Resources and the Environment, the State University of Bolivar, Ecuador. The samples were collected with a frequency of 10 cheeses per week in a period from September 2019 to January 2020.

Collection of samples and isolation of the microorganism

For isolation, a total of 100 fresh cheese samples were taken for 10 weeks from the municipal markets of Guaranda City, Ecuador. These were: 10 de Noviembre market and Bellavista market. The samples were immediately transported to the laboratory and processed within a maximum of three hours. The cheese samples obtained were homogenized by adding 90 mL buffered peptone water (BPW; VM666728, Merck KGaA) to about 10 g cheese sample. All this was combined in a Stomacher bag (BA6141 / STRStomacher®, Seward), and homogenized for a few seconds (García *et al.* 2015). For the individual processing of the samples, 10 mL of each homogenate were taken. Then it was added to 90 mL Arcobacter Broth (OXOID, CM0965B, UK). Afterward, the CAT supplement (cefoperazone-amphotericin-B-teicoplanin) was added to the medium and in culture conditions under microaerophilicity followed by incubating at 37 °C for a period of 24-48 h. Thereafter, 200 µL of each broth was transferred on membrane filters (0.45 pore size) previously placed on *Arcobacter* Agar supplemented with 5% defibrillated sheep blood (Atabay & Corry 1997). It was left to rest for 30 minutes. Then the plates were incubated at 37 °C under microaerophilicity (10% CO_2, 5% O_2, and 85% N_2), using an atmospheric generation system with a CampyGen TM envelope (OXOID, CN0025A, UK). After incubation, colonies showing characteristics apparent to the *Arcobacter* were selected and analyzed by microscopy (Gram stain).

Molecular analysis

Each of the isolates obtained had their DNA extracted using a specific DNA extraction kit (Thermo Scientific ™, K0502, USA). DNA concentration was observed using a micro-spectrophotometer (Thermo Scientific™, NanoDrop™). The amplification reaction was carried out in a final volume of 50 µL, containing 45 µL of the mixture and 5 µL of template DNA, for which, we worked with 1X buffer; 0.2 mM dNTPs each; 1.5 nM $MgCl_2$; Primers: CAH1am: 5'-AACACATGCAAGTCGAACGA-3'; CAH1b: 5'-TTAACCCAACATCTCACGAC-3 ' 0.5 µM of each; and Taq polymerase 5U. The primers using amplify a 1026-bp fragment of the 16S rRNA gene, described by Figueras *et al.* (2012). The conditions for the PCR were: a cycle of 94 °C for 2 minutes; 30 cycles at 94 °C for 30s for denaturation, 52 °C for 30s for primer binding, extension at 72 °C for 90s; finally, a final extension at 72 ºC for 10 minutes. The generated products were analyzed by horizontal agarose gel electrophoresis (Agarose®) (ThermoiScientific ™, BP160-100, USA) at 1.2%, prepared in buffer, with the addition of 5 µL SYBR® Safe DNA Gel Stain (INVITROGEN ™, S33102, USA), and subjected to 100 volts for 45 minutes to estimate the size of the amplified DNA fragments using a 100bp BenchTop 100bp molecular weight marker (G829B, PROMEGA, USA). Finally, the fragments were visualized on a trans-illuminator under ultraviolet (UV) light.

Analysis of antibiotic sensitivity

For the analysis of antibiotic susceptibility, two antibiotics from the Quinolone group (Ciprofloxacin and Levofloxacin) were studied. Disc diffusion methods (Kirby Bauer) were used for both antibiotics.

Preparation of the inoculum and application of the discs

The isolates obtained were placed on *Arcobacter* agar plates + 5% defibrillated sheep blood, and were incubated at 30-37 °C as appropriate for 48 h in microaerophilic conditions. From this growth phase seeding, a 10% saline water suspension was prepared until a turbidity of 0.5 on the McFarland scale (HARDY DIAGNOSTICS, FU-10560, USA) was achieved. Subsequently, by means of a sterile swab, the seeding was carried out in Muller-Hinton Agar plates (Acumedia®, 7101A, USA) in a homogeneous way. Next, the agar was left to rest, and by means of apreviously sterilized forceps, the discs were placed on the surface of the agar. The plates were then incubated under microaerophilic conditions at 37 °C for 48 hours.

Thereafter, the diameters of the inhibition halos of the discs were calculated, which correspond to the inhibitory zone, given that the *Arcobacter* and *Campylobacter* genus correspond to the same family, and that there are no criteria or points for *Arcobacter*. The results were interpreted to the judgments instituted for *Campylobacter* according to the Clinical Laboratory and Standards Institute (CLSI 2010, M45-A2).

RESULTS AND DISCUSSION

Isolation of *Arcobacter* by plate culture

From the culture, up to four colonies with characteristic morphology were taken on a plate by means of Gram stain, for each sample. After a period of selective growth, 66 *Arcobacter* strains were isolated from 47/100 cheese samples tested. In total, 17 out of the 50 samples belonged to the "Bellavista" wholesale market (34%) with 25 isolates; and 30 out of 50 samples belonged to the "10 de Noviembre" market (60%) with 41 isolates. The results of this investigation showed that the culture in selective medium *Arcobacter* agar supplemented with 5% sheep blood (*Arcobacter* Blood Agar) was a satisfactory method for the detection of the pathogen, since it allowed the development of the microorganism at a significant level. Yesilmen *et al.* (2014) worked on cow's milk, buffalo milk and fresh cheese and reported a high prevalence of these bacteria with percentages of 36%, 48% and 56% respectively. Scarano *et al.* (2014) reported that 7 out of 32 samples (21.9 %) of ricotta cheese were positive for *Arcobacter butzleri* by culture examination; all positive samples were collected on the same day and came from the same cheese factory. Research regarding contamination with pathogens of the *Arcobacter* genus is scarce, so we cannot affirm that the differentiations between the norms of contamination and prevalence varied between countries. Since there is no standardized protocol, it could be due to the culture medium used and the procedure chosen for isolation (Merga *et al.* 2011). Giacometti *et al.* (2015) isolated 29 samples (49.5%), of those, 22 were obtained from samples of raw milk (10%), mozzarella cheese (75%), and ricotta cheese (15%). In the area of storage, it is important to consider that the cheeses contaminated with *Arcobacter* coming from a single place is enough to contaminate the entire storage of the product (Scullion *et al.* 2006). Therefore, we can relate the results of the fresh cheese samples from local markets and suggest that the sanitary measures of storage, transport and distribution play a fundamental role in the contamination of cheeses with aforementioned pathogen. In the present study we selected two sites from where the majority of cheeses consumed by the population of the Guaranda City generally come. This is the first work in which *Arcobacter* has been isolated from cheese samples from these markets. In addition, it is the first time that this pathogen has been worked with in Ecuador.

Molecular analysis of the isolates obtained

From the extracted DNA, its concentration was analyzed by Micro-spectrophotometry, where, all cases presented concentrations greater than 5 ng μL^{-1} that is suitable for PCR analysis as described by Guamán (2017). In our study the highest concentration of DNA was 198.3 ng μL^{-1} and the lowest 5.1 ng μL^{-1} , with a mean of 25.49 ng μL^{-1}. The DNA of the isolates was amplified by PCR, of those, 66 cheese isolates obtained by culture. The detection rates by PCR developed as follows: 26 were positive for *Arcobacter* by means of the PCR test, 20 came from the municipal market "10 de Noviembre" and 6 from the wholesale "Bellavista" market, which confirms that a step of enrichment increases the level of viable cells, allowing a higher detection sensitivity also in this type of samples (Table 1). Our result is similar to that obtained by Giacometti *et al.* (2013), who indicated that they were collected from a factory of artisanal ricotta cheese, mozzarella cheese and raw milk. The isolates of these samples were subjected to multiplex PCR and recognized by electrophoresis (PFGE), a prevalence of 46.6%. These data are quite similar to those obtained in our work. By means of PCR amplification and after electrophoresis, a 1026 bp gel band was evidenced (Fig. 1). Several factors may play a fundamental role in this finding, the transport, the cold chain and the place of storage with poor hygiene.

Table 1. Frequency of positive cases for *Arcobacter* in cheese

PCR Positives	Frequency	%
Bellavista	6	23.1
10 de Noviembre	20	76.9
Total	26	100

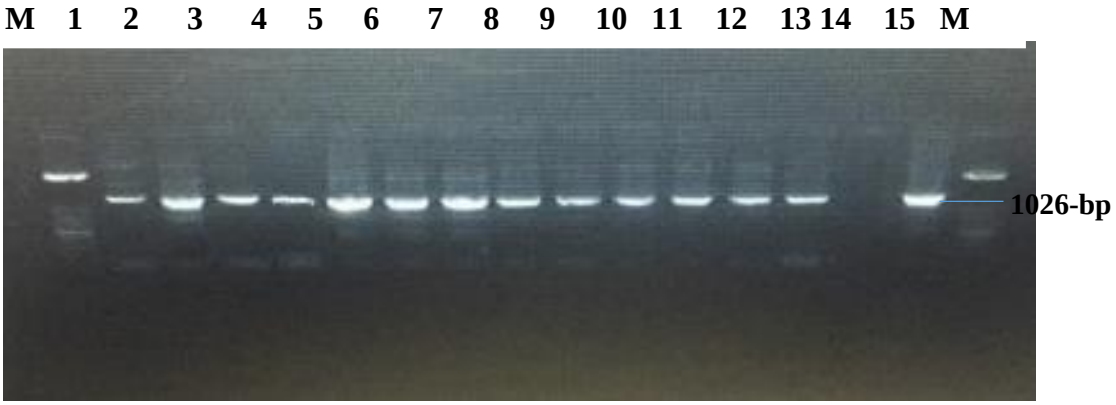

Fig. 1. Electrophoresis of the *Arcobacter* isolates; lanes M: molecular weight marker; C: distribution lanes "isolated positive".

Antimicrobial activity analysis

In the study of antimicrobial activity, Levofloxacin and Ciprofloxacin were used, which are the first-line drugs for the treatment of *Arcobacter* contamination in humans (Houf *et al.* 2004; Vandenberg *et al.* 2006).

Table 2 shows the parameters of the inhibitory zone taken as a reference; where the interpretation of the results was carried out using the recommendations of the European Committee for Susceptibility Testing according to EUCAST (2017) and the Clinical Laboratory Standard Institute (CLSI 2017).

Table 2. *Arcobacter* susceptibility.

	Disc broadcast (mm)					
Interpretation	≤ 6	7-12	13-15	16-19	20-23	≥ 24
CLSI		R	I		S	
EUCAST			R			S
Raised guide (Cip y Lev)		R		I		S

Note: R: Resistant; I: Intermediate; S: Susceptible.

After this analysis, it can be seen that there are 2 strains of *Arcobacter* resistant to Levofloxacin (7.69%); and 6 to Ciprofloxacin (23.08%). Ninteen strains exhibited intermediate resistance to Levofloxacin and 10 to Ciprofloxacin (Table 3). According to preliminary studies, resistance to Quinolones was directly related to a mutation in the QRDR region of the *gyrA* gene, which in previous studies have revealed the presence of a mutation at position 254 of this gene (C-T transition; Abdelbaqi *et al.* 2007; Van den Abeel *et al.* 2016).

Notably, no studies about antimicrobial activity of *Arcobacter* were found with Levofloxacin, however, as they belong to the same family as Ciprofloxacin (Fluoroquinolones), the results can be related. Sauca, (2018) reported antimicrobial susceptibility results exhibiting resistance to nalydixic acid (100%), tetracycline (77.78%), ampicillin (44.45%), ciprofloxacin (44.45%), erythromycin (33.33%) and gentamicin (100%). Therefore, we observed that the percentage of susceptibility of the drug with the pathogen is related to those obtained by Sauca (2018).

CONCLUSION

The results and the finding of *Arcobacter* in this study derived from fresh cheeses that are distributed throughout the city, raises public health concern. Although, little knowledge was found about the pathogenic potential of the genus *Arcobacter* and its presence in food, however, the few studies carried out, exhibited the presence of this genus in a wide variety of foods for human consumption. So, there is a potential risk for the consumer, especially when finding strains resistant to first-line drugs.

Table 3. antimicrobial activity of levofloxacin and ciprofloxacin against isolates of *Arcobacter* spp.

Code	Levofloxacin	susceptibility	Ciprofloxacin	susceptibility
Q5NC1	16	I	13	I
Q6NC1	14	I	12	R
Q6NC2	17	I	14	I
Q8NC1	16	I	19	I
Q8NC4	19	I	20	S
Q18BC2	8	R	2	R
Q19BC2	6	R	2	R
Q27NC1	18	I	19	I
Q43NC2	16	I	20	S
Q44NC2	13	I	9	R
Q49NC2	14	I	12	R
Q51NC2	25	S	24	S
Q54NC1	19	I	20	S
Q57NC1	18	I	20	S
Q58NC1	18	I	17	I
Q59NC1	19	I	18	I
Q59NC2	22	S	20	S
Q59NC3	21	S	20	S
Q60NC1	16	I	19	I
Q60NC2	17	I	19	I
Q61NC2	19	I	20	S
Q62NC1	18	I	19	I
Q71BC2	14	I	11	R
Q74BC1	20	S	19	I
Q89BC1	18	I	24	S
Q94BC1	23	S	24	S

Note: R: Resistant; I: Intermediate; S: Susceptible; Q: Quesos (cheeses); B: Bellavista market; N: 10 de Noviembre market; C: colony.

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