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Research Article

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Abstract

Use of face mask has become an integral part of public life in the post-pandemic era. However, the understanding of the effect of wearing mask on physiology remains incomplete and is required for informing public health policies. Earlier studies indicated changes in breath and blood gas composition upon wearing FFP2 mask. For the first time, we report analysis of the effect of wearing FFP2 mask on metabolic composition of saliva, a proximal matrix to breath. Un-induced saliva was collected from healthy volunteers (n =10) before and after wearing FFP2 mask for 30 minutes and analyzed. Results showed that such short-term mask use did not cause any significant change in heart rate, pulse rate and SpO$_2$. The individuality of overall salivary metabotype was found to be robust and unaffected by mask use. There were marginal increases in relative abundances of L-fucose, 5-aminovaleric acid, putrescine, phloretic acid and benzenepropionic acid. Results indicated that while there were no adverse changes in physiological parameters and salivary metabotype, mask use was associated with changes in microbial metabolic activity. Consequences of such changes remain to be examined. However, these might explain change in odour perception that was reported to be associated with mask use.

Introduction

Use of face masks, particularly, FFP2 masks has been shown to significantly reduce the risk of transmission of several airborne infectious agents including SARS-CoV2 (Cheng et al., 2021). Thus, use of masks has been mandated through public policy (Ford et al., 2021, Martinelli et al., 2021) in several parts of the world during the pandemic. Given that the virus keeps mutating and thriving, masks are expected to remain a part of public life for foreseeable future. However, a significant number of people are reluctant to accept it, particularly, in absence of mandate (Betsch et al., 2020). This can claim significant toll on public health given the recent rise in positivity and caseload in several parts of the world (Ford et al., 2021). In addition to behavioural and socio-political issues(Howard, 2020) (Burger et al., 2022) (MacIntyre et al., 2021) (Esmaeilzadeh, 2022) (Kemmelmeier and Jami, 2021) the reluctance may also be driven by concerns regarding the physiological impact of mask usage. Several studies have documented significant itching, breathlessness, increase in pulse rate and heart rate as well as decrease in SpO$_2$, particularly among elder individuals or those with respiratory conditions (Saccomanno et al., 2022) (Li et al., 2005) (Kisielinski et al., 2021) (Hu et al., 2020) (Fikenzer et al., 2020) (Kyung et al., 2020). Earlier studies have shown that use of masks can lead to significant increase in CO$_2$ levels in both breathe and blood as well as decrease in blood pH (Kyung et al., 2020) (Sinkule et al., 2013) (Rhee et al., 2021) (Kisielinski et al., 2021). Studies have indicated changes in breath odour upon mask use (Fikenzer et al., 2020) (Dirol et al., 2021). A recent study, indeed, has indicated changes in breath volatile organic compounds (VOCs) in breath upon use of FFP2 mask (Sukul et al., 2022). Given the ongoing pandemic and emerging infectious diseases (monkey pox being the latest example), mask usage may become the new normal. So, detailed understanding of physiological impacts of mask use is essential to inform the discourse to formulate optimal public health advisories on mask use. Even otherwise, for healthcare workers, who use masks frequently and for long hours, it is important to gauge the physiological impact.
Saliva is a proximal biological fluid that is constantly exposed to breath that can be easily sampled. Changes in salivary metabolites have been shown to be associated with several diseases and disorders including respiratory pathologies such as COPD, asthma and lung cancer (Wang et al., 2017) (Little et al., 2014) (Lawrence, 2002) (Bel’skaya et al., 2021). Apart from systemic changes, saliva metabolome can also be influenced by changes in oral physiology including the status of oral microbiome (Wei et al., 2011) (Liebsch et al., 2019). Thus, analysis of salivary metabolome may help to noninvasively gauge potential physiological impact of mask use. This pilot study presents results of metabolomic analysis of salivary samples collected from healthy volunteers before and after wearing FFP2 masks.

Materials And Methods

Subjects and sample collection

Ten subjects aged between 25–43 years, including six males and four females, were recruited for the study with informed consent. None of the subjects were known to be suffering from any disease. None of the subjects except one self-reported to be smoker. Subjects were asked not to eat anything for two hours before participating in the study. They were asked to drink water and then allowed to rest for 10 minutes in sitting position upon arrival. Respiratory rate (RR) was manually counted while heart rate (HR) and SpO2 was counted with commercially available pulse oximeter. Subsequently, they were instructed not to ingest saliva for 5 minutes and spit into a 50ml polypropylene tube thereafter. Fresh FFP2 (N95) masks were given to them to wear for 30 minutes and breathe normally. After 25 minutes with the mask on, RR, HR and SpO2 was measured as mentioned before and they were asked not to ingest saliva for 5 next minutes. Saliva was collected in 50 ml polypropylene tube immediately after removal of the mask. All samples were kept on ice, centrifuged at 20000 x g for 20 minutes and supernatant was collected and stored at -80°C until further use. The study protocol was approved by the Institutional Ethics Committee of College of Medicine and Sagore Dutta Hospital, Kolkata, India. Informed consents was obtained from all volunteers prior to enrollment.

Untargeted metabolomic analysis

Saliva samples were thawed on ice and 20ul of saliva was added to 180ul of 80% chilled methanol containing internal standard, vortexed and centrifuged at 20000 x g. 80ul of supernatant was taken into GC vials and evaporated to dryness. Dried samples were derivatized with 30ul of 2% MOX at 50°C for 1hr followed by 50ul MSTFA at 65°C for 1hr. Pooled saliva and extraction blank samples were also prepared following the same procedure for quality control purposes. Samples were analyzed with a 7890B GC fitted with a HP-5MS column (30 m × 0.25 mm × 0.25µm) coupled to 5977B single-quadrupole mass spectrometer (Agilent, USA) with Helium as the carrier gas. The front inlet was used in splitless mode at a temperature of 300°C. The oven temperature was maintained at 70°C for 5 minutes followed by a ramp to 280°C at 5°C/minutes. The temperature was further ramped to 295°C at 10°C/minutes and held at 295°C for 4minutes. MS source and MS quad temperatures were set to 230°C and 150°C respectively. The EI-MS
spectra were acquired in full scan mode in the m/z range of 45–500. Samples were run in randomized order with intermittent QC sample injections.

**Metabolomic data analysis**

Chromatograms were manually inspected to check for column performance and consistency of instrument response using MassHunter qualitative analysis software (Agilent, USA). Data was deconvoluted to extract features using MassHunter quantitative analysis software. One quantifier and at least one qualifier (typically, two) were used to extract features of interest and area under the curves were calculated through integration. The data table comprising area for individual features for respective samples was used for further univariate and multivariate analysis. Coefficients of variation (CV) were calculated for each feature and only those showing CV < 20% in pooled QC samples were used further. Paired (pre- and post-mask) dataset was log transformed and Pareto-scaled following either internal standard- or sum- or endogenous metabolite (myo-inositol)-normalization for multivariate analysis. Unsupervised principal components analysis (PCA) was used to check overall pattern and any inherent clustering of the metabolic signature. Partial least square discriminant analysis (PLS-DA), heat map and volcano plot was used for supervised data mining to identify features showing change in relative abundance upon mask use. Identification of compounds were based on comparative analysis of fragmentation pattern using NIST library and authentic standards.

**Statistical analysis**

Statistical significance was tested using paired two-tailed t-test and p value < 0.05 was considered to be significant. For metabolic features, only those with fold change > 1.5 were considered.

**Results**

**Overall impact on physiology.** None of the subjects reported any significant discomfort during the study. As shown in Table 1, no significant difference in RR, HR or SpO\textsubscript{2} was observed before and after wearing FFP2 mask for 30 minutes.

**Impact on overall metabotype.** The deconvolution of the chromatogram revealed 179 features. Out of these, 114 features showing CV < 20% with a mean of 11.45% in pooled QC samples and were considered for further analysis. The PCA (Supplementary Figure 1) of the sum-normalized data showed that pooled samples were very tightly clustered indicating consistency of sample preparation method and instrument response. In order to check the data quality further, the data was normalized with respect to internal standard as well as an endogenous metabolite, namely, myo-inositol, which showed low CV in pooled samples (<6%) and was completely absent in blank samples. The PCA plots (not shown) showed that while the samples were spread throughout; the QC samples were clustered tightly irrespective of the normalization method indicating good experimental repeatability. However, PCA scores plots didn’t reveal any segregation of the pre-mask and post-mask samples (Figure S1). This indicated that there was no drastic change in overall metabolic signature upon 30 minutes of mask use. Supervised PLS-DA analysis
was performed to unravel specific changes in the metabotype. The 3D scores-scatter plots of sum-normalized, internal standard-normalized and myo-inositol-normalized data are shown in Figure 1A, B and C, respectively. The fact that the pre-and post-mask metabolic signatures were not very clearly segregated even in supervised analysis indicated again that the use of mask did not have any profound effect on the metabotype. However, there were subtle changes in specific features. The VIP scores of top ten features contributing to the PLS-DA model are shown in Figure 1, next to respective scores scatter plot. Heatmap analysis revealed significant inter-individual variability in abundance of was present for many metabolites. However, it also revealed that pre- and post-mask saliva samples from each individual clustered together irrespective of normalization method (Figure S2). In order to examine the robustness of co-clustering of individual pre- and post-mask samples, top ten features that contributed to the difference between pre- and post-mask saliva samples were selected for heatmap analysis. These metabolites also showed significant inter-individual variability. However, even these differential features did not disrupt the co-clustering of pre- and post-mask samples from individual volunteers irrespective of normalization method used (Figure 2). These indicated that the individuality of salivary metabotype was highly conserved irrespective of mask use.

**Impact on specific metabolic signatures.** Heatmap analysis revealed that there were many common metabolites among top ten differential features from three different normalization methods. Particularly, L-fucose, phloretic acid, benzenepropionic acid, 5-aminovaleric acid and aminomalonic acid were common in all three methods (see Figure 2). In order to ascertain if there were small but significant changes in relative abundance of specific metabolites, volcano plot analysis was used to identify features showing at least 1.5 fold and statistically significant (p< 0.05 in paired t-test) change in relative abundance after wearing FFP2 mask for 30 minutes. These features were putatively identified by comparing fragmentation pattern with NIST library and confirmed with authentic standards wherever indicated (see Table 2). Results showed that while 5-aminovaleric acid, phloretic acid, putrescine, fucose (conformer 1 and 2), benzenepropionic acid, aminomalonic acid, Compound 354 (unidentified sugar derivative) and X23a showed slightly elevated abundance in salivary metabolome after wearing FFP2 mask for 30 minutes whereas the citric acid concentration tended to decrease marginally in the internal standard-normalized data. The list of differential features identified in three different normalization methods were compared to select common features that were found to be significantly altered upon wearing mask. Eight such compounds, namely, 5-aminovaleric acid, phloretic acid, putrescine, fucose (conformer 1 and 2), benzenepropionic acid, aminomalonic acid and Compound 354 were found to show significant increase in abundance upon 30 minutes of mask use in all three normalization methods. The relative abundances of these compounds in pre- and post-FFP2 mask use saliva samples are shown in Figure 3 (internal standard-normalized) and Figure S3 (myo-inositol-normalized) for comparison.

**Discussion**

This is the first attempt to analyze the impact of wearing FFP2 mask on salivary metabolome to gauge its potential impact on physiology. The results indicated that there was no profound change in the overall
metabotype. However, marginal increases in relative abundances of eight compounds were noted in salivary metabolome after 30 minutes of wearing FFP2 mask.

Since the number of subjects was modest, extra precaution was warranted while analyzing and interpreting the data. Data normalization is a very important step that can affect the results of metabolomic and well as other omic analysis. The data normalization approach in metabolomics can be very context dependent. Data normalization aims to account for either physiological variabilities (such as, change in water intake or GFR in case of urine) or those associated with operator and instrument. Internal standards are used to account and normalize for variabilities related to method, operator and instrument. While for urine, creatinine normalization is often used, no such consensus endogenous normalization exists for other biofluids like serum or saliva. Thus, sum normalization is widely used in metabolomic data analysis for such samples. However, the sum of feature intensities can be affected by features originating from diet, life style or treatment and may lead to artifacts. An earlier comparative study indicated that sum normalization may perform worse than no normalization (Wulff and Mitchell, 2018). In view of limited sample size, we, therefore, chose to use multiple normalizations methods and compare the results to consensus identify differential signatures. We envisaged that features truly affected by mask use are likely to pop-up in independent normalization approaches. It is worth noting that normalization with respect to an endogenous metabolite would reflect the true ratio of abundance of any metabolite with respect to that metabolite irrespective of dilution or presence of other exogenous compounds that may affect the total ion count. Thus, internal standard-, sum- as well as endogenous feature-normalization were performed and compared. Myo-inositol, which showed very low CV in pooled samples, was chosen as the endogenous metabolite for normalization. The close clustering of pooled QC samples in all these normalizations indicated goodness of the data. It also indicated that a signature showing significant change in multiple normalization methods are less likely to be an artifact of data processing. It was further reassuring to find that internal standard-, myo-inositol- and sum-normalized data all showed tight co-clustering of individual pre- and post-mask samples. These indicated the metabotypic individuality was highly conserved and no drastic change was brought about by 30 minutes mask use. However, it could be noted that the increase in abundance of five confirmed (fucose, putrescine, citric acid, phloretic acid, benzenepropionic acid) and two putatively identified (5-aminovaleric acid, aminomalonic acid) compounds were found to be consistent irrespective of normalization methods. These indicate that salivary abundances of these metabolites are, indeed, elevated in the sample set.

Salivary metabolome has earlier been analyzed to identify biochemical signatures associated with conditions like COPD, lung and oral cancer, periodontitis as well as hepatitis B infection (Mikkonen et al., 2016) (Barnes et al., 2014) (Gilany et al., 2019) (Pozzi et al., 2022). However, no study till date analyzed changes in salivary metabolome in healthy individuals upon wearing mask with or without exercise, which has been shown to affect the respiratory physiology (Pozzi et al., 2022). While the saliva is secreted from the salivary gland, it is mixed with biochemicals released by other mucosal cells as well as the microbes of the oropharyngeal cavity. Saliva is also continuously exposed to breath. Thus, compounds originating from microbial metabolism and breath VOCs can alter salivary composition. The
other side of the same coin is the potential impact of salivary biochemicals on resident microbes or mucosal cells in oropharyngeal cavity and even lungs or other organs.

Earlier studies have indicated that use of FFP2 masks can change the $O_2$, $CO_2$ concentration, respiratory rates and heart rates in individuals with compromised lung capacity such as COPD or healthy individuals performing heavy exercises (Saccomanno et al., 2022) (Li et al., 2005) (Kisielinski et al., 2021) (Hu et al., 2020) (Fikenzer et al., 2020) (Kyung et al., 2020) (Sinkule et al., 2013) (Rhee et al., 2021). But, a recent study (Sukul et al., 2022) reported miniscule changes in SpO$_2$ upon use of FFP2 mask for 30 minutes among individuals below 60 years. This is in agreement with our study that showed no significant change in SpO$_2$ after FFP2 mask use for 30 minutes among healthy young individuals. Given the much younger demography, it is unsurprising that no significant change in pulse or respiratory rates was observed under sitting condition after wearing FFP2 mask for 30 minutes. However, this study indicated that the relative abundance of certain salivary metabolites changed even in absence of any change in these respiratory and physiological parameters. Among these, 5-aminovaleric acid, a product of lysine deamination, is a well known salivary metabolite. It may be formed either by endogenous or microbial metabolism and has been found to be elevated in subjects with chronic periodontitis. It can be formed via cadaverine pathway, which is a polyamine similar to putrescine that was found to be elevated in post-mask saliva. Both putrescine and cadaverine has been found to increase in saliva of ankylosing spondylitis patients (Lv et al., 2021b). Phloretic acid, a product of phenylalanine/tyrosine metabolism, has been shown to be produced by several microbes including commensal and pathogenic ones (Dodd et al., 2017) (Beloborodov et al., 2009). It has been reported to be present in saliva (Sugimoto et al., 2013) and its level was found to be sensitive to oral health (Liebsch et al., 2019). L-fucose, which is a part of several glycans on the cell surface, is the only laevorotatory sugar utilized by mammalian cells. Elevated serum L-fucose has been reported in liver diseases and cancer (Sakai et al., 1990). It was also detected in saliva (Dame et al., 2015). Interestingly, it was shown that commensal microbes like Bacteroides thetaiotaomicron express fucosidases that can release L-fucose from mucosal glycans (Fischbach and Sonnenburg, 2011). It was also shown that E. coli can sense L-fucose concentration in intestine and depends on the downstream signaling pathway that plays a key role in its intestinal colonization (Pacheco et al., 2012). In fact, several oral microbes including streptococci has been shown to possess fucosidase activity (Megson et al., 2015). These indicate that the increase in oral L-fucose upon mask use may be due to degradation of fucosylated mucosal glycoproteins by oral microbiota. Putrescine is well known to affect gene expression and cell cycle (Li et al., 2020). Interestingly, it has been shown to possess anti-inflammatory effect with a recent study demonstrating increase in anti-inflammatory macrophages by putrescine (Nakamura et al., 2021). Taken together, elevation of these compounds indicate alteration in microbial metabolic activity and output upon wearing FFP2 mask. 2-Aminomalonic acid, which has been connected to protein oxidation, was also shown to be elevated in urinary levels in anxiety (Chen et al., 2018) and serum levels in hepatocellular carcinoma (Xue et al., 2008). It has been shown to be a constituent of saliva that increased in ankylosing spondylitis (Lv et al., 2021b) and primary biliary cancer (PBC) patients (Lv et al., 2021a) along with changes in the oral microbiota. A correlation between salivary levels of 2-aminomalonic acid and cytokines were observed in PBC.
Earlier studies have indicated change in odour upon use of FFP2 (Fikenzer et al., 2020) and even surgical masks (Dirol et al., 2021). Compounds such as putrescine, phloretic acid and 5-aminovaleric acid are known odour-forming compounds. Thus, these changes may be responsible for changing odour perception upon use of face masks. Thus, while there seems to be no adverse impact of short-term use of FFP2 masks prima facie, wearing mask may result in alteration in the activity of the oropharyngeal microbiome. The effect of prolonged exposure of oropharyngeal cells to such alterations and elevated levels of aforementioned compounds remains to be determined.

**Conclusion**

The results revealed that even in absence of any tangible changes in respiratory rate or pulse rate or SpO$_2$ under sitting condition, there were subtle alterations in the salivary metabolome upon wearing FFP2 mask. Although overall no drastic changes were observed and individuality of salivary metabotype was maintained irrespective of mask use, compounds including those with potential microbial origin were elevated in saliva upon FFP2 mask use for 30 minutes. Changes in relative abundances of compounds like putrescine, phloretic acid and 5-aminovaleric acid may be responsible for change in odour perception that has been reported to be associated with mask use. However, changes were marginally significant and, hence, warrant validation in larger cohort and assessment of the long-term impact of exposure to these compounds and/or change in biochemical behavior of the oral microbiome.

**Limitations:**

The limitations of the study are small sample size, inclusion of only young and healthy individuals as well as examination only under sitting condition.

**Declarations**

**Acknowledgement:**

The authors thank all the volunteers for participating in the study. The study was supported by intramural research funding at SINP under the aegis of Department of Atomic Energy, Government of India.

**Author contributions:**

RI: sample collection, processing, experimental data collection and analysis, statistical analysis, manuscript preparation and review; DP: sample collection, processing, manuscript preparation and review; RD: sample collection and data processing; SM: data processing, manuscript preparation and review; PC: subject recruitment, ethical supervision and manuscript review; SKM: conceptualization, method development, supervision, interpretation, manuscript preparation and review.

**Compliance with ethical standards:**
The study protocol was approved by the Institutional Ethics Committee of College of Medicine and Sagore Dutta Hospital, Kolkata, India and performed strictly as per guidelines.

Conflict of Interest: None

References


Table 1: Demographics, heart rate, respiratory rate and SpO\textsubscript{2}

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>After</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>31.2±6.3</td>
<td></td>
</tr>
<tr>
<td>Heart rate (per minute)</td>
<td></td>
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<tr>
<td>Before</td>
<td>73.5±10.6</td>
<td></td>
</tr>
<tr>
<td>After</td>
<td>70.4±6.9 (n.s.)\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td>Respiratory rate (per minute)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>13.4±3.9</td>
<td></td>
</tr>
<tr>
<td>After</td>
<td>12.4±3.7 (n.s.)</td>
<td></td>
</tr>
<tr>
<td>SpO\textsubscript{2} (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>98.0±0.8</td>
<td></td>
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<tr>
<td>After</td>
<td>98.0±0.6 (n.s.)</td>
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</table>

\textsuperscript{a} Not significant.

Table 2: Metabolites showing change in abundance upon use of FFP2 mask for 30 minutes.

<table>
<thead>
<tr>
<th>Compound identity \textsuperscript{a}</th>
<th>Retention time (min)</th>
<th>NIST match factor</th>
<th>Fold change \textsuperscript{b}</th>
<th>P value \textsuperscript{b}</th>
<th>Putative biochemical origin</th>
</tr>
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<tr>
<td>L-Fucose (conformer 1)</td>
<td>27.67</td>
<td>&gt; 850</td>
<td>1.62</td>
<td>&lt; 0.005</td>
<td>Fructose and mannose metabolism or microbial fucosidase activity</td>
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<tr>
<td>L-Fucose (conformer 2)</td>
<td>27.87</td>
<td>&gt; 700</td>
<td>1.53</td>
<td>&lt; 0.01</td>
<td>Fructose and mannose metabolism or microbial fucosidase activity</td>
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<tr>
<td>5-Aminovaleric acid</td>
<td>25.03</td>
<td>&gt; 900</td>
<td>1.81</td>
<td>&lt; 0.02</td>
<td>Lysine degradation</td>
</tr>
<tr>
<td>Benzenepropionic acid</td>
<td>19.74</td>
<td>&gt; 500</td>
<td>1.64</td>
<td>&lt; 0.003</td>
<td>Phenylalanine metabolism</td>
</tr>
<tr>
<td>Citric acid</td>
<td>29.53</td>
<td>&gt; 750</td>
<td>-1.5</td>
<td>&lt; 0.03</td>
<td>TCA cycle</td>
</tr>
<tr>
<td>Phloretic acid</td>
<td>27.90</td>
<td>&gt; 500</td>
<td>1.83</td>
<td>&lt; 0.01</td>
<td>Phenylalanine or tyrosine metabolism</td>
</tr>
<tr>
<td>Putrescine</td>
<td>27.47</td>
<td>&gt; 850</td>
<td>1.91</td>
<td>&lt; 0.02</td>
<td>Polyamine biosynthesis</td>
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<tr>
<td>Aminomalonic acid</td>
<td>21.43</td>
<td>&gt; 800</td>
<td>1.80</td>
<td>&lt; 0.003</td>
<td>Protein oxidation</td>
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<td>Compound 354</td>
<td>43.93</td>
<td>2.22</td>
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<td>&lt; 0.005</td>
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<tr>
<td>Unidentified X23a</td>
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<td>1.88</td>
<td></td>
<td>&lt; 0.01</td>
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Those confirmed with authentic standards shown in italics.

Fold change values and raw p values for the change in relative abundance obtained from paired volcano plot analysis.

Figures
Figure 1

3D PLS-DA scores plot and corresponding VIP score plots of (A) sum-normalized, (B) internal standard-normalized and (C) myo-inositol-normalized data. Green and red indicates saliva samples collected before and after mask use, respectively.
Figure 2

Heatmap showing top ten differential features associated with FFP2 mask use in (A) sum-normalized, (B) internal standard-normalized and (C) myo-inositol-normalized data. Alphabets (A, B, ...I, J) represents individual volunteers. Samples collected before and after use of FFP2 masks are indicated by 1 and 2, respectively.
Figure 3

Changes in internal standard-normalized relative abundance of metabolites upon FFP2 mask use.

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

- SupplementaryFigures.docx