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Control of environmental factors on cariogenic *Streptococcus* strains isolated from dental plaque-their interspecies competition

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Abstract

Streptococcus strains isolated from different categories- male, female and children of caries active and caries free mouth, where tested to characterize their tolerance efficiency for growth under varying temperature, pH, moisture, salt concentration, sugar concentration, oxidative stress, and percentage of CO_2 availability. These environmental factors are critical and ubiquitous environmental signals that govern the development and virulence of diverse microbial species. Isolated *Streptococcus* strains from different oral disease condition showed variation in their growth and virulence property under different environmental condition. Isolated *Streptococcus* strains where inhibited by selected *Lactobacilli* and *Bifidobacterium* strains isolated from cow milk, which shows interspecies competitive exclusion.

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Introduction

Consequence of accumulation of heavy plaque biofilm in the oral cavity is still a serious concern in health departments worldwide. Regardless of various measures that are evolving to control and treat dental caries and various other oral diseases as well as other human body infection arising due to cariogenic biofilm formation needs especial focus to develop new methods for treatment. Dental plaque in various studies found to be identified as a biofilm consisting of different microorganisms with different class of bacteria and fungi composed of numerous species [1]. This dental oral biofilm produced by oral health is studied and analyzed deliberately revealing that bacterial oral biofilm are engaged under phase of expansion resulting in different cariogenic oral disease such as dental plaque, gingivitis, early periodontitis, etc. Studies found to explain the consequences and relation of dental plaque biofilm especially isolated from sub gingival and the supragingival site of mouth appeared to be associated with systemic disease and disorders in humans such as deleterious pregnancy outcome, heart disease, diabetes mellitus etc[2].

Streptococcus present in oral health possesses both qualities of causing cryogenic disease as well acting harmless microbial oral flora. A number of research revealed that oral plaque biofilm are responsible and act as the root cause of various critical oral health issues. Over the past 50 years, the philosophical study of understanding and classification of potential property of dental plaque causing caries and tooth decay has undergone consequential growth. In our mouth microflora of about 500 different types reside and proliferate with mutual coordination. Reckoning the strength of oral microflora, it has been impressed that more than 600 verity of species residing with recognizable cadre prevailing at their varying habitats. From group of *Streptococcus* bacteria, *Streptococcus mutans* species found to dominate the dental caries and oral infection more as compared to other group of *Streptococci* species. Studies show the consequences of specialized property of *Streptococci* species for colonizing the different oral sites and withstanding the fluctuating oral environment to fight competing microorganism and to overcome foreign challenges. Initiation of disease by *Streptococci* occurs in oral health as a result of imbalances in the microbial biota. Under some peculiar condition, commensal *Streptococci* can transform to opportunistic pathogens, usher oral disease and other systemic disease too, damaging the host. The required factors include the number and species of bacteria, the type, quantity and frequency of consumption of fermentable carbohydrates and susceptible tooth and sub gingival surfaces [3, 4, and 5].In studies it is shown that tooth decay can be prevented by abolishing the associating factors resulting in dental caries such as acid environment, availability of carbohydrates, etc. Considering water fluoridation for example, has depressed caries by about (50%) without choosing any other extra therapeutic controls [5].

The competence potential of oral microorganism to sustain and proliferate in the available environment such as pH, NaCl, temperature, sugar availability, moisture, etc. will depend on their natural genetic structure, which reveals their property to respond fluctuating local surrounding environment and stress. This competence potential of microbiota locale in the oral biofilm is imperiled to many fluctuating environment, stress, including the availability of nutrients, and various other environmental factors resulting in critical oral health issues involving the major one are dental caries. Considering the form of biofilm as supragingival and subgingival oral plaque are considered as a main etiological agent for dental caries and other oral infections such as periodontitis, Aphthous stomatitis, Burning mouth syndrome , Gingivitisetc.The genus *Lactobacillus* found to consist of a varied group of species of a genetically and physiologically diverse ground. *Lactobacillus* bacteria are rod-shaped, Gram-positive, non-spore forming, non-pigmented, catalase negative and microaerophilic to strictly anaerobic in property[7].

Among lactic acid bacteria (LAB), *lactobacilli* reveals a different group of homo fermentative and hetero fermentative species which can produce a variety of substances such as lactic acid , ethanol, formic acid, acetone, hydrogen peroxide, diacetyl etc. Variety of exopolysaccharides (EPS) is also produced which is an important characteristic of this genus.

These *Lactobacillus* species possess a species specific quality which is diverse within their community of bacteria are found in the most natural microbial ecosystem where they are harboured and sustained competing with their surrounding class of bacteria for space and survival factors. Some studies in the laboratory revealed the potential of pure and mixed culture about reviling many running mechanisms by which bacteria can impair or kill other microbes. In animal and plant, there are a gigantic number of well-studied examples of population, which are held in balance, or driven to Tran by competitive force. Most microorganism experience a persistent battle for available resources, immense number of microbes are present in all but the most esoteric environments, tremendous microbial diversity has been revealed by new microbial tag sequencing[10].

Most research into feuding between bacterial species has entrapped on to explain the bio-chemical mechanism underlying different interaction, and commonly conclude that competition occurs between individual cells of living organisms. The ecological role of compounds that are currently defined as an antimicrobial subset of secondary metabolism has recently been the subject of some controversy. After examining the transcriptional response of sensitive bacteria to sub-inhibitory concentration of antimicrobial several investigators have proposed that the true function of the molecules in nature is treated as single molecules within and between species [11, 12, and 13].

Material and Methods

Isolation of cariogenic streptococcus from Dental plaque

Plaque sampling sited varied depending on the condition and disease diagnose in individual subjects. The plaque sample were collected from sub gingival and supra gingival including region of mouth by using sterile disposable swab stick transferring sample to sterile tube container 1 ml sterile phosphate buffer saline. Sample were stored in cool place and then transported to laboratory. One hundred micro litre of undiluted sample were spreadon the surface of MS- agar plate using sterile swab. Culture were incubated an aerobically for 48 hrs at 37°C. Count of more than 250 colonies (104 cell/ml) was considered as to positive sample [11, 15]. Isolated strains were identified based on colony morphology, characteristics and biochemical test results were performed using Hi strep^{TM}identification kit (code-KB005-10 KT).

Isolated *Streptococcus* strains where assayed for their tolerance characteristic under different environmental condition such as temperature, moisture, pH, and salt NaCl concentration.Tolerance to these envoiromental factors were studied in MS-broth.

Isolation of *Lactobacillus* and *Bifidobacterium*

Strains of *Lactobacillus and Bifidobacterium* were isolated from cow milk of desi indigenous breed known as 'Gir'.The sample were collected from location of Daman city from Kachigam village (Daman and Diu, U.T) . Isolation of strains was done by using pour plate method on MRS agar medium for lactobacilli and Bifidobacterium agar, modified (Hi media M1734) for Bifidobacterium strains. Biochemical test and morphology studies were performed for their further characteristic identifications [16, 17].

Agar overlay interference tests

The "overlay method" of plating bacteria is used when a very uniform lawn of growth is needed. Spread plating often cannot produce a lawn that is homogeneous enough for antibiotic susceptibility testing by the disc diffusion method (Kirby-Bauer) or to form bacterial virus plaque assays. In the overlay method, bacteria are added to a melted agar solution which is poured onto the surface of an agar plate. During incubation, bacteria form micro colonies within the agar top layer. Strains of *Lactobacilli* isolated from raw cow milk and *Bifidobacterium* were tested against strains of *Streptococcus* strains from dental plaque. Strains of *Lactobacilli* were initially cultured for 16-20 hrs on MRS agar; a distinct colony of each bacterium was then transferred to 4.5 ml MRS broth for further 16-20 hrs of incubation. Same test procedure repeated with *Bifidobacterium* using *Bifidobacterium* agar, modified (Hi media M1734) for *Bifidobacterium* strains. Culturing of *Lactobacilli, Bifidobacterium* and *Streptococcus* were performed in anaerobic atmosphere (10% H_2, 5%CO_2 & 85%N_2) in anaerobic chamber at 37°C [17,18].

Lactobacilli isolated from milk were serially diluted by using tenfold serial dilution method, the optical density was measured at 630 nm using spectrophotometer of dilutions. Undiluted suspension and cell suspension corresponding to approximately $10^3$and 10^9 CFU/ml were used in the inhibition experiments. 1 ml of each strain suspension of lactobacilli (producer strain) was added in to 24 ml molten sterile MRS agar and plates were casted. After the agar was set, the plates were incubated at 37°C overnight in anaerobic atmosphere. Broth cultures of streptococci strains grown for 16-20 hrs in tood –Hewitt broth were diluted in the same medium and the O.D was measured at 500 nm . The suspension of *Streptococci* strains were stamped on the plates with steer's replicator. The plates were left at room temperature for 1 hr and were subsequently incubated over night at 37°C in the anaerobic chamber.To test the susceptibility of isolated Streptococcus strains to *Bifidobacterium* , the process repeated by using Bifidobacterium agar, modified (Hi media M1734) for *Bifidobacterium* strains (producer strain). As control isolated *streptococci* strains were stamped onto agar plates without Lactobacilli and *Bifidobacterium* within the first agar layer in each experiment separately with *Lactobacilli* and Bifidobacterium. The were carried out in duplicate and repeated four times on different occasions [24].

Results and Discussion

From 500 selected subject of caries active and caries free mouth, 487 plaque sample showed positive growth of strains. Remaining 13 samples reported no growth. Isolated strains were identified by their morphological and biochemical characteristics by using appropriate procedures and methods as discussed above. Isolated strains of cariogenic *Streptococcus* where exposed to different environmental conditions to assess their ability to survive under different fluctuating condition and factors. Oral biofilm are subjected to considerable alter in environmental condition, including pH, nutrient, carbohydrate source, and oxygen tension, all of which appear to influence the composition and biological activities of the microbial population [18,19]. The ability of oral bacteria to survive and persist in oral biofilm depends on their capacity to respond to environmental change at genetic, physiological, and biochemical levels [20, 21].Consistent with this idea, *S.mutans* has evolved a remarkable capacity to co-ordinate the expression of a variety of genes in response to environmental factor.

Effect of pH variation on cariogenic *Streptococcus* strains

It was investigated that different pH environment can inhibit the metabolism and reduce the growth and viability of cariogenic *Streptococcus* strains. To investigate the viability of these isolated *Streptococcus* strains they were switched to grow at different pH range of 2, 3, 4 and 5 respectively (table: 4).

All strains of *Streptococcus* isolated from different disease condition showed variation in their growth at different pH.

Amongst 4 selected strains *S.Mitis,S.Mutans,S.Sanguis and Enterococci* of oral *Streptococci* showed best growth at pH 5. *S.mutans* isolated from gingivitis and dental plaque showed growth at minimum pH 2, whereas none of isolates showed growth at this pH.Some of isolates showed growth at pH 3, and 4 respectively from different disease condition.

Effect of temperature variation on cariogenic *Streptococcus* strains

Another very important fluctuating factor in oral mouth environment is temperature. Strains were studied under different temperature .The propounded MS medium containing strains were incubated at different temperature (10, 25, 37 and 40).The study revealed the effect of temperature on growth of strains. The result illustrated in (table: 1) and graph indicated that the optimum growth of the isolated strains is 37°C. The growth declined as temperature increases. *S.mutans* showed good growth at 37°C but no growth observed at 40°C, whereas *mitis and enterococci* showed very less growth almost 4% even at 40°C temperature.

Effect of moisture content on cariogenic *Streptococcus* strains

The selected MS agar media contain strains of cariogenic *Streptococcus* was incubated at different moisture concentration(100%,50%,and 0%) at 37°C .The moisture availability affects the growth of strains. Moisture content 100% suppressed the growth of cariogenic *Streptococcus* strains. The best growth of strains were reported at 50% moisture availability as compare to 100% and 0%. None of growth showed growth at 0% moisture content.

Effect of NaCl concentration on cariogenic *Streptococcus* strains

Streptococcus bacteria generally tolerate high salt concentrations. *S.mutans and S.salivarius* strains isolated from dental plaque showed growth at 7%NaCl concentration.Where as, *Enterococci and S.salivarius* isolated from gingivitis and dental plaque revealed growth at 7%NaCl concentration this result shows the tolerance capacity of *S.mutans, Enterococcoi* and *S.salivarius*in fluctuating environment in different disease conditions of mouth. *S.sanguis and S.mitis* did not revealed any growth at 7%NaCl concentration. None of isolates showed growth at 10% NaCl concentration. Maximum growth recovered at 1% and 4%NaCl concentration.

Growth inhibition of Streptococcus strains by *Lactobacilli* strains

The result illustration in Table 5: shows the growth pattern of *Streptococcus* revealing the interspecies competition. At concentration 10^9 CFU/ml, all *Lactobacilli* strains isolated from cow milk inhibited the growth of cariogenic *Streptococcus* strains from dental plaque of different disease conditions of mouth completely, only exception of strains L.1 and L.7 showing that executed only a slight inhibition of some strains at concentration to10^3 CFU/ml. Strains L.1 and L.7 had statistically revealed weaker inhibition capacity in comparison with L.2 and L.5 strains *of Lactobacilli.*

Streptococcus mutans from dental plaque showed low susceptibility towards *Lactobacilli strains* in 10^3 CFU/ml concentration and complete inhibition at 10^9 CFU/ml concentration. Isolates from caries free mouth revealed complete inhibition by *Lactobacilli* strains at given concentration. Same as *S.mutans ,S.mitis, S.sanguis and Enterococci* showed low inhibition at concentration 10^3 CFU/ml of *Lactobacilli.* And good inhibition mark at 10^9 CFU/ml concentration.

Growth inhibition of *Streptococcus* strains by Bifidobacterium Strains

Isolated *Bifidobacterium Strains from cow milk inhibited* the growth of *Streptococcus* strains from dental plaque of different patients with different disease condition of mouth. From Isolated strains of *Bifidobacterium* two strains were selected for investigation in procedure of interspecies completion of survival. Selected two strains of *Bifidobacterium* B.1 and B.4 revealed similar inhibition pattern at same concentration of 10^9 CFU/ml. The inhibition pattern was reduced at 10^3CFU/ml concentration of both strains B.3 and B.6 of *Bifidobacterium* against *Streptococcus* strains from Dental plaque.

Weaker susceptibility was seen of isolates of *Streptococci* from dental plaque towards *Bifidobacterium* strains at 10^3 CFU/ml concentration. Strong inhibition of *Streptococcus* strains by *Lactobacilli* at 10^9 CFU/ml was reported in case of caries free mouth and other disease conditions isolates.

Conclusion

The experiments described above explored the responses of *Streptococci* to rapid fluctuation in their given environment, it revealed that *Streptococcus* strains showed growth variation under different environmental factors like pH, temperature, humidity and salt concentration as well as under different disease condition from which strains where isolated. Still we need further investigation to understand these potential mechanism of *Streptococcus* to tolerate the harsh environmental conditions and proliferate well under fluctuating environment of oral biofilm.

Growth inhibition of *Streptococcus* strains

All tested *Lactobacilli* and the results of the growth inhibition assay are summarized in Table 6 and 7. At concentration of 109 CFU/ml, all selected *Lactobacilli* strains L.2, L.5, inhibited the growth of the *Streptococcus* strains isolated from dental plaque. *S.mitis, S.sanguis and Enterococci* showed higher percentage of susceptibility as compare to *S.mutans.* At concentration 103 strain L.1and L.7 showed less inhibitory action against isolated cariogenic *Streptococcus* strains from dental plaque. Just as *Lactobacilli, Bifidobacterium* also revealed the interspecies competitive exclusion against cariogenic *Streptococcus* strains from dental plaque. Both strains of *Bifidobacterium* showed inhibition result satisfactory against *Streptococcus* strains.

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Table 1. Influence of temperature on growth of cariogenic streptococcus isolated from different oral disease condition.

Strains of Streptococccus	*Disease condition*	10°C	25°C	37°C	40°C
S.mutans	Gingivitis	0	0.468	1.622	0
	Dental Plaque	0.142	0.420	1.753	0.220
	Aphthous stomatitis	0	0.643	1.438	0.0
	Caries free mouth	0	0	0.129	0.0
S.sanguis	Gingivitis	0	0.422	1.524	0.320
	Dental Plaque	0	0.321	1.887	0.212
	Aphthous stomatitis	0	0.340	1.796	0
	Caries free mouth	0	0	0.651	0
S.salivarius	Gingivitis	0	0.204	1.269	0
	Dental Plaque	0	0.239	1.046	0.110
	Aphthous stomatitis	0	0.202	1.280	0
	Caries free mouth	0	0.190	0.240	0
S.mitis	Gingivitis	0	0.171	1.080	0.108
	Dental Plaque	0.120	0.642	1.046	0.310
	Aphthous stomatitis	0	0.753	1.269	0
	Caries free mouth	0	0.342	0.780	0
Enterococci	Gingivitis	0	0.240	0.740	0.244
	Dental Plaque	0.148	0.180	1.790	0.210
	Aphthous stomatitis	0	0.440	1.887	0
	Caries free mouth	0	0.330	0.651	0

Graph: AThe effect of Temperature factor on growth of Streptococcus strains

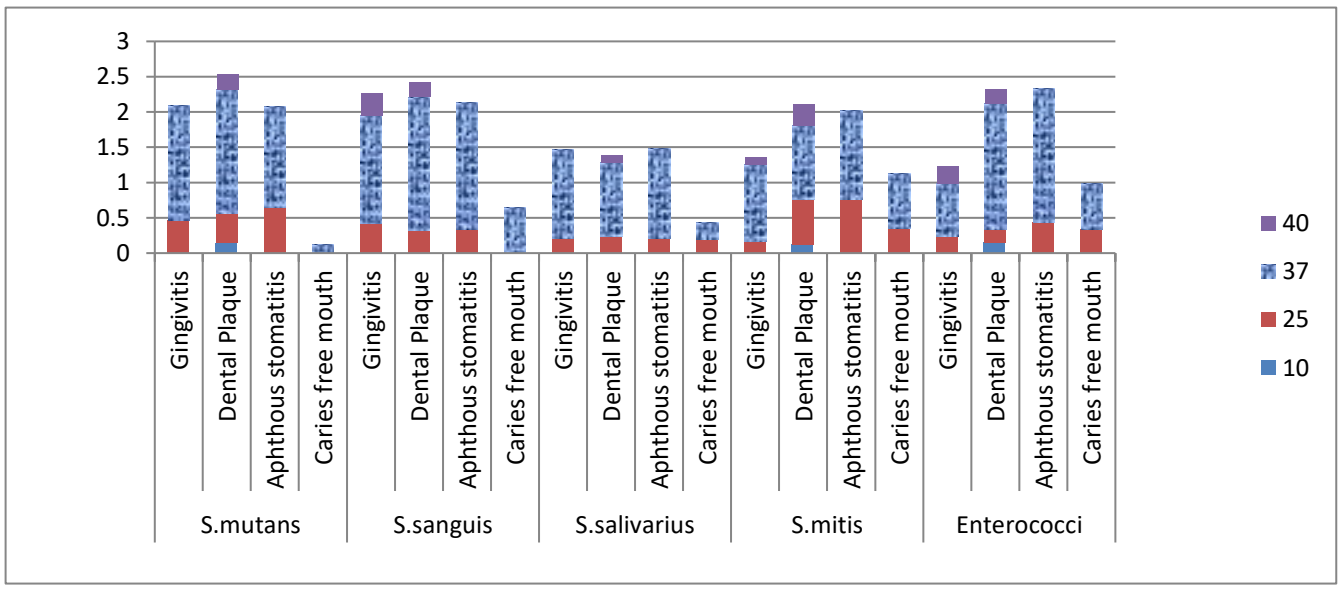

Table 2. Influence of Humidity on growth of cariogenic streptococcus isolated from different oral disease condition

Strains of Streptococccus	***Disease condition***	**0%**	**50%**	**100%**
S.mutans	Gingivitis	0	1.475	0.413
	Dental Plaque	0	1.667	0.321
	Aphthous stomatitis	0	1.786	0.120
	Caries free mouth	0	0.643	0
S.sanguis	Gingivitis	0	1.622	0.799
	Dental Plaque	0	1.803	1.020
	Aphthous stomatitis	0	1.193	0.210
	Caries free mouth	0	0.799	0
S.salivarius	Gingivitis	0	1.426	0.120
	Dental Plaque	0	1.753	0.233
	Aphthous stomatitis	0	1.579	0
	Caries free mouth	0	0.667	0
S.mitis	Gingivitis	0	1.753	0
	Dental Plaque	0	1.786	0.140
	Aphthous stomatitis	0	1.992	0
	Caries free mouth	0	0.882	0
Enterococci	Gingivitis	0	1.989	0
	Dental Plaque	0	1.982	0.122
	Aphthous stomatitis	0	1.720	0
	Caries free mouth	0	1.842	0

Graph: BThe effect of Humidity factor on growth of Streptococcus strains

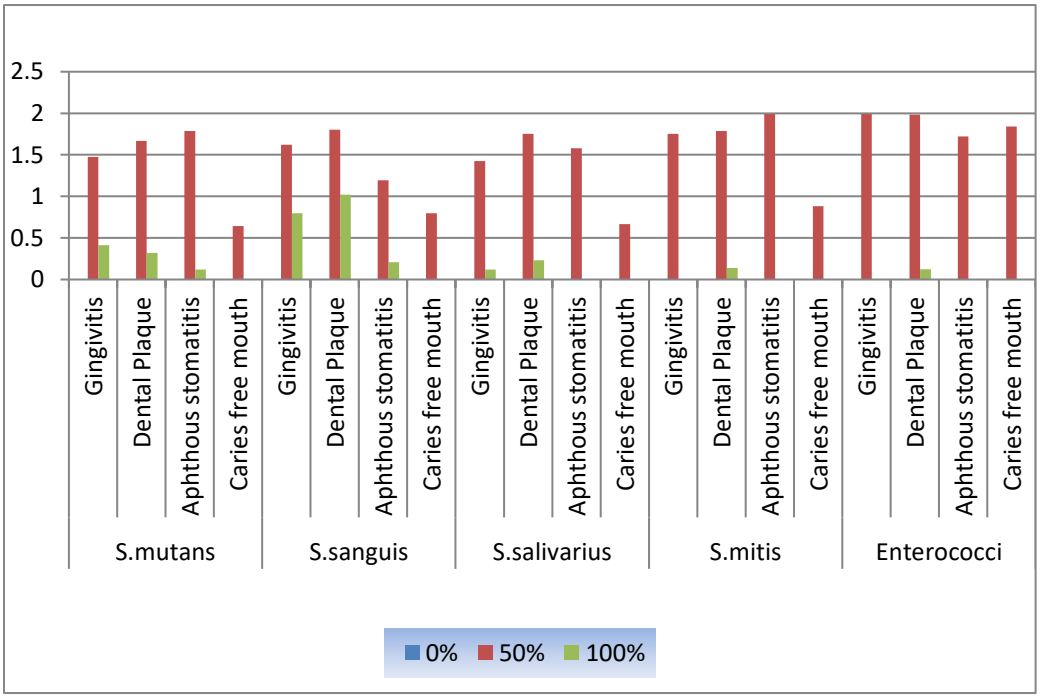

Table 3. Influence of NaCl concentration on growth of cariogenic streptococcus isolated from different oral disease condition

Strains of Streptococccus	Disease condition	1%	4%	7%	10%
S.mutans	Gingivitis	1.428	0.330	0	0
	Dental Plaque	1.210	0.450	0.120	0
	Aphthous stomatitis	1.108	0.230	0	0
	Caries free mouth	0.890	0	0	0
S.sanguis	Gingivitis	1.118	0.420	0	0
	Dental Plaque	1.358	0.193	0	0
	Aphthous stomatitis	1.211	0.268	0	0
	Caries free mouth	0.977	0.120	0	0
S.salivarius	Gingivitis	1.786	0.345	0.220	0
	Dental Plaque	1.426	0.789	0.200	0
	Aphthous stomatitis	1.667	0.220	0	0
	Caries free mouth	0.642	0.110	0	0
S.mitis	Gingivitis	1.887	0.240	0	0
	Dental Plaque	1.667	0.450	0	0
	Aphthous stomatitis	1.456	0.310	0	0
	Caries free mouth	0.230	0.120	0	0
Enterococci	Gingivitis	1.887	0.184	0.120	0
	Dental Plaque	1.765	0.352	0.148	0
	Aphthous stomatitis	1.453	0	0	0
	Caries free mouth	1.010	0	0	0

Graph: C The effect of NaCl concentration on growth of Streptococcus strains

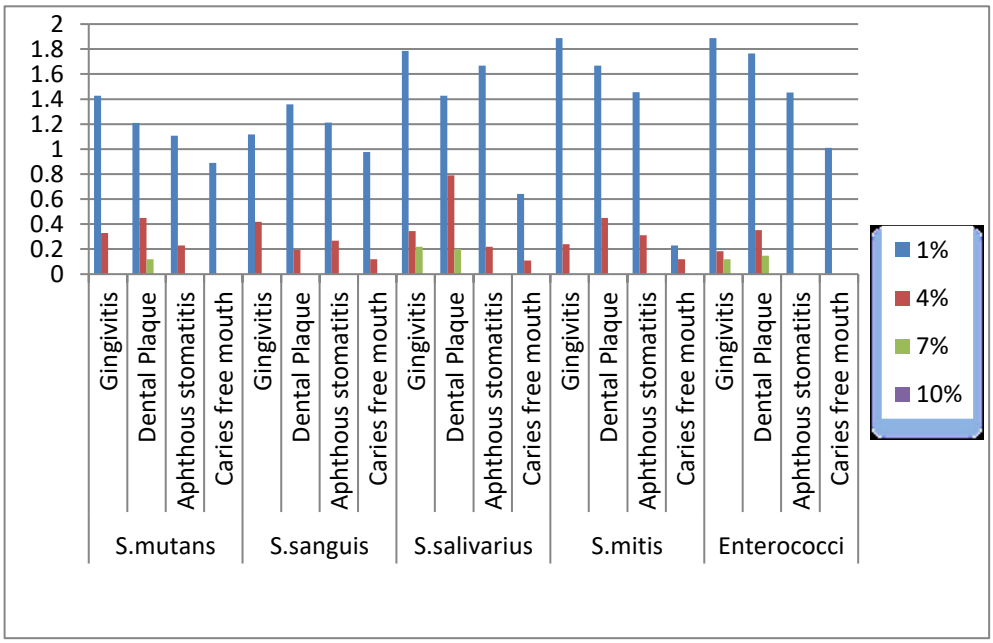

Table 4. Influence of different pH on growth of cariogenic streptococcus isolated from different oral disease condition

Strains of Streptococcus	*Disease condition*	pH 2	pH 3	pH 4	pH 5
S.mutans	Gingivitis	0.120	1.667	1.159	1.013
	Dental Plaque	0.080	0.840	1.887	1.312
	Aphthous stomatitis	0	0.670	1.426	0.992
	Caries free mouth	0	0	0.120	0
S.sanguis	Gingivitis	0	0.643	1.224	1.442
	Dental Plaque	0	0.799	1.148	1.328
	Aphthous stomatitis	0	0.786	1.013	1.482
	Caries free mouth	0	0.074	0.824	0.823
S.salivarius	Gingivitis	0	0.468	1.524	1.669
	Dental Plaque	0	0.321	1.753	1.262
	Aphthous stomatitis	0	0.120	1.492	1.281
	Caries free mouth	0	0	0.220	0.610
S.mitis	Gingivitis	0	0.420	1.753	1.248
	Dental Plaque	0	0.530	1.655	1.622
	Aphthous stomatitis	0	0.320	1.786	1.436
	Caries free mouth	0	0.120	0.430	0.803
Enterococci	Gingivitis	0	0.438	1.667	1.442
	Dental Plaque	0	0.736	1.753	1.148
	Aphthous stomatitis	0	0.365	1.786	1.626
	Caries free mouth	0	0.240	0.672	0.180

Graph: D The effect of Ph factor on growth of Streptococcus strains

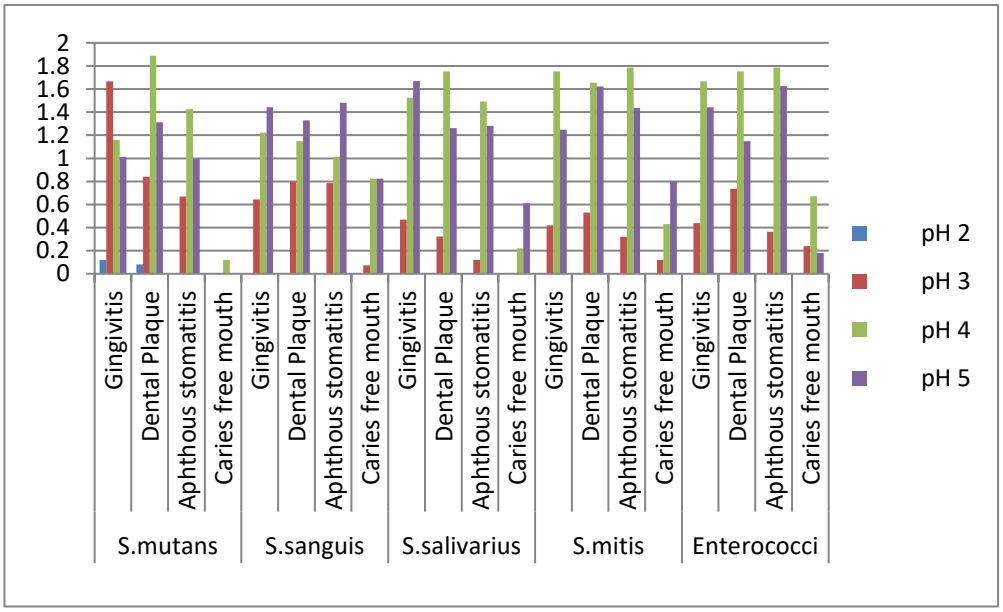

Table 5. Interspecies interaction of lactobacilli strains with streptococcus strains isolated from different oral disease condition.

Strains of Streptococccus	***Disease condition***	***Strains of Lactobacilli***							
		L.1		L.2		L.5		L.7	
		10^3 CFU/ml	10^9 CFU/ml	10^3 CFU/ml	10^9 CFU/ml	10^3 CFU/ml	10^9 CFU/ml	10^3 CFU/ml	10^9 CFU/ml
S.mutans	Gingivitis	+	+	+	0	+	0	0	0
	Dental Plaque	+	0	0	0	+	0	+	+
	Aphthous stomatitis	+	+	+	0	0	0	++	0
	Caries free mouth	0	0	0	0	0	0	0	0
S.sanguis	Gingivitis	++	0	+	0	0	0	+	0
	Dental Plaque	+	0	0	0	0	0	+	0
	Aphthous stomatitis	0	0	0	0	+	0	+	0
	Caries free mouth	+	0	0	0	0	0	0	0
S.salivarius	Gingivitis	0	0	0	0	+	0	+	0
	Dental Plaque	+	+	0	0	0	0	+	0
	Aphthous stomatitis	++	+	0	0	0	0	0	0
	Caries free mouth	0	0	0	0	0	0	0	0
S.mitis	Gingivitis	0	0	+	0	+	+	0	0
	Dental Plaque	+	0	0	0	0	0	+	0
	Aphthous stomatitis	+	0	0	0	0	0	+	+
	Caries free mouth	0	0	0	0	0	0	+	0
Enterococci	Gingivitis	+	0	0	0	+	0	0	0
	Dental Plaque	+	0	+	0	0	0	0	0
	Aphthous stomatitis	0	0	0	0	0	0	0	0
	Caries free mouth	0	0	0	0	0	0	0	0

Disease condition: G- Gingivitis, DP- Dental Plaque, AS-AphthousStomatitis ,CFM- Caries Free Mouth

Inhibition score: 0 = Total inhibition, + = Slight inhibition, ++ = No inhibition

Table 6. Interspecies interaction of Bifidobacterium Strains with streptococcus strains isolated from different oral disease condition.

Strains of Streptococcus	***Disease condition***	***Bifidobacterium Strains***			
		B.3		B.6	
		10^3 CFU/ml	10^9 CFU/ml	10^3 CFU/ml	10^9 CFU/ml
S.mutans	Gingivitis	0	0	0	0
	Dental Plaque	0	0	0	0
	Aphthous stomatitis	0	0	+	0
	Caries free mouth	0	0	0	0
S.sanguis	Gingivitis	+	0	0	0
	Dental Plaque	0	0	+	++
	Aphthous stomatitis	+	0	0	0
	Caries free mouth	0	0	0	0
S.salivarius	Gingivitis	0	0	0	0
	Dental Plaque	+	0	+	0
	Aphthous stomatitis	0	0	+	0
	Caries free mouth	0	0	0	0
S.mitis	Gingivitis	+	0	0	0
	Dental Plaque	+	0	+	0
	Aphthous stomatitis	0	0	0	0
	Caries free mouth	0	0	0	0
Enterococci	Gingivitis	+	0	+	0
	Dental Plaque	0	0	0	0
	Aphthous stomatitis	+	0	0	0
	Caries free mouth	0	0	0	0

Disease condition: G- Gingivitis, DP- Dental Plaque, AS-Aphthous Stomatitis, CFM- Caries Free Mouth

Inhibition score: 0 = Total inhibition, + = Slight inhibition, ++ = No inhibition

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