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Extraction and purification of immunoglobulin y (igy) and assessment of antimicrobial efficacy of igy against dental pathogens- An in-vitro study

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Abstract

Objective: To compare the antimicrobial efficacy of Immunoglobulin (Igy) against Streptococcus Mutans (S. Mutans), Enterococcus Faecalis (E. Faecalis)) and Lactobacillus on day 1-, 15- and 30-day intervals.

Materials and Methods: Igy is extracted and purified by Polyethylene Glycol (PEG) precipitation method and the extracts are added to the culture plates containing the microorganisms by the well diffusion method and measurement of the diameter of the clear zone is done using a calliper.

Results: There is statistically significant result in the effect of Igy against Lactobacillus and E. Faecalis in between the time intervals. There is antimicrobial effect of Igy against S. Mutans but the result is not statistically significant in between the time intervals.

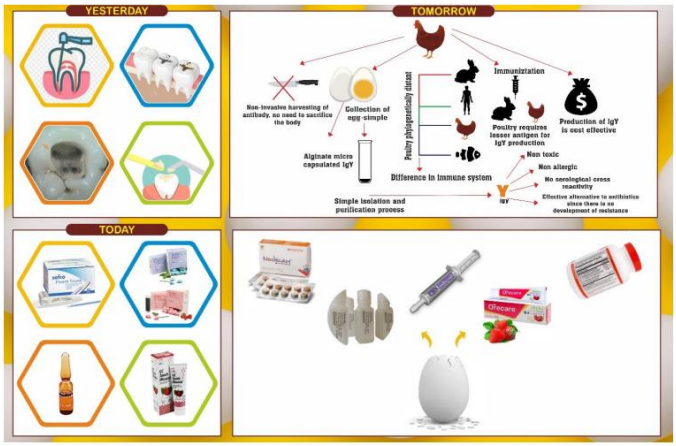
Conclusion: Igy has antimicrobial effect against Streptococcus Mutans, Enterococcus Faecalis and lactobacillus between the time intervals.

Clinical Significance: Extensive studies have been conducted to evaluate the effect of Igy on S. Mutans whereas there are no in-vitro or in-vivo studies

conducted to evaluate effects of Igy on E. Faecalis and Lactobacillus. Thus this study is the first of its kind in the field of Igy and its effects.

Keywords: Immunoglobulin Y(Igy), Streptococcus Mutans, Enterococcus Faecalis, Lactobacillus

Graphical Abstract



Introduction

Dental caries, a chronic disease that is unique among human and is one of the most common preventable diseases which is recognized as the primary cause of oral pain and tooth loss[1]. Biological factors such as cariogenic bacteria play an important role in dental caries initiation, progression as well as tooth decay.3 The development of caries was believed to be caused by only a few grampositive bacterial species, such as S. Mutans, S. Sobrinus and Lactobacillus. [2]

Historically, Lactobacillus was the first known microorganism with dental caries development.[3] S. mutans is the chief etiological agent of dental caries by virtue of its possession of cariogenic determinants such as adhesins, glucosyltransferase enzymes (GTF), mutacin and glucan binding proteins (GBP).[4] Enterococcus Faecalis (E. Faecalis) is associated with different peri radicular diseases and asymptomatic persistent endodontic infections.[7]

Current methods of caries management include traditional intervention, there is a dire need for new alternative and preventive approaches.1,8 The vertebrate immune system's primary goal is to protect the individual against microorganisms. [8] In humans, there are five classes of immunoglobulins: IgG, IgA, IgM, IgD and Ige. Chickens produce three classes: Igy, IgA and IgM. IgG and Igy are the main antibodies found in mammal and avia n's serum, respectively.

Although structurally different, both immunoglobulins are functionally equivalent.[9] Egg yolk antibodies, immunoglobulin Y (Igy) has been recognized as an inexpensive alternative antibody source and passive immunisation with Igy has shown therapeutic value.1 The "European Centre for the Validation of Alternative Methods" (ECVAM) strongly recommends that yolk antibodies should be used as an alternative to mammalian antibodies.[10]

Several studies in the dental field have reported that Igy against CA-GTF is effective in preventing adherence of S. Mutans to tooth surfaces.3 Lactobacilli and E. Faecalis being important dental pathogens, have either been studied very little or no studied at all when it comes to Igy research.

The purpose of this study is to compare the antimicrobial efficacy of immunoglobulin Y (Igy) against dental pathogens such as Streptococcus Mutans, Enterococcus Faecalis and Lactobacillus.

Methodology

Microbial sample preparation

S. Mutans, E. Faecalis and Lactobacillus were revived from stock bacteria in the Laboratory of Microbiology, Dextrose technologies Pvt ltd, Bangalore.

The microbes will be sub-cultured and re-identified prior to commencement of the study. The microbes were cultured on MS agar media then incubated for 18-24 h at 37°C. Colony of bacteria from the subculture media was put into 3 ml of liquid Brain Heart Infusion (BHI) media

and incubated for 18 hours in an incubator at 37°C. The resulting bacterial suspension in BHI media was compared to the turbidity of 0.5 McFarland standards which is equivalent to 108 CFU/ml. PEG

Extraction and purification of IgY

1. The chicken eggshell is cracked; the yolk is transferred to a "yolk spoon" to remove the egg white.

2. To remove remaining egg white the yolk is transferred to a filter paper (Figure 1) and yolk skin is cut with a lancet. The egg volume(V1) is recorded after pouring yolk into a 50ml tube. (Figure 2)

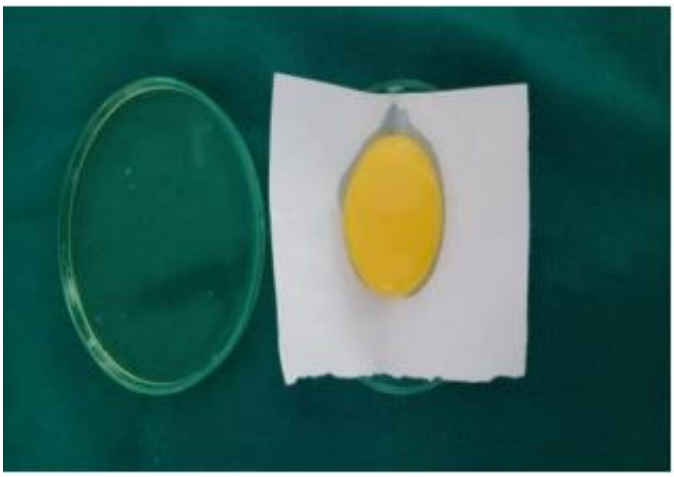


Figure 1: Yolk in filter paper

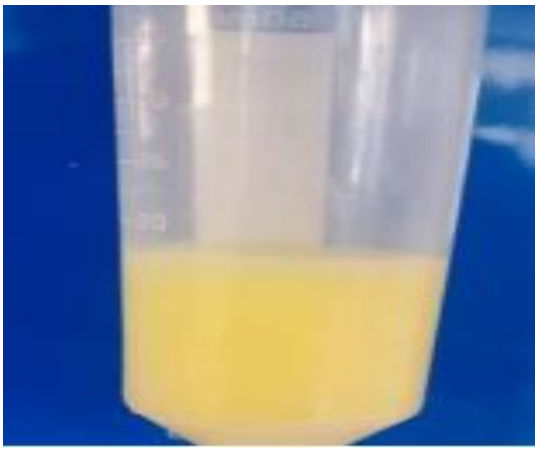


Figure 2: yolk volume is registered

3. Twice V1 volume of Phosphate Buffered Saline (PBS) is mixed with the yolk (∑V1+V2), thereafter 3.5 % Polyethylene Glycol (PEG)6000 (in gram, pulverized) of the total volume and vortexed, for 10 minutes it's rolled on a rolling mixer. The suspension gets separated into 2 phases, one containing yolk solids and fatty substances and the other watery phase with Igy.

4. It is centrifuged at 4°C for 20 min (10,000 rpm). The supernatant (V3) is filtered and transferred to a new tube.

5. 8.5 % PEG 6000 in gram is added to the tube, vortexed and rolled on a rolling mixer as in step 3.

6. Repeat step 4 but the supernatant is discarded in this step.

7. The pellet is dissolved in 1 ml PBS with a glass stick and the vortexer. PBS is added to make a final volume of 10 ml (V4). It is mixed with 12 % PEG 6000 (w/v, 1.2 gram) and treatment in step 3 is repeated.

8. Step 6 is repeated and pellet is dissolved carefully in 800 µL PBS. Pipette the extract to a dialysis capsule. (Figure 3) tube is rinsed with 400 µL PBS and add the volume to the dialysis device (V5).

9. The extract is dialyzed overnight in 0.1 % saline (1,600 ml) and gently stirred by means of a magnetic stirrer. The next morning, the saline is replaced by PBS and dialyzed for another three hours.

Figure 3: extract in dialysis capsule

10. The Igy-extract is pipetted from the dialysis capsule and transferred to 2ml tubes. 2ml is the final volume (V6)
11. The samples were photometrically evaluated for the protein content (mg/mL) at 280 nm with an extinction coefficient of 1.33 for Igy.
12. It is advisable to store the samples in aliquots at -20°C (do not freeze the samples at -70°C).
13. The quality of the final preparations is analysed by simple SDS-PAGE (Figure 4)

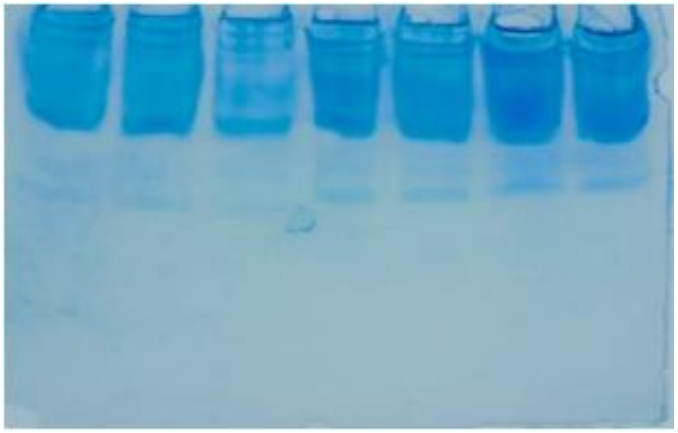


Figure 4: SDS PAGE analysis of preparations

Well diffusion method

The extracted Igy is added to the culture plates of S. Mutans, E. Faecalis and Lactobacillus by the well diffusion method and measurement of the diameter of the clear zone as an indication of bacterial growth inhibitory response by an antibacterial compound is done. (Figure 5, Figure 6, Figure 7) Bacterial growth was observed to see the formation of inhibition zones around the pits. Diameter of inhibition zones, which was clear area (there was no bacterial growth) around the wells were measured using a calliper. The inhibition zones are measured at 1, 15, 30- day intervals.

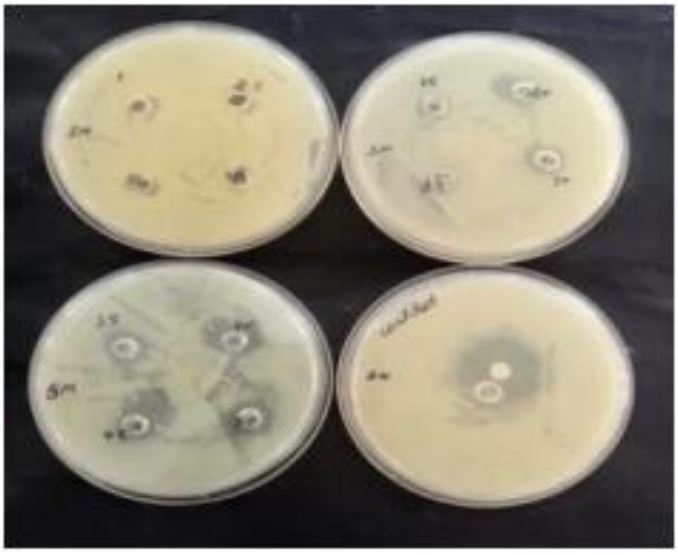


Figure 5: Zone of inhibition Streptococcus Mutans

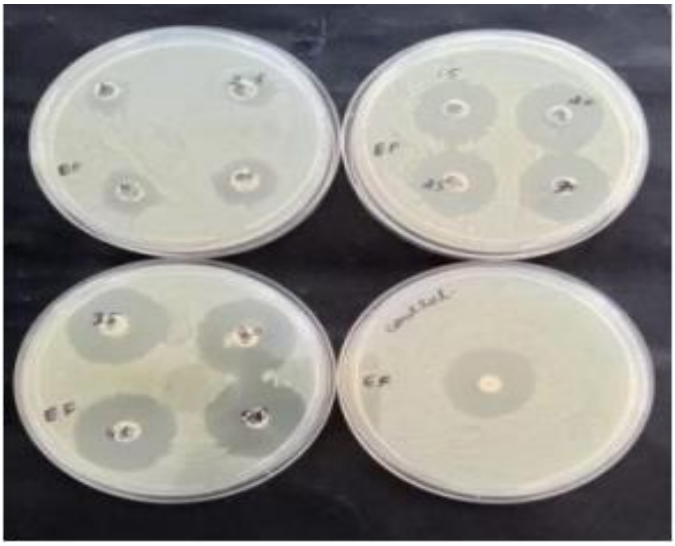


Figure 6: Zone of Inhibition Enterococcus Faecalis

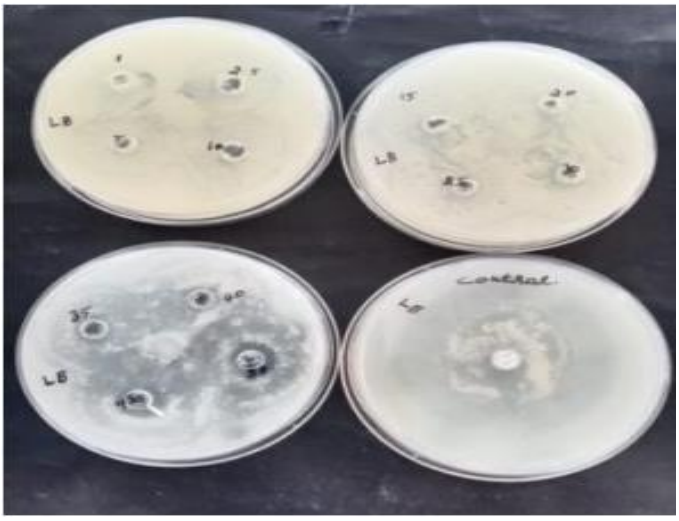


Figure 7: Zone of Inhibition Lactobacillus

Statistical analysis

A repeated measures ANOVA with a Greenhouse-Geisser correction to determine the mean of S. Mutans, E. Faecalis and Lactobacillus between the time points were conducte

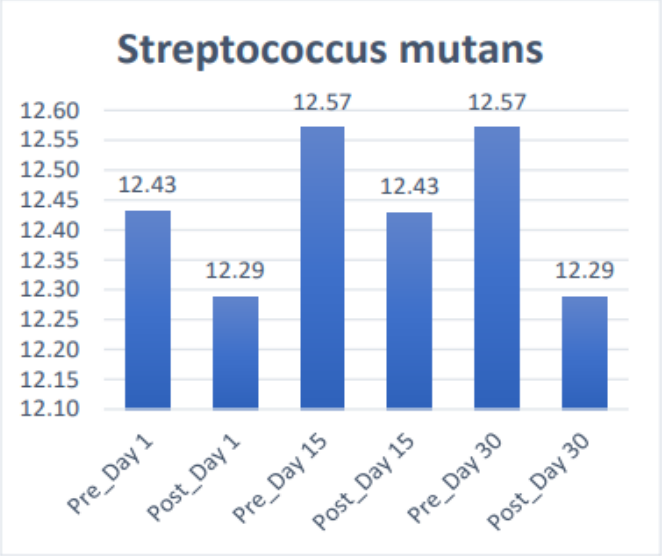
Results

A repeated measures ANOVA with a Greenhouse-Geisser correction determined that mean of S. Mutans between the time points is not statistically significantly (F (2.3, 14.279) = 0.377, P=0.860) (Table 1, Graph 1)

Table 1

Streptococcus Mutans	Mean	Std. Deviation	95% Confidence Interval	
			Lower Bound	Upper Bound
Pre Day 1	12.43	4.35	8.40	16.45
Post Day 1	12.29	4.47	8.15	16.42
Pre Day 15	12.57	4.54	8.37	16.77
Post Day 15	12.43	4.42	8.34	16.52
Pre Day 30	12.57	4.72	8.21	16.94
Post Day 30	12.29	4.32	8.29	16.28

Graph 1

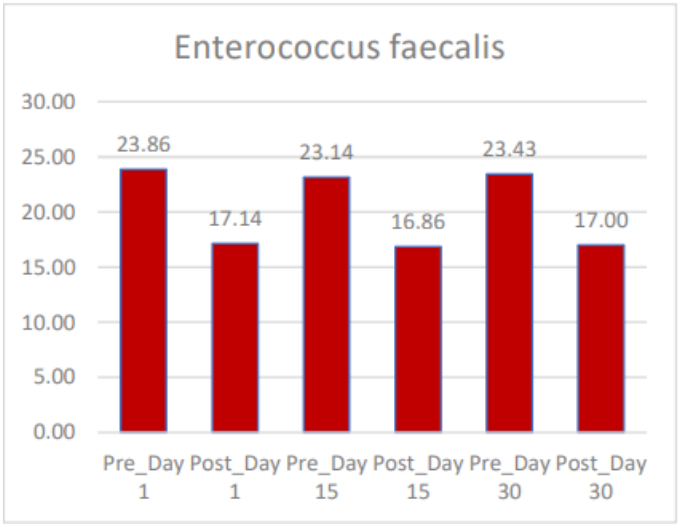


A repeated measures ANOVA with a Greenhouse-Geisser correction determined that mean of Enterococcus faecalis between the time points is statistically significantly (F (1.01, 6.061) = 2.498, P=0.0456). (Table 2, Graph 2)

Table 2

Enterococcus faecalis	Mean	Std. Deviation	95% Confidence Interval	
			Lower Bound	Upper Bound
Pre_Day 1	23.86	0.90	23.03	24.69
Post_Day 1	17.14	11.74	6.29	28.00
Pre_Day 15	23.14	1.46	21.79	24.50
Post_Day 15	16.86	11.54	6.18	27.53
Pre_Day 30	23.43	0.98	22.53	24.33
Post_Day 30	17.00	11.62	6.25	27.75

Graph 2

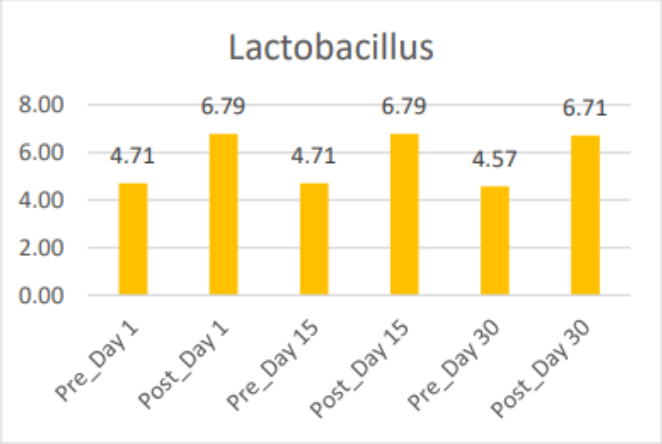


A repeated measures ANOVA with a GreenhouseGeisser correction determined that mean of Lactobacillus between the time points is statistically significantly (F (1.05, 6.333) = 2.80, P=0.034). (Table 3, Graph 3)

Table 3

Lactobacillus	Mean	Std. Deviation	95% Confidence Interval	
			Lower Bound	Upper Bound
Pre_Day 1	4.71	5.91	-0.75	10.18
Post_Day 1	6.79	6.55	0.73	12.84
Pre_Day 15	4.71	5.99	-0.83	10.26
Post_Day 15	6.79	6.57	0.71	12.86
Pre_Day 30	4.57	5.77	-0.76	9.91
Post_Day 30	6.71	6.48	0.72	12.71

Graph 3



There is statistically significant result in the effect of Immunoglobulin Y (Igy) against Lactobacillus and E. Faecalis in between the time intervals. There is antimicrobial effect of Immunoglobulin Y (Igy) against S. Mutans but the result is not statistically significant in between the time intervals.

Discussion

Face is the index of mind and oral cavity is the index of general health.3 Normal flora in the oral cavity are complex and about 350 species of bacteria have been cultured, however still a lot of bacterial species are in the stage of identification.9 Microorganisms found in the human oral cavity have been referred to as the oral microflora, oral microbiota, or oral microbiome.[10]

Dental caries is a microbial, infectious, disease which is multifactorial and can be found in almost all people in the world. Global reduction in dental caries would help in reducing the heavy budget allotment for dental treatment and there is a worldwide need for development and broadening of anti caries measures.[3]

Streptococcus genus in the oral cavity consists of four main species, out of which S. Mutans is the major dental caries causing bacteria.9,10 The molecular pathogenesis of S. Mutans associated dental caries involves a series of binding events that leads to accumulation of cariogenic bacteria thereby causing disease. The initial binding event involves the interaction of bacterial cell adhesins (antigen I/II) with receptors in the salivary pellicle.4After the discovery of the association of S. Mutans with dental caries, various studies have been conducted to determine alternative caries prevention methods using antibacterial agents.[11]

As microbial adherence is a prerequisite for host colonization, any blockade of S. Mutans surface structures before they contact a tooth surface can interfere with the caries formation. A common method for blocking microbial adherence is the administration of vaccine that induces formation of antibodies against surface localised microbial structures. No caries vaccines are available commercially for human use to date and given the hurdles involved in caries vaccine development, passive immunization has received attention. This involves the introduction of antibodies directly to the oral cavity.[12]

Passive immunization cannot become reality unless an inexpensive method for mass production of antibodies are available; and eggs are a convenient source of

polyclonal antibodies in the form of egg yolk immunoglobulins (Immunoglobulin Y(Igy)).[12]

Igy is seen in birds, reptiles, and lung fish and it is the evolutionary predecessor of IgG and IgE. Igy was called IgG due to the structural similarity between the two. Leslie and Clem highlighted the particular differences between Igy and IgG and advocated the use of the term Igy instead of IgG.13 The use of chicken egg yolk can reduce use of experimental animal, as chicken eggs can produce large amount of antibodies.14 In 1893 the specific protective effect off egg yolk extracts from immunised hens, attributed to the transfer of serum chicken antibodies to eggs, was first described.[13]

Igy binds to S. Mutans antigens and neutralizes the biological activity of the antigen thereby prevent colonization of S. Mutans, specific Igy antigen I/II can prevent binding of S. Mutans. this causes the S. Mutans bacteria to fail to proliferate. Virulence of S. Mutans bacteria also due to its intercultural and interspecies communication (quorum sensing).[11]

Study conducted by Hatta et al. evaluated the effect of Igy in mouthwash and demonstrated a decrease in the ratio of S. Mutans percentage after the subjects used a mouth rinse containing Igy and 10% sucrose twice in 4 h.15 A study by Endang Winiati Bachtiar et.al showed that mouthwash containing a combination of Igy anti-ComD S. Mutans + chitosan could decrease the number of S. Mutans in saliva, but not significantly which is in concordance with the results of the present study.[11]

A study conducted by Juni Hand Ajani et al to determine the effect of Igy serum against the growth of dental plaque bacteria was done using diffusion method. It concluded that Igy serum that immunized S. Mutans has antibacterial effect against S. Mutans only.10 In the present study it was seen that there is antimicrobial effect of Immunoglobulin Y (Igy) against S. Mutans but the result is not statistically significant in between the time intervals.

The Lactobacilli comprise a diverse collection of gramspositive bacilli which are associated with dental caries for over a century. After eruption of teeth, the occlusal fissures are ideal retentive sites for Lactobacilli. Modern studies on children below 6 years of age showed a correlation between the presence of Lactobacilli in the oral cavity and dental caries, with few caries-free children positive for Lactobacilli. [16]

Endodontic treatment failure maybe due to persistence of bacteria, poorly cleaned and obturated root canals, improper coronal seal, and untreated canals. Presence of bacteria inside the root canal system like E. Faecalis is the reason for endodontic failure, it is resistant to disinfection agents, leading to persistent intra-radicular or extra radicular infection.[17]

Despite Lactobacilli and E. faecalis being major factors in caries progression and endodontic treatment failures, there are no previous studies evaluating the effect of Igy against Lactobacillus and E. Feacalis. Most of the attention in Igy research in dentistry has been directed towards the study of S. Mutans. This present study shows that there is antimicrobial effect of Immunoglobulin Y (Igy) against Lactobacillus and E. Faecalis, and these results are statistically significant in between the time intervals. Given the various roles of Igy, it is expected that this study could serve as a foundation for further investigations for cariology experiments that can eventually be implemented in the community.

Conclusion

Results of this study shows that Igy has antimicrobial effects against dental pathogens; S. Mutans, E. Faecalis and Lactobacillus. Further in-vivo studies on Igy and its effects can be beneficial in the development of oral

formulations like toothpastes, antimicrobial agents etc. that can be beneficial for the community.

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