Nitrite-producing oral microbiome in adults and children

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Recently, it was suggested that the nitrite (NO₂⁻) produced from NO₃⁻ by oral bacteria might contribute to oral and general health. Therefore, we aimed to clarify the detailed information about the bacterial NO₂⁻-production in the oral biofilm. Dental plaque and tongue-coating samples were collected, then the NO₂⁻-producing activity was measured. Furthermore, the composition of the NO₂⁻-producing bacterial population were identified using the Griess reagent-containing agar overlay method and molecular biological method. NO₂⁻-producing activity per mg wet weight varied among individuals but was higher in dental plaque. Additionally, anaerobic bacteria exhibited higher numbers of NO₂⁻-producing bacteria, except in the adults' dental plaque. The proportion of NO₂⁻-producing bacteria also varied among individuals, but a positive correlation was found between NO₂⁻-producing activity and the number of NO₂⁻-producing bacteria, especially in dental plaque. Overall, the major NO₂⁻-producing bacteria were identified as Actinomyces, Schaalila, Veillonella and Neisseria. Furthermore, Rothia was specifically detected in the tongue coatings of children. These results suggest that dental plaque has higher NO₂⁻-producing activity and that this activity depends not on the presence of specific bacteria or the bacterial compositions, but on the number of NO₂⁻-producing bacteria, although interindividual differences were detected.

Nitrate (NO₃⁻) is abundant in green/yellow vegetables (several tens to thousands ppm), especially in leafy vegetables¹.². Diet-derived NO₃⁻ is partly converted by oral bacteria to NO₂⁻. After being absorbed from the gastrointestinal tract and into the blood, it is gradually oxidized to NO₃⁻, some of which is secreted into the oral cavity in saliva. This is called “the entero-salivary nitrogen oxide cycle”³, and oral bacteria play a major role in the production of NO₂⁻ from NO₃⁻ via this cycle. saliva that is collected directly from the parotid gland contains no NO₂⁻⁴ and the use of antibacterial antiseptic mouthwash reduces the blood NO₂⁻ concentration⁵. These observations indicate that oral bacteria are involved in the production of NO₂⁻ from NO₃⁻ and that oral bacteria are essential for nitrogen oxide production and circulation. In recent years, NO₂⁻ has been reported to have antimicrobial effects, e.g., it suppresses the metabolism and growth of caries- and periodontal disease-related bacteria⁶-⁹, and it is also known to ameliorate cardiovascular disease by improving the systemic blood circulation¹⁰. These findings suggest that NO₂⁻ is relevant to not only oral health, but also general health.

In a previous study, the proportion of NO₂⁻-producing bacteria in the oral biofilm was examined using the metagenomics method¹¹. The metagenomic and metatranscriptomic approaches are comprehensive, while their analytical power depends on comprehensiveness of gene database about bacterial species and nitrate-reducing enzymes. In addition, relatedness of metagenomic data and metatranscriptomic data is quite complicated and it is often difficult to get an answer which bacterium is responsible for the targeted phenotype. The culture method has limitations such as growth selection, while this method has also advantage such as isolating bacteria as live cells and confirming their ability of nitrite production from nitrate. In this study, we would like to focus on the bacterial species that works as nitrate reducer in actual in the oral cavity. From these circumstances, we first tried to isolate the nitrite-producing phenotypic bacteria.

In a report using culture-based method, oral biofilm samples that were collected from the tooth surfaces, tongues, cheeks, and palatal mucosae of adults exhibited NO₂⁻-producing activity, especially those from the tongue surface¹². Veillonella, Actinomyces, Rothia, and Staphylococcus have been identified as the predominant

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NO$_2^−$-producing bacteria in the oral cavity\cite{12}. In addition, these genera were also detected in the saliva of adults and newborns\cite{13}, and the frequency of \textit{Veillonella} was associated with NO$_2^−$-producing activity in saliva\cite{14}. However, no previous study has examined the NO$_2^−$-producing activity of the oral biofilm per unit wet weight, or interindividual differences in the composition of NO$_2^−$-producing bacterial populations at the bacterial species level. Furthermore, the previous findings that the salivary NO$_2^−$ concentration is higher in children\cite{4} and that the composition of the oral bacterial population differs between children and adults\cite{11,15} suggest that the types and proportions of NO$_2^−$-producing bacteria in children are different from those in adults.

Therefore, the purpose of this study was to measure the NO$_2^−$-producing activity of the oral biofilms on the tooth and tongue surfaces of adults and children, and to analyze the bacterial compositions of these biofilms by isolating and identifying NO$_2^−$-producing bacteria using culture-based method for both adults and children.

**Results**

**NO$_2^−$-producing activity detected in dental plaque and tongue coatings.** The NO$_2^−$-producing activity (per mg wet weight) of each sample was determined (Fig. 1a). There was no significant difference in the mean NO$_2^−$ production value between adults and children, although large interindividual differences were seen. The NO$_2^−$-producing activity of dental plaque was significantly higher than that of tongue coatings in both adults (p = 0.017) and children (p < 0.001). On the other hand, the NO$_2^−$-producing activity per unit cell numbers of each sample was determined (Fig. 1b). Similarly, there was no significant difference in the mean NO$_2^−$ production value between adults and children, although large interindividual differences were seen. However, the NO$_2^−$-producing activity of dental plaque was significantly higher than that of tongue coatings in only children (p = 0.002).

**The number and percentage of NO$_2^−$-producing bacteria in dental plaque and tongue coatings.** Dental plaque and tongue-coating samples were cultured on agar plates under aerobic and anaerobic conditions, and the number and percentage of NO$_2^−$-producing bacteria and non-NO$_2^−$-producing bacteria were determined (Fig. 2) using the agar overlay culture method (Fig. 3). The number of NO$_2^−$-producing bacteria (per mg wet weight) was higher in the dental plaque samples than in the tongue-coating samples in both adults and children and under both aerobic and anaerobic conditions. The percentage of NO$_2^−$-producing bacteria was higher in the anaerobic cultures than in the aerobic cultures, except in the case of the adult dental plaque samples (Fig. 2). However, large interindividual differences were again noted, and there were no correlations between the percentage of NO$_2^−$-producing bacteria and oral clinical examination parameters.

**Correlations between the NO$_2^−$-producing activity and the number of NO$_2^−$-producing bacteria in dental plaque and tongue coatings.** The correlation between NO$_2^−$-producing activity and the number of NO$_2^−$-producing bacteria in dental plaque (the number of aerobic cultures, the number of anaerobic cultures, and the total number of cultures) was found to be significant in both adults and children (r = 0.61–0.90, p < 0.05) (Fig. 4). In addition, a significant correlation was detected between these parameters for children's tongue coatings (r = 0.81, p < 0.05), but not adult's tongue coatings (Fig. 4).

**Identification of NO$_2^−$-producing bacteria.** Figure 5 shows the proportion of NO$_2^−$-producing bacteria identified. In both adults and children, \textit{Actinomyces}, \textit{Schaalia} and \textit{Neisseria} accounted for a large proportion of NO$_2^−$-producing bacteria under aerobic conditions, while \textit{Actinomyces}, \textit{Schaalia} and \textit{Veillonella} accounted for a large proportion of NO$_2^−$-producing bacteria under anaerobic conditions. The genus \textit{Actinomyces} was mainly detected in dental plaque than in tongue coating, and the most common species detected were \textit{Actinomyces naeslundii}, \textit{A. oris}, and \textit{A. johnsonii} (Table 1). On the other hand, the genus \textit{Schaalia} was detected higher in tongue coating. The genus \textit{Neisseria} was mainly found under aerobic conditions, and there was no difference in the frequencies of \textit{Neisseria} species between the dental plaque and tongue coating samples. The genus \textit{Veillonella} was only found under anaerobic conditions, and the most common species in the dental plaque samples was \textit{V. parvula}, and the most common species in the tongue coating samples was \textit{V. dispar}, followed by \textit{V. atypica} and \textit{V. parvula} (Table 1).

The samples of adults were characterized by a high proportion of \textit{Neisseria} in tongue coatings, whereas the samples of children were characterized by high proportions of \textit{Rothia} in aerobic bacteria and \textit{Veillonella} in anaerobic bacteria detected in tongue coatings. In addition, \textit{Burkholderia} (\textit{Paraburkholderia}), \textit{Selenomonas}, and \textit{Pseudopropionibacterium} were not observed substantially in adults, while they were relatively common in children.

**Discussion**

**Modified Griess reagent-containing agar overlay method.** Using the Griess reagent-containing agar overlay method, we were able to visually detect colonies of NO$_2^−$-producing bacteria (Fig. 3). The strongest color was seen about 20 min after the application of the Griess reagent-containing agar, and after that the color gradually tended to fade. Therefore, the observations were performed 20 min after the colonies were covered with agar. In addition, normal agar with a melting point of 80–90 °C was replaced by agar with a low melting point of 30–31 °C to prevent heat damage to the bacteria. Furthermore, lactate was added to the covering agar to activate the metabolic activity of lactate-utilizing bacteria, such as \textit{Veillonella}\cite{16,17} and \textit{Neisseria}\cite{18}. These modifications might have allowed us to isolate new groups of NO$_2^−$-producing bacteria as discussed below.
Figure 1. (a) The NO$_2^-$-producing activity of dental plaque and tongue-coating samples (pmol/mg wet weight). (b) The NO$_2^-$-producing activity of dental plaque and tongue-coating samples (pmol/1 × 10$^6$ cells). Subject numbers: 1–11 (adults), 12–22 (children); *significant difference (p < 0.05). $: not available because the wet weight of the sample was below the detectable limit.
**Figure 2.** The numbers of NO$_2^-$-producing and non-NO$_2^-$-producing bacteria detected in dental plaque and tongue-coating samples cultured under aerobic and anaerobic conditions (CFU/mg wet weight of sample). Subject numbers: 1–11 (adults), 12–22 (children); Aerobic: the bacteria on the blood agar plate were cultured aerobically; Anaerobic: the bacteria on the blood agar plate were cultured anaerobically; *significant difference (p < 0.05); a: not available because the wet weight of the sample was below the detectable limit.

**The NO$_2^-$-producing activity and number of NO$_2^-$-producing bacteria in adults and children.** The present study revealed that NO$_2^-$-producing activity per mg wet weight of sample varied among individuals in both adults and children, but overall, it was suggested that the same wet weight of dental plaque made a greater contribution to NO$_2^-$ production than that of tongue coatings (Fig. 1a). On the other hand, NO$_2^-$-producing activity per $10^5$ bacterial cells in each sample varied among individuals in both adults and children similarly, but the tendency that NO$_2^-$-producing activity of dental plaque was significantly higher than that of tongue coatings was observed in only children, but not in adults (Fig. 1b). The bacterial density in same wet weight of sample was higher in dental plaque than tongue coating except only 1 child subject. Such differences in bacterial cell density may affect the results in this study. However, in some adult subjects, the NO$_2^-$-producing activity per same cell numbers was higher in tongue coating than in dental plaque. The inter-individual differences in the bacterial composition of nitrate reducing bacteria may also affect it, although a further study is needed. The number and percentage of NO$_2^-$-producing bacteria also varied among individuals, but they were higher under anaerobic conditions than under aerobic conditions, except in adult dental plaque (Fig. 2), indicating that facultative anaerobic and obligate anaerobic bacteria are the main bacteria involved in oral NO$_2^-$ production.

Positive correlations between NO$_2^-$-producing activity and the number of NO$_2^-$-producing bacteria were detected for the dental plaque of adults and children, and some of the children’s tongue-coating samples (Fig. 4). These results suggest that the NO$_2^-$-producing activity of dental plaque in adults and children and tongue-coating in children is basically determined by the number of NO$_2^-$-producing bacteria. On the other hand, the significant correlation was not detected for tongue coating of adults. We think that multiple nitrite-producing bacteria, rather than a few limited bacteria, may contribute to nitrate reduction in the oral cavity, but we have limited information about the relative nitrate reducing activity of each bacterial species in the oral cavity. To clarify why the correlation was not detected in some cases in Fig. 4, we need to consider the differences of the bacterial composition and the nitrite producing ability of each bacterial species. Furthermore, in a recent research, the NO$_2^-$-producing activity of Veillonella species was much affected by oral environmental factors such as pH,
lactate concentration\(^{19}\). Thus, we maybe should consider such effects when we evaluate the NO\(_2^−\) production in the oral cavity.

In all of the subjects used in this study were in good oral health, and the exclusion criteria included factors such as untreated dental caries and untreated periodontal diseases. Because there was not much differences among subjects in clinical parameters, the correlations between NO\(_2^−\)-producing activity and oral clinical factors may not be fully analyzed. We would like to clarify this correlation as a future research.

\(\text{NO}_2^−\)-producing bacterial species in adults and children.  \(\text{Actinomyces}\) accounted for the largest proportion of NO\(_2^−\)-producing bacteria in both aerobic and anaerobic conditions, particularly in dental plaque (Fig. 5). \(\text{Actinomyces}\) has been reported to be the major type of oral NO\(_2^−\)-producing bacteria\(^{12}\), and the present study supports this finding. The next most common bacterial genus was \(\text{Schaalia}\), especially \(\text{Schaalia odontolytica}\) was detected higher in tongue coatings (Table 1). \(\text{Schaalia odontolytica}\) has been proposed as \(\text{Actinomyces odontolyticus}\), and re-named in 2018\(^{19}\). \(\text{Schaalia odontolytica}\) has been also reported to be the major type of oral NO\(_2^−\)-producing bacteria as \(\text{Actinomyces odontolyticus}\)\(^{12}\). Another most common bacterial genus was \(\text{Veillonella}\), especially in the tongue coatings of children (Fig. 5), which is consistent with the findings of Doel et al. (2005) and Hyde et al. (2014). Mashima et al. (2017) also reported \(\text{Veillonella}\) as a major bacterial genus in children’s saliva.

The present study supports the previous findings that \(\text{Neisseria}\) was one of the predominant NO\(_2^−\)-producing bacteria in the oral cavities of adults\(^ {11}\). Furthermore, it was the first study to show that \(\text{Neisseria}\) accounted for a relatively high percentage of NO\(_2^−\)-producing bacteria in children (Fig. 5). However, \(\text{Neisseria}\) was not detected in a previous study in which the agar overlay method was used to detect NO\(_2^−\)-producing bacteria\(^{12}\). This might have been due to individual differences between the subjects, but it could also have been due to our use of covering agar with a low melting point (30–31 °C). The use of such agar might have helped to prevent the heat damage associated with covering samples with normal agar with a melting point of 80–90 °C, although the heat sensitivity of the NO\(_2^−\)-producing activity of \(\text{Neisseria}\) has not been confirmed. Moreover, the addition of lactate to the covering agar in the present study might have promoted the NO\(_2^−\)-producing activity of \(\text{Neisseria}\), as well as lactate-utilizing bacteria (\(\text{Veillonella}\)), since \(\text{Neisseria}\) was also reported to utilize lactate as an energy source\(^{14}\). The modified agar overlay method used in the current study might be more useful for detecting NO\(_2^−\)-producing bacteria.
**Adults**

### Dental Plaque

- **Correlation**
  - $r = 0.81$
  - $r = 0.90$
  - $r = 0.61$

### Tongue Coating

- **Correlation**
  - $r = 0.004$
  - $r = -0.16$
  - $r = 0.03$

**Children**

### Dental Plaque

- **Correlation**
  - $r = 0.77$
  - $r = 0.73$
  - $r = 0.70$

### Tongue Coating

- **Correlation**
  - $r = 0.51$
  - $r = 0.81$
  - $r = 0.36$

**NO$_2^-$-producing Bacteria (CFU/mg wet weight of sample)**

**Figure 4.** Correlation between NO$_2^-$-producing activity and the number of NO$_2^-$-producing bacteria (aerobic, anaerobic, and aerobic plus anaerobic conditions) in the dental plaque and tongue coating samples from adults and children. Both axes are displayed on logarithmic scales. $r$ correlation coefficient, *Aerobic* the bacteria on the blood agar plate were cultured aerobically, *Anaerobic* the bacteria on the blood agar plate were cultured anaerobically.
Rothia is an NO$_2^-$-producing bacterium that is found in the oral cavity$^{9,12,13}$. The present study showed that Rothia accounted for a high proportion of the NO$_2^-$-producing bacteria in children's tongue coatings (Fig. 5). Mashima et al. (2017) also detected Rothia in the oral cavities of children, although they did not examine NO$_2^-$-producing activity. The finding that Rothia is involved in the NO$_2^-$-producing activity of tongue coatings in children is new, and further research is needed on the activity of this genus.

Paraburkholderia was the first NO$_2^-$-producing bacterium detected in children (Fig. 5). Interestingly, Coenye et al. reported that Burkholderia, which is closely related to Paraburkholderia, displays NO$_2^-$-producing activity$^{21}$. However, Paraburkholderia is not reported yet to exist in the oral cavity, but is reported to be detected in plants$^{22}$. Food debris in samples may contain this bacterium in this study.

The other bacterial genus detected in this study, Streptococcus, Capnocytophaga, Selenomonas, Haemophilus, Prevotella and Eikenella, were also reported to be positive in nitrate reduction in previous reports$^{11,12}$ or to have the related gene of nitrate reduction. Propionibacterium acnes is also reported to have nitrite reductase$^{23}$. Genus Pseudopropionibacterium detected in this study belonged formerly to this genus Propionibacterium, hence this genus also may have nitrate reductase. Fusobacterium nucleatum has been known to have nitrite reductase but not nitrate reductase$^{24}$, therefore we thought the possibility of “false-positive in this method” about the detection of this species.

The culture-based method has a limitation to clarify the comprehensive microbial diversity compared with metagenomics-based method, and also the results may be affected by the differences of culture conditions. The present data basically supported the data in the previous studies$^{11,12}$ at genus and species level, although there is a little difference. These factors on the methods may affect the results, although the difference of subjects may affect it.

**Ecological considerations regarding NO$_2^-$-producing bacteria and their relationships with oral and general health.** Our results indicate that Actinomyces, Schaalia and Veillonella are the main contributor to NO$_2^-$-producing in dental plaque and tongue coatings. In addition, Neisseria is more common in adults' tongue coatings, and Rothia is more common in children's tongue coatings. Actinomyces and Schaalia are facultative anaerobes, and Veillonella is an obligate anaerobe, while Neisseria and Rothia are aerobes. The detection of all of these bacteria suggests that they are segregated in different parts of the biofilm with different oxygen concentrations, but coexist through metabolic networks. The bacterial production of NO$_2^-$ from NO$_3^-$ is usually catalyzed by reducing power, which is supplied by bacterial metabolism. Actinomyces and Schaalia can metabolize carbohydrates, which produces lactate and reducing power, while Veillonella can metabolize lactate$^{25}$, which

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**Figure 5.** The proportion of NO$_2^-$-producing bacterial genera identified in the dental plaque and tongue coating samples from adults and children. All data were adopted from Table 1.
| Adults | Children |
|--------|----------|
|        | Dental plaque | Tongue coating | Dental plaque | Tongue coating |
|        | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic |
| Actinomyces naeslundii | 30 | 18 | 3 | 2 | 24 | 12 | 0 | 0 |
| Actinomyces oris | 5 | 11 | 2 | 0 | 28 | 22 | 0 | 0 |
| Actinomyces johnsonii | 10 | 2 | 0 | 0 | 8 | 9 | 0 | 0 |
| Actinomyces gruvenitzii | 0 | 0 | 2 | 2 | 0 | 0 | 3 | 3 |
| Actinomyces hongkongensis | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Actinomyces georgiae | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| Actinomyces massiliensis | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| Actinomyces linguae | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Actinomyces oris or naeslundii | 5 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| Total Actinomyces | 51 (72.9)* | 33 (53.2) | 7 (12.3) | 6 (8.2) | 61 (71.8) | 45 (52.9) | 3 (8.6) | 4 (6.0) |
| Schaulia odontolytica | 0 | 4 | 21 | 33 | 0 | 2 | 8 | 21 |
| Total Schaulia | 0 (0) | 4 (6.5) | 21 (36.8) | 33 (45.2) | 0 (0) | 2 (2.4) | 8 (22.9) | 21 (31.3) |
| Veillonella parvula | 0 | 8 | 0 | 3 | 0 | 19 | 0 | 3 |
| Veillonella dispor | 1 | 0 | 0 | 6 | 0 | 0 | 0 | 14 |
| Veillonella atypica | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 8 |
| Veillonella tobetsuensis | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 3 |
| Other Veillonella species | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 3 |
| Total Veillonella | 1 (1.4) | 10 (13.9) | 0 (0) | 16 (21.9) | 0 (0) | 19 (22.4) | 1 (2.9) | 31 (44.9) |
| Neisseria flavescens | 0 | 0 | 11 | 0 | 0 | 0 | 0 | 0 |
| Neisseria macacae | 1 | 0 | 0 | 0 | 8 | 0 | 2 | 0 |
| Neisseria mucosa | 1 | 1 | 2 | 0 | 1 | 0 | 2 | 0 |
| Neisseria oralis | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Neisseria subflava | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| Neisseria elongata | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Neisseria sicca | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Other Neisseria species | 3 | 0 | 5 | 0 | 0 | 0 | 0 | 2 |
| Total Neisseria | 11 (15.7) | 1 (1.6) | 21 (36.8) | 0 (0) | 10 (11.8) | 0 (0) | 6 (17.1) | 0 (0) |
| Streptococcus salivarius | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 2 |
| Streptococcus sanguinis | 1 | 1 | 0 | 2 | 0 | 1 | 0 | 0 |
| Streptococcus australis | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 |
| Streptococcus oralis | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| Streptococcus parasanguinis | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| Streptococcus mitis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Streptococcus infantis | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Streptococcus mutans | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Other Streptococcus species | 1 | 2 | 2 | 4 | 0 | 0 | 1 | 0 |
| Total Streptococcus | 2 (2.9) | 3 (4.8) | 4 (7.0) | 12 (16.4) | 3 (3.5) | 3 (3.5) | 3 (8.6) | 5 (7.2) |
| Rothia mucilaginosa | 0 | 0 | 3 | 1 | 0 | 0 | 0 | 1 |
| Rothia dentocariosa | 2 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| Rothia aeria | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 0 |
| Rothia species | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total Rothia | 3 (4.3) | 1 (1.6) | 3 (5.3) | 1 (1.4) | 2 (2.4) | 0 (0) | 9 (25.7) | 1 (1.4) |
| Capnocytophaga ochracea | 1 | 0 | 0 | 0 | 0 | 5 | 0 | 0 |
| Capnocytophaga gingivalis | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Capnocytophaga genospecies | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Total Capnocytophaga | 1 (1.4) | 2 (3.2) | 0 (0) | 0 (0) | 1 (1.2) | 3 (3.4) | 0 (0) | 0 (0) |
| Selenomonas noxia | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Selenomonas flueggei | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Selenomonas artenitis | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 |
| Total Selenomonas | 0 (0) | 1 (1.6) | 0 (0) | 0 (0) | 0 (0) | 5 (5.9) | 0 (0) | 0 (0) |
| Paraburkholderia fungorum | 0 | 0 | 0 | 0 | 3 | 0 | 4 | 1 |
| Fusobacterium nucleatum | 0 | 5 | 0 | 0 | 0 | 2 | 0 | 0 |
| Pseudopropionibacterium propioni- cum | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 |
| Haemophilus parainfluenzae | 0 | 1 | 0 | 2 | 0 | 0 | 1 | 1 |

Continued
produces reducing power and stimulates their metabolism. Lactate can also be produced by most saccharolytic bacteria in the oral cavity, such as streptococci and lactobacilli. Furthermore, *Actinomyces* is also capable of utilizing lactate under aerobic conditions, which produces reducing power, suggesting that it has the potential to produce NO$_2^-$ via a pathway coupled with the aerobic lactate metabolism. *Neisseria* is also known to utilize lactate via a pathway that produces reducing power. However, there is limited information about the metabolic properties of *Rothia*, and further metabolic studies of this genus and the other oral NO$_2^-$-producing bacteria identified in the present study are needed. It was not possible to explain the differences in the composition of the NO$_2^-$-producing bacterial populations between adults and children or among individuals in the current study, but the metabolic characteristics and networks of the oral microbiome might account for these variations. Rosier et al. suggested that nitrite leads to rapid modulation of microbiome in their latest report, various environmental factors such as pH, oxygen concentrations in the oral cavity may also affect the bacterial composition of oral microbiome. Although it is difficult to discuss the detailed effects by these environmental factors on the differences of oral microbiome at the present stage, further study is essential.

NO$_2^-$ has attracted attention due to its antibacterial activity and its ability to suppress cardiovascular disease by improving the systemic blood circulation. A double-blind study found that cardiovascular disease symptoms were ameliorated in individuals that consumed NO$_2^-$-rich foods. Therefore, it might be possible to prevent both oral (such as caries and periodontitis) and cardiovascular disease by using NO$_2^-$-producing bacteria as probiotics and/or NO$_2^-$ as a prebiotic. Furthermore, the associations between NO$_2^-$ and methemoglobinemia or the onset of cancer are unclear and require further study.

### Materials and methods

This study was approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry (approval number: 2018-3-17). All methods were carried out in accordance with relevant guidelines and regulations.

#### Subjects

Twenty-two volunteers (11 adults [subject no. 1–11], 22–43 years; 11 children [subject no. 12–22], 5–12 years) were included in this study, and informed consent was obtained from each subject. The exclusion criteria were as follows: no dental plaque or tongue coating being seen during a visual inspection; toothbrushing after meals; eating or drinking anything other than water or sugar-free drinks after meals; having edentulous jaws, systemic disease, untreated dental caries, untreated periodontal disease, or oral mucosal lesions; having used a mouthwash for disinfection within 24 h; and taking antibiotics within 3 months. DMFT, dmft, and OHI-S were recorded as oral clinical examination parameters.

#### Sample collection and preparation

Samples were collected from adults at the Division of Oral Ecology and Biochemistry, Tohoku University Graduate School of Dentistry, and they were collected from children at the Department of Pediatric Dentistry, Tohoku University Hospital. The volunteers were asked to stop brushing their teeth in the morning, and dental plaque and tongue coating samples were collected ≥ 2 h after meals. Sampling was performed by the same dentist. Dental plaque samples were collected from the full dentition using a sterile toothpick or a sterile excavator and placed in a sterile tube on ice. Tongue coatings were collected from the tongue dorsum using a sterile wooden spatula, suspended in a sterile tube containing 1 mL of ice-cold physiological saline, and centrifuged at 10,000 rpm and 4 °C for 2 min (H-15FR, Kokusan, Tokyo, Japan). The resultant pellets were used as tongue coating samples. The wet weight of the dental plaque and tongue-coating samples was measured, and then the samples were suspended in 1 mL of 40 mM phosphate buffer solution (PPB) (pH 7), homogenized using a sterile glass homogenizer for 5 min, and used for the subsequent experiments.

#### Measurement of NO$_2^-$-producing activity in dental plaque and tongue coating samples

The reaction mixture used to assess NO$_2^-$-producing activity contained 100 μL of the dental plaque or tongue coating samples from adults and children. Bold values indicate the total number for each bacterial genus. *The value in parentheses is percentage in total. Only once detected bacteria, including Aggregatibacter segnis, Staphylococcus epidermidis, Moraxella lacunata, Leptotrichia hofstadii, Gemella morbillorum, Campylobacter concisus, Lachnoanaerobaculum umeaense, and Morococcus cerebrosus.*

|                 | Adults          |          | Children        |          |
|-----------------|-----------------|----------|-----------------|----------|
|                 | Dental plaque   | Tongue coating | Dental plaque | Tongue coating |
|                 | Aerobic         | Anaerobic | Aerobic         | Anaerobic |
| *Prevotella melaninogenica* | 0               | 0         | 0               | 0         |
| *Eikenella corrodens*        | 0               | 0         | 0               | 2         |
| Other species*               | 1               | 1         | 0               | 3         |
| Total others              | 1 (1.4)         | 7 (11.3)  | 0 (0)           | 3 (4.1)   |
| Unknown species           | 0 (0)           | 0 (0)     | 1 (1.8)         | 0 (0)     |
| Total                  | 70 (100)        | 62 (100)  | 57 (100)        | 73 (100)  |

Table 1. The number and proportion of NO$_2^-$-producing bacteria identified in the dental plaque and tongue coating samples from adults and children.
ing suspension, 10 µL of 1 M PPB (pH 7.0), 10 µL of 25 mM KNO₃, and 130 µL of de-ionized water. After being subjected to aerobic incubation at 37 °C for 30 min, the reaction mixture was centrifuged at 10,000 rpm at 4 °C for 3 min, and the supernatants were used for the NO₂⁻ assay.

The NO₂⁻ concentration was measured with the Griess reagent kit (Dojindo Molecular Technologies, Kumamoto, Japan), in which naphthylethylenediamine dihydrochloride and sulphanilamide react with NO₂⁻ to form a purple azo product. The concentration of the azo product was measured colorimetrically at 540 nm using a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Tokyo, Japan).

**Culturing of bacteria**

Dental plaque and tongue coating suspensions that had been diluted ten-fold in sterilized 40 mM PPB (pH 7.0) were prepared, spread onto blood agar plates (CDC anaerobe 5% sheep blood agar, BD Japan, Japan), and cultured at 37 °C aerobically in an incubator (CL-410, Advantec Co. Ltd., Tokyo, Japan) or anaerobically in the anaerobic chamber containing 80% N₂, 10% CO₂, and 10% H₂ (ANB-18-2E, Hirasawa, Tokyo, Japan). After the samples had been cultured for 1 week, the total number of colonies on each plate was counted, and the number of NO₂⁻-producing bacterial colonies on each agar plate were detected using the method described below.

**Detection of NO₂⁻-producing bacteria.** NO₂⁻-producing bacterial colonies were detected by covering the plates with agar containing Griess reagent (Fig. 3). The blood agar plates were covered with agar containing 1 mM KNO₃ and 10 mM sodium lactate. After the agar had hardened, the surface was covered with agar containing naphthylethylenediamine dihydrochloride and sulphanilamide, the components of Griess reagent. In the modified method used in the present study, lactate was added to activate the metabolism of lactate-utilizing bacteria, such as Veillonella and Neisseria, and the covering agar was replaced by agar with a low melting point (30–31 °C) (Nakalai Tesque, Kyoto, Japan) to avoid heat damage to the bacteria. After being incubated for 20 min at room temperature, NO₂⁻-producing bacterial colonies created purple-colored spots, which were easy to identify (Fig. 3).

**Identification of NO₂⁻-producing bacteria.** The detected NO₂⁻-producing bacteria were identified using a molecular biological approach, which was described in a previous report. Genomic DNA was extracted from bacterial colonies using the InstaGene matrix kit (Bio-Rad, California, USA), according to the manufacturer’s instructions. Next, the 16S rRNA gene sequence was amplified using the polymerase chain reaction (PCR) using the universal primers 27F and 1492R and Taq DNA polymerase (HotStarTaq Master Mix, Qiagen, Netherlands), according to the manufacturer’s instructions. The primer sequences were as follows: 27F, 5′-AGA GTTT GATCMTGG CTC AG-3′; and 1492R, 5′-TAGGYYTACCTTTGATCTAG-3′. Amplification was performed using a PCR Thermal Cycler MP (TaKaRa Biomedicals, Japan) and the following program: 15 min at 95 °C for the initial heat activation and 35 cycles of 1 min at 94 °C for denaturation, 1 min at 52 °C for annealing, and 10 min at 72 °C for final extension. The PCR products were purified with the illustra GFX PCR DNA and gel band purification kit (GE Healthcare, Buckinghamshire, UK) and analyzed by Sanger sequencing services (Genewiz Co. Ltd., Saitama, Japan). The DNA sequencing data were analyzed via BLAST searches (performed through the National Center for Biotechnology Information website), and bacterial species were identified based on the highest percentage sequence similarity (> 98%).

**Statistical analyses.** The significance of differences in NO₂⁻-producing activity (per mg wet weight) between dental plaque and tongue coatings or between adults and children were analyzed using Tukey’s test. Similarly, the significance of the differences in the number of NO₂⁻-producing and non-NO₂⁻-producing bacteria were analyzed using Tukey’s test. The correlation between the number of NO₂⁻-producing bacteria and NO₂⁻-producing activity was examined. P-values of <0.05 indicated statistical significance. StatFlex Ver. 6 (Artech Co., Ltd., Osaka, Japan) was used for these statistical analyses.

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**References**

1. Dood, J. E. et al. Protective effect of salivary nitrate and microbial nitrate reductase activity against caries. *Eur. J. Oral Sci.* 112, 424–428. https://doi.org/10.1111/j.1600-0722.2004.00153.x (2004).
2. Noda, H., Minemoto, M. & Noda, A. Occurrence and reactivity of nitrite ion in human environments. *J. UOEH* 3, 425–439. https://doi.org/10.7888/joueh.3.425 (1981).
3. Lundberg, J. O., Weitzberg, E. & Gladwin, M. T. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat. Rev. Drug Discov.* 7, 156–167. https://doi.org/10.1038/nrd2466 (2008).
4. Xia, D., Deng, D. & Wang, S. Alterations of nitrate and nitrite content in saliva, serum, and urine in patients with salivary dysfunction. *J. Oral Pathol. Med.* 32, 95–99. https://doi.org/10.1111/j.1600-0714.2003.00199.x (2003).
5. Kapil, V. et al. Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free Radic. Biol. Med.* 55, 93–100. https://doi.org/10.1016/j.freeradbiomed.2012.11.013 (2013).
6. Xia, D. S., Liu, Y., Zhang, C. M., Yang, S. H. & Wang, S. L. Antimicrobial effect of acidified nitrate and nitrite on six common oral pathogens in vitro. *Chin. Med. J. (Engl)* 119, 1904–1909 (2006).
7. Li, H. et al. Salivary nitrate–An ecological factor in reducing oral acidity. *Oral Microbiol. Immunol.* 22, 67–71. https://doi.org/10.1111/j.1399-3003.2007.00313.x (2007).
8. Rosier, B. T., Marsh, P. D. & Mira, A. Resilience of the oral microbiota in health: Mechanisms that prevent dysbiosis. *J. Dent. Res.* 97, 371–380. https://doi.org/10.1177/0022034517742139 (2018).
9. Rosier, B. T., Buetas, E., Moya-Gonzalez, E. M., Artacho, A. & Mira, A. Nitrate as a potential prebiotic for the oral microbiome. Sci. Rep. 10(1), 12895. https://doi.org/10.1038/s41598-020-6983-x (2020).

10. Lundberg, J. O. & Weitzberg, E. NO-synthase independent NO generation in mammals. Biochem. Biophys. Res. Commun. 396, 39–45. https://doi.org/10.1016/j.bbrc.2010.02.136 (2010).

11. Hyde, E. R. et al. Metagenomic analysis of nitrate-reducing bacteria in the oral cavity: Implications for nitric oxide homeostasis. PLoS ONE 9, e88645. https://doi.org/10.1371/journal.pone.0088645 (2014).

12. Dood, J. J., Benjamin, N., Hector, M. P., Rogers, M. & Allaker, R. P. Evaluation of bacterial nitrate reduction in the human oral cavity. Eur. J. Oral Sci. 113, 14–19. https://doi.org/10.1111/ejos.2004.00184.x (2005).

13. Kanady, J. A. et al. Nitrate reductase activity of bacteria in saliva of term and preterm infants. Nitric Oxide 27, 193–200. https://doi.org/10.1016/j.niox.2012.07.004 (2012).

14. Mitsui, T., Saito, M. & Harasawa, R. Salivary nitrate-nitrite conversion capacity after nitrate ingestion and incidence of Veillonella spp. in elderly individuals. J. Oral Sci. 60, 405–410. https://doi.org/10.2334/josuds.17-0337 (2018).

15. Mashima, L. et al. Exploring the salivary microbiome of children stratified by the oral hygiene index. PLoS ONE 12, e0185274. https://doi.org/10.1371/journal.pone.0185274 (2017).

16. Ng, S. K. & Hamilton, I. R. Lactate metabolism by Veillonella parvula. J. Bacteriol. 105, 999–1005 (1971).

17. Hoshino, E., Yamada, T. & Araya, S. Lactate degradation by a strain of Neisseria isolated from human dental plaque. Arch. Oral Biol. 21, 677–683. https://doi.org/10.1016/0003-9967(76)90142-4 (1976).

18. Wicaksono, D. P., Washio, J., Abiko, Y., Domon, H. & Takahashi, N. Biochemical characterization of nitrate production from nitrate reduction with and without lactate metabolism in oral Veillonella. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.01255-20 (2020).

19. Noutoi, I. et al. Genome-based taxonomic classification of the phylum actinobacteria. Front. Microbiol. 9, 2007. https://doi.org/10.3389/fmicb.2018.02007 (2018).

20. Coenye, T. et al. Burkholderia fungorum sp. Nov. and Burkholderia cedaliae sp. Nov., two new species isolated from the environment, animals and human clinical samples. Int. J. Syst. Evol. Microbiol. 51, 1099–1107. https://doi.org/10.1099/ijs.0.027713-51-3-1099 (2001).

21. Dos Santos, S. G. et al. Development and nitrate reductase activity of sugarcane inoculated with five diazotrophic strains. Arch. Microbiol. 199, 863–873. https://doi.org/10.1007/s00203-017-1357-2 (2017).

22. Allison, C. & Macfarlane, G. T. Dissimilatory nitrate reduction by Propionibacterium acnes. Appl. Environ. Microbiol. 55, 2889–2903. https://doi.org/10.1128/AEM.55.11.2899-2903.1989 (1989).

23. Vanhatalo, A. et al. Nitrate-responsive oral microbiome modulates nitric oxide homeostasis and blood pressure in humans. Free Radic. Biol. Med. 20, 21–30. https://doi.org/10.1016/s0891-5849(00)00407-9 (2000).

24. Gross, E. L. et al. Beyond Streptococcus mutans: Dental caries onset linked to multiple species by 16S rRNA community analysis. PLoS ONE 7, e47722. https://doi.org/10.1371/journal.pone.0047722 (2012).

25. Washio, J., Shimada, Y., Yamada, M., Sakamaki, R. & Takahashi, N. Effects of pH and lactate on hydrogen sulfide production by oral Veillonella spp. Appl. Environ. Microbiol. 80, 4184–4188. https://doi.org/10.1128/aem.00606-14 (2014).

26. Takahashi, N. & Yamada, T. Glucose and lactate metabolism by Propionibacterium acnes strains. J. Bacteriol. 190, 487–503. https://doi.org/10.1128/JB.00606-14 (2015).

27. Takahashi, N. & Washio, J. Oral microbiome metabolism: From “who are they?” to “what are they doing?”. J. Dent. Res. 94, 1628–1637. https://doi.org/10.1177/00220345156006045 (2015).

28. Velmurugan, S. et al. Dietary nitrate improves vascular function in patients with hypercholesterolemia: A randomized, double-blind, placebo-controlled study. Am. J. Clin. Nutr. 103, 25–38. https://doi.org/10.3945/ajcn.115.116244 (2016).

29. Allaker, R. P. & Stephen, A. S. Use of probiotics and oral health. Curr. Oral Health Rep. 4, 309–318. https://doi.org/10.1007/s40496-017-0159-6 (2017).

30. Ward, M. H. et al. Drinking water nitrate and human health: An updated review. Int. J. Environ. Res. Public Health 15, E1557. https://doi.org/10.3390/ijerph15071557 (2018).

31. Yamamoto, Y., Washio, J., Shimizu, K., Igarashi, K. & Takahashi, N. Inhibitory effects of nitrate on acid production in dental plaque in children. Oral Health Prev. Dent. 15, 153–156. https://doi.org/10.3290/ohipd.a37926 (2017).

32. Washio, J., Sato, T., Koseki, T. & Takahashi, N. Hydrogen sulfide-producing bacteria in tongue biofilm and their relationship with oral malodour. J. Med. Microbiol. 54, 889–895. https://doi.org/10.1099/jmm.0.46118-0 (2005).

33. Lane, D. J. 16S/23S rRNA sequencing. In Nucleic Acid Techniques in Bacterial Systematics (eds Stackebrandt, E. & Goodfellow, M.) 115–175 (Wiley, Chichester, 1991).

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Author contributions
Y.S.S. and J.W. contributed equally as the first authors. Y.S.S. contributed to acquisition and analysis, drafted and critically revised the manuscript. J.W. contributed to conception, design, data acquisition, analysis and interpretation, drafted and critically revised the manuscript. D.P.W. contributed interpretation and critically revised the manuscript. T.S. contributed interpretation and critically revised the manuscript. S.F. contributed interpretation and critically revised the manuscript. N.T. contributed to conception, design, analysis and interpretation, drafted and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

Competing interests
The authors declare no competing interests.

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