JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Genetic diversity and characterization of potentially pathogenic *Escherichia coli* isolated from artisanal cheese in Venezuela

Leidys Guillén[1], María Araque[1]

[1] Department of Microbiology and Parasitology, Laboratory of Molecular Microbiology, Faculty of Pharmacy and Bioanalysis, University of The Andes, 5101, Mérida, Venezuela

Abstract

Introduction: Artisanal raw milk cheese can be an important source of bacteria potentially harmful to humans, such as pathogenic *Escherichia coli*. The objective of this study was to determine the genetic diversity, pathogenicity, and antimicrobial resistance of 45 *E. coli* strains isolated from artisanal raw milk cheese in Venezuela.

Methods: These strains were isolated according to the procedures established by the Venezuelan Commission of Industrial Standards (COVENIN) and identified by conventional methods. Antimicrobial resistance was determined by the disk diffusion method, while phylogenetic grouping and detection of 6 virulence genes (*fimH, kpsMT* II*, papAH,* PAI*, fyuA* and *usp*) were performed by PCR amplification. Strain typing was performed by Rep-PCR.

Results: Of the 45 isolates, 73.3% were susceptible, while 26.7% were resistant to at least one of the tested antibiotics. Phylogenetic grouping revealed a relatively homogeneous distribution. Phylogenetic group A dominated in 82.2% of the strains followed by B1 and D (8.9% each). Three major virulence factors, *fimH, fyuA, and kpsMTII*, were genetically encoded in most strains. Rep-PCR typing of *E. coli* strains revealed a heterogeneous population structure.

Conclusions: *E. coli* isolated from artisanal dairy products share characteristics and virulence genes with extraintestinal pathogenic *E. coli* (ExPEC) strains from animals and humans, which represents a public health risk. Thus, it is necessary to increase hygienic and sanitary controls, especially those involved in the production stages, and emphasize the epidemiological surveillance of potentially pathogenic bacterial strains present in unpasteurized artisanal cheese marketed in the city of Mérida, Venezuela.

Key words: Raw milk; cheese; genetic diversity; pathogenicity; virulence; *Escherichia coli*.

J Infect Dev Ctries 2024; 18(11):1726-1733. doi:10.3855/jidc.19441

(Received 24 October 2023 – Accepted 20 January 2024)

Introduction

Milk and its derivatives are important natural foods in the daily diet. Dairy products are readily available even in developing or economically limited countries [1]. Nutritional components such as proteins, carbohydrates, calories, minerals, and water found in milk and its derivatives, represent a complete food for human nutrition. However, it is also a potential vehicle for growth and transmission of various enteric pathogens [1,2]. An important indicator of the sanitary quality of food is the detection and quantification of *Escherichia coli*. Its presence generally indicates direct or indirect fecal contamination, sometimes accompanied by other dangerous pathogens [1,3]. Some dairy products that have not undergone sanitization processes may contain an acceptable level of *E. coli* without necessarily posing a risk to the consumer or degrading the quality of these products [4]. However, levels above the recommended limits for *E. coli* may indicate improper handling and poor sanitary conditions in production, storage, transport, and marketing of these foods [3-5].

E. coli is a facultative anaerobic bacterium that can survive and grow in the environment thanks to its versatility in using different energy sources, besides being a microorganism without special nutritional requirements. This ability to adapt to different conditions favors its integration into microbial communities in a variety of environments [6]. This characteristic of *E. coli* is one of the factors that facilitate food contamination, as well as its persistence at all stages of the food chain [5,6]. Although *E. coli* is one of the microorganisms that make up the intestinal microbiota in humans, its interactions with the host allow it to be classified into three broad groups: commensal, diarrheagenic *E. coli* (DEC), and extraintestinal pathogenic *E. coli* (ExPEC) [1,2,6].

E. coli strains have a wide genetic diversity. Their phylogenetic background and the presence of virulence factors distinguish pathogenic from commensal isolates

[7]. Commensal strains that belong to the A and B1 phylogroups are considered to be of low virulence, while extraintestinal pathogenic strains are mainly of the B2 and D phylogroups and carry the genes responsible for the promotion of virulence factors that affect a wide range of cellular processes. [7,8].

On the other hand, *E. coli* is considered a reservoir of genes to other members of the human or animal microbiota. Consequently, the gastrointestinal tract becomes the ideal niche for large-scale transfer of antimicrobial resistance genes and pathogenicity factors [3,6,8]. In recent years, the number of antibiotic-resistant *E. coli* strains isolated from healthy animals and their products for human consumption has increased significantly [7-9]. This resistance is mainly encoded in mobile genetic elements, facilitating its spread in different eco-environments, including the human ecosystem [7-11]. The World Health Organization (WHO) recommends monitoring antimicrobial resistance using *E. coli* as an "indicator bacterium" to analyze the phenotypes and mechanisms of resistance that could be disseminated through foods of animal origin and their derivatives in different geographical areas [12].

Several studies report the molecular characteristics of *E. coli* strains isolated from clinical samples of humans and sick animals and also analyze the degree of genetic relationship between pathogenic strains found in animals with those of human origin [7,13,14]. In contrast, studies describing the genetic characterization of *E. coli* isolated from artisanal products of animal origin intended for human consumption are scarce [15,16]. In this regard, the aim of this study was to determine the genetic diversity, genes of pathogenicity, and antimicrobial resistance of *E. coli* strains isolated from artisanal raw milk cheese in Venezuela.

Methodology

Sampling and E. coli isolation

Fifteen samples of artisanal dairy products were collected, including five of milk cream, curd, and cottage cheese each. These samples were randomly acquired from different commercial establishments located in the urban area of the Libertador Municipality of Mérida, Venezuela, between January and July 2021. The selected dairy products ranged from 250 to 500 g per presentation and were unrelated to each other. To analyze the samples for *E. coli*, 10 g of each sample was homogenized in 90 mL of 0.1% peptone water (diluted to 10^{-1}), following the procedures established by the Venezuelan Commission of Industrial Standards (COVENIN) [17]. The homogenized solutions were then incubated at 37°C for 2 h to improve the recovery of *E. coli* strains. Three dilutions (10^{-2} to 10^{-4}) were prepared from this solution, and 1 mL from each dilution was inoculated onto rehydratable Petrifilm-type *E. coli*/coliform plates (3M^{TM}, USA). The plates were then incubated at 35 °C for 18 to 24 hours, according to the supplier recommendations. All plates showing growth between 4 to 10 colony-forming units (CFU), suggestive of *E. coli*, were selected. These colonies were recognized by their blue color and their association with gas production. Four colonies from each plate were randomly taken and plated in BHI broth (BBL, Cockeysville, Md, USA) and incubated at 36 °C for 18-24 hours. Subcultures were then plated on Levine or MacConkey agar (Himedia, Mumbai, India) and incubated at 36 °C for 18-24 hours. Lactose-fermenting colonies were picked up, and those with morphology suggestive of *E. coli* were identified by conventional methods. A total of 90 *E. coli* strains were obtained and 45 of these were randomly selected, corresponding to 15 strains for each dairy product group: cream, curd, and cottage cheeses.

Antimicrobial susceptibility tests

Antimicrobial susceptibility profiles of the isolates were determined by the disk diffusion method and the data were interpreted according to the breakpoint values given in the Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. Sixteen antimicrobial agents (Oxoid Ltd., Basingstoke, UK) were tested: ampicillin (10 µg), amoxicillin/clavulanate (20/10 µg), cefazolin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), aztreonam (30 µg), imipenem (10 µg), meropenem (10 µg) ertapenem (10 µg), gentamicin (10 µg), tobramycin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), tetracycline (30 µg) and trimethoprim-sulfamethoxazole (1.25 µg/23.75 µg). *E. coli* ATCC 25922 was used as the quality control strain.

DNA extraction

Bacterial strains were grown overnight on trypticase soy agar (TSA, Oxoid) at 37 °C. A single colony was suspended in 100 µL of sterile deionized water and boiled for 10 min. The suspension was frozen and centrifuged at 14,000 rpm for 5 min, the DNA-containing supernatant was collected and 1 µL was used as a DNA template.

Detection of virulence genes

All 45 *E. coli* were screened for genetic markers of virulence associated with ExPEC by conventional PCR using primers and conditions previously described

[11,19]. The genes selected were: *papAH* (P fimbriae structural subunit), *kpsMT* II (group 2 capsular polysaccharide units), *fimH* (D-mannose specific adhesin, type 1 fimbriae), *fyuA* (yersiniabactin receptor), *usp* (uropathogenic specific protein) and PAI (pathogenicity island: GenBank Nº AF003742). The selection of the six virulence genes was based on their expression in ExPEC strains circulating in the region, as reported by our team in several studies since 2014 [10,11,20]. *E. coli* LMM/E02-ULA (*fimH* +, *fyuA* +, *kpsMTII* + y PAI +), *E. coli* LMM/Sc03-ULA (*papAH* +) and *E. coli* LMM/E02-ULA (*usp* +) were used as positive controls strain.

Phylogenetic grouping

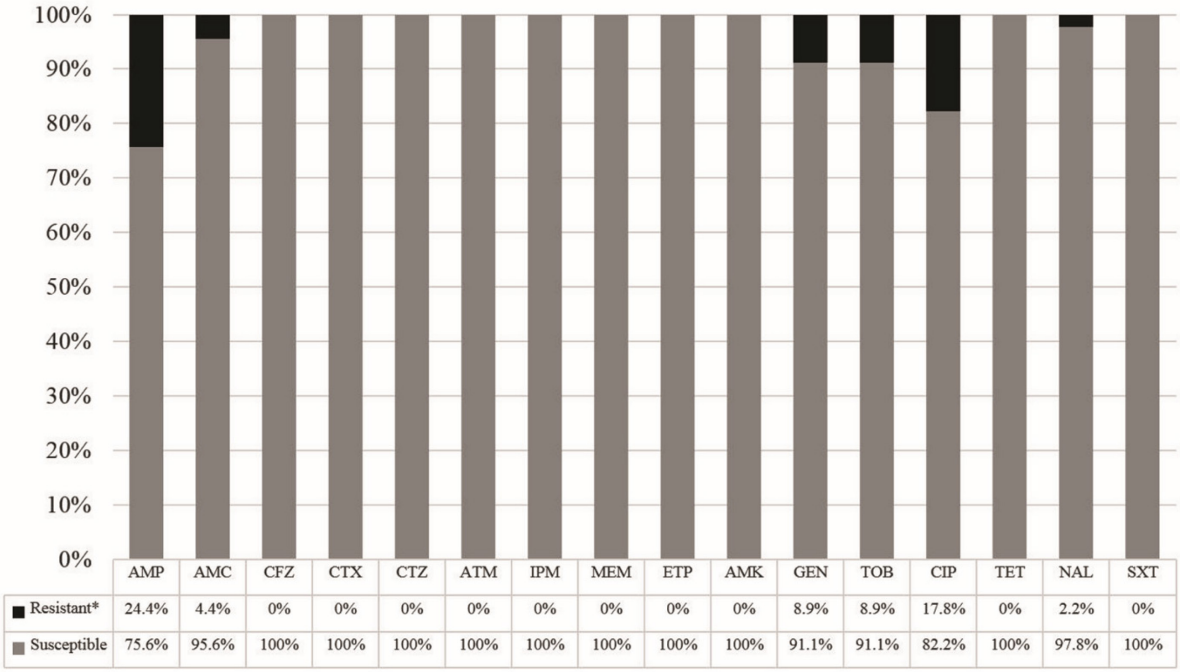
The phylogenetic grouping of *E. coli* isolates was determined by multiplex PCR according to the method described by Clermont *et al.* [21]. Isolates were classified into eight main *E. coli* phylogenetic groups (A, B1, B2, C, D, E, F, and clade I) based on the presence or absence of genes (*chu*A, *yja*A, *arpa*, *trp*A and a non-coding DNA fragment (TspE4.C2). For positive controls, the strains *E. coli* AO38-ULA (*arp*A and *yja*A), UPEC 09-ULA (*chu*A, *yja*A and TspE4.C2) and *E. coli* SC20-ULA (*trpA*) were used.

Repetitive element sequence-based PCR (Rep-PCR) typing

Rep-PCR using the primers Rep-PCR1 (5'-IIIG CGC CGI CAT CAG GC- 3') and Rep-PCR2 (5'-ACG TCT TAT CAG GCC TAC-3') was performed according to previously described protocols [22]. The PCR reaction was accomplished in a 25 µL reaction mixture containing 5 µL of the DNA template, 2.5 µL of buffer (10X; Bioneer, Daejeon, Korea), 2.5 µL of $MgCl_2$ (50 Mm; Bioneer), 3 µL of dNTPs (10 Mm; Bioneer), 3 µL of each of the primers (10 pmol/µL), 0.5 µL of *Taq* polymerase (5 U/µL; Bioneer) and 5.5 µL of sterile milli-Q water. The reaction was carried out using the following cycling procedure: initial denaturation at 94 °C for 2 minutes; 30 cycles at 94 °C for 1 minute, 40 °C for 1 minute, and 65 °C for 8 minutes, and a final extension at 65 °C for 16 minutes. The resulting Rep-PCR patterns were analyzed using TreeCon 1.3b software (http://bioinformatics.psb.ugent.be/software/details/TREECON). Strains with ≥ 90% similarity were classified as genetically related and assigned to the same cluster.

All DNA amplifications were performed in a thermocycler Mastercycler, Eppendorf, Germany. The PCR products were separated by horizontal electrophoresis through 1.5% (w/v) agarose gels (Sigma-Aldrich Co. St. Louis, MO, USA), stained with

Figure 1. Antimicrobial susceptibility of *E. coli* strains isolated from artisanal cream cheese, curd cheese and cottage cheese for the 16 tested antibiotics.



	AMP	AMC	CFZ	CTX	CTZ	ATM	IPM	MEM	ETP	AMK	GEN	TOB	CIP	TET	NAL	SXT
Resistant*	24.4%	4.4%	0%	0%	0%	0%	0%	0%	0%	0%	8.9%	8.9%	17.8%	0%	2.2%	0%
Susceptible	75.6%	95.6%	100%	100%	100%	100%	100%	100%	100%	100%	91.1%	91.1%	82.2%	100%	97.8%	100%

AMP: ampicillin, AMC: amoxicillin/clavulanate, CFZ: cefazolin, CTX: cefotaxime, CTZ: ceftazidime, ATM: aztreonam, IPM: imipenem, MEM: meropenem, ETP: ertapenem, AMK: amikacin, GEN: gentamicin, TOB: tobramycin, CIP: ciprofloxacin, TET: tetracycline, NAL: nalidixic acid, SXT: trimethoprim-sulfamethoxazole. *All resistant strains were isolated from cottage cheese.

ethidium bromide (Sigma-Aldrich) and documented using the UVP Biodoc-it System, California, U.S.A. Amplicons sizes were determined by comparison with a 100-bp DNA ladder (Bioneer).

Results

The results of susceptibility testing for 45 strains of *E. coli* are displayed in Figure 1. Of the strains studied, 12 (26.7%) showed resistance to at least one of the 16 antibiotics tested. However, all the strains were susceptible to third-generation cephalosporins (cefotaxime and ceftazidime), carbapenems, monobactams, amikacin, tetracyclines, and trimethoprim-sulfamethoxazole. Among the resistant strains, 11 (24.4%) showed resistance to ampicillin, and 8 (18%) to ciprofloxacin. A small number of strains were resistant to gentamicin (4/45; 8.8%), tobramycin (4/45; 8.8%), amoxicillin-clavulanate (2/45; 4.4%), and nalidixic acid (1/45; 2.2%). Table 1 shows the susceptibility patterns. Five strains (5/12; 41.6%) showed multidrug-resistant (MDR) phenotypes, with resistance to a combination of three different groups of antibiotics, being the ampicillin and ciprofloxacin resistance markers the most common. The remaining strains showed monoresistance (5/12; 41.6%) or resistance associated with two antibiotics, ampicillin, and ciprofloxacin (2/12; 16.7%). All the resistant *E. coli* strains were obtained from cottage cheese, which accounted for 73.3% (12/15) of the total strains recovered from this product.

Table 1. Distribution of susceptibility patterns of *E. coli* strains isolated from samples of artisanal cheeses.

Susceptibility patterns	N (%)
Sensible strains	33 (73.3)
Resistant strains*	12 (26.7)
Strains resistant to one antibiotic (n = 5)	
AMP	4 (33.3)
CIP	1(8.3)
Strains resistant to two antibiotics (n = 2)	
AMP, CIP	2 (16.7)
Strains resistant to four antibiotics (n = 4)	
AMP, AMC, CIP, NAL	1 (8.3)
AMP, CIP, GEN, TOB	3 (25.0)
Strains resistant to five antibiotics (n = 1)	
AMP, AMC, CIP, GEN, TOB	1 (8.3)

AMP: ampicillin; AMC: ampicillin/clavulanate acid; CIP: ciprofloxacin; NAL: naldixidic acid; GEN: gentamicin; TOB: tobramycin. *All resistant strains were isolated from cottage cheese.

The distribution of phylogenetic groups and virulence genes of *E. coli* isolated from artisanal dairy foods are shown in Table 2. The phylogenetic group analysis revealed that most of the *E. coli* strains isolated from cream cheese, curb cheese, and cottage cheese belonged to group A (37/45; 82.2%), followed by groups B1 and D with the same number of strains (4/45; 8.9%, respectively). Phylogroups B2, C, E, and F were not detected in the strains tested. None of the *E. coli* isolates from cream cheese were classified in phylogroup D, but the distribution of this group was more frequent in strains isolated from curd cheese (20%). Except PAI, all virulence genes investigated were detected. All strains harbored *fimH* followed by *fyuA* genes (31/45; 68.9%), while *usp* was the least frequently detected. The greatest number of virulence

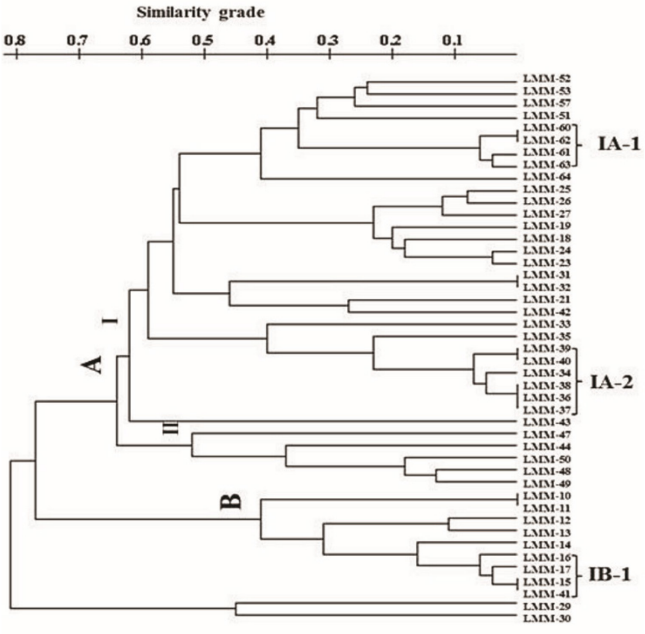
Table 2. Distribution of susceptibility phenotypes according to the phylogenetic groups and virulence genes of *E. coli* strains.

***E. coli* susceptibility phenotypesPhylogenetic group n (%)**	**Virulence genes n (%)**					**PAI**
	fimH	***fyuA***	***kpsMT II***	***papAH***	***usp***	
Sensible strains n = 33						
A 25 (55.6)	25 (75.7)	19 (57.6)	12 (36.4)	11 (33.3)	5 (15.1)	0
B1 4 (8.9)	4 (12.1)	3 (9.1)	1 (3.0)	1 (3.0)	2 (6.1)	0
D 4 (8.9)	4 (12.1)	1 (3.0)	1 (3.0)	0	1 (3.0)	0
Resistant strains n = 12						
A 12 (26.7)	12 (100)	8 (66.7)	12 (100)	8(66.7)	2 (16.7)	0

Table 3. Distribution of phylogenetic groups and presence of virulence genes of *E. coli* according to the type of artisanal cheeses analyzed.

E. coli	**Virulence genes n (%)**					
Phylogenetic group n (%)	***fimH***	***fyuA***	***kpsMT II***	***papAH***	***usp***	***PAI***
Cream cheese, n = 15 (100)						
A 14 (93.3)	14 (93.3)	11 (73.3)	10 (66.7)	9 (60)	3 (20)	0
B1 1 (6.7)	1 (6.7)	0	0	0	1 (6.7)	0
Cottage cheese, n = 15 (100)						
A 13 (86.6)	13 (86.6)	9 (60)	13 (86.6)	9 (60)	3 (20)	0
B1 1 (6.7)	1 (6.7)	1 (6.7)	1 (6.7)	0	0	0
D 1 (6.7)	1 (6.7)	0	0	0	0	0
Curd cheese, n = 15 (100)						
A 10 (66.7)	10 (66.7)	7 (46.7)	1 (6.7)	1 (6.7)	1 (6.7)	0
B1 2 (13.3)	2 (13.3)	2 (13.3)	0	1 (6.7)	1 (6.7)	0
D 3 (20)	3 (20)	1 (6.7)	1 (6.7)	0	1 (6.7)	0

Figure 2. Genetic diversity of *E. coli* strains isolated from artisanal dairy foods based on similarity coefficients calculated from Rep-PCR analysis data. Clusters with similarity of ≥ 95% were designed as: IA-1 (4 strains), IA-2 (6 strains) and IB-1 (4 strains).



genes was identified in phylogroup A strains isolated from cream cheese and cottage cheese. *kpsMT* II was detected more frequently in strains of phylogroup A from cottage cheese (13/15; 86,6%), while the *papAH* gene was not found in the strains belonging to phylogroup D. Overall, most of the *E. coli* strains (43/45) presented three or more virulence genes, regardless of the susceptibility phenotype (Table 3).

Two principal clonal clusters (A and B) were observed in the *E. coli* strains studied (Figure 2). The A cluster concentrated most of the *E. coli* (34/45), while the B cluster consisted of 11 strains. Of the strains analyzed, 14 (31.1%) showed a genetic relationship ≥ 95% and were included in three sub-clusters: IA-1 (4 strains), IA-2 (6 strains) and IB-1 (4 strains). The phenotypic and genetic characteristics of the 14 clonally related *E. coli* strains are shown in Table 4. All strains grouped in each of the clusters belonged to phylogenetic group A. Subcluster IA-1 contained only 4 MDR *E. coli* strains isolated from cottage cheese associated with three or more virulence genes. Among them, strains LMM-60, -62, and -63 have the highest number of resistance markers and virulence genes. Subcluster IA-2 consisted of a total of 6 *E. coli* strains, 4 from curd cheese, that were susceptible to all antibiotics tested, and 2 strains from cottage cheese, one of which was resistant to ampicillin, ampicillin-clavulanate, ciprofloxacin, and nalidixic acid, and the other to ampicillin and ciprofloxacin. All strains in this cluster carried at least 2 virulence genes. Subcluster IB-1 contained only 4 sensible strains isolated from cream cheese. These strains carried at least the combination of 3 or 4 virulence genes.

Discussion

Raw milk is often used in various dairy products. This production, which in many cases is homemade, is gaining popularity among consumers since they are considered healthier foods, especially because they do not contain chemical additives [1-3,23]. On the other hand, the production and marketing of artisanal cheeses represent a local food tradition in different countries [1].

However, the lack of pasteurization during the production chain can allow the establishment of potentially pathogenic bacteria from raw milk obtained from possibly infected animals, contaminants from the

Table 4. Phenotypic and genetic characteristics of the 14 clonally related strains of *E. coli* (IA-1, IA-2, and IB-1).

REP-PCR, Cluster	Nº Strain	Cheese Type	Susceptibility profile	Phylogroup	Virulence Profile
IA-1	LMM-60	Cottage	CIP, AMP, GEN, TOB	A	*fimH, kpsMT II, papAH, fyuA*
	LMM-62	Cottage	CIP, AMP, AMC, GEN, TOB	A	*fimH, kpsMT II, papAH, fyuA*
	LMM-61	Cottage	CIP, AMP, GEN, TOB	A	*fimH, kpsMT II, papAH*
	LMM-63	Cottage	CIP, AMP, GEN, TOB	A	*fimH, kpsMT II, papAH, fyuA, usp*
IA-2	LMM-39	Curd	Sensible	A	*fimH, fyuA*
	LMM-40	Curd	Sensible	A	*fimH, usp, fyuA*
	LMM-34	Cottage	CIP, AMP, AMC	A	*fimH, kpsMT II*
	LMM-38	Curd	Sensible	A	*fimH, fyuA*
	LMM-36	Cottage	CIP, AMP	A	*fimH, kpsMT II*
	LMM-37	Curd	Sensible	A	*fimH, fyuA*
IB-1	LMM-16	Cream	Sensible	A	*fimH, kpsMT II, papAH, fyuA*
	LMM17	Cream	Sensible	A	*fimH, kpsMT II, fyuA*
	LMM-15	Cream	Sensible	A	*fimH, kpsMT II, fyuA*
	LMM-41	Cream	Sensible	A	*fimH, kpsMT II, papAH, fyuA*

CIP: ciprofloxacin; AMP: ampicillin; AMC: amoxicillin-clavulanate; GEN: gentamicin; TOB: tobramycin.

production environment, as well as microorganisms from food handlers [3-5].

E. coli is one of the microorganisms most frequently implicated in the contamination of dairy products made from unpasteurized milk [6-7,13,15,16,24]. In this study, a collection of *E. coli* strains from three groups of artisanal dairy products (milk cream cheese, curd cheese, and cottage cheese) purchased in different commercial establishments in Mérida, Venezuela, was analyzed. A phenotypic study of these strains showed that more than 70% of the strains were sensible to the antibiotics tested, highlighting the excellent activity exhibited by broad-spectrum cephalosporins, carbapenems, amikacin, tetracycline, and trimethoprim/sulfamethoxazole. However, 25% of the strains were resistant to ampicillin and 10% showed low sensibility to amoxicillin/clavulanic acid, gentamicin, tobramycin and nalidixic acid. In this regard, it should be pointed out that within the small group of resistant strains, almost 50% showed resistance to at least three groups of antibiotics. The presence of MDR *E. coli* is not surprising, considering that similar results have been reported in several studies [13-16]. Imre *et al.* [25] found a significant number of MDR *E. coli* strains isolated from raw milk cheese marketed in the Banat region of Romania, and Loeza *et al.* [26] reported that 60% of Mexican, artisanal fresh cheese samples were contaminated with MDR ESBL-producing *E. coli*. In this respect, it has been described that the use of antibiotics as therapeutics, prophylactics, and growth promoters in food animals has contributed to the emergence of resistant bacterial strains [1]. All of the resistant *E. coli* strains analyzed in this study were isolated exclusively from cottage cheese samples, and it is possible that this product was made from raw milk contaminated at its source. However, it cannot be ruled out that this finding is also associated with the nature of the processing itself, since in the draining and kneading stages there is a greater exposure of the raw material to sources of contamination from the environment and food handlers. Cream cheese and curd cheese have shorter production processes that reduce the risk of exposure to contaminants. However, the quality of the raw materials, as well as a good hygiene practice during storage and transportation, also play a significant role in ensuring the microbiological quality of these cheeses.

Several reports indicate that within the genetic diversity of *E. coli*, groups A and B1 gather commensal strains with few or no virulence factors but are reservoirs of resistance genes with a higher frequency than the rest of the phylogroups (B2 and D) [9,24,25]. The results obtained support this statement since 82.2% of the strains analyzed belonged to group A. Ribeiro-Junior *et al.* [27] reported that 63.6% (14/22) of *E. coli* strains isolated from unpasteurized milk and Minas Frescal cheeses in Brazil belonged to phylogroup A, with more than half of these exhibiting resistance to at least one antibiotic. In this regard, several authors agree that the food chain is the vehicle for the transfer of resistance genes, and a food origin has been suggested for the presence of *E. coli* carrying various resistance mechanisms in the intestinal microbiota of humans [6,13,28].

On the other hand, the results obtained are consistent with the hypothesis proposed by Carlos *et al.* [29], pointing out that the distribution of phylogenetic groups of *E. coli* helps in identifying the sources of fecal contamination in animal products. In this regard, A and B1 *E. coli* strains occur in a wide range of herbivorous and carnivorous mammals, whereas B2 and D have a narrow and specialized host range; B2 is a good indicator of human fecal contamination. In this study, A was the dominant phylogroup, corresponding to commensal *E. coli* strains likely present in a bovine host, while the absence of group B2 strains could be an indication that the source of fecal contamination of artisanal cheeses was not of human origin.

E. coli is an important repository of genes encoding various virulence factors that allow this microorganism to evade host defense mechanisms and cause infections in anatomical sites other than the intestinal tract [9,16,25,28]. Indeed, all *E. coli* strains isolated from the three types of cheeses contained at least one virulence gene, regardless of the phylogenetic group or the presence or absence of resistance markers. Thus, most strains were genetically endowed with three major virulence factors: adhesion (*fimH*), iron uptake (*fyuA)* and capsule formation (*kpsMT* II), and less frequently P fimbriae expression (*papAH*) and the production of a bacteriocin (*usp*). This virulence profile is similar to that reported by Millán *et al.* [11] in uropathogenic *E. coli* strains isolated from hospitalized patients in the same geographical area where this study was conducted. Jørgensen *et al*. [30] and Ovi *et al.* [31] state that ExPEC strains isolated from food or animals are genetically related to those causing urinary tract infections (UTIs). Therefore, food is one of the principal sources of *E. coli* strains that carry a high load of genetic information that can be transferred from one strain to another through plasmids or pathogenicity islands [13,24-28]. These strains harbor not only antibiotic resistance genes but also determinants that increase their virulence potential, which favors the onset of extraintestinal infections.

Rep-PCR typing of the 45 *E. coli* strains isolated from the three artisanal cheeses revealed a heterogeneous population structure, with over 95% of the strains distributed in 2 main clusters whose similarity indices did not exceed 60%; however, three sub-clusters (IA-1, IA-2, and IB-1) with a genetically distant relationship stood out with internal similarity ranges of approximately 95%, without any association with phenotypic or genetic characteristics. Particularly, cluster IA-2 was formed by *E. coli* from curd and cottage cheese, suggesting that these strains may have a common origin, probably because they may have shared the same source of contamination, in this case, unpasteurized milk. However, these strains showed important differences in their susceptibility profiles and the presence of virulence genes.

The polyclonal distribution observed in this study has likely been influenced by several uncontrolled intervening factors, such as the origin of the raw milk used in the manufacturing of the artisanal products, the geographical area where the study was carried out, the manufacturing process, the preservation conditions of the product and the randomness in the selection of strains.

Conclusions

The findings obtained in this study show that the *E. coli* strains isolated from dairy foods such as cream cheese, curd cheese, and cottage cheese of artisanal production share characteristics and virulence genes with the ExPEC strains from animals and humans, which constitutes a public health risk. Thus, it is necessary to increase hygienic and sanitary controls, especially those involved in the production stages, and emphasize the epidemiological surveillance of potentially pathogenic bacterial strains present in unpasteurized artisanal cheese marketed in the city of Mérida, Venezuela.

Acknowledgments

This study was partially supported by the Council of Scientific, Humanistic, Technological and Arts of University of The Andes (CDCHTA-ULA), Mérida, Venezuela (grant CVI-ADG-FA-02-97).

Authors' contributions

LG carried out laboratory work, analysis of data, literature review, and wrote the initial draft. MA designed and supervised the study and contributed to the final writing of the paper and its critical review. Both authors have read and approved the final version of the manuscript.

References

1. Garcia SN, Osburn BI, Cullor JS (2019) A one health perspective on dairy production and dairy food safety. One Health 7: 100086. doi: 10.1016/j.onehlt.2019.100086.
2. Górska-Warsewicz H, Rejman K, Laskowski W, Czeczotko M (2019) Milk and dairy products and their nutritional contribution to the average polish diet. Nutrients 11: 1771. doi: 10.3390/nu11081771.
3. Akinyemi MO, Ayeni KI, Ogunremi OR, Adeleke RA, Oguntoyinbo FA, Warth B, Ezekiel CN (2021) A review of microbes and chemical contaminants in dairy products in sub-Saharan Africa. Compr Rev Food Sci Food Saf 20: 1188-1220. doi: 10.1111/1541-4337.12712.
4. Serrano NS, Zweifel C, Corti S, Stephan R (2018) Microbiological quality and presence of foodborne pathogens in raw milk cheeses and raw meat products marketed at farm level in Switzerland. Ital J Food Saf 7: 7337. doi: 10.4081/ijfs.2018.7337.
5. Merchán N, Zurymar S, Niño L, Urbano E (2019) Determination of the microbiological safety of artisanal cheeses according to Colombian technical standards. Rev Chil Nutr 46: 288-294. doi: 10.4067/S0717-75182019000300288
6. Sarowska J, Futoma Koloch B, Jama Kmiecik A, Frej-Madrzak M, Ksiazczyk M, Bugla-Ploskonska G, Choroszy-Krol I (2019) Virulence factors, prevalence and potential transmission of extraintestinal pathogenic *Escherichia coli* isolated from different sources: recent reports. Gut Pathog 11: 10. doi: 10.1186/s13099-019-0290-0.
7. Kaper JB, Nataro JP, Mobley HLT (2004) Pathogenic *Escherichia coli*. Nat Rev Microbiol 2: 123-140. doi: 10.1038/nrmicro818.
8. Curová K, Slebodníková R, Kmetová M, Hrabovsk V, Maruniak M, Liptáková E, Siegfried L (2020) Virulence, phylogenetic background and antimicrobial resistance in *Escherichia coli* associated with extraintestinal infections. J Infect Public Health 13: 1537-1543. doi: 10.1016/j.jiph.2020.06.032
9. Sora VM, Meroni G, Martino PA, Soggiu A, Bonizzi L, Zecconi A (2021) Extraintestinal pathogenic *Escherichia coli*: virulence factors and antibiotic resistance. Pathogens 10: 1355. doi: 10.3390/pathogens10111355.
10. Varela Y, Millán B, Araque M (2017) Genetic diversity of extraintestinal *Escherichia coli* strains producers of beta-lactamases TEM, SHV and CTX-M associated with healthcare. Biomédica 37: 209-217. doi: 10.7705/biomedica.v37i3.3324.
11. Millán Y, Araque M, Ramírez A (2018) Determination of enterobacteria and detection of virulence genes in *Escherichia coli* isolated from raw milk. Rev Soc Venez Microbiol 38: 58-63.
12. Aidara-Kane A, Angulo FJ, Conly JM, Minato Y, Silbergeld EK, McEwen SA, Collignon PJ and for the WHO Guideline Development Group (2018) World Health Organization (WHO) Guidelines on use of medically important antimicrobials in food-producing animals. Antimicrob Resist Infect Control 7: 7. doi: 10.1186/s13756-017-0294-9.
13. Yar A, Choudary MA, Rehman A, Elahi A, Ur Rehman F, Waqar AB, Alshammari A, Alharbi M, Nisar, MA, Khurshid M, Khan Z (2022) Genetic diversity and virulence profiling of multi-drug resistant *Escherichia coli* of human, animal, and environmental origins. Antibiotics (Basel) 11: 1061. doi: 10.3390/antibiotics1108106.
14. Aworh MK, Kwaga JKP, Hendriksen RS, Okolocha, EC, Thakur S (2021) Genetic relatedness of multidrug resistant

Escherichia coli isolated from humans, chickens and poultry environments. Antimicrob Resist Infect Control 10: 58. doi: 10.1186/s13756-021-00930-x.
15. Younis W, Hassan S, Mohamed HMA (2021) Molecular characterization of *Escherichia coli* isolated from milk samples with regard to virulence factors and antibiotic resistance. Vet World 14: 2410-2418. doi: 10.14202/vetworld.2021.2410-2418.
16. de Campos ACLP, Puno-Sarmiento JJ, Medeiros LP, Gazal LES, Maluta RP, Navarro A, Kobayashi RKT, Fagan EP, Nakazato G (2018) Virulence genes and antimicrobial resistance in *Escherichia coli* from cheese made from unpasteurized milk in Brazil. Foodborne Pathog Dis 15: 94-100. doi: 10.1089/fpd.2017.2345.
17. COVENIN (n.d.) Venezuelan Industrial Standards Commission Nº 1126-89. Foodstuffs. Identification and sample preparation for microbiological analysis. Available in: http://www.sencamer.gob.ve/sencamer/normas/1126-89.pdf. Accessed: 10.07.2022. [Standards in Spanish].
18. Clinical and Laboratory Standards Institute (CLSI) (2021) Performance standards for antimicrobial susceptibility testing. 31th ed. CLSI supplement M-100-S27. Available in: https://www.treata.academy/wp-content/uploads/2021/03/CLSI-31-2021.pdf. Accessed: 23.07.2022.
19. Johnson JR, Stell A (2000) Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J Infect Dis 181: 261-272. doi: 10.1086/315217.
20. Guillén L, Millán B, Araque M (2014) Molecular characterization of *Escherichia coli* strains isolated from homemade dairy foods produced in Mérida, Venezuela. Infectio 18: 100-108. doi: 10.1016/j.infect.2014.04.004.
21. Clermont O, Christenson J K, Denamur E, Gordon D M (2013) The Clermont *Escherichia coli* phylo-typing method revisited: Improvement of specificity of new phylogroups. Environ Microbiol Rep 5: 58-65. doi: 10.1111/1758-2229.12019.
22. Versalovic J, KoeuthT, Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19: 6823-6831. doi: 10.1093/nar/19.24.6823.
23. Owusu-Kwarteng J, Akabanda F, Agyei D, Jespersen L (2020) Microbial safety of milk production and fermented dairy products in Africa. Microorganisms 8: 752. doi: 10.3390/microorganisms8050752.
24. Ombarak RA, Hinenoya A, Awasthi SP, Iguchi A, Shima A, Elbagory AR, Yamasaki S (2016) Prevalence and pathogenic potential of *Escherichia coli* isolates from raw milk and raw milk cheese in Egypt. Intern J Food Microbiol 221: 69-76. doi: 10.1016/j.ijfoodmicro.2016.01.009.
25. Imre K, Ban-Cucerzan A, Herman V, Sallam KI, Cristina RT, Abd-Elghany SM, Morar D, Popa SA, Imre M, Morar A (2022) Occurrence, pathogenic potential and antimicrobial resistance of *Escherichia coli* isolated from raw milk cheese commercialized in Banat region, Romania. Antibiotics 11: 721. doi: 10.3390/antibiotics11060721.
26. Loeza-Lara PD, Medina-Estrada RI, Bravo-Monzón AE, Jiménez-Mejía R (2023) Frequency and characteristics of ESBL-producing *Escherichia coli* isolated from Mexican fresh cheese. Food Sci Technol 43: e108222. doi: 10.1590/fst.108222.
27. Ribeiro Júnior JC, Silva FF, Lima JBA, Ossugui EH, Teider Junior PIT, Campos ACLP, Navarro A, Tamanini R, Ribeiro J, Alfieri AA, Beloti V (2019) Molecular characterization and antimicrobial resistance of pathogenic *Escherichia coli* isolated from raw milk and Minas Frescal cheeses in Brazil. J Dairy Sci 102: 10850-10854. doi: 10.3168/jds.2019-16732.
28. Shoaib M, He Z, Geng X, Tang M, Hao R, Wang S, Shang R, Wang X, Zhang H, Pu W (2023) The emergence of multi-drug resistant and virulence gene carrying *Escherichia coli* strains in the dairy environment: a rising threat to the environment, animal, and public health. Front Microbiol 14: 1197579. doi: 10.3389/fmicb.2023.1197579.
29. Carlos CM, Pires MM, Stoppe NC, Hachich EM, Sato MI, Gomes TAT, Amaral LA, Ottoboni LMM (2010) *Escherichia coli* phylogenetic group determination and its application in the identification of the major animal source of fecal contamination. BMC Microbiol 10: 161-171. doi: 10.1186/1471-2180-10-161.
30. Jørgensen SL, Stegger M, Kudirkiene E, Lilje B, Poulsen LL, Ronco T, Pires Dos Santos T, Kiil K, Bisgaard M, Pedersen K, Nolan LK, Price LB, Olsen RH, Andersen PS, Christensen H (2019) Diversity and population overlap between avian and human *Escherichia coli* belonging to sequence type 95. mSphere 4: e00333-18. doi: 10.1128/msphere.00333-18.
31. Ovi F, Zhang L, Nabors H, Adhikari LJP (2023) A compilation of virulence-associated genes that are frequently reported in avian pathogenic *Escherichia coli* (APEC) compared to other *E. coli*. J Appl Microbiol 134: 1-20. doi: 10.1093/jambio/lxad014.

Corresponding author

María Araque, MD, PhD
Laboratory of Molecular Microbiology,
Department of Microbiology and Parasitology,
Faculty of Pharmacy and Bioanalysis,
University of The Andes, Mérida 5101, Venezuela.
Email: araquemc@ula.ve
ORCID ID: 0000-0001-6517-953X

Conflict of interests: No conflict of interests is declared.