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Sustaining elevated levels of nitrite in the oral cavity through consumption of nitrate-rich beetroot juice in young healthy adults reduces salivary pH

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ABSTRACT

Background: Dietary inorganic nitrate (NO_3^-) and its reduced forms nitrite (NO_2^-) and nitric oxide (NO), respectively, are of critical importance for host defense in the oral cavity. High concentrations of salivary nitrate are linked to a lower prevalence of caries due to growth inhibition of cariogenic bacteria.
Objective: *In-vitro* studies suggest that the formation of antimicrobial NO results in an increase of the pH preventing erosion of tooth enamel. The purpose of this study was to prove this effect *in-vivo*.
Methods: In a randomized clinical study with 46 subjects we investigated whether NO_3^- rich beetroot juice exhibits a protective effect against caries by an increase of salivary pH.
Results: Our results show that, in comparison to a placebo group, consumption of beetroot juice that contains 4000 mg/L NO_3^- results in elevated levels of salivary NO_2^-, nitrite NO_3^-, and NO. Furthermore, we determined an increase of the mean pH of saliva from 7.0 to 7.5, confirming the anti-cariogenic effect of the used NO_3^--rich beetroot juice.
Conclusions: Taken together, we have found that NO_3^--rich beetroot juice holds potential effects against dental caries by preventing acidification of human saliva.
Trial registration: C-87-15 (Ethics Commissions of Upper Austria).

1. Introduction

Nitric oxide (NO), a highly reactive gaseous free radical, is involved in several diverse and complex biological processes [1]. NO exerts antimicrobial activity, for example via induction of host immune cells and a subsequent resistance to oral candidiasis [2]. Importantly, being a non-immunological source of nitric oxide, dietary inorganic nitrate (NO_3^-) also contributes to the host defense in the oral cavity [3], and the importance of sustaining a NO-rich environment in the mouth has been widely demonstrated.

Beetroots and leafy green vegetables are a rich source of NO_3^- [4]. Upon ingestion, NO_3^- is absorbed from the small intestine and transported via the entero-salivary circulatory loop where it is subsequently extracted by the salivary glands [5]. Within the saliva glands, NO_3^- is concentrated by at least 10-fold compared to plasma levels [6]. With salivary secretion, the commensal anaerobic microflora in the oral cavity reduces salivary NO_3^- to nitrite (NO_2^-) by bacterial nitrate reductase.

Typically, NO_2^- is further reduced enzymatically to NO in circulation and tissue. NO_2^- is also reduced non-enzymatically to nitric oxide (NO), in the acidified stomach and in capillary beds that may be exhibit hypoxic conditions during intensive exercise, where the pH is lowered [7,8]. In like fashion, NO can be produced by acidification of NO_2^- in the oral cavity. However, low pH conditions in the mouth are often limited to gingivitis, periodontitis, and advanced caries [3,9].

In the past, NO_3^- and NO_2^- have been unsubstantially linked to carcinogenic formation of N-nitroso compounds [10,11]. Epidemiological evidence continues to dismiss these earlier assumptions.

Abbreviations: NO_3^-, nitrate; NO_2^-, nitrite; NO, nitric oxide; IC, ion exchange chromatography; SULF, sulfanilamide; NEED, N-1-(naphthyl) ethylenediamine; VCl_3, Vanadium(III) chloride.

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And isolated reports on infant Methemoglobinemia, which is caused by the interaction of NO_2^- with hemoglobin, was found to be associated with contaminated well water sources [12,13]. In sharp contrast, further clinical studies have clearly shown that NO_3^- rich beet juice and leafy green diets are cardio-protective by reducing blood pressure, enhancing blood flow, and inhibiting platelet aggregation [14–16].

Aside from the regulatory role of nitric oxide in vascular tonality, persistently, high levels concentrated within a cell, such as infected macrophage, or localized region of a microgranuloma, nitric oxide exhibits antimicrobial activity. A wide number of intracellular pathogenic parasites (i.e., malaria, leishmania) and bacteria are sensitive to NO [1]. With that said, patients with high concentrations of salivary NO_3^- and the ability to bio-convert NO_3^- to NO_2^- had significant lower prevalence of caries [17]. Dental caries, which is the most common disease within the oral cavity and particularly found in children, is mainly caused by an acid erosion of tooth enamel resulting from the fermentation of dietary carbohydrates by pathogenic oral bacteria such as *Streptococcus mutans* and *Lactobacillus* spp [18]. These cariogenic bacterial species produce strong acids such as lactic and acetic acid, which lead to the reduction of the salivary pH below the critical value of 5.5, and a subsequent demineralization of tooth surfaces [17,19]. *In-vitro* studies have demonstrated that NO_2^- significantly inhibits bacterial acid production by exerting bactericidal effects on cariogenic bacteria through the formation of antimicrobial NO from NO_2^- under acidic conditions, which results in a subsequent increase of the pH [19,20]. To date, there have been no reports associating that the dietary consumption of a well-characterized, NO_3^--rich plant-based juice results in the bioconversion of NO_3^- to NO_2^- in acidified mouths, likely caused by a cariogenic bacteria, to a corresponding increase in pH.

Here this report addressed this question in a clinical study with 46 subjects by investigating whether NO_3^--rich beetroot juice does exhibit a putative protective effect against caries. These results clearly indicate that NO_3^--rich juice significantly prevents the acidification of the salivary pH if consumed regularly and bioconversion of NO_3^- to NO_2^-occursas determined by a saliva NO test strip.

2. Materials and methods

2.1. Chemicals

Sulfanilamide (SULF), N-1-(naphthyl) ethylenediamine (NEED), Vanadium(III) chloride (VCl_3), sodium nitrite, sodium carbonate, sodium hydrogen carbonate, and sodium nitrate were purchased from Sigma-Aldrich (Schnelldorf, Germany).

2.2. Beetroot juice

A local juice manufacturer (Voglsam, Hofkirchen im Traunkreis, Austria) produced the beetroot juice used for the present study (fitRABBIT) as a single batch. The product consists mainly of beetroot juice and a small part of passion fruit juice. For juice production, a beetroot variety known for its high content of nitrate ("Mona Lisa") was used [21]. Detailed information on juice production and nitrate content measurements can be found in the supplemental appendix.

2.3. Study design

The study protocols were approved by the Ethics Commission of Upper Austria (study C-87-15). This observational study was prepared in agreement with STROBE guidelines. All study participants signed a written informed consent. Forty-six healthy male and female students, 18–35 years of age, were recruited at the University of Applied Sciences Upper Austria. Exclusion criteria were smoking, hypertension, use of any medication and/or dietary supplements that might affect the saliva composition, pregnancy and contagious diseases. All study participants signed a written informed consent. The Ethics Commissions of Upper Austria approved the study protocols. The study was performed as a randomized, single-blinded clinical study at the University of Applied Sciences Upper Austria. All subjects were requested to avoid any mouth wash like Odol® or Listerine® and food and drinks containing beetroot products during the entire study which lasted six weeks. After an overnight fast, each participant took the first saliva sample. From that day on the intake phase of the fitRABBIT/placebo juice started, whereby 100 ml of fitRABBIT corresponding to 400 mg NO_3^- or placebo corresponding to 2 mg NO_3^- (in sequentially numbered containers) were consumed daily in the morning between 7:00 and 10:00 a.m. for two weeks. After these two weeks, an observation phase for four weeks without consuming fitRABBIT or placebo followed in order to detect any long-term effects. Further saliva samples were taken on day 8, 15, 28 and 42 always in the morning before brushing the teeth, having breakfast and drinking the juice. Saliva samples were immediately flash-frozen and stored at −20 °C until analysis. In addition, all participants were asked to keep a dietary record for the first two weeks. During the intake phase, Nitric Oxide Test strips from Berkeley Test LLC (www.berkeleytest.com, Sacramento, California) were used twice a day, two hours after drinking the juice and in the evening between 6:00 and 8:00 p.m., respectively. From day 15–36 the test strips were only used once a day two hours after lunch. Salivary Nitric Oxide Test strips by Berkeley Test are designed to measure the bioconversion of NO_3^-- to NO_2^-- in the mouth. The bioconversion is a critical and necessary step for the subsequent formation of NO.

2.4. Sample preparation

Saliva samples were collected in 50 ml centrifuge tubes (Greiner Bio-One, Kremsmünster, Austria) and immediately frozen. For quantitation, samples were thawed on ice, centrifuged at 3.000 rpm for 10 min, and supernatants were used for further analysis.

2.5. Nitrate and nitrite measurements

Salivary NO_3^- and NO_2^- concentrations were determined according to a method described by *Miranda* et al. [22]. Detailed information on juice production and nitrate content measurements can be found in the supplemental appendix.

2.6. Nitric oxide and pH measurements

Salivary NO levels were determined by using the NO Test Strips from Berkeley according to the following instructions. The test strips do exhibit a saliva absorption pad on one end, and a NO test pad on the other end. First, the saliva collection pad was placed for 5 s on the tongue. The strip was removed and the two ends were gently pressed against each other for 5 s. After releasing the test pad the developing color was observed and compared to the color chart illustrated on the packaging of the test strips. A dark pink color indicated a high NO level, whereas light pink was linked to NO depletion. Berkeley strips are based on the well-established, modified Griess reagent reaction, which is often used in evaluating serum, urine, and saliva NO_2^-. The strips do not measure NO_3^-. Hence, the bioconversion of NO_3^- to NO_2^-, which occurs in the mouth via the nitrate-reducing microflora, is detected by the strips. If bioconversion does not occur, the strips do not display a reaction.

Berkeley Test strips are calibrated as: depleted, low, threshold, target, and high, which translates to 21, 108, 217, 434, and 869 μM NO_2^-, respectively. The pH of all centrifuged saliva samples was measured using a pH meter.

2.7. Statistics

All data are presented as means ± SEM. The differences in the mean values among the placebo and the test group within the same experiment were analyzed by two-way ANOVA grouped analysis and Student's paired *t*-test. Differences between the groups were considered significant at $p \leq 0.05$. Statistical analysis was performed using GraphPad Prism 6 for Windows software package (GraphPad Software Inc.). MS Office Professional Plus 2010 was used for data compilation.

3. Results

3.1. Nitrate content in beetroot juice

The beetroot juice used for this study was found to contain 4000 mg/L NO_3^-. The placebo juice contained less than 20 mg/L nitrate. By drinking 100 mL of juice each subject in the test group ingested about 400 mg of NO_3^- in comparison to the placebo group, which consumed only 2 mg of NO_3^-.

3.2. Changes in salivary nitrate concentration during and after beetroot juice consumption

The averaged NO_3^- content of the overnight fast samples on the first day of the study was found to be 487 μM (mean value obtained from both study groups prior juice consumption). Our collected data show that the salivary nitrate levels of individual subjects within the placebo and the high NO_3^-potency beet juice group vary greatly. As indicated in Table 1, concentrations in the overnight fast samples ranged from 10 to 4000 μM (about 400 fold, Table 1). NO_3^- levels were increasing within the beetroot juice group during juice consumption (Fig. 1). After 8 days the average salivary nitrate concentration reached its maximum at 1425 μM, corresponding to a significant 3-fold increase. There was only a slight increase within the placebo group over the same period. After day 8 the salivary nitrate concentration within the beetroot juice group declined till day 42 and reached a value of 1074 μM on the last day of the study. Salivary NO_3^- concentrations of the placebo group did not significantly vary within the chosen period.

3.3. Changes in salivary nitrite concentration during and after beetroot juice consumption

Basal mean salivary NO_2^- levels after an overnight fast were 102 μM (values taken from both study groups placebo and test group prior juice consumption, Table 2). Average NO_2^- levels within the beetroot juice group showed a steady increase during the intake phase and reached its peak on day 15, the first day after the

Table 1
Overview of average (±SEM), minimal and maximal values for salivary nitrate concentrations within the fitRABBIT group and the placebo group.

	o/n	Day 8	Day 15	Day 28	Day 42
fitRABBIT group – nitrate concentration [μM]					
Average	487 ± 138	1425 ± 646	1114 ± 353	774 ± 300	1074 ± 474
Max	4124	14417	6675	6024	10269
Min	7	19	2	3	1
Placebo group – nitrate concentration [μM]					
Average	487 ± 138	620 ± 230	644 ± 253	589 ± 220	597 ± 294
Max	4124	4696	4768	4380	6535
Min	7	1	1	1	1

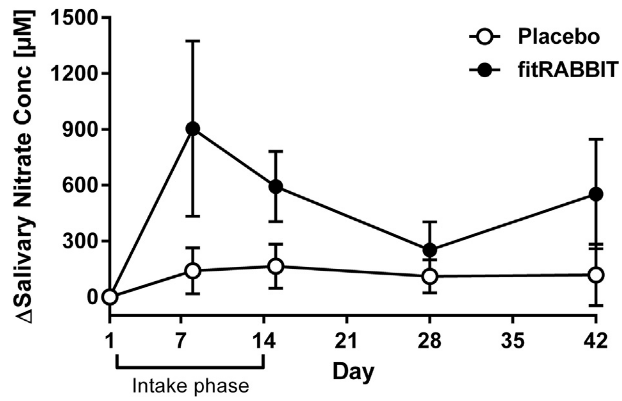

Fig. 1. Temporal changes in salivary nitrate levels during and after drinking fitRABBIT beetroot juice (4000 mg NO_3^-/L) or placebo (20 mg NO_3^-/L), respectively. Average salivary NO_3^- levels were normalized to the overnight fast sample on day 1 (487 μM). Absolute changes in NO_3^- are shown. From day 1 till day 14 the test subjects drank 100 mL fitRABBIT (400 mg NO_3^-) or placebo (2 mg NO_3^-) per day. On day 15 juice consumption was stopped and the subjects were further observed till day 42. Filled circles represent the fitRABBIT group whereas open circles represent the control group. Data are expressed as means ± SEM, n = 23. Statistical significance was determined using two-way ANOVA grouped analysis test.

Table 2
Overview of average (±SEM), minimal and maximal values for salivary nitrite concentrations within the fitRABBIT group and the placebo group.

	o/n	Day 8	Day 15	Day 28	Day 42
fitRABBIT group – Nitrite concentration [μM]					
Average	102 ± 16	125 ± 20	165 ± 33	134 ± 24	123 ± 20
Max	431	176	652	342	330
Min	2	6	15	1	2
Placebo group – Nitrite concentration [μM]					
Average	102 ± 16	83 ± 14	72 ± 26	89 ± 14	82 ± 16
Max	431	224	594	248	326
Min	2	1	1	1	1

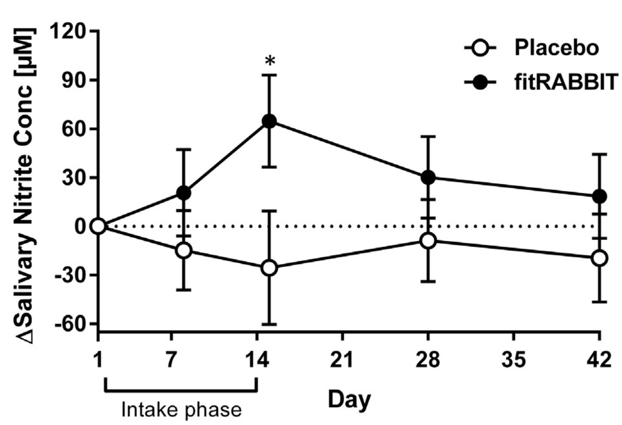

Fig. 2. Temporal changes in salivary nitrite levels during and after drinking fitRABBIT beetroot juice (4000 mg NO_3^-/L) or placebo (20 mg NO_3^-/L), respectively. Average salivary NO_2^- levels were normalized to the overnight fast sample on day 1 (102 μM). Absolute changes in NO_2^- are shown. From day 1 till day 14 the test subjects were drinking 100 mL fitRABBIT (400 ml NO_3^-) or placebo (2 ml NO_3^-) per day. On day 15 juice consumption was stopped and the subjects were further observed till day 42. Filled circles represent the fitRABBIT group whereas open circles represent the control group. Data are expressed as means ± SEM, n = 23. Statistical significance was determined using two-way ANOVA grouped analysis test, *P < 0.05.

juice consumption period. Fig. 2 indicates that the beetroot juice group was characterized by a significantly higher salivary NO_2^- concentration on day 15 than the placebo group. Nitrite levels of the beetroot juice group declined nearly to baseline levels till day 42.

3.4. *Changes in salivary NO or bioconversion of NO_3^- to NO_2^- values during and after beetroot juice consumption*

During the intake phase NO levels were determined twice a day two hours after drinking the juice and 6–10 h after beetroot juice consumption. From day 15 to day 36 NO was measured once a day two hours after lunch. As determined by salivary NO test strips, which is reflective of total bioavailability of NO and assesses the bioconversions of $NO_3^-$to NO_2^-in the mouth, as required and necessary step in the formation of NO. As shown in Fig. 3, peak levels of 100–200 μM where detected with the test strips within the first 2 h, which approximates the time for bioconversion to take place. Basal mean levels of salivary NO after an overnight fast were found to be less than 30 μM. Two hours after drinking beetroot juice levels were increased to 100–200 μM, which was significantly higher than NO levels of the placebo group (30 μM). In the evening NO levels of the beetroot juice group declined, but were still significantly higher than NO levels of the placebo group. Importantly, NO concentrations were found to decrease back to basal levels immediately after the juice consumption period.

3.5. *Changes in salivary pH during and after beetroot juice consumption*

Finally, we tested whether increased salivary NO levels correlate with elevated pH values, which is an important anti-cariogenic parameter. As indicated in Fig. 4 the mean pH value of saliva of all study subjects after an overnight fast was 7.0. Consumption of fitRABBIT juice led to pH increase to ~7.5 on day 7 and 15, respectively. This increase was significantly higher than in the placebo group, whose pH did only change slightly within the study period. The pH value of the beetroot juice group was found to decrease after the juice intake phase. Based on these results we conclude that consumption of nitrate-rich beetroot juice does not only lead to increased nitrate, nitrite and NO levels, but also prevents acidification of the human saliva.

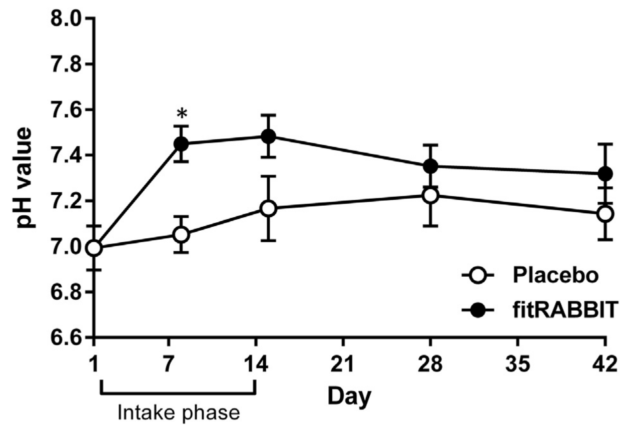

Fig. 4. Temporal changes in salivary pH values of men and women during and after drinking fitRABBIT beetroot juice (4000 mg NO_3^-/L) or placebo (20 mg NO_3^-/L), respectively. From day 1 till day 14 the test subjects were drinking 100 mL fitRABBIT (400 mg NO_3^-) or (2 mg NO_3^-) placebo per day. On day 15 juice consumption was stopped and the subjects were further observed till day 42. The pH on day 1 (7.0) is based on the average value after an overnight fast on day 1. Filled circles represent the fitRABBIT group, whereas open circles represent the control group. Data are expressed as means ± SEM, n = 23. Statistical significance was determined using two-way ANOVA grouped analysis test, *P < 0.05.

4. Discussion

Beetroots provide a major source of inorganic nitrate in daily life [4]. Ingested nitrate is metabolized and recycled in the human body and concentrated in the saliva gland. The bioconversion of salivary gland-derived NO_3^- to NO_2^- is a necessary and required step in the subsequent formation of nitric oxide. The initial reduction of NO_3^- to NO_2^- is strictly dependent upon the commensal microflora that resides on the tongue of the mouth. If this microflora is disrupted by harsh mouthwashes, bioconversion fails to occur and nitric oxide is not formed [23]. Many of these microorganisms such as *Veillonella* spp are anaerobes and require nitrate for respiration. Others such as *Actinomyces* spp and *Staphylococcus* spp are facultative anaerobic bacteria using oxygen as a terminal electron acceptor for respiration, but under conditions of low oxygen nitrate reductase enzymes are induced and nitrate is used instead [17,23,24]. Anaerobic conditions in the oral cavity are found in the deep interpapillary clefts of the tongue where large amounts of NO_2^- are produced [3].

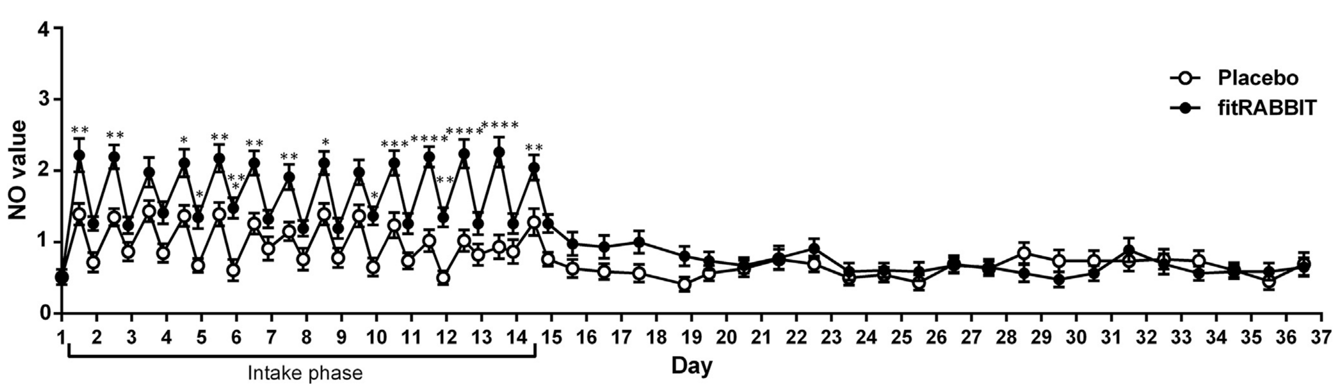

Fig. 3. Effects of a 14-day intake phase of fitRABBIT beetroot juice on salivary nitric oxide levels in healthy male and female volunteers. NO values were measured using Nitric Oxide Test Strips by Berkeley whereby 0 (<60 μM) is the lowest NO level and 5 (>650 μM) the highest one. First measurements on day 1 represent the NO value after an overnight fast. NO measurements were repeated 2 h after drinking the juice and in the evening between 6:00 and 8:00 p.m. From day 15 to day 36 only one NO measurement 2 h after lunch was performed. Filled circles represent the fitRABBIT group (4000 mg nitrate/L), whereas open circles represent the placebo group (20 mg nitrate/L). Data are expressed as means ± SEM, n = 23. Statistical significance was determined using two-way ANOVA grouped analysis test *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

The low pH carious lesions permits the acidification of NO_2^- generating antimicrobial oxides of nitrogen such as NO, which in turn leads to the self-inhibition of acidogenic cariogenic bacteria including *Streptococcus mutans* and *Lactobacillus* spp [19].

In the present study, we examined whether NO_3^--rich beetroot juice is bio-converted to NO_2^- with a corresponding rise in oral pH levels. Although direct assessment of cariogenic activity and a reduction of acid-causing bacteria needs to be measured, this report suggests that NO_3^- to NO_2^- bioconversion results in a subsequent reduction of NO_2^- in an acidified environment likely caused by cariogenic bacteria. We found that the consumption of beetroot juice for two weeks increased levels of NO_3^-, bioconversion to NO_2^- and elevated the pH value in the saliva providing support that beetroot may shift the composition of the microbiome.

The mean NO_2^- level on day 15 of a once a day 400 mg NO_3^- beetroot juice source resulted in about 0.2 mM, which is a concentration known to kill *Streptococcus mutans*, a prominent acidogenic bacterium [19,20]. Assuming bioconversion of beet juice NO_3^- to saliva NO_2^- occurs first, the acid milieu of cariogenic bacteria, will likely reduce NO2 to an adequate antimicrobial response. It is well established that acidified NO_2^- acts bactericidal or bacteriostatic only on those bacteria in the immediate vicinity and those that are NO-susceptible [17]. The major signaling molecule NO within the nitrate-nitrite-NO pathway readily diffuses through cell membranes and has a high affinity for iron-sulfur-containing respiratory enzymes and damages bacterial DNA [9]. We have shown that the intake of fitRABBIT beetroot juice enhanced the salivary NO concentration significantly more than the intake of the placebo product. This finding is suggestive of the anti-cariogenic properties of nitrate-rich beetroot juice. In the field of cardiovascular health it is well established that the administration of NO_3^- supplementation in the form of potassium nitrate or beetroot juice increases cardioprotective and cardioenhancing properties, assuming bioconversion of NO_3^- to NO_2^- occurs in the oral cavity first [25–29].

NO measurements in the evening resulted in lower levels, but those were still higher than the ones of the placebo group. This reduction is similar with other studies showing the cardioprotective effects of beet juice [30]. Thus, dietary NO_3^- needs to be constantly ingested in order to benefit from the antimicrobial effects and other biological functions of NO. Hence, the importance of intra-daily monitoring with salivary nitric oxide test strips. It would not be unreasonable to predict that if oral bioconversion levels were sustained throughout the day, the mouth would remain alkaline, as reflected by a cytocidal effect of the acid-forming bacteria. In this case, the strips would be used to remind the patient to replenish with NO_3^--rich beet juice as the strips levels begin to naturally fall through the day.

In doing so, salivary pH values would seldom become acidic and harmful to the teeth. pH is a prominent indicator for the presence and number of cariogenic bacteria, and elevated pH values result from a decline in the number of acid-producing bacteria [19]. Thus, to shift from a transient cytostatic to a lasting cytocidal effect on cariogenic bacteria, dietary NO_3^--rich natural foods such as a well-characterized, high potency beetroot juice should be incorporated into one's oral health program as regulated by intra-daily readings with a salivary NO test strips. Individuals will vary as to how long their NO_3^-/NO_2^-/NO, hence, it will be critical to monitor frequently with test strips and, if levels are falling, replenish with beet juice before acidogenic bacteria reestablish. An important aspect in this regard is the efficacy of commercial antibacterial mouthwash products in comparison to the one of nitrate-rich beetroot juice: These products reduce the growth of the entire oral microbiome including nitrate-reducing bacteria and thus inhibit the bioactivation of NO_3^- and all its health beneficial functions. Contrary, beetroot juice may lead to a selective cytostatic or cytocidal effect of acidogenic bacteria assuming antimicrobial levels are sustained as determined by test strips.

In conclusion, we have found that nitrate-rich beetroot juice holds potential effects against dental caries. The ingestion of nitrate leads to a change of the oral microbiome via the NO_3^--NO_2^- bioconversion, with the subsequent acidification of NO_2^- resulting in elevating pH levels which is predictably a reduction of acidogenic cariogenic bacteria. Although this report does not show a persistent anti-cariogenic effect which is likely due to the clearance of NO_3^-/NO_2^- which is similar to the blood pressure lowering effect if beet juice is replenished based on test strip outcomes. We believe that the oral health beneficial effects can be sustained by replenishing based on intra-daily monitoring of NO_3^--NO_2^- bioconversion.

In view of the high sugar content of beetroot juice, a potential source of bacterial nutrition, our findings might appear contradictory. However, we conclude that the bioconversion of NO_3^- to NO_2^- via NO_3^- reduction coupled by an acidic environment drives antimicrobial activity, out weighing the sugar source.

Statement of conflict of interest

There are no patents, products in development or marketed products to declare. The authors declare that there is no conflict of interest regarding the publication of this paper, and disclose any existing financial arrangements.

Author contributions

BH assisted in manuscript preparation and performed the experiments. OH and JW conceived and designed the experiments. BH, RH, UM and PL analyzed the data. JW supervised all aspects of this work and wrote the paper. All authors drafted and approved the final manuscript.

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