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In Vitro Comparison of Four Listerine Mouthwash Formulations on the Inhibition of *Lactobacillus reuteri DSM 17938* and Human Oral Microbiota

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Abstract

Listerine mouthwashes are the most widely used over-the-counter and common daily supplies for people to maintain oral health and hygiene. However, few study reveals the differences between types of mouthwash and compares the influence they each might bring to oral probiotics and general oral bacteria. In this experiment, *Lactobacillus reuteri DSM17938* and human saliva samples of different concentrations are set and each cultivated to represent significant oral probiotics and potential bacteria in human saliva, including pathogens, for further comparison. Mouthwashes are added onto agar plates and into liquid saliva samples to reflect differential inhibitory effects possibly due to variations in compositions. Results from gar plate diffusion assay have shown that cosmetic mouthwash is the most effective in inhibiting both *Lactobacillus reuteri DSM17938* and oral bacteria's growth, indicating both an advantages and risk of the application of cosmetic mouthwash. While liquid culture analysis suggest that alcohol mouthwashes have the most immediate antibacterial effects but tend to be a short-term treatment for inhibition and alleviation, lacking the ability for complete, long-term solution.

Keywords

Oral Bacteria, Probiotic, Listerine Mouthwashes, Antibacterial Effects

1. Introduction

This study aims to investigate and compare the effect of four different types of Listerine mouthwashes on the growth of different strains of bacteria, including *Lactobacillus reuteri DSM17938,* an oral probiotic that is able to avoid black stains

in children and young adults, and possible bacteria existing in saliva samples obtained from six random volunteers.

Oral diseases are widely spread among humans, with 3.5 billion people being e22stimated to suffer from such diseases [1]. Common oral diseases include dental caries (tooth decay), periodontal diseases, dental plaque, oral cancers and so on. Dental caries, dental plaque, periodontal diseases are caused mainly due to the existence of bacteria [1]. Dental caries, as an example, is caused by bacteria, especially strains of streptococci and lactobacilli, dimineralizing tooth enamel by secreting acid [2]. Dietary carbohydrates are being converted into acid through the metabolism of bacteria, which further generates an acidic environment (low pH) that is adverse to the growth of strains related to health maintenance of the enamel [3].

To address oral diseases and maintain oral health, mouthwashes are widely utilized for oral hygiene, with Listerine being a prominent commercial brand [4]. Listerine mouthwashes are found to possess broad-spectrum antibacterial activity and can effectively kill bacteria within 30 seconds [5]. It has been classified into multiple types, each being composed of different ingredients, such as essential oils, fluoride compounds, hydrogen peroxide etc. [6] [7]. It is proven that these ingredients do have inhibitory effect on bacterial growth [6]-[8]. Essential oils, one of the basic component of most mouthwashes, are able to disrupt bacterial enzymes responsible for energy production at low concentrations, and at higher concentrations, causing proteins to denature [9]. Fluoride compounds show obvious inhibitory effects against plaque bacteria. Effect of fluoride compounds targets on weakening acidity of oral cavity. By increasing the permeability of bacterial membrane towards protons and damaging F-ATPases' function of proton transport, fluoride compounds further induce the acidifying of bacterial cytoplasm and acid inhibition of glycolytic enzymes [10]. Generally, despite differences in the composition of mouthwashes, most mouthwashes aim at decomposing bacterial cell wall and further kill bacteria [11].

However, the effects of these ingredients and mechanisms are not targeted specifically on pathogenic bacteria [6]. Previous study has also justify that mouthwashes may cause dysbacteriosis, where certain types or groups of bacteria, possibly including both pathogens and probiotics, are being killed, and the survived microbes including bacteria, fungi etc. dominates human oral cavity, leading to a decrease in diversity [6]. As a result, while mouthwashes are performing their functions to inhibit the growth of pathogens, there is a possibility that patients will also suffer from a loss of beneficial bacteria.

In this experiment, *Lactobacillus reuteri DSM17938,* an oral probiotic known for its ability to improve intestine-related illnesses and properties, including enhancing gut barrier, reduce infantile colic, improve gut peristalsis and so on is used to evaluate the effect of mouthwashes on oral probiotic [12]-[14]. It is found that *Lactobacillus reuteri DSM17938* can inhibit the colonization of other pathogenic microbes within a certain area by the production of its by product such as reuterin, a type of antibiotic that is able to inhibit the growth of both gram positive

and negative microbes (bacteria, yeast, fungi), which plays an significant role in promoting oral health and preventing diseases such as dental plaque [15]. Such properties of *Lactobacillus reuteri DSM17938* also make it an promising method to treat periodontal diseases [15].

This study aims to investigate whether four types of commercial mouthwashes (fluoride, original, antiseptic, alcohol) [16]-[19] inhibit the growth of *Lactobacillus reuteri DSM17938*, a probiotic critical for oral micro-biome balance and oral health, and general bacteria in saliva samples, assessing the duration of antimicrobial effects to evaluate risks to oral microbiome balance, and further provide comparison between effects of probiotic and possible pathogens.

2. Materials and Methods

All procedures are conducted in aseptic conditions in the clean bench, maintained by regular ultraviolet disinfection, alcohol sterilization, autoclaving, sterilized equipment etc.

2.1. Biological Materials

Lactobacillus reuteri DSM17938 lyophilized powder (1×10^9 CFU/g, JINGBIO).

MRS agar plates (REBIO).

Blood agar plates (REBIO).

Fluoride mouthwash: LISTERINE® TOTAL CARE Anticavity Fluoride Mouthwash FRESH MINT.

Antiseptic mouthwash: LISTERINE® Antiseptic Oral Care Mouthwash ORIGINAL.

Alcohol mouthwash: LISTERINE® Antiseptic Mouthwash COOL MINT®.

Cosmetic mouthwash: LISTERINE® Alcohol-Free Mouthwash COOL MINT®.

2.2. Preparation

2.2.1. Equipment

Autoclaving for the equipment before the experiment, in a condition of 121˚C for 15 minutes, including petri dishes, shaking flask, test tubes, tweezers, MRS broth, distilled water, to provide an as sterile condition as possible. Equipment is then dried under a laminar flow hood for 20 minutes prior to the experiment.

2.2.2. Saliva

6 17-year old volunteers are chosen randomly from the campus. Prior to the extraction of saliva, volunteers are informed about the experiment and signed informed consents. They are asked to wear gloves before extracting saliva. Volunteers first accumulate saliva in the oral cavity and then expectorate about 1 mL of unstimulated saliva into sterilized, dried tubes provided. An addition self-collected 20 mL of saliva is also obtained using the same methodology.

2.3. Bacteria Cultivation

Types of agar plates selected to cultivate bacteria in each experiment group are

based on previous studies and data [20] [21].

2.3.1. Probiotic Plate Inoculation

Lactobacillus reuteri DSM17938 lyophilized powder was reconstituted in 0.2 mL sterile water (pH 7.4). A 0.1 mL aliquot was spread onto MRS agar plates, containing 10 g/L peptone, 8 g/L beef extract powder, 4 g/L yeast extract, 20 g/L glucose, 2 g/L dipotassium phosphate, 2 g/L dipotassium hydrogen citrate, 5 g/L sodium acetate, 0.2 g/L magnesium sulfate, 0.04 g/L manganese sulfate, and 1 g/L tween80, using a sterile L-shaped spreader and incubated anaerobically (37˚C, 48 h). Single colonies were inoculated into 8 mL liquid MRS broth and incubated (37˚C, 160 rpm, 24 h).

Strain is placed and revived in the shaker at 37˚C, 160 rpm for 30 minutes and inoculated onto petri plates containing solid MRS medium with a sterile spreading rod in a clean bench. The plates are then being placed up-side-down in an incubator at 37˚C for *Lactobacillus reuteri DSM17938* strain and oral bacteria to reproduce.

2.3.2. Saliva Plate Inoculation

Saliva samples collected from 6 volunteers are used in this part of the experiment. The samples are divided into 4 identical groups each being diluted according to ratios of 1:5, 1:10, 1:20, 1:40 with sterile water (to better mimic different amount of microbiota across individuals and better evaluate the effects of mouthwashes under different pressure), generating solutions of 200 μL, to evaluate the inhibitory effects of mouthwashes under different concentrations of saliva samples . The solutions are inoculated onto blood agar using sterile spreading rod. Cultivation of bacteria in incubator is conducted under the same conditions as probiotic group.

2.4. Inhibitory Effects Test

2.4.1. Addition of Mouthwash Tablets in Probiotic Group

A total of 15 plates are being prepared, being composed of 3 groups for repeated experiments and 5 plates in each group (four plates for four types of mouthwashes and one for control group). Each plate is marked into four sections and labeled with names of mouthwashes (LISTERINE® TOTAL CARE Anticavity Fluoride Mouthwash FRESH MINT, LISTERINE® Antiseptic Oral Care Mouthwash ORIGINAL, LISTERINE® Antiseptic Mouthwash COOL MINT®, LISTERINE® Alcohol-Free Mouthwash COOL MINT®). Four tablets (sterile filter paper discs with diameter of 6 mm saturated with 10 μL of mouthwashes) are placed into the according sections on each plate where *Lactobacillus reuteri DSM17938* strain is being cultured. Plates added with filter paper discs containing no mouthwashes are being set as negative control.

2.4.2. Addition of Mouthwash Tablets in Saliva Group

Each volunteer's sample is given four plates labeled with different concentrations

(1:5, 1:10, 1:20, 1:40), mouthwash tablets are added into each plate applying the same methodology as the probiotic group.

2.4.3. Inhibitory Zone Test

The growth of bacteria and size of inhibition zones in both the probiotic group and the saliva samples group are observed at vertical direction and measured every 12 hours using a ruler (±0.05 mm) at three equidistant points.

2.4.4. Saliva Growth Curve

20 mL of self-collected saliva is used in this part of the experiment. Prepare 100 1.5 mL tube, each being added with 190 uL of self-collected saliva. Add another 10 uL of mouthwash into the tubes to produce solutions of 200 uL. Each type of mouthwash accounts for 25 tubes. The tubes added with mouthwashes are cultivated in a shaker at 37˚C. Growth of bacteria is measured and calculated using a ultraviolet spectrophotometer under wavelength of 600 nm at time periods of 0 min, 15 min, 30 min, 60 min, and 120 min after the addition of mouthwashes.

3. Results and Discussion

3.1. Inhibition Zone Test of Probiotic Group

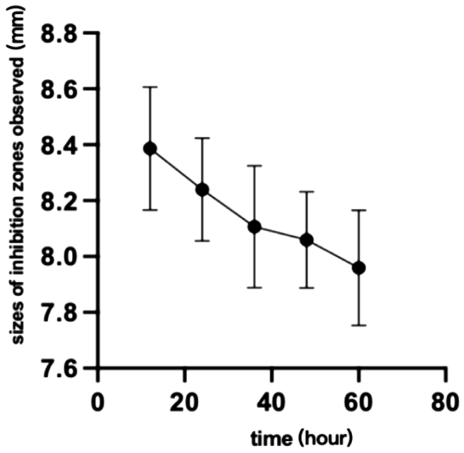

Figure 1. Mean sizes of inhibition zones generated by cosmetic tablets observed in 15 plates of the probiotic group.

As shown in Figure 1, sizes of the inhibition zones caused by cosmetic mouthwash on plates cultivating *Lactobacillus reuteri DSM17938* showcased a decreasing trend: mean sizes of inhibition zones is around 8.3 mm at 12 hours after the addition of mouthwashes, as the time reaches 60 hours, an approximately 0.5 mm of reduction is observed, ending at around 7.9 mm. This indicates a time-dependent decrease in the inhibitory effects of cosmetic mouthwash, possibly due to evaporation of mouthwash and continuous reproduction of cosmetic-resistant bacteria. However, the other three types of mouthwashes (fluoride, antiseptic, and alcohol) exhibit no antibacterial effects on the probiotic's growth throughout the repeated groups (0 mm), and is therefore insignificant for the investigation.

3.2. Inhibition Zone Test for Saliva Samples Group

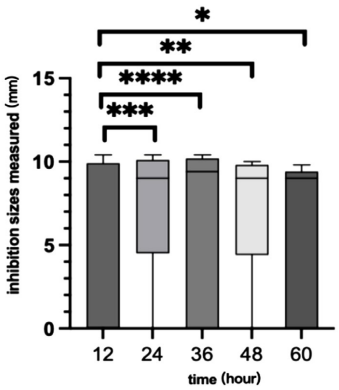

Figure 2. Sizes of inhibition zones of cosmetic tablets on plates of saliva samples at a concentration of 1:5 measured at five time periods (****: $p \leq 0.0001$, ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, ns: not significant).

Through measurements of the sizes of cosmetic inhibition zones of saliva samples measured at a 1:5 concentration, it is found that at such concentration, zone sizes remain relatively stable around 10 mm with fluctuations, lacking a clear increasing or decreasing trend (Figure 2). However, measurements conducted at 12 hours period reflects most tablets performing no antibacterial effects, which indicates a possible delay for the mouthwash to perform its antibacterial effects caused by time required for the active ingredients to diffuse in the agar. Notably, the multiple asterisks between different time points signify statistical significance; for instance, the high significance difference between 12 and 36 hours indicates that the inhibition zone size at 36 hours after the addition of mouthwash are distinctly larger than that of the 12 hours group, and other pairs of time points also show significant differences with varying numbers of asterisks representing different significance levels. In summary, at a 1:5 concentration, while the overall inhibition zone sizes do not consistently trend up or down, there are significant differences between the sizes at different time periods.

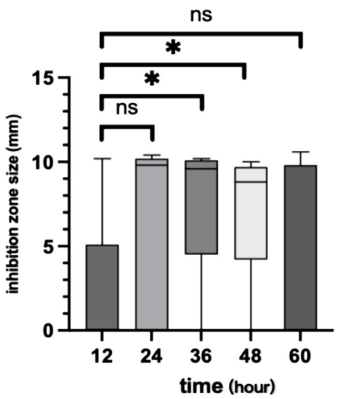

Figure 3. Sizes of inhibition zones of cosmetic tablets on plates of saliva samples at a concentration of 1:10 measured at five time periods (****: $p \leq 0.0001$, ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, ns: not significant).

In Figure 3, sizes of the cosmetic inhibition zones measured at 12 hours are the smallest among all time points, with most of the tablets generating no inhibition zones (0 mm). In contrast, the inhibition zone sizes measured at 24, 36, 48, and 60 hours are relatively larger, also supporting the possibility of a delay for mouthwash to function. Significance value calculated also suggest that cosmetic mouthwash is the most effective at around 24 - 48 hours (p value < 0.05), as the there are significant differences in the sizes of inhibition zones. Whereas, it might lose its ability of inhibition when time reaches 60 hours because of the rise in bacteria concentration and higher prevalence of resistance, represented by the difference in sizes inhibition zone being insignificant.

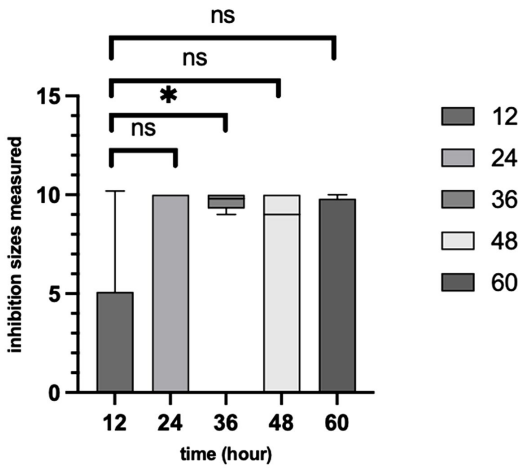

Figure 4. Sizes of inhibition zones of cosmetic tablets on plates of saliva samples at a concentration of 1:20 measured at five time periods (****: p ≤ 0.0001, ***: p ≤ 0.001, **: p ≤ 0.01, *: p ≤ 0.05, ns: not significant).

Data in Figure 4 shows following similar trend with that of Figure 5, the inhibition zone sizes at 12 hours is obviously smaller than those at the other time points. From 24 to 60 hours, the inhibition zone sizes are relatively close, fluctuating around 9 and 10 mm. Notably, comparing to the data from 1:10 concentration group, differences in the sizes of inhibition zone are less significant, with only data from 12 ans 36 hours presenting a significant difference in size, which can possibly attribute to the rising ration of dilution, reducing the pressure of mouthwash to function and inhibit bacterial growth. In summary, at a 1:20 concentration, the inhibition zone size at 12 hours is an outlier. While some time points demonstrate a statistically significant divergence, others show no significant difference in inhibition zone sizes.

As the concentrations of saliva samples become lower, the inhibition zone sizes across these time points are relatively more similar, with values generally around 10 mm. (Figure 5) Insignificant differences between inhibition zone sizes measured at different time periods further strengthen the trend: reducing concentration of saliva samples is likely to increase and stabilize mouthwash's antibacterial effects. The inter-individual variability observed in each level of dilution condi-

tions also suggests the complexity of oral microbiomes and individual differences in salivary composition. Furthermore, under all dilution conditions, the inhibitory effect of cosmetic mouthwash are weakened, which might be a result of resistance.

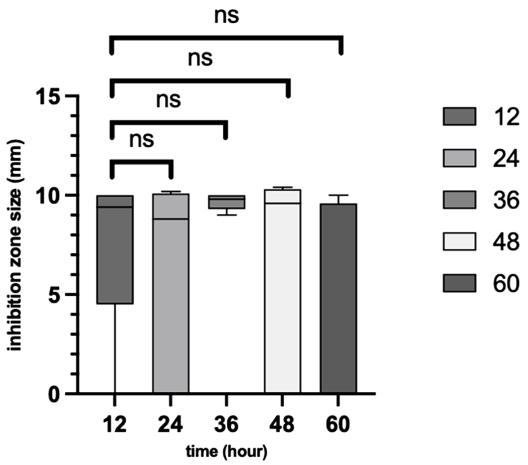

Figure 5. Sizes of inhibition zones of cosmetic tablets on plates of saliva samples at a concentration of 1:40 measured at five time periods (****: $p \leq 0.0001$, ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, ns: not significant).

In both experiment cultivating *Lactobacillus reuteri DSM17938* and saliva samples, cosmetic mouthwash exhibit obvious antibacterial effects when compared to the other three types of mouthwashes (fluoride, antiseptic, and alcohol). The obvious inhibitory effects of cosmetic mouthwash may come from extra surfactant, flavouring agents etc., which is sensitive to *Lactobacillus* strains [22]. Further comparison of ingredients contained by four types of mouthwashes reflects that cosmetic mouthwash lacks the presence of strong antibacterial ingredients such as chlorhexidine but is added with propylene Glycol, a type of flavouring agent to provide a sweet taste. However, propylene Glycol is proven to present bactericidal activity [23], suggesting a potential source of the inhibitory effects of cosmetic mouthwashes. The inhibitory effects presented by cosmetic mouthwash may also be attribute to ingredients such as thymol, eucalyptol, methyl salicylate and menthol, which are proven to have certain inhibitory effects on bacteria [2] [24]-[26]. However, other types of mouthwash also contained such ingredients but did not exhibit obvious antibacterial effects, therefore their ability to persistently inhibit bacterial growth may be relatively weak.

To clarify the difference in effectiveness and provide a better comparison between different mouthwashes, the total number of inhibition zones generated by each type of mouthwash is counted. As shown in **Figure 6**, compared to cosmetic mouthwash, the inhibitory effects of other three types against saliva samples are relatively weak. However, they did generate few inhibition zones throughout the experiment. As being recorded in **Figure 7**, fluoride mouthwash generates the higher number of inhibition zones (17) with mean sizes of about 10 mm, while

antiseptic exhibit the weakest inhibitory effects, producing only 3 inhibition zones across all samples. The effects of alcohol mouthwash remain medium among the three types, producing a total of 7 inhibition zones. However, number of inhibition zones generated by antiseptic and alcohol mouthwash are insufficient to be considered as significant and to determine their effectiveness. Calculations of significance value also implies that there are significant difference when comparing fluoride, antiseptic and alcohol mouthwash to cosmetic mouthwash.

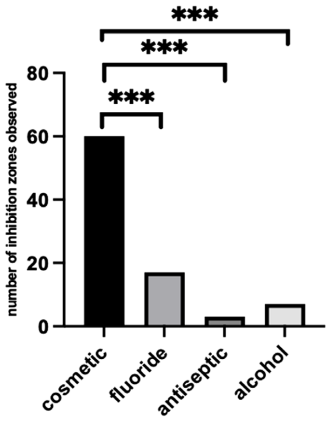

Figure 6. Number of inhibition zones measured in 24 plates of the saliva sample group.

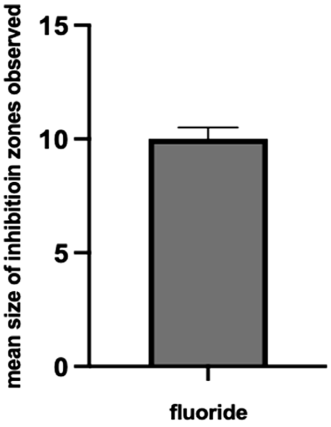

Figure 7. Mean size of inhibition zones generated by LISTERINE® TOTAL CARE Anticavity Fluoride Mouthwash FRESH MINT.

Growth curve analysis

Plate cultivation provides abundant nutrients for cultivated bacteria and therefore might not accurately reflect the actual oral condition of humans. To better mimic oral conditions in-vitro and investigate effects of different mouthwashes after the treatment, growth curve analysis test is applied.

From the data obtained (Figure 8), cosmetic mouthwash showed an initial increase in absorbance at 15 min, reaching its peak, followed by a decline at 30 min and 60 min, before slightly recovering at 120 min. This suggests a temporary stimulation of bacterial growth before a delayed inhibitory effect.

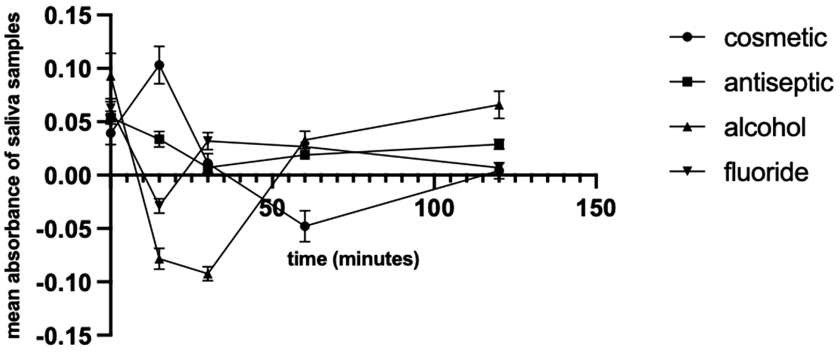

Figure 8. Mean absorbance of saliva samples measured at 5 time periods.

Alcohol mouthwash initially maintained a stable absorbance but then exhibited a sharp decline at 15 min, reaching its lowest point slightly higher than −0.1. This indicates a strong bactericidal effect at the early stage. However, after 30 min, the absorbance gradually increased again, suggesting a weakening in antibacterial effects.

Fluoride mouthwash displayed an initial decrease in absorbance at 15 min, suggesting early bacterial inhibition, followed by a gradual increase, stabilizing after 30 min, which might indicate a moderate antibacterial effect that diminishes over time.

Antiseptic mouthwash exhibited relatively mild change in absorbance over time. After an initial drop for the first 30 min, the absorbance gradually increased, stabilizing around 60 min. This suggests that its effect on bacterial growth is mild compared to the other mouthwashes.

Overall, the results indicate that alcohol mouthwash had the most obvious and most immediate antibacterial effect, but its efficacy declined over time. Fluoride mouthwash showed moderate inhibition, with bacterial growth recovering relatively quickly. Cosmetic mouthwash exhibited an irregular pattern, possibly due to its formulation, while the antiseptic mouthwash had the least impact on bacterial growth.

Differences in the data relevant to the efficacy of mouthwashes in growth curve test when compared to the inhibition zone test, specifically between the effect of alcohol and cosmetic mouthwashes, may be caused by factors such as difference in volatility of ingredients. With alcohol being much more volatile, alcohol mouthwash tends to present short-term inhibitory effects. However, sizes of inhibition zones were tested at intervals of 12 hours, alcohol may have evaporated therefore producing no positive effects in inhibition zone tests. Whereas in growth curve analysis, absorbance was measured at times of 15, 30, 60, and 120 minutes, better reflecting alcohol mouthwash's immediate effects. In addition, more sufficient (direct) contact of mouthwash with saliva samples also provided continuous and higher level of antibacterial effects. For propylene glycol in cosmetic mouthwash, its lower volatility enables it to be retained on agar plates for longer periods, producing long-lasting inhibition zones. Though proven to exhibit antibacterial

effects, flavouring agent such as propylene glycol may still not be as effective as direct active ingredients such as alcohol, therefore cosmetic mouthwash presented a relatively mild antibacterial effect in growth curve analysis, weaker than that of alcohol.

There are weaknesses and limitations regarding to the experiments, mainly referring to the representativeness of the selection of bacteria. For example, despite the significance of *Lactobacillus reuteri DSM17938* to human oral health, one strain of probiotic lacks representativeness of all potential probiotic existing in human oral cavity. Similarly, only six samples of unstandardized saliva are collected, which is also insufficient to represent all possible bacteria in human saliva, limiting the generizability of the experiment. However, the obtained data can act as a reference and provide relevant suggestions on the purchase and usage of mouthwashes.

4. Conclusions

In conclusion, three tests in this experiment suggest that cosmetic mouthwash possess the most obvious effects in inhibiting oral bacteria, whereas fluoride mouthwash, antiseptic mouthwash and alcohol mouthwash have relatively mild effects. In fact, growth curve analysis indicated the alcohol formulation had the most immediate bactericidal effect in liquid culture. The weaker effects of such mouthwash may be attributed to the resistance possessed by bacteria used in this experiment, for example it is found certain *Lactobacillus* strains are tolerant to alcohol at low concentrations etc. [27]. For all four types of mouthwashes, concentration of bacteria, interval time between usage of mouthwashes can both affect the actual efficacy of mouthwashes: increasing concentration and interval duration may lead to invalidation of mouthwashes and fail to inhibit bacterial growth.

The study of mouthwash's inhibitory effects on oral bacteria, including both pathogens and probiotics, has important implications for oral and systemic health. Oral probiotics, like *Lactobacillus*, help maintain a balanced oral microbiome, preventing conditions like caries and periodontal disease. If mouthwashes containing antimicrobial agents disrupt these beneficial bacteria, it could lead to dysbiosis (Brookes et al., 2023), increasing the risk of oral diseases and potentially fostering resistant pathogens. Dysbiosis is also related to various systematic diseases including obesity, diabetes, Alzheimer's disease and so on [28].

Understanding this effect could guide the development of mouthwashes that selectively target harmful bacteria while preserving probiotics, supporting long-term oral health. Additionally, the results may inform clinical recommendations on mouthwash use, ensuring oral hygiene practices do not inadvertently harm beneficial microbial populations.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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