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Periodontal treatment causes a longitudinal increase in nitrite-producing bacteria

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Abstract

Background: The oral microbiome-dependent nitrate (NO_3^-)–nitrite (NO_2^-)–nitric oxide (NO) pathway may help regulate blood pressure. NO_2^--producing bacteria in subgingival plaque are reduced in relative abundance in patients with untreated periodontitis compared with periodontally healthy patients. In periodontitis patients, the NO_2^--producing bacteria increase several months after periodontal treatment. The early effects of periodontal treatment on NO_2^--producing bacteria and the NO_3^-–NO_2^-–NO pathway remain unknown. The aim of this study was to determine how periodontal treatment affects the oral NO_2^--producing microbiome and salivary NO_3^- and NO_2^- levels over time.

Methods: The subgingival microbiota of 38 periodontitis patients was analysed before (baseline [BL]) and 1, 7 and 90 days after periodontal treatment. Changes in NO_2^--producing bacteria and periodontitis-associated bacteria were determined by 16s rRNA Illumina sequencing. Saliva samples were collected at all-time points to determine NO_3^- and NO_2^- levels using gas-phase chemiluminescence.

Results: A significant increase was observed in the relative abundance of NO_2^--producing species between BL and all subsequent timepoints (all $p < 0.001$). Periodontitis-associated species decreased at all timepoints, relative to BL (all $p < 0.02$). NO_2^--producing species negatively correlated with periodontitis-associated species at all timepoints, with this relationship strongest 90 days post-treatment ($\rho = -0.792$, $p < 0.001$). Despite these findings, no significant changes were found in salivary NO_3^- and NO_2^- over time (all $p > 0.05$).

Conclusions: Periodontal treatment induced an immediate increase in the relative abundance of health-associated NO_2^--producing bacteria. This increase persisted

Abbreviations: ASV, amplicon sequence variant; AUC, area under curve; BOP, bleeding on probing; BP, blood pressure; CAL, clinical attachment loss; CRP, c-reactive protein; GCF, gingival crevicular fluid; NO_3^-, Nitrate; NO, nitric oxide; NO_2^-, nitrite; OUT, operational taxonomic unit; PISA, periodontal inflamed surface area; PPD, probing pocket depth; SDCEP, Scottish Dental Clinical Effectiveness Programme; TNFα, tumour necrosis factor α.

throughout periodontal healing. Future studies should test the effect of periodontal treatment combined with NO_3^- intake on periodontal and cardiovascular health.

KEYWORDS
16s rRNA sequencing, nitrate, nitrite, oral nitrate reduction, periodontitis

1 | INTRODUCTION

Over the past decade, the interplay between oral and systemic health has gained increasing attention. Periodontitis is estimated to affect 62% of dentate adults (Trindade et al., 2023). In addition to its oral effects, it is associated with worse clinical outcomes in systemic conditions, including cardiovascular diseases (Sanz et al., 2020), diabetes mellitus (Preshaw & Bissett, 2019) and Alzheimer's disease (Desta, 2021). Therefore, it is apparent that periodontitis has far-reaching health and economic implications which extend beyond the oral cavity.

The subgingival microbiome in periodontal health is dominated by commensal facultative anaerobic species, which coexist alongside host immunosurveillance (Lamont et al., 2018). During periodontitis, the increase in periodontitis-associated species at diseased sites is accompanied by decreases in these health-associated species (Chen et al., 2022). In addition to the deregulated inflammation, this change has implications for the host as some bacteria carry out functions which aid in the maintenance of oral and systemic health – such as nitrate (NO_3^-)-reducing bacteria.

Nitrate-reducing bacteria form an essential part of the enterosalivary NO_3^-–nitrite (NO_2^-)–nitric oxide (NO) pathway (Hezel & Weitzberg, 2015). The NO_3^-–NO_2^-–NO pathway provides an alternative source of bioactive NO, complimenting the breakdown of L-arginine by human NO synthases enzymes (Förstermann & Sessa, 2012). NO is a signalling molecule with multiple physiological functions. These include the maintenance of endothelial function and control of blood pressure (BP) (Bryan et al., 2008; Kapil et al., 2020), regulation of platelet function (Webb et al., 2008), enhancement of exercise performance (Senefeld et al., 2020), improved efficiency of muscle contraction (Bailey et al., 2009), wound healing (Luo & Chen, 2005), insulin sensitivity and glucose homeostasis (Gilchrist et al., 2013), attenuation of oxidative stress and inflammation (Carlström et al., 2011), immune function (Bogdan, 2001), the maintenance of brain (Wightman et al., 2015) and oral health (Kapil et al., 2013; Moran et al., 2024; Rosier et al., 2022). Although NO_3^--reducing bacteria are commonly classified by their capacity to produce NO_2^-, this may be a misnomer as the NO_2^- may be generated from other substrates, such as the oxidation of ammonium (Rosier, Moya-Gonzalvez, et al., 2020). In this study, we therefore refer to these bacteria as NO_2^- producing bacteria. Bacteria which demonstrate NO_2^--producing capacity include members of the genera *Rothia*, *Neisseria*, *Veillonella*, *Actinomyces*, *Corynebacterium*, *Haemophilus* and *Prevotella* (Doel et al., 2005; Hyde et al., 2014; Rosier et al., 2022). Of these, genera with oral representatives that were confirmed to reduce NO_3^- (by physiological measurements of NO_3^-) include *Rothia*, *Neisseria*, *Veillonella* and *Actinomyces* (Rosier et al., 2022).

An overview of the NO_3^-–NO_2^-–NO pathway is shown in Figure 1. Inorganic NO_3^- is found in dietary sources and is abundant in certain vegetables (e.g. leafy greens, beetroots and radishes), a food group clearly associated with health benefits (Hord et al., 2009). Once absorbed from the small intestine, approximately 25% of NO_3^- is transported via the circulation to the oral cavity and actively secreted from the salivary glands (Qin et al., 2012). Salivary NO_3^- can be converted to NO_2^-, and in some instances, further reduced to NO, by denitrifying oral bacteria (Hyde et al., 2014; Schreiber et al., 2010).

Nitrite and NO generated by oral bacteria have direct benefits for systemic health. Swallowed NO_2^- is converted to NO in the acidic environment of the stomach or absorbed and then stored in the blood and tissues where it forms NO following reaction with haemoglobin and other NO_2^- reductases (Kadach et al., 2023). Additionally, NO_2^- and NO can pass through the oral mucosa to reach the systemic circulation directly (Aerts et al., 1997). In light of this, decreased cardiometabolic disease risk is associated with greater oral NO_2^--production capacity by subgingival plaque (Goh et al., 2022), suggesting links between NO_2^- production in the mouth and systemic NO availability. The administration of commonly used antimicrobial mouthwashes has been shown to disrupt oral NO_2^- production, with subsequent increases in BP (Bondonno et al., 2015; Brookes et al., 2020). Collectively, this shows that the enterosalivary NO production is dependent on the actions of commensal oral bacteria.

In addition to the systemic effects of the NO_3^-–NO_2^-–NO pathway, common NO_2^--producing bacteria and their products are implicated in the maintenance of oral health (reviewed by Rosier et al., 2022). Most relevant to periodontitis, the addition of NO_3^- to subgingival biofilm samples from periodontitis patients decreases biofilm growth rates and reduces the subgingival microbial dysbiosis index, alongside a reduction in the abundance of periodontitis-associated species (Mazurel et al., 2023). In vivo patients who consumed NO_3^--rich lettuce juice – compared with patients receiving placebo NO_3^--depleted lettuce juice – demonstrated reduced gingival inflammation following professional mechanical plaque removal (PMPR) (Jockel-Schneider et al., 2016, 2021).

These results suggest that oral NO_2^- production may have a protective effect against periodontitis. Several mechanisms may contribute to this finding. Certain periodontitis-associated bacteria are sensitive to oxidative stress, so are vulnerable to the antibacterial effects of NO (Backlund et al., 2015). NO is produced by denitrification by some oral

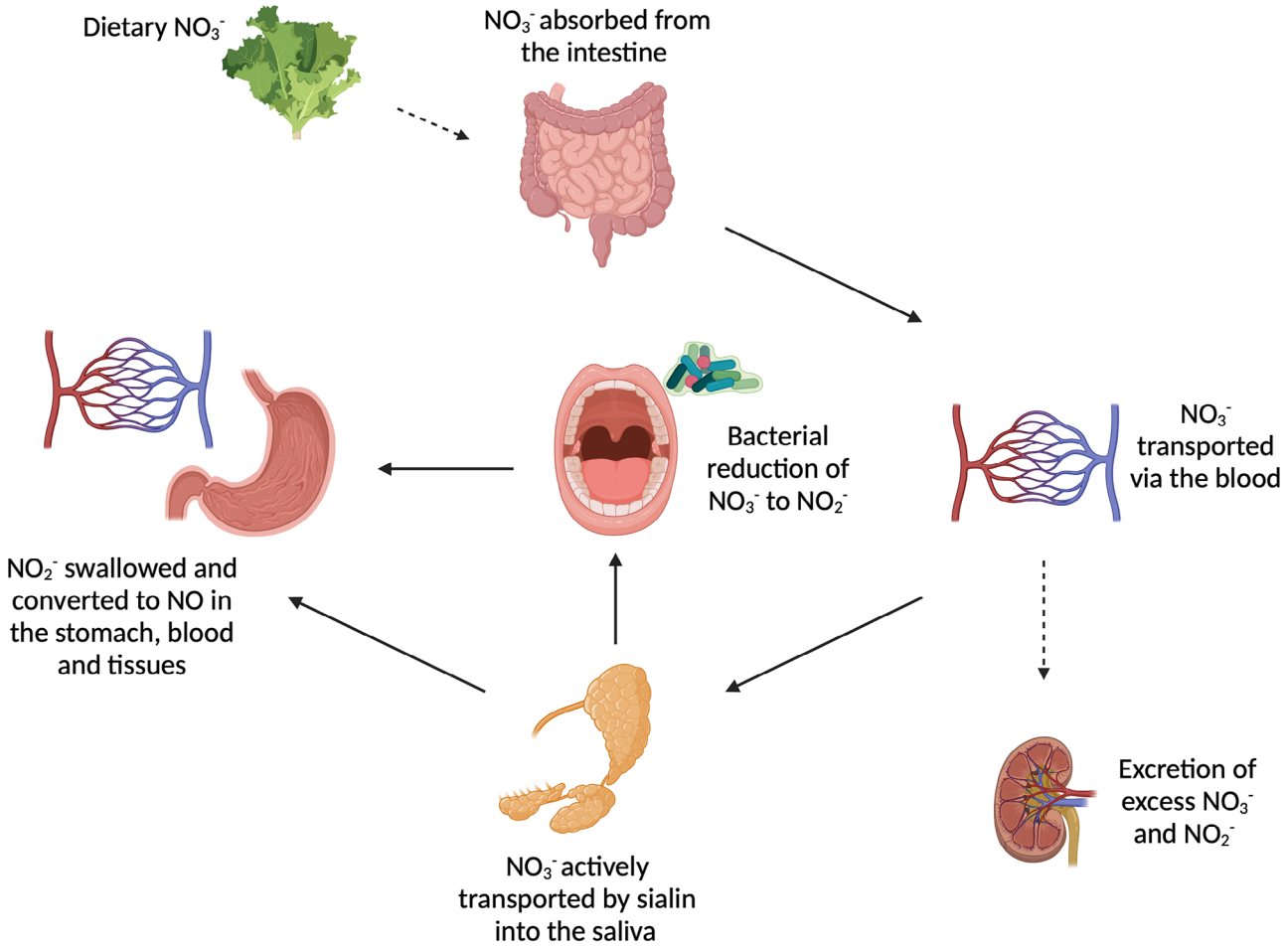

FIGURE 1 The stages of the enterosalivary NO_3^-–NO_2^-–NO pathway. Dietary NO_3^- is converted to NO_2^- by the actions of commensal NO_3^- reducing bacteria in the oral cavity. *Source*: Created in biorender.com.

bacteria, which may impair pathogen growth. Following supplementation with dietary NO_3^-, *Rothia* and *Neisseria* consistently increase in abundance (Burleigh et al., 2019; Jockel-Schneider et al., 2021; Vanhatalo et al., 2018). A greater abundance of *Rothia* species are associated with limited inflammation during experimental gingivitis (Huang et al., 2021) while *Neisseria* rapidly declines after oral hygiene is halted (Hall et al., 2023) and, when found in greater abundance, are associated with improved gingival recovery from experimental gingivitis (Joshi et al., 2014). Finally, an increase in NO availability is likely to benefit other elements of the immune response, for example, by increasing blood flow (Hezel & Weitzberg, 2015) and mucous production (Lanas, 2008) at barrier sites.

Recently, evidence of a lower abundance of NO_2^--producing bacteria in subgingival plaque and impaired oral NO_3^--reduction capacity in untreated disease was observed (Rosier et al., 2024). The same study demonstrated that periodontal treatment increases the level of NO_2^--producing bacteria and restores the NO_3^- reduction capacity of the salivary microbiome to a level comparable to dental health (Rosier et al., 2024). However, this observation was found 90 days after periodontal treatment, allowing the healing of the subgingival tissue and reestablishment of the subgingival community (Darcey & Ashley, 2011), and it currently remains unknown how the NO_2^--producing microbiota and its activity changes between treatment and Day-90. Early changes in health-associated species and functions may be amenable to therapeutic manipulation and may determine clinical outcomes at later stages, and this should be explored (Johnston et al., 2023).

The aim of this study was to determine how the NO_2^--producing subgingival microbiota and its activity change over time, during the reestablishment of subgingival plaque after periodontal treatment (i.e. oral hygiene instruction, PMPR and subgingival instrumentation). For this, the levels of NO_2^--producing species in subgingival plaque were determined at baseline (BL) (before treatment) and 1, 7 and 90 days after treatment using 16S rRNA gene data of 38 periodontitis patients. Additionally, saliva samples of these patients were obtained to measure the levels of NO_3^- and NO_2^- and determine the effect of periodontal treatment on the NO_3^-–NO_2^-–NO pathway over time. Finally, the relationship between the NO_2^--producing microbiota and periodontitis-associated parameters (i.e. disease-associated bacteria and inflammation) was explored.

2 METHODS

2.1 Ethical approval

All patients gave written, informed consent prior to enrolment. The study was approved by the NHS Greater Glasgow and Clyde Health Board Research Ethics Committee (REC reference number 18/NI/0059) and the Office for Research Ethics Committee Northern Ireland (ClinicalTrials.gov identifier NCT03501316). All procedures were carried out in accordance with the WMA Declaration of Helsinki.

2.2 Participants

The patients were enrolled in the Inflammatory Response after Periodontal Treatment trial at the University of Glasgow Dental Hospital, investigating the systemic inflammatory response to non-surgical periodontal treatment. The inclusion and exclusion criteria, patient demographics and clinical signs of periodontitis are previously described; 42 patients were recruited, aged 32–65 and 38 followed to completion (Johnston et al., 2020).

2.3 Study procedures

Figure 2A shows a timeline of study procedures. Patients attended a BL appointment and returned at Day-1, Day-7 and Day-90 after completion of subgingival instrumentation (full mouth subgingival instrumentation completed in 24 h). At BL, patients were provided with detailed oral hygiene instruction, dental health education and full mouth supragingival PMPR (Step 1 of treatment) (treatment provided according to current guidelines at time of study, Scottish Dental Clinical Effectiveness Programme, 2014, equivalent to Steps 1 and 2 of S3 Guidelines, West et al., 2021), following the relevant treatment guidelines. All clinical treatment was carried out by an experienced calibrated dental hygienist or specialist trainee in restorative dentistry (as described Johnston et al., 2020).

Clinical disease indicators were recorded at BL and Day-90, and the periodontally inflamed surface area (PISA) was calculated to quantify local inflammation (Nesse et al., 2008). Samples of subgingival plaque saliva were collected at BL, Day-1, Day-7 and Day-90. Systolic BP (SBP) and diastolic BP (DBP) were recorded at each appointment as a health screening measure, using an automated device. Mean arterial pressure (MAP) was then calculated using the following equation $MAP = (2 \times DBP + SBP)/3$.

2.4 Sample collection and processing

Four plaque samples were collected, one from each quadrant, from sites ≥ 5 mm, at all timepoints, as described previously (Johnston et al., 2023). In brief, samples were collected using a curette and transferred to a sterile Eppendorf containing 500 μL of sterile phosphate-buffered saline solution (Sigma-Aldrich). Bacterial cells were harvested via non-refrigerated centrifuge (approximately 14,800 g for 10 min). Saliva was collected with the passive drool method and clarified via centrifuge (approximately 13 700 g for 5 min). All samples were processed immediately in laboratories adjacent to the Glasgow Dental Hospital Clinical Research Facility and stored at −80 ° C prior to analysis.

2.5 Nitrite analysis

A solution of 2.5 mL acetic acid, 0.5 mL of deionised water and 25 mg sodium iodide was placed into a glass purge vessel heated to 50°C and connected to a NO analyser (Sievers NOA 280i, Analytix). A standardisation curve was constructed by injecting 100 μ L of NO_2^- solution to achieve a maximum concentration of 3000 nM. Samples were thawed at 37 ° C in a water bath before being diluted with deionised water at a ratio of 1:100 prior to injection. The NO_2^- content was calculated using the area under the standard curve.

2.6 Nitrate analysis

Vanadium reagent (24 mg of vanadium tri-chloride and 3 mL of 1 M hydrochloric acid) was added to the purge vessel and heated to 90°C. A standard curve was constructed by adding injections of 10 μ L NO_3^- solution up to a concentration of 100 μ M. Samples were thawed at 37 ° C in a water bath before being diluted with deionised water at a ratio of 1:100 prior to injection. The NO_3^- content was calculated as described above.

2.7 Bacterial 16S rRNA sequencing

DNA was isolated from the plaque samples using the MagNA Pure LC DNA isolation kit (Roche Diagnostics) and sequenced using an Illumina MiSeq sequencer as described by Johnston et al. (2023). The sequences were classified into genera and species using the DADA2 pipeline in R, and different downstream analyses were performed (e.g. Shannon's index and Chao1 index) as described by Johnston et al. (2023). An ANCOM-BC2 filter was applied to the relative abundance values, and analysis of the microbiome was carried out on the transformed dataset unless stated otherwise. During analysis, two samples (one at Day-7 and the other at Day-90) had <5000 total reads so were excluded from statistical analysis at these timepoints. Bacteria were grouped as periodontal pathogens (Pérez-Chaparro et al., 2014; Socransky et al., 1998) or as NO_2^- producers (Rosier et al., 2022) (Tables S1–S5).

2.8 Statistical analysis

This is a novel, secondary, exploratory analysis of previously published data (explained in Figure 2b). Johnston et al. (2020, 2023) divided their population into patients treated using hand or ultrasonic

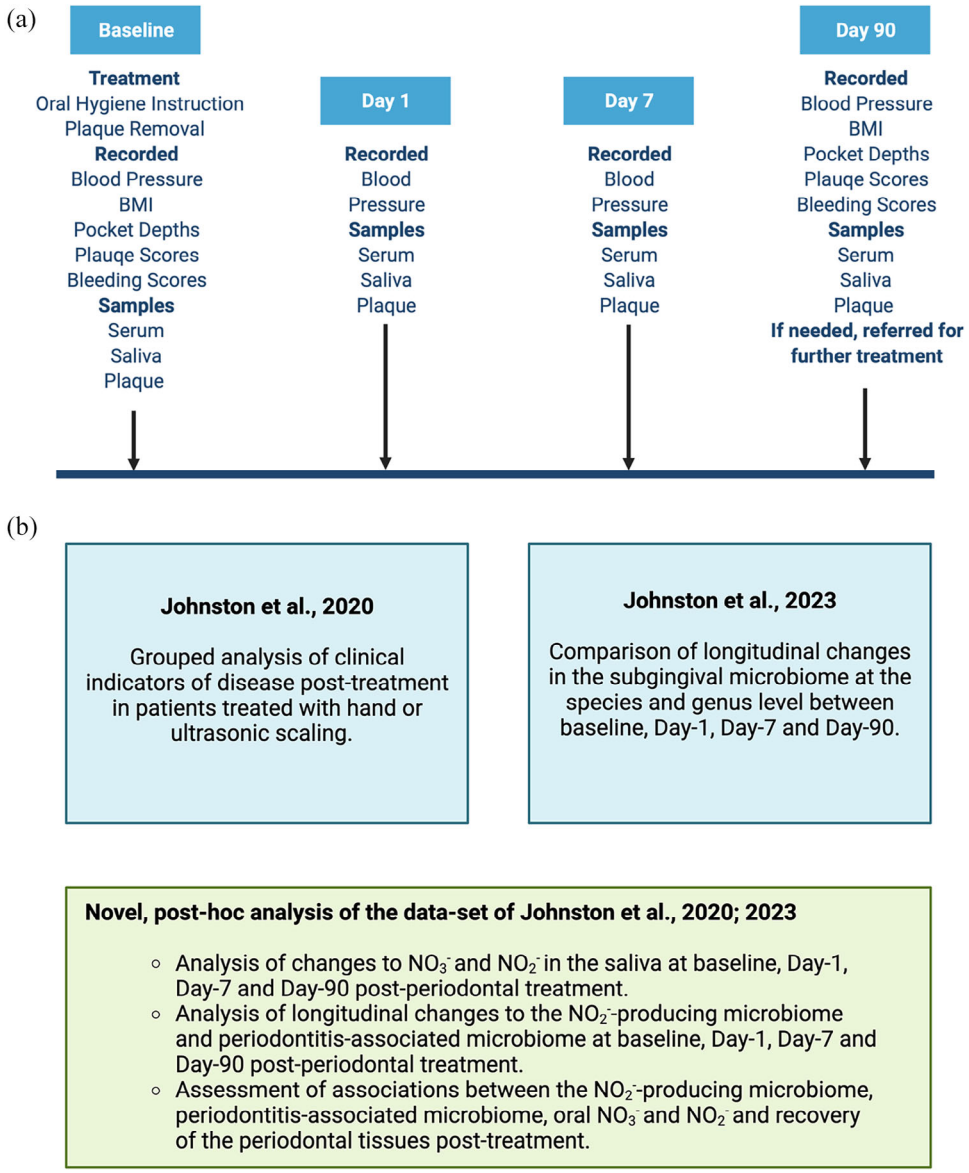

FIGURE 2 (a) A schematic of the study timeline, showing the data and sample collection procedures at each timepoint. (b) A description of the novel analysis carried out on the dataset of Johnston et al. (2020, 2023). *Source*: (a) Adapted scheme from Johnston et al. (2020, 2023). (b) Created in biorender.com.

instrumentation. However, they found no significant differences between both groups; therefore, we grouped all individuals together for analysis. SPSS (Version 29.1.01) and R were used for statistical analysis. Figures were constructed in GraphPad PRISM. Data were assessed for normality using the Shapiro–Wilk test and by examination of boxplots. Non-parametric tests were used where appropriate. Where appropriate, outliers out with three times the inter-quartile range were removed from analysis. One-way repeated measures ANOVAs or Friedman's tests were used to detect changes in clinical disease indicators, BP, NO_3, NO_2^- and in relative abundance of bacteria between Day-1, Day-7 and Day-90. Post hoc testing consisting of paired samples *t* tests or Wilcoxon signed rank tests with Bonferroni adjustment applied was used to determine differences between timepoints where indicated. Associations among clinical disease indicator, BP, NO_3^-, NO_2^- and groups of bacteria were assessed with Pearson's correlation coefficient or Spearman's rank correlation coefficients used where indicated. A $p \leq 0.05$ was taken to indicate statistical significance. Unless otherwise stated, values are reported as mean ± SD.

3 | RESULTS

3.1 | Patient demographics

BL demographic characteristics of the full cohort of (Johnston et al., 2020) are summarised in Table 1. In summary, 45% of patients had generalised Stage 4 Grade C periodontitis, 42% had generalised Stage

TABLE 1 Patient demographics at baseline ($N = 38$).

Age (years)	44.66 (8.65)		
Sex (%)	Female 50	Male 50	
Smoking status (%)	Never 39.5	Former 31.6	Current 28.9
BMI grouping (%)	Normal 28.9	Overweight 31.6	Obese 39.5
BP grouping (%)	Normal 23.7	Pre-high 55.3	High 21.1
Disease classification (%)	Generalised Stage 4 Grade C 45.0	Generalised Stage 3 Grade B 42.0	Generalised Stage 3 Grade C 13.0

Note: Variables presented as mean (SD) or percentage. Data previously published in Johnston et al. (2020, 2023) as subgroups. Data show total amalgamated population.
Abbreviation: BP, blood pressure.

TABLE 2 Comparison of clinical indicators of disease severity between baseline and Day-90 ($N = 38$).

Variable	Baseline	Day-90	Change in variable (%)	*p* value
CAL (mm^2)	4.14 (3.63, 4.95)	3.81–9.42 (3.10, 4.57)	−9.42 (−2.43, −18.42)	<0.001‡
PISA (mm^2)	979.56 (487.91, 1994.77)	134.85 (60.44, 289.30)	−83.01 (−74.24, −91.09)	<0.001‡
Sites with plaque (%)	50.96 (25.00, 65.83)	7.50 (4.05, 13.25)	−80.00 (−63.01, −89.17)	<0.001‡
Sites with BOP (%)	39.23 (21.52, 69.31)	7.78 (3.88, 12.08)	−81.29 (−65.68, −87.53)	<0.001†
Total PPD (mm per site)	26.72 (3.19, 4.32)	2.81 (2.40, 3.28)	−24.72 (−17.39, −30.41)	<0.001‡
Pockets ≥5 mm (%)	26.73 (20.83, 42.83)	11.44 (3.81, 19.82)	−56.05 (−47.02, −79.64)	<0.001‡

Note: Presented as median and inter-quartile ranges. Data previously published in Johnston et al. (2020, 2023) as subgroups. Data show total amalgamated population.
Abbreviations: BOP, bleeding on probing; CAL, clinical attachment loss; PISA, periodontal inflamed surface area; PPD, probing pocket depth.
†Paired samples *t* test.
‡Wilcoxon Signed-Rank test.

3 Grade B periodontitis and 13% had generalised Stage 3 Grade C periodontitis (Papapanou et al., 2018). Statistically significant improvements were observed following treatment for all investigated clinical indicators of periodontitis (Table 2). No significant changes were found in any of the recorded BP measurements following treatment (Figure S1). The full mouth plaque score (FMPS) was the only clinical parameter that was also determined at Day-7 (Figure 3c), decreasing clearly from BL. However, a small but significant increase in FMPS was found from Day-7 to Day-90.

3.2 | Salivary NO_3^- and NO_2^- do not change following periodontal treatment

To investigate the effects of periodontal treatment and subsequent healing on local and salivary NO_3^- and NO_2^- levels, changes in the concentration of both molecules were evaluated between study timepoints. At BL NO_3^- and NO_2^-, levels were 641 ± 258 and 216 ± 188 μM, respectively. On Day-90, salivary NO_3^- and NO_2^- levels were 630 ± 281 and 194 ± 299 μM. No significant differences were observed in salivary NO_3^- and NO_2^- levels between any investigated timepoints (all $p > 0.05$, Figure 3a,b). Dental plaque has previously demonstrated NO_2^- production capacity (Schreiber et al., 2010) but the significant alterations to FMPS in this study were not accompanied by changes in salivary NO_3^- and NO_2^- levels (Figure 3c). There were also no significant associations detected between FMPS and salivary NO_3^- and NO_2^- levels (Figure S2).

3.3 | Saliva NO_3^- and NO_2^- are not associated with levels of inflammation in periodontitis

To investigate if a decrease in inflammation after periodontal treatment is associated with salivary NO_3^- and NO_2^- levels, Spearman-Rho correlations were performed between salivary NO_3^- and NO_2^- levels and PISA scores at BL and Day-90. No significant associations were detected at any timepoint (all $p > 0.09$, Figure 4).

3.4 | The periodontitis-associated microbiome changes following treatment

Historically, periodontitis associated bacteria have been collated into complexes, depending on their association with clinical signs of disease (Socransky et al., 1998). When changes in relative abundance with treatment were examined, there were significant reductions in the relative abundance of Socransky's red complex between BL and all subsequent timepoints (all $p < 0.02$, Figure 5a). Bacteria of Socransky's orange complex were also found to decrease in relative abundance between BL and Day-1 ($p = 0.001$, Figure 5b). Socransky's

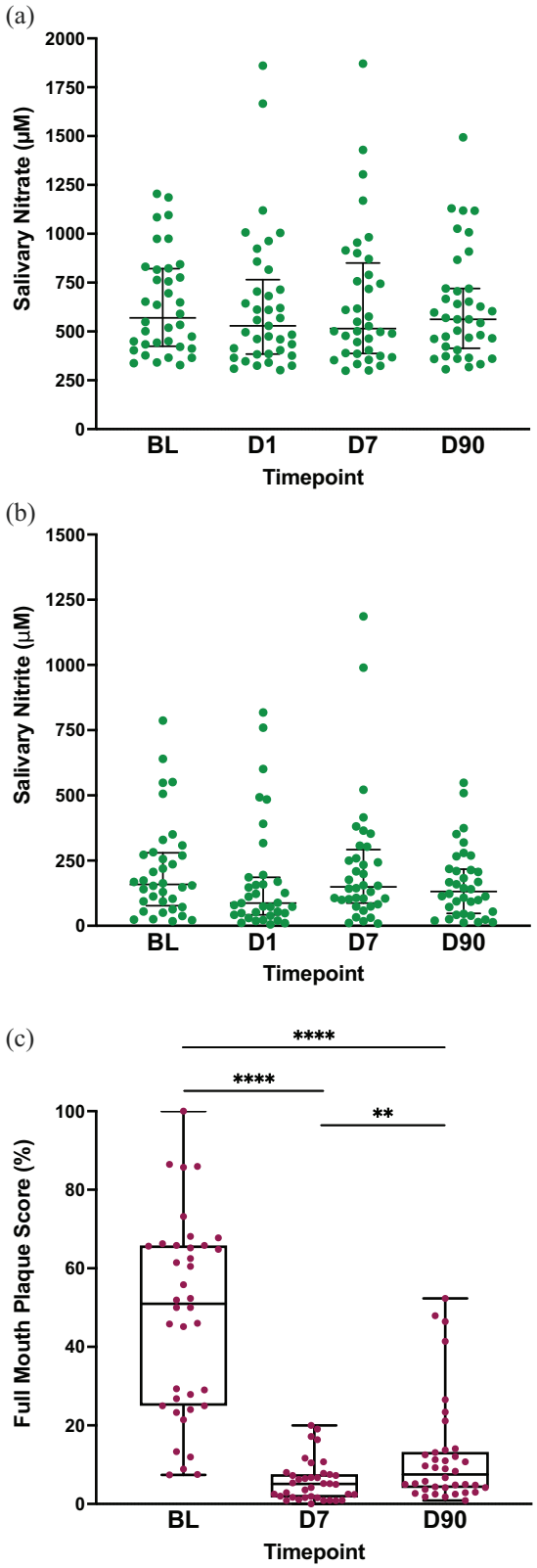

FIGURE 3 Salivary NO_3^- and NO_2^- in patients with periodontitis before, baseline (BL) and 1, 7 or 90 days post-treatment. (a) Salivary NO_3^- ($N = 36$ at BL, $N = 37$ at Day-1, $N = 36$ at Day-7, $N = 37$ at Day-90), (b) Salivary NO_2^- ($N = 36$ at BL, $N = 35$ at Day-1, $N = 36$ at Day-7, $N = 36$ at Day-90). Each point represents a patient. Error bars show mean ± SD. (c) Changes in plaque levels assessed by full mouth plaque score ($N = 38$). Data are presented with the box encompassing the 25th–75th percentiles and the black horizontal line representing the median value. Differences between timepoints were assessed Friedman's test with Bonferroni corrected post hoc testing.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.00001$.

yellow complex consists of early colonising streptococci. The yellow complex increased in relative abundance between BL and all subsequent timepoints (all $p < 0.0001$, Figure 5c). Further advancements in sequencing technology have revealed a greater number of bacterial species to be associated with periodontitis (Pérez-Chaparro et al., 2014). When all species currently known to be associated with periodontitis were collated, there were reductions between BL and all subsequent post-treatment timepoints (all $p < 0.013$, Figure 5d).

To test if the presence of periodontitis-associated bacteria within the subgingival biofilm was impacted by the levels of salivary NO_3^- and NO_2^-, Spearman-Rho correlations were performed between these molecules and the relative abundance of disease-associated bacteria and between NO_2^--producing bacteria and salivary NO_3^- and NO_2^- levels. No significant associations were detected at any timepoint (data not shown).

3.5 | The NO_2^--producing microbiome changes following treatment

NO_2^--producing bacteria are generally associated with dental health. Bacterial species of the oral microbiome which are known to be capable of NO_2^- production have been previously described (Rosier et al., 2022). The overall composition of the NO_2^- producing microbiome at each timepoint is shown in Figure S3. To investigate the impact of periodontal treatment on the NO_2^--producing microbiome, changes to the summed abundance of all known NO_2^- producing species were assessed (Figure 6a). NO_2^--producing bacteria increased in abundance between BL and all subsequent timepoints (all $p < 0.001$). To investigate the relationship between the relative abundance of NO_2^--producing bacteria and the periodontitis-associated microbiome within subgingival plaque, Spearman-Rho correlations were performed between the relative abundances of both groups of bacteria at each timepoint (Figure 6b–e). An initial moderate negative association at BL ($\rho = -0.596$, $p = 0.002$) became progressively stronger as the subgingival biofilm reformed ($\rho = -0.673$, $p < 0.001$ at Day-1 and $\rho = -0.687$, $p < 0.001$ at Day-7). This negative association was strongest at Day-90 ($\rho = -0.792$, $p < 0.001$). NO_2^--producing bacteria have previously demonstrated an inverse relationship with inflammation in experimental gingivitis. To investigate the impact of differing levels of inflammation in periodontitis on the NO_2^--producing microbiome, we calculated Spearman-Rho correlations between NO_2^--producing bacteria and PISA. No significant associations were detected at any timepoint. There were also no significant correlations between NO_2^--producing bacteria and salivary NO_3^- or NO_2^- (data not shown).

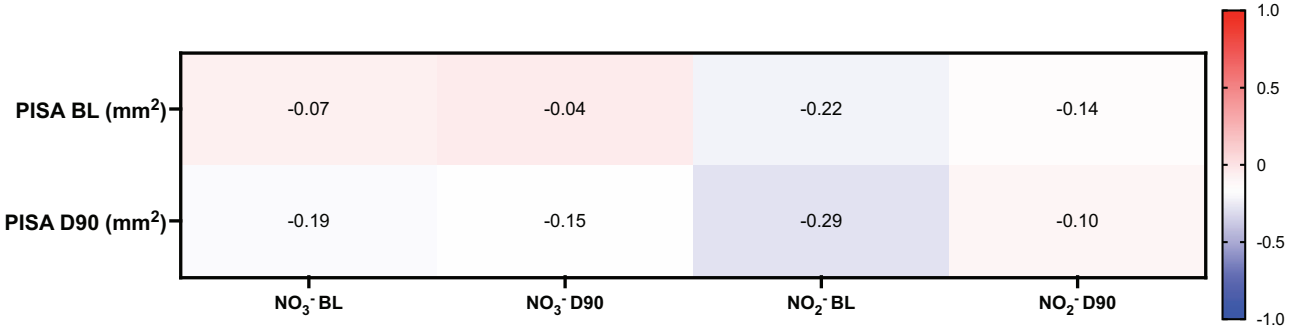

FIGURE 4 Correlations between NO_3^- and NO_2^- in the saliva and periodontal inflammation periodontally inflamed surface area (PISA). PISA and NO_3^- at baseline (BL) ($N = 36$). PISA and NO_2^- at BL ($N = 36$). PISA and NO_3^- at Day-90 ($N = 37$). PISA and NO_2^- at Day-90 ($N = 36$). Correlations were assessed by calculating Spearman-Rho. These correlations were not significant.

4 | DISCUSSION

To our knowledge, this is the first study to quantify salivary NO_3^- and NO_2^- levels in vivo during periodontal healing and include multiple post-treatment timepoints. Although salivary NO_3^- and NO_2^- levels remained unchanged, we demonstrate an increase in NO_2^--producing bacteria 1, 7 and 90 days after treatment. Commensurate with Johnston et al. (2023), we show extensive disruptions to the subgingival biofilm by periodontal treatment decreased the relative abundance of periodontitis-associated bacteria at all post-treatment timepoints. The abundance of NO_2^--producing species and periodontitis-associated species were found to be negatively associated at all timepoints, with this relationship strongest at Day-90.

4.1 | The longitudinal impact of periodontal treatment on the subgingival plaque biofilm

This cohort demonstrated significant improvements in all recorded clinical indicators of periodontitis. Johnston et al. (2023) evaluated the cohort as two separate treatment groups (hand vs. ultrasonic instrumentation) and found that the treatment outcome was identical. In our current study, both treatment types were therefore grouped, leading to a cohort of 38 individuals, and new measurements and analyses were performed. First, the dramatic decrease in plaque level (quantified by FMPS) at Day-7 indicated successful removal of the dysbiotic biofilm during treatment. There was a modest increase in FMPS at Day-7; however, plaque levels were still far lower than pre-treatment. This means that the relative abundance data collected from Day-1 onwards come from a much smaller quantity of biofilm compared to BL. There was a marked decrease in the oral inflammatory load, as quantified by PISA. This will have further altered the environment of the periodontal pocket, shifting conditions away from those which suit the development of the inflammation-tolerant periodontitis-associated microbiome (Mira et al, 2017; Rosier et al., 2018).

Following treatment and subsequent healing, the subgingival microbiota seen in untreated periodontitis changes to resemble the composition seen in periodontal health (Chen et al., 2018; Johnston et al., 2021; Shi et al., 2015). The formation of this stable community takes time (Li et al., 2023) and recovery of the periodontal tissues post-treatment takes around 12 weeks (Darcey & Ashley, 2011). We demonstrate changes in the NO_2^--producing species, as well as their relationship with the periodontitis-associated microbiota and salivary NO_2^- and NO_3^-, at multiple timepoints during healing.

4.2 | The longitudinal impact of periodontal treatment on the NO_2^--producing microbiome

The increase in NO_2^--producing bacteria between BL and Day-90 is in agreement with the results of our recent multicentre study (Rosier et al., 2024). This study revealed that the relative abundance of NO_2^--producing bacteria was consistently lower in periodontitis compared with periodontal health, in five different cohorts of patients with periodontitis (Rosier et al., 2024). It can thus be concluded that the cluster of NO_2^--producing bacteria is a marker of periodontal health that increases after periodontal treatment. The participants of our current study are the second independently recruited cohort of periodontitis patients from Scotland to show a significant increase in NO_2^--producing bacteria 90 days post-treatment (Johnston et al., 2021; Rosier et al., 2024). We show a significant increase in NO_2^--producing bacteria at Day-1 and Day-7 post-treatment when biofilm reformation is incomplete. Importantly, we show that at all time points, there is a significant negative correlation between NO_2^--producing species and periodontitis-associated species, which was strongest at Day-90. It should thus be explored if stimulating NO_2^--producing bacteria with pre- or probiotic treatments could improve periodontal treatment outcomes and/or delay redevelopment of periodontal inflammation after treatment.

Due to mechanical disruptions within the periodontal pocket, levels of inflammation within the oral cavity are high immediately post-treatment (Johnston et al., 2020; Morozumi et al., 2018). Increased levels of inflammation in experimental gingivitis studies are associated with decreases in levels of *Rothia* (Corrêa et al., 2019; Huang et al., 2021) and *Neisseria* (Joshi et al., 2014). Additionally, representatives of these genera can further reduce NO_2^- to NO, which is an antimicrobial

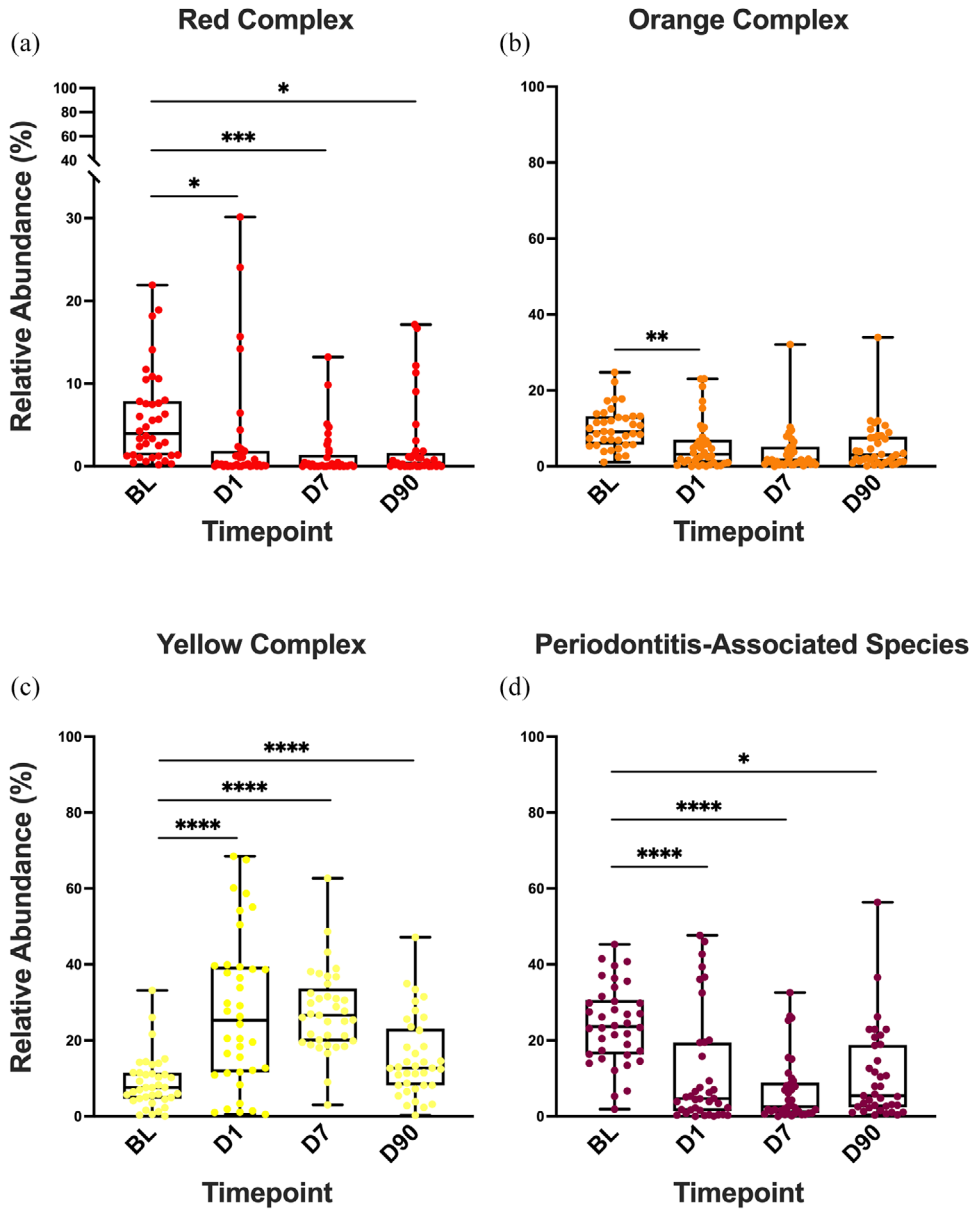

FIGURE 5 Changes in the relative abundance of disease associated bacterial complexes in the subgingival plaque of patients with periodontitis before, baseline (BL) and 1, 7 or 90 days post-treatment and associations between periodontitis-associated species and salivary NO_3^- and NO_2^-. (a–c) Changes in relative abundance of Socransky's complexes. (d) Changes in relative abundance of all known periodontitis-associated species. $N = 38$ at BL and Day-1 and Day-37 at Day-7 and Day-90. Data are presented with the box encompassing the 25th–75th percentiles and the black horizontal line representing the median value. For this analysis, data from all 38 individuals from Johnston et al. (2023) were grouped together. Bacterial species were classified as red complex, orange complex, yellow complex or periodontitis-associated. Each data point represents a patient in the study. Differences between timepoints were assessed using Friedman's test with Bonferroni corrected post hoc testing of compositional data standardised by ANCOM-BC. *p*-Values adjusted for multiple testing are shown. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.00001$.

molecule. The negative correlation between NO_2^--producing bacteria and periodontitis-associated bacteria in our study could in part result from NO production that can kill sensitive bacteria, such as anaerobes associated with periodontitis (Rosier et al., 2022).

Changes in the relative abundance of NO_2^--producing bacteria in this cohort at the species level have been previously published (Johnston et al., 2023). Not all species capable of NO_2^--production increased in abundance following treatment. At BL, *Fusobacterium nucleatum*, a member of Socransky's orange complex, was the most abundant NO_2^--producing species. Interestingly, *F. nucleatum* is periodontitis-associated, which is in contrast with most NO_2^--producing species and has been found to decrease in the presence of NO_3^- (Mazurel et al., 2023). The contribution of *F. nucleatum* and other individual NO_2^--producing species to NO_2^- and NO availability should be tested in future studies. Notably, *F. nucleatum* decreased at all post-treatment timepoints relative to BL (Johnston et al., 2023). This meant that *Rothia dentocariosa*, which increased in abundance relative to BL at Day-7 and Day-90, was the most

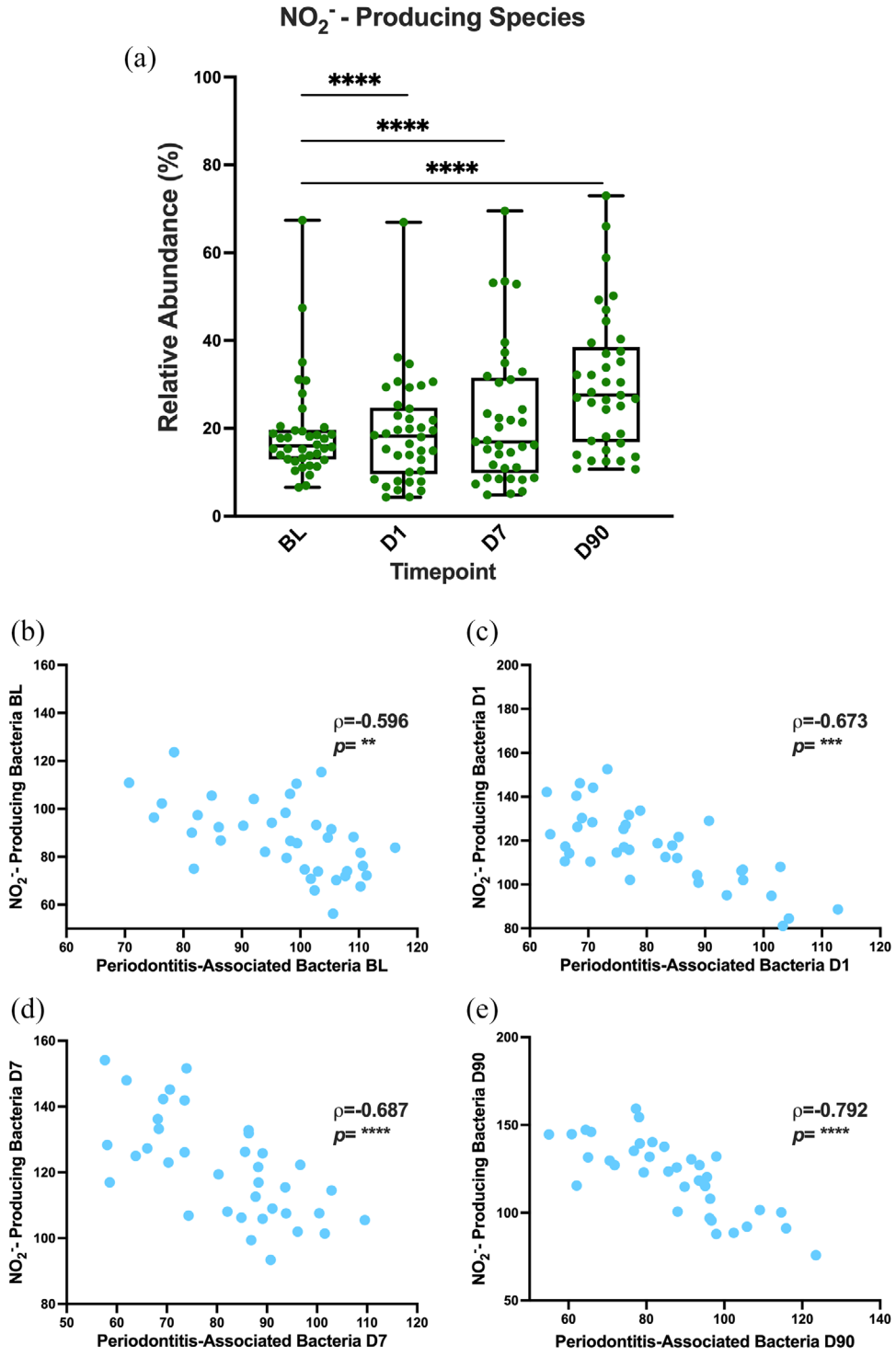

FIGURE 6 Changes in NO_2^--producing bacteria in the subgingival plaque of patients with periodontitis before, baseline (BL) and 1, 7 or 90 days post-treatment, associations between the NO_2^--producing microbiome and periodontitis-associated species and associations between the NO_2^--producing microbiome and levels of periodontal inflammation. $N = 38$ at BL and Day-1 and Day-37 at Day-7 and Day-90. (a) Relative abundance of known NO_2^--producing species. Data are presented with the box encompassing the 25th–75th percentiles and the black horizontal line representing the median value. Each data point represents a patient in the study. Differences in relative abundance were assessed using Friedman's test with Bonferroni corrected post hoc testing of compositional data standardised by ANCOM-BC. (b–e) Correlations between periodontitis associated bacteria and NO_2^--producing bacteria at Day-90. $N = 38$ at BL and Day-1 and Day-37 at Day-7 and Day-90. The correlation strength and significance were determined by calculating Spearman-Rho of standardisation of compositional data with ANCOM-BC. (f) Correlations between the relative abundance of NO_2^--producing bacteria and periodontal inflammation (quantified by periodontally inflamed surface area [PISA]). The correlation strength and significance were determined by calculating Spearman-Rho of standardisation of compositional data with ANCOM-BC. *p*-Values adjusted for multiple testing are shown. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.00001$.

abundant NO_2^--producing species at all subsequent timepoints. *Rothia mucilaginosa* also increased in relative abundance at all post-treatment timepoints. Supporting the growth of these species is likely to be of interest in the reduction of residual periodontal inflammation. Both *R. dentocariosa* and *R. mucilaginosa* have previously been proposed as probiotic bacteria, capable of increasing oral NO_3^- reduction capacity and supporting oral health (Rosier, Moya-Gonzalvez, et al., 2020). The administration of supplementary NO_3^- is a suitable strategy to increase the abundance of these, and other NO_2^--producing bacteria within the biofilm (Burleigh et al., 2019; Jockel-Schneider et al., 2021; Rosier et al., 2021; Rosier, Moya-Gonzalvez, et al., 2020; Vanhatalo et al., 2018; Velmurugan et al., 2016), while decreasing the abundance of periodontal pathogens (Burleigh et al., 2019; Mazurel et al., 2023; Rosier, Buetas, et al., 2020; Rosier, Moya-Gonzalvez, et al., 2020; Vanhatalo et al., 2018).

4.3 | The longitudinal impact of periodontal treatment on groups of bacteria associated with periodontal health and disease

An increase was observed in Socransky's yellow complex at Day-1, Day-7 and Day-90, compared to BL. Some yellow complex members are capable of NO_2^- production (Rosier et al., 2022) and *streptococci* increase in abundance at the genus level on the dorsal surface of the tongue following NO_3^-supplementation (Burleigh et al., 2019). Although no individual sequences assigned to specific *Streptococcal* species changed in abundance, there was an increase in unclassified *streptococci* between BL and all subsequent timepoints. *Streptococcal* species have a relatively similar 16S rRNA gene (Gao et al., 2014), limiting the number of species which could be classified using Illumina sequencing of part of the 16S rRNA gene. Future studies should determine what species of *Streptococcus* increase using full 16S rRNA gene sequencing or metagenomic approaches. It is feasible that some of these unclassified *streptococci* were capable of NO_2^- production. To better understand the nuances within the NO_2^--producing microbiome, future studies should consider the use of higher resolution microbiome sequencing where feasible, especially at earlier timepoints during biofilm reformation.

4.4 | Periodontal treatment does not alter levels of NO_3^- and NO_2^- in the saliva

Despite the cohort all suffering from periodontitis, salivary NO_3^- and NO_2^- levels were similar to those seen among these dentally healthy individuals, in the absence of NO_3^- supplementation (Burleigh et al., 2018, 2023). This is in agreement with our previous study, where no significant differences in BL levels of salivary and plasma NO_3^- and NO_2^- levels were found between health and periodontitis, but differences between the salivary NO_3^- reduction capacity were found when incubating saliva with 8 mM NO_3^- (a concentration found in saliva after vegetable intake) (Rosier et al., 2024). Moreover, NO_3^- reduction capacity was revealed to have improved post-treatment. It could thus mean that differences in the levels of NO_2^--producing microbiota found in our study after treatment would result in differences in NO_3^- after dietary NO_3^- intake. Alternatively, the decrease in plaque levels after treatment could have resulted a higher relative abundance of NO_2^--producing bacteria, but not quantity. Finally, it should be noted that although BL levels of NO_3^-, NO_2^- and NO can be comparable between periodontitis and health, the source of these molecules could shift. For example, iNOS expression has been found to be higher in periodontitis (Lappin et al., 2000), whereas the NO_3^--reduction capacity has been found to be higher in health, with both pathways leading to NO production. Future studies should test how the balance between host and microbiota NO production could shift between health and disease.

The use of conventional adjunctive antimicrobial therapies, such as chlorhexidine mouthwashes, could be reconsidered. Although these products reduce the overall oral microbial load, they also disturb the commensal oral microbiome (Bescos et al., 2020; Brookes et al., 2020). The effects of conventional antimicrobial therapies on BP control are becoming apparent (Bondonno et al., 2015; Joshipura et al., 2020), and their impacts on the actions of the NO_2^--producing oral cavity and subsequent long-term effects on recovery from periodontitis should be investigated.

Periodontitis-associated species and NO_2^--producing bacteria were inversely associated with one another at all timepoints. The association was weakest at BL but became progressively stronger as healing took place and was strongest at Day-90 when levels of periodontal inflammation were lowest. Previously, negative associations have only been reported between NO_3^- -reducing bacteria and periodontal pathogens 90 days after treatment (Rosier et al., 2024).

The negative association between NO_2^--producing species and periodontitis-associated species at all timepoints may indicate NO_2^- produced by health-associated bacteria oppose the growth of disease-associated bacteria. Bacteria found in dental plaque are capable of further reduction of NO_2^- to form NO (Schreiber et al., 2010). Upon reaction with oxygen intermediates, NO forms oxidative and nitrosative species. These cause oxidative damage to bacterial cells, including DNA alterations and disturbances to cell membranes and functional proteins (Jones et al., 2010). Furthermore, NO acts as a biofilm dispersal signal (Rosier et al., 2018), reducing the mass of biofilm accumulation in in vitro models of periodontitis (Mazurel et al., 2023).

Previous work suggests that increases in NO_3^- bioavailability would further enhance the growth of health-associated NO_2^--producing species and oppose the development of the periodontitis-associated microbiota. Increasing levels of salivary NO_3^- are associated with reductions in periodontitis relevant dysbiosis within the oral microbiome (Chen et al., 2022). *Prevotella* (Burleigh et al., 2019; Vanhatalo et al., 2018) and *Fusobacterium* (Vanhatalo et al., 2021) decrease in the salivary microbiome following NO_3^- supplementation in vivo and concentrations of 5 mM NO_3^- decrease the abundance of *Porphyromonas gingivalis* and other periodontitis-associated in an in vitro periodontal plaque biofilm model (Mazurel et al., 2023). Therefore, future studies

should consider the administration of a suitable NO_3^- supplement to investigate possible beneficial changes in the subgingival microbiome during biofilm reformation following treatment of periodontitis.

4.5 | A role for oral NO_2^- production in the link between periodontitis and cardiovascular disease

A recent systematic review found links between reduced BP in the months following completion of periodontal treatment in five out of the 12 studies included. The mechanism responsible for longer term reductions in BP in some cohorts following periodontal treatment remains to be fully elucidated (Muñoz Aguilera et al., 2020). In our study, we may not have found an effect of periodontal treatment on BP as this was an otherwise healthy population, and simple rapid screening hypertension measurements in a dental setting are likely subject to inaccuracies.

Impaired oral NO_2^- production and the resulting decrease in systemic NO availability is another factor which may link cardiovascular disease and periodontitis (Rosier et al., 2024). Previous studies show dietary NO_3^- supplementation works effectively to reduce BP (Bahadoran et al., 2017; Li et al., 2020). Current research suggests that these protective effects would be impaired among hypertensive cohorts with untreated periodontitis and that the recovery in NO_2^--production capacity could also have protective effects for hypertension (Rosier et al., 2024). Future research should investigate the effect of NO_3^- intake on BP in patients with periodontitis before and after periodontal treatment.

4.6 | Strengths and limitations

We used a species-level analysis, which was obtained by Illumina sequencing of part of the 16S rRNA gene, to group species based on their function (NO_2^- production) or association with periodontitis. To further improve the resolution of the oral microbiome, full-length sequencing of the 16s rRNA gene or metagenomic approaches should be considered, to increase certainty of the exact species implicated in community-wide microbiome interactions. For instance, this will be useful in determining the role of streptococci in NO_2^- production during the early stages of periodontal healing. Additionally, 16s rRNA sequencing identifies changes in the relative abundance of types of bacteria but does not identify changes in gene expression (Paster et al., 2006). The same species may fill different functional niches in health compared to disease, so a multi-omics approach, including metatranscriptomics, would have enabled a better understanding of changes in bacterial functions (Kuboniwa et al., 2012). At BL, the volume of biofilm collected would have been far larger compared to all other timepoints. Changes in relative abundance after treatment may be affected by a decrease in the number of bacterial cells sequenced. To confirm changes in the microbiome, future studies may wish to consider the use of qPCR to establish changes in the quantity of NO_2^--producing bacteria at healing sites.

Different areas of the oral cavity house different biofilms (Xu et al., 2015). The subgingival pocket is of most interest in periodontitis, but is a different environment compared to the dorsal surface of the tongue, where the largest quantity of NO_2^- producing bacteria are found and where the most effective NO_2^- production takes place. However, it is known that species from one habitat can correlate with species from another habitat (e.g. *P. gingivalis* levels in subgingival plaque correlate with its levels in saliva) (Jiang et al., 2021), indicating that changes in subgingival plaque will affect other communities to some extent. Nevertheless, the effect of periodontitis and periodontal treatment across multiple oral habitats should be evaluated.

Few studies investigate changes to salivary NO_3^- and NO_2^- levels. This study effectively assesses these changes and how they relate to local inflammation and the oral microbiome. By including only individuals, who had not recently received antibiotics, we can be confident that changes were likely caused by mechanical disruption, not pharmacological intervention or systemic disease.

The study sample size was similar to that of other studies examining changes in the oral environment after treatment for periodontal disease (Belstrøm et al., 2018; Johnston et al., 2021). Despite this, as a post hoc analysis, it was not powered to detect changes in the NO_2^--producing microbiome, nor NO_3^- or NO_2^- levels. To enable generalisation of results to other groups of patients receiving treatment, future studies should be specifically designed to test for these changes.

5 | CONCLUSIONS

Our data demonstrate successful treatment of periodontitis result in increases in the relative abundance of NO_2^--producing bacteria at Day-1, Day-7 and Day-90 post-treatment, which was accompanied by a decrease in known clusters of periodontitis-associated bacteria. In addition, disease-associated species and NO_2^- producing species were negatively associated with each other at all timepoints, with this relationship strongest following healing of the periodontal tissues at Day-90. The role of these NO_2^- producing bacteria in oral and systemic health is becoming more apparent and NO_3^- has previously demonstrated efficacy as an adjunctive aid in the reduction of reversible gingival inflammation. Such adjuncts may ultimately offer much needed realistic alternatives to systemic and local antimicrobials. Future studies should consider the use of supplementary dietary NO_3^- as an adjunctive aid in the treatment of periodontal disease, enabling improvements in clinical indicators of disease and reducing the re-establishment of a disease associated, inflamophilic microbiome.

AUTHOR CONTRIBUTIONS

Annabel Simpson, Mia Burleigh, Bob T. Rosier drafted the manuscript. Annabel Simpson, William Johnston, Bob T. Rosier, Mia Burleigh and Shauna Culshaw contributed to the design of work. Shauna Culshaw, Chris Easton, Fiona L. Henriquez and Alex Mira revised the manuscript. William Johnston did most experimental work, and Miguel Carda-Diéguez and Bob T. Rosier did most bioinformatic work. Shauna

Culshaw and William Johnston contributed to data acquisition and analysis and all authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

A. Mira and B. T. Rosier are coinventors in a pending patent application owned by the FISABIO Institute, which protects the use of nitrate as a prebiotic and certain nitrate-reducing bacteria as probiotics. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supporting Information of this article (Supplementary Datasheet).

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PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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