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1 **The Role of Dietary Nitrate and the Oral Microbiome on Blood Pressure and Vascular tone**

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16
17 Running title: Dietary nitrate, oral bacteria and vascular tone

18 **Abstract:**

19 There is increasing evidence for the health benefits of dietary nitrates including lowering blood
20 pressure and enhancing cardiovascular health. Although commensal oral bacteria play an important
21 role in converting dietary nitrate to nitrite, very little is known about the potential role of these
22 bacteria in blood pressure regulation and maintenance of vascular tone. The main purpose of this
23 review is to present the current evidence on the involvement of the oral microbiome in mediating
24 the beneficial effects of dietary nitrate on vascular function and to identify sources of inter and
25 intra-individual differences in bacterial composition. A systematic approach was used to identify
26 the relevant articles published on PubMed and Web of Science in English from January 1950 until
27 September 2019 examining the effects of dietary nitrate on oral microbiome composition and
28 association with blood pressure and vascular tone. To date, only a limited number of studies have
29 been conducted, with n=9 in humans and n=3 in animals focusing mainly on blood pressure. In
30 general, elimination of oral bacteria with use of a chlorhexidine based antiseptic mouthwash
31 reduced the conversion of nitrate to nitrite and was accompanied in some studies by an increase in
32 blood pressure in normotensive subjects. In conclusion, our findings suggest that oral bacteria may
33 play an important role in mediating the beneficial effects of nitrate-rich foods on blood pressure.
34 Further human intervention studies assessing the potential effects of dietary nitrate on oral bacteria
35 composition and relationship to real time measures of vascular function are needed, particularly in
36 individuals with hypertension and those at risk of developing cardiovascular diseases.

37
38
39
40 **Key words:**

41 Nitrate, nitrite, nitric oxide, oral microbiome, blood pressure, mouthwash,

42

43

44 **Introduction**

45 Cardiovascular diseases (CVDs), including coronary heart disease and stroke, are one of the leading
46 causes of death globally. In 2017, the World Health Organization (WHO) reported that 18 million
47 people had died from CVDs worldwide which represents 31% of deaths¹. Abnormally raised blood
48 pressure, defined as greater than 140 (systolic)/90 (diastolic) mmHg, is an independent risk factor
49 for CVDs and this silent killer is associated with a three-fold higher risk of having a stroke or
50 developing heart failure^{2,3}. High blood pressure affects more than 1 in 4 adults in England, around
51 12.5 million people. However, the prevalence of hypertension appears to differ between sexes, with
52 31% reported amongst men and 26% amongst women⁴. Dysfunction of the endothelium, which
53 controls vascular tone and strongly associated with hypertension, is now recognised as an early, but
54 potentially reversible, step in the development of CVDs⁵.

55 The control of vascular function is known to be influenced by dietary factors, with nitrate-
56 rich vegetables considered an important modulator^{6,7,8}. This has been demonstrated in many
57 observational and cohort studies which have shown consumption of nitrate and nitrite-rich foods to
58 significantly improve cardiovascular health⁹ such as lowering blood pressure¹⁰ in both healthy¹¹ and
59 hypertensive individuals¹², reducing endothelial dysfunction^{13,14 15,16,17} and inflammation¹⁸,
60 protection from ischemia reperfusion injury¹⁹, and improved exercise performance in patients with
61 heart failure²⁰. A prospective cohort study has also concluded that an increased adherence to a diet
62 high in nitrate is accompanied by a significant reduction in the risk of suffering both cardiovascular
63 complications and death due to any cause²¹. Clinically, nitrate supplementation or use of nitrate as a
64 medication to increase the bioavailability of nitrite and nitric oxide (NO) can reduce blood
65 pressure²². The interest in using dietary nitrates as a treatment for lowering blood pressure is
66 growing but mechanisms underlying the effects are unclear which limits their current application as
67 a dietary treatment for hypertension²². Furthermore, there is some evidence to suggest that high
68 dietary nitrate intakes are associated with negative effects on health, which has led to the
69 development of the Acceptable Daily Intake (ADI) for nitrate of 3.7 mg/kg body weight/day and for

70 nitrite of 0.07 mg/kg body weight/day²³. The ADI for nitrate is based on the risk of
71 methaemoglobinaemia commonly known as blue baby syndrome, which can occur following high
72 nitrate intake in some babies, and can be fatal ²⁴. In addition, some epidemiological studies have
73 reported an association between dietary nitrite intake and colorectal cancer. However, the weight of
74 evidence only supports a significant relationship between cancer and red and processed meat²⁵, with
75 little known about vegetables and drinking water. The nitrate and nitrite within processed meat may
76 be a contributing factor in the association with cancer, although this needs further confirmation.

77 Humans are naturally colonised by an array of microorganisms, such as commensal or
78 symbiotic communities, whose metabolic activity is important for host physiology and health.
79 Commensal oral bacteria and those residing in the gastrointestinal (GI) tract play an important role
80 in converting dietary nitrate to nitrite and the potent vasodilator NO^{26,27,28,29,30,31}. Up to 85% of
81 ingested nitrate is reduced to nitrite by the nitrate-reducing bacteria in the oral cavity³² raising the
82 salivary nitrite concentration to 1000 times that of plasma²⁸. A cohort study conducted in 281
83 volunteers found that the high abundance of nitrate reducing bacteria was associated with blood
84 pressure in normotensive individuals, although this association was not found in those with
85 hypertension³³. To date, very little is known about the role of these oral bacteria in the control of
86 vascular function, and the variation in composition that exists between individuals. The aim of this
87 review is to present the current evidence on the potential role of dietary nitrate and the oral
88 microbiome on vascular function including blood pressure and vascular tone. Important
89 determinants of the number and composition of the oral bacteria will also be described. However,
90 the impact of dietary nitrate interventions on vascular function only will not be specifically
91 addressed in this instance due to the large number of review articles which already exist in this
92 research area^{13,14 15,16,17}. Before presentation of the methodology and results of the literature
93 review, we provide a general overview of dietary nitrate sources, the pathways for the conversion of
94 dietary nitrate and nitrite to NO, location and type of nitrate-reducing bacteria in the oral cavity and
95 their potential role in regulating vascular tone.

97 Nitrate, nitrite and nitric oxide sources and nitric oxide pathway

98 NO, the most effective form of nitrate, was first recognised in 1998 as an important signalling
99 molecule in the cardiovascular system³⁴. NO plays a significant role in virtually all organs in the
100 body, and higher circulating concentrations are associated with a lower CVD risk³⁵. In addition to
101 the dietary (exogenous) sources of nitrate and nitrite which leads to the production of nitrite, and
102 subsequently NO, via the oral bacteria, the body can also derive NO endogenously (figure1). The
103 endogenous pathway can occur in a number of different tissues in the body using three forms of NO
104 synthase (NOS) enzyme, neuronal (nNOS), endothelial (eNOS) and inducible NOS (iNOS). eNOS
105 was initially discovered in endothelial cells and is important in modulating vascular tone and
106 upholding endothelial integrity. However, eNOS can also be expressed in various tissues and
107 requires the presence of oxygen, calcium and calmodulin to be activated³⁶. Within the endothelium,
108 L-arginine undergoes a 5-electron oxygen dependent oxidation to produce NO and L-citrulline,
109 catalysed by the synthase enzymes. Five cofactors required by the NOS enzymes are flavin adenine
110 dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH₄), reduced
111 nicotinamide-adenine dinucleotide phosphate (NADPH) and heme iron²⁸. Once produced in the
112 endothelial cell, NO rapidly diffuses to the underlying smooth muscle layer where it mediates blood
113 vessel vasodilation. Any NO remaining in the circulation is rapidly converted to nitrate by
114 oxyhaemoglobin or superoxide before it enters the enterosalivary pathway. Therefore, the NO
115 produced has a relatively short half-life in the order of seconds to minutes³⁷.

116

117 Nitrate metabolism, enterosalivary circulation and gastrointestinal tract

118 High levels of inorganic nitrate are found in vegetables (such as beetroot and spinach) as well as
119 drinking water, and these dietary sources accounts for 80% of the daily intake. In contrast, the
120 intake of dietary nitrite is very low, being approximately 100 times lower³⁸ than that of nitrate ³⁹.
121 Although the process of re-circulation of nitrates in the body has been known since 1970s, the

122 importance of the oral nitrate-reducing bacteria in the enterosalivary circulation has only recently
123 been recognised²⁷ (Figure 2). The key role these bacteria play in nitrate reduction was supported by
124 a previous human study in which a significant correlation was found between high abundance of
125 oral nitrate-reducing bacteria and nitrite level in saliva⁴⁰. Nitrate secretion from the salivary glands
126 leads to a 10 fold rise in salivary nitrate levels⁴¹ and this nitrate enriched saliva appears to be a
127 supportive environment for the growth of the oral bacteria particularly the nitrate-reducing bacteria
128 on the tongue⁴². These bacteria are mostly facultative anaerobes which use nitrate as an alternative
129 electron acceptor for their respiration⁴³. A symbiotic relationship therefore exists between the oral
130 commensal bacteria in which they receive nitrate from the host for their own respiration and in
131 return produce nitrites required by the host⁴². This relationship is particularly important for nitrite
132 bioavailability since humans are unable to complete this process independent of the nitrate-reducing
133 bacteria, with 80% of nitrates swallowed and present in the stomach produced by the oral
134 commensals⁴⁴. Once in the stomach, contact with the gastric acidity leads to the protonation of
135 nitrites to form nitrous acid (HNO₂), which then decomposes into not only NO but also several
136 other nitrogen oxides⁴⁵ which have localised benefits on maintaining the gastric mucosa layer⁴⁶ and
137 enhancing mucosal blood flow⁴⁵ which increases the thickness of the mucosal layer⁴⁷. This process
138 is referred to as non-enzymatic conversion which does not require bacteria. However, the presence
139 of *Helicobacter pylori* can contribute to a more acidic environment within the stomach and increase
140 non-enzymatic conversion⁴⁸. Residual nitrates and nitrites are then absorbed in the small intestine
141 with the half-life of circulating nitrate in the blood stream of around 5-6 hours⁴⁹. In contrast, plasma
142 nitrite concentrations start to increase within 15 minutes of nitrate ingestion and reach a peak level
143 in 2 hours⁵⁰. A large portion, approximately 70-75% of the plasma nitrate, is excreted in the urine
144 whereas the remaining 25% is stored in the salivary gland and then recycled in the enterosalivary
145 pathway⁵¹.

146 The role of the nitrate-reducing bacteria can persist past the oral cavity as most of these
147 bacteria move into the stomach with both swallowed food and saliva. Limited studies have

148 investigated the existence of these bacteria in the stomach and have confirmed that the gastric
149 acidity is not a germ-free environment⁵². Although the gastric pH is below 5, some bacteria species
150 can tolerate the stomach acidity, with a culture based study reporting *Clostridium* spp,
151 *Veillonella* spp and *Lactobacillus* spp as the most predominant gastric species⁵³, with
152 *Veillonella* spp identified as the most abundant nitrate reducing bacteria⁴³. There are many factors
153 that can influence gastric acidity such as inflammation and long-term use of proton pump inhibitors.
154 The pH level has been found to have a positive impact on nitrate and nitrite concentration in the
155 gastric juice. In a study conducted in 99 patients with dyspepsia, results showed that when the pH
156 level of gastric mucosal surface increased there was a comparable increase in both nitrate and nitrite
157 concentrations. Findings from another study conducted in participants with achlorhydria, in which
158 gastric pH ranged from 6-8, reported three genera of nitrate reducing bacteria: *Streptococci* and
159 *Neisseriae* to be responsible for the nitrite accumulation in the gastric secretions⁵⁴.

160 The small intestine and colon contain many different species of bacteria including both
161 facultative and obligate anaerobes which are involved in the bioconversion of nitrite to NO,
162 although they are not necessarily the same as the nitrate reducing bacteria found in the oral cavity⁵⁵.
163 A study conducted in germ-free and normal rats has shown that NO can be produced by the bacteria
164 resident in the small intestine of normal rats, but not in germ free rats⁵⁶. Furthermore, two studies
165 have identified *Lactobacilli*, *Bifidobacteria*⁵⁶, *Escherichia coli* and *Shigella* as the predominant
166 nitrate reducing bacteria in the large intestine⁵⁷. However, an *in-vitro* study which used pure strains
167 of gut bacteria incubated in agar media with nitrate then nitrite, found that in the presence of nitrite,
168 both *Bifidobacterial* and *Lactobacilli* generated large amounts of NO, up to 5000 parts per billion
169 (ppb), but only approximately 35 ppb of nitrate⁵⁸. Interestingly, Sobko et al reported that the NO
170 formed was being utilised by *Escherichia coli* and *Staphylococcus aureus*⁴⁶. These authors
171 speculated that these gut bacteria may consume NO in order to help adapt to their environment in
172 this *in vitro* experiment. Therefore, it appears that the presence of NO and other nitrate metabolites
173 in the large intestine may be dependent on the relevant abundance of these bacteria species and their

174 production and utilisation of NO⁵⁹. Localised effects of the NO could include altering blood flow
175 which could potentially increase the uptake of nitrate and nitrite in the proximal small intestine
176 where the majority are absorbed⁴⁴. However, the NO level in the GI tract could also be influenced
177 by other factors such as pH level, inflammation, oxygen tension and the level of dietary nitrate
178 intake of an individual. Further studies are need to determine the direct effects of nitrate and nitrite
179 on gut bacteria composition and nitrate metabolism.

180

181 **Bacterial nitrate reduction in the oral cavity, composition and location**

182 A continuous flow of saliva, specialized mucosal surfaces and teeth in the human oral cavity
183 provide a unique microbial habitat for bacteria. Most of these bacteria are found on the dorsum
184 (surface) of the tongue and around the teeth where a wash of 1 ml of saliva can contain up to 10⁷ –
185 10⁸ microorganisms⁴⁴. However, only 700 species have currently been identified⁴⁴. The majority of
186 these bacteria shelter in the gingival crevices between teeth which represents a conducive anaerobic
187 environment. Here, the gingival crevicular fluid bathes the bacteria within a nutritionally rich
188 medium supporting their proliferation⁶⁰. In contrast, the smoother surfaces of teeth have much
189 lower levels of bacteria due to the forces that act on these areas during eating and drinking.
190 However, the nitrate-reducing bacteria are found predominately on the rear dorsum of tongue, with
191 a higher proportion of gram-negative bacteria found within the papillae of the tongue compared to
192 the surface. Some studies have identified the genus and species of these bacteria that can produce
193 nitrate reductases and nitrite reductases that aid in the production of nitric oxides. These include:
194 *Veillonella atypical Veillonella dispar*, *Actinomyces eslundii*, *A. odontolyticus*, *Staphylococcus*
195 *epidermids*, *Neisseria flaescens*, *Haemophilus*, *Porphyromonas*, *Rothia mucilaginosa*, *Rothia*
196 *dentocarisa*, *Prevotella* and *Leptotrichia*^{42 43}. The two major groups of oral nitrate-reducing
197 bacteria are the strict anaerobes such as *Veillonella atypica* and *Veillonella dispar* and the
198 facultative anaerobes such as *Actinomyces odontolyticus* and *Rothia mucilaginosa*⁴². Facultative
199 anaerobes are mostly prevalent on the surface of the tongue, with a study stratifying participants

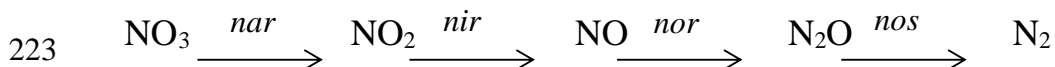
200 according to oral nitrate reduction capacity observing a higher abundance of *Streptococcus*,
201 *Granulicatella*, *Prevotella*, *Neisseria*, and *Haemophilus* on the posterior surface of the tongue
202 compared to *Actinomyces*⁴³. Interestingly, although lower in prevalence, *Actinomyces* have been
203 reported to be more efficient reducers of dietary nitrates under anaerobic conditions.

204

205 **Mechanisms by which bacteria may convert nitrate to nitrite**

206 The three mechanisms through which nitrates are converted to nitrites and other components by
207 bacteria are denitrification, assimilation and dissimilation. The first process, denitrification, occurs
208 in the oral cavity under aerobic conditions⁶¹ and is also called the respiratory nitrate reduction
209 process. During microbial respiration, oxygen is replaced by nitrogen oxides as terminal electron
210 acceptors and ultimately reduces nitrate to nitrous oxide or free nitrogen⁶². Most of the bacteria
211 which have genes for respiratory nitrate reductases (*nirS* and *nirK*) prefer aerobic conditions⁶³ such
212 as *Rothia spp* and *Neisseriae spp*. However, some denitrification species of bacteria also reside in
213 anaerobic conditions⁴⁴ such as *Veillonella*. The specialised surface of the tongue dorsum therefore
214 represents a microaerophilic environment which allows denitrification to occur under both aerobic
215 and anaerobic conditions. In the oral cavity, nitrite (NO₂) is initially formed from salivary nitrate
216 (NO₃) by some oral bacteria such as *Actinomyces*⁴³ that are considered to possess the nitrate
217 reductase enzyme (*nar*) and further converts nitrite to NO through either enzymatic (*nir*) or non-
218 enzymatic denitrification. The latter process is a well-established step in the gastric environment of
219 the stomach. NO is then converted to nitrous oxide (N₂O) by nitric oxide reductase (*nor*) and finally
220 to nitrogen (N₂) by nitrous oxide reductase (*nos*). The nitrogen oxides and enzymes that participate
221 in the process of denitrification are as follows:

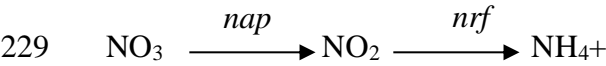
222



224

225 In the second pathway known as dissimilation, nitrate is reduced to ammonia (NH₄⁺) by
226 periplasmic nitrate reductase (*nap*), with the intermediate product being nitrite⁶⁴. This two-step
227 process is strictly anaerobic and occurs in the human gut by the facultative anaerobes⁵⁵.

228



230

231 Assimilation, which occurs predominantly in plants, water and soil⁶⁵, is the third pathway. Similar
232 to denitrification, the conversion of nitrate to ammonia occurs but during this pathway, the enzyme
233 cytoplasmic nitrate reductase (*nas*) is used⁶⁵. In this biosynthetic anabolic pathway, nitrite is further
234 reduced to ammonia, which can then undergo ammonium assimilation by incorporating the amino
235 acid glutamine⁴⁴. The assimilation and dissimilation processes are therefore important in the
236 utilization of nitrates. Nitrifying bacteria (including *Nitrobacter*, *Nitrococcus* and *Nitrosomonas*)⁶⁶
237 are responsible for the dissimilation and ammonification of nitrates and oxidises ammonium salts
238 and nitrites to nitrates in a process called nitrification. It has been hypothesised that this process
239 might happen in the gut, but to date, this has not been described⁶⁷.

240 In humans, nitrate reduction seems to occur either directly, such as in assimilatory nitrate
241 reduction, or during a series of reactions during respiratory nitrate reduction. Notably, the latter
242 process needs more than one enzyme for further reduction which is mediated by the bacterial
243 communities⁴⁴. This suggests that nitrate reducing capacity of nitrate-reducing bacteria is related to
244 the bacterial species, cellular location of enzymes and environmental conditions such as oxygen
245 level. Therefore, dissimilation would occur more in the gut and denitrification in the oral cavity⁶⁷.
246 Although the role of oral bacteria in mediating the beneficial effect of nitrate on vascular function is
247 poorly understood, this review aims to address this knowledge gap by focussing on studies that used
248 antibacterial mouthwash and toothpaste to determine the importance of the presence of oral
249 microbiome on blood pressure and vascular tone.

250

251 **Methods**

252 A systematic approach was used to identify the relevant human and animal studies which investigated
253 the role of dietary nitrate and the oral microbiome on blood pressure. PubMed and Web of Science
254 were used for the literature search which included all relevant articles published in English from
255 January 1950 until September 2019. There were three stages in the selection process. The
256 combinations of the key terms used in the search strategy were as follows: (“Nitrate” OR “Nitrite”
257 OR “Nitric Oxide”) AND (“Oral Bacteria” OR “Oral Microbiom” OR “Nitrate-Reducing Bacteria”)
258 AND (“Blood Pressure” OR “Hypertension” OR “Cardiovascular” OR “Vascular Function”) AND
259 (“Mouth Wash” OR “Antiseptic” OR “Antibacterial”). The titles and abstracts of the identified papers
260 were screened by one member of the review team (HA) who identified potentially relevant papers.
261 This review was restricted to animal studies and human studies which used antibacterial mouthwash
262 or toothpaste to determine the effects on oral nitrate reduction on blood pressure and vascular tone.
263 Only published peer-reviewed literature was considered and ‘grey’ literature such as dissertations,
264 conference proceedings, reports, letters to editors and other non-peer-reviewed research, was
265 excluded. After duplicates were removed, the abstract and full papers were screened for eligibility.
266 In addition, a hand-search of the bibliographies of the articles found from the electronic database
267 searches was also conducted. An overview of the literature search is shown in Figure 3.

268 The quality of the included human RCTs and animal studies were assessed for the risk of
269 bias using the Cochrane risk of bias tool⁶⁸ for human studies and SYRCLE’s tool⁶⁹ for animal
270 studies.

271

272 **Results and Discussion**

273 The systematic search identified 160 publications. Of these, 11 relevant publications were included,
274 with 9 describing studies conducted in humans and 3 in animals. The risk of bias assessment
275 summaries for each study are presented in Supplementary Tables 1 and 2, respectively. Animal
276 studies will be discussed before studies including human participants. This will be followed by

277 discussion of the non-modifiable and modifiable factors affecting intra-individual variability in
278 number and composition of oral bacteria, with potential mechanisms of action.

279

280 **Animal studies**

281 Of the 14 animal studies which have investigated the effect of nitrate on blood pressure, only 3
282 studies have determined whether oral bacteria are important in mediating the improvements in
283 blood pressure and endothelial function (Table 2). Formation of bioactive NO takes place within the
284 gastric environment of the stomach as a result of the enterosalivary circulation of nitrate, as well as
285 systemically in the blood vessels. In 2009, Petersson and his colleagues⁷⁰ reported daily mouthwash
286 treatment for 7 days in rats to attenuate both the gastroprotection provided by NO and the diastolic
287 blood pressure lowering effect of sodium nitrate. A similar pattern was also evident for the mean
288 arterial pressure in the rats treated with mouthwash and nitrate, but the lack of an effect in the rats
289 treated with mouthwash and nitrite suggested that oral bacteria play an important role in the
290 metabolism of nitrate to NO and mediated vasodilation. Furthermore, these rats also had reduced
291 oral bacteria suggesting that nitrite could bypass the reduction step by the oral bacteria and was
292 being reduced in the circulation or within endothelial cells to NO, or via effects on the formation of
293 the intermediate nitrosothiols⁷⁰. However, dietary nitrite intake is generally lower than that of
294 nitrate, and the half-life in plasma shorter (seconds versus hours) which suggests that even if nitrite
295 directly stimulates NO signalling, the quantity and kinetics of nitrite versus nitrate indicates that the
296 critical aspect of this mechanism is the reduction of nitrate. Therefore, the role that dietary nitrite
297 plays in blood pressure lowering may be more limited relative to nitrate.

298 In agreement, Hyde et al²⁹ also reported a significant reduction in diastolic blood pressure
299 and increase in plasma nitrite concentrations following the addition of sodium nitrate to drinking
300 water in male Wistar rats. However, in this study, mouthwash treatment was unable to diminish the
301 blood pressure lowering effects of the nitrate supplementation. The authors speculated that the
302 direct application of the chlorhexidine-based mouthwash (Vedco, St. Joseph, MO) to the tongue

303 surface using a swab might not have enabled sufficient time for the mouthwash to exert its full
304 extent on the bacteria relative to mouthspray²⁹. A novel aspect of this longer-term supplementation
305 study was the focus on the changes in the microbiota composition on the rat tongue in response to
306 the treatments. Compared with baseline, there was a greater relative abundance of nitrate reducing
307 bacteria (*Haemophilus spp* and *Streptococcus spp*) after 6 days of sodium nitrate consumption, and
308 of these *Haemophilus parainfluenzae* has also been identified as 1 of 14 species contributing to
309 nitrate reduction in the oral cavity of healthy adults. Co-supplementation of mouthwash with nitrate
310 was found to increase the diversity of the oral bacteria present relative to nitrate intake only, with
311 increases found in the low abundance taxa such as Enterobacteriaceae, *Corynebacterium*, and
312 *Morganella*. Therefore, the use of mouthwash appeared to disturb the oral microbiome by reducing
313 the abundance of the normally dominant taxa but not completely to impact nitrate reduction. These
314 findings suggest that the lower abundance taxa which were evident after mouthwash treatment may
315 be functionally important in the bioactivation of dietary nitrate. However, the authors did caution
316 against translating these findings on the oral bacteria composition to humans since the oral human
317 microbiome has been shown to be more diverse and of a differing composition compared with the
318 rat²⁹.

319 The impact of mouthwash on chronic changes in blood pressure in response to nitrate or
320 nitrite supplementation was further examined by Pinheiro et al⁷¹ in both control and hypertensive
321 rats. After 4 weeks, significant reductions in mean arterial pressure and systolic blood pressure were
322 evident in both the nitrate and nitrite groups, with concordant increases found in circulating plasma
323 nitrate and nitrite levels. Interestingly, co-supplementation with mouthwash attenuated the rise in
324 plasma nitrite levels by 25-30% in both groups but was only found to blunt the blood pressure
325 lowering effect of nitrate, with little impact found on blood pressure in the mouthwash and nitrite
326 group. In agreement with Petersson et al⁷⁰, these findings suggested that anti-hypertensive effects of
327 nitrite were potentially occurring via non-enzymatic reactions within the gastric environment after
328 swallowing this ion independently of the enterosalivary pathway and potentially via non-enzymatic

329 reactions within the gastric environment after swallowing this anion. Analysis of the endogenously
330 produced vasodilatory compound S-nitrosothiol and levels of vascular nitrosylation revealed
331 mouthwash to reduce nitrosylation responses to nitrate only, leading the authors to speculate that S-
332 nitrosylation was an important mediator of the blood pressure lowering effects of both nitrate and
333 nitrite^{70,71}. Studies have also reported that the foods consumed with dietary nitrites, such as
334 conjugated fatty acids, are also a target of nitrating species in the stomach leading to the formation
335 of nitro-fatty acids (such as nitro-conjugated linoleic acid). These electrophiles have been shown to
336 have anti-hypertensive effects independent of S-nitrosothiols suggesting that they may also play a
337 role in mediating the effects of nitrate and nitrite on blood pressure⁷². Antiseptic mouthwash was
338 proposed to attenuate the beneficial effects of dietary nitrate intake on blood pressure by reducing
339 the amount of nitrite formation by the oral bacteria and therefore reaching the stomach, inhibiting
340 gastric formation of S-nitrosothiols. However, the positive benefits on blood pressure of raised S-
341 nitrosothiols was only found in the antihypertensive rats, supporting previous observations in both
342 animals and humans that raised blood pressures often show a greater sensitivity to the anti-
343 hypertensive effects of medication and/or dietary modification.

344 Studies performed in animals may provide useful insights into the mechanisms underlying
345 the effects of oral bacteria in the bioactivation of nitrate. However, findings in rats and mice need to
346 be interpreted with caution due to differences in physiology and dependence on nitrate as a source
347 of NO between organisms. In contrast to humans, rats and mice do not recirculate nitrate in saliva⁷³
348 and so salivary nitrate concentrations never exceed those levels found in plasma⁷⁴ and they also
349 have other nitrate reducing mechanisms that may work in tandem with nitrate reduction by the oral
350 bacteria to control nitrite and NO level⁷³.

351

352 **Human Studies**

353 The publications describing the human studies were divided into those which examined 1) the
354 association between oral bacteria with nitrate/nitrite levels and/or blood pressure (n=5; Table 2) and

355 2) the combined effects of nitrate ingestion and oral bacteria on nitrate/nitrite levels and/or blood
356 pressure (n=4; Table 3). The role of the oral bacteria in mediating systemic nitrite production after
357 nitrate intake has been primarily investigated with the use of an antiseptic mouthwash to remove the
358 bacteria prior to the measurement of the outcomes of interest. The type of mouthwash has been
359 shown to be important, with the strong antibacterial chlorhexidine-based mouthwash (Corsodyl)
360 found to be more effective at reducing *Veillonella dispar* (nitrate reducing bacteria) in the oral
361 cavity than Listerine (mixture of essential oils), Isodine and Cepacol (antibacterial) in healthy
362 adults⁷⁵. In support of these findings, gargling with 10 ml of chlorohexidine mouthwash (Corsodyl)
363 twice for 1 min was also found to reduce the bacterial count of nitrate reducing bacteria by
364 approximately 80% and virtually abolish the oral nitrate reducing capacity compared with no
365 mouthwash in healthy subjects²⁷. Although nitrate accumulated in saliva after ingestion of sodium
366 nitrate in both studies, a significant reduction in the conversion of salivary nitrate to nitrite after
367 mouthwash was associated with 30% lower plasma nitrate concentrations at 3 h post-ingestion,
368 compared with no prior use of mouthwash. In contrast, a randomised cross-over study found an
369 antibacterial toothpaste to have no effect on salivary or plasma nitrate concentrations in 16 women
370 after consuming 400 mg of nitrate before brushing their teeth with antibacterial toothpaste (0.3%
371 triclosan) or toothpaste containing no antibacterial agent⁷⁶. The lack of an effect observed with the
372 antibacterial toothpaste may reflect either the lower prevalence of the nitrate reducing bacteria on
373 the surface of the teeth, relative to the tongue, or the less efficient removal of the bacteria sheltering
374 within the gingival crevices between the teeth compared with mouthwash.

375 Four studies have determined the impact of mouthwash on changes in oral nitrate reducing
376 capacity and blood pressure (Table 2). Compared with no mouthwash, Kapil et al⁴¹ reported that
377 using 0.2% chlorhexidine twice daily for 7 days significantly increased systolic and diastolic blood
378 pressure measured using 3 different techniques (clinic, ambulatory and home measurements) by
379 approximately 3 and 2 mmHg respectively in 19 healthy normotensive subjects. Interestingly, the
380 effects of mouthwash treatment on blood pressure was evident after only a single use of the

381 chlorhexidine mouthwash and was maintained for the following 6 days. The rise in blood pressure
382 was significantly correlated with the significant reduction in plasma nitrite levels, with only a trend
383 for a relationship with the salivary nitrite, highlighting the potential importance of the oral nitrate-
384 reducing bacteria in blood pressure modulation.

385 In 15 subjects treated with anti-hypertensive medication, the attenuation found in oral nitrate
386 reducing capacity after daily use of chlorhexidine mouthwash for 3 days was associated with an
387 increase in systolic blood pressure of 2.3 mmHg, but only a trend for a decrease in plasma nitrite
388 concentrations compared with the control (tap water)⁷⁷. The lack of a significant effect on the
389 plasma nitrite response relative to Kapil et al⁴¹ was thought to be due to the study visit being
390 performed 12 h after prior use of the mouthwash treatment or related to the age or medication use of
391 the hypertensive participants. In order to determine the mechanism underlying the effects of dietary
392 nitrate intake on blood pressure, plasma cGMP, a mediator of NO-dependant smooth muscle
393 relaxation in the endothelium and a good marker of NO production, can be measured. Although
394 increases in plasma nitrite and cGMP after dietary nitrate intake have been previously associated
395 with blood pressure lowering, no effects were evident on cGMP concentrations after 3 days of using
396 mouthwash. This may be related to the lack of a nitrate challenge on the study visit (which provides
397 an important source of NO under hypoxic conditions) but could also suggest that dietary nitrate may
398 impact on vascular tone via direct effects on smooth muscle function.

399 In contrast to these two studies, Tribble et al⁷⁸ reported use of chlorhexidine mouthwash
400 twice daily for 7 days to be associated with a highly variable effect on clinic systolic blood pressure
401 (an increase of at least 5 mmHg found in n=9 subjects whereas a decrease was observed in n=4) in
402 an orally healthy cohort. Post-hoc data analysis revealed the inclusion of tongue cleaning as part of
403 the daily dental hygiene routine to play a significant role in the responses observed both on blood
404 pressure and the diversity of the oral bacteria at baseline and during the study. Specifically, regular
405 tongue cleaning was associated with a greater ability to reduce nitrite to NO whereas the lack of
406 tongue cleaning resulted in an oral microbiome composition which favoured conversion of nitrite to

407 ammonia and not NO. The authors speculated the use of chlorhexidine mouthwash was having a
408 chemo-stimulatory effect on the oral bacteria, with the temporary loss of bacterial numbers
409 proposed to stimulate a rapid population recovery and increase in bacterial nitrate reductase activity.
410 However, these effects may also reflect a protective upregulation of the nitrate, nitrite and NO
411 regulating mechanisms in the microbiota suddenly detached from their biofilms during tongue
412 cleaning and warrants further investigation.

413 In a cross-over study, treatment with chlorhexidine (0.2%) for 3 days was shown to have no
414 effect on clinic or 24 h ambulatory blood pressure in 17 young females compared with a placebo
415 mouthwash ⁷⁹. Although a reduction in salivary nitrite and oral nitrate reducing capacity was found
416 after the antibacterial mouthwash, comparable changes were not evident in either the plasma or
417 urine samples collected. The lack of effects observed relative to other studies may reflect the short
418 intervention time with the mouthwash treatments or inclusion of female participants only. Based on
419 a previous study conducted by the same research group in athletes, they speculated that cross-talk
420 may exist between the enterosalivary nitrate-nitrite-NO pathway and eNOS, with a greater intake of
421 dietary nitrate associated with a lower eNOS activity. However, whether a reduction in nitrate-
422 nitrite-NO with antibacterial mouthwash leads to an upregulation in eNOS is yet to be established.

423 In the studies presented in Table 3, measures of blood pressure have been related to salivary
424 and plasma nitrate/nitrite levels following nitrate intake and use of mouthwash. In agreement with
425 previous findings, Woessner et al ³⁰ found antibacterial mouthwashes to attenuate postprandial
426 salivary and plasma nitrite concentrations following dietary nitrate intake (concentrated beetroot
427 juice) compared with the weaker antiseptic mouthwash and control. Although changes in clinic
428 systolic blood pressure 0-3 h after the treatments were not related to plasma/salivary nitrite or
429 nitrate levels, systolic blood pressure at 4 h was 2-5 mmHg higher after Chlorhexidine and Cepacol
430 mouthwashes compared with control and Listerine mouthwash. These findings potentially suggest
431 an important role of the nitrate-nitrite-NO enterosalivary pathway, but should be interpreted with
432 caution due to the small sample size, inclusion of male subjects only and the short duration of the

433 study visit relative to the expected peak in plasma nitrite concentrations (approximately 3 h).
434 Furthermore, these findings may have been influenced by the large inter-individual variability
435 observed in blood pressure responses following the mouthwash treatments.

436 In the study of McDonagh and co-workers ⁸⁰, consumption of 2 x 70 ml shots of
437 concentrated beetroot juice and daily use of strong or weak antibacterial mouthwash for 6 days were
438 found to have limited effects on baseline blood pressure and salivary and plasma nitrate/nitrite
439 levels compared with the control (water). However, differences were evident 2-4 h after drinking
440 the beetroot juice, with the rise in plasma nitrite found to be attenuated after use of the strong and
441 weak mouthwash for 6 days. These changes were associated with a reduced oral nitrate reducing
442 capacity after the strong mouthwash, with lower nitrite levels compared with both the weak and
443 placebo mouthwashes. Although changes in resting measures of blood pressure (supine and seated)
444 and pulse wave analysis (arterial stiffness) after the juice were not influenced by the strength of the
445 mouthwash used, differences were evident in blood pressure during low-intensity activity on the
446 treadmill. In particular, there was a greater increase in systolic blood pressure and mean arterial
447 pressure after rinsing with the strong (Chlorhexidine) compared with the control (water)
448 mouthwash. The lack of effect on arterial stiffness even in the presence of lower salivary and
449 plasma nitrite levels after the strong mouthwash indicates that either the availability of NO was not
450 altered sufficiently over the 4 h acute test period in these young active participants or that their
451 higher physical active level may have masked any effects of the mouthwash on the vascular
452 function measures. However, this is one of the only studies to incorporate a measure of blood vessel
453 elasticity to determine the role of oral bacteria in mediating the beneficial effects of beetroot juice
454 on vascular function, and so further studies are needed in which to compare these findings and
455 determine the underlying mechanisms.

456 As highlighted in the human studies, oral bacteria composition appears to vary between
457 individuals, with both non-modifiable (e.g. age, sex, genetics and tongue physiology) and
458 modifiable (e.g. diet, health conditions, life style and dental hygiene routine) factors considered to

459 impact on the abundance and prevalence of nitrate reducing bacteria in the oral cavity. These factors
460 are important to consider during interpretation of the study findings and for informing the design of
461 future studies exploring the role of oral nitrate reducing bacteria on the regulation of vascular
462 function. The following section summarises the main factors identified from the human studies.

463

464 **Intra-individual variability in number and composition of oral bacteria**

465 **Non-modifiable factors**

466 Geographical location and culture have all been suggested to impact on oral bacteria composition.
467 Findings from a study including participants from Northern and Southern Europe, reported a higher
468 abundance of *Rothia* and unclassified *Gemellaceae* in Finnish populations compare to Spanish
469 while *Lactococcus*, *Fusobacterium* and *Porphyromonas* genus were significantly higher in Spanish
470 compare to Finnish groups⁸¹. Comparing findings of this study with another study which
471 investigated the differences in oral bacteria between people living in Africa, Alaska and Germany
472 showed that oral bacteria composition is highly variable between countries⁸². These differences may
473 represent the sex and age distributions of these different populations, genetic make-up and habitual
474 food preferences^{83,82}.

475 Moreover, the dorsal surface of the tongue plays a major role in nitrate reduction and
476 represents a highly papillated surface area. The papillary structure of the human tongue is unique in
477 nature and supports a higher bacterial density than the mucosal surface, accumulating oral debris
478 and anaerobic bacteria on the rear of tongue⁴². There are three kinds of papillae on the tongue:
479 fungiform, circumvallate and foliate papillae. The fungiform papillae have a mushroom shape and
480 are found predominately on the dorsal surface of the tongue covering up to two-thirds of the
481 surface. Their shape supports a higher bacterial density⁸⁴. However, the shape and number of
482 papillae varies between individuals which has been related to differences in oral bacteria
483 composition. Studies have shown that a number of factors can affect the papillary number on the

484 tongue including ageing (with lower number of papillae observed in those individuals over 60
485 years), genetic make-up, ethnicity⁸¹, demographics and environment⁸⁴.

486 Within the oral cavity, the presence of teeth increases the bacterial density compared to
487 those with permanent tooth loss since the gingival crevices between teeth represent a greater surface
488 area and environment for bacterial growth⁸⁵. Other important factors considered to impact on the
489 variety of nitrate reduction bacteria present in the oral cavity are ageing and sex. However, in a
490 recent human study conducted in n=9 participants < 22 years and n=9 > 70 years, a similar salivary
491 microbiome at baseline and after placebo beetroot juice was found in both groups. Comparable
492 changes in bacterial composition (increases in *Rothia* and *Neisseria*) were also evident in both age
493 groups in response to consuming 70 ml of beetroot juice (\approx 6.2 mmol nitrate) daily for 10 days⁸⁶
494 suggesting that age was not an important modulator of the oral bacteria composition in this study.
495 Few studies have determined differences in oral bacteria composition between men and women. In
496 order to address this knowledge gap, Kapil and colleagues⁸⁷ examined the impact of sex on nitrate
497 reducing bacteria abundance in 13 male and 13 females age 18-45 years. Oral bacteria samples were
498 collected before and after nitrate supplementation and all samples were analyzed by 16S rRNA
499 sequencing. Significant sex dependent effects on oral nitrate reducing bacteria composition were
500 not found in this study. However, sub-group analysis indicated females to have a non-significant
501 tendency for a higher activity of nitrate reducing bacteria than men^{87,74} but these findings need to be
502 confirmed in a suitably powered study.

503

504 **Modifiable factors**

505 Several modifiable factors have been reported to influence and change the oral nitrate reducing
506 bacteria composition, with dietary nitrate intake considered to be one of the most important
507 factors^{27,88}. In a recent cross-over study conducted in 18 volunteers assigned to receive a nitrate
508 supplement or a placebo for 10 days, an increase in the abundance of some nitrate reducing bacteria,
509 particularly *Rothia* and *Neisseria* was linked with the ability of an individual to reduce the nitrate

510 supplement. However, changes were not observed with the *Prevotella* and *Veillonella* species⁸⁶.
511 Interestingly, these results corroborate findings from another study which reported the reduction in
512 *Prevotella* and *Veillonella* species in the oral cavity of elderly adults following dietary nitrate intake
513 to be associated with a lower mortality risk in this population⁸⁸. Furthermore, the increased
514 prevalence of *Rothia* and *Neisseria* species relative to the *Prevotella* and *Veillonella* species was
515 linked to higher NO bioavailability in both saliva and plasma⁸⁶. These findings imply that the oral
516 bacteria community is responsive to changes in the level of dietary nitrate intake⁸⁹. However, the
517 authors also reported that individuals with a higher abundance of *Campylobacter concisus* and
518 *Prevotella melaninogenica* in their oral cavity at baseline may not be as responsive to dietary nitrate
519 intake than those with a lower proportion of these bacteria⁸⁶. This might reflect the fact that both
520 *Campylobacter. concisus* and *Prevotella. melaninogenica* are predominately nitrite, but not nitrate,
521 reducers in the oral cavity. Therefore, dietary nitrate availability may affect the growth and
522 composition of particular groups of oral bacteria which can be related to improved cardiovascular
523 health⁸⁹. Of particular note, drinking beetroot juice rich in dietary nitrate can increase the oral cavity
524 pH from 7.0 to 7.5 which is close to the optimal pH of 8 required for nitrate reductase activity⁹⁰.
525 Therefore, the effect of pH is also important in terms of the proliferation and inhibition of different
526 populations within the oral bacterial community⁸⁶.

527 In a similar fashion, some health conditions have also been reported to influence the oral
528 bacterial composition, with a lower density of nitrate reducing bacteria and a different bacterial
529 composition found in people with raised blood pressure (hypertensives) than normotensive
530 subjects³¹. A recent novel study has provided further evidence on the relationship between
531 differences in oral bacteria composition with hypertension in postmenopausal women (n=446). This
532 study analysed oral bacterial samples by using 16S RNA sequencing and found that the abundance
533 of *Prevotella oral species 317* and *Streptococcus oralis* were significantly lower in women with
534 elevated blood pressure compared with those with normal blood pressure⁹¹. Furthermore, the
535 differences in the oral bacteria communities between groups also seemed to be associated with the

536 severity and progression of the hypertension³³. Conversely, a higher abundance of nitrate reducing
537 bacteria were observed in individuals who suffer from migraines (a vascular driven process
538 associated with changes in NO). Interestingly, the dominant nitrate reducing bacteria in these
539 individuals were *Pseudomonas* and *Streptococcus* which are not common in subjects who did not
540 suffer with migraines. Oligotyping (the technique for differentiation between closely related
541 microbial taxa)⁹² was performed for both genera to investigate the strain-level differences across the
542 bacterial population. *Pseudomonas* decompose to 2 oligotypes (different strains of the same
543 species) and has differential abundance patterns with significantly higher abundance in oligotype 2
544 in those suffering from migraines compared with non-sufferers⁹³. These results suggest that the type
545 of these oral bacteria may be more prevalent in people with migraines. However, more work is
546 needed to find the link and the mechanism to explain how these bacteria adapt genetically to their
547 host environment.

548 Therefore, there may be an optimum number and composition of nitrate reducing bacteria
549 which has beneficial effects, and a greater level may have a negative impact on conditions
550 associated with blood vessel dilation such as migraine. However, it should be acknowledged that
551 nitrate reduction and metabolism cannot be attributed to single bacterial species as they are unlikely
552 to express all of the enzymes required to decompose nitrate simultaneously. More likely, these
553 individual nitrate reducing bacteria are considered to work in synergy with other members of the
554 microbial community. This has been demonstrated by Hyde et al,⁴³ who found that mixed colonies
555 of high and low nitrate reducers showed a greater capacity for nitrate reduction than mixes of either
556 multiple high reducers or individual nitrate reducing bacteria. This highlights the complexity of the
557 oral microbiome and the impact on dietary nitrate metabolism.

558 Cardiometabolic diseases including obesity, the metabolic syndrome and type II diabetes are
559 major contributors to global CVD disease burden. Whilst some studies have reported plasma
560 nitrate/nitrite levels to be negatively associated with waist circumference⁹⁴, obesity⁹⁵ and blood
561 pressure, others have observed positive associations between plasma nitrite and BMI, fasting blood

562 glucose⁹⁶, systolic blood pressure and the fasting lipid profile. In support of these findings, Akram
563 et al (2018)⁹⁷, found plasma nitrite levels to be higher in individuals with both obesity and the
564 metabolic syndrome followed by those with obesity alone, with the lowest levels in those with
565 normal weight. Whether high plasma nitrite levels play a role in the worsening of the
566 cardiometabolic risk markers is a public health issue since higher dietary nitrate intakes may also
567 cause higher levels of plasma NO_x (sum of nitrate and nitrite levels). Furthermore, these data are
568 associations, do not indicate whether cardiometabolic risk markers change in response to varying
569 nitrate/nitrite intakes and do not prove cause and effect. Interestingly, a review of the evidence
570 suggests the contrary, with dietary nitrate supplementation found to reverse or improve some of the
571 features of the metabolic syndrome and be protective against the development of CVD⁹⁸. Although
572 these beneficial effects may be related to improvements in NO metabolic pathways and glucose
573 control, we cannot discount that favourable changes in the gut microbiota in response to dietary
574 nitrate intake may also represent an important mechanism since dysbiosis (a term to describe
575 microbial imbalance) is a common feature of the cardiometabolic diseases. However, very few
576 studies have determined the impact of dietary nitrate supplementation on the gut microbiota in
577 humans, with a very short-term study with nitrate-rich fruit and vegetable juice suggesting a
578 reduction in the Firmicutes to Bacteroides ratio after 3 days which was related to higher plasma
579 nitrate/nitrite levels⁹⁹. Furthermore, a one-year intervention with the Mediterranean diet, rich in
580 vegetables, was associated with increased abundance of specific taxa that were inversely associated
581 with inflammatory markers¹⁰⁰. More studies are needed to address this research gap which also
582 include analysis of the oral microbiome to determine whether increases in the abundance of nitrate
583 reducing bacteria are related to improvements in cardiovascular health.

584 Oral hygiene habits, including daily use of an antibacterial mouthwash or tongue scraper
585 have been found to not only reduce acute bacterial infection, but also numbers of bacteria present³⁰.
586 On the other hand, poor oral hygiene contributes to dysbiosis by accumulating a plaque biofilm
587 which contains large number of microbes including nitrate reducing bacteria¹⁰¹. This can cause

dental infections and gingivitis by increasing pathogenic bacteria such as (*porphyromonas gingivalis*)¹⁰². Studies have shown that patients with periodontal disease to have higher levels of salivary nitrite which may be partly derived from the reduction of nitrates by the oral bacteria. Since nitrite has been shown to have an antimicrobial effect against gastrointestinal and oral pathogens, it has been speculated that the salivary glands may respond to the periodontal infection by enhancing the secretion of nitrate and production of nitrite by the nitrate reducing bacteria as a host defence mechanism¹⁰³. This is thought to reduce the prevalence of the acidogenic bacteria which contribute to the development of dental caries. In agreement, Doel et al ⁴² has reported a significant reduction in dental carries in study participants with high salivary nitrate concentration. Epidemiological studies have reported an association between periodontal disease with CVD. Although the cause and effect relationship has not been proven, studies have suggested that inflammation caused by the oral infection may contribute to the development and progression of the atherosclerotic plaque. Interestingly, periodontal pathogens have been identified in the atherosclerotic plaque suggesting a direct role in CVD. However, to date, periodontal disease has not been considered to be a CVD risk marker¹⁰⁴. Lifestyle habits such as smoking can also influence oral bacteria composition⁷⁴. In a study conducted in 9 non-smokers aged 20-45 y and n=5 healthy active smokers (>20 cigarettes per week) aged 30-60 years, nitrate reduction activity was found to be over 80% lower in smokers compared to non-smokers⁷⁴. However, the low numbers of individuals within each group may have influenced the results observed.

As previously mentioned, dietary nitrates have been shown to interact with other food components such as lipids⁷², with similar reports for polyphenols¹⁰⁵, alcohol¹⁰⁶ and proteins ²⁵. In particular, foods and beverages rich in polyphenols including apple, tea and orange juice have been shown to lead to a 3 fold increase in NO production in the stomach¹⁰⁷ and reduce endogenous N-nitrosamine formation. Along with polyphenols, the content of ethanol in red wine can also interact with nitrite forming ethyl nitrite which works as a nitrosation agent and may mediate NO effects.

613 These interactions with other dietary components may therefore play a role in modulating the
614 circulating NO levels and bioavailability of the nitrate and nitrite contained within foods.

615 In summary, a systematic approach was used to identify the studies that have determined the
616 impact of oral bacteria on blood pressure in response to nitrate intake, from dietary sources or
617 supplements. However, only a very limited number of human (n=2) and animal (n=3) studies have
618 addressed this research question, with the remaining studies examining the importance of the oral
619 bacteria on the nitrate reducing capacity on circulating nitrite concentrations and blood pressure.
620 Based on our observations from these studies, there is accumulating evidence to suggest that
621 absence of nitrate-reducing oral bacteria was associated with increasing blood pressure even when
622 accompanied by a high nitrate intake. However, some of the studies failed to see any effects, which
623 may be due to type of mouthwash used in the human studies or the method of application of the
624 mouthwash in the animal study²⁹. Sex, hypertension, and tongue cleaning were all found to be
625 important potential determinants of the variability in the responses between participants. Of these,
626 the dental hygiene practice of tongue cleaning, which is recommended by the American Dental
627 Association, appeared to promote oral microbiota diversity and be associated with a greater ability
628 to recover the tongue microbiome after mouthwash use. Potential mechanisms to explain the blood
629 pressure lowering effects of dietary nitrates included increases in plasma nitrite, S-nitrosothiols,
630 nitro-fatty acids and vascular nitrosylation and cross-talk between the enterosalivary nitrate-nitrite-
631 NO pathways and eNOS activity in the endothelial cells. However, the limited number of studies
632 performed make it difficult to draw any firm conclusions from this literature review.

633

634 **Conclusions**

635 With the increasing prevalence of non-communicable diseases there is an urgent need for further
636 studies to investigate the role of the oral bacteria on cardiovascular health in response to dietary
637 nitrate intake, and to determine the underlying mechanisms. With vascular function now recognised
638 as an important prognostic marker for future CVD risk, studies incorporating real time measures of

639 vascular reactivity and tone are required. Furthermore, the use of rigorous methods to determine
640 changes in the abundance and composition of the oral bacteria in response to intake of dietary
641 nitrate would help to identify important nitrate-reducing bacteria related to changes in vascular
642 function and determine whether these bacterial groups are also evident in the gut microbiome, a
643 proposed modulator of chronic disease risk. Diets containing nitrate-rich foods may contain other
644 bioactive components which could also contribute to CVD risk reduction, including fibres,
645 vitamins, minerals and flavonoids. Such diets may offer a number of advantages over nitrate/nitrite
646 supplemental use, not only due to the availability of other bioactive components, but also because
647 of reports of vascular adaptation and risk of marked acute hypotension after supplemental nitrate
648 use, not found with nitrate-rich diets¹⁰⁸. With hypertension a major risk factor for CVD, more
649 studies are needed to determine whether diets higher in nitrate-rich foods can be recommended for
650 blood pressure lowering and disease prevention in healthy individuals and those at greater CVD
651 risk.

652

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655 conception of the literature search strategy. H.S.A. undertook the literature review. D.A.H., K.G.J.
656 and J.A.L. provided feedback and guidance on previous drafts of the review and J.A.L. was
657 responsible for final content. The authors have no conflicts of interest to declare.

658

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Tables

Table1: Commonly reported nitrate reducing bacteria species found in the oral cavity

Bacteria species	Condition	Change in abundance in response to nitrate intake	Location in the oral cavity
<i>Veillonella dispar</i> ^{42, 43}	Anaerobic	↑	Tongue
<i>Actinomyces odontolyticus</i> ^{42, 43}	Facultative anaerobic	↑	Tongue
<i>Prevotella salivae</i> ^{42, 43}	Anaerobic	↑	Tongue
<i>Rothia mucilaginosa</i> ^{42, 14}	Aerobic	↑↑	Tongue
<i>Neisseria flavescens</i> ^{43, 14}	Aerobic	↑↑	Tongue

Table 2: Animal studies investigating the importance of oral nitrate reducing bacteria on blood pressure in response to nitrate intake.

Reference	Animals	Study design and duration	Intervention	Measurement	Outcome measures
Petersson 2009 ⁷⁰	n= 4-7 Male Sprague Dawley rats each group (190-360 g, B and K, Sollentwia, Sverge).	Parallel groups with 7 day treatment periods: 1) No treatment (control). 2) NaNO ₃ only 3) Mouthwash 4) Mouthwash + NaNO ₃ or NaNO ₂	Water supplemented with 10 mM NaNO ₃ or 1 mM NaNO ₂ Mouthwash groups: Chlorhexidine mouthwash spray (0.3 ml), 2X daily.	Plasma	Δ NO ₂ ↓ after mouthwash + NaNO ₃ vs control p<0.05.
				HR	NS
				SBP	NS
				DBP	↓ after NaNO ₃ . DBP lowering absent in mouthwash treated rats
				MAP	↓ after NaNO ₃ and mouthwash + NaNO ₂ vs mouthwash only. MAP lowering absent in mouthwash + NaNO ₃ rats
Hyde 2014 ²⁹	n= 8 Male Wistar rats 7 weeks old	19 day sequential intervention: 0-5 control (water) 6-12 NaNO ₃ , 13-19 NaNO ₃ + mouthwash Blood collected at day 1, 5, 6, 12, 13 & 19. BP (telemetry) and tongue swab every day	NaNO ₃ (1 g/L) in drinking water Mouthwash regime: 0.3 ml of chlorhexidine applied 2X daily to tongue dorsal surface (days 13-19)	Oral bacteria	↓ viable bacteria on tongue after mouthwash
				SBP	NS
				DBP	↓ after NaNO ₃ and mouthwash + NaNO ₃ vs control
				Plasma NOx	NS

Pinheiro 2016 ⁷¹	n = 10, Male Wistar rats each group (190-210 g) 2 kidney, 1 clip (2K1C) hypertensive group. Sham operated control group	6 weeks – 2 weeks baseline followed by 4 weeks treatment Experiment 1 Vehicle NaNO ₂ Mouthwash Mouthwash + NaNO ₂ Experiment 2 Vehicle NaNO ₃ Mouthwash Mouthwash + NaNO ₃ 6 h after last treatment, blood and tongue swab collected.	15 mg NaNO ₂ /kg or 140 mg NaNO ₃ /kg (gavage) Mouthwash groups: Daily mouth clean with Chlorhexidine (0.12%) soaked swab.	Plasma	Δ NO ₂ ↓ 25–30% after mouthwash vs NaNO ₂ and NaNO ₃ groups (P < 0.05) Δ NO ₃ ↓ 45% after mouthwash vs NaNO ₂ group (P < 0.05)
				BP	↓ SBP (40 mmHg) and MAP with NaNO ₂ and NaNO ₃ (P= 0.01). Mouthwash blunted MAP and SBP lowering effect of NaNO ₃ (p <0.05) but not NaNO ₂
				Oral bacteria	↓CFU 50-70% with mouthwash

Abbreviations: DBP: Diastolic Blood Pressure, HR: Heart Rate, MAP: Mean Arterial Pressure, NS: Not Significant, NO₂: Nitrite Concentration, SBP: Systolic Blood Pressure, NO₃: Nitrite Concentration, CFU: Colony Forming Unit (number of viable bacteria)

Table 3: Human studies determining the effects of oral bacteria on salivary and plasma nitrite concentrations, and/or blood pressure in response to nitrate intake.

Reference	Subject characteristics	Study design and duration	Nitrate dose	Type of mouthwash	Measurement	Significant outcomes
ACUTE STUDIES						
Mitsui et al., 2017 ⁷⁵	n=12 (6M/6F) Normotensive, Age 19-44 y Non-smoking,	Acute, RCT, CO 4 visits 10 h in duration with 1 wk washout. Saliva and oral bacteria collected 0, 1 and 10 h.	100 g lettuce (110 mg NO ₃) with breakfast. Lunch at 5 h.	1. Water (control) 2. Listerine (antiseptic) 3. Isodine (povidone- iodine, 0.35%) 4. Chlorhexidine 0.0025% Treatment for 3 min prior to nitrate ingestion	Saliva Oral bacteria	Relative to baseline: ↑ NO ₃ and NO ₂ after each treatment (P < 0.05) ↓ nitrate reducing bacterium <i>V. Dispar</i> at 1 and 5 h after Chlorhexidine
Govoni et al 2008 ²⁷	n=7 Normotensive Age 24-51y BMI 23 kg/m ² Non-smoking	Acute, RCT, CO 2 visits of 3 h in duration. Blood and saliva samples collected before and for 3 h after nitrate intake. Oral bacteria collected in n=4 after mouthwash only.	10 mg/kg NaNO ₃ in 100 ml water	Mouthwash vs no mouthwash	Saliva	↑NO ₃ on both visits ↓ NO ₂ vs no mouthwash
				Corsodyl (Chlorhexidine) gargled twice for 1 min, 15 min before nitrate ingestion.	Plasma	NO ₃ ↓ 29 nM and NO ₂ ↓ 250 nM at 3 h vs no mouthwash
					Oral bacteria	↓ bacteria count and (80%) and nitrate reducing capacity after mouthwash.

Woessner et al 2016 ³⁰	n=12 (M) Normotensive \bar{x} age 36 y and BMI 24 kg/m ² Non-smoking	Acute, RCT, CO 4 visits, 4 h in duration with 1 wk washout. BP, blood and saliva collected before and for 4 h after juice consumption	140 ml of concentrated beetroot juice (8.4 mmol nitrate)	1) Water (control) 2) Listerine (antiseptic) 3) Cepacol (antibacterial) 4) Chlorhexidine (0.12%) Treatment 15 min after beetroot juice for 60s.	SBP	↓ Listerine and control vs Cepacol and Chlorhexidine ($P \leq 0.05$)
					DBP	NS
					Saliva	↑ NO ₃ all treatments ↑ NO ₂ control vs all mouthwashes and ↓ NO ₂ Chlorhexidine and Cepacol vs antiseptic ($P \leq 0.05$)
					Plasma	↑ NO ₃ all treatments ↓ NO ₂ Chlorhexidine vs all treatments and Cepacol vs control ($P \leq 0.05$)

Bondonno et al 2012 ⁷⁶	N=16 F Normotensive \bar{x} age 52±11y (F) Non-smokers,	Acute, RCT, CO 5 visits of 3 h in duration. 1 wk washout. Blood and saliva samples collected before and for 3 h after nitrate intake	0, 100, 200, 400 mg NaNO ₃ in water	1) Antibacterial toothpaste (0.3% triclosan) 2) Toothpaste without antimicrobial agent (control)	Saliva Plasma	↑ NO ₃ all treatments ↑ NO ₃ all treatments
ACUTE WITHIN CHRONIC						
McDonagh et al 2016 ⁸⁰	n=12 (6M/6F) Normotensive \bar{x} age 22±2y (F) and 24±2 y (M). Non-smokers,	Acute within chronic, RCT, double blind 6 visits over 8 weeks Each treatment 6 days, with acute visits (4 h) on days 0 and 6. Acute visits: Rinse with mouthwash 15 min before ingesting 2 x 70 ml beetroot juice. Measurements at 0, 2 and 4 h. BP and PWA measured at rest and during 10 min of treadmill walking. Saliva and plasma samples collected.	70 ml of beetroot juice (6.2mmol nitrate) twice a day	1) Strong - Corsodyl (Chlorhexidine) 2) Weak - Vademecum med (non-chlorhexidine- containing antibacterial mouthwash) 3) Deionised water (con) 3X daily 15 mins before beetroot juice and meals, for 6 days	SBP DBP MAP HR PWA	Relative to baseline (0 h): Resting - NS After 10 min exercise, ↑ 3 mmHg after strong mouthwash vs control (P = 0.07) 4 h after beetroot juice Resting and during exercise – NS Resting - NS After 10 min exercise, ↑ after strong mouthwash vs control (P<0.05) at 4 h. During exercise ↑ after strong vs control and weak (P<0.05). NS

					Plasma	↑NO ₃ all treatments Δ NO ₂ ↓ after strong vs other treatments at 2 and 4 h, and weak vs control (P<0.05) at 2 h
					Saliva	Δ NO ₃ ↑ and Δ NO ₂ ↓ after strong vs weak and control (P<0.05) at 4 h.

Abbreviations: DBP: Diastolic Blood Pressure, HR: Heart Rate, MAP: Mean Arterial Pressure, RCT: Randomized Controlled Trial, NS: Not

Significant, PWA: Pulse Wave Analysis, SBP: Systolic Blood Pressure, CO: Cross Over.

Table 4: Chronic human studies investigating the involvement of oral bacteria in the blood pressure lowering effect of nitrate.

Reference	Subject characteristics	Study design and duration	Oral nitrate reducing capacity	Mouthwash regime	Measurement	Significant outcome between treatment
Tribble et al., 2019 ⁷⁸	n=26 (16F/10M) Normotensive Age 22-71 y	Sequential 4 visits over 14 days: days 1 (baseline), 7 (post mouth wash), 10 (recovery) and 14 (recovery) Clinic BP and oral bacteria at each visit. n=6 oral nitrate reducing capacity for 8 h after 30 s mouthwash	Mouth rinse with 1 mM NaNO ₃ for 2 min	Chlorhexidine (0.12%) 2 x daily for 30 sec	SBP DBP Oral bacteria Oral nitrate reducing capacity	In response to mouthwash, ↑ 5mmHg (n=9) and ↓ (n=4) NS ↓ Species diversity and abundance with mouthwash for 7 days. ↑ bacterial metabolic activity at day 14. ↓ NO ₃ :NO ₂ ratio for 6-8 h after mouthwash.

Sunqvist et al 2016 ⁷⁹	n=17 (F) Normotensive \bar{x} age 23 y BMI = 22 kg/m ² Non-smoking.	RCT, CO, double blind Each treatment 3 days with a 28 day washout 4 visits (days 3 and 4 of each treatment) 24 h ABP and urine sample. Clinic BP, saliva and plasma samples and oral nitrate reducing capacity	Mouth rinse with 10 mM NaNO ₃ for 5 min	Chlorhexidine (0.2%) or placebo mouthwash 3 x daily after meals for 60s.	BP	No difference in ABP or clinic BP
					Saliva	↑ NO ₃ and ↓ NO ₂ after mouthwash (P ≤ 0.01)
					Plasma Urine Oral nitrate reducing capacity	No change in NO ₃ and NO ₂ with mouthwash vs placebo excretion of NO ₃ with mouthwash vs placebo ↓NO ₂ after mouthwash (8 μM) vs placebo (234 μM)(P<0.001)
Bondonno et al 2015 ⁷⁷	n=15 (8M/7F) Hypertensives taking medication	RCT, CO Each treatment 3 days with a 10-12 day washout.	Ratio of NO ₂ and NO ₃ measured in saliva.	Chlorhexidine or tap water (control)	SBP	↑ 2.3 mmHg after mouthwash vs water (P= 0.01)
					DBP	NS

	BP 120-159/100 mmHg. Age 53-69 y and BMI 20-35 kg/m ² . Non-smokers	Visits at day 0 and 3 of each treatment. Saliva sample, oral nitrate reducing capacity and plasma sample. BP measured at home.		2x daily with 20 ml for 30 sec after brushing teeth	Saliva Plasma Oral nitrate reducing capacity	↑ NO ₃ and ↓ NO ₂ after mouthwash vs control (P= 0.001) ↓ NO ₂ after mouthwash vs control (P= 0.09). NO ₃ - NS ↓ nitrate reductase ratio after mouthwash
Kapil et al 2013 ⁴¹	n=19, Normotensive, Age 18-45y, BMI 18-40 kg/m ² , Non-smokers, No self-reported use of	Sequential 2 visits (0 and 14 days). At each visit, clinic BP, blood, urine and saliva samples and oral nitrate reduction capacity.	Mouth rinse after holding 3 doses of KNO ₃ (0, 0.8 and 80 μmol) in the mouth for 5 min.	Chlorhexidine (0.2%) 2x daily days 8-14 only.		Relative to baseline, use of mouthwash
					Clinic SBP	↑ 3.5mmHg (P = 0.003)
					Clinic DBP	↑ 2.2mmHg (P = 0.038)
					A-SBP	↑2.4 mmHg (P= 0.017)
					A-DBP	↑2.2 mmHg (P= 0.014)
					Home SBP	↑2.9 mmHg (P< 0.001)
					Home DBP	↑2.0 mmHg (P< 0.001)
					HR	NS

	mouthwash or antibiotic	Fitted with ABP unit for 24 h and BP measured at home.			Saliva	↑NO ₃ and ↓NO ₂ 90% (P< 0.001)
					Plasma	↑NO ₃ and ↓NO ₂ 25% (P= 0.001)
					Urine	↑NO ₃ and ↓NO ₂
					Oral nitrate reducing capacity	At baseline, NO ₂ in mouth rinse dose dependent (0<0.8<80 μmol KNO ₃) After mouthwash, ↓ 90% NO ₂ in mouth rinse for 0.8 and 80 μmol KNO ₃ .

Abbreviations: DBP: Diastolic Blood Pressure, HR: Heart Rate, MAP: Mean Arterial Pressure, RCT: Randomized Controlled Trial, NS: Not

Significant, PWA: Pulse Wave Analysis, SBP: Systolic Blood Pressure, CO: Cross Over, ABP: Arterial Blood Pressure

FIGURE LEGENDS

Figure 1: Diagram of the endogenous generation of nitric oxide (NO) by NO synthase (NOS) (right panel highlighted in pink), and exogenous generation of NO from the diet (left panel highlighted in blue)²⁸. In biological fluids, NO is oxidized to nitrite (NO₂) and nitrate (NO₃) (dashed arrows).

Figure 2: Overview of the nitrate enterosalivary circulation and nitrate metabolism in humans.

Ingested inorganic nitrate is converted to nitrite in the oral cavity by nitrate reducing bacteria with reduction to NO and nitrogen oxides occurring within the acidic environment of the stomach.

Remining nitrate and other nitrate components are then rapidly absorbed into the bloodstream via the small intestine. A large proportion of nitrate is then excreted by the kidneys into the urine, with up to 25% being recycled by the salivary glands and then concentrated in saliva.

Figure 3: Flow of information through the different phases of the literature review

FIGURE 1

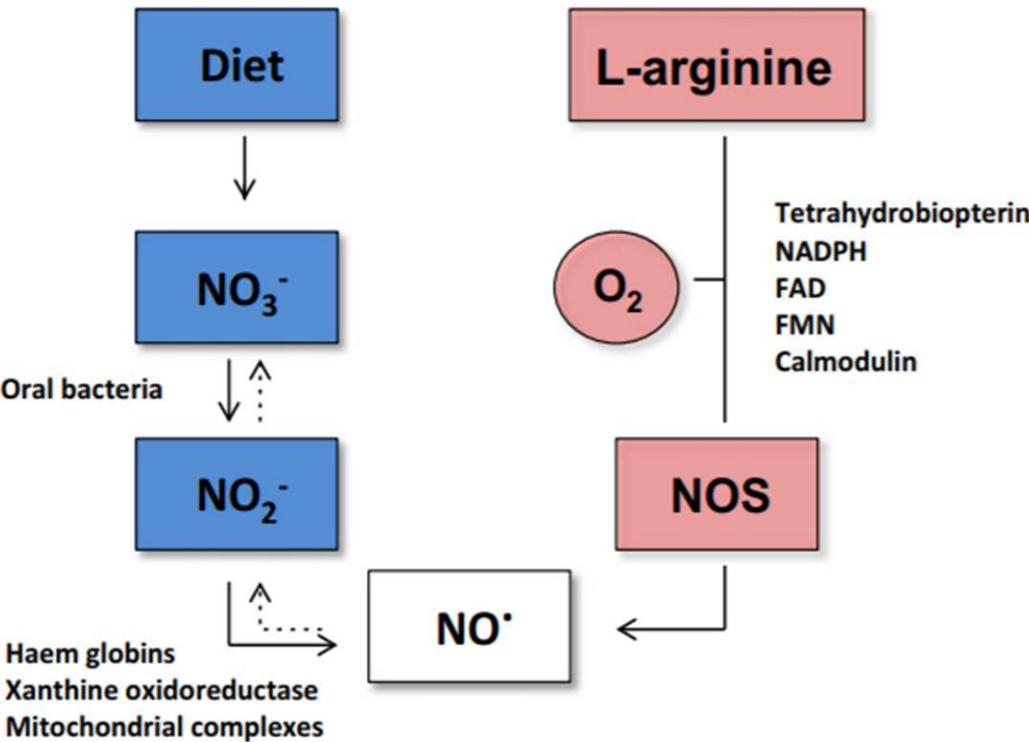


FIGURE 2

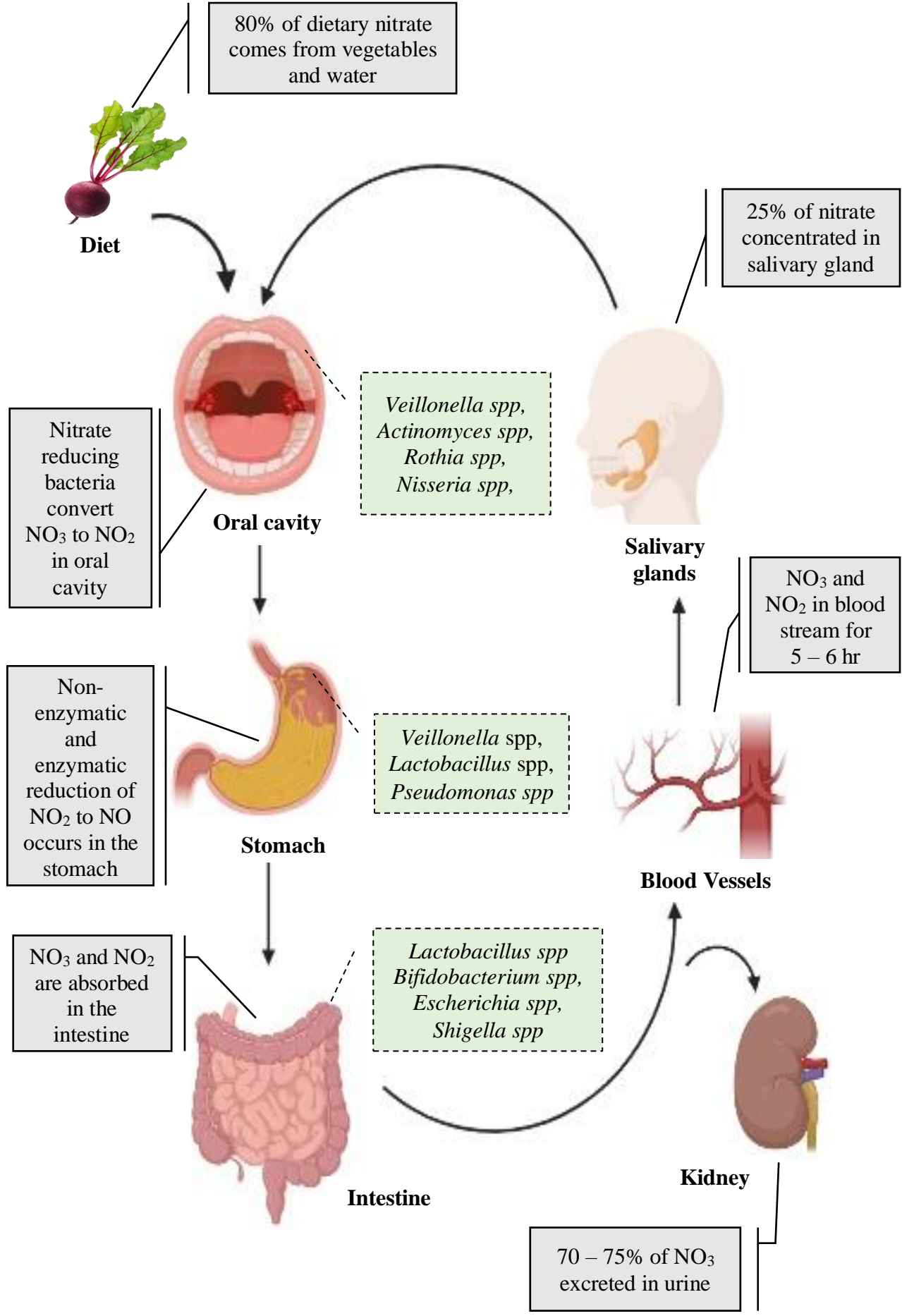


FIGURE 3

