Phylogenetic grouping and probiotics antibacterial studies on Escherichia coli isolates obtained from calves' excrement in an Industrial slaughterhouse of Mashhad city

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Abstract

Diarrhea in calves caused by *Escherichia Coli (E. Coli)* poses an economic risk to livestock farms. Classifying new pathogen strains through genetic recombination aids in infection prevention and treatment. This study aims to identify *E. Coli* strains in calf feces and examine the antibacterial effects of probiotics on them. 85 samples were prepared from healthy and diarrheal calves' excrement at Mashhad industrial slaughterhouse to isolate E. *Coli* strains. Then, they were phylogenetically grouped using multiplex polymerase chain reaction (PCR) on *yjaA*, *chuA*, *arpA*, and *TspE4.C2* genes, based on the new *Claremont* method, and were classified using the ERIC-PCR method based on genetic diversity. Also, double-layer culture and plate-well methods investigated *Lactobacilli's* antibacterial and anti-adhesion effects (*L.*) *Casei* and *Plantarum* and their aggregation effects with isolates were done using the Coaggregation method. Based on the PCR results of 70 *E. Coli* strains, the phylogenetic grouping was classified as A(40%), B1(17.14%), B2(14.3%), E(7.14%), F(5.71%), D(4.29%), C(0%), and unknown(11.42%). Their genetic diversity consisted 3 main clusters including subclusters: G1(2 isolates), G2(4 isolates), G3(6

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isolates), G4(3 isolates), G5(18 isolates), G6(3 isolates). Probiotics' antibacterial and anti-adhesion effects were confirmed against pathogenic and non-pathogenic *E. Coli* strains, and these effects were more impressive about *L. Casei* than *L. Plantarum*. To effectively prevent diarrhea in calves, it is essential to understand the phylogenetic grouping and genetic diversity of the bacterial causes. Additionally, probiotics can expedite the treatment of diarrhea.
Keywords: Calf, Diarrhea, ERIC, Phylogenetic Grouping, Probiotic

1. Introduction

Calf diarrhea in the livestock industry poses significant economic risks due to treatment costs, weight loss, stunted growth, and potential fatalities that can lead to irreparable financial losses [1]. Many infectious agents causing diarrhea in animals are zoonotic, highlighting the public health implications for domestic animals. Additionally, drug residues from antibiotic treatments used to control diarrhea in livestock pose a significant challenge [2]. Diarrhea in newborn calves is caused by both infectious and non-infectious agents. Key infectious agents include bacteria from the *Escherichia*, *Salmonella*, and *Clostridium* species, as well as *rotavirus* and *coronavirus*. Non-infectious factors are linked to animal feeding and environmental conditions [3]. *Escherichia Coli* (*E. Coli*) is a significant infectious agent due to its high prevalence. While it normally inhabits the intestines of warm-blooded animals, certain strains can cause enteritis in calves during their early weeks [4]. Enterotoxigenic strains enter the digestive system via surface fimbriae, binding to specific ganglioside receptors on small intestine enterocytes, leading to diarrhea by increasing water and solute secretion [5]. This issue is crucial for understanding how microorganisms spread through diarrheal excrement at the herd level, contaminating inanimate objects and exposing other calves to pathogenic strains [6].

Phylotyping analysis and phylogenetic grouping of new isolates help determine bacterial evolution and pathogenicity. Research on the evolution and genetic changes of *E. Coli* indicates that this bacterium has undergone slight genetic structural changes due to recombination, making it a valuable tool for studying intraspecies polymorphism [7]. Understanding the causes of diarrhea in different regions and breeding units is essential for developing prevention and treatment policies [6]. Enterobacterial Repetitive Intergenic Consensus (ERIC) is a rapid and effective method for identifying, classifying, and analyzing bacterial diversity. ERIC sequences are dispersed

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throughout the genomes of pathogenic bacteria and may significantly influence genomic organization. By mapping these sequences, researchers can infer the structure and evolutionary history of bacterial genomes. Additionally, analyzing intergenic repetitive sequences aids in epidemiological studies [8].

Probiotics, non-pathogenic living microorganisms, are prescribed to enhance the microbial balance in the digestive system [9]. Adding probiotics to water or animal feed can enhance animal weight by promoting growth-stimulating compounds and inhibiting pathogenic microorganisms [10]. Probiotics can be a suitable alternative to antibiotics and their problems, such as antibiotic resistance and remaining drugs in animal products [10]. *Lactobacillus Plantarum* (*L. Plantarum*) and *Lactobacillus Casei* (*L. Casei*) are considered the most important probiotics with therapeutic potential to prevent or treat infections [11].

L. casei and *L. plantarum* were selected in this study on calf diarrhea because they are natural members of the gut microbiota, exhibit well-known probiotic effects, can inhibit pathogenic bacteria, and contain genetic markers (such as the 16S rRNA gene) suitable for reliable phylogenetic analysis. Their evaluation helps clarify how the gut flora changes during diarrhea and how these beneficial bacteria are associated with disease conditions.

This study also aims to trace, identify, and phylogenetically classify *E. Coli* isolates from the feces of healthy and diarrheal calves at the Mashhad industrial slaughterhouse (2022). We employed New Clermont phylotyping and ERIC-PCR fingerprinting to better understand the infection's origin and to inform potential control measures. Additionally, the antibacterial and anti-inflammatory effects of probiotic lactobacilli on these isolates were assessed.

2. Materials and Methods

2.1. Materials

All bacterial and differential-biochemical culture media, glycerol, glacial acetic acid, and crystal violet were obtained from Merck, Germany. The DNA extraction kit was purchased from Cinaclon (EX6021C), and the Polymerase Chain Reaction (PCR) kit was purchased from Cinagen. The primers were designed by Sina Gene Company, Iran, and Mastermix (BIOFACT, South Korea). Research Ethics Committees of Islamic Azad University, Damghan Branch has approved the experimental protocols (IR.IAU.DAMGHAN.REC.1403.001).

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2.2. Sample collection and biochemical confirmation of isolated bacteria

This step was taken to isolate the *E. Coli* strain. Over eight months, 85 samples of healthy and diarrheal feces from 2- to 60-day-old calves were collected from the Mashhad slaughterhouse. All samples were cultured on MacConkey agar and EMB media for 24 hours at 37 °C. Pink colonies on MacConkey medium or those with a metallic sheen on EMB were phenotypically tested using the IMViC test (++--), confirming them as *E. Coli* isolates. The verified isolates were stored in microtubes with BHI medium and 10% glycerol at -70 °C for future molecular testing.

2.3. Phylogenetic studies

2.3.1. DNA extraction from isolates

After defreezing the microtubes containing the isolates, they were cultured on a Nutrient agar medium for 24 hours at 37 °C. Pure-grown colonies were transferred to microtubes containing 350 µl of sterile distilled water and placed in a hot block machine for 11 minutes at 98 °C. After 5 min, the microtubes were cooled in a -20 °C freezer, vortexed for 10 sec and then centrifuged (12000 rpm, 2 minutes). Finally, 250 µl of the DNA extract supernatant was transferred into new sterile microtubes for further studies [12].

2.3.2. PCR of isolates

Based on Farzin et al.'s study, primer sequences used in the developed quadruple phylotyping method for four genes *yjaA*, *chuA*, *arpA*, and *TspE4.C2* were designed as shown in Table 1 [7]. For the PCR, 10 µl of prepared master mix, 3 µl of bacterial DNA extracted from the previous step, and 20 picomoles of each of the primers (except for AceK.f (40 pmol), ArpA1.r (40 pmol), trpBA. f (12 pmol), and trpBA.r (12 pmol)) and the rest volume up to 20 µl of sterile distilled water was added. The PCR was performed using a thermocycler (Corbett research model, Australia) and according to Table 2. Then, the PCR products were electrophoresed on 1% agarose gel (Major Science, Taiwan), and the results were analyzed using Dock gel (Optigo ISOGENE, Netherlands). The standard strain 62ECOR was used as the positive control, and a sample without DNA was used as the negative control [13].

Table 1: Primer sequences used in the developed quadruple phylotyping method

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PCR reaction	Primer ID	Target	Primer sequence	PCR product (bp)
Quadruplex	chuA.1b	*chuA*	5-ATGGTACCGGACGAACCAAC-3	288
	chuA.2		5-TGCCGCCAGTACCAAAGACA-3	
	yjaA.1b	*yjaA*	5-CAAACGTGAAGTGTCAGGAG-3	211
	yjaA.2b		5-AATGCGTTCCTCAACCTGTG-3	
	TspE4C2.1b	*TspE4C2*	5-CACTATTCGTAAGGTCATCC-3	152
	TspE4C2.2b		5-AGTTTATCGCTGCGGGTCGC-3	
	AceK.f	*arpA*	5-AACGCTATTCGCCAGCTTGC-3	400
	ArpA1.r		5-TCTCCCCATACCGTACGCTA-3	
Group E	ArpAgpE.f	*arpA*	5-GATTCCATCTTGTCAAAATATGCC-3	301
	ArpAgpE.r		5-GAAAAGAAAAAGAATTCCCAAGAG-3	
Group C	trpAgpC.1	*trpA*	5-AGTTTTATGCCCAGTGCGAG-3	219
	trpAgpC.2		5-TCTGCGCCGGTCACGCCC-3	
Internal control	trpBA.f	*trpA*	5-CGGCGATAAAGACATCTTCAC-3	489
	trpBA.r		5-GCAACGCGGCCTGGCGGAAG-3	

Table 2: Steps, schedule, and number of PCR cycles

Steps	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	94	4 min	1
Denaturation	94	5 sec	30
Annealing for E group	57	20 sec	30
Annealing for C and Quadruplex groups	59	20 sec	30
Extension	72	60 sec	30
Final Extension	72	5 min	1

2.3.3. Phylogenetic grouping

According to the results obtained from the PCR stage and Table 3 [14], the strains were phylogenetically classified into A, B1, B2, C, D, E, and F groups. Due to the similarity of the pattern of group A with C and also D with E, in the case of the isolates that were identified as group A or D, a specific PCR for C and E groups was performed, and if the reaction was negative, they were classified as group A or D.

Table 3: Interpretation of developed quadruple phylotyping (14)

GROUPS	TSPE4.C2 (152BP)	YJAA (211BP)	CHUA (288BP)	ARPA (400BP)
A	-	-	-	+
B1	+	-	-	+
F	-	-	+	-
B2	-	+	+	-
B2	+	+	+	-
B2	+	-	+	-
A/C	-	+	-	+
D/E	-	-	+	+
D/E	+	-	+	+

2.4. Determination of genetic diversity of isolates

The ERIC-PCR method was used to determine intergenic repetitive sequences [15]. *E. Coli* DNA was isolated from diarrheal samples (36 samples) using a DNA extraction kit and according to the manufacturer's instructions (Cinagen Company, Iran), which are briefly as follows: After adding

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400 µl of lysis buffer to each microtube containing the precipitate of freshly cultured bacteria, were microfuged for 2 minutes at 12000 rpm. Then, the supernatants were transferred to new microtubes, and 300 µl of sedimentation solution was added to each tube. After 20 minutes, they were microfuged again (5 min, 10000 rpm) to obtain sediment. Next, 50 µl of Tris-EDTA buffer was added to each microtube for the PCR. According to Table 4, ERIC-PCR was performed using a device (Corbett research thermocycler, Australia) in a volume of 20 µl containing 10 µl of ready master mix (master mix 2X of the Danish Ampliqon brand, including dNTP nucleotide mixture, $MgCl_2$, Taq polymerase enzyme, buffer), 1 µl of primer according on Table 5, 5 µl of DNA extract related to the isolates, and remain volume was adjusted to 4 µl with distilled water. The PCR product was electrophoresed on 1% agarose gel (Major Science, Taiwan), and the gel obtained by UV light was photographed by documentation gel (Uvi Pro-Uvi Tec, England) to obtain the genetic classification of isolates.

Table 4: Steps, schedule, and number of PCR cycles

Steps	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	94	6 min	1
Denaturation	94	30 sec	30
Annealing	52	35 sec	30
Extension	72	60 sec	30
Final Extension	72	5 min	1

Table 5: Primer sequences used in the ERIC-PCR method

Method	Sequence
ERIC1	5-ATGTAAGCTCCTGGGGATTCAC-3
ERIC2	5-AAGTAAGTGACTGGGGTGAGCG-3

2.4.1. Drawing phylogenetic tree

A phylogenetic tree is a diagram that illustrates how microorganisms have originated from a common ancestor and diverged over time into different evolutionary lineages. Drawing a phylogenetic tree is very useful for classifying and understanding biodiversity. The ERIC-PCR gel electrophoresis images were analyzed, and the phylogenetic tree was drawn using the NTSYS software (version 2.02e). For this purpose, gel electrophoresis results were coded as zero or one in a matrix (the presence or absence of bands). Then, the matrix was analyzed with NTSYS software, and the dendrogram was drawn using the Jaccard and Dice similarity coefficient. The UPGMA algorithm (Unweighted Pair Group Method using arithmetic Averages) was used based on the

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similarity coefficient with the highest Cophentic correlation coefficient. The effectiveness of the ERIC-PCR method was also determined using Simpon's Diversity Index (Eq. (1)).

Eq. (1): $$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j \left(n_{j-1}\right)$$

D: Simpon's Diversity Index, N: total number of strains in the ERIC-PCR, S: number of calculated genetic types, and nj: the number of type j strains.

2.5. Probiotics antibacterial studies

The strains of *L. Casei* (ATCC 393) and *L. Plantarum* (ATCC 700211) were used for probiotics antibacterial studies. The *E. Coli* strains were studied included in 3 groups: Pathogenic *E. Coli* strains (P-EC), the isolated strains from diarrheal samples Non-pathogenic *E. Coli* strains (NP-EC), the isolated strains from non-diarrheal samples, and *E. Coli* strains (ATCC10536) (ST-EC) as a standard control in all tests.

2.5.1. Bilayer culture

The bilayer culture method is used to isolate and accurately count specific pathogenic bacteria in calf diarrhea samples. By placing the sample between two layers of agar, bacterial growth becomes more uniform and controlled, allowing clear colony formation and easier identification of organisms such as *E. coli* or *Salmonella*.

First, *L. Plantarum* and *L. Casei* were inoculated in the center of the *Man–Rogosa–Sharpe* agar (MRS agar) plate and incubated for 24 hours at 37 °C. Then Moller Hinton's agar medium (MHA) was poured on MRS agar, and after coagulation, the suspensions (0.5 McFarland) containing *E. Coli* strains were thickly cultured and were again incubated (24 h, 37 °C) to measure an aura of no-growth [11].

2.5.2. Well Diffusion Method

The well diffusion method is used to evaluate the antibacterial activity of antibiotics or other antimicrobial agents against bacteria isolated from calf diarrhea. By placing the drug in wells on an inoculated agar plate, the inhibition zones around the wells show how effective each antibiotic is, helping determine the best treatment option.

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The suspensions containing *E. Coli* strains (0.5 McFarland) were thickly cultured on MHA medium. Then, wells (6 mm) were drilled on the MHA, and 30 µl of the supernatant of probiotics of *L. Plantarum* and *L. Casei* were inoculated into the wells and incubated for 24 h at 37 °C to measure an aura of no-growth [11].

2.5.3. The anti-adhesion effect of probiotics supernatant

To prepare the probiotics supernatant, *L. Casei* and *L. Plantarum* were cultured in two MRS broth mediums and were incubated for 24 hours at 37 °C. Then, the MRS mediums were centrifuged (4 °C, 5 min, 10000 RPM) and passed through a filter (0.22 µm) to eliminate microbial load. Next, 75 µl of probiotic supernatants and 75 µl suspensions of *E. Coli* strains (0.5 McFarland) were added to the wells of a 96-well microplate and incubated at 37 °C (triplicate). After 24 h, the wells were washed with sterile phosphate buffer (×3) and fixed with 96% ethanol for 15 min. The wells were dried and stained with 200 µl of 2% crystal violet for 10 min. After wells washing, 200 µl of 33% acetic acid as a solvent was added to each well, and their light absorption was read with a plate reader (BIOTEK, USA) at 490 nm. In the end, the adhesion reduction percentage was calculated by the Eq. (2) [16].

Eq. (2): $Adhesion\ reduction\ percentage = \frac{ODa-ODb}{ODa} \times 100$

ODa: the optical absorbance of the control well, and ODb: the optical absorbance of the tested well.

2.5.4. Antagonistic effect of probiotics

The antagonistic effect of probiotics on *E. Coli* strains was assessed by coaggregation. First, *Lactobacilli* were cultured in MRS broth medium and incubated at 37 °C for 24 h. After centrifugation (5 min, 10000 RPM, 20 °C) and washing by sterile phosphate buffer (×3), the probiotic suspensions (0.5 McFarland) were prepared. 500 µl of probiotic suspension with 500 µl of *E. Coli* strains were mixed and incubated for 24 h at 37 °C (triplicate). After again centrifugation of tubes (5 min, 1600 RPM, 20 °C), the optical absorbance of the supernatant was read using a UV/Vis spectrophotometer equipment (Jenwey 6305, England) at a wavelength of 660 nm in two steps immediately and after 4 hours culture and the amount of accumulation percentage was calculated based on Eq. (3) [16].

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203 $$\text{Eq. (3): Accumulation percentage} = \frac{A_1 - A_2}{A_1} \times 100$$

204 A1: the amount of light absorption immediately after mixing the probiotics, A2: the amount of
205 light absorption of the supernatant after 4 hours.

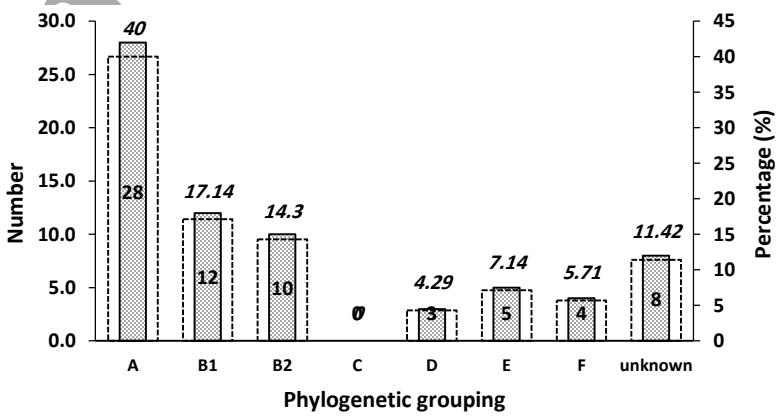
206 **2.6. Data analysis**

207 The data was analyzed using SPSS version 19 software. Central and dispersion indices were used
208 to describe quantitative variables, and frequency tables were used to describe qualitative variables.
209 Chi-square statistical tests and Fisher's exact test were used for data analysis to compare groups.
210 Also, *p*-value <0.05 was considered.

211 **3. Results**

212 **3.1. Isolation and phylogenetic grouping**

213 The results of differential and biochemical cultures of 85 samples from calves' excrement showed
214 that 70 samples (82.35%) had *E. Coli* strains. 36 isolates of *E. Coli* (51.4%) were separated from
215 diarrheal samples, and 34 isolates (48.6%) were separated from non-diarrheal samples.
216 Based on the PCR results of 70 *E. Coli* strains, the phylogenetic grouping was classified as
217 A(40%), B1(17.14%), B2(14.3%), E(7.14%), F(5.71%), D(4.29%), C(0%), and
218 unknown(11.42%). The highest frequency was related to group A (28 isolates), and then B1 (12
219 isolates), and the lowest frequency belonged to group C (zero), as shown in Figure 1.
220



221
222 **Figure 1:** Number and percentage phylogenetic grouping of *E. Coli* strains isolated from calves' excrement
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The results of ERIC-PCR from diarrhea samples which were done to investigate genetic diversity were consisted 3 main clusters including subclusters: G1(2 isolates), G2(4 isolates), G3(6 isolates), G4(3 isolates), G5(18 isolates), G6(3 isolates). The analysis of the shape and dendrogram of ERIC-PCR of diarrhea-causing *E. Coli* serotypes are shown in Figure 2. By examining the different clusters obtained, it was observed that the samples were primarily gathered in cluster 3.

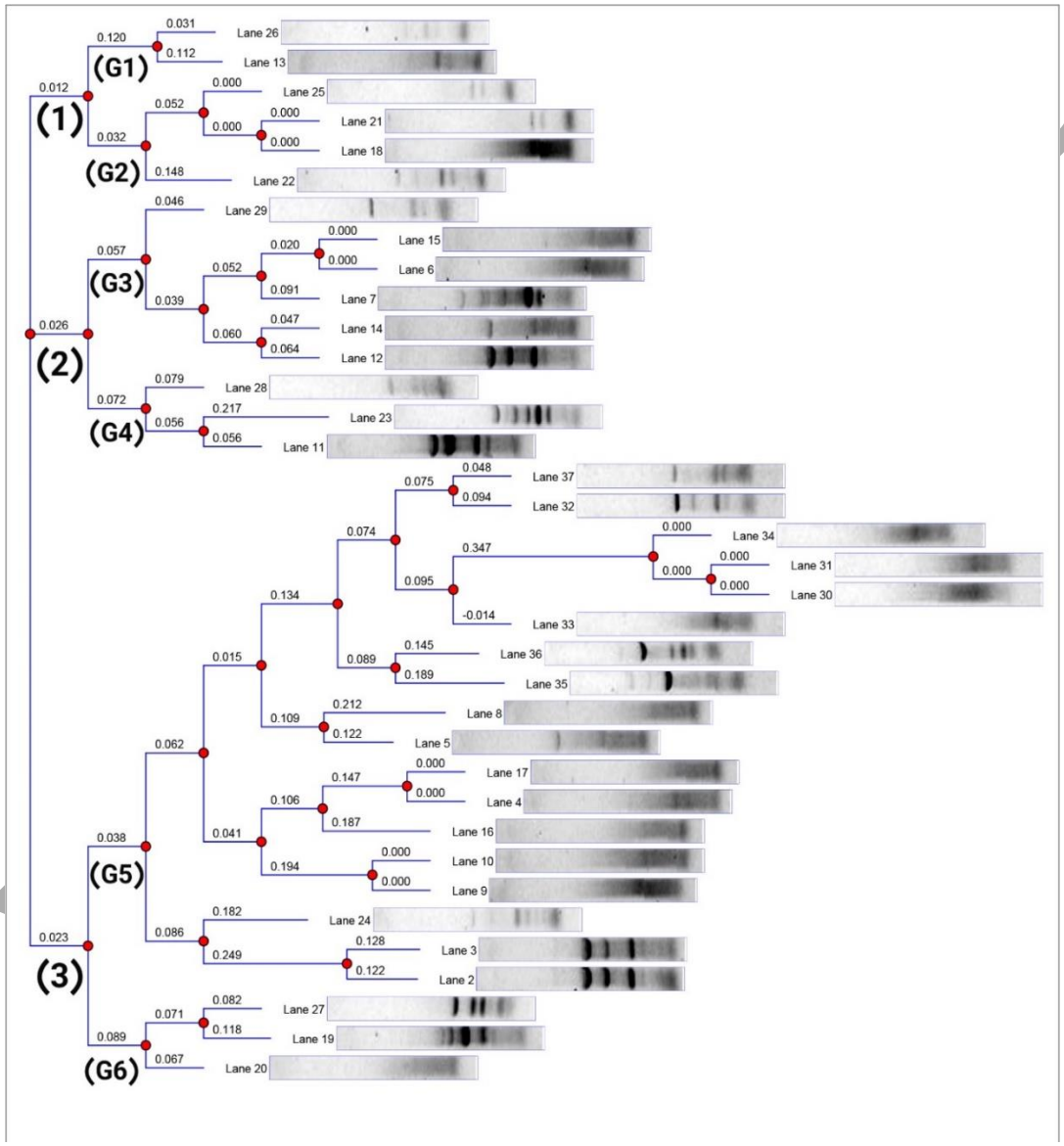

Figure 2: The results of ERIC-PCR and dendrogram of *E. Coli* serotypes causing diarrhea

3.2. Probiotics antibacterial studies

3.2.1. Bilayer cultivation

Figure 3 shows the results of the modified bilayer culture. The average diameters of the aura of no-growth by *L. Casei* were 38.2, 34.3, and 34 mm for P-EC, NP-EC, and ST-EC strains, respectively. The average diameters of the aura of no-growth by *L. Plantarum* were 29.3, 26.9, and 23 mm for P-EC, NP-EC, and ST-EC strains, respectively. The results showed that *L. Casei* probiotic had a more significant inhibitory effect than *L. Plantarum* on three pathogenic, non-pathogenic, and control groups. The results showed a significant difference between the inhibitory effect of *L. Casei* and *L. Plantarum* on the growth of pathogenic *E. Coli* strain than standard *E. Coli* strain.

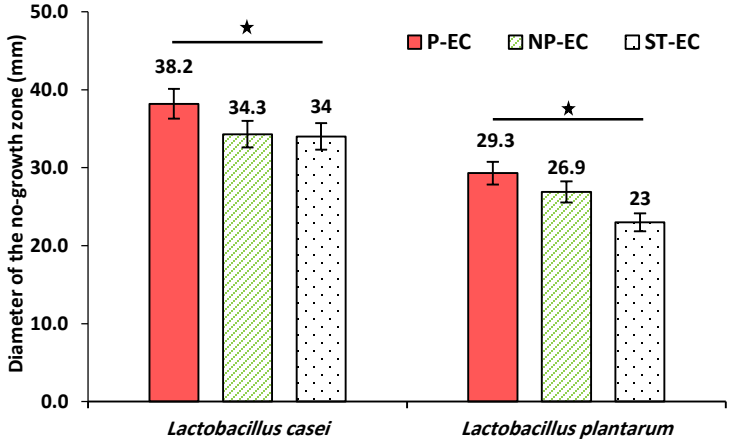

Figure 3: The average diameter of the aura of no-growth (mm) of *Lactobacillus Casei* and *Lactobacillus Plantarum* on the Pathogenic (P-EC), Non-pathogenic (NP-EC), Standard *E. Coli* (ST-EC) in the two-layer culture test. *: $p<0.05$

3.2.2. Well diffusion method

Figure 4 shows the results of the inhibitory activity of the supernatant of probiotics on the *E. Coli* strains. Although there was no significant difference between the results, the average diameter of the aura of no-growth by *L. Casei* was more than 18 mm for P-EC than for the ST-EC strain (as a control), which was 15.5 mm. Also, these numbers were more (14.8 and 12.5 mm) than the ST-EC strain, which was 11.7 mm in the case of *L. Plantarum*.

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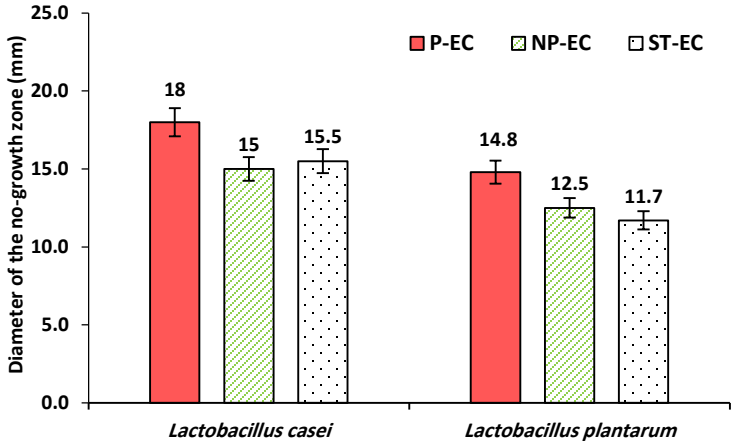

Figure 4: The average diameter of the aura of no-growth (mm) of *Lactobacillus Casei* and *Lactobacillus Plantarum* on the Pathogenic (P-EC), Non-pathogenic (NP-EC), Standard E. Coli (ST-EC) in the Well Diffusion method.

3.2.3. Anti-adhesion effect of probiotics supernatant

Figure 5 shows anti-binding effect of probiotic *lactobacilli* supernatant. The most impact was for *L. Casei* supernatant about pathogenic *E. Coli* isolates (P-EC: 67.5%) with a significant difference compared to the control group. Also, there was a significant difference in the effect of *L. Plantarum* supernatant on the pathogenic *E. Coli* isolates (P-EC: 25.2%). The results show that the Anti-adhesion effect of probiotics supernatant for non-pathogenic *E. Coli* isolates (NP-EC) was less than the control strain: 40% for *L. Casei* supernatant and 12.6% for *L. Plantarum.*

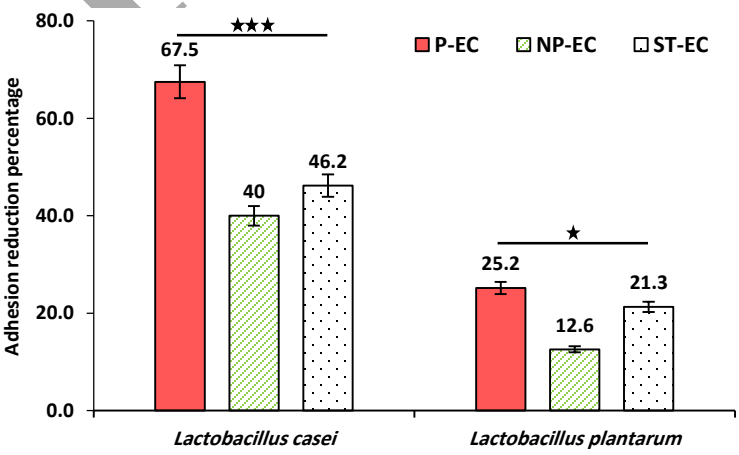

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Figure 5: The Anti-adhesion effect (%) of *Lactobacillus Casei* and *Lactobacillus Plantarum* on the Pathogenic (P-EC), Non-pathogenic (NP-EC), Standard E. Coli (ST-EC), *: p<0.05, ***: p<0.001

3.2.4. Antagonistic effect in coaggregation method

The results of the *L. Casei* and *L. Plantarum* aggregation method on the E. Coli strains showed that both probiotics distinctly could accumulate bacteria. There was a significant difference in the effects of *L. Casei* on the pathogenic *E. Coli* isolates (P-EC: 62.3%) compared to the standard strain. There was also a significant difference in the accumulation effect of *L. Plantarum*, which was 58.6%. These effects of probiotics were not significant in the case of NP-EC strains (See Figure 6).

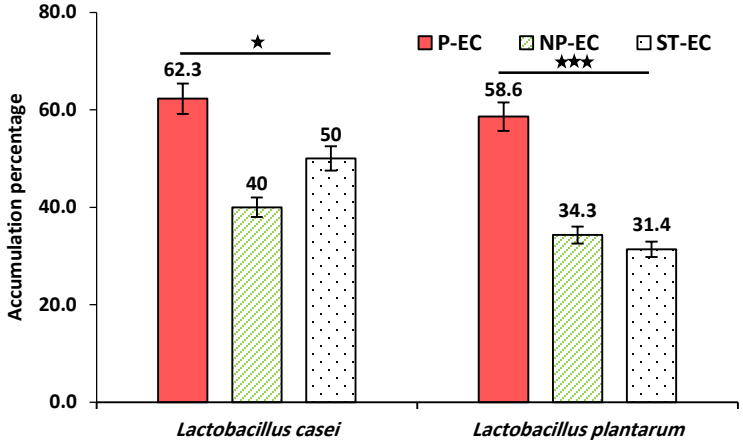

Figure 6: Accumulation percentage of *Lactobacillus Casei* and *Lactobacillus Plantarum* on the Pathogenic (P-EC), Non-pathogenic (NP-EC), Standard *E. Coli* (ST-EC) by Coaggregation method, *: p<0.05, ***: p<0.001

4. Discussion

Diarrhea in calves can lead to significant economic losses in animal husbandry due to treatment costs and potential fatalities. Since *E. Coli* is part of the natural flora in the intestines of warm-blooded animals, calves are particularly susceptible to diarrhea caused by this bacterium, making it crucial to address this issue [13, 17]. Samples of excrement from 85 calves at the Mashhad industrial slaughterhouse revealed that 70 (82.35%) tested positive for *E. Coli*. In contrast, a study by Fanedian et al. found a prevalence rate of 96% of verotoxigenic strains of *E. Coli* in cow feces from cattle farms near Shahrekord [18]. Despite the results of our study, in Moradi et al.'s study, the second cause of diarrhea in calves' feces was *E. Coli* bacteria, with a prevalence of 24.39%

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[19]. Also, in another study (2019), *E. Coli* was ranked third after Listeria monocytogenes and *Salmonella* enterica with a prevalence of 23% [20].

E. Coli bacteria significantly contribute to economic losses on cattle farms beyond causing diarrhea. A key measure for controlling *E. Coli* is identifying and phylogenetically classifying isolates from cattle samples using the Claremont method. Phylogenetic analysis of fecal samples from healthy and diarrheal calves revealed that 40% of the isolates were classified in group A, followed by 17.14% in group B1. A similar study in three regions of Mexico using the Claremont method found that 82.9% of *E. Coli* isolates belonged to phylogroups A and B1 [17]. Also, in another study in Tanzania, most *E. Coli* isolates belonged to B1 phylogroups [21]. The phylogenetic analysis in Murcati et al.'s (2019) study on *E. Coli* from non-diarrheic and diarrheal water buffalo calves revealed that 58.95% of the isolates belonged to phylogroup B1 [22]. In the Euzin study in Romania, the most frequently isolated *E. Coli* belonged to phylogenetic group A, followed by group B2 and then B1 [23]. While studies have highlighted the pathogenicity of groups B2 and D [24], particularly B2's association with intestinal diseases [14], , our analysis reveals that phylogroups A and B1 dominated in the samples examined. Calves with diarrhea serve as significant reservoirs for spreading these strains, although the difference in *E. Coli* isolation rates between diarrheal (51.4%) and non-diarrheal (48.6%) samples lacks statistical significance. Nonetheless, non-diarrheal excrement may contribute to a lower dissemination of these strains and reduce contagion among calves. Phylogroups A and B1 are more closely associated with commensal strains [14]. Analysis of diarrheal and non-diarrheal samples, compared with other studies, indicates that the prevalence rate and distribution of various phylogroups are influenced by geographical location and sampling time. Additionally, factors such as the host's health, nutrition, antibiotic use, and genetic characteristics also impact this grouping [24].

The ERIC-PCR method was employed for the genetic classification of 36 *E. Coli* isolates from diarrheal samples. After confirming the presence of amplified fragments in the PCR, this method effectively distinguished the isolates, classifying them into three clusters and indicating the cluster with the most pathogenic samples. A high sequence similarity among the bacterial isolates typically shows they share a common ancestor, reflecting their phylogenetic relationship [8]. ERIC-PCR results indicated that the screened samples exhibited significant genetic diversity, with only minor differences among isolates from different species.

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Probiotic antibacterial studies revealed that *L. Casei* and *L. Plantarum*, along with their supernatants, significantly inhibited pathogenic *E. Coli* strains. *L. Casei* demonstrated a stronger antibacterial effect and growth inhibition on *E. Coli* isolates compared to *L. Plantarum*. These effects were more pronounced in pathogenic strains than in non-pathogenic ones, consistent with findings from other studies. Antibacterial studies of probiotics demonstrated that *L. Casei* and *L. Plantarum*, along with their supernatants, significantly inhibited pathogenic *E. Coli* strains. *L. Casei* exhibited stronger antibacterial effects and growth inhibition against *E. Coli* isolates than *L. Plantarum*. These effects were more pronounced in pathogenic strains compared to non-pathogenic ones, aligning with findings from previous studies [25]. Growth inhibition occurs through various mechanisms against diseases caused by pathogenic *E. Coli*, with one approach involving the ability to bind specifically and non-specifically to the surfaces of target epithelial cells, thus competing with pathogenic microorganisms [11].

The new Claremont technique allows for more precise and sensitive separation of *E. Coli* isolates. Given the high prevalence of *E. Coli* in this study, prompt identification and treatment of diarrheal calves or detection of commensal strains is crucial for preventing disease-related damage. Analyzing ERIC PCR method results can enhance routine monitoring of *E. Coli* in cattle farm diarrheal samples. Antibacterial studies of *L. Plantarum* and *Cazei* probiotics demonstrated their ability to inhibit and accumulate with pathogenic *E. Coli* in laboratory conditions. Thus, using probiotics during illness, such as calf diarrhea, may improve treatment outcomes and reduce antibiotic use.

This study highlights the role of *L. casei* and *L. plantarum* as beneficial members of the gut microbiota with probiotic potential in calves. The bilayer culture and well diffusion methods allowed for accurate isolation and assessment of pathogenic bacteria, including *E. coli* and *Salmonella*. Phylogenetic analysis using updated Clermont classification provided detailed and sensitive grouping of *E. coli* isolates, contributing to a better understanding of microbial dynamics during diarrhea.

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Authors' Contribution

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Ethics:

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Conflict of Interest

The authors of this study have no financial interests or personal relationships that could potentially influence the results. The study was not funded by any company or for profit organization, and the authors maintain full independence in their research and conclusions.

Data Availability

The data that underpin the findings of this study are available upon request from the corresponding author.

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