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Suppressive Effect of *Bifidobacterium bifidum* Probiotic Supernatant on Tigecycline Resistance Gene Expression in Clinical Isolates of *Streptococcus pyogenes*

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ABSTRACT

Background: The rapid rise in antibiotic resistance is a serious worldwide concern, with tigecycline being a key treatment. Therefore, scientists are looking for natural alternatives such as probiotics. This study aimed to investigate whether *Bifidobacterium bifidum*-derived cell-free supernatant could modulate the expression of key tigecycline resistance-associated genes, including *tet*(M), *tet*(O), and *tet*(X1), in clinical *Streptococcus pyogenes* isolates.
Materials & Methods: A total of 60 *S. pyogenes* isolates were recovered from 100 clinical specimens and identified using standard phenotypic and biochemical assays. Susceptibility to tigecycline was evaluated according to established antibiogram guidelines. The presence of tigecycline resistance-associated genes was assessed by multiplex polymerase chain reaction (PCR). The antimicrobial potential of *B. bifidum* supernatant was assessed by MIC and MBC determination. Real-time PCR was used to detect changes in gene expression following treatment with the supernatant.
Findings: About 58.3% (35 of 60) of isolates were resistant to tigecycline. The most common resistance-associated gene was *tet*(M) (16.67%), followed by *tet*(O) (11.67%) and *tet*(X1) (1.67%). No isolate carried all three genes together. MIC and MBC values of probiotic supernatant were within the range of 8-128 and 16-128 µg/mL, respectively. Crucially, real-time PCR showed that the supernatant significantly reduced the expression of all three resistance genes in treated isolates.
Conclusion: These results suggest that *B. bifidum* supernatant may help suppress tigecycline resistance in *S. pyogenes* by downregulating the expression of critical resistance genes. This highlights its potential as a natural, supportive strategy to combat antibiotic resistance and paves the way for future clinical or therapeutic research.

Keywords: *Streptococcus pyogenes*, Antibiotic resistance, Tigecycline, *Bifidobacterium bifidum*, Probiotic supernatant, Gene expression

CITATION LINKS

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Introduction

Antimicrobial resistance (AMR) represents a growing global public health challenge, with increasing reports of resistance even to last-resort antibiotics such as tigecycline. *Streptococcus pyogenes*, a Lancefield Group A Gram-positive β-hemolytic bacterium, is a major human pathogen responsible for a broad spectrum of infections, ranging from mild diseases such as pharyngitis to severe and life-threatening conditions, including necrotizing fasciitis and streptococcal toxic shock syndrome (STSS) [1]. Although penicillin remains the first-line treatment for *S. pyogenes* infections, therapeutic limitations arise in cases of macrolide treatment failure or penicillin allergy, necessitating the use of alternative antimicrobial agents such as tigecycline [2]. Tigecycline exerts its antibacterial activity by binding to the 30S ribosomal subunit and inhibiting the entry of aminoacyl-tRNA into the A site, thereby suppressing protein synthesis [3]. However, resistance to tigecycline in *S. pyogenes* has been increasingly reported, which is primarily associated with the acquisition and expression of tetracycline resistance genes, particularly *tet*(M), *tet*(O), and the flavin-dependent monooxygenase gene *tet*(X1) [4].

These resistance determinants are frequently located on mobile genetic elements, including transposons and integrative conjugative elements (ICEs), which facilitate rapid bacterial adaptation under selective pressure and promote horizontal gene transfer [5]. The *tet*(M) gene encodes a ribosomal protection protein displacing tetracycline from its binding site on the 30S ribosomal subunit, thereby restoring protein synthesis. Similarly, *tet*(O) might protect the ribosome in a similar manner, with a GTPase-dependent action. On the other hand, *tet*(X1) as a flavin-dependent monooxygenase inactivates tetracyclines by oxidative modification and confers a broader resistance phenotype, including tigecycline resistance [6].

Hence, considering the increased resistance to common antibiotics, there has been a surge in interest in natural and adjuvant treatments, mainly probiotics. *Bifidobacterium bifidum* is the main commensal bacterium found in the human gut and breast milk, which is known not only for immunomodulation but also for producing antimicrobial products such as bacteriocins, organic acids, and hydrogen peroxide [7]. The supernatant of this strain has been reported to inhibit the growth of several pathogens, probably through alteration of pH, production of reactive metabolites, or interference with bacterial gene expression.

Evidence suggests that metabolites produced by probiotic bacteria could modulate antimicrobial resistance mechanisms, including regulation of resistance gene expression and biofilm formation in Gram-positive pathogens. Despite these advances, the impact of probiotic-derived metabolites on resistance determinants in *S. pyogenes* remains poorly characterized, particularly with respect to tigecycline resistance genes.

Objectives: The objective of the present study was to investigate the effect of Bifidobacterium bifidum-derived cell-free supernatant on the transcriptional expression of tigecycline resistance-associated genes, including tet(M), tet(O), and tet(X1), in clinical isolates of Streptococcus pyogenes. Furthermore, this study aimed to evaluate the antimicrobial activity of the probiotic supernatant and to determine its potential as an adjunctive strategy for mitigating antimicrobial resistance [8].

Materials and Methods

The present study aimed to evaluate the effect of *B. bifidum*-derived supernatant on the transcriptional regulation of tigecycline resistance-associated genes, including *tet*(M),

tet(O), and *tet*(X1), in clinical isolates of *S. pyogenes*. All microbiological and molecular studies, such as bacteriology, genomic extraction, RNA extraction, and quantitative expression studies, were performed under certified biosafety level 2 (BSL-2) conditions. The experiment was done in a molecular microbiology laboratory equipped with a laminar flow hood, CO_2 incubators, calibrated microcentrifuges, and HEPA-filtered PCR workstations. During all procedures, proper aseptic techniques were employed to prevent contamination and ensure data reproducibility and reliability [9].

Sample collection and bacterial identification: Throat swab specimens were obtained from patients presenting with clinical symptoms of pharyngitis at regional medical centers. Samples were immediately placed in Amies transport medium and transferred to the laboratory under cold-chain conditions. Each specimen was inoculated onto blood agar plates supplemented with 5% sheep blood and incubated aerobically at 37 °C for 24 hrs. Colonies exhibiting β-hemolysis were selected for further analysis. Microscopic examination revealed Gram-positive cocci arranged in chains. Phenotypic identification was further confirmed using standard biochemical tests, including catalase negativity, bacitracin susceptibility (0.04-unit disk with a clear inhibition zone), and CAMP test negativity. Confirmed *S. pyogenes* isolates were preserved in brain heart infusion (BHI) broth containing 15% glycerol and stored at −70 °C until further use [10, 11].

Cultivation of *B. bifidum* and preparation of cell-free supernatant: The probiotic strain *B. bifidum* was cultured anaerobically in de Man, Rogosa, and Sharpe (MRS) broth at 37 °C for 48 hrs using GasPak anaerobic systems. Following incubation, the culture was centrifuged at 6000 rpm and 4 °C for 10 min to pellet bacterial cells. The resulting supernatant was carefully collected and filtered through a sterile 0.22-µm syringe filter to ensure complete removal of viable bacterial cells. The sterile cell-free supernatant (CFS) was stored at 4 °C and used within one week to preserve bioactive metabolites [11].

Preparation of culture medium control: To exclude any antimicrobial or gene-modulatory effects attributable to the culture medium itself, a sterile MRS broth control was prepared in parallel. Uninoculated MRS broth was incubated under identical anaerobic conditions (at 37 °C for 48 hrs), followed by centrifugation and filtration through a 0.22-µm membrane filter using the same procedure described for the probiotic culture. This sterile MRS filtrate was used as a negative control in all MIC, MBC, and qRT-PCR assays to confirm that the observed biological effects were specifically associated with metabolites produced by *B. bifidum*.

Assessment of antibacterial variables (MIC and MBC): The sub-inhibitory concentration (sub-MIC) used for gene expression analysis was defined as one-half (½) of the MIC value determined individually for each isolate. Fresh sub-MIC solutions were prepared for each experiment to ensure accuracy and consistency.

The antibacterial activity of *B. bifidum* cell-free supernatant was evaluated by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the broth microdilution method. Briefly, two-fold serial dilutions of the supernatant were prepared in sterile 96-well microtiter plates to obtain final concentrations ranging from 1 to 128 µg/mL. Each well was inoculated with a standardized *S. pyogenes* bacterial suspension adjusted to 0.5 McFarland turbidity (approximately 1.5×10^8 CFU/mL). Following incubation at 37 °C for 24 hrs, the MIC was defined as the lowest concentration at which no visible bacterial growth was observed. To determine the

MBC, aliquots (10 µL) from wells showing no visible growth were sub-cultured onto Mueller Hinton agar (MHA) plates and incubated at 37 °C for an additional 24 hours. The MBC was recorded as the lowest concentration at which no bacterial colonies were detected [12].

Extraction of genomic DNA and detection of resistance genes: Genomic DNA was extracted from bacterial isolates using the SinaClon Genomic DNA Extraction Kit. Prior treatment of bacteria with lysozyme and Proteinase K was necessary for proper lysis. Next, DNA purity was checked with a NanoDrop spectrophotometer based on the 260/280 nm ratio, while DNA integrity was analyzed using 1% agarose gel electrophoresis. Detection of *tet*(M), *tet*(O), and *tet*(X1) genes was carried out in PCR reaction using specific primers. PCR products were observed under UV (ultraviolet) light after staining with ethidium bromide on 1.5% agarose gel [13, 9].

RNA extraction and quantitative gene expression analysis: To understand the gene expression response of *S. pyogenes* to *B. bifidum* metabolites, isolates were treated with sub-MIC concentrations of the probiotic supernatant. Total RNA was extracted with RNX-Plus solution, and the obtained RNA was treated with DNase I to avoid any genomic DNA contamination. The quantity and quality of RNA were evaluated using a NanoDrop device and 1% agarose gel, respectively.

A RevertAid First Strand cDNA Synthesis Kit was used for cDNA synthesis. qRT-PCR was performed using the SYBR Green system. Cycling conditions for PCR consisted of denaturation at 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 55.5 °C for 30 s, and 72 °C for 30 s. Relative gene expressions were normalized against 16S rRNA and analyzed using the 2^-ΔΔCt method.

RNA purity was confirmed by spectrophotometric analysis, with A260/280 ratios ranging between 1.82.0- and A260/230 values above 1.8. RNA integrity was further verified by agarose gel electrophoresis prior to cDNA synthesis.

Statistical analysis: All assays were conducted in triplicate with results reported as means ± SD. SPSS and GraphPad Prism were used to conduct statistical analyses. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was carried out to compare means between groups. Differences were considered statistically significant when $p < .05$ [14, 15].

Findings

Identification and phenotypic characterization of *S. pyogenes*: Among the 100 throat swab specimens collected from patients clinically diagnosed with pharyngitis, 60 bacterial isolates were conclusively identified as *S. pyogenes* based on classical microbiological criteria complemented by confirmatory phenotypic assays. On blood agar medium, all 60 isolates formed colonies demonstrating clear β-hemolysis, indicative of complete red blood cell lysis, which is a hallmark feature of Group A β-hemolytic streptococci.

Microscopic examination of Gram-stained smears revealed purple-stained cocci arranged in linear chains, consistent with the morphology of *S. pyogenes*. All isolates were catalase-negative, which helps distinguish streptococci from catalase-positive genera such as staphylococci. Furthermore, each isolate displayed sensitivity to bacitracin (0.04 U) and tested negative in the CAMP assay, effectively excluding Group B streptococci. The phenotypic and biochemical profiles of all isolates are summarized in Table 1, which collectively confirm their classification as *S. pyogenes*. This precise identification was considered a prerequisite to ensure the validity of subsequent molecular detection and expression analysis [16,21].

Table 1) Phenotypic and biochemical test results for identification of *S. pyogenes* isolates

Test Name	Observed Result	Interpretation
Gram staining	Presence of purple-stained cocci arranged in chains	Gram-positive cocci with characteristic chain formation
Catalase test	No bubble formation in the presence of hydrogen peroxide	Catalase-negative
Bacitracin susceptibility	Clear zone of inhibition observed around the bacitracin disk	Bacitracin-sensitive (indicative of Group A *Streptococcus*)
CAMP test	No enhanced hemolysis at the intersection line	CAMP-negative (excluding Group B *Streptococcus*)

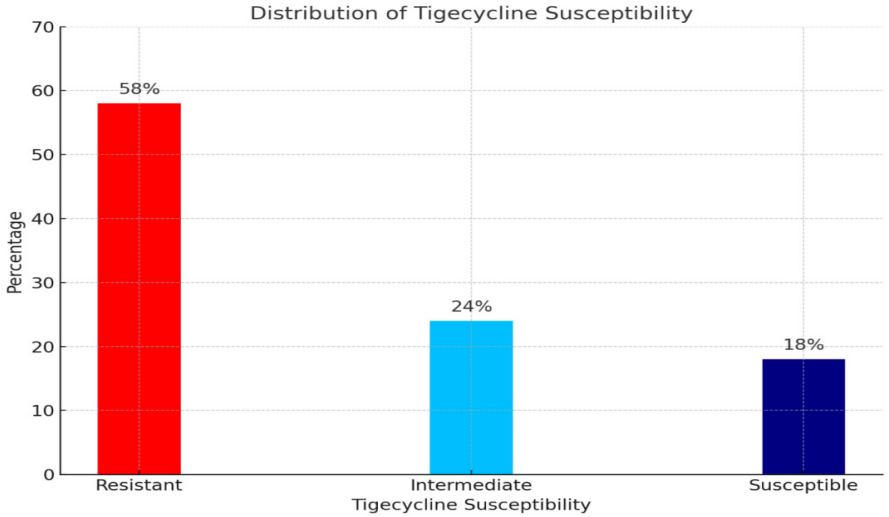

Figure 1) Distribution of tigecycline susceptibility profiles among clinical isolates of *S. pyogenes* (n = 60). Antimicrobial susceptibility testing was performed according to CLSI guidelines using the disk diffusion method. Among the tested isolates, 35 (58.3%) were resistant, 14 (24%) showed intermediate susceptibility, and 11 (18.3%) were susceptible to tigecycline. The results indicate a high prevalence of tigecycline resistance among the clinical isolates investigated in this study.

Antibiotic susceptibility testing: Antibiotic susceptibility testing revealed elevated resistance to tigecycline. Antimicrobial susceptibility of the 60 confirmed isolates against tigecycline was evaluated using standardized interpretive criteria. The results demonstrated an alarming prevalence of resistance, with 35 (58.3%) isolates being fully resistant to tigecycline, 14 (24%) isolates exhibiting intermediate susceptibility, and only 11 (18%) isolates showing full susceptibility to this drug.

These results, graphically depicted in Figure 1, suggest that a significant proportion of isolates have acquired mechanisms conferring reduced responsiveness to tigecycline, a last-resort antibiotic for multidrug-resistant Gram-positive pathogens. Such resistance patterns are especially concerning in the case of *S. pyogenes*, traditionally considered highly susceptible to most antimicrobial agents. These implications extend beyond treatment challenges; they raise serious public health concerns regarding the increasing circulation of resistant clones in the community, potentially driven by selective pressure resulting from empirical antibiotic use [17, 18].

Molecular detection of tetracycline resistance determinants via multiplex PCR: To elucidate the genetic basis of phenotypically-observed tigecycline resistance, multiplex PCR assays were employed to detect three specific genetic determinants linked

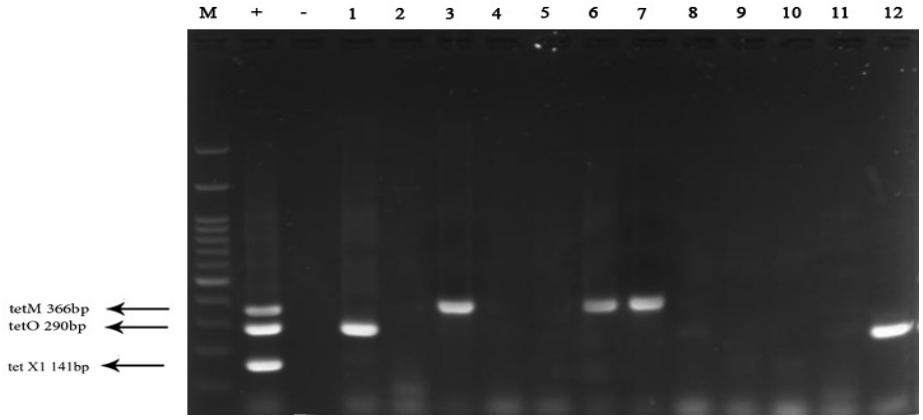

Figure 2) Agarose gel electrophoresis analysis of multiplex PCR products for detection of tet(M), tet(O), and tet(X1) resistance genes in clinical isolates of *S. pyogenes* (isolates 1-12).
PCR products were separated on 1.5% agarose gel stained with ethidium bromide and visualized under UV illumination. Lane M: 100-bp DNA ladder molecular size marker; Lane PC: positive control containing known tet-positive DNA; Lane NC: negative control without template DNA. Expected amplicon sizes were 366 bp for tet(M), 290 bp for tet(O), and 141 bp for tet(X1). Lanes corresponding to positive isolates showed distinct amplification bands at the expected molecular sizes.

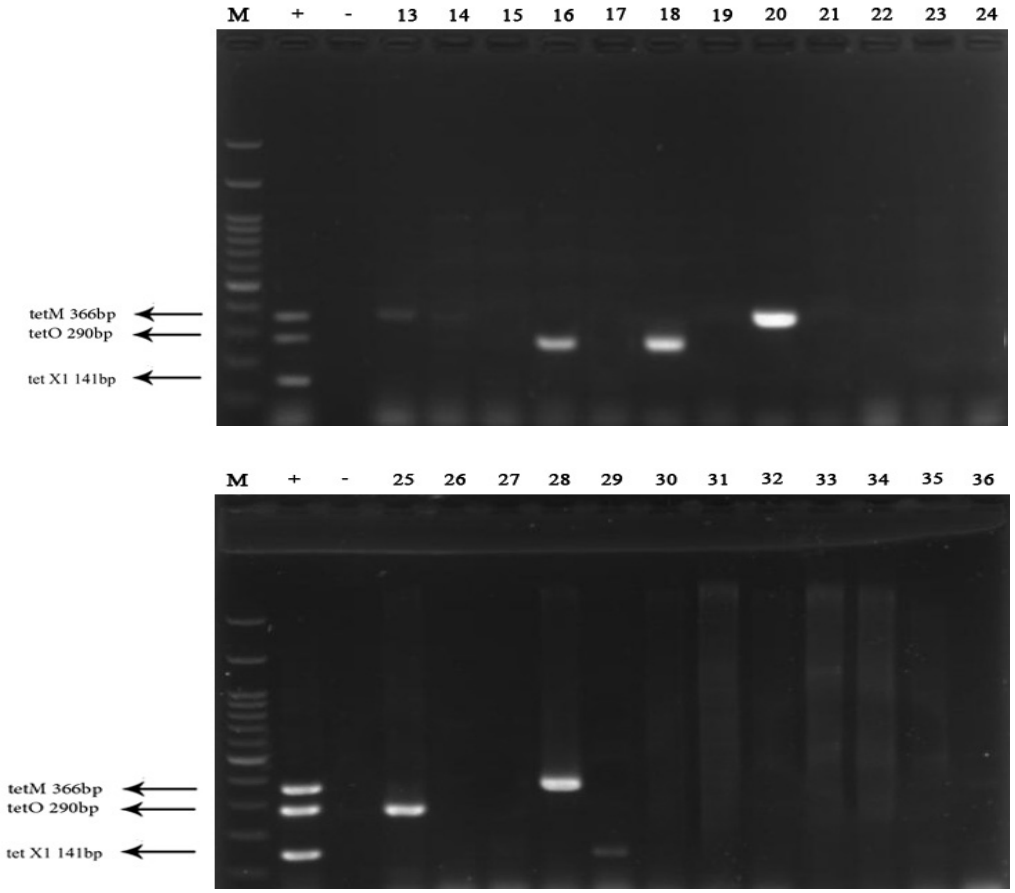

Figure 3) Representative multiplex PCR amplification profiles of tet(M), tet(O), and tet(X1) genes in *S. pyogenes* clinical isolates 13-36.
Electrophoresis was performed using 1.5% agarose gel containing ethidium bromide. Lane M represents the 100-bp DNA molecular weight marker. Positive and negative controls were included to validate amplification specificity and exclude contamination. Bands corresponding to tet(M), tet(O), and tet(X1) were identified according to their expected fragment sizes of 366 bp, 290 bp, and 141 bp, respectively.

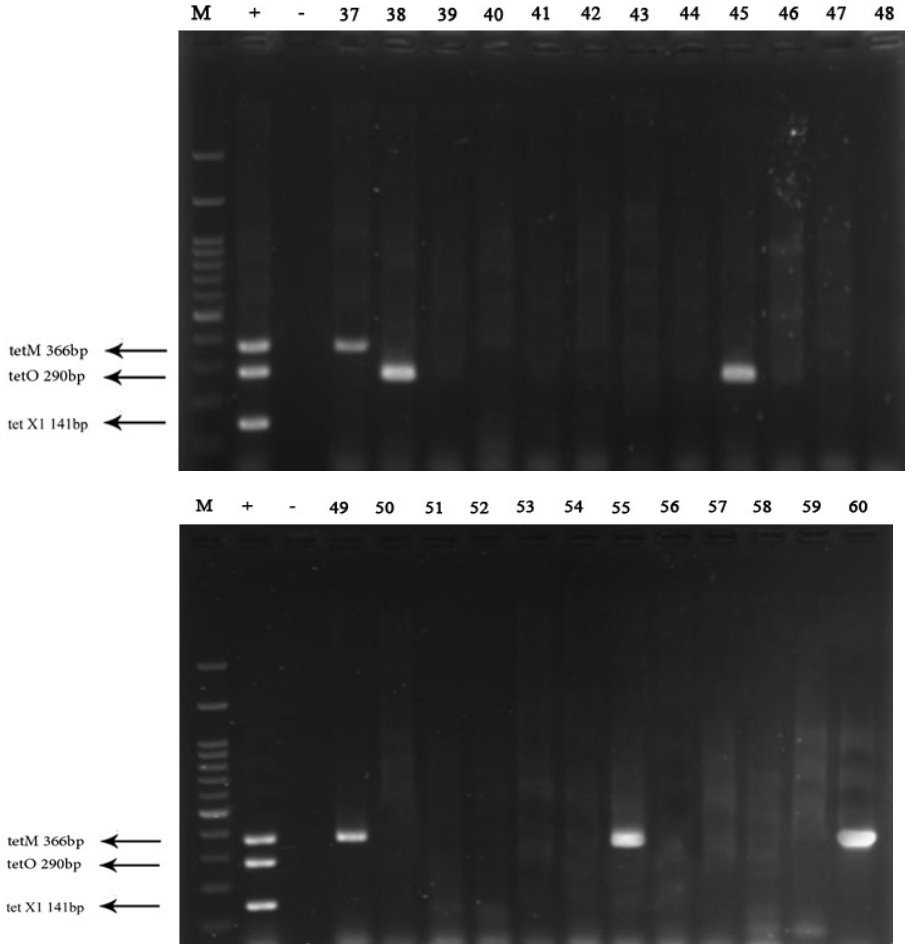

Figure 4) Multiplex PCR detection of tigecycline resistance-associated genes among *S. pyogenes* isolates 37-60. Lane M: 100-bp DNA ladder; PC: positive control; NC: negative control. PCR amplification products were resolved by electrophoresis on 1.5% agarose gel. The presence of tet(M), tet(O), and tet(X1) genes was confirmed based on the appearance of bands corresponding to 366 bp, 290 bp, and 141 bp, respectively.

Table 2) Prevalence of tigecycline resistance genes in *S. pyogenes* clinical isolates

Target Gene	tet(M)	tet(O)	tet(X1)	tet(M), tet(O), tet(X1)
Amplicon size (bp)	366	290	141	–
Number of positive isolates	10	7	1	0
Gene prevalence (%)	16.67%	11.67%	1.67%	0%

to tigecycline resistance, including *tet*(M), *tet*(O), and *tet*(X1), each encoding different mechanisms for resistance to tetracyclines. Electrophoretic analysis of the PCR products confirmed the expected fragment sizes, including 366 bp for *tet*(M), 290 bp for *tet*(O), and 141 bp for *tet*(X1).

The distribution of these resistance genes among the 60 isolates was as follows: ten (16.67%) isolates were positive for *tet*(M), seven (11.67%) isolates were positive for *tet*(O), and one (1.67%) isolate was positive for *tet*(X1).

No isolate exhibited co-expression of multiple resistance genes. The absence of multi-gene carriage may reflect the biological cost of harboring multiple mobile genetic elements or incompatibility between plasmids and transposons involved in horizontal gene transfer. The gel electrophoresis results

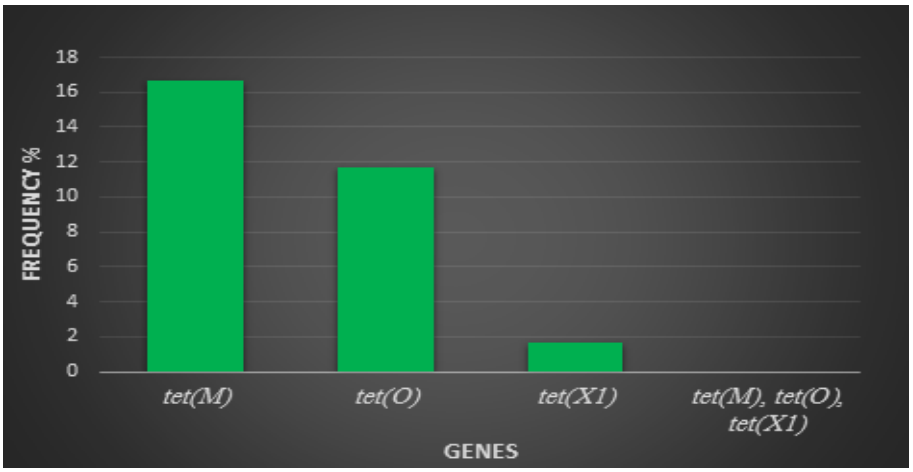

Figure 5) Frequency distribution of tigecycline resistance-associated genes among S. pyogenes clinical isolates.

The prevalence rates of tet(M), tet(O), and tet(X1) genes were determined by multiplex PCR analysis. tet(M) was the most prevalent resistance determinant detected among the isolates, followed by tet(O), whereas tet(X1) was identified in only one isolate.

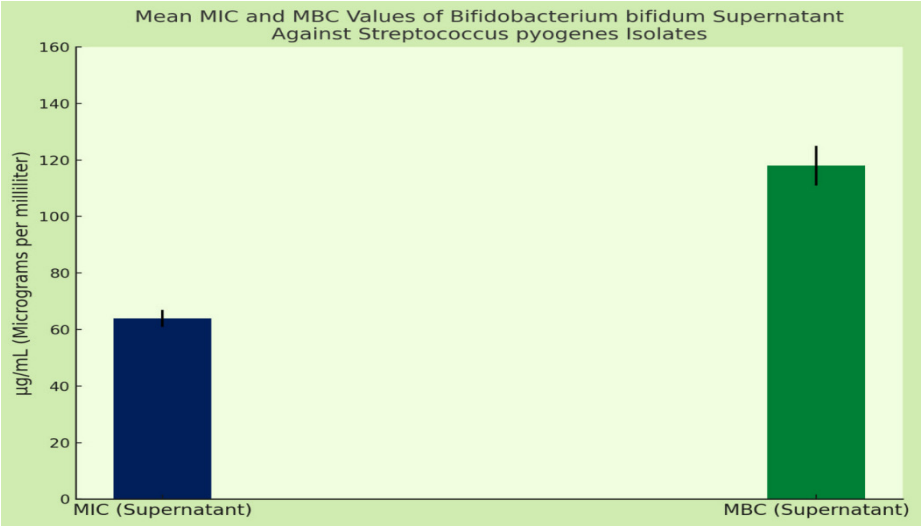

Figure 6) Mean MIC and MBC values of *B. bifidum* supernatant against *S. pyogenes* isolates

Although MIC values ranged from 8 to 128 µg/mL, the arithmetic mean MIC was 64 µg/mL, while the mean MBC was 128 µg/mL (n = 60).

for each group of isolates are displayed in Figures 2-4, corresponding to isolates 1-12, 13-36, and 37-60, respectively. A consolidated graphical representation of gene frequency is presented in Figure 5, while the full distribution of PCR results is tabulated in Table 2 [19, 21].

Evaluation of antimicrobial properties of *B. bifidum* supernatant: To assess the biological activity of probiotic-derived compounds, cell-free supernatant (CFS) of *B. bifidum* was subjected to antimicrobial testing against resistant isolates using broth microdilution methods. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values exhibited substantial variability among the tested isolates. Specifically, MIC values ranged from 8 to 128 µg/mL, while MBC values extended from 16 to below 128 µg/mL. The mean MIC and MBC values were calculated as 64 and 128 µg/mL, respectively.

These data, illustrated in Figure 6, demonstrate that metabolites within CFS possess

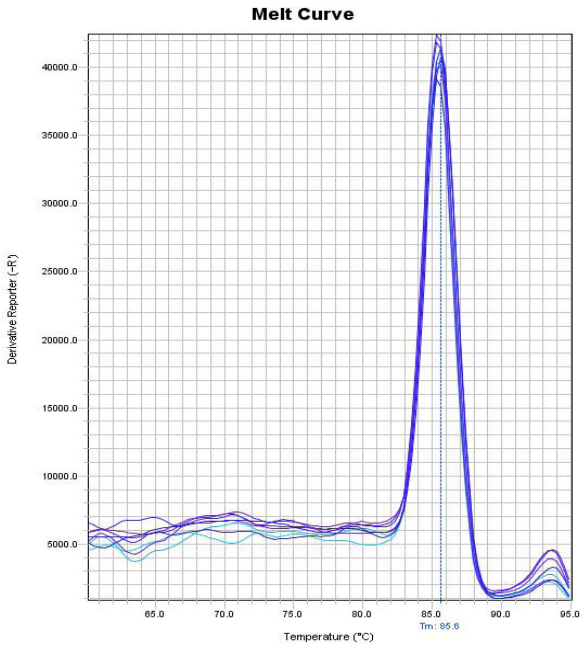

Figure 7) Real-time PCR melting curve analysis of tet(M) amplification products in *S. pyogenes*.
Melting curve analysis was performed following SYBR Green-based qRT-PCR amplification to verify product specificity. A single sharp melting peak at Tm = 86.6 °C confirmed the absence of nonspecific amplification and primer-dimer formation.

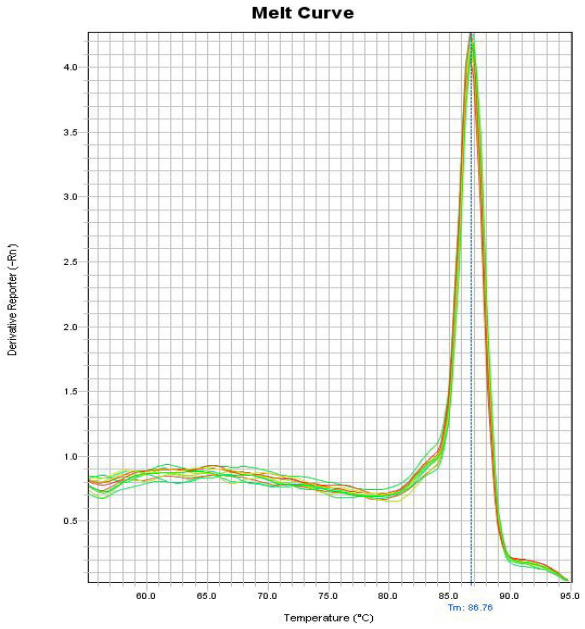

Figure 8) Real-time PCR melting curve analysis of tet(O) amplification products in *S. pyogenes*.
The melting curve demonstrated a single specific amplification peak at Tm = 86.76 °C, indicating high specificity of the qRT-PCR reaction and absence of nonspecific products.

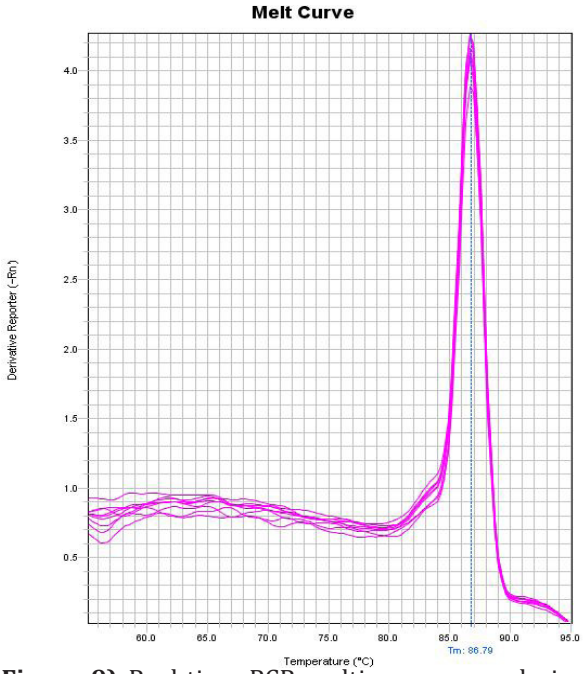

Figure 9) Real-time PCR melting curve analysis of tet(X1) amplification products in *S. pyogenes*.
SYBR Green melt curve analysis revealed a single distinct melting peak at Tm = 86.79 °C, confirming specific amplification of the tet(X1) target gene.

substantial inhibitory and bactericidal capabilities even against *S. pyogenes* strains harboring resistance determinants. This supports the potential of non-antibiotic modalities, such as probiotic-derived secondary metabolites, to exert antimicrobial effects through novel mechanisms such as membrane disruption, intracellular pH modulation, or quorum sensing interference [20].
Quantitative real-time PCR analysis revealed a marked reduction in the transcription levels of *tet*(M) (p= .013), *tet*(O) (p= .021), and *tet*(X1) (p= .004) in *S. pyogenes* isolates treated with *B. bifidum* cell-free supernatant compared with untreated controls (Figure 11).
Impact of CFS on gene expression: Real-time PCR analysis: To explore whether exposure to *B. bifidum* CFS modulates the expression of resistance genes, quantitative real-time PCR (qRT-PCR) was performed on resistant isolates treated with sub-MIC levels of the supernatant.
Melting curves confirmed amplification specificity for each target gene (Figures 7-9).

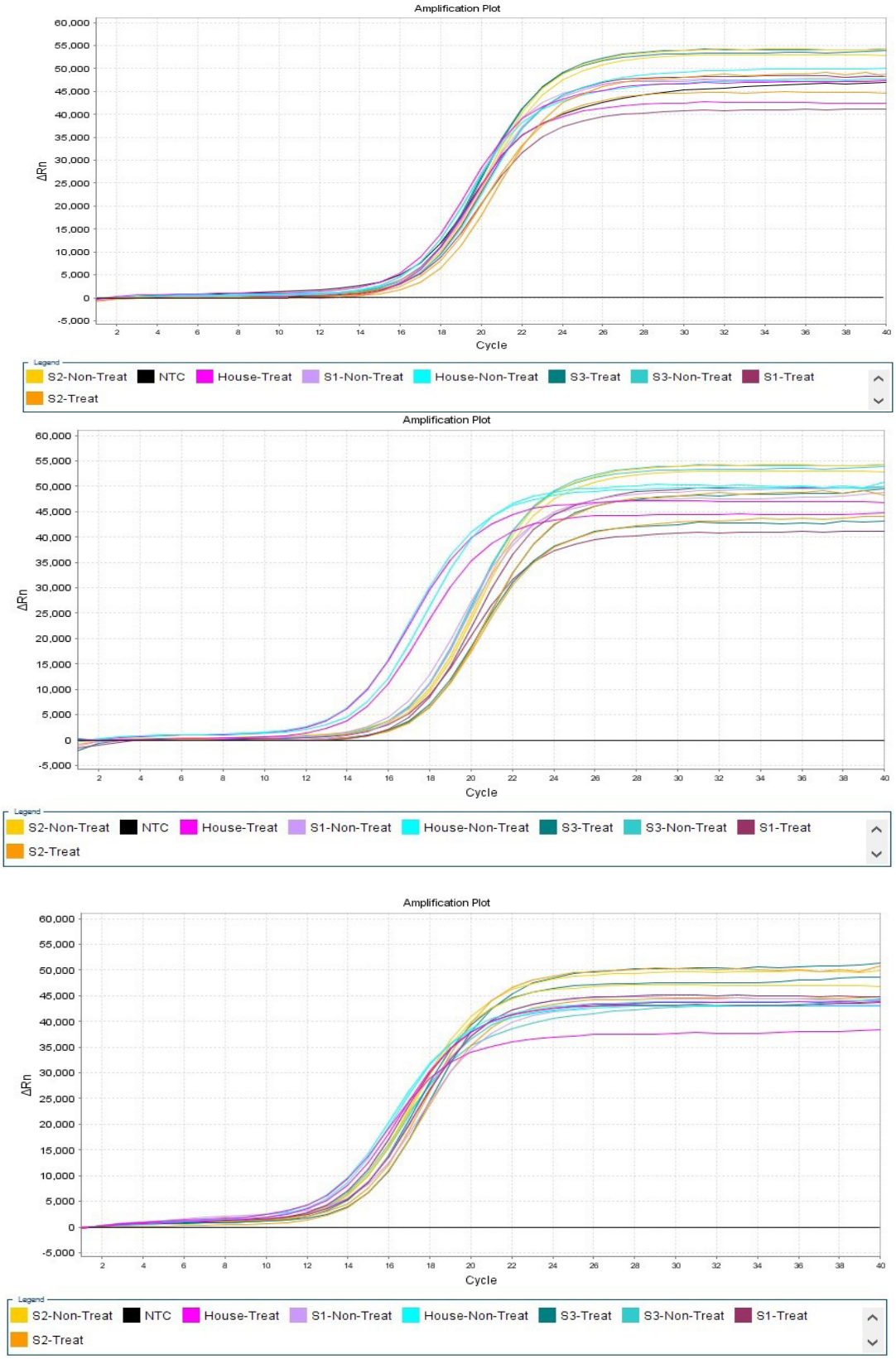

Figure 10) Representative qRT-PCR amplification curves of *tet*(M), *tet*(O), and *tet*(X1) genes in *S. pyogenes*. Amplification was performed using SYBR Green chemistry under standardized cycling conditions. Threshold cycle (Ct) values were determined automatically using instrument-defined baseline settings. Curves represent treated and untreated control samples from three independent experiments performed in triplicate.

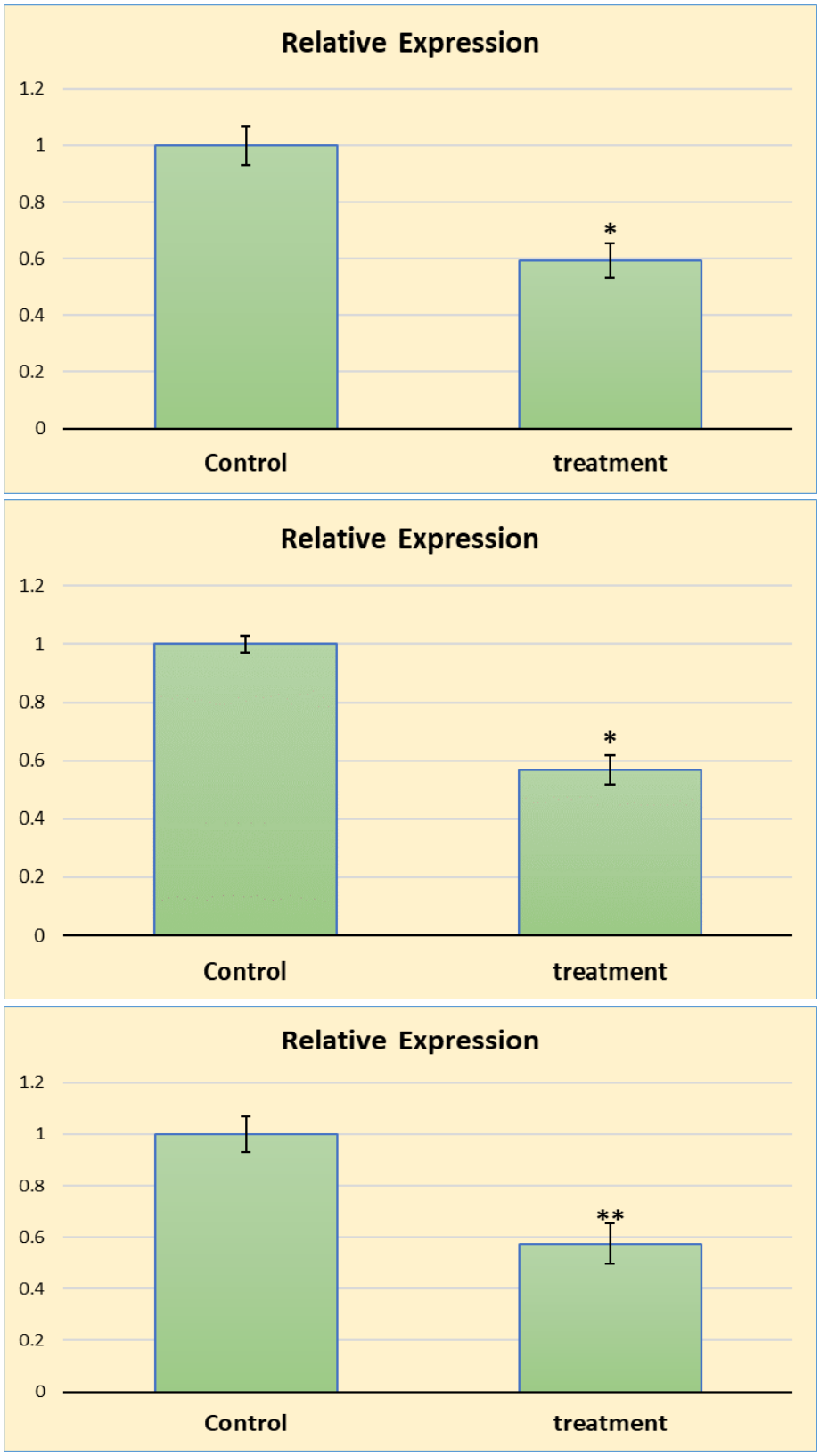

Figure 11) Relative transcription levels of *tet*(M), *tet*(O), and *tet*(X1) genes in *S. pyogenes* following exposure to sub-MIC concentrations of *B. bifidum* cell-free supernatant. Gene expression was normalized to 16S rRNA and calculated using the 2^−ΔΔCt method. Data represent mean ± SD of three independent experiments performed in triplicate. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test (*tet*(M): p= .013; *tet*(O): p= .021; *tet*(X1): p= .004).

The amplification plot (Figure 10) indicated high-quality and reproducible signal generation. The gene expression data, after normalization by the 2^-ΔΔCt approach, showed the following fold reductions in comparison with untreated controls: *tet*(M) was downregulated by ~2.7-fold, *tet*(O) was downregulated by ~2.3-fold, and *tet*(X1) was downregulated by ~3.6-fold.

The most significant reduction in expression was observed for *tet*(X1), a gene encoding an enzyme that enzymatically modifies tigecycline. These results are visualized in Figure 11, which compares gene expression levels pre- and post-treatment.

This transcriptional suppression suggests that CFS contains molecules capable of interfering with resistance gene regulatory circuits, possibly via inhibition of transcription factor binding or destabilization of mRNA transcripts [20, 22].

Integrated analysis: Functional, phenotypic, and molecular correlation: Taken together, the data present a cohesive narrative in which *B. bifidum* supernatant acts through dual functional mechanisms: 1) direct inhibition of bacterial growth (phenotypically demonstrated via MIC and MBC assays) and 2) suppression of genetic resistance pathways (confirmed by qRT-PCR downregulation of *tet* genes) [23].

The most compelling aspect of this dual action is the pronounced inhibition of *tet*(X1), a gene of high clinical relevance due to its role in tigecycline inactivation. The synergistic effect of growth suppression and gene expression regulation underscores the translational potential of probiotic metabolites as adjunctive therapeutic strategies in the management of resistant infections. Such findings necessitate further investigation into the specific bioactive molecules responsible as well as in vivo assessments to evaluate safety, delivery mechanisms, and clinical efficacy [24,25].

Discussion

The findings show that *B. bifidum* cell-free supernatant (CFS) significantly suppresses the expression of tigecycline resistance genes *tet*(M), *tet*(O), and *tet*(X1)) in *S. pyogenes* clinical isolates. This is of significant clinical importance, especially given the growing prevalence of tigecycline-resistant strains linked to the mobilization of ribosomal protection proteins and enzymatic inactivators such as *tet*(X) [19]. Among the target genes, *tet*(M) was the most prevalent, aligning with previous epidemiological studies across Gram-positive pathogens. The simultaneous suppression of *tet*(O) and *tet*(X1) indicates that *B. bifidum* CFS may interfere with regulatory or transcriptional pathways shared among resistance genes, suggesting the involvement of a broad-acting bioactive profile [29, 31].

Mechanistically, it is postulated that acidic metabolites present in CFS (such as lactic acid and acetic acid) lower the pH and thereby destabilize resistance gene mRNA or alter the activity of DNA-binding regulators. Additionally, bacteriocins, hydrogen peroxide, and redox-active compounds present in bifidobacterial supernatants have been reported to disrupt bacterial stress response networks and inhibit resistance-associated quorum sensing signals [26, 27]. The lack of co-expression of all three resistance genes in any single isolate may indicate metabolic constraints or incompatibility in the co-carriage of plasmid-encoded determinants, consistent with recent genomic surveillance of multidrug-resistant streptococci [28].

Importantly, our experiments using sub-MIC concentrations demonstrate that even low levels of CFS are sufficient to modulate gene expression. This offers translational potential, potentially supporting optimized antibiotic use strategies and minimizing adverse effects, an increasingly essential consideration in antimicrobial stewardship [29]. Nev-

ertheless, this study was conducted under in vitro conditions. Chemical characterization of the active components and in vivo trials are essential next steps. Incorporating metabolomics and transcriptomics could illuminate the precise molecular targets, while combination therapy models may help validate CFS as an effective antibiotic adjuvant [30, 31].

Conclusion

This study demonstrates that *B. bifidum* cell-free supernatant significantly reduces the transcription levels of tigecycline resistance-associated genes, including *tet*(M), *tet*(O), and *tet*(X1), in clinical isolates of *S. pyogenes*. The consistent downregulation observed among resistant strains suggests that probiotic-derived metabolites may influence resistance gene expression at the transcriptional level. These findings support the emerging concept that probiotic metabolites could extend their biological impact beyond gut microbiota modulation and potentially contribute to strategies aimed at mitigating antimicrobial resistance in Gram-positive pathogens. However, further mechanistic and in vivo investigations are required to clarify the molecular pathways involved.

The results not only reinforce the feasibility of using probiotic supernatants at sub-inhibitory concentrations but also advocate for the exploration of metabolite-based therapeutics to restore antibiotic susceptibility; however, clinical application requires further validation through comprehensive chemical characterization of active metabolites, elucidation of the exact molecular mechanisms involved, and in vivo testing in relevant infection models.

The integration of such natural bioactive substances into antimicrobial stewardship programs could reduce reliance on conventional antibiotics, mitigate resistance spread, and improve therapeutic outcomes in the post-antibiotic era.

In conclusion, *B. bifidum* supernatant represents a promising bioresource in the global effort to overcome antibiotic resistance, which warrants further investigation as a next-generation antimicrobial adjuvant.

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