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Molecular Typing of Potentially Pathogenic *Escherichia coli* Isolated from Fresh Pasta Filata Venezuelan Cheeses

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HIGHLIGHTS

- All strains of *Escherichia coli* carried virulence genes and were susceptible to the antibiotics tested.
- *E. coli* strains carried at least two virulence genes, *fimH* (type 1 fimbriae), and *fyuA* (yersiniabactin receptor).
- The profile of virulence factors of *E. coli* was similar to the pathotypes Uropathogenic *E. coli*.
- About 80% of the *E. coli* strains were grouped within the phylogroups A and D.
- Rep-Polymerase Chain Reaction typing of *E. coli* strains revealed a heterogeneous and genetically diverse population structure.

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Acronyms and abbreviations
ExPEC=Extraintestinal Pathogenic *Escherichia coli*
PAI=Pathogenicity Island
PCR=Polymerase Chain Reaction

ABSTRACT

Background. Fresh pasta filata cheese is considered as one of the most important foods in the Venezuelan diet. It is typically produced by small-scale producers using raw milk. The objective of this research was to molecularly characterize the pathogenic potential of *Escherichia coli* strains isolated from pasta filata cheese manufactured and marketed in Venezuela.

Methodology. In the period between January and March of 2019, a total of 36 strains of *E. coli* were isolated from a variety of pasta filata cheeses including 17 samples of mozzarella, 16 of telita, and 3 of guayanés. These strains were isolated according to the Venezuelan Commission of Industrial Standards (COVENIN) and identified by conventional methods (biochemical and phenotypic tests). Antimicrobial susceptibility was determined using the disk diffusion technique. Phylogenetic grouping and detection of virulence genes were performed by Polymerase Chain Reaction amplification. Diversity and genetic relationships were determined by Rep-PCR.

Results: All strains were susceptible to the tested antibiotics. Phylogroup A (n=19) was the most frequent (52.8%), followed by groups D (n=11; 30.6%), and B1 (n=2; 5.6%). The majority of isolates carried at least two virulence genes, one coding for adhesion mechanisms (*fimH*) and the other for iron uptake (*fyuA*). Only one strain of phylogroup A presented a profile consisting of four virulence genes (*fimH, fyuA, kpsMT II,* and *papAH*). Four strains that could not be classified according to Clermont's scheme carried resistance genes as well. A heterogeneous population structure was observed by Rep-PCR of the strains.

Conclusion: Results support the hypothesis that the *E. coli* strains isolated from the three types of pasta filata cheeses manufactured and marketed in Venezuela have identical characteristics and virulence factors to Extraintestinal Pathogenic *E. coli* strains observed in animals and humans, posing a potential health risk. Therefore, it is essential to improve hygienic and sanitary controls at all stages of cheese production and to implement measures for epidemiological surveillance of potentially pathogenic bacterial strains present in Venezuelan, artisanal pasta filata cheeses.

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Introduction

Pasta filata cheese, also referred to as spun paste or stretched-curd cheese is one of the most popular and significant food in the Venezuelan diet. It is typically made by small-scale producers who frequently consume raw milk, which contains the optimal microbiota that contributes to the organoleptic characteristics of the final product (Perdomo et al., 2015). Fresh pasta filata cheeses, containing telita, guayanés, and mozzarella, are firmly embedded in artisanal traditions that have been transmitted from one generation to the next. These practices exhibit considerable regional variation, resulting in notable differences in the texture, flavor, and organoleptic characteristics of the resulting cheeses. For instance, alterations in the stretching technique and coagulation temperature may influence the ultimate quality of the product. The production process is greatly influenced by the climatic and environmental conditions in each region. Other factors including temperature, humidity, and the quality of the raw milk, exert an influence on the natural microbiota and, consequently, on the fermentation and ripening of the cheese (Maldonado Gómez et al., 2011). These cheeses are in high demand due to their ready availability for human consumption following artisanal production, delicate flavor, medium fat content, and smooth texture. Furthermore, these cheeses have high pH and humidity levels with a low salt content (Márquez and García R, 2007; Rodríguez et al., 2009).

Artisanal pasta filata cheeses are typically observed in markets, open-air food fairs or from street vendors. They are frequently immersed in whey, which can promote the growth of microorganisms (Maldonado Gómez et al., 2011; Rodríguez et al., 2009). Additionally, the consumption of raw milk, as well as unsophisticated manufacturing processes, transportation, and storage, are critical points that can result in contamination of the product with harmful microorganisms (Perdomo et al., 2015). Previous studies have suggested that consumption of artisanal cheeses is associated with an increase in food-borne disease outbreaks (Koski et al., 2022; Sebastianski et al., 2022).

Escherichia coli is a microorganism commonly observed in the intestines of humans and animals as part of the normal microbiota. Its presence in food is typically indicative of direct or indirect fecal contamination, which may occasionally be accompanied by other intestinal pathogens (Bujnáková et al., 2021). However, the genomic plasticity of *E. coli* allows it to survive and evolve in various environments, casing it adaptable to different ecological niches, including food, and enabling its presence at any stage of the food chain. Detection and quantification of *E. coli* is an important factor in assessing food hygiene standards (Ombarak et al., 2016; Sarowska et al., 2019).

Although *E. coli* is a natural member of the human intestinal microbiota, its interactions with the host and the presence of virulence factors permit it to be classified into three main groups: commensal, Diarrheagenic *E. coli* (DEC), and Extraintestinal Pathogenic *E. coli* (ExPEC) (Braz et al., 2020). Phylogenetically, *E. coli* can be divided into eight phylogroups: A, B_1, B_2, C, D, E, F, and the cryptic clade I, which can be utilized to estimate its pathogenic potential (Clermont et al., 2013). However, the majority of *E. coli* strains are classified in groups A, B_1, B_2, and D (Beghain et al., 2018). Commensal strains are regarded to be of low virulence and comprise phylogroups A and B_1, whereas ExPEC are principally grouped in phylogroups B_2 and D, which commonly carry genes encoding virulence factors that act on a wide range of cellular processes (Sarowska et al., 2019).

Several studies conducted in Latin America have reported the prevalence of ExPEC in various types of cheese made with unpasteurized milk (Guillén et al., 2014; Pineda et al., 2021). In Venezuela, fresh pasta filata cheese is one of the most common carriers of food-borne diseases due to its poor microbiological quality (Maldonado Gómez et al., 2011; Márquez and García R, 2007; Perdomo et al., 2015; Rodríguez et al., 2009). However, studies describing the genetic characterization of ExpEC in artisanal dairy products for human consumption in the country are scarce (Guillén et al., 2014; Millán et al., 2018). In this regard, the purpose of this research was to molecularly characterize pathogenic *E. coli* strains isolated from fresh pasta filata cheeses manufactured and marketed in Venezuela.

Materials and methods

This study employed an observational, cross-sectional, descriptive methodology which was conducted between January and March 2019.

Sampling and E. coli isolation

A total of 75 *E. coli* strains were isolated from a variety of pasta filata cheeses. Of these, 36 strains were randomly selected for analysis. This sampling approach was based on proportionality for each of the selected products and product availability during sampling. The selected sample included unpasteurized soft pasta filata cheeses. Mozzarella, telita, and guayanés were collected from local markets and informal vendors in the urban area of the Caroní municipality of the city of Puerto Ordaz, Bolívar State, Venezuela.

Two hundred and fifty g of the substance were

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collected into a sterile sample collection bag, transferred to the laboratory in a chilled container, and processed within 24 h of collection.

For *E. coli* isolation, 10 g of each cheese were 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), (1989). Briefly, the homogenized solutions were subsequently incubated at 36 °C for 2 h to enhance the detection 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™, USA) and incubated at 35 °C for 18 to 24 h, according to the supplier's recommendations (Guillén et al., 2014). All plates indicating growth between 4 to 10 Colony Forming Units (CFU), suggestive of *E. coli*, were selected. These colonies were recognized by their blue color and gas production which manifested as bubbles. Four colonies from each plate were randomly selected and plated in Brain Heart Infusion (BHI) broth (BBL, Cockeysville, Md, USA) and incubated at 36 °C for 18-24 h. The subcultures were then plated on Levine or MacConkey agar (Himedia, Mumbai, India) and incubated at 36 °C for 18-24 h. Lactose-fermenting colonies were collected, and those with a morphology typical of *E. coli* were identified by conventional methods (biochemical and phenotypic tests).

Although we were not aware of any clinical cases associated with the consumption of the dairy products under investigation during the course of this study, we additionally investigated the presence of *E. coli* O157:H7. All *E. coli* strains were tested for sorbitol fermentation using biochemical methods. Sorbitol-negative phenotypic variants were subjected to agglutination assays with particular antisera (AntiColi O157:K-, Sifin Berlin, Germany). *E. coli* ATCC 25922 and *E. coli* O157:H7 (CVCM1931) were utilized as control strains according to the supplier's recommendations (Guillén et al., 2014; Millán et al., 2018).

Antimicrobial susceptibility tests

The disk diffusion method was applied to consider the antimicrobial susceptibility profiles of the isolates and the data were interpreted based on the breakpoint values presented in the Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines. Fifteen 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), tetracycline (30 µg), and trimethoprim-sulfamethoxazole (1.25 µg/23.75 µg). *E. coli* ATCC 25922 was applied as a quality control strain (CLSI, 2023).

DNA preparation

For DNA extraction, the strains were primarily streaked onto Trypticase Soy Agar (TSA; Oxoid, Ltd., Basingstoke, UK) and incubated for 24 h at 36 ^{0}C. A single colony was suspended in 100 µl of sterile deionized water and placed in a thermal block (Eppendorf, Germany) at 100 ^{0}C 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 utilized as a DNA template (Guillén et al., 2014; Millán et al., 2018).

Detection of virulence genes

All 36 *E. coli* isolates were subjected to conventional Polymerase Chain Reaction (PCR) screening for genetic markers of virulence related to ExPEC, employing primers and conditions previously depicted (Johnson and Stell, 2000). The selected genes 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 Pathogenicity Island (PAI; GenBank Nº AF003742). These six virulence genes were selected on the basis of their expression in ExPEC strains circulating in the region. Previous studies have identified the prevalence of these genes in strains isolated from dairy products and clinical samples, denoting a potential connection with outbreaks of infections in humans and animals, as reported by our team since 2014 in various investigations (Guillén et al., 2014; Millán et al., 2018). *E. coli* LMM/E02-ULA (*fimH*+,*fyuA*+,*kpsMTII*+, and PAI+), *E. coli* LMM/Sc03-ULA (*papAH*+), and *E. coli* LMM/E02-ULA (*usp*+) were used as positive controls strain.

Phylogenetic grouping

Phylogenetic grouping of *E. coli* strains was determined by multiplex PCR, following the procedure outlined by Beghain et al. (2018). Isolates were classified into eight major *E. coli* phylogenetic groups (A, B_1, B_2, C, D, E, F, and clade I) in accordance with the presence or absence of genes (*chu*A, *yja*A, *arpa*, *trp*A, and a non-coding DNA fragment (TspE4.C2). 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 as positive controls.

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

Rep-PCR analysis was conducted using the primers Rep-PCR1 (5'-IIIG CGC CGI CAT CAG GC- 3') and Rep-

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PCR2 (5'-ACG TCT TAT CAG GCC TAC-3') according to the established protocols described by Versalovic et al. (1991). Briefly, the amplification was performed in 25 µl of reaction mixture including 5 µl of the DNA template, 2.5 µl of buffer (10X; Bioneer, Daejeon, Korea), 2.5 µl of $MgCl_2$ (50 Mm; Bioneer, Daejeon, Korea), 3 µL of dNTPs (10 Mm; Bioneer, Daejeon, Korea), 3 µl of each of the primers (10 pmol/µl), 0.5 µl of *Taq* polymerase (5 U/µL; Bioneer, Daejeon, Korea), and 5.5 µl of sterile milli-Q water. The resulting Rep-PCR patterns were analyzed using the TreeCon 1.3b software (http://bioinformatics.psb.ugent.be/ software/details/TREECON). A minimum of 95% genetic similarity was exploited to classify strains as genetically related and to assign them to the identical cluster.

All DNA amplifications were executed in a thermocycler Master cycler (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 ethidium bromide (Sigma-Aldrich, Co. St. Louis, MO, USA) and documented by using the UVP Biodoc-it system (California, USA). Amplicon sizes were compared with a 100-bp DNA ladder (Bioneer, Daejeon, Korea).

Statistical analysis

Data were analyzed using the IBM SPSS Statistics software, version 21 (IBM Corporation, NY, USA). Continuous variables were characterized using mean and Standard Deviation (SD), whereas nominal and ordinal variables were expressed as percentages. The Chi-square test was employed to ascertain associations between categorical variables. A p-value<0.05 was considered statistically significant.

Results

A total of 75 *E. coli* strains were isolated from three different types of fresh pasta filata cheese: mozzarella, telita, and guayanés. From these strains, 36 *E. coli* isolates were randomly selected and distributed as illustrated in Table 1. None of the selected strains tested positive for the pathogenic serotype *E. coli* O157:H7. All strains were susceptible to the 15 antibiotics tested (Table 2).

Table 1: Distribution of 36 selected strains of *Escherichia coli** isolated from the pasta filata cheeses

Type	Number of *Escherichia coli* strains	%
Mozzarella	17	47.2
Telita	16	44.4
Guayanés	3	8.3
Total	36	99.9

* The total *E. coli* population was 75 strains.

Table 2: Susceptibility of 36 strains of *Escherichia coli* against 15 antimicrobial agents isolated from mozzarella, telita, and guayanés cheeses

Antibiotics	Antimicrobial susceptibility disk diffusion method
Ampicillin	sensible
Amoxicillin/clavulanate	sensible
Cefazolin	sensible
Cefotaxime	sensible
Ceftazidime	sensible
Aztreonam	sensible
Imipenem	sensible
Meropenem	sensible
Ertapenem	sensible
Amikacin	sensible
Gentamicin	sensible
Tobramycin	sensible
Ciprofloxacin	sensible
Tetracycline	sensible
Trimethoprim-sulfamethoxazole	sensible

Table 3 presents the phylogenetic grouping of the *E. coli* strains found in pasta filata cheeses. Approximately half of the strains were classified in phylogroup A, followed by groups D and B_1. Four strains isolated from telita cheese could not be distributed. The *E. coli* strains of phylogroup A were observed in all studied cheeses, while those of group D were discovered in mozzarella and telita cheeses, and group B_1 in telita and guayanés cheeses. Phylogroups B_2, C, E, F, and I clade were not detected.

Table 3: Distribution of phylogenetic groups of *Escherichia coli* according to the type of pasta filata cheese

Type of artisanal cheese *Escherichia coli strains*	*E. coli* phylogenetic group	n (%)
Mozzarella	A	10 (58.8)
	D	7 (41.2)
Telita	A	7 (43.7)
	B_1	1 (6.3)
	D	4 (25.0)
	UC*	4 (25.0)
Guayanés	A	2 (66.7)
	B_1	1 (33.3)

UC*=Unclassified

The relationship between phylogenetic group and virulence gene profile of *E. coli* strains is illustrated in Table 4. All strains had at least one virulence gene, with *fimH* being the most abundant. Regardless of the phylogenetic group, most of the strains unveiled various virulence genes associations. The majority of profiles were formed by the combination of two or three virulence genes. Only one strain from phylogroup A presented a profile consisting of four virulence genes (*fimH, fyuA, kpsMT II*, and *papAH*). Table 5 reveals the distribution of phylogroups and virulence factors according to the type of cheese analyzed. Although most *E. coli* strains were

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isolated from mozzarella cheese, two phylogroups, A (10) and D (7), were exclusively identified. The strains isolated from telita cheese uncovered the greatest diversity of phylogroups, including A, B_1, and D, and the four unclassified strains. The B_1 phylogroup was detected in strains isolated from telita and guayanés cheeses, whereas D group was observed in strains from mozzarella and telita cheeses. Regardless of the type of cheese analyzed, 91.7% of the strains carried the virulence gene *fimH*, which was the only statistically significant virulence factor (p=0.008). The frequency of *fyuA* was greater than 50% in the strains isolated from these cheeses. *kpsMT II* was not detected in any of the strains isolated from guayanés cheese, nor in group D strains from mozzarella cheese or in *E. coli* phylogroups B_1 and D isolated from telita cheese. Furthermore, the PAI was absent in all the studied strains.

Table 4: Distribution of the number and profile of virulence genes according to phylogenetic group of *Escherichia coli* from pasta filata cheeses

***Escherichia coli* phylogenetic group *n* (%)**	**No. virulence factors**	**Profile of virulence genes**	**n (%)**
A=19 (52.8)	1	*fimH*	6 (31.5)
	2	*fimH; papAH*	3 (15.8)
		fimH; usp	2 (10.5)
		fimH; fyuA	1 (5.3)
		fyuA; usp	1 (5.3)
	3	*fyuA;usp; kpsMT* II	1 (5.3)
		fimH; kpsMT II; *papAH*	1 (5.3)
		fimH; fyuA; papAH	1 (5.3)
		fimH; papAH; kpsMT II	1 (5.3)
		fimH; fyuA; kpsMT II	1 (5.3)
	4	*fimH; fyuA; kpsMT* II; *papAH*	1 (5.3)
B_1=2 (5.6)	1	*fimH*	1 (50.0)
	2	*fimH; fyuA*	1 (50.0)
D=11 (30.5)	1	*fimH*	1 (9.1)
		usp	1 (9.1)
	2	*fimH; fyuA*	2 (18.2)
		fimH; usp	1 (9.1)
	3	*fimH; fyuA; papAH*	3 (27.3)
		fimH; fyuA;usp	2 (18.2)
		fimH; papAH;usp	1 (9.1)
UC*=4 (11.1)	2	*fimH; fyuA*	3 (75.0)
	3	*fimH; fyuA; kpsMT* II	1 (25.0)

UC*=Unclassified

Table 5: Distribution of phylogenetic groups and presence of virulence genes of *Escherichia coli* according to the type of pasta filata cheese

***Escherichia coli* Phylogenetic group**	**Virulence genes *n* (%)**					
	fimH	***fyuA***	***kpsMT* II**	***papAH***	***usp***	**PAI**
Mozzarella cheese						
n=17	**14 (82.4)**	**6 (35.3)**	**4 (23.5)**	**8 (47.0)**	**5 (29.4)**	**0**
A=10	8 (80)	3 (30)	4 (40)	4 (40)	2 (20)	0
D=7	6 (85.7)	3 (42.9)	0	4 (57.1)	3 (42.9)	0
Telita cheese						
n=16	**16 (100)**	**11 (68.8)**	**2 (12.5)**	**2 (12.5)**	**3 (24.0)**	**0**
A=7	7 (100)	2 (28.6)	1 (14.3)	2 (28.6)	1 (14.3)	0
B_1=1	1 (100)	1 (100)	0	1 (100)	0	0
D=4	4 (100)	4 (100)	0	0	2 (50)	0
UC*=4	4 (100)	4 (100)	1 (25)	0	0	0
Guayanés cheese						
n=3	**3 (100)**	**2 (66.6)**	**0**	**1 (33.3)**	**1 (33.3)**	**0**
A=2	2 (100)	1 (50)	0	1 (50)	1 (50)	0
B_1=1	1 (100)	1 (100)	0	0	0	0
Total n=36	**33 (91.7)**	**19 (52.8)**	**6 (16.7)**	**12 (33.3)**	**9 (25.0)**	**0**
***p*-value**	0.008	0.138	0.502	0.226	0.733	-

PAI=Pathogenicity Island; UC*=Unclassified

A total of 30 Rep-PCR profiles were detected among the 36 *E. coli* strains analyzed (Figure 1). Two major clusters A and B were observed. A was the dominant cluster, concentrating most of the *E. coli* (33/36; 91.7%), while the B cluster consisted of three strains. Out of the analyzed *E. coli* strains, 6 (16.7%) showed a genetic relatedness of ≥95% and were grouped into three sub-clusters (A-I, A-II, and B-I), containing 2 strains each. The remaining profiles

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were represented by a single isolate. The phenotypic and genetic features of the six clonally related *E. coli* strains are displayed in Table 6. Two clonally related strains were identified for each type of analyzed cheese. Sub-cluster A-I involved two *E. coli* strains belonging to group D, which were isolated from guayanés cheese. Both strains harbored two and three virulence genes, respectively, with the presence of *fimH* and *fyuA* in both strains. Sub-cluster A-II comprises two groups A *E. coli* strains isolated from mozzarella cheese. These two strains had associations of three and four virulence genes, respectively, with *fimH*, *fyuA*, and *kpsMT II* being common to both. Sub-cluster B-I was formed by two strains of *E. coli* isolated from telita cheese. One of these strains, belonging to group A, had a single virulence gene (*fimH*), whereas the other, from phylogroup B_1, had two virulence genes (*fimH* and *fyuA*).

Table 6: Genetic characteristics of the six clonally related strains of *Escherichia coli* (A-I, A-II and B-I)

Rep-PCR Cluster	Nº Strain *Escherichia coli*	Cheese type	Phylogenetic group	Virulence genes
A-I	N30-15	Guayanés	D	*fimH, fyuA*
	N32-16	Guayanés	D	*fimH, fyuA, usp*
A-II	N21-12	Mozzarella	A	*fimH, kpsMT* II, *fyuA*
	N22-13	Mozzarella	A	*fimH, fyuA, kpsMT* II, *papAH*
B-I	N4-2	Telita	A	*fimH,*
	N5-2	Telita	B_1	*fimH, fyuA*

PCR=Polymerase Chain Reaction

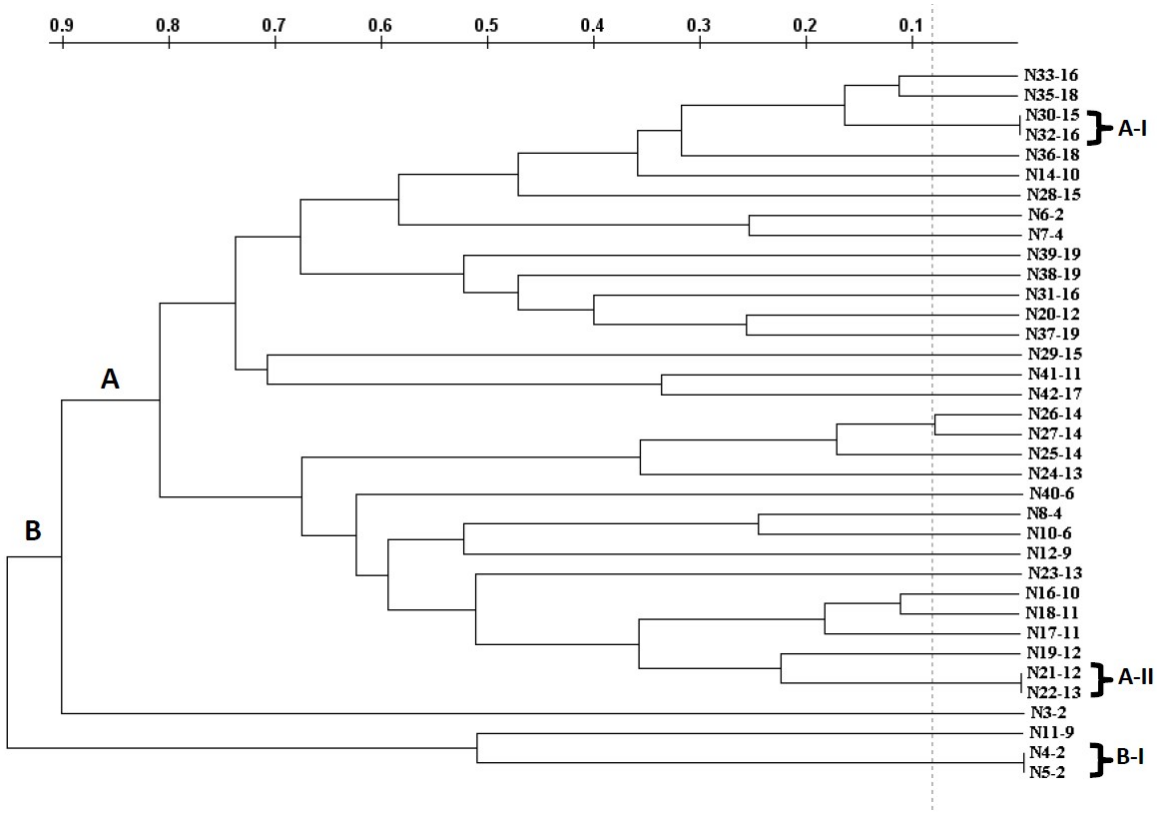

Figure 1: Genetic diversity of *Escherichia coli* strains isolated from fresh pasta filata cheeses based on similarity coefficients calculated from Repetitive element sequence-based Polymerase Chain Reaction (Rep-PCR) analysis data. Clusters with similarity of ≥95% were designed as: A-I (2 strains), AII (2 strains), and B-I (2 strains)

Discussion

One of the most appreciated fresh white cheeses in Venezuela is pasta filata. In this investigation, a collection of 36 *E. coli* strains was analyzed from three types of artisanal pasta filata cheeses, mozzarella, telita, and guayanés, purchased from different local popular markets and informal vendors in the urban area of the Caroní municipality of the city of Puerto Ordaz, Bolívar State, Venezuela, were analyzed. According to previous studies (Bagel and Sergentet, 2022; D'Amico, 2014) dairy

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products that have not undergone sanitization processes may contain an acceptable levels of *E. coli* without necessarily posing a risk to the consumers or degrading the quality of these products. However, levels above the recommended limits for *E. coli* may indicate fecal contamination, inappropriate handling, and poor hygiene practices (Pineda et al., 2021). Although it is frequently assumed that the production and marketing of pasta filata cheeses imply deficiencies in hygienic practices, the 36 *E. coli* strains isolated were susceptible to the tested antibiotics. These results may be connected to the healthy dairy animals that provided the milk. The use of antibiotics in dairy cows, which exerts selective pressure, is directly associated with bacterial resistance. However, if *E. coli* strains in dairy products carry virulence genes, they could potentially harm consumers.

Carlos et al. (2010) recommend that the distribution of *E. coli* phylogenetic groups can assist to identify sources of fecal contamination and the virulence potential of these strains in animal products. In this regard, A and B_1 *E. coli* strains appear in a wide range of herbivorous and carnivorous mammals, whereas B_2 and D have a narrow and specialized host range; B_2 is an appropriate indicator of human fecal contamination. In this study, group A was the dominant phylogroup, corresponding to commensal *E. coli* strains probably present in a bovine host, while the absence of group B_2 could indicate that the source of fecal contamination of artisanal cheeses was not of human origin. The obtained results are in agreement with those of Bujnáková et al. (2021), De Campos et al. (2018), and Ombarak et al. (2016). They reported that *E. coli* isolated from unpasteurized ovine cheeses in Slovakia, Minas cheeses in Brazil, and raw milk cheeses in Egypt, respectively, were predominantly from the A phylogenetic group. On the other hand, Beghain et al. (2018) reported that only 1% of *E. coli* strains cannot be assigned to one of the eight recognized phylogroups. However, in this work, the number of unclassified strains was ten times higher than what might normally be expected. It is possible that these 4 (11.1%) unclassified strains are the result of recombination between various phylogroups.

Although *E. coli* strains classified into phylogroups A and B_1 are typically considered to be of low virulence, in this study it was recognized that most of the strains carry at least two virulence genes encoding adhesion (*fimH*) and iron uptake (*fyuA*) mechanisms. It is crucial to highlight that the only strain carrying four (*fimH*, *fyuA*, *kpsMT II*, and *papAH*) of the six virulence genes studied, belonged to phylogroup A. In addition, four strains that couldn't be classified according to the Clermont's scheme also contained resistance genes (*fimH*, *fyuA*, *kpsMT II)* (Clermont et al., 2013). These results demonstrate the heterogeneity in the distribution of virulence factors among *E. coli* strains isolated from pasta filata cheeses, highlighting the multifactorial and complex nature of their pathogenic potential. In bacterial pathogens which infect mucosal tissues, such as *E. coli*, the expression of a single adhesin is necessary and sufficient for pathogenesis. However, if this adhesin is linked to genes for iron uptake, a critical factor for survival, it may allow the bacterium to establish itself in a competitive environment (Sarowska et al., 2019).

In recent years, researchers have postulated that food, including unpasteurized dairy products, can serve as a reservoir for numerous virulence factors responsible for extra intestinal infections and play a significant role in the transmission of ExPEC strains (Bujnáková et al., 2021, Ovi et al., 2023). The *E. coli* isolated in this study showed a virulence factor load identical to that reported by Millán et al. 2020 and Quijada-Martínez et al. 2017 in Uropathogenic *E. coli* (UPEC) isolated from hospitalized patients in Mérida, Venezuela. Sarowska et al. (2019) describe Food-borne Urinary Tract Infection (FUTI) as UTIs caused by contaminated food. The specific pathotypes of *E. coli* that are responsible for FUTI have not yet to been precisely defined. However, Jakobsen et al. (2010) evaluated the presence of ExPEC-associated virulence genes in *E. coli* isolated from UTI patients, in dairy derivatives and meat. Similarly, De Campos et al. (2018) isolated *E. coli* strains in unpasteurized cheeses in Brazil with virulence patterns comparable to the ones detected in *E. coli* causing Meningitis Neonatal *E. coli* (MNEC). Presumably, this phenomenon is based on the observation that the virulence genes of *E. coli* are located in transmissible genetic elements, including genomic islands, bacteriophages, Insertion Sequences (ISs), integrons, plasmids, and transposons; therefore, these elements can be readily transferred among various bacterial species and induce genetic rearrangements. As a result, the horizontal transfer between different *E. coli* strains facilitates the emergence of novel pathogenic strains, designated as Hybrid Pathogenic *E. coli.* (HyPEC) (Braz et al., 2020). Although, in this work, approximately 80% of the *E. coli* strains isolated in pasta filata cheeses were assigned to the low virulence phylogroups (A and B_1), their virulence factor content was remarkably comparable to the profile of UPEC pathotypes of clinical origin recorded in the region (*fimH, fyuA, kpsMT II, papAH,* and *usp*), making these HyPEC strains represent a highly serious threat that requires further study.

Rep-PCR typing of the 36 *E. coli* strains isolated from the three pasta filata cheeses revealed a heterogeneous population structure, which was clustered into 30 profiles. The strains were distributed in two main clusters (A and B) with similarity indices not exceeding 10%; however, three sub-clusters (A-I, A-II, and B-I) with a genetically distant

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relationship emerged with internal similarity ranges of >95%, without any association with phenotypic or genetic characteristics. Each subcluster contained two strains from the identical type of pasta filata cheese, implying a frequent source of contamination, presumably raw milk. The genetic diversity and polyclonal distribution of the *E. coli* strains observed in this research may have been influenced by various uncontrolled factors, including the origin of the raw milk, the manufacturing process, the preservation conditions of the final product, the randomness in the selection of the strains and the geographical area where the study was implemented.

Conclusion

The results of the study confirm the hypothesis that the *E. coli* strains isolated from the three types of fresh pasta filata cheeses share characteristics and virulence factors compatible with ExPEC strains from animals and humans, and therefore pose a health risk. However, the presence of *E. coli* in these cheeses not only indicates contamination of fecal origin, which could potentially be associated with other enteropathogens, but also suggests that these cheeses may serve as a means of transmission and selection of virulent clones causing intestinal and extra intestinal diseases. This study highlights the need to strengthen hygienic and sanitary controls at all stages of cheese production and to implement measures for epidemiological surveillance of potentially pathogenic bacterial strains found in fresh, artisanal pasta filata cheeses sold in the state of Bolívar, Venezuela. Although this study was limited to a specific geographical area, the obtained results may be representative of a common issue detected in other regions of the world with similar socio-economic and cultural characteristics.

Author contributions

Y.Y.V.-R. and L.G. investigated, analyzed data, and participated in the experimental development of the study; C.C.-S. investigated, analyzed data, and conducted the experimental work; M.A. investigated, analyzed data, conducted the experimental work, corrections, and critical review of the manuscript. All authors have read and approved the final manuscript.

Conflicts of interest

We declare that there is no conflict of interest.

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Ethical consideration

No ethical approval is required. No human or animal was used for the study.

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