IN VITRO ANTIMICROBIAL ACTIVITY OF *Lactobacillus parabuchneri* NU14 AS A PROBIOTIC

Nouralhuda A. Hussein **Khalid J. K. Luti**
Researcher **Professor**
Dept. Biotech, Coll. Sci. University of Baghdad
Nouralhuda.abbas.hussein@gmail.com **khalid.kadhum@sc.uobaghdad.edu.iq**

ABSTRACT

The present study was designed to select a suitable isolate of *Lactobacillus* sp. in order to use it as a probiotic in a toothpaste for the prevention of dental disease. For this purpose, 30 *Lactobacillus* isolates were collected from different sources and subjected to a screening program to evaluate their antagonism activities against three oral pathogens: *Staphylococcus aureus, E. coli* and *Streptococcus mutans*. Eight *Lactobacillus* isolates were selected from primary screening according to their ability to inhibit the growth of three indicators which then exposed to a secondary screening to detect their ability to produce high amount of bacteriocin. Based on results, the isolate *Lactobacillus parabuchneri*.Nu14 was selected as a highest bacteriocin producing isolate. Next, several tests were performed to study the properties of *Lactobacillus parabuchneri* Nu14, in particular its biosafety and its suitability to be a successful probiotic. Results showed that *Lactobacillus parabuchneri* was resistance to β-lactams group such as penicillin and ampicillin whereas, it was sensitive to amoxicillin-clavulanate. In addition, results revealed that this bacterium was strongly adherent and good biofilm producer, has the ability to survive in pH 2 for 2hr; has the ability of surviving in the presence of 85.2% of lysozyme after 90 min; tolerant to 0.3% of bile salts for 2hr; had high auto-aggregation capability and high ability of adhering with *S. mutans*. Two toothpaste formulas were selected and optimized to choose the most useful one that can be used in this study. Based on results, *L. parabuchneri* Nu14 was successfully survive in formula II over 21 days.

Keywords: toothpaste formula, oral diseases, bacteriocin.

مجلة العلوم الزراعية العراقية -2023 :54(6):1647-1658 **حسين ولوتي**

تقييم النشاط المضاد لبكتريا *Lactobacillus parabuchneri* Nu14 كمعزز حيوي

نور الهدى عباس حسين **خالد جابر كاظم لوتي**
باحث **استاذ**
قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق.

المستخلص

صممت الدراسة الحالية لاختيار عزلة مناسبة من *Lactobacillus sp* من أجل استخدامها كمعزز حيوي محتمل في معجون اسنان للوقاية من أمراض الفم. تم جمع 30 عزلة من *Lactobacillus* من مصادر مختلفة وخضعت لبرنامج غربله لتقييم نشاطها المضاد ضد ثلاثة من مسببات أمراض الفم: *Staphylococcus aureus* و *E. coli* و *Streptococcus mutans*. تم اختيار ثمانية عزلات من *Lactobacillus* من االغربله الأوليه وفقًا لقدرتها على تثبيط نمو ثلاثة مؤشرات والتي اخضعت بعد ذلك لغربله ثانويه للكشف عن قدرتها على إنتاج كمية عالية من البكتيريوسين. تم اختيار العزلة*L. parabuchneri* Nu14 كأعلى عزلة منتجة للبكتيريوسين. تم إجراء العديد من الاختبارات لدراسة خصائص *L. parabuchneri* Nu14 ، ولا سيما سلامتها الحيوية ومدى ملاءمتها لتكون معزز حيوي ناجح حيث أظهرت النتائج أن *L. parabuchneri* كانت مقاومة لمجموعة β−lactam مثل البنسلين والأمبيسلين بينما كانت حساسة للأموكسيسيلين− clavulanate. بالإضافة إلى ذلك ، أوضحت النتائج أن هذه البكتيريا كانت شديدة الالتصاق ومنتج بيوفيلم جيد ولديها القدرة على البقاء في الظروف الحامضية ولديها القدرة على البقاء على قيد الحياة في وجود اللايسوزايم بنسبة 85.2% ومقاومه لـ0,3% للأملاح الصفراوية بنسبة 75,6% لمدة ساعتين ولديها قدرة عالية على التجمع التلقائي والتجمع مع *S. mutans*. تم اختبار تركيبتين من معجون الأسنان لاختيار الأكثر فائدة التي يمكن استخدامها في هذه الدراسة. بناءً على النتائج ، اظهرت خلايا *L. parabuchneri* Nu14 قدرة على البقاء على قيد الحياة بنجاح في التركيبة II ولمدة 21 يومًا ، ولذلك تم اختيارها للاستخدام لتخزين ونقل الخلايا القابلة للحياة من بكتريا *L. parabuchneri*

الكلمات مفتاحية: تركيبة معجون اسنان, امراض الفم, بكتريوسين.

Received:1/11/2021 Accepted:13/2/2022

INTRODUCTION

The dental plaque is a bacterial biofilm which has been considered as the major etiological factor for most oral diseases such as dental caries and periodontal diseases. Biofilm of dental plaque cannot be removed, but it can be decreased and controlled by daily brushing teeth. Recently, there is an urgent need to use living organisms as probiotic for treatment of oral diseases where antibiotics are no longer useful due to the evolution of pathogens resistance. In fact, the treatment with probiotic is now, considered as a new strategy to compete several pathogens (3, 24). The WHO was defined a probiotic as a "live microorganisms that have health benefits on animal host when they administered in enough amounts". The first probiotics introduced in research work were *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. The probiotic can be bacteria, molds or yeast, but the most probiotics are bacteria. The most common probiotics are belonged to the genera *Lactobacillus* and *Bifidobacterium* (33). A probiotic may involve single strain or more, and it can be in powder or liquid form, gel, paste granules, capsules as well as sachets. In this context, different bacterial probiotic cells, in particular *Lactobacillus* sp., were utilized in formula to treat several diseases, caused by different pathogens including *Staphylococcus aureus* (27) and *E. coli* (48), such as *Lactobacillus acidophilus* cells in an emul gel formula against some skin pathogens (35). In addition, Israa and Luti, used *Lactobacillus crispatus* in an emul gel formula against some vaginal pathogens (19). The first toothpaste manufactured and called "Plidenta Pro-t-action" was contained *L. paracasei* probiotic which had the ability to co-aggregate with *S. mutans* to reduces oral caries (30). Jose and colleagues reported the effect of probiotic toothpaste on *S. mutans* in plaque where they confirmed that *lactobacillus, bifidobacterium* and *streptococci* which were genetically designed, had high ability to adhere and inhibit *S. mutans* (22). Probiotics have the ability to compete other microorganisms using different mechanisms such as inhibiting pathogens via competing them on the limited substrates necessary for their metabolites. Some probiotics preventing adherence of pathogens to the host cell. Other probiotics have the ability to secrete metabolites with antimicrobial activity such as bacteriocins, H_2O_2, and organic acids (10). With increasing resistance to the most antibiotics in use today, it becomes vital to develop and find new therapeutic strategies to combat microbial infections without effecting the patient. One of the suggested strategies, in this context, is to select bacteria from different sources and evaluate its probiotic potential in a suitable toothpaste for the prevention of dental disease.

MATERIALS AND METHODS

Collection of *Lactobacillus* isolates

Thirty-five samples were collected from dairy products including sheep milk and Homemade yoghurt using MRS agar plates after serial dilutions. Plates were incubated at 37^{o}C for 48hrs in anaerobic jar then recultured as single colony on MRS (De Man, Rogosa and Sharpe) agar plates in the same conditions (23). All isolates were subjected to biochemical, morphological and physiological tests (18).

Collection of oral pathogens

Twenty-five swabs from unhealthy oral patients were cultured on mannitol salt agar and MacConkey agar to isolate both of Gram-positive *S. aureus* and Gram-negative *E. coli* which then already subjected to morphological and biochemical tests to prove their species (4,6). In addition, oral *S. mutans* was obtained ready from Department of Biotechnology, Collage of Science, University of Baghdad which was checked by VITEK2 system to ensure its genus and specie.

Screening of *Lactobacillus* isolates for bacteriocin production: All *Lactobacillus* isolates were subjected to a screening program to select an isolate that produce an active bacteriocin against oral indicators pathogens: *S. aureus, E. coli* and *S. mutans.* First, 30 isolates were subjected to primary screening by using agar plug diffusion method (1). The inhibition zones around plugs were used as a measure of antagonistic activity of each *Lactobacillus* isolate. Then, the selected isolates were subjected to secondary screening by using well diffusion method to detect the highest bacteriocin producing isolate (1, 25) The bacteriocin activity was determined based on the method described in the following

section. Next, the selected *Lactobacillus* isolate was identified by using VITEK 2 system to determine species of isolate.

Determination of bacteriocin activity

A 2% (10^8 cells/ml) of an overnight culture of *Lactobacillus* isolates was used to inoculate 25ml of MRS broth and incubated at 37 ^oC for 24 hrs. Then each culture broth was centrifuged at 10000 for 15 min and cell-free supernatant (CFS) was collected and filtered by using 0.2µm sterile filter paper (37). Few drops of 1 N NaOH and catalase solution were added to CFS for neutralize effect of organic acids and H_2O_2 respectively. Two-fold dilution series of CFS were prepared and well diffusion method was used to detect the presences of bacteriocin in each dilution. Arbitary unit (AU) was calculated by detecting the highest dilution that produce inhibition zone against *S. mutans* according to the following equation (3):

$$\textbf{AU/ml}= \frac{1}{DF} \times \frac{1000}{volums\ spotted\ in\ \mu l}$$

Where *DF* represents dilution factor

Characterization of the selected *Lactobacillus* isolate as probiotic: Antibiotic Susceptibility: The antibiotics susceptibility of the selected *Lactobacillus* isolate was assessed on Mueller-Hinton agar (MHA) plate using antibiotic disc method (29). The antibiotic discs utilized were: Pencillin, Ampicillin, Amoxicillin - clavulanate, Cefotaxime, Ceftriaxone and Tetracycline (39).

Biofilm formation: Biofilm formation was investigated by using two methods: Congo red method (14) and Microtiter plate methods (8)

Acid tolerance: An overnight growth of *Lactobacillus* isolate was harvested by centrifugation (7000 rpm, 10 min) and re-suspended in a fresh MRS broth with pH (2.0). Samples were withdrawn at 0 time and after 1 and 2 hrs of incubation at 37 ◦C. For each sample, serial dilution was performed in phosphate buffer saline (PBS) and plated on MRS agar which then incubated at 37◦C for 48 hrs. MRS broth with pH 6.5 was used as a control. viable cell count was performed to check the capability of cells to resist acidic pH (12).

Lysozyme resistance: Evaluation of lysozyme resistance was performed as following: 6 ml of an overnight growth of isolate was harvested by centrifugation at 7000 rpm for 10 min and re-suspended in 10 ml of sterile saline solution in presence of 100 mg L^{-1} of lysozyme. Isolate suspended in sterile saline solution without lysozyme was utilized as control. Samples were incubated at 37^oC and viable cell count was performed after 30 and 90 min (41).

Bile salt : An overnight growth of the selected isolate was harvested and re-suspended in 5 ml of MRS medium with 0.3% bile salt. Then, samples were withdrawn at 0 time and after 1 and 2hr of incubation at 37◦C. Serial dilution and viable cell count were performed on MRS agar (40).

Auto-aggregation assay: Cells of an overnight of *Lactobacillus* culture were centrifuged and washed twice in PBS pH 6.0 to give 10^8 CFU.ml^{-1}. A 4 ml of bacterial suspension were mixed by vortex for 10 seconds and auto-aggregation was calculated during 5hrs of incubation at room temperature as follow: 0.1 ml of the upper suspension was transferred to another tube containing 3.9 ml of PBS and the absorbance was measured at 600 nm (26). The percentage of auto-aggregation was calculated according to the following equation:

$$\textbf{Auto-aggregation} = \frac{[ODi - ODf-]}{ODi} \times \mathbf{100}$$

Where OD_i is the OD at initial time (t=0 h) of auto-aggregation assay, and OD_f is the OD at t=1,2,3,4 and 5 h

Co-aggregation assay: Cells of an overnight of *Lactobacillus* culture were centrifuged and washed twice in PBS pH 6.0 to give 10^8 CFU.ml^{-1}. Then, 2 ml of *Lactobacillus* suspension and indicator (*S. mutans*) were mixed together with vortex for 10 seconds. A 4 ml of each bacterial suspension was used as a control. The absorbance was measured at 600 nm during 5h of incubation at room temperature (26). The percentage of co-aggregation was calculated based on the following equation:

$$\textbf{\% Co-aggregation} = \frac{\frac{Ax+Ay}{2} - \frac{A(x+y)}{Ax+Ay}}{2} \times \mathbf{100}\%$$

Where: *x* represents *Lactobacillus* isolate, *y* represent indicator, *Ax* represent the absorbance of *Lactobacillus*, *Ay* represent the absorbance of indicator, *A(x+y)* represent the absorbance of mixture of both bacteria.

Probiotic toothpaste formula contains viable *Lactobacillus* cells : In this work, two types of formula of toothpaste were prepared and tested to check the ability of *Lactobacillus* cells to stay viable, as the following:

Formula 1 probiotic toothpaste

This formula was prepared based on Gaurav and Jayesh (15), the components of this formula was listed as the following: Calcium carbonate 6gm, Sodium lauryl sulphate 0.1gm, Glycerin 2.2ml, Arabic gum 0.15gm, D.W 2ml and Saccharin sodium 0.01gm. This formula was prepared by using dry gum method. At the end of preparation, the mixture was autoclaved at 121^{o}C for 15 min then water containing viable *Lactobacillus* cells (10^{8} cells/ml) was added to the sterile mixture of toothpaste. Viable cell count method was used to test the ability of *Lactobacillus* cells to stay viable in this formula.

Formula 2 probiotic toothpaste

This formula was prepared by the modification of Formula 1, the components of this formula was listed as the following: Calcium carbonate 0.5gm, Sorbitol 3gm, Sodium bicarbonate 3gm, Sodium lauryl sulphate 0.1gm, Glycerin 1ml, Arabic gum 0.15gm, D.W 3ml and Saccharin sodium 0.01gm. This formula was prepared by using dry gum method. At end of preparation, the mixture was autoclaved at 121^{o}C for 15 min then water containing viable *Lactobacillus* cells (10^{8} cells/ml) was added to the sterile mixture of toothpaste. Viable cells count method was used to test the ability of *Lactobacillus* cells to stay viable in this formula.

RESULTS AND DISCUSSION

In this work, thirty *Lactobacillus* isolates were collected from different sources which already subjected to morphological, biochemical and physiological identification tests. These tests involved growing on the *Lactobacillus* selective medium MRS agar as white, mucoid, convex, opaque and smooth without pigment colonies after 24 to 48hr at 37^{o}C under anaerobic conditions (43) Furthermore, the microscopic examination which showed that all isolates were Gram-positive with rod-shaped. In addition, catalase and oxidase tests were performed where all isolates showed negative results. Moreover, on blood agar, all the examined isolates appeared as gray colonies with alpha hemolysis. In order to select a suitable isolate of *Lactobacillus* that can be used in this study, all isolates were subjected to screening program in two steps, primary and secondary screening. Three different bacteria (*Staphylococcus aureus, Streptococcus mutans* and *E. coli*) to be used as indicators due to them were selected with some oral infections, in particular, *S. mutans* which was considered as the main oral pathogen that cause caries (28). In the primary screening, 30 isolates were tested using agar plug diffusion method to detect the antagonistic activity between the exanimated *Lactobacillus* isolates and the indicators. Two criteria were used to select *Lactobacillus* isolates: First: the isolate being an active against two or three indicators (more than 15 mm inhibition zone). Second: the isolate being an active against indicator with more than 20 mm inhibition zone. According to results, out of 30, only 8 isolates were met these criteria and therefore, they were selected for further experiments in the secondary screening. In the secondary screening, the well diffusion method was used to detect the ability of *Lactobacillus* isolates to produce bacteriocin in liquid culture. The highest bacteriocin – producing isolate was detected based on its performance through forming an inhibition zone around the well in agar plate which containing cell free supernatant. As mentioned earlier, 8 isolates were selected and subjected to secondary screening program. These isolates showed the higher antimicrobial activity against indicators used in the primary screening compared with the other isolates. Based on results presented in (Table 1), five isolates had the ability to inhibit three indicators, while the rest were able to inhibit two indicators. In addition, results revealed that the isolate *Lactobacillus* Nu14 showed the highest bacteriocin production with 16, 19, 18 mm inhibition zone against *S. aureus. S. mutans* and *E. coli* respectively (Figure 1).

Table 1. Secondary screening of *Lactobacillus* isolates for bacteriocin production

No. of isolate	Diameter Zone of inhibition (mm)		
	S. aureus	*S. mutans*	*E. coli*
Nu 13	15	14	17
Nu 14	18	19	18
Nu 15	16	15	16
Nu 16	16	-	15
Nu 18	-	16	17
Nu 20	11	-	15
Nu 24	16	15	18
Nu 27	18	16	17

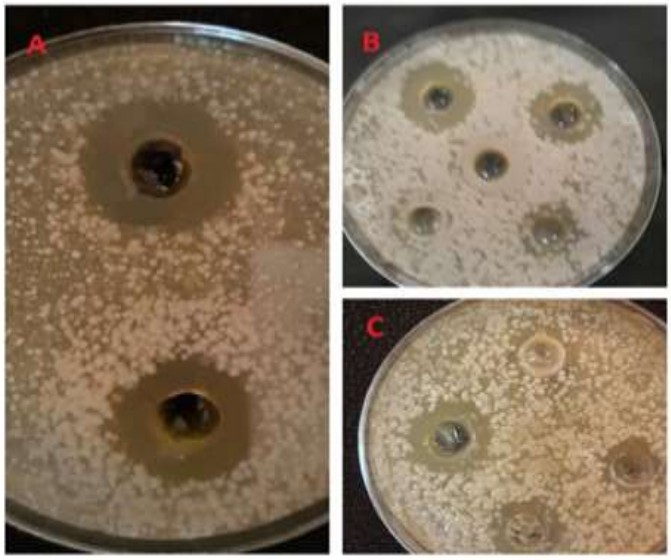

Figure 1. Secondary screening of *Lactobacillus* Nu14 isolate against three indicators (A: *S. aureus*, B: *S. mutans* and C: *E. coli*) by using well diffusion method

Based on results, the isolate *Lactobacillus* Nu14 was chosen to be used to achieve this work. Next, this isolate was subjected to identification method using VITEK2 system which revealed that this isolate was *Lactobacillus parabuchneri.* In addition, for the next experiments, *S. mutans* was selected to be used as the indicator to detect the activity of the selected *Lactobacillus parabuchneri* Nu14. This selection was due to the important of this bacterium as one of the main oral pathogens that cause caries and also because of its high sensitivity to the bacteriocin produced by the selected isolate. In order to use *Lactobacillus parabuchneri* Nu14 as oral probiotic, several tests were performed to provide some necessary information about this isolate, in particular its biosafety as well as its suitability to be a successful probiotic. It was useful to take the antibiotic sensitivity profile of the isolate into consideration. For this purpose, six different antimicrobial drugs were tested. Based on results, *Lactobacillus parabuchneri* was resistant to β-lactams group such as penicillin and ampicillin whereas, it was sensitive to amoxicillin-clavulanate because of this antimicrobial drug can bind to β-lactamase inhibitor (38, 47). In addition, *Lactobacillus parabuchneri* Nu14 showed an intermediate resistance to Cefotaxime, Ceftriaxone and Tetracycline. The next test performed was biofilm formation. Biofilm-producing microorganisms have some advantages that are not found in planktonic ones such as protection and resistance to drugs (e.g., antibiotics), adhesion capability, mechanical properties, avoiding immune system and cellular communication (4,2). In this study, Congo red agar (CRA) and Microtiter culture plate methods were used. The results of both methods revealed that *L. parabuchneri* Nu14 was a strongly adherent and good biofilm producer. These results were agreed with Maria *et al.* (32) who confirmed the ability of *L. parabuchneri* to produce biofilm. In addition, many studies reported the ability of different species of *Lactobacillus* to produce

biofilm, such as *L. crispatus* (19**)** and *L. plantarum* (16). Tolerance of *Lactobacillus* to acid is an important test to determine the ability of isolate to survive in acidic conditions that may occur in mouth due to gastroesophageal reflux that may occur when stomach content rise up into esophagus (30). Based on results presented in (Figure 2), the number of viable *Lactobacillus parabuchneri* Nu14 cells was decreased to 63.12% after 2 hrs. These results indicated that *L. parabuchneri* Nu14 cells have the ability to survive at acidic conditions for a suitable period, if the gastroesophageal reflux was occurred. The obtained results were agreed with Nouralhuda and Luti (34) who reported that *L. plantarum* can survive at acidic condition (pH 2) by 94.4% after 2hrs.

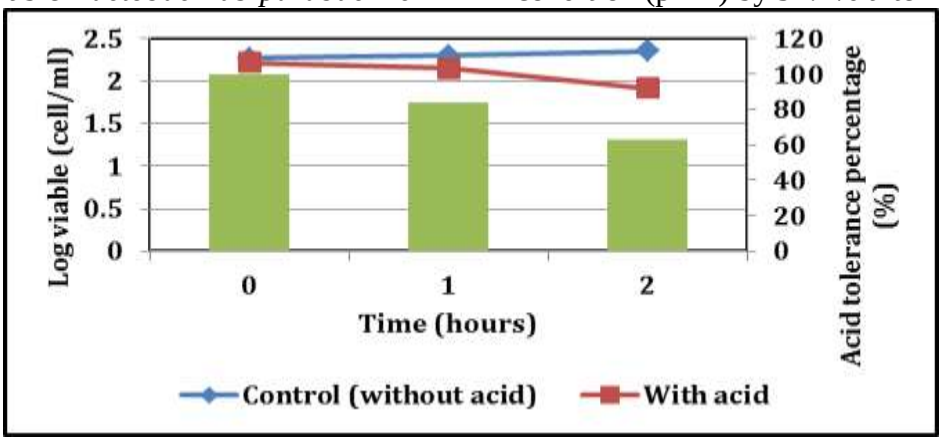

Figure 2. Effect of HCl on *Lactobacillus parabuchneri* Nu14 through 2hrs

Lysozyme is an antimicrobial enzyme produced by animals that forms part of the innate immune system. This enzyme catalyzes the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, the major component of Gram-positive bacterial cell wall. Therefore, the presence of this enzyme in the mouth may cause lysis of *Lactobacillus* cells (30). Consequently, testing the ability of *Lactobacillus parabuchneri* Nu14 to survive in the mouth was one of the important experiments that performed to demonstrate its suitability to be a successful probiotic. As can be seen in (Figure 3), *Lactobacillus parabuchneri* Nu14 cells showed a considerable ability of surviving in the presence of lysozyme with a percentage of tolerance for approximately 85.2% after 90 min. This result showed the ability of *L. parabuchneri* Nu14 to surviving with presence of lysozyme in saliva of the oral cavity for a suitable period. The obtained result is in agreement with Nouralhuda and Luti (34) who reported that *L. plantarum* had a good percentage of 97.8% cells in presence of lysozyme.

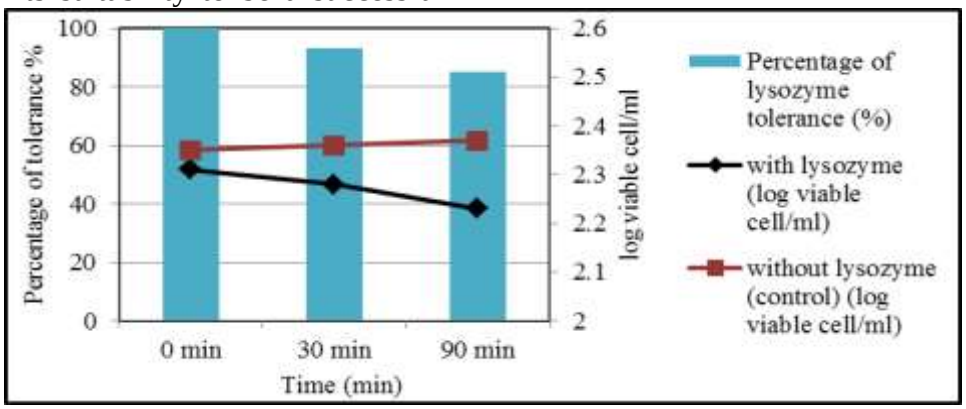

Figure 3. The effect of lysozyme on *Lactobacillus parabuchneri* Nu14 after 90 min

Bile salt is one of components of bile which is the digestive liquid produce naturally in the liver. There is a possibility of bile reflux occur when backs up stomach and may reach into esophagus and oral cavity (2). Therefore, study the ability of *Lactobacillus parabuchneri* Nu14 cells to tolerant bile salt is necessary. (Figure 4) shows that number of viable cells of *L. parabuchneri* Nu14 was slightly decreased in the presence of bile salt

after two hours of incubation. Based on results, the percentage of tolerance to bile salts was 75.6% after two hours of incubation. The obtained results are in agreement with both Nouralhuda and Luti (34) and Shokryazdan *et al.* (37) who reported that *L. plantarum* can tolerate 0.3% of bile salt. In addition, Zhang *et al.* (7) reported that *L. buchneri* has bile tolerance.

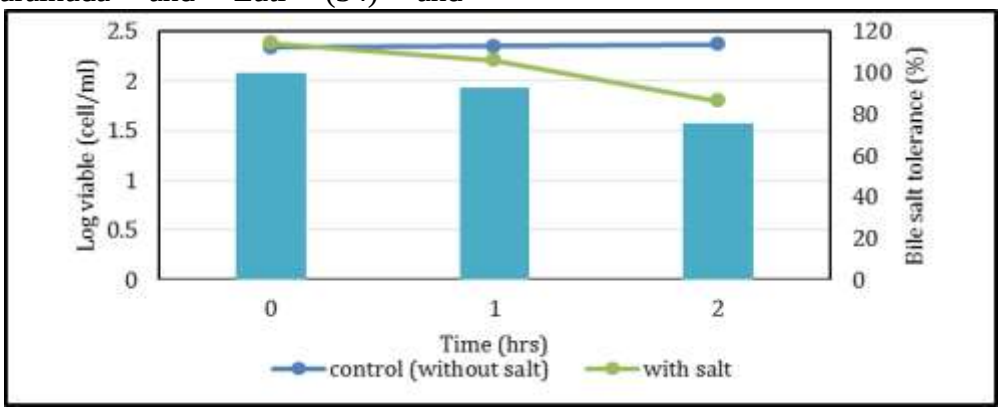

Figure 4. Effect of bile salt on *Lactobacillus parabuchneri* Nu14 during two hours of incubation

Auto-aggregation and co-aggregation are an important property of probiotics (26). Some *Lactobacillus* strains have the ability to inhibit adherence of pathogens either by forming a barrier via auto aggregation or by direct co-aggregation with the pathogens **(**9,13,42**).** Therefore, this test is important to determine the possibility of using *Lactobacillus parabuchneri* Nu14 cells as a prospective probiotic. According to results presented in (Figure 5), *Lactobacillus parabuchneri* Nu14 cells had high auto-aggregation capability of 86.76% after 5 hrs. This test clearly prove that this isolate has a good ability to adhere to epithelial tissue and hence, may inhibit adherence of pathogens. Some parameters were influencing both auto and co-aggregation, such as presence of bile salt and hydrophobicity (46). *Lactobacillus* sp. are usually found on the mucous surfaces of oral cavity and it can be collected from deep caries lesions (19). *Lactobacillus* strains have the ability to survive in saliva and hence, effect on the oral ecology by preventing the adherence of other bacteria as well as modifying protein composition of saliva (17). Based on the result presented in (Figure 5), the ability of *Lactobacillus parabuchneri* Nu14 to adhere with *S. mutans* was high and reached to 71.66% after 5 hrs. This result provides evidence for the possibility of using *L. parabuchneri* Nu14 as a probiotic to protect oral cavity. The obtained result was in agreement with Israa and Luti (19) and Younes *et al.* (45) who reported the ability of *L. crispatus* to co-aggregate with pathogens.

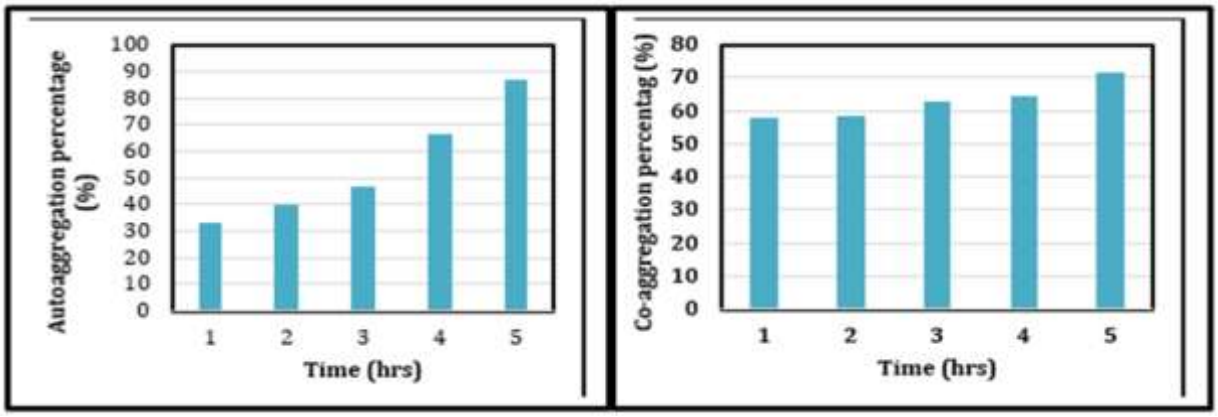

Figure 5. Auto-aggregation of *Lactobacillus parabuchneri* Nu14 and Co-aggregation between *Lactobacillus parabuchneri* Nu14 and *S. mutans*

Probiotic toothpaste formula including viable *Lactobacillus parabuchneri* Nu14

Toothpaste is a mixture of specific components. Actually, the challenge in this study was to select a useful toothpaste which can act as a vector to store and transport viable cells of *Lactobacillus parabuchneri* Nu14 into the mouth of unhealthy patient.

Therefore, several types of toothpaste formula were used and tested. Some of those toothpaste formulas were failed in keeping *Lactobacillus parabuchneri* Nu14 as viable cells therefore, they were excluded. After several tests, two formulas of toothpaste were selected and optimized to choose the most useful one that can be used in this study.

Toothpaste formula 1

This formula was described by Gaurav and Jayesh (15). The first observation revealed that this formula had a strong antagonistic activity against oral pathogens that used as indicators (*S. aureus, S. mutans, E. coli* and *S. mutans*) (Figure 6). Where, 2µl of 2% for each indicator was cultured on MHA (Muller Hinton agar) then 500µl of formula was added to wells of plates. However, this formula was excluded based on the results of viable cell count which was used to test the ability of *Lactobacillus* cells to stay viable in this formula. As can be seen in (Figure 7), the number of viable cells of *Lactobacillus parabuchneri* Nu14 in the formula was significantly decreased after three days. At the fourth day, no viable cells were observed suggesting that *Lactobacillus parabuchneri* Nu14 cells cannot survive after 3 days in this formula and therefore, this formula was inappropriate to use in this study. A possible explanation for these results might be the presence of calcium carbonate at a high concentration (6gm/10gm formula). This compound may act as neutralizing agent that regulate the pH of medium which allow to increase lactic acid production. This may lead to increase the solubility of calcium carbonate causing the strong antagonistic activity observed with formula I. In addition, lactic acid may react with calcium carbonate to produce calcium lactate which can negatively effect on the viability of *Lactobacillus parabuchneri* Nu14 (44).

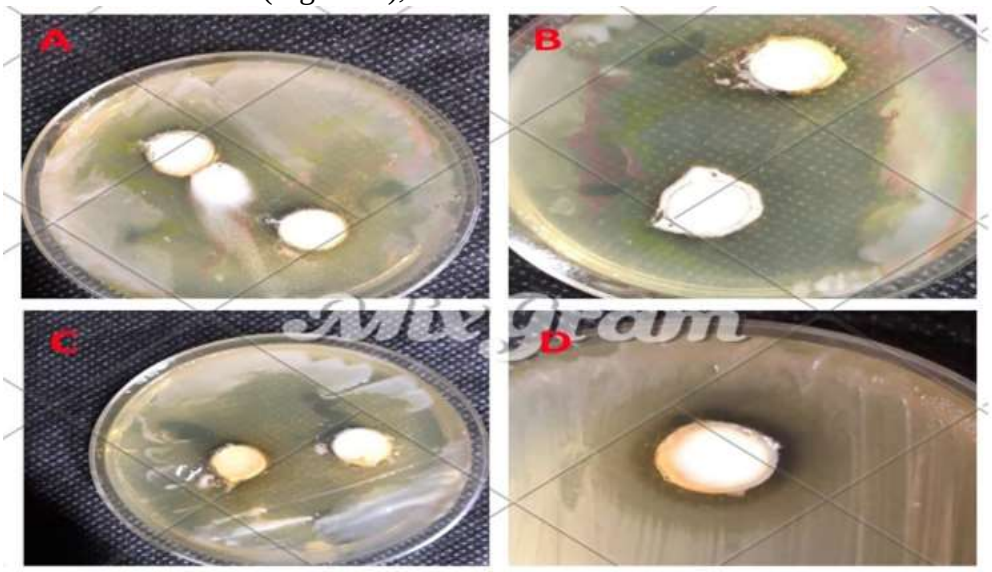

Figure 6. Effect of formula 1 on (A: *S. aureus*, B: *E. coli*, C- D: *S. mutans*)

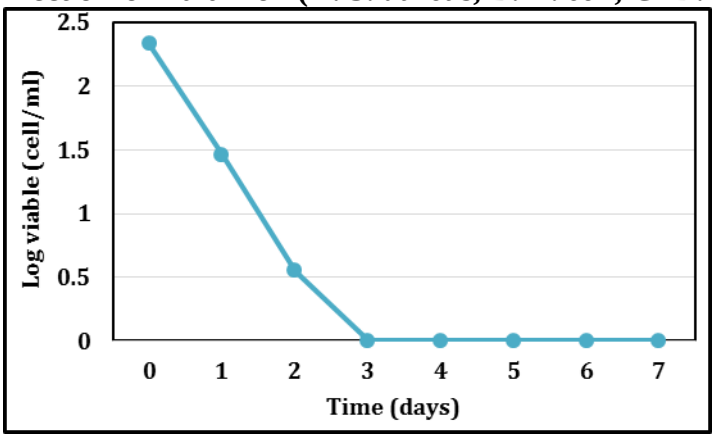

Figure 7. Monitoring of viability of *Lactobacillus parabuchneri* Nu14 in the toothpaste formula 1

Toothpaste formula 2

This formula was prepared by the modification of Formula 1 via decreasing calcium carbonate to 0.5gm/10gm with the addition of sorbitol. Based on results of viable cell count method, *L. parabuchneri* Nu14 was successfully survive in this formula over 21 days as shown in (Figure 8). The percentage of viable cells was slightly decreased to 51.3% after three weeks. Therefore, this formula was selected to use in this study which can be utilized as a vector to store and transport viable cells of *Lactobacillus parabuchneri* Nu14. On the other hand, this formula may provide good medium to keep and survive *Lactobacillus parabuchneri* Nu14. For example, sorbitol which was already used as humectant to toothpaste, can also utilized as a carbon source for *Lactobacillus parabuchneri* Nu14 (5). In addition, the presence of glycerol in the formula may play an important role to store bacteria for long term at 4^{o}C (11), and hence, may participate in maintaining *Lactobacillus parabuchneri* Nu14 cells viable for long time.

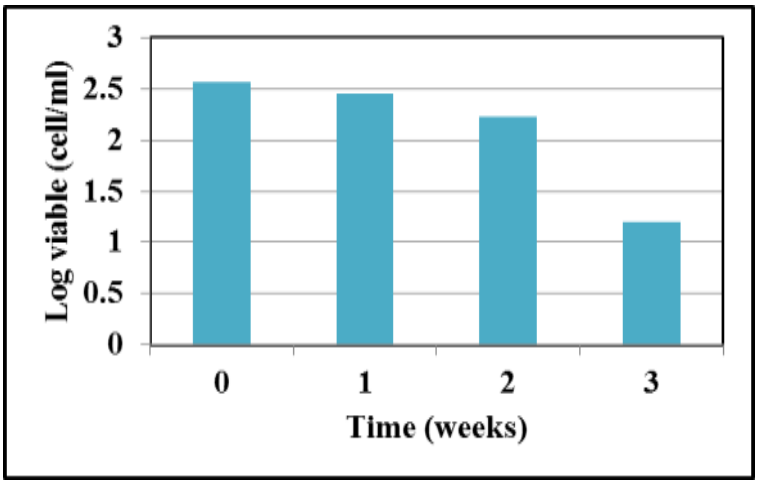

Figure 8. Monitoring of viability of *Lactobacillus parabuchneri* Nu14 in toothpaste formula 2 for three weeks

CONCLUSION

It is necessary to find new strategies to fight pathogens due to the increase in the resistance to antibiotics. This work contributes to this field via suggesting the use of viable cells of *Lactobacillus* as a useful strategy to combat oral pathogens. Probiotic toothpaste is composed from safe formula containing *Lactobacillus* cells which generally recognized as safe organism and can be effective in fighting some oral pathogens, in particular, *S. mutans*. This work confirms the ability of *L. parabuchneri* Nu14 to be survived in toothpaste formula with their viability for long time as well as production of bacteriocin without being affected by the components of toothpaste.

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