Bacterial and Fungal Isolation from Face Masks: Newly Emerged Hygiene Issues Under COVID-19 Pandemic

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Abstract

The COVID-19 pandemic has led people to wear face masks daily in public. Although the effectiveness of the face masks against viral transmission has been extensively studied, there is no report on potential hygiene issues due to bacteria and fungi attached to the face masks. We aimed to demonstrate 1, information of the usage duration and types of masks; 2, the number of bacteria and fungi attaching to masks; and 3, identification of the bacteria and fungi attaching to masks. We conducted a survey of 109 volunteers on their mask usage and lifestyles, and cultured bacteria and fungi on their masks. The colony numbers of bacteria were greater in the face-side than outer-side and those of fungal were fewer in the face-side than in the outer-side. A longer mask usage significantly increased the fungal colony counts, but not the bacterial colony counts. Although most identified microbes were non-pathogenic; S. epidermidis, S. aureus and Cladosporium, there were several pathogenic microbes; B. cereus, S. saprophyticus, Aspergillus, and Microsporum. We also investigated the associations between mask-attached microbes and individual lifestyles such as transportation and gargling. These findings suggest that people, especially compromised hosts, should avoid repeated use of masks to prevent microbial infection.

Introduction

The rapid global spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the resulting coronavirus disease 2019 (COVID-19) pandemic have led to urgent efforts to prevent the virus transmission. The most traditional and reasonable method to prevent respiratory infections is to wear face masks; several research groups have demonstrated its effectiveness against the respiratory viral transmission before the COVID-19 pandemic [1,2]. During the COVID-19 pandemic, increasing lines of evidence have supported the effectiveness of wearing face masks against SARS-CoV-2 and the droplets [3,4], although the World Health Organization (WHO) claims that face masks are effective only when used in the combinations with hand hygiene, the proper use, and disposal of masks [5].

Three types of face masks are commercially available for our daily lives: 1) non-woven masks, 2) polyurethane masks, and 3) gauze or cloth masks (**Figs. 1a, b**). Non-woven masks are commonly used around the world to prevent droplet infections by most respiratory microbes, including SARS-CoV-2 (**Fig. 1c**). Polyurethane masks have been used to protect against hay fever particularly in Asian countries. Since polyurethan masks are breathable and washable the masks have become popular and been reused several times during the COVID-19 pandemic. Although gauze masks are less popular, the masks can be washed, reused, and effective in preventing infections. Thus, the Japanese government distributed gauze masks to all citizens because of the shortage of non-woven masks in the early stage of the COVID-19 pandemic.

Although the effectiveness of face masks against viral transmission has been extensively studied [3,4], the hygiene issues in mask usage remain unclear. The standard mask usage is disposable non-woven masks. In some cases, however, people may use non-woven masks repeatedly or use different types of

masks in different situations depending on their socioeconomic cultures. For example, in Japan, the short supply of non-woven masks had led to the repeated use of disposable non-woven masks and the usage of other types of face masks, such as handmade masks and polyurethane masks [6]. Even after the shortage of mask supply has been resolved, some people have used disposable non-woven masks repeatedly or other types of face masks.

Among environmental pathogens, viruses cannot replicate without infecting host cells; most bacteria and fungi can survive and grow on a variety of materials depending on the conditions. Bacteria and fungi are widely present on the surface of the materials used in our daily lives (e.g., currency notes and in public transportation systems), where we can detect pathogenic bacteria and fungi [7–10]. However, there has been no study on what and how many microbes adhere to masks used on a daily basis; this is the neglected hygiene issue under the COVID-19 pandemic. Since masks can be a direct source of infection to the respiratory tract, digestive tract, and skin, it is crucial to maintain their hygiene in order to prevent bacterial and fungal infections that can exacerbate COVID-19. These are the neglected hygiene issues under the COVID-19 pandemic; it is crucial to maintain their hygiene in order to prevent bacterial and fungal infections that can exacerbate COVID-19. Thus, in this study, following a survey of 109 volunteers on their mask usage and lifestyles, we aimed to quantify and identify the bacteria and fungi attached to face masks by culturing microbes isolated from the masks.

Results

Mask types, gender differences, and duration of mask usage

During the study period, although the numbers of COVID-19 patients were relatively low in Japan, most people wore face masks in public places and all survey participants wore face masks. First, we collected information about the mask types and duration of mask usage from 109 participants: 63 male (58%) and 46 female (42%). The majority (78% in total) of the participants used non-woven masks (**Fig. 2a**); the percentage of the non-woven mask users was significantly higher than that of the participants wearing the other mask types ($P < 0.001$, most of them were polyurethane mask users except a few gauze or cloth mask users). Regarding the duration of mask usage, we found that 75% of non-woven mask users wore the masks for a single day. In contrast, 58% of the participants using the other-mask-types wore the same masks for two days or more (**Fig. 2b**). This could be due to the fact that other mask types, including polyurethane, gauze, and cloth masks, are all reusable. We found no significant differences between genders regarding the mask types and usage duration (**Figs. 2a and 2c**).

Microbial counts on the face-side and outer-side of masks

Microbes on the masks were cultured by pressing the face-side and outer-side of the masks onto agar plates (two plates per participant: the face-side and the outer-side). We incubated the agar plates for 18 hours and 5 days for bacterial and fungal propagation, respectively, and conducted colony-counting.

Bacteria (**Fig. 2d**): We observed bacterial colonies in 99% of the samples on the face-side and 94% on the outer-side; no colony was seen in one sample on the face-side and six samples on the outer-side. The colony counts of the face-side and outer-side were 168.6 ± 24.7 and 36.02 ± 7.0 [mean ± standard error of the mean (SEM)], respectively. We compared the colony counts between the face-side and outer-side in each individual, and found that the mean colony counts were 13.4-times higher on the face-side of masks (paired *t*-test, $P < 0.001$). To evaluate the influence of the mask types and duration of mask usage, we compared the colony counts among those who used the mask for one day (3–6 hours), two days, and longer based on the mask types [non-woven, others, and all (non-woven and others combined)]. We found no significant differences in the colony counts among the different mask types, regardless of the duration of usage.

Fungi (**Fig. 2e**): We observed fungal colonies in 79% of the samples on the face-side and 95% on the outer-side. The colony counts of fungi were fewer than those of bacteria. The colony counts on the face-side and outer-side were 4.6 ± 1.9 and 6.1 ± 1.9 (mean ± SEM), respectively. In contrast to the bacterial colonies, the fungal colony counts in each individual were 2.4-times higher on the outer-side than on the face-side (paired *t*-test, $P < 0.05$). When the participants used the same masks for more than two days, the fungal colony counts were increased, particularly on the outer-side of masks, compared with the one-day usage. We found no statistical differences in the colony counts between non-woven and “others” mask users except for the fungal colony counts of the outer-side of masks after one-day usage.

Since females preferentially make up their faces, we examined whether the bacterial and fungal colony counts could be different between males and females. Only the bacterial colony counts in the face-side samples of one-day users were significantly different, being lower in females (**Fig. S1**).

Microbial colonies and lifestyles: gargling, transportation, and natto consumption

We determined whether individual lifestyles could affect microbial counts on the masks that originate from the host (i.e., human) or the environment. One of the environmental factors that seemed to affect the levels of microbes on the masks is transportation to commute (**Fig. 3a**). Here, we classified into three transportation systems: 1) public transportation including trains and buses; 2) private vehicles such as cars and trucks; and 3) walking, biking, and motor bikes. We found no differences in the bacterial or fungal colony counts on both sides of the masks among the transportation systems.

Next, we evaluated two habits that are popular in Japan: gargling and natto consumption. Gargling (also known as mouth/throat wash) is a Japanese custom that has been believed to prevent respiratory infections [11]. Of the participants, 67% gargled at least once a day and usually gargled when they returned home. There were no differences in the bacterial or fungal colony counts among the participants regardless of gargling (**Fig. 3b**).

Natto is a traditional Japanese fermented food that is sticky when eaten and clings to the mouth and chopsticks(**Fig. 3c**). Natto is made by fermenting soybeans with the spore-forming bacterium *Bacillus subtilis*, which can survive in dry conditions. As expected, in this study, we observed the large white

colonies formed by *B. subtilis*. According to the questionnaire, 9% and 27% of the participants have eaten natto daily and weekly, respectively; 19% (21 of 109) of the participants ate natto during the experimental period. The participants who ate natto had a significantly higher incidence of large white *B. subtilis* colonies on both sides of the masks than those who did not.

Bacterial colony morphologies and identification

In the bacterial cultures, we observed a variety of colonies on the agar plates (**Fig. 4a**). We morphologically classified the colonies into four major colony forms and the other forms: 1) small white, 2) large white, 3) small yellow, 4) medium white, and the other forms including medium to large in size with yellow or pink in color, based on the colony size (small < 2 mm, medium 2–10 mm, and large 10 mm <), color, and frequencies (**Figs. 4a and 4b**). The frequency of colonies was calculated in two formulas: I) colony incidence = number of plates containing the colony of interest/total plate number (n = 109) × 100; and II) % total = counts of colonies of interest/total counts of colonies in each plate × 100 (then, the mean of % total from all plates was calculated). As shown in Fig. 4A, most participants had more than one colony form. The dominances of the four colony forms regarding the colony incidence and the mean % total of each colony were overall similar on the face-side and the outer-side (**Fig. 4b**). The small white colonies were most frequently observed, with their incidence and % total exceeding 80% and 70%, respectively.

To further determine bacteria composing each colony, we conducted Gram staining and 16S ribosomal RNA (rRNA) sequencing. The 16S rRNA sequencing showed that the small white colonies consisted mainly of *Staphylococcus epidermidis,* or *S. aureus*; the major bacteria species forming the small yellow colonies was *S. aureus*. The large white colonies were the second most observed ones and consisted of *B. subtilis*, a component of natto (as shown in **Fig. 3c**). The medium white colonies consisted of *Bacillus cereus* and *Bacillus simplex*; *B. cereus* was identified only on the outer-side of masks. Among the colonies, we also identified other bacterial species by 16S rRNA sequencing (**Fig. 4c**). Although most identified bacteria were non-pathogenic, there were several potential pathogenic bacteria in humans as follow: *S. aureus* (a commensal bacterium, but its overgrowth can cause various diseases); *B. cereus* (intestinal bacteria, causing food poisoning); *Staphylococcus saprophyticus* (urinary tract infection); and *Pseudomonas luteola* (opportunistic pathogen) [12–14].

Fungal colonies and identification

Following the quantification of fungal colonies, we further incubated them for another 2 days at 37°C to induce spore formation. Then, using lactophenol cotton blue staining, we identified fungi on face masks by their colony morphology macroscopically as well as the hyphae and spore morphology microscopically. Although some fungi could not be identified due to lack of spore formation, we identified a total of 13 fungal genera (**Fig. 5**). Among them, more than 20% of the participants had the four fungal genera, namely *Cladosporium, Fonsecaea, Mucor,* and *Trichophyton*, in common on both sides of the masks. The latter three of which are potentially pathogenic in humans (**Fig. 5)**.

Discussion

In this study, we demonstrated the associations between several factors and microbial contaminations of face masks commonly used around the world during the COVID-19 pandemic. Although some of our findings were what we had anticipated, there were several unpredicted findings, which need to be addressed as essential hygiene issues. In **Table 1**, we summarized the major findings and showed in bold the results with statistical differences ($P < 0.05$). The colony counts of face masks were higher in bacteria than in fungi; the bacterial and fungal colony counts were larger on the face-side and outer-side, respectively. The longer duration of mask usage correlated with increases in the fungal colony counts, but not the bacterial colony counts. We also found that non-woven masks had fewer fungi on the outer-side than other mask types. Although the bacterial colony counts were comparable in all mask types, those on the face-side were lower in females than in males.

We further conducted an ROC analysis to see the associations among the data obtained in this study shown in **Table 2**, where positive and negative associations were shown by the area under the curve (AUC) (**Figs. 2e and S1)**. The genus *Cladosporium*, the most frequently detected fungus in this study, was more frequently detected in females (58% females and 29% males). *B. subtilis* was more frequently detected on the masks used by the participants who ate natto at least once a month. In contrast, the transportation systems were not associated with the colony counts of bacteria or fungi. These results were consistent with our findings in **Fig. 3**, where neither usage of public transportation nor gargling did not alter the bacterial or fungal colony counts. On the other hand, eating natto strongly increased the *B. subtilis* colony counts on the masks. Although *B. subtilis* multiplies quickly and forms colonies large enough to outcompete other bacterial colonies, the presence of *B. subtilis* did not affect the counts of *S. epidermidis*, the most frequently detected bacteria in this study. The counts of white medium colonies seemed to be negatively affected by the presence of *B. subtilis* (AUC=0.65). This is consistent with the previous report [15] that *B. subtilis* inhibited the growth of *B. simplex*, which was a major component of a medium-sized white colony in the current study.

Although we anticipated that the counts of bacterial colonies could increase due to the duration of mask usage, this was not the case. This may be explained by the moisture requirement of bacteria [16,17]. While we wear a face mask, the humidity under the mask space becomes approximately 80%, in which bacteria can survive and grow [18,19]. In contrast, when a used mask is not worn for a long time, particularly at night, it dries out overnight and bacteria on the mask are likely to die due to the dry conditions. On the other hand, since fungi and their spores are resistant to drying, they can survive under the condition where masks dry out. This explains why fungi tended to accumulate and increase with longer mask usage. When we compared the microbial colony counts by mask types, there were no substantial differences in the microbial colony counts between non-woven and other mask types. These findings suggest that the higher fungal colony counts on the outer-side of masks would be due to the duration of mask usage, but not the mask types. Although most fungi isolated in this study were opportunistic pathogens rather than pathogenic **(Fig. 5)**, immunocompromised hosts should be advised to wear non-woven masks on a daily basis. We detected *B. cereus*, a foodborne pathogen, on the outer-side of masks in 5% of the

participants (**Fig. 4c**), suggesting that *B. cereus* might adhere to the face masks through hands from feces. Intensive handwashing is recommended since handwashing is effective in reducing the incidence of diarrhea [20].

The bacterial colony counts on the face masks were higher in males than in females among the daily users (**Fig. S1**). To test whether this difference was due to the fact that females apply foundation, we further recruited volunteers and ask them to wear the mask for 4 hours with foundation applied to only the left half of their faces. We found no differences in the bacterial colony counts between the left and right halves of the face masks (**Fig. S2a**). We also performed a principal component analysis (PCA), using the survey data based on a daily facial skincare routine (face-wash, lotion, sunscreen, and apply foundation) as well as the bacterial and fungal colony counts on the face-side of masks worn for 4 hours (**Fig. S2b**). The proportion of variance of principal component (PC) 1 was 44%; PC1 values reflected more intensive facial skincare. The colony counts of bacteria and fungi contributed to PC1 values negatively and positively, respectively. These findings suggest that facial skincare may decrease bacteria but increase fungi on the face masks. This is consistent with our findings that the participants who washed their faces with facial cleansers in the morning had lower bacterial and higher fungal colony counts than those who did not wash their faces or washed without facial cleansers in the morning (data not shown). Although we did not examine other factors that may contribute to the gender difference in the bacterial colony counts, these factors may include the higher facial temperature in males [21] and the gender difference of sweat and sebum [22].

There were several limitations in this study. First, the survey of face masks in this study was not comprehensive. Although the face masks were classified into three major types, they can be further subdivided according to the thickness, fabric coating, and other factors that may affect microbial growth. Second, in all the experiments, since the face masks were put on and taken off with bare hands, there was a possibility that microbes on the hands could be transferred to the face masks. Furthermore, we intentionally did not instruct the participants to wear gloves during the experimental period, since our objectives were to examine bacteria and fungi on the face masks under our normal lifestyles. Lastly, there is an argument that the face masks need to be thoroughly washed with detergent broth for better isolation of microbes on masks [23]. In this study, however, we decided to collect microbes on the face masks by simply pressing them onto the agar plates. Although this method may leave substantial amounts of microbes on the mask materials, we believe that easily detachable microbes are more relevant to respiratory infections. This simple method may also reduce the variability in the bacterial colony counts, compared with the labor-intensive washing method.

In this study, we focused on a newly emerged-hygiene issue in the current lifestyles of wearing face masks during the COVID-19 pandemic. These results will provide new insights into the face mask usage to prevent potential pathogenic infections.

Methods

Study design

This study was conducted between September and October, 2020. The participants were 109 medical students, 63 males (aged 22.4 ± 0.43) and 46 females (aged 21.2 ± 0.33, no significant difference between genders) at Kindai University Faculty of Medicine, Osaka, Japan. All experimental protocols were approved by the institutional Biosafety Committee of Kindai University and performed in accordance with the institutional guidelines. The informed consent was obtained from all participants. The survey for the participants was as follows: age, gender, type of mask, duration of mask usage, transportation, gargling habit, and natto consuming habit. We confirmed that no participants were treated with antimicrobial drugs during the experimental periods.

Sample collection, microbial culture, and colony count

To isolate and culture the microbes adhered to face masks, the face-side and outer-side of the face masks were pressed onto the agar plates (8.6 cm in diameter, 58 cm^2 in area), separately, which were covered with the lids immediately to avoid contamination. The culture conditions were as follows: for the bacterial cultures, the brain heart infusion (BHI) agar plates (Eiken chemical Co., LTD, Tokyo, Japan) were used and incubated at 37°C under the aerobic condition for 18 hours; for the fungal cultures, the Sabouraud dextrose agar plates (Nissui pharmaceutical Co., LTD, Tokyo, Japan) were used and incubated at 25°C under the aerobic condition for 5 days. Following the primary incubation, we evaluated the colony morphology and conducted colony counting.

Identification of microbial colonies

Bacteria: we collected 94 colonies from the cultured plates, isolated DNA, and conducted 16S ribosomal RNA (rRNA) sequencing by the MiSeq (Illumina, San Diego, CA) at the Center for Oral Microbiota Analysis (Takamatsu, Japan). We also prepared a smear of bacteria on a glass slide for Gram-staining (Fujifilm Wako, Osaka, Japan) and took the microscopic images, using the CX33 Microscope with the CCD Camera DP22 (Olympus, Tokyo, Japan).

Fungi: we selected representative plates containing different types of colonies from all cultured plates. We further incubated the cultured plates at 37°C for 2 days to induce the spore formation. We stained fungi with lactophenol cotton blue (Muto pure chemical Co., LTD, Tokyo, Japan) and identified them by their colony morphology and microscopically[34].

Data analyses

We conducted principal component analysis, using the software RStudio (version 1.4.1106) and Exploratory (Exploratory, Inc., CA). For statistical analyses, we conducted the paired *t*-test, Student's *t*-test, and χ^2 test. To determine the correlations between the data obtained in this study, we conducted a receiver operating characteristic (ROC) analysis to evaluate the association between the factors and

outcomes by calculating the area under the curve (AUC). AUC close to 1 indicates a strong association, and less than 0.5 indicates no association.

Declarations

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Author contribution

Conceptualization: AMP, IT

Methodology: AMP, SK

Investigation: AMP, SK, MF, FS, SO

Visualization: AMP, KH, IT

Funding acquisition: AMP, IT

Project administration: AMP, IT

Supervision: AMP, IT

Writing – original draft: AMP, IT

Writing – review & editing: SK, MF, FS, SO

Data availability statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: All authors have no competing interest.

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Tables

Table 1 Factors associated with microbial colony counts on face masks.

	bacteria	**fungi**
colony count / plate	1 - 1600	1 - 22
Face-side / Outer-side	**high on the face-side**	**high on the outer-side**
duration of usage	no effect	**high in 2 days~**
mask type	no effect	low in non-woven outer-side
gender	**low in female (face-side)**	**high *Cladosporium* in female**

Boldface indicates a significant difference ($P < 0.05$).

Table 2 Receiver operating characteristic (ROC) analysis.

factor	variable	AUC	association
mask type, non-woven	outer-side fungal count	**0·77**	negative*
gender, female	face-side bacterial count	**0·71**	negative†
usage 2 days	outer-side fungus count	**0·65**	positive*
gender, female	*Cladosporium* positive	**0·65**	positive
B. subtilis, inside	white medium colony	**0·65**	negative
natto once/month	*Bacillus subtilis*	**0·61**	positive
public transportation	bacterial or fungal count	0·50	no
B. subtilis, inside	*Staphylococcus epidermidis*	0·42	no

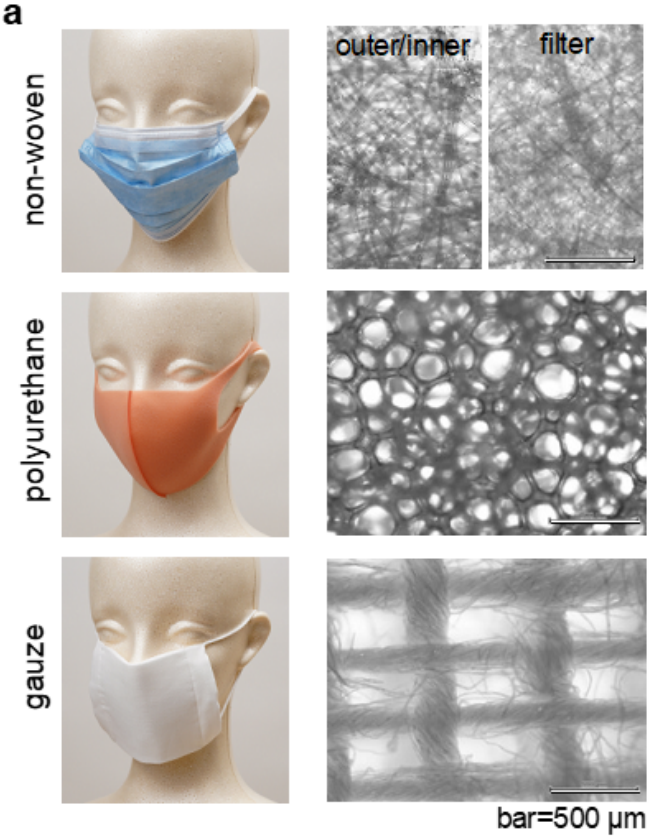
Boldface shows AUC higher than 0.6

AUC: 0.5–0.6, unsatisfactory; 0.6–0.7, satisfactory; 0.7–0.8, good; 0.8–0.9, very good; 0.9–1, excellent

*, †: Associations were consistent with statistical differences shown in *, Figs 2; †, S1.

Figures

a



b

mask type	pore size (μm)	thickness (mm)	layer number	intended use
non-woven	outer/inner, 50-150 middle filter, 5-30	0.25	3	infection
polyurethan	100-400	2	1	hey fever
gauze	200x450	4	15	infection

c

	standard size (μm)
virus	0.02-0.2
aerosol	< 5
droplet	> 5
bacteria	0.8-10
fungal spore	2-50
fungal hyphae	10-50
pollen	20-30

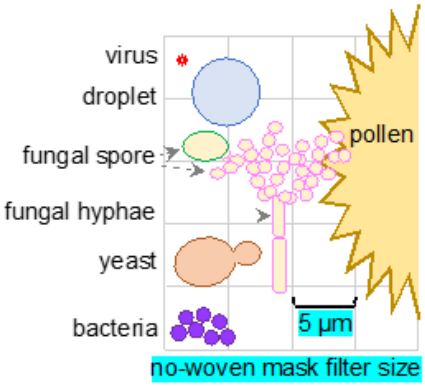


Figure 1

Face mask types and the sizes of microbes. a. Macroscopic and microscopic images of three different types of face masks that are commercially available. Non-woven masks have three layers: the pore size of the outer and the inner layers are identical (50-150 μm); the pore size of the middle layer (considered as a filter) is smaller (5-30 μm). Microscopic images were taken by the Olympus Microscope CX33 with the CCD Camera DP22 (bar = 500 μm). **b.** Pore size, thickness, layer, and intended use of three mask types.

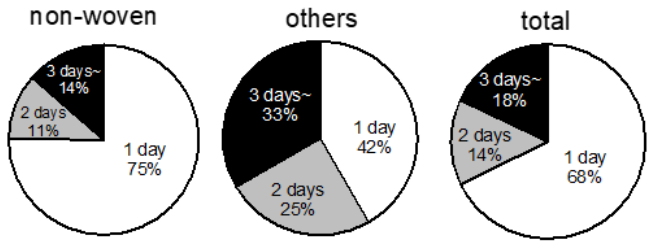
The pore size of face masks from manufacturers' instruction was confirmed using the microscopic images shown in A (right panels). **c.** The standard size of microbes and particles (left panel) and their comparisons with the pore size (5 μm) of the middle filter of non-woven masks (right schema).

a

mask type	gender % (n)		total % (n)
	male	female	
non-woven	80% (51)	75% (34)	*78% (85)
others	20% (12)	25% (12)	*22% (24)
total	58% (63)	42% (46)	100% (109)

* percentage of users: non-woven vs others, $p<0.001$

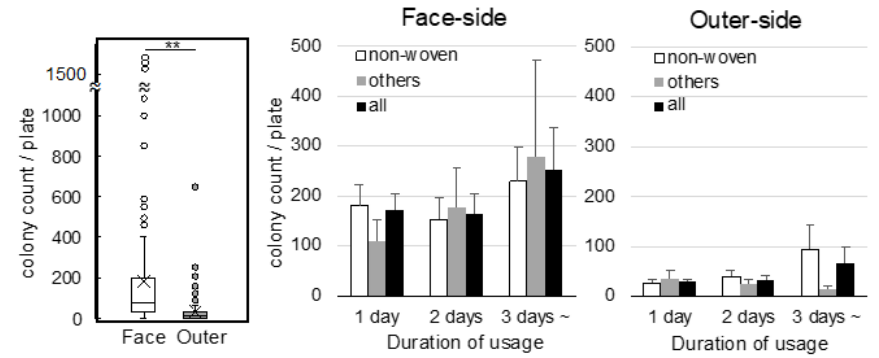
b



c

duration of mask usage	gender (n)	
	male	female
1 day	45	28
2 days	8	8
3 days~	10	10

d Bacteria



e Fungi

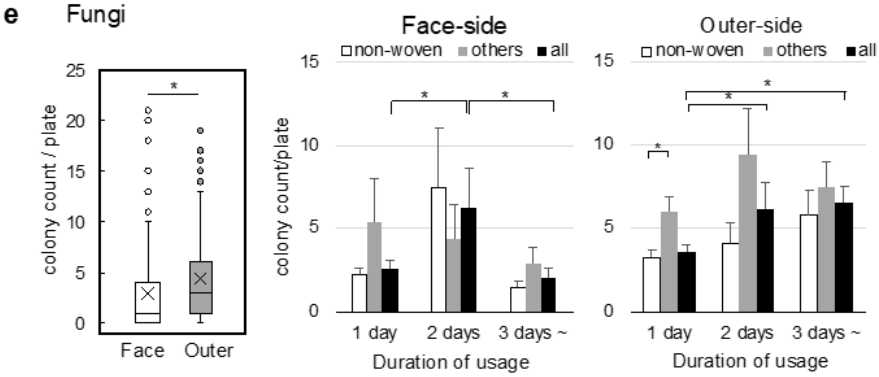
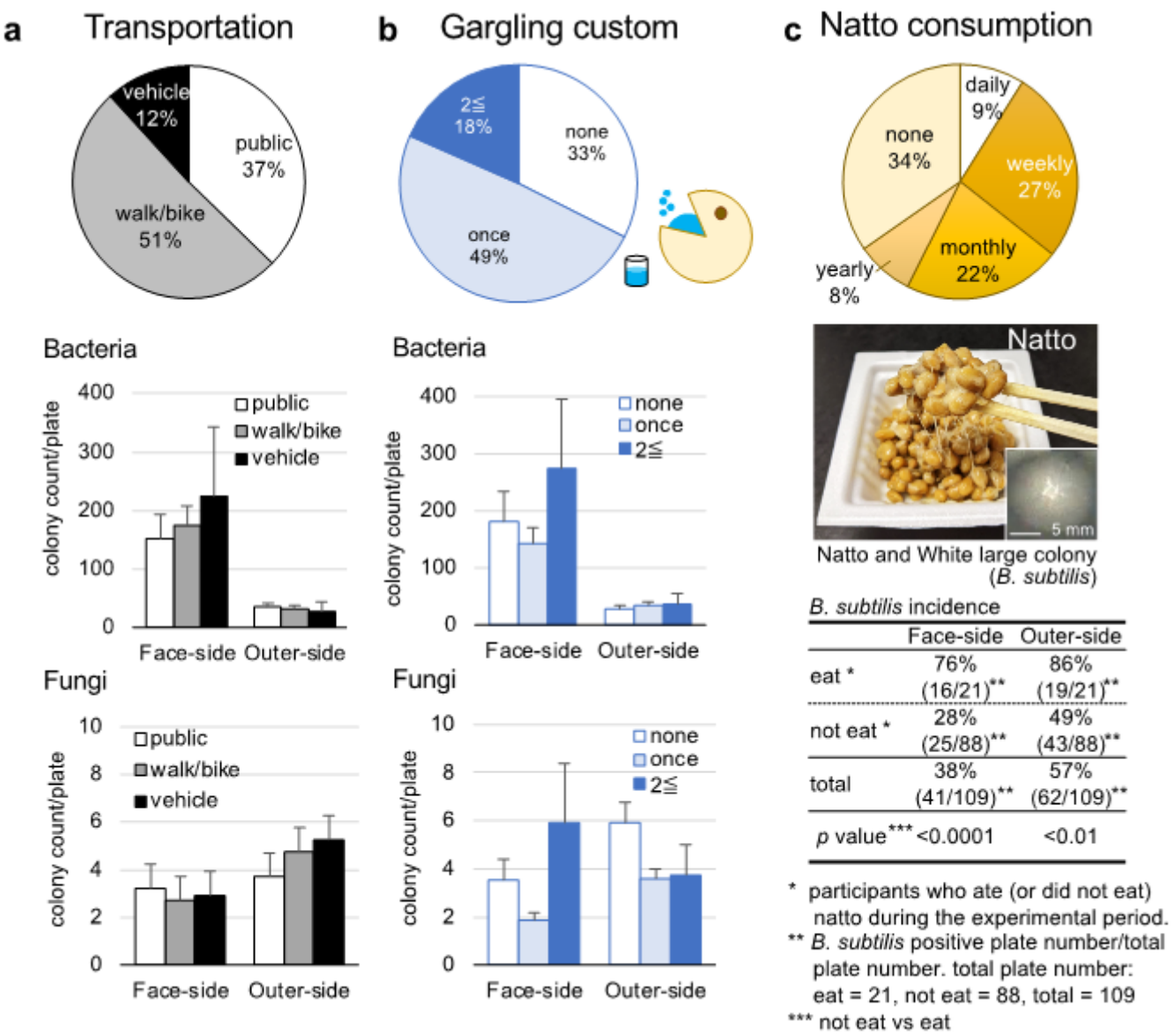


Figure 2

Survey results about the mask usage and microbe colony counts on the face-side and outer-side of the face masks. a. Usage of non-woven masks and other mask types (others) among male and female participants (n = 109). Most "others" were polyurethane masks except a few gauze or cloth masks. **b.** Duration of usage in non-woven, other mask types, and total (non-woven and others combined). The percentage of "others" wearing the same masks for two days or more (58%) was significantly higher than that of non-woven mask users ($P < 0.001$). **c.** Duration of mask usage in each gender (no significant difference). **d, e.** Bacteria (**d**) and fungi (**e**) on the face-side and outer-side masks were cultured separately after pressing each mask surface onto the agar plates. Microbial colony counts/plate (left panels); in boxplots, the cross symbols, bars, and dots indicate the mean, median, and outliers, respectively. Microbial colony counts on the face-side (middle panels) and outer-side (right panels) were compared based on the mask types and duration of mask usage. Mean + standard error of the mean (SEM). The paired *t*-test and Student's *t*-test were used for statistical analyses. * , $P < 0.05$; ** , $P < 0.001$.



B. subtilis incidence

	Face-side	Outer-side
eat *	76% (16/21)**	86% (19/21)**
not eat *	28% (25/88)**	49% (43/88)**
total	38% (41/109)**	57% (62/109)**
p value***	<0.0001	<0.01

* participants who ate (or did not eat) natto during the experimental period.
** *B. subtilis* positive plate number/total plate number. total plate number: eat = 21, not eat = 88, total = 109
*** not eat vs eat

Figure 3

Lifestyles and microbial colonies: transportation, gargling, and natto consumption. a. We categorized three transportation systems to commute: 1) public transportation: trains and/or buses; 2) private vehicles: cars and trucks; and 3) walk/bike: walking, bicycles, and motorbikes. We found no differences in bacterial and fungal colony counts on the face-side or outer-side of masks among the three transportation categories. **b.** Microbial colony counts and the gargling habit. The pie chart showed the percentage of participants' gargling frequency; 67% of the participants had gargling at least once a day. We found no differences in bacterial or fungal colony counts among the participants regardless of the gargling frequency. **c.** Natto consumption and *Bacillus subtilis* colonies. Natto is a traditional Japanese food made from soybeans fermented with *B. subtilis* that forms large white colonies on the agar plates. According to the survey, 9% and 27% of the participants have eaten natto daily and weekly, respectively; 19% (21 of 109) of the participants ate natto during the experimental period. The participants who ate natto had a significantly higher percentage of *B. subtilis* colonies than those who did not eat natto.

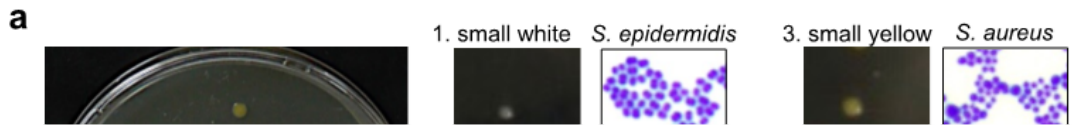


Figure 4

Bacterial colony morphologies and identification. a. We observed a variety of colonies on the agar plates and classified the colonies into four major colony forms, morphologically. Representative bacteria composed of each colony were visualized with their Gram-stain images. **b.** Major colony forms and their identified bacteria, and frequencies (incidence and % total). **c.** Identified bacteria, their localization, and pathogenicity in humans.

Figure 5

Identification of fungal colonies. We identified fungi by their colony morphology macroscopically as well as the hyphae and spore morphology microscopically. Ten representative fungal images were shown. The white and yellow bars are 10 mm and 5 mm, respectively. Identified fungi, the incidence in this study, localization, and pathogenicity were listed.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supf12.pdf