DOI: 10.24996/ijs.2026.67.4.26

ISSN: 0067-2904

Utilization of *Lactobacillus* cells as a probiotic in chewing gum against multi-drug-resistant pathogens associated with gingival inflammation

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Received: 13/ 12 /2024 Accepted: 10/4/2025 Published: 30/4/2026

ABSTRACT

This study was Conceived to choose an appropriate isolate of *Lactobacillus* sp. To use it as a probiotic in chewing gum for the Prophylaxis of gingivitis. Due to the above reason, 20 *Lactobacillus* isolates were collected from various sources and then primarily enrolled in ascreening program to assess their activities of antagonism against two oral pathogens, *Streptococcus pluranimalium* and *Staphylococcus aureus*. Twenty *Lactobacillus* isolates were selected from the initial screening based on their efficacy to suppress the growth of the two chosen indicators which was then followed by another step of secondary screening to detect their production of the high amount of bacteriocin and their effectiveness. The key findings show, that the isolate of *Lactobacillus rhamnosus* T was chosen it's the producing isolate of the highest bacteriocin. Then, several *in vitro* experiments were run to determine the characteristics of *Lactobacillus rhamnosus* T, mostly, its biosafety considerations and its validity for acting as a successful probiotic which all revealed the suitability of this isolate to use as aprobiotic. Five types of natural and artificial gums were tested to select one that can be utilized in producing chewing gum containing *Lactobacillus rhamnosus* T cells which were encapsulated before integrating into the sterile prepared chewing gum. Based on the results, the chewing gum base, water Iraqi gum, xanthan gum [XG], and Arabic gum showed good viability based on the high number of colonies revealed on the MRS agar after 21 days whereas the minimum viability result was gained for the Frankincense.

Keywords: probiotics, chewing gum, *Lactobacillus rhamnosus,* gingivitis, encapsulation.

استخدام العصيات اللبنية كمعزز حيوي ضد بعض الممرضات ذات المقاومة المتعددة للمضادات المسببة لالتهاب اللثة

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الخلاصه

تم تصميم هذه الدراسة لاختيار عزلة مناسبة من *Lactobacillus sp*. لاستخدامها كبروبيوتيك في علكة المضغ للوقاية من التهاب اللثة. نظرًا للسبب المذكور أعلاه، تم جمع 20 عزلة من Lactobacillus من مصادر مختلفة ثم تم تسجيلها في برنامج الفحص لتقييم أنشطتها المضادة ضد مسببات الأمراض الفموية،

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Streptococcus pluranimalium و*Staphylococcus aureus*. تم اختيار عشرين عزلة من Lactobacillus من الفحص الأولي بناءً على فعاليتها في قمع نمو المؤشرين المختارين والتي تلت ذلك خطوة أخرى من الفحص الثانوي للكشف عن إنتاجها لكمية عالية من البكتيريا وفعاليتها. تظهر النتائج الرئيسية أن عزلة *Lactobacillus rhamnosus* T تم اختيارها لأنها العزلة المنتجة لأعلى نسبة من البكتيريا. بعد ذلك، أجريت عدد من التجارب المعملية لتحديد خصائص *Lactobacillus rhamnosus* T، وخاصة اعتبارات السلامة البيولوجية وصلاحيتها للعمل كبروبيوتيك ناجح والتي كشفت جميعها عن ملاءمة هذه العزلة لاستخدامها كبروبيوتيك. تم اختبار خمسة أنواع من الصمغ الطبيعي والاصطناعي لاختيار النوع الذي يمكن استخدامه في إنتاج علكة تحتوي على خلايا *Lactobacillus rhamnosus* T والتي تم تغليفها قبل دمجها في علكة المضغ المعقمة المحضرة. بناءً على النتائج، أظهرت قاعدة العلكة وصمغ الماء العراقي وصمغ الزانثان (XG) والصمغ العربي قابلية جيدة للبقاء بناءً على العدد الكبير من المستعمرات التي تم الكشف عنها على أجار MRS بعد 21 يومًا، بينما تم الحصول على الحد الأدنى من نتيجة قابلية البقاء للبان الذكر .

1. Introduction

Gingivitis represents a prevalent oral health disorder that is defined by the inflammatory response of the gingival tissues. The etiology of this condition is predominantly attributed to the accumulation of dental plaque, which is a viscous biofilm composed of various bacterial species that adheres to the dental surfaces [1]. In the absence of appropriate mediation, gingivitis may advance to periodontal disease, a more severe pathological state that has the potential to result in the loss of dental structures. Nevertheless, the persistent variant of gingivitis induced by plaque is regarded as the most prevalent form. Clinically, the gingival tissues exhibit features including edema, erythema, sensitivity, a glistening surface, and hemorrhaging upon light probing. Gingivitis may lead to spontaneous hemorrhage and is typically devoid of pain; in this regard, a considerable portion of patients are unaware of the condition and refrain from seeking medical attention [2]. The mean causes of gingivitis are due to several aspects such as plaque accumulation on dental surfaces which is the principal factor contributing to this condition. In addition, Lack of adequate brushing and flossing practices facilitates the hardening of plaque into calculus. Moreover, tobacco consumption, whether by smoking or chewing, significantly increases the risk of periodontal disease. Physiological changes, such as those occurring during puberty, the menstrual cycle, or pregnancy, may adversely influence periodontal health. Furthermore, specific medical conditions like certain illnesses, including diabetes, can compromise blood circulation to the periodontal tissues [3].

The World Health Organization defines a probiotic as "live micro-organisms that confer a therapeutic advantage to the host when administered in adequate quantities". The initial probiotics studied were Bifidobacterium and *Lactobacillus acidophilus*. However, probiotics can include molds, bacteria, or yeast, with bacteria being the predominant type. The most commonly utilized probiotics are from the genera Bifidobacterium and Lactobacillus. According to recent studies, the clinical effectiveness of several probiotics, such as Bifidobacterium, *Lactobacillus*, and *Bacillus* species, has been evaluated in gingivitis. According to this research, probiotics effectively decreased bleeding, gingival indices, and plaque [4]. Recently, encapsulation has emerged as the most extensively researched technique for enhancing the survival of probiotics and the delivery of bioactive compounds. It is well known that probiotic encapsulation improves stability, makes handling and storage of probiotic cultures easier, and shields delicate probiotic lactic acid bacteria from freezing, oxygen, and acidic environments during manufacture and storage [5].
Numerous factors have been recognized to influence the effectiveness of encapsulation in safeguarding probiotics. These include the encapsulation method, the materials used for the

wall, the pH level, the initial population of cells, the specific probiotic strain, and the food matrix, among others [6,7] Previous studies, were reported that Probiotic chewing gum has more effect to decrease the amount of plaque and gingival inflammation with adjunct scaling when compared to the control group [8]. Chewing gum has progressively gained recognition as an effective medium for delivering active ingredients over the years. A variety of components are now integrated into chewing gum formulations. The objective of the current study was to produce chewing gum containing encapsulated probiotics cells of *Lactobacillus* isolate and evaluate its viability for a certain time.

2. Material and methods

Collection of *Lactobacillus* isolates

Twenty samples were derived from dairy products such as sheep milk and commercial yogurt as well as from vaginas using MRS (De Man, Rogosa, and Sharpe) agar plates after serial dilutions. These agar plates were incubated at 37ºC for 48 hours in an anaerobic incubator, then re-cultured as MRS agar as single colony plates in the same conditions [10]. All isolates obtained were introduced to biochemical, physiological, and morphological tests.

Collection of *oral pathogens associated with gingivitis*

Samples taken by swabs of subgingival and supragingival were taken from gingival patients from private different dental clinics. Swabs were kept in sterile transport media and then transported safely to the laboratory and cultured on different media including blood agar, MacConkey agar, and MSA agar (Mitis Salivarius Agar) to obtain all possible Gram-positive and negative pathogens in the mouth. Inoculated plates were then incubated in an anaerobic jar overnight for 24 hours at 37ºC, for the subgingival samples and aerobically for the supragingival samples at 37ºC for 24 hours. Then, colonies representing different morphologies were identified using several techniques including biochemicals test and vitek test.

Screening of *Lactobacillus* isolates for bacteriocin production.

Twenty samples were exanimated under screening programmed to select the *Lactobacillus* isolate-producing bacteriocin against oral pathogens, *Streptococcus pluranimalium,* and *Staphylococcus aureus,* that were isolated were then selected in the previous step. First, the twenty isolates of *Lactobacillus* were introduced into primary screening by using the agar plug diffusion method. *Lactobacillus* isolates were grown in MRS broth, then streaked onto MRS agar and incubated anaerobically. After incubation, small plugs of this growth were placed on MHA plates pre-streaked with indicator bacteria (*Staphylococcus aureus* and *Streptococcus pluranimalium*). The pathogenic bacteria were grown in BHI broth to a specific concentration. After overnight incubation at 37°C, the inhibition zones around the plugs were measured. These zones indicated antimicrobial activity; larger zones suggested stronger inhibition of the oral pathogenic growth by the isolate. This process helped determine the effectiveness of each isolate as a potential antibacterial agent. The selected isolates from the result of primary screening were introduced into the follow-up screening using a well diffusion method to select the producing isolate of the highest bacteriocin in liquid culture [11].

Universal tubes with 25 ml of MRS broth and 2% of an overnight culture of each *Lactobacillus* isolate containing approximately (10^8 cells/ml) were incubated for 48 hours at 37°C. Then, the broth culture was centrifuged at 10000 rpm for 15 minutes. The cell-free supernatant (CFS) was harvested and filtered. NaOH and acatalase solution were added to neutralize the effect of H_2O_2 and organic acid.

In this experiment, 200 µL of an overnight growth culture, containing approximately 1×10^8 cells/mL of the chosen oral pathogenic strains was streaked on a petri dish of MHA, Circular wells (5 mm in diameter) were made in the solidified agar, via using a sterile cork borer, and the bottoms of the wells were sealed with low-melting MHA agar. Next, 100 µL of the filtered cell-free supernatant (CFS) was added to each well, and the plates were incubated at 37°C for 24 hours. After incubation, the diameter of the inhibition zones around each well was measured to assess bacteriocin activity.[31]
The selected isolate was identified genetically by using PCR(Polymerase chain reaction)

Determination of bacteriocin activity

A total of 25 milliliters of MRS broth (De Man, Rogosa, and Sharpe) were inoculated with 2% (10^8 cells/ml) of culturing *Lactobacillus* isolates overnight, followed by incubation at 37 °C for 24 hours. Thereafter, 15-minutes centrifugation at 10,000 rpm was performed to collect the cell-free supernatant (CFS), then filtered by using 0.2-micron filter paper (sterile)[12]. To neutralize the effects of organic acids and hydrogen peroxide, a few drops of base 1 N NaOH and a catalase solution were added to the CFS, respectively. In a dilution of a two-fold series, the CFS was prepared through a well-diffusion method utilized to assess the bacteriocin presence in each dilution [32], The arbitrary unit (AU) was calculated using the following equation by identifying the highest dilution that resulted in an inhibitory zone against the oral pathogens indicator, *Streptococcus pluranimalium,* and *Staphylococcus aureus* [13].

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

Characterization of the selected *Lactobacillus* as a probiotic

Multiple Antibiotic Resistance Index [MAR index]

The profiles of antibiotic resistance for the selected *Lactobacillus* spp. were evaluated through the method of Kirby–Bauer disc diffusion on Mueller–Hinton agar, following the protocol established by Bauer et al., [14] via utilizing commercially available discs. A selection of 12 commonly used antibiotics from different families was employed to determine the susceptibility of isolates to different classes of antimicrobial agents. 100µl of the *Lactobacillus* isolate was streaked over the MHA agar, and then antibiotic discs were put on the surface of the MHA plate. The plates containing the antibiotic discs presented in Table 1 were kept at room temperature for a minimum of 20 minutes before being incubated at 37°C for a duration of 24 to 48 hours. Following the incubation period, the diameters of the inhibition zones were measured. The obtained outcomes were expressed as moderately susceptible (I), susceptible (S), or resistant (R) to antibiotics.

Auto-aggregation

For twenty-four hours at 37 °C, *Lactobacillus* was cultured in MRS broth and then bacterial centrifuged the cells at 7000× g for 10 min. Next, cells were two times washed with PBS (phosphate buffer saline) (pH 7.2). At 600 nm, the absorbance of bacterial cell suspension was measured to Three milliliters of the cell suspension divided among three glass tubes, and the tubes were vortexed to quantify the auto-aggregation. After a period of one to five hours, absorbance was measured at 600 nm once more, [15], and analyzed the auto aggregation by using the equation followed below:

Auto-aggregation [%] = 1 – [At / A0]× 100

Where:
At and A0 represent the absorbance at different time intervals [2, 5, and 24 h] and initial absorbance, respectively.

Co- aggregation

The pathogenic strains alongside *Lactobacillus* were maintained at 37 °C for 24 hours in BHI (brain heart infusion broth) and MRS broth respectively. After centrifuging the bacterial cells at 5000 for 15 minutes, cells were two times rinsed with the PBS at pH 7.2. The absorbance of the bacterial cell suspension was then calibrated to 0.5 at a wavelength of 660 nm. 2 ml of *Lactobacillus* cell suspension was combined with 2 ml of each of the two oral pathogenic strains, specifically *Streptococcus pluranimalium* and *Staphylococcus aureus*. After the mixing process, absorbance was re-evaluated at 660 nm after one to five hours [15]. Co-aggregation was then quantified utilizing the formula below.

$$\text{Coaggregation (\%)} = \frac{((Ax + Ay)/2) - A(x + y)}{Ax + Ay/2} \times 100$$

Where: the x and y represent each of the two strains in the control tube and the [x+y] is the mixture.

Antagonistic activity

Spot-on lawn antimicrobial assay

The agar spot antimicrobial assay involved the following steps: MRS broth was prepared and inoculated with the selected isolate of *Lactobacillus*. The broth was subsequently incubated at 37°C for 48 hours under anaerobic conditions to promote heavy growth culture. Muller-Hinton agar plates were prepared and inoculated at its center with a 75 µl drop of *Lactobacillus* broth culture. The oral pathogens indicators, *Staphylococcus aureus* and *Streptococcus pluranimalium*., were cultured in BHI broths and then after incubation maintained at a concentration of 1×10^8 cells/ml. Later, *Staphylococcus aureus* and *Streptococcus pluranimalium* broths were applied in a sterilized spray bottle which was used to spray the inoculated Muller-Hinton agar plates. The plates were subsequently incubated anaerobically, and after 24 hours of incubation, the inhibition zone was observed. The presence of a distinct zone extending at least 1 mm from the location signifies a positive result [19].

Acid tolerance

MRS broth adjustment with HCL to pH of 2.0 was used to re-suspend an overnight culture of the isolate of *Lactobacillus*, which was centrifuged (at 7000 rounds per minute (rpm), for 10 min). Then collected culture was incubated at 37 ˚C and samples were taken at 0 time and after for 1 and 2 hours. PBS was used for serial dilution of each sample, and the samples were plated on MRS agar for each hour and incubated for 48 hours at 37°C. As a control, MRS broth with a pH of 6.5 was utilized. The count of viable cells was conducted to assess the cells' ability to withstand high pH which refers to acidic [16].

Lysozyme resistance

The assessment of resistance to lysozyme was experimented as follows: 6 ml of an overnight culture of the isolate was collected via 7000 rpm for 10 minutes of centrifugation and subsequently 10 ml to re-suspended in a sterile saline solution containing 100 mg L^{-1} of lysozyme. *Lactobacillus* isolates suspended without lysozyme in a sterile saline solution served as the control. The samples were streaked on an MRS agar plate for 30 minutes and 90 minutes. Plates were incubated at 37°C, and then the viable cell count for *Lactobacillus* isolate was determined compared to control [17].

Bile salt

The selected isolate was cultivated overnight, after which it was collected and re-suspended in MRS medium in 5 ml containing 0.3 percent bile salt. By graduation, samples

were taken at the initial time point (zero time) and after 1 and 2 hours of incubation at 37°C. Serial dilutions and viable cell counts were conducted on MRS agar [16]

Preparation of chewing gum containing encapsulated probiotic
Encapsulation of probiotics

A bacterial suspension of *Lactobacillus* isolate was introduced into a 5 millitorrs solution of 1% lecithin and inulin which was subjected to 121 C° for 15 min, then 20 mL mixed with a sterile 2% sodium alginate solution already subjected to 121 °C for 15 minutes. The cell suspension was then administered into a container containing a 0.05 molar solution of calcium chloride that was sterile. Thereafter a sterile 1 mL syringe (30G 8mm), and the formed capsules were maintained in the solution for 30 minutes at room temperature. Afterward, the capsules experienced a washing method twice with PBS and were kept in 0.1 percent peptone sterile liquid at 4 °C for not more than one hour [18].

Preparation of chewing gums

Five different gums including Arabic gum, water gum, and xanthan glue(gum), Frankincense and the base of gum were used and tested in this study. All these were purchased from the local market except for the base of gum which was imported from China. Additional ingredients like sucrose, lecithin, glycerin, glucose powder, and inulin were purchased from local Iraqi markets. Each gum was carefully measured in precise quantities and then sterilized by autoclave for only 10 minutes at 121ºC. To obtain texture and flexibility for the dough of the gums, a sterilized glycerol was added. The next step was transferring the sterilized dough to a laboratory mixer and puddled it at 45–50 ºC which was then left overnight at room temperature to achieve a soft consistency. Dry ingredients, such as inulin, glucose, lecithin, were gradually added to the base gum at a 1% ratio and mixed well until all ingredients were solved or mixed with gum dough. Followed by careful mixing until it a homogenous by using a blender or manual mixer. In the final step of preparation, encapsulated probiotics, previously dried from peptone water using filter paper to form bead-like clusters, were added to each gum. To obtain a refreshing flavor, edible mint essence was added as the finishing touch for each type of gum according to Qaziyani *et al.,*[9] as previously in this study prepared encapsulated chewing gum which shows probiotics survival and good texture.

3. Result and discussion

In this study, twenty isolates of *Lactobacillus* were collected from various origins and underwent biochemical (oxidase, catalase, indole) morphological (colony size, colony shape and color, smell), and physiological identification tests. The tests involved cultivation on the selective medium MRS agar the *Lactobacillus* bacteria.

This resulted in convex, mucoid, white, opaque, and smooth colonies with no pigment after 24 to 48 hours at 37°C under anaerobic conditions [20] which could be a primary indicator for *Lactobacillus* isolation. Moreover, the result of the previous tests shows the microscopic scrutiny disclosed that each isolate was marked as Gram-positive and presented a rod-form morphology. When cultured on blood agar, tested isolates manifested as colonies providing a gray color with alpha hemolytic activity. In addition, catalase and oxidase assays were conducted, yielding negative outcomes for all isolates.

On the other hand, many samples of subgingival and supragingival were taken from the gingiva of patients collected from different dental clinics in Baghdad city.
Based on the results, the two prevalent bacterial species associated with gingival infections were *Staphylococcus aureus* and *Streptococcus pluranimalium*, therefore were chosen as indicators for this study.

To identify a suitable isolate of *Lactobacillus* for utilization in this research, all isolates underwent a two-tiered screening process, encompassing both primary and secondary screening experiments. Two distinct properties were employed for the selection of *Lactobacillus* isolates: Firstly, the isolate was considered active if it demonstrated efficacy against two indicators used in this study (*Staphylococcus aureus, Streptococcus pluranimalium*)characterized by an inhibition zone exceeding 15mm shown as a result from the agar plug diffusion method. Secondly, an isolate was classified as active if it exhibited inhibitory effects in the good diffusion method against an indicator with an inhibition zone surpassing 20 mm.
Firstly, for the primary screening, 20 isolates were subjected to the experiment utilizing the agar plug diffusion technique to assess the antagonistic character produced by the *Lactobacillus* isolates with the oral pathogenic bacteria. Based on the findings, from the initial 20 isolates, only 4 isolates met these predetermined criteria and, as a result, were chosen for subsequent following screening experiments.

The method of good diffusion was employed in the secondary screening to find out if the *Lactobacillus* isolates could produce bacteriocin in liquid culture. Based on its ability to establish an inhibitory zone around the well on the containing cell-free supernatant agar plate, the isolate that produced bacteriocin was identified. The four isolates chosen from the previous experiment were subjected to a secondary screening program, as was previously described.

In comparison with other isolates, the isolate *Lactobacillus rhamnosus* T showed the highest antimicrobial activity against the indicators employed in the screening. The result displayed that, this isolate had the greater inhibitory zone against both MDR indicators, *S. pluranimalium* and *Staphylococcus aureus,* which is certainly due to the highest bacteriocin production [160 AU/ml]. Later, this isolate was identified genetically using PCR and results showed that it was *Lactobacillus rhamnosus*T.

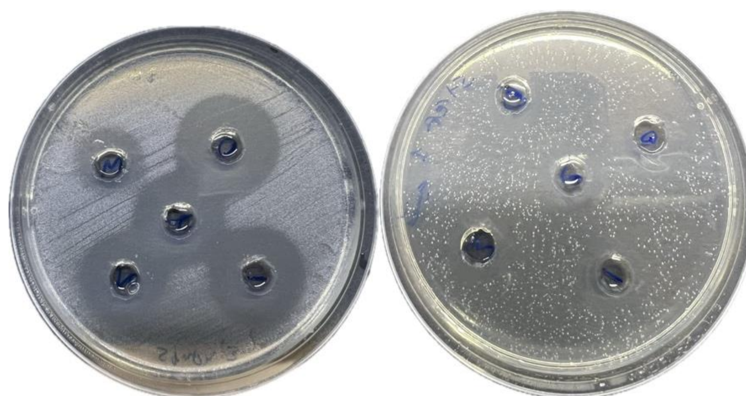


Figure 1: Secondary screening of *Lactobacillus* T isolates against two indicators [*S. aureus*, *S. pluranimalium*] by using well diffusion method

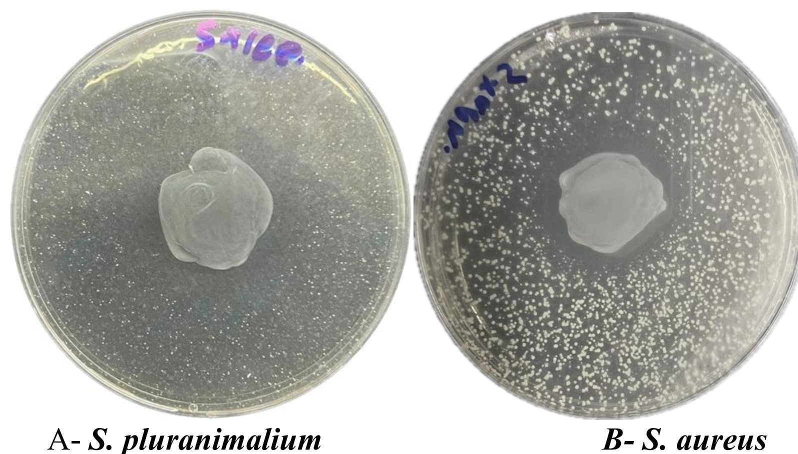
Following the selection of T isolate, several tests were performed to evaluate its safety and assessment of activity to be used as oral probiotics. Multiple antibiotic resistance index (MAR index) was done by disc diffusion method using 12 antibiotic discs as shown in the table [1].
According to results, *Lactobacillus rhamnosus*T showed resistance to only two antibiotics, the first one is cefepime which represented the fourth generation of the cephalosporine family, and the third generation of cephalosporine, ceftriaxone, whereas it was sensitives to 10 types of antibiotics according to Adamski *et al.,*[27] which used 26 antibiotic resistance profiles for all isolates, the result of this study showed all isolate were sensitive to

chloramphenicol and meropenem. In addition, other group strains were mostly sensitive to multiple antibiotics such as gentamicin, Cefoxitin, vancomycin, and trimethoprim/sulfamethoxazole.

Table 1: MAR index test assessment of *Lactobacillus rhamnosus* T using 12 antibiotics discs.

Antibiotic	SYMBOL	Conc. [µg]	Antimicrobial Activity
Tetracycline	TE	30	S
Cefepime	CPM	30	R
Gentamycin	GEN	10	S
Levofloxacin	LE	5	S
Clindamycin	CD	2	S
Ceftriaxone	CTR	30	R
Rifampicin	RIF	5	S
Sulfa\trimethyl	COT	1.25/23.75	S
Erythromycin	E	15	S
Cefoxitin	CX	30	S
Chloramphenicol	C	30	S
Azithromycin	AZM	15	S

The antagonistic activity of *Lactobacillus rhamnosus* T was evaluated using spot-on-lawn assay. This method is usually used to assess the antimicrobial activity of probiotics by investigating their antagonistic ability against pathogenic indicators. This antagonistic activity reflects the ability of probiotic cells to produce bacteriocin, and organic acids, lowering the pH by hydrogen peroxide, immune modulation, and activation host defense mechanism. Certainly, all these characteristics could explain the antagonistic activity of the probiotics when competing with other micro-organisms. It can be said that, by using this method, the patient's oral environment can be mimicked when probiotics display antagonistic effects against pathogenic indicators. As can be seen from the results shown in Figure 2 the Inhibition zone surrounding the spot of *Lactobacillus rhamnosus* T cells against *Streptococcus pluranimalium* was 38 mm, the spot diameter was 20mm.
Whereas the inhibition zone observed against *Staphylococcus aureus* was 33 mm with a spot diameter of 18mm. Certainly, this high antagonistic activity against the two indicators shows a good probiotic tendency for the isolate *Lactobacillus rhamnosus* T. This result agreed with Darweesh and Luti [31] who used emulgel containing *Lactobacillus fermentum* on Muller Hinton agar sprayed with *P. aeruginosa* and reported the antimicrobial activity of *Lactobacillus fermentum.*



A- ***S. pluranimalium*** ***B- S. aureus***

Figure 2: Antimicrobial activity of *Lactobacillus rhamnosus* T spot on Muller Hinton agar sprayed with *Strep. pluranimalium* [A] *Staph. Aureus* [B].

In addition, *Lactobacillus rhamnosus* T showed a tendency to associate with the same species to cluster together. This property can aid in the generation of biofilms by *Lactobacillus* strains, increasing their survival and protecting them from threatening oral environments. Additionally, by physically obstructing the attachment sites of pathogens, auto-aggregation can aid in their exclusion. According to the key findings in Figure 3, *Lactobacillus rhamnosus* T cells had a high-pitched auto aggregation tendency of 60% after 5 hr. of incubation.

This experiment illustrated that the isolate *Lactobacillus rhamnosus* T has a strong capacity of epithelial tissue to adhere, potentially preventing pathogen adhesion. These results showed outcomes agreed with Li et al. [26], who reported that many strains of *Lactobacillus* can autoaggregate, and the percentage of autoaggregation increased over time.

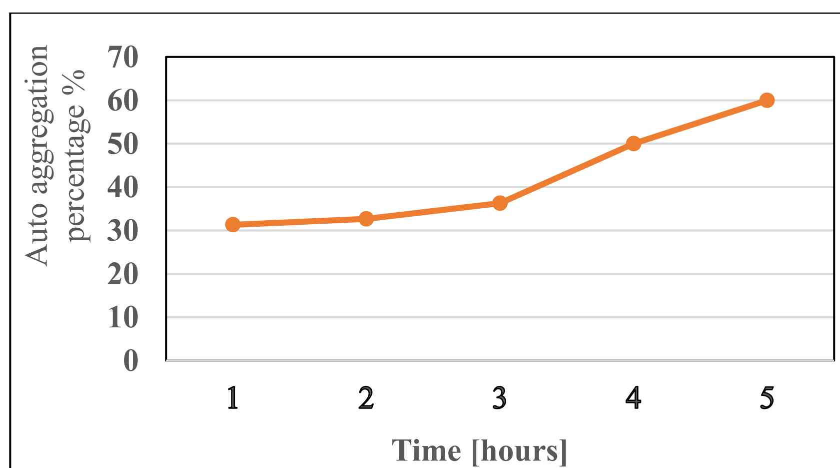


Figure 3: The percentage of autoaggregation of *Lactobacillus rhamnosus*T in five hours.

Moreover, the ability of *Lactobacillus rhamnosus* T to co-aggregate with *Staphylococcus aureus* and *Streptococcus pluranimalium* was proven.

The capacity of *Lactobacillus rhamnosus* T to aggregate with *Streptococcus pluranimalium* was 44.4% while the ability to adhere with *Staphylococcus aureus* was higher reaching 71.27% after 5 hours (Figure 4).

Co-aggregation refers to the capacity of various bacterial strains to adhere to another one. This characteristic is crucial for the development of intricate microbial communities within the oral cavity, which may contribute to the preservation of oral health.
*Lactobacillus rhamnosus*T,in conjunction with pathogenic microorganisms, can also impede their proliferation and inhibit their colonization within the oral environment.
This evidence approves the use of *Lactobacillus rhamnosus* as oral probiotics. These outcomes were agreed with Hussein and Luti, [22].

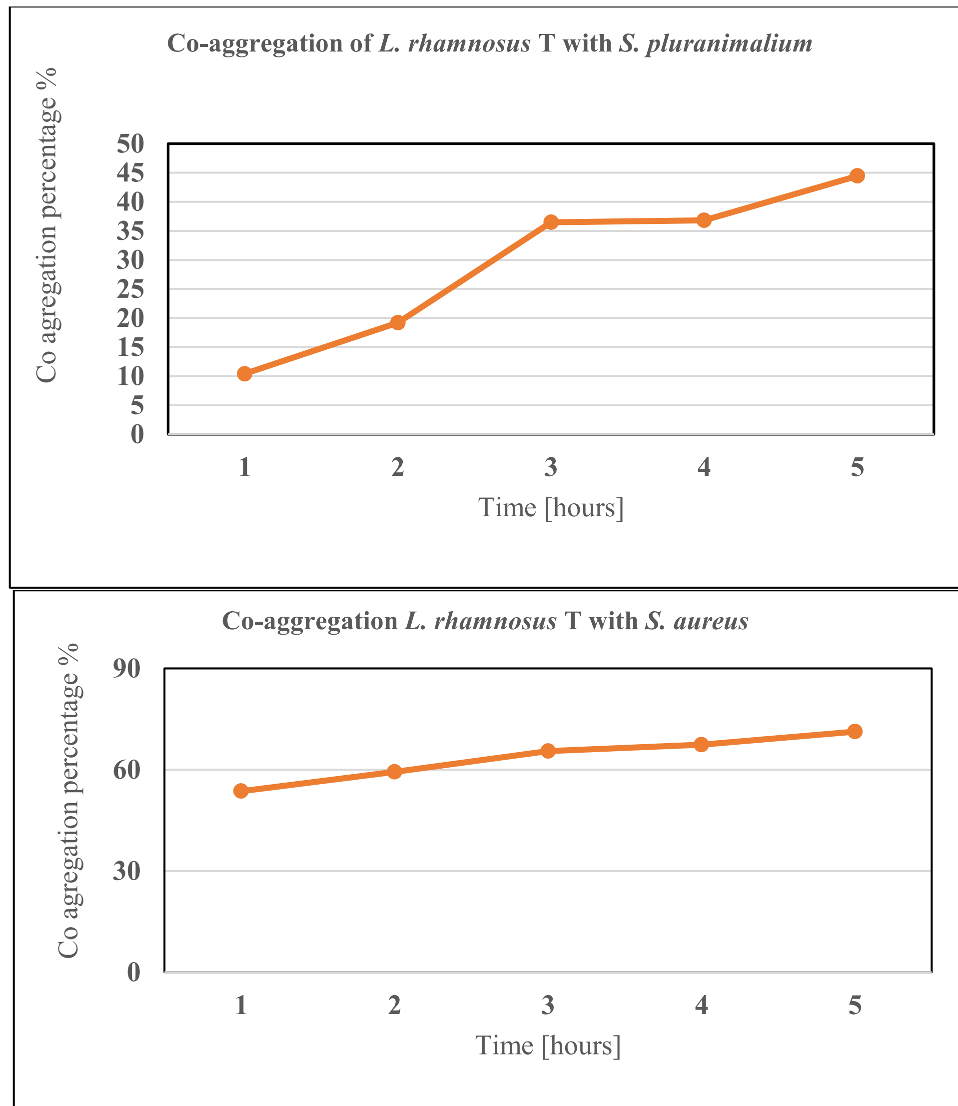


Figure 4: The coaggregation of *Lactobacillus rhamnosus* T with the two indicators *S. aureus* and *S. pluranimalium* in five hours

The capacity of *Lactobacillus rhamnosus* T to withstand acidic environments constitutes a critical assessment for evaluating the isolate's proficiency in enduring acidic conditions that may arise within the oral cavity as a consequence of gastroesophageal reflux, which transpires when gastric contents ascend into the esophagus.
According to the data illustrated in (Figure 5), the viability of *Lactobacillus rhamnosus* T cells exhibited a decline by 22.5% after 2 hours, which is considered a good tolerance of acidity.

These findings suggest that *Lactobacillus rhamnosus* T cells possess the capability to endure acidic conditions for an appropriate length of time, contingent upon the occurrence of

gastroesophageal reflux. These results that obtained agreed with Nouralhuda and Luti [23] who reported the survival of the *lactobacillus* strain they were working on at acidic conditions(pH 2) called *L. plantarum* can by 94.4% after 2 hours.

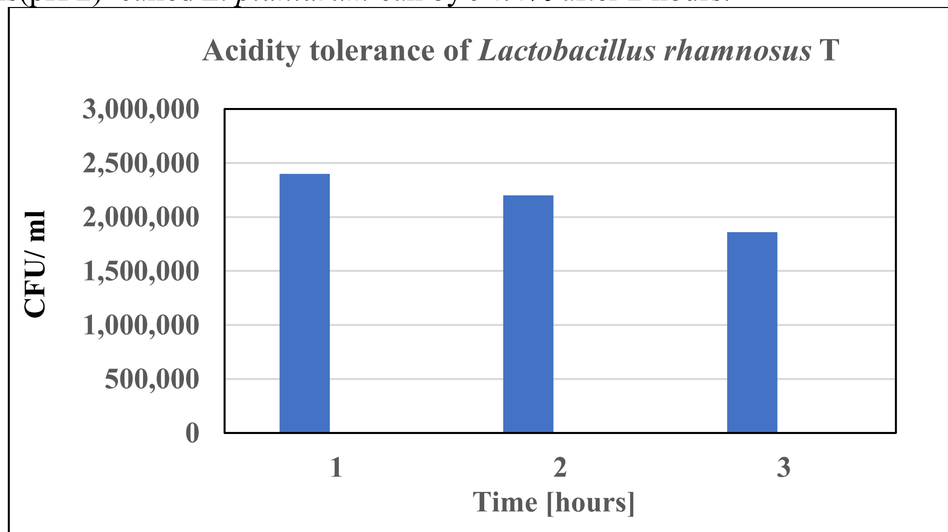


Figure 5: Acidity tolerance evaluation of *Lactobacillus rhamnosus*T in 3 hours

Lysozyme is an antimicrobial enzyme synthesized by various animal species, which constitutes a vital component of the innate immune system. Consequently, the enzyme presence within the oral cavity may induce lysis to the cells of *Lactobacillus* cellular structures.Thus, evaluating the resilience of *Lactobacillus rhamnosus* T in the oral environment constituted a pivotal experiment designed to ascertain its viability as an effective probiotic. As illustrated in Figure 6, *Lactobacillus rhamnosus* T demonstrated a significant capacity for survival in the presence of lysozyme, exhibiting a percentage tolerance of approximately 81.28% after 90 minutes. This finding indicates the capability of *Lactobacillus rhamnosus* T to endure the lysozyme presence in the oral cavity with saliva for an adequate duration. These key findings also agreed with Hussein and Luti [22].

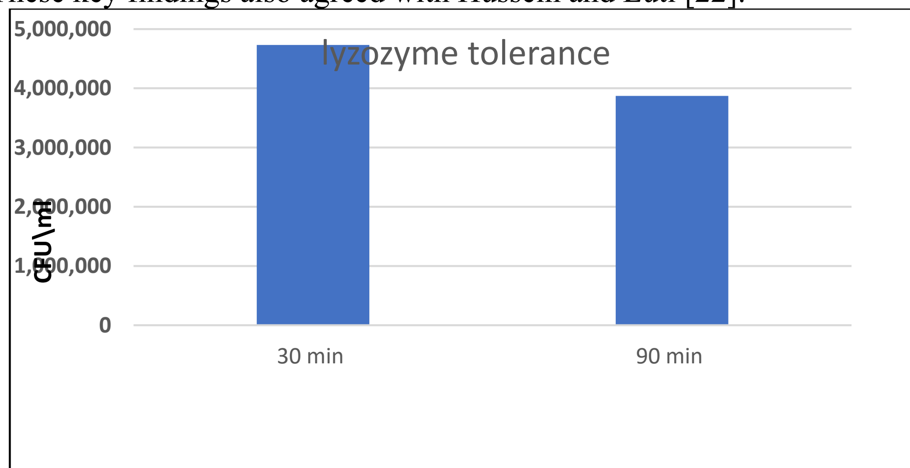


Figure 6: Lysozyme tolerance of *Lactobacillus rhamnoses* by colony number in 30 minutes and 90 minutes

One of the constituents of bile, the digestive fluid that the liver naturally produces, is bile salt. When the stomach backs up, it can cause bile reflux, which can spread to the esophagus and oral cavity. Thus, research on the tolerance of *Lactobacillus rhamnosus* T to bile salt is required. After two hours of incubation, the number of viable *Lactobacillus rhamnosus* T cells was partially reduced where bile salt was present. Figure (7) shows the bile salt tolerance of *Lactobacillus rahmnosus* from zero time to 3 hours.

According to the outcomes, following hours of incubation, 75% of the key findings showed tolerance to bile salts. The results observed are consistent with the findings of Shokryazdan et al. [24] and Nouralhuda and Luti [23], who claimed that *Lactobacillus rhamnosus* can withstand 0.3% of bile salt. Furthermore, bile tolerance was reported by Zhang et al. [25].

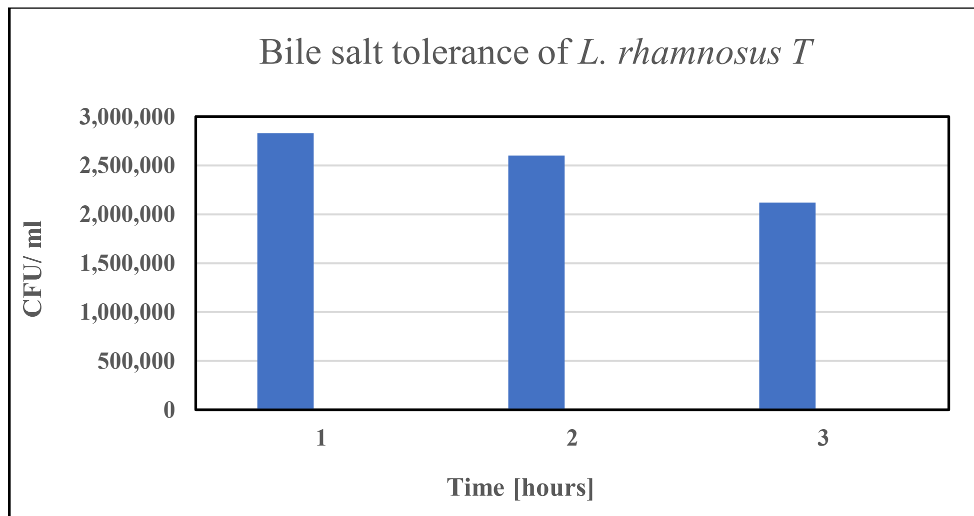


Figure 7: Bile salt tolerance of *Lactobacillus rhamnosus* in 3 hours

As mentioned earlier, the target of this study was to produce chewing gum containing probiotics that have antimicrobial properties against gingival pathogenic bacteria.
To achieve this target, the first step was to prepare encapsulated probiotic from the isolate *Lactobacillus rhamnosus* T and the next step was to integrate the encapsulated probiotic into the sterile prepared chewing gum.

The encapsulation process of the probiotic cells showed different characteristics, firstly the quantity of *Lactobacillus rhamnosus* T present within the encapsulated probiotic chewing gum was elevated in comparison to the probiotic without encapsulation. Quantitative microbial analysis of the probiotics indicated that the probiotics remained viable within the capsules, and the encapsulation procedure did not diminish their population. This phenomenon illustrated the protective influence of capsules on the probiotic cells to adverse environmental conditions; Capsules shown suspended in peptone water in Figure 8.

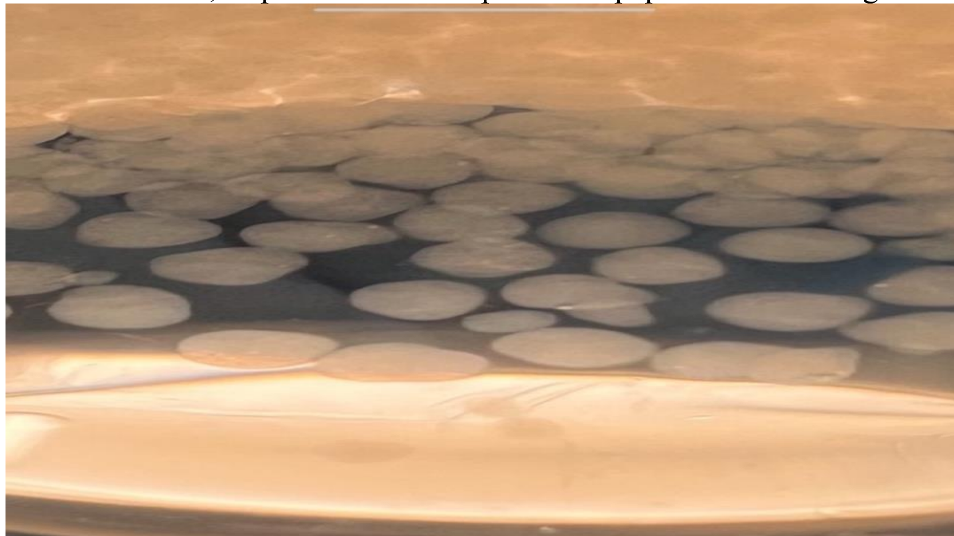


Figure 8: The encapsulation of *Lactobacillus rhamnosus* T with alginate and calcium chloride

Upon completion of the maintenance period, the concentration of *Lactobacillus rhamnosus* T within the encapsulated probiotic chewing gum after 24 hours surpassed that of the free probiotic. However, a slight decrease was observed in the concentration of *Lactobacillus rhamnosus* T within the chewing gum that contained encapsulated probiotics after 24 hours and continued to decrease gradually after one week. The observed reduction in bacterial numbers within the encapsulated forms may be ascribed to the accumulation of metabolites produced through the encapsulate activity of bacteria, which exerted an inhibitory effect on bacterial proliferation and consequently led to the decline in their numbers during the storage phase [21].

Edgar and Geddes [21] reported increasing probiotic survival by the presence of plenty of lecithin and inulin in cell walls. In addition, their investigations revealed that employing calcium alginate for probiotic encapsulation did not impact bacterial levels. The assessment of bacterial viability within capsules over a 21-day duration demonstrated an increase in the concentrations of inulin and lecithin within the walls of encapsulated seeds which positively influenced the bacterial survival rates of the samples Donthidi et al., [28]. In this study, the percentage of adding both inulin and lecithin was 1% which is considered a high concentration that can provide maximum viability.

In this study, five different types of chewing gums were tested to identify the most efficient one for producing chewing gum containing *Lactobacillus rhamnosus* T cells. The first gum was selected based on the study of Qaziyani et al. [9]. The base gum was composed of pure mixed ingredients, lacking any antimicrobial properties or inhibitory effects, and was considered a junk food appropriate for use in the delivery system. Its main component is sugar such as sucrose, glycerin, lecithin, glucose the other types of sugar, sorbitol, and inulin.

The second type was Iraqi water gum which is produced from a special type of tree called *Boswellia sacra*. This type of gum has an antimicrobial effect, it's also available in local stores at low cost, and it has a special test that releases small shots of water with every chew, this property could be a benefit for this study after adding the encapsulation probiotics with the gum, so with every shot of water the capsule and bacteria and other component will mixed well together.

Additionally, Arabic gum was the third type of gum examined in this study. It is a dried exudate collected from the Acacia trees stems and branches; primarily *Acacia senegal* and *Acacia seyal*. Arabic gum possesses many biological properties, including antioxidant and antimicrobial effects, therefore influencing renal function, blood glucose levels, and intestinal absorption, as reported by Baien et al. [29]. Furthermore, it is recognized as a prebiotic for probiotics.

Moreover, xanthan gum was also tested in this study which is a polysaccharide of high molecular weight created by strains of Xanthomonas spp. Via various fermentation processes. This type of gum or glue is known as a stabilizing agent and nontoxic gelling that does not affect probiotics viability or effects.

The last type of gum used was frankincense resin-like water gum which is easily available in Iraqi stores with low economic cost. It is extracted from Boswellia serrata and Boswellia carteri which are known to be particularly useful. This resin is known to have a wide range of pharmacological characteristics such as analgesic, tranquilizing, and anti-bacterial effects Al-Yasiry and Kiczorowska, [30].

After integrating the encapsulated probiotics in each type of chewing gum, the result indicated that encapsulation of *Lactobacillus rhamnosus* T improved its viability in chewing gums, such that after 21 days, the survival rate of the encapsulated cells reached the standard threshold for probiotics. Furthermore, this research suggested that a product specifically encapsulated chewing gums could represent a viable option for the intake of safe and efficient probiotics.

Based on results obtained in this study, the chewing gum base, water Iraqi gum, xanthan gum (XG) and Arabic gum showed good viability based on the high number of colonies revealed on the MRS agar media for twenty-one days. Figure (9) that presented the viability of probiotic inside all types of chewing gums for 3 weeks of preservation in sterile conditions. However, a minimum viability result was gained for the Frankincense, as the number of bacterial cells was reduced which may attributed to the powerful antimicrobial properties of this type of gum.

All these results showed clearly the viability and efficiency of chewing gum containing *Lactobacillus rhamnosus* T cells prepared in this study for 3 weeks. This viability and efficiency may be due to the presence of the prebiotics (the sugar glucose) and the encapsulation that contributed to this process by protecting bacterial cells from the outer harsh environment conditions and trapping the bacteria in.

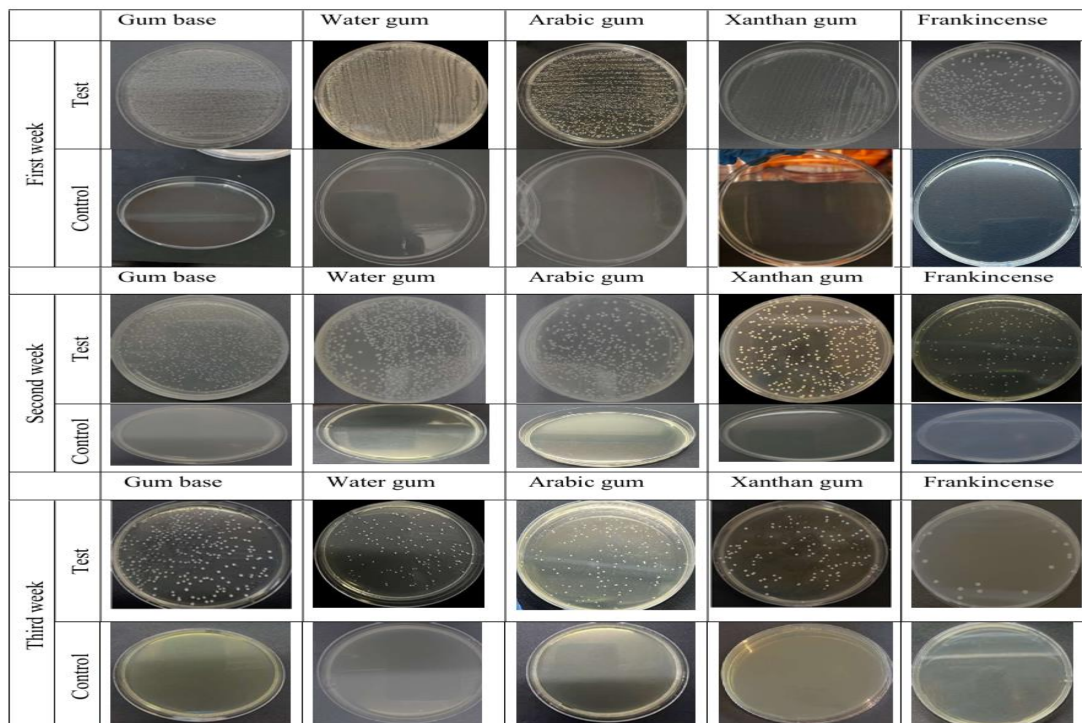


Figure 9: the viability of encapsulated *Lactobacillus rhamnosus* T cells in five types of probiotic chewing gums after 21 days.

4. Conclusion

The results of this study highlight the potential of five types of probiotic chewing gums as a simple yet effective solution for improving oral health. By successfully inhibiting oral pathogenic bacteria that cause gingivitis and reducing inflammation, these five chewing gums offer a promising alternative for treating gingivitis rather than using antibiotics and mouthwashes that contain harsh chemicals for the mouth environment. Not only are they an affordable option, but they also provide a quick way to prevent and control gum disease, making them valuable in promoting long-term oral health.

Ethical Clearance This research was ethically approved according to the reference number CSEC/1224/0121 by the ethical committee of the University of Baghdad College of Science accepted this work.

Conflict of Interest:

The authors declare that they have no conflict of interest.

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