Nitrate as a prebiotic and nitrate-reducing bacteria as probiotics for oral health

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Thesis summary

It has been estimated that we obtain over three quarters of dietary nitrate from vegetables and fruits. Nitrate-rich vegetable types include leafy greens and certain root vegetables (e.g., beetroots and radishes). The salivary glands actively concentrate plasma nitrate, leading to high salivary nitrate concentrations (5–8 mM) after a nitrate-rich meal. Nitrate is an ecological factor that can induce rapid changes in structure and function of polymicrobial communities. However, the effects on the oral microbiota have not been clarified, whilst a limited number of previous studies did indicate that nitrate is likely to be beneficial for oral health. The aim of this thesis was therefore to study nitrate-induced microbiome changes and identify potential mechanisms for nitrate-induced homeostasis, in order to determine if nitrate can be considered a prebiotic compound for oral health. A second aim was to isolate nitrate-reducing isolates and test their probiotic potential *in vitro*. In chapter 1, an *in vitro* study was set up testing the effect of 6.5 mM nitrate on oral communities grown from saliva of 12 healthy individuals. In chapter 2, fifty-three nitrate-

reducing isolates were obtained and the effect of six probiotic candidates was tested on healthy oral communities grown from saliva of different donors with or without 6.5 mM nitrate. In chapter 3, the effects of nitrate-rich beetroot extracts on oral acidification after sugar rinsing was tested in 24 individuals without active caries. Supernatants (chapters 1 and 2) or saliva samples (chapter 3) were taken for nitrate, nitrite, ammonium, lactate and pH measurements. Additionally, the bacterial composition of *in vitro* biofilms and salivary pellets were determined using 16S rRNA gene Illumina sequencing and/or qPCR of the nitrate-reducing genus *Rothia*. We showed that nitrate stimulates the growth of the beneficial genera *Rothia* and *Neisseria* in our *in vitro* model, while potentially decreasing caries-, halitosis- and periodontal disease-associated bacteria. Additionally, the *in vitro* and *in vivo* data presented in this thesis indicate that nitrate can limit or prevent pH drops when sugars are fermented by the oral microbiota – a mechanism of resilience that could be stimulated by the consumption of nitrate-rich vegetable extracts. The main pH buffering mechanisms of nitrate were lactic acid usage during denitrification (observed both *in vivo* and *in vitro*) and during the reduction of nitrite to ammonium, as well as the potential production of ammonia (observed *in vitro*). In this thesis, the effects of nitrate were observed after short periods, i.e., after 5-9 h incubation *in vitro* and/or after 1-4 hours after nitrate supplement intake *in vivo*. Future studies should focus on the longitudinal effects of daily nitrate intake. In chapter 2, nitrate-reducing species belonging to the genera *Rothia* and *Actinomyces* were isolated. A selection of *Rothia* isolates increased lactate usage and nitrate reduction capacities of oral communities, potentially benefitting dental health and systemic health, respectively. The *in vitro* and *in vivo* data presented in the current thesis suggest that nitrate can modulate the oral microbiota in ways that are beneficial for the host and could thus be considered a prebiotic substance for the oral microbiota. Additionally, nitrate-reducing isolates can stimulate certain beneficial effects of nitrate metabolism. Nitrate and nitrate-reducing bacteria are thus promising components for future oral care products to prevent or treat oral diseases and this should be further investigated.

Resumen de la tesis

Se ha estimado que obtenemos más de las tres cuartas partes del nitrato que ingerimos de la fruta y la verdura. Los vegetales ricos en nitratos incluyen

verduras de hoja verde y ciertos tubérculos (p. ej., remolachas y rábanos). Las glándulas salivales concentran activamente el nitrato plasmático, lo que da lugar a concentraciones elevadas de nitrato en la saliva (5 a 8 mM) después de una comida rica en nitratos. El nitrato es un factor ecológico que puede inducir cambios rápidos en la estructura y función de las comunidades polimicrobianas. Sin embargo, los efectos sobre la microbiota oral no se han estudiado en detalle, mientras que un número limitado de estudios previos a esta tesis indican que es probable que el nitrato sea beneficioso para la salud bucal. El objetivo de esta tesis es, por tanto, estudiar los cambios microbiológicos inducidos por nitratos e identificar posibles mecanismos de homeostasis generados por este compuesto, con el fin de determinar si el nitrato puede considerarse un prebiótico para la salud bucal. Un segundo objetivo fue aislar cepas reductoras de nitrato y probar su potencial probiótico *in vitro*. En el capítulo 1, se realizó un estudio *in vitro* para testar el efecto del nitrato 6,5 mM en comunidades orales cultivadas a partir de la saliva de 12 individuos sanos. En el capítulo 2, se obtuvieron 53 aislados de bacterias reductoras de nitrato y se probó el efecto de seis candidatos a probióticos en comunidades orales sanas cultivadas a partir de saliva de diferentes donantes con o sin nitrato 6,5 mM. En el capítulo 3, se estudió el efecto de un extracto de remolacha rico en nitrato sobre la acidificación oral después de un enjuague con azúcar en 24 individuos sin caries activas. Se tomaron sobrenadantes (capítulos 1 y 2) o muestras de saliva (capítulo 3) para mediciones de nitrato, nitrito, amonio, lactato y pH. Además, la composición bacteriana de la biopelícula *in vitro* y del pellet salivar se determinó usando secuenciación Illumina del rRNA 16S y/o qPCR del género nitratorreductor *Rothia*. Los datos demuestran que el nitrato estimula el crecimiento de los géneros beneficiosos *Rothia* y *Neisseria* en nuestro modelo *in vitro*, mientras que potencialmente disminuye las bacterias asociadas a la caries, la halitosis y la enfermedad periodontal. Además, los datos *in vitro* e *in vivo* presentados en esta tesis indican que el nitrato puede limitar o prevenir caídas de pH cuando los azúcares son fermentados por la microbiota oral, un mecanismo de resiliencia que podría ser estimulado por el consumo de extractos vegetales ricos en nitratos. Los principales mecanismos de amortiguación del pH por parte del nitrato son el uso de acido láctico durante la desnitrificación (observado tanto *in vivo* como *in vitro*) y durante la reducción de nitrito a amonio, así como la producción potencial de amoníaco (observado *in vitro*). En esta tesis, los efectos del nitrato se observaron después de períodos cortos, es decir, después de 5-9 h

de incubación *in vitro* y 1-4 horas después de la ingesta del suplemento de nitrato *in vivo*. Los estudios futuros deberían centrarse en los efectos longitudinales de la ingesta diaria de nitratos. En el capítulo 2, se aislaron bacterias reductoras de nitrato pertenecientes a los géneros *Rothia* y *Actinomyces*. Una selección de aislados de *Rothia* aumentó el uso de lactato y la capacidad de reducción de nitratos de las comunidades bucales, lo que potencialmente beneficiaría la salud dental y la salud sistémica, respectivamente. Los datos *in vitro* e *in vivo* presentados en esta tesis sugieren que el nitrato puede modular la microbiota oral en aspectos que son beneficiosas para el huésped y, por lo tanto, podría considerarse una sustancia prebiótica para la microbiota oral. Además, los aislados reductores de nitratos pueden estimular los efectos beneficiosos del metabolismo del nitrato, sobre todo en personas con bajos niveles de estas bacterias. El nitrato y las bacterias reductoras de nitrato son, por lo tanto, componentes prometedores para futuros productos de salud oral para prevenir o tratar enfermedades bucales, lo cual debería investigarse más a fondo.

Resum de tesi

S'ha estimat que obtenim més de les tres quartes parts del nitrat que ingerim de la fruita i la verdura. Els vegetals rics en nitrats inclouen verdures de fulla verda i uns certs tubercles (p. ex., remolatxes i raves). Les glàndules salivals concentren activament el nitrat plasmàtic, la qual cosa dona lloc a concentracions elevades de nitrat a la saliva (5 a 8 mm) després d'un menjar ric en nitrats. El nitrat és un factor ecològic que pot induir canvis ràpids en l'estructura i funció de les comunitats polimicrobianes. No obstant això, els efectes sobre la microbiota oral no s'han estudiat detalladament, mentre que un nombre limitat d'estudis previs a aquesta tesi indiquen que és probable que el nitrat siga beneficiós per a la salut bucal. L'objectiu d'aquesta tesi és, per tant, estudiar els canvis microbiològics induïts per nitrats i identificar possibles mecanismes d'homeòstasi generats per aquest compost, amb la finalitat de determinar si el nitrat pot considerar-se un prebiòtic per a la salut bucal. Un segon objectiu va ser aïllar soques reductores de nitrat i provar el seu potencial probiòtic *in vitro*. En el capítol 1, es va realitzar un estudi *in vitro* per a testar l'efecte del nitrat 6,5 mm en comunitats orals cultivades a partir de la saliva de 12 individus sans. En el capítol 2, es van obtindre 53 aïllats de bacteris reductors de nitrat i es va provar l'efecte de sis

candidats a probiòtics en comunitats orals sanes cultivades a partir de saliva de diferents donants amb o sense nitrat 6,5 mm. En el capítol 3, es va estudiar l'efecte d'un extracte de remolatxa ric en nitrat sobre l'acidificació oral després d'un glopeig amb sucre en 24 individus sense càries actives. Es van prendre sobrenadants (capítols 1 i 2) o mostres de saliva (capítol 3) per a mesuraments de nitrat, nitrit, amoni, lactat i pH. A més, la composició bacteriana de la biopel·lícula *in vitro* i del pèl·let salivar es va determinar usant seqüenciació Illumina del RNAr 16S i/o qPCR del gènere nitratorreductor Rothia. Les dades demostren que el nitrat estimula el creixement dels gèneres beneficiosos Rothia i Neisseria en el nostre model *in vitro*, mentre que potencialment disminueix els bacteris associats a la càries, l'halitosi i la malaltia periodontal. A més a més, les dades *in vitro* i *in vivo* presentades en aquesta tesi indiquen que el nitrat pot limitar o previndre caigudes de pH quan els sucres són fermentats per la microbiota oral, un mecanisme de resiliència que podria ser estimulat pel consum d'extractes vegetals rics en nitrats. Els principals mecanismes d'amortiment del pH per part del nitrat són l'ús de àcid làctic durant la desnitrificació (observat tant *in vivo* com *in vitro*) i durant la reducció de nitrit a amoni, així com la producció potencial d'amoníac (observat *in vitro*). En aquesta tesi, els efectes del nitrat es van observar després de períodes curts, és a dir, després de 5-9 h d'incubació *in vitro* i 1-4 hores després de la ingesta del suplement de nitrat *in vivo*. Els estudis futurs haurien de centrar-se en els efectes longitudinals de la ingesta diària de nitrats. En aquesta tesi es van aïllar bacteris reductors de nitrat pertanyents als gèneres Rothia i Actinomyces. Una selecció d'aïllats de Rothia va augmentar l'ús de lactat i la capacitat de reducció de nitrats de les comunitats bucals, la qual cosa potencialment beneficiaria la salut dental i la salut sistèmica, respectivament. Les dades *in vitro* i *in vivo* presentats en aquesta tesi suggereixen que el nitrat pot modular la microbiota oral en aspectes que són beneficiosos per a l'hoste i, per tant, podria considerar-se una substància prebiòtica per a la microbiota oral. A més, els aïllats reductors de nitrats poden estimular els efectes beneficiosos del metabolisme del nitrat, sobretot en persones amb baixos nivells d'aquests bacteris. El nitrat i els bacteris reductors de nitrat són, per tant, components prometedors per a futurs productes de salut oral per a previndre o tractar malalties bucals, la qual cosa hauria d'investigar-se més a fons.

Introduction

Introduction

Part of the introduction (page 16–34) is a repository copy of the literature review published in the Journal of Dental Research:

Rosier, B. T., Marsh, P. D., & Mira, A. (2018). Resilience of the Oral Microbiota in Health: Mechanisms That Prevent Dysbiosis. *Journal of Dental Research*, 97(4), 371–380*

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The burden of oral diseases

The most common oral diseases include caries, periodontal diseases (gingivitis and periodontitis) and halitosis. These diseases have a significant impact on population health and/or wellbeing and, particularly caries and periodontitis, generate a financial burden for healthcare budgets.

Dental Caries

Caries is globally considered the most common health condition with an estimated 2.3 billion people suffering from caries of permanent teeth in 2017[1]. The cost of treating caries is expensive and believed to consume 5-10% of healthcare budgets in industrialized countries[2,3]. Caries lesions start with reversible demineralization of the enamel, but can progress to irreversible enamel damage and cavitation into the dentin and pulp[4]. When reaching the nerves inside the pulp, the cavitated lesion becomes painful, which can lead to difficulty in eating, sleeping and talking[5,6]. This can severely reduce the quality of life of a person. Additionally, caries are associated to infections and abscesses, while unproper mastication could lead to gastrointestinal complications[6].

Periodontal diseases – gingivitis and periodontitis

Gingivitis is a mostly reversible inflammation of the gums, which can be found in ~80% of different adolescent populations[7]. Long-lasting or repeated episodes of gingivitis can lead to periodontitis, which is a chronic and destructive inflammatory disease. Periodontitis is also highly prevalent, affecting up to 50% of the adult population, with ~10% suffering from severe disease[8]. Inside the oral cavity, periodontitis can be painful, cause halitosis and ultimately lead to tooth loss. Importantly, emerging evidence suggests that the consequences of periodontitis extend beyond the oral cavity. For example, periodontitis is associated with increased risk of diabetes[9], rheumatoid arthritis[10], atherosclerosis[11], and hypertension[12]. Current treatments of periodontitis are resource intensive, time consuming and often only partially successful[13].

Halitosis

Nearly 90% of halitosis (bad breath) has an intra-oral origin with the main causative factor being tongue coating[14,15]. Intra-oral halitosis (IOH) can also be caused by pathological conditions (e.g., periodontal diseases)[15]. According to previous studies, IOH can be detected in 10-30% of the total population[14]. Oral

malodour can have a large impact on the psychosocial wellbeing of an individual by limiting social interactions and relationships[16]. Due to the psychological problems associated to halitosis, this disease can require psychological support apart from treatment by dentists[16].

Reducing the global burden of oral diseases

Initial enamel demineralization can be arrested and possibly reversed[17]. However, dental cavities are irreversible and need expensive operative care to stop disease progression[18,19]. Similarly, gingivitis is often reversible, but periodontitis is a chronic disease that needs to be treated repeatedly over time[20,21]. Therefore, to reduce the global health and financial burdens of oral diseases, preventive strategies are important to reduce the prevalence of irreversible gum and tooth damage. Regarding this, the oral microbiota has a key role in the development of caries and periodontal diseases, as well as halitosis[22,23]. Appropriate preventive strategies can therefore focus on the modulation of the oral microbiota to limit disease development in susceptible individuals before irreversible symptoms. For this, it is crucial to understand how the oral microbiota changes from health to disease[24].

Oral disease susceptibility [a]

In healthy individuals with the right dietary and oral hygiene habits, the oral microbiota lives in symbiosis with the host, preventing the colonization of foreign pathogens and contributing to host physiology[25]. In this thesis, we define symbiosis as a microbial composition, activity and ecology that keeps a balanced relationship with the host, resulting in a healthy state. Nevertheless, perturbations in the microbiome caused by certain stress factors, such as carbohydrate consumption or plaque accumulation, can lead to the development of oral diseases, e.g. caries or periodontal diseases, respectively[22,26]. In these oral diseases a shift of species and functions associated with the diseases, i.e. dysbiosis, is observed[27-30].

Importantly, people do not develop similar levels of oral diseases under identical circumstances. In the Vipeholm study, mentally-challenged subjects received high amounts of fermentable carbohydrate snacks over 5 years[31]. Most individuals developed caries, but 20-30% did not, "although they had a frequent intake of between meal sweets for long periods"[31]. Likewise, in a Sri Lankan

[a] The repository copy of Rosier et al., 2018 (Journal of Dental Research, 97(4), 371–380) starts here.

population with no oral hygiene habits nor dental care, while 89% of the population had moderate to fast progression of periodontal breakdown, the other 11% had no periodontal disease beyond gingivitis (i.e., an inflamed gingiva)[32]. Low and high responders are also observed in experimental gingivitis studies[33].

Several disease drivers have been identified which can potentially induce disease. When populations are exposed to a certain level of disease drivers, a unique opportunity is provided to retrospectively discriminate between susceptible and tolerant individuals. In the last century, the focus of research has mainly been on oral diseases in susceptible individuals. Accordingly, mechanisms that limit disease development in tolerant individuals when disease drivers are present remain relatively uninvestigated. However, a clearer understanding of these health-maintaining mechanisms might allow their active stimulation in susceptible individuals and thus open up new avenues for disease prevention and treatment.

The oral microbiota in health

Bacteria are the main inhabitants of the oral cavity. In healthy adults, the majority of species belong to the bacterial phyla Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes and Fusobacteria[34]. In addition, archaea, protozoa, viruses, and fungi are present. Of the 700+ species of oral bacteria identified[35], a healthy individual is colonized by between 100-200+ bacterial species[30,36]. Inter-individual variation in microbiota composition results from differences in the environment, genetics, age and lifestyle of the host[37] (figure 1A and B). Importantly, certain microbiota functions can be fulfilled by groups of different microbes (functional redundancy)[28,38]. For instance, some core functions of the oral microbiota are conserved between healthy individuals even when there are large differences on a taxonomic level[34].

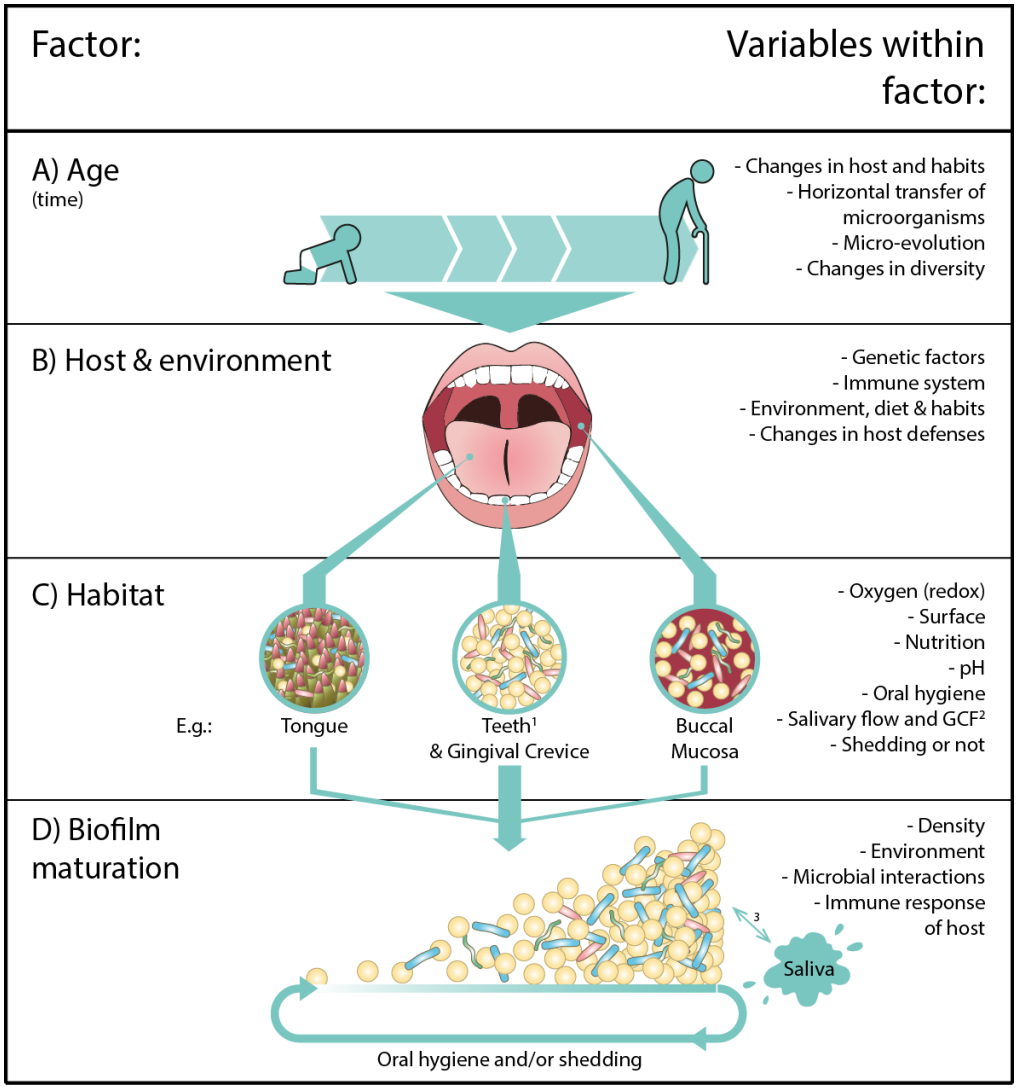

Figure 1: Factors that determine the composition of the oral microbiota. The main factors that determine the composition of the oral microbiota are shown in the left column. Examples of different variables within these factors are listed in the right column and can vary within the same individual (i.e., over time) and between different individuals. A) Age (time). The diversity of the microbiota changes as the host ages (e.g., due to tooth eruption and age-related changes in hormones and the immune system). Additionally, horizontal transfer of microorganisms and micro-evolution of the oral microbiota takes place over time. B) Host & Environment. Differences among hosts, such as genetics and the (integrity of the) immune system affect the microbiota composition. Additionally, the environment in which the host is present further affects the composition by e.g. influencing host habits and diet. C) Habitat. The habitats of the oral cavity differ in environmental conditions such as oxygen levels, pH, and nutrition.

Importantly, the oral mucosal surfaces undergo desquamation (e.g., the buccal mucosa sheds frequently preventing biofilm accumulation), while the dental surfaces do not. Three examples of habitats are given of the many different habitats inside the oral cavity. Changes due to biofilm maturation are most relevant to the teeth and gingival crevice in light of the most common oral diseases (i.e., caries and periodontal diseases), indicated by the thicker arrow in the middle going to row D. D) Biofilm Maturation. Physical and chemical perturbations from food, oral hygiene and, in the case of the oral mucosa, shedding remove biofilms from surfaces. Young biofilms differ from mature ones due to changes in density that affect the internal environment (e.g., the interior of the biofilm becomes more anaerobic as it becomes thicker), microbial interactions (e.g., quorum sensing) and the immune response that is triggered by the host. [1]Teeth are only present after certain age and extractions and denture wearing cause variability during the lifespan of a person. [2]GCF: Gingival crevicular fluid. [3]The two way arrow between saliva and maturing biofilms means that the saliva inoculates clean surfaces, while detached microbes from colonized surfaces enter saliva.

In the oral cavity, indigenous species can reach all surfaces via the flow of saliva, but the corresponding environments determine which species are able to adhere and colonize successfully. As a result, the biofilms that form at different habitats of the oral cavity (e.g., teeth, gingival crevice, tongue and buccal mucosa) have a distinct microbial composition[34] (figure 1C). The composition changes as the biofilm matures (e.g., the hours after oral hygiene)[39], which is most relevant for the tooth surfaces that do not shed (figure 1D). Biofilms on the teeth, called dental plaque, are associated with the most common oral diseases—caries and periodontal diseases. The oral epithelia shed several times a day, which restricts biofilm accumulation.

Compared to the other microbial communities of the body, the oral microbiota in health is often considered most stable over time[40]. Several studies demonstrated that a stable core microbiota is maintained in the oral cavity of healthy individuals over periods up to 7 years[41,42]. When saliva of a single adult was sampled daily for over a year, only 195 OTUs in saliva—a fraction of the total found in one year—were stable (i.e., present in 95% of samples) but these comprised 99.7% of total bacteria detected[41]. The other 0.3% of bacteria detected consisted of a high variety of low abundance species, which seem to appear and disappear over time. In addition, oral microbial communities have been found to recover after the use of antibiotics in adult individuals, whereas the gut

microbiota could suffer longer term alterations in composition[43]. The continuous presence and the composition of saliva appears to have crucial roles in maintaining the stability of the oral microbiota.

Role of saliva in ecological stability

A healthy adult produces approximately 1L of saliva per day[25]. The importance of saliva is reflected by the fact that individuals with salivary deficiencies are prone to oral diseases[44]. Saliva contains a broad range of antimicrobial components (e.g., lysozyme, lactoferrin, histatins, defensins, and secretory IgA)[45], provides pH buffering, and is continuously refreshed, while swallowing discards food remains, detached cells and microbial waste products.

Salivary glycoproteins regulate attachment of different types of microbes to oral surfaces by either stimulating or blocking their adherence[46,47]. Additionally, they provide a consistent source of nutrients for the oral microbiota[45]. Mucins, which are large and complex glycoproteins, make up around 25% of the total protein content and are broken down by mixed consortia of microorganisms in a concerted manner which promotes the characteristic diversity and stability of the oral microbiota[48].

The salivary glands also concentrate plasma components like nitrate into the saliva resulting in high salivary nitrate concentrations (100–500 μM during fasting, which is ~10 times higher than in plasma, and 5-8 mM after a nitrate-containing meal)[25]. Nitrate is an electron acceptor used in the respiration of nitrate-reducing bacteria, leading to the production of nitric oxide, a molecule with antimicrobial properties[49].

Perturbations by food intake are generally relatively short compared to fasting periods in which the saliva is refreshed creating a constant environment. The salivary components thereby confer long term stability to the composition and activity of the oral microbiota.

Resilience to disease drivers

In ecology, resilience is the capacity of an ecosystem to deal with perturbations without shifting to an alternative state in which core species and key functions are lost[50,51]. Resilience can be divided into 'resistance' and 'recovery': the

'resistance' determines the magnitude of perturbation that an ecosystem can handle before its state changes, while the 'recovery' is the rate at which it returns to its original state. These ecological concepts can be applied to the oral ecosystem to help understand how a stable microbial community is maintained over time.

Recently, the term resilience was applied in the oral cavity as the capacity to recover from perturbations caused by gingivitis in smokers vs non-smokers[52]. After the recovery period, smokers had higher levels of disease-associated species and, accordingly, a higher pro-inflammatory response. This indicates that smokers had a decreased resilience (recovery) from experimental gingivitis. In another recent study, a metatranscriptomic approach was used to observe the active oral microbiota before and after a carbohydrate meal[39]. Even though the group size was small (5 subjects), the microbiota of each individual changed in its own way. Interestingly, virtually no changes were observed in one subject who had never suffered from dental caries, indicating a strong resilience (resistance). Resilience could thus discriminate between susceptible and tolerant individuals when disease drivers are present and can be described as a capability to cope with stress factors (resistance) and recover from perturbations that are potentially triggered (recovery, figure 2). Perturbations can lead to oral diseases when disease drivers are strong or persistent enough.

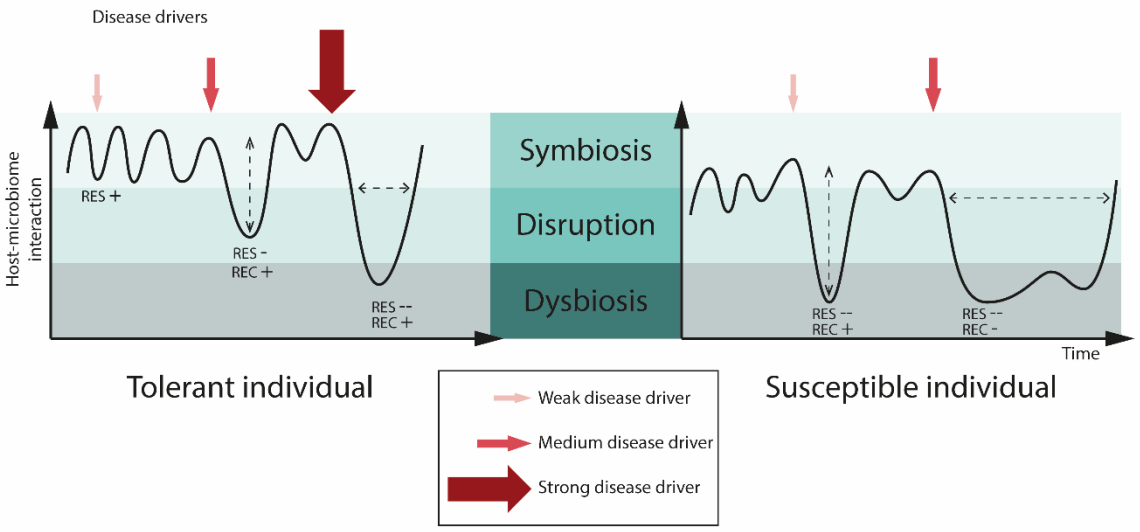

Figure 2: Disease drivers (i.e., stress factors that can potentially induce disease), perturbations and resilience. In this hypothetical graph, the y-axis indicates host-microbiome interactions, which can be divided in three zones (symbiosis, disruption or dysbiosis). Symbiosis keeps healthy interactions between host and microbiota, whereas a disruption is a reversible situation in which microbiome species or functions are altered. Dysbiosis is a host-microbiome interaction that leads to adverse symptoms for the host (e.g., gingival inflammation). Time is shown on the x-axis. Here we give an example of how a tolerant individual could differ from a susceptible individual when disease drivers have the same magnitude (i.e., weak or medium). The thickness of the disease driver arrows (on top) represents its magnitude that is also determined by the duration and frequency of its presence. Disease drivers can trigger perturbations towards dysbiosis in some cases. The resistance (RES) and recovery (REC) determine the impact of the disease driver and the potentially triggered perturbation, respectively. When the resistance is strong (+), the disease driver does not cause a perturbation. When the resistance is weak (- or --), a perturbation is caused and the time it will be present depends on the recovery rate (- or +). Note that recovery can also be the result of active interference such as removing plaque by oral hygiene, which could be presented as an arrow in the opposite direction than the disease drivers.

'Regime shifts' towards oral disease

The disruption of resilience during the development of oral disease can be compared to the ecological phenomenon of 'regime shifts' (i.e., large, abrupt, persistent changes in function and structure)[51]. In ecological systems, 'regime

shifts' take place when perturbations pass a certain threshold. A classic example is given by shallow clear water lakes (reviewed by Folke et al. in 2004[51]). When the lake receives a high phosphorus input (perturbation driver), e.g. from agricultural waste, phytoplankton can overgrow. The shading by phytoplankton makes the environment less suitable for higher aquatic plant beds. Additionally, bottom-feeding fish, which feed on phytoplankton, increase in number and damage the plant beds. As the plant beds decrease, phosphorus from the sediment becomes available, which further stimulates phytoplankton overgrowth. Altogether, these events reinforce themselves in a positive feedback loop driving change from a clear water regime to a turbid one.

To draw a parallel to the oral cavity, more than two decades ago, a chain of self-reinforcing processes was proposed that can lead to disruption of the oral microbiota and increase the risk of disease[22,26]. For example, the development of caries and periodontitis can be represented as positive feedback loops[23,24] (Figure 3 and 4). While positive feedback loops triggered by disease drivers can cause microbial regime shifts towards oral disease (i.e., dysbiosis) in susceptible individuals, health-maintaining mechanisms prevent this and could enhance resilience in tolerant individuals.

Carbohydrate consumption: regime shift to caries vs resilience

Regime shift: caries development due to carbohydrate consumption and acidification

In caries, the disease drivers are mostly fermentable carbohydrates, when consumed in high amounts and frequencies[4]. The microbiota ferments these carbohydrates into organic acids. If the acid surpasses the buffer capacity of dental plaque and saliva, then the local pH will fall. Acid-producing (acidogenic) species that are adapted to the acidic conditions will gain a selective advantage[22,24,53]. Over time, the microbiota shifts towards a community that is more efficient at fermenting carbohydrates (i.e., saccharolytic) and more adapted to growth and metabolism at a low pH (i.e., aciduric)—a shift towards a cariogenic microbiota[22]. These include, but are not limited to, aciduric representatives of *Lactobacillus*, *Streptococcus*, *Veillonella*, *Bifidobacterium*, *Actinomyces* and certain yeasts[27,53]. As the pH reaches a critical level (below around pH 5.5), enamel demineralization exceeds remineralization. If the acidic

conditions persist, or are repeated frequently without sufficient time for remineralization, then a caries lesion can develop [4]. Frequent carbohydrate intake can therefore lead to a positive feedback loop causing a shift to a saccharolytic, acidogenic and aciduric microbiota that can cause irreversible dental caries over time.

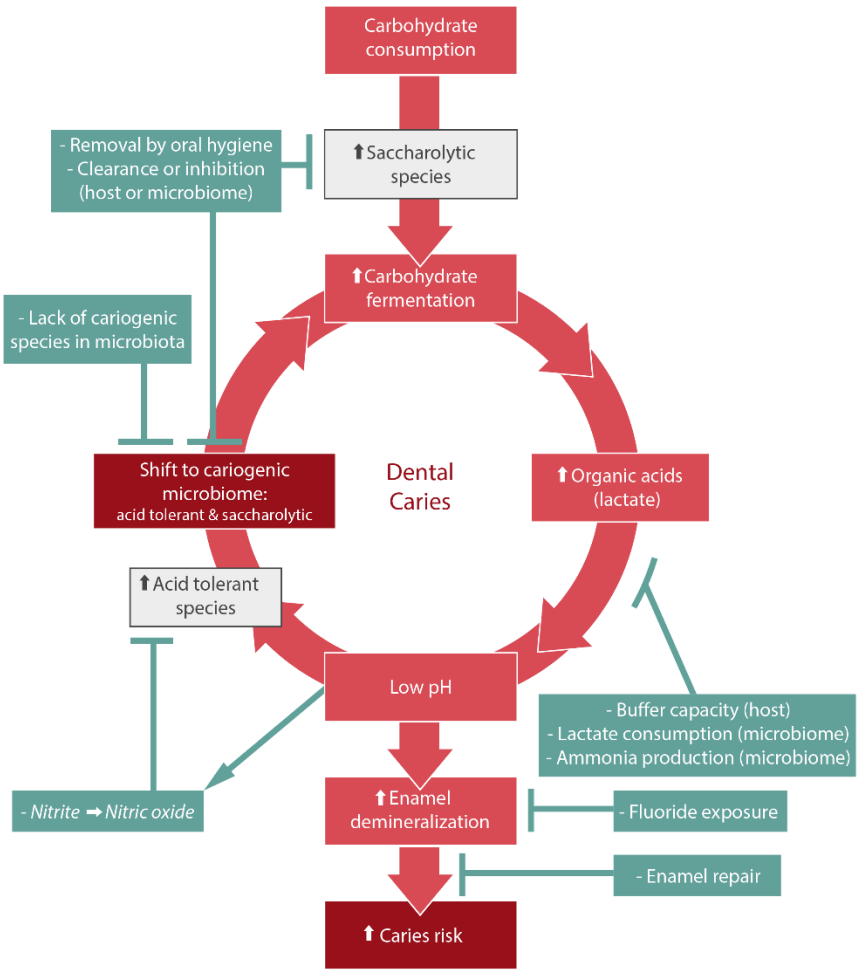

Figure 3: Positive feedback loop leading to caries. In this positive feedback loop, carbohydrate consumption is the disease driver that can cause a regime shift towards a cariogenic microbiota. The health-maintaining mechanisms that could prevent various

stages of the loop are listed in green boxes. Some of these mechanisms are likely to contribute to resilience and be enhanced in individuals that are more tolerant to carbohydrate consumption. Italicized text indicates hypothetical involvement.

Resilience to carbohydrate consumption and acidification

Saliva plays an important role in preventing a regime shift to caries. Salivary characteristics, such as flow rate and buffer capacity, differ among individuals, which can lead to differences in resilience towards acidification[54].

Compared to the prevention of plaque accumulation associated with periodontal diseases, the role of the immune system in caries is often neglected[24]. However, several studies showed differences in host genes involved in immune response or salivary immune components between caries active and caries free individuals[55-57]. For instance, a classical observation is that low caries susceptibility is associated with a high amount of total antigen-specific S-IgA against *Streptococcus mutans*[56]. In accordance with this, the saliva of caries-free individuals was recently shown to have higher concentrations of S-IgA and proportions of S-Ig-coated bacteria than samples from caries-active patients[57,58]. This indicates that the immune system of caries-free individuals clears bacteria, including those involved in caries development, more efficiently.

The microbiota itself provides resilience to acidification in several ways. Certain species (e.g., *Veillonella* spp.) metabolize lactic acid into weaker acids (i.e., with a lower pKa)[59]. Additionally, there are species (e.g., *Streptococcus* spp. and *Actinomyces* spp.) that generate the alkali, ammonia, by the catabolism of arginine or urea, increasing the local pH[60,61]. Salivary nitrate and the capacity of the microbiota to reduce nitrate to nitrite also appear to have an anti-caries effect, possibly due to the resulting production of ammonia and antimicrobial nitric oxide or the consumption of lactic acid by nitrate reducing species[62,63]. Additionally, at pH 5 or lower, acidic decomposition of nitrite to nitric oxide takes place[49], which could provide negative feedback to acidification (figure 3, blue box "nitrite → nitric oxide").

Finally, antimicrobial peptides (like bacteriocins) were significantly over-represented in the metagenomes of caries free individuals, compared to caries-experienced subjects[27]. Furthermore, bacteriocins produced by *Streptococcus*

dentisani, isolated from caries-free individuals, inhibited the growth of several cariogenic species[61] and other health-associated microorganisms have also been found to have antimicrobial activity against caries pathogens[64]. Host and microbiota functions that prevent a fall in pH, promote pH recovery or inhibit cariogenic species can thus contribute to resilience against caries. In addition, there are external components that can enhance resilience, such as fluoride, which increases resistance to demineralization and recovery by remineralization (see, for example, Pitts et al. 2017[4]).

Plaque accumulation: regime shift to periodontal diseases vs resilience

In periodontal diseases, the first disease driver is accumulation of dental plaque as a result of poor oral hygiene[22] (figure 4). Firstly, the conditions within the plaque biofilm slowly become more anaerobic over time as the biofilm becomes thicker, which increases the levels of anaerobic species. Additionally, to clear the accumulated microbes, the host responds with gingival inflammation[52]. This includes an increase in temperature[65] and increased flow of gingival crevicular fluid (GCF, i.e., a serum-like exudate). GCF contains components of host defenses (e.g., immune cells and antibodies), but also many (glyco)proteins. Unintentionally, the GCF proteins can act as a novel source of nutrients for proteolytic species that increase in number during periodontal diseases[22,26]. As a result of the metabolism of asaccharolytic proteolytic species, the pH of the environment stays neutral[23] or becomes slightly alkaline[66]. Another component present in GCF is iron, which is essential for bacterial growth, and triggers potential pathogenic mechanisms in oral bacteria associated with periodontal disease[67].

In the new environment, inflammation-tolerant, anaerobic, proteolytic, alkaliphilic species (i.e., a periopathogenic microbiota) have a selective advantage and increase in number[22,24,26]. The host responds with more inflammation and a positive feedback loop is formed. This may be further stimulated by bacterial manipulation and subversion of the immune system[67]. For instance, *Porphyromonas gingivalis* can instigate a crosstalk between the C5a receptor (C5aR) and Toll-like receptor 2 (TLR2) that increases an inflammatory response, but impairs bacterial killing, which in mice facilitates survival of the entire microbial community[68]. In respect to this, it appears that periodontitis-

associated communities have not only evolved to endure the inflammation, but also take advantage of the new environment with more nutrients in the form of tissue-breakdown products (e.g., peptides and heme-containing compounds)[69]. These so called 'inflammophilic' biofilms increase with inflammation, while anti-inflammatory treatments diminish the bacterial load in animal models, and could contribute to the positive feedback loop leading to periodontal diseases.

In conclusion, plaque accumulation and the resultant inflammatory host response, can lead to a positive feedback loop that causes gingivitis and, in some cases, if the host is susceptible, periodontitis. The composition of the microbiota associated with periodontitis is diverse and can include different species from the phyla *Bacteroidetes*, *Candidatus Saccharibacteria*, *Firmicutes*, *Proteobacteria*, *Spirochaetes* and *Synergistetes*[70].

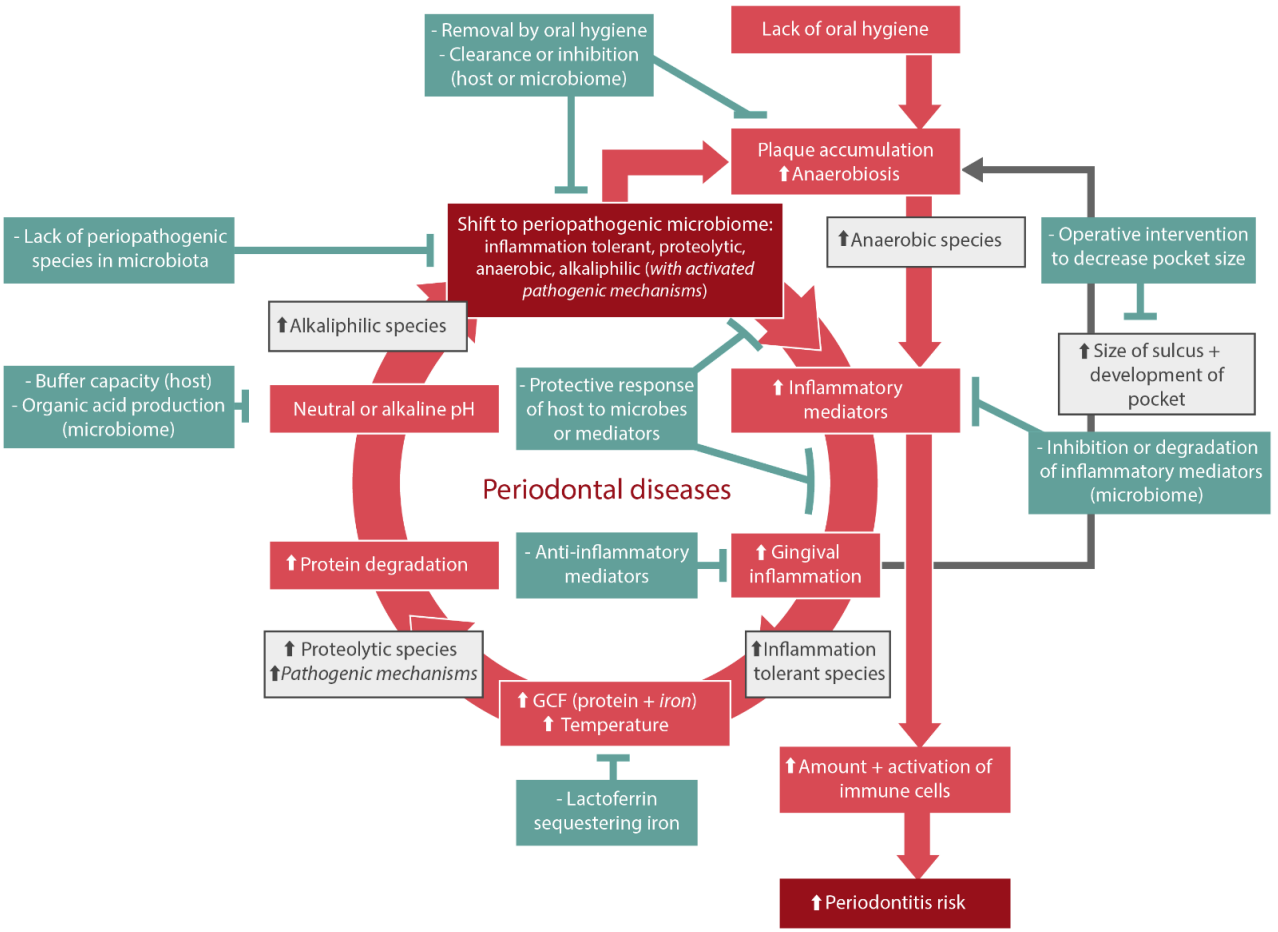

Figure 4: Positive feedback loop leading to periodontal diseases. In this positive feedback loop, plaque accumulation as a result of poor oral hygiene is the disease driver that can cause a regime shift towards a periopathogenic microbiota. The grey arrow represents another chain of self-reinforcing events, in which an increase in sulcus size or the formation of a pocket allows for more plaque accumulation. The health-maintaining mechanisms that could prevent various stages of the loop are listed in green boxes. Some of these mechanisms are likely to contribute to resilience and be enhanced in individuals that are more tolerant to a lack of oral hygiene. Italicized text indicates hypothetical involvement.

Resilience to plaque accumulation

In ecological systems, species can keep themselves in balanced numbers by negative feedback mechanisms, which can allow long-term stability. A simple example is the cycle of a predator and its prey that allows both species to survive over time: if owls increase, mice will decrease, but if mice keep decreasing, owls will decrease, allowing mice to increase again, and so forth[50]. Microbial examples of these negative feedback mechanisms are the observed increase of bacteriophages specifically predating a given bacterial taxon[38] and the density-dependent regulation of bacterial populations by their corresponding phage predators[71]. Regarding this, the vast majority of oral viruses are bacteriophages that could provide negative feedback[72]. Wang et al. (2016) showed that the levels of certain phages were negatively correlated with periodontal-disease associated bacteria[73], suggesting a role in shaping the microbial community. Other negative feedback mechanisms that can decrease the abundance of a member of the human microbiota after it exceeds a certain threshold are the lack of nutrition or essential growth factors in its habitat or the accumulation of a specific toxic product of metabolism[38] (figure 5).

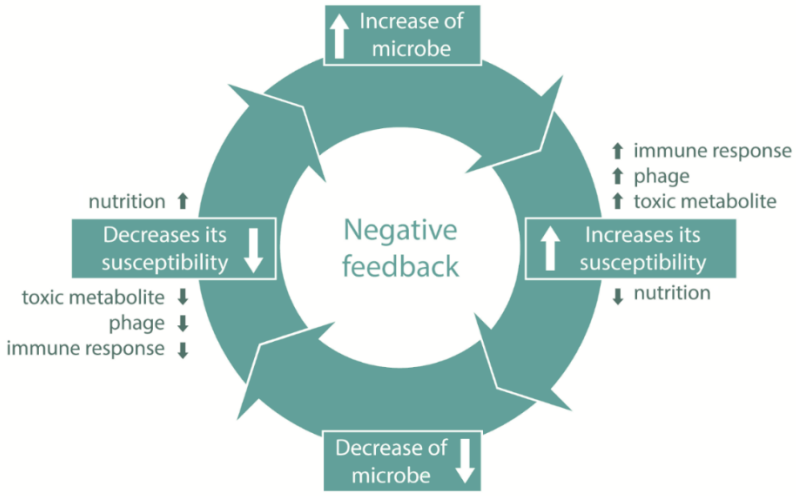

Figure 5: Negative feedback loop preventing plaque accumulation. This negative feedback loop is based on ecological studies and could prevent the accumulation of members of the microbiota, for instance, on the tooth surface.

The complex roles of the immune system in preventing microbial accumulation could be enhanced in tolerant individuals, but this falls outside the scope of this review. In short, different immune components actively kill, inhibit and agglutinate microbes (lyzosyme, defensins, histatins, S-IgA), deprive them of iron (lactoferrin), prevent their adhesion (S-IgA, IgG, IgM), or act as opsonins (complement, IgG, IgM) that increase phagocytosis by immune cells[45].

Species of the microbiota also produce antimicrobial compounds (e.g., bacteriocins and toxic compounds such as hydrogen peroxide and nitric oxide) that suppress the growth of other species [74], providing resistance to plaque accumulation, and genes involved in hydrogen peroxide metabolism have been associated with periodontal health[29]. Nitric oxide is also a signaling molecule that triggers dispersal of various types of bacterial cells from biofilms[75,76]. Salivary nitrate concentrations could generate nitric oxide concentrations that decrease biofilm formation by susceptible species[75], which could provide resistance to, and recovery from, plaque accumulation in health.

Altogether, resilience to plaque accumulation can be provided by negative feedback mechanisms that are also present in macro-ecosystems. Additionally, the host has several strategies to limit microbial accumulation, and the microbiota itself produces antimicrobials and biofilm dispersal signals.

Resilience to destructive inflammation

Inflammation can result from plaque accumulation when certain receptors are triggered in a complex interaction of the immune system with the microbiota[77]. Differences in immune system phenotypes are likely to be detected in susceptible hosts compared to tolerant hosts[78].

The host regulates inflammation in several ways, e.g. by adjusting cytokine expression levels and complex cytokine-receptor interactions and signaling, depending on the types and amounts of microbes that are detected. Consequently, some species correlate with anti-inflammatory mediators (e.g., *Streptococcus*, *Neisseria* and *Veillonella*), while others correlate with pro-inflammatory mediators (e.g., *Selenomonas*, *Parvimonas* and *Campylobacter*)[52].

Apart from being the trigger of inflammation, the microbiota can also actively suppress immune activation. This could be a mechanism enabling microbes to evade the immune system and accumulate to levels that could induce periodontal diseases[77]. An example is *Porphyromonas gingivalis* which expresses a type of lipopolysaccharide (LPS) that decreases Toll-like receptor 4 response and secretes a serine phosphatase that inhibits the secretion of IL-8 (i.e., a pro-inflammatory cytokine), which is thought to impair inflammation[77]. Alternatively, in health, the suppression of the immune response by indigenous species may contribute to homeostasis. In light of this, *P. gingivalis* is also present in health, albeit in lower numbers. Additionally, health-associated commensal species can have comparable mechanisms; for example, *Streptococcus salivarius* inhibits IL-8 secretion[79]. The microbiota can thus correlate with anti-inflammatory mediators and also actively prevent inflammation by inhibiting pro-inflammatory cytokines. This could contribute to homeostasis and resilience in health by preventing unnecessary and destructive inflammation.

Mechanisms that prevent dysbiosis

In summary, certain health-maintaining mechanisms (green boxes in figure 3 and 4) may prevent a shift to dysbiosis when disease drivers are present. Some of these mechanisms can be triggered by the disease drivers (e.g., acidification that could lead to more nitric oxide production) and act as negative feedback (figure 5). Other mechanisms are continuously present (e.g., the buffering effect of saliva) or take place in episodes (e.g., oral hygiene and fluoride exposure). Altogether, complex interactions between disease drivers, health-maintaining mechanisms and feedback loops will determine the relationship between the microbiota and the host (Figure 6).

Our hypothesis is that certain health-maintaining mechanisms that prevent disease-associated positive feedback loops are enhanced in tolerant individuals. These mechanisms can be microbial, which was the emphasis of this review, but can also be at the host level (e.g., genetic and epigenetic differences) and human genome association studies could shed light on this issue[78]. By identifying markers that are involved in resilience, susceptible individuals that lack them could be identified before disease develops. Additionally, health-maintaining mechanisms could be actively enhanced in novel strategies of disease treatment (enhancing health rather than reducing disease).

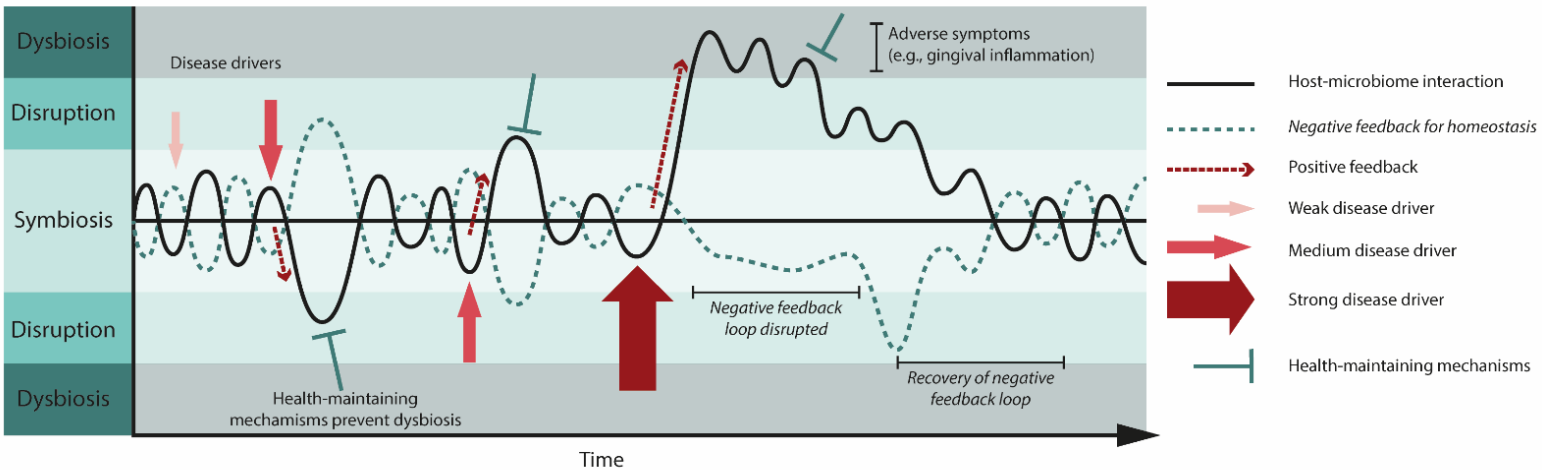

Figure 6: Example of how disease drivers (i.e., stress factors that can potentially induce disease), negative and positive feedback, and health-maintaining mechanisms may act over time. For the descriptions of symbiosis, disruption and dysbiosis, see Figure 2. In this simplified graph of the complex oral ecosystem, where in reality many different symbiotic and dysbiotic states may exist, we show how disease drivers could act in a systemically healthy individual. If the driver is weak (weak disease driver arrow), no perturbation by positive feedback is triggered (i.e., resistance, also see Figure 2). In the case of a medium disease driver (medium disease driver arrow), some positive feedback might destabilize the host-microbiota interaction leading to a short perturbation into disruption, but negative feedback and other health-maintaining mechanisms shortly counteract it (i.e., recovery, also see Figure 2) and a symbiotic homeostasis is restored. However, if the disease driver is strong or persistent enough (strong disease driver arrow), the positive feedback might temporary surpass negative feedback and other health-maintaining mechanisms as shown in this graph, leading to dysbiosis (e.g., gingival inflammation). Over time, the health-maintaining mechanisms (e.g., the immune response or oral hygiene) may allow recovery in which adverse symptoms disappear and disease progression stops. However, in contrast to this graph, in a more susceptible host or after frequent exposure to a disease driver, at some point dysbiosis can become stable (e.g., periodontitis is a chronic infection), and new, disease-associated (feedback) mechanisms could contribute to this stability.

Prospects: preventive dentistry

Identification of markers of resilience

Several decades ago, the ecologist C.S. Holling mentioned that when ecosystems are studied, there is a "tendency to emphasize the quantitative (i.e., a single time point) rather than the qualitative (i.e., fluctuations over time)", while only the latter informs about resilience[50]. For instance, the numbers of some species fluctuate enormously, and others seem to disappear and reappear over time—a single time point does not provide information about this. The same holds for studies involving the oral microbiota. Until now, most OMICS studies have focused on comparison between healthy and diseased individuals from whom samples were taken at a single time point. This does not provide information about their resistance to disease drivers and recovery rate after potential perturbations. For instance, to measure the microbiota's resilience against a sugar pulse, it is necessary to observe its activity before and several time points after the sugar pulse. Only a few recent (small scale) OMICS studies have assessed the response of the oral microbiota to disease drivers, detecting individuals with different susceptibilities[39,41,52]. More and larger qualitative OMICS studies, which measure fluctuation over time, will provide new insights into microbial and host markers that lead to resilience[78]. Markers of resilience may include health-associated bacterial species or functions, genetic polymorphisms associated to protection from disease[24], certain levels of salivary compounds that prevent disease[57] or specific tests directed towards detecting resilience capacity (e.g., pH buffering capacity or Ig-coating levels).

Enhancement of resilience with pre- and probiotics [a]

Prebiotics: arginine and nitrate

Just like a shift of species and functions is observed after a period of stress, long periods of rest may allow microbiota recovery, which could be enhanced by the frequent administration of a prebiotic—in this review referring to compounds that stimulate beneficial microorganisms or beneficial microbial mechanisms. In a recent *in vitro* study, the continuous administration of arginine enhanced oral microcosm (i.e., *in vitro* oral microbiota) resilience toward acidification and suppressed outgrowth of the opportunistic pathogen *Candida*[80]. Similarly, 1.5% and 8% arginine toothpaste enhanced ammonia production and decreased lactate production in clinical trials[81,82], both of which reduce acidification. Furthermore, the microbiota changed towards having a more health-associated

[a] This is the last section of the repository copy of Rosier et al., 2018 [Journal of Dental Research, 97(4), 371–380], containing minor modifications for this thesis.

composition from a caries point of view[82]. Interestingly, the production of ammonia by the metabolism of arginine or urea is induced by a low pH (negative feedback)[60,61], and therefore it is initially unlikely that arginine has an effect on periodontal pockets where the pH is already neutral or alkaline.

Another potential prebiotic is nitrate, but current *in vivo* evidence in humans is limited. In a recent clinical trial focusing on the cardiovascular benefits of dietary nitrate, oral bacterial profiles were measured [83]. After six weeks of daily nitrate-rich beetroot juice consumption, 78 bacterial taxa were affected and two nitrate reducing species, *Rothia mucilaginosa* and *Neisseria flavescens*, increased notably. *Rothia* spp. and *Neisseria* spp. have both been associated with dental and periodontal health[27,30,52]. Furthermore, two weeks of nitrate-rich lettuce juice consumption in another recent clinical study reduced gingival inflammation[84].

In conclusion, prebiotics can drive beneficial changes in the oral microbiota and could increase resistance to dysbiosis and recovery of health.

Probiotics

The addition of probiotics—microorganisms that confer a health benefit on the host—with beneficial functions (e.g., preventing acidification, plaque accumulation or harmful inflammation) may further contribute to resilience. A recent systematic review of 50 studies (3247 participants) concluded that the current evidence is insufficient for recommending probiotics for managing dental caries, but supportive towards managing gingivitis or periodontitis[85]. The identification of new probiotic species which inhabit the oral cavity—as opposed to dairy products or gut-associated bacteria[61] —and the development of personal rather than general treatments could improve these results in the future. In respect to this, Kort proposed the triple-A model (acquirement, alteration and administration of the microbiota) for the vaginal microbiota, in which strong selective media enable a person's own beneficial bacteria to be grown for subsequent re-application[86]. A comparable idea could work for the oral cavity to obtain indigenous probiotic species or communities with certain beneficial functions (e.g., arginolytic pathways to produce ammonia or denitrification pathways to produce nitric oxide).

Probiotics and prebiotics are thus promising compounds in oral disease prevention and treatment. A promising prebiotic candidate to prevent or treat caries and periodontal diseases is nitrate – a dietary component that is mostly obtained from vegetables. To a lesser extent, fruits and other plant-based foods contain nitrate. A literature review was performed to determine the amounts of nitrate in these food groups.

Nitrate in edible plants

Most dietary nitrate is obtained from vegetables

It has been estimated that over 80% from dietary nitrate is obtained from vegetables, a food group generally associated with health benefits and increased longevity[87] as well as decreased disease prevalence (including but not limited to cardiovascular diseases, cancer and diabetes)[88-90]. Nitrate present in soil and water accumulates in different plant tissues, leading to higher levels of nitrate in vegetables[91] (Table 1). For example, in one study, the levels of nitrate were between 12.36 and 33.14 mg/l in irrigation water and 4.35 and 9.7 mg/kg in soil[92]. In a large vegetable analysis by the EFSA, it was found that >41000 samples of different vegetables had a mean nitrate content of 336 mg/kg[93] (Table 2). The nitrate content of vegetable organs can generally be listed in descending order (most to least) as petiole > leaf > stem > root > influorescence > tuber > bulb > fruit (in the case of fruited vegetables) > seed[91,94]. Factors that further affect the nitrate content are the plant genotype and soil conditions, as well as the conditions for crop growth, storage and transportation[91]. All these factors together lead to different levels of nitrate detection within the same vegetable species (Table 1). Nevertheless, when comparing different vegetable groups, differences in the mean nitrate content can be observed. For example, it was found that leafy green vegetables have most nitrate (mean of 1614 mg/kg), whilst fruiting and bulb vegetables have least nitrate (means of 149 and 159 mg/kg, respectively, Table 2). Some of the vegetables with most nitrate (often in the 1000-2000+ mg/kg range) are rocket, radishes, spinach, lettuce, chard and beetroots (Table 1, Supplementary Table T1).

Table 1: Nitrate content of in vegetables [combined and adapted tables of Lidder & Webb (2013)[95] and Roila et al. (2018)[96]]

Food	Nitrate content (mg/kg)			Source
	Mean	Min	Max	
Rocket / Rucola	2597			Lidder & Webb (2013)[95]
	4415	1463	6724	Roila et al. (2018)[96]
Radish	1868	1060	2600	Lidder & Webb (2013)[95]
	3817	3650	3985	Roila et al. (2018)[96]
Spinach	2036	96	3559	Roila et al. (2018)[96]
	2137	965	4259	Lidder & Webb (2013)[95]
Lettuce	1079	<d.l.	3660	Roila et al. (2018)[96]
	1893	970	2782	Lidder & Webb (2013)[95]
Chard	1728	1026	2430	Roila et al. (2018)[96]
Beetroot	1459	644	1800	Lidder & Webb (2013)[95]
Chinese cabbage	1388	1040	1859	Lidder & Webb (2013)[95]
Zucchini / Courgette	736	15	973	Roila et al. (2018)[96]
Turnip	624	307	908	Lidder & Webb (2013)[95]
Kidney beans*a	535*b	194	1030	Zhou et al. (2000)[97]
Cabbage	513	333	725	Lidder & Webb (2013)[95]
Green beans	496	449	585	Lidder & Webb (2013)[95]
Red chicory	496	281	711	Roila et al. (2018)[96]
Eggplant	399	<d.l.	420	Roila et al. (2018)[96]
Leek	398	56	841	Lidder & Webb (2013)[95]
Spring onion	353	145	477	Lidder & Webb (2013)[95]
Cucumber	240	151	384	Lidder & Webb (2013)[95]
Carrot	222	121	316	Lidder & Webb (2013)[95]
	238	<d.l.	299	Roila et al. (2018)[96]
Potato	96	73	223	Roila et al. (2018)[96]
	220	81	713	Lidder & Webb (2013)[95]
Garlic	111			Roila et al. (2018)[96]
	183	34	455	Lidder & Webb (2013)[95]
Sweet pepper	81	<d.l.	163	Roila et al. (2018)[96]
	117	93	140	Lidder & Webb (2013)[95]
Onion	69			Roila et al. (2018)[96]
	87	23	235	Lidder & Webb (2013)[95]
Tomato	69	27	170	Lidder & Webb (2013)[95]

<d.l. = below detection limit in Roila et al. (2018)

*[1]Data added from Zhou et al. (2000)[97]. Kidney beans are considered legume seeds.

*[2] In Zhou et al. (2000)[97], other means are giving for kidney beans from different studies (i.e., 120, 412 and 1145 mg/kg). New studies need to determine exact mean.

Table 2: Nitrate content in different groups of vegetables, herbs and fungi (data from EFSA, 2008)[93]

Vegetable group*[a]	Total samples	Nitrate content (mg/kg)			
		Mean	Median	P5	P95
Leafy vegetables	25306	1614	1140	66	4556
Herbs	492	1240	791	10	4040
Stem vegetables	1379	698	302	3	2923
Roots and Tubers	7579	506	152	15	2302
Brassica vegetables	3192	411	241	7	1383
Legumes	882	221	56	1	748
Bulb vegetables	243	159	60	1	601
Fruiting vegetables	2822	149	83	1	486
Fungi	12	59	41	31	100
Total*[b]	41415	336	255	27	851

*[a] See Supplementary Table T1 for vegetables, herbs and fungi in different groups.
*[b] Total given by EFSA (2008), excluding herbs.
P5 and P95 are confidence intervals of 5% and 95%, respectively.

Fruits, cereal grains and oil seeds

Nitrate can also be detected in fruits, cereal grains and oil seeds, but (compared to vegetables) limited information is available[98]. However, based on current literature, it can be concluded that the nitrate levels in these food groups are generally lower than in vegetables.

Fruits are often grouped together with vegetables as the main source of nitrate in European countries[93]. However, in different common fruits (e.g., apples, pears and oranges), nitrate has been detected in low levels (<15 mg/kg) (Table 3). In some fruits, including melons, bananas and strawberries, relatively more nitrate accumulation has been detected. For example, nitrate levels of 45 and 153 mg/kg have been detected in bananas[99]. It should be noted that some authors consider strawberries and melons, fruited vegetables[99], but the exact botanical classification falls outside of the scoop of this thesis.

Cereal grains and oil seeds often have a nitrate content similar or slightly lower than fruits. For example, in wheat grains, a nitrate range of 0.4-11 mg/kg has been detected, whilst a range of 8.6-22.9 mg/kg was detected in soybeans[98,100]. Among cereal grains, it has been observed that oats can accumulate the highest levels of nitrate and, in a limited amount of 6 samples, a

mean of ~820 mg/kg nitrate was reported (with a median ~12 mg/kg)[98]. Future studies should confirm this.

Table 3: nitrate content in a convenience sample of fruits (data from Colla et al., 2018)[99]

Food	**Nitrate content (mg/kg)**			**Source**
	Mean	**Min**	**Max**	
Melon	48	41	56	Colla et al. (2010)[101]
	221			Temme et al. (2011)[102]
Banana	45			Hord et al. (2009)[91]
	153			Temme et al. (2011)[102]
Strawberry	18			Yordanov et al. (2001)[103]
	55	<30	111	Tamme et al. (2006)[104]
	94	9	360	Susin et al. (2006)[105]
Apple	3.3	0.2	15	Susin et al. (2006)[105]
	11			Temme et al. (2011)[102]
Pear	2.8	1.4	4.5	Susin et al. (2006)[105]
	14			Temme et al. (2011)[102]
Orange	9			Hord et al. (2009)[91]
	13			Temme et al. (2011)[102]

In conclusion, among edible plants, vegetables generally contain most nitrate, especially leafy green vegetables and certain root vegetables (e.g., beetroots and radishes). Lower amounts of nitrate are found in fruits, cereal grains and oil seeds, but some (e.g., bananas and oats) can reach similar nitrate levels as certain types of vegetables (e.g., fruiting and bulb vegetables).

Western diets and nitrate content

Western diets are high in domesticated animal protein, saturated fats, refined grains, sugar and salt, while lacking fresh vegetables in addition to fruits, whole cereal grains, legume seeds, oil seeds, and nuts[106,107]. Inside the gut, which contains most of the microbial load of the human microbiota, these dietary habits can contribute to dysbiosis and inflammatory disease development, both associated to a broad range of systemic diseases[106]. Conversely, vegetable molecules, such as fiber and polyphenols, are associated to gut microbiome homeostasis and gut health[108]. For example, some beneficial gut bacteria, like *Bifidobacteria*, thrive on fiber and metabolize this into Short Chain Fatty Acids

(SCFAs), which have a myriad of positive effects, ranging from local epithelial barrier function and anti-inflammatory effects to systemic cardio-metabolic benefits and immune system regulation[109,110].

Similarly, it has become clear that consuming nitrate-rich vegetables can affect the microbiome residing in the oral cavity, leading to positive effects for the host[83]. Evidence obtained in the last decade shows that the metabolization of nitrate by oral bacteria can improve cardio-metabolic health[25,111] and also appears to be potentially beneficial for oral health[62,63,84]. These benefits start with the reduction of nitrate to nitrite by oral bacteria, which is the first step in the so-called nitrate-nitrite-nitric oxide pathway.

The nitrate-nitrite-nitric oxide pathway

When chewing on vegetables with nitrate or drinking a vegetable juice, the salivary nitrate increases directly, allowing denitrifying oral bacteria to produce nitrite. Additionally, part of the nitrate that is swallowed is recycled back into the saliva by the salivary glands[112] (Figure 7). This mechanisms leads to fasting salivary nitrate in the 100–500 μM range (i.e., approximately 10 times higher than in plasma) (reviewed by Hezel & Weitzberget, 2015)[25]. The fasting levels of nitrate result from exogenous production by the oxidation of nitric oxide. This nitric oxide is produced by human nitric oxide synthases (NOS)[25]. After a nitrate-rich meal, salivary nitrate concentrations further increase into the millimolar range (e.g., 5–8 mM), which remain elevated many hours due to the long half-life circulation of nitrate.

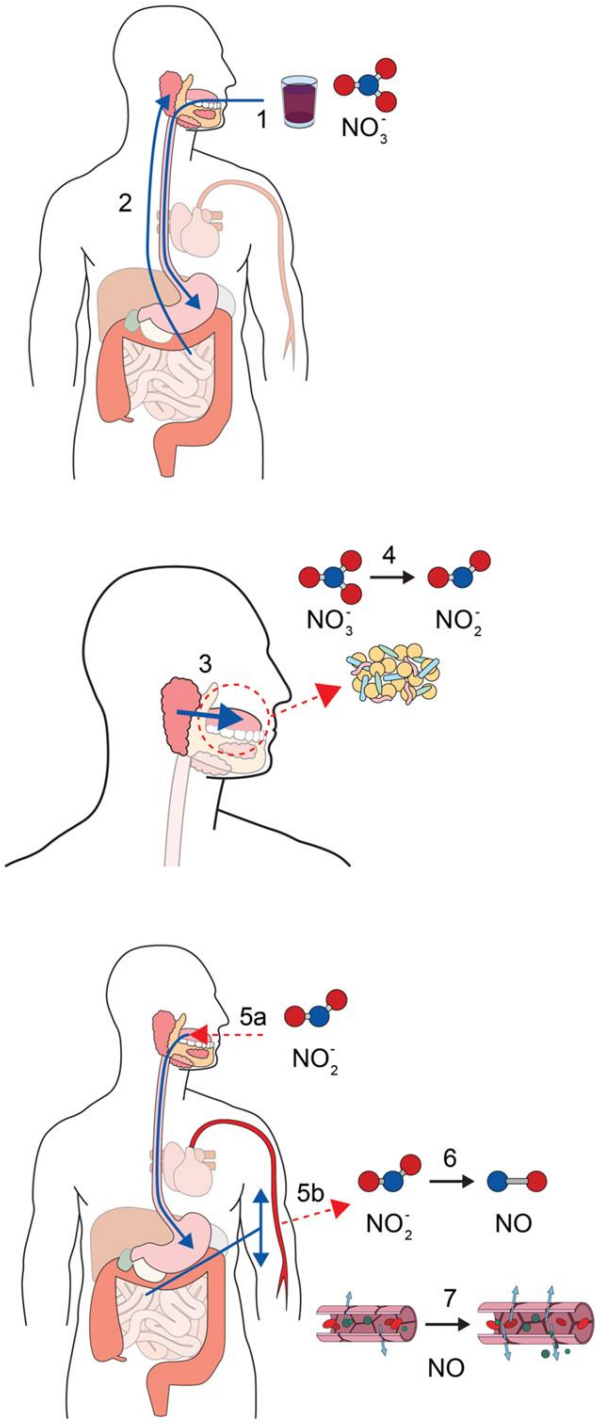

Figure 7: the nitrate-nitrite-nitric oxide pathway. (1) Nitrate-rich beetroot juice is swallowed. (2) The nitrate (NO_3^-) content is taken up by the intestine into the blood stream. (3) When the blood passes by the salivary glands, the nitrate is actively concentrated to high levels (5-8 mM) into your saliva. (4) Oral bacteria reduce nitrate to nitrite (NO_2^-). (5a) The saliva, which is now nitrite rich, is swallowed. (5b) This nitrite also enters the blood stream via the gut. (6) In the blood and tissues, there are several processes that further reduce nitrite into nitric oxide (NO) – an important cardio-metabolic regulator of the human body. (7) One of the many effects that nitric oxide has on the body is vasodilation. This means that nitric oxide expands our blood vessels thereby potentially decreasing blood pressure. © Bob T. Rosier

Nitrate reducing bacteria, such as (representatives of) *Neisseria*, *Rothia*, *Veillonella*, *Actinomyces* and *Kingella*[113,114], thus have the opportunity to use nitrate as an electron donor during respiration throughout the day. Oral bacteria further denitrify nitrite to nitric oxide, nitrous oxide and nitrogen[49], but a significant part of the nitrite is swallowed by the host[111]. The human body cannot metabolize nitrate itself, but provides several enzymatic and non-enzymatic processes to convert nitrite into nitric oxide[25]. For example, in the acidic gastric juice, part of the nitrite is decomposed to reactive nitrogen intermediates, such as nitric oxide (NO), which enhances the bactericidal effects of gastric juice[115]. In the blood vessels, nitrite reacts with hemoglobin and myoglobin to form nitric oxide, which apart from being antimicrobial, is also an important cardio-metabolic regulator of the human body[25,116]. Nitric oxide is involved in vasodilation, endothelial function, anti-aggregation of platelets, nerve transmission and mitochondrial function, among other physiological functions[117].

Cardio-metabolic benefits

It was shown that an antiseptic mouthwash acutely increases blood pressure in fasting individuals by disrupting nitrate reduction by oral bacteria[111]. Nitrate-rich vegetables and vegetable extracts can, in turn, stimulate nitrate reduction by the oral microbiota resulting in a lowering of blood pressure[118,119]. This oral microbiota dependent pathway (i.e., the nitrate–nitrite–nitric oxide pathway) can also increase sport performance, improve vascular endothelial function, inhibit platelet aggregation and promote the release of circulating angiogenic cells from the bone marrow[118,120,121]. Additionally, nitrate can reverse metabolic syndrome and have anti-diabetic effects in animal models[122]. Regarding this, over-the-counter mouthwash usage correlates with pre-diabetes and diabetes development[123]. It thus appears that nitrate consumption can improve different cardio-metabolic processes, whilst killing too many oral bacteria with mouthwash can interfere with these processes. It should be noted that epidemiological evidence indicates that, among vegetables, leafy green vegetables are particularly protective against cardiovascular disease and type 2 diabetes, indicating that the nitrate content may be related[25]. For a full list of potential systemic benefits see reviews, for example, by dos Santos Baião et al., (2021)[124], Johnes et al. (2021)[125], Carlstrom and Montenegro (2018)[126] and Lundberg et al. (2018)[122].

Nitrate and oral health

Compared to the cardiovascular benefits of nitrate reduction by the oral microbiota, much less research has explored the oral effects. This is striking in light of the high concentrations of salivary nitrate, which stay elevated over many hours after a nitrate-rich meal, as it has been known for several decades that nitrate affects microbial community composition and metabolism in environmental microbial communities[127]. Nitrate metabolism beyond nitrate reduction to nitrite has been detected inside the oral cavity and indicates it could stimulate oral health.

Nitrate and nitrite metabolism inside the oral cavity

One of the main nitrate reductase enzymes that reduced nitrate to nitrite is NarG, and Schreiber et al. (2010) identified the presence of *narG* genes in dental plaque[49] (Figure 8). Another common nitrate reductase enzyme is NapA, which could be present in oral species. Other genes identified in the oral microbiome are nitrite reductases *nirS* and *nirK*, nitric oxide reductase *qnorB* and nitrous oxide reductase *nosZ*[49]. Finally, genes involved in dissimilatory nitrate reduction to ammonium (DNRA) (e.g., *nirB* and *nirD*) are likely to be present. DNRA generally happens in the absence of oxygen or at low oxygen concentrations[128] and could thus take place inside oral biofilms on the tongue and teeth. Finally, genes involved in nitrogen fixation or assimilatory reduction of nitrate to ammonium could be present, but this remains unexplored. Importantly, small amounts of ammonium, which can be found in saliva, could inhibit or limit assimilatory nitrate reduction[63].

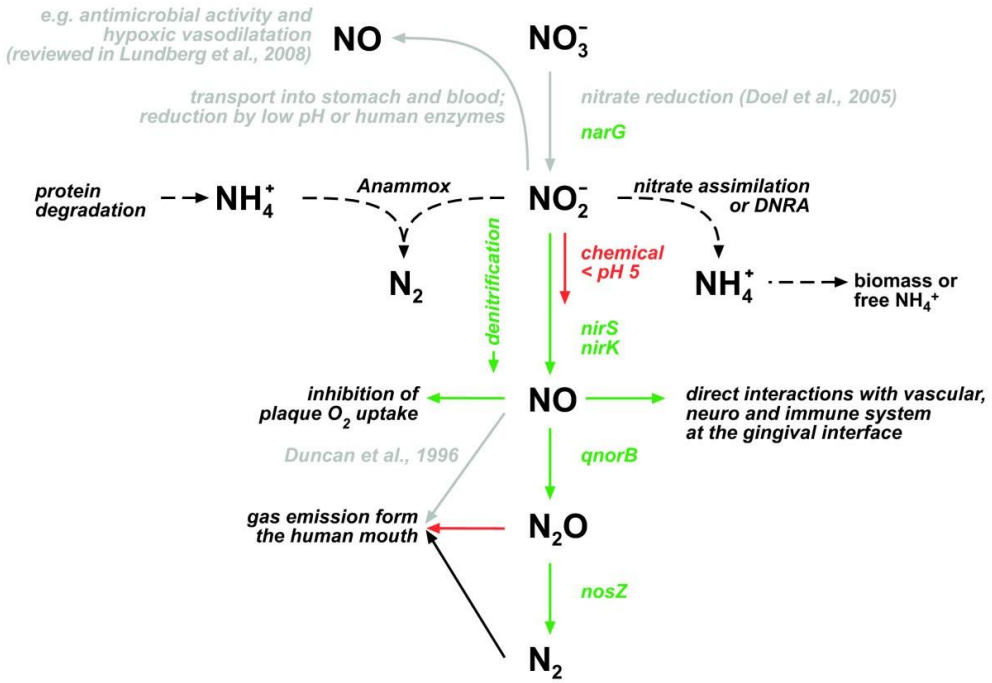

Figure 8. Microbial conversions of salivary nitrate (NO_3^-) inside the oral cavity. Figure from Schreiber at al (2010)[49]. Grey lines show pathways that have been reported previously. Dotted lines show potential pathways that have not been reported to occur in dental plaque in their or other studies. Coloured lines show pathways that are suggested to occur in the study by Schreiber et al. (2010)[49]. Green lines show biologically-mediated pathways and red lines show chemically-mediated or physically-mediated pathways. Genes encoding for enzymes that mediate individual steps of denitrification are depicted if detected in dental biofilms via polymerase chain reaction (PCR). Genes of the cytochrome c-dependent NO reductase (cNorB) were not detected. Anammox = anaerobic oxidation of ammonium; DNRA = dissimilatory nitrate reduction to ammonium.

Potential beneficial effects of nitrate on oral health

In 2004, it was observed that a lower caries incidence was found in patients with high salivary nitrate and nitrate-reducing ability[62]. Some years after this, it was shown that nitrate can limit acidification when incubating oral bacteria with sugar, but the exact mechanisms have yet to be identified[63]. Acidification by organic acids generated from sugar fermentation is the main factor in enamel demineralization and caries development[4]. Based on environmental microbiology studies, it was hypothesized that nitrite reduction to nitric oxide

(antimicrobial compound), the usage of lactic acid as an electron (as well as carbon) donor and alkali production by nitrate-reducing bacteria could limit caries-associated dysbiosis development[63].

More recently, nitrate in the form of lettuce juice has been shown to reduce gingival inflammation compared to a placebo (nitrate-depleted lettuce juice) in patients with chronic gingivitis[84]. Gingivitis is an inflammation of the gingiva and long or repeated episodes of gingivitis can lead to periodontitis (i.e., a chronic and destructive inflammation in which host tissue is lost)[24].

Finally, nitrate could inhibit halitosis. Firstly, it is interesting to note that nitrate is used as a biological agent to treat malodour from sewer networks by limiting microbial volatile sulphur compounds (VSCs) production resulting from sulfate reduction[129]. As long as more energy-efficient denitrification takes place, sulfate reduction is inhibited and this could also be the case in the oral cavity, where sulfate reduction is associated to halitosis[130]. Secondly, similar to what was hypothesized for lactic acid, nitrate-reducing bacteria could use hydrogen sulfide (one of the main VSCs involved in halitosis) as an electron donor.

Nitrate thus appears to potentially limit the development of common oral diseases, that is, caries and periodontal diseases (gingivitis and periodontitis), and a systematic program to test and validate these initial data is required to evaluate its potential as an oral health-promoting factor and to fully understand the mechanisms underlying its beneficial effects.

Objectives and hypotheses

Objective 1: To test the potential prebiotic effects of nitrate in an *in vitro* model and determine how oral community composition and fermentation capacity can be affected.

Hypotheses objective 1:

Based on the literature review, our hypotheses are that:

- The addition of nitrate will lead to health-associated changes in oral microbiota composition.
- Nitrate would limit acidification when sugars are fermented by stimulating lactic acid usage during denitrification and nitrite reduction to ammonium.
- Nitrate would affect biofilms' growth by nitric oxide production and/or stimulation of nitrate reducing species

Objective 2: To isolate nitrate-reducing species from individuals with good oral and systematic health and test their probiotic potential *in vitro*.

Hypotheses objective 2:

- Nitrate-reducing species from healthy individuals are associated to oral health.
- Nitrate-reducing isolates can increase the nitrate reduction capacity of oral communities.
- Nitrate-reducing isolates can limit acidification when sugars are fermented by oral communities in the presence of nitrate.

Objective 3: test the potential prebiotic effects of nitrate *in vivo* and determine how oral community composition and fermentation capacity can be affected.

Hypotheses objective 3:

- The addition of nitrate will lead to health-associated changes in the oral microbiota.
- Nitrate would limit oral acidification when sugars are consumed, resulting from lactic acid usage during denitrification and nitrite reduction to ammonium.

Results of thesis

Chapter 1.

This chapter was published in Scientific Reports:

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In chapter 1-3, minor modifications were made to few sentences compared to the original published versions to clarify that nitrite is reduced to ammonium (the ion of ammonia) and organic acids can be used during this process and during denitrification, which increases the pH. Additionally, ammonium is converted into ammonia (a weak base) under some conditions. Another minor modification was changing "lactate" to "lactic acid" where relevant. It is a common error in literature that lactic acid and its ion, lactate, as well as ammonia and its ion, ammonium, are used interchangeably as synonyms, while they have different properties.

**This article was added to the collection "Top 100 in Microbiology" of Scientific Reports, being in the top 25 of most downloaded articles in 2020.*

1. Nitrate as a potential prebiotic for the oral microbiome

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1.1 Abstract

The salivary glands actively concentrate plasma nitrate, leading to high salivary nitrate concentrations (5-8 mM) after a nitrate-rich vegetable meal. Nitrate is an ecological factor that can induce rapid changes in structure and function of polymicrobial communities, but the effects on the oral microbiota have not been clarified. To test this, saliva of 12 healthy donors was collected to grow *in vitro* biofilms with and without 6.5 mM nitrate. Samples were taken at 5h (most nitrate reduced) and 9h (all nitrate reduced) of biofilm formation for ammonium, lactate and pH measurements, as well as 16S rRNA gene Illumina sequencing. Nitrate did not affect biofilm growth significantly, but reduced lactate production, while increasing the observed ammonium production and pH (all $p<0.01$). Significantly higher levels of the oral health-associated nitrate-reducing genera *Neisseria* (3.1×) and *Rothia* (2.9×) were detected in the nitrate condition already after 5h (both $p<0.01$), while several caries-associated genera (*Streptococcus*, *Veillonella* and *Oribacterium*) and halitosis- and periodontitis-associated genera (*Porphyromonas*, *Fusobacterium*, *Leptotrichia*, *Prevotella*, and *Alloprevotella*) were significantly reduced ($p<0.05$ at 5h and/or 9h). In conclusion, the addition of nitrate to oral communities led to rapid modulation of microbiome composition and activity that could be beneficial for the host (i.e., increasing eubiosis or decreasing dysbiosis). Nitrate should thus be investigated as a potential prebiotic for oral health.

1.2 Introduction

The salivary glands actively concentrate plasma nitrate into the saliva, which leads to fasting levels of salivary nitrate in the 100–500 μM range (i.e., approximately 10 times higher than in plasma) [reviewed by Lundberg et al. (2004)[131] and Hezel & Weitzberg (2015)[25]]. After a nitrate containing meal, this mechanism causes a further increase of salivary nitrate concentrations up to 5-8 mM[131], which remain elevated many hours due to the long half-life circulation of nitrate[132]. The food groups that naturally contain most nitrate are vegetables and fruits, both generally associated with health benefits, lower disease prevalence and longevity[87,122]. Certain oral bacteria reduce nitrate (NO_3^-) mostly to nitrite (NO_2^-), but also further to more reactive nitrogen intermediates such as nitric oxide (NO) in a process called denitrification[49].

Importantly, a significant amount of the produced nitrite is swallowed and taken up into the blood circulation of the host, where it is converted into nitric oxide—a signaling molecule involved in cardiovascular and metabolic regulation[111,122]. This is referred to as the nitrate-nitrite-nitric oxide pathway and provides an oral microbiome-dependent way of obtaining bioactive nitric oxide in addition to the classical NO synthases of the host[25]. Described systemic effects from nitrate supplementation include lowering of blood pressure, improved endothelial function, increased exercise performance, reversal of metabolic syndrome and anti-diabetic effects[122]. The importance of nitrate-reducing oral bacteria is reflected by the observation that blood pressure acutely increases in fasting individuals after using chlorhexidine mouthwash resulting from the loss of oral nitrate reduction[111]. Additionally, chlorhexidine mouthwash interferes with post-exercise hypotension[133] and over-the-counter mouthwash usage correlated with pre-diabetes and diabetes development[123].

Importantly, inside the oral cavity, salivary nitrate, nitrite and the nitrate reducing capacity of the oral microbiome have been proposed to be beneficial to prevent caries[62,63,134]. Additionally, two weeks of nitrate-rich lettuce juice consumption improved gingival health in a recent clinical study[84]. Finally, in other recent clinical intervention studies[83,135], 1-4 weeks of high doses of nitrate in the form of concentrated beetroot juice increased the number of health-associated nitrate-reducing bacteria in saliva [136]. It is therefore important to

determine experimentally if nitrate can be considered a dietary component associated with oral health.

While different studies have shed light on the molecular mechanisms involved in the systemic effects of nitrate reduction by the oral microbiota[25,111,122,137], mechanisms leading to the apparent health-associated roles inside the oral cavity remain largely hypothetical. To unravel these mechanisms, the effect of nitrate on oral communities must be studied. Oral microorganism in saliva form biofilms on tooth surfaces (i.e., dental plaque) and the tongue (i.e., tongue coating)[37,136]. These biofilms are involved in the development of the most common oral diseases, including caries, periodontitis and halitosis.

It must be born in mind that nitric oxide has antimicrobial properties and planktonic cultures of certain oral species associated to periodontitis have shown to be sensitive to this highly oxidative compound[138]. It would therefore be crucial to determine if the reduction of nitrate affects the composition of oral communities by inhibiting the growth of disease-associated species, while increasing health-associated nitrate-reducing species. Furthermore, the capacity of oral bacteria to reduce nitrite to ammonium (the ion of ammonia) and to use lactic acid as electron donor and carbon source during this process and throughout denitrification could prevent acidification, which is responsible for caries development[49,63].

In relation to this, in a pioneering study, Koopman et al. (2016)[139] applied 5 mM nitrate pulses of 6 minutes to 1-4 week old oral microcosms (i.e., complex, large *in vitro* oral biofilms) from two individuals, and each of them responded differently to nitrate. However, an effect on pH buffering was not detected and the number of participants was too low to conclude how the biofilm composition may be affected by nitrate.

In our current study, the effect of a single dose of nitrate on freshly sampled oral communities was tested *in vitro*. In short, saliva of 12 healthy donors was incubated in nutrient-rich medium with or without the physiologically relevant concentration of 6.5 mM nitrate in an impedance-based system (xCELLigence) that monitors real-time biofilm growth[140,141]. Samples were taken after 5h, when most nitrate was reduced and some nitrite was produced, and at 9h, when all

nitrate and most nitrite were metabolized. Supernatant samples were taken for the measurements of nitrate, nitrite, ammonium, lactate and pH. The remaining biofilms were collected for protein and DNA quantification, as well as Illumina sequencing of the 16S rRNA gene. By this experimental protocol, we aimed to study the short-term effect of a single dose of nitrate on pH, oral biofilm growth and bacterial composition.

1.3 Materials and methods

1.3.1 Unstimulated saliva sampling

For this study, adults who reported to be systemically healthy were recruited as saliva donors at the FISABIO Institute (Valencia, Spain). Individuals were excluded if frank cavitation was detected at the moment of sampling (following the criteria of Rosier et al., 2017[142]), which was assessed visually by an experienced dentist, or any history of periodontitis (following the criteria of Camelo-Castillo et al., 2015[143]), as well as if they had used antibiotics or regularly used oral antiseptics in the previous month.

Twelve healthy donors were instructed to have a normal breakfast and abstain from oral hygiene before saliva collection in the morning. Five ml of unstimulated saliva were collected at least one hour after breakfast by drooling[144] in a sterile tube in a quiet room. The saliva was used for *in vitro* growth and biofilm quantification. The procedure was repeated another time for biofilm sequencing and supernatant analysis.

To determine the effect of nitrate on acidification due to glucose fermentation, 9 healthy donors were asked to donate saliva under fasting conditions (abstaining from breakfast and oral hygiene) to avoid the presence of dietary-derived salivary nitrate.

The fresh unstimulated saliva was always directly used in the experiments or kept at 4°C for <1 h before usage. All donors gave informed consent prior to sample collection and the protocol was approved on 2016/05/23 by the Ethical Committee of DGSP-FISABIO (Valencian Health Authority) with the reference BIO2015-68711-R2. This study was carried out according to the relevant guidelines and regulations of the Declaration of Helsinki.

1.3.2 *In vitro* oral biofilm growth and impedance-based quantification

Unstimulated saliva of twelve donors was grown in 'E-Plate 96' 96-well plates in the xCELLigence system (ACEA Biosciences, San Diego, California, US). Each E-Plate is coated with a golden layer at the bottom of the wells that is connected to microelectrodes, allowing the measurement of biofilm growth in real-time[140,141,145,146]. The impedance formed by biofilm adherence has been shown to be proportional to biofilm mass, which is provided by a corresponding Cell Index and expressed in arbitrary units[147]. Previous sequencing of biofilms grown under these circumstances show that bacterial composition is representative of different oral niches, depending on the sample type used for inoculation[141].

BHI medium (Biolife, Deerfield, Illinois, US) with an additional 0.05 mg/L haemin, 0.005 mg/L menadione and 0.2 mM vitamin K (all Sigma-Aldrich, St. Louis, Missouri, US) was prepared of which 100 µl was added to each well for background impedance measurements. Additional 25 µl of 65 mM nitrate ($NaNO_3$, Sigma-Aldrich) in water or just water was added to each well of the nitrate or control condition, respectively. Then, 125 µl freshly collected saliva was added, leading to a final concentration of 6.5 mM nitrate (within the 5-8 mM physiological range of saliva after a nitrate containing meal) in the nitrate condition. The E-Plate 96 was placed in the xCELLigence system inside an incubator at 37°C. Every 10 minutes, a Cell Index measurement was taken. All experiments were performed without agitation and anaerobic conditions were favored by sealing the wells with adhesive aluminum foil (VWR, Radnor, Pennsylvania, US), which previously allowed the growth of strictly anaerobic bacteria[141]. All conditions (control 5h, control 9h, nitrate 5h, nitrate 9h) were grown in duplicate.

For 0h measurements, 1:1 medium and saliva mixtures were used. After 5h and 9h of growth, the supernatant was sampled and stored at -20°C until pH, nitrate, nitrite, ammonium and lactate measurements were performed in duplicate. A PBS washing step was then performed to remove unattached cells and, after this, the remaining cells were removed with a pipette and resuspended in 100 µl PBS. Biofilm duplicates were resuspended together in 200 µl PBS for storage at -20°C until DNA isolation for sequencing.

The washing step removed part of the (slightly attached) bacteria that had accumulated after 5h or 9h at the bottom of the well. Therefore, for protein and DNA quantification, the PBS washing step was not performed to quantify the entire microbial community that affected the physiological measurements in our *in vitro* system (e.g., pH, lactate and ammonium). After removing supernatant, the biofilms were resuspended in 75 µl PBS. It was observed that nitrate affects the impedance of the xCELLigence system and this effect depended on the saliva of the donor. Therefore, controls with microorganism-free filtered saliva were used to normalize the cell-index measurements. For this, the saliva was first filtered with a 5 µm filter and then with 0.1 µm filter.

1.3.3 Incubating saliva with nitrate and glucose

The unstimulated saliva of nine donors collected under fasting conditions was used to test the effect of different concentrations of nitrate on a pH drop caused by 0.2% of glucose after 5 h of incubation. For each donor, 187 µl of saliva and 22 µl of glucose (2% diluted in water) was added per well of a standard 96-well plate. Then, 11 µL of water without or with different concentrations of nitrate was added, leading to final concentrations of 0 mM, 0.5 mM, 1 mM, 1.5 mM, 2.5 mM, 3.5 mM, 4.5 mM, 5.5 mM, 6.5 mM ,7.5 mM and 8.5 mM of nitrate. The plate was sealed with adhesive aluminum foil and incubated during 5 h at 37°C. After incubation, the samples were stored at -20°C until pH measurements.

1.3.4 Nitrate, nitrite, ammonium, lactate and pH measurements

For the nitrate, nitrite, ammonium, lactate and pH measurements, the RQflex 10 Reflectoquant (Merck Millipore, Burlington, Massachusetts, US) reflectometer was used. This method is based on the intensity of reflected light by two reactive pads on test strips that change in color intensity based on the concentration of a specific substance[148].

The test strips (Reflectoquant, Merck Millipore) for pH had a range from pH 4-9, the strips for nitrate a range of 3-90 mg/l, the strips for nitrite a range of 0.5-25 mg/l, the strips for ammonium a range of 5-20 mg/l, and the strips for lactate a range of 3-60 mg/l. Accuracy of all reflectometer methods was confirmed by the use of standard solutions (nitrate, nitrite, ammonium, all Merck Millipore; lactate, BioVision, Milpitas, California, US) with known concentrations of the different compounds.

A method was used based on Hemke et al. (2009) and Ferrer et al. (2020)[149,150]. In short, undiluted supernatant was used for pH measurements and, for the rest of the measurements, 10× or higher dilutions were made to obtain a concentration within the detection threshold of the test strips. Then, 15 µl of (diluted) supernatant was added to each of the two reactive patches on a strip, and excess liquid was removed by tipping the side of the strip on a tissue. Before nitrate measurements, the diluted supernatants in which 0.5 mg/l or more nitrite was detected were treated with amidosulfuric acid (Sigma-Aldrich) based on the manufacturer's instructions. For this, 35 µl of diluted supernatant was mixed with 1.5 µl amidosulfuric acid solution (10%).

For ammonium measurements, aliquots were made of 50 µl diluted supernatant to which 10 µl of reagent 1 (ammonium 5-20 mg/l test strip kit, Reflectoquant, Merck Millipore) was added first and resuspended well. Then, 15 µl of a freshly made mixture of reagent 2 dissolved in 1.25 ml water was added and resuspended. This solution was then directly added to the strips and incubated according to the manufacturer's instructions.

1.3.5 Biofilms protein and DNA quantification

Biofilms grown for 5h and 9h were resuspended in 75 µl PBS of which 30 µl was used for protein quantification and the rest for DNA isolation. For protein quantification, the Bradford protein assay was applied, which is based on the colour change of the Coomassie Brilliant Blue dye (G-250) when bound to proteins. Duplicates of 15 µl of resuspended pellet were added to different wells of a standard 96-well plate. Then, 240 µl of Bradford Reagent (Sigma-Aldrich), containing G-250, was added and, after 5 minutes of incubation in the dark, the absorbance was measured with an Infinite F200 plate reader (TECAN, Männedorf, Switzerland) at 600 nm. Protein concentrations were determined using a calibration curve with known concentrations of BSA (range: 0 to 1.5 mg/ml, Sigma-Aldrich) on each plate. For DNA quantification, DNA was extracted as described in the next section. Then, the DNA concentration was measured using the Qubit 1X dsDNA HS Assay Kit and a Qubit 3 Fluorometer (both Thermo Scientific, Waltham, Massachusetts, US), according to manufacturer’s instructions.

1.3.6 Biofilm composition determined by 16 rDNA Sequencing

DNA extraction for sequencing

For DNA extraction, the biofilm duplicates (or salivary pellets from 250 µl saliva for inoculum sequencing) were resuspended in 100 µl PBS and disaggregated 30 s in a sonicator bath (model VCI-50, Raypa, Barcelona, Spain) at low ultrasound intensity. After this, DNA was isolated by MagNA Pure LC 2.0 Instrument (Roche Diagnostics, Risch-Rotkreuz, Switzerland), using the MagNA Pure LC DNA Isolation Kit III for Bacteria and Fungi (Roche Diagnostics) following the manufacturer's instructions with an additional enzymatic lysis step: to a bacterial pellet in 100 µl PBS, 130 µl lysis buffer and 2.5 µl of enzyme mix, containing 20 mg/ml lysozyme (Thermomixer comfort, Eppendorf, Hamburg, Germany), 5 mg/l lysostaphin (Sigma-Aldrich) and 0.625 mg/ml mutanolysin (Sigma-Aldrich), were added and incubated for 60 min at 37°C. DNA was resuspended in 100 µl elution buffer and frozen at -20°C until further analysis. To determine the amount of DNA for sequencing, the Quant-iT PicoGreen dsDNA Assay Kit and a Qubit 3 Fluorometer (both Thermo Scientific) were used, according to manufacturer's instructions.

16 rDNA Sequencing

A pre-amplification step of the V1-V5 regions of the 16S rRNA gene was performed, following Dzidic et al. (2018)[151]. An Illumina amplicon library was then performed following the 16S rRNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Part #15044223 Rev. A), targeting the 16S rRNA gene V3 and V4 regions, resulting in a single amplicon of 460 bp. Amplicons were sequenced on a MiSeq Sequencer according to manufacturer's instructions (Illumina, San Diego, California, US) using the 2x300 bp paired-ends protocol.

Taxonomic classification

The sequences were analyzed according to Boix-Amorós et al. (2016)[152]. In short, the reads were quality-filtered and end-trimmed in 10 bp windows with Prinseq. The PCR chimeras were removed with UCHIME according to Edgar et al. (2011)[153]. Given that taxonomic assignment accuracy decreases dramatically in reads shorter than 200 bp[154], single reads were discarded and only joined reads were used to be taxonomically assigned at the genus level with the classifier of the Ribosomal Database Project[155], using a confidence interval of 80%. Operational Taxonomic Unit (OTU) selection was performed using

VSEARCH[156] at a 97% of sequence identity. Given that taxonomic accuracy gets reduced at species level, especially in some genera with a highly similar 16S rRNA gene among species, only sequences >400 bp were used for species-level classification[157]. We aligned each OTU centroid using BLAST at 97% of identity and 100% query coverage and retrieved only those species that agreed with the previous classification of the centroid at genus level provided by RDP classifier[158].

1.3.7 Statistical analysis

We used overall R programming language for statistical computing[159] to perform downstream analyses. Only those taxa with an abundance of >0.001% in more than three samples in at least one condition were selected for the analyses. For multivariant analysis, an Adonis test (Permutational Multivariate Analysis of Variance Using Distance Matrices), provided by the Vegan library of R[160], was used to compare groups. To visualize groups and their differences in a two-dimensional map, we computed constrained principal components via constrained correspondence analysis (CCA) which is also part of Vegan library[160]. For univariate analyses, paired non-parametric Wilcoxon tests (i.e., "wilcox.test" function of stats library of R[159]) were performed to test the differences in genera and all other parameters between groups. Correlations within and between genera and other parameters were determined with Spearman's rho, along with associated p-value using the "cor.test" function of the stats library of R[159]. Finally, to see if the observed taxonomic changes were supported by a standard compositional data analysis (CODA) technique, an ANCOM-II analysis was performed according to Kaul et al. (2017)[161], which also controls the false discovery rate at a desired level of significance. For all taxonomic comparisons and correlations, only adjusted p-values were used.

1.4 Results

1.4.1 Effect of nitrate on biofilm growth

The addition of 6.5 mM nitrate (i.e., 403 mg/L) did not show significant changes in real-time impedance measurements of biofilm formation compared to the control condition (Figure 1A). In agreement with this, total protein measurements of formed biofilms did not differ significantly between the different conditions (Figure 1B). There was a positive correlation between DNA and protein of the communities ($R=0.62$, $p<0.01$). The amount of DNA,

however, was 21% higher in the nitrate condition at 5h ($p<0.01$, Figure 1C), suggesting that the number of cells could be higher under nitrate supplementation or that there is an increase in extracellular DNA. Additionally, 56% and 58% more DNA were detected in the control and nitrate conditions, respectively, at 9h compared to 5h (both $p<0.05$).

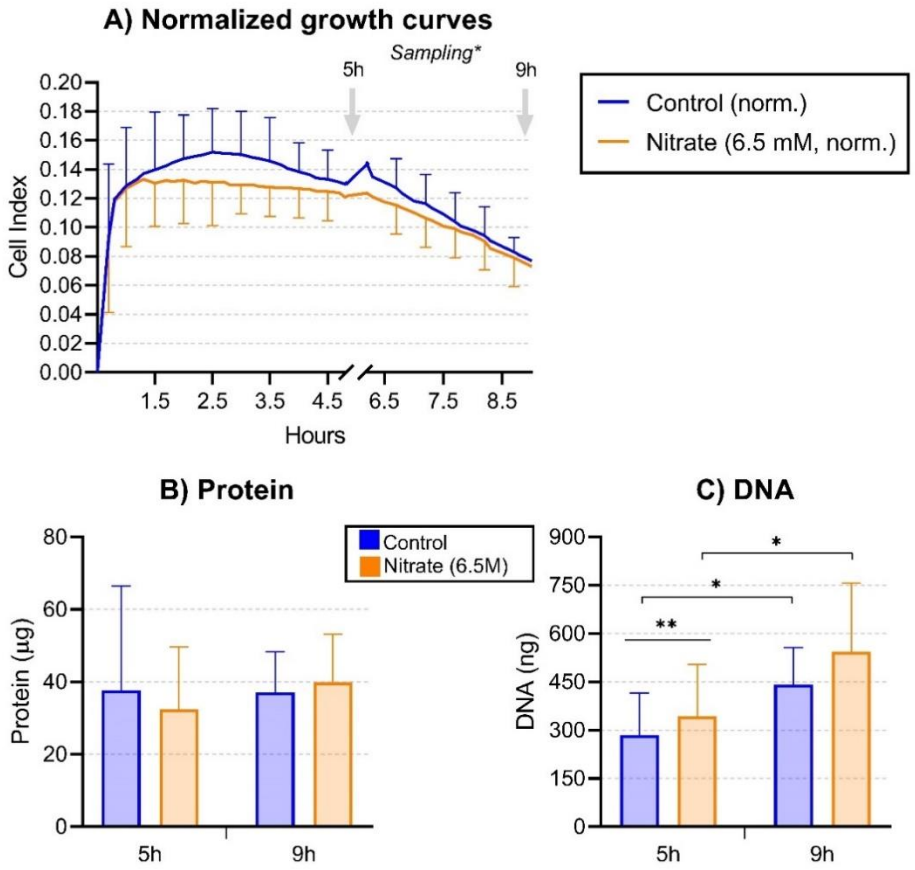


Figure 1. Effect of nitrate on biofilm formation. Biofilms were grown with saliva as inoculum in a 6.5 mM nitrate condition (orange) and a control condition (blue). A: plot shows averages ±SE of biofilm mass, measured by Cell Index values, as indicated by impedance measurements, after normalization (norm.) with microorganisms-free filtered saliva. Measurements were taken every 10 minutes. Error bars are only shown at half an hour intervals for clarity. Sampling: samples were collected at 5h and 9h for different measurements (grey arrows). B and C: Protein and DNA quantification of the biofilms harvested at 5h and 9h. Bars represent averages of 12 donors with their corresponding standard deviations. * $p<0.05$, ** $p<0.01$ according to a Wilcoxon test.

1.4.2 Changes in nitrate, nitrite, ammonium, lactate and pH during biofilm growth

Mixtures of saliva and BHI medium with or without 6.5 mM nitrate before growth (0h) and the supernatants after 5h and 9h of growth were analyzed. At baseline (0h), there were differences in the measured parameters between donors due to person-specific saliva properties (Figure 2A-E; for measurements in individual donors, see Supplementary Spreadsheet).

In the condition with an additional 6.5 mM nitrate (i.e., 403 mg/l), most nitrate was used up after 5h (Figure 2A): in 7 individuals there was no nitrate detectable after 5h, while for the other 5 donors, 76-85% of the nitrate had been reduced. In accordance with this, nitrite increased from an average of 1.64 mg/l at 0h to 64.68 mg/l at 5h. However, at 9h the nitrite levels had dropped significantly, indicating further reduction to other compounds (Figure 2B).

After 5h, the mean of ammonium had increased 1.72× in the control condition and 2.21× in the nitrate condition compared to 0h (both $p<0.005$). Ammonium was significantly higher in the nitrate condition compared to the control conditions at 5h ($p<0.01$, Figure 2C) and 9h ($p<0.005$). The mean of lactate increased notably after 5h in both conditions compared to 0h (4.74× in the control condition and 3.40× in the nitrate condition, both $p<0.005$). After 9h, the lactate had decreased in both conditions compared to 5h ($p<0.005$), indicating lactate was being metabolized. Importantly, lactate was significantly lower in the nitrate condition compared to the control condition at 5h and 9h ($p<0.005$, Figure 2D). There was a negative correlation between lactate and ammonium at 9h that was more evident in the control condition (at 9h, $r=-0.87$, $p<0.005$ in control and $r=-0.71$, $p<0.05$ in nitrate condition, Table 1A and Table 1B).

In accordance with a higher amount of ammonium production and lower amounts of lactate, pH was significantly higher in the nitrate condition at 5h and 9h (both, $p<0.005$, Figure 2E). In this regard, the pH dropped significantly after 5h compared to 0h in the control condition ($p<0.005$), but not in the nitrate condition ($p=0.056$). Interestingly, at 5h, there was a negative correlation between pH and nitrite in the nitrate condition ($r=-0.82$, $p<0.005$, Table 1B): individuals with 0 mg/L nitrite, possibly all used up, had the highest pH.

Likewise, in the nitrate condition, ammonium correlated negatively with nitrite at 5h ($r=-0.64$, $p<0.05$), indicating that part of the nitrite was further reduced to ammonium. In agreement with this, there was a negative correlation between the nitrate reduction capacity of communities (determined by the nitrate left after 5h in the nitrate condition) and ammonium detected in both the nitrate and the control conditions (both $r=-0.717$, $p<0.01$, Table 1B and Supplementary Table 1). This suggests that communities with the best nitrate reduction capacity produced most ammonium/ammonia (in the presence or absence of nitrate), indicating a possible link between the two processes.

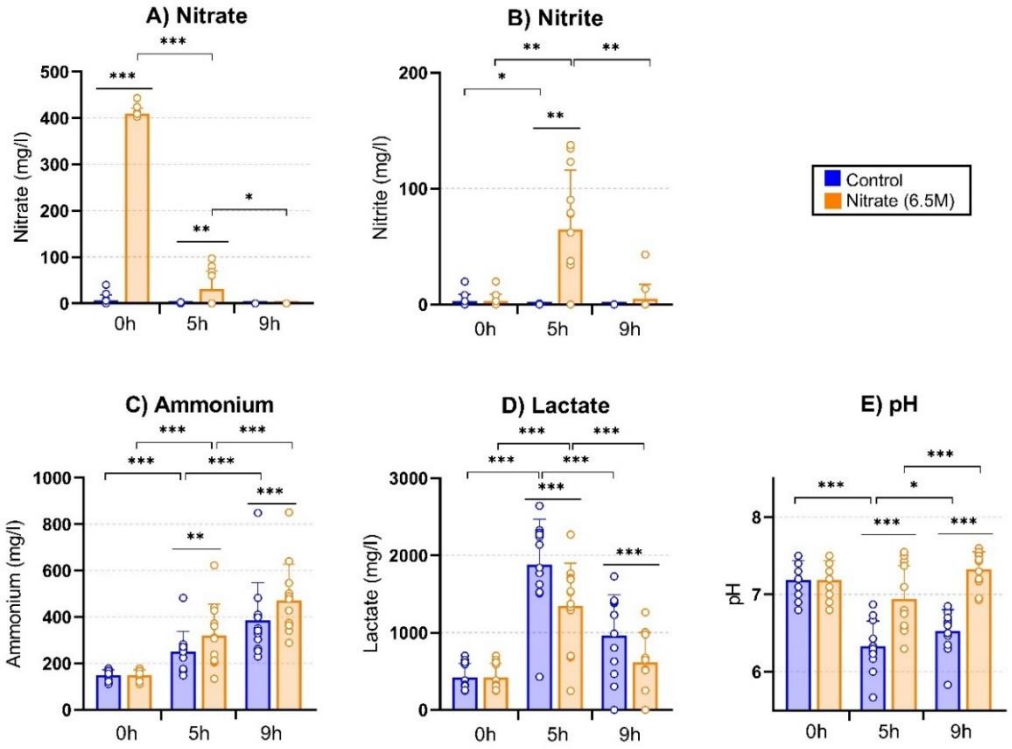


Figure 2. Effect of nitrate supplementation on *in vitro* oral biofilm metabolism. Barplots show averages and standard deviations of measurements in supernatant samples from 12 donors under 6.5 mM nitrate (orange) and control (blue) conditions at different times of biofilm growth (0h is the 1:1 mixture of saliva and medium, and 5h and 9h are supernatant measurements after *in vitro* growth). A) Nitrate (mg/l), B) nitrite (mg/l), C) ammonium (mg/l), D) lactate (mg/l) and E) pH. *** $p<0.005$ ** $p<0.01$, * $p<0.05$, according to a Wilcoxon test.

Table 1A: correlations of physiological parameters in control condition after 5h and 9h (n = 12)

			Control condition					
			Ammonium (mg/l)		Lactate (mg/l)		pH	
			5h	9h	5h	9h	5h	9h
Control condition	Ammonium (mg/l)	5h	1.000	0,860***	-0.566	-0,769***	0.217	-0.147
		9h	0,860***	1.000	-0,720**	-0,874***	0.350	0.196
	Lactate (mg/)	5h	-0.566	-0,720**	1.000	0,860***	-0.203	-0.434
		9h	-0,769***	-0,874***	0,860***	1.000	-0.196	-0.399
	pH	5h	0.217	0.350	-0.203	-0.196	1.000	0.427
		9h	-0.147	0.196	-0.434	-0.399	0.427	1.000

* p<0.05
** p<0.01
***p< 0.005

Table 1B: correlations of physiological parameters in nitrate condition after 5h and 9h (n = 12)

			Nitrate condition							
			Nitrate (mg/l)[a]	Nitrite (mg/l)[a]	Ammonium (mg/l)		Lactate (mg/l)		pH	
			5h	5h	5h	9h	5h	9h	5h	9h
Nitrate condition	Nitrate (mg/l)[a]	5h	1.000	0.322	-0,717**	-0.530	0.405	0,711**	-0.336	-0.016
	Nitrite (mg/l)[a]	5h	0.322	1.000	-0,641*	-0.528	0,655*	0,578*	-0,822***	-0.394
	Ammonium (mg/l)	5h	-0,717**	-0,641*	1.000	0,888***	-0.573	-0,694*	0,651*	0.035
		9h	-0.530	-0.528	0,888***	1.000	-0,594*	-0,708*	0,641*	0.091
	Lactate (mg/l)	5h	0.405	0,655*	-0.573	-0,594*	1.000	0,753***	-0.571	-0.308
		9h	0,711**	0,578*	-0,694*	-0,708*	0,753***	1.000	-0.461	-0.070
	pH	5h	-0.336	-0,822***	0,651*	0,641*	-0.571	-0.461	1.000	0,588*
		9h	-0.016	-0.394	0.035	0.091	-0.308	-0.070	0,588*	1.000

[a] After 9h, there was no nitrate and nitrite detected in supernatants of 12/12 and 9/12 donors, respectively.
* p<0.05
** p<0.01
*** p<0.005

1.4.3 The effect of different nitrate concentrations on acidification by sugar metabolism

To investigate if nitrate would have an effect on salivary acidification by sugar without the presence of culture medium, unstimulated saliva was incubated with 0.2% glucose and a concentration range of nitrate from 0.5-8.5 mM during 5h (Figure 3). The salivary pH before growth was 7.17 (SD 0.41). After 5h of incubation with 0.2% glucose without nitrate, the pH dropped to pH 4.71 (SD 0.29, LQ 4.49, UQ 4.96). All nitrate concentrations from 1 mM to 8.5 mM resulted in a significantly higher pH after 5h compared to 0 mM nitrate ($p<0.05$ for 1 mM and 1.5 mM, $p<0.01$ for higher concentrations up to 8.5 mM). Interestingly, 3.5 mM nitrate resulted in pH 4.92 (SD 0.36, LQ 4.75, UQ 5.2) and the pH levels did not further increase significantly when adding more nitrate.

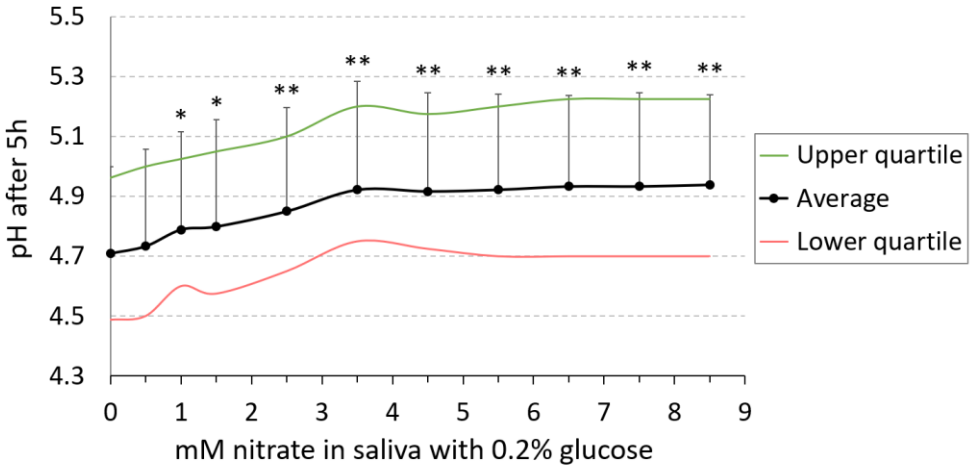


Figure 3: Salivary acidification is inhibited by nitrate. Saliva of 9 donors was incubated for 5h with 0.2% glucose and a given concentration of nitrate (0.5-8.5 mM), which is within the physiological range of human saliva. In this plot, averages (black dots) with standard deviations, upper quartiles (green line) and lower quartiles (red line) are shown. All the different concentrations of nitrate were compared with 0 mM nitrate and significance was marked with * for $p<0.05$ and ** for $p<0.01$ according to a Wilcoxon test.

1.4.4 Nitrate strongly affects biofilm composition

The addition of nitrate had a significant effect on the bacterial composition of *in vitro* oral communities, explaining a large proportion of data variability regardless of biofilm sampling time (Figure 4). The control and nitrate conditions differed significantly at 5h and 9h (individual CCA and Adonis p-values ≤ 0.005, Supplementary Figure 1). The CCA p-value between the control condition at 5h and 9h was not significant, but the Adonis p-value was (<0.05). The difference between the nitrate condition at 5h and 9h was significant (CCA and Adonis p-value < 0.05), indicating that oral communities can change rapidly (in a matter of a few hours) under certain conditions, especially in the presence of nitrate.

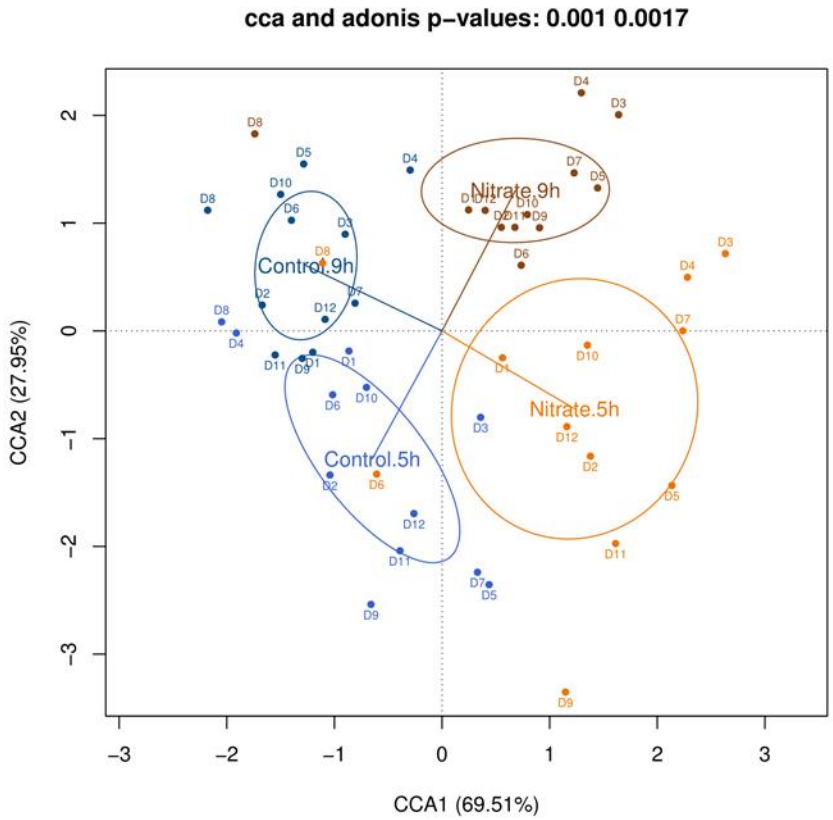


Figure 4: Effect of nitrate on oral biofilm composition at 5h and 9h of *in vitro* growth. Both Adonis and CCA p-values suggest statistically significant differences in bacterial composition on a genus-level between the control and nitrate conditions at 5h and 9h (for two-group comparisons, see Supplementary Figure 1). In this plot, the first

constrained component clearly separates the two experimental conditions (control and nitrate), whereas the second one reflects variability due to time (5h and 9h), showing that both biofilm formation time and nitrate influence bacterial composition.

The relative abundances of individual donors at 5h and 9h were plotted as percentages in Figure 5B and Supplementary Figure 2B, respectively (individual data can be found in the Supplementary Spreadsheet). Based on medians, in the control condition, the five most common genera after 5h of biofilm growth were *Streptococcus* (50.17%), *Veillonella* (19.14%), *Neisseria* (6.62%), *Haemophilus* (6.16%) and *Granulicatella* (1.94%). In the nitrate condition, the most abundant genera after 5h were *Streptococcus* (43.81%), *Neisseria* (20.27%), *Veillonella* (10.63%), *Haemophilus* (6.64%) and *Gemella* (2.17%). After 9h, *Streptococcus* remained the dominant genus (38.94% and 32.78% in control and nitrate conditions, respectively) followed by *Veillonella* (28.85%), *Haemophilus* (8.75%), *Neisseria* (6.27%) and *Porphyromonas* (1.77%) in the control condition and by *Neisseria* (20.55%), *Veillonella* (19.53%), *Haemophilus* (8.22%) and *Gemella* (1.48%) in the nitrate condition. The saliva used as inoculum of 4 randomly selected donors was sequenced (Supplementary Figure 2A) and comparable dominant genera were found as in the *in vitro* biofilms (Figure 5A and Supplementary Figure 2B): with a similar percentage of *Streptococcus* (43.88%) on the first place in the inoculum and then *Veillonella* (7.19%), *Neisseria* (6.97%), *Gemella* (6.44%) and *Granulicatella* (5.27%).

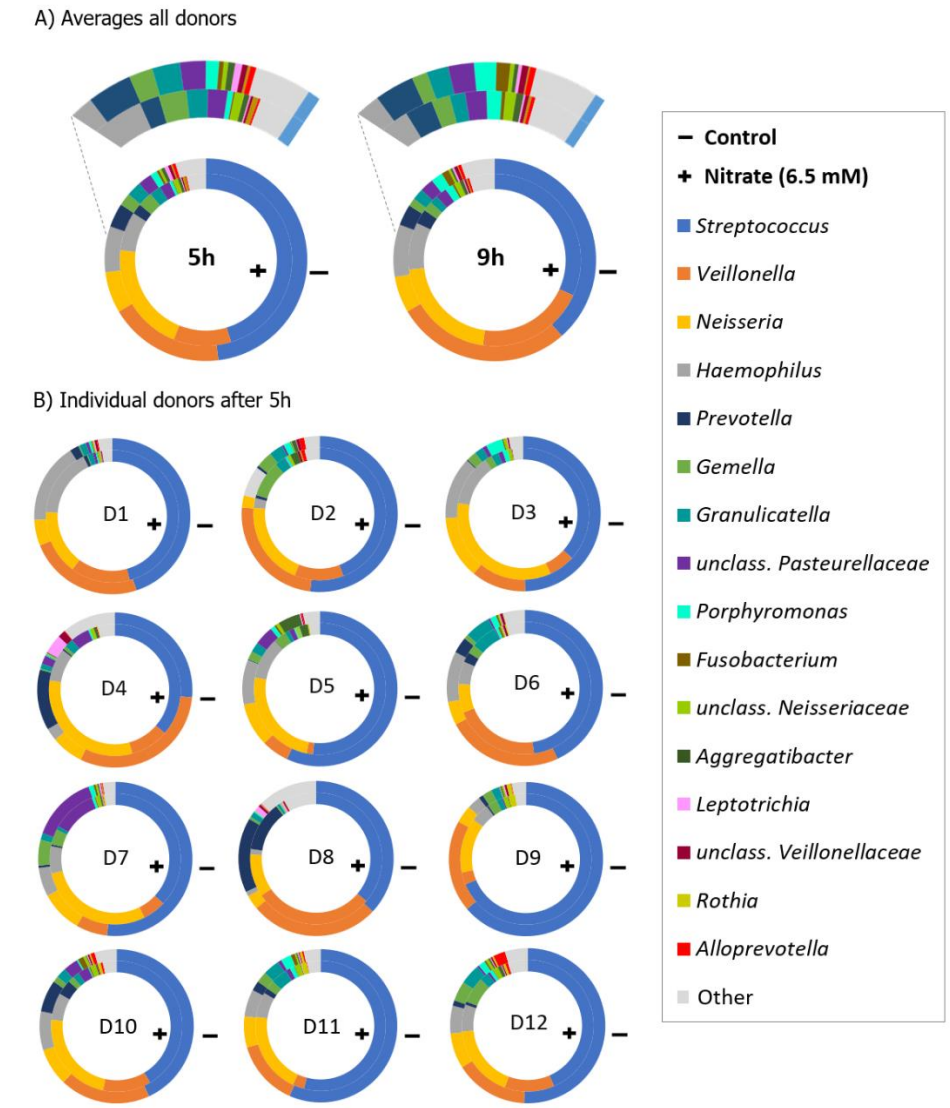


Figure 5: Bacterial composition in oral biofilms as determined by 16S rRNA sequencing. A) Ring charts of the average relative abundance of genera in biofilms of 12 donors at 5h and 9h of growth. Above the ring charts, there is a zoom-in showing the low-abundance genera between *Haemophilus* (dark grey) and *Steptococcus* (blue). B) Relative abundance of genera in biofilms of individual donors at 5h. In this figure, the outer rings are the control condition (-) and the inner rings the condition with 6.5 mM nitrate (+). Genera at <0.1% abundance are indicated as "Other" for clarity. The genera are sorted based on their maximum average abundance in one of the conditions from most to least abundant. Data for the inoculum and 9h can be found in Supplementary Figure 2.

To study the effect of nitrate supplementation, significant changes in genera between the nitrate and the control conditions at 5h or 9h were analyzed (see Supplementary Spreadsheet for all p-values). The lower abundance of *Veillonella* (a bacterium that uses lactate as carbon source) in the nitrate condition compared to the control condition was significant at 5h and 9h ($p<0.05$ and $p<0.01$, respectively, Figure 6) as well as the lower percentage of *Streptococcus* (a genus known to produce lactic acid) at 9h ($p<0.05$). The genus *Prevotella* at 5h was lower in the control condition (1.51% compared to 0.78% in the nitrate condition, $p<0.05$). No significant differences were observed between *Haemophilus and Gemella* in the two conditions. The dominant OTUs within each genera are listed in Supplementary Tables 2 and 3.

The 3.1× increase at 5h and 3.3× at 9h of the median of *Neisseria* in the nitrate condition compared to the control condition were significant (both $p<0.01$, Figure 6). Identified *Neisseria* OTUs included *N. flavescens*, *N. subflava*, *N. bacilliformis*, and *N. elongata*. *Rothia*, another nitrate-reducing genera dominated by an OTU classified as *R. aeria* or *R. dentocariosa*, was at low abundance (i.e., median in all conditions 0.14%, range 0.01-1.7%), but the median was 2.9× higher in the nitrate condition (0.35%) than in the control condition at 5h (0.12%, $p<0.01$). A third nitrate-reducing bacterium, *Kingella*, was present at low abundance but also at higher levels in the nitrate condition. However, the difference was not significant ($p=0.15$).

Other genera significantly lower in the nitrate condition were periodontitis and/or halitosis-associated *Porphyromonas* (including the OTU *P. endodontalis* or *oral taxon 285*), *Fusobacterium* (including *F. periodonticum* and *F. nucleatum*), *Leptotrichia* (including the OTU *L. wadei* or oral taxon 417, and *L. hongkongensis*) and *Avoprevotella* (including *A. rava* and *A. tannerae*) at 5 h ($p<0.05$), and the caries-associated *Oribacterium* (including *O. parvum* and *O. sinus*) at 9h ($p<0.05$). Decreases were also observed for the periodontitis-associated "red complex" bacteria *Tannerella* and *Treponema* (Figure 6), but these differences were not significant using Wilcoxon adjusted p-values.

When using ANCOM-II adjusted p-values (Supplementary Figure 3 and Supplementary Spreadsheet), the significant changes in genera between the control and nitrate conditions were consistent (i.e., genera that differed

significantly between the nitrate and control conditions at 5h and/or 9h using Wilcoxon adjusted p-values, still differed significantly at 5h and/or 9h using ANCOM-II adjusted p-values). However, several additional genera decreased significantly in the nitrate condition compared with the control condition when using ANCOM-II. These were *Tannerella* at 9h, and *Granulicatella*, *Atopobium*, *Actinomyces*, *Lachnoanaerobaculum* and *Dialister* at 5h.

In the nitrate condition, *Veillonella* and *Actinomyces* correlated negatively with pH at 9h (r=-0.77 and -0.72, respectively, both p<0.05, Supplementary Figure 4). In contrast, *Neisseria* correlated positively with pH at 9h in the nitrate condition (r=0.84, p<0.01), as well as at 5h in the control condition (r=0.75, p<0.05).

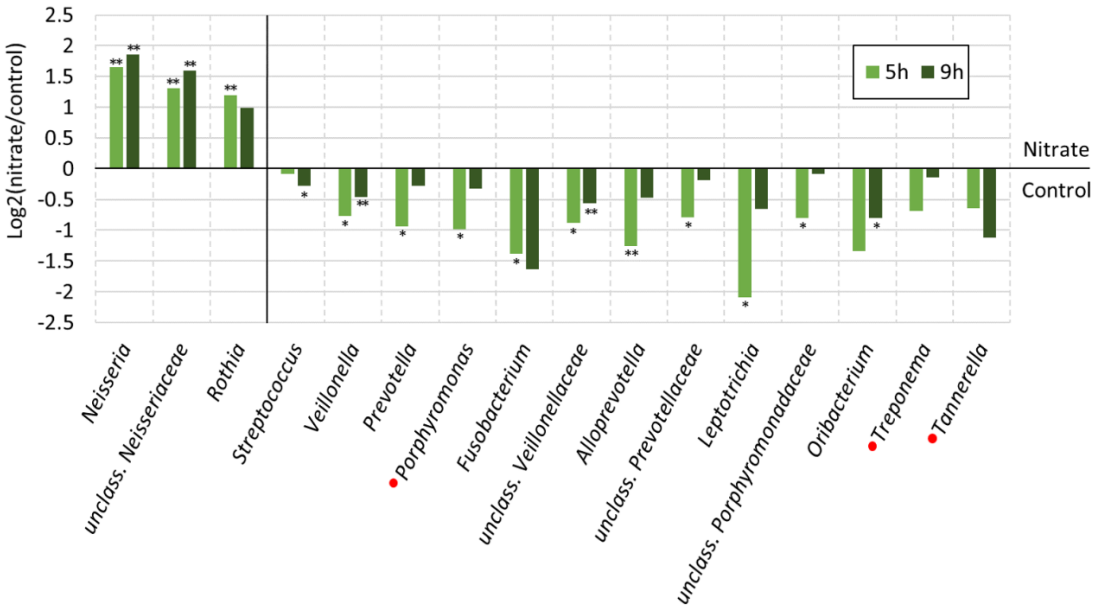


Figure 6: Changes in biofilm bacterial composition under nitrate conditions. Bar graphs show the log2 value of the ratio [average abundance nitrate condition]/[average abundance control condition] of 12 donors. Genera shown are those significantly different between the 6.5 mM nitrate and control conditions at 5h or 9h and, additionally, *Treponema* and *Tannerella* -two clinically relevant genera- were added. The genera that are higher in the nitrate condition are listed first and sorted by their highest average abundance in one of the conditions. After the vertical black stripe, the genera that are lower in the nitrate condition are listed, sorted by their highest average abundance in one of the conditions from highest to lowest (all taxa after unclass. *Veillonellaceae* had an average abundance of <0.5% in all conditions). Red circles are

placed before the genera of periodontitis-associated "red-complex" bacteria. unclass. = unclassified (only shown at family level); *adjusted $p<0.05$ and **adjusted $p<0.01$ between the control and nitrate conditions, according to a Wilcoxon test (Supplementary Spreadsheet).

1.5 Discussion

The salivary glands contain electrogenic sialin $2NO_3^-/H^+$ transporters, which after a nitrate-containing meal increase salivary nitrate to millimolar levels, resulting in elevated nitrate concentrations over many hours[112]. The data presented in the current manuscript indicate that nitrate supplementation at the physiological levels found in saliva is able to prevent or reduce bacterial dysbiosis and stimulate eubiosis by elevating health-associated genera and reducing the levels of disease-associated bacteria. In addition, our work provides some of the mechanisms underlying the potential beneficial effect of nitrate for oral health, including lactic acid usage during nitrite reduction to ammonium and denitrification (Figure7).

It is surprising that nitrate metabolism in the oral cavity has scarcely been studied as a potential factor influencing biofilm composition and activity, as nitrate is an important ecological factor influencing the composition and functioning of microbial communities in natural environments[49,127]. There is evidence suggesting that nitrate reduction is also relevant for the oral cavity, where nitrate appears to lead to health-associated changes in the oral microbiota. For instance, nitrate metabolism has been associated with lower caries abundance[62] and in a recent clinical study, gingival inflammation in patients with chronic gingivitis was significantly reduced after 14 days of nitrate intake in the form of lettuce juice[84].

Our data show that less lactate and more ammonium was produced after 5h and 9h of *in vitro* oral biofilm growth in the presence of 6.5 mM nitrate, and, accordingly, the pH was higher than in the control condition without nitrate (all $p<0.01$). Additionally, there was a strong negative correlation between lactate and ammonium produced after 9h. This supports the hypotheses by Li et al. (2007)[63] that lactic acid consumption by nitrate-reducing communities limit a drop in pH when carbohydrates are fermented. *In vivo* this could potentially

reduce the time that the dental tissue is under demineralizing pH[162]. In our study, we found that nitrate concentrations from 1 mM prevented salivary acidification due to glucose fermentation, while no additional pH buffering was observed for concentrations above 3.5 mM. Li et al.[63] only used a nitrate concentration of 1.5 mM in modified saliva samples and also observed that nitrate prevented a drop in pH due to sugar fermentation by oral microorganisms under anaerobic conditions, but not under aerobic conditions. In addition, Burleigh et al. (2020)[163] observed an increase in salivary pH after 7 days of nitrate-rich beetroot juice supplementation, which could be explained by the mechanisms detected in our *in vitro* study.

In our study, biofilm growth curves and protein levels were not significantly different between the control and the nitrate condition. Notably, biofilms grown with nitrate contained several times higher levels of *Neisseria* and *Rothia*, which are nitrate-reducing genera[113,164], already after a short period of 5h ($p<0.01$) and *Neisseria* remained significantly higher after 9h ($p<0.01$). This included an increase of total abundance of *Rothia mucilaginosa* and *Neisseria flavescens*, which was also observed *in vivo* by Velmurugan et al. (2016)[83] in saliva after 6 weeks of daily beetroot juice intake. Vanhatalo et al. (2018)[135] showed that 10 days of daily beetroot intake increased *Rothia* and *Neisseria*, while decreasing *Veillonella* and *Prevotella*, in saliva, which is in accordance with our results in newly formed *in vitro* oral biofilms. Putting these results together, it appears that species of *Rothia* and *Neisseria* have a selective advantage in the presence of nitrate.

In the majority of recent sequencing studies, different *Neisseria* and *Rothia* species have been associated with disease-free individuals. For example, *Rothia* spp. and *Neisseria* spp. were more abundant in subgingival plaque of periodontally healthy individuals compared to patients with periodontitis[30,165-167]. Apart from relative abundance, also the prevalence of *Rothia* and *Neisseria* is higher in healthy subgingival plaque compared to periodontitis samples[168]. Periodontitis is a chronic and destructive inflammation of the gingiva and can result from repeated or long-lasting episodes of gingivitis (i.e., reversible inflammation of the gingiva). In one study, the genus *Neisseria* correlated with anti-inflammatory mediators and was associated with a better recovery of the gingiva after experimental gingivitis[52]. In another study, *Rothia aeria* negatively

correlated with inflammatory cytokines IL-17 and TNF-α[169], whereas *Rothia dentocariosa* has been found to induce TNF-α production in a human cell line *in vitro*[170]. Given the high dynamic nature observed for many oral bacteria, nitrate-reducing isolates that show promising features as oral probiotics should be individually tested to confirm their systemic safety[171].

Additionally, both *Rothia*[27,172] and *Neisseria*[172,173] species were more abundant in supragingival plaque of caries-free individuals compared to individuals with active caries. In a recent study, *Rothia dentocariosa* was also found to be more abundant on the tongue of halitosis-free individuals compared to halitosis patients[174]. In conclusion, *Neisseria* and *Rothia* are associated with oral disease-free individuals and an increase in these genera can be considered a positive change in the microbiota related to general oral health (i.e., eubiosis).

The DNA of biofilms in our study was 21% higher in the nitrate condition compared to the control condition after 5h ($p<0.01$), but not significantly different after 9h. The small increase in DNA after 5h could have resulted from a higher number of cells from, e.g., *Rothia* and *Neisseria*. Alternatively, the higher DNA amount could be derived from a larger production of extracellular DNA, which has previously been shown to affect biofilm integrity and adhesion[175]. Given that extracellular DNA could affect 16S rRNA sequencing results, in future experiments the samples could be treated with DNAse prior to DNA isolation to remove the effect of this extracellular DNA on bacterial composition assessment. Additionally, possible differences in biofilm properties due to extracellular DNA should be tested.

Regarding disease-related bacterial composition, we observed a decrease in *Veillonella*, *Streptococcus* and *Oribacterium*, which are genera associated with lactate, acidification and caries[24,176,177], in the nitrate condition after 9h ($p<0.05$). Future studies performed with longer sequences should focus on species-level analyses, because even when a given genus is generally associated with disease (i.e., a consistent increase in disease is observed when compared with health in different studies), species[61,178] and probably even strains within species could be associated with health.

Another important observation in our experiments was that periodontal-disease associated *Porphyromonas*, *Fusobacterium*, *Prevotella, Leptotrichia* and *Alloprevotella* were significantly lower in biofilms grown with nitrate after 5h ($p<0.05$). *Porphyromonas*, *Fusobacterium,* and *Prevotella* contain species of the classic 'red and orange complexes' identified by Socransky et al., which are strongly associated with periodontitis[179]. These include *Fusobacterium periodonticum*, *Prevotella intermedia* and *Prevotella nigrescens* that were also identified in our study. Similarly, *Leptotrichia* has a strong association with periodontitis[180], while very recently it was confirmed that *Alloprevotella* is more abundant in disease as well[178]. Nitric oxide-releasing material and metal oxides have been shown to have antimicrobial activity against several periopathogenic species, including *Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum*[181]. Nitric oxide resulting from nitrate reduction could have killed slow-growing anaerobic bacteria in the inoculum, explaining their decrease after 5h.

It is interesting to note that *Porphyromonas*, *Fusobacterium*, *Leptotrichia* and *Prevotella* are also associated with halitosis - bad breath resulting from microbial production of volatile sulfur compounds (VSCs)[14]. It must be kept in mind that nitrate is used as a biological agent to treat malodour from sewer networks by limiting microbial VSCs production resulting from sulfate reduction[129]. As long as more energy-efficient denitrification takes place, sulfate reduction is inhibited and this could also be the case in the oral cavity, where sulfate reduction is associated to halitosis[130] (Figure 7). The effect of nitrate supplementation on sulfate reduction and halitosis should be tested in future clinical studies.

The metabolism of nitrite and the production of ammonium in our study indicate the presence of Dissimilatory Nitrate Reduction to Ammonium (DNRA) activity by oral species. The observation that nitrite correlated negatively with ammonium and pH at 5h (biofilms that metabolized all nitrite produced most ammonium and had the highest pH) further supports this. In the nitrate condition, the amount of ammonium after 9h was 4.75 mM higher than in the control condition. Stoichiometrically, this could account for 73.1% of the 6.5 mM added nitrate, while (part of) the other 26.9% of nitrate may have been denitrified into nitric oxide and other nitrogenous products. It is unlikely that nitrate was used in assimilatory pathways as low concentrations of ammonium, which were present in all cultures, inhibit nitrate assimilation[127,182].

Interestingly, several environmental conditions, including pH and the protein:carbohydrate ratio[183], direct the conversion of nitrate into ammonium or nitric oxide, and their role in regulating nitrate reduction in the oral cavity should be further investigated.

Arginine has received much attention as a prebiotic that is converted by certain oral microorganisms into ammonia (a weak base), increasing the local pH and thereby having an anti-caries effect *in vivo*[81,82]. Urease activity by some oral bacteria has also been shown to buffer acidic pH by ammonia production and was shown to correlate with caries status[184,185]. We provide evidence that nitrate stimulates ammonium (the ion of ammonia) production and lactic acid consumption and suggest that all three metabolic activities (i.e. arginine deaminase, urease and nitrate reduction) are considered when estimating the oral biofilm's pH buffering capacity.

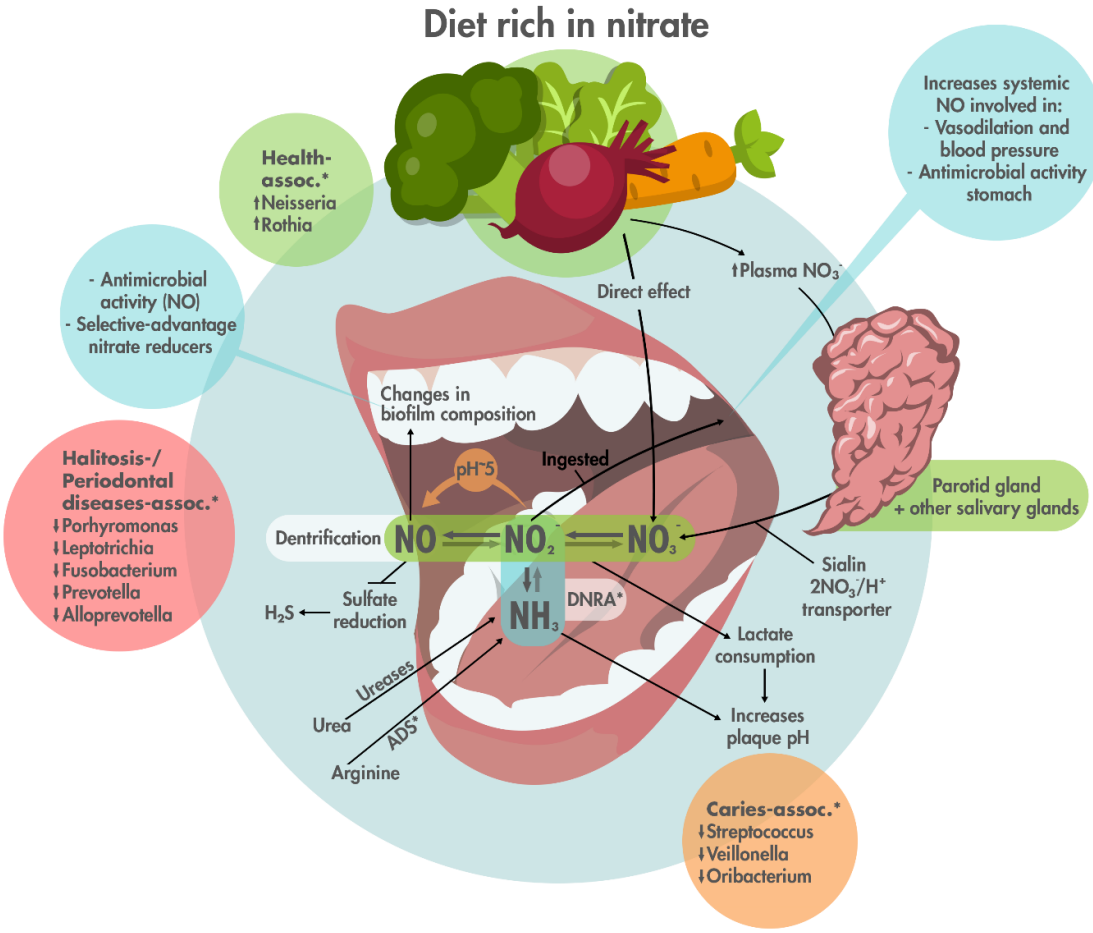


Figure 7: Overview of potential effects of nitrate inside the oral cavity. This graphical summary of the discussion is based on this study and current literature. Nitrate (NO_3^-) from food, such as vegetables (e.g., leafy greens, beetroots and carrots) and fruits, enters the blood stream and plasma nitrate is concentrated into saliva by sialin transporters in the salivary glands. There is also a direct effect of nitrate when the foods are chewed or pass through the mouth. Nitrate is reduced into nitrite (NO_2^-) and further to nitric oxide (NO) by denitrifying oral bacteria. Nitric oxide is an antimicrobial molecule that could limit the growth of certain species (e.g., periopathogenic species have been shown to be sensitive to NO) and thereby affecting the composition of oral biofilms. Additionally, at a pH of 5 and lower, acidic decomposition of nitrite to nitric oxide takes place (orange arrow), which could stimulate the antimicrobial effect when the pH drops due to sugar fermentation. Nitrite can also be reduced to ammonium (NH_4^+, the ion of ammonia, NH_3) by the bacterial Dissimilatory Nitrate Reduction to Ammonium (DNRA) pathway, increasing the local pH by proton consumption. The effect of nitrate on ammonium and ammonia levels should be further investigated in future studies.

Additionally, nitrate-reducing species can use lactic acid as electron donor and carbon source, which further prevents a drop in pH. Other bacterial enzymes that lead to ammonia production are ureases using urea as a substrate and arginine deiminase system (ADS) enzymes using arginine. Denitrification is more energy efficient than sulfate reduction and, therefore, the presence of nitrate should limit hydrogen sulfide (H_2S) production. In our study, we observed an increase of the health-associated genera *Neisseria* and *Rothia*, while caries-associated genera *Streptococcus*, *Veillonella* and *Oribacterium* decrease, as well as the anaerobic periodontal diseases- and halitosis-associated genera *Porphyromonas*, *Fusobacterium*, *Leptotrichia*, *Prevotella*, and *Alloprevotella*. It should be noted that these genera may also contain health-associated representatives. However, generally, the total abundance of these genera increases in the associated diseases. *assoc. = associated genera, ADS = arginine deiminase system

1.5.1 Nitrate: from disease-associated to neutral compound with health benefits

Nitrate has had a bad reputation for decades, because under certain conditions, its bacterial reduction product, nitrite, can react with other molecules and form potentially carcinogenic N-nitroso compounds (e.g., nitrosamine)[186,187]. This has been reported on processed meats, where nitrate salts are added as preservatives, resulting from bacterial and chemical reactions over time. However, humans obtain more than 80% of dietary nitrate from vegetables, which is a food group unequivocally associated with health benefits[122], longevity and lower prevalence of diseases[87], including cancer[89]. Anti-oxidants in fruits and vegetables prevent the formation of N-nitroso compounds from nitrite and stimulate the formation of nitric oxide[188,189]. In relation to this, different safety agencies stated that epidemiological studies do not suggest that nitrate intake from diet or drinking water is associated with increased cancer risk[93] (reviewed by Lundberg et al., 2018[122]). In contrast, evidence has accumulated that the oral microbiota-dependent increase of systemic nitric oxide resulting from dietary nitrate can have several beneficial cardio-metabolic effects[122].

The current acceptable daily intake (ADI) of nitrate is 3.7 mg/kg of body weight (222 mg for an adult of 60 kg). In recent clinical studies focusing on cardiovascular effects, which observed an increase in *Neisseria* and *Rothia* in saliva, high daily doses of nitrate were given to individuals in the form of beetroot juice (i.e., 372-770 mg per day, which is 1.7-3.5× the ADI) during

periods of 1-4 weeks[83,135,163]. In our study, we show that a single dose of 101 µg nitrate to obtain 6.5 mM in a volume of 250 µl was enough to increase these nitrate-reducing genera in a short period of 5 hours. To draw a parallel to the oral cavity, where volumes of around 0.5-1 ml of saliva are often found, topical doses far below the ADI would suffice to maintain a 6.5 mM concentration over time.

1.6 Conclusions

The results in this study showed that nitrate caused rapid structural and functional shifts in oral communities *in vitro* that would be of benefit to the human host. Based on our results, we conclude that nitrate could be an ecological factor stimulating health-associated oral genera, with the potential to decrease caries-, periodontitis- and halitosis- associated genera (Figure 7). Additionally, we conclude that nitrate metabolism provides resilience to acidification resulting from sugar metabolism, by increasing lactic acid consumption during ammonium production and denitrification, and future studies should test this possibility *in vivo*.

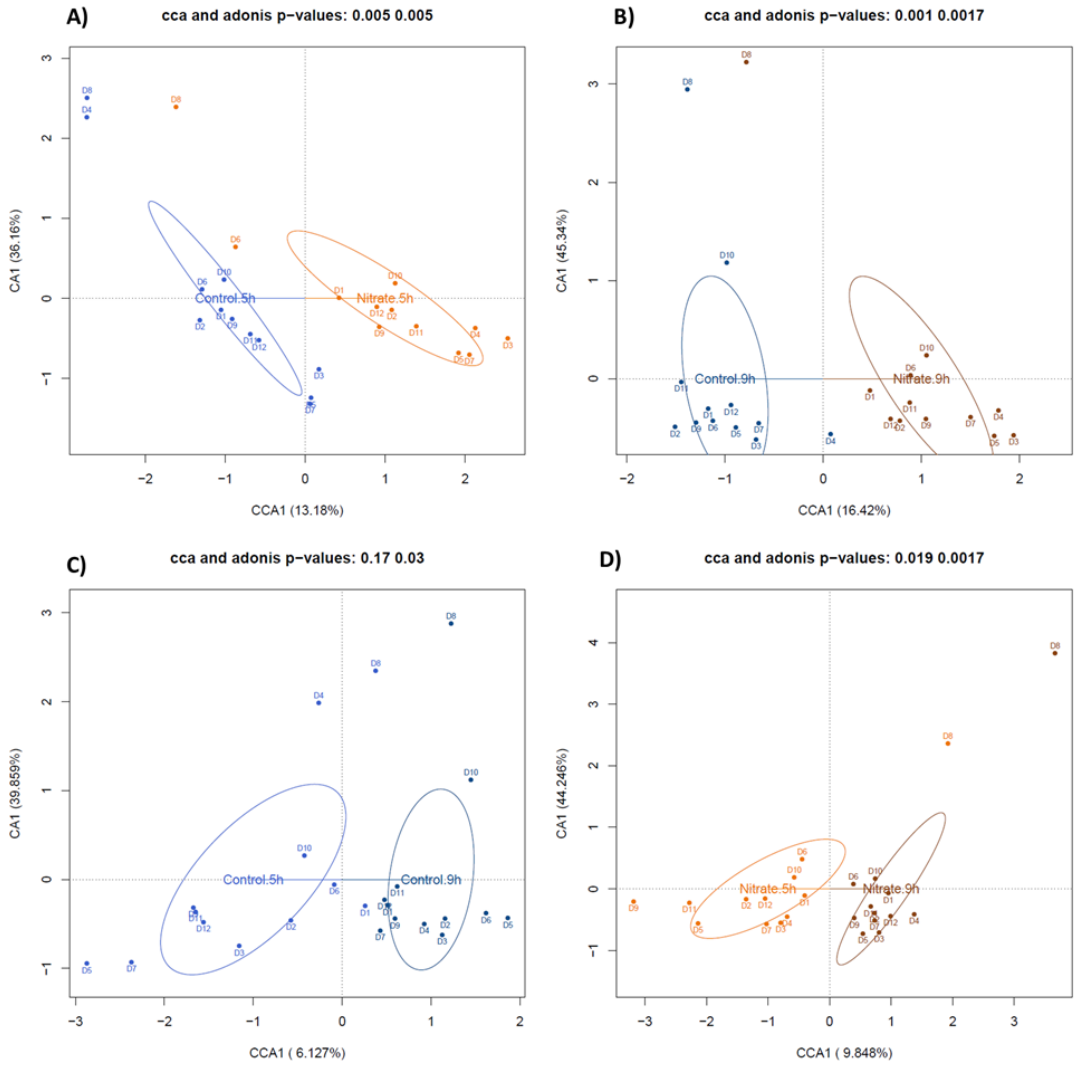
In biofilms grown with nitrate, *Veillonella* -a genus that uses lactate as carbon source- correlated negatively with pH and *Neisseria* positively. Due to the high *in vivo* prevalence of *Neisseria* and *Rothia* in different oral habitats, we argue that these genera could have essential roles in maintaining a healthy symbiotic relationship between the oral microbiota and the host by the reduction of salivary nitrate.

Taking into account our findings and other studies focusing on nitrate and oral health[62,63,84,136], we propose that nitrate is a health-associated molecule in the oral cavity. We therefore suggest that nitrate could be used as a prebiotic (e.g., vegetable extracts or nitrate combined with anti-oxidants), and nitrate-containing vegetables tested in dietary interventions, in order to stimulate eubiosis or reduce dysbiosis in the oral cavity. Representatives of *Neisseria*, *Rothia* and other nitrate-reducing genera (e.g. *Kingella*) have traditionally been associated with oral health in many studies and we propose that this is, in part, due to their capacity to reduce nitrate. We therefore suggest that certain nitrate-reducing strains could be used as probiotics to stimulate the benefits of nitrate metabolism. We hope that, although preliminary, our *in vitro* data stimulate

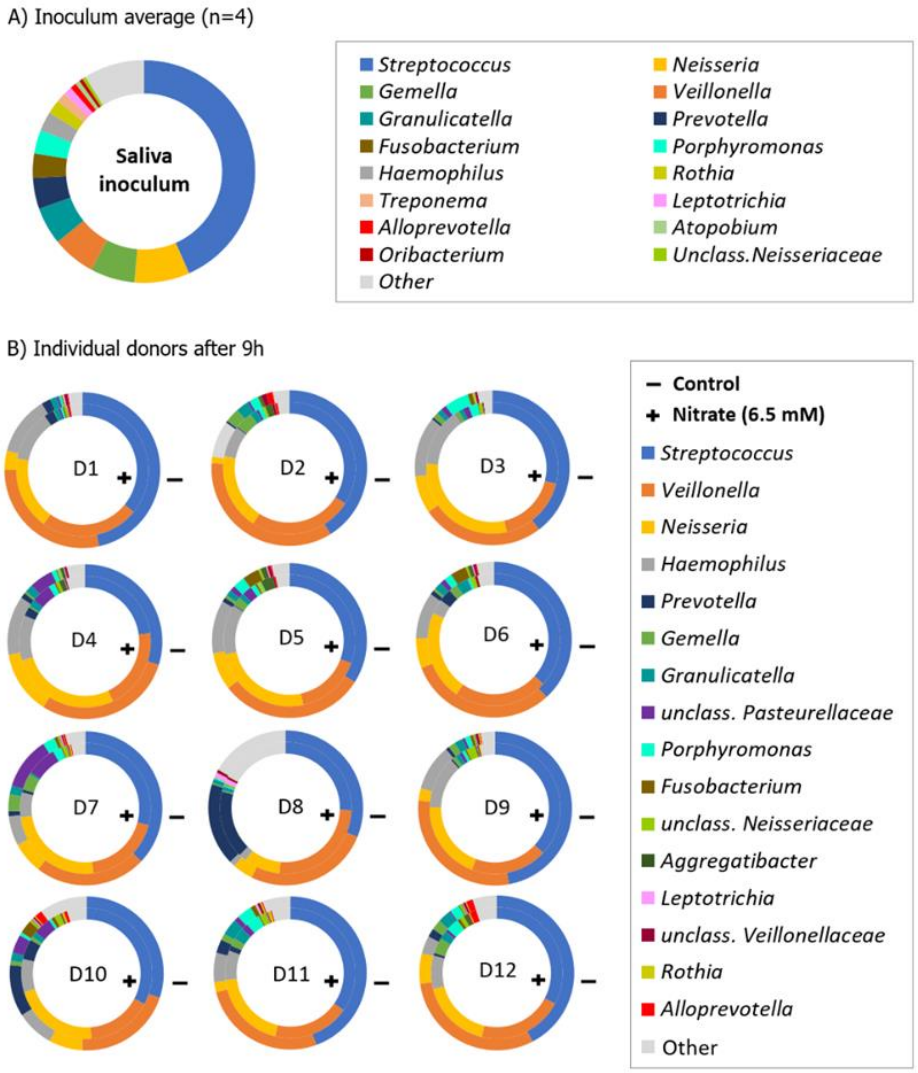
further research to test the potential effect of nitrate and nitrate-reducing bacteria on oral health in human subjects.

1.7 Supplementary figures and tables

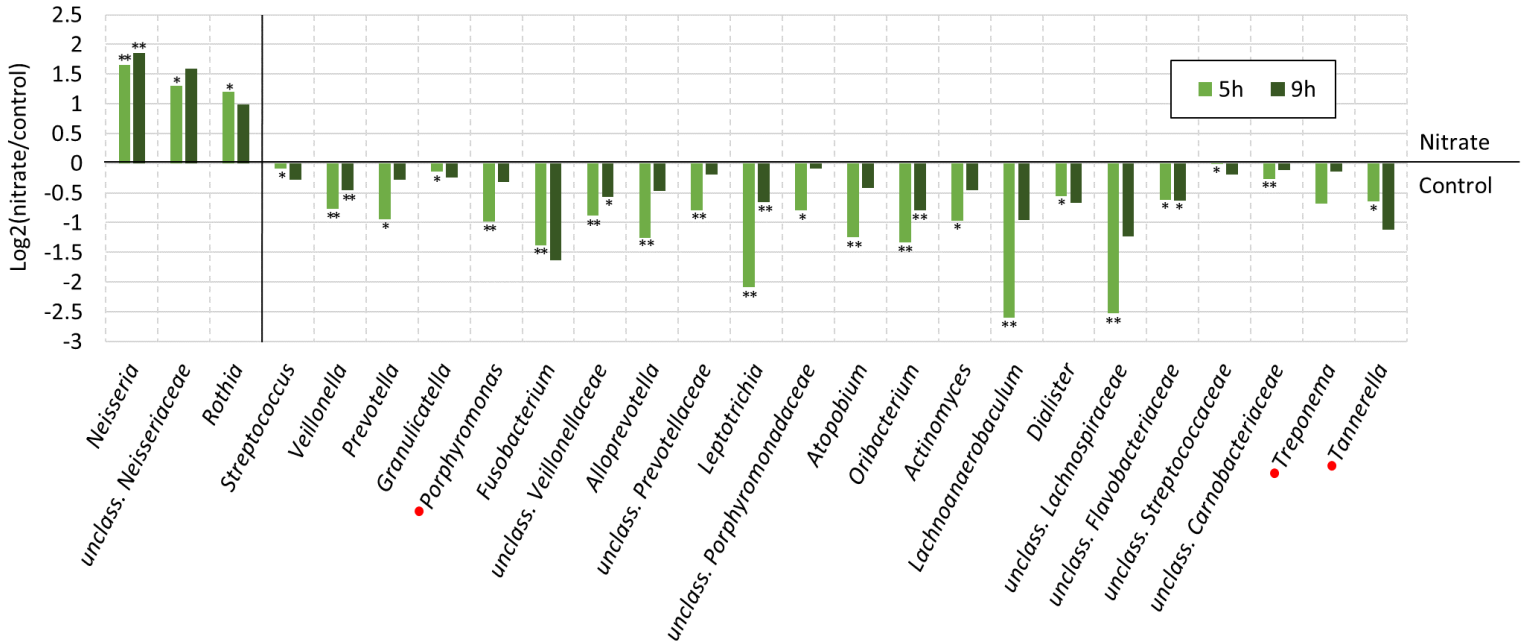
Supplementary figures



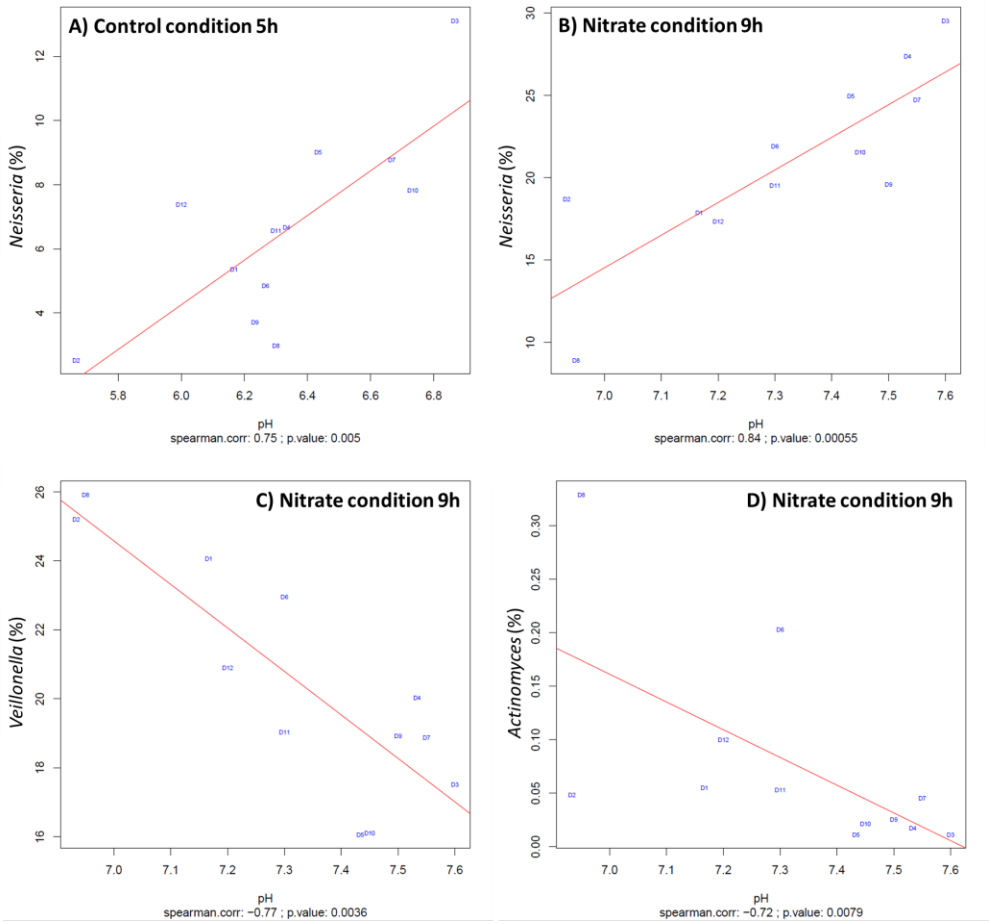
Supplementary Figure 1: Effect of nitrate on oral biofilm composition at 5h and 9h of *in vitro* growth (two-group comparisons). Both Adonis and CCA p-values are significant between the control and nitrate conditions at 5h (A) and 9h (B). There were also significant differences between 5h and 9h in the control condition (CCA p-value only, C) and nitrate condition (Adonis and CCA p-values, D).



Supplementary Figure 2: Bacterial composition in inoculum and oral biofilms as determined by 16S rDNA sequencing. A) Ring chart of the average relative abundance of genera in the saliva used as inoculum of donors 2, 3, 4 and 6 (n=4), which were randomly selected. B) Relative abundance of genera in biofilms of individual donors at 9h. In this figure, the outer rings are the control condition (-) and the inner rings the condition with 6.5 mM nitrate (+). Genera at <0.1% abundance are indicated as "Other" for clarity. The genera are sorted based on their maximum average abundance in one of the conditions from most to least abundant.



Supplementary Figure 3: Changes in biofilm bacterial composition under nitrate conditions (based on Standard Compositional Data Analysis, CODA). Bar graphs show the log2 value of the ratio [average abundance nitrate condition]/[average abundance control condition] of 12 donors. Genera shown are those significantly different between the 6.5 mM nitrate and control conditions at 5h or 9h and, additionally, *Treponema* - a clinically relevant genera- was added. The genera that are higher in the nitrate condition are listed first and sorted by their highest average abundance in one of the conditions. After the vertical black stripe, the genera that are lower in the nitrate condition are listed, sorted by their highest average abundance in one of the conditions from highest to lowest (all taxa after unclass. Veillonellaceae had an average abundance of <0.5% in all conditions). Red circles are placed before the genera of periodontitis-associated "red-complex" bacteria. unclass. = unclassified (only shown at family-level); *adjusted p≤0.05 and **adjusted p<0.01 between the control and nitrate conditions, according to an ANCOMII analysis (for the complete dataset, see Supplementary Spreadsheet).



Supplementary Figure 4: Significant correlations between genera and pH. All significant correlations (adjusted $p < 0.05$) between classified genera and pH are shown. A) Correlation between *Neisseria* and pH in the control condition at 5h. In the other sections, the correlations between *Neisseria* (B), Veillonella (C) and *Actinomyces* (D) are shown in the nitrate condition at 9h. In the graphs, unadjusted p-values are shown. The adjusted p-values for A, B, C and D were 0.025, 0.007, 0.016 and 0.017, respectively.

Supplementary tables

Supplementary table 1: correlations of physiological parameters between control and nitrate conditions after 5h and 9h (n = 12)

			Control condition					
			Ammonium		Lactate		pH	
			5h	9h	5h	9h	5h	9h
Nitrate condition	Nitrate[a]	5h	-0,717**	-0,710**	0.491	0,795***	-0.094	-0.140
	Nitrite[a]	5h	-0,641*	-0.535	0.401	0.500	-0.563	0.007
	Ammonium	5h	0,937***	0,923***	-0,615*	-0,790***	0.371	-0.021
		9h	0,811***	0,909***	-0,762***	-0,734**	0.427	0.077
	Lactate	5h	-0.545	-0,594*	0,776***	0,734**	-0.566	-0.503
		9h	-0,718**	-0,771***	0,883***	0,932***	-0.165	-0.277
	pH	5h	0.550	0.553	-0.424	-0.448	0,792***	0.172
		9h	-0.007	0.154	0.000	-0.084	0,776***	0.517

[a] After 9h, there was no nitrate and nitrite detected in supernatants of 12/12 and 9/12 donors, respectively.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.005$

1.8 Data availability

The FASTA sequences of this study have been uploaded into MG-RAST with project number mgp97421. Kindly find the Supplementary Spreadsheet by opening Supplementary Information 2 (https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-020-69931-x/MediaObjects/41598_2020_69931_MOESM2_ESM.xlsx) and Supplementary tables 2A, 2B and 3 in Supplementary Information 1 (https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-020-69931-x/MediaObjects/41598_2020_69931_MOESM1_ESM.pdf) of the Scientific Reports website.

Chapter 2.

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2. Isolation and characterization of nitrate-reducing bacteria as potential probiotics for oral and systemic health

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2.1 Abstract

Recent evidence indicates that the reduction of salivary nitrate by oral bacteria can contribute to prevent oral diseases, as well as increase systemic nitric oxide levels that can improve conditions such as hypertension and diabetes. The objective of the current manuscript was to isolate nitrate-reducing bacteria from the oral cavity of healthy donors and test their *in vitro* probiotic potential to increase the nitrate-reduction capacity (NRC) of oral communities. Sixty-two isolates were obtained from five different donors of which 53 were confirmed to be nitrate-reducers. Ten isolates were selected based on high NRC as well as high growth rates and low acidogenicity, all being *Rothia* species. The genomes of these ten isolates confirmed the presence of nitrate- and nitrite reductase genes, as well as lactate/lactic acid utilization genes, and the absence of antimicrobial resistance, mobile genetic elements and virulence genes. The pH at which most nitrate was reduced differed between strains. However, acidic pH 6 always stimulated the reduction of nitrite compared to neutral pH 7 or slightly alkaline pH 7.5 ($p<0.01$). We tested the effect of six out of 10 isolates on *in vitro* oral biofilm development in the presence or absence of 6.5 mM nitrate. The integration of the isolates into *in vitro* communities was confirmed by Illumina sequencing. The NRC of the bacterial communities increased when adding the isolates compared to controls without isolates ($p<0.05$). When adding nitrate (prebiotic treatment) or isolates in combination with nitrate (symbiotic treatment), a smaller decrease in pH derived from sugar metabolism was observed ($p<0.05$), which for some symbiotic combinations appeared to be due to lactic acid consumption. Interestingly, there was a strong correlation between

the NRC of oral communities and ammonium production even in the absence of nitrate (R=0.814, p<0.01), which indicates that bacteria involved in these processes are related. As observed in our study, individuals differ in their NRC. Thus, some may have direct benefits from nitrate as a prebiotic as their microbiota naturally reduces significant amounts, while others may benefit more from a symbiotic combination (nitrate + nitrate-reducing probiotic). Future clinical studies should test the effects of these treatments on oral and systemic health.

2.2 Introduction

The salivary glands contain electrogenic sialin $2NO_3^-/H^+$ transporters to concentrate plasma nitrate into the saliva[112]. This leads to salivary nitrate concentrations that are around ten times higher than plasma during fasting (100-500 µM compared to 10-50 µM), which can go up to 5-8 mM after a nitrate-containing meal (reviewed by[25] and[131]). Foods that naturally contain significant amounts of nitrate are fruits and vegetables, which are both unequivocally associated with health benefits. It is estimated that we obtain more than 80% of nitrate from vegetables[122].

Nitrate-reducing oral bacteria, including representatives of *Neisseria*, *Rothia*, *Veillonella*, *Actinomyces*, *Corynebacterium*, *Haemophilus* and *Kingella* reduce nitrate to nitrite[113,114,164]. Human cells cannot reduce nitrate, but there are several enzymatic and non-enzymatic processes that convert nitrite into nitric oxide[25]. For example, in the acidic gastric juice, nitrite is decomposed to nitrogen oxides, such as nitric oxide (NO), which is essential for the antimicrobial activity of the stomach[131]. It should be noted that anti-oxidants and polyphenols in vegetables and fruits prevent the formation of carcinogenic N-nitroso compounds from nitrite, while stimulating nitric oxide production[188,189].

In the blood vessels, nitrite reacts with hemoglobin to form nitric oxide, which apart from being antimicrobial, is also an important vasodilator of the human body [25]. It was shown that an antiseptic mouthwash acutely increases blood pressure by disrupting nitrate reduction by oral bacteria[111]. Nitrate-rich supplements, in turn, stimulate nitrate reduction by the oral microbiota resulting in a lowering of blood pressure[135]. This pathway (i.e., the nitrate-

nitrite-nitric oxide pathway) can also increase sport performance and has apparent antidiabetic effects[122]. In light of this, antiseptic mouthwash has shown to interfere with post-exercise hypotension[133] and over-the-counter mouthwash correlated with diabetes and pre-diabetes development[123].

Nitrate in the form of lettuce juice has also been shown to reduce gingival inflammation compared to a placebo (nitrate-depleted lettuce juice)[84]. Additionally, nitrate prevented acidification by oral bacteria[63] and the nitrate reduction capacity (NRC) of the oral microbiota correlated negatively with caries abundance[62]. Recently, Rosier et al. (2020) have proposed that nitrate reduction stimulates eubiosis (i.e., an increase in health-associated species and functions) of the oral microbiota[190]. Specifically, nitrate reduction prevented acidification and the resulting overgrowth of cariogenic bacteria by increasing lactic acid consumption during denitrification and ammonium production. Additionally, nitrate increased health-associated nitrate-reducing genera, while decreasing strictly anaerobic periodontal diseases- and halitosis-associated bacteria, which could be sensitive to oxidative stress caused by nitric oxide. Nitrate reducing bacteria, such as representatives of *Neisseria*, *Rothia*, *Actinomyces* and *Kingella* have been associated to oral health in many 16S rRNA sequencing studies and this could be related with their capacity to reduce nitrate[136,190,191]. In conclusion, current data suggest that nitrate-reduction of the oral microbiota contributes to a healthy host physiology and appears to stimulate oral health.

The amount of nitrate-reducing species varies among individuals and, accordingly, the NRC as well[192]. Individuals with low baseline levels of nitrate-reducing species could use nitrate as a prebiotic to increase the levels of these bacteria over time. For example, after 1-4 weeks of beetroot consumption (a vegetable with high nitrate levels), the salivary levels of *Neisseria* and *Rothia* increased significantly[83,135]. Alternatively, in individuals lacking nitrate-reducing species, a direct increase could be achieved by the addition of nitrate-reducing probiotics. In a seminal study, Doel et al. isolated 99 oral bacteria that produced nitrite in the presence of nitrate under anaerobic conditions and 33 under aerobic conditions, but did not further test the effects of these isolates on oral communities[113]. Apart from their ability to produce nitrite, nitric oxide or ammonium, oral probiotics should not be acidogenic (a feature associated with

dental caries) and from a technological point of view should ideally be capable of fast aerobic growth to enable large-scale production[193].

The aim of our current study was therefore to isolate nitrate-reducing oral strains under aerobic conditions and make a selection of isolates that were most suitable from a biomedical and industrial point of view. To achieve this, a protocol was applied to obtain isolates with a high NRC, fast growth rate and low acidogenicity. Additionally, their genomes were sequenced and analyzed for functional predictions and for the detection of potentially harmful genes. To test our hypothesis that oral communities could benefit from nitrate-reducing isolates, six selected isolates were added to *in vitro* oral biofilms grown from saliva of different individuals that varied in NRC. The NRC was determined and changes in nitrate-related metabolism (e.g., ammonium and lactate production) monitored. Oral biofilm colonization was tested by 16S rRNA sequencing. All experimental data were used to assess the *in vitro* probiotic potential of nitrate-reducing oral bacteria as a first step to evaluate their possible use for oral and systemic health.

2.3 Materials and methods

2.3.1 Donor selection and sample procedure

For probiotic isolation, five young adults (1 male, 4 female, age 23-32) were selected with all teeth and good oral health, which was assessed by an experienced dentist. Individuals were excluded if they showed bleeding on probing or a periodontal pocket below 3 mm; a cavitated lesion or filling in any tooth surfaces. The absence of nitrate reduction capacity, as determined by lack of salivary nitrite, was as an exclusion criterion (the salivary nitrite and pH measured in the morning before breakfast for all donors are shown in Supplementary Table 1). All individuals had a healthy blood pressure (i.e., systolic between 90-120 mmHg, and diastolic between 60-80 mmHg), which was measured with an Automatic Blood Pressure Monitor Model M6 Comfort IT after sitting down in a quiet room for 15 minutes (OMRON Healthcare Europe B.V., Hoofddorp, the Netherlands). Plaque and tongue coating samples were collected by the dentist following Simon-Soro et al. (2013) and resuspended in 1 mL of PBS[194].

For saliva donor selection, individuals were recruited at the FISABIO institute if they reported not to have active caries during their last dental visit, nor any history of periodontitis. Nine individuals (3 male, 6 female, age 23-45) were selected and asked to donate saliva, which was used for *in vitro* oral community growth with and without nitrate-reducing isolates. Unstimulated saliva was collected by drooling in a sterile tube in a quiet room in the morning, at least half an hour after eating or drinking. Donors were instructed to have a normal breakfast and abstain from oral hygiene before saliva collection.

The fresh unstimulated saliva was always directly used in the experiments or kept at 4ºC for less than 1 h before usage. All donors signed an informed consent form prior to sample collection and the protocol was approved by the Ethical Committee of DGSP-FISABIO (Valencian Health Authority) with code 27-05-2016. This study was carried out according to the relevant guidelines and regulations of the Declaration of Helsinki.

2.3.2 Isolation of nitrate-reducing bacteria

Plaque or tongue samples in PBS were diluted 10^2 to 10^7 times and plated on Brain Heart Infusion (BHI) 1.4% agar plates (Merck Millipore, Burlington, Massachusetts, United States). Plates were incubated at 37ºC under aerobic conditions during 2 days to obtain separated colonies in some of the dilutions. A protocol adapted from Doel et al., 2005 was employed to detect nitrate-reducing activity by individual colonies[113]. This protocol consists of a double agar overlay method based on the Griess reaction that stains nitrite. Briefly, a plate with separated colonies grown from plaque or tongue samples was overlaid with 10 mL of 2.5% w/v agar with 1 mM sodium nitrate ($NaNO_3$, Sigma-Aldrich, St. Louis, Missouri, US) and incubated at 37ºC for 10 min in which nitrate-reducing colonies would produce nitrite. Then, the first layer was overlaid with 10 mL 2.5% w/v agar containing the Griess reagents and incubated at room temperature for 10 min. Colonies with nitrate-reducing capability produced a red color due to the presence of nitrite (Figure 1). These colonies were then transferred to new BHI agar plates and incubated during 2 more days at 37ºC. The nitrite-producing capability of the isolates was confirmed by repeating the double overlaid agar method for each isolate. Subsequently, one colony was passed to 5 mL of liquid BHI and incubated aerobically for 2 days at 37ºC. After that, 0.5 ml of the medium was used to create a glycerol stock of each isolate for

future experiments. The rest of the cells were resuspended in PBS and used for DNA extraction and sequencing.

2.3.3 Nitrate reduction screenings of bacterial isolates

To evaluate the nitrate-related metabolism of the isolates, the concentrations of nitrate, nitrite, and ammonium, as well as pH levels, were measured in spent medium. Isolates were incubated in 5 mL BHI liquid medium overnight at 37°C. The next day, isolates were diluted in BHI to an OD of 0.01 and a final concentration of 6.5 mM nitrate, which is within the physiological range of salivary nitrate after a nitrate-containing meal (i.e., 5-8 mM)[25]. The tubes were then incubated for 7 hours and 1 mL was taken at 4 and 7 hours, after vortexing and, from this volume, 0.5 ml was used to measure the OD and 0.5 ml for the other measurements. A similar experiment was performed with 10 isolates selected as probiotic candidates, which were grown for 5h in three types of buffered medium (100 mM MES, pH 6.0; 100 mM HEPES, pH 7.0; 100 mM HEPES pH 7.5, all Sigma-Aldrich) with 6.5 mM nitrate to keep a stable pH and evaluate the effect of different pH levels on the NRC of those isolates. All samples were frozen at -20°C before analysis of the supernatants.

2.3.4 Effect of isolates on *in vitro* biofilms

Six out of 10 selected nitrate-reducing isolates were studied *in vitro* to define their effect when added to an oral microcosm community. These isolates were tested by growing them with saliva of nine different donors in 96-wells plates in which oral communities form biofilms on the bottom of wells[141]. For each experiment, there were 4 conditions: control, nitrate (final concentration: 6.5 mM nitrate), control with isolate, and nitrate with isolate. For all samples, prepared in duplicate, 100 µl of BHI (with 0.05 mg/L haemin, 0.005 mg/L menadione and 0.2 mM vitamin K) were added to each well. Then, 100 µL of saliva (or BHI for negative controls) was added and, for the nitrate conditions, 10 µL of nitrate solution 162.5 mM was added (or 10 µL of BHI for control conditions). Before being added to the 96-well plate, the isolates were grown for 24h. Then, 40 µL isolate in BHI solution with OD 1.5 was added (or 40 µL of BHI in conditions without isolates) to each well. The final concentration of nitrate was 6.5 mM and the starting OD of the isolate was 0.24. The 96-well plate was sealed to stimulate anaerobic conditions by preventing new oxygen from entering the wells, and incubated during 7 h at 37°C. After that, the supernatant

was collected and stored at -20ºC until measurements were performed. The remaining biofilms were resuspended in PBS for DNA isolation.

2.3.5 DNA isolation for sequencing

DNA was extracted from pure cultures of the 53 confirmed nitrate-reducing isolates and also from the *in vitro* communities grown with isolates of two donors (D6 and D11). Pure cultures of isolates grown for 48h in 4.5 ml BHI were centrifuged (15 min at 4000 rpm) and the pellet resuspended in 100 µl PBS. After supernatant removal, duplicates of *in vitro* communities were resuspended together in 100 µl PBS and disaggregated for 30 s in a sonicator bath (model VCI-50, Raypa, Barcelona, Spain) at low ultrasound intensity. Total DNA was extracted with the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA), following the manufacturer's instructions, with the addition of lysozyme[27]. DNA was resuspended in 30 µl elution buffer and frozen at -20ºC until further analysis.

2.3.6 Taxonomic classification of nitrate-reducing isolates

For the taxonomic classification of the isolates, concentrations of DNA isolated from pure cultures were measured using a NanoDrop 1000 spectrophotometer (ThermoScientific, Waltham, Massachusetts, United States). A PCR was performed to amplify the 16S rRNA gene of each isolate, using universal primers 8-F and 785-R for the 16S rRNA gene, comprising the hypervariable regions V1-V2-V3-V4. The PCR products were then purified using flat 96 well filter plates (NucleoFast 96 PCR; Macherey-Nagel, Düren, Germany) and sequenced at both ends by Sanger technology at the Sequencing Unit of the University of Valencia (Valencia, Spain). To taxonomically assign the isolates, the sequences were compared by BLASTn[195] against 16S ribosomal RNA sequences at NCBI nr database. Species assignment was confirmed by Average Nucleotide Identity (ANI) values using JSpeciesWS software[196].

2.3.7 Genome sequencing

Illumina libraries for all 10 selected isolates were generated using the Illumina XT Nextera library prep kit (catalog number FC-131-1024) starting from 0.2 ng/ul of purified gDNA measured by a Qubit double-stranded DNA (dsDNA) high-sensitivity assay kit (catalog number Q32851). Libraries were sequenced

using a 2× 150-bp paired-end run MiSeq reagent kit v2 (catalog number MS-102-2002) on an Illumina MiSeq sequencer.

Oxford Nanopore libraries were obtained from the same DNA extracted samples following the manufacturer's standard protocol. Nanopore libraries were indexed and sequenced using type R9.4.1 (catalog number FLO-MIN106D) in a ONT MinION sequencer for 48h. Both sequencing approaches were performed at the Sequencing Service of FISABIO-Public Health (Valencia, Spain).

Long ONT fast5 reads were base-called and transformed into fastq files by ONT Albacore Sequencing Pipeline Software[197] and generated approximately four billion total bases in more than 850,000 reads (fastq-stats version 1.01, http://expressionanalysis.github.io/ea-utils). These ONT long reads as well as the Illumina short reads were quality filtered and trimmed using Prinseq-lite[198]. Long ONT reads were assembled with Canu v1.8 using the nanopore preset parameters[199] into one single contig per genome. Their errors were corrected using Illumina quality-filtered and trimmed reads with Pilon software v1.23[200]. After three rounds of corrections, genomes were annotated by Prokka v1.13.3[201] and the downstream analysis was performed with these ten annotated genomes.

2.3.8 Genomic analysis

In order to identify the presence of possible mobile genetic elements (MGEs), the annotated genomes were compared against the latest version of the ACLAME database[202] where different kinds of MGEs are collected and classified at gene and protein levels. Sequences were compared by similarity using the program BLASTx[203] identifying potential hits after filtering with the following criteria: minimum sequence identity higher than 80% of the gene length and coverage greater than 50%.

Potential antibiotic resistance genes (ARGs) were searched in the latest version of CARD database[204]. The detection of possible genes conferring pathogenicity was performed using the latest version of the virulence factors database (VFDB) which currently contains DNA sequences from 1,067 virulence factors from 951 bacterial strains having 32.252 virulence factor-related non-redundant genes information[205]. Sequences were compared by similarity against both databases

using the program BLASTn[195], identifying potential hits with E-value $<10^{-5}$, sequence identity >80% and >50% sequence length as thresholds.

2.3.9 Determination of bacterial composition of *in vitro* communities from two donors

From the *in vitro* biofilms of donors 6 and 11, DNA was isolated as described in chapter 1 and 16S rRNA Illumina sequencing and data processing as described in chapter 3.

2.3.10 Supernatant analysis: nitrate, nitrite, ammonium, lactate and pH measurements

Nitrate, nitrite, lactate and pH were measured in supernatants with a Reflectoquant (Merck Millipore, Burlington, Massachusetts, US) reflectometer. This method is based on the intensity of reflected light by two reactive pads on test trips that change in color intensity based on the concentration of a specific substance [148]. The test strips (Reflectoquant, Merck Millipore) for pH had a range from pH 4-9, the strips for nitrate a range of 3-90 mg/l, the strips for nitrite a range of 0.5-25 mg/l and the strips for lactate a range of 3-60 mg/l. A method was used based on Helmke et al. (2009) [150] and Ferrer et al. (2020)[149] as described by Rosier et al. (2020)[190]. The concentration of ammonium in supernatants was measured spectrophotometrically by the Nessler Method[206]. Accuracy of all procedures was confirmed by using standard solutions with known concentrations of the different compounds.

2.3.11 Statistical analysis

Statistical analysis was performed with SPSS 25 UK software (SPSS, Inc.). A Mann-Whitney U Test was applied to compare parameters between different (groups of) species. The Wilcoxon test was used to compare different conditions and the Spearman's rank correlation coefficient was calculated for different parameters. Significant changes ($p < 0.05$) and trends ($p < 0.1$) were presented.

2.4 Results

2.4.1 Isolation of nitrate-reducing bacteria and testing their nitrate-reduction capacity

Tongue and dental plaque samples were plated from 5 different healthy donors (D1-D5). Potential nitrate-reducing colonies were detected by a red tone, produced by the Griess reaction resulting from nitrite production (Figure 1). In total, 33 nitrite-producing isolates were obtained from tongue (T) samples and 29 from dental plaque (P). Most isolates (74%) were obtained from two donors (D1 and D4, Supplementary Table 1).

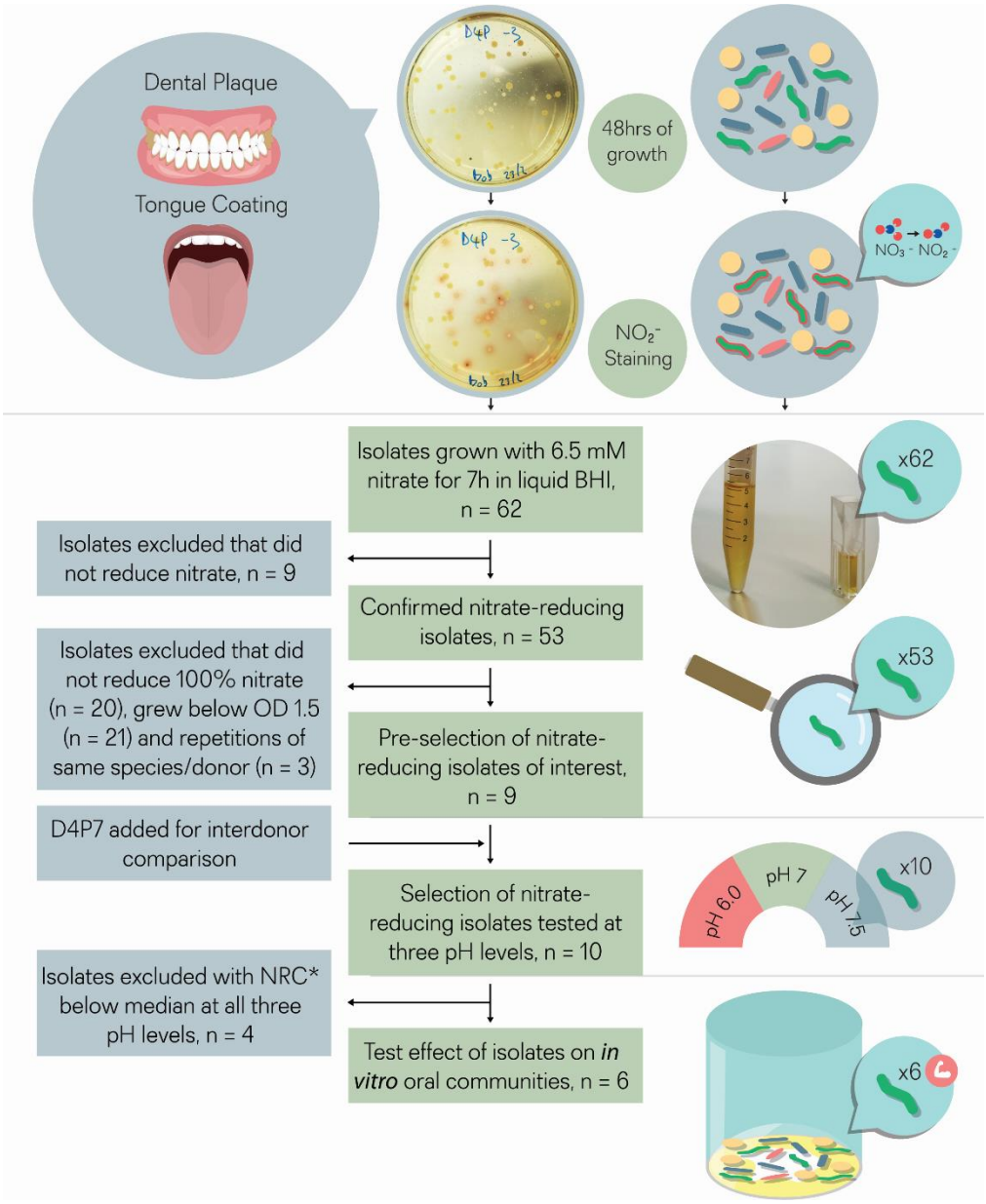


Figure 1: Isolate selection overview. Tongue coating and/or dental plaque samples were obtained from 5 different donors. Sixty-two colonies that produced a red color after adding Griess reagent, which stains nitrite, were isolated. The 62 isolates were incubated with 6.5 mM nitrate for 7h for double confirmation. Nine out of 62 isolates did not reduce nitrate and were excluded, while the other 53 isolates did reduce (part of) the nitrate and were considered as confirmed cases. From these 53 isolates, nine isolates were selected that reduced all nitrate and grew well under aerobic conditions (OD >1.5), a relevant feature for future large-scale production. One isolate (D4P7, *R. dentocariosa*)

was added to compare nitrate reducing capacity with the three selected *R. dentocariosa* isolates between different donors. None of the 10 selected isolates acidified the pH of the glucose-containing medium (starting pH 7.3) below pH 6.8 after 7h, which indicated that they were suitable probiotics from a caries point of view. The nitrate-reduction capacity of the final selection of 10 isolates was tested at three different pH levels (pH 6.5, pH 7 and pH 7.5) in buffered medium. The six isolates that reduced the nitrate-best at different pH levels were added to oral communities (from saliva) to test their effect on *in vitro* oral biofilm metabolism. *NRC: nitrate-reduction capacity

In an initial screening to quantify NRC, all 62 isolates were incubated with 6.5 mM nitrate during 4 and 7 hours under aerobic conditions (Figure 2, Table 1). Nine isolates, including strains of *Streptococcus salivarius, S. cristatus* and *S. mitis*, did not reduce any of the nitrate and were excluded. The other 53 confirmed nitrate-reducing isolates were mostly classified as *Rothia* (i.e., 23× *R. mucilaginosa*, 21× *R. dentocariosa* and 4× *R. aeria*) and five as *Actinomyces* (3x *Actinomyces viscosus* and 2× *A. oris*, Table 1). All *R. mucilaginosa* isolates originated from the tongue, while all *R. dentocariosa* and *R. aeria* isolates were obtained from dental plaque (except for one *R. dentocariosa* isolate, D3T1, from the tongue). After 7h, the average nitrite detected ranged from 1.20-10.39 mM (average 6.15 mM, SD 2.17 mM, Supplementary Table 2), suggesting that some bacteria produced (part of the) nitrite by other pathways than nitrate reduction.

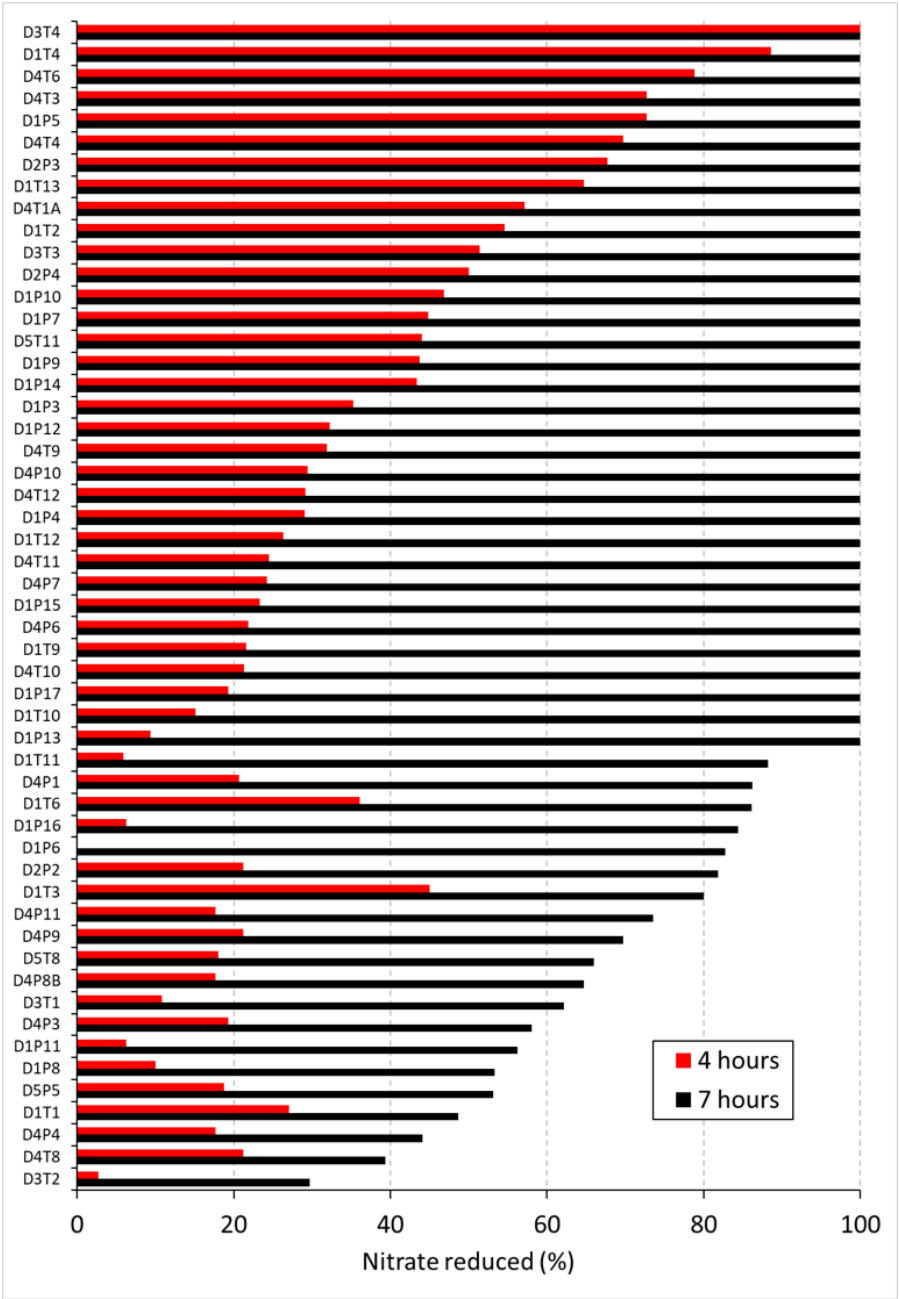


Figure 2: Nitrate Reduction Capacity of 53 isolates (1st screening). Bars show the percentage of nitrate reduced by 53 isolates after 4 hours (red bars) and 7 (black bars) hours of incubation at 37 °C under aerobic conditions with starting OD 0.01. Values represent the percentage of initially added nitrate (6.5 mM) that had been used up after 4h or 7h of incubation. Codes of the 53 isolates are shown on the right, where D relates to the donor, T refers to tongue coating and P to dental plaque samples.

Table 1: Confirmed nitrate-reducing isolates sorted by % nitrate reduced and optical density (OD) at 7h

Isolate	Species (16S BLAST)	Nitrate reduced (%)		OD		pH spent med.*[2]	
		4h	7h	4h	7h	4h	7h
D1P10*	*Rothia dentocariosa* ATCC17931	46.88	100.00	0.77	2.56	7.10	6.90
D3T4*	*Rothia mucilaginosa* DSM20746	100.00	100.00	1.00	2.16	7.10	6.80
D1P7*	*Rothia aeria* A1-17B	44.83	100.00	0.64	2.00	7.30	7.00
D4T4*	*Rothia mucilaginosa* DSM20746	69.70	100.00	0.65	1.97	7.20	6.80
D4T3	*Rothia mucilaginosa* DSM20746	72.73	100.00	0.69	1.96	7.20	6.70
D1P17*	*Rothia dentocariosa* ATCC17931	19.35	100.00	0.54	1.88	7.20	7.10
D4T6*	*Rothia mucilaginosa* DSM20746	78.79	100.00	0.79	1.87	7.20	6.80
D1P9	*Rothia dentocariosa* ATCC17931	43.75	100.00	0.57	1.76	7.20	7.20
D1P15*	*Rothia dentocariosa* ATCC17931	23.33	100.00	0.41	1.63	7.25	7.10
D4T9*	*Rothia mucilaginosa* DSM20746	31.91	100.00	0.57	1.60	7.20	7.10
D1P14	*Rothia aeria* A1-17B	43.33	100.00	0.48	1.56	7.30	7.10
D5T11*	*Rothia mucilaginosa* DSM20746	44.00	100.00	0.59	1.56	7.25	7.10
D1P12	*Rothia aeria* A1-17B	32.26	100.00	0.44	1.42	7.30	7.10
D1T4	*Rothia mucilaginosa* DSM20746	88.57	100.00	0.65	1.38	7.00	6.80
D1T13	*Rothia mucilaginosa* DSM20746	64.71	100.00	0.68	1.34	7.10	6.70
D4T12	*Rothia mucilaginosa* DSM20746	29.17	100.00	0.49	1.27	7.20	7.10
D1P13	*Rothia dentocariosa* ATCC17931	9.38	100.00	0.45	1.27	7.30	7.20
D1T9	*Rothia mucilaginosa* DSM20746	21.62	100.00	0.44	1.22	7.10	6.90
D1T12	*Rothia mucilaginosa* DSM20746	26.32	100.00	0.35	1.12	7.20	7.20
D4T10	*Rothia mucilaginosa* DSM20746	21.28	100.00	0.50	1.07	7.20	7.10
D4T1A	*Rothia mucilaginosa* DSM20746	57.14	100.00	0.44	1.04	7.20	7.00
D1T10	*Rothia mucilaginosa* DSM20746	15.15	100.00	0.29	1.03	7.30	7.10
D4T11	*Rothia mucilaginosa* DSM20746	24.49	100.00	0.41	0.96	7.30	7.10
D3T3	*Rothia mucilaginosa* DSM20746	51.35	100.00	0.33	0.85	7.20	7.00
D4P10	*Rothia dentocariosa* ATCC17931	29.41	100.00	0.35	0.81	7.10	7.00
D4P7*	*Rothia dentocariosa* ATCC17931	24.24	100.00	0.26	0.73	7.20	7.10
D4P6	*Rothia dentocariosa* ATCC17931	21.88	100.00	0.23	0.67	7.30	7.10
D2P4	*Rothia dentocariosa* ATCC17931	50.00	100.00	0.27	0.61	7.10	6.70
D1T2	*Rothia mucilaginosa* DSM20746	54.55	100.00	0.20	0.59	7.00	6.80
D1P3	*Actinomyces oris* ATCC27044	35.29	100.00	0.18	0.53	7.00	6.50
D2P3	*Rothia aeria* A1-17B	67.74	100.00	0.18	0.44	7.00	6.80
D1P5	*Rothia dentocariosa* ATCC17931	72.73	100.00	0.18	0.43	7.00	7.00
D1P4	*Rothia dentocariosa* ATCC17931	29.03	100.00	0.19	0.41	7.00	6.90
D1T11	*Rothia mucilaginosa* DSM20746	5.88	88.24	0.20	0.82	7.20	7.00
D4P1	*Rothia dentocariosa* ATCC17931	20.69	86.21	0.53	1.85	7.20	6.90
D1T6	*Rothia mucilaginosa* DSM20746	36.11	86.11	0.43	0.79	7.10	7.00
D1P16	*Rothia dentocariosa* ATCC17931	6.25	84.38	0.32	1.11	7.20	7.20
D1P6	*Rothia dentocariosa* ATCC17931	0.00	82.76	0.36	1.00	7.30	7.10
D2P2	*Rothia dentocariosa* ATCC17931	21.21	81.82	0.06	0.19	7.00	6.90

D1T3	*Rothia mucilaginosa* DSM20746	45.00	80.00	0.42	0.74	7.20	7.00
D4P11	*Rothia dentocariosa* ATCC17931	17.65	73.53	0.24	0.61	7.30	7.20
D4P9	*Rothia dentocariosa* ATCC17931	21.21	69.70	0.21	0.54	7.30	7.20
D5T8	*Rothia mucilaginosa* DSM20746	18.00	66.00	0.23	0.81	7.20	7.20
D4P8B	*Rothia dentocariosa* ATCC17931	17.65	64.71	0.24	0.45	7.20	7.20
D3T1	*Rothia dentocariosa* ATCC17931	10.81	62.16	0.25	0.48	7.20	7.10
D4P3	*Actinomyces viscosus* JCM8353	19.35	58.06	0.44	1.25	7.30	7.10
D1P11	*Rothia dentocariosa* ATCC17931	6.25	56.25	0.32	0.92	7.30	7.30
D1P8	*Rothia dentocariosa* ATCC17931	10.00	53.33	0.33	0.90	7.30	7.20
D5P5	*Actinomyces viscosus* JCM8353	18.75	53.13	0.24	0.57	7.20	7.10
D1T1	*Rothia mucilaginosa* DSM20746	27.03	48.65	0.29	0.50	7.20	7.10
D4P4	*Actinomyces viscosus* JCM8353	17.65	44.12	0.24	0.47	7.20	7.10
D4T8	*Actinomyces oris* JCM16131	21.21	39.39	0.24	0.49	7.20	7.10
D3T2	*Rothia mucilaginosa* DSM20746	2.70	29.73	0.18	0.23	7.20	7.10
Average (*SD*)	-	33.74 (*23.11*)	86.95 (*19.92*)	0.40 (*0.19*)	1.06 (*0.56*)	7.19 (*0.10*)	7.02 (*0.17*)

*final selection of 10 nitrate-reducing isolates of interest

*[2] pH spent med.: pH spent medium

After 4 hours, the average nitrate reduced by the 53 isolates was 33.74% (SD 23.11%) and after 7h this value increased to 86.95% (SD 19.92%). The starting OD of the medium was 0.01 and went up to an average OD of 0.40 (SD 0.19) after 4h and OD 1.06 (SD 0.56) after 7h. Isolates with acidogenic properties were excluded from the selection of potential probiotics, as acid production of isolates is associated with dental caries risk [207]. Only one nitrate-reducing isolate (i.e., D1P3 *Actinomyces oris*) acidified the pH of the glucose-containing BHI medium (starting pH 7.3) to pH 6.5 after 7 hours of growth and the average of all isolates was pH 7.02 (SD 0.17), indicating there was little caries-associated acidification. With regard to this, one *Streptococcus salivarius* isolate, which was excluded because it did not reduce nitrate, lowered the pH to 5.5 under the same conditions.

When comparing all 23 *R. mucilaginosa* isolates and 21 *R. dentocariosa* isolates, *R. mucilaginosa* grew 1.39× more ($p < 0.05$, Figure 3B) and reduced 1.79× more nitrate ($p < 0.01$, Figure 3A) after 4h. After 7h there were no significant difference in OD and nitrate-reduction between the two species, but *R. mucilaginosa* had significantly decreased the pH by 0.1 points ($p < 0.05$, Figure 3C). This pattern of pH difference was consistent when comparing *R.*

mucilaginosa and R. *dentocariosa* of donor 1 ($p < 0.05$) and donor 4 ($p = 0.094$, Supplementary Figure 1), which were the only two donors with enough isolates for an intra-donor comparison.

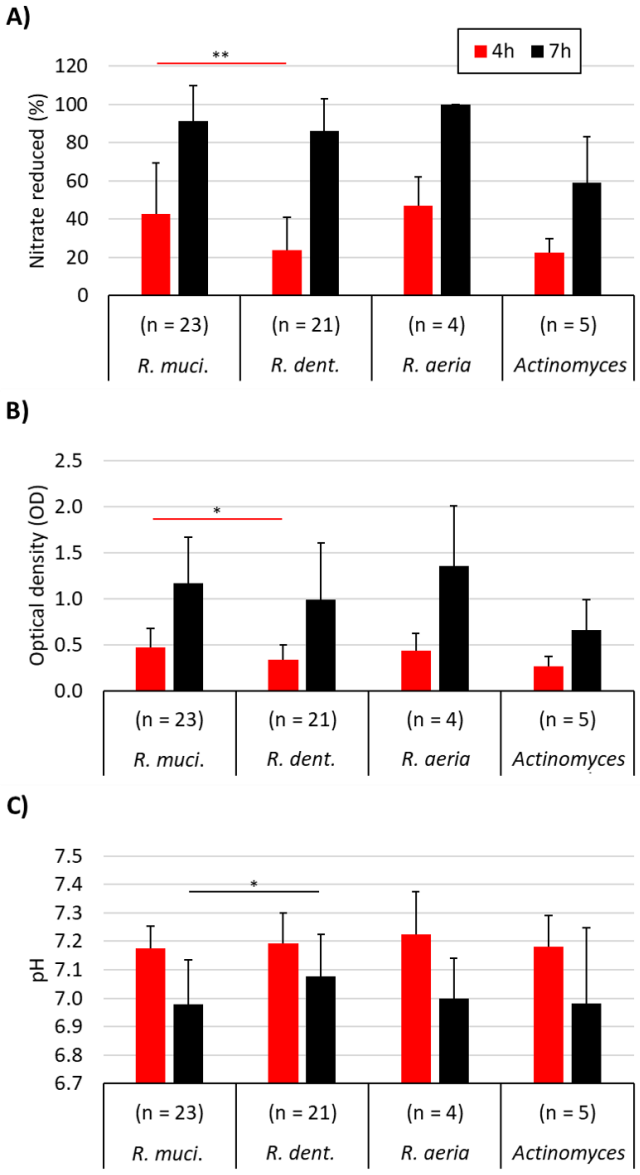


Figure 3: Comparison of different nitrate-reducing species isolated in the current work after 4h and 7h of growth with 6.5 mM nitrate. The different *Rothia* species are grouped separately (23 *R. mucilaginosa* isolates, 21 *R. dentocariosa* isolates, four *R. aeria* isolates) and all five *Actinomyces* isolates are represented together. Bars represent the nitrate reduced (A), optical density (B) and medium pH (C) after 4h and 7h of growth. Only *R. mucilaginosa* (*R. muci.*) and *R. dentocariosa* (*R. dent.*), to which most isolates belonged, were statistically compared (Mann–Whitney U test). *$p < 0.05$, **$p < 0.01$.

2.4.2 Selection of probiotic candidates

Thirty-three isolates reduced 100% nitrate after 7h (Table 1) and from these bacteria, 9 isolates were selected that grew to a final OD >1.5 after 7h (i.e., they grew well under aerobic conditions relevant for large-scale production, Table 1) for further analysis. These corresponded to three *R. dentocariosa* isolates from donor 1 (D1P10, D1P17, D1P15) and three *R. mucilaginosa* isolates from donor 4 (D4T4, D4T6, D4T9), which allowed to study strain differences. The other three isolates were *R. aeria* isolate D1P7 and *R. mucilaginosa* isolates D3T4 and D5T11. Additionally, an isolate from another donor (D4P7, *R. dentocariosa*) was added to the selection for inter-donor comparison of *R. dentocariosa* with the isolates of donor 1. These 10 selected isolates were further studied by whole genome sequencing, genome analysis, nitrate reduction quantification under different pH levels and their effect on *in vitro* oral communities was tested.

2.4.3 Genome analysis and identification of possible virulence genes

After the whole genome sequencing procedure, sequences obtained by Illumina and Oxford Nanopore Technologies (ONT) procedures were quality-filtered, corrected and combined to produce a final assembly (Table 2). The genome sequences of all 10 isolates could be assembled into one single contig, except the sequences of D5T11 (Supplementary Table 3), which had less ONT sequences that passed the quality filter (only 8.73%).

Average nucleotide identity (ANI) values for the whole genomes confirmed that the five isolates from plaque corresponded to *Rothia dentocariosa* (D1P10, D1P17, D1P15 and D4P7) and *Rothia aeria* (D1P7), while all isolates from the tongue were closely related to the reference strain of *Rothia mucilaginosa* ATCC2296. Interestingly, when comparing the 10 genomes with each other, it appeared that isolates from the tongue of donor 4 (D4T4, D4T6 and D4T9) could belong to the same strain (sequence similarity at homologous regions > 99.9%, Table 2) and all *R. dentocariosa* studied were clearly different strains (ANI values 95-97%), indicating that within the same individual there may be substantial intra-specific genetic heterogeneity, confirming the phenotypic heterogeneity previously detected (Figure 2 and Table 1).

Table 2. Whole genome sequence analysis, assembly information and species identification of the 10 selected isolates

Isolate	Illumina Sequences	ONT Sequences	ONT mean length (bp)	Estimated genome size (bp)	Contigs	Mean Coverage	Closest sequenced genome	ANI value*
D1P7	1.427.028	43.207	8.085	2.654.231	1	74x	*Rothia aeria* C6B	97,4
D1P10	1.649.834	34.991	9.775	2.486.471	1	93x	*Rothia dentocariosa* NCTC10917	96,58
D1P15A	195.232	19.010	9.637	2.496.211	1	33x	*Rothia dentocariosa* NCTC10917	96,17
D1P17	259.928	52.125	12.066	2.566.927	1	39x	*Rothia dentocariosa* NCTC10917	96,52
D3T4	2.206.376	23.696	7.854	2.296.981	1	226x	*Rothia mucilaginosa* ATCC2296	95,41
D4P7	263.877	114.000	8.649	2.511.012	1	42x	*Rothia dentocariosa* NCTC10917	96,31
D4T4	3.773.501	19.976	11.083	2.283.903	1	390x	*Rothia mucilaginosa* ATCC2296	93,87
D4T6	207.423	27.874	10.981	2.276.594	1	35x	*Rothia mucilaginosa* ATCC2296	93,87
D4T9	3.731.259	70.368	8.875	2.297.550	1	382x	*Rothia mucilaginosa* ATCC2296	93,87
D5T11	365.174	941	9.342	924.739*2	30	55x	*Rothia mucilaginosa* ATCC2296	94,54

* An ANI value >94% to the reference genome is considered to correspond to the same species [208].

*2 In this case, the genome size could not be estimated and the sum of the contigs is presented.

According to a FAO/WHO report (2002), newly registered probiotic strains must be examined in pathological, genetic, toxicological, immunological and microbiological aspects that could be relevant for human safety[209]. Thus, mobile genetic elements (MGEs), antimicrobial resistance genes (ARGs) and virulence factors databases were used to determine if the 10 genomes contained any of these genetic elements, which could make them unsuitable for probiotic usage.

No virulence factors nor ARGs were found in any of the studied genomes (Supplementary Table 4). Several MGEs were found in the genomes of D1P7

and D1P17. These MGEs corresponded to methyltransferases or transposases previously identified in *Corynebacterium* species, which could correspond to horizontal gene transfer events from this genus, which is a common inhabitant of the oral cavity. Specifically, D1P7 had a double insertion of a transposase (tnp1249) in two close genomic regions and both D1P7 and D1P17 had three insertions of three different 23 rRNA methyltransferases: erm(X), ermCX and ermLP. Based on the genome analysis all 10 isolate could be suitable strains for probiotics due to their absence of known virulence factors and antibiotic resistance genes.

Genome annotation revealed that all selected isolates contained genes encoding nitrate transport proteins, nitrate to nitrite reduction, denitrification and DNRA (dissimilatory nitrate reduction to ammonium) enzymes (Figure 4). In addition, the gene encoding further reduction of nitric oxide to nitrous oxide (N_2O) was also found, but no gene for reduction of N_2O to nitrogen (N_2) was detected, nor for fixation of N_2 to ammonium. A large set of molybdenum (Mb) transport proteins and molybdopterins was also found, in agreement with molybdenum being a vital cofactor for bacterial nitrate reduction enzymes. A diverse array for genes involved in lactate/lactic acid metabolism was also present in all fully sequenced strains. A full list of genes involved in nitrate metabolic pathways and lactate/lactic acid utilization are shown in Supplementary Data Sheet 1.

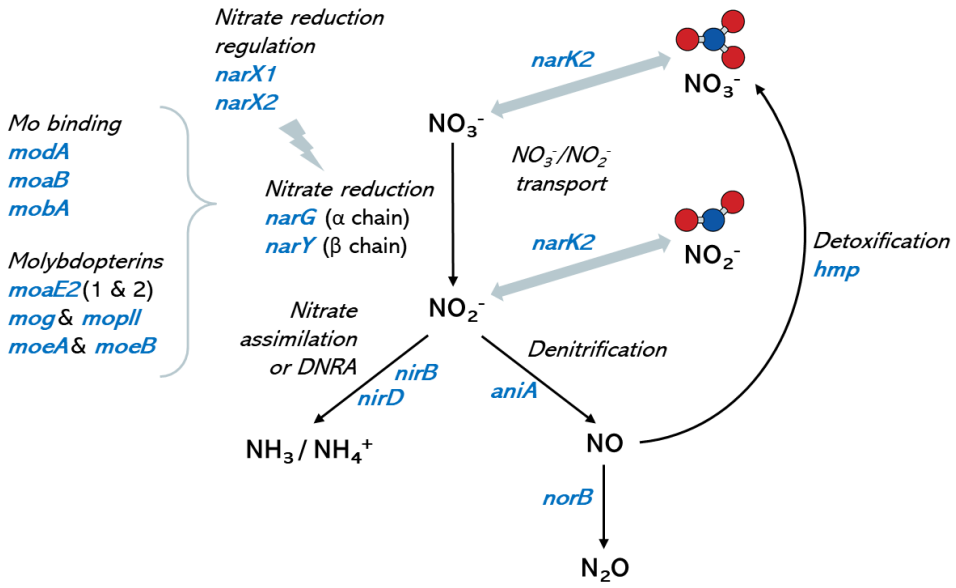


Figure 4. Nitrate metabolism genes in oral *Rothia* isolates. Graph shows a schematic representation of the nitrate metabolic pathways (depicted in black italic text) and the corresponding coding genes (in blue) that have been identified in the genomes of the oral isolates sequenced in the current manuscript. Molybdenum is a cofactor in nitrate reductase enzymes and identified molybdenum-related genes are included for reference. No genes for the reduction of nitrous oxide (N_2O) to nitrogen (N_2), nor for the oxidation of ammonium to nitrite were found. DNRA: dissimilatory nitrate reduction to ammonium. Ammonia production, denitrification and nitrite reduction to ammonium are proposed as mechanisms of pH buffering in the oral cavity, and nitric oxide is an antimicrobial molecule. A full list of genes in the different isolates can be found in Supplementary Data Sheet 1. To the version of this figure in this thesis, "nitrate assimilation" was added as, apart from DNRA, this is also possible with the *nirB* and *nirD* genes. Additionally, ammonia (NH_3) – a weak base – was added as this can be formed from NH_4^+ under certain conditions.

2.4.4 Isolate-specific effect of pH on nitrate-reduction

The 10 selected isolates were incubated during 5 hours with 6.5 mM of nitrate at three different pH levels (pH 6.0, 7.0 and 7.5, Figure 5). There were some isolate-dependent effects of the pH on nitrate reduction. For example, D1P7 reduced 100% of nitrate after 5 hours of incubation at pH 7.5 and pH 7, but reduced 51.72% of nitrate when grown at pH 6.0. Opposite to D1P7, D4T6 reduced

76.67% of nitrate when pH was 6.0, but it reduced only 35.19% of nitrate at a pH of 7.5.

The amount of nitrite detected was normalized by the amount of nitrate reduced, taking into account a 1:1 molar conversion of nitrate to nitrite (nitrite detected, Figure 5C). Interestingly, at pH 6.0 more nitrite appeared to be further reduced to other compounds compared to pH 7 and pH 7.5 (both $p < 0.01$). Specifically, 65.46% (SD 11.48%) of the reduced nitrate was detected as nitrite, which means that the other 34.54% had been converted to other compounds (e.g., reduction to nitric oxide or ammonium). It should be noted that no ammonium was detected in any of the cultures. At pH 7 and 7.5, the average percentage of nitrite detected was 116.16% (SD 16.76%) and 128.62% (SD 25.75%). This implies that 16.16% and 28.62%, respectively, of nitrite detected could not be explained by reduction of the 6.5 mM added nitrate, indicating that nitrite is, in part, being produced by other pathways than nitrate reduction.

Six isolates (D1P7, D3T4, D4T4, D4T6, D4T9 and D5T11) reduced nitrate equal to or above the median at two or three of the pH levels (Supplementary Table 5) and were selected to test their effects when added to *in vitro* complex oral communities.

2.4.5 Effect of isolates on *in vitro* oral community metabolism

The effect of the *R. aeria* isolate D1P7 and five *R. mucilaginosa* isolates (D3T4, D4T4, D4T6, D4T9, and D5T11) on oral community metabolism was tested *in vitro.* For this, biofilms were grown from saliva of nine different donors during 7h with and without 6.5 mM nitrate (Figure 6). In these nine independent experiments, the levels of reduced nitrate were higher in the presence of any of the six isolates compared to the "no isolate" condition ($p < 0.05$, Figure 6A). Furthermore, the addition of isolates to the *in vitro* oral communities led to more nitrite production (Figure 6B), which was significant for D4T4, D4T6, D4T9 and D5T11 ($p < 0.05$), but not D1P7 ($p = 0.11$) and D3T4 ($p = 0.051$). Interestingly, D1P7 addition led to 100% of nitrate reduction in all donors, which indicates that nitrite was further metabolized into other compounds (e.g. ammonium or nitric oxide) when adding this isolate.

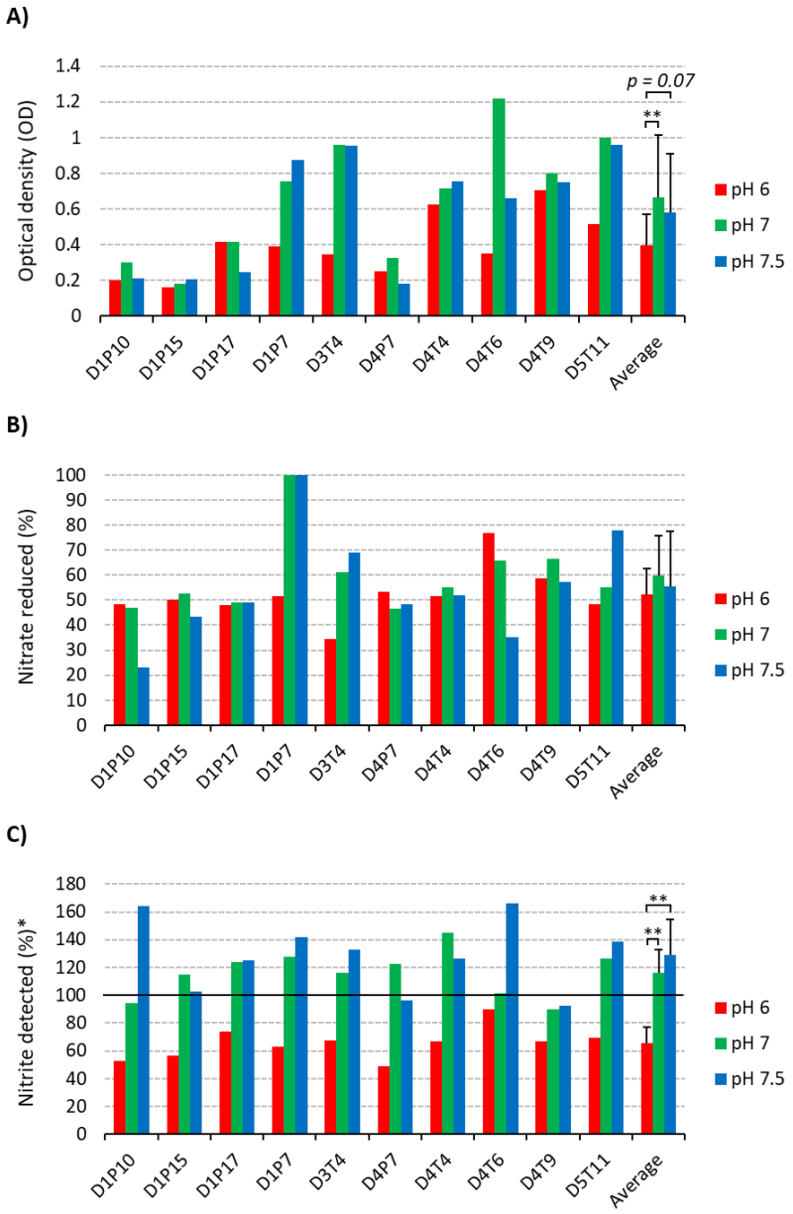


Figure 5: Nitrate Reduction Capacity of isolates at 3 pH levels (2nd screening). Bars show results after 5h of incubation (37 °C) at pH 6 (red bars), pH 7 (green bars) and pH 7.5 (blue bars) under aerobic conditions with starting OD 0.01. A) Final optical density values. B) Percentage of initially added nitrate (6.5 mM) that had been used up after 4 and 7 hours of incubation. C) Amount of nitrite detected, represented as a percentage of nitrate reduced (100 x mM nitrite detected / mM nitrate reduced). Reference codes of the 10 isolates are shown at the bottom, where D relates to the donor, T refers to tongue coating and P to dental plaque samples. **$p < 0.01$ according to a Wilcoxon test comparing all 10 isolates at different pH levels.

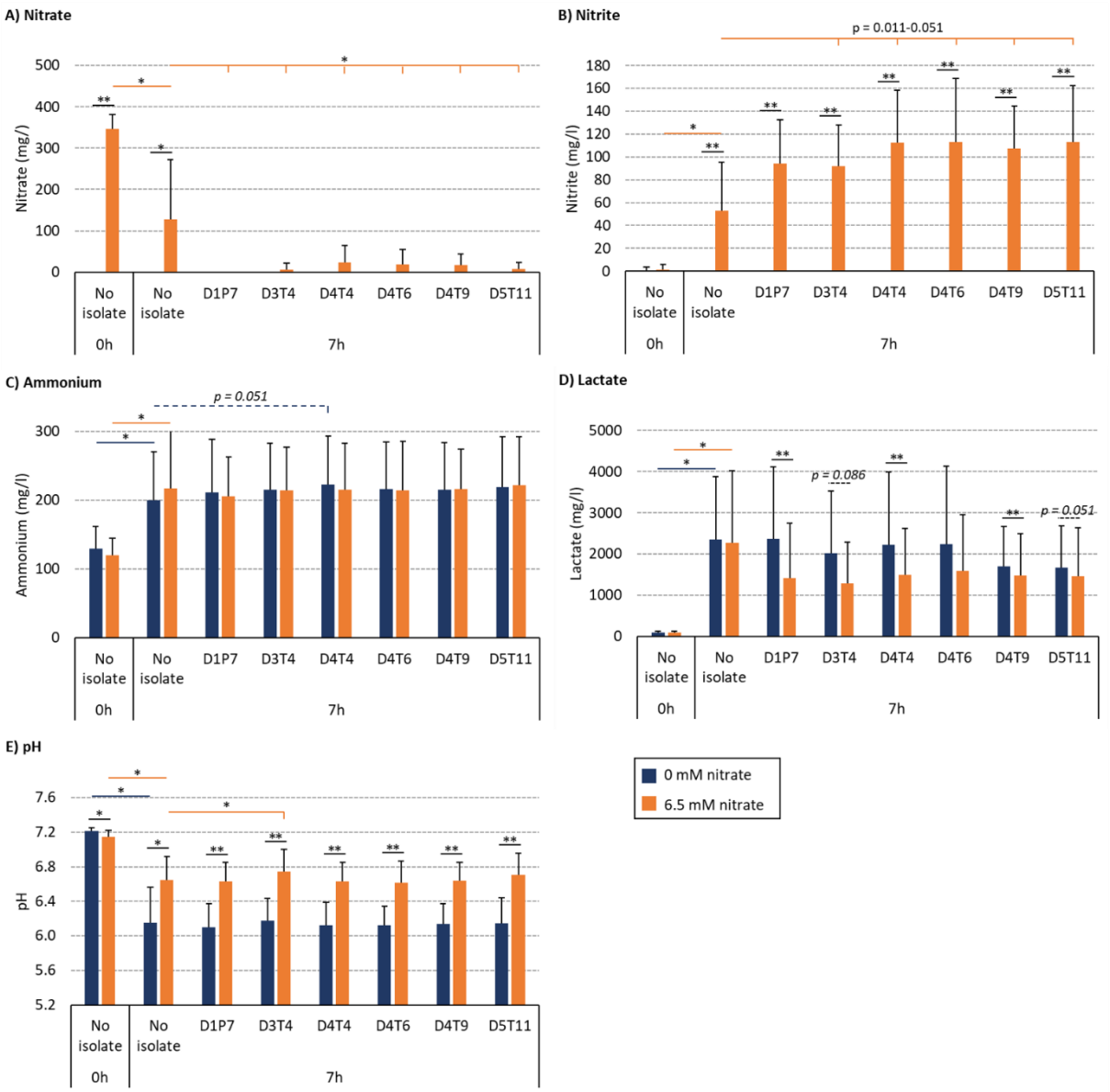


Figure 6. Effect of nitrate and six nitrate-reducing isolates on oral microcosm community metabolism. Bar plots show averages and standard deviations of supernatant measurements. Saliva of 9 donors was incubated with nutrient-rich medium to form *in vitro* oral biofilm with 6.5 mM nitrate (orange) or without nitrate (blue). Values correspond to measurements before incubation (0h, which is the initial mixture of saliva and medium) and after 7h of incubation. A) Nitrate (mg/l), B) nitrite (mg/l), C) ammonium (mg/l), D) lactate (mg/l) and E) pH. All conditions with and without nitrate were compared (black lines with p-values). Additionally, conditions with isolates (D3T4, D4T4, D4T6, D4T9, and D5T11) were compared with the conditions without an isolate

(i.e., no isolate): dark blue lines with p-values compare 0 mM nitrate conditions and orange lines with p-values compare 6.5 mM nitrate conditions. *p<0.05 and **p<0.01 according to a Wilcoxon test.

The BHI medium contains glucose (2 g/l) and this leads to acidification by oral communities over time. Regarding this, lactate correlated negatively with pH ($R = -0.820$, $p < 0.01$ in the control condition and -0.778, $p < 0.05$ in the nitrate condition). Importantly, 6.5 mM nitrate supplementation always led to a smaller decrease in pH after 7h (Figure 6E) compared to 0 mM nitrate ($p < 0.05$). In communities with added isolates, this appeared to be, at least partly, due to lactic acid consumption: lower amounts of lactate were detected when combining nitrate with isolates compared to isolates without nitrate (Figure 6D, $p = 0.008$-0.110), which was significant for D1P7, D4T4 and D4T9 ($p < 0.05$), whereas there were no significant differences in the detected levels of ammonium. Only one isolate (D3T4), when combined with nitrate (i.e., symbiotic combination), significantly prevented the pH drop due to sugar metabolism more than when just adding nitrate without any of the isolates (i.e., a prebiotic treatment, $p < 0.05$). Another isolate, D4T4, showed a trend of increasing ammonium production without nitrate addition (i.e., a probiotic treatment, $p = 0.051$).

Nitrate reduction and ammonium production in communities without isolates

In communities without isolates, there was no significant difference in lactate levels between the nitrate and control conditions after 7h. Additionally, the ammonium detected did not differ significantly ($p = 0.170$). Nevertheless, the two individuals that produced the largest increase of ammonium resulting from nitrate supplementation (D5 and D13) reduced 100% of the nitrate after 7h (Figure 7). Additionally, there was a clear correlation between the NRC of communities and ammonium production ($R = -0.833$, $p < 0.01$, between nitrate left and ammonium produced after 7h, Figure 7A) which, unexpectedly, was also found in the control condition ($R = -0.814$, $p < 0.01$, Figure 7B). This indicates that communities with a better NRC are able to produce more ammonium or ammonia even in the absence of nitrate, while nitrate can further increase ammonium accumulation in some individuals.

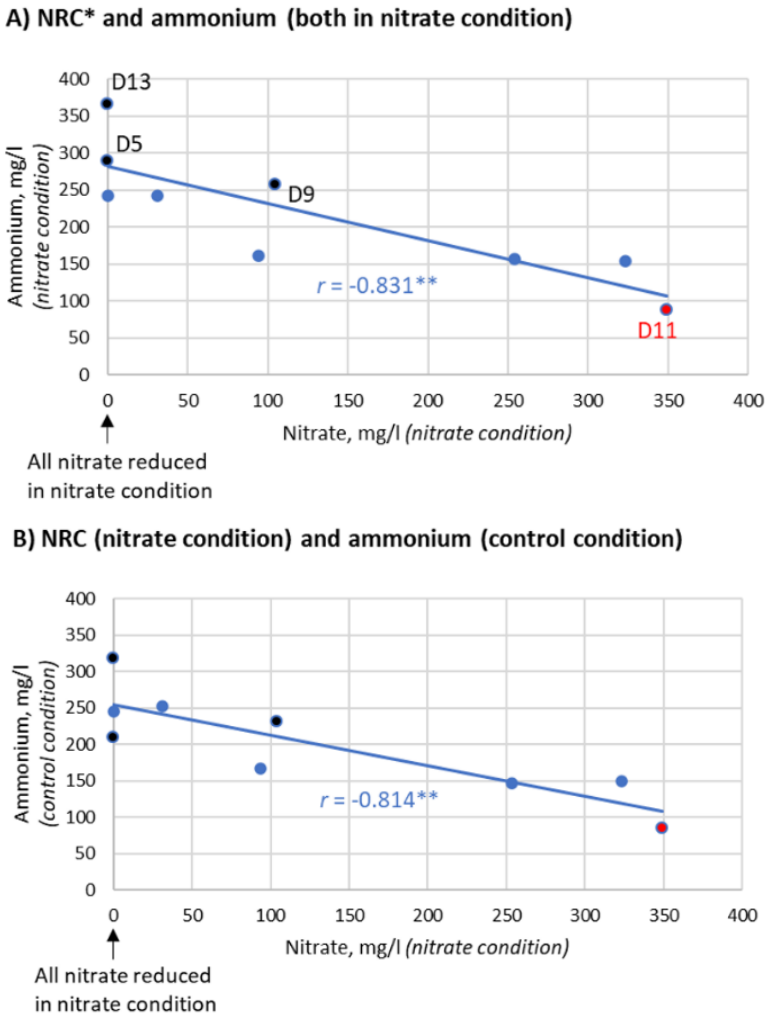


Figure 7. Correlation between nitrate reduction capacity (NRC) and ammonium production after 7h. A) Correlation between the nitrate reduction capacity (NRC), as determined by how much nitrate is left after incubation, and the ammonium detected in the nitrate condition ($R = -0.831$, $p < 0.01$). B) Correlation between NRC and the ammonium produced in the control condition ($R = -0.814$, $p < 0.01$). This correlation indicates that the bacterial communities of donors that reduce nitrate efficiently are also capable of producing more ammonium or ammonia in the absence of nitrate. The three donors that clearly produced more ammonium in the nitrate condition compared to the control condition are marked in black (from most to least extra ammonium production: D5 > D13 > D9). D5 and D13 were also two out of the three donors that reduced 100% of nitrate after 7h. D11 had little to no nitrate reduction activity (marked in red).

The DNA of the 7h biofilms of two donors (D6 and D11, corresponding to a donor with above average and the lowest NRC, respectively) was analyzed by 16S rRNA Illumina sequencing. The isolates, which were added together with the saliva inoculum, appeared to colonize the initial biofilms successfully, as indicated by a higher relative abundance of the corresponding species in 16S rRNA sequencing data (Supplementary Table 6). The *Rothia* species increased more when adding the isolate and nitrate than when only adding the isolate ($p < 0.05$), which is an indication of active growth stimulated by nitrate.

The effect of nitrate and probiotic addition in donors with different nitrate reduction capacities (NRC)

When growing *in vitro* biofilms with the saliva of donor D11 in the presence of nitrate, nitrate did not decrease compared to the baseline (Figure 6 and 7) and virtually no nitrite was produced, showing that the oral microbiota of this donor had a dramatically low NRC. However, a large percentage of nitrate was reduced when adding any of the isolates and the concentration of nitrite also increased notably (Figure 6B). This indicates that the addition of a probiotic was able to compensate the lack of NRC in this donor. When the same experiment was performed with the saliva of donors D5, D9 and D13, all added nitrate was reduced after 7h, even in the absence of isolates. This suggests that these donors have a high NRC and the addition of nitrate alone is enough to promote nitrate reduction.

2.5 Discussion

The nitrate-nitrite-nitric oxide pathway, which depends on nitrate-reduction by oral bacteria, contributes significantly to systemic nitric oxide levels and appears to be important for oral and systemic health[25,62,84,111,122,136,190]. Apart from the stimulation of this pathway with nitrate supplementation, which can result in beneficial cardio-metabolic and oral effects[122], nitrate reduction could also be promoted with the aid of nitrate-reducing probiotic bacteria[136,190]. In the current *in vitro* study we isolate and select potential nitrate-reducing oral probiotics and demonstrate that the NRC of oral communities can be enhanced with newly isolated *Rothia* strains even in communities with little to no NRC. Our data show that nitrate by itself (prebiotic treatment) or combined with a nitrate-reducing isolate (symbiotic combination) prevented pH drops due to sugar

metabolism, and some of the symbiotic combinations increased lactate consumption. A higher local pH and lactic acid consumption can prevent the development of cavities, suggesting a possible anti-caries potential of these isolates. Under certain conditions, nitrite was further reduced by isolates, but no ammonium was detected in the medium, suggesting that part of it may be converted to nitric oxide. Thus, future work should evaluate the potential contribution of some of these isolates to improve conditions that benefit from nitric oxide availability. This could be provided either by direct bacterial nitric oxide production or by production of nitrite and subsequent transformation into nitric oxide by the acidity of the stomach or at other body sites by human encoded pathways[25].

Some authors have criticized the use as oral probiotics of bacteria isolated from dairy products or from the gut, which may hamper their ability to colonize the oral cavity or provide undesired effects[61,210]. We therefore isolated oral strains from healthy individuals and potentially probiotic candidates were selected based on their efficiency to perform an important function of the oral microbiota, namely nitrate reduction. Other selection criteria included a fast aerobic growth rate, lack of acidogenicity (a feature associated to tooth decay), ability to grow when added to *in vitro* oral communities and lack of virulence and antibiotic resistance genes. In total, 53 oxygen-tolerant nitrate-reducing bacteria were isolated from dental plaque or tongue coating samples from orally and systemically healthy individuals. In a first nitrate-reduction screening, 10/53 isolates were selected as potential probiotic candidates. In a second nitrate-reduction screening under three different pH levels, a final list of 6/10 candidates was completed. The effect of these six *Rothia* isolates on oral microbiota communities was studied, allowing us to evaluate their effect when added to a complex ecosystem, as a first step towards future animal or clinical studies.

Effect on oral communities

The six *Rothia* isolates in the final selection were added to saliva and grown together in nutrient-rich medium for 7 hours with or without 6.5 mM nitrate. In 7 hours, initial biofilms grow with a highly similar bacterial composition to natural oral biofilms[141] and, together with planktonic cells, effect the pH and metabolite content of the supernatant. Metabolism of sugar in the medium

leads to a decrease in pH, but both nitrate (prebiotic treatment) and the combination of any of the 6 isolates with nitrate (symbiotic combination) limited this decrease in pH compared to the control condition ($p < 0.05$). Li et al. (2007)[63] added 1.5 mM nitrate to bacteria from saliva and also observed a limited sugar-derived pH drop. In a recent study, 6.5 mM nitrate under similar *in vitro* conditions as our study, prevented a pH drop after 5h and 9h and this was shown to be due to lactic acid consumption during denitrification and ammonium production[190]. In our current study, ammonium production and lactate consumption after nitrate supplementation was only observed in some cases, and therefore depended on the specific isolate under study or the donor's saliva. Importantly, in both studies, a correlation was found between the NRC of oral communities and ammonium production with or without nitrate. This indicates that bacteria or bacterial processes involved in nitrate reduction are linked to ammonium or ammonia production under these conditions.

The analysis of the genomes sequenced in the current manuscript reveals that all isolates also contain the gene encoding the nitrite reductase, which metabolizes nitrite into nitric oxide, and this gene is also present in other sequenced *Rothia* isolates. Some authors have determined that environmental factors like pH, the nitrogen:carbohydrate ratio[183] and carbon source[211] influence whether nitrite is converted to ammonium or to nitric oxide by aquatic or soil bacteria and future work should establish which factors switch the metabolic machinery of oral communities towards DNRA or denitrification. It is also notable that a wide array of genes involved in molybdenum transport and capture are present in the genomes of the oral *Rothia* isolates (Figure 4, Supplementary Data Sheet 1). Thus, the strong molybdenum-related genomic content could partly explain the lower dental caries prevalence in areas of high molybdenum soil concentrations [212,213], as the dietary availability of this vital cofactor for nitrate reductase enzymes could contribute to the oral health benefits associated with nitrate reduction[139].

An interesting finding in our study was that one isolate (D3T4), when combined with nitrate (i.e., symbiotic combination), significantly prevented the pH drop due to sugar metabolism more efficiently than when just adding nitrate without any of the isolates (i.e., a prebiotic treatment, $p < 0.05$). Another isolate, D4T4, showed a trend ($p = 0.051$) of increasing ammonium production without nitrate

addition (i.e., a probiotic treatment). In relation to this, arginine is effectively used as a prebiotic to stimulate ammonia production and increase the local pH to prevent caries[60]. The isolates D3T4 and D4T4 could therefore provide similar benefits and this should be further investigated *in vivo*. It should be noted that differences in ammonium detection can also be affected by ammonium consumption (an isolate may produce ammonium but metabolize it into other compounds or incorporate it as a nitrogen source). In fact, the detected levels of nitrite concentrations were often higher than the expected stoichiometric conversion of nitrate to nitrite, suggesting that nitrite could also be produced by other pathways (e.g., ammonium oxidation or nitrification). Our genome analysis however, did not detect known ammonium oxidizing pathways or the presence of genes transforming nitrogen into nitrite. Additionally, we did not detect bacterial nitric oxide synthases (NOS) that convert arginine in nitric oxide. Thus, future genetic and experimental studies with controlled carbon, nitrogen and oxygen supplies should shed light on the complex nitrogen cycle that starts to be envisaged in oral communities.

Our data also show that, in the presence of nitrate, *Rothia* isolates can consume lactic acid, an organic acid which is produced by oral communities and associated to caries development[214], as less lactate was detected compared to the conditions of the isolates without nitrate ($p = 0.008$-0.110). On the one hand, nitrate has been shown to increase the salivary pH *in vivo*[163] and different *in vitro* studies, including this current work, show that nitrate provides resilience against acidification and lactic acid accumulation[63,190]. On the other hand, nitrate-rich beetroot juice consumption has been shown to increase *Rothia* levels significantly compared to nitrate-depleted beetroot juice[83,135]. This indicates that *Rothia* species could contribute to the increase in pH and potentially also to resilience against acidification resulting from nitrate supplementation *in vivo*.

Importantly, many periodontitis-associated pathobionts are alkaliphiles and the effect of an increased salivary pH, which can result from nitrate supplementation[163], on oral communities *in vivo* should be investigated. Notably, previously, an increase in pH but a decrease in periodontitis-associated genera (some of which have been shown to be sensitive to nitric oxide[138]) was observed after 5 hours of incubation of oral communities with nitrate *in vitro*[190]. Additionally, nitrate-rich lettuce juice reduced gingival inflammation compared

to nitrate-depleted lettuce juice in patients with chronic gingivitis[84]. This provides preliminary evidence that nitrate supplementation could be beneficial for gum diseases, but this should be further investigated in clinical studies.

Isolates' habitat comparison and pH preference

From all 53 nitrate-reducing isolates obtained in this study under aerobic conditions, forty-eight were *Rothia* species and five corresponded to *Actinomyces* species. Interestingly, all 23 *R. mucilaginosa* isolates originated from the tongue, while 20/21 *R. dentocariosa* isolates and all 4 *R. aeria* isolates originated from dental plaque. Recently, Wilbert et al. (2020)[215] used Human Microbiome Project data analysed by Eren et al., (2014)[216] and concluded that *R. mucilaginosa* is strongly associated to the tongue (~100-fold more abundant than on teeth), while *R. aeria* and *R. dentocariosa* appear to be strongly associated to teeth surfaces (>100-fold more abundant there than on the tongue). Our cultivation-based results confirm that *R. mucilaginosa* mostly lives on the tongue surface and *R. dentocariosa* and *R. aeria* on the teeth. Doel et al. (2005)[113] also obtained 8 *Rothia* isolates from the tongue under aerobic conditions and most (6 out of 8) were *R. mucilaginosa*. In our study, after 4h of incubation, *R. mucilaginosa* isolates had significantly grown to a higher optical density ($p < 0.05$) and reduced more nitrate than *R. dentocariosa* isolates ($p < 0.01$). Additionally, after 7h, *R. mucilaginosa* isolates had reduced the pH 0.1 point more ($p < 0.05$), indicating possible differences in metabolism of these species.

The nitrate-reduction capacity of ten probiotic candidates was evaluated at three different pH levels (pH 6, 7 and 7.5). Some isolates appeared to reduce nitrate at the same rate at all pH levels (e.g., D1P17 or D4T4), while others had a clear preference for a neutral and/or slightly alkaline pH (e.g., D1P7, D3T4 and D5T11) and one isolate reduced most nitrate at an acidic pH (D4T6). Regarding this, differences in oral pH levels among donors can result from host-specific factors such as salivary pH and dietary habits. Additionally, within a single donor, different habitats have different environmental conditions and pH gradients can be found within oral biofilms[194], which could explain intra-donor differences in the pH preference for nitrate-reduction of different stains.

Importantly, our data show that nitrite reduction was consistently stimulated by pH 6 compared to pH 7 and pH 7.5. Nitrite reduction of microbial

communities can increase as the pH decreases[217]. In the mouth this could have an important consequence for oral health, namely the prevention of acidification which is clearly associated with caries development. Arginine deiminase in oral probiotics has been shown to be activated by a low pH [61], leading to ammonia production and providing a self-regulatory feedback mechanism against acidification[136]. Likewise, nitrite reduction could be stimulated to increase the local pH by lactic acid consumption during ammonium production (DRNA) or antimicrobial nitric oxide release (denitrification). Similarly, lactic acid stimulates nitrite consumption which is performed in parallel with lactic acid consumption[63]. Importantly, non-enzymatic nitrite decomposition into nitric oxide occurs below pH 5.0[49,127] and this was not the case of this experiment, indicating that enzymatic nitrite conversion of *Rothia* was stimulated by pH 6.0.

The associations with health and disease of Rothia and nitrate

The clear association of *Rothia* with oral health has been discussed previously[190]. In short, *Rothia* species are found in higher relative abundance in oral biofilms when comparing healthy individuals with caries active, periodontitis or halitosis patients (see, for example references[27,30,168,174]). Related to this, *R. aeria* correlated negatively with inflammatory cytokines IL-17 and TNF-α in humans[169]. Thus, multiple studies indicate that this is genus generally associated with oral health.

Rothia isolates have occasionally been isolated from, and associated with, endocarditis and other systemic disease samples[171], indicating that under certain conditions, there are strains that can translocate to other human niches. This is an extended feature of representatives of many oral species, which appear to be pre-adapted to attach to distant human tissues by their ability to adhere to oral mucosa components like collagen and fibronectin[218]. As a consequence of that, many oral bacteria have been isolated from endocarditis samples, including classic commensals generally recognized as safe ("GRASS" organisms) such as *Streptococcus salivarius*[219], and different species of oral *Gemella*, *Granulicatella* or *Prevotella*, among others[218]. Likewise, probiotic Lactobacilli (also considered GRASS) have been isolated from the blood of immunosuppressed patients in intensive care units[220]. It is therefore crucial with all oral probiotics to select strains without virulence genes potentially involved in endocarditis or other diseases, which were shown to be absent in all 10 probiotic candidates selected

in our study, and future work should perform animal trials to confirm their safety.

The safety and beneficial effects of dietary nitrate in general[122] and for the oral cavity[190] were recently discussed. It should be noted that nitrate salts added to processed meat are associated with cancer[221,222]. This nitrate is reduced to nitrite by bacteria in the meat and can further react with other molecules, such as heme, amines and amides, forming potentially carcinogenic N-nitroso compounds[186,187]. However, we obtain most nitrate (>80%) from vegetables that are generally associated with health benefits and considered anticarcinogenic[87,89,223]. Antioxidants and polyphenols in fruits and vegetables prevent the formation, and possibly damage, of N-nitroso compounds[188,189,224,225]. In relation to this, different safety agencies concluded that epidemiological studies do not indicate that nitrate intake from diet or drinking water is associated with increased cancer risk[93]. The concentrations of nitrate used in this study can be obtained in saliva by vegetable consumption. The application of topical doses of nitrate far below the acceptable daily intake (ADI, 3.7 mg/kg of body weight) would also be sufficient[190]. These could be obtained by oral products containing vegetable extracts or low amounts of nitrate salts in combination with antioxidants. The effect of nitrate-reducing probiotics and nitrate in combination with different dietary compounds (e.g. antioxidants and polyphenols) should be explored to make sure that potential future products do not result in harmful N-nitroso compounds formation.

Experimental conditions and limitations

The aim of our study was to isolate aerobic fast-growing bacteria with a high NRC. All experiments were performed under aerobic conditions with BHI medium that contains 0.2% glucose as a carbon source. Doel et al.[113] used a different medium (including tryptone, horse serum and 0.5% glucose) under aerobic and anaerobic conditions. In their study, most nitrite-producing isolates obtained in the presence of oxygen were *Rothia* (from the tongue) and *Actinomyces* (from saliva and plaque), but without oxygen, most nitrite-producing bacteria were *Veillonella* and *Actinomyces*, while no *Rothia* was detected. Other isolates in their study included *Staphylococcus*, *Corynebacterium* and *Haemophilus*, all including species obtained with or without oxygen. The identification of these other species under aerobic conditions may have resulted

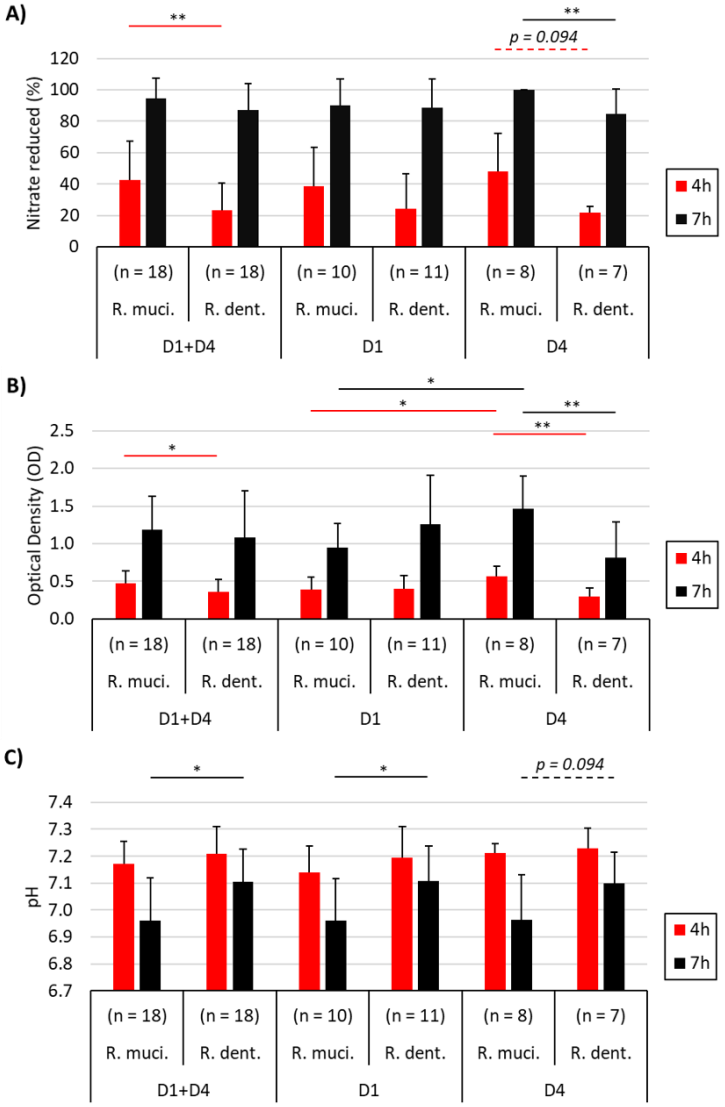
from differences in medium composition or donor microbiota. Furthermore, in a recent study, it was shown that different carbon sources enrich different nitrate reducers of terrestrial environments[211]. Therefore, future work should focus on the isolation of a more diverse set of nitrate-reducing bacteria from the oral cavity using different growth conditions. Additionally, the effect of different nitrate-reducers and nitrate reduction rates on oral communities should be determined. In our study, we tested 6 *Rothia* isolates separately, but combining a variety of isolates, even from different species, could lead to mutualistic interactions and enhanced beneficial effects.

2.6 Concluding remarks

Efficient nitrate reduction in the oral cavity has been clearly associated to human health but this metabolic pathway cannot be performed by humans, which lack the necessary enzymes. Thus, oral bacteria capable of nitrate reduction arise as a fascinating example of symbiosis by which the microbiome provides a health-associated benefit to the human host, which in exchange, recycles nitrate by actively concentrating plasma nitrate into the saliva[25]. In fact, disruption of oral microbial communities by over-use of antiseptics or antibiotics will interfere with the nitrate-nitrite-nitric oxide pathway[111], as well as food habits with low dietary nitrate[119]. As a result of these, humans appear to vary widely in their NRC[62,192], with important potential consequences for diseases or conditions that are influenced by a deficit in nitric oxide, ranging from cardiovascular diseases to reduced sport performance or diabetes development, among others[122]. The current work shows that, in oral bacterial communities with a slightly reduced NRC, the supplementation of nitrate may suffice to restore and promote efficient nitrate reduction; however, our data also show that in individuals with extremely low NRC, the addition of a nitrate-reducing probiotic could be instrumental for a recovery of the function. Thus, future work should be performed to further characterize nitrate-reducing probiotics and test their potential efficacy in animal and clinical studies.

2.7 Supplementary figures and tables

Supplementary figures



Supplementary Figure 1. Comparison of two *Rothia* species from two donors. In the graph *R. mucilaginosa* and *R. dentocariosa* isolates of donor 1 and donor 4 are compared after 4h and 7 h of growth. Bars show the percentage of nitrate reduced (A), optical density (B) and medium pH (C) . *$p < 0.05$, **$p < 0.01$ according to a Mann–Whitney U test.

Supplementary tables

Supplementary Table 1: salivary properties and isolates from donors 1-5

	Salivary pH*[1]	Salivary nitrite (mg/l)*[1]	Original sample	Nitrite-producing isolates*[2]	Confirmed nitrate-reducing isolates
Donor 1	7.3	21	Plaque	15	15
			Tongue	12	10
			Total	27	25
Donor 2	6.8	64	Plaque	3	3
			Tongue	0	0
			Total	3	3
Donor 3	7.1	14	Plaque	0	0
			Tongue	6	4
			Total	6	4
Donor 4	7.3	31	Plaque	10	9
			Tongue	11	9
			Total	21	18
Donor 5	7.5	21	Plaque	1	1
			Tongue	4	2
			Total	5	3
All (sum)			Plaque	29	28
			Tongue	33	25
			Total	62	53

*[1]Measured on a different day than sampling of plaque and tongue bacteria.

*[2]Colonies were selected that produced a red tone caused by a Griess reaction that stains nitrite.

Supplementary Table 2: nitrite detected after 4h and 7h growth with 6.5 mM nitrate (continuation of table 1)

Isolate	Species (by 16S rRNA)	Nitrite detected (mM)	
		4h	7h
D1P10*	*Rothia dentocariosa* ATCC17931	4.00	7.09
D3T4*	*Rothia mucilaginosa* DSM20746	5.43	1.32
D1P7*	*Rothia aeria* A1-17B	3.35	8.17
D4T4*	*Rothia mucilaginosa* DSM20746	8.65	4.00
D4T3	*Rothia mucilaginosa* DSM20746	5.04	4.15
D1P17*	*Rothia dentocariosa* ATCC17931	1.41	8.43
D4T6*	*Rothia mucilaginosa* DSM20746	10.61	3.26
D1P9	*Rothia dentocariosa* ATCC17931	3.28	6.52
D1P15*	*Rothia dentocariosa* ATCC17931	1.78	8.72
D4T9*	*Rothia mucilaginosa* DSM20746	3.20	8.61
D1P14	*Rothia aeria* A1-17B	2.93	7.13
D5T11*	*Rothia mucilaginosa* DSM20746	4.64	9.22
D1P12	*Rothia aeria* A1-17B	2.17	7.22
D1T4	*Rothia mucilaginosa* DSM20746	5.43	2.72
D1T13	*Rothia mucilaginosa* DSM20746	5.43	4.43
D4T12	*Rothia mucilaginosa* DSM20746	3.54	6.74
D1P13	*Rothia dentocariosa* ATCC17931	0.70	9.13
D1T9	*Rothia mucilaginosa* DSM20746	3.20	5.43
D1T12	*Rothia mucilaginosa* DSM20746	2.09	5.43
D4T10	*Rothia mucilaginosa* DSM20746	3.52	7.87
D4T1A	*Rothia mucilaginosa* DSM20746	3.30	4.74
D1T10	*Rothia mucilaginosa* DSM20746	1.59	5.43
D4T11	*Rothia mucilaginosa* DSM20746	3.06	8.87
D3T3	*Rothia mucilaginosa* DSM20746	4.11	5.24
D4P10	*Rothia dentocariosa* ATCC17931	1.63	8.00
D4P7*	*Rothia dentocariosa* ATCC17931	1.41	8.61
D4P6	*Rothia dentocariosa* ATCC17931	1.13	8.30
D2P4	*Rothia dentocariosa* ATCC17931	5.22	10.30
D1T2	*Rothia mucilaginosa* DSM20746	4.87	6.52
D1P3	*Actinomyces oris* ATCC27044	2.63	10.39
D2P3	*Rothia aeria* A1-17B	6.48	7.69
D1P5	*Rothia dentocariosa* ATCC17931	6.13	7.04
D1P4	*Rothia dentocariosa* ATCC17931	6.56	7.30
D1T11	*Rothia mucilaginosa* DSM20746	0.89	5.43
D4P1	*Rothia dentocariosa* ATCC17931	1.59	6.96
D1T6	*Rothia mucilaginosa* DSM20746	2.89	5.43
D1P16	*Rothia dentocariosa* ATCC17931	0.54	7.13
D1P6	*Rothia dentocariosa* ATCC17931	0.74	7.09
D2P2	*Rothia dentocariosa* ATCC17931	2.35	7.69

D1T3	*Rothia mucilaginosa* DSM20746	2.61	5.43
D4P11	*Rothia dentocariosa* ATCC17931	0.70	6.17
D4P9	*Rothia dentocariosa* ATCC17931	0.98	4.83
D5T8	*Rothia mucilaginosa* DSM20746	1.48	7.22
D4P8B	*Rothia dentocariosa* ATCC17931	0.93	4.02
D3T1	*Rothia dentocariosa* ATCC17931	1.02	4.87
D4P3	*Actinomyces viscosus* JCM8353	0.96	5.26
D1P11	*Rothia dentocariosa* ATCC17931	0.61	4.87
D1P8	*Rothia dentocariosa* ATCC17931	0.43	4.35
D5P5	*Actinomyces viscosus* JCM8353	1.04	3.91
D1T1	*Rothia mucilaginosa* DSM20746	2.02	5.17
D4P4	*Actinomyces viscosus* JCM8353	0.72	3.39
D4T8	*Actinomyces oris* JCM16131	0.91	1.20
D3T2	*Rothia mucilaginosa* DSM20746	0.50	1.76
Average (*SD*)	-	2.88 *(2.22)*	6.15 *(2.17)*

*Selection of final 10 nitrate-reducing isolates of interest

Supplementary Table 3: Annotation results using Prokka v1.13.3 for the ten sequenced and assembled genomes for this study.

Sample	Genes	Coding Regions	tRNA	rRNA	tmRNA
D1P7	2364	2304	49	8	1
D1P10	2190	2131	50	8	1
D1P15A	2194	2137	48	8	1
D1P17	2274	2216	49	8	1
D3T4	1879	1819	49	10	1
D4P7	2203	2144	50	8	1
D4T4	1878	1817	50	10	1
D4T6	1871	1811	49	10	1
D4T9	1899	1835	53	10	1
D5T11*	744	719	17	7	1

*Incomplete genome

Supplementary Table 4: Presence of possible Mobile Genetic Elements according to the ACLAME Database in the studied genomes of potential nitrate-reducing probiotics.

Sample	Gene Symbol	Gene Origin	Tax Name	Start	End	Identity (%)	Description
D1P7	erm(X)	plasmid	*Corynebacterium diphtheriae*	1.586.366	1.587.390	99.90	23S rRNA methyltransferase
D1P7	ermCX	plasmid	*Corynebacterium striatum*	1.586.536	1.587.298	99.87	23S rRNA methyltransferase
D1P7	ermLP	plasmid	*Corynebacterium striatum*	1.586.389	1.586.436	100	23S rRNA methyltransferase
D1P7	tnp1249	plasmid	*Corynebacterium striatum*	1.585.112	1.586.320	100	Transposase
D1P7	tnp1249	plasmid	*Corynebacterium striatum*	1.588.252	1.589.460	100	Transposase
D1P17	erm(X)	plasmid	*Corynebacterium diphtheriae*	603.130	604.153	99.80	23S rRNA methyltransferase
D1P17	ermCX	plasmid	*Corynebacterium striatum*	603.299	604.061	99.87	23S rRNA methyltransferase
D1P17	ermLP	plasmid	*Corynebacterium striatum*	603.152	603.199	100	23S rRNA methyltransferase

Supplementary table 5: nitrate reduced (%) by 10 selected isolated at 3 pH levels

	pH 6	pH 7	pH 7.5
D1P10	48.28	46.88	23.08
D1P15	50.00	52.54	43.33
D1P17	48.15	49.15	49.02
D1P7	51.72*	100.00*	100.00*
D3T4	34.48	61.29*	68.97*
D4P7	53.33*	46.43	48.28
D4T4	51.72*	55.17*	52.00*
D4T6	76.67*	65.63*	35.19
D4T9	58.62*	66.67*	57.14*
D5T11	48.28	55.17*	77.78*
MEDIAN	*50.86*	*55.17*	*50.51*

*Isolate reduced a percentage equal to or higher than the median at this pH level

Supplementary table 6: Percentage of isolates in biofilms of two donors (D6 and D11) at 5h of growth

Isolate	Condition	Donor 6		Donor 11	
		R. mucilaginosa (%)	*R. aeria* (%)	*R. mucilaginosa* (%)	*R. aeria* (%)
None	Control	2.33	0.06	5.11	0.03
	Nitrate	4.48	0.09	7.94	0.03
D1P7 *R. aeria*	Control	0.85	10.13	0.15	32.34
	Nitrate	2.64	17.49	0.36	41.09
D3T4 *R. mucilaginosa*	Control	22.08	0.02	37.15	0.00
	Nitrate	26.36	0.04	45.83	0.00
D4T4 *R. mucilaginosa*	Control	17.14	0.02	47.43	0.00
	Nitrate	27.95	0.04	58.63	0.00
D4T6 *R. mucilaginosa*	Control	15.23	0.03	33.74	0.00
	Nitrate	24.53	0.05	58.14	0.00
D4T9 *R. mucilaginosa*	Control	19.96	0.03	42.43	0.00
	Nitrate	34.12	0.04	54.53	0.00
D5T11A *R. mucilaginosa*	Control	5.14	0.04	35.15	0.00
	Nitrate	14.43	0.04	40.72	0.00

2.8 Data availability

The Supplementary Spreadsheet for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.555465/full#supplementary-material. The genomes of the isolates are publicly available and can be found in NCBI, under accession number PRJNA658327.

Chapter 3

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3. A single dose of nitrate increases resilience against acidification derived from sugar fermentation by the oral microbiome

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3.1 Abstract

Tooth decay starts with enamel demineralization due to an acidic pH, which arises from sugar fermentation by acidogenic oral bacteria. Previous *in vitro* work has demonstrated that nitrate limits acidification when incubating complex oral communities with sugar for short periods (e.g., 1-5 h), driven by changes in the microbiota metabolism and/or composition. To test whether a single dose of nitrate can reduce acidification derived from sugar fermentation *in vivo*, 12 individuals received a nitrate-rich beetroot supplement, which was compared to a placebo in a blinded crossover setting. Sucrose-rinses were performed at baseline and 2 h after supplement or placebo intake, and the salivary pH, nitrate, nitrite, ammonium and lactate were measured. After nitrate supplement intake, the sucrose-induced salivary pH drop was attenuated when compared with the placebo ($p < 0.05$). Salivary nitrate negatively correlated with lactate production and positively with ΔpH after sucrose exposure ($r= -0.508$ and 0.436, respectively, both $p < 0.05$). Two additional pilot studies were performed to test the effect of sucrose rinses 1 h ($n = 6$) and 4 h ($n = 6$) after nitrate supplement intake. In the 4 h study, nitrate intake was compared with water intake and bacterial profiles were analysed using 16S rRNA gene Illumina sequencing and qPCR detection of *Rothia*. Sucrose rinses caused a significant pH drop ($p < 0.05$), except 1 h and 4 h after nitrate intake. After 4 h of nitrate

intake, there was less lactate produced compared to water intake ($p < 0.05$) and one genus; *Rothia*, increased in abundance. This small but significant increase was confirmed by qPCR ($p < 0.05$). The relative abundance of *Rothia* and *Neisseria* negatively correlated with lactate production ($r = -0.601$ and -0.669, respectively) and *Neisseria* positively correlated with pH following sucrose intake ($r = 0.669$, all $p < 0.05$). Together, these results show that nitrate can acutely limit acidification when sugars are fermented, which appears to result from lactic acid usage by nitrate-reducing bacteria. Future studies should assess the longitudinal impact of daily nitrate-rich vegetable or supplement intake on dental health.

3.2 Introduction

When sugars are fermented by the oral microbiota, lactate and other organic acids are produced that can decrease the local pH [22]. The presence of sugars and acidic conditions select for saccharolytic and acid-tolerant microorganisms, increasing the fermentation capacity of the microbial community. This leads to the formation of a vicious circle that can result in the acidification of dental plaque to pH levels at which enamel is demineralized (approximately pH 5.5) [136]. When demineralization exceeds remineralization, a caries lesion can develop (reviewed in reference[4]). In the early stages, caries can be arrested or remineralized with preventive non-invasive therapy (e.g., changes in dietary practices or fluoride availability)[4,226]. However, in the later stages when the microbial activity has cavitated the lesion, the damage is mostly irreversible, and the lesion can only be restored through operative therapy.

According to the Global Burden of Disease 2017, untreated caries in permanent teeth was the most common health condition among those evaluated[1]. It is estimated that 2.3 billion people suffer from caries of permanent teeth and 530 million children from caries of primary teeth. As such, the cost of treating caries is expensive and believed to consume 5-10% of healthcare budgets in industrialized countries[2,3].

To reduce the global health and financial burden of caries, preventative care is essential. Two behavioural changes that are recommended to prevent caries are reducing the frequency of fermentable sugar intake and brushing twice daily with fluoridated toothpaste[226]. Fluoride increases enamel remineralization and

resistance against acidic conditions[4,136], whilst inhibiting bacterial carbohydrate fermentation and thereby limiting acidification[227]. Another preventive approach is using arginine as a prebiotic substrate that oral bacteria convert to ammonia – a weak base that increases the local pH[60]. Combining arginine and fluoride has been found to be more efficient at preventing plaque acidification in the presence of sugar when compared with fluoride alone [81].

A second potential anti-caries prebiotic is nitrate, but current *in vivo* evidence is limited[136]. It is estimated that humans obtain more than 80% of dietary nitrate from vegetables – a food group unequivocally associated with health benefits[87,89,122,223]. The salivary glands contain electrogenic sialin $2NO_3^-/H^+$ transporters that concentrate plasma nitrate into the saliva[112], leading to high salivary nitrate concentrations (100–500 μM during fasting, which is ~10x higher than in plasma, and 5-8 mM after a nitrate-containing meal)[25,131]. The human body is incapable of metabolizing nitrate, however, certain oral bacteria can effectively reduce salivary nitrate to nitrite[49]. Some of this nitrite is swallowed and converted into nitric oxide (an antimicrobial and bioactive molecule) by enzymatic and non-enzymatic processes inside the human body[25]. This process is called the nitrate-nitrite-nitric oxide pathway and is associated with various cardiovascular and metabolic benefits, including blood pressure reduction and antidiabetic effects[25,122].

Along with systemic benefits, nitrate appears to contribute to oral health. Salivary nitrate and the nitrate reduction capacity of the oral microbiota have been shown to negatively correlate with caries incidence[63]. Additionally, physiologically relevant nitrate concentrations (1–8.5 mM) can attenuate a pH drop when sugars are fermented by complex oral communities *in vitro*[63,190,228]. In a recent *in vitro* study, nitrate decreased the levels of cariogenic genera and lactic acid production, whilst increasing nitrite and ammonium production and limiting a pH drop caused by sugar fermentation[190]. These results may be explained by the use of lactic acid by nitrate-reducing bacteria during denitrification and the reduction of nitrite to ammonium, and/or the bacterial production of antimicrobial nitric oxide through denitrification[49,63]. However, these mechanisms have not been confirmed *in vivo*.

The aim of our current study was therefore to test whether nitrate limits pH acidification when sugar is consumed and study the potential mechanisms involved in this process *in vivo*. The pH buffering effect of nitrate has been observed as soon as 1 h after incubation *in vitro*[63]. Furthermore, the addition of the reduction product, nitrite, to *ex vivo* dental plaque of children limited acidification after 10 minutes of incubation[229]. We therefore hypothesized that a single dose of nitrate could acutely limit the oral pH drop that results from sugar consumption.

To test this, 12 individuals without active caries received a nitrate-containing supplement which was compared with a placebo in a crossover blinded setting. The individuals rinsed their mouth with a sucrose rinse before and 2 h after the supplement intake. To test the effect of supplement on acidification, salivary pH levels and concentrations of nitrate, nitrite, ammonium and lactate were measured. To confirm the pH buffering effect and study the time frame in which this process occurs, two additional pilot studies were performed to measure the effects 1 h and 4 h after supplementation and changes in microbiota composition were monitored. With this experimental protocol, we aimed to evaluate whether nitrate may be utilised to reduce oral acidification derived from sugar fermentation.

3.3 Materials and methods

3.3.1 Experimental supplements

For the main blinded crossover study (Figure 1, study 1), a soluble nitrate-rich supplement powder was used containing dry beetroot extract (*Beta vulgaris*, 3% nitrate), vitamin C and molybdenum (Supplementary Table 1a). The supplements were provided by NutriSpain S.A. (Llíria, Valencia, Spain). One 11.5 g dose contained 250 mg nitrate and the recommended daily amounts of Vitamin C (80 mg) and molybdenum (50 µg). This was compared to a placebo (Supplementary Table 1b), consisting of the same ingredients, but instead of nitrate-containing beetroot powder, it contained nitrate-poor orange powder (one 11.5 g dose contained <6 mg of nitrate). For the other two pilot studies (Figure 1, study 2 and 3), nitrate-containing beetroot extract was used without other ingredients. The powder supplements were weighted to obtain the desired dose of nitrate and dissolved in mineral water (Aguas Cortes) immediately before consumption.

3.3.2 Study population

Twelve adults who reported to be systemically healthy were recruited at the FISABIO Institute (Valencia, Spain) to participate in a blinded crossover study (study 1). Individuals could participate with healthy fillings, but were excluded if enamel breakdown or cavitation (i.e., caries with ICDAS scores >2)[230] was detected at the moment of sampling, which was visually assessed by an experienced dentist. Other exclusion criteria were the usage of antibiotics in the previous month and weekly usage of mouthwash.

Twelve additional adults were recruited if they reported not to have active caries during their last dental visit. These individuals were divided into two groups of six for the two pilot studies (study 2 and 3).

Unstimulated saliva samples were collected by drooling[144] in the morning. Individuals were instructed to avoid mouthwash usage in the week(s) of sampling and to have breakfast and brush their teeth with water at least 1 h before arrival on sampling days. For breakfast, they were asked to exclude any vegetable or fruit-derived products, as well as processed meats or other products containing nitrate as a preservative. If measurements were taken on two days, individuals were asked to have the same breakfast twice. All included subjects signed a written informed consent prior to their participation. The study protocol was approved on 2016/05/23 by the Ethical Committee of DGSP-FISABIO (Valencian Health Authority) with the reference BIO2015-68711-R2.

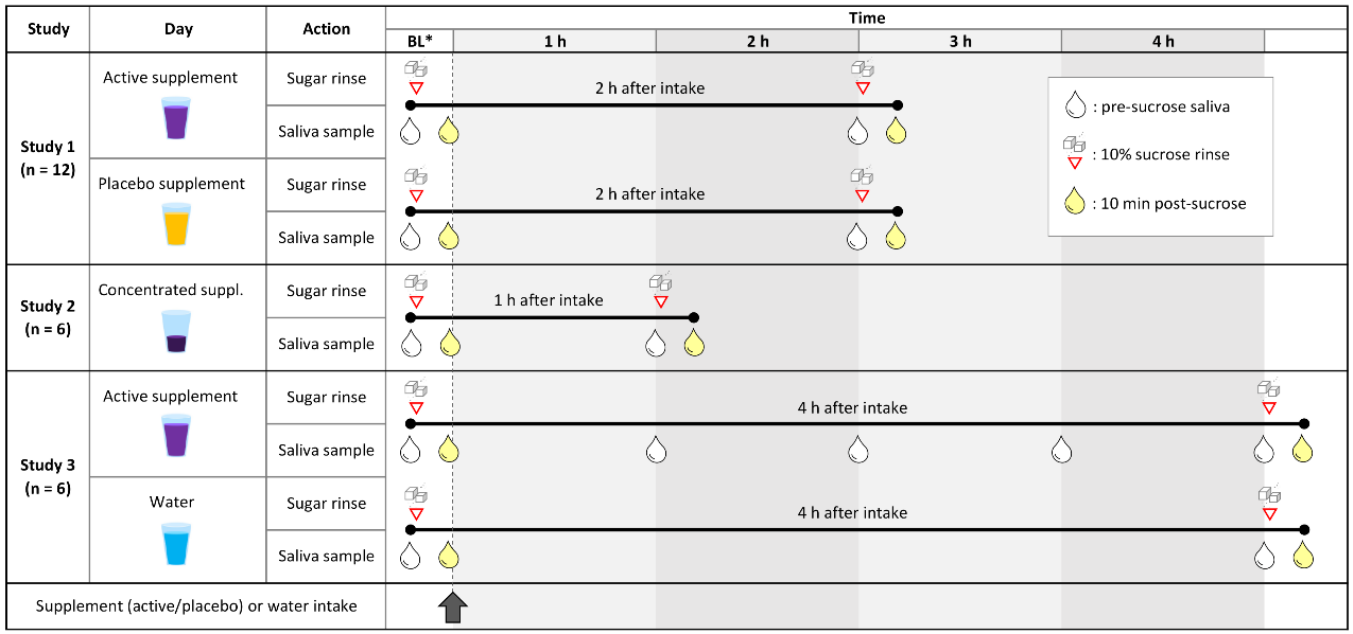


Figure 1. Design of the different clinical studies included in this article. Study 1: Cross-over study design in which 12 individuals received a nitrate-rich supplement (250 mg nitrate in 200 ml) on one day and a nitrate-poor placebo (<6 mg nitrate in 200 ml) on the same day a week earlier (individuals 1-6) or later (individuals 7-12). The effects of both supplements on sucrose rinses were compared. Study 2: Six individuals received a concentrated beetroot extract (300 mg nitrate in 70 ml) to compare the effect of a sucrose rinse before and 1 h after supplement intake. Study 3: Another six individuals received a nitrate rich-supplement (220 mg nitrate in 200 ml) and saliva was taken every hour to monitor changes in physiological parameters. Additionally, the effect of a sucrose rinse after 4 h of supplement intake was compared to a sucrose rinse 4 h after water intake (200 ml) on the previous day. BL = baseline (before supplement intake). Red triangles with sugar cubes show when 10% sucrose rinses were performed, and the drops represent saliva sampling. White drops are saliva samples taken right before a sucrose rinse or without a subsequent sugar, and yellow drops are 10-minute post-sucrose rinse saliva samples.

3.3.3 Sucrose rinses

Solutions were made of 10% sucrose (Laboratorios Conda, Madrid, Spain) in mineral water. Individuals were instructed to donate 2 ml of saliva (for pre-sucrose measurements), rinse their mouth with sucrose solution for 1 minute, sit down on a chair for 10 minutes, and donate another 2 ml of saliva (for post-sucrose measurements). In all studies, individuals were asked not to consume any food in the period between donating the first and the last saliva samples.

3.3.4 Study designs

Study 1: Effect of nitrate-rich supplement 2 h after intake compared to placebo (blinded crossover design)

Twelve individuals took a nitrate-rich supplement dissolved in mineral water (250 mg nitrate in 200 ml) and a nitrate-poor placebo dissolved in mineral water (<6 mg nitrate in 200 ml) in a blinded crossover setting (Figure 1, study 1). Specifically, 6 individuals took the active supplement on one day and the placebo on the same day a week later, while the other 6 individuals took the supplement and placebo in the reversed order. The two vegetable extracts had a different colour but participants were unaware of the active ingredient of interest (nitrate). On each day, a 10% sucrose rinse was performed at baseline (before supplement intake) and 2 h after supplement intake.

Study 2: Effect of nitrate-rich supplement 1 h after intake compared to baseline

In a first pilot study including six individuals, the immediate effect of a concentrated beetroot extract (300 mg nitrate in 70 ml) was tested 1 h after intake (Figure 1, study 2). A sucrose rinse was performed before (baseline) and 1 h after supplement intake.

Study 3: Effect of nitrate-rich supplement 4 h after intake compared to water intake

In a second pilot study including another six individuals, the effect of a beetroot extract (220 mg nitrate in 200 ml) was tested for 4 h after intake (Figure 1, study 3). A sucrose rinse was performed at baseline and 4 h after supplement intake. Additionally, 1 ml saliva was collected at 1 h, 2 h and 3 h after supplement intake to monitor physiological parameters over time. As a control, the same 6

individuals consumed 200 ml of mineral water on another day and sugar rinses were performed at baseline and 4 h after water intake.

3.3.5 Physiological measurements in saliva

Salivary concentrations of nitrate and nitrite, and pH levels were measured with a Reflectoquant reflectometer (Merck Millipore, Burlington, Massachusetts, US) as described by Rosier et al.[190]. The concentration of ammonium in saliva was measured spectrophotometrically by the Nessler Method[206]. The concentration of lactate in saliva was measured with the Lactate Colorimetric/Fluorometric Assay Kit (BioVision, Milpitas, California, US) following the manufacturer's instructions. Accuracy of all procedures was confirmed by using standard solutions with known concentrations of the different compounds.

3.3.6 DNA isolation

DNA was isolated from 24 saliva samples of study 3, corresponding to the baseline and 4 h pre-sucrose samples of both days (supplement and water intake). To obtain a bacterial pellet, 250 µl were centrifuged and the supernatant was removed. The pellet was dissolved in 100 µl PBS and DNA was extracted from the samples using the MagNA Pure LC DNA isolation kit (Roche Diagnostics, Mannheim, Germany) with the addition of a chemical lysis step with an enzymatic cocktail containing lysozyme, mutanolysin and lysostaphin, following Dzidic et al. (2018) and Rosier et al. (2020a)[151,190]. DNA concentrations were measured using a QubitTM 3 Fluorometer (Thermofisher, Loughborough, UK).

3.3.7 16S rRNA gene Illumina sequencing

An Illumina amplicon library was prepared following the 16S rRNA gene Metagenomic Sequencing library preparation Illumina protocol (Part #15044223 Rev. A). The primer sequences used in this protocol were; 16S Amplicon 341F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 805R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC), which amplify the V3-V4 hypervariable regions of the gene. Following 16S rDNA gene amplification, DNA was sequenced with an Illumina

MiSeq Sequencer according to manufacturer's instructions using the 2x300 bp paired-ends protocol.

3.3.8 Taxonomic classification using DADA2

To process the paired-end fastq files, an amplicon sequence variant (ASV) table was obtained using the DADA2 pipeline (v1.8) in R[231]. In short, the forward and reverse reads were trimmed, removing the v5-v6 primer sequences and low-quality bases at the end of reads. Reads with any ambiguous N base or exceeding 5 expected errors were also discarded. The forward and reverse pairs were merged together, with a minimum overlap of 12 bases and a maximum mismatch of 1 base in the overlapping region, to obtain the single denoised variants. After chimeric variant removal, the final amplicon sequence variants (ASVs) were mapped onto the Homo sapiens genome (assembly GRCh38.p13), using Bowtie2[232] (v2.3.5.1), in order to remove reads from the host. The Silva database[233,234] (v138) was set as reference to assign taxonomy to each ASV. Genus classification was achieved using the DADA2 naive Bayesian classifier method. The ASVs with an assigned genus but without exact species, were aligned using the Blastn tool[235] (v2.10.0+) against the Silva database with a minimum of 97% of identity.

3.3.9 qPCR of *Rothia*

The total amount of *Rothia* cells in saliva were analysed through quantitative PCR (qPCR) amplification of the *Rothia* nitrate reductase *narG* gene. Primers sequences were designed to be specific for the *Rothia* genus, using conserved regions of *narG* from *Rothia mucilaginosa*, *R. dentocariosa* and *R. aeria*. The forward sequence was 5'-ACA CCA TYA AGT ACT ACGG-3' and the reverse 5'-TAC CAG TCG TAG AAG CTG-3'. Reactions of 20 µl were added per well of a qPCR plate, consisting of 10 µl of Light Cycler 480 SYBR Green I Master mix (Roche Life Science, Penzberg, Germany), 0.4 µl of each specific primer (10 µM), 6.7 µl water and 2.5 µl of template DNA (DNA isolated from saliva and pre-diluted to 2 ng/l). Each sample was added in duplicate and measurements were performed using a Light Cycler 480 Real-Time PCR System (Roche Life Science) with the following conditions: 95°C for 2 min, and 40 cycles of 95°C for 30 s, 60 °C for 20 s, and 72 °C for 25 s. Negative controls were added, as well as a standard curve, consisting of a series dilution of an equimolar DNA mix of three *Rothia* species (*R. mucilaginosa* DSM-20746, *R. dentocariosa* DSM- 43762, *R.*

aeria DSM-14556) quantified with a QubitTM 3 Fluorometer (Thermofisher). Based on genome sizes, the cell number was calculated, assuming a single copy of the *narG* gene per cell.

3.3.10 Data analysis

Physiological parameters and qPCR data at different time points were compared with SPSS (v27) using Wilcoxon sign rank tests. Correlations between physiological parameters were explored using Spearman-rho in SPSS.

The salivary microbiota composition of six individuals was determined before and 4 h after nitrate intake. For analysis of the bacterial composition, R programming language[159] was used as described by Johnston et al. (2021)[13]. In short, Wilcoxon sign rank (wilcox.test function) and Spearman's rho (cor.test function) tests were performed. Additionally, the Vegan library of R[160] was used for Adonis tests (Permutational Multivariate Analysis of Variance Using Distance Matrices) and the visualization of bacterial composition in a two-dimensional map using constrained correspondence analysis (CCA). For these analyses, a species or genus was included if it was present in 70% of the samples from at least one of the two groups (relevant for Wilcoxon tests) or from the given group under study (relevant for Spearman's correlations) with an abundance superior to ten times the smallest percentage above zero. Only cases of genera with a median abundance >0.1% were discussed. Data were visualized with GraphPad PRISM (v9).

3.4 Results

3.4.1 pH-buffering effect 2 h after nitrate intake compared to placebo

In the first study, including twelve individuals (5 males and 7 females, age 25-60), the consumption of a nitrate-rich beetroot supplement dissolved in mineral water (250 mg nitrate in 200 ml) significantly increased salivary nitrate and nitrite after 2 h ($p < 0.05$, Figure 2A and 2C, or see Supplementary Spreadsheet for all physiological parameters and participants' information). In contrast, 2 h after the placebo supplement dissolved in mineral water (<3 mg nitrate in 200 ml), nitrate levels had decreased significantly, whilst nitrite levels did not vary. Following sucrose rinses, the pH dropped significantly in all cases (Figure 2B). However, on average, the pH dropped 0.23 points less when using the nitrate-rich supplement compared with the placebo ($p < 0.05$).

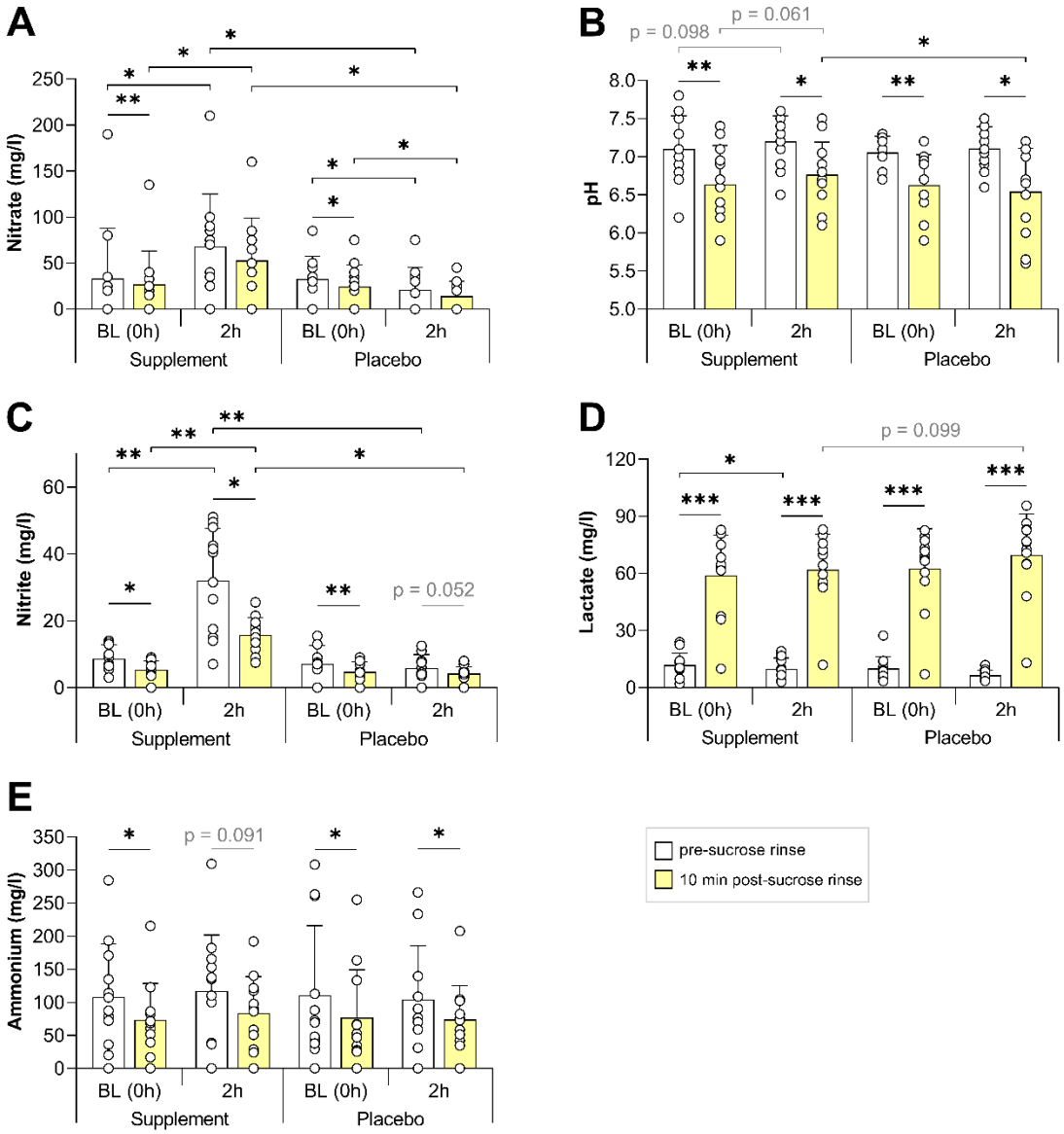


Figure 2. The effect of a nitrate-rich supplement on sucrose rinses 2 h after intake compared with a placebo. In 12 individuals, salivary nitrate (A), pH (B), nitrite (C), lactate (D) and ammonium (E) were measured. Sugar rinses were performed at baseline (BL, 0 h) on the two different days (supplement day and placebo day) and 2 h after intake of a nitrate-rich supplement (250 mg nitrate in 250 ml) or nitrate-poor placebo (<6 mg nitrate in 250 ml). Saliva samples were collected immediately prior to the sugar rinse (pre-sucrose, white bars) and 10 min after the sugar rinse (post-sucrose, yellow

bars). The bars and small white circles represent the averages and individual donor's data, respectively. Wilcoxon tests were used to compare the pre-sucrose with the post-sucrose measurements. Additionally, the BL pre- and post-sucrose measurement were compared with 2 h pre- and post-sucrose measurement, respectively. Finally, every measurement on the supplement day was compared with the same measurement on the placebo day. Significant changes (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$) and trends (grey text, $p = 0.05$ - 0.1) are shown.

There was also a trend for lower lactate concentrations 2 h after the nitrate supplement compared with the placebo (Figure 2D, $p = 0.099$). Furthermore, there was a trend for higher salivary pH 2 h after nitrate supplement intake compared with baseline, both in the pre-sucrose rinse pH ($p = 0.098$), and after the sucrose rinse ($p = 0.061$). These trends were not found when taking the placebo ($p = 0.439$ and $p = 0.339$, respectively).

Nitrite levels dropped after all sucrose rinses ($p < 0.05$ on the supplement day and $p = 0.052$ on the placebo day), indicating possible metabolization of this compound or dilution by rinsing, whilst nitrate only dropped after sucrose at the baseline rinses ($p < 0.05$). Lactate increased after all sucrose rinses ($p < 0.005$), whereas ammonium dropped significantly after all sucrose rinses ($p < 0.05$), except 2 h after nitrate supplement intake ($p = 0.091$, Figure 2E).

The change in pH after the sugar rinse (ΔpH) at 2 h negatively correlated with the lactate detected post-sucrose (Figure 3A) and with the the change in lactate after the sugar rinse (Δlactate, Figure 3B), indicating that lactate/lactic acid production is a good proxy for the magnitude of the pH drop. It is therefore interesting that the salivary nitrate (pre-sucrose) correlated negatively with the lactate produced ($r = -0.508$, $p < 0.5$, Figure 3D). In line with these results, the nitrate levels showed a significant positive correlation with the ΔpH ($r = 0.436$, $p < 0.05$, Figure 3C), indicating that nitrate availability at 2 h was associated with lower pH drops. No evidence of pH buffering by ammonium or ammonia production was obtained as changes in ammonium after the sugar rinse (Δammonium) did not correlate significantly with the ΔpH (Figure 3F). An unexpected positive correlation was found between salivary ammonium (pre-sucrose) and the lactate detected after sugar rinsing ($r = 0.552$, $p < 0.01$, Figure 3E). Similar correlations were observed when analysing the baseline

measurements together with measurements after supplement intake of all individuals that participated in the three studies of this manuscript (Supplementary Figure 1).

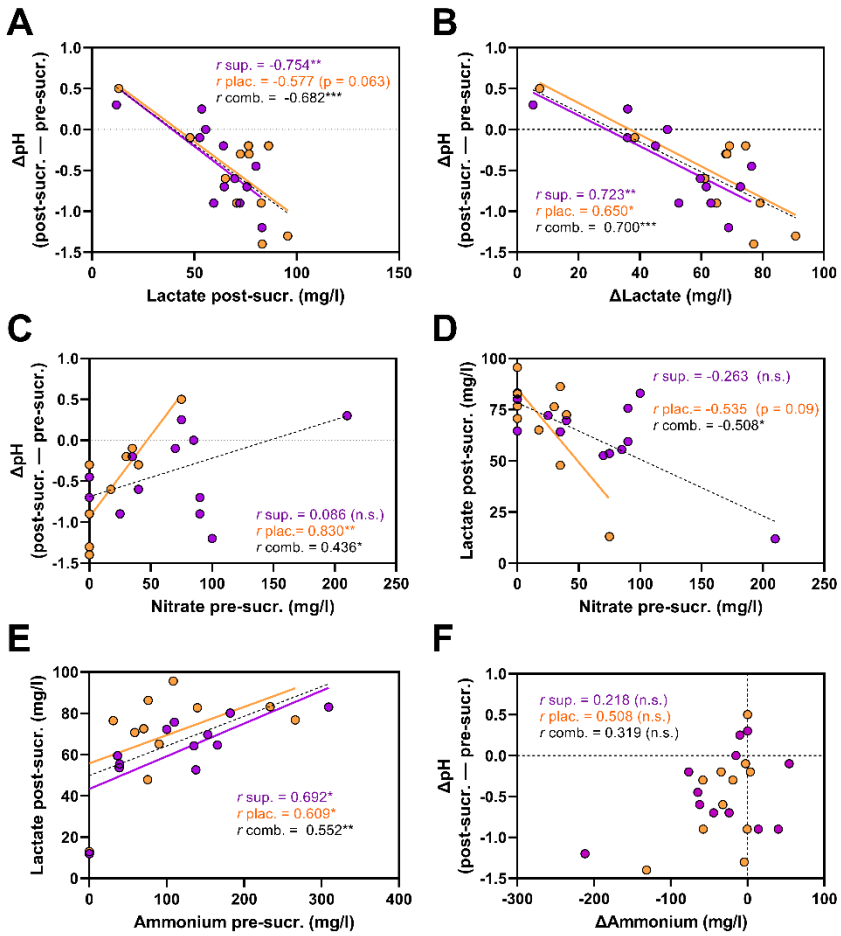


Figure 3. Correlations between physiological parameters 2 h after nitrate-rich supplement and placebo intake. In A-F, correlations between different physiological parameters are shown. The purple dots are measurements 2 h after nitrate-rich supplement intake (n = 12) and the orange dots 2 h after placebo intake on a different day (n = 12). The black dotted lines are the linear regression curves when combining the measurements after supplement and placebo intake (total n = 24). The ΔpH is the pH difference between the pre-sucrose measurement and the post-sucrose measurement (negative values are a pH drop). A: ΔpH and lactate detected post-sucrose. B: ΔpH and Δlactate. C: ΔpH and salivary nitrate (pre-sucrose) D: lactate detected post-sucrose and salivary nitrate (pre-sucrose). E: salivary ammonium (pre-sucrose) and lactate detected

post-sucrose. F: ΔpH and Δammonium. Abbreviations: sup. = supplement, plac. = placebo, comb. = combined, pre-sucr. = pre-sucrose measurements, post-sucr. = 10 min post-sucrose measurement. Spearman-Rho correlations (*r*) were calculated 2 h after supplement (sup.) or placebo (plac.) intake, or both combined (comb.). P-values and linear regression curves are shown if trends were found (p 0.05-0.1). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n.s. = not significant. Δ = post-measurement – pre-measurement.

3.4.2 Immediate pH-buffering effect 1 h after nitrate intake

In the second study, including six individuals (3 males and 3 females, age 24-46), the potential effect of a more concentrated nitrate supplement (300 mg nitrate in 70 ml) was tested only 1 h after intake (Figure 4). The sucrose rinses led to an average pH drop of 0.37 points at baseline, which was significant ($p < 0.05$, Figure 4A), and 0.23 points after supplement intake, which showed a trend towards significance ($p = 0.084$). However, there was some heterogeneity in this outcome: in 4 out of 6 participants, the nitrate supplement limited or fully prevented the pH drop caused by the sucrose rinse and in the other two participants, the pH drop was as strong as at baseline (Supplementary Spreadsheet). Lactate also increased significantly after the sucrose rinse at baseline ($p < 0.05$), but not 1 h after taking the supplement (Figure 4D). Ammonium levels dropped after the sugar rinses, but the difference was not significant (Figure 4E). When combining the data of the three studies in this manuscript, significant drops in nitrate, nitrite and ammonium after sucrose rinses were confirmed ($p < 0.05$, Supplementary Figure 3).

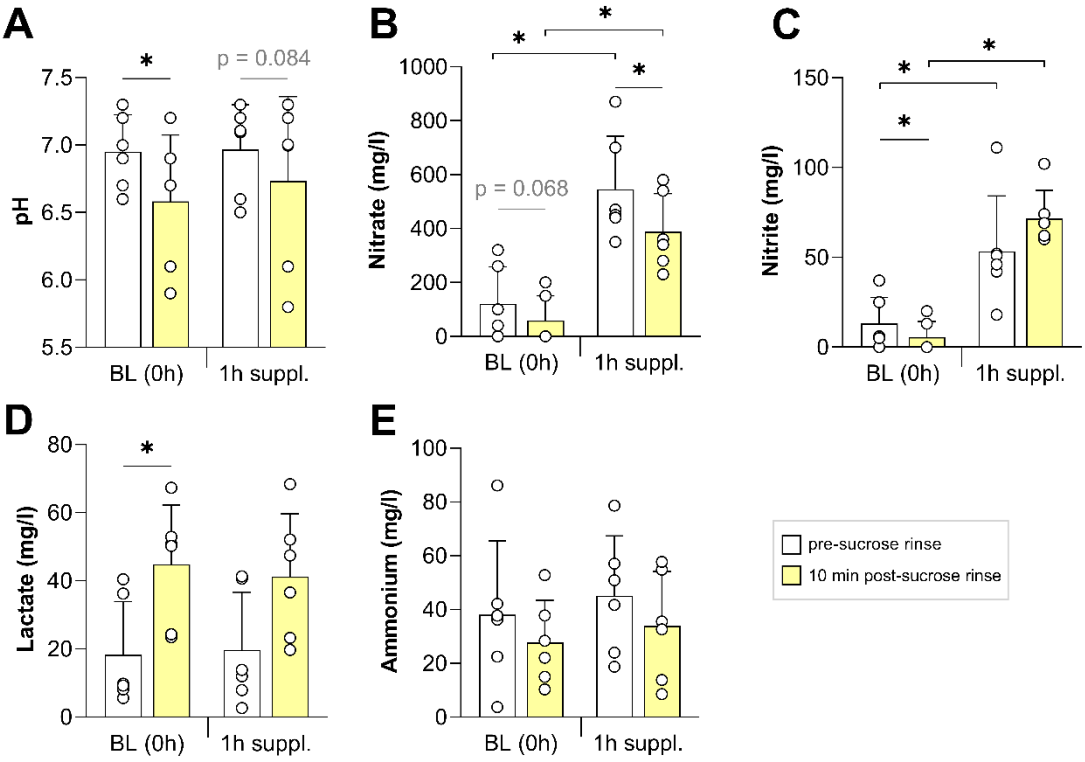


Figure 4. The effect of a concentrated nitrate-rich supplement on sucrose rinses 1 h after intake. In six individuals, the salivary pH (A) and concentrations of nitrate (B), nitrite (C), lactate (D) and ammonium (E) were measured. Sugar rinses were performed at baseline (BL, 0 h) and 1 h after intake of a nitrate-rich supplement (300 mg nitrate in 70 ml). Saliva samples were collected immediately prior to the sugar rinse (pre-sucrose, white bars) and 10 min after the sugar rinse (post-sucrose, yellow bars). The bars and small white circles represent the averages and individual donor data, respectively. Wilcoxon tests were used to compare the pre-sucrose with the post-sucrose measurements. Additionally, the BL pre- and post-sucrose measurement were compared with 2 h pre- and post-sucrose measurements, respectively. Significant changes (* $p < 0.05$) and trends (grey text, $p = 0.05$ - 0.1) are shown.

3.4.3 pH-buffering effect 4 h after nitrate intake compared to water intake

In the third study, including another six individuals (3 males, 3 females, age 25-33), the effect of a nitrate supplement (220 mg nitrate in 200 ml) on pH and the

salivary microbiota composition was tested 4 h after intake and this was compared with water intake. On the day of nitrate intake, saliva samples were collected every hour for physiological measurements, including nitrate, nitrite, pH, lactate and ammonium (Figure 5). After supplement intake, salivary nitrate and nitrite levels peaked at 1 h and stayed elevated over the entire study period of 4 h (Figure 5A and B, $p < 0.05$). In contrast, lactate levels dropped and stayed lower over the entire period compared with the pre-sucrose baseline measurement (Figure 5G). The pH was higher at all time points after supplement intake ($p < 0.05$ when comparing 0 h with 1-4 h, Figure 5E).

The salivary pH dropped significantly after a sucrose rinse at baseline and also 4 h after water intake (Figure 6B), and a trend ($p = 0.075$) towards a pH decrease was observed 4 h after the nitrate-rich supplement intake. Four hours after nitrate supplement ingestion, there was also a trend ($p = 0.072$) towards the post-sucrose pH being less acidic, whereas lactate production was significantly lower ($p < 0.05$), compared with water intake.

Regardless of supplement or water intake, the pre-sucrose salivary pH increased after 4 h (both $p < 0.05$, Figure 6), which may be due to the natural buffering effect of saliva over time after breakfast. Specifically, 4 h after supplement intake, the pH increased from an average of 6.98 (SD 0.66) to 7.32 (SD 0.41), whilst 4 h after water intake, it increased from 6.95 (SD 0.63) to 7.18 (SD 0.55).

3.3.4 Changes in microbiota composition 4 h after nitrate intake

Preliminary data on bacterial composition changes was obtained by comparing the salivary communities of six individuals before (0 h) and 4 h after nitrate supplement or water intake (Figure 7). The general bacterial community structure was not affected significantly by the nitrate supplement compared to water intake (no significant changes based on Adonis and CCA p-values, Supplementary Figure 2). There was a trend towards *Rothia* being elevated 4 h after nitrate supplement intake compared with water intake ($p = 0.063$, Figure 7B). When comparing 0 h and 4 h ($p < 0.05$), *Rothia* increased significantly after supplement intake and showed a trend of increase following water intake ($p = 0.063$). A larger increase in *Rothia* cells after nitrate supplement intake was confirmed by qPCR ($p < 0.05$, Figure 7C).

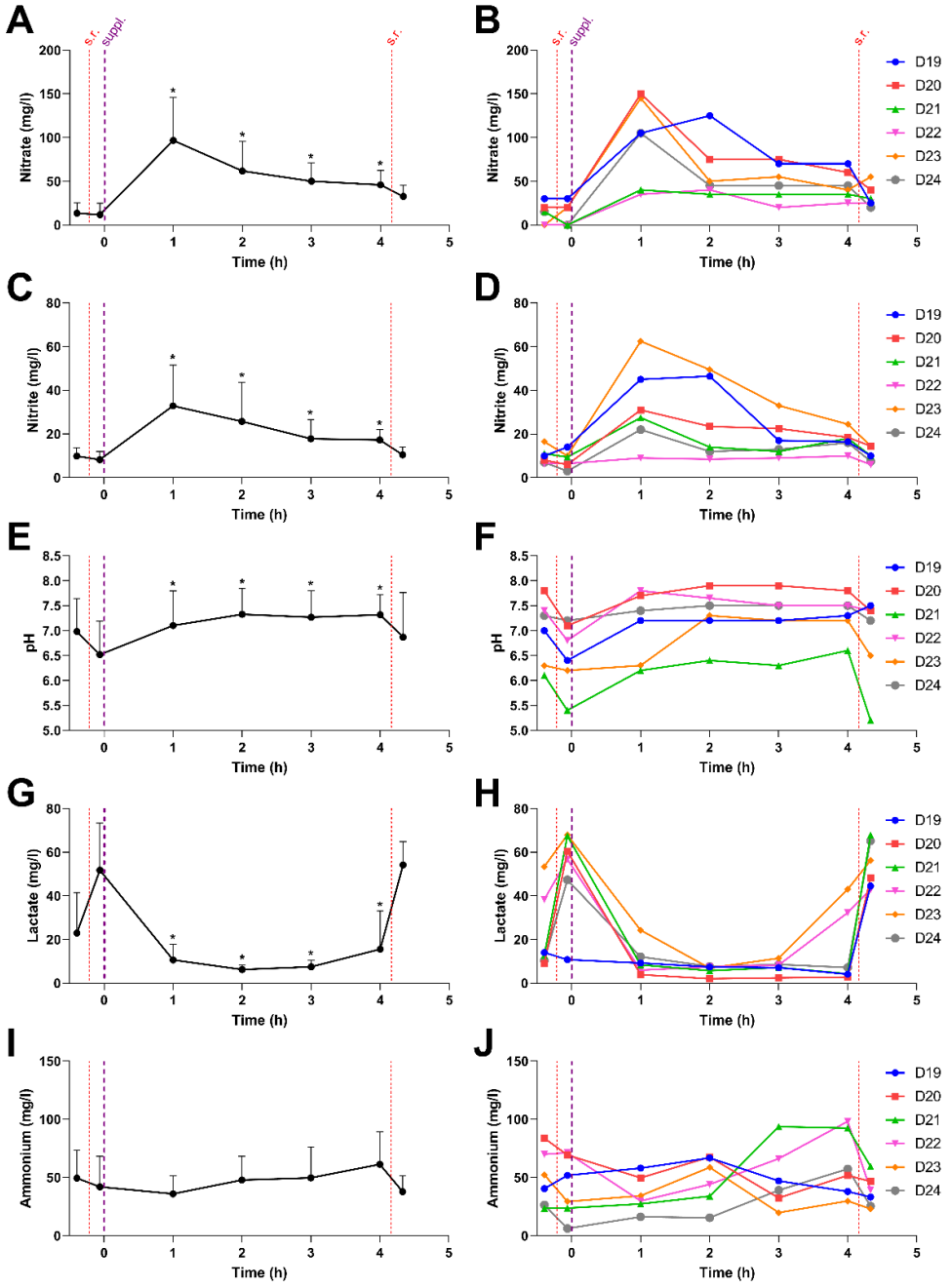


Figure 5. Changes in physiological parameters during the 4 h after taking a nitrate-rich supplement. In this figure, the salivary nitrate (A-B), nitrite (C-D), pH (E-F), lactate (G-H) and ammonium (I-J) are shown over time. The red lines are sugar rinses dividing pre- and post-sucrose measurements at baseline (0 h) and after 4 h of supplement intake. The

thicker purple line represents when the nitrate-rich supplement was consumed. On the left side, the averages are shown with standard deviations. The time points 1 h, 2 h, 3 h and 4 h (pre-sucrose) were compared to 0 h (pre-sucrose). * = compared to 0 h (pre-sucrose), the p-value was < 0.05. On the right side, the individual donors are shown. S.r. = sugar rinse. Suppl. = supplement intake. In Figure 6, the pre- and post-sucrose measurements are compared.

On a species taxonomic level (Supplementary Figure 4A), *R. dentocariosa*, increased significantly from 0 h to 4 h after nitrate supplement intake ($p < 0.05$), but not after water intake (Supplementary Figure 4C). Another *Rothia* species, *R. mucilaginosa* increased significantly from 0 h to 4 h after both nitrate supplementation and water intake (both $p < 0.05$, Supplementary Figure 4B). However, there was a trend of *R. mucilaginosa* being more abundant 4 h after supplementation compared with water intake ($p = 0.063$), whilst two low abundance species (median abundance <0.15%); *Prevotella salivae* and *Corynebacterium durum*, were significantly less abundant ($p < 0.05$, Supplementary Figure 4D and 4E).

When combining the measurements from all samples collected 4 h after supplement and water intake, the relative abundance of the *Rothia* and *Neisseria* negatively correlated with lactate production post-sucrose ($r = -0.601$ and -0.669, respectively, both $p < 0.05$, Figure 7D). *Neisseria* also positively correlated with the post-sucrose pH ($r= 0.621$, $p < 0.05$), but not with the pre-sucrose pH (Figure 7E). *Peptostreptococcus*, which had a median abundance of only 0.22% after 4 h of supplement and water intake, positively correlated with post-sucrose lactate production ($r = 0.734$, $p < 0.01$) and negatively correlated with post-sucrose pH ($r = -0.825$, $p < 0.001$, Supplementary Spreadsheet).

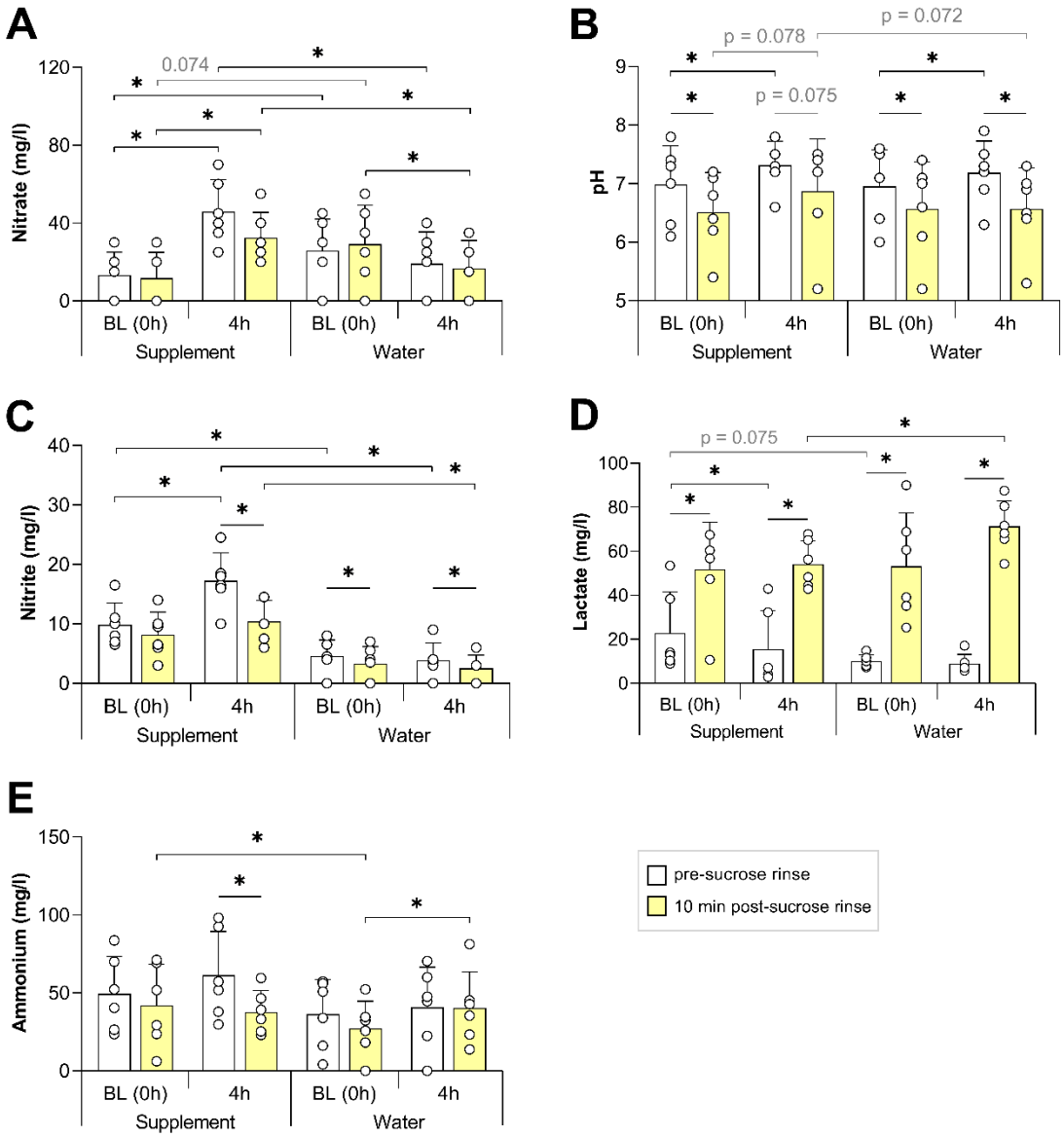


Figure 6. The effect of a nitrate-rich supplement on sucrose rinses 4 h after intake compared with water. In 6 individuals, salivary nitrate (A), pH (B), nitrite (C), lactate (D) and ammonium (E) were measured. Sugar rinses were performed at baseline (BL, 0 h) of the two different days (supplement day and water day) and 2 h after intake of a nitrate-rich supplement (220 mg nitrate in 200 ml) or water (200 ml). Saliva samples were collected immediately prior to the sugar rinse (pre-sucrose, white bars) and 10 min after the sugar rinse (post-sucrose, yellow bars). The bars and small white circles represent the averages and individual data, respectively. Wilcoxon tests were used to

compare the pre-sucrose with the post-sucrose measurements. Additionally, the BL pre- and post-sucrose measurements were compared with 2 h pre- and post-sucrose measurements, respectively. Finally, every measurement of the supplement day was compared with the same measurement on the water day. Significant changes (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$) and trends (grey text, $p = 0.05$) are shown.

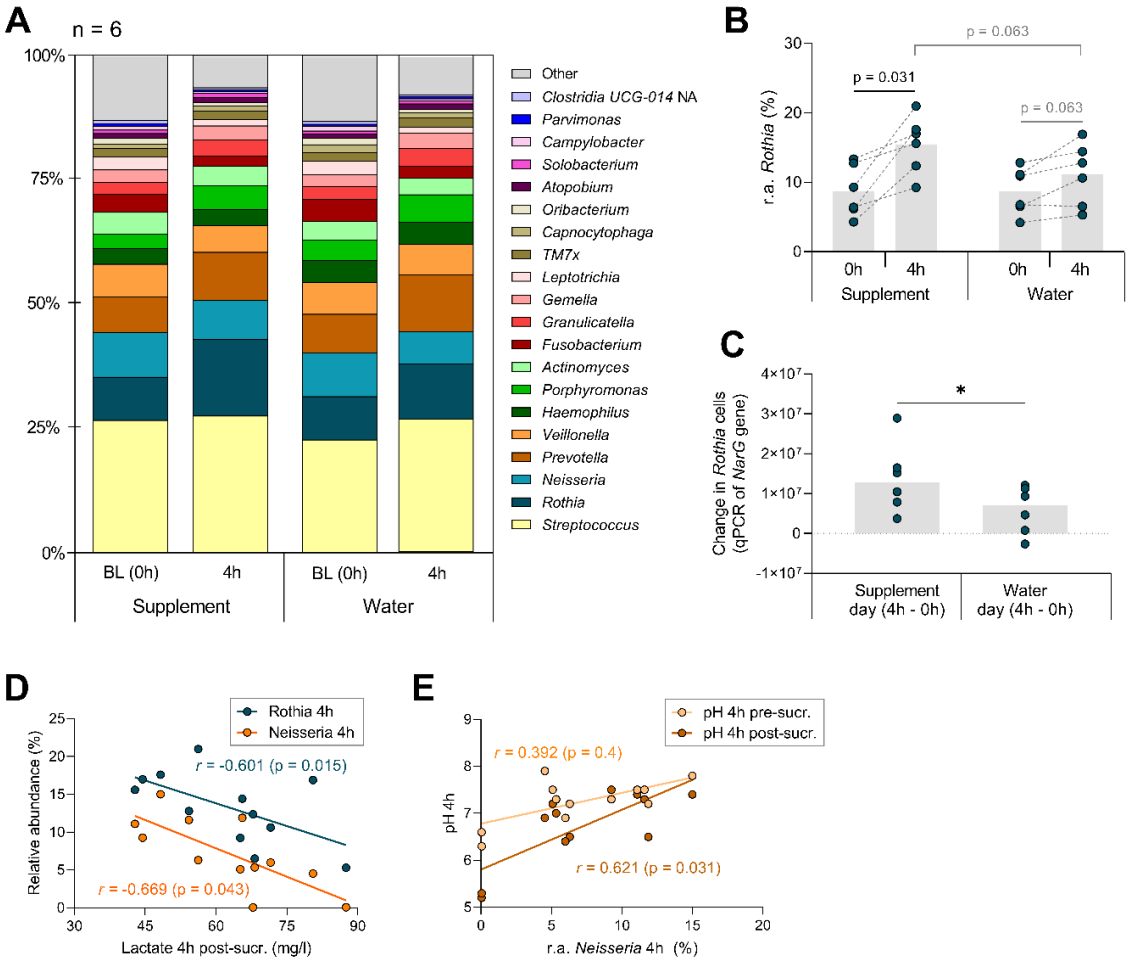


Figure 7. Genus level analysis of study 3 (comparing a nitrate-rich supplement intake with water intake). In A, the relative abundance of the genera detected in the 6 individuals of study 3 are represented in bar charts. Based on the median, the 20 most abundant genera are shown. The grey boxes with "other" contain genera < 0.34% abundance grouped together. In B, the relative abundance (r.a.) of the genus *Rothia* is shown, which showed the strongest trend towards an increase when comparing BL (0 h)

with 4 h after nitrate-rich supplement intake (p value = 0.031, adjusted p-value = n.s.). The grey bars represent the averages and the dark cyan dots the data from individual participants. The higher increase of *Rothia* after taking the supplement compared with water intake was confirmed by qPCR in C: the increase of *Rothia* cells from 0 h to 4 h, based on the number of copies of the *Rothia* nitrate reductase *NarG* gene, was higher after taking the supplement than after taking water (*p < 0.05). D: the measurements 4 h after water intake and 4 h after supplement intake were combined (n = 12) and a lactate post-sucrose correlated negatively with the abundance of *Rothia* and *Neisseria*. In E, the correlation with *Neisseria* between pH pre-sucrose and pH post-sucrose is shown. Because of the small sample size, unadjusted p-values are shown.

3.5 Discussion

Our data show that a single dose of nitrate around the acceptable daily intake (i.e., 3.7 mg/kg, which would imply 222-296 mg for an adult of 60-80 kg) can attenuate acidification following sugar fermentation by the oral microbiota in individuals with good dental health (without enamel breakdown or untreated cavities). The different studies presented in the current manuscript indicate that increased protection against sucrose can happen as early as 1 h, and last for at least 4 h after nitrate-rich beetroot supplementation. We identify that, in this timeframe, the main pH buffering mechanism after sucrose rinsing was lactic acid usage. Preliminary evidence was also obtained suggesting that the bacteria involved in pH buffering are *Neisseria* and *Rothia* – known nitrate reducers – as their abundance in saliva was negatively associated with lactate production after sucrose rinsing. *Rothia* appears to react quickly to nitrate, increasing in number 4 h after nitrate intake, whereas *Neisseria* may act at a transcriptomic level.

In total, 24 healthy individuals participated in the three studies, performing mouth rinses with a 10% sucrose solution at baseline (0 h) and 1 h (n = 6), 2 h (n = 12) or 4 h (n = 6) after nitrate-rich beetroot supplementation. Immediately prior to sucrose rinsing (pre-sucrose) and 10 minutes after (post-sucrose), salivary pH was measured to determine the sucrose-induced pH drop (ΔpH). In all samples, the lactate produced post-sucrose showed a strong negative correlation (p < 0.0001) with the ΔpH, confirming its role in acidification after sugar rinsing[236].

In the 12 individuals for which pH was monitored 2 h after nitrate intake, the effect of the sucrose rinse was compared to a sucrose rinse 2 h after placebo intake on another day (blinded cross-sectional design). After nitrate rich-supplementation, the post-sucrose pH drop was significantly lower ($p < 0.05$), and there was a trend towards less lactate production post-sucrose ($p = 0.099$), compared with placebo intake. Specifically, the pH drop was limited by 0.23 points on average, which could prevent or reduce the time during which enamel is exposed to critical, demineralizing pH levels (around pH 5.5) in some individuals. In our study, excluding caries active patients, only 3 out of 84 sucrose rinses caused a salivary pH decrease below pH 5.5. Therefore, ensuing studies would benefit from studying these effects in a population with active caries, as well as focusing on plaque pH, which is expected to undergo a larger pH drop. In the individuals without active caries in our current manuscript, salivary nitrate correlated negatively with lactate production and positively with the ΔpH (the more nitrate, the smaller the pH drop) (both $p < 0.05$). This data supports previous *in vitro* studies showing that nitrate (at concentrations found in saliva) prevented or limited a pH drop when incubating oral communities with sugar or sugar-rich medium during periods of 1 to 9 h[63,190,228].

In the six individuals monitored for 4 h after nitrate intake, the effect of the sucrose rinse was compared with a sucrose rinse 4 h after water intake on the previous day. Compared with water intake, there was significantly less lactate produced post-sucrose ($p < 0.05$), and a trend was observed for a lower post-sucrose pH drop ($p = 0.072$) after nitrate intake. Lactate is the ion of lactic acid, which is the main organic acid involved in caries development[4]. A decrease of lactate can result from nitrate-reducing bacteria which use lactic acid as an electron donor and carbon source during nitrate reduction[237]. The negative correlation between salivary nitrate pre-sucrose and the lactate produced post-sucrose in this study ($p < 0.05$) suggests that lactic acid was used in the presence of nitrate. In a previous *in vitro* study, we incubated saliva in sugar-rich medium and observed that nitrate (6.5 mM = 403 mg/l) decreased the lactate production and prevented a pH drop after 5h and 9h of incubation[190]. In another recent clinical study, the use of chlorhexidine mouthwash impaired nitrate-reduction, whilst increasing lactate production and decreasing the pH buffering capacity of saliva[238]. This indicates that nitrate reduction is an important regulator of lactic acid levels in the oral cavity, a hypothesis which fits with environmental

microbiology studies, where nitrate reduction has also found to increase lactic acid usage [63,127,139]. However, it cannot be excluded that (in parallel) lactic acid production is decreased (e.g., by inhibition of lactic acid-producing bacteria or enzymes) and this possibility should be tested in future *in vitro* studies.

In our previous *in vitro* study, we also observed an increase in *Rothia* and *Neisseria* 5 h after incubating saliva with 6.5 mM nitrate[190]. Four hours after nitrate intake in our current manuscript, a significantly larger increase in *Rothia* cells (measured with qPCR) was observed when compared with water intake ($p < 0.05$). Compositional data, determined by 16S rRNA gene Illumina sequencing in the samples of six individuals, showed similar trends: an increase in the genus *Rothia* and the species *R. mucilaginosa* and *R. dentocariosa* after nitrate intake. However, only moderate *Rothia* increases were detected after 4 h and therefore we expect that an important fraction of this immediate pH buffering mechanism is due to transcriptomic changes. Previous clinical studies have found higher levels of *Rothia* and *Neisseria* after 10 days[135], and *Rothia mucilaginosa* and *Neisseria flavescens* after 6 weeks[83], of daily nitrate-rich beetroot juice intake. Additionally, Vanhatalo and colleagues found that after 10 days, *Veillonella* and *Prevotella* had decreased[135]. In our study, we did not find an increase in *Neisseria*, nor decreases in any genera. Only two species, *Prevotella salivae* and *Corynebacterium durum*, appeared to be less abundant in saliva 4 h after nitrate intake compared with water intake. Thus, preliminary composition data of our study suggest that an initial increase in *Rothia* can happen as soon as 4 h after intake of the first nitrate dose, whereas *Neisseria* and other taxonomic changes may require more time to develop in the presence of nitrate. To better understand the response to sugar in the presence of nitrate, future clinical studies should focus on metagenomic and metatranscriptomic changes directly measured in dental plaque. Additionally, the accumulative effect of nitrate should be determined when taking daily doses over several days. Regarding this, pH buffering effects were observed after 2-4 weeks of daily toothbrushing with an arginine-containing dentifrice[206], suggesting that continuous exposure to arginine induced an ecological shift in plaque bacterial populations, and future studies should address this possibility in relation to nitrate exposure and oral health parameters.

The relative abundance of *Rothia* and *Neisseria* in saliva negatively correlated with the lactate produced after sugar rinsing. Additionally, the levels of *Neisseria* positively correlated with the post-sucrose pH, but not with the pre-sucrose pH. This indicates that *Rothia* and *Neisseria* may be involved in the nitrate-mediated pH buffering mechanism observed throughout the current study. We recently showed that oral communities to which a *Rothia* isolate (*R. mucilaginosa* or *R. aeria*) was added produced less lactate in the presence of nitrate *in vitro* [228]. This nitrate-dependent difference was not found in the same oral communities without *Rothia*, indicating that the added *Rothia* consumed lactic acid in the presence of nitrate. Likewise, Wicaksono and colleagues found that nitrate-reduction by *Veillonella* atypica or *Veillonella parvula* was linked to lactate usage[237]. Interestingly, both *Rothia* and *Neisseria* have been associated with oral disease-free individuals (previously discussed in references[136,190,228]), including caries-free individuals[27,172,173], and their ability to use lactic acid may be linked with dental health.

In a recent and pioneering clinical study, Burleigh et al. (2020) showed that a single high dose of nitrate (770 mg) in the form of concentrated beetroot juice limited acidification 5-15 minutes after intake of a citric acid- and sugar-rich sport drink, compared to a nitrate-depleted beetroot juice[239]. In their study, the acidification induced by the sport drink was likely a combination of the effects of citric acid and sugar. The sucrose rinses in our study consisted of mineral water with 10% sucrose, confirming that nitrate limited pH drops caused by sucrose fermentation. In our pilot experiment with six individuals, we showed that whilst the pH dropped significantly by 0.37 points after sucrose rinsing ($p < 0.05$), 1 h after a nitrate rich supplement (300 mg nitrate) intake, the pH decrease reached only 0.23 points after sucrose rinsing ($p = 0.084$). Similarly, the post-sucrose lactate significantly increased prior to nitrate-rich supplement intake ($p < 0.05$), but did not 1 h after intake ($p = 0.116$). This indicates that immediately after the supplement intake, nitrate can induce resilience against lactic acid production when sugar is consumed, limiting a pH drop in some individuals.

Along with lactic acid usage, denitrification and the reduction of nitrite to ammonium may contribute to the pH buffering effect of nitrate by proton consumption or the possible formation of ammonia (a weak base)[49,63]. This idea

is supported by our previous *in vitro* data, showing elevated ammonium production after 5h and 9h by oral biofilm communities in the presence of nitrate[190]. In the current manuscript, we did not find a significant increase of salivary ammonium in any of the clinical studies. In all participants, the ammonium detected at baseline was 75.99 mg/l, while 1-4 h after nitrate supplement intake, it was 85.23 mg/l, but this difference was not significant ($p = 0.114$, Supplementary Figure 3E). Additionally, 10 minutes after sugar rinsing, we did not detect an increase in salivary ammonium in individuals who consumed a nitrate-rich supplement. Thus, in these *in vivo* conditions, ammonium production did not appear to be a main pH buffering mechanism in saliva. It is possible that ammonium is produced and accumulates in dental plaque as DNRA is usually a strictly anaerobic process[49], but may be diluted in saliva by salivary clearance and swallowing. Additionally, ammonium production may be an important buffering mechanism in saliva with different amounts and/or frequencies of nitrate intake. For example, Burleigh and colleagues (2019) observed an increase in salivary pH after 7 days of daily nitrate-rich beetroot juice supplementation[163]. In future clinical experiments, ammonium should be measured after long-term (chronic) nitrate consumption to elucidate its role in salivary pH elevation.

Instead of finding a significant increase in ammonium after nitrate consumption as hypothesized, an unexpected strong correlation was found between ammonium in saliva (pre-sucrose) and lactate produced after sucrose. This correlation was strong and consistent in different samples ($p < 0.05$). In addition to ammonium production by DNRA, ammonia can be produced by proteolysis, and through metabolism of arginine and urea[60]. Thus, lactate and ammonium/ammonia may be linked by lactic acid producing bacteria (e.g., *Streptococcus*, *Lactobacillus* and *Actinomyces*) which can convert arginine into ammonia[60]. Additionally, lactic acid can be used during, and stimulate, DNRA[240]. We hypothesize that the salivary ammonium/ammonia levels under the *in vivo* conditions of this study may partly reflect the amount of lactic acid producing bacteria, or that they were metabolically linked to lactate in a different way. In dental plaque, where the clearance effect of saliva will be limited, ammonium could accumulate locally with time as previously observed *in vitro*[190].

The acceptable daily intake of nitrate is currently at 3.7 mg/kg of body weight [241]. This limit is set because under certain conditions nitrate can form N-nitroso compounds, some of which cause cancer. This has been reported on processed meats to which nitrate salts are added as preservatives, resulting from bacterial reduction of nitrate to nitrite in meat and chemical reactions of nitrite with meat molecules[186,187]. These chemical reactions are further stimulated in the acidic stomach. Another source of nitrate is drinking water and high nitrate levels in water, resulting from agricultural contamination, have been associated with cancer and other adverse health effects[242]. However, we obtain over 80% of nitrate from vegetables, which are considered protective against cancer and other diseases[87,89,223]. This includes nitrate-rich vegetables such as lettuce and spinach, which are considered protective against different types of cancer (mouth, pharynx, larynx, oesophagus and stomach) [88]. Therefore, nitrate outside of its natural context appears to be potentially harmful, whereas eating vegetables which provide the most nitrate, is beneficial for health and reduces the risk of cancer. Natural combinations of anti-oxidants and polyphenols in vegetables (and fruits) prevent N-nitroso compound formation and possibly damage[188,189,224,225]. Future assessments of nitrate ADI should take the source of nitrate into account.

Limitations of this work include the small number of participants for different experiments, the exclusion of caries active patients, the analysis of saliva as the only oral sample and monitoring only *Rothia* among nitrate-reducing bacteria by qPCR. Future studies should confirm these results in dental plaque samples of a larger group of participants, including caries active patients, ideally under fasting conditions to avoid the effect of dietary nitrate and other food components on physiological outcomes. Additionally, changes in other nitrate-reducing species (e.g., representatives of *Neisseria*) should be measured by qPCR for quantitative assessment. It should also be noted that the effect of nitrate was tested in combination with other potentially active molecules present in beetroot, such as polyphenols or antioxidants, and therefore nitrate from other vegetable sources or nitrate salts could have a different effect. Along with the acute protective effects of nitrate, long term nitrate intake should be studied as nitrate-reducing genera and enzymes may increase over time. The resilience against acidification when sugars are fermented could thus increase over time

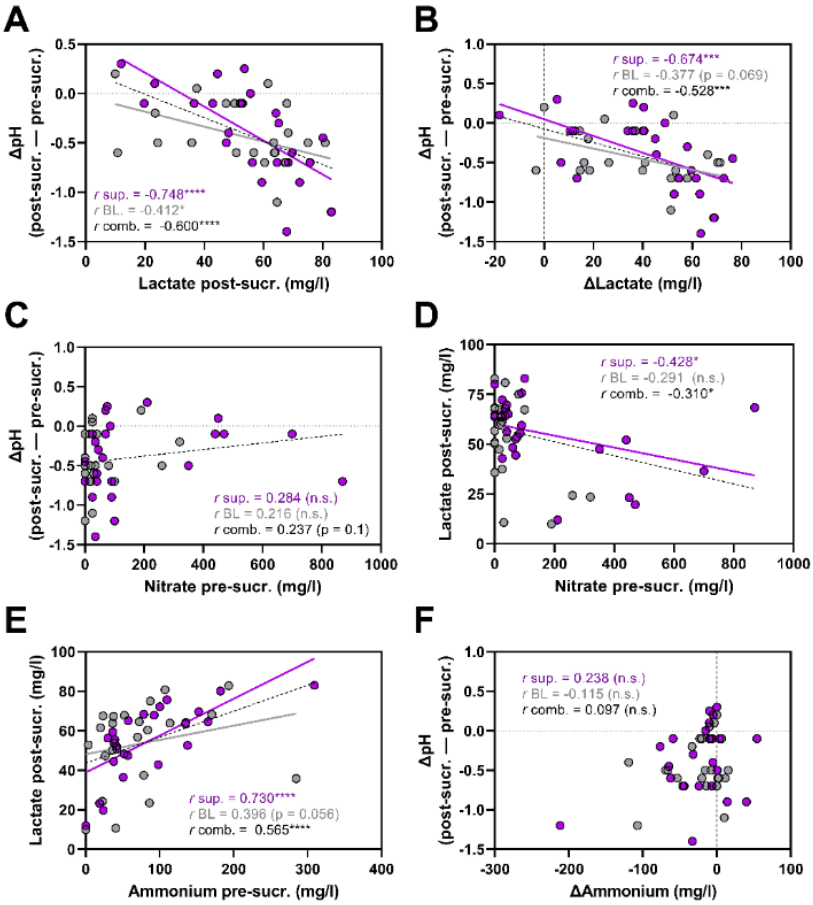
when nitrate-rich vegetables are consumed on a daily basis, and future studies should test this possibility.

3.6 Concluding remarks

To our knowledge, this is the first study to confirm a pH buffering effect of nitrate when sugars are fermented *in vivo*. The data demonstrate an acute effect measurable after 1-4 h of nitrate intake, and future studies should investigate the effect of daily nitrate exposure over longer periods of time. The main underlying mechanism appeared to be lactic acid usage by nitrate reducing bacteria, including *Rothia* and *Neisseria*. Thus, our data suggest that the amount of nitrate in nitrate rich-vegetables could act as an important anti-caries agent. In this respect, it is interesting to note that dental caries have been associated with diets low in fruits and vegetables[243] – the food groups which naturally contain the most nitrate. In one study, children that ate fewer than five servings of fruit and vegetables per day had more caries in primary teeth[244]. Additionally, in a study with 3689 Japanese children, the habit of eating vegetables before a meal was associated with a lower caries incidence in primary teeth[245]. In addition to reducing the frequency of fermentable sugar intake and brushing twice per day with fluoridated toothpaste[226], we hypothesize that increasing the amount of dietary nitrate could be an effective way to prevent caries development. The results in this study support the hypothesis that nitrate could be used as a prebiotic and certain nitrate-reducing bacterial strains as probiotics to prevent caries development. Furthermore, our results suggest that the addition of a nitrate source (e.g., vegetable extract or nitrate salt in combination with anti-oxidants) to oral hygiene products would be beneficial in this regard.

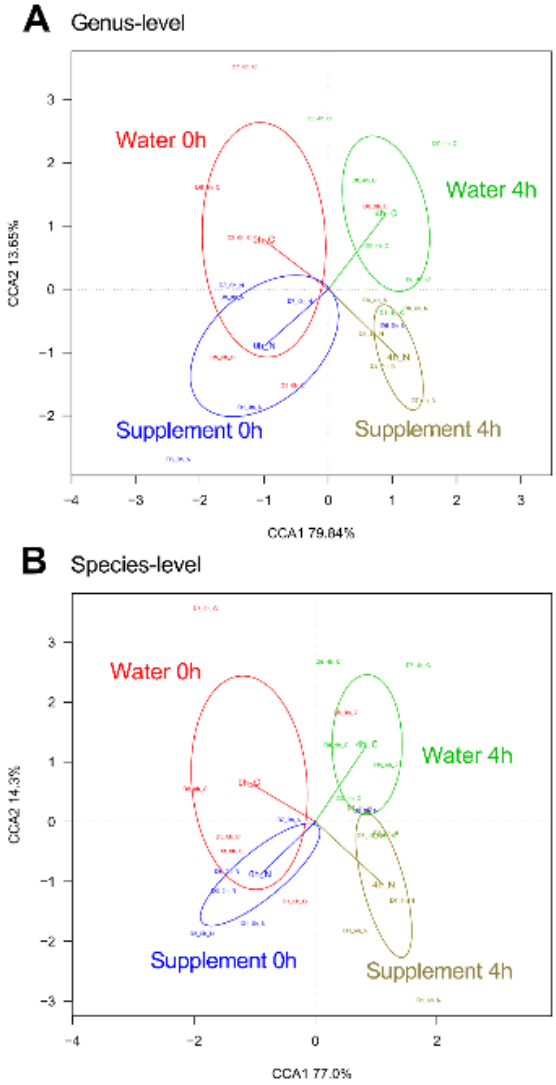
3.7 Supplementary figures and table

Supplementary figures



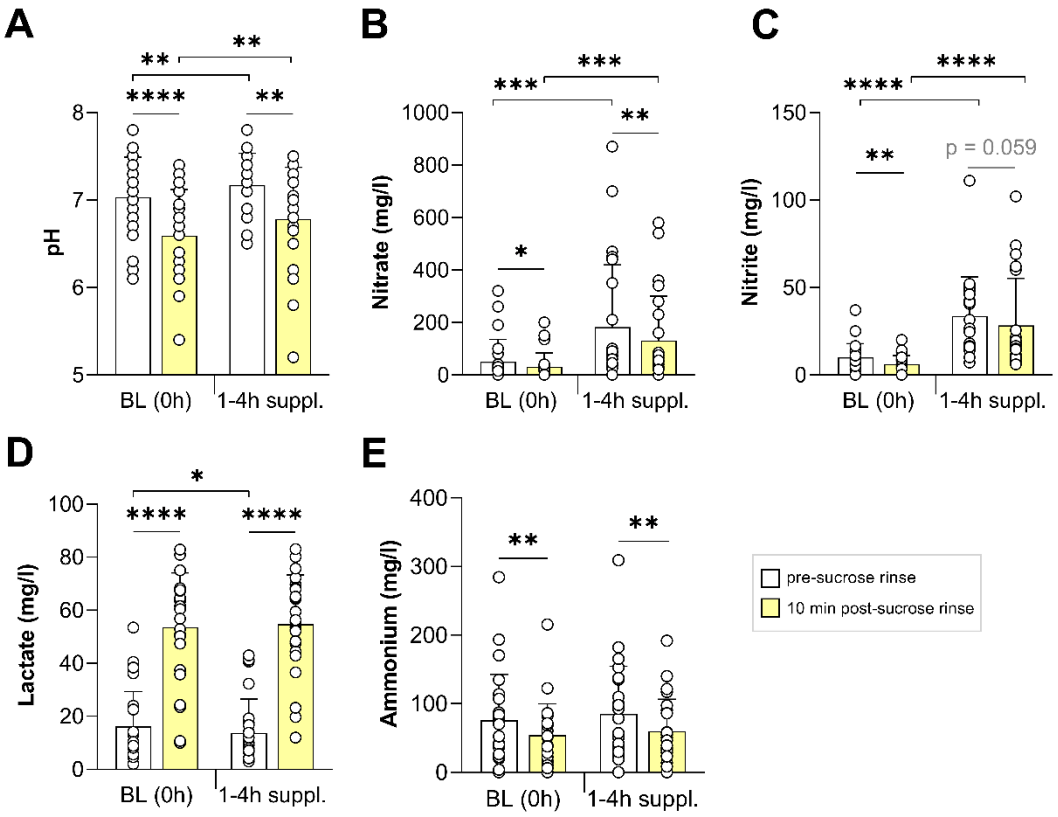
Supplementary Figure 1. Correlations between physiological parameters in all participants of this study at baseline and after supplement intake. In A-F, correlations between different physiological parameters are shown. The purple dots represent data at 1-4 h after nitrate-rich supplement intake (n = 24) and the grey dots at the baseline measurements on the same day (n=24). The black dotted lines are the linear regression curves when combining the baseline measurements with the measurements after supplement intake (total n = 48). The ΔpH is the salivary pH difference between the pre-sucrose measurement and the post-sucrose measurement (negative values indicate a pH drop). A: ΔpH and lactate detected post-sucrose. B: ΔpH and Δlactate. C: ΔpH and salivary nitrate (pre-sucrose) D: lactate detected post-sucrose and salivary nitrate (pre-sucrose). E: salivary ammonium (pre-sucrose) and lactate detected post-sucrose. F: ΔpH and Δammonium. Abbreviations: sup. = supplement, BL = baseline (0 h), comb. =

combined, pre-sucr. = pre-sucrose measurements, post-sucr. = 10 min post-sucrose measurement. Spearman-Rho correlations (*r*) were calculated 1-4 h after supplement intake (sup.), at baseline (BL) or both combined (comb.). P-values and linear regression curves were shown if trends were found (p 0.05-0.1). *p < 0.05, *** = p <0.001, ****p < 0.0001, n.s. = not significant. Δ = post-measurement – pre-measurement.



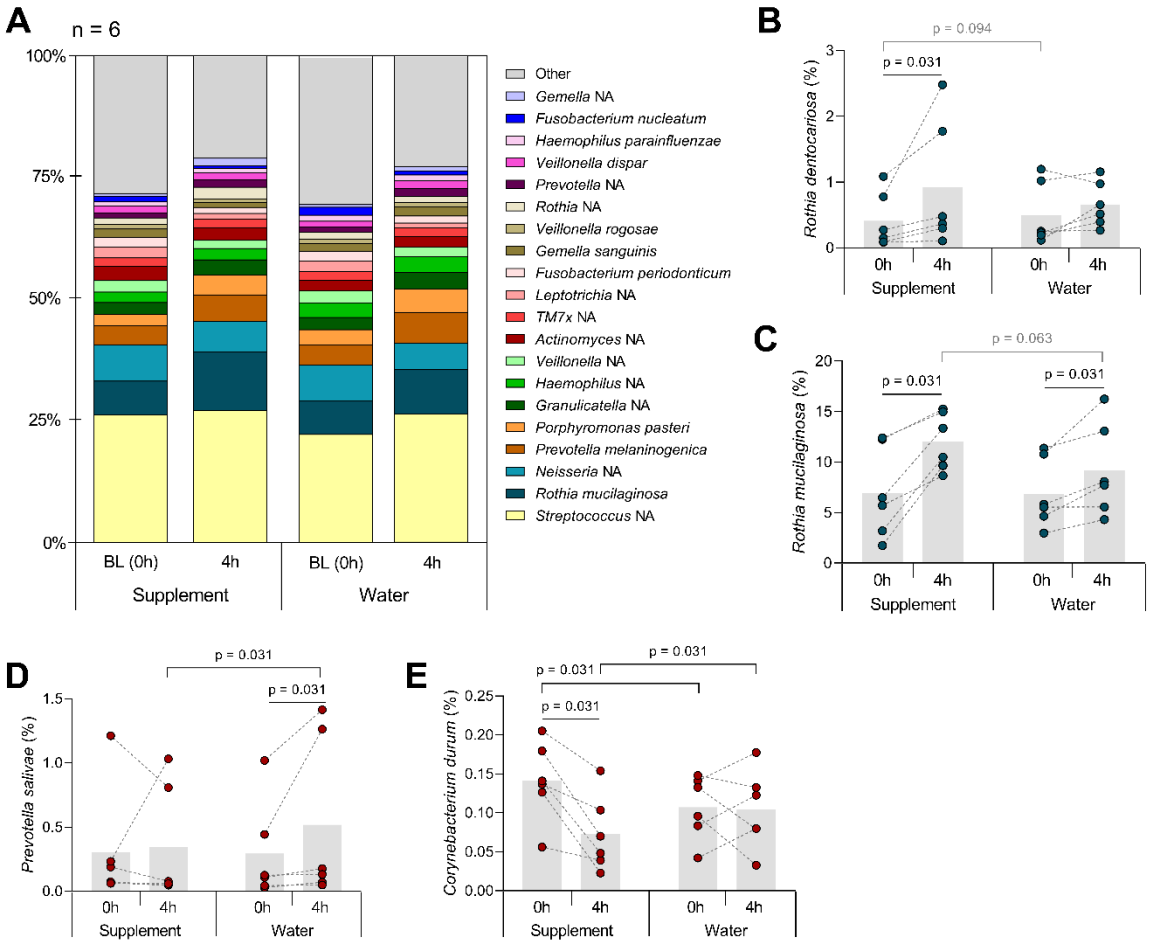
Supplementary figure 2. Comparing the communities of 6 individuals at baseline (0 h) and 4 h after nitrate supplement or water intake. In A, bacterial composition data at the genus-level is shown and in B, at the species-level. Water 0 h and supplement 0 h are the

baseline communities before intake of water or supplement, respectively. Water 4 h and supplement 4 h are the bacterial communities 4 h after intake of water or the supplement, respectively. The Adonis and CCA p-values between the different groups were not significant ($p > 0.6$). This suggest that the general community structures are not significantly different 4 h after nitrate intake compared to 4 h after water intake, although some differences in specific bacteria were detected (see Figure 7 and Supplementary Figure 4).



Supplementary figure 3: The effect of nitrate-rich supplements 1-4 h after intake on sucrose rinses compared to baseline measurements (n = 24). In this figure the 24 individuals from studies 1-3 are combined. The salivary pH (A) and concentrations of nitrate (B), nitrite (C), lactate (D) and ammonium (E) are shown. Sugar rinses were performed at baseline (BL, 0 h) and 1-4 h after intake of a nitrate-rich supplement. Saliva samples were collected right before the sugar rinse (pre-sucrose, white bars) and 10 min after the sugar rinse (post-sucrose, yellow bars). The bars and small white circles represent the averages and individuals donors, respectively. Wilcoxon tests were used to

compare the pre-sucrose with the post-sucrose measurements. Additionally, the BL pre- and post-sucrose measurement were compared with 1-4 h pre- and post-sucrose measurement, respectively. Significant changes (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) and trends (grey text, $p = 0.05$ - 0.1) were shown.



Supplementary figure 4. Species level analysis of study 3 (comparing a nitrate rich supplement intake with water intake). In A, the species detected in the 6 individuals of study 3 are represented in bar charts. Based on the median, the 20 most abundant species are shown. The grey boxes with "other" contain species < 0.5% abundance grouped together. Changes in relative abundances of different species are shown in B (*Rothia dentocariosa*), C (*Rothia mucilaginosa*), D (*Prevotella salivae*) and E (*Corynebacterium durum*). The single dark cyan or red dots represent individual participants. Because of the small sample size, unadjusted p-values are shown.

Supplementary tables

Supplementary Table 1a: nitrate-rich beetroot supplement

Ingredient	mg per dose of 11.5 g
Beetroot extract 3% nitrate* (*Beta vulgaris*)	8333 (= 250 mg nitrate)*
Apple Pectin E-440	2883
Natural Red Fruits Flavor	200
Vitamin C, L-Ascorbic Acid	80
Sucralose E-955 (richness 98% - 102%)	4
Ammonium molybdate (54.32% Mo) (Ammonium heptamolybdate)	0.09205 (= 0.050 mg molybdenum)
Total	11500

*HPLC measurement provided by manufacturer

Supplementary Table 1b: nitrate-poor placebo supplement

Ingredient	mg per dose of 11.5 g
Orange powder	8333 (with <6 mg nitrate)*
Apple Pectin E-440	2883
Natural Red Fruits Flavor	200
Vitamin C, L-Ascorbic Acid	80
Sucralose E-955 (richness 98% - 102%)	4
Ammonium molybdate (54.32% Mo) (Ammonium heptamolybdate)	0.09205 (= 0.050 mg molybdenum)
Total	11500

*the analytical sensitivity of the colorimetric assay started at 6 mg, because of the orange color of the placebo

3.8 Data availability statement

All measurements of this study can be found in the Supplementary Spreadsheet. The sequencing reads are deposited in the Sequence Read Archive under BioProject PRJNA725996.

General discussion of thesis

General discussion

The research presented in this thesis shows that nitrate can modulate the oral microbiota in ways that appear to be beneficial for the host and can thus be considered a potential prebiotic substance. Specifically, by growing saliva *in vitro* under different conditions, we confirmed that nitrate stimulates the growth of the beneficial genera *Rothia* and *Neisseria*. Additionally, we showed that nitrate can potentially decrease caries-, halitosis- and periodontal disease-associated bacteria. This thesis also contains the first *in vivo* results confirming that salivary nitrate can limit or prevent pH drops when sugars are fermented by the oral microbiota – a mechanism of resilience that can be stimulated by the consumption of nitrate-rich vegetables. Regarding this, the main pH buffering mechanisms that we identified were lactic acid usage and proton consumption (Figure D1) during denitrification (observed both *in vivo* and *in vitro*) and during nitrite reduction to ammonium, as well as potential ammonia (a weak base) production (observed *in vitro*). Finally, we showed that adding *Rothia* isolates to saliva grown with nitrate *in vitro* increased the community's nitrate reduction and lactic acid usage capacities. This may be especially relevant in individuals with a limited nitrate reduction capacity and could improve not only dental health but also systemic conditions that benefit from nitric oxide availability. Therefore, the probiotic potential of these isolates should be tested in future *in vivo* studies.

The oral microbiota is strongly affected by ecological factors such as carbohydrate availability, oxygen and pH[22,26]. Given the well-known impact of nitrate on environmental microorganisms, it is surprising that the effect of nitrate on oral communities has been neglected over many decades[49]. One the one hand, nitrate is the most efficient electron acceptor after oxygen that bacterial communities use for respiration, affecting their metabolism and composition[127]. On the other hand, the nitrate recycling activity of the salivary glands lead to high salivary nitrate concentration throughout the day, which is stimulated by the consumption of nitrate-rich foods (e.g., leafy greens, beetroots

and radishes). In 2010, Schreiber and colleagues showed that dental plaque denitrifies nitrate all the way to nitrogen and identified several denitrification genes, including the nitrate reductase *narG*, the nitrite reductases *nirS* and *nirK*, the nitric oxide reductase *cnorB* and the nitrous oxide (N_2O) reductase *nosZ* (Figure D1, brown boxes)[49]. In this thesis, we identified several additional genes in *Rothia* species related to nitrogen metabolism, including the DNRA and/or nitrate assimilation genes *nirB* and *nirD* and the flavohemoglobin *hmp* gene that converts nitric oxide to nitrate (Figure D1, blue boxes). Additionally, in a collaboration that did not form part of this thesis[246], a metatranscriptomic approach was used to identify several other genes involved in nitrogen metabolism of tongue biofilms, including other genes involved in nitrate reduction (*narH*, *narI*, *narJ* and *narW*), the DNRA gene *nrfA* and the nitrification gene *nifX* (Figure D1, yellow boxes). Together, these results indicate that nitrate metabolism inside the oral cavity goes beyond the denitrification and DNRA pathways, which were the focus of this thesis, and other processes that could affect oral microbiota homeostasis include nitrate assimilation, nitric oxide detoxification and possibly nitrification by fixation of N_2. I therefore anticipate that many more nitrogen metabolism (and related) genes are expected to be identified in future studies.

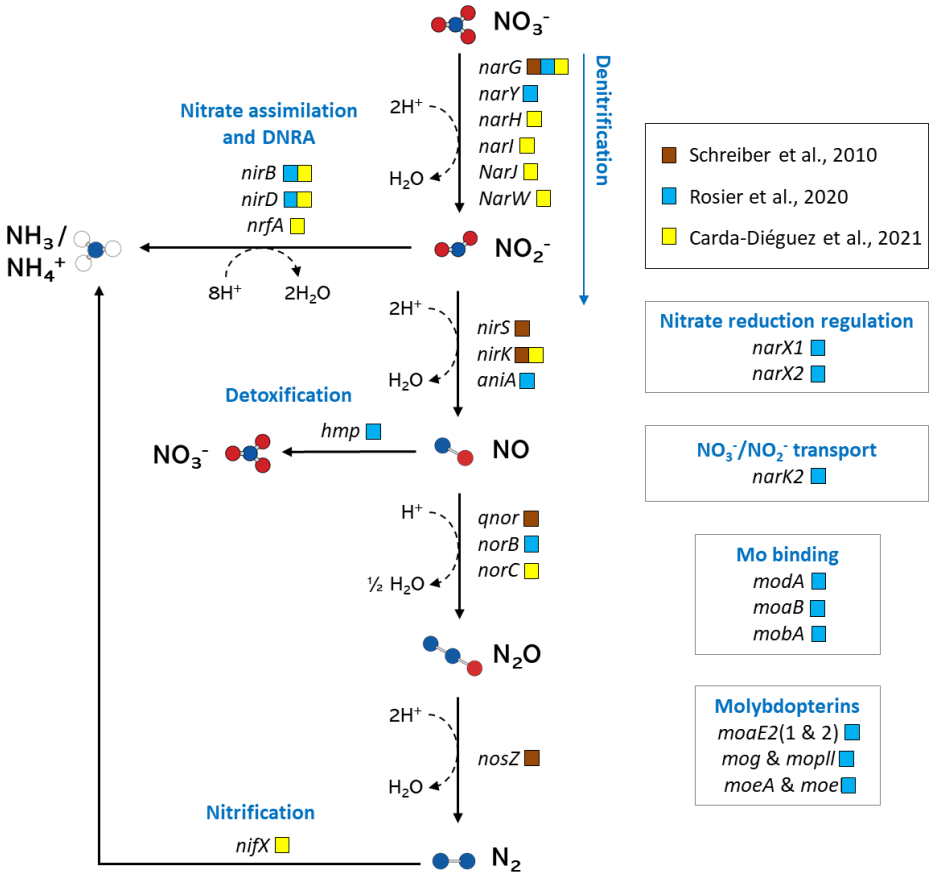


Figure D1: Nitrogen metabolism and related genes of the oral microbiota. The graph represents genes identified in three different studies. Brown boxes are genes identified by Schreiber et al. (2010)[49], blue boxes by Rosier et al. (2020)[228] and yellow boxes by Carda-Diéguez et al. (2021)[246]. The metabolites in this figure are nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), nitrogen (N_2), ammonium (NH_4^+) and ammonia (NH_3). The genes in the boxes on the right are related to nitrogen metabolism (discussed in chapter 2) and include nitrate reduction regulation, nitrate/nitrite transport and genes encoding proteins that bind and transport molybdenum – an essential cofactor of nitrate reductase enzymes. Nitric oxide, nitrous oxide and nitrogen are gasses released from the oral cavity. DNRA: Dissimilatory nitrate reduction to ammonium. Mo: molybdenum. H+ represent the protons consumed during different reduction steps (note that electron usage is not shown, but happens in parallel), leading to the production of water (H_2O) as described by Shi et al. (2020)[247]

The studies focusing on nitrate and oral health prior to this thesis were limited in number[62,63,84,138], but clearly indicated that nitrate was likely beneficial, which warranted further research. Firstly, in 2004, Doel and colleagues found that salivary nitrate and the nitrate reduction capacity of the oral microbiota was associated to a lower caries incidence in children[62]. Secondly, Li and colleagues (2007) found that nitrate could attenuate acidification when incubating bacteria obtained from saliva with glucose for short periods of (1-3 hours)[63]. Thirdly, Backlund and colleagues (2014) found that periopathogenic *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* were sensitive to nitric oxide (one of the products of denitrification)[138], whilst Schreiber and colleagues found that nitric oxide was produced by oral bacteria in dental plaque[49]. Finally, Jockel-Schneider and colleagues (2016) found that nitrate-rich lettuce juice can reduce inflammation in patients with chronic gingivitis[84]. This previous work indicated that nitrate is likely to be beneficial for oral health, but did not look into nitrate-induced changes in bacterial composition or specific functions of the oral microbiota, and it did not identify the mechanisms underlying the observed benefits. The aim of this thesis was therefore to study nitrate-induced microbiome changes and identify potential mechanisms for nitrate-induced homeostasis, in order to determine if nitrate can be considered a prebiotic compound for oral health. A second aim was to isolate nitrate-reducing isolates and test their probiotic potential *in vitro* and *ex vivo*.

In chapter 1, an *in vitro* study was set up testing the effect of 6.5 mM nitrate on healthy oral communities grown from saliva. In chapter 2, nitrate-reducing strains were isolated and the effect of six probiotic candidates was tested on healthy oral communities grown from saliva of different donors with or without 6.5 mM nitrate. In chapter 3, the effects of nitrate-rich beetroot extracts on oral acidification after sugar rinsing was tested in 24 individuals without active caries. Each of the three chapters in this thesis has been published in a Q1 journal and contains its own discussion sections, including limitations and future research ideas. A combination of the main observations and further discussion is provided below.

Nitrate as a prebiotic for oral health

One of the main results of this thesis is the confirmation that 6.5 mM nitrate (a physiologically relevant concentration), in the form of sodium nitrate, stimulates the growth of the health-associated genera *Rothia* and *Neisseria*. In other clinical studies, increases in (representatives of) *Rothia* and/or *Neisseria* were observed after nitrate-rich beetroot juice consumption in saliva[83,135] and tongue coating[163], and after nitrate-rich lettuce juice consumption in subgingival plaque[248]. These vegetable juices were compared to nitrate-depleted placebo juices, giving a strong indication that nitrate was responsible for the observed changes. However, vegetables contain a broad range of molecules, including sugars, polyphenols and anti-oxidants. It is known that nitrate affects sugar metabolism[63] and anti-oxidants can affect nitrate metabolism[249]. Our *in vitro* data with a nitrate salt complement these clinical results, confirming that nitrate stimulates the growth of *Rothia* and *Neisseria*. It was surprising that already after 5h, *Rothia* and *Neisseria* had increased significantly in the presence of nitrate under the *in vitro* conditions of our experiment (i.e., nutrient-rich medium mixed with saliva grown in oxygen limited conditions at 37°C). Conversely, in previous studies, individuals consumed nitrate-rich vegetable juices on a daily basis for one to four weeks[83,135,163,248]. In chapter 3, we show that *Rothia* in saliva started increasing significantly already 4h after nitrate-rich beetroot supplement intake, confirming that rapid shifts in nitrate reducers can also occur *in vivo*. The strong association of *Rothia* and *Neisseria* with oral health have been discussed in chapter 1 and 2. Taking these results together, it can be concluded that nitrate increases health-associated nitrate-reducing bacteria in oral communities and these changes can happen as soon as several hours after nitrate exposure, highlighting the dynamic potential of the structure of the oral microbiota. The current thesis only tests the immediate, short-term effect of nitrate, demonstrating an acute impact on oral communities. However, given that similar trends have been found after longer-term nitrate ingestion by other authors, it is expected that positive shifts in bacterial composition would take place after routine use of nitrate-containing oral care products. I hope this thesis

stimulates future work to test this possibility, for example after the use of nitrate-rich dentifrices.

Nitrate as an anti-caries prebiotic

Another important finding was the verification that nitrate can limit or prevent pH drops when sugar is fermented by the oral microbiota. To our knowledge, it was the first time that this was confirmed *in vivo* by letting individuals rinse their mouth with sugar before and after 1-4 h after a nitrate-rich supplement. In these clinical experiments, an average nitrate concentration of 2.9 mM (Q25-Q75 = 0.6-4 mM) was detected at the moment of sugar rinsing. In the *in vitro* experiments of this thesis (chapters 1 and 2), 6.5 mM nitrate prevented a pH drop when incubating saliva with glucose-containing medium after 5, 7 and 9 h of incubation. In chapter 1, 1-8.5 mM nitrate also limited a pH drop when incubating saliva with just glucose for 5 h. Furthermore, in chapter 2, the symbiotic combination of 6.5 mM nitrate and a nitrate-reducing *Rothia* isolate prevented a pH drop after 7 h of incubation. One of the isolates (D3T4) in combination with nitrate increased the pH more than just nitrate, making this an interesting symbiotic combination to limit caries development, which should be further studied.

Li and colleagues (2007)[63], who were the first to observe that nitrate can limit a pH drop when incubating bacteria from saliva with sugar, hypothesized that the pH buffering effects of nitrate could result from lactic acid consumption and/or alkali production by nitrate-reducing bacteria. However, these hypotheses were never confirmed using oral microbiota samples. In chapter 1, we show that the pH buffering effect of nitrate happened in parallel with significantly more ammonium and less lactate detection. Nitrate and nitrite reduction can happen in parallel with lactic consumption as the latter provides electrons needed for denitrification[237]. The observation that nitrate and nitrite were reduced, while *Rothia* and *Neisseria* increased, and that lower lactate levels were detected, indicated that lactic acid was being consumed. This was further supported the preliminary results in chapter 3, showing that *Rothia* and *Neisseria* in saliva

correlated negatively with the lactate produced after sugar rinsing. Additionally, 1-4 h after nitrate supplement intake, less lactate was detected after sugar rinsing compared to baseline ($p < 0.05$), water intake ($p < 0.05$) and placebo intake ($p = 0.099$). Finally, in chapter 2, lower levels of lactate were detected when adding *Rothia* isolates to salivary communities *in vitro*, but only in the presence of nitrate. Given that the *Rothia* isolates also increased the nitrate-reduction capacity of the communities and lactate/lactic acid usage genes were detected in all isolates, it was likely that lactic acid was consumed by these isolates in parallel.

In chapter 1, the possibility of alkali production was also confirmed. Specifically, ammonium levels were significantly higher after 5 and 9 h of incubation of saliva with 6.5 mM nitrate *in vitro*. In chapter 2, after 7 h saliva incubation with 6.5 mM nitrate *in vitro*, ammonium appeared to be produced by some individuals, particularly individuals that reduced most nitrate, but the mean increase was not significant. In chapter 3, ammonium production did not increase significantly either 1-4h after nitrate supplement intake, but the significance level was close to a trend of increase ($p = 0.114$). During the reduction of nitrite to ammonium, lactic acid can be used and protons are consumed. We therefore hypothesize that nitrate-derived ammonium production could be a relevant pH buffering mechanism *in vivo* under certain conditions (e.g., after a higher dose of nitrate or longer periods of daily nitrate intake), which may only be observed in some individuals, and future research should test this. It must be kept in mind that ammonium in the *in vitro* system would be accumulated through time, making more plausible to detect differences in their production, whereas in the clinical study, saliva samples were used, where ammonium produced by oral biofilms could be diluted and also continuously swallowed. Thus, future ammonium measurements *in vivo* should also be directed towards plaque samples instead of saliva. The exact effects of nitrate on ammonium and ammonia levels needs to be further investigated in future studies.

A third hypothesis by Li and colleagues (2007) and Doel and colleagues (2004) was that nitrate could limit a pH drop and hamper caries development, resulting from the anti-microbial activity of the denitrification product, nitric oxide, thereby limiting acid-producing bacteria[63]. In chapter 1, we observed that *Streptococcus*, *Veillonella* and *Oribacterium* decreased in the presence of 6.5 mM nitrate when using a Wilcoxon test. As discussed in chapter 1, these genera are found in higher levels in caries and also associated to acidification and lactic acid production. When using an ANCOM-II test, two other caries-associated genera, *Atopobium* and *Actinomyces*, also decreased significantly (in addition to *Streptococcus*, *Veillonella* and *Oribacterium*). The decrease in these five genera can unequivocally be considered a positive change in microbiota composition from a caries point of view. Apart from potential nitric oxide-induced inhibition, representatives of these genera have a selective advantage at lower pH levels[22,53,214], which could explain their decrease in the nitrate condition in which pH levels were higher. Future research should test this possibility and determine nitric oxide, higher pH levels or other factors (e.g., anti-microbial peptide production by the genera that increased) limited their growth. Our results are supported by clinical studies showing a decrease in *Streptococcus* and *Actinomyces* in tongue coating[163], and *Veillonella* in saliva[135], after beetroot juice consumption. The effect of nitrate on bacteria in dental plaque, which is the community directly involved in caries development, should be further explored in future research. In summary, the metabolic and compositional changes induced by nitrate limit or prevent acidification when sugars are fermented by the oral microbiota. Nitrate is therefore a promising anti-caries agent, which should be further studied.

Nitrate as a promising prebiotic to limit periodontal disease development

In subgingival plaque of patients with periodontal diseases, higher amounts of alkaliphilic, proteolytic, anaerobic and inflammation tolerant species are found[22,136]. These include representatives of *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Tannerella*, *Treponema*, *Eubacterium* and *Aggregatibacter* among others[28,30,70,165]. Nitrate and other compounds that increase the salivary pH (e.g.,

arginine or urea) could give a selective advantage to alkaliphiles associated to periodontitis. For example, in a clinical study, the use of 8% arginine toothpaste arginine led to an increase of OTUs of *Prevotella*, *Treponema* and *Eubacterium* in dental plaque[82]. Conversely, in chapter 1, we showed that the bacterial reduction of (most of) 6.5 mM nitrate happened in parallel with the decrease the levels of *Porhpyromonas*, *Prevotella* and *Fusobacterium* when using a Wilcoxon test (and also *Tannerella* when using the ANCOM-II test) *in vitro*. Nitric oxide-releasing material and/or metal oxides have been shown to have antimicrobial activity against several anaerobic periodontitis-associated species, including *Porphyromonas gingivalis*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum*[138,181], indicating sensitivity against oxidative stress. The production of nitric oxide in parallel to the pH buffering effects of nitrate could explain why the pH increased, but a decrease of alkaliphilic anaerobic genera was observed in chapter 1. In clinical studies, it has been consistently observed that *Prevotella* decreases after nitrate-rich beetroot juice intake in saliva[135,163]. Preliminary results in 6 individuals in chapter 3 showed that *Prevotella salivae*, together with *Corynebacterium durum,* decreased 4 h after nitrate-rich beetroot supplement intake compared to water intake, but these results should be confirmed in larger population sizes. In a recent study, 10 days of daily beetroot supplement intake led to a decrease of Firmicutes, Bacteroidetes and Fusobacteria in saliva, which are phyla that increase in periodontitis[30,165]. Nitric oxide is also a biofilm dispersal signal and could cause sensitive species to disperse from dental plaque, limiting plaque accumulation[76,136,250]. Thus, the mechanisms for the reduction of periodontitis-associated bacteria in dental plaque, which could reduce the inflammatory response, are still to be clarified.

Apart from a decrease of periodontal disease-associated bacteria, an increase in Proteobacteria and health-associated *Rothia* and *Neisseria*[248] in subgingival plaque in the presence of nitrate could further benefit the gingiva. In one study, the genus *Neisseria* correlated with anti-inflammatory mediators and was associated with a better recovery of the gingiva after experimental gingivitis[52]. In

another study, *Rothia aeria* negatively correlated with inflammatory cytokines IL-17 and TNF-α[169]. It should be investigated if these genera actively reduce inflammation, like some commensal species[79]. Additionally, the production of other antimicrobial components (e.g., bacteriocins) by these genera could cause decreases in other bacteria as has been observed for some oral probiotic species (e.g., *S. dentisani* or *S. salivarius*)[61].

Finally, nitric oxide can signal to human cells, affecting processed that are important in periodontal disease development such as inflammation and blood flow[25]. It was found that dental plaque produces nitric oxide levels that are involved in host signaling[49]. Future studies should thus determine the effect of nitric oxide produced by oral bacteria on inflammation in periodontal diseases.

The above-mentioned study, which looked at the effect of nitrate intake by patients with chronic gingivitis, found that gingival inflammation was reduced[84] and *Neisseria* and *Rothia* increased[248]. Based on the results in this thesis, another potential mechanism behind the reduction of inflammation could be the inhibition of periopathogenic species (e.g., by the antimicrobial properties of nitric oxide). Additional mechanisms that should be explored in future studies are a decrease in pro-inflammatory mediators and bacterial nitric oxide signaling to the biofilm or to host cells.

Nitrate as potential prebiotic against halitosis

It is interesting to note that some of the genera that decreased in the presence of 6.5 mM nitrate in chapter 1 are also associated to halitosis, namely *Prevotella*, *Porphyromonas* and *Fusobacterium*[174,246]. These genera contain VSC producing representatives that contribute to halitosis[14]. As discussed in chapter 1, nitrate is used as a biological agent to treat malodour from sewer networks by limiting microbial VSCs production resulting from sulfate reduction[129]. As long as more energy-efficient denitrification takes place, sulfate reduction is inhibited and this could also be the case in the oral cavity, where sulfate reduction is associated to halitosis[130]. Additionally, hydrogen sulfide can be used as an electron donor

when nitrate is reduced[251]. The increase in *Rothia* and *Neisseria* observed in the presence of nitrate could thus reduce hydrogen sulfide levels similarly to lactic acid levels as observed in chapters 1-3. In a recent metatranscriptomic study, nitrate reducing species *R. mucilaginosa* and *V. dispar* were higher in halitosis free individuals, while nitrate-reduction genes were overexpressed in halitosis free individuals[246]. I therefore suggest that the effect of nitrate supplementation on sulfate reduction and halitosis should be tested in future clinical studies.

In summary, the data in this thesis support the idea that nitrate is important for oral health. Nitrate has an enormous potential as a prebiotic compound to increase health-associated bacteria, prevent caries development and treat or prevent periodontal diseases and halitosis. This makes it the first prebiotic compound that could contribute to improve all three types of main oral diseases. Other prebiotics such as arginine, have very solid evidence demonstrating an improvement from a caries point of view[80-82,206], but its mechanism of action (ammonia production) make it unlikely that this compound could also provide an advantage against gum diseases or halitosis. Because of the promising application possibilities of nitrate, it is mandatory to evaluate the potential toxicity of this compound, given the traditional view of nitrate as a potentially toxic compound. The literature was therefore reviewed to evaluate the safety and risks of increasing oral nitrate levels with foods, drinking water and nitrate-containing oral hygiene products.

Nitrate safety and risks

Toxic N-nitroso compound formation

Nitrate had a bad reputation for many decades as its reduction product, nitrite, can form potentially carcinogenic N-nitroso compounds (NOCs) under certain conditions[249]. For example, during the conversion of nitrite to nitric oxide inside the acidic stomach, reactive nitrogen intermediates are formed. Specifically, nitrite is immediately protonated in the acidic gastric milieu forming nitrous acid (HNO_2)[122,249] (Figure D2). Nitrous acid spontaneously forms nitric oxide

through disproportionation via the intermediate formation of dinitrogen trioxide (N_2O_3). Dinitrogen trioxide is a nitrosating agent that can react with amines or (after protonation to HNO_2) with amides, forming nitrosamines and nitrosamides, respectively [252]. Nitrosamines and nitrosamides are subgroups of NOCs that, in turn, can form reactive intermediates that bind to DNA[252,253]. If the DNA is not repaired, mutations can occur that potentially increase the risk of cancer development[253,254]. In animal models, the administration of different NOCs have shown to cause cancer[255]. Metabolization of nitrate inside the human body can therefore lead to beneficial nitric oxide production, but also to potentially carcinogenic NOC formation. Apart from amines and amides, different dietary components can affect these processes, which could explain why vegetables do not increase the risk of cancer.

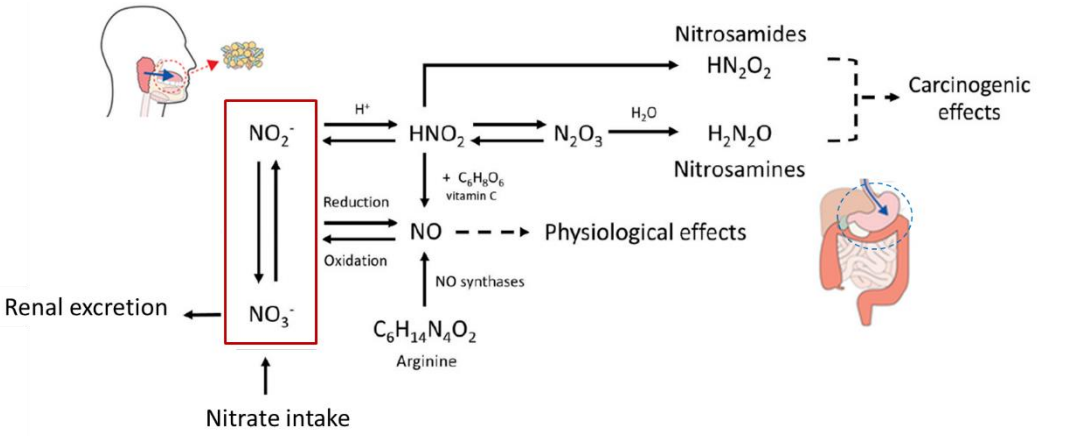


Figure D2. Metabolic pathway of nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), nitrosamines and the effect of vitamin C (modified from Berends et al. 2019[252]). Nitric oxide (NO) is mainly responsible for the physiological effects of nitrate. The body uses arginine as a source to form NO, and for this reaction oxygen is needed. However, NO can also be formed after intake of nitrate-rich products such as beetroot juice. Ingested nitrate (NO_3^-) will be partly reduced to nitrite (NO_2^-) by microflora in the oral cavity (red box). In oxygen-poor environments nitrate and nitrite can be reduced into NO. NO can also be oxidized back into nitrate and nitrite which are water soluble and can therefore be excreted in urine. Under acidic conditions, such as in the human stomach,

nitrite is protonated into HNO_2 (nitrous acid). Also in the stomach, two molecules of HNO_2 can form N_2O_3 (dinitrogen trioxide), by proton catalysis. N_2O_3 plays a role in the N-nitrosation rate. Increasing the amount of nitrate will therefore lead to an increase in the N nitrosation rate. Subsequently, HNO_2 can react with amides to form nitrosamides, and N_2O_3 can react with amines to form nitrosamines. Both nitrosamides and nitrosamines are N-nitroso compounds and potentially carcinogenic. Vitamin C (and other anti-oxidants) can inhibit the nitrosation process because it reacts faster than the amine with N_2O_3. Vitamin C reduces $2HNO_2$ to NO and is itself oxidized to dehydroascorbic acid. This will reduce the amounts of N-nitroso compounds that can be formed. The corresponding author from Berends et al., 2019[252] gave permission for the use of this figure (Creative Commons Attribution CC BY license).

Fresh vegetables and fruits: safe and healthy

Different dietary molecules can prevent the formation of NOCs. For example, it has been shown that vitamin C can inhibit nitrosation reactions inside the stomach (Figure D2) when nitrite and amines are present[252,253]. Vitamin C reduces HNO_2 to NO and reacts faster with N_2O_3 than amines, diminishing NOC formation while increasing NO generation. Similarly, other anti-oxidants, such as vitamin E and dietary-derived polyphenols, also inhibit nitrosation and enhance NO formation from HNO_2[122,249,256]. Natural combinations of nitrate, polyphenols and (other) anti-oxidants in fruits and vegetables could explain why these food groups are not carcinogenic[249]. As a matter of fact, fruits and vegetables are generally considered anticarcinogenic[89,223]. There is strong evidence for vegetables, including nitrate-rich spinach and lettuce, decreasing cancer risk (mouth, pharynx, larynx, oesophagus and stomach)[88]. Fruits and vegetables are strongly associated with longevity and health benefits, leading to the general dietary recommendation to consume plenty of them (e.g., "the more vegetables, the better", according to Harvard Health)[257]. It should be noted that this refers to fresh fruits and vegetables (raw or cooked), while other recommendations may apply to modified products (e.g., fermented products, extracts and processed products).

In contrast to fruits and vegetables, not all dietary nitrate sources contain high amounts and varieties of anti-oxidants and polyphenols. Nitrate can be found in drinking water in different levels and nitrate is added to processed meats as a preservative.

Nitrate in drinking water

The levels of nitrate in rainwater can be up to 5 mg/l in industrial areas, but tend to be somewhat lower in rural areas[258,259]. The nitrate concentration in surface water is normally low (0-18 mg/l) and in most countries, nitrate levels in drinking-water derived from surface water do not exceed 10 mg/l[258,259]. However, nitrate can reach high levels in drinking water resulting from agricultural runoff, refuse dump runoff or contamination with human or animal wastes[259].

A limit for nitrate in drinking water was set based on the association between high nitrate concentrations in well water and infantile methaemoglobinaemia (also known as blue baby syndrome)[95]. This limit is 45 mg/l in the USA and 50 mg/l in Europe as cases of methaemoglobinaemia were rarely observed below 44 mg/l[260]. It should be noted that methaemoglobinaemia is highly unlikely in the absence of contaminating bacteria found in wells, which are needed to reduce sufficient nitrate to nitrite[95,261]. Additionally, gastric infections increase the chance of methaemoglobinaemia[259].

Nitrite can react with hemoglobin and form methemoglobin, which cannot transport oxygen. Normally, this reaction is directly reversed by the enzyme methemoglobin reductase[262,263]. However, this enzyme is less active in babies younger than 3 months. Current water regulations have made methaemoglobinaemia a rare condition in Western countries (for example, the last case reported in the UK was in 1972)[262]. Notably, a 2010 article by the EFSA stated that there was no increase in methemoglobin in babies 3 months and older that consumed up to 15 mg/kg nitrate daily, a rate 4 times the ADI (3.7 mg/kg)[264]. Additionally, the NHS recommends to include spinach and cabbage

(both nitrate-rich vegetables) in the first vegetable meals of weaned infants (6 months and older)[265].

The nitrate limit in drinking-water also prevents an unnecessary increase of NOC formation. When nitrate levels in drinking-water exceed 50 mg/l, drinking-water can easily become the major source of total nitrate intake in many individuals[259]. In some cases, a significant correlation has been found between high concentrations of nitrate in drinking water and the prevalence of gastric caner[252,266]. For example, when comparing a population in the UK with 93 mg/L nitrate in their drinking water, significantly more gastric cancer was detected compared to a UK population with 15 mg/L nitrate in their drinking water[252]. In another study, a correlation by gastric cancer and drinking water was only found in individuals that consumed vitamin C below the median (131.8 mg/day)[267], indicating that other factors in addition to nitrate may be involved. In light of this, nitrate can be a marker for other contaminants (e.g., pesticides)[268], whilst dietary vitamin C is often an indication of the amount of vegetables and fruits an individual consumes. These factors, together with the fact that nitrate levels are kept low in drinking water in many countries, could explain why safety authorities have stated that there is no or inadequate evidence for the association between nitrate in drinking water (and diet) with cancer[93,266].

Nitrate in processed meat

Processed meats (e.g., bacon, ham, chorizo, hot dogs, and canned meat) were classified as group 1 carcinogens (known to cause cancer in humans)[222]. One of the potential mechanisms behind their carcinogenicity is N-nitroso compound formation from nitrate[269]. Nitrate is added to processed meats (mostly in the form of a salt) to prevent the growth of microorganisms and/or to modify the taste and colour of the meat [reviewed by Skibsted, 2011; and Sindelar & Milkowski, 2012][186,187]. Nitrate itself does not have these antimicrobial or taste and colour modifying effects. However, nitrate is reduced to nitrite by bacteria on meat and further reactions of nitrite with meat molecules lead to the observed effects. Specifically, nitrite can react with haemoglobin, amines, and

amides on meat, forming nitrogen oxides, like nitric oxide, and NOCs[186,187]. Just like inside the stomach, the production of these molecules is favoured by an acidic environment, which in this case results from the metabolisms of bacteria in meat[270]. The heat generated during cooking further stimulates the reactions between nitrite and meat molecules[186]. The nitrogen oxides kill bacteria, preventing their growth and accumulation, thereby delaying the rotting of meat[270]. However, the NOCs formed on meat are potentially carcinogenic[255], which could (in part) explain their association with cancer[269]. In contrast to fruits and vegetables, meat has low levels of anti-oxidants. Inside the stomach, more N-nitroso compounds can form, especially if meat and nitrite are not consumed in combination with vegetables, fruits or anti-oxidants, which limit NOC formation[225,271] and possibly damage[224]. This could further explain why the association between nitrate and cancer is found particularly (or, in some studies, only) in subgroups (e.g., high meat intake or low vitamin C intake)[267,272,273].

Acceptable Daily Intake of Nitrate

The Acceptable Daily Intake (ADI) set by the European Food Safety Authority for nitrate is 3.7 mg/kg of body weight (0.06 mmol/kg), which is ~220-290 mg for adults of 60-80 kg[241]. This limit was first set by the WHO in 1962 based on the observation that ~500 mg sodium nitrate (= ~370 mg nitrate) had been found harmless to rats and dogs[95]. This number was divided by 100 to obtain an ADI of 3.7 mg/kg nitrate and has not changed since[274] with the purpose to limit NOC formation inside the body. In chapter 3, we chose to use beetroot supplement doses with 220-300 mg nitrate, which are around the ADI, taking into account that future products taken by ingestion should respect the official limit.

However, different authors have questioned the rationale behind the current ADI[91,95,249]. Firstly, vegetables, which provide over 80% of dietary nitrate, are strongly associated to low cancer incidence, including gastric cancer. Vegetarians often consume 4 times more nitrate per day than individuals with a

non-vegetarian diet and can thus more easily surpass the ADI[95]. Nevertheless, the vegetarian (and vegan) diet has been associated to a lower cancer incidence, including gastric cancer[275]. These observations do not support the conclusion of some authors that attention should be paid to nitrate intake via vegetables in vegetarians to avoid "unsafe nitrate levels"[96]. Also the Dietary Approaches to Stop Hypertension (DASH) diet high in vegetables and fruits is associated to lower cancer incidence, including gastric and breast cancer[276]. An individual following a DASH dietary pattern with high-nitrate vegetable and fruit choices would exceed the current Acceptable Daily Intake (ADI) of nitrate by ~550-700%[91,249]. In fact, a portion of spinach commonly consumed in one serving of salad can exceed the ADI for nitrate, whilst spinach is included in the list of vegetables considered to be protective against cancer[95].

In conclusion, the fact that typical consumption patterns of vegetables and fruits (generally considered the most health-associated food groups and protective against gastric cancer) exceed regulatory limits for dietary nitrate calls into question the rationale behind current nitrate regulations. Future assessment of the ADI should thus take the source of nitrate into account, as well as molecules formed after bacterial and chemical reactions. Consuming vegetables and fruits is a safe and healthy strategy to increase nitrate levels, which could promote oral health.

New potential applications of nitrate and nitrate-reducing probiotics to stimulate oral health

Nitrate in oral hygiene products

In oral hygiene products, low amounts of nitrate would be sufficient to reach physiological concentrations of nitrate in saliva that can have beneficial oral effects. For example, in chapter 3 it was found that an average salivary nitrate concentration of 3 mM (Q25-Q75: 0.6-4 mM) was associated to beneficial oral effects. As discussed in chapter 1 and 2, inside the oral cavity, volumes of 0.5-1 ml saliva are often found and adding 202-404 µg to these volumes leads to a

concentration of 6.5 mM, which caused potential beneficial effects *in vitro*. Amounts of few to several mg (far below the ADI) per toothpaste or mouthwash dose could thus be sufficient to activate oral health-associated mechanisms. These products are not swallowed, leading to an even lower amount of nitrate entering the stomach. Saliva contains several endogenous anti-oxidants[277], which could prevent local NOC formation (e.g., in dental biofilms with a low pH). The salivary anti-oxidant capacity could be further stimulated by adding vitamin C and other reducing agents to nitrate-containing oral hygiene products. Current potassium toothpastes (for gum sensitivity) often contain 5% potassium nitrate, which results in a dose of 30.7 mg nitrate in 1 g of toothpaste (without antioxidants), which is still 10 times below the ADI but leads to a concentration of 258-495 mM in 0.5-1 ml saliva (or 83-165 mM applying the standard 1/3 salivary dilution of toothpaste components[278]). This may not be the optimum nitrate source and dose to stimulate eubiosis as high concentration of nitrate (e.g., 100 mM) can inhibit certain oral bacteria[237], while potassium is associated with dysbiosis[279]. Future studies should test this.

Possible oral care products with nitrate would include dentifrices with low amounts of a nitrate salt, together with antioxidants to increase safety and even other compounds to maximize the desired effect. This could include the enzymes cofactor (e.g., molybdenum for nitrate reductases as discussed in chapter 2) or molecules that affect the balance between DRNA and denitrification. For example, ammonium produced during DNRA could prevent caries development (by lactic acid usage and proton consumption), while nitric oxide production could be particularly helpful to inhibit strictly anaerobic species involved in periodontal diseases and halitosis. In respect to this, it has been shown that the available carbon source[183] and nitrogen:carbohydrate ratio[211] can cause shifts in nitrate metabolism and this should be further explored in future projects. These studies could include *in vitro* experiments, similar to the ones in chapter 1 and 2, modifying the variables of interest. Additionally, the effect of other environmental factors such as pH and oxygen levels should be investigated (e.g., a nitrate-rich gel in an anaerobic

periodontal pocket may have different effects than toothpaste on the aerobic tooth surface). Apart from nitrate salts, nitrate-rich vegetable extracts, containing both nitrate and natural antioxidants, could be included to oral hygiene products as discussed in chapters 1-3. Such natural formulations may be demanded by current societies with an increased interest to consume healthy, organic and plant-based foods.

Apart from classical oral hygiene products, such as mouthwash and toothpaste, nitrate could also be part of products that are swallowed. In one individual that donated saliva in chapter 2, we observed a dramatically low nitrate reduction capacity, which provided an excellent opportunity to monitor nitrate recycling dynamics. When this individual consumed a beetroot supplement, two clear peaks were observed (Figure D3): one directly after intake (topical effect) and one two hours after intake (ingested effect). Additionally, a third small peak was observed after 5 hours, potentially also resulting from nitrate recycling. Products like capsules and pills that are directly swallowed would only trigger an ingested-derived effect and may not be an interesting choice to stimulate oral eubiosis immediately by a direct, topical effect. However, some products can trigger both and immediate and a delayed effect, like the beetroot supplements used in chapter 3, especially if the supplement is first rinsed in the mouth before swallowing. On the other hand, a nitrate-rich mouthwash would provide mainly a topical, immediate effect. One example of a potentially disruptive product would be an anti-caries tablet, which one can chew on for a direct topical dose of nitrate and then swallow to increase salivary nitrate for several hours while plasma nitrate is recycled by the salivary glands[25]. Other disruptive products could include a nitrate-rich tongue paste to stimulate systemic nitric oxide formation and dental floss with nitrate to increase eubiosis at interproximal sites.

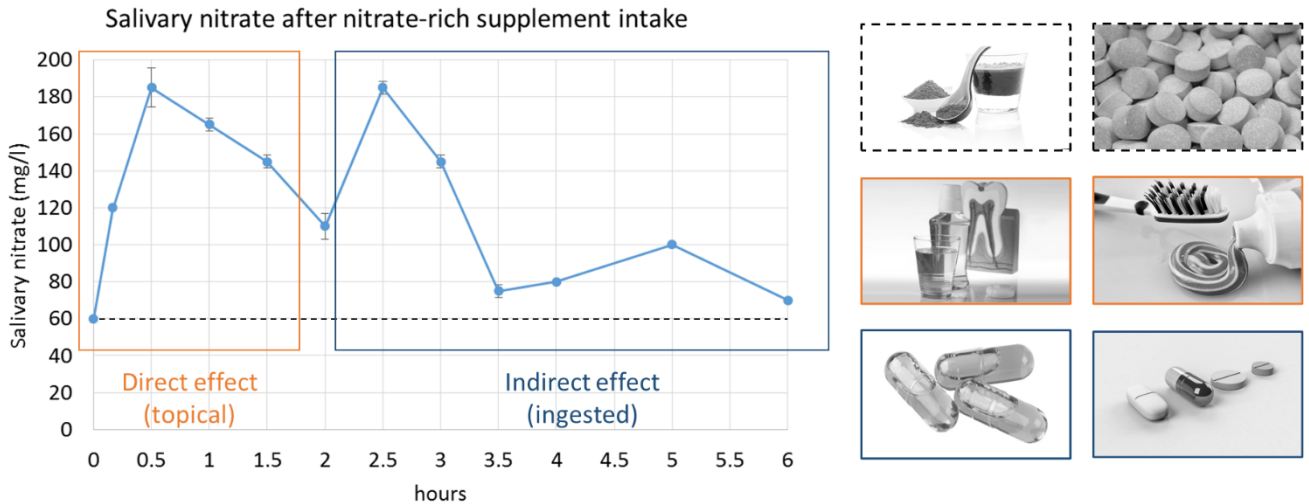


Figure D3: Direct and indirect peaks of salivary nitrate after ingestion of a nitrate-rich supplement. One donor of chapter 2 (D11) had a dramatically low nitrate-reduction capacity: virtually no decrease in nitrate was detected after 7 h of incubating the donor's saliva with 6.5 mM nitrate. As a comparison, other donors reduced all nitrate after 7 h. When this donor (D11) took a single dose of 250 mg nitrate in the form of a beetroot supplement as described in chapter 3, a direct increase could be observed after intake (direct topical effect). Additionally, the activity of the salivary glands (without a large effect of bacterial nitrate reduction) could be observed over time (indirect ingested effect). The baseline (0 h) sample was taken right before supplement intake and the second measurement was 10 minutes after supplement intake. Throughout the entire period of 6 hours, elevated nitrate levels were detected compared to baseline. Some products only have a direct topical effect (e.g., mouthwash and toothpaste) indicated by orange squares in the right panel, while other products only have an indirect ingested effect (e.g., capsules and pills) indicated by blue squares. Other products have both direct and indirect effects (e.g., food supplements, fresh vegetables and chewing tablets) indicated by black dotted lines. To stimulate oral health, products without a topical effect may be least interesting, while from a safety point of view when nitrate salts are used, products with only a topical effect may be most interesting as low doses of few to several milligrams (far below de acceptable daily intake) could be used.

Nitrate-reducing isolates as potential probiotics

As shown and discussed in chapter 2, among healthy individuals, some have a low NRC capacity. Given that nitrate-reduction is associated with a large amount of systemic and oral benefits, nitrate-reducing probiotics are a promising treatment to increase the NRC. As discussed in chapter 1, it has been shown that nitrate-reducing bacteria, such as *Rothia* and *Neisseria*, are consistently lower in oral communities of patients with periodontitis, caries and halitosis. In a recent literature review, focusing on metagenomic studies, it was confirmed that *Rothia*, *Neisseria* and other genera with nitrate reducing representatives (e.g., *Actinomyces*, *Kingella*, *Veillonella* and *Corynebacterium*) are less abundant in subgingival plaque of periodontitis patients compared to health individuals[280]. It could therefore be that individuals with a lower NRC are more common among patients with oral diseases, which should be tested in future studies. It should be noted that periodontitis is associated to a broad range of systemic conditions[13]. These include conditions that can be improved with nitrate supplementation, such as hypertension and diabetes[25,122]. I therefore hypothesize that the decrease of nitrate-reducing bacteria in patients with periodontitis could contribute to the development of certain systemic conditions. This would be another mechanism to take into account in the oral-systemic axis, in addition to periopathogenic bacteria, such as *Porphyromonas gingivalis*, entering the blood stream and affecting other body sites, as well as inflammatory mediators produced in gingiva that can cause systemic inflammation[281]. Consequently, nitrate supplementation and nitrate-reducing probiotics, of which the latter could be essential in patients with a low NRC to experience any benefits from nitrate supplementation, could potentially improve these conditions. The effect of these treatments on parameters related to systemic disease development in patients with periodontitis should be explored in future studies.

The safety of nitrate-reducing probiotics should be carefully monitored. Under some conditions, nitrate-reducing bacteria could stimulate beneficial effects (e.g., individuals consuming high amounts of vegetables)[122], while in others it

could increase NOC formation (e.g., individuals consuming water with high nitrate levels or high amounts of processed meats)[242]. It could therefore be that nitrate-reducing probiotics should always be combined with appropriate vegetable and fruit intake. Nitrate-reducing probiotics could be particularly interesting for individuals with systemic diseases related to low nitric oxide levels[228], especially if the potential risk outweighs the benefits, but more research is needed. The effect of nitrate-reducing probiotics in combination with different dietary compounds (e.g., nitrate, antioxidants and polyphenols) should be explored to make sure that potential future products do not result in harmful NOC formation.

As discussed in chapter 2, another safety issue with probiotics is their potential to reach other parts of the body and cause infection. For example, many oral bacteria have been isolated from endocarditis samples, including representatives of *Rothia*[171], *Gemella*, *Granulicatella* and *Prevotella*[218], as well as classic commensals generally recognized as safe ("GRASS" organisms) such as *Streptococcus salivarius*[219] and *Lactobacillus* strains used a probiotics[218]. This appears to be an extended feature of representatives of many oral species, which appear to be pre-adapted to attach to distant human tissues by their ability to adhere to oral mucosa components like collagen and fibronectin[218]. It is therefore crucial with all oral probiotics to select strains without virulence genes potentially involved in endocarditis or other systemic complications. Specifically, according to a FAO/WHO (2002), newly registered probiotic strains must be examined in pathological, genetic, toxicological, immunological, and microbiological aspects that could be relevant for human safety[209]. We confirmed that in all 10 probiotic candidates of the *Rothia* genus in chapter 2 there were no genes for mobile genetic elements, antimicrobial resistance and virulence factors, which could make them otherwise unsuitable for probiotic usage from a genetic point of view. From an oral health point of view, probiotics should not induce acidification, volatile sulfide compound production or inflammation. Our *Rothia* isolates did not lead to acidification when combined with saliva in sugar-rich medium in chapter 2. Additionally, the

strong association of *Rothia* with periodontal health, as well as the lower abundance of *Rothia* species in the absence of halitosis, indicate that this genus does not contribute to these diseases. Potential periodontal products with oral probiotics could include gels to apply inside periodontal pockets and toothpastes (e.g., dry toothpastes with lyophilized strains) combined with nitrate and other molecules discussed above. It is also important to underline that apart from *Rothia* species, we also isolated *Actinomyces* species in chapter 2, while other teams have isolated different nitrate-reducing bacteria from the oral cavity such as *Neisseria* and *Schaalia*[282], whose probiotic potential should be tested in the future.

In conclusion, high vegetable intake is a recommended strategy to increase salivary nitrate levels and oral hygiene products with low amounts of nitrate are promising products to stimulate beneficial bacteria and functions of the oral microbiota. More research is needed to determine the efficacy, colonization, safety and potential risks of nitrate-reducing probiotics, as well as their biotechnological aptitudes like scale-up growth or viability and stability in a final product. In addition, the development of "symbiotics" (i.e. the combination of a nitrate source and a nitrate-reducing bacteria) should also be explored. I hope that the work presented in this thesis stimulates further basic and applied research directed towards understanding the role of nitrate in oral homeostasis and towards the development of oral care products that contribute to improve oral health.

Future prospects

The research field about the contribution of nitrate to oral health is still in its infancy. Future research avenues should include clinical studies to test the longitudinal effect of daily nitrate intake (e.g., vegetables or vegetable extracts) or application (e.g., toothpaste, mouthwash or chewing tablets). Apart from healthy individuals, which was the focus of this thesis, the effect of nitrate on patients with caries, gingivitis, periodontitis and halitosis should be evaluated.

Nitrate reduction could also differ in systemic diseases and be affected by hormone fluctuations (e.g., during the menstrual cycle). Apart from microbiota and physiological measurements, the effect on host processes, such as inflammation and salivary flow should be measured. Additionally, *in vitro* studies should test the effect of nitrate and nitrate-reducing isolates on the oral microbiota composition and activity under different conditions. These include different carbon:nitrogen ratios, different carbon sources, the addition of other prebiotics (e.g., arginine), different environmental conditions (e.g., oxygen and pH levels), using different samples as inoculum (e.g., dental plaque and tongue coating) as well as using dysbiotic communities as inoculum (e.g., from patients with periodontitis or caries), and using different isolates apart from *Rothia* (e.g., species of *Neisseria* and *Kingella*). For more accurate nitrate and nitrite measurements other methods (e.g., HPLC or chemiluminescence) should be considered. These experiments would clarify the mechanisms underlying nitrate metabolism by oral microbial communities, paving the road to efficient modulation of nitrate-reduction pathways.

In future studies, the effect of the *Rothia* isolates obtained in chapter 3 should also be tested *in vivo* to confirm their safety in animal models and to see if systemic nitric oxide levels and the associated benefits could be stimulated. Additionally, it should be explored if oral acidification and caries development are prevented, which we hypothesize based on the results of this thesis. *Rothia* and/or other nitrate-reducing isolates may also have a positive impact on inflammation (e.g., by local nitric oxide production or the production of anti-inflammatory mediators) and bad breath (e.g., hydrogen sulfide consumption), which should be explored. Finally, it should be determined if individuals with different oral and systemic diseases have differences in their NRC, which could be measured with a diagnostic test. Such a test could for example be based on the ability to reduce nitrate by salivary bacteria or through qPCR of nitrate reduction genes. Based on the dietary nitrate, the NRC and the oral health status of individuals, the most effective treatment for oral and systemic conditions could vary. Potential treatments include nitrate as a prebiotic (e.g., for

individuals with good oral health and NRC), nitrate-reducing species as probiotics (e.g., for individuals with a low NRC and a diet rich in vegetables) or the combination of both with antioxidants (a symbiotic treatment, e.g., for individuals with oral diseases and/or a low NRC). I expect that in the coming years the interest in nitrate as a prebiotic and nitrate-reducing species as probiotics will increase, leading to an increase of research directed towards these potential treatments and the development of several new diagnostic and therapeutic products.

Conclusions of thesis

Conclusions

- Nitrate stimulates the growth of the genera *Rothia* and *Neisseria in vitro*, both associated to oral health.
- The observed effects of nitrate in an *in vitro* model suggest that it can potentially decrease caries-, halitosis- and periodontal disease-associated bacteria.
- Nitrate did not significantly affect total biofilm growth in an *in vitro* model when using saliva from healthy individuals as inoculum.
- The *in vitro* and *in vivo* data presented in this thesis indicate that nitrate can limit or prevent pH drops when sugars are fermented by the oral microbiota – a mechanism of resilience that could be stimulated by the consumption of nitrate-rich vegetable extracts.
- The main pH buffering mechanisms of nitrate are lactic acid usage during denitrification (observed both *in vivo* and *in vitro*) and during nitrite reduction to ammonium, as well as potential ammonia production (observed *in vitro*).
- The effects of nitrate are acute and were observed after 5-9 h incubation *in vitro*, and/or 1-4 hours after nitrate supplement intake *in vivo*.
- The nitrate-reducing species that we isolated were associated to general oral health (*Rothia dentocariosa*, *R. mucilaginosa* and *R. aeria*) or periodontal health (*Actinomyces oris* and *A. viscosus*). Modifying the culture conditions should allow isolation of other nitrate-reducing genera.
- *Rothia* isolates can increase lactic acid usage and nitrate reduction capacities of oral communities, potentially benefitting dental health and systemic health, respectively. Such *Rothia* isolates can thus be considered potential probiotic candidates if they fulfill safety requirements.
- The absence of genes for mobile genetic elements, antimicrobial resistance and virulence factors in 10/10 tested *Rothia* isolates made them suitable probiotic candidates from a genetic point of view.

- One symbiotic combination (the *R. mucilaginosa* isolate D3T4 combined with nitrate) prevented pH drop more than just nitrate, making this an interesting probiotic candidate to prevent caries.
- The *in vitro* and *in vivo* data presented in the current thesis suggest that nitrate can modulate the oral microbiota in ways that are beneficial for the host and can thus be considered a prebiotic substance for the oral microbiota.
- Due to the physiological ability to recycle nitrate into saliva, nitrate-containing products can have not only a direct effect on oral microbial communities, but also an indirect, longer-term effect after the nitrate is ingested, offering interesting new possibilities for disruptive oral care products.

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Supplementary table of thesis

Supplementary table T1: vegetables in different groups (data from EFSA, 2008)*

Vegetable group*[1]	**Included vegetables (number of samples: mean nitrate content in mg/kg)**
Leafy vegetables	Amaranth (12: 2167 mg/kg), beet (12: 1852 mg/kg), Belgian endive (1006: 1465 mg/kg), butterhead lettuce (3426: 2026 mg/kg), cos lettuce (124: 1105 mg/kg), curled lettuce (301: 1601 mg/kg), dandelion (23: 605 mg/kg), escarole (73: 523 mg/kg), Iceberg lettuce (1980: 875 mg/kg), lamb's lettuce (710: 2104 mg/kg), lettuce (7749: 1324 mg/kg), mixed lettuce (89: 2062 mg/kg), oak-leaf lettuce (470: 1534 mg/kg), radicchio (40: 355 mg/kg), rucola (1943: 4677 mg/kg), chard (666: 1690 mg/kg), spinach 6657: 1066 mg/kg), water cress (25: 136 mg/kg)
Herbs	Basil (68: 2292 mg/kg), borage (15: 1918 mg/kg), chives (83: 748 mg/kg), coriander (20: 2445 mg/kg), dill (57: 1332 mg/kg), parsley (249: 958 mg/kg)
Stem vegetables	Asparagus (260: 209 mg/kg), celery (387: 1103 mg/kg), fennel (116: 1024 mg/kg), leek (558: 345 mg/kg), rhubarb (58: 2943 mg/kg)
Roots and Tubers	Artichokes (65: 174 mg/kg), beetroot (1013: 1379 mg/kg), black radish (19: 1271 mg/kg), black salsify (12: 43 mg/kg), carrot (2383: 296 mg/kg), celeriac (41: 390 mg/kg), parsnip (22: 83 mg/kg), potato (2795: 168 mg/kg), radish (788: 967 mg/kg), turnip (241: 663 mg/kg), white radish (200: 1416 mg/kg)
Brassica vegetables	Broccoli (227: 279 mg/kg), brussels sprouts (130: 24 mg/kg), cabbage (1198: 311 mg/kg), cauliflower (289: 148 mg/kg), Chinese cabbage (469: 933 mg/kg), curly kale (169: 537 mg/kg), kohlrabi (135: 987 mg/kg), red cabbage (196: 281 mg/kg), sauerkraut (37: 66 mg/kg), savoy cabbage (342: 324 mg/kg).
Legumes	Beans (48: 392 mg/kg), French beans (52: 756 mg/kg), green beans (362: 323 mg/kg), peas (407: 30 mg/kg), string beans (13: 618 mg/kg)
Bulb vegetables	Garlic (13: 69 mg/kg), onions (230: 164 mg/kg)
Fruiting vegetables	Aubergine (182: 314 mg/kg), capsicum (455: 108 mg/kg), chili pepper (152: 67 mg/kg), courgette (159: 416 mg/kg), cucumber (898: 185 mg/kg), gherkin (88: 69 mg/kg), pumpkin (32: 894 mg/kg), tomato (856: 43 mg/kg)
Fungi	Mushroom (12: 61 mg/kg)

*This is a continuation of Table 2 in the introduction

Conflict of interest

Together with Alex Mira, Mariam Ferrer and Arantxa Lopez, I am a co-inventor of a patent owned by our institute (the FISABIO Foundation) protecting the use of nitrate and certain nitrate-reducing bacteria in products to stimulate oral eubiosis.