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Prevention of the proliferation of oral pathogens due to prolonged mask use based on α-cyclodextrin and hydroxytyrosol mouthwash

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Abstract. – OBJECTIVE: Face masks help contain the aerosol-mediated transmission of infectious viral particles released from individuals via cough and sneezes. However, the prolonged use of face masks has raised concerns regarding oral hygiene. Here, we present a mouthwash formulation based on α-cyclodextrin and hydroxytyrosol that can maintain healthy oral microbiota.

MATERIALS AND METHODS: We isolated and cultured *Candida albicans*, *Staphylococcus aureus*, and a mix of *Streptococcus sp.*, *Staphylococcus sp.* and *Neisseria sp.* from oral and throat swabs. The microorganisms were cultured in a standard medium with or without the mouthwash. To evaluate the effect of the mouthwash on the oral microbiota, the DNA from the saliva of 3 volunteers that used the mouthwash was extracted. Then, the DNA was amplified using primer pairs specific for bacterial and fungal DNA. Twelve further volunteers were offered to use the mouthwash and a questionnaire was submitted to them to assess the possible beneficial effects of mouthwash on halitosis and other oral disturbances.

RESULTS: The bacteria and fungi cultured in media containing the mouthwash showed a growth reduction ranging from 20 to 80%. The PCR amplification of fungal and bacterial DNA extracted from volunteers that used the mouthwash showed a reduction of both bacteria and fungi. Volunteers that used the mouthwash reported a tendency towards a reduction of halitosis, gingival and mouth inflammation, and dry mouth.

CONCLUSIONS: The use of a mouthwash containing α-cyclodextrin and hydroxytyrosol is not aggressive against oral mucosa; it is safe and effective to reduce the bacterial and fungal load due to the continuous use of face masks.

Key Words:
Face mask, Mouthwash, α-cyclodextrin, Hydroxytyrosol.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), like other respiratory viruses, such as influenza virus, rhinovirus, adenovirus, respiratory syncytial virus, spreads through inhalation of infected droplets, released by coughing/sneezing of an infected person[1,2]. Looking at the highly infectious nature of the virus, the only way to avoid immediate contact is to prevent the inhalation of and exposure to the viral particles suspended in aerosols and contaminated surfaces. The World Health Organization has emphasized on the compulsory use of respiratory protective devices, such as face masks[3]. Respiratory masks or face masks are protective devices that cover a part of the face thus protecting both the users and the people around them from breathable aerosols. Many governments have implemented the use of face masks, and this has resulted in

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reducing the spread of the infection[4]. However, prolonged use of facial masks has resulted in many problems, such as enhanced perspiration and temperature alterations in perioral region, reduced heat loss from the body thus creating a difference in the temperature of the facial area covered by the masks and the external environment. Furthermore, the warmth and moisture in exhaled air trapped inside the mask increases the heat burden and humidity in area surrounding the mouth leading to sweating and as the mask covers both mouth and nose it results in a decrease in cooling impact of the facial temperature causing significant discomfort to the users[5-9]. In addition, a mask induced dampness around the mouth and promotes growth of fungi, such as *Trichophyton rubrum* causing *Tinea faciei*[10]. Therefore, although the use of face masks in the current pandemic situation is inevitable and necessary to reduce the chances of being infected, the face mask use may have some negative consequences[11]. For instance, mask wearers may experience halitosis. This originates when the oral cavity presents a suitable environment for the growth of Gram-negative and proteolytic obligate anaerobes bacteria capable to produce odorous compounds by degrading organic substrates[12,13]. The inflammation associated with poor oral hygiene of gingival and periodontal tissues is the main cause of oral malodors[14]. The severity of halitosis is affected by plaque-related periodontal conditions. Another important factor in halitosis is the flow of saliva. In fact, saliva influences the composition and growth of oral bacterial populations, and reduction of the salivary flow has negative effects on self-cleaning of the mouth and inadequate cleaning of the mouth causes halitosis[15-17].

Here we suggest that a mouthwash formulation for daily use based on natural compounds isolated from olives, such as hydroxytyrosol, and α-cyclodextrin may reduce oral dryness and prevent "mask mouth".

Materials and Methods

Mouthwash Composition

The mouthwash that we tested has the following composition for 100 g: demineralized water q.s. for 100 g, ethanol (96%) 4.29 g, α-cyclodextrin 0.20 g, xanthan gum 0.05 g, European olea extract (Momast 30 plus) 1.00 g, sodium benzoate 0.20 g, potassium sorbate 0.20 g, sucralose 0.10 g, lemon flavour 0.20 g, soluble mint flavour 0.05 g, green colouring 0.0005 g.

Bacterial Growth Assay

We isolated and cultured the following microorganisms collected from lingual and throat swabs, and pus culture: *Candida albicans* isolated from lingual swab; *Staphylococcus aureus* isolated from pus culture; mix of bacteria from throat swab composed of *Streptococcus sp.*, *Staphylococcus sp.* and *Neisseria sp.* The microorganisms were inoculated in the standard Brain Heart Infusion (BHI, Liofilchem) liquid medium. Media with microorganisms were incubated at 37°C for 4 hours in order to reach the logarithmic growth phase.

Four tubes containing 2 ml of BHI medium and 4 tubes containing 1 ml of BHI and 1 ml of mouthwash were then prepared and 100 µl of liquid medium from logarithmic growth phase cultures were inoculated in all tubes. We thus obtained the following microorganism/medium combinations: one tube with 2 ml of BHI medium and one tube with 1 ml of BHI medium + 1 ml of mouthwash both inoculated with 100 µl of *Candida albicans*; one tube with 2ml of BHI medium and one tube with 1ml of BHI medium + 1 ml of mouthwash both inoculated with 100 µl of *Staphylococcus aureus*; one tube with 2 ml of BHI medium and one tube with 1 ml of BHI medium + 1 ml of mouthwash inoculated with 100 µl of bacterial mix from a patient's throat swab; one tube with 2 ml of BHI medium and one tube with 1ml of BHI medium + 1 ml of mouthwash inoculated with 100 µl of bacterial mix from another patient's throat swab.

After an overnight incubation of all cultures at 37°C, 1 µl of these cultures was inoculated in solid media in a Petri dish divided in two sides: on one side the culture with BHI medium and on the other the one with medium BHI + mouthwash. Sabouraud Dextrose Agar (Biolife) was used for *Candida albicans* and Columbia Blood Agar (Biolife) for all other cultures. After incubation at 37°C overnight we evaluated the growth differences reported in the results.

Amplification of Bacterial and Fungal DNA

To evaluate the effect of the mouthwash we extracted DNA from the saliva of 3 volunteers that were offered to use the mouthwash: without using the mask, after using the mask for 8 hours,

Table I. Primers used for the amplification of the bacterial and fungal genomic universal regions.

Primer name	Forward sequence (5′->3′)	Reverse sequence (5′->3′)	Fragment length
Universal 16S rRNA bacterial primers 27F	AGAGTTTGATCCTGGCTCAG	GGTTACCTTGTTACGACTT	1440 bp
Universal Panfungal ITS	GCATCGATGAAGAACGCAGC	TCCTCCGCTTATTGATATGC	500 bp

after they had used the mask for 8 hours and then used the mouthwash. Saliva extraction and DNA isolation and amplification were performed using standard protocols. We then amplified with universal primers the extracted DNA for the amplification of bacterial and fungal DNA (Table I), to evaluate possible differences in microbial growth with or without the use of the facial mask and with or without the use of the mouthwash.

Questionnaire

Twelve further volunteers were offered to use the mouthwash and were administered a questionnaire to assess the possible effects of mouthwash on oral disturbances reported by the volunteers themselves, due to prolonged use of face masks. We performed a 10-day study. In the first 5 days, 12 volunteers did not use the mouthwash, while, in the remaining 5 days, the same volunteers used the mouthwash. As a basic assumption, we considered that all volunteers use toothbrushes and toothpaste after each meal and wear the mask for 8 hours a day. After that, these volunteers were given a questionnaire to assess whether they had experienced halitosis, and other oral disturbances, at the beginning of the study and if they perceived improvements after the use of the mouthwash for 5 days.

Results

Microorganisms' Growth

The bacteria and fungi cultured in media containing the mouthwash in addition to the standard medium showed a reduction in the growth rate ranging from 20 to 80% (Figure 1). In particular, the mixed culture from throat swab is important because it represents the most similar bacterial composition of the oral microbiota.

Bacterial and Fungal Oral Microbiota

The DNA extracted from the saliva of 3 volunteers was amplified using universal primers specific for bacterial and fungal DNA showed the following results. There is an increase in bacterial and fungal growth when those 3 volunteers wore the mask for 8 hours, while when they wore the mask for 8 hours then used the hydroxytyrosol and α-cyclodextrin-based mouthwash, a reduction in the presence of bacteria and fungi was found (Figure 2).

Mouthwash Users Questionnaire

Twelve further volunteers were offered to use the mouthwash and were administered a questionnaire to assess the possible effects of mouthwash on oral disturbances reported by the volunteers

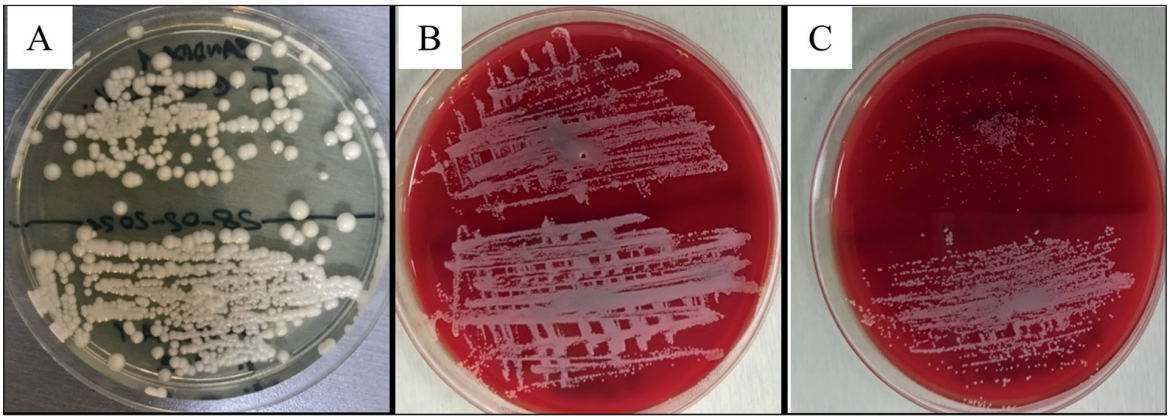

Figure 1. The figure shows the growth inhibition, ranging from 20 to 80%, in cultures of *Candida albicans* (**A**), *Staphylococcus aureus* (**B**) and a mix of bacteria (S*treptococcus sp*., *Staphylococcus sp.* and *Neisseria sp.*) from throat swab (**C**), grown in standard culture medium (below) and in standard culture medium with the addition of mouthwash (above).

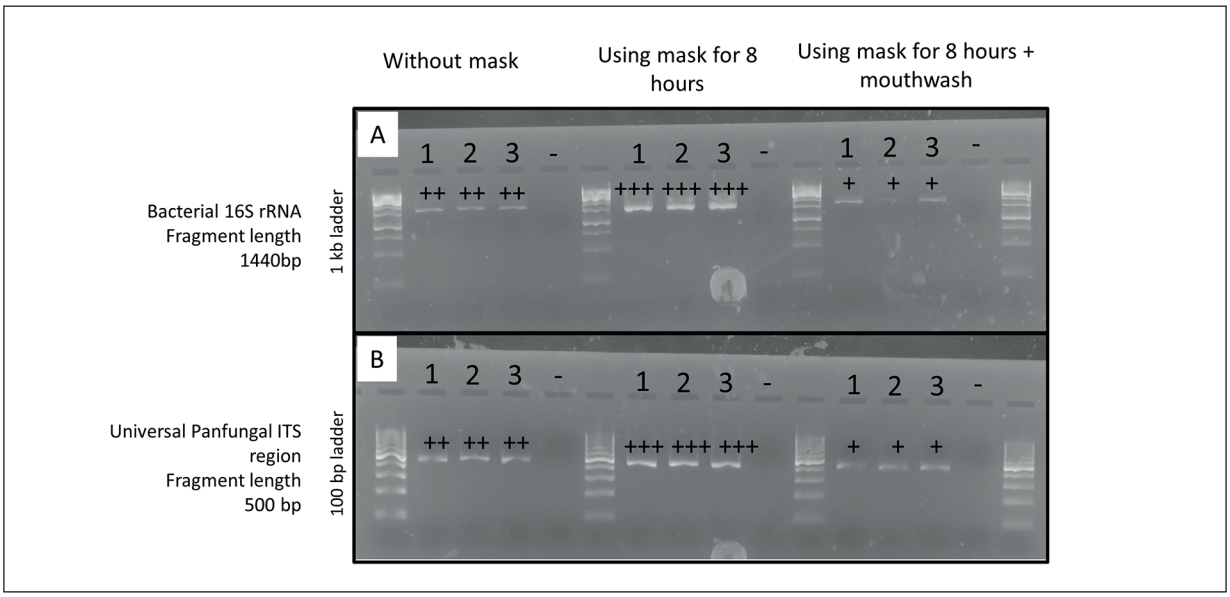

Figure 2. The figure shows the reduction of DNA amplification by PCR of bacterial (**A**) and fungal (**B**) DNA after using the mouthwash (the three rightmost wells in A and B panels) in individuals who have worn the mask continuously for 8 hours, while it is highlighted a greater presence of bacteria and fungi after using the mask for 8 hours without using mouthwash.

themselves, due to prolonged use of face masks. We performed a 10-day study. In the first 5 days, the volunteers did not use the mouthwash, while, in the remaining 5 days, the same volunteers used the mouthwash. As a basic assumption, we considered that all volunteers use toothbrushes and toothpaste after each meal and wear the mask for 8 hours a day. After this 10-day period, these volunteers were given a questionnaire to assess whether they felt halitosis and other oral disturbances due to prolonged use of face mask after the use of the mouthwash for 5 days. At the end of the study, we found a strong tendency towards a reduction of halitosis (-78%), gingival inflammation (-67%), mouth inflammation (-75%), and dry mouth (-40%) (Table II).

Discussion

Mouthwashes are widely used for routine oral and dental hygiene for cosmetic and therapeutic purposes. However, most of them give only a temporary relief[18,19]. Alternatively, therapeutic mouthwashes have active ingredients designed to amend the oral microbiota for improving conditions, such as gingivitis, caries, plaque, and bad breath. The most common ingredient of these mouthwashes is the disinfectant and antiseptic chlorhexidine[18,19]. However, mouthwashes that claim antiseptic or anti-bacterial properties may kill bacteria indiscriminately but only a few of the oral bacteria are harmful, whereas the rest partly form first line of defence against pathogens[20]. This mouth-

Table II. Answers to the questionnaire on the use oral disturbances and possible effects of mouthwash usage.

Disturbance	Value	Reduction (%)
Halitosis	Before: 9/12 After: 2/12	78%
Gingival inflammation	Before: 6/12 After: 2/12	67%
Mouth inflammation	Before: 8/12 After: 2/12	75%
Dry mouth	Before: 5/12 After: 3/12	40%

wash could lead to mucosal inflammation on the long term. For instance, chlorhexidine is effective against gingivitis, periodontitis, the development of oral biofilm, and in post-surgery periodontal disease[21], but it has some side effects like tooth tartar, discoloration of teeth and mouth, taste impairment[22], allergic reactions[23] and can cause irritation or nausea if ingested[24]. In addition, the Food and Drug authority of USA has recommended to limit the use of chlorhexidine gluconate-based formulations to a maximum period of 6 months[25-28]. Furthermore, the use of antiseptic mouthwash may increase blood pressure in healthy individuals[29] and may abolish the blood pressure lowering effects of nitrate[30-32]. This deleterious effect is associated with the removal of the oral bacteria that normally reduce nitrate to nitrite[33,34].

For these reasons, we developed a mouthwash containing well-tolerated active ingredients to deal with halitosis and other mouth disturbances associated with the prolonged use of face masks. This preliminary study shows that using a mouthwash based on α-cyclodextrin and hydroxytyrosol a bacteriostatic and micostatic effect may obtained. This seems sufficient to reduce the so-called mask mouth effect without causing oral dysbiosis by killing good bacteria. In addition, we have recently reported that the administration of hydroxytyrosol and α-cyclodextrins as active ingredients may help in providing a possible defence against viral infections[35].

Molecular docking analysis revealed that hydroxytyrosol and α-cyclodextrin might interact with viral and human proteins involved in the entry of SARS-CoV-2 into the cells, thereby potentially affecting the entry rate of SARS-CoV-2 into the cells[36]. Similarly, in another preliminary study[37] we evaluated the cytotoxic activity and safety of an oral spray containing hydroxytyrosol and α-cyclodextrin in two human cell lines. Our results indicated that hydroxytyrosol and α-cyclodextrin in combination have neither any cytotoxic activity or side effects when administered in healthy volunteers. It is worth mentioning here that both studies are preliminary. There are some indications that hydroxytyrosol and α-cyclodextrin might also be able to help reduce the viral burden.

Conclusions

The use of a mouthwash based on natural compounds, such as α-cyclodextrin and hydroxytyrosol is less aggressive against oral mucosa, is safe and possibly effective to reduce the bacterial and fungal load due to the continuous use of face masks.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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