Evaluate the efficacy of Probiotic Lactobacilli on growth and biofilm formation in *Streptococcus mutans* isolated from gingivitis

Abbas Mayar Hezam

*abbas.hezam@qu.edu.iq*

Biology Department, College of Science, University of Al-Qadisiyah, Iraq

**Abstract**

The present study aimed to assess the antibacterial effects of Probiotic Lactobacilli against *Streptococcus mutans* isolates. A total of 13/52 (25%) *S. mutans* isolates were collected from patients suffering from gingivitis, which were identified using morphological characteristics, biochemical tests and PCR technique. Micro titer plate method was used to detect the biofilm formation in tested bacterial isolates by measuring optical density (OD) using ELISA reader at a wavelength 640 nm. The results revealed that only 10/13 (77%) isolates produced the biofilm. Based on the results obtained in this study, the isolates were classified according to their ability to the biofilm formation as follows: the isolates with thick biofilm (OD ≥ 0.300 nm), the isolates with moderate biofilm (OD 0.2-0.3 nm), the isolates with thin biofilm (OD 0.1-0.2 nm) and Non-productive isolates of biofilm (OD < 0.1 nm). On the other hand, the agar well diffusion method was used to evaluate the effectiveness of Probiotic Lactobacilli against the growth of bacterial isolates by measuring the inhibition zones around the wells containing of probiotics (50, 100 mg/ml and stock). The results indicate that inhibition zones of the tested isolates were weak at 50 mg/ml, moderate at 100 mg/ml and strong at stock solution. A modified crystal violet test in well micro-titer plates was used to detect the ability of isolates to the biofilm formation after treatment with various concentrations of Probiotic Lactobacilli by measuring optical density (OD). The results indicate a decrease in the ability of the tested isolates to the biofilm formation compared to control where the isolates treated with probiotics at 50 mg/ml formed a strong biofilm (OD 0.300 - 0.341 nm) and the isolates treated with probiotics at 100 mg/ml formed moderate biofilm (OD 0.209 - 0.263 nm), while the isolates treated with the stock solution of probiotics formed a thin biofilm (OD 0.144 - 0.198 nm). In conclusion, the results of the current study indicate that the Probiotic Lactobacilli have inhibitory effect on growth and biofilm formation in *Streptococcus mutans* isolates and the antibacterial activity of Probiotic Lactobacilli is increased with increasing concentration.

**Keywords:** *Streptococcus mutans*, Biofilm, Optical density, Probiotic, Gingivitis.
Introduction
Gingivitis is a one of common disease in humans that occurs because the colonization of the oral cavity by *Streptococcus mutans*. Pathogenesis of *Streptococcus mutans* is due to their ability to biofilm formation and synthesize extracellular polysaccharides on living surfaces (1). Most individuals colonized by *Streptococcus mutans* have higher gingivitis than uninfected individuals. *Streptococcus mutans* forming biofilms have gained high resistance to antibiotics, where the biofilm formed leads to a loss of antibiotic effectiveness in the treatment of infections (2).

Therefore, recent studies have focused on the use of Probiotic Lactobacilli as a therapeutic agent rather than antibiotics to reduce infections, especially oral diseases caused by *Streptococcus mutans* associated with the biofilm formation such as gingivitis and oral candidiasis (3). The antibacterial activity of probiotics acts on morphological and physiological changes in the microbial plasma membrane and thus the contents of the cytoplasm are released out of the cell and eventually the death of the microbial cells as well as reduce the biofilm formed in bacterial cells (4). Probiotic Lactobacilli can be defined as beneficial viable bacteria that when given in sufficient quantities inhibit the growth of pathogenic bacteria, improve digestion, stimulate the immune system, increase resistance to infection and neutralize toxins (5,6). The *Lactobacillus acidophilus* in Fermented foods have many of the trait that make it as Probiotic, It is highly competitive for adhesion to cells and is characterized by the production of many antibacterial substances such as lactic acid, Bacteriocins, and hydrogen peroxide and other substances that have antibacterial activity on pathogenic bacteria (7). The present study revealed the effect of Probiotic Lactobacilli in most of *Streptococcus mutans* isolates. Therefore, the aim of the current study was to evaluate the antimicrobial activity of Probiotic Lactobacilli on biofilm formation and bacterial growth.

Materials and Methods
Collection of samples:
Fifty-two specimens (swabs) were collected from patients suffering from gingivitis during the period from January to June 2018. All specimens were suspended in Phosphate buffer saline and transported in ice containers to the laboratory for the purpose of testing.
Isolation and identification of \textit{S. mutans}

\textit{Streptococcus mutans} were isolated and diagnosed through streaking of \textit{Mitis-Salivarius Bacitracin Agar (MSB)} with specimens and incubated at 37ºC for 24 hrs. The colonies were examined and diagnosed on MSB Agar as pale-blue colonies. Catalase test, Hydrolysis of bile esculin and PCR also used as confirmation test of \textit{S. mutans} (8,9).

Biofilms Formation
The ability of isolates for biofilms formation were detected by using Micro titer plate method according (10,11): inoculate 3 ml of Tryptic soy broth with isolates and incubated at 37ºC for 24 hours. Then, added 200ul of bacterial suspension to each well of micro plate and incubated at 25 ºC for 24 hours. The bacterial growth was removed from wells by wash with normal saline, incubate the micro plate at 60 ºC for 1 hrs. to fixation membrane, and dye the wells with crystal violet for 5 min and washing with tap water to eliminate surplus dye. The biofilms were measured using ELISA reader at 640 nm (12)

Probiotic Lactobacilli Preparation
Inoculated the MRS broth with 1% of the lactic acid bacteria (LAB), the bacterial culture was incubated under anaerobic conditions at 37º C for 18-24 hours. Then, the bacterial growth was centrifuged at 6,000 cycles / min for 15 minutes. The Supernatant was collected and adjusted to the pH at 6.5 using NaOH. After that, the Supernatant was filtered through Millipore filter 0.22 µm (13,14).

Antibacterial activity of Probiotics by agar well diffusion method
The test was achieved according to (18) using modified agar-well diffusion test, the overnight culture of \textit{S. mutans} was spread on Mueller-Hinton agar, then made wells in media using a cork borer, add 50 µl of the probiotic to each well and incubated at 37 C for 24-48 h. The result was revealed by measurement the inhibition zone surrounding the well and recorded as follows: none (< 10), weak (11–14 mm), moderate (15–19 mm), strong (≥20 mm). (19,20).

The Probiotic Lactobacilli activity of anti-biofilm
The antimicrobial activity of probiotic on biofilm formation was performed by using modified crystal violet test in well micro-titer plates. The results were recorded by measurement optical density using an ELISA reader (15,16,17).

Polymerase chain reaction
PCR was performed to detect of \textit{16S rRNA} gene using specific primer as in table (1). Genomic DNA Mini Kit used to extract of genomic DNA from \textit{S. mutans}. Genomic DNA concentration was measured by a Nano drop spectrophotometer. PCR master mix was performed in 20μl total volume according to kit instructions (AccuPower® PCR PreMix kit. Bioneer. Korea) by adding 5μl of genomic DNA,1.5μl of forward primer and 1.5μl of reverse primer in to PCR premix tube, then complete the to 20μl
with deionizer PCR water. The reaction was completed in a thermo cycler by the following steps; initial denaturation at 95 °C for 5 min, followed by 30 cycles at denaturation at 95 °C for 30 s, annealing at 58°C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. The results were revealed by electrophoresis at agarose gel (1.5%), and used the UV light (21).

Table (1): DNA primers

| primers | product | Sequence                      |
|---------|---------|-------------------------------|
| 16S rRNA| 216 bp  | TTGCTCGTGTCAGTTCTGGG F        |
|         |         | CCTCAACATTTACCATGGGC R        |

Results

Isolation and diagnosis of S. mutans

A total 52 specimens were collected from patients suffering from gingivitis. The findings revealed that 13/52 (25%) isolates were diagnosed as S. mutans by using morphological characteristics, biochemical tests. The colonies appeared pale-blue in color on MSB agar. The results of the biochemical tests revealed that all tested isolates were negative for catalase test and positive for hydrolysis of bile esculin. The PCR technique was used as a confirmation test to detect 16S rRNA gene in S. mutans isolates where the results revealed presence 16S rRNA gene in 13/13 (100%) isolates as in (figure 1).

![Figure 1](image)

Figure (1): Electrophoresis of agarose gel (1.5%), which shows the PCR product results for the 16S rRNA gene in S. mutans isolates at 216 bp PCR product size, where M: Marker (100 -2000) bp, Lane (1-13) are positive results.

Biofilm Formation

Micro titer plate method was used to detect S. mutans's ability to the biofilm formation by measuring optical density (OD) with ELISA reader at a wavelength 640 nm. The results revealed only 10/13 (77%) of tested isolates produced the biofilm as in table (2). The optical density values of formed biofilm between (0.062-0.422 nm).
The isolates were classified according to their ability to the biofilm formation as follows: the isolates with thick biofilm (OD ≥ 0.300 nm), the isolates with moderate biofilm (OD 0.2-0.3 nm), the isolates with thin biofilm (OD 0.1-0.2 nm) and Non-productive isolates of biofilm (OD < 0.1 nm).

Table (2): Biofilm formation of *Streptococcus mutans* using Micro titer plate method.

| No. of isolates | Biofilm formation (OD<sub>640 nm</sub>) |
|-----------------|----------------------------------------|
| S 1             | 0.321 +++ve                            |
| S 2             | 0.402 +++ve                            |
| S 3             | 0.309 +++ve                            |
| S 4             | 0.401 +++ve                            |
| S 4             | 0.380 +++ve                            |
| S 6             | 0.422 +++ve                            |
| S 7             | 0.279 ++ve                             |
| S 8             | 0.221 +ve                              |
| S 9             | 0.243 +ve                              |
| S 10            | 0.121 –ve                              |
| S 11            | 0.075 –ve                              |
| S 12            | 0.096 –ve                              |
| S 13            | 0.062 –ve                              |

- -ve: no biofilm formation
- +ve: thin biofilm formation
- ++ve: moderate biofilm formation
- +++ve: thick biofilm formation
- S: *Streptococcus mutans* isolates

**Inhibitory effect of Probiotic Lactobacilli on the growth of S. mutans.**

The agar well diffusion method was used to evaluate the antibacterial activity of Probiotic Lactobacilli against the growth of bacterial isolates by measuring the inhibition zones around the wells containing of probiotics (50, 100 mg /ml and stock). The results indicate that inhibition zones of the most of tested isolates were weak (+) at 50 mg /ml, moderate (++) at 100 mg /ml and strong (+++) at the stock solution as shown in the table (3) where antimicrobial activity of Probiotic Lactobacilli was more effective with high concentration.

Table (3): sensitivity of bacterial isolates for Probiotic Lactobacilli.

| No. of isolates | Zone of Inhibition (mm) |
|-----------------|-------------------------|
|                 | 50 mg / ml              | 100 mg / ml | Stock     |
| S1              | 10 (+)                   | 15 (++)     | 22 (+++)  |
The Probiotic Lactobacilli activity on the biofilm formation

A modified crystal violet test in well micro-titer plates was used to detect the ability of isolates to the biofilm formation after treatment with various concentrations of Probiotic Lactobacilli by measuring optical density (OD). The test was performed by using Probiotic Lactobacilli at (50, 100 mg/ml and stock) concentration and Streptococcus mutans with a strong biofilm. The results indicate a decrease in the ability of the tested isolates to the biofilm formation compared to control where the isolates treated with Probiotic Lactobacilli at 50 mg/ml formed a strong biofilm (OD 0.300 - 0.341 nm) and the isolates treated with Probiotic Lactobacilli at 100 mg/ml formed moderate biofilm (OD 0.209 - 0.263 nm), while the isolates treated with the stock solution of Probiotic Lactobacilli formed a thin biofilm (OD 0.144 - 0.198 nm).

Table (4): shows Biofilm formation of Streptococcus mutans after treatment with different concentrations of Probiotic Lactobacilli.

| No. of isolates | Biofilm formation (OD 640 nm) |
|-----------------|--------------------------------|
|                 | - Probiotics (control) | + probiotics |
|                 | 50 mg / ml | 100 mg / ml | Stock |
| S 1             | 0.321 (+++ve) | 0.300 (+++ve) | 0.209 (++ve) | 0.164 (+ve) |
| S 2             | 0.402 (+++ve) | 0.340 (+++ve) | 0.261 (++ve) | 0.198 (+ve) |
| S 3             | 0.309 (+++ve) | 0.305 (+++ve) | 0.230 (++ve) | 0.144 (+ve) |
| S 4             | 0.401 (+++ve) | 0.330 (+++ve) | 0.260 (+++ve) | 0.193 (+ve) |
| S 5             | 0.380 (+++ve) | 0.321 (+++ve) | 0.261 (++ve) | 0.165 (+ve) |
| S 6             | 0.422 (+++ve) | 0.341 (+++ve) | 0.263 (++ve) | 0.190 (+ve) |

- Probiotics: biofilm formation in presence of Probiotic Lactobacilli.
- - Probiotics: biofilm formation in absence of Probiotic Lactobacilli
Discussion

Gingivitis is a major public health challenge. *Streptococcus mutans* plays an important role in oral cavity diseases (22) and is pathogenic through forming biofilms on the tissues of the oral cavity. The ability of *Streptococcus mutans* to biofilm formation may be due to synthesize extracellular polysaccharides. The formation of biofilm is one of the virulence factors, which plays role to protect the bacteria from host defenses, It also gives bacteria many traits such as antibiotic resistance and phagocytosis and as well as increases the adhesion of bacteria to the surfaces (23). Recently, *Streptococcus mutans* have acquired resistance to most antibiotics, therefore probiotics were used as therapeutic agents to target the biofilm and inhibition the oral cavity pathogens (24). The antimicrobial properties of probiotics may be due to the production of bacteriocins, hydrogen peroxide or organic acids (25). The data obtained in this study confirmed a significant decrease in the biofilm formed and as well as increased in inhibition zone around colonies of *Streptococcus mutans* after treatment with different concentrations of probiotics. This is due to the effect of inhibitors and acidic pH in the probiotics (23,25). The results were identical to previous results as the results obtained by Tahmourespour *et al* (23), Miller *et al* (26), Aween *et al* (27) confirmed that probiotics have an inhibitory activity against many bacterial isolates. On the other hand, the results differ from those obtained from Hawaz (28), Jose *et al* (29), where they revealed that probiotics do not affect the growth of most bacterial species.

Conclusions

The study concluded that the antimicrobial activity of Probiotic Lactobacilli against the growth of *Streptococcus mutans* increases with increasing concentration. Probiotic Lactobacilli can be used instead of antibiotics as a therapeutic alternative to inhibit of growth and biofilms formation in *Streptococcus mutans* causing dental diseases, especially gingivitis.

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