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The influence of diet, saliva, and dental history on the oral microbiome in healthy, caries-free Australian adults

Sonia Nath[1]✉, Peter Zilm[1], Lisa Jamieson[1], Pedro Henrique Ribeiro Santiago[1], Don Hasan Kevin Ketagoda[1] & Laura Weyrich[2,3]

Oral microbiome transplantation (OMT) has the potential to serve as a therapeutic approach for managing dental caries; however, it is essential to identify suitable donors. The aim of this study was to analyse the relationship between dietary (i.e., energy, water, carbohydrate and sugar intake), saliva quality (i.e., saliva flow rate and salivary pH), and clinical factors (i.e., past caries experience and fluoride exposure) on the oral microbiome composition of potential OMT donors. For this cross-sectional study, a comprehensive dental examination was conducted for 93 healthy, caries-free adults (aged 18–85 years) without systemic or oral disease. All participants completed questionnaires on food frequency and socio-economic, lifestyle, and behavioural factors. Supragingival plaque samples were collected, and bacterial 16S rRNA genes were amplified, sequenced, and assigned to bacterial taxa. Stimulated saliva samples were collected for salivary flow rate and pH measurements. Constrained partial ordination analysis revealed that dietary factors, such as carbohydrate and sugar intake, had strong directional influences on microbial composition, while salivary factors like flow rate and pH showed opposing effects. Correlation analysis linked high sugar intake and reduced salivary pH to increased *Streptococcus* abundance. Differential abundance analysis identified significantly higher abundance of *Streptococcus* species among low water and high carbohydrate and sugar consumers. In mediation analysis, sugar consumption was directly and indirectly linked to reduced salivary pH, with *Streptococcus* showing a significant negative mediation effect (mean: -0.198; 95% CI: -0.387 to -0.010). High carbohydrate and sugar intake significantly influenced alpha diversity metrics ($p < 0.05$). Beta diversity permutational multivariate ANOVA revealed that covariates explained 11.45–12.52% of microbial variation ($p < 0.05$). This study emphasises that OMT donors with diverse oral microbiomes, low sugar and carbohydrate intake, and reduced levels of acidogenic taxa, such as *Streptococcus*, which significantly mediate salivary pH reduction, may be preferred for caries prevention.

Keywords Dental caries, Mediation analysis, Microbiota, Oral health, 16S ribosomal RNA, Sequencing, Microbiome

According to the Global Burden of Disease Data[1], dental caries is one of the most prevalent chronic disease worldwide, affecting individuals across all age groups and disproportionately impacting minority populations[2]. Dental caries are primarily caused by consuming dietary free sugars, such as sucrose, glucose, and fructose, which provide the essential substrate for cariogenic oral bacteria, enabling them to produce acids that demineralise tooth enamel and lead to cavity formation[3]. In recent years, there has been growing interest in the role of the oral microbiome in maintaining oral health and its potential as a therapeutic target for caries management. The oral microbiome, a complex ecosystem of microorganisms, is now recognised as a key player in maintaining oral health. Modulating this microbial community offers a promising strategy for creating an environment less conducive to caries development by fostering a healthy and diverse microbiome that naturally resists caries-causing pathobionts[4]. This approach marks a shift from traditional caries management by prioritising non-invasive strategies, such as fluorides, chemical agents for biofilm control, and dietary management, and invasive

[1]Australian Research Centre of Population Oral Health, Adelaide Dental School, The University of Adelaide, Adelaide, SA 5000, Australia. [2]School of Biological Sciences, University of Adelaide, Adelaide, SA, Australia. [3]Present address: Department of Anthropology and Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA 16802, USA. ✉email: Sonia.nath@adelaide.edu.au

procedures such as placement of restorations[5]. Oral Microbiome Transplantation (OMT) is an intervention being studied to reduce caries risk by transferring a microbial community from a healthy donor to a recipient at risk of developing caries[6,7]. The goal of OMT is to modulate the recipient's oral microbiome, shifting its composition toward a more protective state against caries. For OMT to be successful, it is crucial to identify donors who pose minimal risk for future caries development. In the context of fecal microbiome transplantation, such individuals are referred to as "super donors" due to their highly beneficial microbial profiles[8].

Key factors affecting the oral microbiome include diet, salivary pH and flow rate, fluoride exposure, and past caries history. Frequent consumption of carbohydrates and sugars promotes the proliferation of acidogenic and aciduric bacteria, such as *Streptococcus mutans*, *Lactobacilli*, *Scardovia*, *Bifidobacteria*, and *Actinomyces*[9,10]. Salivary pH and flow rate are also critical determinants of oral microbial dynamics. A higher salivary flow rate enhances the buffering capacity of saliva, diluting acids and facilitating remineralisation by maintaining a neutral pH conducive to oral health[10,11]. Conversely, low salivary pH and reduced flow rates are associated with increased bacterial adhesion and metabolic activity of cariogenic species, contributing to caries progression[10]. Fluoride exposure further influences microbial composition by selectively reducing the abundance of cariogenic bacteria, such as *Streptococcus*, and *Scardovia*[11,12]. The microbial profiles of individuals with a history of dental caries but no active lesions may differ from those who have never experienced caries[13]. Even after treatment, residual cariogenic bacteria can persist in the oral environment, maintaining their metabolic activity, increasing the risk of secondary caries or degradation of restorations, and increasing the risk of developing new caries lesions.

It is also important to consider the ecological plaque hypothesis[14], which posits that during disease, intrinsic homeostasis within the microbial community is disrupted, often due to environmental changes such as increased exposure to fermentable carbohydrates, and this leads to a lower pH in plaque, altering the competitiveness and stability of individual microbial species. Such changes are thought to facilitate the development of caries by favouring acidogenic and aciduric bacteria, even after caries treatment, as the ecological niche may still support recolonisation by cariogenic species. We hypothesise that a combination of factors, including consistent low fermentable carbohydrate intake, adequate water consumption, and the absence of caries experience, are crucial for donor selection. The complex interactions between these factors remain an open question in determining whether they can reliably predict an ideal donor profile for OMT. Therefore, this paper aims to investigate the diversity and composition of oral microbiota in healthy individuals' diet, salivary and clinical factors and define a comprehensive profile of a "super donor" for OMT. The findings may contribute to developing evidence-based donor selection criteria and improving the efficacy of OMT as a preventive strategy against dental caries.

Results

Participant characteristics

This study collected samples from 93 healthy adults with no dental or systemic conditions and revealed several significant associations between sociodemographic, lifestyle factors and dietary, salivary, and clinical covariates (Table 1). Water intake showed statistically significant differences ($p = 0.02$), with the 26–35 age group having the highest mean intake (3190 ± 1143 ml/day). Australian-born and employed participants had a higher mean daily sugar intake (92.8 ± 32.5 g/day, $p = 0.03$; 91.9 ± 39.9 g/day, $p = 0.04$). The Missing + Filled (MF) score significantly differed among age groups ($p < 0.01$). The > 35y age group had the highest mean MF score (4.85 ± 4.12). Mental well-being correlated with differences in caries scores ($p = 0.01$), saliva pH ($p = 0.04$), and fluoride application rates ($p = 0.04$). Regular alcohol consumers had higher water intake compared to occasional or non-consumers ($p = 0.01$). Lastly, recent dental visits were associated with higher MF scores and saliva flow rates ($p = 0.02$ and $p = 0.05$), possibly indicating more frequent visits for those with active dental issues. These findings underscore the complex interplay between demographic factors, lifestyle choices, and oral health outcomes.

Dietary factors and salivary factors drive the oral microbial composition

The constrained partial ordination analysis identified statistically significant associations between dietary factors, salivary parameters, and microbial composition after controlling for age. The dietary factors, such as high energy, carbohydrate, and sugar intake, demonstrated strong directional influences on microbial community variation among Australian-born individuals, as indicated by the longer arrows in Fig. 1A. In contrast, saliva flow rate and salivary pH post-glucose had opposing effects on microbial composition. The correlation heatmap (Fig. 1B) further identified high sugar consumption correlated positively with *Veillonella* and negatively with *Leptotrichia*, *Prevotella*, and *Cardiobacterium*. High energy and carbohydrate consumption were negatively associated with *Leptotrichia*, *Fusobacterium*, *Prevotella*, *Selenomonas*, *Capnocytophaga*, and *Campylobacter*. Salivary parameters also played a role: lower salivary pH post-glucose (more acidic condition) is associated with higher *Streptococcus* (negative correlation) abundance and lower abundance of *Alloprevotella* (positive correlation), while a high saliva flow rate correlated positively with *Lautropia*. Interestingly, fluoride application exhibited a suppressive effect and was negatively associated with most taxa's abundance. The differential abundance analysis further supports these findings (Fig. 1C, Table S1). High carbohydrate intake was associated with a higher abundance of *Comamonadaceae*, *Actinomyces*, *Corynebacterium*, *Streptococcus*, and *Veillonella*, while *Parvimonas* showed a lower abundance. Similarly, low sugar intake was linked to a lower abundance of *Stomatobaculum* and *Streptococcus* and a higher abundance of *Peptococcus* and *Treponema*. Lower water consumption was associated with higher abundance of *Streptococcus* and lower abundance of *Aggregatibacter*. The salivary pH post-glucose was associated with lower abundance of *Neisseria*, *Comamonadaceae*, *Corynebacterium*, and *Lautropia*. The genus *Parvimonas* was abundant in caries experience. This suggests that dietary factors like high sugar and carbohydrate intake significantly influence the oral microbiome, altering the abundance of specific microbial genera. Saliva pH and flow rate impact microbial composition, and fluoride suppresses most genera.

Category	Subgroups	*N*	Energy, kJ/day	Water (ml/day)	Carbohydrate (g/day)	Sugar (g/day)	Caries score (missing + filled teeth)	Saliva pH, pH unit	Saliva flow rate (ml/min)	Number of fluoride application, *n* (%)
Age	18–25	33	8960 ± 4017	2647 ± 1215	210 ± 105	83 ± 45.5	0.39 ± 0.89	6.19 ± 0.82	1.98 ± 0.77	19 (57.5%)
	26–35	40	9151 ± 2700	3190 ± 1143	202 ± 68.4	90 ± 40.8	1.58 ± 2.51	6.13 ± 0.86	2.07 ± 0.70	22 (55%)
	> 35	20	8971 ± 3748	3093 ± 912	195 ± 88.8	95.5 ± 38.3	4.85 ± 4.12	6.47 ± 0.85	2.12 ± 0.74	11 (55%)
	p-value		0.6	**0.02**	0.64	0.12	< **0.01**	0.25	0.63	0.97
Gender	Male	32	9186 ± 4101	2972 ± 934	201 ± 83.8	88.1 ± 39.4	1.56 ± 2.61	6.21 ± 0.93	2.10 ± 0.63	17 (53.1%)
	Female	61	8970 ± 3003	2979 ± 1243	204 ± 88.8	89.4 ± 43.4	2.02 ± 3.24	6.23 ± 0.80	2.02 ± 0.78	35 (57.3%)
	p-value		0.72	0.83	0.85	0.94	0.67	0.85	0.48	0.86
Country of birth	Australian	36	9182 ± 3098	3047 ± 960	209 ± 74.8	92.8 ± 32.5	2 ± 2.88	6.16 ± 0.88	2.09 ± 0.75	25 (69.4%)
	Other	57	8962 ± 3603	2932 ± 1248	199 ± 93.8	86.5 ± 46.9	1.77 ± 3.15	6.26 ± 0.83	2.02 ± 0.72	27 (47.3%)
	p-value		0.55	0.28	0.25	**0.03**	0.83	0.39	0.66	0.06
Level of education	Tertiary education	57	9028 ± 3126	3091 ± 1026	203 ± 74.4	92.5 ± 40.6	2.14 ± 2.94	6.21 ± 0.93	1.99 ± 0.68	29 (50.8%)
	Secondary education or less	36	9072 ± 3843	2796 ± 1297	203 ± 104	83.3 ± 43.7	1.42 ± 3.17	6.23 ± 0.80	2.15 ± 0.80	23 (63.8%)
	p-value		0.86	0.07	0.68	0.07	0.14	0.75	0.38	0.3
Employment	Employed	61	9351 ± 3795	3045 ± 1131	209 ± 93.7	91.9 ± 39.9	1.97 ± 3.17	6.28 ± 0.88	2.13 ± 0.76	39 (63.9%)
	Not working	32	8473 ± 2466	2846 ± 1167	192 ± 71.2	83.3 ± 45.6	1.66 ± 2.79	6.12 ± 0.77	1.90 ± 0.66	13 (40.6%)
	p-value		0.4	0.17	0.28	**0.04**	0.81	0.47	0.14	**0.05**
Physical activity	Minimal activity (< 600 met min)	35	8440 ± 2802	2878 ± 1170	203 ± 80.3	93.9 ± 43.6	1.97 ± 3.38	6.28 ± 0.89	2.15 ± 0.75	20 (57.1%)
	Moderate activity (600–1500 met min)	25	9722 ± 3897	3044 ± 1038	213 ± 106	86.7 ± 38.5	1.56 ± 2.89	6.19 ± 0.80	1.89 ± 0.73	19
	High activity (> 1500 met min)	33	9156 ± 3565	3031 ± 1209	196 ± 78.9	85.4 ± 43.2	1.97 ± 2.82	6.20 ± 0.86	2.06 ± 0.71	13 (39.3%)
	p-value		0.43	0.69	0.62	0.65	0.74	0.94	0.35	0.02
Mental well being	Low distress	42	8451 ± 2553	3086 ± 1267	187 ± 63.1	86.5 ± 43.0	2.48 ± 3.46	5.99 ± 0.81	2.09 ± 0.63	19 (45.2%)
	Moderate distress	19	8039 ± 2335	2974 ± 916	189 ± 59.8	86.1 ± 37.7	0.63 ± 2.09	6.46 ± 0.68	2.04 ± 0.87	15 (78.9%)
	Severe distress	32	10,468 ± 4443	2835 ± 1102	232 ± 116	93.8 ± 43.5	1.78 ± 2.74	6.39 ± 0.92	2.00 ± 0.78	18
	p-value		0.07	0.9	0.11	0.74	**0.01**	**0.04**	0.73	**0.04**
Alcohol consumption	>= 1 standard drink/ week	34	8616 ± 3138	3270 ± 1004	196 ± 62	88.3 ± 32.2	2.71 ± 3.63	6.15 ± 0.90	2.13 ± 0.78	24 (70.5%)
	Occasional/non-alcohol consumers	59	9285 ± 3547	2808 ± 1188	207 ± 98.4	89.3 ± 46.4	1.37 ± 2.54	6.27 ± 0.82	2.00 ± 0.70	28 (47.4%)
	p-value		0.23	**0.01**	0.99	0.39	0.17	0.56	0.39	**0.05**
Smoking	Smoker	18	9530 ± 3839	3183 ± 1002	213 ± 89.5	97.6 ± 42.0	3.06 ± 3.35	6.33 ± 0.74	2.13 ± 0.75	12 (66.6%)
	Non-smoker	75	8936 ± 3316	2927 ± 1172	201 ± 86.4	86.9 ± 41.9	1.57 ± 2.90	6.20 ± 0.87	2.03 ± 0.75	40
	p-value		0.57	0.17	0.5	0.14	0.07	0.82	0.56	0.44
Tooth brushing frequency	Once	21	9117 ± 2283	3172 ± 1291	208 ± 77.7	99.2 ± 50.2	1.52 ± 2.34	6.16 ± 0.96	1.87 ± 0.72	12 (57.4%)
	Twice or more	72	9026 ± 3668	2920 ± 1096	202 ± 89.5	85.9 ± 39.0	1.96 ± 3.22	6.24 ± 0.82	2.10 ± 0.73	40 (55.5%)
	p-value		0.38	0.66	0.72	0.28	0.63	0.59	0.19	0.99
Flossing frequency	Once or more everyday	25	8692 ± 2752	3009 ± 710	185 ± 53	80.4 ± 29.4	2.04 ± 2.85	6.40 ± 0.90	2.24 ± 0.78	14 (56%)
	Occasionally/never	68	9170 ± 3616	2965 ± 1267	210 ± 95.5	92.1 ± 45.4	1.79 ± 3.12	6.16 ± 0.82	1.98 ± 0.70	38 (55.8%)
	p-value		0.82	0.46	0.52	0.37	0.36	0.22	0.17	0.99
Last dental visit	Less than 12 months	52	9482 ± 3814	2930 ± 1046	217 ± 102	91.7 ± 40.9	2.65 ± 3.64	6.27 ± 0.82	2.17 ± 0.72	32 (61.5%)
	More than 12 months	41	8502 ± 2762	3036 ± 1261	186 ± 59.6	85.4 ± 43.3	0.85 ± 1.56	6.17 ± 0.89	1.89 ± 0.72	20 (48.7%)
	p-value		0.25	0.83	0.1	0.28	**0.02**	0.65	**0.05**	0.3

Table 1. The socio-demographic data of the study participants based on eight selected covariates. Mean and standard deviations were calculated for energy intake, water consumption, carbohydrate intake, sugar intake, caries score, saliva pH, and saliva flow rate, and prevalence of fluoride application was calculated. Kruskal Wallis test was used for multiple group comparisons, followed by post hoc Dunn's test, and for two group comparisons, the Wilcoxon test was used. The *p-value* of < 0.05 was considered significant.

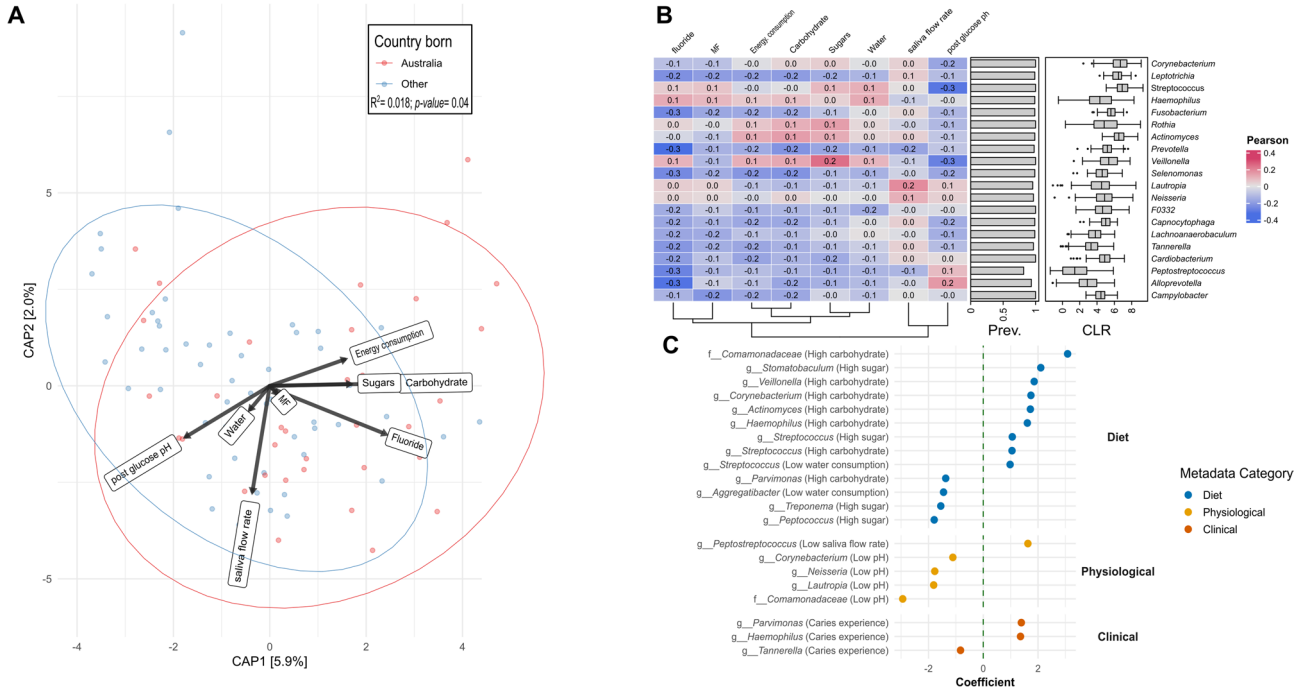

Fig. 1. (**A**) Constrained partial ordination plot: constructed using Euclidean distance, controlling for age. The arrows represent constraint vectors for key explanatory variables, including energy intake, water consumption, carbohydrate intake, sugar intake, saliva flow rate, fluoride application, salivary pH post-glucose, and past caries experience score (MF). Samples are coloured by country of birth (red for Australia, blue for Other). The plot was generated using the *microViz* package. (**B**) Correlation heatmap: constructed with centre-log ratio transformation, using Pearson's method for the top 20 genera to observe an association between continuous variables and the oral microbiome. The variables tested were coefficients between microbial genera (rows) and host variables (columns), including fluoride application, past caries score (MF), energy intake, carbohydrate intake, sugar intake, water consumption, saliva flow rate, and salivary pH post-glucose. The colour gradient represents the strength and direction of the correlation, with red indicating positive correlations and blue indicating negative correlations. The plot was generated using the *microViz* package. (**C**) Differential abundance analysis: The significantly abundant taxa were mapped according to their standard coefficient. The plot was created using *MaAsLin2* and *ggplot2* package.

Daily carbohydrate and sugar intake have a significant impact on oral microbial diversity

The alpha diversity of daily carbohydrate intake showed significant differences in both observed features (OF) (χ^2 = 3.90; $p = 0.05$) and Shannon's index (SI) (χ^2 = 4.69; $p = 0.03$) (Fig. 2A), and daily sugar intake exhibited highly significant differences in both metrics (OB: χ^2 = 9.14, SI: χ^2 = 13.32; $p < 0.01$) (Fig. 2B). Professional fluoride application status was associated with a significant difference in the SI (χ^2 = 3.77; $p = 0.05$) (Table S2). For the linear regression model (Table S3), significant associations were identified for carbohydrate intake (OF: $R^2 = 0.10$, $p = 0.02$; SI: $R^2 = 0.11$, $p = 0.01$) and sugar intake (OF: $R^{2=}$ 0.08, $p = 0.01$; SI: $R^2 = 0.07$, $p = 0.04$), indicating that these dietary factors influence microbial diversity. Daily energy intake was significantly associated with the SI ($R^2 = 0.08$, $p = 0.02$). Other variables did not demonstrate significant associations. These findings suggest that dietary factors, mainly carbohydrate and sugar intake, influence microbial diversity.

For the PERMANOVA model (Table S4) using Euclidean distance, the covariates significantly explained 11.45% of the variation ($R^2 = 0.11$; $p = 0.02$) (Fig. 2C). Similarly, for the Bray-Curtis model, the results showed that the covariates collectively explained 12.52% of the variation in microbial community composition ($R^2 = 0.12$; $p < 0.01$) (Fig. 2D). Carbohydrate intake significantly impacted Euclidean distances ($R^2 = 0.02$, $p = 0.02$) and Bray-Curtis distances ($R^2 = 0.02$, $p = 0.02$) (Fig. 2E). Sugar intake showed similar significant effects for Euclidean ($R^2 = 0.02$, $p = 0.01$) and for Bray-Curtis ($R^2 = 0.01$, $p = 0.04$) (Fig. 2F). Saliva pH post-glucose and fluoride application influenced Bray-Curtis metrics only ($R^2 = 0.022$, $p < 0.01$), which is associated with changes in the relative abundances of taxa.

High sugar consumption mediated by *Streptococcus* is linked to reduced salivary pH

A mediation model was constructed where sugar consumption directly and indirectly influences the saliva pH, mediated through changes in the oral microbiome, accounting for confounding factors (Fig. 3A). Results indicated a negative direct effect of sugar consumption on salivary pH, with a mean estimate of -0.02 (0.65) and −0.40 (0.65) (Fig. 3B, Table S5), suggesting that sugar consumption, directly and indirectly, lowers salivary pH through its influence on the microbiome. Certain microbial taxa play varying roles in mediating the relationship between sugar consumption and salivary pH post-glucose (Fig. 3C, Table S6). *Streptococcus* demonstrated a

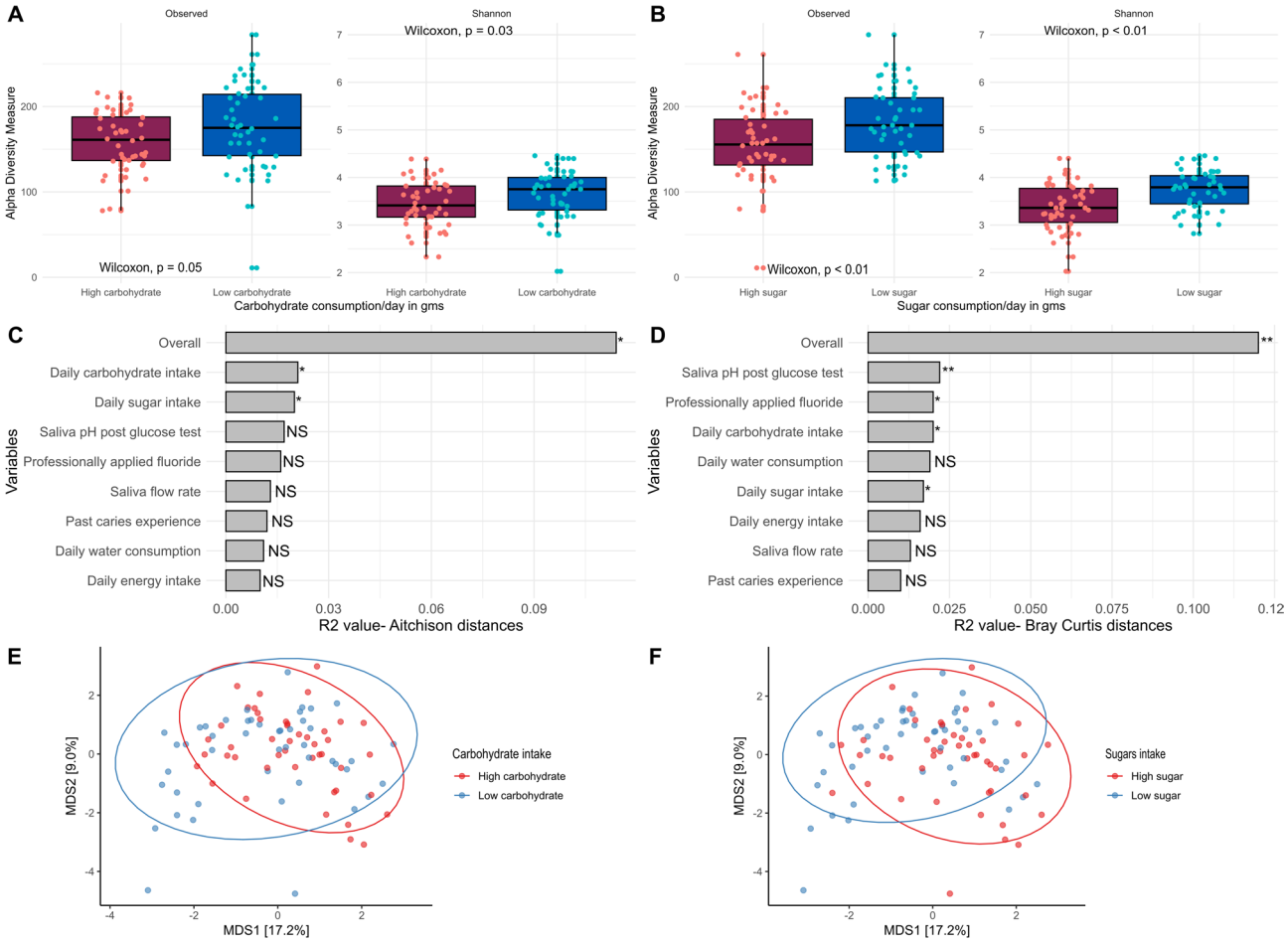

Fig. 2. (**A-B**) Alpha diversity box plot: for the significant variable (carbohydrate and sugar intake stratified as high vs. low based on median value) using the observed feature metrics and Shannon's diversity index. The Wilcoxon test was used for comparison. Each dot in the boxplot represents a donor. (**C-D**) PERMANOVA bar plots: the beta diversity metrics were tested for all the variables using the Euclidean distance and Bray-Curtis metrics using the PERMANOVA *adonis2* function from the *vegan* package. The R^2 is the amount of variation explained, while the star represents the significant variables associated with oral microbiome composition (* $p < 0.05$, ** $p < 0.01$). (**E-F**) MDS (Multidimensional Scaling) plots: based on carbohydrate and sugar intake, significant in Euclidean distance. The PCoA plots with the ellipses represent the clustering of microbial communities, showing partial separation between high and low groups for each measured parameter.

statistically significant negative mediation effect (mean: -0.198; 95% CI: -0.387 to -0.010), highlighting its critical role as an acidogenic taxon contributing to pH reduction in response to sugar intake. Other taxa, such as *Neisseria* and *F0332* (family *Actinomycetaceae)*, exhibit negative mediation effects, and *Veillonella* and *Actinomyces* show positive mediation effects.

Discussion

The findings of this study underscore the significant influence of dietary, salivary, and clinical factors on oral microbiome diversity and composition, with important implications for donor selection in OMT for caries prevention. High carbohydrate and sugar intake were strongly associated with reduced microbial diversity and shifts in community composition, particularly increasing the abundance of acidogenic taxa like *Streptococcus*, demonstrating a significant negative mediation effect on salivary pH. Therefore, potential good donors are individuals who follow low sugar dietary patterns, maintain a balanced intake of nutrients, and have low caries experience score. By carefully selecting donors based on these criteria, OMT could effectively modulate the recipient's oral microbiome toward a caries-protective state[7]. While most oral microbiome research primarily focuses on dysbiosis associated with disease, it is crucial to understand the factors that drive oral health and the role of commensal microbiomes in maintaining a healthy state. This cohort may be utilised to establish foundational baselines for a healthy oral microbiome, thereby enhancing the understanding of how specific characteristics promote oral health.

The constrained partial ordination analysis revealed strong directional influences of dietary factors on microbial community variation. Our analysis indicates that while Australian-born individuals had higher

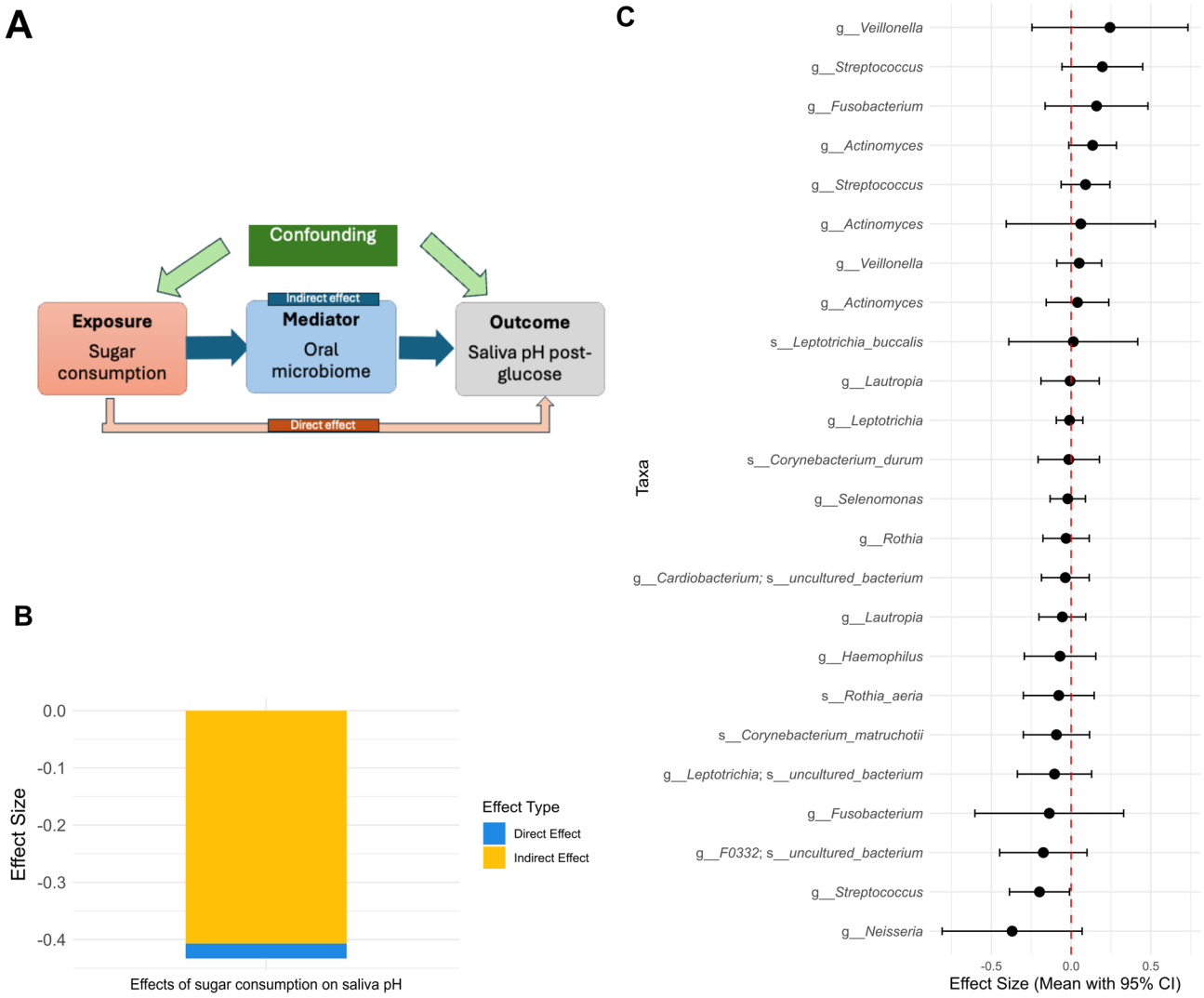

Fig. 3. (**A**) Directed Acyclic Graph (DAG): a conceptual framework of the mediation analysis model. Sugar consumption (exposure) influences salivary pH post-glucose (outcome) and the oral microbiome (mediator). Confounding factors accounted for in the analysis included age, dental visit frequency, fluoride exposure, saliva flow rate, country of birth, and education level. The direct effect represents the pathway from sugar consumption to salivary pH, while the indirect effect captures the influence mediated by changes in the oral microbiome. (**B**) The stacked bar graph quantifies the total effect of sugar consumption on salivary pH, partitioned into direct and indirect effects, highlighting the dominant role of microbial mediation. (**C**) Forest plot showing component-wise mediation effect of individual microbial taxa on the relationship between sugar consumption and salivary pH. Each point represents the mean effect size, with error bars indicating 95% confidence intervals. The red dashed line indicates no mediation effect (effect size = 0). The analysis was conducted using the *SparseMCMM* package.

sugar intake, and sugar intake was significantly associated with beta diversity. When included in multivariate models, sugar intake remained a significant driver of microbial composition and suggests that the impact of site of birth on beta diversity is primarily mediated through dietary behaviours, particularly sugar consumption. The mediation analysis investigating the effect of sugar consumption on salivary pH revealed a complex relationship mediated by the oral microbiome. This finding aligns with its well-established association with cariogenic activity and its ability to create an acidic oral environment conducive to dental caries development[15]. The differential abundance analysis further supported this, identifying significant associations between specific microbial taxa and various dietary variables. For instance, high carbohydrate intake was positively correlated with taxa such as *Comamonadaceae*, *Actinomyces*, *Corynebacterium*, *Streptococcus*, and *Veillonella*, while high sugar intake was linked to changes in *Stomatobaculum* and *Streptococcus* abundance. These findings align with previous studies that have implicated these genera in caries development and progression, highlighting the potential role of diet in modulating the oral microbiome's cariogenic potential[15–17]. There was also a negative association between high energy and carbohydrate consumption and the abundance of genera such as *Leptotrichia*, *Fusobacterium*, *Prevotella*, *Selenomonas*, *Capnocytophaga*, and *Campylobacter*. These genera

are often associated with periodontal health, suggesting that high carbohydrate diets may promote cariogenic bacteria and suppress beneficial microbes, as previously suggested[16,18]. This dual effect could exacerbate the risk of oral diseases and underscores the importance of dietary moderation in maintaining oral health. Our analysis of alpha diversity metrics further emphasises the significant impact of carbohydrate and sugar intake on oral microbial diversity. The differences in observed features and Shannon's index suggest that these dietary factors influence the abundance of specific taxa and affect the overall community structure and evenness, as observed by others[15]. This finding has important implications for understanding how diet shapes the oral microbiome and potentially influences its resilience and stability. However, the relationship between sugar intake and caries risk is not always consistent, and the resilience of the oral microbiome can modulate this relationship. Interventions like OMT may help restore or enhance this microbial resilience, supporting the reestablishment of a balanced and robust microbial ecosystem even in dietary or environmental challenges[19]. Overall, these results emphasise the significant role of *Streptococcus* in mediating the acidogenic effects of sugar consumption on salivary pH while highlighting the need for further research to clarify the roles of other taxa with borderline or uncertain effects, especially in healthy people without active caries.

Significant associations exist between salivary parameters and specific microbial taxa and the correlation between genera such as *Haemophilus* and *Parvimonas* with caries experience. Other studies also found a higher abundance of *Haemophilus* in a healthy group than those with past caries experience[19]. The observed suppressive effect of fluoride application on the abundance of most genera is noteworthy and aligns with fluoride's known antimicrobial properties[12]. This finding supports the continued use of fluoride as a preventive measure in oral health care while highlighting the need for further research into its long-term effects on oral microbial ecology[20]
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This study's strength is its comprehensive analysis of the oral microbiome of a healthy, caries-free population of potential donors for OMT in Australia. Nevertheless, there are some limitations. The population was skewed towards younger age groups, and there was no representation from the Aboriginal population, potentially limiting the generalizability of the findings to a broader and more diverse population, highlighting the need for future research to enhance representativeness[21]. Another important limitation of our study was that it focused exclusively on bacterial components of the oral microbiome, without analysis of other microorganisms such as viruses, fungi, and archaea. These non-bacterial components constitute a significant part of the oral microbiota and may play important roles in oral health and disease. Also species level profilling was not possible with 16S sequencing platform. We excluded low-abundance taxa for statistical analysis to ensure robustness and interpretability. While this approach minimises data sparsity and noise, it may overlook ecologically relevant low-abundance species, including potential pathobionts that could be important OMT.

In conclusion, our study provides a comprehensive view of the factors shaping the oral microbiome in healthy people, with diet emerging as a primary driver of microbial composition and diversity. The complex interactions between dietary factors, host physiology, and the oral microbiome in influencing salivary pH and potentially oral health outcomes emphasise the need for personalised approaches to oral health care. Future research should focus on longitudinal studies to elucidate the temporal dynamics of these relationships and explore potential interventions targeting diet and the oral microbiome to promote oral health.

Materials and methods

The data used in this study is part of the OMT study conducted at the School of Dentistry, University of Adelaide, Adelaide, Australia, from June 2021 to July 2022. This study recruited healthy participants to identify a suitable donor for microbiota transplantation. The study was approved by the University of Adelaide Human Research Ethics Committee (H-2020-34609). All study participants gave informed consent before participating, and the research was conducted under the declaration of Helsinki. A detailed protocol for the study has been published[7] and follows the STORMS guideline (Supplementary file).

Participant recruitment and data collection

This cross-sectional study included adults (> 18–85 years) without active dental caries or periodontal disease. Caries activity was assessed using the Decayed, Missing, and Filled Teeth (DMFT) index[22]. Participants were considered free of active caries if they had no teeth with untreated carious lesions (D = 0) at the time of examination and periodontally healthy if they exhibited probing depths (PD) of ≤ 4 mm, no clinical attachment loss, and less than 20% of sites with bleeding on probing (BOP). All oral examinations were conducted by two calibrated dental examiners (SN & KK) who underwent training and calibration before the study to ensure consistency and reliability in the assessments. Individuals with systemic diseases (e.g., diabetes, cardiovascular disease, cancer), pregnant or lactating women, or those who have used medications that may impact the oral microbiome (e.g., antibiotics, corticosteroids), or had dental treatment in the last three months were excluded. Our previous publication detailed participant screening, exclusion, and inclusion criteria[6,7]. A self-administered questionnaire was used to capture sociodemographic data, general health status, oral health behaviours, and dental hygiene using *REDCap* (Research Electronic Data Capture) software. Dietary information was collected using a Dietary Questionnaire for Epidemiological Studies (DQES v3.2)[23].

Sample collection

Plaque samples were collected from six combined sites using a sterile curette, pooled in a phosphate buffer solution (PBS), immediately transported to the Oral Microbiology Laboratory, University of Adelaide, and stored at -80 °C for microbial analysis. The six sites were selected: the mesio-buccal surfaces of a right maxillary molar, a left mandibular molar, a right mandibular molar, and a mesio-palatal surface of a maxillary molar. Curette wash (CW) samples were collected each collection day to create an instrumental control for microbiome sampling. Stimulated saliva samples were collected to measure the salivary flow rate and baseline and post-

glucose challenge pH. A detailed description of the sample collection, saliva sample testing, and categorisation of covariates is provided in Supplementary Text S1.

DNA extraction, Microbiome sequencing and data-preprocessing

DNA was extracted from samples using the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), with extraction blank controls included for every 12 samples. DNA was extracted from dental plaque, CW, and extraction blank controls (EBCs) and negative controls (NTCs) for contamination control. In total, 93 samples and 48 controls were sequenced (14 EBCs, 17 NTCs, and 17 CWs). The V4 region of the bacterial 16 S rRNA gene was amplified in each sample, uniquely barcoded[24]. PCR was performed with Invitrogen Platinum High Fidelity DNA polymerase, and products were quantified using the Qubit dsDNA BR assay. Amplicons were pooled, purified with AxyPrep Mag PCR Clean-up beads, and quantified using TapeStation D1000 reagents (Agilent). Sequencing was performed using the Illumina MiSeq 2300 platform (Illumina, San Diego, CA) at the Pennsylvania State University Genomics Core Facility. Raw paired-end reads were processed in QIIME2 (v2024.11)[25], including quality filtering, denoising, and amplicon sequence variant (ASV) inference using the DADA2 plugin[26]. Taxonomy was assigned with the SILVA 138 database. The entire process, including contaminant assessment and filtering, is described in Supplementary Text S2. From 93 samples, 5,702,771 DNA sequences were obtained, with averages of 61,320 and 56,091 and min/max reads of 1,017 and 304,058. The total number of ASVs detected was 968.

Statistical analysis

Statistical analysis was conducted in R Statistical software (v 4.4.2, R Core Team, 2024). All analyses were based on eight covariates, including dietary factors (energy, water, carbohydrate, and sugar intake), salivary measures (saliva flow rate and saliva pH post-glucose), and clinical variables (MF score and professional fluoride application status). The codes used for analysis can be accessed on GitHub (https://github.com/sonianath/Caries_Factors_OMT.git).

Participant co-variables

The mean and standard deviation were reported for continuous variables, and a count and a percentage were reported for categorical variables. Initially, a Shapiro-Wilk test and histograms were plotted, and the data followed a non-normal distribution. Kruskal-Wallis test was used, followed by a post hoc Dunn's test with Bonferroni's correction for pair-wise comparison, or the Wilcoxon test was used to assess statistical significance. A $p\text{-}value < 0.05$ was considered significant.

Microbiota community analysis

After pre-processing, a *phyloseq*[27] object was created, checked for ASV distribution, read depth, and missing ASVs, and then used for subsequent analysis. First, a constrained partial ordination analysis explored how multiple covariates influenced microbial community composition while accounting for age as a conditioning variable based on the Euclidean distance. Next, a correlation heatmap using Pearson's association was constructed to examine the relationship between continuous variables and the 20 most abundant taxa. Both analyses were done using *the MicroViz*[28] package. Then, the *MaAsLin2*[29] package utilised a negative binomial regression model for differential abundance analysis. All sociodemographic and lifestyle variables listed in Table 1 were included as covariates in differential analysis.

Alpha diversity was assessed using two metrics: observed features and Shannon's Index, with results visualised through boxplots created using the *ggplot2*[30] and *ggpubr*[31] packages. These boxplots compared the alpha diversity indices across eight covariates to explore variations in microbial diversity. Histograms and Shapiro-Wilk tests were performed to assess the normality of the alpha diversity distribution metrics. If non-normal distributions were observed across covariates, the Wilcoxon test would be used to compare alpha diversity between the two groups. Multiple linear regression models were used with observed features and Shannon's Index as dependent variables to explore the relationship between environmental factors as independent variables. Each model included one primary predictor variable alongside age and gender as covariates to account for potential confounding effects. Beta diversity was analysed using Euclidean and Bray-Curtis distances in Principal Coordinate Analysis (PCoA) and visualised using multidimensional scaling (MDS) plots. The PERMANOVA test from the *vegan*[32] package was used to evaluate the significance of beta diversity. This test assessed the impact of eight covariates on microbial community composition, with both Euclidean and Bray-Curtis distance methods employed to calculate dissimilarities[33].

Next, a mediation analysis was conducted to explore the potential direct and indirect effects of sugar consumption (exposure) on salivary pH (outcome), with the oral microbiome acting as a mediator using the *SparseMCMM*[34] package. The relative abundance table was prepared at genus rank, and a filtering threshold was set to 0.1% for abundance and 5% for prevalence. Before mediation analysis, propensity score matching was conducted as a preprocessing step, following suggestions by Austin et al.[35]. The propensity score matching balanced the characteristics of high and low-sugar consumption (exposure) groups before mediation analysis. Using the *MatchIt*[36] package, the nearest neighbour matching with a 1:16 ratio and replacement was conducted, focusing on the Average Treatment Effect on the Treated (ATT). The matching was based on several covariates, including age (squared), dental visit frequency, fluoride exposure, saliva flow rate, country of birth, and education level. A logistic regression model estimated the propensity scores. The balance of covariates between groups was assessed using summary statistics and visualised through a love plot (Figure S1), which displays standardised mean differences before and after matching. This matching process reduces confounding effects to create comparable groups for analysing the impact of sugar consumption on salivary pH via the oral microbiome. The

matched data set was then used for mediation analysis, incorporating 100 random data splits to calculate the direct, indirect, and total effects. A component-wise effect of each taxon was calculated as a mediator.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

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Author contributions
S.N: contributed to conception and design, contributed to the acquisition, drafted manuscript, and critically revised the manuscript and gave final approval. P.Z: contributed to conception and design, contributed to interpretation, drafted manuscript, and critically revised the manuscript and gave final approval. L.J: contributed to design, contributed to interpretation, drafted manuscript and critically revised manuscript. PHRS: contributed to design, contributed to analysis and interpretation, drafted manuscript, and critically revised the manuscript and gave final approval. DHKK: contributed to design, contributed to analysis, drafted manuscript, and critically revised the manuscript and gave final approval. LW: contributed to conception and design, contributed to acquisition, analysis, and interpretation, drafted manuscript, and critically revised manuscript. All authors gave their final approval and agreed to be accountable for all aspects of the work and reviewed the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to S.N.

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