MOLECULAR CHARACTERIZATION OF ARCOBACTER SPP. FROM SAMPLES OF DAIRY SERUM FROM THE CHEESE FACTORIES OF THE SALINAS, GUARANDA (ECUADOR).

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Abstract

Bacteria of the Arcobacter genus are associated with intestinal diseases, both in people and in animals, it has also been included in the group that causes foodborne diseases, since it is frequently found in the food chain. For this reason, in our research, the objective was to characterize Arcobacter spp isolates molecularly from milk serum samples from the cheese factories of the Salinas parish, Guaranda, Ecuador, for which the pathogen was isolated from serum samples. milk in media and specific culture conditions in a plate, the microorganism was characterized by Gram staining and biochemical tests, after which the DNA was extracted and amplified by Polymerase Chain Reaction, finally, the antimicrobial activity against to two flouroquinolones. After the analysis, of the 50 samples of milk serum, 28 samples presented translucent colonies and the majority were Gram (-), after carrying out the biochemical tests, the behavior of the microorganism coincides with the pathogen of interest, finally, after the amplification was carried out. of the genetic material by PCR, it was confirmed that 26 of the 28 (52%) isolated strains belonged to the Arcobacter genus, in antimicrobial activity, the microorganism was susceptible to the control antibiotic levofloxacin, while some samples presented resistance to ciprofloxacin. In conclusion, since the microorganism is present in dairy serum, cheeses could act as a vehicle for transmission to humans and represent a potential risk to public health.

Keywords: Arcobacter spp, Molecular characterization, Biochemical tests, antimicrobial activity.

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1. Introduction

In the last 30 years, a large number of diseases in humans have been identified, most of these are of infectious origin caused mainly by bacteria, viruses, parasites, there are even unclassified diseases such as spongiform encephalopathies, many of these are of animal origin, due to the transmission of pathogens from animals to humans, such is the case of Arcobacter spp, an emerging bacterium of great importance in public health (**FAO & PAHO, 2017**). The genus Arcobacter spp is a Gram - bacterium, it has a polar flagellum on one or both sides of the cell, it grows in aerobic and anaerobic conditions in a wide range of temperatures from 15 to 42°C, belonging to the Campylobacteraceae family, where the species Arcobacter butzleri, has been classified as an emerging intestinal pathogen and potential zoonotic agent (**Montes, 2020; Fernández, 2018**). Currently this genus comprises 74 species, isolated from a great diversity of hosts and ecological niches, they are generally transmitted through contaminated food and water sources (**Sauca, 2018**). Where, the species most related to diseases in humans are: Arcobacter butzleri and Arcobacter cryaerophilus and to a lesser extent Arcobacter skirrowii and Arcobacter thereius, although most cases are asymptomatic (**Vidal, 2017**). Therefore, the bacteria of this genus are widely distributed in the environment, as well as their presence in the intestinal microflora of various animals. (**Fernández & Jaramillo, 2016**). This pathogen has also been included in the group that causes foodborne diseases, since it is considered an emerging pathogen with global distribution in food (**Ferreira et al., 2019**).

The mechanisms of pathogenicity of the different species of Arcobacter spp are little known, adhesion, invasion and cytotoxicity contribute to the pathogenic potential of these bacteria, causing acute diarrhea in people, the main contagion of these bacteria is caused by consumption of beef. poorly cooked bird and is more frequent in young children, in Latin America there are few studies on the prevalence of this bacterium, in Ecuador there are also few studies (**Paguanquiza, 2016**). Indeed, there is only one study in dairy products that demonstrated the presence of the pathogen in food, especially in cheeses (**Bayas-Morejón et al., 2022**).

Knowing that the dairy industry produces about 200 million tons of whey annually through cheese production. Statistics show that a large part of this waste is discharged as sewage, which reduces aquatic life by depleting dissolved substances (**Rodríguez et al., 2020**). In Ecuador, in the Bolívar province, mainly the Parish Salinas of the canton Guaranda, where 18% of its milk production is destined for the production of cheeses, of which whey represents 80 to 90% of the total volume of milk, and this contains 50% of the nutrients (**Jiménez et al., 2016; Amador, 2020**). Dairy serum is used in a wide range of dairy beverages, in addition, the microorganisms that are present in these wheys from the cheese factories of the Salinas parish and its communities are unknown, given that no isolation and identification studies have been carried out on the whey. genus Arcobacter spp, since it is an emerging bacterium, associated with food and of great importance in public health.

For the identification of microorganisms present in derivatives and dairy sub-products, molecular techniques are generally applied, where the study of DNA and RNA genetic material makes it possible to recognize and identify genetic characteristics (**Pérez et al., 2019**). Within these techniques, PCR (Polymerase Chain Reaction) is the most used and specific, which allows exponential amplification of the DNA of a single species in different cycles and temperatures from a pair of primers (**Arias et al.,2018**).

It is important to know if the isolated and identified microorganisms present resistance or susceptibility to antibiotics for clinical use, in this sense, the best and last study option, especially for microorganisms of the Campylobacteraceae family, is the use of antibiotics from the fluoroquinolone group, which are a group of commonly prescribed broad-spectrum antibiotics that work by inhibiting enzymes responsible for bacterial DNA replication, transcription, repair, and recombination (**Gonzáles et al., 2018**). The classification of these antibiotics is: First generation (Nalidixic acid, Lomefloxacin, Norfloxacin), second generation (Ofloxacin, Ciprofloxacin), Third generation (Levofloxacin, Gemifloxaxin, Gatifloxacin), Fourth generation (Moxifloxacin Trovafloxacin), others (Grepafloxacin, Cinoxacin, Enoxaxin) (**Chávez, 2020**).

Finally, an antibiotic susceptibility test identifies the pattern of susceptibility or resistance of an organism to a group of ATM (antimicrobial) based on the determination of the MIC (minimum inhibitory concentration), which is defined as the lowest concentration of an antibiotic. to inhibit microbial growth after incubation (**Hilarión, 2020**).

With everything previously described, the objective of this study was to molecularly characterize the Arcobacter spp. from samples of dairy serum from the cheese factories of the Salinas, canton Guaranda canton, province of Bolívar.

2. Materials and Methods

This research was carried out in the General Laboratory of the Faculty of Agricultural Sciences and in the facilities of the Research Department of the State University of Bolívar in the city of Guaranda (Ecuador).

Obtaining the sample

The samples were obtained randomly from the different cheese makers of the Salinas parish and its communities belonging to the canton Guaranda of the province Bolívar (Ecuador), as evidenced in Table 1.

Table 1. Sample collection locations

Nº	**Locations**	**Codification**	**# samples**
1	Salinas	S	6
2	Capina	C	1
3	Verdepamba	V	11
4	Pambabuela	P	1
5	Mercedes de Pumin	M	4
6	Apahua	A	4
7	San Vicente	Sv	3
8	Ramos	R	3
9	Natahua	N	3
10	Pachancho	Pch	3
11	Arrayanes	Arr	2
12	Yavubiana	Y	3
13	Yurauksha (Andes)	A	3
14	Gramalote	G	3
	Total		50

Note: Of the 28 communities that make up Salinas, serum samples were collected from 14 communities, of which one community belongs to a warm climate (Gramalote).

Dairy serums were collected in plastic containers previously sterilized, labeled and transferred in a cooler to the Laboratory in a period of 6 hours, finally placed in refrigeration until further study.

Isolation of the bacterium Arcobacter spp.

For the isolation of Arcobacter spp, a total of 50 samples of milk serum were taken from the different cheese factories of the Salinas communities. The collected whey samples were homogenized in 90 mL of BPW (Buffered Peptone Water) + 10 mL of whey, which were placed in Eppendorf tubes and stored for 24 h under refrigerated conditions.

Development conditions of Arcobacter spp.

For microbial development, it was taken from the research carried out by **Vázquez** (**2021**). Aliquots of milk serum plus APT were placed on a membrane filter (Oxoid, 1789, Spain) previously placed in Petri dishes of Arcobacter spp agar with 5% sheep blood (blood agar) with CAT supplement (cefoperazone-amphotericin-B- teicoplanin), of which each of the 50 samples were pipetted into 150 µL of the enriched medium (APT + serum) and later placed on a membrane filter, to finally incubate at 37ºC for 5 min. After this time, the membrane filters were removed with sterile forceps and the media were left to incubate in an anaerobic jar under microaerophilic conditions (10% CO_2, 5% O_2, 85% N_2) generated using the Campy-Gem and incubated at 37ºC for 48 h. Finally, the boxes with growth of translucent colonies of Arcobacter spp. were transferred to refrigeration to be analyzed by microscopy and biochemical tests (catalase, urease, nitrate reduction).

Molecular characterization by PCR

DNA extration

Bacterial colonies were taken and centrifuged at 5000 xg for 10, later for the extraction of genetic material (DNA) the kit (GeneJET Genomic DNA Purification) (Invitrogen, 2337897, USA) was used and the manufacturer's specifications were followed. This system combines the advantages of a silicon base with a micro-column format. To measure the concentration of the DNA obtained, a Nanodrop UV spectrophotometer (Thermo Scientific, AZY17073808, USA) was used.

DNA amplification using the PCR technique.

For the characterization of the Arcobacter spp isolates, a PCR assay was carried out, in which a final volume of 50 µL was used, which contains 45 µL of the mixture and 5µL of template DNA, for said reaction the following were used: Buffer 1X, 0.2 mM/each DNTPs, 1.5 mM $MgCl_2$, 0.5 µM/each Primers and 5U Taq polymerase. For PCR amplification, the primers CAH1am: 5'-TTAACCCAACATCTCACGAC-3' (Forward) and CAH1b: 5'- AACACATGCAAGTCGAACGA-3' (Reverse) were used, which amplify a 1026-bp fragment of the 16S rRNA gene, previously 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 30 s for denaturation, 52°C for 30 s for primer ligation, and extension at 72°C for 90 s; finally, a final extension at 72ºC for 10 minutes. The resulting PCR products were analyzed by electrophoresis in 1.2% agarose gel (Agarose®) (ThermoiScientific™, BP160-100, USA) in 1X TAE buffer (Invitrogen™ 24710-030, USA), with the addition of 1 µL of Green GoTaq, which was exposed to 100 volts for 60 min; 1 µL of PCR product previously mixed with 2 µL of loading buffer was used to estimate the size of the amplified DNA fragments. A 100-bp Bench Top 100-bp molecular weight marker was included in each gel (Promega, G829B, USA), finally the fragments were observed in a transilluminator under ultraviolet (UV) light.

Antimicrobial activity against fluoroquinolones

For the analysis of the antimicrobial activity, it was carried out with the antibiotics Ciprofloxacin and Levofloxacin from the fluoroquinolones group against the isolates of Arcobacter spp, for the antibiotics, the plate disk diffusion method of (Kirby Bauer) was used. In an eppendorf tube, 1000 µL of distilled water was placed and a portion of the Arcobacter spp bacteria was added until a whitish color was obtained, later the sowing was carried out in the Mueller Hinton agar medium, and we proceeded to make pores in the center of the medium to place antibiotics as appropriate at concentrations of 10-15-20 mg/1ml, were subsequently incubated at 37ºC for 24 h. Finally, the diameters of the inhibition zones of the discs were measured and the results were interpreted according to the criteria established for Campylobacter according to the Clinical Laboratory and Standards Institute (**CLSI, 2020**) given that the Arcobacter spp and Campylobacter genera belong to the same family. The following table details the sensitivity criteria according to the CLSI.

Table 2. Interpretation criteria.

Antibiotic	**Concentration**	**Diameter (mm)**			**MIC (µg/mL)**		
		S	**I**	**R**	**S**	**I**	**R**
Ciprofloxacin	5 µg	26	22-25	21	≤ 1	2	≥ 4
Levofloxacin	5 µg	26	-	16	≤ 1	2	≥ 4

S: sensitive; R: resistant; I: intermediate.

3. Results and Discussion

Result of the isolation of Arcobarter spp

The following table shows the results of microbial growth.

Table 3. Description of the identification of the isolates obtained.

N°	**Code**	**Origin**	**Initial culture**	**Translucent colonies**	**N° colonies**
1	M1	S	+	?	0
2	M2	C	+	?	1
3	M3	M	+	?	1
4	M4	P	-	-	0
5	M5	V	+	-	0
6	M6	An	+	-	0
7	M7	S	+	?	0
8	M8	M	+	+	2
9	M9	V	+	+	1
10	M10	An	+	?	1
11	M11	M	+	-	0
12	M12	An	+	+	0
13	M13	V	-	-	0
14	M14	M	+	?	0
15	M15	V	-	-	0
16	M16	A	+	+	2
17	M17	V	+	+	0
18	M18	V	+	?	0
19	M19	V	+	?	1
20	M20	V	+	+	0
21	M21	V	+	+	2
22	M22	S	+	-	1
23	M23	S	+	+	1

24	M24	S	+	-	0
25	M25	S	+	-	0
26	M26	Sv	+	-	0
27	M27	Sv	+	+	1
28	M28	Sv	+	+	1
29	M29	R	+	-	0
30	M30	R	+	+	1
31	M31	R	+	-	0
32	M32	N	+	-	0
33	M33	N	+	+	1
34	M34	N	+	+	2
35	M35	Pch	-	-	0
36	M36	Pch	+	+	1
37	M37	Pch	+	+	1
38	M38	V	-	-	0
39	M39	V	-	?	1
40	M40	Arr	-	-	0
41	M41	Arr	-	-	0
42	M42	Y	-	+	0
43	M43	Y	-	-	0
44	M44	Y	+	+	1
45	M45	A	-	-	0
46	M46	A	+	+	1
47	M47	A	+	+	1
48	M48	G	+	+	1
49	M49	G	-	-	0
50	M50	G	-	-	0

In table 3, the results obtained from the 50 samples, it was evidenced that the 11 samples did not grow in the initial culture (-) from (P, V, Arr, A, G), while 10 samples showed multiple growth, although no translucent colonies from (M, V, A, N, S, Sv, Pch, Arr, Y) were identified. On the other hand, 20 samples showed absolute growth (+) from (S, C, M, V, A, R, Sv, N, Pch, Y, G) and 9 samples were of possible growth (?) from (S, C, M, V).

The Arcobacter spp colonies presented specific morphological characteristics such as small or punctate size and a translucent appearance, the same characteristics that **Vásquez** (**2021**) identified. It is important to highlight that in the works of **Hualpa et al.** (**2020**) and **Bayas-Morejón et al.** (**2022**), they demonstrated that the CAT supplement is a preponderant factor when isolating bacteria of the genus Arcobacter spp.

Results of characterization by microscopy and biochemical tests

Biochemical tests for urease, catalase, and nitrite reduction confirmed the identification of this pathogen.

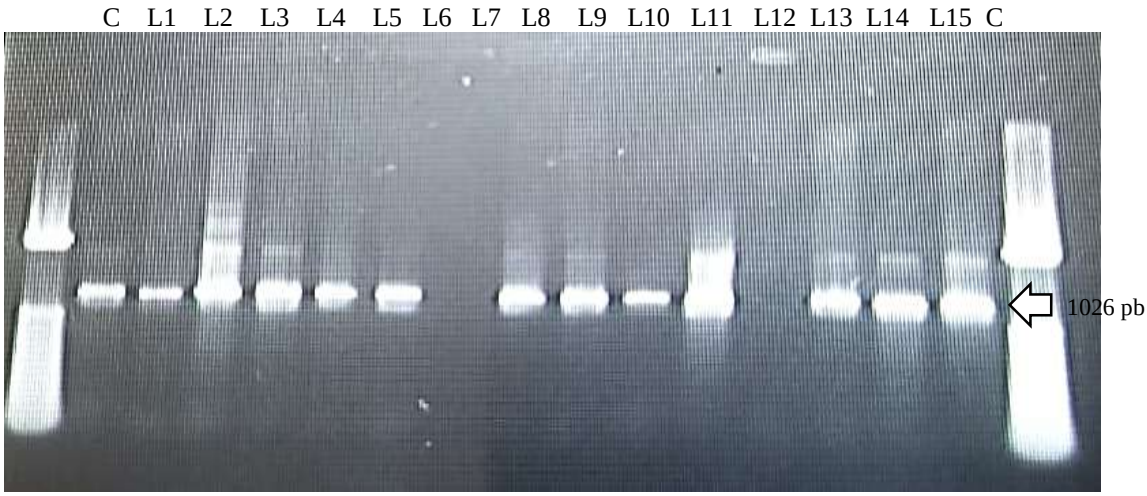
Upon Gram stain analysis, 28 samples with their isolates were shown to be Gram -. In the biochemical tests, of the 28 presumptive isolates of Arcobacter spp, the genomic reaction of catalase, urease and nitrate reduction is evident. Arcobacter spp colonies lack pigments, present oxidative activity and react negatively to methyl red; most species of this pathogen reduce nitrates (**Alveal, 2019**). **Catalase:** 23 positive isolates (+) for since index bubble formation was observed, which shows that it is positive and 5 negatives (-) since said reaction did not exist. **Urease:** 20 isolates were positive (+) since their coloration was cherry red and 8 negatives (-) maintained the initial yellow color of the medium. **Nitrate reduction:** 24 isolates showed a slightly yellow or whitish index of being positive (+) and 4 negatives (-) did not change color.

Molecular Characterization.

Before carrying out the PCR, the concentration of the DNA extracted from those extracted from *Arcobacter* was measured, where the concentration of this in all the isolates was higher than 12.1 ng/µL, it should be noted that the minimum concentration recommended according to Guamán et al. (2018) should be 5.1 ng/µL, therefore, the concentrations obtained are ideal for DNA amplification by PCR.

Arcobacter spp DNA amplification by PCR.

The DNA extracted from the bacterial isolates was analyzed and amplified using the PCR technique, where the fragments were observed in a transilluminator under ultraviolet (UV) light, as shown in Figure 1.



Note. Amplification of the 16S rRNA gene of Arcobacter spp isolates with agarose gel, C: 50 bp molecular weight marker; L1- L15: DNA of the bacterial isolates analyzed.
Figure 1. Electrophoresis of PCR results of Arcobacter spp isolates.

The figure details the analysis of the PCR observed by electrophoresis where L represents the number of bacterial isolates, after this analysis 26 isolates turned out to be positive for the Arcobacter spp microorganism and 2 isolates were negative for the microorganism of interest, therefore, the presence of the pathogen is evident in the milk serum extracted from the different communities of Salinas, with the exception of samples M16 (Apahua) and M23 (Salinas), but that does not mean that it is free of other microorganisms, because the analysis carried out was specifically for Arcobcater spp.

In the research carried out by **Bayas-Morejón et al.** (**2022**), he confirmed by PCR the presence of Arcobacter spp in cheeses from Guaranda markets with 26 positive cases for the microorganisms. On the other hand, **Mena** (**2018**), affirms that the identification and detection of the presence of Arcobacter spp, is carried out more reliably using molecular techniques, PCR being one of the most specific in the detection of Arcobacter spp species., In another investigation carried out by **Mottala et al.** (**2021**), they detected a wide presence of Arcobacter spp in soil, water, feces, ready-to-eat foods and processing plants, their contamination occurs due to poor processing and poor handling, It can be said that the microorganism is present in the whey from most of the samples taken from the cheese makers of the Salinas parish and its communities.

Analysis of antimicrobial activity.

From the 26 isolates confirmed as Arcobacter spp, the antimicrobial activity against Levofloxacin and Ciprofloxacin was performed. The interpretation of results was carried out according to the recommendations of the European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2020) and the Clinical Laboratory Standard Institute (**CLSI, 2019**) for the genus Campylobacter spp.

Table 4. Antimicrobial activity of levofloxacin and ciprofloxacin against Arcobacter spp.

Code	**Levofloxacin**			**Ciprofloxacin**	
	mg/mL	**Halo (mm)**	**Susceptibility**	**Halo (mm)**	**Susceptibility**
M1S	10	34	S	31	S
	15	38	S	28	S
	20	37	S	20	R
M2C	10	39	S	32	S
	15	38	S	27	S
	20	39	S	29	S
M3M	10	34	S	16	R
	15	37	S	16	R
	20	38	S	20	R
M8M	10	36	S	33	S
	15	37	S	27	S
	20	35	S	32	S
M9V	10	33	S	26	S

	15	37	S	31	S
	20	39	S	28	S
M10An	10	36	S	30	S
	15	38	S	35	S
	20	40	S	36	S
M18V	10	36	S	31	S
	15	39	S	30	S
	20	38	S	28	S
M19V	10	37	S	20	R
	15	41	S	22	I
	20	40	S	28	S
M21V	10	37	S	24	I
	15	36	S	29	S
	20	38	S	34	S
M22S	10	34	S	34	S
	15	35	S	31	S
	20	37	S	37	S
M27Sv	10	33	S	25	I
	15	36	S	25	I
	20	37	S	24	I
M28Sv	10	32	S	32	S
	15	36	S	31	S
	20	31	S	32	S
M30R	10	36	S	30	S
	15	39	S	34	S
	20	38	S	20	R
M31R	10	36	S	30	S
	15	38	S	35	S
	20	40	S	36	S
M33N	10	37	S	28	S
	15	37	S	30	S
	20	39	S	25	I
M34N	10	32	S	29	S
	15	37	S	26	S
	20	37	S	29	S
M36Pch	10	36	S	24	I
	15	38	S	20	R
	20	39	S	21	R
M37Pch	10	36	S	19	R
	15	37	S	25	I
	20	36	S	30	S
M38V	10	35	S	32	S
	15	34	S	33	S
	20	36	S	32	S
M39V	10	36	S	30	S
	15	38	S	35	S
	20	40	S	36	S
M43V	10	35	S	23	I
	15	36	S	30	S
	20	35	S	26	S
M45A	10	38	S	30	S
	15	39	S	27	S
	20	38	S	29	S
M46A	10	37	S	30	S
	15	37	S	25	I
	20	39	S	34	S
M47A	10	34	S	31	S

	15	33	S	30	S
	20	35	S	33	S
M48G	10	37	S	24	I
	15	38	S	26	S
	20	36	S	21	R
M49G	10	38	S	27	S
	15	39	S	25	I
	20	38	S	26	S

R: Resistant, I: Intermediate, S: Susceptible.

Table 4 shows the inhibition halos of fluoroquinolones at different concentrations against isolates of *Arcobacter* spp, where it is detailed that the 26 samples resulting from the PCR are all susceptible to the control antibiotic levofloxacin, while for ciprofloxacin showed that in some samples there was intermediate resistance to this antibiotic. **EUCAST (2020),** considers the susceptibility of the *Campylobacter* genus against groups of fluoroquinolone antibiotics with a halo diameter ≥26 mm, in the same way as the **CLSI (2019); CLSI (2020)**.

In another study carried out by **Abbasi et al.** (**2018**), they reported the susceptibility of 65.6% of the antibiotic ciprofloxacin against the microorganism, therefore, considering our results and the bibliographical background, we can say that the antibiotics ciprofloxacin and levofloxacin are of high efficiency for the control of bacteria of the genus *Arcobacter* spp present in milk whey, for first and second generation antibiotics it is necessary to perform antibiotic susceptibility against these microorganisms.

4. Conclusions

As the microorganism is present in dairy serum, cheeses could act as a vehicle for transmission to humans and represent a potential risk to public health. It was also shown that PCR is the most sensitive and reliable technique for the characterization of the microorganism.

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