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Original Article

Microbial Communities on the Inner Surfaces of Face Masks and in Saliva During the COVID-19 Pandemic

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ABSTRACT

During the COVID-19 pandemic, the use of face masks was widely recommended. This study sought to characterize the oral microbiome present on the inner surface of masks, evaluate the abundance of specific bacterial species, and assess whether xerostomia influenced microbial composition. The study involved 55 generally healthy adults (45 females and 10 males; mean age 38.18 ± 12.49 years). For each participant, unstimulated (UFR) and stimulated (SFR) saliva flow rates were measured, and saliva samples were collected. Fourteen major oral bacterial species—including Porphyromonas gingivalis (P. gingivalis), Lactobacillus casei (L. casei), Tannerella forsythia (T. forsythia), and Treponema denticola (T. denticola)—were quantified on both the inner surface of masks and in saliva using real-time PCR. The findings indicated that total bacterial DNA was significantly greater in both UFR and SFR than on the mask surface ($p < 0.001$). Among bacteria on the mask, P. gingivalis was the predominant Gram-negative species, while L. casei dominated among Gram-positive species. Microbial profiles on the mask differed from those in saliva samples. Shannon's diversity index was markedly higher in UFR and SFR (2.64 ± 0.78 and 2.66 ± 0.76, respectively) compared with the mask (1.26 ± 1.51, $p < 0.001$), and a strong positive correlation existed between UFR and SFR diversity ($r = 0.828$, $p < 0.001$), whereas no significant relationship was observed with mask diversity. The abundance of Red Complex bacteria (P. gingivalis, T. forsythia, and T. denticola) was greater in UFR than on masks. Notably, xerostomia did not significantly impact bacterial counts, total DNA, or diversity measures ($p > 0.05$).In summary, oral bacteria were transferred to and persisted on the inner surfaces of masks, but xerostomia had no discernible effect on microbial composition. Although masks harbored a unique oral microbiome, both bacterial load and diversity were lower than in unstimulated or stimulated saliva.

Keywords: Microbial, COVID-19, Face masks, Saliva

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Introduction

Coronavirus disease 2019 (COVID-19), which emerged in December 2019, was declared a global pandemic by the World Health Organization (WHO) on January 30, 2020. By early May 2023, more than 688 million confirmed cases and approximately 6.87 million deaths had been reported worldwide [WHO Coronavirus (COVID-19) Dashboard, https://covid19.who.int]. During the pandemic, wearing face masks became mandatory in many countries, though public mask mandates have since been lifted [1]. Evidence from epidemiologists and virologists indicates that COVID-19 primarily spreads from infected individuals through respiratory droplets and aerosols, with the mouth and nose serving as principal routes of transmission [2, 3]. Public health guidelines from the Centers for Disease Control and Prevention (CDC) and WHO emphasize the use of masks to reduce infection risk [4], and combining mask use with vaccination, hand hygiene, and physical distancing has been shown to further limit viral spread [5]. Consequently, masks play a crucial role in

preventing transmission of SARS-CoV-2, especially during periods without effective therapeutic interventions.

Despite their protective benefits [6], prolonged mask use has been linked to several health challenges. Physiological and psychological burdens, decreased work performance, and impaired cognitive function have been reported [7, 8]. Physically, masks can induce headaches, dyspnea, and discomfort due to restricted oxygen intake [9]. Tight-fitting masks alter thermal balance and increase CO2 retention, which can stimulate respiration and modify lung ventilation, skin temperature, and humidity [10]. CO2 buildup may also contribute to cognitive disturbances and confusion. Additionally, extended mask use can provoke dermatological issues, including dryness, itching, rashes, and acne, often resulting from duct blockage and humid conditions beneath the mask [11, 12]. However, the potential for bacterial growth in this warm, moist environment and the origins of such microbes remain underexplored.

The oral cavity, naturally coated in saliva, provides a unique, nutrient-rich, and humid environment supporting a diverse microbiome of over 700 species [13]. Saliva is primarily water (>98%) with electrolytes, mucins, enzymes, nutrients, and antibacterial factors, all of which influence microbial homeostasis [14, 15]. Proper salivary hydration is crucial for maintaining oral microbial balance, and disruptions may contribute to dysbiosis. Few studies have directly examined the microbiome of face masks. Au *et al*. (2022) found that continuous mask use over two months did not significantly alter the salivary microbiome, though their study focused solely on saliva and included only young dental students (mean age 26.36 ± 1.58 years) [16]. Park *et al*. analyzed bacteria and fungi from masks via culture-based methods, finding no significant changes with prolonged mask use [17]. Nonetheless, comprehensive quantitative and qualitative analyses across different age groups are needed to validate these findings.

We hypothesized that oral bacteria could colonize the inner surface of masks and sought to determine which bacterial populations proliferate under these conditions. We also examined the relationship between bacteria present in saliva under unstimulated (UFR) and stimulated flow rates (SFR) and those found on mask surfaces. This study employed PCR techniques to analyze microbial communities in saliva and masks from participants of various ages. Additionally, we considered xerostomia, a common complaint during extended mask use, which can result from reduced salivary flow or decreased water intake [18]. Our findings provide insights into maintaining mask hygiene, explain potential skin complications related to oral microbes under masks, and support recommendations for frequent mask replacement.

Materials and Methods

Study population

This study included 55 healthy volunteers (45 females and 10 males; mean age 38.18 ± 12.49 years) recruited at Kyung Hee University Dental Hospital between September 1 and October 31, 2021. The study protocol adhered to the Declaration of Helsinki and received approval from the Institutional Review Board of Kyung Hee University Dental Hospital (IRB No-KH-DT21023). Written informed consent was obtained from all participants.

Participants were assessed for xerostomia and categorized into a xerostomia group (n = 14; 12 females; mean age 36.43 ± 12.99 years) and a non-xerostomia group (n = 41; 33 females; mean age 38.78 ± 12.42 years). They completed questionnaires detailing gender, age, mask-wearing habits, salivary pH, buffer capacity, and xerostomia status. Clinical evaluation included periodontal tissues, buccal mucosa, and general oral health. A related study examining halitosis using the same methods has been previously published [19].

Inclusion criteria encompassed medically healthy adults with intact permanent dentition (loss of fewer than two teeth), healthy periodontal status, and ability to comprehend and consent to study procedures. Exclusion criteria included use of medications affecting salivation (e.g., psychiatric drugs, antibiotics), pregnancy or lactation, systemic diseases or disabilities affecting oral care or salivary function, and presence of partial dentures or fixed orthodontic appliances. Participants with incomplete data or those unable to complete sample collection were also excluded.

Strict protocols were followed to prevent contamination during saliva and mask sampling. Researchers wore masks, disinfected hands, and used sterilized gloves replaced for each participant. Laboratory surfaces and equipment were cleaned with alcohol, and all consumables contacting samples were sterilized and single-use. Aerosol contamination was minimized with covered centrifuges, and PCR reagents were prepared under sterile conditions on a clean bench.

Collection of unstimulated and stimulated saliva

Participants were asked to avoid caffeine and nicotine for at least four hours prior to saliva collection and to abstain from alcohol for 24 hours. To reduce variability due to circadian rhythms, all samples were collected between 9:30 and 11:30 a.m., approximately three hours after waking. Before sampling, participants refrained from eating, drinking, or performing oral hygiene. Unstimulated saliva (UFR) was gathered over a ten-minute period using the spitting technique. Subsequently, stimulated saliva (SFR) was collected for five minutes while participants chewed 1 g of gum base, following a two-minute pre-stimulation phase to clear retained saliva. Flow rates for both UFR and SFR were recorded in milliliters per minute (mL/min).

Assessment of salivary pH and buffering capacity

Salivary pH and buffering properties were assessed using GC Saliva Check Buffer kits (GC, Tokyo, Japan). After UFR collection, a pH test strip was submerged in the resting saliva for 10 seconds, and the resulting color was matched to the kit's reference chart. Saliva with pH ≥ 6.8 was considered within the normal range, whereas pH < 6.6 was classified as acidic. To evaluate buffering capacity, stimulated saliva was applied to three designated areas of the test strip using a pipette. After two minutes, the color response was scored as follows: green = 4 points, green/blue = 3 points, blue = 2 points, red/blue = 1 point, and red = 0 points. Total scores were interpreted according to the manufacturer's scheme: 0–5 indicated very low, 6–9 low, and 10–12 normal buffering capacity.

Oral bacteria sampling and identification

Bacterial presence and abundance were assessed in UFR, SFR, and mask samples. Fourteen key oral bacteria were analyzed in saliva, including Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Prevotella nigrescens, Eikenella corrodens, Campylobacter rectus, Fusobacterium nucleatum, Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia, Lactobacillus casei, Streptococcus mutans, Streptococcus sobrinus, Parvimonas micra, and Eubacterium nodatum.

For mask samples, the inner surface of KF94 masks worn continuously for over three hours was washed with 20 mL of preservation solution to collect adherent microbes. KF94 masks are capable of filtering approximately 94% of airborne particles, including microorganisms.

Bacterial DNA extraction

Samples from masks and saliva were vortexed to ensure thorough mixing. From each sample, 500 µL was combined with 500 µL of lysis buffer (5 mM EDTA, 5 M guanidine hydrochloride, 0.3 M sodium acetate) and incubated at 65°C for 10 minutes. S2 buffer (0.25 g/mL silicon dioxide; Merck KGaA, Darmstadt, Germany) was then added (20 µL) and mixed by vortexing, followed by a 5-minute incubation at room temperature with periodic inversion. Samples were centrifuged at 5,000 rpm for 30 seconds, and the supernatant was discarded. DNA purification involved adding 1 mL of activated PureLink PCR purification buffer 1 (50 mM MOPS, pH 7.0, 1 M NaCl, with 160 µL ethanol), followed by vortexing and centrifugation. Wash buffer 2 (1,000 µL ethanol) was applied to the pellet, vortexed, and centrifuged again. DNA was eluted with 100 µL of elution buffer (100 mM Tris-HCl, pH 7.5, 1 M EDTA) and incubated at 65°C for 10 minutes. For PCR analysis, samples were centrifuged at 13,000 rpm for 5 minutes, and the supernatant was transferred to sterile tubes.

Real-Time PCR amplification

Quantitative PCR (qPCR) was performed to determine the abundance of the 14 target bacterial species in UFR, SFR, and mask samples using species-specific primers. Total bacterial load was quantified using universal 16S rRNA primers, as in our previous study [19]. Total DNA copy numbers were calculated using conservative 16S rRNA primer probes for each sample **(Table 1).**

Table 1. Bacteria 16s RNA primer probe for quantifying total bacteria.

No	Name	Sequence	Bp
1	Forward	CTCAAAKGAATTGACGGGG	19
2	Reverse	GTCATCCMMACCTTCCTC	18
3	Probe	5′Cy5-CATGGYTGTCGTCAGCTCGTG-3′BHQ2	21

Quantitative PCR setup

For each real-time PCR assay, a reaction mixture was prepared containing 5 µL of extracted DNA, 2.5 pM of both forward and reverse primers, and 10 µL of a 2X master mix (GeNet Bio, Daejeon, Korea), yielding a total volume of 20 µL per reaction. Thermal cycling began with an initial denaturation at 95°C for 10 minutes, followed by 45 amplification cycles consisting of 95°C for 15 seconds and 60°C for 1 minute for combined annealing and extension [20]. Each run included plasmid DNA corresponding to the targeted bacterial species as positive controls and DNase/RNase-free water as negative controls to validate assay performance.

Estimation of bacterial DNA copy number

Saliva samples were prepared by combining 2 mL of the sample with 2 mL of stock preservation solution. Mask samples were processed by submerging the mask in 20 mL of preservation solution to recover attached bacteria. For DNA extraction, 500 µL of the prepared solution from each sample type (UFR, SFR, mask) was used, with DNA eluted in 100 µL. Five microliters of this eluted DNA was applied to qPCR, and bacterial DNA copy numbers were calculated using a standard curve. Final results were expressed per milliliter of saliva or per 20 mL of preservation solution. The preservation solution contained Tris-HCl, urea, sodium acetate, SDS, EDTA, sodium ascorbate, and ethanol.

Microbial α-diversity assessment

To evaluate bacterial diversity within each sample, the Shannon diversity index was calculated, providing a measure of both species richness and evenness. Total bacterial load, represented by the sum of DNA copies, was used as an indicator of richness. Shannon index values were computed for all samples to compare α-diversity across saliva and mask microbiomes [21]:

$$H = -\sum pi \times ln(pi) \quad (1)$$

The Shannon diversity index (H) was used to evaluate α-diversity within the microbial communities. In this calculation, the relative abundance of each species (p_i) is expressed as the proportion of cells of that species (n) relative to the total number of bacterial cells (N) in the community, i.e., $p_i = n/N$. The index ranges from zero, indicating no diversity, to higher values reflecting greater diversity. In natural microbial ecosystems, Shannon index values typically fall between 1.5 and 3.5, with values rarely reaching 4.5 [22].

Statistical analysis

Descriptive statistics, including absolute counts, percentages, means, and standard deviations, were calculated for all categorical and continuous variables. Differences in oral bacterial abundance among the three sample types (UFR, SFR, and mask) were assessed using analysis of variance (ANOVA). Comparisons between participants with and without xerostomia were conducted using the Mann–Whitney U test. For categorical data, χ^2 tests, Fisher's exact test, and Bonferroni-adjusted tests were applied to examine differences in proportions. Relationships between continuous variables were evaluated using Spearman's correlation analysis, with correlation strength indicated by r values approaching ±1 [23]. Multiple linear regression models were constructed to explore associations between the total bacterial load (dependent variable) and individual bacterial species (independent variables), with age included as a covariate. Beta coefficients (β), standard errors (SE), and 95% confidence intervals (CI) were reported. Statistical significance was defined as $p < 0.05$. Analyses were performed using IBM SPSS Statistics (version 24.0; IBM Corp., Armonk, NY, USA), and Shannon diversity indices were computed in R (version 4.0.2; R Foundation for Statistical Computing, Vienna, Austria).

Results and Discussion

Among the 55 participants, 14 (25.5%) reported xerostomia. Unstimulated salivary flow rate was significantly lower in this group compared to participants without xerostomia (0.92 ± 0.13 vs. 1.10 ± 0.37 mL/min, $p < 0.05$). No significant differences were observed between the two groups in terms of age, gender distribution, salivary pH, buffer capacity, or mask-wearing duration **(Table 2).** The overall mean salivary pH (7.16 ± 0.47) and buffering capacity (10.01 ± 0.95) for all participants remained within the normal physiological range.

Table 2. Demographics, clinical characteristics, and volatile sulfite compounds levels of participants

executar	Non-xerostomia (n = 41)	Xerostomia (n = 14)	Total (n = 55)	p-value (Non-xerostomia vs. Xerostomia)
Epidemiology				
Age (years)[a]	38.78 ± 12.42	36.43 ± 12.99	38.18 ± 12.49	0.560
Sex (female)[b]	33 (80.5%)	12 (85.7%)	45 (81.8%)	1.000
Saliva				
UFR (ml/min)[a]	**1.10 ± 0.37**	**0.92 ± 0.13**	1.06 ± 0.33	**0.048***
SFR (ml/min)[a]	1.43 ± 0.44	1.38 ± 0.34	1.41 ± 0.42	0.573
Salivary pH[a]	7.15 ± 0.51	7.17 ± 0.34	7.16 ± 0.47	0.868
Buffer capacity[a]	10.00 ± 1.04	10.07 ± 0.62	10.01 ± 0.95	0.743
Mask wearing duration (hours)[a]	5.76 ± 2.89	6.36 ± 3.20	5.91 ± 2.95	0.541

Data are presented as mean ± SD or n (%). [a] Mann–Whitney U test; [b] Chi-square test (two-sided). *p < 0.05 was considered statistically significant. Significant results are shown in bold.

Quantification of bacterial DNA and red complex species

Of the fourteen bacterial species examined, nine were classified as Gram-negative (A.

actinomycetemcomitans, P. intermedia, P. nigrescens, E. corrodens, C. rectus, F. nucleatum, P. gingivalis, T. denticola, and T. forsythia), and five were Gram-positive (L. casei, S. mutans, S. sobrinus, P. micra, and E. nodatum). When comparing sample types, total DNA levels were substantially higher in saliva—both unstimulated (UFR: 43,214,244.45 ± 86,900,936.03) and stimulated (SFR: 41,015,254.15 ± 109,947,416.90)—than on the inner surface of face masks (299,449.14 ± 1,356,728.10; $p < 0.001$).

Analysis of mask microbiota revealed that P. gingivalis was the dominant Gram-negative species, followed by F. nucleatum, P. nigrescens, E. corrodens, T. forsythia, and T. denticola. Among Gram-positive bacteria, L. casei had the highest prevalence, with P. micra and E. nodatum detected at lower levels **(Figure 1).** Notably, while total bacterial DNA and Gram-negative species were significantly more abundant in both UFR and SFR compared with masks, Gram-positive species showed no significant differences across the three sample types.

The "red complex," which includes P. gingivalis, T. forsythia, and T. denticola, is recognized for its role in severe periodontal pathology [24]. Consistent with this, red complex bacteria were found in significantly higher amounts in unstimulated saliva than on masks, indicating that the microbial composition on the inner surface of face masks is distinct and exhibits lower abundance of pathogenic oral species compared to saliva.

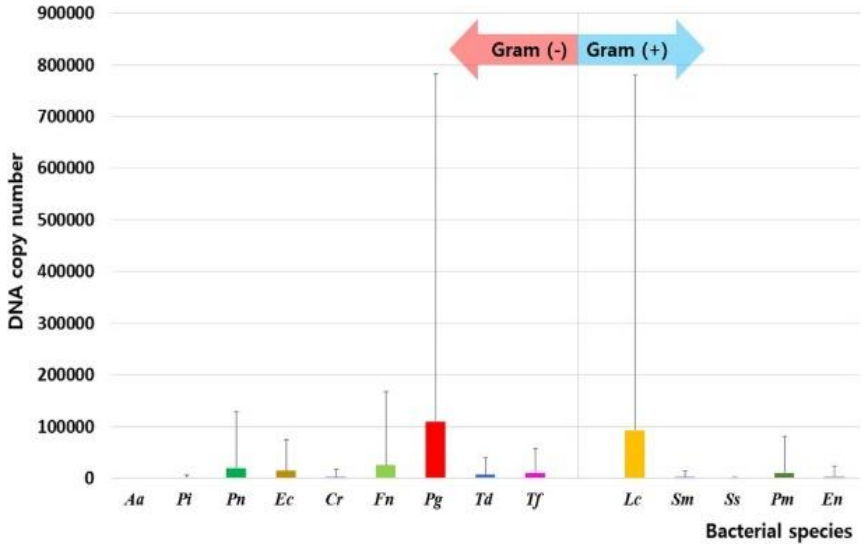


Figure 1. Composition of Oral Bacteria on the Inner Surface of Face Masks. Aa= Aggregatibacter actinomycetemcomitans; Pi= Prevotella intermedia; Pn= Prevotella nigrescens; Ec= Eikenella corrodens; Cr= Campylobacter rectus; Fn, Fusobacterium nucleatum; Pg= Porphyromonas gingivalis; Td= Treponema denticola; Tf= Tannerella forsythia; Lc= Lactobacillus casei; Sm= Streptococcus mutans; Ss= Streptococcus sobrinus; Pm= Parvimonas micra; En= Eubacterium nodatum.

When comparing sample types, Gram-negative bacteria such as P. intermedia, E. corrodens, P. gingivalis, and T. forsythia were significantly more abundant in unstimulated saliva (UFR) than on masks, while no statistically meaningful difference was observed between stimulated saliva (SFR) and mask samples. In contrast, F. nucleatum, P. nigrescens, C. rectus, and T. denticola showed significantly higher DNA counts in both UFR and SFR compared with masks (all $p < 0.05$). Among Gram-positive bacteria, only P. micra demonstrated a significantly elevated presence in UFR relative to mask samples **(Table 3).**

Table 3. Comparison of DNA copies of each bacterium, red complex species, and Shannon's diversity index of 14 bacterial species

Species / Group	Mask (Mean ± SD)	SFR (Mean ± SD)	UFR (Mean ± SD)	p-value	ANOVA post-hoc
Gram (−)					
Aggregatibacter actinomycetemcomitans (Aa)	0.0 ± 0.0	134,356.69 ± 540,986.39	84,755.60 ± 578,052.88	0.299	–
Prevotella intermedia (Pi)	1,009.07 ± 4,756.38	2,621,023.11 ± 6,569,457.88	3,252,431.72 ± 10,798,693.98	**0.049***	UFR > Mask

Prevotella nigrescens (Pn)	20,065.93 ± 108,399.48	5,776,950.63 ± 9,490,677.27	7,788,593.60 ± 13,760,420.11	**0.000*** **	UFR, SFR > Mask
Eikenella corrodens (Ec)	14,441.06 ± 60,449.39	1,559,684.78 ± 2,837,115.07	2,922,048.32 ± 7,027,864.73	**0.003** **	UFR > Mask
Capnocytophaga rectus (Cr)	2,420.45 ± 14,756.47	321,813.35 ± 1,056,329.93	806,903.23 ± 2,686,562.33	**0.041***	UFR, SFR > Mask
Fusobacterium nucleatum (Fn)	25,462.62 ± 141,779.44	7,175,748.41 ± 10,887,153.63	10,531,300.90 ± 17,370,158.89	**0.000*** **	UFR, SFR > Mask
Porphyromonas gingivalis (Pg)	109,153.86 ± 673,306.71	7,136,010.12 ± 19,615,461.35	13,088,432.59 ± 37,707,571.96	**0.023***	UFR > Mask
Tannerella forsythia (Td)	6,748.89 ± 33,845.44	372,568.44 ± 738,112.41	477,025.28 ± 989,482.40	**0.002** **	UFR, SFR > Mask
Treponema denticola (Tf)	10,335.07 ± 47,508.19	1,055,919.86 ± 2,316,593.77	1,749,732.47 ± 3,972,943.97	**0.003** **	UFR > Mask
Gram (+)					
Lactobacillus crispatus (Lc)	93,236.38 ± 687,696.80	13,235,096.48 ± 98,117,235.79	14,219.64 ± 95,547.16	0.373	–
Streptococcus mitis (Sm)	2,256.01 ± 12,020.02	171,988.60 ± 619,753.35	140,262.82 ± 726,608.70	0.232	–
Streptococcus sanguinis (Ss)	310.76 ± 1,671.51	10,774.20 ± 72,600.66	1,413.51 ± 7,256.32	0.361	–
Parvimonas micra (Pm)	10,823.97 ± 70,561.99	1,176,769.25 ± 3,274,469.32	1,844,955.69 ± 5,906,186.05	**0.047***	UFR > Mask
Enterococcus faecalis (En)	3,185.06 ± 20,219.21	266,550.33 ± 758,378.21	512,169.07 ± 1,918,624.16	0.084	–
Total bacteria	299,449.14 ± 1,356,728.10	41,015,254.15 ± 109,947,416.90	43,214,244.45 ± 86,900,936.03	**0.000*** **	UFR, SFR > Mask
Gram (−) total	164,174.34 ± 935,102.95	18,978,326.97 ± 35,550,304.76	30,169,922.80 ± 66,728,696.32	**0.002** **	UFR, SFR > Mask
Gram (+) total	135,274.81 ± 726,509.83	22,036,927.28 ± 99,405,273.61	13,044,321.63 ± 22,908,382.41	0.150	–
Red complex (Pg + Td + Tf)	126,237.82 ± 753,796.99	8,564,498.42 ± 22,077,308.09	15,315,190.34 ± 41,966,894.16	**0.016***	UFR > Mask
Shannon's diversity index	1.26 ± 1.51	2.66 ± 0.76	2.64 ± 0.78	**0.000*** **	UFR, SFR > Mask

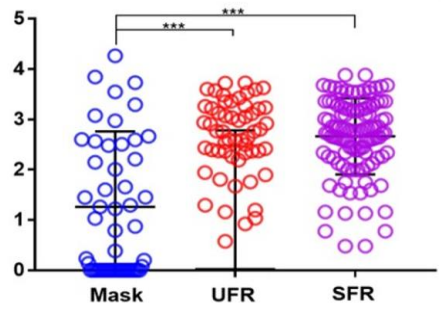
Data were analyzed using ANOVA followed by post hoc comparisons, with statistical significance set at $p < 0.05$. Significant differences are highlighted in bold. Abbreviations used include: Aa= Aggregatibacter actinomycetemcomitans; Pi= Prevotella intermedia; Pn= Prevotella nigrescens; Lc= Lactobacillus casei; Fn= Fusobacterium nucleatum; Sm= Streptococcus mutans; Ss= Streptococcus sobrinus; Td= Treponema denticola; Pg= Porphyromonas gingivalis; Tf= Tannerella forsythia; Ec= Eikenella corrodens; Pm= Parvimonas micra; Cr= Campylobacter rectus; En= Eubacterium nodatum.

Mask samples represent bacterial DNA collected from the inner mask surface, while UFR and SFR correspond to bacterial DNA in unstimulated and stimulated saliva, respectively. Gram-negative species include Aa, Pi, Pn, Ec, Cr, Fn, Pg, Td, and Tf; Gram-positive species include Lc, Sm, Ss, Pm, and En. The "red complex" refers to the pathogenic periodontal group consisting of Pg, Td, and Tf. Significance levels are indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

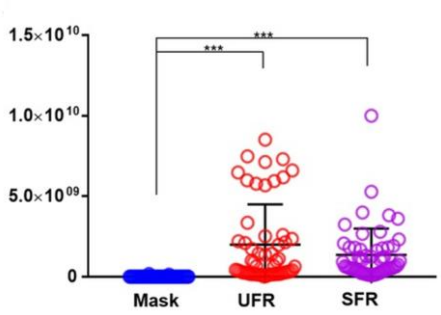
Notably, there were no significant differences in the DNA copy numbers of any of the 14 bacterial species, total bacterial load, or Shannon diversity index between participants with or without xerostomia. The presence of xerostomia did not significantly alter bacterial abundance in UFR, SFR, or mask samples.

Shannon diversity index

Shannon diversity was substantially higher in saliva compared with mask samples, with values of 2.64 ± 0.78 for UFR, 2.66 ± 0.76 for SFR, and 1.26 ± 1.51 for masks ($p < 0.001$) **(Figure 2).** A positive correlation was observed between total bacterial DNA recovered from masks and the Shannon index for mask samples ($r = 0.510$, $p < 0.01$). Shannon indices of UFR and SFR were strongly correlated with each other ($r = 0.828$, $p < 0.001$), but no significant association existed between saliva diversity and mask diversity **(Figure 3).** These findings indicate that the bacterial diversity on the inner mask surface operates independently from the oral microbiome present in saliva.

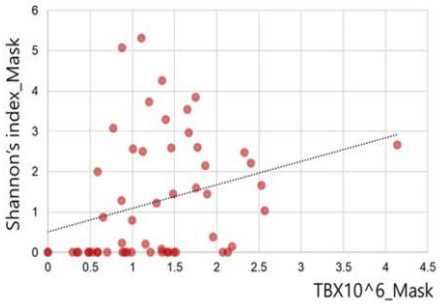


a)

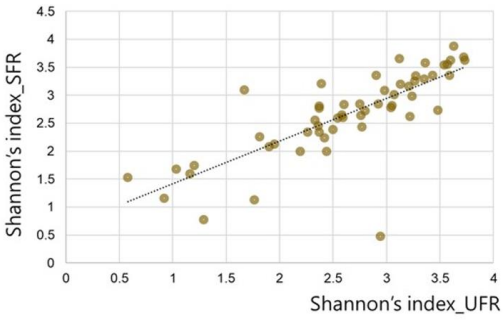


b)

Figure 2. Assessment of oral microbial diversity and overall bacterial quantity in samples from the mask, under unstimulated salivary flow (UFR), and under stimulated salivary flow (SFR). (a) Shannon diversity index, (b) Total bacterial DNA copies. Statistical comparisons were conducted via ANOVA, with significance defined as $p < 0.05$ (***$p < 0.001$).



a)



b)

Figure 3. Relationships between saliva diversity and inner mask microbiota. (a) Association of Shannon's diversity index with total bacterial DNA copies (TB) on the inner mask. (b) Comparison of Shannon's index between SFR and UFR.

Determinants of oral bacterial load on the inner mask

Advancing age of participants was significantly linked to elevated levels of P. intermedia ($r = 0.185$), C. rectus ($r = 0.204$), P. gingivalis ($r = 0.021$), T. denticola ($r = 0.185$), and F. nucleatum ($r = 0.113$) in mask samples (all $p < 0.05$), along with a rise in total bacterial DNA copies ($r = 0.162$, $p < 0.05$). Lower UFR values were associated with increased S. mutans ($r = -0.268$, $p < 0.05$), whereas reduced SFR corresponded to higher quantities of P. intermedia ($r = -0.301$), L. casei ($r = -0.270$), and P. micra ($r = -0.270$) (all $p < 0.05$). In contrast, salivary pH, buffer capacity, duration of mask use, and xerostomia showed no significant correlation with total bacterial load, individual species, or the red complex bacteria (P. gingivalis, T. denticola, T. forsythia) **(Table 4).**

Table 4. Correlation between clinical indicators and oral bacteria of inner surface of the mask.

r	*Aa* Mask	*Pi* Mask	*Pn* Mask	*Ec* Mask	*Cr* Mask	*Pg* Mask	*Td* Mask	*Tf* Mask	*Lc* Mask	*Fn* Mask	*Sm* Mask	*Ss* Mask	*Pm* Mask	*En* Mask	TB Mask	Red complex	Shannon's diversity
Age		**0.185**	−0.017	0.206	**0.204**	**0.021**	**0.081**	−0.251	0.234	**0.113**	−0.072	0.105	**−0.098**	−0.164	**0.162**	**−0.011**	0.233
UFR		−0.144	−0.042	0.017	−0.011	−0.023	−0.130	0.019	−0.155	0.013	**−0.268**	−0.022	0.063	0.234	−0.162	−0.077	−0.055

SFR		**−0.301**	−0.081	−0.070	−0.265	−0.140	−0.034	−0.163	**−0.270**	−0.230	−0.070	−0.127	**−0.270**	−0.091	−0.217	−0.114	−0.028
Salivary pH		0.027	−0.012	−0.015	−0.042	−0.037	−0.039	−0.132	−0.055	0.018	0.060	−0.016	−0.128	−0.068	−0.088	−0.133	−0.032
Buffer capacity		−0.116	0.090	0.128	0.090	0.084	0.216	0.094	−0.019	−0.176	0.121	0.135	0.050	0.190	0.102	0.013	0.055
Mask wearing time		0.153	0.080	0.064	0.005	0.057	0.127	−0.093	0.194	0.064	0.012	−0.097	−0.041	0.028	0.087	0.013	0.035
Xerostomia		−0.185	−0.017	0.206	−0.204	−0.021	0.081	−0.251	0.234	−0.113	−0.072	0.105	−0.098	−0.164	−0.058	−0.011	0.098

Spearman correlation analysis was conducted to assess relationships, with statistical significance defined as $p < 0.05$ (*$p < 0.05$). Significant findings are indicated in bold. Key abbreviations include: r for correlation coefficient; "Bacterium name Mask" for bacterial load in mask samples; TB representing total bacterial DNA copy number; Red complex denoting the combined DNA of periodontitis-associated pathogens (Pg, Td, Tf); Shannon's diversity indicating the diversity index of the total bacterial population; and UFR and SFR for unstimulated and stimulated whole saliva, respectively. Bacterial species evaluated were Aa (Aggregatibacter actinomycetemcomitans), Pi (Prevotella intermedia), Pn (Prevotella nigrescens), Lc (Lactobacillus casei), Fn (Fusobacterium nucleatum), Sm (Streptococcus mutans), Ss (Streptococcus sobrinus), Td (Treponema denticola), Pg (Porphyromonas gingivalis), Tf (Tannerella forsythia), Ec (Eikenella corrodens), Pm (Parvimonas micra), Cr (Campylobacter rectus), and En (Eubacterium nodatum).

For the multiple linear regression, total bacterial quantity in masks was the outcome variable, while the presence of individual bacterial species served as predictors. F. nucleatum showed the strongest association with total bacterial abundance in masks (β = 1,292.72; 95 percent CI = 947.34–1,638.11), followed by P. gingivalis (β = 133.82; 95 percent CI = 81.16–186.48) and E. corrodens (β = 84.80; 95 percent CI = 10.85–158.75), resulting in a highly predictive model ($R = 0.996$, adjusted $R^2 = 0.989$). In contrast, for UFR and SFR saliva samples, although F. nucleatum remained the primary contributor to total bacterial DNA levels, the influence and significance of other bacterial species differed from the mask samples (UFR: $R = 0.956$, adjusted $R^2 = 0.884$; SFR: $R = 0.967$, adjusted $R^2 = 0.913$) **(Table 5).**

Table 5. Linear regression analysis regarding total amount of oral bacteria as independent variable.

Dependent variable	**Total bacteria_Mask**					**Total bacteria_UFR**					**Total bacteria_SFR**				
	β	**SE**	***p*-value**	**95% CI Lower**	**95% CI Upper**	**β**	**SE**	***p*-value**	**95% CI Lower**	**95% CI Upper**	**β**	**SE**	***p*-value**	**95% CI Lower**	**95% CI Upper**
Gram (−)															
Aa						190.436	363.822	.604	−544.876	925.748	15.802	154.631	.919	−296.719	328.324
Pi	**−745.70**	120.54	**0.00**	−989.14	−502.26	29.19	34.76	0.41	−41.05	99.44	40.89	45.90	0.38	−51.88	133.66

Pn	−147.95	75.41	0.06	−300.25	4.35	**72.48**	29.55	**0.02**	12.75	132.21	−0.40	19.77	0.98	−40.35	39.56
Ec	**84.80**	36.62	**0.03**	10.85	158.75	20.54	36.61	0.58	−53.46	94.53	36.02	39.58	0.37	−43.98	116.01
Cr	681.98	1,013.72	0.50	−1,365.27	2,729.24	60.55	177.14	0.73	−297.46	418.56	98.27	240.92	0.69	−388.64	585.18
Fn	**1,292.72**	171.02	**0.00**	947.34	1,638.11	**168.89**	13.73	**0.00**	141.13	196.64	**138.64**	20.18	**0.00**	97.85	179.42
Pg	**133.82**	26.07	**0.00**	81.16	186.48	**56.57**	20.56	**0.01**	15.01	98.13	**−66.88**	31.13	**0.04**	−129.79	−3.98
Td	114.71	128.68	0.38	−145.16	374.57	−419.27	316.79	0.19	−1,059.52	220.97	−336.28	219.68	0.13	−780.26	107.70
Tf	146.46	108.63	0.19	−72.94	365.85	−239.98	186.53	0.21	−616.97	137.00	**373.41**	174.11	**0.04**	21.53	725.29
Gram (+)															
Lc	0.33	0.67	0.63	−1.03	1.69	−699.21	1,231.24	0.57	−3,187.64	1,789.21	−0.70	0.71	0.33	−2.14	0.74
Sm	10.00	281.40	0.97	−558.30	578.31	240.32	171.26	0.17	−105.81	586.45	**367.37**	110.64	**0.00**	143.76	590.99
Ss	−91.44	271.14	0.74	−639.02	456.15	15,661.22	13,553.27	0.41	−22,621.20	53,943.64	−1,664.79	5,381.93	0.76	−12,542.08	9,212.51
Pm	**−563.08**	113.83	**0.00**	−792.97	−333.20	**−546.16**	81.58	**0.00**	−711.04	−381.27	158.19	138.54	0.26	−121.80	438.18
En	**−1,991.18**	386.89	**0.00**	−2,772.51	−1,209.85	−71.09	159.11	0.66	−392.66	250.48	−351.32	348.03	0.32	−1,054.72	352.07

The outcomes were derived using linear regression modeling, with statistical relevance determined when the p-value fell below the 0.05 threshold, and any variables meeting this criterion are highlighted in bold. In this context, β corresponds to the estimated beta coefficient, SE represents the standard error, and CI denotes the confidence interval. Total bacteria_Mask refers to the DNA copy count of all bacterial species detected on the inner surface of the mask, whereas Total bacteria_UFR indicates the overall bacterial DNA quantity measured under unstimulated salivary flow rate conditions, and Total bacteria_SFR reflects the total bacterial DNA load identified during stimulated salivary flow assessments. Gram (−) denotes Gram-negative organisms, while Gram (+) signifies Gram-positive taxa. The abbreviations for individual bacterial species are maintained as follows: Aa= Aggregatibacter actinomycetemcomitans; Pi=

Prevotella intermedia; Pn= Prevotella nigrescens; Lc= Lactobacillus casei; Fn= Fusobacterium nucleatum; Sm, Streptococcus mutans; Ss= Streptococcus sobrinus; Td= Treponema denticola; Pg= Porphyromonas gingivalis; Tf= Tannerella forsythia; Ec, Eikenella corrodens; Pm= Parvimonas micra; Cr, Campylobacter rectus; En= Eubacterium nodatum.

Airborne spread of COVID-19 can occur when individuals infected with SARS-CoV-2 emit droplets or fine aerosol particles during normal breathing as well as during more forceful respiratory actions. Because therapeutic options remain limited [25], mask use has served as an accessible and practical strategy to curb transmission. In the present investigation, we examined how bacterial communities accumulate on the inner surface of worn masks and explored their connection to the salivary microbiome. Particular attention was given to nine Gram-negative taxa [A. actinomycetemcomitans, P. intermedia, P. nigrescens, E. corrodens, C. rectus, F. nucleatum, P. gingivalis, T. denticola, T. forsythia] and five Gram-positive organisms [L. casei, S. mutans, S. sobrinus, P. micra, E. nodatum], using both unstimulated and stimulated saliva samples. Our findings revealed clear associations between bacterial abundances in saliva and those detected on the inside of the mask. Although the total bacterial load and diversity were consistently lower on the mask than in UFR and SFR, the mask still exhibited a distinct pattern of oral microbial colonization.

Because participants used sterile masks at baseline, it is reasonable to assume that the microbes recovered from the inner surface originated from the oral cavity. Yet, the microbial configuration within the mask environment did not simply mirror that of saliva. Among Gram-negative species, the sequence of abundance was P. gingivalis > F. nucleatum > P. nigrescens > E. corrodens > T. forsythia > T. denticola, while Gram-positive bacteria appeared in the order L. casei > P. micra > E. nodatum. Oral microorganisms often adhere firmly to many niches in the mouth, embedding themselves into the native community in ways that encourage persistence [26], but their adherence and proliferation on mask surfaces likely vary considerably among species. P. gingivalis—an anaerobic Gram-negative pathogen recognized as a keystone organism in periodontitis [27]—can be detected even among individuals without periodontal disease, with reports showing occurrence in roughly 25% of healthy subjects [28]. Members of the red complex, such as P. gingivalis, T. forsythia, and T. denticola, are known to strengthen the biofilm's resilience and alter surrounding microbial composition [29], contributing to periodontal decline. Notably, P. gingivalis was the leading Gram-negative species identified on mask interiors. In our earlier work, elevated P. gingivalis levels inside masks were linked to increased volatile sulfide compounds—primary contributors to halitosis—along with other anaerobic species including T. denticola, T. forsythia, P. intermedia, and P. nigrescens [19]. Interestingly, xerostomia did not influence the mask-associated microbiome. Prior research among dental students similarly reported no significant relationship between mask-wear duration and xerostomia severity [16]. Although P. gingivalis and F. nucleatum have been associated with dry mouth following radioiodine therapy [30], it remains unclear how mask use might influence xerostomia-related microbial shifts in the general population. In our data, red complex abundance was significantly reduced in mask samples when compared with UFR but did not differ from SFR. L. casei, a Gram-positive species frequently used in probiotic formulations and known to antagonize P. gingivalis [31], also appeared on mask surfaces, underscoring the complexity of microbe–microbe interactions within this unique environment.

A subset of oral bacteria seems particularly capable of attaching to and multiplying on mask materials. Total bacterial DNA copy numbers in UFR and SFR were markedly higher—approximately 130-fold greater—than those obtained from masks. Shannon's diversity index, which quantifies richness as well as distributional evenness [32], showed strong concordance between UFR and SFR ($r = 0.828$, $p < 0.001$), but neither saliva condition correlated with mask diversity. Mask samples exhibited considerably reduced diversity (1.26 ± 1.51) relative to UFR (2.64 ± 0.78) and SFR (2.66 ± 0.76). Earlier investigations comparing oral sites found median Shannon's index values of 2.308 in saliva, 1.413 in buccal mucosa, and 2.095 on the tongue, all higher than tooth surfaces but lower than subgingival plaque, which showed substantially greater diversity [33]. Why suspended salivary communities and subgingival environments harbor more diverse microbial assemblages is not entirely understood. Healthy gingiva typically supports a mixture of Gram-positive cocci, limited Gram-positive bacilli, and minimal Gram-negative cocci [34]. Because this is the first study to characterize mask-associated oral microbiota, comparisons with prior literature are not possible, and further work is required to determine which parts of the oral cavity best correspond to the composition found on used masks.

The microbiological profile of a mask might also shift in connection with demographic or physiological factors. Although population-level data remain limited,

age and salivary flow are two variables expected to influence oral bacterial dynamics. In this investigation, increasing age showed a weak but positive correlation with several Gram-negative species—P. intermedia, C. rectus, P. gingivalis, and T. denticola—all of which hold pathogenic relevance for adult periodontal disease [35–37]. Since periodontitis affects roughly 14% of individuals and becomes more prevalent with age [38], age-related increases in these organisms are not unexpected. Conversely, higher salivary flow corresponded to diminished representation of certain bacteria within the mask microbiome, including S. mutans, a Gram-positive anaerobic coccus strongly associated with dental caries [39], as well as P. intermedia, L. casei, and P. micra. Saliva contains antimicrobial components such as lysozyme and lactoferrin, which can disrupt bacterial integrity or limit nutrient availability [40], suggesting a biological rationale for reduced pathogen levels when salivary output increases. However, research assessing salivary flow effects on either cariogenic pathogens or probiotic species such as L. casei is lacking.

Microbial accumulation on mask interiors may also influence the skin beneath the mask. Park *et al*. documented that prolonged mask use produced substantial changes in skin characteristics—including temperature, erythema, hydration, and sebum secretion—after 1 and 6 hours [41], and these alterations differed when comparing covered versus uncovered areas [41]. Because participants in the current study began with sterile KF94 masks, the bacteria later recovered from mask surfaces likely originated from oral secretions or adjacent perioral skin [42]. The enclosed, warm, and humid microenvironment created during mask wear may facilitate microbial growth, potentially contributing to dermatological complications. Staphylococcus aureus and Streptococcus pyogenes account for the majority of skin infections, though anaerobes such as Prevotella and Bacteroides species are also implicated [43, 44]. P. gingivalis, a principal driver of oral dysbiosis and periodontal inflammation [45], is capable of surviving under low-oxygen conditions [46], an environment similar to that found inside a mask. Its predominance in our samples raises questions about whether similar organisms might irritate or infect facial skin. Recent reports have noted cutaneous manifestations involving P. gingivalis [47]. Although no participants in this study presented with clinically evident skin disorders—and no specific dermatological assessments were performed—the potential link between mask-associated oral bacteria and skin disturbances warrants further targeted investigation to identify which taxa might contribute to such conditions.

This investigation set out to characterize how the oral microbiome behaves in relation to mask use by examining the bacterial diversity present on the inner surface of worn masks, quantifying the abundance of individual taxa, and identifying factors that may shape these microbial patterns. In addition, the findings were assessed against those obtained from whole-saliva samples to determine how closely mask-associated communities reflect salivary profiles. Although the use of masks dates back to the 17th-century European epidemics [48], their role has once again become indispensable, as they greatly contributed to reducing coronavirus transmission during the COVID-19 outbreak. From the current results, maintaining the cleanliness of the mask's inner surface appears prudent to limit possible bacterial-related complications associated with prolonged mask wear. The data also shed light on potential mechanisms underlying mask-related skin disturbances, reinforcing the need for regular mask replacement. One constraint of this study is the imbalance in participant sex distribution, as more men than women enrolled due to recruitment being conducted through hospital advertisements. In addition, no children, adolescents, or elderly individuals participated, which means age- or sex-related influences on microbiome composition may be under-represented. Because volunteers were enrolled sequentially based on their willingness to participate, the proportions of individuals with xerostomia versus those without were unequal. Another limitation lies in the fact that only 14 major bacterial taxa commonly associated with the oral microbiome were analyzed. To more fully understand how mask wearing influences the entire microbial community, future research should involve larger cohorts and apply next-generation sequencing and multi-omics approaches capable of capturing a broader range of bacteria, as well as fungi and viruses.

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Ethics Statement: The research protocol for this study was reviewed in compliance with the Declaration of Helsinki and approved by the Institutional Review Board of Kyung Hee University Dental Hospital in Seoul, South Korea (KHD IRB, IRB No-KH-DT21023). Informed consent was obtained from all the participants. The patients/participants provided their written informed consent to participate in this study.

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