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Research Article

In Vitro Antagonistic Activity of Lactobacillus Spp. Isolates Against Some Pathogenic Bacterial Oral Cavity

Tebarek Al-Taei1, a, Ali A. Al-Hisnawi2, b
University of Kerbala, Collage of science, kerbala, Iraq

Article Info
Article history:
Received 29 -1-2026
Received in revised form 16-2-2026
Accepted 22-2-2026
Available online 31 -3 -2026

Keywords:
Lactobacillus acidophilus, Lactobacillus plantarum, oral cavity, antagonistic activity, bacterial pathogens

Abstract

Oral bacteria can be exhibited by antagonistic activity of *Lactobacillus spp*. isolated from different sources. The current study antagonistic activities was investigated in vitro of *Lactobacillus acidophilus* and *Lactobacillus plantarum* isolated from against bacterial oral cavity . One hundred fifty samples were collected from patients with problems of oral cavity from September 2024 to December 2024. The isolates were cultured and identified biochemically. Molecular identification for Lactobacillus spp. was performed by PCR technique. Thereafter, cell-free supernatant (CFS) of those species was prepared from MRS culture and used in an antimicrobial assay that was conducted by agar well diffusion method. Results showed that both species revealed a significant ($P < 0.05$) antibacterial effect against tested pathogens including *Sphingomonas spp*., *Streptococcus spp.*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *and E. coli.* In conclusions, cell-free supernatant produced by isolated *Lactobacillus spp*. exhibited antagonistic activity against a wide range of Gram-positive and Gram-negative oral cavity pathogens suggesting its potential application in new functional probiotic products for oral healthcare.

Corresponding Author E-mail: tabarek.f@s.uokerbala.edu., iq Ali.alhisnawi@uokerbala.edu.iq
Peer review under responsibility of Iraqi Academic Scientific Journal and University of Kerbala.

INTRODUCTION

Oral cavity is an important complicated organ that forms the lower part of the face and the upper end of the digestive tract. The oral cavity is made up of both hard and soft tissues [1].

The oral cavity has multiple lines of defense, the most notable of which is first, the mucosal barrier which is an anatomical barrier that blocks the entry of hazardous bacteria while providing a habitat for normal microbes. Second, intraoral lymphoid tissue ,which plays a role in immune surveillance and mucosal integrity [2].

Gingivitis and periodontitis are caused by buildup of microbial biofilm (dental plaque) on the teeth and gums. Dynamic interaction between bacteria found in dental plaque and immune system of hosts cause these disease to progress. Progression from gingivitis to periodontitis involves the formation of periodontal pockets, progressive tissue destruction, alveolar bone loss, and tooth loss [3].

The causative agents of periodontal disease are distributed between aerobic and anaerobic bacteria. However, the most common aerobic species are *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Streptococcus* spp. . The first three are considered as opportunistic species [4,5].

Bacterial resistance towards antibiotics is a growing global health issue that threaten human health. Bacteria acquire resistance through horizontal gene transfer by conjugation, bacteriophage transfer and bacterial transformation [6,7]. Probiotics as beneficial live microorganisms are an alternative or complementary option that provides benefits to the host by promoting a balanced microbial environment. This contributes to long-term microbial stability and oral health by reducing plaque and gingivitis while inhibiting pathogenic bacteria responsible for bad breath and oral infections, with minimal side effects [8,9].

Aim of Study

The aim of the present study is to investigate the antagonism activity of *Lactobacillus* spp. against some bacterial pathogens isolated from patients with mouth bacterial disease.

MATERIAL AND METHOD

1. Collection of Samples

About of 150 samples were collected from patients with oral cases, ulcers, gingivitis, and periodontitis, who were referred to the Specialized Dental Center/ Periodontology Department and outpatient clinics in different areas of Babylon Governorate. The patients ages ranged from 11 to 75 years old, during the period from September 2024 to December 2024. Samples were collected using sterile swabs with a carrier medium, which were passed over the entire inflamed area in cases of gingivitis. For periodontitis, a Periodontal Probe was used and introduced into the inflammatory area to collect a sample. Swabs were then subsequently taken to the microbiology lab for isolation and microbial diagnosis. Additionally, 15 samples were gathered, from dairy, milk and fish products for isolation and identification of *Lactobacillus planatrium* and *Lactobacillus acidophilus*, and their qualities and effects were examined in the present study.

1.2 Isolation and Diagnosis of Microorganisms

Samples collected from the oral cavity were cultured on Mannitol salt agar(*S.aureus*), Citrimide agar(*P.auroginosa*), Mitis salivaruis agar(*Streptococcus spp*.) and EMB agar(*E.coli*) (HiMedia),for the isolatating Lactobacillus spp.,they were cultured on MRS, after which plates were incubated at 37°C for 24-48 h, followed by microscopic examination and biochemical analysis. Pure colonies were identified and diagnosed on the

basis of cultured, microscopic characteristics, and VITEK 2 system.

1.3 Antibiotics Susceptibility Test

Following the manufacturer's guidelines, Vitek 2 System was used to assess the bacteria's resistance activities against a variety of antibiotics in the present study.

1.4 Molecular Diagnosis of *Lactobacillus spp.*

Isolates of *Lactobacillus* spp. were chosen for molecular diagnosis through polymerase chain reaction (PCR) technology. A genomic DNA extraction kit (Microgen- Korea) was used to obtain DNA extraction from LAB pure colonies. After that, PCR reactions were carried out using primers that were acquired from Macrogen, Korea (show in Table 1) . A final volume of 25 µL was used for the reaction, which contained 5 µL Master Mix, 3 µL of forward and reverse primers at 10 pmol, 3 µL DNA, and 14 µL deionized water. PCR process consists of fundamental steps, beginning with an initial denaturation at 95°C for 3 min, followed by 32 cycles repeated denaturation at 94°C for 30 sec each cycle. The primers bind to the complementary strand during the annealing stage, which occurs at an 54 ◦C for *L. acidophilus* and 57 ◦C for *L. plantarum* temperature. Following that, elongation is carried out at 72°C for 55 sec. Final elongation was conducted at 72°C for 5 min. The PCR products were loaded onto electrophoresis plate on agarose gel 1% in TAE buffer 1x at 90 v for 60 min. DNA ladder 100 bp was run alongside with the samples to check the size of PCR products [10].

TABLE 1: The sequence of primers used to diagnose *Lactobacillus* spp.

Name of the bacteria	Nitrogenous base sequence		Product size	Reference
L. acidophillus	TCATGTTGGGATGCAATGAG	F	828bp	11
	TTTCAAAACTTGTCCTGCTG	R		
L. plantarum	AGGGTGAAGTCGTAACAAGTAGCC	F	428bp	12
	CTAGTGGTAACAGTTGATTAAAACTGC	R		

Antimicrobial Activity of *Lactobacillus* spp. Against Bacterial Isolates

2.1 Cellular Filtration Preparation of *Lactobacillus spp*.:

Colonies of *Lactobacillus* spp. were cultured on MSA broth and then incubated at 37°C for 24 h under anaerobic conditions. Following the incubation period, samples were centrifuged at 6000 rpm for 20 min to separate the cell-free filtrate. Finally, the liquid was filtered to sterilize it and remove any microbiological contamination [13].

2.2 Inhibitory Activity Test of Lactobacillus spp.

Well diffusion technique was applied to evaluate the inhibitory activity of *L. Planetarium* and *L. acidophilus*. A volume of 0.1 mL of pathogenic bacteria including *Staphylococcus aureus*, *Streptococcus mitis*

and *Streptococcus pseudoporcinus* , *Escherichia coli*, *Pseudomonas aeruginosa*, and *Sphingomonas* spp. (At a concentration of 0.5 McFarland's solution of 1.5x10^8)were cultured on Muller-Hinton medium and spread using a sterile swab and left for 5-10 min to dry. After that, a cork drill was used to make 5 mm diameter holes on the surface of the plate. Separately, each well was filled with 50 µL of MRS-cultured *Lactobacillus* spp. filtrate in triplicate for each sample, and incubated at 37°C for 24 hours to measure the zone of inhibition [14,15] .

STATISTICAL ANALYSIS

The current data were presented as means and standard deviation (M±SD). One-way analysis of variance (ANOVA), followed by post hoc LSD, was used to test for significant differences between diameter of resulted inhibition zones by each species of *Lactobacillus* spp. The social sciences statistical software package (MiniTab statistical software version17, IBM (Pennsylvania, USA) was applied. The accepted level of significance was $P < 0.05$.

RESULTS

One hundred and fifty oral swabs were collected from gingivitis and periodontitis. Six different bacterial genera were identified by a number of biochemical tests, as the following 24.5% (43) isolates of *Sphingomonas* spp., 13.1% (23) isolates of *Streptococcus* (*S. mitis, S. pseudoporcinus, S. pneumoniae, S. sanguinis, S. salivarius, S. pluranimalium*), 12.5% (22) isolates of *Staphylococcus aureus*, 4% (7) isolates of *Pseudomonas aeruginosa*, 3.4% (6) isolates of *E.* coli. The remaining percentage made up the other bacterial genera as demonstrated in Fig (1).

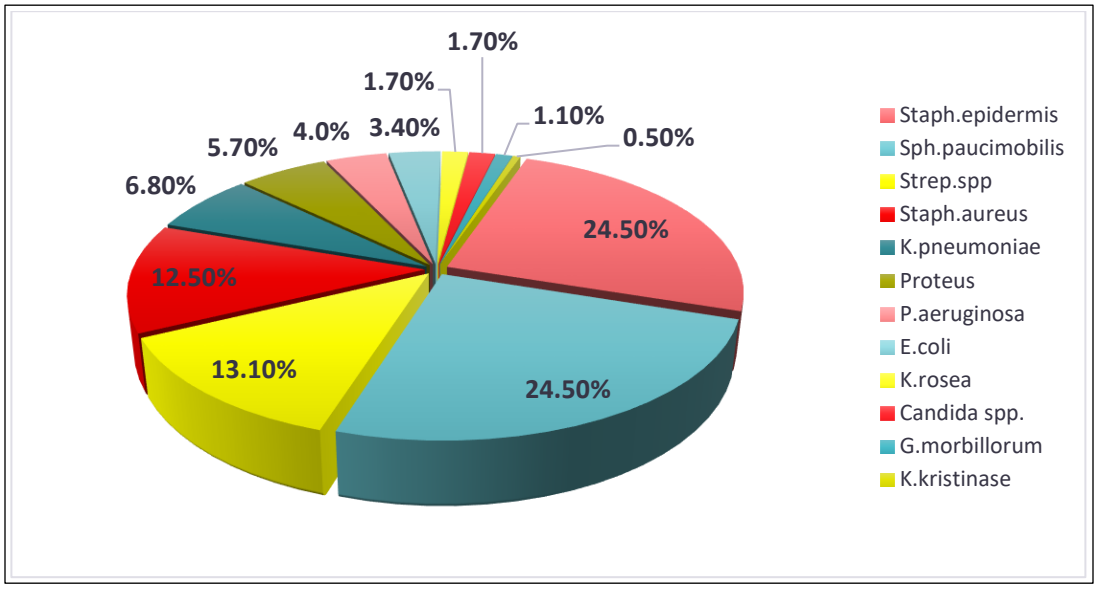
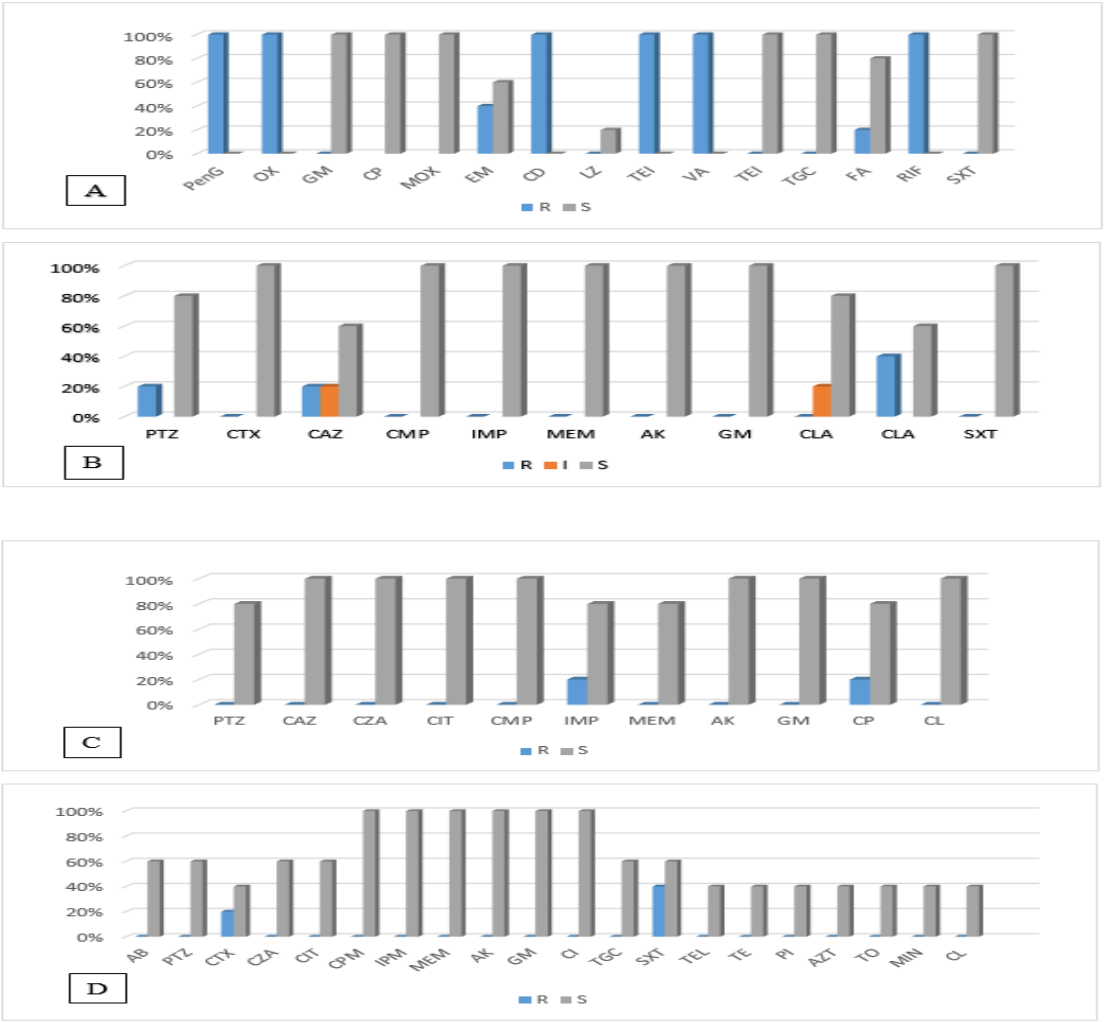


FIGURE 1. Distribution of microorganisms in oral cavity

Bacterial resistance detection by using Vitek2 system revealed that *S. aureus* isolates were 100% resistant to oxacillin, clindamycin, and vancomycin. On the other hand *Streptococcus* spp. isolates were 80% resistant to erythromycin and tetracycline. Moreover, *P. aeruginosa* , *E. coli*, and *Sphingomonas* spp. isolates were susceptible to most anibiotics. Antibiotic susceptibility results are summarized in Fig (2).



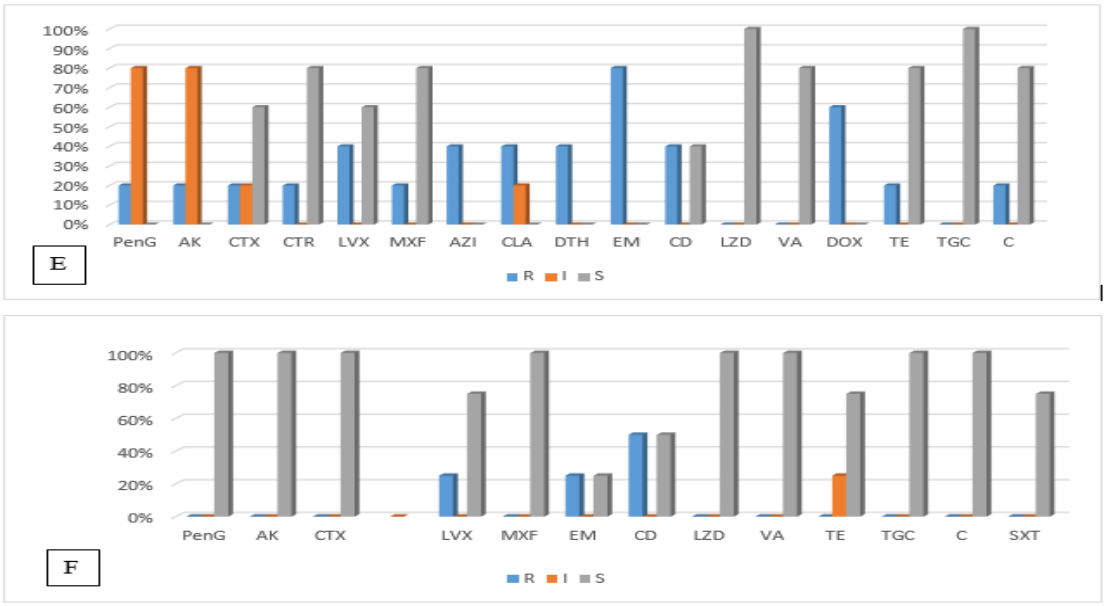


FIGURE 2. Antibiotic susceptibility of pathogenic bacteria (A) *S. aerues* (B) *Sphingomonas spp*. (D) *P.aeruginosa* (E) *E.coli* (F) *St. mitis* (F) *St. pseudoporcinus*.

Data obtained from the present study showed that all isolates of *S. aureus* were resistant to oxacillin, clindamycin, vancomycin, and rifamycin in rate of 100%. Resistance to erythromycin was 40%, and resistance to fusidic acid was 20%.

In addition, *Streptococcus spp*. revealed varying resistance based on the species. *St. mitis* were resistant to tetracycline and levofloxacin. On the other hand, *St. pseudoporcinus* were resistant to clindamycin and showed no resistance to vancomycin or moxifloxacin.

Moreover, *P. aeruginosa* were highly sensitive to antibiotics, with no resistance to amikacin, gentamicin, ceftazidime, or piperacillin/tazobactam, indicating that they are effective in treating this bacterium's infections.

The results obtained from antibiotics susceptibility showed that *E. coli* revealed resistance to trimethoprim and cefotaxime , respectively. Also, *Sphingomonas* spp. showed a resistance to colistin, piperacillin/tazobactam and ceftazidime, but was sensitive to other antibiotics.

In molecular diagnosis , PCR technique was used with a pair primers specific for *L. acidophilus* and *L. plantarum*. The results of electrophoresis of PCR products for diagnosis of *L. actobacillus* and *L. plantarum* were (828) bp and 428, respectively as illustrated in Fig (3 and 4).

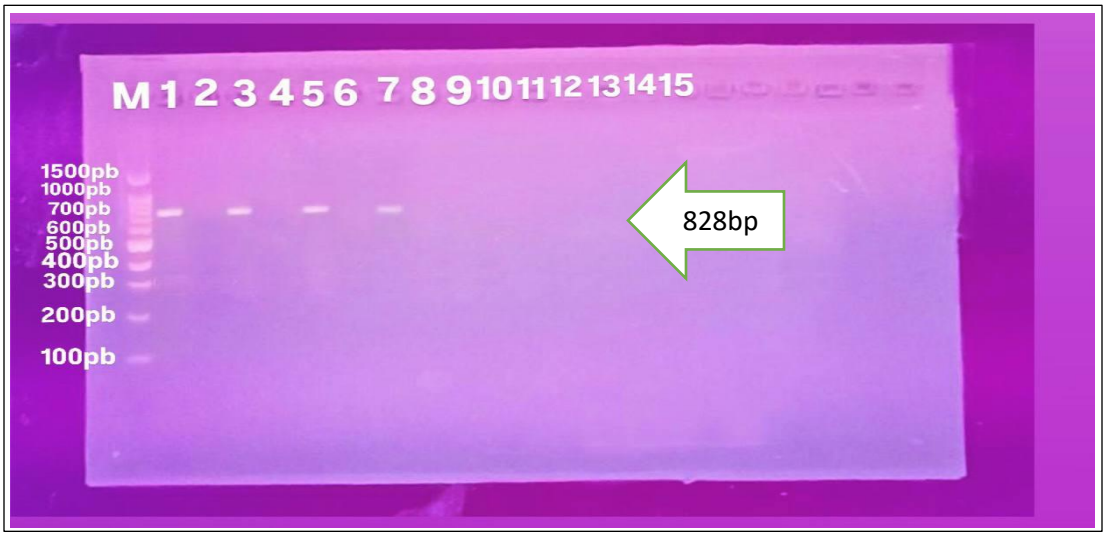


FIGURE 3: Electrophoresis of PCR products of *L. acidophilus* isolated from food sources using specific primer to yield (828 bp) in 1% agarose gel at 50V for 1 hour.

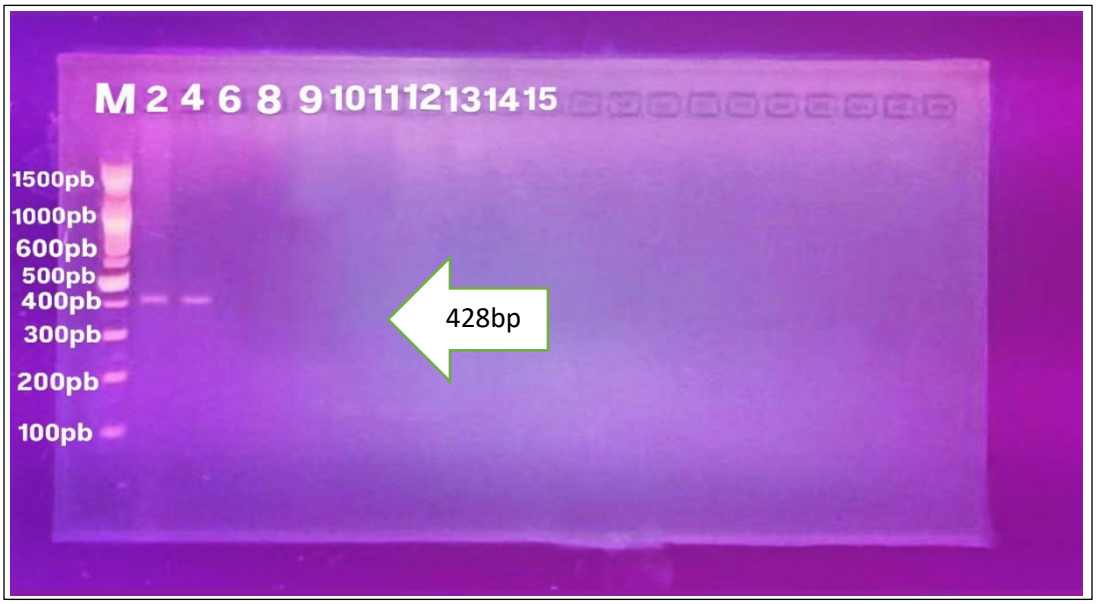


FIGURE 4: Electrophoresis of PCR products of *L. plantarum* isolated from food sources using the specific primer to yield (428bp) in 1% agarose gel at 50V for 1 hour.

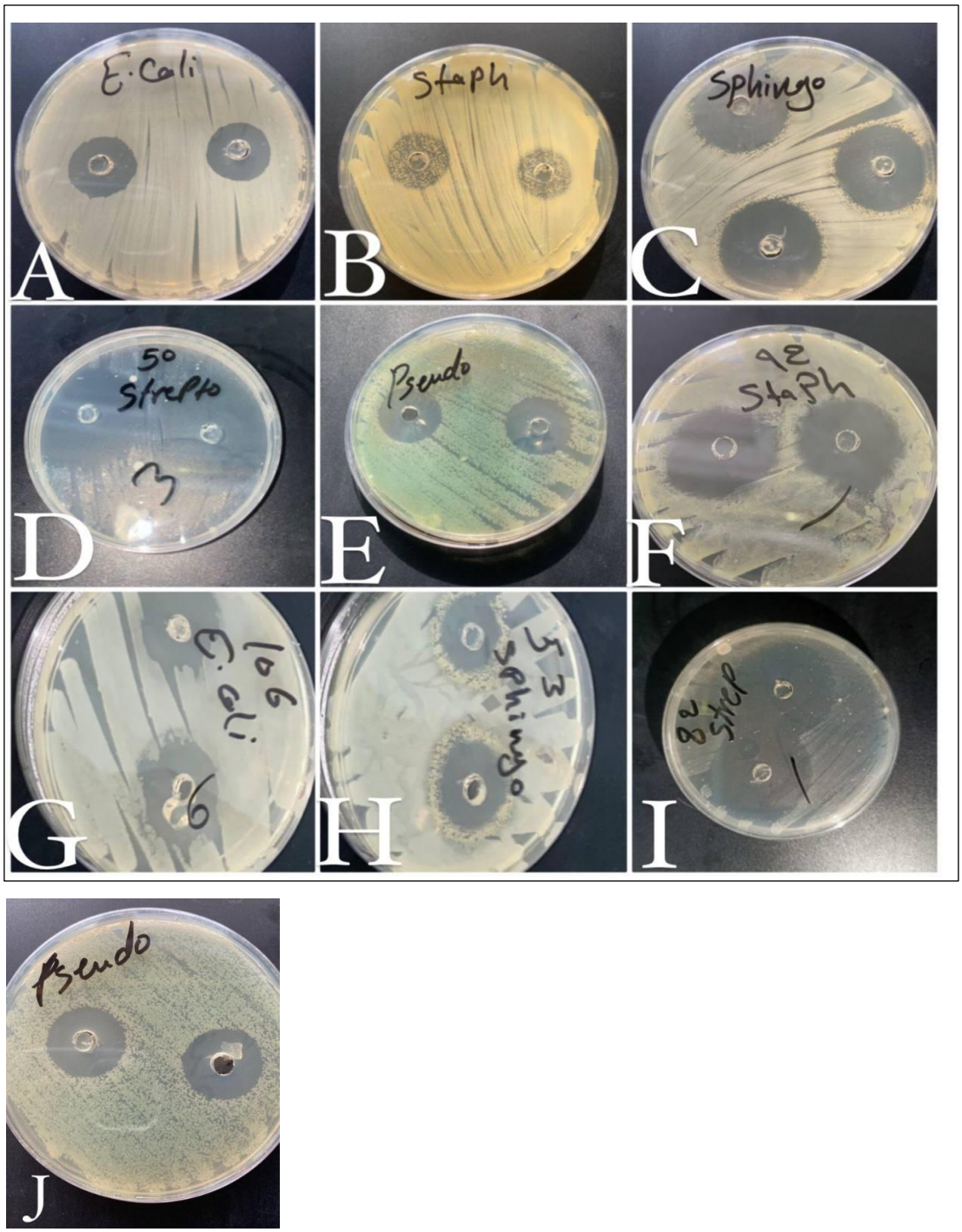


FIGURE 5: Evaluation of the antagonistic activity of lactic acid bacteria against pathogenic bacteria .

(A)*L.acidophilus* vs *E.coli*; (B)*L.acidophilus* vs *S.aureus* ; (C)*L.acidophilus* vs *Sphingomonas spp.*; (D)*L.acidophilus* vs *S.mitis*; (E) *L.acidophilus* vs *P.aeruginosa*, (F)*L.plantarum* vs *S.aureus*; (G) *L.plantarum* vs *E.coli*; (H) *L.plantarum* vs *Sphingomonas spp.*; (I) *L.plantarum* vs *S.mitis*; (J) *L.plantarum* vs *P.aeruginosa*

An antagonistic test was conducted between the first isolate of *L. acidophillus* isolated from dairy which called dairy 1 and pathogenic bacteria Fig (6&5).

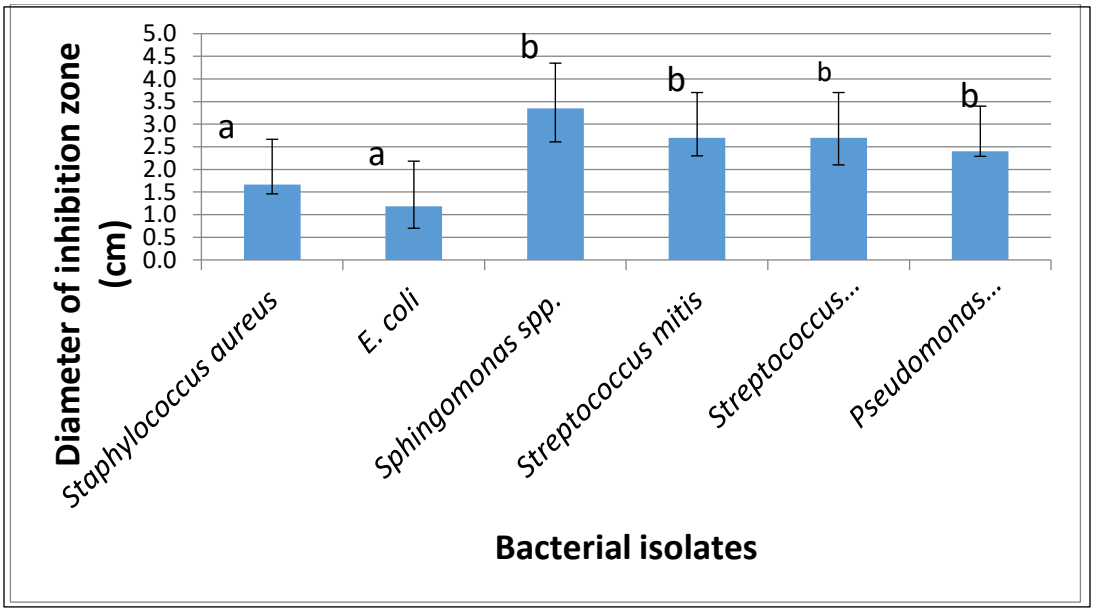


FIGURE 6: Effect of *L. acidophilus* isolated from dairy1 on the growth of bacterial pathogen isolated from oral cavity. Columns with different letters are significantly different (P≤0.05).

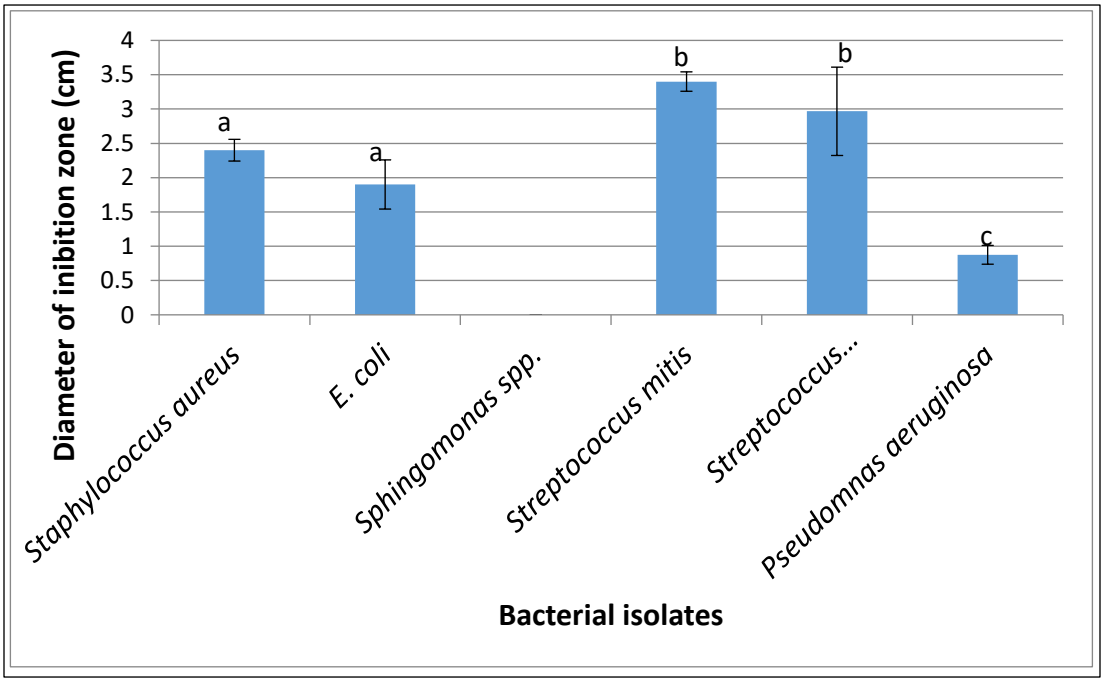


FIGURE 7: Effect of *L. acidophilus* isolated from dairy2 on the growth of bacterial pathogen isolated from oral cavity. Columns with different letters are significantly different (*P*≤0.05).

These results clearly indicate that growth of all tested bacteria were influenced by *L. acidophilus* where the diameter of inhibition zone was significantly higher for *Sphingomonas spp., S. mitis, S. pseudoporcinus,* and *P. aeruginosa* in comparison to *E. coli* and *St. aureus* ($P \leq 0.05$).

Additionally, the inhibitory activity of the second dairy isolate of *L. acidophilus* was tested against the aforementioned bacterial pathogen Fig (7).

Data were demonstrated that *L. acidophilus* were revealed antagonistic activity against almost all tested pathogen with significant differences between them ($P \leq 0.05$). Nonetheless, the diameter of inhibition zone of *P. aerugionsa* 0.8 cm was significantly lower compared to other bacterial pathogen. In addition, there were no significant differences between two species of *Streptococcus*, but diameter of inhibition zone for them was significantly affected by *L .acidophilus* in comparison to other bacteria ($P \leq 0.05$), Fig (7).

Furthermore, the inhibitory activity of *L. acidophilus* isolated from milk was tested against the aforementioned bacterial pathogen Fig (8 &5).

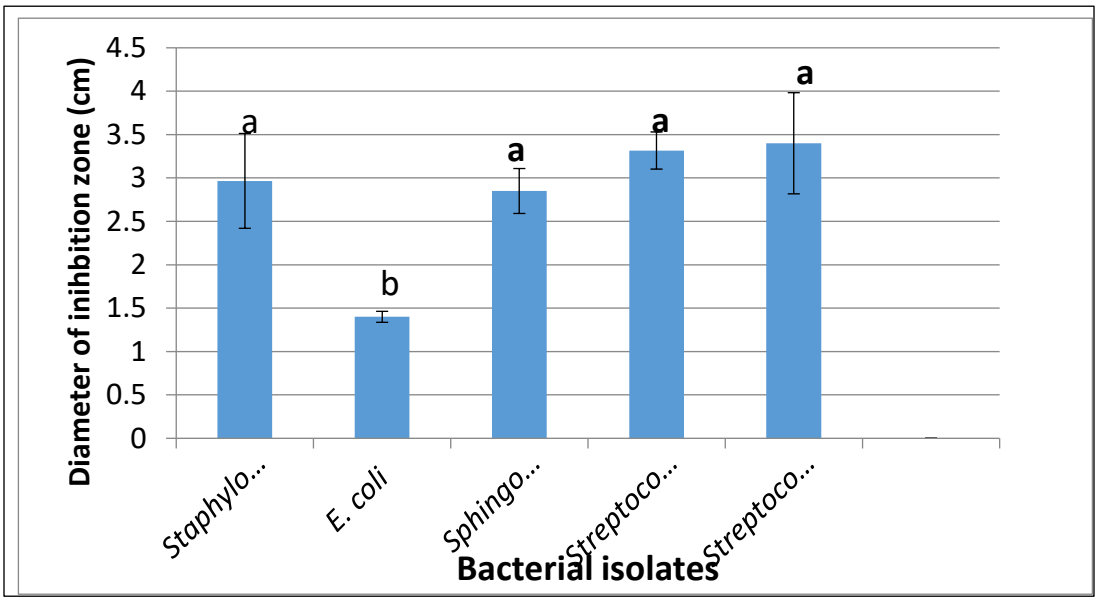


FIGURE 8: Effect of *L. acidophilus* isolated from milk on the growth of bacterial pathogen isolated from oral cavity. Columns with different letters are significantly different ($P \leq 0.05$).

L. acidophilus exerted antagonism activity against all pathogenic bacteria, but the diameter of inhibition zone for *E. coli* 1.3 cm was significantly lower in comparisons to other bacteria.

Ultimately, fish-derived *L. acidophilus* demonstrated a significant differences on antimicrobial activity among the tested bacterial pathogens ($P \leq 0.05$) Fig (9&5).

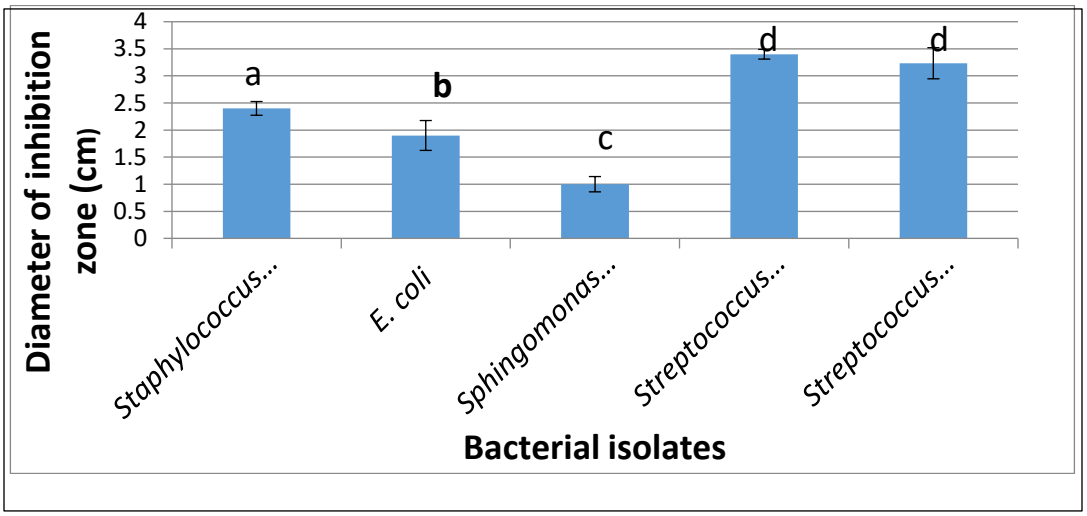


FIGURE 9. Effect of *L. acidophilus* isolated from fish on the growth of bacterial pathogen isolated from oral cavity. Columns with different letters are significantly different ($P \leq 0.05$).

Although, all bacteria were influenced by the antimicrobial activity of *L. acidophilus*, *Sphingomonas spp*. were significantly lowest to effect by probiotic (1 cm in diameter).

On the other hand, the antimicrobial activity of *L. planetarium* isolated from dairy and fish towards bacterial pathogens was investigated Fig (10&5). Significant differences on antimicrobial activity of *L. planetarium* isolated from dairy among the tested bacterial pathogens were demonstrated ($P \leq 0.05$).

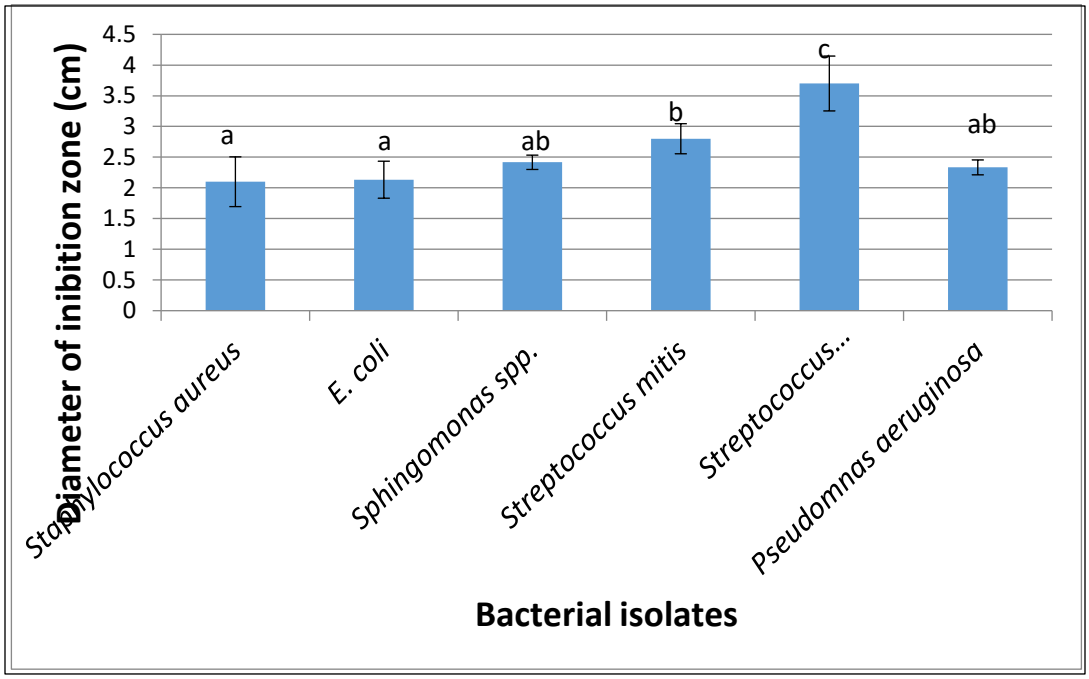


FIGURE 10: Effect of *L. plantarum* isolated from dairy on the growth of bacterial pathogen isolated from oral cavity. Columns with different letters are significantly different ($P \leq 0.05$).

The diameter of inhibition zone for *Streptococcus pseudoporcinus* 3.7 cm was significantly higher compare to other pathogens.

Although the growth of bacterial pathogens was inhibited by *L.plantarum* isolated from fish, *Streptococcus* spp. were the more bacterial genus affected ($P \leq 0.05$) Fig 11.

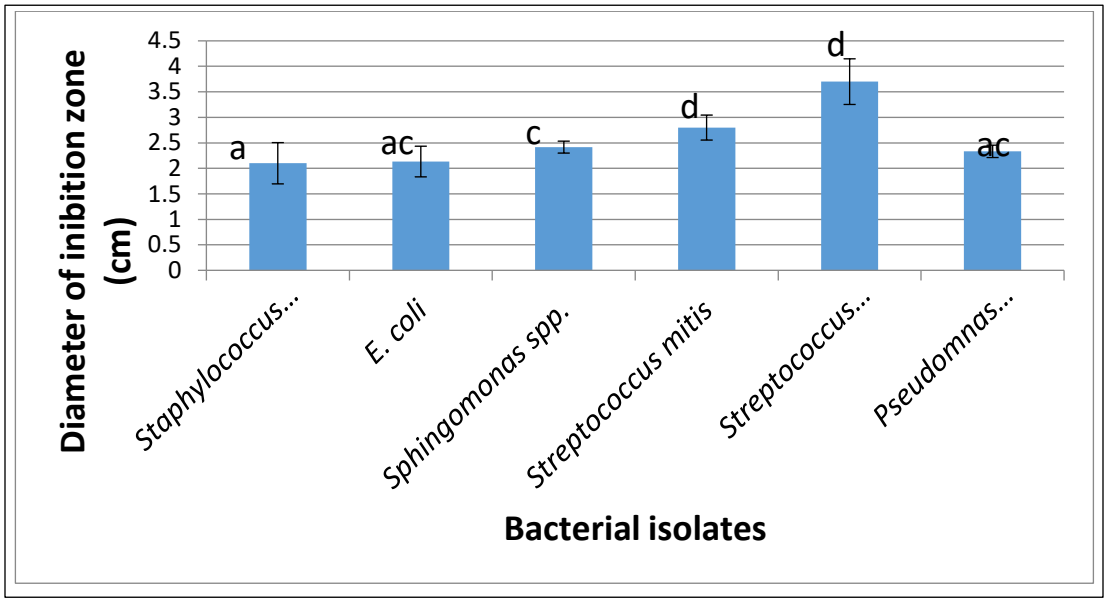


FIGURE 11: Effect of *L. plantarum* isolated from fish on the growth of bacterial pathogen isolated from oral cavity. Columns with different letters are significantly different ($P \leq 0.05$).

DISCUSSION

The present study showed that the presence of non-oral bacteria in oral swabs of patients with oral diseases, frequently brought on by inadequate oral hygiene. These results are consistent with earlier research in Germany (72.2%) and India (77%) [16,17], whereas it is not similar to the percentages found in Brazil and America [18,19]. This could be because of variations in economic and geographic factors as well as various analytical techniques.

The results of the current study concerning the resistance of *Staphylococcus aureus* isolates to antibiotics are consistent with the findings of earlier studies [20] which showed that *S. aureus* were 90% resistant to at least two drugs. In contrast, the present results are not in agreement with the results of [21], which indicated that *S. aureus* was sensitive to most antibiotics. Antibiotics resistance could be increased as a result of indiscriminate use of antibiotics without consulting the dentist or private hospitals, as well as the indiscriminate use of antibiotics without consulting the dentist or private hospitals. Regarding *Streptococcus spp.* antibiotic resistance, strains of *S. mitis* shown resistance to both levofloxacin and tetracycline, which was in line with findings from other investigations [22]. *S. pseudoporcinus* strains showed resistance to clindamycin. These findings are consistent with data obtained from [23] which demonstrated that *St. pseudoporcinus* samples collected from the female reproductive system. The present study are in agreement with study conducted by [24] that there was a high rate of resistance to tetracycline and erythromycin. Antibiotic resistance has increased due to gene

exchange during horizontal gene transfer between bacterial species, as well as the intake of sugars and refined carbohydrates, creating an ideal environment for the establishment of resistant strains [25]. Furthermore, the results of resistance to *Pseudomonas aeruginosa* isolates were consistent with a study conducted in 2020 at the Universidad Autónoma de Asunción [26]. *Escherichia coli* resistance tests revealed considerable susceptibility to certain drugs. This demonstrates that non-oral bacteria isolated from the mouth cavity have genetic differences from those collected from other parts of the body [27]. While, *Sphingomonas spp.* isolates from gingivitis and periodontitis cases were resistant to colistin and ceftazidime but sensitive to several other antibiotics. These findings were similar with a prior study carried out in Babylon governorate, where bacteria were isolated from the oral cavity and shown to be very sensitive to most antibiotics [28].

An antigenicity test was performed between pathogenic bacteria (*S.aureus, S.mitis, S. pseudoporcinus, E. coli, P.aeruginosa, and Sphingomonas spp*.) isolated from the mouth and Lactobacillus bacteria obtained from various dietary sources. The results showed that Lactobacillus lactis isolated from dairy 1 clearly affected the pathogenic bacteria tested, with the largest zone of inhibition in *S. mitis* and *Sphingomonas spp*.. These results are in line with the results of previous studies that confirmed the efficacy of these metabolites against pathogenic bacteria [29,30]. The results of the current study were not consistent with the results of a study conducted on *S. aureus* by agar well diffusion , where no antagonistic effect was observed [31]. Furthermore, the current investigation found that Lactobacillus acidophilus isolated from dairy products2 had antimicrobial action against pathogens, albeit the degree of inhibition varied between bacterial species. *P. aeruginosa* showed the lowest inhibitory response, with an inhibition zone diameter of 0.8.This study showed similarity to a previous study of *L. acidophilus* against *S.aureus* and *E. coli* by agar well diffusion [32,33]. These findings were also congruent with a local study of *P. aeruginosa* isolates from clinical sources, which found that *L. acidophilus* and *L. plantarum* were highly effective against this pathogenic bacterium [34]. Furthermore, *L. acidophilus* isolated from milk demonstrated significant pathogen inhibition. All pathogens were affected to varied degrees, with the lowest zone of inhibition observed against *E.coli*. These results aligned with a study of *Staphylococcus* spp. isolated from surgical wounds, intravascular catheters, urine, and blood [,35]. In addition, *Lactobacilli*'s ability to inhibit pathogen growth was attributed to their bactericidal activity, which included the production of lactic acid, propionic acid, decyl acid, and acetic acid, which have the ability to break down the bacterial cell wall and prevent it from growing[36]. Not only that, the results also aligned with a study that obtained *P. aeruginosa* from burns in Iraq, where *L. acidophilus* clearly showed an impact on the isolated bacterium [37]. On the other hand, these findings are not in agreement to the study performed by [38] regarding clinical strains of *E. coli* isolated from burns, which found bacteria resistant to both type of lactobacillus. This resistance could be attributed to the acquisition of several resistance genes, as well as the pathogen's isolation from various location.

Regarding *L. plantarum*, exhibiting clear efficacy against all pathogenic bacterial isolates isolated from the oral cavity, recording the biggest zone of inhibition for *S.pseudoporcinus* compared to the rest of the pathogenic bacterial species used in the study. This study is consistent with the results of a previous study of *L. plantarum*

isolated from other food sources [39]. Furthermore, the results of *L. plantarum's* effect on pathogenic bacteria, including S. *aureus, E. coli,* and *P. aeruginosa*, showed consistency with previous studies that confirmed their effectiveness against these pathogens [40,41]. In contrast to these findings, a study that examined the impact of *L. plantarum* on *E. coli* in Iran revealed that the latter exhibited a high level of resistance to *Lactobacillus* spp. [42]. Ultimately, the efficacy of fish-isolated *L. plantarum* was tested against oral cavity infections, with the *Streptococcus* genus showing the greatest impact. In support of these results, a study of *L. plantarum* inhibited the growth of *S. aureus* and *P. aeruginosa*. This finding is related to the bacteria's ability to create a large amount of bacteriocins [43]. These findings are also consistent with a prior study, which found that *L. plantarum* were be able to suppress growth of *E. coli* and *Pseudomonas aeruginosa*. As a result, the bacterium produces 40 chemicals compounds that suppress these dangerous bacterial pathogen [44]. According to the study by Munoz and colleagues, all types of LAB especially *L. plantarum* were not revealed any antibacterial activity without the addition of FOS (fructo-oligo-saccharide) [45].

No literature were supported the current study regarding examined the efficacy of *L.acidophilus* and *L.plantarum* against *Sphingomonas spp, S. mitis* and *S. pseudoporcinus* isolated from gingivitis and periodontitis.

CONCLOSION

Both *Lactobacillus spp.* isolates (LAB) showed strong antagonistic activity against the majority of the pathogens tested in the oral cavity. Furthermore, the CFS generated by these isolates inhibited a wide range of Gram-positive and Gram-negative oral infections. These data suggest that LAB's antimicrobial metabolites play an important function in inhibiting harmful bacteria. As a result, LAB and their bioactive substances may be a viable alternative or supplement to traditional antibiotics for the treatment of some oral microbial illnesses. This supports their potential use in the creation of innovative functional probiotic products targeted at improving oral health.

ACHKNOWLEDGMENTS

The author expresses gratitude to Dr. Salah Rasool Muhammad, Director of the Periodontology Unit in Babylon Governorate, and all contributors to this research.

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