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研究題目：Enhancement of caries –preventive nitrite（NO_2^-）production by oral Veillonella

Aim：

The oral cavity is an important part of our body, which acts as the first door/ gateway for every substrate to the body. It also plays an important role for mastication, aesthetic, and phonetic, hence maintaining the health in the oral cavity is essential for our health and Quality of Life（QOL）. However, the prevalence of oral diseases, notably dental caries in children is relatively high, especially in the developing countries such as Indonesia. Dental caries is initiated by exposure of acid production such as lactic acid produced by carbohydrate metabolism of some acidogenic microorganism such as *Streptococcus mutans*. This acid production furthermore causes loss of enamel structure and known as a demineralization. In 2013, the prevalence of dental caries is 76.2% in Indonesia（Fig.1）. Since this number is high, many researchers seek the efficient and effective way to suppress it. Then, optimizing beneficial bacteria in the oral cavity through controlling the daily intake of food, drink and snack, as well as promoting the oral hygiene through the oral health education, seem one of the promising options as a caries-preventive strategy to improve this situation.

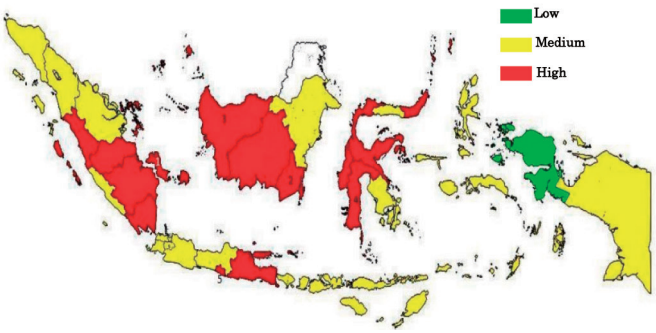

Fig 1. The prevalence of dental caries in Indonesia（Rikesda, 2013）

More than 700 bacterial species are living in the oral cavity by utilizing a kind of substrates that available in the oral environment. *Veillonella* species is known as one of major oral bacteria and detected at high frequency on the tongue surface, buccal mucosa, dental surface, and also have been found in severe early childhood caries. Recently, several *Veillonella* species have been identified in children and healthy young adults such as *V. atypica, V. dispar, V. rogosae, V. tobetsuensis, V. parvula,* and *V. denticariosi*. These bacteria possess capability to produce NO_2^- by reducing nitrate（NO_3^-）. NO_3^- as an essential compound is easily found in the oral cavity due to supply from green leafy vegetables such as spinach, lettuce and cabbage. Furthermore, 25% of ingested NO_3^- is secreted in saliva, therefore NO_3^-

is always available in the oral cavity (Fig 2).

NO_2^- is known as a beneficial compound and has been used widely in our daily life in food preservation. In dental and medical field, NO_2^- is known to inhibit the growth and metabolism of oral pathogenic bacteria such as *Streptococcus mutans, Porphyromonas gingivalis*. NO_2^- is not only effective to inhibit the growth and metabolism of single bacterium, but also inhibit the acid production of dental plaque. In addition, NO_2^- is known to be able to contribute general health to normalize the blood pressure. After NO_3^- was ingested from daily intakes, it comes into contact with NO_3^- reducer bacteria in the oral cavity such as *Veillonella*, then be reduced into NO_2^- as describe above (fig 2). The NO_2^- then enters to the circulation and converts into nitric oxide (NO) by mammalian nitrite reductase or acidic stomach, resulting vasodilatation and lowering the blood pressure.

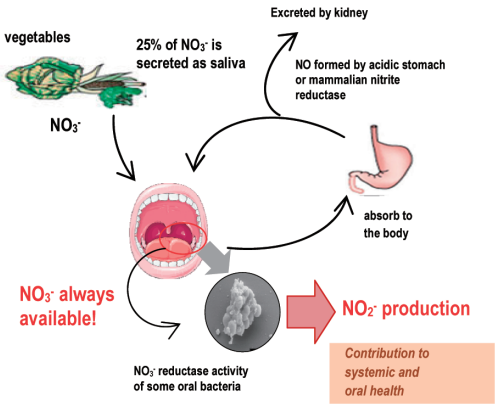

Fig 2. NO_3^- - NO_2^- - NO regulation in the body

However, the regulation of NO_2^- production mechanism of oral *Veillonella* is still unknown. Therefore, the aim of this study is to investigate how the environmental factors affect the growth and NO_2^- production of oral *Veillonella*.

Materials and Methods :

Effects of NO_3^- and NO_2^- on the growth activity of oral *Veillonella*

In this research, *Veillonella atypica and Veillonella parvula* were used as representative oral *Veillonella*. *Veillonella* species were pre-cultured in a complex medium containing 0.5% tryptone, 0.3% yeast extract and 1.26% sodium lactate in 50 mM potassium phosphate buffer (PPB, pH 7)(TYL) with and without 1mM sodium nitrate (KNO_3) or 1mM sodium nitrite (KNO_2) under anaerobic condition. At the logarithmic phase of growth, these pre-cultured bacteria were transferred to the new TYL medium with or without 1mM KNO_3 or 1mM KNO_2, and then bacterial growth was estimated by monitoring the optical density (OD) of culture medium at 660nm using spectrophotometer.

NO_2^- production of oral Veillonella

Veillonella species were anaerobically cultured in TYL medium with or without 1mM KNO_3 or 1mM KNO_2. After harvested at late logarithmic phase, the bacterial cells were washed twice and re-suspended in the buffer containing 75mM potassium chloride, 75mM sodium chloride, 2mM magnesium chloride in 2mM PPB (pH 7) as a bacterial cell suspension. Reaction mixtures containing bacterial cell suspension (OD=1), sodium lactate (0-50mM), and 1mM KNO_3 in 40mM PPB (pH 7 or 5) were prepared, then incubated at 37℃ in aerobic

for 30min. The amount of NO_2^- in the supernatant was measured by using Griess Reagent kit.

Results and Discussion :

In the presence of NO_3^- or NO_2^- , the length of lag phase was shortened, however the growth speed in the logarithmic growth phase and final OD growth was similar. This effect became clearer when the pre-cultured condition was modified. The lag phase of *Veillonella atypica* in the absence of NO_3^- or NO_2^- became longer when pre-cultured with NO_3^-, while that of *Veillonella parvula* became longer only when pre-cultured with NO_3^- (fig. 3). These observations suggest that NO_3^- and NO_2^- are tightly linked to the physiological properties of *Veillonella* species that can adapt flexibly to the growth condition and establish the optimum system for the logarithmic phase of growth.

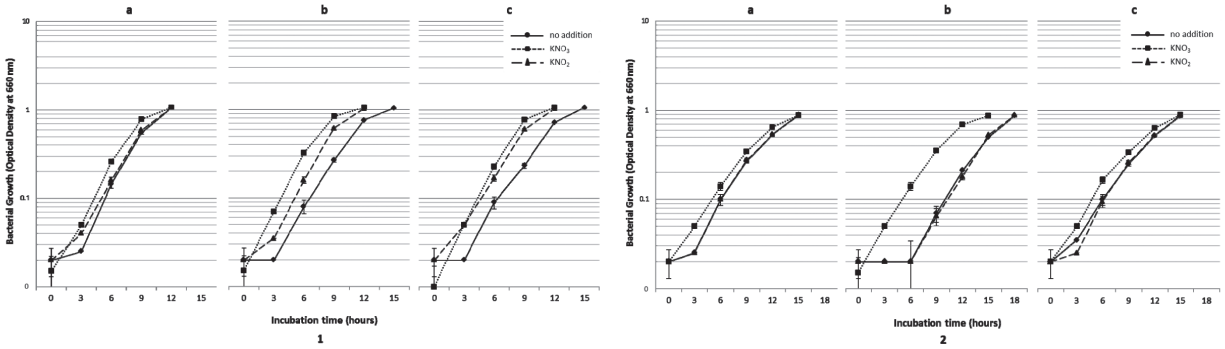

Fig 3. Effects of NO_3^-, NO_2^- on the growth of *Veillonella atypica* (1) and *Veillonella parvula* (2) pre-cultured without KNO_3 or KNO_2 (a), with KNO_3 (b), and with KNO_2 (c)

NO_2^- production was observed by the addition of lactate and it became higher with a concomitant increase of the lactate amount. Furthermore, this NO_2^- production was higher under acidic conditions. The enhancement of NO_2^- production was also observed in bacterial cells grown with NO_3^- and NO_2^- (fig. 4). Based on these results, lactate is obviously required for NO_2^- production and the acid condition might play a role to turn dissociated form of lactate ($lactate^- + H^+$) to undissociated form (lactate-H) that can penetrate bacterial cell membrane more efficiently. The increase in NO_2^- production by bacterial cells grown in the presence of NO_3^- and NO_2^- suggests that these compounds induce an enzymatic system responsible for NO_2^- production, although detailed mechanisms remain to be elucidated.

These results indicate that oral *Veillonella* species are responsible for NO_2^- production, especially in the presence of lactate and under acidic conditions. Considering that caries initiates under acidic conditions that are induced by bacterial lactate production, these physiological properties of oral *Veillonella* to produce NO_2^- from NO_3^- seem to be advantageous for preventing caries through inhibiting the growth and metabolism of acid-producing bacteria. Furthermore, NO_2^- is enzymatically and/or non-enzymatically converted to NO in the stomach, resulting in vasodilation and hypotension. Hence, **the enhancement of**

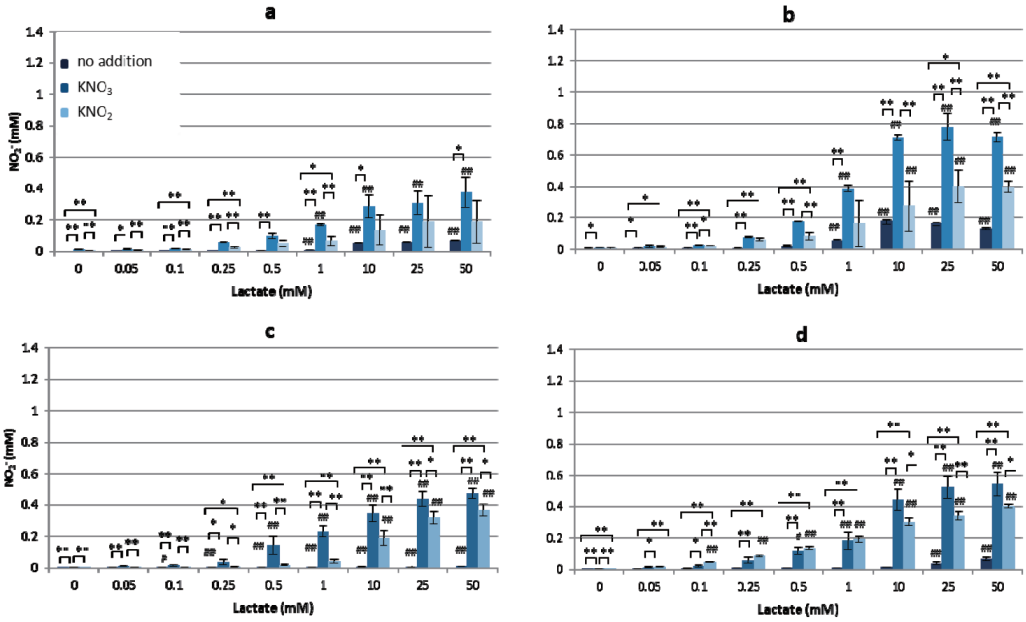

Fig 4. NO_2^- production of *Veillonella atypica* at pH 7 (a), pH 5 (b) and *Veillonella parvula* at pH 7 (c), pH 5 (d)
*p<0.05, **p<0.01 (comparing the groups between bacterial cell grown only with TYL, KNO_3, KNO_2), by tukey's test.
#p<0.05, ##p<0.01 (comparing the groups in the same grown condition with 0 mM of lactate) by dunnett's test.

NO_2^- production by oral Veillonella might be a way to maintain not only the oral health but also the general health.

Presentation：

Dimas Prasetianto Wicaksono, Jumpei Washio, Nobuhiro Takahashi：Nitrate/nitrite promote the growth and nitrite production of oral Veillonella The 61th Annual Meeting of Japanese Association for Oral Biology, Tokyo Dental University, Tokyo, Japan, 2019/10/12-14

Published Journal：in the progress