



A pilot study of caries preventive effect of xylitol against plasmid born biofilm producing *Lactobacillus acidophilus* isolated from human dental caries

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Abstract

Today, tooth decay usually happens slowly over a period of years. Decay begins when the bacterial population increases in the mouth. A cavity forms when acids accumulate and cause more damage to the tooth. The cariogenic infection is caused from predominant pathogen *Lactobacillus acidophilus*. So the plaque samples were collected from different childhood caries around varied dental clinics around Tirupur Dt. The organisms confirmed by phenotypic and genotypic characterization. After the organism was screened for biofilm production to check the resistant ability of cariogenic pathogen. Next the resistant was confirmed by plasmid analysis. In this study 10 biofilm producing cariogenic isolates (MTVG02, MTVG06, MTVG17, MTVG36, MTVG38, MTVG75, MTVG85, MTVG92, MTVG95 and MTVG120) having 10000 – 600bp. Due to this resistance ability of organism urgently need therapeutic drug to fight the predominant pathogen. Currently, xylitol has a powerful mechanical action to eliminate a cariogenic pathogen around oral cavity. Different concentration of xylitol (3%, 8%, 12% and 18%) was performed by Agar Well Diffusion assay. Among the concentration the maximum inhibitory activity was recorded 3% concentration of xylitol against *Lactobacillus acidophilus*.

Keywords: dental caries, *Lactobacillus acidophilus*, xylitol, antimicrobial activity

1. Introduction

Dental caries is one of the main oral problems suffered by the human population. It is a very destructive disease of teeth. The resulting in destruction of tooth structure by acid forming bacteria found in dental plaque, an intraoral biofilm, in the presence of sugar. The infection can progress through the dentin to the pulp, ultimately compromising the vitality of the tooth.

A Variety of literatures have proved that *Streptococcus mutans* and *Lactobacillus acidophilus* was the main causative agent for dental caries (Fitzgerald and Keyes, 1960) [1]. The reduced oral hygiene due to lack of proper knowledge of dental care has led to increased prevalence of dental caries around the world. Its treatment is costly and requires the expertise of highly skilled professionals. In developing countries where dental care facilities are out of reach of most of people, there is need to educate them about dental care and to promote conventional means of teeth cleaning.

At present, xylitol promotes mineralization by increasing the flow of saliva, which is a common effect of all polyol sweeteners. The unique in xylitol is that it is practically nonfermentable by oral bacteria which counter acts low pH-values in the oral cavity (Havenaar *et al.* 1978) [2]. The caries-preventive effect of xylitol in clinical studies could not be explained by the exclusion of fermentable sugars from the diet (Ly *et al.* 2008) [3]. In controlled clinical trials xylitol has performed better than sorbitol (Hayes, 2002) [4].

Recently, it was shown that xylitol syrup management to children banned early childhood caries, while no such effect was detected in the control group receiving high-sorbitol syrup (Milgrom *et al.* 2009) [5]. This study also demonstrated that not only chewing gum but also other xylitol vehicles can be effective in delivering xylitol has given a good promise in the pharmaceutical line to treat cariogenic pathogen around oral cavity.

2. Materials and methods

2.1 Sample Collection

Plaque sample was collected from different dental clinics around Tirupur district using transport medium. After, the collected sample was kept in laboratory incubator at 37°C, 24h for enrichment process. Finally, the samples were taken for dilution technique to obtain specific dilution for isolating cariogenic pathogen *Lactobacillus acidophilus* using selective medium like Man Rogosa Sharpe agar.

2.2 Identification of *Lactobacillus acidophilus*

The samples were identified by morphological, gram staining, biochemical tests and 16Sr-DNA gene sequencing.

2.3 Physiological properties of *Lactobacillus acidophilus*

2.3.1 Tolerance of NaCl

All the isolates were grown in MRS broth supplemented with different concentration of NaCl (1%, 2%, 3%, 4%, 5%, 6%,

7%, 8%, 9% and 10%). The broth were inoculated with 100 μ l overnight culture of the isolates and incubated in anaerobic condition at 37°C for 24 h. After 24 h incubation, growth was determined by using a spectrophotometer and reading optical density at 600 nm.

2.3.2 Nephelometric method for measurement of bacterial growth in different sugar

Growth of control culture (glucose) and of test cultures (different sugars with test organisms) were monitored after 24h, 48h and 72h of incubation by measuring optical density at 600 nm using the spectrophotometer.

2.3.3 Determination of supernatant pH after the cultivation of oral pathogen

The bacterial cells were centrifuged after 48 hours (15 mins, 5000 rpm at 4°C). The pH of the supernatant was measured using digital pH meter.

2.4 Screening of Cariogenic Dental Biofilm

2.4.1 Micro Titer Plate Assay

An overnight culture of bacterial strain was prepared with chemically defined media. After vortexing the overnight, 50 μ l of volume were transferred in to Micro titer plate. The basal medium MRS broth was substituted with different sugar (glucose, lactose, fructose, maltose, raffinose and sucrose) at different concentration (1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%). 200 μ l of these media were transferred to each micro plate. After it was kept in 24h of incubation at 37°C. The micro titer plate wells were washed two times with sterile distilled water to remove loosely associated bacteria. Each well was stained with 200 μ l of ethanol 95%. The ethanol was removed after 15 mins. After the plates were air dried for 45 mins. The each well was stained with 150 μ l of 1% Crystal violet solution for 5 mins. After staining plates were washed with sterile distilled water. At this end point biofilm were visible as purple rings formed on the side of each well. The wells were left to dry and after drying each well was stained with acetic acid 33% and the absorbance was read in an ELIZA reader.

2.5 Isolation of cariogenic plasmid from *Lactobacillus acidophilus*

The isolation of plasmid from *L. acidophilus* was done by using the method of rapid boiling preparation technique (Holmes & Quigley, 1981 [6]; modified by Riggs & McLachlan, 1986) [7].

2.6 Preparation of pharmaceutically active xylitol stock solution

Preparation of xylitol stock solution 30.43% of 2M xylitol stock solution was prepared by dissolving 30.43g xylitol in 100 ml distilled water. 30.43% aqueous xylitol solution was made by dissolving 30 g of xylitol in 100 ml distilled water. The stock solution was autoclaved at 121°C for 15 min.

2.7 Transfer of bacterial culture with different concentration of xylitol

Take 2.72 ml, 2.22 ml, 1.83 ml and 1.23 ml of cariogenic *L. acidophilus* cultures were added in 0.29 ml, 0.78 ml, 1.17 ml

and 1.77 ml of 2M (30.43%) xylitol stock solution. The mixture provided 3 ml of bacterial growth medium with 3%, 8%, 12% and 18% of xylitol. Likewise, 3 ml of bacteria in broth was prepared to serve as control. All the solutions were stored in a 37°C incubator.

2.8 Antimicrobial efficacy of xylitol against *Lactobacillus acidophilus*

The antimicrobial activity of xylitol was done by well diffusion method. The solid medium was prepared with using Muller Hinton agar. After solidification of medium the 0.1ml overnight culture was spread over the surface of agar. After puncture was made by using different range of Cork borer (50 μ l, 100 μ l and 150 μ l). Finally the hole was filled with 3%, 8%, 12% and 18% concentration of xylitol at different μ l (50, 100 and 150). After it was kept in incubator at 37°C, 24h for its antimicrobial activity. Measure the zone of inhibition around the well.

3. Results

Preface

ECC is a public health problem that continuous to affect babies and pre-school children worldwide. A comprehensive review of the epidemiology of ECC showed that its prevalence varies from population to population. This disease exerts a social, physical, mental and financial burden on global scale, with developing countries being the most affected.

Isolation and Characterization of Biofilm Producing *Lactobacillus acidophilus*

Totally one fifty caries plaque samples were collected from different dental clinic hospitals from rural area of Tirupur city, Tamilnadu, India. 129 isolates of *Lactobacillus acidophilus* were isolated from the samples. Selective media: Man Rogosa Sharpe media were used to isolate the cariogenic pathogen *Lactobacillus acidophilus*. The colonies were cream, little sticks and smooth round white color colony respectively. These colonies were isolated and stored for further experiment. The decay causing predominant cariogenic pathogen *Lactobacillus acidophilus* strains were identified by comparing the results with standard biochemical test of *Lactobacillus acidophilus* such as gram positive in rod shape as result of gram staining. In liquid MRS broth it produced uniform turbidity. It was homofermentative and it showed positive reaction in following tests include fermentation of Glucose, Sucrose, Lactose, Raffinose, Galactose, Carbohydrate, Tribble sugar iron, Simmon citrate, Urease, Oxidase, Nephelometric detection, pH after cultivation of bacteria and Tolerance of NaCl negative results were observed in case of Starch hydrolysis, Catalase, Motility. Hence to identify and confirm the *Lactobacillus acidophilus* at molecular level, 16S-rDNA gene region was amplified and sequenced.

Tolerance of NaCl

NaCl is an inhibitory substance which may inhibit growth of oral bacteria. In the present study all the cariogenic isolates MTVG02, MTVG20, MTVG25, MTVG38, MTVG46, MTVG54, MTVG66, MTVG72, MTVG87 and MTVG92 showed the good tolerance over the range of 1-10% w/v

concentration of NaCl in the MRS broth. The result indicated that the strain MTVG72 was showed highest growth in the salt concentration among other isolated *Lactobacillus acidophilus*. However significant decrease in the growth with the increase in the salt concentration was observed (Fig. 1).

Nephelometric Method for Measurement of Bacterial Growth in Different Sugar

In this study different sugars were used for growth of cariogenic pathogen. Among different sugars the maximum optical density was recorded in glucose at 24h, 48h and 72h of incubation (Fig.3).

Determination of Supernatant pH after the Cultivation of Oral Pathogen

The result was obtained in this study the cultivation of bacterial cells in pH 7. After cultivation the organism was centrifuged. Finally, the pH of the supernatant was measured using digital pH meter. Suddenly, the rate of pH was increased after cultivation of bacteria. It indicates the test organisms are able to emit their predominant growth using utilizing of food ingredients in culture medium to degrade the neutral pH (Fig. 2).

Screening of Cariogenic Dental Biofilm

Among the different carbon sources at different concentration (1-10%) used in this study the biofilm formation was increased to a certain level of glucose concentration in the medium at 37°C (Fig. 4).

Isolation of Cariogenic Plasmid

Electrophoretic analysis of the plasmid DNA was prepared and carried out by agarose gel electrophoresis on 0.7%. 10 biofilm producing cariogenic isolates (MTVG02, MTVG06, MTVG17, MTVG36, MTVG38, MTVG75, MTVG85, MTVG92, MTVG95 and MTVG120) which was showed more than 100% resistance against antimicrobial tested (Na F) were selected for plasmid isolation (Fig. 5).

Antimicrobial Activity of Xylitol against Decay Pathogen

Antibacterial activity of 3%, 8%, 12% and 18% concentration of xylitol was performed by Agar Well Diffusion assay against cariogenic *Lactobacillus acidophilus*. Three different concentrations (50µl, 100µl and 150µl) were used in this assay against oral *Lactobacillus acidophilus*. The maximum zone of inhibition 25 mm, 35mm and 32mm was observed in strain no. MTVG75 followed by the minimum zone of inhibition 24mm, 28mm and 30mm was observed in strain no. MTVG06 at different µl concentration of (50,100 and 150) xylitol (Fig. 6).

4. Discussion

Isolation, Identification and Antimicrobial production of *Lactobacillus acidophilus*

The present study, *Lactobacillus acidophilus* was isolated from tooth decay of rural patient around Tirupur city, which were identified on the basis of their colony morphologies and some biochemical tests. Sodium chloride is an important factor which dramatically affects bacterial growth. In this study experimental design we have observed the growth of isolated *Lactobacillus* in various NaCl value range was to determine whether LAB species can grow in 1-9% concentration and also to predict the optimum NaCl value for good growth. This results concordance with the author Hoque *et al.* (2010) [8] studied, *Lactobacillus* spp. were isolated from two regional yoghurts in Bangladesh, which were identified on the basis of their colony morphologies and some biochemical tests. It was observed that isolated *Lactobacillus* spp. were resistance to inhibitory substances like phenol (0.4%), NaCl (1-9%) and bile acid (0.05-0.3%).

Screening of Cariogenic Dental Biofilm

In this study, enhanced biofilm formation was observed with increasing concentration of glucose in the medium at 37°C. This indicates that biofilm formation was increased to a certain level of glucose concentration in the medium and the gradual diminished may be an effect of glucose concentration on the microbe. This result is in concurrence with the observation laid down by Christensen *et al.* (1985) [9] found that bacterial adherence and glycocalyx formation was enhanced with the supplementation of glucose in the culture media.

Isolation of Plasmid from Biofilm Producing *Lactobacillus acidophilus*

The result presented in this research, the convenience of the technique makes this rapid mini-prep procedure suitable for routine plasmid isolation from biofilm producing *Lactobacillus acidophilus* having 1000kb in size. But, the author O'Sullivan and Klaenhammer, (1993) [10] who reported a simple, rapid plasmid mini-prep procedure for *Lactococci* and *Lactobacilli* which gives high yields large plasmids were isolated from both LAB strains, including a 70kb plasmid from *Lactobacillus acidophilus*.

Antimicrobial Activity of Xylitol against *Lactobacillus acidophilus*

The present study indicates that the addition of xylitol drastically can enhance the effect of antibacterial agent to treat caries infections. Also, the study demonstrated that different concentration (3%, 8%, 12% and 18%) of xylitol consumption affect the oral flora by a decrease in *L. acidophilus* and inhibits the growth of LAB. Similar report was obtained in the previous reports of the author Helanto *et al.* (2006) [11] state that the subsequent accumulation of xylitol result of their study also confirms that *S. mutans* and *L. acidophilus* possess the fructose pathway and could thus be inhibited by xylitol.

5. Figures

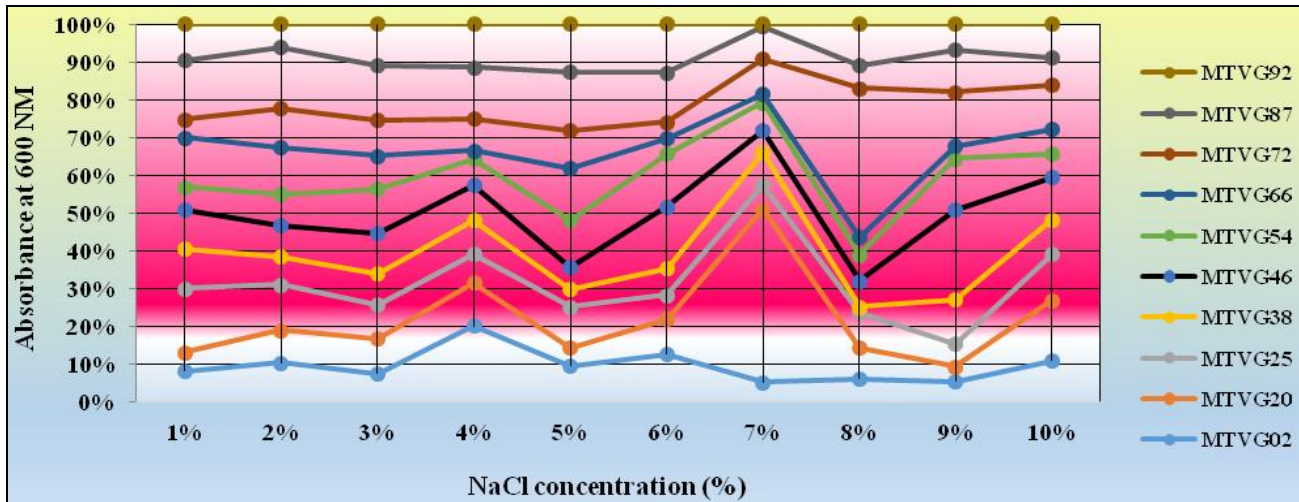


Fig 1: Effect of NaCl on the growth of cariogenic *Lactobacillus acidophilus*

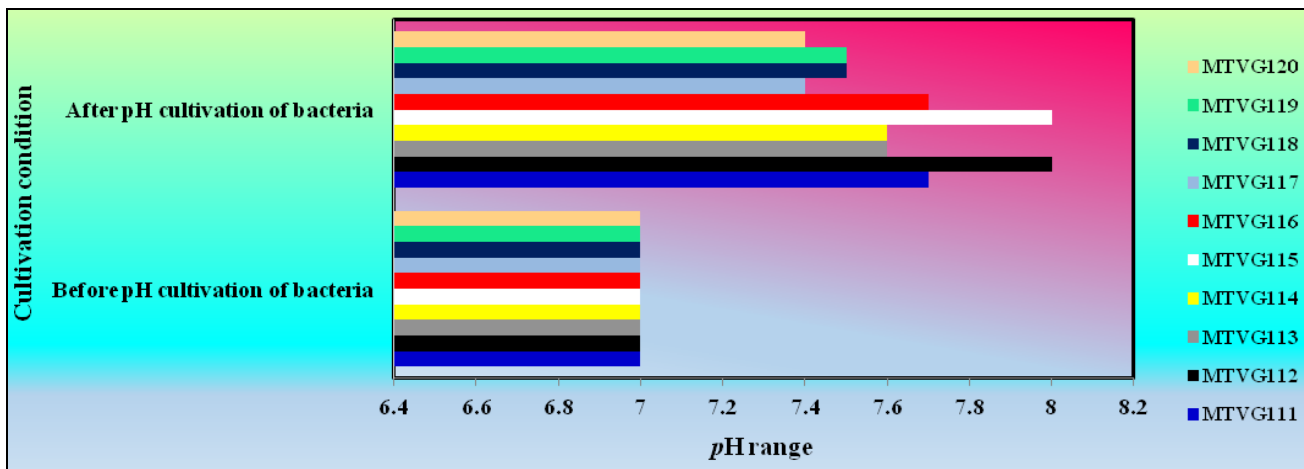


Fig 2: Determination of pH subsequently cultivation of *Lactobacillus acidophilus*

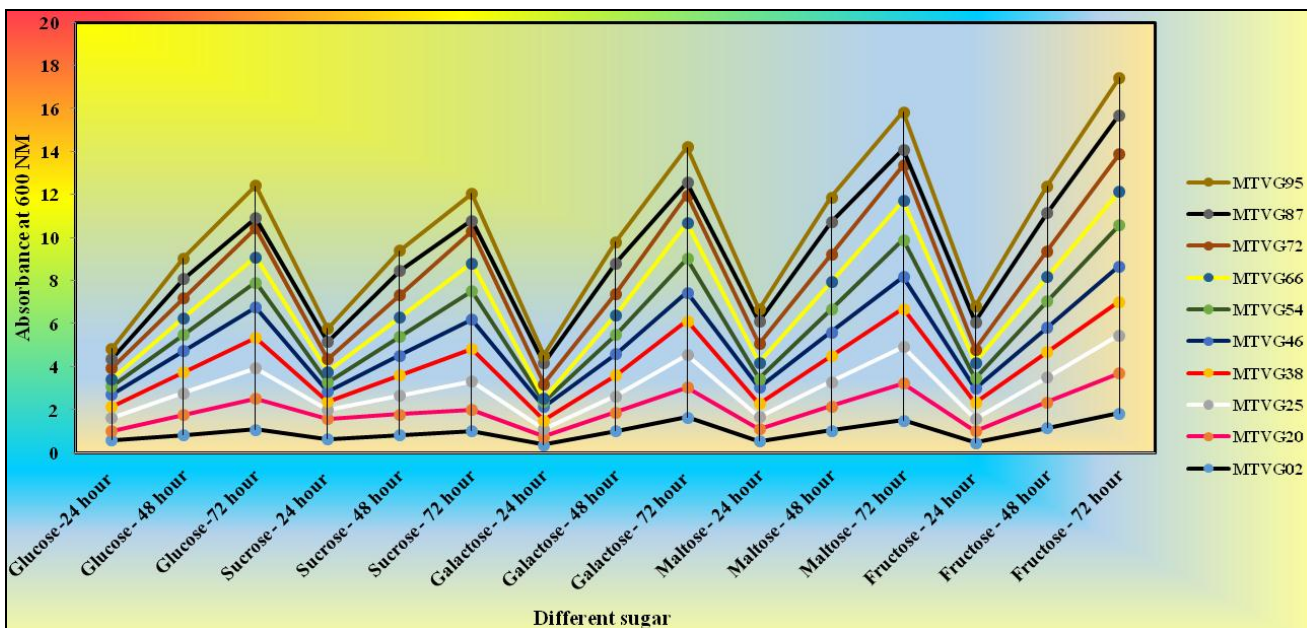


Fig 3: Nephelometric detection of measurement of bacteria growth on different sugar by LAB

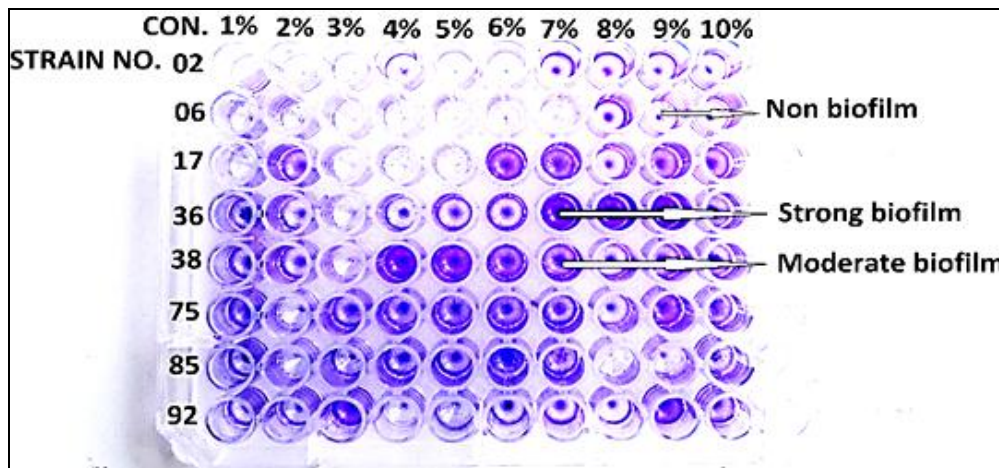


Fig 4: Detection of biofilm by cariogenic pathogen *L. acidophilus*

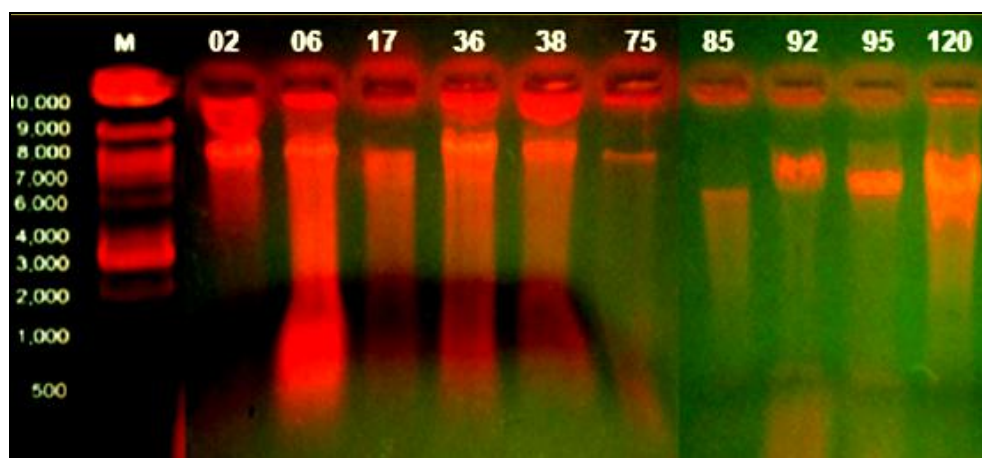


Fig 5: Isolation of cariogenic plasmid from *Lactobacillus acidophilus*

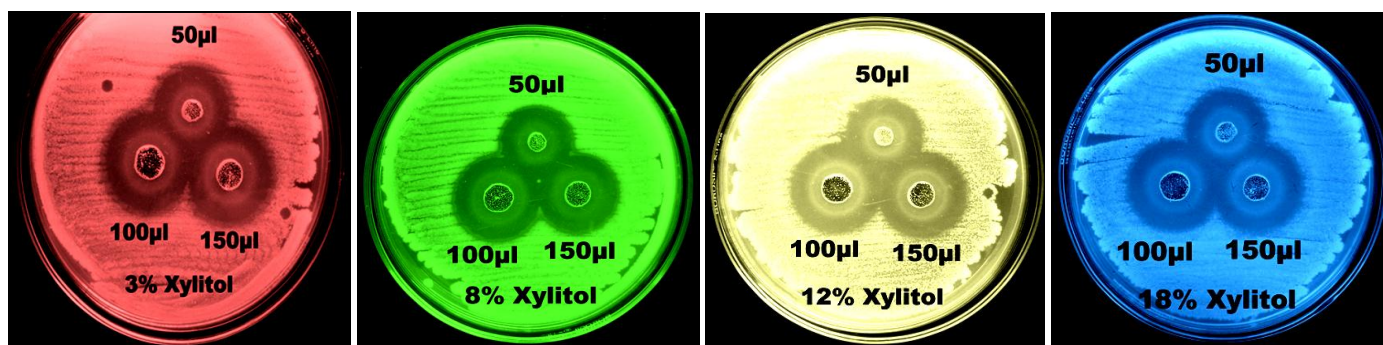


Fig 6: Antimicrobial Effect of Xylitol against *Lactobacillus acidophilus*

6. Conclusion

In this present study concluded that the isolation of LAB strains from various dental clinics and after identified by morphological and genomic characterization. Further the isolates able to tolerant maximum sodium chloride concentration and able to survive at different sugars it consequently confirm that the test organism is able to cause sweeter eating children's leads to decay. Currently so many drugs used to treat the cariogenic organisms but some organism it able to tolerate drug because they have biofilm mechanism to deliver the resistance characteristics. The

investigation confirms the drug resistance character using biofilm technique. After the resistance isolates was confirmed by isolating plasmid from cariogenic strain. Recurrently the five pentahydroxy alcohol, broadly occurring in nature to posses' antimicrobial activity against dental pathogens. The consumption of 3% xylitol it able to reduce the *L. acidophilus* growth. This study confirms that even in low concentrations of xylitol has act a vital role in inhibitory effect against main oral cavity inducer bacteria. This agent is act as next weapon to treat predominant drug resistance cariogenic microorganisms around oral cavity of children's.

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