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Original Article

Inhibitory potential of probiotic strains against pathogens associated with osteomyelitis of the jaws: a quantitative MIC and MBC analysis

Moayad Baazeem[1,2], Abdurahman Niazy[3*], Sameh Rabea[4], Abdulaziz A. Alomiery[5]

[1] Department of Oral and Maxillofacial Surgery, College of Dentistry, King Saud University, Riyadh, Saudi Arabia
[2] Department of Oral and Maxillofacial Surgery Ministry of Health, Third Cluster, Diriyah Hospital Riyadh, Saudi Arabia
[3] Department of Oral Medicine and Diagnostic Sciences, College of Dentistry, King Saud University, Director of College of Dentistry Research Center (CDRC), Director of the Molecular and Cell Biology Laboratory, Administrative Director, Prince Naif Health Research Center (PNHRC), Riyadh, Saudi Arabia
[4] Department of Pharmaceutical Sciences, College of Pharmacy, Al-Maarefa University, Riyadh, Saudi Arabia
[5] Basic Science Department, Prince Sultan College for Emergency Medical Services, King Saud University, Riyadh, Saudi Arabia

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Abstract

Osteomyelitis of the jaw is a significant health burden, often associated with biofilm formation, antimicrobial resistance, and complex bacterial interactions. This study aimed to evaluate the antibacterial efficacy of two probiotic strains, Lactobacillus reuteri DSM 17938 and ATCC PTA 6475, against three key pathogens: Fusobacterium nucleatum, Actinomyces israelii, and Staphylococcus aureus. The focus was on their potential as adjunctive treatments for osteomyelitis of the jaws. This in-vitro study employed tailored standard protocols to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the probiotic strains against the tested pathogens. Probiotic suspensions were prepared in serial dilutions and bacterial strains were inoculated into brain heart infusion media for exposure. The growth inhibition and eradication were confirmed through subculture techniques and microscopic examination of Gram-stained samples. Both probiotic strains exhibited significant antibacterial activity against Fusobacterium nucleatum and Actinomyces israelii at low concentrations, achieving MIC of 0.625 µl and MBC of 1.25 µl for both pathogens. In contrast, Staphylococcus aureus demonstrated greater resistance, with MIC and MBC recorded at 100 µl and 200 µl, respectively. The differential response underscores the variable susceptibility of bacterial species to probiotics and highlights the potential for selective therapeutic application. Within the scope of this study, two Lactobacillus reuteri strains demonstrated promising antibacterial effects against Fusobacterium nucleatum and Actinomyces israelii, suggesting their potential utility in managing osteomyelitis of the jaws. However, higher concentrations or synergistic approaches may be needed to combat Staphylococcus aureus. Further studies including preclinical and clinical trials are essential to translate these findings into effective therapeutic strategies.

Keywords: Probiotics, Osteomyelitis, Biofilm.

1. Introduction

Chronic wounds are commonly inhabited by microorganisms that play a key part in wound healing [1]. In chronic wounds, Edward and Harding [2004] determined that low numbers of microorganisms in the injury site had a potentially favorable influence on the healing process, in contrast to those involving the presence of large numbers of microbiota, which hindered the healing process. Osteomyelitis of the maxillofacial region, particularly involving the maxilla and mandible, may be acute or chronic in origin, but most cases are chronic and most linked with the spread of odontogenic infections. Other factors include injury, malignant tumors, malnutrition, diabetes, chronic systemic disorders, and infectious diseases resulting in hypovascularized bone [2].

The pathophysiology of osteomyelitis may be hematogenous in origin or stem from local infection, and its treatment requires eradication of bone sequestra, wound debridement, and bone decortication. In addition, an adequate microbiological diagnosis combined with prolonged antibiotic therapy is necessary, especially in cases that are deemed refractors [3,4]. The prevalence, type, aggressiveness, and clinical prognosis of osteomyelitis rely on multiple variables, including the infecting pathogen's characteristics and virulence, the host's immune response, and the source of infection [5]. Most oral anaerobes rely on ideal environmental circumstances and subtle ecological interactions with diverse microbial species to acquire their ability to attack and colonize other parts of the host. The presence of distinct oral anaerobic organisms in complex

* Corresponding author.
E-mail address: aaniazy@ksu.edu.sa (A. Niazy).
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infections shows that ecological relationships may be crucial in the development of osteomyelitis of the jaws [2,6].

The meeting of host and bacterial organisms is a calibrated symbiotic exchange in which they cause a restricted degree of damage to each other. Although numerous microorganisms coexist in the oral cavity, health is the most prevalent status. On the cellular level, illness will only arise when the balanced interplay between host and microorganism is interrupted [1].

The presence of bacteria, which is located either in the superficial surface or deep tissue of the wound, could interrupt any phase of wound healing, and it is the host reaction to the bacteria that defines the clinical manifestation [7]. Infection is regarded as a local factor that inhibits wound healing and is defined as the presence of reproducing organisms in the host injury [8]. Interestingly, the bacterial population inside an injury does not correspond directly to the likelihood of an infection [7]. *P.gingivalis* has been shown to release byproducts that actively impair the migration of oral epithelial cells in an in-vitro scratch experiment, eventually lengthening the time of wound closure [9].

Unicellular bacteria often create biofilms as a means of adapting to environmental stress. These biofilms provide a protective and resistant community that enables survival against host immune responses and antibiotic treatments. Biofilms provide prolonged bacterial cell viability in challenging conditions in many ways. Biofilms serve as a form of defense for bacterial cells by creating a physical barrier that restricts the entry of immune cells, therefore avoiding phagocytosis and (ROS) killing. Moreover, the presence of bacteria in biofilms is very harmful due to the significant phenotypic variation among them, which facilitates the development of antimicrobial resistance [10]

The microbiology of chronic osteomyelitis is directly associated with the cause of the infection. In cases of hematogenous origin, oxygen-tolerant organisms, such as enteric rods and staphylococci, predominate whereas osteomyelitis attributed to previous odontogenic infections depends on the bacteria from the origin of the infectious process, typically causing mixed infections, most of them originating with oral anaerobes. *Fusobacterium*, *Porphyromonas, Prevotella, Parvimonas,* and *Eikenella* were found to be frequently associated with actinomycetes and staphylococci [2,11,12]. In a study by Gaetti-Jardim et al. [2] to evaluate the bacteria linked with osteomyelitis of the jaws in some Brazilian patients, bacterial cultivation and PCR analysis showed that the most frequently cultivated microorganisms were *Parvimonas micra, Staphylococcus aureus, Fusobacterium nucleatum*, and *actinomycetes*.

The challenge of antimicrobial resistance has been attributed to the overuse of these agents and the unavailability of newer medications related to strict regulatory requirements and decreasing financial inducements. Extensive efforts are required to decrease the rate of resistance by understanding emerging bacteria, resistance mechanisms, and antimicrobial agents [13].

Although antibiotic resistance is a natural process due to genetic mutations in the bacteria after antibiotic exposure, the process is being expedited via the overuse of prescription medications. Overuse of antibiotics causes vulnerable microorganisms to be destroyed and permits drug-resistant bacteria to emerge [14]. In contrast, bacterial impacts may enhance the healing process, in which an increase in granulation tissue development, angiogenesis, and tensile strength of the wound have been observed [7].

Alternatives to antibiotics, such as probiotics and lytic bacteriophages, can help decrease the burden of antibiotic resistance. The spread of antibiotic resistance can be contained with the rational use of antibiotics, infection control, immunization, awareness, and education among health practitioners [13].

At the proper dose, probiotics may decrease the risk of some infections and thus minimize the demand for antibiotics. Furthermore, probiotics do not contribute to the development of antibiotic resistance and could reduce the use of antibiotics [15,16].

Possible mechanisms of probiotic effect in the oral cavity may be inferred from prior investigations conducted on the gastrointestinal system. Barzegari et al [17] showed that the introduction of probiotics from microorganisms as a therapeutic measure for the management of oral and dental disease will result in direct interaction, in which a disruption of dental plaque results from biding site competition and nutritional competition. It also might have an indirect interaction that involves modulation of innate and adaptive immunity to the infectious process [18].

Lukic et al. (2017) reported multiple mechanisms of how probiotics exert their beneficial effects, including direct competition with pathogenic bacteria for nutrients and binding sites on the host cell; toxin and metabolite inactivation; production of antimicrobial substance production, which affects the growth of pathogenic microorganisms; and host immune system activation. Clinical outcomes have been demonstrated to be significantly enhanced when probiotics are used in conjunction with clinical periodontal therapy, as opposed to clinical treatment alone [18]. Moreover, probiotics inhibit the colonization of pathogenic bacteria by acting as a competitive adhesion to human tissues or medical equipment by decreasing the pH of the surrounding environment and the biofilm's biomass [17].

Probiotics primarily affect the inflammation phase, which plays a significant role in wound healing impairment. When applied topically or systematically in recent studies on humans and animals, they demonstrate a clear-cut benefit in wound healing, affecting the inflammatory response in an oxytocin-mediated fashion [19]. The probiotic *Lactobacillus reuteri*, together with mechanical therapy, produced an additional improvement over treatment with mechanical therapy alone in the general clinical parameters of patients with mucositis (bleeding on probing) and at the level of implants with mucositis (probing pocket depth) or peri-implantitis (bleeding on probing and probing pocket depth) [20]. The treatment group with the combination of scaling and root planing probiotic extracts demonstrated a significant reduction of plaque index compared to scaling and root planning (SRP) and probiotic effects individually [21]. Therefore, the use of probiotics enhanced the plaque reduction SRP caused. Probiotics and SRP alone were similarly efficacious in plaque reduction, i.e., no difference in mean plaque reduction was observed [21]. Other studies have also shown the benefits of various probiotic supplements [22–24]

This study aimed to investigate the effects of two types of probiotics on three different pathological bacteria, namely *Staphylococcus aureus, Fusobacterium nucleatum*, and *Actinomyces israeli*.

2. Materials and Methods

2.1. Bacterial strains

All bacterial strains *S. aureus* ATCC 25923*, A. Israeli* ATCC 12102 *and F. nucleatum* ATCC 25586 were obtained from The American Type Culture Collection (ATCC, USA), and samples were prepared in the College of Dentistry, Department of Botany and Microbiology, King Saud University.

Bacterial sample preparation: for aerobic bacteria, plates are kept in an incubator for 24 h at 37 °C. For anaerobic bacteria, plates are kept in a gas pack anaerobic jar for 5–6 days at 37 °C. Each bacterial strain was cultured in a Mueller Hinton broth (MHB).

2.2. Probiotics

Probiotic bacteria were obtained from generic probiotics (2 products namely: BioGaia Osfortis and BioGaia Immune Active; BioGaia, Sweden). Every product contains different strains of *Lactobacillus reuteri* (*L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 6475). Probiotics were grown in de Man, Rogosa, and Sharpe (MRS) medium, a selective medium optimized for lactic acid bacteria.

2.3. Estimating bacterial cell counts using McFarland standard

The bacterial count was standardized using the McFarland standard. In this study, the count of each bacterial suspension was adjusted to match a McFarland standard of 0.5 MFU (approximately 1.5×10^8 CFU/ml).

2.4. Preparation of bacterial suspensions

Bacterial suspensions were prepared by culturing each strain in thioglycolate broth under aerobic or anaerobic conditions as specified for each organism. After incubation, the cultures were suspended in sterile saline to obtain 200,100, 50, 30, 20, 10, 5, 2.5,1.25 and 0.625 µl of 0.5 MFU. The McFarland standardized bacterial counts were verified by subculture and viable counting and obtaining a standard calibration curve (O.D. Vs. CFU) Figure S1.

2.5. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) of each probiotic against each bacterial strain was determined by the serial dilution method. Probiotic suspensions were prepared individually in a range of concentrations (200,100, 50, 30, 20, 10, 5, 2.5, 1.25, 0.625 µl) of 0.5 McFarland standard (1.5×10^8 CFU/ml). Each pathogenic bacteria (*Staphylococcus aureus, Actinomyces israelii, Fusobacterium nucleatum*) was inoculated into brain heart infusion media (BHI) with a cell count of 10 µl of 0.5 MFU. Challenge test: Each concentration of probiotic suspension was inoculated into BHI broth and then inoculated with the standardized pathogen suspension. *Staphylococcus aureus* inoculated broth was incubated at 37°C in aerobic conditions for 24 hrs. *Actinomyces israelii* and *Fusobacterium nucleatum* inoculated broths were incubated at 37°C in anaerobic conditions for 72 hrs. After incubation, A standard 5 µl loopful from each BHI broth was streaked on Mueller Hinton agar plates.

All inoculated plates were compared with control plates with no probiotics to determine the inhibitory and bactericidal effects. Gram staining was performed on obtained bacterial samples from each plate to confirm the identity of the bacterial cells after exposure to probiotics. The MIC was determined as the minimum probiotic count concentration that inhibits the growth of most of the challenged pathogens. The inhibitory effect could be confirmed by microscopic investigation of the Gram-stained smears. The MBC was determined as the minimum probiotic count concentration that prevents the growth of the challenged pathogens and investigated under the optical microscope. Additionally, the presence or absence of bacterial colonies was recorded to determine the MBC of each strain.

2.6. Ethical approval

The study was conducted in accordance with King Saud University's ethical standards and protocols after obtaining institutional review board (IRB) approval and the College of Dentistry Research Center (CDRC) 's approval.

2.7. Statistical analysis

Measurement data were collected and tabulated in Excel 2022 (Microsoft, Redmond, USA). GraphPad Prism version 9.4.1 (681) (GraphPad Software Inc, San Diego, USA) was used for all graphical representations. SPSS version 24 (IBM, Armonk, USA) statistical package was used for all statistical data analysis.

3. Results

3.1. Estimating bacterial cell counts using McFarland standard

A calibration curve for bacterial cell counts versus O.D. was conducted for each strain and then matched with the standard 0.5 McFarland. The standardized bacterial counts were used for determination of MIC and MBC.

3.2. Determination of MIC & MBC of Probiotics on *Fusobacterium Nucleatum*

The bacterial growth was significantly reduced using the lowest bacterial count concentration (0.625µl) of both tested probiotics strains *Lactobacillus reuteri* (*L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 6475). As the lowest concentration significantly inhibits the growth of *Fusobacterium Nucleatum*, we consider it the MIC for both strains, Figure 2. The growth inhibition was confirmed using microscopic investigation of Gram-stained samples and subcultures growth (Figure 2S, Table S1). The next larger bacterial count concentration (1.25 µl) of both probiotic strains eradicated all coexisting *Fusobacterium nucleatum* cells. Accordingly, we consider it MBC,

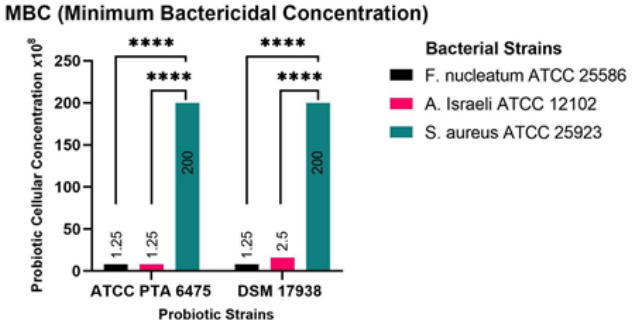

Fig. 1. Minimum bactericidal concentration (MBC) of both probiotic strains (*Lactobacillus reuteri* DSM 17938 and ATCC PTA 6475 against three oral pathogens *Fusobacterium nucleatum*, *Actinomyces israelii*, and *Staphylococcus aureus*.

Figure 1.

3.3. Determination of MIC & MBC of probiotics on *Actinomyces israelii*

L. reuteri DSM 17938 probiotic strain showed inhibitory effect on *Actinomyces israelii* growth on bacterial count concentration of 1.25µl (MIC), and full eradication on 2.5 µl (MBC), Figure 1&2. The other probiotic strain *L. reuteri* ATCC PTA 6475 showed lower MIC that was 0.625 µl and lower MBC that was 1.25 µl, Figure 1. All results were confirmed by microscopic investigation and subculture results (Figure 3S, Table 2S).

3.4. Determination of MIC & MBC of Probiotics on *Staphylococcus aureus*

Staphylococcus aureus showed higher resistance against all tested probiotic strains. For both probiotic strains, there was an inhibitory effect at 100 µl (MIC) and full eradication at 200 µl (MBC), Figure 1&2. All results were confirmed by microscopic investigation and subculture results (Figure 4S, Table 3S).

4. Discussion

Osteomyelitis of the jaws is a challenging health burden. Its prevalence is higher in the developing countries [25]. The key virulence factors are biofilm formation by some opportunistic floral bacteria [26], poor oral hygiene, previous dental extraction, trauma, and compromised blood supply [27]. Systemic comorbidity also can be attributed to jaws osteomyelitis for instance diabetes mellitus, immunosuppression, osteoporosis [27], and COVID-19 [28]. The scarce epidemiological studies of this condition acquire the need for further research in this area. More studies are needed to explore advanced therapeutic and diagnostic strategies [29]. Probiotics play a crucial role in oral health by competing with oral pathogens, changing oral pH, and reducing inflammation [30]. In this study, we evaluated the effectiveness of two probiotics at various concentrations on the growth of three pathogenic bacteria: *Staphylococcus aureus, Fusobacterium nucleatum*, and *Actinomyces Israelii*.

In our study, the results demonstrate large difference in the sensitivity of the tested bacteria to probiotic treatment. Both Fusobacterium and Actinomyces showed significant susceptibility to the probiotics at all tested concentrations. The bacterial count concentration (625 µl) of *L. reuteri* ATCC PTA 6475 which represents approximately 9.375 * 10^4 CFU was able to inhibit *Actinomyces israelii* in concentration of 10 µl which represents approximately 1.5 * 10^6 CFU, and the concentration of 1.25µl (1.87500 * 10^4 CFU) was able to kill the same number of the pathogen. The same previous concentration of *L. reuteri* DSM 17938 was able to inhibit also the same number of pathogen cells, while 2.5µl (3.75* 10^5 CFU) was able to kill the same counts of the pathogen. These potent probiotic concentrations suggest the preventive power of the probiotic against *Actinomyces israelii* and potential adjunctive therapy. Nine lactobacillus species showed significant antagonistic activity against different pathogens: namely *Staphylococcus epidermidis*, *Staphylococcus epidermidis*, *Enterobacter cloacae*, *Listeria monocytogenes*, *Helicobacter pylori*, *Klebsiella pneumonia*, and *Escherichia coli*. The antagonistic activity varied according to the challenging strain. Bacteriocins, hydrogen peroxide, and organic acids

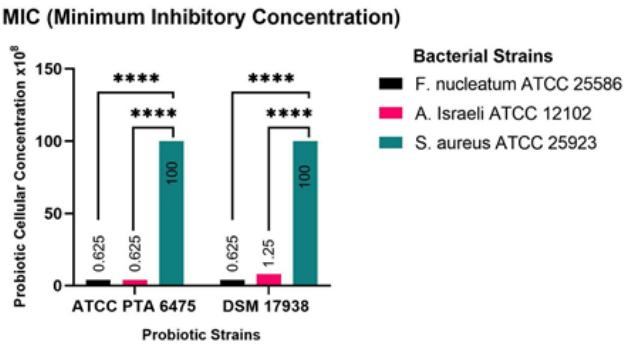

Fig. 2. Minimum inhibitory concentration (MIC) of both probiotic strains (*Lactobacillus reuteri* DSM 17938 and ATCC PTA 6475 against three oral pathogens *Fusobacterium nucleatum*, *Actinomyces israelii*, and *Staphylococcus aureus*.

[31] were detected in the probiotic's media supernatant and were correlated to the competitive activity [32]. Five strains of lactobacillus spp. showed the minimum inhibitory concentration at 10^8 CFU/mL against carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* after 24 hr. incubation. *Escherichia coli* was totally eradicated after 48 hr. incubation at all tested concentrations of lactobacillus spp. according to kill-time test [33]. In a dynamic oral in-vitro model, bacteriocin producing probiotics (*S. salivarius* K12 and *S. salivarius* M18, alone or combined) antagonize the growth of *Streptococcus mutans*. Both biofilm and growth of this oral opportunistic flora were inhibited when they were co-cultivated for 48 hr. The growth and biofilm formation was measured by counting CFU and scanning electron microscope, respectively [31].

In this study, both probiotic strains inhibited the growth of the anaerobic oral pathogen *Fusobacterium nucleatum* at the lowest used concentration (0.625 µl/mL). They eradicate the pathogen at bacterial count concentration of 1.25 µl/mL. In an in-vitro study with mimic to human saliva media, *Lactobacillus reuteri* DSM 17938 showed significant antibiofilm activity against *Prevotella intermedia* and *Fusobacterium nucleatum*. The bacterial suspension concentration was 10^8 of the probiotics. The antibiofilm activity varied according to the pH degrees (4.5 to 7). The lower pH augmented the probiotic antibiofilm activity [34]. The heat-killed probiotic Lactobacillus acidophilus was able to prevent biofilm formation *Fusobacterium nucleatum* on the oral epithelium. The suggested mechanism was the co-aggregation and further suppression of virulence gene *fap*-2. The suppression of proinflammatory cytokines release in the oral epithelium was also recognized [35]. *Lactobacillus reuteri* AN417 is recently identified probiotic with antibacterial activity against some oral pathogens. The free-cell lactobacillus culture supernatant MIC was 20% v/v against *Fusobacterium nucleatum*. Although LR AN417 strain lacked reuterin, reuteran, and reutericyclin encoding genes, they showed antibacterial activity. The reduction of its antibacterial activity was recognized with α-amylase and lipase suggesting the potential activity of carbohydrates and fatty acids metabolites [36].

Staphylococcus aureus showed resistance to the probiotics at the previous low concentrations. The MIC and MBC of both lactobacillus strains were 100 and 200 µl/mL, respectively. This suggests that intrinsic factors confer a higher resistance to probiotic antibacterial and

potential antibiofilm formation. The inherited capsule or slime layer, lytic enzymes, and intercellular wall efflux pumps might be responsible for such resistance [37]. Various probiotics, namely, *L. acidophilus*, *L. casei*, and *L. plantarum* exhibited in-vitro synergistic activity against multi-drug-resistant staphylococci, including numerous clinical MRSA isolates [38]. The supernatant of their cultures (*Lactobacillus fermentum*, *Bifidobacterium longum*, and *Bifidobacterium animalis* subsp. lactis) showed antibacterial activity and biofilm-disturbing ability and suggested production of potentially bioactive molecules [39]. Another study emphasized the effect of produced lactic acid from two probiotics, *L. plantarum* K.F. and *L. casei* Y1. The significant amount of produced acid contributed to the MRSA growth inhibition, as well as the biofilm building [40]. Investigation of antimicrobial activity of 77 probiotic strains obtained from some pharmaceutical preparations, and homemade probiotic products resulted in exploring variable antibacterial activity against clinical and standard *Staphylococcus aureus* isolates. Lactic acid was the suggested key active constituent [41].

The difference in susceptibility to probiotics suggests variable mechanisms of antibacterial, antibiofilm actions, and sensitivity of the investigated pathogens to probiotics. However, for Staphylococcus, higher probiotic concentrations or perhaps the use of synergistic combinations with other antimicrobial agents may be necessary to achieve a comparable inhibitory effect. These findings contribute to previous understanding of the potential use of probiotics as a therapeutic intervention against specific pathogens [42]. They highlight the importance of considering bacterial species-specific responses when formulating probiotic-based treatments. For *Fusobacterium nucleatum* and *Actinomyces Israelii*, the probiotics at lower concentrations will be sufficient to achieve a therapeutic effect, potentially reducing the risk of adverse effects and maintaining beneficial microbiota balance.

There are several requirements for being a prospective probiotic: survival in low pH and enzyme-rich environments, adhesion to the epithelium for host-probiotic interaction, competition with pathogenic microbes, and, most importantly, safety of *L. reuteri* [43]. Reuterin, which is a mixture of different forms of 3-hydroxypropionaldehyde (3-HPA), is a well-known antibacterial molecule that can be produced and excreted by the majority of L. reuteri strains. In addition to reuterin, strains of *L. reuteri* also generated organic acid and H_2O_2 with profound antibacterial activity [44]. *S. mutans* growth was totally suppressed by the acid product of *L. reuteri*, whereas *S. mutans* growth was at least 46% inhibited by *L. reuteri's* H_2O_2 and bacteriocin-like compound (BLC) [44,45].

In this study, more investigations are needed to explore the bioactive components that manifest the antibacterial activities of the tested probiotics. More probiotic strains should be tested, especially the traditionally used ones and naturally present in our nutrition. Preclinical and clinical trial conductions must be accomplished to translate this work into clinical practice. Increasing the healthcare workers, specifically dentist's awareness and perception of probiotics preventive and curative roles is highly recommended.

The antibacterial activity of both probiotic strains *L. reuteri* ATCC PTA 6475 and DSM 17938 showed relatively high potency against *Fusobacterium nucleatum* and *Actinomyces Israelii*. These two pathogens have a crucial role in jaw osteomyelitis and oral periodontitis. The two probiotics exhibited relatively weak antibacterial activity against *Staphylococcus aureus*. Our findings enlighten the potential role in the treatment and protective strategies against jaw osteomyelitis and oral periodontitis. The protective measures utilizing the probiotic administration are important and recommended.

Conflict of interest

The authors declare no conflict of interest for this manuscript.

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Ethical approval

This project got IRB committee approval. (NO. 24/1222/IRB)

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