Original Article

Isolation and Characterization of Staphylococcus aureus from Raw Cow's Milk and Investigating the Effect of Bifidobacterium bifidum Probiotic Cell Free Supernatant on Their Enterotoxins Genes Expression

Jalaliani, H[1], Anvar, SAA[1*], Amini, K[2], Karim, G[3]

1. Department of Food Hygiene, Science and Research Branch, Islamic Azad University, Tehran, Iran
2. Department of Microbiology, Faculty of Basic Science, Islamic Azad University, Saveh Branch, Saveh, Iran
3. Department of Food Hygiene, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

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Corresponding Author's E-Mail:
Saaa4824@gmail.com

ABSTRACT

The present reserach aimed to detect and isolate the genes involved in the staphylococcal enterotoxins (SEs) production in strains isolated from unprocessed cow's milk and to examine the impact of *Bifidobacterium bifidum* probiotic cell-free supernatant (CFS) on their expression. Standard techniques were used for isolation and identification of Staphylococci strains in unprocessed milk. The PCR was used to identify strains carrying enterotoxin genes. The *B. bifidum* CFS was applied to strains containing the target genes, and the genes expression levels were quantified using Real-time PCR. Using 16SrDNA sequencing, the phylogenic relationship of the isolated strains was determined. Analysis revealed that bacteria such as Staphylococcus species were found in the 72% of the samples. The PCR test showed the presence of various SE superantigens, including *SEA* (16.7%), *SEC* (11.7%), *SED* (8.3%), *SEE* (6.7%), and *SEB* (1.7%) in isolated strains. The *B. bifidum* CFS had obvious antimicrobial activity against strains 24, 51, 54, and 35 of Staphylococcus species, and the minimum inhibitory concentration and minimum bactericidal concentration values for these strains treated with *B. bifidum* CFS were in the range of 31.25-125 µg/ml. Strains 51 and 24 were clustered with *S.aureus* ATCC 25923, and strains 54 and 35 were clustered with *S.aureus* ATCC 12600, respectively. The RT-PCR exhibited that probiotics CFS suppressed the expression of SEA, SEB, SEC, and SEE genes ($P<0.05$). The average fold change for *SEA*, *SEB*, *SEC*, and *SED* genes was -1.681, -1.28, -1.52, and -0.84, respectively. The research demonstrated that probiotic bacteria can lower enterotoxin production by downregulating the expression of SEs genes.

Keywords: *Bifidobacterium bifidum*, Enterotoxin, *Staphylococcus aureus*

1. Introduction

Bovine mastitis, an inflammatory condition, arises from the confluence of factors pertaining to the animal, its environment, and the pathogenic agent involved. Clinical mastitis can be identified through the observation of symptoms such as inflammation, redness, warmth, discomfort, and the presence of unusual milk (such as clumps, color changes, or flakes) in one or more sections of the udder. Conversely, subclinical mastitis does not exhibit any discernible indication (1, 2). Subclinical mastitis, caused by the common pathogen *S. aureus*, can lead to significant financial losses as it reduces milk quality and production. During milking, the pathogen can spread among animals, and a collaborative effort is necessary to prevent its transmission to healthy cows. The *S. aureus* from bovine sources can also cause foodborne diseases in humans through bulk tanks and raw milk products (3) (4).

This microorganism produces various pathogenic factors, including enzymes, cytotoxins, exotoxins, and exfoliative toxins. The main role of these toxins and enzymes is to convert host components into nutrients that can be used for bacterial growth. Exotoxins, such as staphylococcal enterotoxins (SEs), are among the factors secreted by the pathogen and are the subject of this investigation. These substances disrupt the host's immune defenses and trigger significant reactions (5) (6).

Staphylococcal enterotoxins are part of a family of over twenty related exotoxins secreted by staphylococcal and streptococcal bacteria. These toxins share similar sequences and functions and are known to cause fever, toxic shock syndrome, and food poisoning. Although *S. aureus* is the main producer of these toxins, other species have also been found to produce enterotoxins (6-8).

The SEA and SEB are the most studied and reported SEs. The genes that code for these toxins are typically found on mobile genetic elements, such as plasmids, pathogenicity islands, bacteriophages, or transposons. These agents are the most frequently found toxins in cases of staphylococcus-related food poisoning (9). The SED is the second most prevalent toxin linked to staphylococcal food poisoning globally. Research indicates that even minimal quantities of this toxin can result in disease (10).

Antimicrobial resistance is a growing problem in both the healthcare and agricultural industries. This phenomenon occurs when microorganisms such as bacteria and fungi develop the ability to resist the effects of antimicrobial treatments. As a result, infections become more difficult to treat, and the number of effective treatment options decreases (11), presenting a challenge to farmers in the dairy industry and posing a risk to public health. To combat this issue, there has been growing interest in using natural antimicrobials such as probiotics, nanoparticles, and herbal sources (12). Probiotics, including Bifidobacteria, are helpful bacteria that naturally reside in the intestines and stomachs of mammals. They assist the human body in carrying out crucial functions like digestion and fighting off pathogenic microorganisms. Investigations have shown that probiotics can decrease the number of antibiotic-resistance genes and affect the pathogenesis gene expression in the gut microbiota of individuals susceptible to colonization who have not been exposed to antibiotics (13). Decrease in abovementioned genes indicates that probiotics may be a helpful tool in reducing the spread of antibiotic-resistant pathogens within the dairy industry (14).

Current study aimed to identify and isolate the genes responsible for producing SEs in fresh and unprocessed cow's milk and investigate the effect of *Bifidobacterium bifidum* probiotic cell-free supernatant (CFS) on their mRNA expression levels. This investigatation will contribute to our understanding of the pathogenesis of *S. aureus* and the potential use of probiotics as a natural alternative to antibiotics in the dairy industry. Ultimately, this study may lead to the development of new strategies for reducing the risk of foodborne

illnesses caused by *S. aureus*.

2. Materials and Methods

2.1. Sampling and Staphylococcus Identification

In March 2023, 100 fresh cow milk samples were collected over one week. During each collection, 200 mL of milk was aseptically obtained and kept at 4℃. Milk samples (n=5) were collected from the bulk tanks of industrial companies in Iran with the highest dairy product production: Pegah, Kale, Pak, Damdaran, and Mihan. The remaining 95 samples were randomly selected from five groups of 19 farms in different regions of Tehran province, Iran, with each group being covered by one of the industrial companies' collection centers. Milk storage tanks were tested in compliance with Iran's national standard number 326. Gram staining and conventional biochemical techniques were utilized to detect bacteria in collected samples. Then, 16srDNA sequence analysis was performed using universal primers (8F and 1391R) to verify the differential diagnosis of specific Staphylococcus species, as previously described (15). The identified isolates were preserved in Brain Heart Infusion Broth (BHI, HiMedia) with 18% glycerol at -70°C.

2.2. Multiplex PCR

A multiplex PCR was used to identify bacteria harboring SE genes. DNA was extracted using a QIAamp kit according to the manufacturer's instructions. The quality of extracted DNA was verified using a NanoDrop™ 2000/2000c Spectrophotometer (Thermofisher). Specific primers for Staphylococcal enterotoxin genes were designed using Oligo7 software, and their specificity was confirmed by blasting on the National Institutes of Health (NIH) website (Table 1). The PCR reaction condition included an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C denaturation for 30 sec and primer binding at 56°C for 30 sec. The propagation step was carried out at 72°C for 1 min, and after 35 cycles, a final propagation step was performed at 72°C for 10 min. The PCR products were subjected to electrophoresis in a 1.5% agarose gel with positive and negative controls. The gel was stained with erythrogel and photographed using a gel documentation device.

2.3. Determination of Probiotic Cell-free Supernatant Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

To determine the impact of probiotics secondary metabolites on the expression levels of staphylococcal enterotoxin genes, strains carrying target genes were exposed to CFS from probiotic bifidobacteria. *Bifidobacterium bifidum* BGN4 was obtained from the

Table 1. Primers used to isolate *Staphylococcus aureus* strains harboring entrotoxin genes

Primer	Sequence (5´-3´)	Size (bp) PCR
SEA	F5′-AAAATACAGTACCTTTGGAAACGGTT R5′-TTTCCTGTAAATAACGTCTTGCTTGA	92
SEB	F: 5′-ACACCCAACGTTTTAGCAGAGAG R5′-CCATCAAACCAGTGAATTTACTCG	81
SEC	F5′-AATAAAACGGTTGATTCTAAAAGTGTGAA R5′-ATCAAAATCGGATTAACATTATCCATTC	541
SED	F5′-TGATTCTTCTGATGGGTCTAAAGTCTC R5′-GAAGGTGCTCTGTGGATAATGTTTT	306
SEE	F5′-TGATTCTTCTGATGGGTCTAAAGTCTC R5′-GAAGGTGCTCTGTGGATAATGTTTT	209
rpoB	F5′-TTCTTACCGATCTATGCATG' R5´ CCTTACATGCGTTAAAACCA	258

Pasteur Institute in Tehran, Iran, and its CFS was prepared according to previous studies (16, 17). The *B. bifidum* BGN4 was cultured in MRS broth for 24 h at 37°C. Cell free supenatant was obtained by centrifuging the culture at 3000 g for 15 min at 4°C. The supernatant's pH was adjusted to 6.5 using 1 M NaOH, and the bacteria were removed by filtration through a 0.22 µm Minimart filter (Germany).

The minimum inhibitory concentration (MIC) of each bacterium exposed to the CFS of probiotic was determined according to the standard method recommended by the National Committee for Clinical Laboratory Standards. MIC determination involved using two 96-well microplates, each containing 100 µl of tryptic soy broth. In the first step, 100 µl of serially diluted probiotics CFS (56 to 109 gr/ml) were added to each well. The microplates were then incubated in a shaker incubator at 200 rpm and 37°C for up to 24 h and placed at 37°C for 18-24 h. The turbidity of each well was then checked. The lowest concentration of CFS that prevented visible growth of a microorganism was considered the MIC.

To determine the minimum bactericidal concentration (MBC), 100 µL of a portion of the broth was taken from each well, and transferred to BHI medium, and incubated at 37°C overnight. Twenty mL of cultures with no turbidity were inoculated on BHI agar and incubated at 37°C for 24 h. The lowest concentration at which the incubated microorganisms were completely killed (based on the absence of colonies on the plate) was defined as MBC. Gentamicin was used as a positive antimicrobial control.

2.4. Real-time PCR

The expression levels of SE and rpoB genes were measured by quantitative real-time PCR (qRT-PCR). The total bacterial RNA of the isolates in sub-MIC wells was extracted using an RNeasy Mini Kit (QIAamp) and quantified through spectrometry (NanoDrop, Thermo Scientific, USA).

The relative expression of SE genes was calculated using the rpoB housekeeping gene. The threshold cycle numbers were confirmed by the detection system software, and data were analyzed using the ΔΔCt method. The expression levels of target genes were determined and compared. Each reaction was performed in triplicate, and statistical analysis was carried out using SPSS (version 16). Correlations were calculated and means were compared using a t-test with a significance level of $P < 0.05$.

The cDNA was synthesized using a Prime Script RT Reagent Kit (CinnaGen, Iran) and qRT-PCR was performed using an SYBR Premix Ex Taq II Kit (CinnaGen, Iran) on a thermocycler system (StepOneplus, Thermo Fisher Scientific, Germany) with an initial incubation at 94°C for two min seconds, followed by 40 cycles of 15s at 94°C and 1 min at 62°C. The relative expression of staphylococcal entrotoxin genes was determined using the rpoB housekeeping gene. The threshold cycle numbers were confirmed by the detection system software, and the fold change of target gene expressions were determined using ΔΔCT method. Each reaction was performed in triplicate, and statistical analysis was performed using SPSS (version 16). Correlations were calculated, and means were compared using a t-test with a significance level of $P < 0.05$.

2.5. Molecular Identification

The genomic DNA of the strains was extracted from an overnight bacterial culture. The conserved region of the 16S rDNA gene sequence was amplified using PCR with universal primers pair 27FYM and 1492R. The PCR conditions were initial denaturation at 95°C for 4 min, followed by 30 cycles of 95°C for 60 sec, 56°C for 60 sec, and 71°C for 65 sec. The final step was conducted at 72°C for 6 min. The amplified fragments were purified and sent to Macrogen Company in South Korea for sequencing. The sequences were compared to those in the GenBank database using the BLAST application (Zhang et al., 2022). Isolates with over 95% similarity to the reference were classified as the same species. A phylogenetic tree was constructed

using MEGA7 software, the Kimura 2-parameter model, and the neighbor-joining method. The *S. aureus* ATCC 25923 and *S. aureus* ATCC 1260 were used as reference strains, while *Bifidobacterium longum* 1714 was used as an outgroup to construct the phylogenetic tree.

3. Results

3.1. Isolated Bacteria

Analysis of 100 raw milk samples revealed that 28 (28%) were sterile, while bacteria such as Staphylococcus species (n=60), *Escherichia coli* (n=5), and *lactococcus* (n=2) were found in the remaining 72 (72%) samples. This study focused on the isolation of staphylococci species; therefore, samples containing other strains were not included.

3.2. Multiplex PCR

The analysis of milk sample bacteria revealed the existence of various SE superantigens in 27 (45%) strains. Specifically, SEA superantigen was found in 10 bacteria, SEC superantigen in 7 bacteria, SED in 5 bacteria, SEE in 4 bacteria, and SEB superantigen in 1 isolate. Two isolates carried both SEC and SEA superantigens, and one of the isolates carried both SEE and SEA superantigens (Figure 1).

3.3. Antimicrobial Activity of Bifidobacteria Cell-free Supernatant (CFS)

In this step, the antimicrobial activity of the probiotic CFS was examined against isolated strains. Findings showed that out of 60 isolated carrying target genes, bifidobacteria CFS showed antimicrobial activity against strains 24, 51, 54, and 35. The MIC obtained for Staphylococcus species treated with *B. bifidum* CFS in isolates 24, 51, 54, and 35 were 125 µg/ml, 62.5 µg/ml, 62.5 µg/ml, and 125 µg/ml, respectively. The MBC results obtained for Staphylococcus species treated with probiotic cell free supernatant in isolates 24, 51, 54, and 35 were 62.5 µg/ml, 31.25 µg/ml, 31.25 µg/ml, and 62.5 µg/ml, respectively.

3.4. Real-time PCR

We studied how probiotic bacteria's CFS affects gene

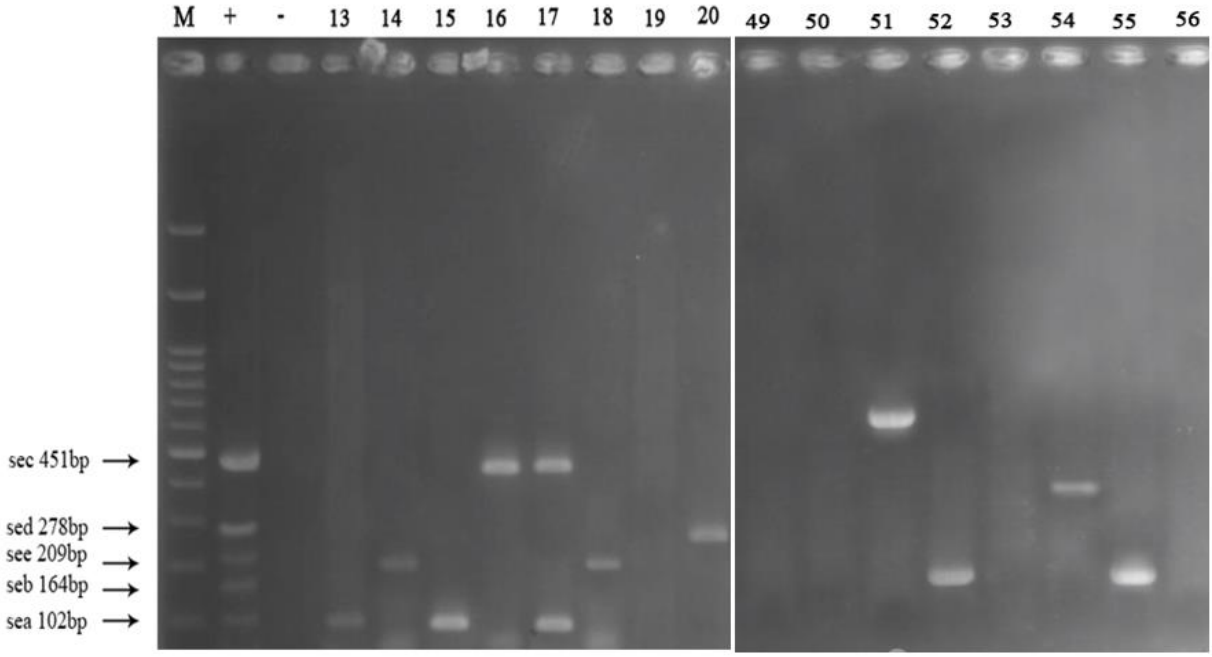

Figure 1. Agarose gel electrophoresis of PCR products of random samples. The M Lane:100bp ladder, +: positive control, -: negative control

expression to understand its antimicrobial activity and suppression of SE production. Using RT-PCR, we analyzed the gene encoding for SEs production after 24 h of exposure to sub-MIC concentrations of probiotics CFS. Our results showed that probiotics CFS suppressed the expression of SEA, SEB, SEC, and SEE genes (P<0.05). In samples containing Staphylococcus species treated with *B. bifidum* CFS, the expression of target genes was significantly reduced. The average fold change for *SEA*, *SEB*, *SEC*, and *SED* genes was -1.681, -1.28, -1.52, and -0.84, respectively (Figures 2 and 3).

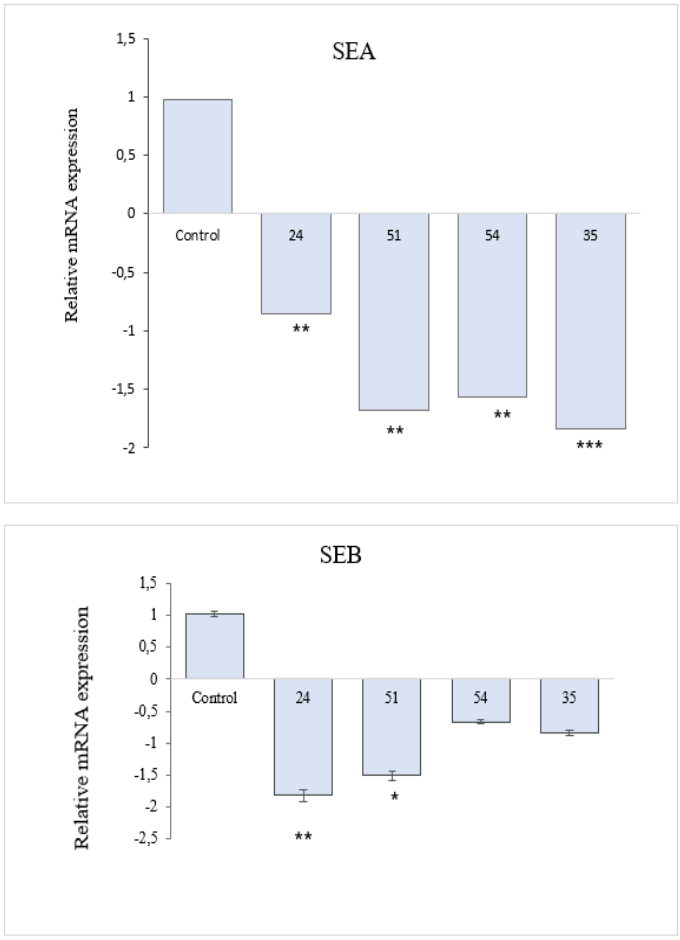

Figure 2. Quantitative RT-PCR demonstrating fold changes of *SEA* and *SEB* genes in CFS-treated strains. Strain numbers were expressed within the columns. Data represent the averages from three independent experiments. Error bars represent the standard deviations from the means. *: $P < 0.05$; **: $P < 0.01$, ***: $P < 0.001$ versus control group.

3.5. Phylogenic Relationship

The 16SrDNA sequences of the four selected *S.aureus* isolates were used to establish a phylogenetic tree, and the result is exhibited in Figure 4. Strains 51 and 24 were clustered with *S.aureus* ATCC 25923, and strains 54 and 35 were clustered with *S.aureus* ATCC 12600, respectively (Figure 4).

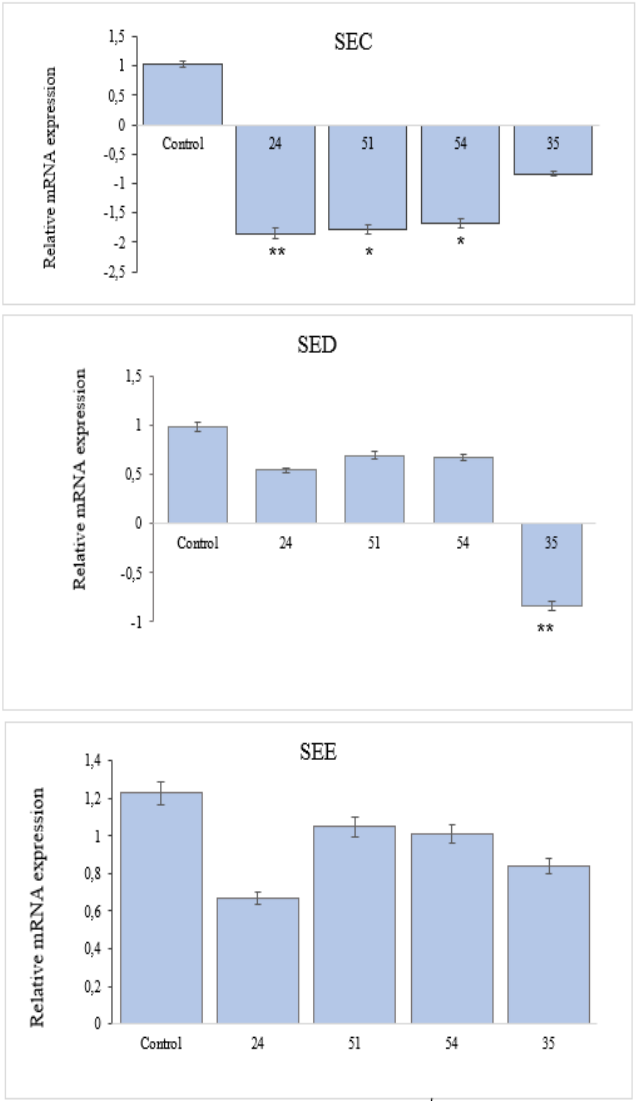

Figure 3. Quantitative real-time PCR showing fold changes of *SEC*, *SED*, and *SEE* genes in CFS-treated strains. Strain numbers were expressed within the columns. Data represent the averages from three independent experiments. Error bars represent the standard deviations from the means. *: $P < 0.05$; **: $P < 0.01$, ***: $P < 0.001$ versus control group.

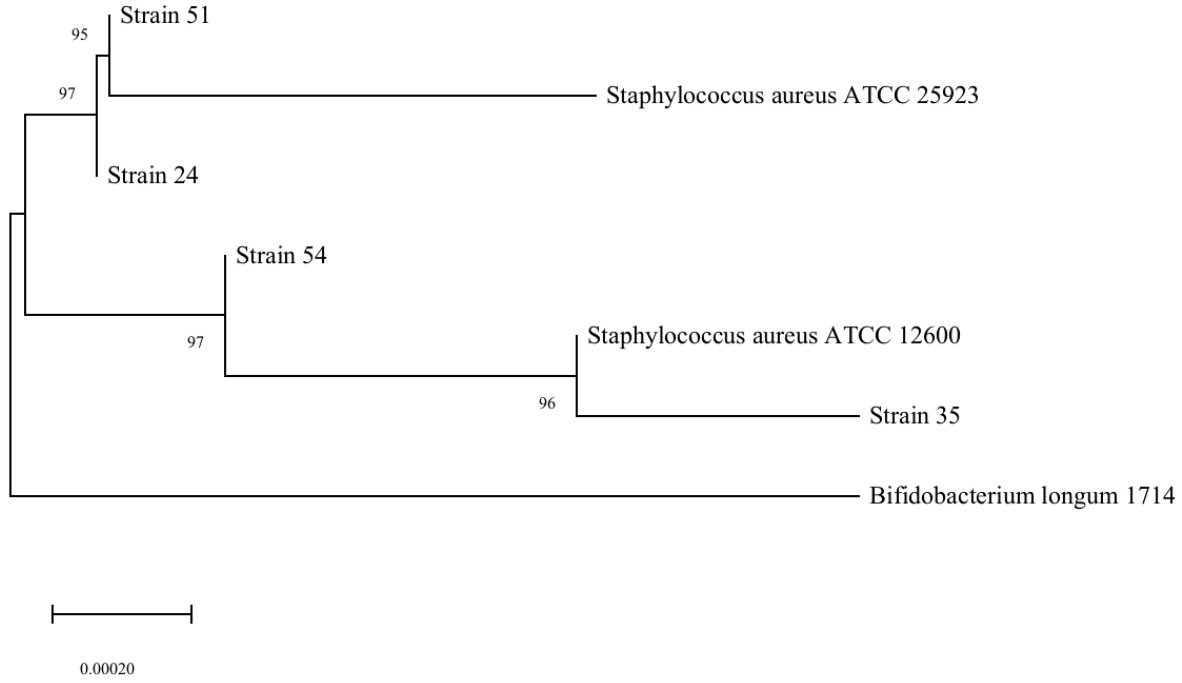

Figure 4. Phylogenetic relationship of strains based on 16S rDNA sequences of the four isolates, outgroup, and type strains

4. Discussion

Bovine mastitis is an inflammatory disease caused by factors such as *S. aureus*, which produces pathogenic factors like SEs. Antimicrobial resistance is a growing problem, leading to interest in natural antimicrobials like probiotics. This study examined the influence of *Bifidobacterium bifidum* probiotic on the gene expression of SEs isolated from raw cow's milk. Analysis of unprocessed milk samples revealed that 28% were sterile while bacteria such as Staphylococcus species were found in the remaining 72%. This study focused on the isolation of staphylococci species. The analysis of milk sample staphylococci revealed the existence of various SE superantigens, including *SEA* (16.7%), *SEC* (11.7%), *SED* (8.3%), *SEE* (6.7%), and *SEB* (1.7%).

In line with our results, Nazari et al. studied the enterotoxin gene profiles of *S.aureus* isolated from Iranian raw milk and found that the gene encoding enterotoxin A (SEA) was the most common (16 isolates, 30.7%), followed by SEB (14 isolates, 26.9%) and SED (8 isolates, 15.37%) (18). The evidence revealed the existence of various SE genes in 27 (45%) strains. Similarlly, in Bianchi et al. study (19), of 481 analysed *S. aureus* strains, 255 (53%) were positive for one or more SE genes, and while this ratio was about 80.7% in Nazari et al. study. The *SED* was previously reported as the most frequent isolated toxin type, after SEA, in staphylococcal food-poisoning outbreaks involving dairy products (20) (21), which is in line with the findings of the present study. Although considerable variation in the prevalence of enterotoxin genes in *S. aureus* isolates found in different foods and biovars, SEA is consistently the most frequently observed enterotoxin in this microorganism.

Findings showed that bifidobacteria CFS had prominent antimicrobial activity against strains 24, 51, 54, and 35 of Staphylococcus species, and the MIC and MBC results obtained for these strains treated with *B. bifidum* CFS were in the range of 31.25 µg/ml

to 125 µg/ml. The CFS of probiotics has demonstrated antimicrobial capabilities (22-24).

Probiotics have the ability to inhibit the growth of harmful bacteria by producing antimicrobial metabolites in their CFS. A study investigated the effectiveness of CFS from probiotics such as Bacillus spp. and Pediococcus spp. in inhibiting the growth of pathogenic bacteria found in fresh boar semen. The results showed that CFS from Lactobacillus spp. and Pediococcus spp. were more effective in inhibiting the growth of pathogenic bacteria with antimicrobial resistance genes (25). Our results showed that *B. bifidum* probiotic CFS suppressed the expression of *SEA, SEB, SEC*, and *SEE* genes. Following our findings, several studies have shown that the CFS of probiotic bacteria can affect gene expression in bacteria (26-29). To understand its antimicrobial activity and suppression of biogenic amine production, Moghadam et al. examined the effects of cell free supernatant of *Lactococcus lacts* and *B. bifidum* on target genes expression. They reported that *L. lactis* and *B. bifidum* probiotics CFS suppressed the expression of genes encoding for biogenic amine production (15). Even et al. studied the effect of *Lactobacillus lactis* on the expression of *S. aureus* virulence factors when cocultured (30). They found that the expression of several regulators, including *agr* and *sarA* loci, as well as virulence factors, such as enterotoxins, were strongly influenced. Similarly, Parsaeimehr et al. found that a mixed culture of *Lactobacillus acidophilus* and *Lactobacillus casei* reduced the expression of the sea gene in *S. aureus* (31). In contrast, Saidi et al. found that while the *S. cerevisiae* S3 lysate extract had no inhibitory effect on the expression of *sea* and *hla* genes in *S. aureus*, the supernatant extract of this yeast was able to reduce the expression of both genes (32). The present study revealed a probable antimicrobial mechanism of probiotic bifidobacteria against *staphylococcus aureus*.

Even et al. investigated the impact of *Lactobacillus lactis* on the expression of *S. aureus* virulence factors when grown together (26). They found that the expression of several regulators, including the *agr* and *sarA* loci, as well as virulence factors, such as enterotoxins, were strongly affected. Similarly, Parsaeimehr et al. found that coculture of *Lactobacillus acidophilus* and *Lactobacillus casei* reduced the expression of the *SEA* gene in *S. aureus* (31). In contrast, Saidi et al. demonsterated that while the *S. cerevisiae* S3 lysate extract had no inhibitory effect on the expression of *SEA* and *HLA* genes in *S. aureus*, the supernatant extract of this yeast was able to reduce the expression of both genes (32). The present study revealed a potential antimicrobial mechanism of probiotic bifidobacteria against *S. aureus*. In conclusion, the SEs prevalence in raw cow's milk is a concerning issue, especially with the growing problem of antimicrobial resistance. By analyzing the distribution of enterotoxin genes in *S. aureus*, valuable epidemiological information can be obtained to improve public health and food safety. The use of natural antimicrobials like probiotics has shown promising results in preventing the growth of pathogenic bacteria and suppressing the toxin genes expression. The present study specifically focused on the effect of the *B. bifidum* probiotic on staphylococcal enterotoxin gene expression, and the results demonstrated its potential as a natural antimicrobial. Further research is needed to explore the effectiveness of probiotics in preventing staphylococcal food poisoning in humans and other related diseases.

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None.

Authors' Contribution

Study concept and design: H. J and S.A.A. Acquisition of data: H. J Analysis and interpretation of data: S.A.A. Drafting of the manuscript: H.J. Critical revision of the manuscript for important intellectual content: K.A. Statistical analysis: G.K. Administrative, technical, and material support: K.A and S.A.A.

Ethics

Not Applicable.

Conflict of Interest

The authors declare that they have no conflict of interest.

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