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Salivary nitrate – An ecological factor in reducing oral acidity

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Background/aims: Human oral cavities represent a novel environment with a constant supply of concentrated nitrate. For humans, over eighty percent of dietary nitrate originates from fruit and vegetables. With a healthy, balanced diet rich in fruit and vegetables, the concentration of nitrate in saliva can reach up to more than three times the European drinking water standard. The physiological function of the active excretion of salivary nitrate is unknown. Furthermore, little is known of the ecological function of oral nitrate and the effect on the oral environment during its subsequent oral microbial conversions. The objectives of the research were to investigate the effect on salivary pH coupled with oral microbial nitrate and/or nitrite reduction.

Methods: Human saliva samples were incubated anaerobically in the presence of 111.0 mM glucose (2%), with and without 1.5 mM nitrate/nitrite, and with pH and nitrate/nitrite consumption measured during the time course of the incubations.

Results: We found that anaerobic incubation of saliva containing a mixture of oral bacteria in the presence of nitrate/nitrite substrates and glucose resulted in a higher pH than controls in the

absence of nitrate/nitrite. These results suggest that the presence of these electron acceptors either repressed acid fermentation, or increased alkali production, or consumed acid produced, thus reducing salivary acidity.

Conclusion: This finding identifies salivary nitrate as a possible ecological factor in reducing oral acidity. The possibility that a symbiotic relationship between host nitrate excretion and nitrate reducing microorganism might help protect against tooth decay should be explored further.

Key words: salivary nitrate-oral nitrate reduction-pH-tooth decay

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Human oral cavities represent a novel environment with a constant supply of concentrated nitrate (5, 19, 24). Nitrate availability in other natural environments, such as the oceans, on the other hand, is limited by a series of biological processes (12). Human dietary nitrate is absorbed into the bloodstream from the stomach and small intestine, and then concentrated by a factor of 10 to 20 by salivary glands (2, 5, 19, 24). Resultant salivary nitrate concentration ranges between 0.3 to 2.6 mM depending on the diet (5, 19, 24). Salivary nitrate is dose dependent on dietary nitrate (5, 19, 24) and more than eighty percent of dietary nitrate originates from fruits and vegetables (32). As a result, humans with a healthy, balanced diet rich in fruit and vegetables may have a constant nitrate flow into the oral cavity and its concentration reaches up to more than three times higher than that of the European drinking water standard (50 mg/l)(31). Most mammals, including humans, also synthesize nitrate (2, 6, 7). This indigenous nitrate arises from oxidation of nitric oxide (NO), which is a signal molecule and links to numerous biological functions (9). Therefore, even with a nitrate-free diet, there will be significant nitrate concentrations in plasma (2). The physiological function of active salivary nitrate excretion is as yet unknown.

Nitrate is the next most favourable electron acceptor in the environment for micro-organisms conducting energy yielding metabolism when oxygen becomes limited and it is reduced into different nitrogen species in the process (3, 10, 30, 33). This is crucial in the global nitrogen cycle, such as in the removal of nitrate in the marine ecosystem (12). The reduction of nitrate in anaerobic environments is dominated by two dissimilatory processes: respiratory denitrification and dissimilatory nitrate reduction to ammonium (DNRA) (29, 30). Salivary nitrate is reduced to

nitrite and nitrous oxide by oral micro-organisms in humans and animals (17, 19, 23, 28), indicating nitrate respiration is operating in the oral cavity. There have been concerns regarding the harmful effect of salivary nitrite. For example, it has been associated with the formation of nitrosamines, known to be carcinogenic to animals (18, 20, 27). More recently, increasing evidence suggests that salivary nitrite has beneficial effects to the host, such as anti-microbial activity, which can be enhanced under acidified conditions to prevent stomach and oral infections (19).

When oxygen becomes limited, nitrate respiration takes precedence over the next energy-inefficient, fermentation process (10, 33). Some bacterial niches in human hosts, such as the interior of dental plaques, are anaerobic (21, 22). Nitrate respiration in such niches is energetically less favourable than aerobic respiration, but more favourable than fermentative breakdown of carbohydrates. In fact, most bacteria which carry out DNRA, are also fermentative (29). For oral bacteria with both DNRA and fermentation metabolisms, the supply of nitrate in the oral cavity may activate their nitrate respiration and repress fermentation and, as a result, buffer salivary acidity. Repressing fermentation is not the only effect contributing to a saliva pH increase due to nitrate respiration. Other mechanisms relating to nitrate reduction, such as hydroxyl ion generation (8, 26) and the scavenging of organic acid (3) may further contribute to the reduction of oral acidity. Human saliva contains a high density of micro-organisms detached from oral biofilms (15, 16, 21, 22). When human saliva is incubated with glucose, the pH has been found to fall due to fermentative metabolism by salivary bacteria (15, 16). This has been used as a conventional approach to studying factors affecting oral pH and tooth decay.

Materials and methods

Sampling: A total of 13 volunteers (6 males, 7 females) were selected across the age span 20 to 48. Saliva samples were collected by spitting saliva into 20-ml sterile plastic tubes without any stimulation. The total volume for each sample was between 8 to 10 ml. For experiments with no pre-incubation, the sample tubes were placed in ice-water during collection. For other experiments, samples were collected at room temperature (20 ± 3 °C). Samples were stored at 4-8 °C if not analysed immediately, but were never stored for more than 8 h.

Degassing and pre-incubation: Equal volumes of Milli Q water were added to each saliva sample and mixed thoroughly to reduce viscosity. Aliquots (2 ml) of the diluted saliva samples were then sub-sampled into six 40-ml, sterile, amber vials (triplicate for each treatment) with polypropylene hole caps, and sealed with PTFE/silicone septa (Supelco 27121U). The samples were then degassed with pure nitrogen through a sterile stainless needle (Fisher SZR-370-135X), which was pierced through the septum into the vial, and the tip submerged in the sample. Gas outlet during degassing was carried out via a sterile disposable syringe needle (TERUMO 0411080). To stop contamination during degassing, nitrogen gas was passed through a 0.2 µm filter. The degassing procedure was of 10 min duration, at a rate of approximately 200 ml min^{-1} . After degassing, the samples were incubated at 30 °C for 12 h where pre-incubation was applied.

Nitrate reduction assay: Glucose (BDH) and glucose plus nitrate /nitrite (added as potassium nitrate (Sigma) or potassium nitrite (Fisher) respectively) substrate solutions were prepared in the

sample vials as for the sample preparation from stock solution in Milli Q water. The pH of the solution was adjusted to 7.0 by addition of 2 M NaOH and/HCl. The solutions were then degassed as described previously.

Saliva samples and substrate solutions were both placed in a 37 °C water for 5 min before starting the reaction. The reactions were started by piercing the septa and injecting 3 ml aliquots of substrate solution into saliva sample vials to the final glucose concentration of 111.0 mM (2%) and nitrate/nitrite of 1.5 mM. Aliquots (0.8 ml) were taken by piercing the septa using a 1 ml sterile, disposable syringe, needle at the time indicated.

Determination of pH and nitrate/nitrite: Sample pH was measured in a 1 ml Eppendorf tube, using a micro pH probe (VWR 6621767), attached to a pH meter (JENWAY 3305, UK). Nitrate and nitrite concentrations were determined using an ion chromatography system (Dionex, UK), with conductivity detector and anion exchange column (AS9-HS). The mobile phase was 1.8 mM NaCO₃ and 1.7 mM NaHCO₃ in water, at a flow rate of 1.5 ml min⁻¹.

Aeration: Where aeration was applied, a similar method was used as for degassing, but air replaced nitrogen during incubation. The air was supplied by an air pump (Whisper 6000, UK) at a rate of approximately 100 ml min⁻¹.

Statistical analysis: All statistical analysis was carried out using MINITAB-14 software. T-tests were carried out using both the pH value at 3h and slopes of pH change between treatments during incubation.

Results

pH fluctuation coupled with nitrate consumption. The pH fall decreased during incubation with nitrate (1.5 mM) compared with controls (without nitrate) ($P < 0.001$, Paired T-Test, $N = 13$). A significant relative pH increase in the nitrate treatment compared to the control during incubation was observed ($N=13$, Figure 1 (a)). There was a very strong negative correlation between the average nitrate concentration and average relative pH increase during incubation ($R = -0.952$, $P = 0.012$, $N = 13$, Figure 1 (b)). Figure 1 (c) represents an empirical Cumulative Distribution Function plot (CDF). It highlights the pH differences between the nitrate treatment and control ($N=13$) for all saliva samples and at all incubation times, indicating no pH difference for pH 7 and above for both treatments, but suggesting pH was generally lower in control than that in the nitrate treatment during incubation. Approximately 22 percent of data points ($N=195$) were less than pH 6 in the control, with only 15 percent of data points ($N=195$) less than pH 6 in the nitrate treatment. There were 20 percent of data points less than pH 5.8 in the control, with the same percentage of data less than pH 6.3 in the nitrate treatment.

There were 4 volunteer's samples out of 13 in which pH fall was not reduced to a significant extent during incubation. Interestingly, nitrate was not consumed in a significant quantity during incubation for these four individuals (data not shown).

pH fluctuation coupled with nitrite consumption. Nitrous oxide has been detected in human exhaled air (23). In this study, during the reduction of nitrate, the accumulation of nitrite did not account for the loss of nitrate in terms of stoichiometry (data not shown), indicating nitrite is further reduced during microbial nitrate reduction. Indeed, when replacing nitrate with nitrite in a primary experiment using three saliva samples, under the same conditions, the fall in salivary pH declined significantly in the presence of nitrite. Figure 2 shows the relative pH increase and nitrite concentration at 3h (N=3). There was no significant pH difference in one out of 3 samples in the presence of nitrite. However, in this experiment, nitrite was not consumed in significant quantities (data not shown). The remaining two samples demonstrated progressive consumption of nitrite, and the relative pH increase was coupled to nitrite consumption (data not shown).

Factors influencing nitrate consumption and pH fluctuation. When using saliva from one individual, no reduced acidity was observed when nitrate was added and saliva was incubated aerobically, without anaerobic pre-incubation, and there was no consumption of added nitrate. Even nitrate carried over from the original saliva samples, in the control experiment, was not consumed in significant quantities (Figure 3 (a)). The reduced salivary acidity was also observed after addition of nitrate to washed saliva pellets, during anaerobic incubation (Figure 3 (b))

Discussion

Whole human saliva usually contains 2.6 mM ammonium (11), which is higher than the concentration required to suppress assimilatory nitrate reduction (29). This ensures salivary nitrate is used as an electron acceptor, to support anaerobic nitrate respiration in buffering salivary pH, rather than consumed by assimilatory nitrate reduction by the oral microflora. The

results suggest that the function of active concentration and excretion of salivary nitrate by humans and animals might have been selected to support anaerobic microbial nitrate respiration that competes for carbon with processes that lead to fermentative acid production; the subsequent nitrate respiration generates hydroxyl ions and scavenges organic acids which have been produced.

Experiments with saliva from one individual also supported the suggestion that the consumption of nitrate is unlikely to have been due to assimilatory nitrate reduction as aeration inhibited nitrate consumption, and assimilatory nitrate reduction is not sensitive to oxygen (29). The washed cell experiment demonstrated that the effect of nitrate on saliva pH is unlikely to be linked chemically to the supernatant, and is more likely to be associated with the activity of microbial populations in the pellets. In figure 1 (c), differences are mainly apparent below pH 7. The lack of difference above pH 7 between treatments is because data for pHs above 7 mainly reflect the initial incubation conditions (all saliva samples were initially above pH 7).

The primary experiment showed nitrite also mediated the reduction in acidity during anaerobic incubation, suggesting nitrate was consumed via an anaerobic nitrate respiration pathway. However, the final reduction pathway needs to be further elucidated. Of course one could also expect selective enrichment of denitrifiers (or nitrate-respiring bacteria) when nitrate is available and oxygen is limited (29). Salivary nitrate might have selected an anaerobic, nitrate-respiring microbial community to compete for carbon with obligate, fermentative bacteria. One of the ecological functions of dissimilatory nitrate reduction is to scavenge fermentative products, such as organic acids (3). Salivary nitrate may support the consumption of organic acids, which have

already been produced in the oral cavity, as electron donors through DNRA and the resultant pH increase in the oral cavity. The reason for the lack of nitrate reduction in the four out of 13 saliva samples might have been the lower microbial activity in saliva or individual differences in saliva microflora. This is not surprising since samples were collected without control, such as after brushing of teeth, and prior to fluid or food consumption. The same reason might be responsible for one individual result obtained in the experiment with nitrite, which did not indicate either nitrite reduction or acidity reduction. The reason for lack of nitrate/nitrite reduction in these samples should be explored in the future. The lack of acidity and nitrate/nitrite reduction capacities in all these experiments also supports the hypothesis that the slower pH decrease is linked to microbial metabolism of nitrate or/and nitrite.

It is commonly believed that lactobacilli and streptococci are the main lactic acid producers responsible for tooth decay (21, 22). Nitrate may not affect their metabolism as most of them do not reduce nitrate. However, species of *Actinomyces* are common in human sub-gingival plaque (25), are the main nitrate reducers in the human oral cavity (4) and are carbohydrate fermenting bacteria (14). For these bacteria, suppression of acid production could be theoretically expected when nitrate is available.

In the oral cavity, streptococci ferment carbohydrates to produce lactic acid, which is a principal fermentation substrate used by veillonellae (16). It has been shown that veillonellae from the human oral cavity can reduce nitrate (4). Lactic acid is also produced in the rumen (1). The importance of lactate fermenting bacteria with nitrate reducing capacity in protecting lactate accumulation in rumen (referred to as lactic rumen acidosis) has been postulated. The mechanism

is based on supplying nitrate to enhance the utilisation of lactate by the nitrate reducing *Selenomonas ruminantium* (1). In fact, lactate can stimulate nitrate and nitrite reduction to a greater extent than glucose in these bacteria (13). It will be very interesting to investigate the effect of salivary nitrate on the metabolic communication between lactic acid producing communities and lactic acid utilising communities, such as occurs in the rumen. The mechanism of nitrate reduction related oral acid reduction should be further explored.

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Figure legends

Figure 1. Fluctuations of salivary pH and nitrate concentration during anaerobic incubation.

Figure (a) shows relative pH increases in nitrate treatment during incubation (N = 13). The values of “relative pH increase” are calculated from the pH values in the nitrate treatment deducting the pH values in control (without nitrate) for all experiments of 13 samples at different times. All samples were pre-incubated and incubated anaerobically during the reaction. (b) Shows the correlation between average nitrate concentration and relative pH increase (N=13). (c) Shows the empirical Cumulative Distribution Function (CDF) plots (N = 13, each line was plotted from 195 pH data points). It calculates the proportion of data points less than each x value (pH value), and then plots the proportion (y value) as a function of x . The y -axis scale represents the percentage of data points less than each x (pH) value, not a probability scale for a specific distribution. Bars are standard errors of the mean in all figures.

Figure 2. Effect of nitrite reduction on pH during anaerobic incubation

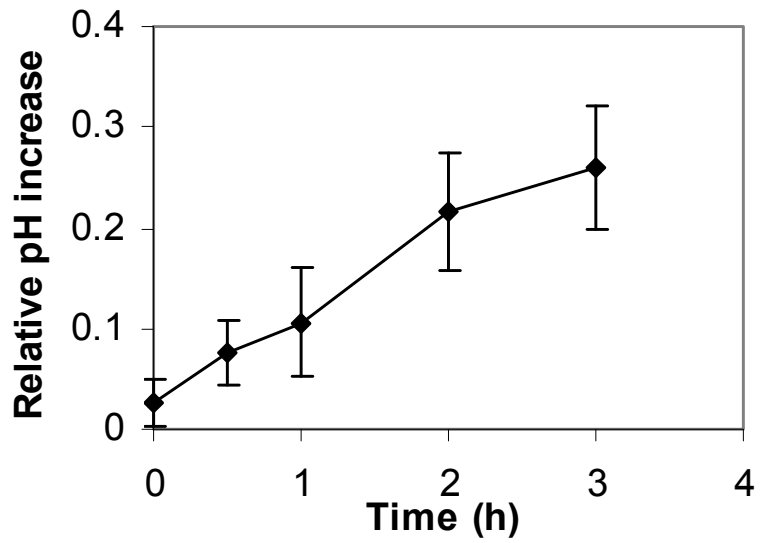
All conditions are the same as described in Figure 1, except nitrate was replaced by nitrite. Only relative pH difference and nitrite concentration at 3h were plotted (N=3).

Figure 3. Effects of aeration and saliva supernatant on pH and nitrate consumption

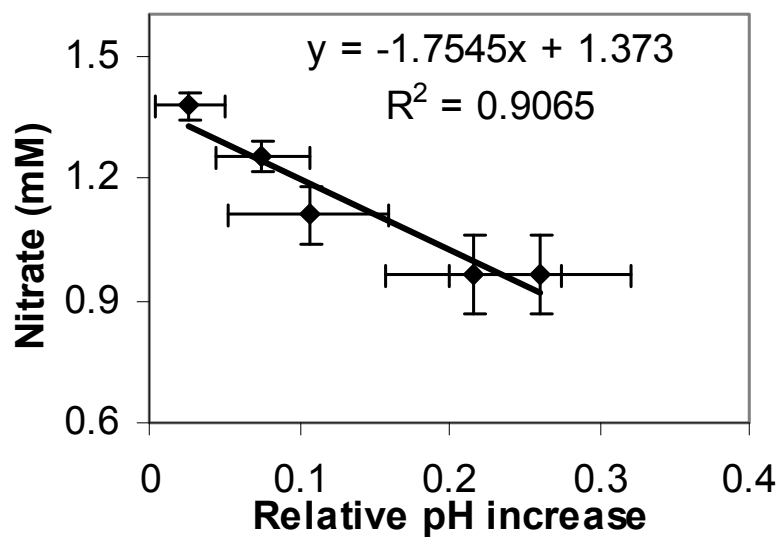
The saliva incubation conditions were: (a) no pre-incubation and incubated aerobically; Saliva was washed three times using 1x PBS (phosphate buffered saline), and re-suspended to the original volume. Other procedures were the same as described in methods except without pre-incubation (b). Each data point represents means from triplicate experiments from a volunteer's saliva sample.

Figure 1

a



b



c

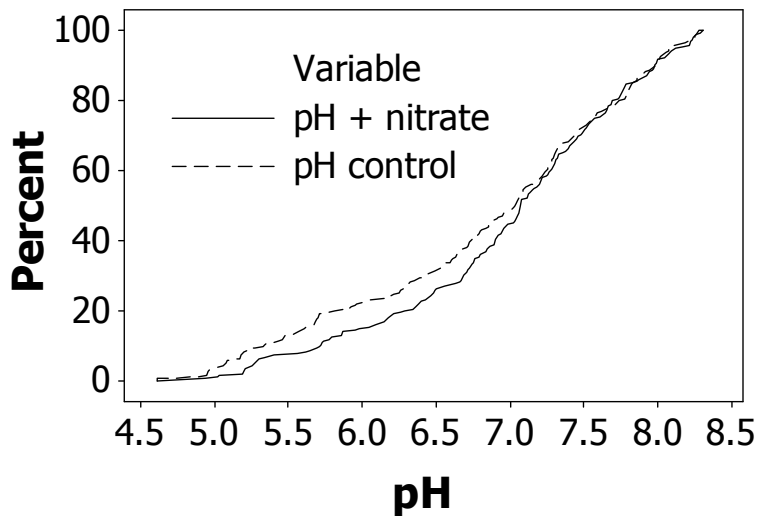


Figure 2

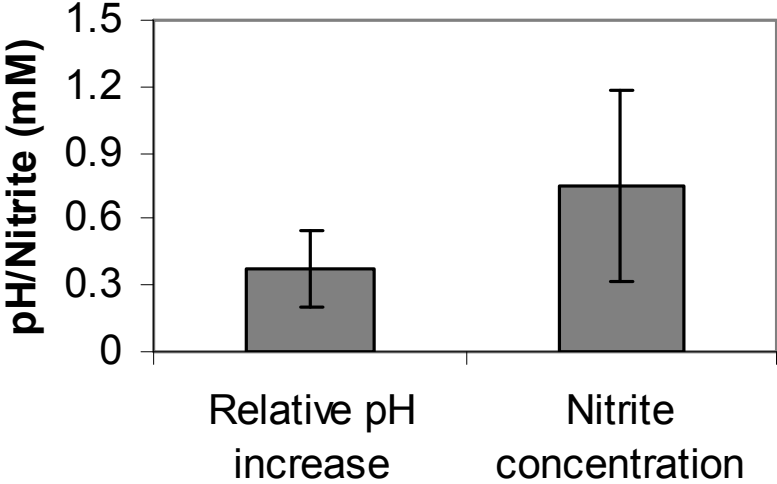
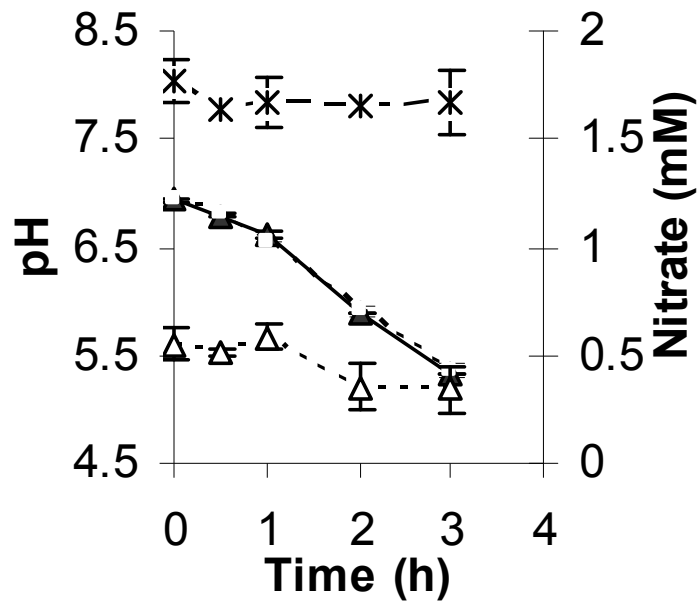
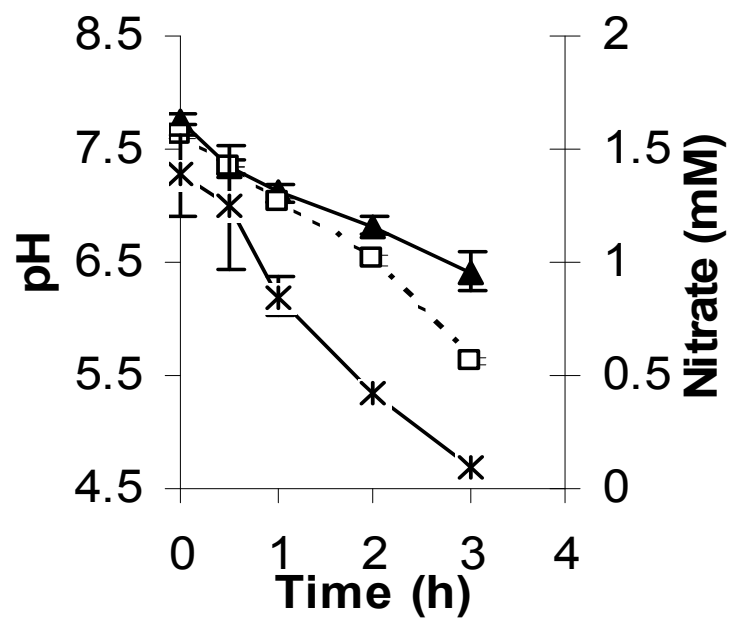


Figure 3

a.



b.



—▲— pH + nitrate

---□--- pH control

--✕-- nitrate concentration in nitrate treatment

---△--- nitrate concentration in control