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Selection of oral Lactobacillus with high adherence capacity from Syrian Children

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ABSTRACT

Objective: Dental caries is a common problem threating most children and adults in Syria. Lactobacillus is one of the bacteria that could contribute in dental caries, but this genus is considered safe and it is commonly used as probiotic. Moreover, new studies have showed that some oral lactobacillus can highly adhere to teeth and prevent the adherence of pathogenic bacteria especially streptococcus mutans. Accordingly, our research was set to study the adherence properties of some strains of lactobacillus isolated from sound children saliva which could be used in future as probiotics for oral health.

Methods: Test of the adherence capacity of 42 strains of oral lactobacillus isolated from saliva by the microtiter dish assay. Study of the adherence capacity on saliva-coated hydroxyapatite discs of 20 adherent strains according to microtiter dish assay after identification by API 50 CHL.

Results: The microtiter dish assay results showed 20 strains of lactobacillus with high and moderate adherence capacity and 22 strains with low or very low adherence capacity. All of the 20 strains formed biofilms on HA discs, but 3 strains (1.L. pentosis, 2.L.fermentium 1, 3. L.fermentium 1) formed the highest biofilm. These 3 strains could be used as probiotic in future if they achieved other probiotic conditions.

KEY WORDS: Lactobacillus, saliva, adherence capacity, biofilm, probiotic, Syrian children.

1. INTRODUCTION

Dental caries is a common problem threating people worldwide. (Karpinski and Szkaradkiewicz, 2013). The cause of dental caries is the adherence of some bacteria to the teeth surface which produce acids that solubilize the teeth mineral and then form the dental plaque. (Loesche, 1996). Streptococcus mutans is the first bacteria that cause caries. Caries can also be caused by other bacteria, including members of the mitis, anginosus and salivarius groups of *streptococci*, *Enterococcus faecalis*, *Actinomyces* and *Prevotella* (Karpinski and Szkaradkiewicz, 2013). Various lactobacilli are related with progression of the lesion. When the lesion reaches the advanced clinical stage, only opportunistic bacteria such as lactobacilli can appeared (Loesche, 1996).

Lactobacilli are commonly considered safe and non-pathogenic. Occasionally, isolates of Lactobacillus are related with opportunistic infectious diseases in humans. In most cases the infections associated with lactobacilli have preceded predisposing microbial infections and the pathogenesy of lactobacillus in a healthy host is considered very low. (Adams and Marteau, 1995; Husni, 1997). Lactobacilli appear in the mouth after birth especially at the first year of a child's life. In children without caries, the rate of oral lactobacilli varied among the different studies depending on many factors like the ecological niches (Badet and Thebaud, 2008), breastfeeding or formula-feeding (Vestman, 2013) and the carbohydrate intake (Slavin, 2013).

Recently, some reports suggested that oral lactobacilli could play a beneficial role by inhibiting some cariogenic microorganisms such as streptococcus mutans (Ahumada, 2001). Some species isolated from the mouth of healthy subjects have an antimicrobial activity against *S.mutans*, *Porphyromonas gingivalis* and some other bacteria (Koll-Klais, 2005). Another effect is the inhibition of adherence of streptococcus mutans (Chung, 2004; Ahmed, 2014). According to these properties, lactobacillus could be used as probiotic.

Probiotics are "live micro-organisms which when administered in adequate amounts confer a health benefit on the host" (Rastogi, 2011). Probiotic first needs to attach to the surfaces of oral cavity in order to avoid or reduce its rapid exclusion from the mouth, and then they compete with other bacteria for carbohydrates, growth factors and site of adhesion (Jain and Sharma, 2012). According to these reports, the high adhesive capacity of lactobacilli is required to achieve its purpose. Our research was set to study the adherence properties of some strains of lactobacillus isolated from sound children saliva which could be used in future as probiotics for healthy teeth.

2. MATERIALS AND METHODS

Sampling and culturing: 94 saliva samples were collected in the early morning before eating or drinking or teeth cleaning from sound children (2-6 years old) without dental caries or gingivitis.

All samples were cultured in Man, Rogosa and Sharpe Medium (MRS) (HiMedia, India) in (5-10% CO_2) and 37°C for 24-48 hours. Identification of isolated bacteria in Lactobacillus genus was done by studying specifications according to referenced methods (gram negative rods or coccobacilli, catalase negative, none motile, none forming spores). 42 strains of Lactobacillus were isolated and cultured in MRS and cryopreserved at -80 °C.

Test of the adherence capacity by microtiter dish assay: according to the following protocol:

a) An overnight culture of each isolate was grown in MRS medium. The suspensions were adjusted with MRS broth to 0.5 McFarland turbidity standards.

b) Add 100 µL of the dilution per well in a 96 well dish. (Use 5 replicate wells for each isolate)
c) Incubate the microtiter dish for 48 hrs at 37°C in (5-10% CO2).
d) Turn the dish over and throw out the liquid.
e) Wash the dish softly with water to remove unattached cells.
f) Add 150 µL of a 0.1% solution of crystal violet in water to the wells.
g) Wait for 20 minutes.
h) Wash the dish with water.
i) Wait a few hours until the dish drying.
j) Add 150 µL of ethanol 95% to each well of the microtiter dish to solubilize the crystal violet.
k) Wait for 20 minutes.
l) Transfer 125 µL of the solubilized crystal violet to a new flat bottomed microtiter dish.
m) Quantify absorbance in a dish reader at 550 nm using ethanol 95% as the blank.

The strains which have high and moderate adherence capacity were identified by APE 50 CHL strips (Biomerieux, France) (a biochemical method depends on fermentation 50 sugars).

Adherence of bacteria to hydroxyapatite (HA) discs:

Saliva collection: Saliva was collected from sound children at least 2 h after eating, drinking, or teeth cleaning into sterile tubes and clarified by centrifugation (16 g for 30 min). The supernate was pasteurized (30 min, 65°C) and re-centrifuged in sterile tubes; the resulting supernate was dispensed into sterile tubes and stored at -20 °C. The success of pasteurization was evaluated by culturing saliva samples on blood agar aerobically and anaerobically.

Biofilm formation on (HA) discs: (3D bioteck, USA): Adherence of bacteria to HA discs was assessed on the 20 strains exhibiting high and moderate adherence capacity by microtiter dish assay.

a) In each well of a sterile 24-well cell culture plate, HA disc was put with a mixture of 1000 ml of saliva and 200 ml of bacterial inoculum.
b) The plate was incubated in (5-10% CO2) and 37°C for 48 hours.
c) To remove unattached bacteria, physiological saline was used to wash the surface of HA discs.
d) The surface of each disc was scrapped after putting it in a sterile Petri dish.
e) 1000 ml of physiological saline serum was used to wash the petri dish which contains the disc surface (the bacterial biofilm).
f) The bacterial biofilm were diluted many times (1m bacterial biofilm + 9 ml physiological saline) and then cultured on MRS agar.
g) After 48 h of incubation at 37 °C in (5-10% CO2), colony-forming units (CFU) were counted.

3 replicate discs were used for each strain, and then the CFU per population were averaged.

3. RESULTS

Adherence capacity by microtiter dish assay: Lactobacilli adherence capacity is shown in Table 1. Among 42 strains, 9 strains scored high adherence capacity and 11 strains scored intermediate adherence capacity, while 22 strains did not form clear biofilm on the dish surface.

Identification of adherent strains by API 50 CHL strips: The species of 20 adherent strains is identified by API 50 CHL, and the result is shown in Table 2. 40% of strains were L. Fermentum, and the others were L.salivarius, L. pentosus, L.Fermentum2, L. delbrueckii and L.rhamnosus.

Adherence of bacteria to hydroxyapatite discs: The adherence capacity of 20 strains to HA discs is shown in Table 3. Biofilms formed on HA discs were recovered for all 20 strains. 3 strains (L. pentosis, L.fermentium, L.fermentium) formed the highest biofilm (CFU/mm^2 ≤ 1*10^6).

Table.1. Adherence capacity of 42 lactobacillus strains

Adherence Capacity	Absorbance	Number of adherent strains	Percentage %
High	0.2 <	9	21.43%
Intermediate	0.15 – 0.2	11	26.19%
low	0.1 – 0.15	15	35.71%
Very low (no adherence)	0.1 >	7	16.67%

Table.2. Species of the adherent strains of isolated oral lactobacilli

Species of the strains	Number of the strains with the same species
L. Fermentum 1	8
L.salivarius	4
L. Pentosus	3
L. Fermentum 2	2
L. delbrueckii	2
L.rhamnosus	1

Table.3.Adhesion of salivary lactobacilli on hydroxyapatite discs. The CFU per population for triplicate discs were averaged

Species of the strain	CFU/mm^2	Species of the strain	CFU/mm^2	Species of the strain	CFU/mm^2
L. Pentosus	$1.8*10^6$	*L. delbrueckii*	$1.6*10^5$	*L. Fermentum 1*	$1.3*10^4$
L. Fermentum 1	$1.6*10^6$	*L. Fermentum 1*	$1.2*10^5$	*L. Fermentum* 2	$1.3*10^4$
L. Fermentum 1	$1.1*10^6$	*L. Pentosus*	$1*10^5$	*L.salivarius*	$1.2*10^4$
L. Fermentum 2	$6*10^5$	*L.salivarius*	$6*10^4$	*L. Fermentum 1*	$1.2*10^4$
L.rhamnosus	$3*10^5$	*L. Fermentum 1*	$4*10^4$	*L.salivarius*	$2*10^3$
L. delbrueckii	$3*10^5$	*L. Fermentum 1*	$1.8*10^4$	*L.salivarius*	$1*10^3$
L. Pentosus	$3*10^5$	*L. Fermentum 1*	$1.4*10^4$		

DISCUSSION

Lactobacilli are the most common supplemental flora in the mouth. (Patil, 2013). Saliva is a link between the different tissues and structures of the oral cavity. Its composition reveals the oral microbiological characteristics (Badet and Thebaud, 2008). Therefore, samples were collected from sound children saliva to isolate oral flora lactobacilli. Adherence and forming a biofilm is the first stage in the bacterial action. Lactobacillus adhesive properties were investigated by using two methods: microtiter dish assay and adhesion on saliva-coated hydroxyapatite discs.

The microtiter dish assay play an important role in evaluating the first stages in the biofilm formation, while its role in studying the mature biofilms is very low. However, this assay is necessary to evaluate many factors contributed in initiation of biofilm formation and well as genes involved in extracellular polysaccharide production. (O'Toole, 2011). By this assay, we were able to perform quick screening of the biofilm formation capacity of our strains. Our results showed that some strains of lactobacillus were able to biosynthesize polysaccharides that allow them to attach to a dish surface, so we continued the study on the 20 most adhesive strains using an HA model.

The composition of synthetic hydroxyapatite discs is similar to the composition of the bone and the teeth and their crystallographic properties are similar to the dental enamel (Al-Sanabani, 2013). The 20 strains of lactobacillus which we studied were able to adhere to saliva-coated hydroxyapatite discs. These results agree with (Samot, 2011) study which showed that 13 oral lactobacillus strains can adhere to saliva coated HA discs, but Lactobacillus buchneri formed the highest biofilm. Our results also agree with (Stamatova, 2009) study which demonstrated that Lactobacillus rhamnosus GG and *L. delbrueckii bulgaricus* can adhere to saliva coated HA discs, but they were isolated from yoghurt.

These 3 strains (*L. pentosis, L.fermentium 1, L.fermentium 1*) formed the highest biofilm because they have more ability to t biosynthesize of extracellular polymers. These molecules are the major factor influencing the microbial biofilm formation process because they contribute in irreversible adhesion phase (Myszka and Czaczyk, 2011).

4. CONCLUSION

The adhesion ability of the strains (*L. pentosis, L.fermentium 1, L.fermentium 1*) was very high, so they could be used as probiotic in future if they achieved other probiotic conditions especially the safety and the capacity of stimulating the immune system.

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