Original Article

HEALTH SCIENCES
MEDICINE

DOI: 10.32322/jhsm.1680759

J Health Sci Med. 2025;8(3):483-488

Evaluation of antibiofilm effect of vancomycin, melatonin, and boric acid combination on caries due to microleakage under prosthetic restoration

Süha Kuşçu[1], Yeliz Hayran[2], Ali Aydın[3]

[1]Department of Prosthodontics, Faculty of Dentistry, Yozgat Bozok University, Yozgat, Turkiye
[2]Department of Prosthodontics, Faculty of Dentistry, Bursa Uludağ University, Bursa, Turkiye
[3]Department of Basic Medical Sciences, Faculty of Medicine, Yozgat Bozok University, Yozgat, Turkiye

Cite this article as: Kuşçu S, Hayran Y Aydın A. Evaluation of antibiofilm effect of vancomycin, melatonin, and boric acid combination on caries due to microleakage under prosthetic restoration. *J Health Sci Med.* 2025;8(3):483-488.

Received: 21.04.2025 ◆ **Accepted**: 12.05.2025 ◆ **Published**: 30.05.2025

ABSTRACT

Aims: Drug-resistant oral bacteria causing dental caries under prosthetic restorations have become a significant clinical challenge that needs to be addressed. This study aims to demonstrate the antibiofilm effect of vancomycin (VAN) combined with melatonin (MEL) and boric acid (BOR) against biofilm formation caused by *Escherichia coli (E. coli)* and *Lactobacillus acidophilus (L. acidophilus)* on dentin of tooth surfaces under restorations due to microleakage.

Methods: Extracted human teeth, free from caries, resorption, or fractures, were collected by slicing 2 mm dentin discs. A total of 64 dentin discs were inoculated with *E. coli* and *L. acidophilus* and randomly assigned to five experimental groups: MEL, BOR, MEL+VAN, BOR+VAN, and MEL+BOR+VAN, along with a control group. Antibiofilm activity and combination indices were analyzed using the MTT viability test and the Chou-Talalay method, respectively. The biofilm structure was examined using scanning electron microscopy.

Results: MEL and VAN demonstrated antimicrobial effects against *E. coli* and *L. acidophilus* bacteria in unary combinations, while BOR showed ineffectiveness. However, a notable synergistic interaction was observed in the binary combinations of VAN. Interestingly, a significant synergistic effect (CI<1) was noted in the triple combination against both bacterial species ($p<0.05$). Upon examining this effect within the triple combination, it became apparent that VAN exhibited a Favorable Dose Reduction Index (DRI>1) ($p<0.05$). When evaluating these synergistic and dose reduction results alongside scanning electron microscopy image analysis, it can be concluded that the triple combination of MEL+BOR+VAN likely induces the most optimal antibiofilm effect on dental caries bacteria.

Conclusion: The tendency of cements used in the luting of prosthetic restorations to microbial colonization and their tendency to dissolve in oral fluids increases the formation of caries under crowns and bridges due to microleakage, which may lead to tooth loss. Combining melatonin and boric acid with vancomycin may offer a potential treatment option for preventing this problem, as it exhibits strong antimicrobial properties. Thanks to this combination, the microbial load in the areas under prosthetic restorations can be reduced, and both the development of caries and the progression of existing active caries can be stopped. Thus, oral infections resulting from microleakage and their associated systemic complications can be reduced, and the longevity of restorations, as well as patient comfort, can be increased.

Keywords: Melatonin, boric acid, vancomycin, dentin, caries

INTRODUCTION

The oral cavity is a complex ecosystem where a dynamic balance exists between microbial communities and the host's defense mechanisms. Disruption of this balance can lead to various oral diseases, including dental caries and root decay. Among these, biofilm formation on dentin surfaces is a critical factor in the progression of these conditions. *Escherichia coli (E. coli)* and *Lactobacillus acidophilus (L. acidophilus)* are known to play pivotal roles in biofilm development, contributing to microbial colonization and persistence.[1,2] These biofilms pose a significant challenge in prosthetic dental treatments, where microleakage around crowns and bridges can serve as a nidus for bacterial infiltration, leading to secondary caries and root decay.[3,4]

To address this issue, antimicrobial agents such as melatonin (MEL), boric acid (BOR), and vancomycin (VAN) have garnered attention for their potential efficacy in oral healthcare. These compounds have shown promising antibacterial and anti-inflammatory properties in various contexts.[5,6] Melatonin,

Corresponding Author: Süha Kuşçu, suha.kuscu@yobu.edu.tr

a known antioxidant and antimicrobial hormone, has been shown to inhibit bacterial growth and biofilm formation in the oral cavity.[7] Boric acid, a compound with well-established antibacterial activity, has been reported to disrupt bacterial membranes and biofilm integrity, making it a valuable adjunct in caries prevention.[8] Vancomycin, a glycopeptide antibiotic, remains a gold standard for treating gram-positive bacterial infections, including *E. coli* and *L. acidophilus*, which can contribute to caries and root decay in compromised dental environments.[9] However, the application of these agents in managing biofilms on dentin surfaces, particularly within the scope of prosthetic dentistry, remains underexplored. By targeting bacterial populations responsible for microleakage-related complications, these agents could significantly enhance the longevity and success of prosthetic restorations. When these three agents are combined, the bactericidal effect of vancomycin, the cell permeability-increasing structure of boric acid, and the biofilm-inhibiting property of melatonin may create a synergistic effect. This combination offers a potential strategy to prevent the colonization of microorganisms such as *E. coli* and *L. acidophilus*, which play a role in the development of secondary caries under prosthetic restorations, especially due to microleakage.[10-12]

The current study investigates the antimicrobial effects of MEL, BOR, and VAN on biofilms formed by *E. coli* and *L. acidophilus* on dentin surfaces. Using established methodologies such as the MTT assay and scanning electron microscopy (SEM), we evaluated the viability and structural integrity of biofilms following treatment. This research aims to elucidate the potential of these agents for integration into oral care products, such as mouthwashes, toothpaste, or dissolvable tablets, to prevent biofilm-related complications in prosthetic dentistry. By reducing the bacterial load associated with microleakage, these interventions could mitigate the risk of secondary caries and root decay, thereby improving patient outcomes and prosthesis durability. The null hypothesis of the study was that the combination of vancomycin, melatonin, and boric acid would not have a significantly greater antibiofilm effect compared to single or dual agent applications against caries-associated biofilms caused by *E. coli* and *L. acidophilus* on dentin surfaces affected by microleakage under prosthetic restorations.

METHODS

Ethics

This study received approval from the ethics committee of the Yozgat Bozok University Rectorate Non-interventional Clinical Researches Ethics Committee (Date: 09.04.2025, Decision No: 2025-GOKAEK-257_2025.04.09_443), and all experiments were conducted in accordance with the ethical guidelines of the Helsinki Declaration.

Preparation of Dentin Discs

This study utilized 64 permanent molars extracted for periodontal or orthodontic purposes, all of which maintained intact crowns. To avoid external contamination, the teeth underwent mechanical cleaning using a curette, ensuring no organic or inorganic residues remained on their surfaces. To maintain their biological integrity, the cleaned teeth were stored at room temperature in a 0.01% thymol solution. They were then embedded in 3 cm high and 2 cm wide polyvinyl chloride (PVC) cylinder molds with auto polymerized acrylic resin (Ortho-Jet Resin Acrylic; Lang Dental Manufacturing Co, Illinois, USA). Before completing the polymerization of acrylic resin, the teeth were positioned in molds, set 1 mm above the cementoenamel junction. During sample preparation, a large, water-cooled, low-speed diamond cutting saw (Metkon Microcut 201, HTP High Tech Products, İstanbul, Turkiye) was used to expose the dentin layer. Dentin discs, each 2 mm thick, were created by cutting 3 mm and 5 mm below the occlusal surface. The surfaces of these dentin discs were then polished using a circular medium-grained rotary abrasive tool (Model 902; Brasseler USA). Dentin discs were marked with an acetate pen, utilizing a metal mold that features a 6 mm diameter circular cavity at its center. After marking, a skilled dentist produced standard samples, measuring 6 mm in diameter and 2 mm in thickness, with a water-cooled aerator (Bien-Air Tornado; Bien-Air Dental, Bienne, Switzerland). A digital caliper (Mitutoyo 500-196-30; Mitutoyo Corp., Kawasaki, Japan) was used to verify the accuracy of the measurements. Finally, all prepared samples were rinsed with distilled water and then treated with isopropyl alcohol for 3 minutes to remove any organic and inorganic residues from the surface. To minimize inter-group bias in the study, the samples were randomly assigned to the experimental groups.

Minimum Inhibitor Concentration (MIC) Determination

MIC values of the compounds against bacterial strains were determined using a micro-well dilution method. To determine the MIC values, *E. coli* (ATCC 11229) and *L. acidophilus* (ATCC 11975) in a 12-h Luria-Bertani (LB) broth and DE MAN, ROGOSA and SHARPE (MRS) broth culture, respectively, were adjusted to 0.5 McFarland. Each substance was dissolved in dimethyl sulfoxide (DMSO), and serial twofold dilutions were made in a concentration range of 4 to 512 µg/ml in microplate wells containing nutrient broth. The growth of microorganisms was visually determined after incubation for 24 hours at 35°C. The lowest concentration at which no visible growth (turbidity) was taken as the MIC. Unary, binary, and ternary combinations of MEL, BOR, and VAN were added to the wells in increasing doses and incubated for 24 hours. The well with no turbidity at the end of the period was selected as the MIC value.

Minimal Biofilm Inhibitory Concentration (MBIC)

Firstly, dental discs were transferred into wells to form biofilm on them. Dental discs in a 24-well plate were treated with 500 µl of a 0.5 McFarland bacterial sample and then incubated overnight for 16 hours. At the end of the period, the dental disc samples were incubated for 72 hours to promote biofilm formation, with the medium changed every 24 hours. After the dental disc surface is covered with a bacterial biofilm layer, bacteria that could not adhere were gently washed with DPBS and removed from the medium. Samples were thoroughly vortexed with 500 µl of DPBS and then plated in a new 24-

well plate. Unary, binary, and ternary combinations of MEL, BOR, and VAN were added to the wells in increasing doses and incubated for 24 hours. An MTT assay gives an accurate estimate of the number of viable cells. Thus, we performed an MTT assay according to AFST-EUCAST guidelines. During the experiment, one-part MTT is mixed with nine parts medium (LB Broth for *E. coli* and MRS broth for *L. acidophilus*) and used. The MTT solution, prepared with fresh medium, was added and incubated in the dark for at least 4 hours in the incubator. Then, the MTT solution was withdrawn, and DMSO and 100 µl Sorenson's glycine buffer (glycine 0.1M, NaCl 0.1M, pH 10.5) were added to the medium and left on the mixer in the dark for 15-20 minutes. Samples were loaded onto a 96-well plate without a lid and read at 570-630 nm on a microplate reader. Unary, binary, and triple combinations were evaluated using the obtained absorbance values with the Calcusyn synergy analysis program.

Synergy Model

Synergy measurement by microplate synergy analysis was used to determine the effect of unary, binary, and triple combinations of MEL, BOR, and VAN on potency compared to their activities (Table 2). The antibiofilm effects of MEL, BOR, and VAN were studied for the first time on *E. coli* and *L. acidophilus* dentin biofilm. The MTT cell proliferation assay was used to evaluate the results of the in vitro pharmacodynamic drug interaction analysis of the selected drugs, employing different unary, binary, and ternary drug combinations.[13] Absorbance data (CLARIOstar microplate reader) were loaded for automated calculation of the slope of the median-effect plot (m), the dose that produces 50% effect such as IC50 (Dm), and the linear correlation coefficient of the median-effect plot (r) parameters, as well as the Combination Index (CI) and Dose Reduction Index (DRI) using CalcuSyn software, version 2.11, commonly used to study drug interactions described by Chou[14] and Chou and Talalay.[15]

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was conducted using *E. coli* and *L. acidophilus* biofilms formed on the surface of dentin discs. The samples were washed twice with DPBS and then fixed in 2.5% glutaraldehyde in phosphate buffer for 16 hours. Shortly after, they were refixed in 2% osmium tetroxide for an additional two hours. Then, they were dehydrated through ethanol rinses (30%, 50%, 90%, 95%, and 100%) and mounted and sputter-coated with gold. Sample surfaces were examined using a scanning electron microscope (SEM) (Zeiss LEO 440, Cambridge, UK).

Statistical Analysis

The statistical significance of differences was determined by the one-way analysis of variance (one-way ANOVA) followed by Tukey's test. The SPSS for Windows computer program was used for statistical analyses. The results of test values were reported as mean values ±SD of three independent assays, and differences among groups were considered to be significant at $p<0.05$.

RESULTS

Susceptibility Testing and Synergy Analysis

For the antibacterial activity studies of the test substances, the selected pathogenic gram (-) *E. coli* (ATCC 11229) and *L. acidophilus* (ATCC 11975) bacterial species were used. The plate-well technique was used to calculate the MIC values of single molecules and combinations. Accordingly, the MIC values of the BOR molecule could not be calculated since they were >512 µg/ml ($p<0.05$). MEL MIC values were measured as 16-64 µg/ml ($p<0.05$). VAN MIC value was measured as 16-128 µg/ml ($p<0.05$). When we examined the binary combinations, we found that the concentrations were 16-64 µg/ml for BOR+VAN and 8 µg/ml for MEL+VAN ($p<0.05$). When we examined the triple combinations, the concentration was measured as 4 µg/ml (Table 1).

MTT test was performed to measure the minimal biofilm inhibitory concentration (MBIC) effects of single molecules and combinations. The ratios and effect values used for combinations are explained in Table 2-4. The activity values of the combinations were determined by the Chou-Talalay CI (mass-action law) method. After performing the MTT cell proliferation test for each substance alone against bacteria, CompuSyn software was used to calculate the mass-action law parameters (Dm), (m), and (r). Accordingly, the Dm values (IC50) of the tested substances in *L. acidophilus* were found to be between 78.00, 127.00, and 15.00µg/ml for MEL, BOR, and VAN, respectively. In *E. coli*, the Dm values (IC50) of the tested substances were between 35.00, 172.00, and 112.00µg/ml for MEL, BOR, and VAN, respectively. The % inhibition of the tested substances in *L. acidophilus* ranged from 77.35%, 70.21%, and 6.42% for MEL, BOR, and VAN, respectively. In *E. coli*, the % inhibition of the tested substances ranged from 54.54%, 148.49%, and 13.58% for MEL, BOR, and VAN, respectively (Table 5, 6). The Dose Reduction Index (Fa-DRI) for MEL, BOR, and VAN combinations are presented in Table 7, 8 respectively. The Chou-Talalay method for drug combination is based on the median effect equation, which provides the theoretical basis for the CI, which allows the quantitative determination of drug interactions where CI <1, =1, and >1 indicate synergy, additive effect, and antagonism (Table 4). Accordingly, in *L. acidophilus*, the Dm values (IC50) of the tested binary and triple combinations were between 9.41-13.56 and 6.25 µg/ml, respectively. In *E. coli*, the Dm values (IC50) of the tested binary and triple combinations were between 14.86-59.78 and 5.68 µg/ml, respectively.

Table 1. MIC value of unary, binary, and triple combinations of the agents

							One-way ANOVA	
MIC (µg/ml)	**BOR**	**MEL**	**VAN**	**BOR VAN**	**MEL VAN**	**MEL BOR VAN**	**F**	**Sig.**
E. coli	ND	16^{a*}	128^{c}	64^{b}	8^{a}	4^{a}	344.41	.000
L. acidophilus	ND	64^{c}	16^{b}	16^{b}	8^{a}	4^{a}	339.69	.000

*Values followed by the same letter in the row are not significantly different, MIC: Minimum inhibitor concentration, ANOVA: Analysis of variance, BOR: Boric acid, MEL: Melatonin, VAN: Vancomycin, Sig.: Significance, *E. coli: Escherichia coli, L. acidophilus: Lactobacillus acidophilus,* ND: Not detected

Table 2. Concentrations of substances used (µg/ml)		
MEL	BOR	VAN
25	37.5	12.5
50	75	25
100	150	50
200	300	100
400	600	200

MEL: Melatonin, BOR: Boric acid, VAN: Vancomycin

Table 3. Combination ratios used in the study		
MEL 2	MEL+VAN 2/1	MEL+BOR+VAN 2/3/1
BOR 3	BOR+VAN 3/1	
VAN 1		

MEL: Melatonin, BOR: Boric acid, VAN: Vancomycin

This study evaluated the synergistic-antagonistic effects of MEL, BOR, and VAN combinations with CI values for fa=0.5. Accordingly, when the binary and triple combinations tested in *L. acidophilus* were examined at fa=0.5, MEL+VAN (0.83) and MEL+BOR+VAN (0.77) showed a moderate synergistic effect, and BOR+VAN (0.94) displayed an additive impact (Table 5). When the binary and triple combinations tested in *E. coli* were examined at fa=0.5, MEL+VAN (0.78) and MEL+BOR+VAN (0.56) showed synergistic effects, and BOR+VAN (0.64) exhibited a moderate synergistic impact (Table 6).

Table 4. CI method			
Range of CI	Description	Range of CI	Description
<0.1	Very strong synergy	1.10-1.20	Mild antagonism
0.1-0.3	Strong synergy	1.20-1.45	Moderate antagonism
0.3-0.7	Synergy	1.45-3.3	Antagonism
0.7-0.85	Moderate synergy	3.3-10	Strong antagonism
0.85-0.90	Light synergy	10>	Very strong antagonism
0.90-1.10	Additive		

CI: Combination Index

Table 5. Parameters were calculated from the median effect equation and median effect plot. 'm' is the slope, and m=1, >1 and <1 indicate hyperbolic, sigmoidal, and flat sigmoidal shape, respectively; 'Dm' denotes power; and 'r' is the linear correlation coefficient					
	CI values at				
L. acidophilus	ED50	Dm	m	r	% inhibition
MEL	N/A	78.00	0.83	0.95	77.35
BOR	N/A	127.00	0.68	0.94	70.21
VAN	N/A	15.00	0.75	0.99	6.42
MEL+VAN	0.83	9.41	1.06	0.96	
BOR+VAN	0.94	13.56	1.27	0.97	
MEL+BOR+VAN	0.77	6.25	1.15	0.98	

L. acidophilus: Lactobacillus acidophilus, CI: Combination Index, MEL: Melatonin, BOR: Boric acid, VAN: Vancomycin

Table 6. Parameters were calculated from the median effect equation and median effect plot. 'm' is the slope, and m=1, >1 and <1 indicate hyperbolic, sigmoidal, and flat sigmoidal shape, respectively; 'Dm' denotes power; and 'r' is the linear correlation coefficient					
	CI values at				
E. coli	ED50	Dm	m	r	% inhibition
MEL	N/A	35.00	1.05	0.95	54.54
BOR	N/A	172.00	1.04	0.96	148.49
VAN	N/A	112.00	1.11	0.95	13.58
MEL+VAN	0.78	14.86	1.15	0.95	
BOR+VAN	0.64	59.78	1.09	0.94	
MEL+BOR+VAN	0.56	5.68	0.90	0.98	

E. coli: Escherichia coli, CI: Combination Index

This study also focused on determining the appropriate Dose Reduction Index (DRI) for the dual and triple drug combinations based on actual experimental data points. The Fa-DRI table shows the results. DRI, DRI=1, >1 and <1 indicate no dose reduction, appropriate dose reduction, and inappropriate dose reduction for each drug in the combination, respectively. Typically, the primary objective of combination therapy is to achieve synergistic effects (CI<1) by reducing the dose of specific toxic drugs (DRI>1) and, consequently, to minimize the likelihood of drug resistance. Accordingly, when the Fa-DRI table was examined in detail at fa=0.5 (Table 7, 8), at 50% inhibition (fa=0.5) in *L. acidophilus*, the binary combinations (1.41-3.15) showed an appropriate dose reduction (DRI>1). The triple combinations MEL/BOR/VAN (3.25/3.14/1.46) showed an appropriate dose reduction (DRI>1). At 50% inhibition (fa=0.5) in *E. coli*, the binary combinations (1.44-4.82) showed an appropriate dose reduction (DRI>1), while the triple combination MEL/BOR/VAN (4.59/9.11/1.51) showed an appropriate dose reduction (DRI>1). These results suggest that the combined use of MEL/BOR has the potential to significantly enhance the effectiveness of dental caries treatment.

Table 7. DRI, DRI=1, >1, and <1 indicate no dose reduction, appropriate dose reduction, and inappropriate dose reduction for each drug in the combination, respectively						
L. acidophilus	Drug alone			DRI		
Fa	MEL	VAN		MEL	VAN	
0.5	78.00	15.00		3.15	1.44	
Fa	BOR	VAN		BOR	VAN	
0.5	127.00	15.00		2.77	1.41	
Fa	MEL	BOR	VAN	MEL	BOR	VAN
0.5	78.00	127.00	15.00	3.25	3.14	1.46

DRI: Dose Reduction Index, *L. acidophilus,* MEL: Melatonin, VAN: Vancomycin, BOR: Boric acid

SEM Analysis

When the SEM images in Figure are evaluated using ImageJ software, it becomes clear that BOR does not exhibit antibiofilm properties on its own. In the control dentin disc surface images for both *E. coli* and *L. acidophilus*, it is evident that the area is covered with a greater number of bacteria.

Table 8. DRI, DRI=1, >1, and <1 indicate no dose reduction, appropriate dose reduction, and inappropriate dose reduction for each drug in the combination, respectively

E. coli	Drug alone		DRI			
Fa	**MEL**	**VAN**	**MEL**	**VAN**		
0.5	35.00	112.00	1.55	1.44		
Fa	**BOR**	**VAN**	**BOR**	**VAN**		
0.5	172.00	112.00	4.82	2.26		
Fa	**MEL**	**BOR**	**VAN**	**MEL**	**BOR**	**VAN**
0.5	35.00	172.00	112.00	4.59	9.11	1.51

DRI: Dose Reduction Index, *E. coli: Escherichia coli,* **MEL: Melatonin, VAN: Vancomycin, BOR: Boric acid**

When the SEM images of BOR and MEL are compared with BOR+VAN and MEL+VAN for *E. coli* and *L. acidophilus*, it is seen that the single combinations are less effective.

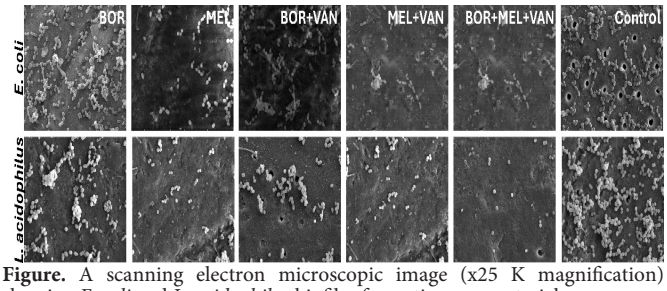

Figure. A scanning electron microscopic image (x25 K magnification) showing *E. coli* and *L. acidophilus* biofilm formations on materials

E. coli: Escherichia coli, L. acidophilus: Lactobacillus acidophilus

When both bacterial biofilms are evaluated for BOR+MEL+VAN, it is determined that the highest antibiofilm effect occurs. In accordance with the MIC and Synergy tests, when the SEM images are examined, the potency order of BOR+MEL+VAN > MEL+VAN > BOR+VAN > MEL > BOR is revealed. When SEM analysis images for E. coli were examined in five different areas, biofilm removal was 50%, 10%, 15%, 5%, and 3% on the disk surfaces applied with BOR, MEL, BOR+VAN, MEL+VAN, and BOR+MEL+VAN, respectively (Figure). Similarly, when SEM analysis images for L. acidophilus were examined in five different areas, biofilm removal was 40%, 7%, 10%, 5%, and 2% on the disk surfaces applied with BOR, MEL, BOR+VAN, MEL+VAN, and BOR+MEL+VAN, respectively (**Figure**).

DISCUSSION

This study aims to investigate the antimicrobial activity of melatonin and boric acid against *L. acidophilus* and *E. coli* bacteria that cause dental caries in human dentin tissue. Based on the findings of our study, our null hypothesis was rejected. Vancomycin was determined as the active ingredient in combination studies and used as the reference antimicrobial agent.[16] The findings revealed that the triple combination showed the highest antimicrobial activity, while single molecules showed lower inhibition. Previous studies have demonstrated that *L. acidophilus* plays a crucial role in the progression of dental caries, producing acidic metabolites that demineralize the dentin matrix.[17] *E. coli*, on the other hand, is not a dominant microorganism in the normal oral flora, has been associated with dental infections and periodontal diseases, and has been reported to trigger tissue damage by secreting bioactive molecules.[18] The antimicrobial activity of melatonin can be attributed to its effects on oxidative stress and bacterial metabolism. In addition to its antioxidant and immunomodulatory properties, melatonin is reported to have antibacterial effects by affecting bacterial virulence factors of oxidative stress.[19,20] It is well established that oxidative stress plays a significant role in the pathogenesis of periodontal diseases, and that melatonin promotes healing in gingival tissues.[21] Boric acid is defined as an element that disrupts bacterial membrane stability and inhibits metabolic activity.[22,23] Additionally, it has been reported that boric acid promotes remineralization in dental tissues and contributes to stabilizing the hydroxyapatite crystal structure.[24] Considering the clinical and academic importance of this study, the potential effects of combining melatonin and boric acid applications should be evaluated in the future.

The SEM analysis of the dentin disc surfaces revealed significant differences in the antibiofilm efficacy of the various treatment combinations. BOR alone showed minimal antibiofilm activity, with SEM images indicating a high bacterial density for both *E. coli* and *L. acidophilus* on the treated surfaces. This was further confirmed by the low biofilm removal percentages, which were recorded as 3% for *E. coli* and 2% for *L. acidophilus*. In contrast, the combination of BOR with MEL+VAN demonstrated a stronger antibiofilm effect, though still inferior to the triple combination. The highest biofilm removal efficacy was observed with the triple combination of BOR+MEL+VAN, with biofilm removal rates of 50% for *E. coli* and 40% for *L. acidophilus*, indicating a significant synergistic effect. These findings align with the MIC and synergy tests, which revealed that the efficacy of the combinations followed the order: BOR+MEL+VAN > MEL+VAN > BOR+VAN > MEL > BOR. In particular, further research is required on the pharmacokinetic profile, dosage, and administration methods to optimize the antibacterial activity of MEL+VAN and BOR+MEL+VAN combinations.

The substantial reduction in biofilm formation observed with the triple combination emphasizes its potential as an effective therapeutic option for preventing caries associated with microleakage under prosthetic restorations. Future in vivo and clinical studies will more clearly demonstrate the usability of these bioactive compounds in the prevention and treatment of dental caries.

Limitations

This study was conducted in vitro, which may limit the generalizability of the results obtained to clinical practice. The oral cavity is a complex environment where numerous variables, including saliva, pH fluctuations, enzymatic activity, mechanical stress, and microbial diversity, interact. Therefore, it is not guaranteed that the antimicrobial effects obtained under laboratory conditions will be observed at the same level in vivo. In addition, although an expert dentist performed the preparation of dentin discs according to standard protocols, it is essential to consider that patient-derived biological samples may exhibit individual variations.

Confirmation of these findings with future animal models or clinical studies will further strengthen the scientific validity of the study.

CONCLUSION

Today, the robust antimicrobial agents investigated in this study are crucial in dentistry because of their beneficial properties. They are particularly significant in clinical settings. Maintaining tooth integrity is critical for oral health. Therefore, it is crucial to minimize the microbial load from pathogens such as *L. acidophilus* and *E. coli* on dentin discs. The findings from this study will aid in the development of effective antimicrobial agents for oral hygiene and their implementation in clinical practice.

ETHICAL DECLARATIONS

Ethics Committee Approval

The study was carried out with the permission of the Yozgat Bozok University Rectorate Non-interventional Clinical Researches Ethics Committee (Date: 09.04.2025, Decision No: 2025-GOKAEK-257_2025.04.09_443).

Informed Consent

Since extracted human teeth without caries, resorption or fractures are used, informed consent is not required.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request. All data analyzed during this study are included in this published article as tables and figures.

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