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ORIGINAL ARTICLE

Pseudomonas clearance from the oropharyngeal cavity and healing of mouth ulcers using a selenium-enriched *Lactobacillus brevis* Lse mouthwash: a randomized, double-blind, controlled clinical trial

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ABSTRACT

Background and Objectives: A selenium (Se) enriched *Lactobacillus brevis* was applied as mouthwash to relieve mouth ulcers and regulate oral pathogens. The persistence of four frequent oral pathogens (*Pseudomonas*, *Klebsiella*, *Proteus*, and *Enterobacter*) and the quantity of mouth lesions were to be measured after exposure to the above probiotic. Susceptibility of the pathogens was also examined.

Materials and Methods: In this randomized double-blind controlled clinical trial, 60 ICU patients were allocated into three groups equally (to receive normal saline, chlorhexidine, and Se-probiotic) every 6 hours for 1 week. The utilized *Lactobacillus brevis* was previously separated from a traditional dairy product and its enrichment was accomplished through streaking on MRS plates supplemented with sodium selenite.

Results: *Klebsiella* and *Pseudomonas* were the most frequent initial pathogens, from which *Pseudomonas* were totally omitted from the probiotic group. The Se-probiotic existed in the mouth cavity of the test group six hours after the last dose. In

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the probiotic group, the number of oral lesions were significantly reduced.

Conclusion: Clearance of *Pseudomonas* from mouth cavity and significant decrease in the quantity of mouth ulcers are considerable achievements. Hence, the applied Se-supplemented mouthwash exhibited promising capability to be considered as a potential oral hygiene formulation.

Keywords: Selenium; Probiotics; *Lactobacillus*; Ulcer; *Pseudomonas*

INTRODUCTION

The oropharyngeal space is considered the prime entryway of the body, through which food and air are taken in. It is also a suitable site for colonization by a wide range of microorganisms, either benign or pathogenic. From this cavity, microorganisms may migrate to other parts of the body, most frequently the respiratory and gastrointestinal tract (1). Consequently, it is reasonable to implement measures to regulate the microbiome of the oropharyngeal cavity.

The mouth of patients admitted to the intensive care unit (ICU) represents a pool of respiratory pathogens associated with nosocomial pneumonia (2). In comparison to normal population, obviously the mouth microbial flora of ICU patients differs greatly with the healthy individuals (3). Four microbial pathogens are frequent in the oropharyngeal cavity of patients admitted to ICU wards. These include *Pseudomonas*, *Klebsiella*, *Enterobacter*, and *Proteus* (4). Some of these pathogens are able to produce biofilms, mechanical disruption of which (i.e., brushing) may result in dislocation to the posterior oropharynx and causing pneumonia. Mouth washing with antiseptic solutions such as chlorhexidine is, therefore, an effective alternative (5). However, there are reports that chlorhexidine usage may bring about adverse effects such as xerostomia, pain and numbness in the mouth and tongue, and teeth discoloration (6).

Mouthwashes are aqueous formulas widely found on the market, used for various purposes, including plaque control and antiseptic effects (7). Literature is rich in reports addressing the usefulness of mouthwashes in attenuating oral pathogens and improving oral health (8). In this sense, it is the active ingredients of mouthwashes that exhibit the pharmacological effect. Hence, current commercial mouthwashes contain various agents like chlorhexidine, thymol, benzalkonium chloride, and hydrogen peroxide which are known for their antibacterial features (7). The inclusion of probiotics in mouth rinse products is a recent approach in oral healthcare, leveraging the beneficial properties of bioactive microorganisms (9-12). This provides an alternative attempt in oral healthcare that helps avoid adverse effects of chemical mouthwashes, the superiority of which is endorsed in the literature (13, 14). Notably, a decrease in the quantity of mutans streptococci in saliva or dental plaque has been attributed to the adequate use of probiotics (15). One feature that enables oral probiotics to exhibit their bio-efficacy is their ability to adhere to the buccal surface and stay durably active for a reasonable period. This characteristic has been reported for Lactobacilli (10).

Besides, oral ulcers are usual complications of ICU patients due to difficulty in managing their oral hygiene, their medications, and interventions (16). Here again, the application of mouthwashes has been practiced as an effective procedure.

We recently reported a successful trial of Selenium-Enriched *Lactobacillus brevis* LSe probiotic (17) in bedsore healing of ICU patients (18). This motivated us to evaluate the clinical relevance of a mouthwash containing our probiotic with the management of the oropharyngeal pathogen microflora and mouth ulcer healing.

MATERIALS AND METHODS

Trial design and ethics statements. This randomized, double-blind, controlled clinical trial was approved by the ethics committee of Kerman University of Medical Sciences (IR.KMU.REC.1401.529), Kerman, Iran. It was then submitted to the Iranian website for registration of clinical trials (IRCT20221221056887N1). The trial was executed in the ICU wards of Bahonar Medical Center, Kerman, Iran. Signed informed consent declaring that all personal details will be kept private and not published was obtained in all cases. This project was financed via grant No. 401000681 by Kerman University of Medical Science, Kerman, Iran.

Trial registration. IRCT, IRCT20221221056887N1. Registered 6 March 2023, https://irct.behdasht.gov.ir/trial/68726.

Probiotic preparation. *Lactobacillus brevis* Lse was previously isolated and characterized from a traditional dairy product in a western city of Kerman, Iran (17). Morphological and biochemical identification, followed by 16S rDNA sequencing and NCBI-BLAST comparison (19) against the NCBI GenBank accession No. JX317077, was repeated to confirm the strain identity with the reference strain. The bacterium was cultured on MRS agar (Merck, Germany) overnight, and loopfuls were streaked onto MRS plates supplemented with sodium selenite (Cat. No. S5261, Sigma-Aldrich, USA), then incubated anaerobically at 37°C for 48 h (17). Probiotic suspensions were freshly prepared under aseptic conditions by suspending bacterial colonies from MRS agar cultures for daily use (18).

Selenium content. Similar to our previous report (18), the selenium content of the bacterial suspension was measured by Zarazma Company, Tehran, Iran. Briefly, a 15 mL Se-enriched *Lactobacillus* suspension (3 McFarland) was autoclaved and sent to the above company to be measured by Inductively Coupled Plasma Mass Spectrometry (ICP MS or ICP mass).

Study design and sample size. The ICU wards where the study was executed belonged to a main trauma center in the region and therefore the main reason for admissions was trauma. According to the criteria presented in (Table 1), 60 patients were randomly selected and divided into three groups (to receive normal saline, chlorhexidine, and Se-probiotic) based on the block randomization with the blinding process.

Each trial group included 20 patients (60 in total) who received normal saline (30 ml), chlorhexidine 0.12% (30 ml), or the Se-probiotic suspension (30 ml, 10^9 CFU/ml) every 6 hours for 1 week. To administer this, each patient's mouth cavity was rinsed with the desired mouthwash for 30 seconds and suctioned (6, 20-22). A throat swab of each patient was examined at the beginning and the end of the trial (6 hours after the last mouthwash) for the presence of the four known pathogens (*Pseudomonas*, *Klebsiella*, *Enterobacter*, and *Proteus*) via plate culture, followed by antibiogram analysis against a selection of antibiotics. In addition, the existence of mouth ulcers at commencement and their fate at the end of the trial was investigated.

Data collection. Medical documents from the ICU wards were reviewed for available data. Other ward routine data were obtained from the hospital laboratory and recorded. Cut-off points and relevance of data included: age [≥65 years], body mass index (BMI) [≥25], albumin (Alb) [<3.4 g/dL], gender, past medical history (diabetes mellitus, DM), and smoking (23). Other parameters, including white blood cell count (WBC), hemoglobin (Hb), platelet count (Plt), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and hospitalization period [mean length of hospital stay], were likewise measured (18). Throat culture results and mouth ulcer quantities were recorded at the beginning and the end.

Statistical methods. Data were analyzed using GraphPad Prism 8 (GraphPad Software, USA). A minimum sample size of 17.4 participants per group was calculated based on a previous study's mean and standard deviation, with $\alpha = 0.05$ and a 95% confidence interval. Including 20 participants per group aligns with this calculation, ensuring adequate statistical power. Figures were generated using the same software. The significance level was set at $P < 0.05$. Initially, all data were evaluated for normality using the Kolmogorov–Smirnov test. Results were expressed as mean ± SE for those distributed normally ($P > 0.05$). Unpaired t-tests and chi-square tests were carried out to compare the control and case groups. A two-way ANOVA was conducted to assess the effect of confounding factors, followed by Tukey's post hoc test for group comparisons. Results that failed normality distribution ($p < 0.05$ in the Kolmogorov–Smirnov test) were reported as median and interquartile range (scatter dot plots or box-and-whisker plots expressed as median [interquartile range]). This information was analyzed using a Mann–Whitney U test (18).

RESULTS

Strain authentication. Morphological identifications and biochemical assays confirmed the strain as a rod-shaped Gram-positive bacillus belonging to the *Lactobacillus* family. Further, the BLAST comparison of the obtained sequence of the probiotic's 16S rDNA gene to the GenBank accession No. JX317077 established the strain with 100% identity (17).

Table 1. The trial inclusion and exclusion criteria

Inclusion criteria	Above 18 years old
	Not receiving oral antibiotics or other probiotics
	Not having sepsis, other active infections, or known immunodeficiency
	Not pregnant or lactating
	Hospitalized in the ICU wards of Bahonar Hospital, Kerman, Iran
Exclusion criteria	Patient exiting from the ICU wards and anesthesia service of Bahonar Hospital (discharge for any reason or death)

Se content. ICP mass analyses indicated that each portion of 10^9 cells of the probiotic encompasses 23.1×10^{-8} gr Se (equal to 231 ng Se).

Demographic analyses. The demographic parameters (age, gender, BMI, and hospitalization period) of the three groups were analysed and summarized in (Table 2). The age range of the whole population was 21-86 years, there were 34 males and 26 females, the BMI ranged from 18 to 29, and the pre-trial hospitalization period (days hospitalized at the start of the clinical trial) spanned from 5 to 42 days. None of the above results was found to be significantly different.

Biochemical parameters. Table 3 shows the laboratory variables, including CRP, ESR, WBC, Hb, Plt, and Alb. No significant difference was observed between the groups in these results.

Throat culture. The quantitative results of the throat cultures are tabulated in Table 4, and the antibiogram data are shown in Table 5. While all four pathogens were frequent in the samples, klebsiella was the most dominantly detected pathogen. In addition, both *Klebsiella* and *Proteus* were found to be resistant to all of the seven applied antibiotic disks, whereas the *Pseudomonas* and *Enterobacter* showed sensitivity to some antibiotics. Nonetheless, the antibiogram patterns for all pathogens were consistent throughout the study. The Se-probiotic omitted *Pseudomonas* from throat cultures in the Se-probiotic group (P-value = 0.0133), whereas insignificant increases in *Pseudomonas* incidence were seen in the members of the other two groups, (Fig. 1). The applied probiotic (*L. brevis* Lse) was recovered from the throat cultures of all Se-probiotic patients at the end of the study.

Mouth ulcer and factors. At the start of the trial, mouth ulcers were present in 25 of 60 patients (41.7%), divided among normal saline (40%), chlorhexidine (45%), and Se-probiotic (4%). A significant reduction was seen in the mouth ulcer cases in the Se-probiotic group compared to the other two ($p = 0.0051$; Fig. 2 and Table 6). No significant correlation was seen between demographic, laboratory, or lifestyle factors and mouth ulcer healing (Table 7).

DISCUSSION

In this study, the edible *L. brevis* LSe was initially enriched with selenium via growing on Se-supplemented MRS agar, accumulating 231 nanograms of Se in roughly 10^9-cell portions. In the clinical phase, three groups of 20 patients each received normal saline, chlorhexidine, or Se-probiotic suspension as a 30-second mouthwash every 6 hours for one week. Mouth ulcers and laboratory parameters were monitored as the outcome.

Efficient oral hygiene maintenance by washing or brushing is key to reducing the hazard of ventilator-associated pneumonia (VAP) in ICU patients and has been found to delay the onset of VAP (23). Of the four renowned oral pathogens frequently found in the buccal cavity (23), *Pseudomonas* was omitted entirely from throat cultures in the Se-probiotic group despite their slight initial majority. This pathogen, however, showed insignificant increases in the other two trial groups (Fig. 1A). On the other hand, no significant difference was observed in the frequency of the other three microbes (*Klebsiella*, *Enterobacter*, and *Proteus*) in all three trial groups (Figs. 1B-D). Recovery of *L. brevis* Lse from all of the Se-probiotic group clearly indicates its ability to stay viable in the buccal cavity at least over a dose interval period (six hours), possibly via adhesion to surfaces.

While *Pseudomonas* spp. are well-known causes of mortality and morbidity in general (24), they are

Table 2. Patient demographic parameters

Parameters	Normal saline	Chlorhexidine	Se-probiotic
Number	20	20	20
Age (years) (mean ± SE)	50.8 ± 3.09	55.05 ± 3.83	54.35 ± 3.91
Gender (male%)	50%	60%	60%
BMI (mean ± SE)	22.78 ± 0.59	22.48 ± 0.55	22 ± 0.36
Hospitalization period (days) (mean ± SE)	16.85 ± 2.01	15.5 ± 2.71	16.35 ± 1.54

Table 3. Laboratory parameters

Parameters	Normal saline	Chlorhexidine	Se-probiotic
WBC (mean ± SE)	10258 ± 517.5	12205 ± 75	10260 ± 550
Hb (g/dl) (mean ± SE)	10.99 ± 0.23	11.01 ± 0.13	10.38 ± 0.13
Plt (mean ± SE)	252925 ± 4825	238200 ± 4200	233650 ± 1700
Alb (g/dl) (mean ± SE)	2.73 ± 0.09	2.96 ± 0.12	2.83 ± 0.11
ESR (positive)	2 patients	1 patient	2 patients
CRP (positive)	2 patients	1 patient	2 patients

Table 4. Throat culture results

Bacterial types	Stage	Normal saline (Number of patients)	Chlorhexidine (Number of patients)	Se-probiotic (Number of patients)
Klebsiella	First	16	15	19
	Last	17	19	18
Pseudomonas	First	4	4	6
	Last	7	5	0
Enterobacter	First	3	2	2
	Last	2	2	1
Proteus	First	0	1	0
	Last	1	1	1
L. brevis Lse	First	0	0	0
	Last	0	0	20

Table 5. Antibiotic susceptibility of detected mouth pathogens.

Bacterial types	Antibiotic-resistance
Pseudomonas	cefotaxime, co-trimaxazol
Klebsiella	ciprofloxacin, amikacin, co-trimaxazol, meropenem, cefepime, cefotaxime, ampicillin-sulbactam
Enterobacter	ciprofloxacin, co-trimaxazol, ampicillin-sulbactam
Proteus	ciprofloxacin, amikacin, co-trimaxazol, meropenem, cefepime, cefotaxime, ampicillin-sulbactam

famous cariogenic and biofilm-producing bacteria in the oral cavity (25, 26). Consequently, the clearance of pseudomonades from the oropharyngeal samples highlights a considerable achievement. However, future trials with expanded patient population would lead to a stronger judgement.

It is well-accepted that the inclusion of antibacterial agents in mouth products can reduce the population of oral bacteria non-specifically, let alone increase the risk of antibiotic resistance. This fact favours the application of probiotics as benign bacteria able to enhance a healthy equilibrium of the mouth microor-

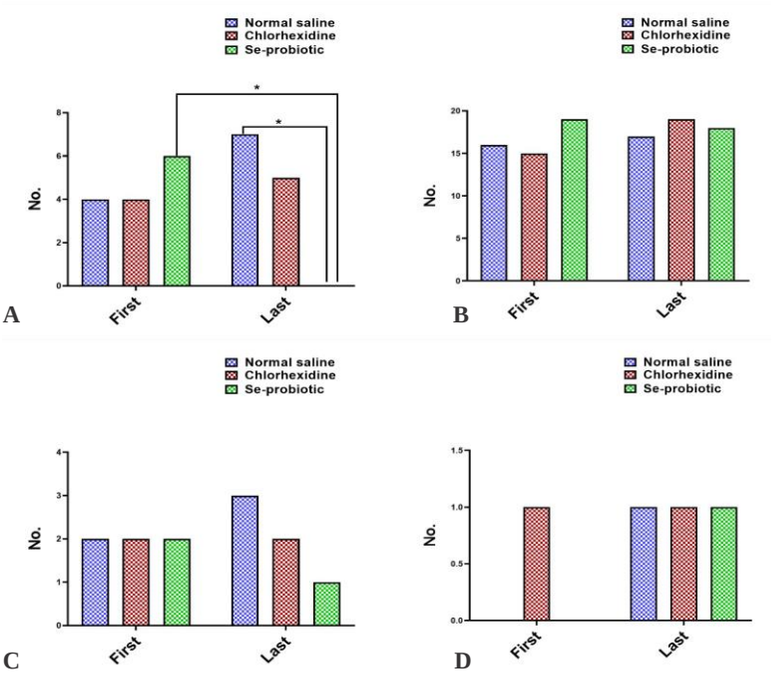

Fig. 1. Comparison of throat culture results at study onset and conclusion. Histograms show the number of patients positive for each bacterium. (*: p-value <0.05). A: *Pseudomonas*; B: *Klebsiella*; C: *Enterobacter*; D: *Proteus*

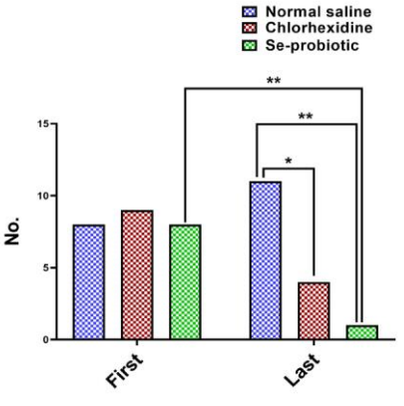

Fig. 2. Comparison of mouth ulcer results at study onset and conclusion. Histograms show the number of patients affected. (*: p-value <0.05, **: p-value <0.01)

ganisms (27, 28). In addition, probiotic mouth products have been highlighted as alternative bio-remedies to overcome multi-drug resistance (29). Mechanistically, probiotic lactobacilli have been found to inhibit other bacteria's adhesion to oral surfaces by alteration of the salivary proteins (30). In line with this, Marcos et al. reported that *Bifidobacterium* probiotics decreased the probing depth and improved clinical attachment significantly and meaningfully reduced the pathogens of periodontal importance as a measure to treat chronic periodontitis (31). In another study, patients who received a probiotic mouthwash benefitted from a significant decline in plaque and gingival indices, as well as oral bacterial counts (32). Utilization of biogenic selenium nanoparticles in the combat against pathogens is well documented. For instance, Selenium nanoparticles synthesized by the probiotic *L. acidophilus* have shown strong antibacterial properties against drug-resistant bacteria and have been proposed to degrade bacterial biofilm (33). Based upon the above findings, using a selenium-supplemented probiotic in the present trial is a highlight of the current article.

Considering the second main objective of this project, the prevalence of end-of-study mouth ulcers was significantly declined in the Se-probiotic group. In addition, a considerable though insignificant decrease in the number of mouth ulcers was observed in the chlorhexidine group. Comparing the three trial groups, reductions of end-of-study mouth ulcers in the chlorhexidine and Se-probiotic groups were significantly varied from the normal saline group ($p<0.05$ and $p<0.01$, respectively) (Fig. 2).

Oral ulcers are usual but challenging complications of ICU patients due to limitations in oral hygiene management and necessary interventions.

Table 6. Comparison of the mouth ulcer results at the beginning and the end of the study. Table represent the number of patients with mouth ulcers.

Groups	Normal saline (Number of mouth ulcers)	Chlorhexidine (Number of mouth ulcers)	Se-probiotic (Number of mouth ulcers)
First	8	9	8
Last	11	4	1

Table 7. Patient demographic, clinical, and preclinical factors versus mouth ulcer

Factors		Normal saline	Chlorhexidine	Se-probiotic
Age (years)	>65	1	2	1
	65≥	10	2	0
Gender	Male	4	1	0
	Female	7	3	1
BMI	≥25	3	1	0
	<25	8	3	1
Hospitalization period (days)	≥17	5	2	0
	<17	6	2	1
Albumin (g/dl)	≥3.4	2	1	1
	<3.4	9	3	0
Diabetes Mellitus (DM)	DM	3	1	0
	No-DM	8	3	1
Smoking	Smoker	2	1	0
	Non-Smoker	9	3	1

To address this challenge, chlorhexidine solutions have been used along with tooth brushing (16). Acknowledging chlorhexidine effectiveness, its adverse effects should not be ignored (6). In addition, tooth brushing is also suspected of disruption of biofilms and inducing nosocomial pneumonia (5). These point to the advantages of probiotic mouthwash as a potential alternative.

This project follows upon our previous study (18), where we reported that the same probiotic taken Per Os considerably reduced the bedsore healing period. Formulated in an oral rinse applied three times a day for one week, *L. reuteri* exhibited potency to notably decrease the size of aphthous stomatitis and severity of pain (34). Another study reports the oral application of a liquid probiotic formulation containing a soil-dwelling *Bacillus* as an auxiliary treatment for aphthous ulcers, significantly improving symptoms (35). Dou X et al. conducted a robust study probing the bioactivity of *L. rhamnosus* entrapped in hydrogel patches to enhance oral ulcers' healing. They correlated the effectiveness of the applied probiotic to its antimicrobial capabilities as well as regulating of deposition of collagen fiber, induction of cell migration, epithelial reconstruction, and angioneogenesis. Their finding well supports the efficacy of probiotics´ promising properties in managing oral medical conditions (36).

CONCLUSION

Mouthwashes and mouth rinses are routine oral hygiene products nowadays, in addition to flossing and brushing (37). Besides, the utilization of probiotics signifies an innovative approach to improving oral health and combating harmful microbes by employing edible bacteria. Dental products infused with probiotics enjoy superior features due to augmented bioactive and safe microorganisms and attire excellent customer interest (38). It is considered to have notable effectiveness in improving two interrelated oral conditions, namely oral microbiome and mouth ulcers, which strongly affect patient nutrition

and general well-being. The present study introduces such a beneficial product that deserves to appear in the market and is subject to further clinical and formulation studies. Innovative formulations such as toothpaste, chewing gums, buccal patches, lozenges, etc., containing this probiotic and adding anti-halitosis flavoring agents could also be suggestible ideas for a larger spectrum of applications.

Limitations. 1. It was not possible to follow up the patients after the ICU discharge.

2. The research team observed that the probiotic group patients exhibited apparent senses of halitosis, with no known adverse health effect. However, investigation of the nature of this side effect was beyond this project.

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