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RESEARCH ARTICLE

EFFECT OF LACTOBACILLUS ACIDOPHILUS AND STREPTOCOCCUS SALIVARIUS ON GROWTH OF PERIODONTAL PATHOGENS – AN IN-VITRO STUDY.

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Keywords:-

Probiotics, pathogens, in-vitro, periodontal disease.

Abstract

Background: Probiotics are defined as “live bacteria that naturally occur in certain foods as live micro-organisms which, when administered in adequate amount, confer a health benefit on the host.” They are safe for human consumption only when ingested in certain quantity, have beneficial effects on human health. Literature pertaining to the antimicrobial activity of probiotics is very limited. So this study was conceptualized to comprehensively report the antimicrobial potential of probiotics against periodontal pathogens.

Objectives: To determine the effect of Lactobacillus Acidophilus and Streptococcus Salivarius probiotics on periodontal pathogens, (Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans and Prevotella intermedia) in-vitro using well diffusion method.

Methods: Actinobacillus actinomycetemcomitans was cultured using tryptic soy-serum-bacitracin-vancomycin-fluoride (TSBVF) agar, Porphyromonas gingivalis was cultured using modified Wilkins-Chalgren (MWC) medium and Prevotella intermedia was cultured using brain heart infusion agar and incubated in Brewer-like anaerobic jars. Plates were inoculated with the probiotic strain of the lactobacillus acidophilus and Salivarius. Zone of inhibition was measured and compared with unaided eye using a Vernier caliper measuring the zone sizes.

Results: Comparison of Zone of Inhibition after 24 hours and 48 hours using one way ANOVA test showed statistically significant results with inoculation of probiotics. Post hoc Tukey test compared within groups that showed the statistically significant difference between all organisms except for within A. actinomycetemcomitans and Prevotella intermedia.

Conclusions: Lactobacillus and S. salivarius had inhibitory effect on periodontal pathogens in the oral microflora.

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Introduction:-

Probiotics are defined as “live micro-organisms which, when administered in adequate amount, confer a health benefit on the host.”¹ They are relatively safe for human when consumed and ingested in sufficient quantities, have beneficial effects on human health.²

Several mechanisms have been proposed to explain the mode of action of probiotics. These bacteria secrete various antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins, which have bacteriostatic effect on pathogens. Probiotics also compete with pathogenic agents for adhesion sites on the mucosa and competitively inhibit them from their adhesion sites. Probiotics can modify the surrounding environment by modulating the pH or the oxidation reduction potential, which compromises the ability of pathogens to establish in the oral cavity. Finally, probiotics may provide beneficial effects by stimulating nonspecific immunity and modulating the humoral and cellular immune response which in-turn will activate the mediators of phagocytosis.²

Gingivitis is “inflammation of the gingival tissues surrounding the tooth. Bacterial attachment on the tooth surface and co-adhesion of initial colonizers with other species initiate the formation of biofilm.” Mature biofilm is also known as dental plaque, triggers a series of events that leads to gingival inflammation. When the bacterial biofilm is removed the gingival tissues return to their healthy state.³ Thus, it is called a reversible disease as since there is no loss of hard tissues that supports the teeth.

Given the widespread emergence of bacterial resistance to antibiotics, the concept of probiotic therapy has been applied for benefit of oral health. For a probiotic microorganism to represent a of interest for the benefit of oral health is its ability to adhere to tooth surfaces and colonize in the oral cavity.⁴

Literature pertaining to the antimicrobial activity of probiotics on periodontal bacteria has been very limited. Therefore, the present study was conceptualized as an initial step to comprehensively report the antimicrobial potential of probiotics against periodontal pathogens in-vitro using well diffusion method.

Materials and Method:-

The study was an in-vitro study. The study was conducted in the Department of Public Health Dentistry, MCODS Mangalore and Department of Microbiology, Maratha Mandal Nathajirao G Halgekar Institute of Dental Sciences and Research Centre Belgaum. Ethical approval was obtained from institutional ethics committee MCODS Mangalore. (Protocol number 17072)

Collection of materials:-

Commercially available Probiotic strains of *S. salivarius* and *Lactobacillus acidophilus* were obtained from online Neoflora Company in Mangalore city. Authenticity of the organisms was confirmed by from the Department of Microbiology, Maratha Mandal's NGH Institute of Dental Sciences & Research Centre, Belgaum, Karnataka, India. Pure cultures of *P. gingivalis*, *P. intermedia* and *Actinobacillus actinomycetemcomitans* were obtained from the Department of Microbiology, Maratha Mandal's NGH Institute of Dental Sciences & Research Centre, Belgaum, Karnataka, India. Culture media was used for organisms *Actinobacillus actinomycetemcomitans* was tryptic soy-serum-bacitracin-vancomycin-fluoride (TSBVF) agar. *Porphyromonas gingivalis* was cultured using modified Wilkins-Chalgren (MWC) medium and *Prevotella intermedia* was cultured using brain heart infusion agar and incubated in Brewer-like anaerobic jars for 48 hours.

Antimicrobial Tests:-

About 15-20 ml Mueller-Hinton agar was poured and allowed to solidify on glass petro plates of same size. All plates had agar surfaces, which was streaked by a sterile cotton swab with the known bacterial strain. Plates were punched with a sterile cork borer of 4 mm size. About 100 µL of each sample were poured with micropipette into the bore. For 30 min plates were kept. Then plates were incubated at 37°C for 48 hours. That's how Lawn cultures were made for these pathogens. Three culture plates were done per organism, resulting in a total of 12 plates for the four pathogens. The first plate was inoculated with the strain of the probiotic *Lactobacillus acidophilus*, the second plate was inoculated with the strain of the probiotic *S. salivarius* and the third plate was kept as the control. This procedure was carried out for all the four pathogens. They were then incubated for 24 and 48 hours. After incubation, the zone of inhibition was measured and compared with unaided eye using a Vernier caliper measuring the zone sizes to the nearest millimeter.

Results:-

When *Lactobacillus* was inoculate, comparison of Zone of Inhibition (ZOI) was done after 24 hours using one way ANOVA test showed that the mean of Zone of Inhibition value is highest in *P. gingivalis* (1.224) than *A. actinomycetemcomitans* (1.188) and *P. intermedia* (table no 1). This difference is statistically significant with a test value of 59.13(p <0.001). Post hoc Tukey test showed that the difference between *P. gingivalis* and *A.*

actinomycetumcomitans was statistically significant with a mean difference of 0.188 ($p < 0.001$). When Zone of Inhibition of *P. intermedia* and *P. gingivalis* was compared it was found to be statistically significant with a mean difference of -0.224 ($p < 0.001$).

Comparison of ZOI was done after 48 hours using lactobacillus, one way ANOVA test showed that the mean Zone of Inhibition value is highest in *A. actinomycetumcomitans* (1.416) followed by *P. gingivalis* (1.304) least in *P. intermedia* (1.086). This difference in Zone of Inhibition was statistically significant with a test value of 124.578 ($p < 0.001$). Post hoc Tukey test showed that the difference between *P. gingivalis* and *A. actinomycetumcomitans* was statistically significant with a mean difference of 0.33 ($p < 0.001$). The difference in Zone of Inhibition between *Actinomycetumcomitans* and *P. gingivalis* was statistically significant with a mean difference of 0.112 ($p < 0.001$). The difference between *P. intermedia* and *P. gingivalis* was also statistically significant with a mean difference of 0.218 ($p < 0.001$).

When *S. salivarius* was inoculated, comparison of ZOI mm was done after 24 hours using one way ANOVA test showed that the mean Zone of Inhibition value was highest in *A. actinomycetumcomitans* (0.81) followed by *P. gingivalis* (0.714) least in *P. intermedia* (0.598). This difference was statistically significant with a test value of 184.8 ($p < 0.001$). The difference between mean Zone of Inhibition of *A. actinomycetumcomitans* and *P. gingivalis* was statistically significant with a mean difference of 0.096 ($p = 0.003$). The difference between *A. actinomycetumcomitans* and *P. intermedia* was statistically significant with a mean difference of 0.212 ($p < 0.001$). The difference between Zone of Inhibition of *P. gingivalis* and *P. intermedia* was statistically significant with a mean difference of 0.116 ($p < 0.001$).

Comparison of ZOI mm 48 hours was measured for *S. salivarius* using one way ANOVA test showed that the mean value of Zone of Inhibition is highest in *A. actinomycetumcomitans* (0.97) followed by *P. gingivalis* (0.914) least in *P. intermedia* (0.826). The difference in Zone of Inhibition between *P. gingivalis* and *A. actinomycetumcomitans* was statistically significant with a mean difference of 0.056 ($p < 0.001$). The difference between *actinomycetumcomitans* and *P. intermedia* was statistically significant with a mean difference of 0.144 ($p < 0.001$). The difference between *P. intermedia* and *P. gingivalis* was statistically significant with a mean difference of 0.088 ($p < 0.001$).

Group A (*Lactobacillus acidophilus*)

Table no 1:-zone of inhibition in millimeters using *Lactobacillus acidophilus* as probiotic

		N	Mean	Std. Deviation	p value
ZOI mm 24 hours	<i>A. actinomycetumcomitans</i>	5	1.18	0.02	<u><0.001</u>
	<i>P. gingivalis</i>	5	1	0.02	
	<i>P. intermedia</i>	5	1.	0.03	
ZOI mm 48 hours	<i>A. actinomycetumcomitans</i>	5	1.41	0.02	<u><0.001</u>
	<i>P. gingivalis</i>	5	1.08	0.03	
	<i>P. intermedia</i>	5	1.30	0.06	

(P value $< .05$ reflects statistical significance)

Table no 2:-comparison within the organisms using *Lactobacillus acidophilus* as probiotic

Dependent Variable	COMPARISON GROUP	COMPARED WITH	MEAN DIFFERENCE	Std. Error	P VALUE
ZOI mm 24 hours	<i>A. actinomycetumcomitans</i>	<i>P. gingivalis</i>	0.18	0.02	<u><0.001</u>
		<i>P. intermedia</i>	-0.03	0.02	0.441
	<i>P. gingivalis</i>	<i>P. intermedia</i>	-0.22	0.02	<u><0.001</u>
ZOI mm 48 hours	<i>A. actinomycetumcomitans</i>	<i>A. actinomycetumcomitans</i>	0.33	0.02	<u><0.001</u>
		<i>P. intermedia</i>	0.11	0.02	<u>0.004</u>
	<i>P. gingivalis</i>	<i>P. intermedia</i>	-0.21	0.03	<u><0.001</u>

(P value $< .05$ reflects statistical significance)

Group B (*S. salivarius*)**Table no 3:-**zone of inhibition in millimeters using *Streptococcus salivarius* as probiotic

		<u>N</u>	<u>Mean</u>	<u>Std. Deviation</u>	<u>p value</u>
<u>ZOI mm</u> 24 hours	<u>A. actinomycetumcomitans</u>	<u>5</u>	<u>0.81</u>	<u>0.02</u>	<u><0.001</u>
	<u>P. gingivalis</u>	<u>5</u>	<u>0.71</u>	<u>0.08</u>	
	<u>P. intermedia</u>	<u>5</u>	<u>0.59</u>	<u>0.03</u>	
<u>ZOI mm</u> 48 hours	<u>A. actinomycetumcomitans</u>	<u>5</u>	<u>0.97</u>	<u>0.02</u>	<u><0.001</u>
	<u>P. gingivalis</u>	<u>5</u>	<u>0.91</u>	<u>0.02</u>	
	<u>P. intermedia</u>	<u>5</u>	<u>0.82</u>	<u>0.01</u>	

(P value <.05 reflects statistical significance)

Table no 4:-comparison within the organisms using *Streptococcus salivarius* as probiotic

<u>Dependent Variable</u>	<u>COMPARISON GROUP</u>	<u>COMPARED WITH</u>	<u>MEAN DIFFERENCE</u>	<u>Std. Error</u>	<u>p value</u>
	<u>A. actinomycetumcomitans</u>	<u>P. gingivalis</u>	<u>0.09</u>	<u>0.02</u>	<u><0.001</u>
		<u>P. intermedia</u>	<u>0.21</u>	<u>0.02</u>	<u>0.001</u>
	<u>P. gingivalis</u>	<u>P. intermedia</u>	<u>0.11</u>	<u>0.02</u>	<u>0.012</u>
	<u>A. actinomycetumcomitans</u>	<u>P. gingivalis</u>	<u>0.05</u>	<u>0.01</u>	<u><0.001</u>
		<u>P. intermedia</u>	<u>0.14</u>	<u>0.01</u>	<u><0.001</u>
	<u>P. gingivalis</u>	<u>P. intermedia</u>	<u>0.08</u>	<u>0.01</u>	<u><0.001</u>

(P value <.05 reflects statistical significance)

Discussion:-

Inter-microbial interactions, and colonization in the oral cavity are considered to be of major significance for probiotic actions when they are integrated into the oral biofilm in the oral cavity.^{9, 10} In this study, our aim was to compare zone of inhibition of the inoculated probiotics in vitro and to compare their effects on the growth of other oral bacteria. *Lactobacillus acidophilus* and *S. salivarius* are important members of a healthy oral microflora bacteria as shown by previous study.¹³ Therefore, the role of probiotics bacteria in the microbiota is important for the oral colonization of *Lactobacillus acidophilus* and *S. salivarius*, and our study supports the fact that the colonization of oral indigenous bacteria is affected by probiotic bacteria.

In our study, the growth of all pathogens *P. gingivalis*, *P. intermedia* and *A. actinomycetumcomitans* were reduced significantly, this was probably due to a combination of competition for nutrients and result of the acidic conditions generated by *Lactobacillus acidophilus*. The growth inhibition of *P. gingivalis*, *P. intermedia*, *A. actinomycetumcomitans* by oral *Lactobacillus* was related to the ability of *Lactobacillus* to reduce the mutual growth factor vitamin K.¹⁷ Zhu et al in 2010 performed growth-inhibition study¹³ showed probiotic *Bifidobacterium* inhibited the growth of *P. gingivalis* when inoculated first there was inhibition initially, but later no inhibition was seen when both *Bifidobacterium* and *P. gingivalis* were inoculated together at the same time.¹³ The reason for inhibition was the result of the of inhibitory substances produced by probiotic bacteria or competition for nutrients.¹³ In our study, the increased zone of *P. gingivalis* in culture containing *Lactobacillus* might be due to a result of the increased level of probiotics.¹⁸

Piwat et al¹⁹ conducted an in-vitro study which explains how adhesion, aggregation and co-aggregation mechanisms of probiotic bacteria can influence the growth of other periodontal pathogens. These organisms attach to the oral hard tissues more strongly than pathogens, by competing for the adhesion surfaces. In our study zone of inhibition may have formed because the probiotics have inhibited the periodontal pathogen's active multiplication the same way.^{19, 20} A study by Morales A, et al showed the additional use of *L. Rhamnosussachets* provided similar results when compared with scaling and root planning, thus showing benefit of probiotics.²¹ One of the suggested mechanism stated by Hojo et al says that *Lactobacillus acidophilus* and *P. gingivalis* compete with each other for vitamin K and inhibits the growth of latter by possibly competing for the growth factor in a co-culture.¹⁷ Gruner D, et al²² included clinical trials which measured the influence of *Lactobacilli* for probiotic therapy in periodontal pathogens and there was no significant differences when compared with the control group.²² This shows the need of further studies to establish our positive findings.

Conclusion:-

With the available evidence, it is easy to conclude whether probiotics offer many clinical benefit in the treatment of periodontal disease. Most studies show a limited and temporary improvement in periodontal parameters when probiotics are given. *Lactobacillus* and *S. salivarius* had an inhibitory effect on the growth of periodontal pathogens when inoculated like our results suggested. Further studies should be done comparing with oral bacterial species/strains and using in vivo models will enable to establish our findings even more. Well-designed clinical studies with larger sample sizes and long-term follow-ups are required. Future studies will also have to adhere to a uniform methodology to avoid heterogeneity.

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