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EVALUATION OF ANTIBACTERIAL EFFICACY OF *LACTOBACILLUS* PROBIOTIC ON *STREPTOCOCCUS MUTANS* – AN IN VITRO STUDY

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ABSTRACT

The oral cavity consists of a highly diverse microbiome that plays significant role in maintaining oral and systemic health. Disruption in the microbial balance, known as dysbiosis, is a key factor in the development of dental caries. This study explores the concept of bacteriotherapy using probiotic strains of *Lactobacillus* to suppress the cariogenic pathogen *Streptococcus mutans*. We assessed the antibacterial efficacy and determined the Minimum Inhibitory Concentration (MIC) of three commercially available probiotics *Lactobacillus rhamnosus GG*, *Lactobacillus plantarum*, and *Lactobacillus casei Shirota* against *S mutans* MTCC-497. The results may help us in incorporating the probiotic-based strategies for oral health management and caries prevention.

Introduction

The human body harbours a vast and diverse microbiota. These microorganisms inhabit anatomical regions like the skin, oral cavity, and gastrointestinal tract, each offering distinct microenvironments defined by specific physicochemical parameters such as pH, oxygen tension, and nutrient availability [1]. This ecological partitioning facilitates region-specific colonization, emphasizing the microorganisms' evolutionary adaptations to their habitats. Disruptions to the balance of these bacteria, called dysbiosis, can be linked to diseases like obesity, malnutrition, various inflammatory conditions and also oral diseases such as dental caries [2]. The Ecological Plaque Hypothesis, proposed by Marsh in 1994[3] and refined by Takahashi in 2008, emphasizes the role of plaque's metabolic activity in promoting

microbial imbalance. If acid production surpasses the buffering capacity of basic metabolites, the environment becomes acidic. This favours acidogenic and acid tolerant bacteria facilitates caries development [4].To counteract the imbalance, bacteriotherapy uses harmless bacteria or probiotics to combat pathogenic bacteria such as S.mutans. Probiotics are live microorganisms, which when administered in adequate amounts, confer health benefits on the host [5]. Their primary role is to rebalance and maintain a healthy microbiome, by inhibiting the growth of pathogenic microorganisms. They produce antibacterial compounds like lactic acid, hydrogen peroxide, bacteriocins, and biosurfactants [6, 7]. These molecules act by lowering pH, competing for binding sites, modulating host immunity, and even influencing gene expression. Oral probiotics are primarily administered in children as their nascent dental biofilm presents a more malleable microbial milieu, facilitating probiotic colonization and competitive exclusion of pathogens. But they face challenges in gaining widespread acceptance. Factors such as limited public awareness, lack of standardization in strains and dosages, insufficient clinical evidence, cost, scepticism from traditional dental practices, and focused marketing primarily on gut health hinder their popularity. Increased awareness, more research, and better regulation could help them gain more mainstream acceptance. Species of *Lactobacillus*, including *L. rhamnosus*, *L. plantarum*, and *L. casei*, are among the most studied probiotics for oral health [8]. These Gram-positive, catalase-negative rods are known for their lactic acid production and have been classified as "Generally Recognized As Safe" GRAS by the Food and Drug Administration. Despite their safety, their efficacy in the oral environment must be validated through microbiological assays. The crucial parameters in determining effectiveness is the antibacterial efficacy and the Minimum Inhibitory Concentration (MIC).MIC is defined as the lowest concentration required to inhibit visible bacterial growth after incubation. Taking these factors into consideration, the present study was done with these aim and objectives.

Materials and Methods

Aim:

To examine the antibacterial efficacy and determine the MIC of three commercially available *Lactobacillus* probiotics against *S. mutans* MTCC-497.

Probiotics Tested:

Group 1: The Good Bug - Lactobacillus rhamnosus GG
Group 2: The Good Bug - Lactobacillus plantarum
Group 3: Yakult - Lactobacillus casei shirota

Bacterial Strain:

Streptococcus mutans MTCC-497

Methods:

1. **Agar Well Diffusion Assay** - To assess the antibacterial efficacy of the probiotic strains against *S. mutans*. Zones of inhibition were measured to compare effectiveness.

2. **Micro-broth Dilution Assay** - To determine the MIC values, indicating the lowest concentration of probiotic required to inhibit bacterial growth.

RESULTS

AGAR WELL DIFFUSION ASSAY AGAINST *S.MUTANS* MTCC-497

Table 1:

GROUPS	PROBIOTICS	ZONE OF INHIBITION (mm) IN PETRI PLATES			
		50 µL	100 µL	150 µL	AMPICILLIN (disc)
Group 1	*L. rhamnosus GG 1*	8 mm	8 mm	8 mm	35 mm
	L. rhamnosus GG 2	8 mm	8 mm	8 mm	35 mm
Group 2	*L. plantarum 1*	8 mm	8 mm	8 mm	35 mm
	L. plantarum 2	8 mm	8 mm	8 mm	35 mm
Group 3	*L. casei shirota 1*	14 mm	13mm	15 mm	35 mm
	L. casei shirota 2	12 mm	15mm	15 mm	35 mm

Distribution of zone of inhibition (in millimeter) for three commercially available probiotics and Ampicillin (positive control) against *Streptococcus mutans* MTCC-497

Table 2: Mean and standard deviation of zone of inhibition diameter in three different groups of probiotics at different concentrations

Descriptive Statistics						
Groups	**Concentration (µL)**	**N**	**Minimum (mm)**	**Maximum (mm)**	**Mean**	**Std. Deviation**
Group 1	50	2	8	8	8.00	.000
	100	2	8	8	8.00	.000
	150	2	8	8	8.00	.000
Group 2	50	2	8	8	8.00	.000
	100	2	8	8	8.00	.000
	150	2	8	8	8.00	.000
Group 3	50	2	12	14	13.00	1.414
	100	2	13	15	14.00	1.414
	150	2	15	15	15.00	.000
Ampicillin		2	35	35	35.00	.000

Table 3: Comparison of three groups of probiotics at 50, 100 and 150 µl concentrations - Kruskal Wallis test

Concentration (µL)		**N**	**Mean (mm)**	**Std. Deviation**	**p-value**
50	**Group 1**	2	8.00	.000	0.091
	Group 2	2	8.00	.000	
	Group 3	2	13.00	1.414	
100	**Group 1**	2	8.00	.000	0.091
	Group 2	2	8.00	.000	
	Group 3	2	14.00	1.414	
150	**Group 1**	2	8.00	.000	0.082
	Group 2	2	8.00	.000	

	Group 3	2	15.00	.000	

MICRO BROTH DILUTION ASSAY AGAINST *STREPTOCOCCUS MUTANS* MTCC 497

Table 4: Minimum Inhibitory Concentration (MIC) for three commercially available probiotics against *Streptococcus mutans* MTCC-497

GROUPS	PROBIOTICS	MIC VALUES
GROUP 1	*L. rhamnosus GG 1*	400 µl
	L. rhamnosus GG 2	400 µl
GROUP 2	*L. plantarum 1*	50 µl
	L. plantarum 2	50 µl
GROUP 3	*L. casei shirota 1*	25 µl
	L. casei shirota 2	100 µl

Table 5: Pairwise comparison of zone of inhibition mean values between groups - Post Hoc Tests – Tukey Honestly Significant Difference (HSD) test

Concentration	(I) Groups	(J) Groups	Mean Difference (I-J)	Sig.
50	Group 1	Group 2	.000	1.000
		Group 3	-5.000*	.018*
	Group 2	Group 1	.000	1.000
		Group 3	-5.000*	.018*
	Group 3	Group 1	5.000*	.018*
		Group 2	5.000*	.018*
100	Group 1	Group 2	.000	1.000
		Group 3	-6.000*	.011*
	Group 2	Group 1	.000	1.000
		Group 3	-6.000*	.011*
	Group 3	Group 1	6.000*	.011*
		Group 2	6.000*	.011*
150	Group 1	Group 2	.000	1.000
		Group 3	-7.000	.016*
	Group 2	Group 1	0.000	1.000
		Group 3	-7.000	.016*
		Group 1	7.000	.016*

		Group 2	7.000	**.016***

DISCUSSION

Oral microbiome is a dynamic polymicrobial consortium that maintains host homeostasis through competitive exclusion of pathogenic microorganisms, quorum-sensing-regulated biofilm dynamics and immune optimization [9]. Oral dysbiosis refers to an imbalance or disruption in the oral microbial community; arise from factors such as neglected oral care, improper diet and usage of medications. This in turn can lead to dental caries, periodontitis and oral cancers [10]. In dysbiosis, certain microorganisms multiply excessively, initiating biofilm formation. This aligns with the ecological plaque hypothesis, which suggests that oral disease isn't caused by a single pathogen but rather by an imbalance where some microbial species outnumber others within the oral ecosystem. Therefore, the therapeutic strategies should prioritize on regulating and controlling the environment that causes the microbiome shift instead of depending primarily on antimicrobial therapy. This insight has led to research targeted on interventions that restore and maintain the microbial balance in the oral microbiome in a non-invasive natural way. Dental caries is defined as a dysbiosis-regulated biofilm-mediated disease, considered a significant public health concern [11]. Fluoride being the benchmark for prevention of caries, the efficacy of fluoride on dental biofilm is limited. Recent developments for combating caries focus on maintaining the ecology of oral cavity with an objective of preventing dysbiosis. The oral cavity has a complex intricate ecological system consisting of more than thousand bacterial species which is responsible for maintaining the homeostasis [12]. Dental caries is triggered by the interaction between cariogenic microbiota, diet and the host. Disrupting any one of these factors can halt the progression of dental caries [13]. This study focuses on minimizing the population of cariogenic bacteria, particularly S.mutans. The disequilibrium in the numbers of native bacteria and pathogenic strains can be emphasized as key element for the onset of caries. Therefore, to maintain the microbial stability, competitiveness among microorganisms appears to be an innovative method in preventing the cariogenic microflora from initiating its niche (hard palate, soft palate, tongue, floor of the mouth, saliva, gingival sulcus and teeth) within the oral ecosystem. Such intervention approaches which enhance the growth of health promoting bacteria called as bacteriotherapy or microbiome therapy or probiotic therapy. This paradigm is grounded in the concept of sustaining or reconstituting the indigenous oral microbiota through strategic microbial interference, targeted suppression of pathogenic species, or a synergistic interplay of both mechanisms [14].

Microorganisms such as *Lactobacilli, Bifidobacterium* and *Bacillus* species are identified as probiotic agents. The European Food Safety Authority (EFSA) and FDA approved these probiotic agents and renowned GRAS. Bifidobacterium and Bacillus have no documented support for use as oral probiotics. In this study, the choice of lactobacilli strains- Group I: L. rhamnosus GG (The Good bug-supplement), Group II: L. plantarum (The Good bug-supplement) and Group III: L. casei shirota (Yakult-fermented milk) for oral probiotics was driven by their ability to produce lactic acid, adhere to oral surfaces, modulate immune responses, inhibit specific pathogenic bacteria such as S. mutans and the selection of species was done based on the literatures. In this study, we use products which consist of single probiotics rather than the combination of probiotics. The use of such single-strain probiotic products allows for a precise evaluation of their antibacterial effectiveness and enables the identification of the most effective probiotic. By focusing on a single strain, we can accurately determine its specific impact on pathogenic bacteria, such as S. mutans, without interference from other probiotic species. This approach helps in understanding its mechanism of action, optimal dosage, and potential for clinical applications in preventing dental caries.

In the oral cavity, biological mechanism of *Lactobacillus* includes the production of antibacterial substances that suppresses the growth, the co-aggregation and inhibition of adhering capacity of S. mutans to the tooth surface, also modulates the virulence-related genes in S. mutans and host immune response. Biosurfactant- like substances and bacteriocin-like substances secreted by Lactobacillus penetrates and disintegrates the biofilm formed by S.mutans. They can sustain a pH of 3.5 because of their aciduric nature [15]. This accounts for its heightened recognition and scholarly focus within the scope of our study on dental caries prevention [16].

This above study evaluated the antibacterial efficacy of three probiotic strains - *L. rhamnosus GG, L. plantarum, and L. casei Shirota* —against *S. mutans* MTCC 497, a primary etiological agent in dental caries pathogenesis. To ascertain the antibacterial potential of these probiotics, a standardized agar well diffusion assay was employed, wherein the diameter of the zone of inhibition served as a determinant of susceptibility profiling. Additionally, two cardinal microbiological parameters, the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC), were evaluated. The MIC evaluated helps to determine the minimal concentration required to effectively suppress the proliferation of bacteria. The MBC was designated to ascertain the minimal inhibitory threshold required for complete bacterial eradication over an extended period of 24 hours. A 24 - hour period ensures that any delayed bactericidal effects can be observed under standardized conditions, minimizing

variability and enhancing the reproducibility of results. Here, in this study, supernatant of the probiotic was used. The supernatant of a probiotic refers to the liquid portion obtained after centrifuging the probiotic culture, which was incubated for a period of three weeks, separated the bacterial cells from the surrounding fluid. This supernatant consists of bioactive compounds. Among the three probiotic strains examined, Group III (*L. casei Shirota*) exhibited the most profound antibacterial efficacy against S. mutans, compared to the performance of Group II (*L. plantarum*) and Group I (*L. rhamnosus GG*). Experimental findings indicated a mild zone of inhibition of about 15 mm (Table 1) by Group III (*L.casei shirota*) at 50 µl concentration and bactericidal effect was found at 25 µl (Table 4), thereby exhibiting superior bacteriostatic and bactericidal activity. Conversely, L. plantarum demonstrated no antibacterial efficacy at all concentrations 50, 100, 150 µl and comparatively modest bactericidal effect at 50 µl. Similarly, *L. rhamnosus GG* demonstrated no antibacterial efficacy and required a substantially higher MBC of 400 µl to achieve bactericidal effect. These results, summarized in Table 4, affirm that Group III (*L. casei Shirota*) exhibited significantly higher antibacterial efficacy compared to Group I (*L. rhamnosus GG*) and Group II (*L. plantarum*). The underlying mechanism of action of these lactic acid bacteria is hypothesized to be multifaceted, primarily involving the biosynthesis of organic acids predominantly lactic acid and antimicrobial peptides. The release of lactic acid contributes to a localized reduction in environmental pH, thereby fostering an inhospitable milieu for the proliferation of cariogenic bacteria.

Concurrently, antimicrobial peptides disrupt the bacterial cell walls, culminating in cellular membrane destabilization and bacterial lysis. Corroborating these findings, Chen Huizhen's research delineates that L. casei Shirota exerts inhibitory effects on the virulence gene expression of S. mutans, thereby mitigating formation of biofilm and acidogenicity in vitro. Furthermore, the supernatant of L. casei Shirota has been demonstrated to contain bacteriostatic and bactericidal compounds, which exhibit a pH-dependent yet thermally stable antibacterial activity against S. mutans within oral biofilm communities [17].

The clinical efficacy of probiotic interventions is inherently contingent upon multiple critical factors, including strain specificity, duration of therapeutic administration, optimal concentration thresholds, and the compatibility of delivery vehicles. The findings of this study corroborate the notion that probiotics serve as an adjunct in mitigating *S. mutans* colonization, thereby exerting a prophylactic effect against dental caries development. Importantly, probiotics do not cause any side effects associated with antimicrobial therapies, making them a viable alternative. Their ability to regulate inflammatory responses and to maintain microbiota

balance has garnered attention for their use as adjunctive therapies in preventing dental caries and other diseases. A significant advantage of probiotic therapy is its safety profile, allowing for long-term use without the risk of adverse effects [18].

Notably, among the probiotic strains tested, Group III (*L. casei Shirota*) exhibited the most pronounced antibacterial efficacy and bactericidal potential compared to other probiotics, reinforcing its therapeutic plausibility in precision-driven microbiome modulation strategies for the restoration of oral microbial homeostasis and the attenuation of cariogenic biofilm progression.

CONCLUSION

L. casei Shirota group III exhibited the largest zone of inhibition of about 15 mm, suggesting stronger antibacterial activity compared to group I (L. rhamnosus GG) and group II (L. plantarum), which had no antibacterial efficacy. The MBC values indicate that Group III (*L. casei Shirota)* required a lower concentration of about 25 µL to achieve complete bacterial elimination. In contrast, Group I (*L. rhamnosus GG)* and Group II (*L. plantarum)* required higher concentrations of about 400 µL and 50 µL respectively, suggesting weaker bactericidal effects compared to Group III (*L. casei shirota).*

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