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ORIGINAL ARTICLE

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Oral microbiota of patients with phenylketonuria: A nation-based cross-sectional study

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Abstract

Aim: The oral microenvironment contributes to microbial composition and immune equilibrium. It is considered to be influenced by dietary habits. Phenylketonuria (PKU) patients, who follow a lifelong low-protein diet, exhibit higher prevalence of oral diseases such as periodontitis, offering a suitable model to explore the interplay between diet, oral microbiota and oral health.

Materials and Methods: We conducted 16S rDNA sequencing on saliva and subgingival plaque from 109 PKU patients (ages 6–68 years) and 114 age-matched controls and correlated oral microbial composition and dental health.

Results: PKU patients exhibited worse dental health, reduced oral microbial diversity and a difference in the abundance of specific taxa, especially *Actinobacteriota* species, compared to controls. PKU patients with poor periodontal health exhibited higher alpha diversity than the orally healthy ones, marked by high abundance of the genus *Tannerella*. Notably, the observed taxonomic differences in PKU patients with normal

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indices of decayed/missing/filled teeth, plaque control record, gingival bleeding index and periodontal screening and recording index generally differed from microbial signatures of periodontitis.

Conclusions: PKU patients' reduced microbial diversity may be due to their diet's metabolic challenges disrupting microbial and immune balance, thus increasing oral inflammation. Higher alpha diversity in PKU patients with oral inflammation is likely related to expanded microbial niches.

KEYWORDS

16s rDNA sequencing, periodontitis, phenylketonuria, saliva, subgingival plaque

Clinical Relevance

Scientific rationale for study: Phenylketonuria (PKU) patients, who must follow a lifelong low-protein diet, show high prevalence of periodontal disease. To explore the interplay of diet, microbiota and oral health in PKU patients, we sequenced subgingival and salivary microbiota, correlating it with dental health.

Principal findings: The observed microbial patterns suggest that periodontitis-associated bacteria benefit from novel niches due to diet-induced microbial dysbiosis, triggering the host's immune response.

Practical implications: Findings of this study may aid clinicians in customizing oral care strategies, including microbial balance maintenance. Monitoring oral microbial diversity in PKU patients can signal niche expansion, prompting early intervention to prevent oral health issues.

1 | INTRODUCTION

The oral microbiome, the human body's second most diverse ecosystem after the gut, comprises numerous microorganisms co-evolved in homeostasis with the host's immune system (Dewhirst et al., 2010; The Human Microbiome Project Consortium, 2012). Imbalances within this complex ecosystem often precipitate oral diseases such as periodontitis, for which clinical attachment loss of >2–3 mm at two or more non-adjacent teeth is considered indicative (Papapanou et al., 2018). The 'polymicrobial synergy and dysbiosis model' relates oral microbial interactions with periodontal diseases (Hajishengallis & Lamont, 2012); yet, defining the precise causal relationships between pathogens and disease remains challenging. Diet significantly impacts oral health, evident in the well-established association between sugar consumption and caries development, and recent research suggests that a diet rich in saturated fatty acid promotes gingivitis (Woelber et al., 2019). Oral microbial communities organize into biofilms, which adhere to surfaces and embed in a self-produced matrix, resulting in plaque. Diet shapes biofilm composition and metabolism, influences pH levels and extracellular matrix dynamics and impacts oral health by favouring the growth of beneficial or pathogenic species (Marsh & Devine, 2011). For instance, in caries, acidogenic species in high-sugar diets, such as *Streptococcus mutans*, lower pH levels through fermentation, fostering an acidic environment favouring acid-tolerant and acidogenic bacteria, further stimulating the formation of an adhesive matrix and promoting a shift towards a pathogenic microbial profile of the oral biofilm (Liu et al., 2023).

In contrast, the health-beneficial properties of microbial communities that evolved with an ancestral diet can be attributed to the production of beneficial metabolites influencing pH, re-mineralizing enamel, regulating inflammation or contributing to maintain a protective biofilm (Franck et al., 2022).

At this time, comprehensive studies on the interaction between diet, microbiome and oral health are scarce, likely due to logistical challenges in long-term dietary interventions. Additional challenges arise from high inter- and intra-individual microbial variability, which requires larger sample sizes (Costello et al., 2009).

Groups of probands that must adhere to specific diets to avoid health consequences represent a promising study sample to investigate the influence of diet on oral microbiota and oral health. Patients with phenylketonuria (PKU; OMIM261600) lack a functioning phenylalanine (PHE) hydroxylase and require a lifelong diet low on proteins to avoid PHE, which otherwise would cause severe disease symptoms (van Spronsen et al., 2017). They rely on protein-free or low-protein foods in their diet, which increases their total fat or carbohydrate intake. In addition, their diet contains special products with a low protein content and nutritional supplements to prevent malnutrition, which often contain large quantities of sugar (Cleary et al., 1997). This could have effects on the oral microenvironment, triggering changes in microbial composition and activity, as suggested by Hajishengallis and Lamont (2012).

PKU patients exhibit deteriorated oral health, including periodontitis, caries and enamel hypoplasia (Abola et al., 2022; Bingol et al., 2023). However, although high sugar consumption is causal for

higher caries prevalence, the relationship between specific PKU diet and susceptibility to periodontitis is unclear. Although protein-calorie malnutrition impairs host immunity (Daly et al., 1990), PKU-related comorbidities primarily involve metabolic or cardiovascular diseases, suggesting other aetiological processes (Trefz et al., 2019). PKU patients exhibit significant changes in their gut microbiota, sometimes with reduced microbial diversity (Pinheiro de Oliveira et al., 2016; Timmer et al., 2021) and depletion of health-beneficial bacteria and metabolites (Bassanini et al., 2019).

We hypothesize that microbial communities in PKU patients differ from those in individuals with common dietary habits. The resulting quantitative and qualitative changes in microbial composition may trigger the immune system, creating a pro-inflammatory environment, with a positive feedback cycle amplifying the inflammatory response with additional changes to the microbiome composition (Sedghi et al., 2021).

The aim of this study was, therefore, to investigate the oral microbiological signatures in PKU patients and their correlations with oral disease. To this end, we performed 16S rDNA sequencing from saliva (S) and subgingival plaque (SP) samples from PKU patients and age-matched healthy controls, and integrated the microbial profiles with dental clinical data and participant questionnaires.

2 | MATERIALS AND METHODS

One-hundred and nine PKU patients, aged 6–68 years, and 114 metabolically healthy age-matched controls, 15 of whom were siblings of PKU patients from the cohort, were recruited and clinically examined as previously described (Bingol et al., 2023), according to the European guidelines on PKU (van Wegberg et al., 2017). This study followed the Declaration of Helsinki and STROBE protocol (Supplementary Methods) and was approved by the medical ethical committees of the Charité - Universitätsmedizin Berlin (EA2/036/18) and the University of Leipzig (369/18-Ik), registered in the German Clinical Trials Register and International Clinical Trials Registry Platform (DRKS00027482). For each participant, health status, dietary habits and oral hygiene were recorded and the questionnaire was completed. The following health parameters were collected: the decayed/missing/filled teeth (DMFT) index (Broadbent & Thomson, 2005), plaque control record (PCR) (O'Leary et al., 1972), gingival bleeding index (GBI) (Carter & Barnes, 1974), periodontal screening and recording index (PSI, also referred to as PSR) (Landry & Jean, 2002) and developmental enamel defects (DDE) index (Clarkson & O'Mullane, 1989). Stimulated S and SP samples were collected. A detailed description of sample collection, processing, 16S rDNA sequencing and data analyses is given in the Supplementary Methods.

3 | RESULTS

3.1 | PKU patients exhibit worse dental health

PKU patients exhibited worse dental health than controls, which was in part described for this cohort earlier (Bingol et al., 2023), with significantly higher PCR, GBI, PSI and DMFT values, the latter two applying only to the adult group (Table 1). Children with PKU had a significant higher prevalence of DDE than their age-matched controls and PKU adults. No difference in oral hygiene (e.g., frequency of dentist visits, oral care routine) was observed. Thirty percent of the adult PKU patients had vitamin D deficiency, but this did not correlate with worse dental health. Fifteen underaged healthy controls were siblings of 16 PKU patients (Table S1).

3.2 | Salivary microbiota of PKU patients is less diverse

We found a clear reduction of alpha diversity and divergent microbial structure in saliva of PKU patients (PKU-S) compared to controls (Ctrl-S, Figure 1a,d, Tables S2 and S3). Of the 2663 amplicon sequence variants (ASVs) detected, 394 were exclusive for PKU patients, whereas 967 out of 3236 were found only in controls (Figure S1).

We observed an age effect, with less diverse and distinct microbiome in children compared to adults (Figure 1b, Figures S3 and S4). The microbial composition of healthy siblings of PKU patients was similar to that observed in PKU children, with significantly decreased alpha diversity and a different microbial structure compared to control children unrelated to PKU patients (Figure 1c, Figures S5 and S6).

Abundances of the most common phyla Proteobacteria (25.8%), Bacteroidota (25.5%) and Firmicutes (24.9%) were comparable between PKU-S and Ctrl-S (Figure 2a). The most significant differences in PKU-S corresponded to a depletion of the phylum Actinobacteriota, while the phyla Campylobacterota, Patescibacteria and Spirochaetota were significantly enriched (Table S4).

At the genus level, the most abundant genera in Ctrl-S were *Neisseria* (12.5%) and *Prevotella* (10.7%), which were also abundant in PKU-S. In PKU-S, *Haemophilus* had the highest relative abundance (13.7%) and was significantly enriched compared to Ctrl-S (Figure 2b,c, Figure S2, Table S5). The most significant difference in abundance in PKU-S corresponded to a depletion of *Actinomyces*. In general, more species were depleted in PKU-S than enriched.

In Ctrl-S, the strongest correlations of bacterial abundances related to positive correlations of *Treponema* × *Tannerella*, *Prevotella* × *Veillonella* and *Corynebacterium* × *Cardiobacterium* (Figure 3a, Table S6). In PKU-S, these were *Corynebacterium* × *Cardiobacterium*, *Corynebacterium* × *Kingella* and *Prevotella* × *Campylobacter* (Figure 3b, Table S7).

3.3 | PKU-associated loss of oral microbial diversity in SP

The subgingival plaque microbiota of PKU patients (PKU-SP) was less diverse and differed significantly from controls (Ctrl-SP), but less

TABLE 1 Cohort characteristics.

	Controls ($n = 114$)		PKU ($n = 109$)		*p*-Value PKU versus controls		*p*-Value adults versus children	
	Adults (≥18 years, $n = 52$)	Children (<18 years, $n = 62$)	Adults (≥18 years, $n = 50$)	Children (<18 years, $n = 59$)	Adults	Children	Controls	PKU
No. of saliva samples	52	62	50	59				
No. of plaque samples	52	61	45	51				
General information								
Males, *n* (%)[a]	20 (38.5)	30 (48.4)	13 (26.0)	33 (55.9)	n.s.	n.s.	n.s.	1.6×10^{-3}
Mean age (SD)[b]	29.8 (7.7)	11.0 (3.1)	31.4 (10.6)	11.4 (3.4)	n.s.	n.s.	NA	NA
Smokers, *n* (%)[a]	14 (26.9)	1 (1.6)	6 (12.0)	2 (3.4)	n.s.	n.s.	1.0×10^{-4}	n.s.
General/PKU health parameter								
Body mass index (BMI), mean (SD)[b]	23.7 (3.2)	18.2 (3.1)	26.0 (5.1)	18.9 (3.8)	.011	n.s.	NA	NA
Vitamin D deficiency, *n* (%)[a]	4 (7.7)	1 (1.6)	15 (30.0)	2 (3.4)	4.8×10^{-3}	n.s.	n.s.	1.0×10^{-4}
Sweet food intake (≥2 times per day), *n* (%)[a]	9 (17.3)	15 (24.2)	12 (24.0)	15 (25.4)	n.s.	n.s.	n.s.	n.s.
Current phenylalanine tolerance (mg), mean (SD)[b]	NA	NA	852.6 (481.2)	562.4 (366.0)	NA	NA	NA	$<10^{-5}$
Daily amount of PHE-free amino acid mixture (g), mean (SD)[b]	NA	NA	59.5 (26.0)	55.1 (24.8)	NA	NA	NA	n.s.
Dental health parameter								
Plaque control record (PCR), mean (SD)[b]	0.17 (0.07)	0.16 (0.08)	0.30 (0.18)	0.21 (0.10)	$<10^{-5}$	1.4×10^{-3}	n.s.	1.6×10^{-3}
PCR ≥ 20%, *n* (%)[a]	14 (26.9)	12 (19.4)	35 (70.0)	27 (45.8)	10^{-5}	1.9×10^{-3}	n.s.	0.011
Gingival Bleeding Index (GBI), mean (SD)[b]	0.07 (0.11)	0.04 (0.04)	0.14 (0.14)	0.05 (0.04)	8.2×10^{-4}	6.6×10^{-4}	1.9×10^{-4}	8.0×10^{-5}
GBI ≥ 15%, *n* (%)[a]	4 (7.7)	1 (1.6)	19 (38.0)	2 (3.4)	3.0×10^{-4}	n.s.	n.s.	$<10^{-5}$
Number of sextants with periodontal screening index (PSI) = 3 or 4, mean (SD)[b]	0.37 (1.10)	0.06 (0.51)	2.00 (2.12)	0.14 (0.66)	$<10^{-5}$	n.s.	n.s.	$<10^{-5}$
Periodontitis (≥2 sextants with PSI ≥3), *n* (%)[a]	4 (7.7)	1 (1.6)	24 (48.0)	2 (3.4)	$<10^{-5}$	n.s.	n.s.	$<10^{-5}$
Decayed/missing/filled teeth (DMFT), mean (SD)[b]	4.8 (4.3)	1.5 (2.3)	10.0 (7.2)	1.9 (2.3)	8.0×10^{-5}	n.s.	1.0×10^{-4}	$<10^{-5}$
Developmental enamel defects (DDE), *n* (%)[a]	8 (15)	15 (24)	9 (17)	40 (68)	n.s.	$<10^{-5}$	n.s.	$<10^{-5}$

[a]Fisher exact-or Chi-square test, where applicable.

[b]Mann–Whitney *U* test. Adults ≥18 years, children <18 years. SD, standard deviation; n.s, not significant; NA, not applicable. Distribution of PSI and DMFT values had been analysed in a pooled case–control setting in this cohort before (Bingol et al., 2023).

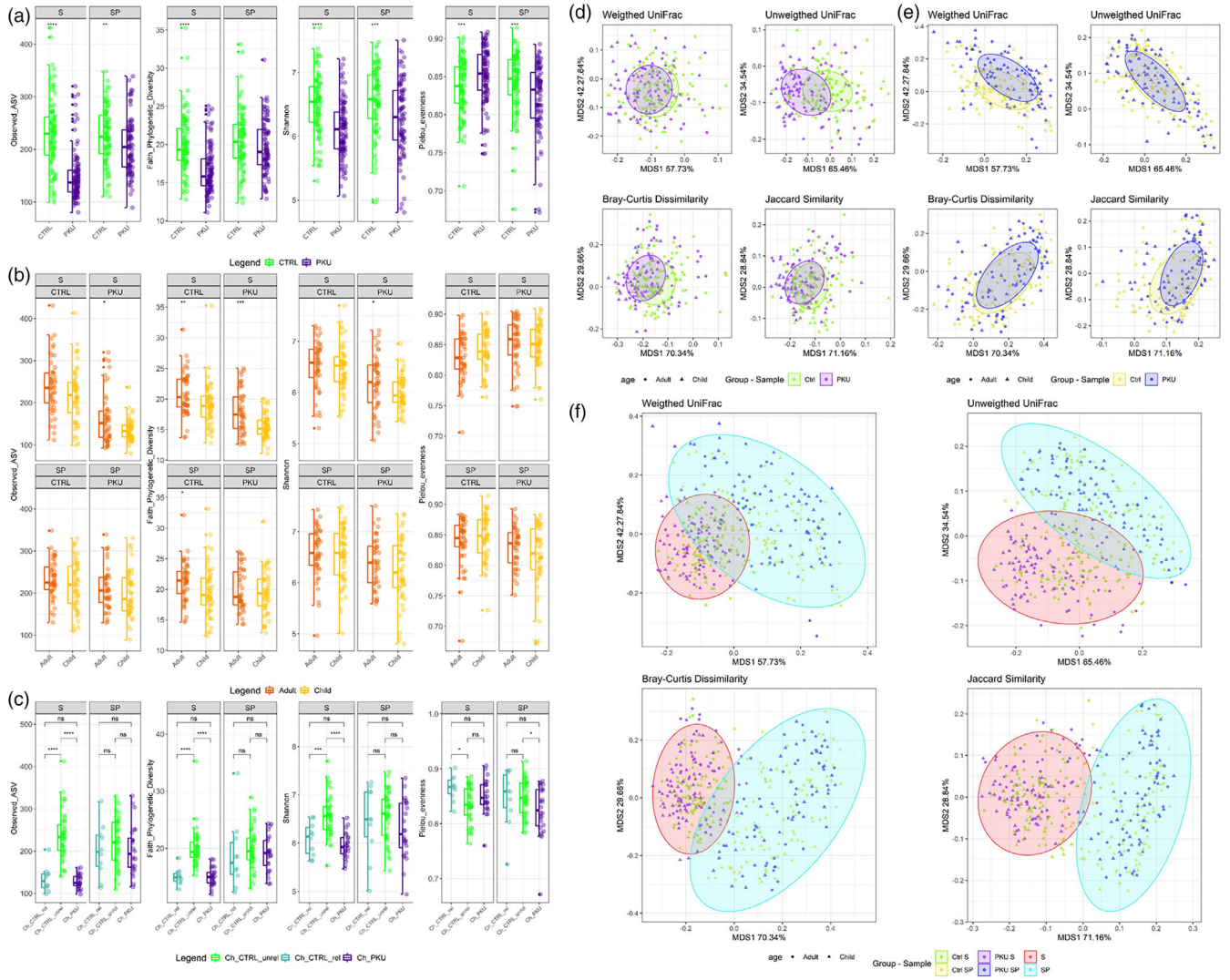

FIGURE 1 Microbial diversity in saliva and subgingival plaque of phenylketonuria (PKU) patients and healthy controls. (a) Alpha diversity in PKU patients compared to controls. (b) Alpha diversity in PKU patients compared to controls stratified by age. (c) Underage healthy siblings of PKU patients compared to PKU children and unrelated control children. (d) Beta diversity in saliva. (e) Beta diversity in subgingival plaque. (f) Beta diversity in saliva and subgingival plaque. Ad, adults; Ch, children; Ctrl, healthy controls; Unrel, unrelated to PKU patients; Rel, related to PKU patients. ****p <.0001; ***p <.001; **p <.01; *p <.1.

pronounced than in saliva (Figure 1a,e, Tables S2 and S3). PKU-SP exhibited 3047 ASVs compared to 3380 in Ctrl-SP, with 366 exclusively detected in PKU patients and 699 in controls (Figure S1). We observed only a weak age effect (Figure 1b, Figures S3 and S4). SP microbiota from healthy siblings of PKU patients did not differ from that in Ctrl-SP (Figure 1c, Figures S5 and S6).

The most relatively abundant phylum in PKU-SP was Actinobacteriota (21.9%, Figure 2a), whereas the most abundant phyla were Proteobacteria (20.1%) and Bacteroidota (19.8%) in Ctrl-SP. Actinobacteriota were significantly over-represented in PKU-SP, while Bacteroidota and Fucobacteriota were significantly depleted (Table S4).

At the genus level, *Fusobacterium* was the most abundant species in Ctrl-SP (9.9%), similarly abundant in PKU-SP (Figure 2b). PKU-SP exhibited the highest abundance for *Actinomyces* (11.4%), significantly enriched compared to Ctrl-SP (Figure 2c, Figure S2, Table S5). The most significantly depleted genus in PKU-SP was *Capnocytophaga*. Taxonomic differences between PKU-SP and Ctrl-SP were less pronounced than in saliva, with significant differences in abundance only in 7 genera, compared to 16.

The strongest correlations between genera in Ctrl-SP were positive correlations of *Streptococcus* × *Haemophilus* and *Neisseria* × *Bergeyella*, and a negative correlation of *Selenomonas* × *Streptococcus* (Figure 3c, Table S8).

In PKU-SP, these were positive correlations of *Streptococcus* × *Veillonella* and *Prevotella* × *Fusobacterium*, and a negative correlation of *Selenomonas* × *Streptococcus* (Figure 3d, Table S9).

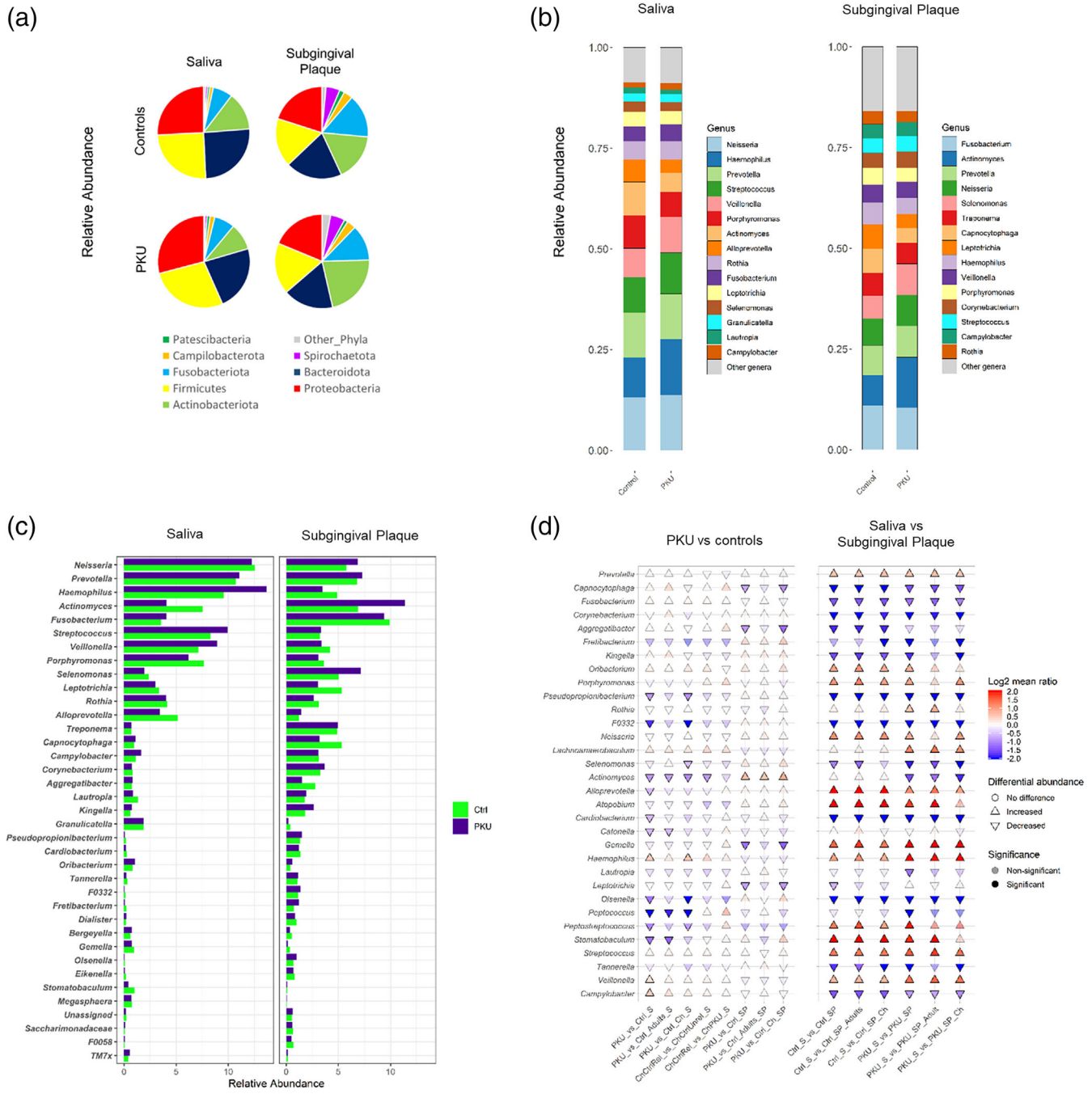

FIGURE 2 Relative taxon abundances in saliva and subgingival plaque of phenylketonuria (PKU) patients and controls. (a) Relative phyla abundances. (b) Relative abundance proportions of the most abundant genera. (c) Relative genus abundances. (d) Differences in most abundant genera by PKU status and sample type. Ch, children; Ctrl, controls; Rel, related to PKU patient; S, saliva; SP, subgingival plaque; Unrel, unrelated to PKU patient. The significance threshold was defined as $\alpha = .05$.

3.4 | Sample-type-specific oral microbial signatures differ between PKU patients and controls

In PKU patients, but not in controls, a significantly higher mean alpha diversity was observed in subgingival plaque compared to saliva (Figure 1a, Table S2). Microbial composition was significantly less variable in saliva than subgingival plaque and differed significantly between sample types (Figure 1f, Table S3). The strongest shift in phylae corresponded to an enrichment of Fusobacteriota in Ctrl-SP (Figure 2a), while in PKU-SP, Actinobacteriota were significantly enriched. In Ctrl-S, three most significantly enriched genera were *Alloprevotella*, *Streptococcus* and *Stomatobaculum*, while in Ctrl-SP, the three most significantly enriched genera were *Fusobacterium*, *Capnocytophaga* and *Campylobacter* (Figure 2c, Table S10).

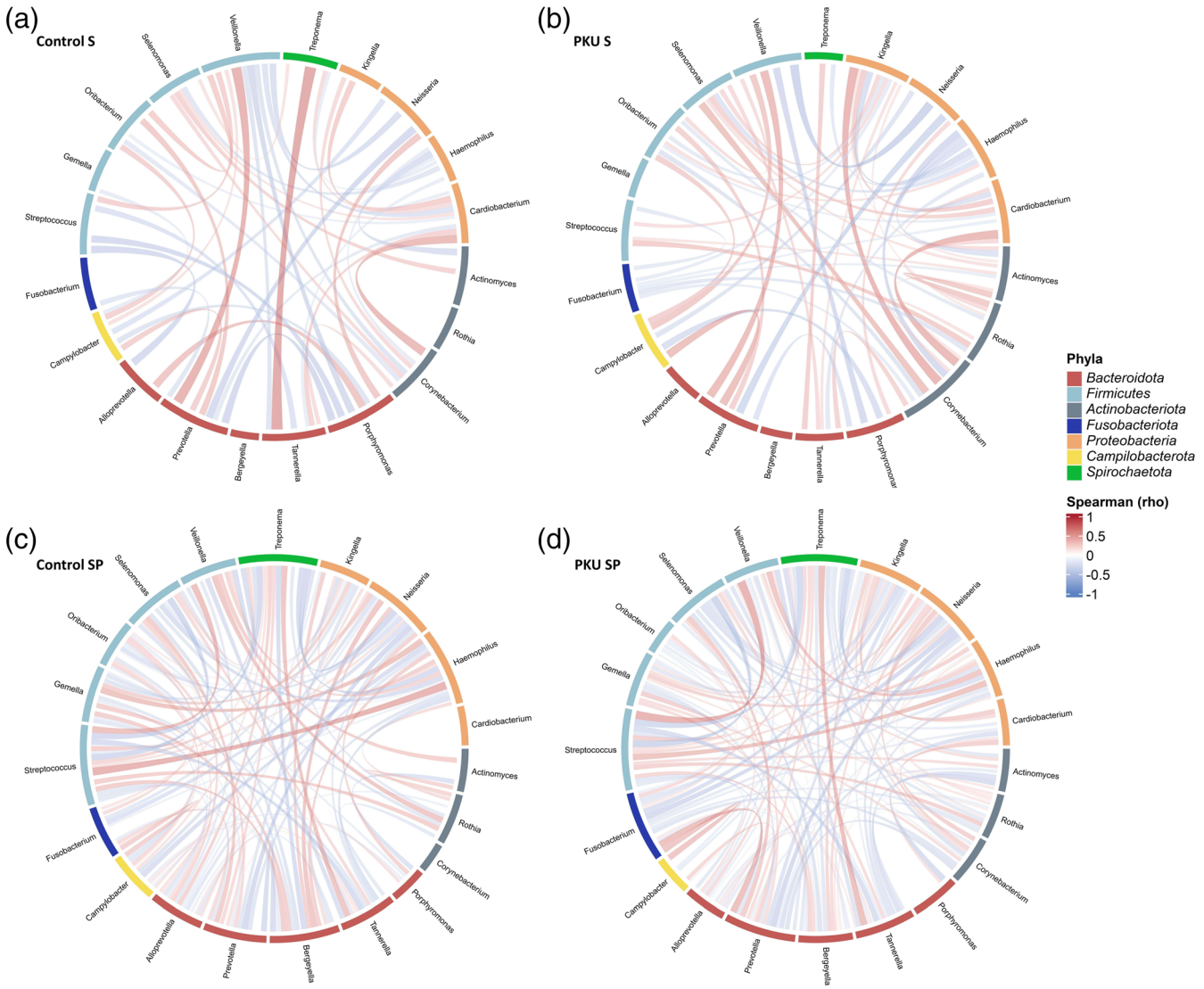

FIGURE 3 Correlation network of taxon abundances. Spearman correlations are shown for (a) saliva in controls, (b) saliva of phenylketonuria (PKU) patients, (c) subgingival plaque of controls and (d) subgingival plaque of PKU patients.

3.5 | Worse dental health in PKU patients is associated with distinct microbial patterns in S, but not SP

In an analysis stratified for PKU affection status, we evaluated the association of different health parameters with oral microbiota.

In PKU-S, a higher frequency of sweet food intake (≥2 times per day) correlated weakly with a decrease in alpha diversity (Figure 4a, Table S11). A sub-analysis revealed that this effect was primarily driven by the children (Figure S7). In Ctrl-S, BMI correlated positively with alpha diversity and the abundance of *Olsenella* and *Atopobium* (Figure 4b,d, Tables S12 and S14) and negatively with *Neisseria* abundance. In PKU-S, BMI correlated positively with *Olsenella* and *Tannerella* abundances, but not with microbial diversity.

Notably, in PKU-S, parameters of worse oral health (PCR, GBI, PSI and DMFT) and suspected periodontitis (indicated by the presence of more than two sextants with a PSI score ≥3) correlated positively with alpha diversity, compared to Ctrl-S (Figure 4a,b, Tables S11 and S12). Specifically, PKU-S of individuals with suspected periodontitis showed a strong decrease in alpha diversity compared to controls, while in PKU patients without suspected PD, reduction of microbial diversity was only marginal (Figure S8).

At the taxonomic level, especially *Tannerella* and *Olsenella* were enriched and *Haemophilus* was depleted in PKU-S with poor dental health. Specifically, *Tannerella* abundance correlated positively with GBI, PSI, PCR and DMFT and *Olsenella* abundance with PSI and DMFT. For GBI, enrichment of *Streptococcus* was also observed, and GBI and DMFT correlated with a depletion of *Haemophilus* (Figure 4c,d, Tables S13 and S14). In Ctrl-S, the association between dental health parameter and microbial diversity was minor, with only positive correlation of *Prevotella* and *Atobobium* and negative correlations of *Neisseria* with DFMT. No microbial correlations were observed for DDE or vitamin D deficiency.

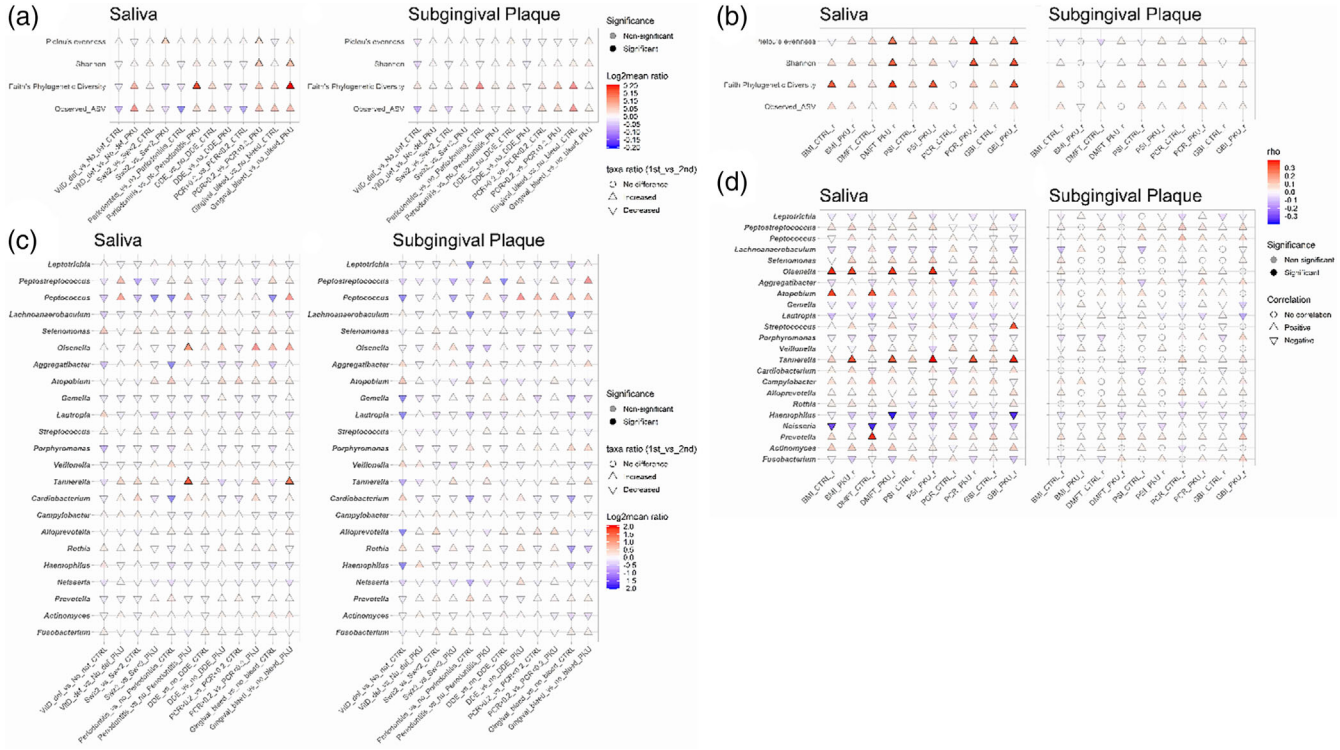

FIGURE 4 Oral microbial changes with clinical parameters in phenylketonuria (PKU) patients and controls. (a) Correlation of alpha diversity with categorical clinical parameters. (b) Correlation of alpha diversity with ordinal clinical parameters. (c) Correlation of relative genus abundances with categorical clinical parameters. (d) Correlation of relative genus abundances with ordinal clinical parameters. Wilcoxon signed-rank test was performed to obtain log2mean ratios for categorical clinical traits. For correlation with ordinal traits, Spearman's rank correlation test was performed. The significance threshold was defined as $\alpha = .05$. BMI, body mass index; Ctrl, controls; DDE, presence of developmental enamel defects; DMFT, decayed, missing or filled teeth; GBI, gingival bleeding index; Gingiva_bleed, gingival bleeding index ≥15%; No_def, normal vitamin D blood levels; no_Periodontitis, no indication for periodontitis based on PSI records; PCR, plaque control record; PCR, plaque control record; Periodontitis, diagnosed with suspected periodontitis based on PSI records; PSI, periodontal screening index; Sw < 2, consumption of sweet food <2 times/day; Sw ≥ 2, consumption of sweet food ≥2 times/day; VitD_def, diagnosed with vitamin D deficiency.

In subgingival plaque, none of the clinical parameters was significantly associated with differences in microbial abundance (Tables S15–S18).

4 | DISCUSSION

In the current study, we analysed the oral microbiota of PKU patients on a strict low-protein diet and compared it with that of healthy controls. Additionally, we correlated dental health parameters with microbial compositions. This is the first study that combines oral microbiota and dental health data in PKU patients.

We observed a reduction of alpha diversity in PKU patients compared to controls, with a stronger effect in saliva than in subgingival plaque. PKU patients rely on food low in protein and rich in carbohydrates and fat, which renders their diet less varied. This likely contributes to a reduction of bacterial species that evolved with traditional diets and depend on the omitted nutrient sources. In contrast, bacteria that primarily ferment carbohydrates and fat can thrive. It is unlikely that new species would profit from reduced nutrient availability, explaining the loss of bacterial diversity. Furthermore, interactions between members of microbial communities occur sequentially, as species depend on the mutual supply of nutrients in a cascade-like manner. Thus, depletion of nutrient availability also affects compound synthesis in bacterial communities, reducing the availability of essential by-products such as glucose and short-chain fatty acids. This, consequently, deteriorates growth conditions for other bacteria, further impacting the broader microbial community and reducing its diversity (Zengler & Zaramela, 2018). Findings on the relationship between diet and salivary microbiome are conflicting; however, a correlation between a greater variety in diet with increased microbial diversity has been suggested (De Filippis et al., 2014; Li et al., 2014; Nasidze et al., 2011). Interestingly, saliva of healthy PKU siblings showed similarly reduced alpha diversity, which is likely due to dietary patterns shared within households where family members might adapt to specific dietary needs. Notably, these siblings also showed a comparable increase in DMFT (Bingol et al., 2023), suggesting a combined influence of shared dietary habits on the oral microbiota and dental health.

In contrast to the reduced bacterial diversity in orally healthy PKU patients and their siblings, we found that the diversity, including the abundance of taxa that colonize periodontal pockets, was enriched in PKU patients with higher indices for DMFT, PCR, GBI and

PSI. This may be explained by the presence of additional ecological niches resulting from gingival pocket formation and gingival bleeding, favouring new species.

In general, the presence of abundant species and age effects in saliva mirrored observations from the literature (Handsley-Davis et al., 2021; Lif Holgerson et al., 2020; Nasidze et al., 2009; Willis et al., 2018).

However, in PKU-S, aerobic and facultatively anaerobic *Haemophilus* was significantly enriched, rendering it the most abundant genus. *Haemophilus* is a commensal with varying abundances in saliva, with *H. parainfluenzae* being the most prevalent species (Eren et al., 2014; Mark Welch et al., 2016; Segata et al., 2012). *Haemophilus* abundances vary with subsistence strategies, but a clear and consistent pattern with diet has not yet emerged (Clemente et al., 2015; Lassalle et al., 2018). Of note, oral *Haemophilus* species possess highly effective ureolytic capabilities, breaking down urea into ammonium and CO_2 (Burne & Marquis, 2000). Although speculative, this may, in a protein-deprived diet with limited access to nitrogen-containing amino acids, confer growth advantages over other species by efficiently utilizing urea as an alternative nitrogen source.

In PKU-SP, taxonomic differences were less pronounced. The enrichment of Actinobacteriota, mainly *Actinomyces*, was contrary to its depletion in PKU-S. *Actinomyces* is part of the normal oral microbiome (Sutter, 1984). *Actinomyces*, a major early colonizer of dental biofilms following aerobic Streptococcus, is associated with dental plaque. It is known to thrive in dental pockets and dominant in subgingival plaque and over-represented in periodontitis (Slots, 1979; Ximenez-Fyvie et al., 2000). However, in our study, no association with PSI was observed. *Actinomyces* is influenced by various factors, including substances from other bacteria, fluoride use and eating habits (Dworkin et al., 2006). For example, sugar intake correlates with higher abundances in plaque, while a health-optimized diet is found to reduce abundances in plaque and, notably, saliva (Pfister et al., 1984; Tennert et al., 2020). Hence, the variations in our study, not perfectly aligning with the literature, underscore the intricate influence of parameters such as nutrient availability, biofilm complexity, host immunity, sample size, species heterogeneity and individual variability on microbial communities and the need for more in-depth studies elucidating these factors.

Comparing saliva and subgingival plaque of the sampling sites, PKU-SP showed significantly higher microbial diversity than PKU-S, whereas no such difference was observed in controls. Increased alpha diversity in subgingival plaque compared to saliva was reported earlier in healthy subjects (Caselli et al., 2020), which was likely attributed to the formation of anaerobic niches. The discrepancy between PKU patients and controls could be explained by the greater diet-induced reduction in diversity in PKU-S compared to subgingival plaque, such that diversity remained relatively high in PKU-SP compared to PKU-S.

Higher alpha diversity correlated with worse dental health in PKU-S, matching with observations in a large cohort representative of the population (Takeshita et al., 2016). Furthermore, in PKU-S, dental health indices were correlated with abundance of *Tannerella*, an anaerobic genus best known for its species *T. forsythia*, which is described to be associated with chronic periodontitis (Griffen et al., 2012; Socransky et al., 1998). In PKU-S, suspected periodontitis, PSI and DMFT, and in Ctrl-S and PKU-S, BMI, correlated with anaerobic *Olsenella*, which is enriched in the saliva of periodontitis patients (Toyama et al., 2021).

Furthermore, in PKU-S, a negative correlation of DMFT and GBI with *Haemophilus* was observed. This finding is intriguing, as its ureolytic activity has a significant impact on maintaining pH balance within oral biofilms by alkali generation, counteracting acidification and allowing for remineralization and restoration of enamel integrity (Burne & Marquis, 2000). It is considered to be a major contributor to urease activity in the plaque, which is significantly lower in caries (Morou-Bermudez et al., 2015; Nascimento et al., 2009; Shu et al., 2007). Thus, in PKU-S, the abundance of *Haemophilus* may prevent certain individuals from developing caries and help maintain oral health, resulting in low DMFT.

Notably, elevated ammonia levels, which may result from ureolytic activity, can promote calculus formation and periodontitis (D'Souza L et al., 2023).

Generally, we found the subgingival microbiota to be more stable against factors such as diet, age or clinical parameters, likely because the main nutritional source for subgingival plaque bacteria is gingival crevicular fluid, in contrast to salivary microorganisms that largely depend on the host's nutrient intake. Likewise, the stability of subgingival plaque was identified before in healthy individuals (Tamashiro et al., 2021).

A limitation of this study was that, because of the good dental, especially periodontal, health of the age-matched controls (mean age 30 years), statistical analyses were constrained, which is why association of clinical parameters mainly related to PKU-S. The only index with significant taxa correlation in controls was DMFT, addressing caries prevalence. Notably, associations in Ctrl-S with DMFT differed from those observed in PKU-S. The marked difference of DMFT-associated taxa between controls and PKU patients underlines the significant manifestation of oral dysbiosis linked to dietary constraints.

Further limitations of this study were the restriction of analyses to the genus level by 16S rDNA sequencing data instead of metagenomics sequencing, thereby hampering direct functional interpretation of bacterial shifts at the species level, and the use of PSI as a proxy for suspected periodontitis, which impedes comparability between different studies.

In summary, our data indicate the presence of a distinct oral microbiota in PKU patients and their healthy siblings, characterized by substantially reduced bacterial diversity and taxonomic differences. However, the oral microbiome diversity of PKU patients with periodontitis showed a high diversity as found in non-PKU periodontitis patients, including bacteria that generally colonize periodontal pockets.

Though speculative, the loss of bacterial species unadapted to a specific diet may induce shifts in the functional dynamics of microbial communities, potentially including an elevation of virulence factor or inflammatory mediator release. Consequently, the oral mucosal barrier

may be compromised, facilitating increased penetration of bacteria and their by-products, triggering the host's immune response. Synergistic relationships or competitive dynamics among bacteria may exacerbate this inflammatory response, perpetuating a self-reinforcing cycle. Further studies are warranted to elucidate the precise functional dynamics at the transition of diet-induced oral dysbiosis to oral inflammation.

This study demonstrates that long-lasting changes of the diet, which substantially differ from the composition of the natural human diet, correlate with reduced bacterial diversity. Over time, these dietary changes correlate with worsened periodontal health. However, worsened periodontal health is paralleled by increased bacterial diversity, which did not differ from the bacterial groups commonly found in periodontal pockets. The results suggest more complex effects that go beyond dysregulated growth of specific bacterial strains. They likely also apply in different contexts, where specific dietary habits deviate from a natural diet and affect oral health, irrespective of the genetic predisposition. The outcomes of this study have the potential to guide efforts towards more comprehensive approaches for prevention and treatment. These may include exploring holistic strategies like probiotic supplementation, microbiome restoration therapies or personalized dietary interventions.

AUTHOR CONTRIBUTIONS

Memduh Bingöl conceived and designed the study, coordinated and performed the patient recruitment, performed sample collection, gathered participant data, performed dental examinations, contributed to data analysis and interpretation and drafted and critically revised the manuscript. Alessio Cardilli performed bioinformatic and statistical analyses and contributed to data analysis and interpretation and drafting and critically revising the manuscript. Anne Carolin Bingöl coordinated and performed the patient recruitment, contributed to sample collection, gathering of participant data and dental examinations and critically revised the manuscript. Ulrike Löber performed bioinformatic and statistical analyses and critically revised the manuscript. Corinna Bang and Andre Franke were responsible for sample preparation and sequencing and critically revised the manuscript. Theodosia Barzela and Skadi Beblo coordinated and performed the patient recruitment and critically revised the manuscript. Eberhard Mönch and Simone Stolz conceived and designed the study, coordinated and performed the patient recruitment and critically revised the manuscript. Arne S. Schaefer designed the study, contributed to data analysis and interpretation and drafted and critically revised the manuscript. Sofia Kirke Forslund designed the study, performed bioinformatic and statistical analyses, contributed to data analysis and interpretation and drafted and critically revised the manuscript. Gesa M. Richter designed the study, contributed to data analysis and interpretation and drafted and critically revised the manuscript. All authors gave their final approval and agreed to be accountable for all aspects of the work.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The present study was approved by the medical ethical committees of the Charité - Universitätsmedizin Berlin (EA2/036/18) and the University of Leipzig (369/18-Ik), registered in the German Clinical Trials Register and International Clinical Trials Registry Platform (DRKS0 0027482).

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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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