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Strain-level profiling reveals the impact of prolonged areca nut chewing on oral microbiome

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ABSTRACT: The areca nut is a widely consumed psychoactive substance linked to an elevated risk of oral diseases, including oral cancer. Although it is well-established that areca nut chewing disrupts oral microbiota composition, its impact on microbial functions, specifically the underlying strain-level diversity remains elusive, calling for advanced exploration to uncover hidden dynamics. In this study, we constructed a well-defined cohort of 80 long-lived individuals, comprising habitual areca nut chewers and age-matched non-chewers, to investigate the long-term effects of areca nut exposure on the oral microbiome. Species and function-level profiling revealed significant alterations in microbial community structure, including marked shifts in *Streptococcus* associated with disruption of the thiamine diphosphate salvage pathway. Strain-resolved analysis further identified extensive single nucleotide variations in *Neisseria* genes *btuD* and *bacA*, which are essential for cobalamin transmembrane transport. Notably, similar strain-level alterations were observed in oral cancer patients, suggesting that areca nut chewing may disturb oral vitamin homeostasis and contribute to disease development. Moreover, the combined exposure to areca nut chewing and smoking was associated with more pronounced functional shifts in the oral microbiome, particularly in metabolic pathways related to sulfur and nitrogen cycling. Together, these findings demonstrate that chronic areca nut use induces profound remodeling of the oral microbiome at both functional and genomic levels, providing novel insights into microbial mechanisms potentially linking lifestyle exposures to oral disease, cancer, and broader impacts on human health.

Keywords: Areca nut, Metagenomic, Thiamine, Cobalamin, Strain diversity

1. Introduction

The areca nut, derived from the *Areca catechu L. palm*, is a small, fibrous fruit endosperm known for its psychoactive properties. Historically, areca nut has been used in traditional herbal medicine and as a snack for social activities [1]. The Indian traditional medicine system uses the areca nut as a popular digestive, astringent and emmenagogue agent [2]. Since 1953, the areca nut has been included in the Pharmacopoeia of

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the People's Republic of China, and over 100 prescriptions containing areca nut have been utilized to treat parasitic diseases, dyspepsia, and abdominal distension [3]. In addition, the areca nut is also consumed regularly in certain populations for relaxation, enhanced concentration, and a sense of euphoria [4].

However, habitual areca nut chewing poses significant health risks. Approximately 700 million people worldwide are regularly chewing areca nut, with adult usage rates ranging from 2.3% in China to 47.8% in Indonesia. This makes areca nut the fourth most commonly used addictive substance after tobacco, alcohol, and caffeine [5,6]. Evidence suggests that areca nut can adversely affect nearly all organs in the human body, contributing to a range of diseases such as myocardial infarction, cardiac arrhythmias, and hepatotoxicity [7]. Notably, its impact on oral health is particularly severe, often resulting in cancerous lesions within the oral cavity [8]. The International Agency for Research on Cancer has identified areca nut as a Group I carcinogen [9]. Epidemiological studies have established a strong link between areca nut abuse and potentially malignant oral conditions, with nearly half of all reported oral cancers being associated with its abuse [10]. Moreover, the areca nut is often chewed with substances like tobacco and slaked lime, which further heightens the health risks [11].

Areca nut chewing exerts such a pronounced carcinogenic effect on the oral cavity, driven not only by mechanical damage from crude fibers [12] but also by its chemical constituents, including arecoline, alkaloids, and polyphenols [13]. Experimental studies have demonstrated that these extracts possess potent genotoxic and cytotoxic properties, impairing cellular DNA repair mechanisms, inducing oxidative stress and inflammation, and stimulating autophagy, which enhances cancer cell survival and resistance to apoptosis, thereby promoting the progression of oral submucosal fibrosis and squamous cell carcinoma [14,15]. Interestingly, areca nut use has also been found to alter the composition of the oral microbiota, disrupting the balance and stability of the microbial community [7]. The human oral cavity harbors over 700 bacterial species, forming complex and distinct communities on the teeth, tongue, hard palate, and epithelial tissues, with saliva representing their composite mixture [16]. Although the oral microbiome remains remarkably stable within an individual over time, factors such as dietary habits, tobacco use, and oral hygiene practices significantly influence its composition and metabolic functions [17]. Given the critical roles of the oral microbiota in defending against pathogens, regulating inflammatory responses, and neutralizing reactive nitrogen species [18], these findings highlight the potential importance of exploring the mechanisms by which areca nut consumption alters microbial communities and contributes to health risks.

Several descriptive studies employing 16S rRNA sequencing have identified substantial alterations in the oral microbiota associated with areca nut chewing, characterized by significant shifts in microbial diversity and disruptions in commensal bacteria essential for maintaining oral homeostasis [19–21]. In long-term areca chewers, significantly lower alpha diversity has been observed, with an even more pronounced reduction in chewers with oral lesions compared to both chewers and non-chewers without lesions [19]. Notably, while the overall abundance of ***Streptococcus*** typically decreases in areca nut chewers, levels of ***Streptococcus infantis*** are up to four times higher in current chewers compared to non-chewers [20]. These findings underscore the

complex and selective impact of areca nut chewing on oral microbiota populations. Additionally, the oral dysbiosis observed in areca nut chewers is associated with an increased relative abundance of potentially pathogenic bacteria, such as *Porphyromonas gingivalis*, *Treponema denticola*, and *Fusobacterium nucleatum* [21]. These pathogens are known to promote inflammation, tissue destruction, and the colonization of other opportunistic pathogens, collectively contributing to the progression of oral diseases, including periodontitis and oral cancer.

However, in health conditions linked to microbial communities, specific physiological phenotypes are often associated with distinct bacterial strains that perform unique functional roles [22]. Investigating the functional dynamics of these communities is crucial for uncovering how areca nut chewing-induced microbiota alterations contribute to disease progression. Here, we performed a metagenomic analysis of saliva samples from 80 elderly people (> 80 years old) in Hainan Province, China, as a valuable model for the prolonged effects of areca nut chewing (median duration of 50 years). Subsequent species and functional profiling revealed significant effects of areca nut chewing on the composition of *Streptococcus*, which are involved in thiamine diphosphate (vitamin B1) salvage. Strain-level analysis revealed significant SNV alterations in *Neisseria* genes *btuD* and *bacA*, which are involved in cobalamin (vitamin B12) transport [23], with similar SNV patterns also detected in oral cancer patients [24,25]. Notably, co-exposure to areca nut and smoking was associated with more pronounced alterations in microbial functions, particularly affecting metabolic pathways involved in sulfur and nitrogen cycling [26]. Collectively, our findings reveal how areca nut chewing reshapes the oral microbiome at both compositional and functional levels, and may provide a mechanistic basis for future microbiome-informed strategies aimed at restoring microbial equilibrium and mitigating oral health risks.

2. Materials and Methods

2.1 Study design and sample collection

This study was approved by the Ethical Committee of Hainan Provincial People's Hospital Medical Ethics Committee (approval number: 2017-004), and all participants provided written informed consent. We included 80 individuals from a longevous population recruited from seven sites in Wanning, located on the southeast coast of Hainan Province, between September 2021 and January 2022 (Figure 1A). The inclusion criteria were an age > 80 years and no prior treatment or current diagnosis of cancer in the head and neck area. The participants' covariate information was obtained from questionnaires administered upon induction into the study. The demographic characteristics including age, gender, ethnicity, height, and weight were recorded. Body mass index was calculated from participants' height and weight. The information about areca nut chewing, smoking, drinking was provided by the participants, and all these covariates were classified as current and non-current groups. Overall, 21 current areca nut chewers and 59 non-current areca nut chewers were included in the study as the control group.

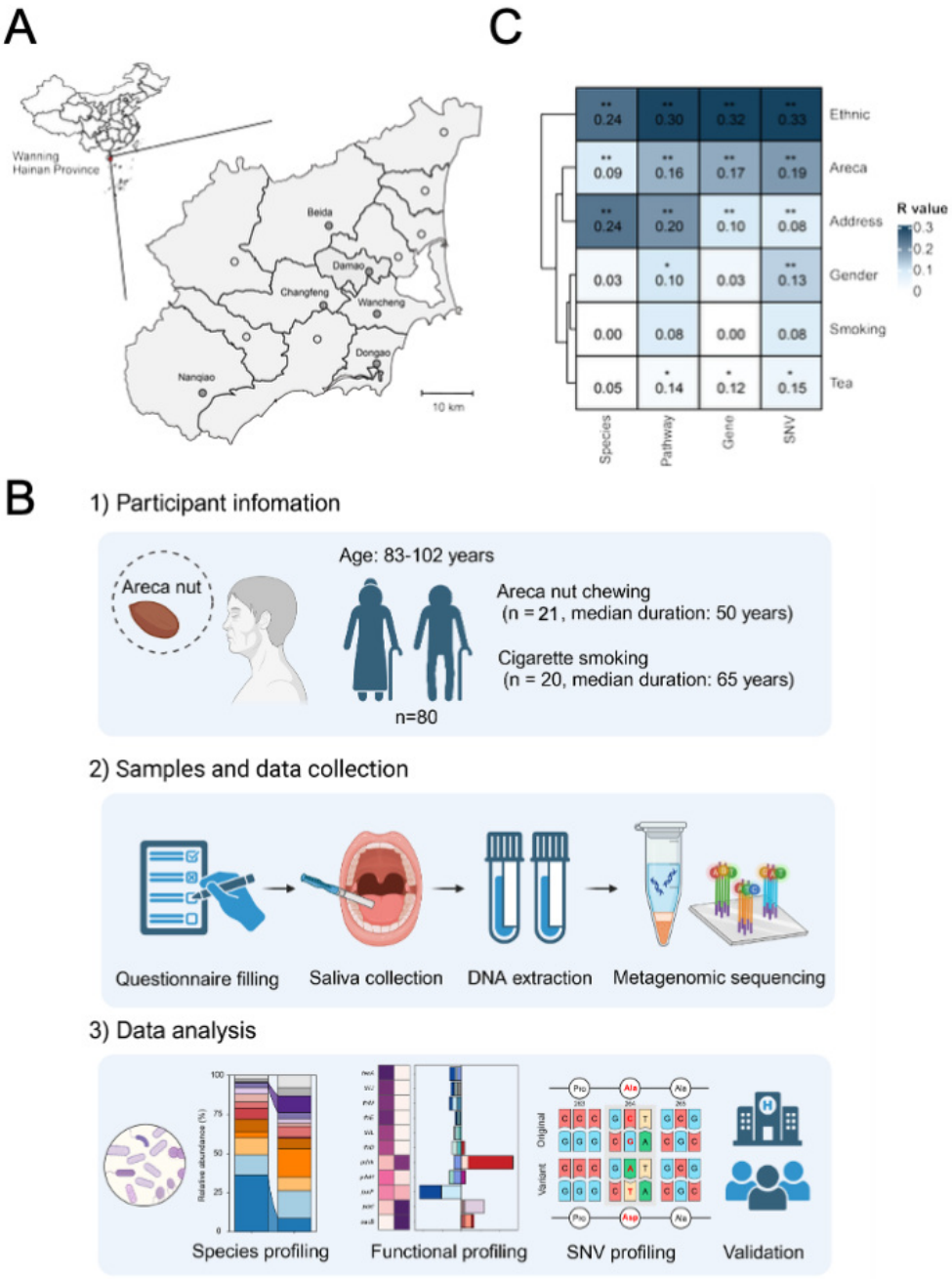

Figure 1 Experimental design and integrated analysis of areca nut chewing-associated oral microbiome. (A) Map showing the locations of six sampling sites in Wanning, Hainan Province, China. **(B)** Workflow of oral microbiome study. **(C)** The PERMANOVA test identified the significance of different confounders. Asterisks: statistical significance (*$P < 0.05$; **$P < 0.01$).

Paraffin-stimulated whole saliva samples were collected from all participants in a forward-leaning sitting position with a 30-s pre-stimulation by the paraffin-wax chewing followed by 5-min continued stimulation and collection by spitting into a sterile Nunc™ conical test tube (Thermo Scientific™, Thermo Fisher Scientific Inc., Waltham, MA USA). All samples were immediately stored on ice after collection and delivered to the laboratory for further processing.

2.2 DNA extraction and shotgun metagenomic sequencing

Total genomic DNA of the saliva samples was extracted using the cetyltrimethyl ammonium bromide buffer-based method as previously reported [27]. All DNA samples were submitted to the Novogene

Company (Beijing, China) for shotgun metagenome sequencing. NEBNext Ultra™ II DNA Library Prep Kit for Illumina (New England Biolabs, MA, USA) was used for the generation of sequencing libraries, and Qubit 4.0 Fluorometer (Thermo Fisher Scientific) and Agilent 4200 (Agilent, Santa Clara, CA, USA) system were used for library quality assessment. The libraries were then sequenced on the Illumina HiSeq platform with 150-bp paired-end reads. Following the acquisition of raw sequencing data, the adaptor trimming, and quality control process was performed using the fastp [28] software. Subsequently, the sequencing reads were aligned to human reference genome (GRCh38) using KneadData (https://github.com/biobakery/kneaddata) to remove host-derived sequences.

2.3 Taxonomic and functional profiling

Taxonomic classification of the filtered reads was conducted using MetaPhlAn4 (v4.4.1) [29], which employs a database of species-level genome-specific unique marker genes to assign microbial taxa classification. Functional profiling was conducted using HUMAnN 3.9.0 [30]. For each sample, taxonomic profiling identified detectable organisms, and reads were mapped to sample-specific pangenomes using Bowtie2 [31]. Unmapped reads were aligned against the UniRef90 database using DIAMOND [32]. Gene family abundances were normalized based on alignment quality, gene length and gene coverage. Both nucleotide and protein-level abundances were mapped to Enzyme Commission (EC) classifications and integrated into MetaCyc pathways [33]. A custom script was used to combine the results from different samples.

The alpha diversity of microbial species was evaluated using the Shannon diversity index, and group differences were analyzed using the Wilcoxon signed-rank test. Beta diversity was evaluated using the Bray-Curtis measure and compared with the anosim function in R package vegan [34], followed by visualization with a principal coordinate analysis (PCoA) using R package ggplot2. Linear Discriminant Analysis Effect Size (LEfSe) [35] was conducted to identify taxonomic units with significantly different abundances between current and non-current areca nut chewers. The P-values were corrected using Benjamini-Hochberg procedure, with a significance threshold set at 0.05. The phylogenetic tree was visualized using tvBOT web server [36]. Classification was achieved using the random forest classifier using the abundance table. Random forest classification was performed by “trans_diff” class of microeco package [37] and the fixed seed number was used. A total of six classification models were conducted, generating characterization tables for species-level, genus-level, and species-level abundance focusing solely on the four main genera (*Streptococcus*, *Neisseria*, *Porphyromonas*, and *Prevotella*) respectively. The performance was evaluated by calculating the value of area under the curve (AUC). Twenty-fold cross-validation was performed subsequently, and the mean and standard deviation of AUC was calculated.

For functional profiling, we employed the multivariate general linear models (MaAsLin2) for identifying the differentially abundant MetaCyc pathways [38]. P-values were corrected using the Benjamini-Hochberg procedure, with a significance threshold set at 0.05, and a **coefficient value** greater than 1.5. Beta diversity was also evaluated using the Bray-curtis measure and compared through anosim function in R package vegan

[34]. The relative abundances of specific pathways were converted to z-scores and visualized using the pheatmap package [39].

To determine the effect of areca nut chewing and smoking with the species and pathways, we performed a distance-based redundancy analysis (db-RDA) [40]. Spearman correlation was employed for network inference. The co-presence and mutually exclusive networks were analyzed using the Network analyzer tool of Cytoscape (v3.8.0) [41] and outputted for further analysis, together with node and edge tables representing the full association network.

2.4 Strain-level community profiling

The Metagenomic Intra-Species Diversity Analysis System 2 (MIDAS2) was used for profiling SNV frequency [42]. As described in previous study, the database containing 258,405 genomes comprising 47,893 species provided by GTDB v202. First, we obtain species-level abundance per sample by aligning the quality-filtered paired-end sequence reads to the reference by using HS-BLASTN with the "run_midas.py species" module. Then, the results across all samples were combined using "merge_midas.py species". The resulting alignment files were subsequently processed by "run_midas.py snps" module to profile SNVs against the selected bacterial representative genome. Specifically, the 90 taxonomic units with the highest prevalence were selected for downstream SNV profiling analysis using the "run_midas.py snps" with "--aln_mapid 85 --aln_cov 0.5" parameters. The results were merged using the "merge_midas.py snps" module to generate core genomic Single Nucleotide Polymorphism (SNP) matrices to compare nucleotide variants in genomic loci in different samples. We focused on the bi-allelic SNVs that were present in more than 50% of the samples, requiring a minimum of two reads per genomic site and a maximum site depth to genome vertical depth ratio of 2. SNPs were called based on their frequency in the bacterial population, and only those with a minor allele frequency of at least 1% were retained. We included a total of 90,975 SNV sites that passed quality control criteria. Strain-level diversity within species clusters in each sample was estimated by quantifying the fraction of SNVs in protein-coding genes (number of SNVs/length of genes). We also generated a Bray–Curtis distance matrix and performed principal coordinate analysis and visualization based on the pairwise fractions of shared SNVs for different samples using the vegan package.

2.5 Prediction of SNV effect on the protein stability

The structures of BtuD and BacA were predicted using SWISS-MODEL web server [43]. The template library was searched in parallel with both BLAST and HHblits against the PDB-based SMTL and AlphaFold DB to identify templates and generate target–template alignments [44]. The AlphaFold DB model of gene F2BBG8_9NEIS from *Neisseria bacilliformis* ATCC BAA-1200 was selected as the template for homology modeling of BtuD_1, with a sequence identity of 74.56% and a global model quality estimate (GMQE) score of 0.79. For BtuD_2, the AlphaFold DB model of Q5F634_NEIG1 (Q5F634_NEIG1 from *Neisseria gonorrhoeae* ATCC 700825) was used (GMQE = 0.91). Moreover, the AlphaFold DB model of A0A1F1GMW2 was selected as the template of BacA with GMQE of 0.90. The variant site locations were submitted to the DDMut web server for predicting mutation effects on protein stability [45]. The method

primarily utilizes a Siamese network to predict changes in Gibbs Free Energy (ΔΔG) caused by single and multiple point mutations to evaluate the change in protein stability.

2.6 Validation cohort and single nucleotide variant identification

We also utilized de-identified data from participants who had consented to the use of their anonymized information for research, sourced from two previously published studies. The protocols for these studies were approved by their respective institutional review boards Jiang_2021 (Chongqing Cohort) [24] received approval from the Ethics Committee of Chongqing Medical University (ethical approval number: CQHS-IRB-2017-01). The study included 133 patients with salivary adenoid cystic carcinoma and 10 healthy controls, from whom unstimulated saliva samples were collected. Liu_2022 (Shanghai Cohort) [25] was approved by the Ethics Committee of Shanghai Ninth People's Hospital, involving 40 oral squamous cell carcinoma patients and 10 healthy controls.

To analyze the SNV composition of the specified genes in the samples, we developed custom scripts as previously described [43]. Specifically, the BWA mem algorithm was used with the "-x intractg" option to map quality-controlled reads to reference genomes, resulting in a high mapping fraction (> 95%). Duplicated reads and those mapped to multiple positions were removed using samtools fixmate and samtools view with the "-bq 1" option. Variant calling was conducted using LoFreq with the following options: lofreq viterbi, lofreq indelqual --dindel, and lofreq call-parallel --sig 1e-4 --min-cov 25 --min-bq 25 --min-alt-bq 25 --min-mq 60 -d 10000. The tidyverse and vegan packages were employed to organize and visualize the SNV frequency data. PCoA of Bray-Curtis distances based on SNV-level composition of *btuD*_1, *btuD*_2, and *bacA* across different patients and controls from Chongqing Cohort and Shanghai Cohort.

3. Results

3.1 Study design and analysis of the saliva microbiome

We studied long-lived elderly populations, as these individuals have experienced prolonged exposure over extended periods, offering a distinct perspective compared to younger or more heterogeneous populations. We specifically recruited 80 elderly individuals aged 80 to 102 years between September 2021 and January 2022 from seven sites in Wanning, a city located on the southeastern coast of Hainan Province (Figure 1A). To minimize the influence of confounding variables related to demographic and lifestyle factors, participants were selected based on strict inclusion criteria. Specifically, we focused on individuals with simple and consistent dietary habits, excluding those with significant medical conditions, a history of head and neck cancer, or recent medical treatments. Each participant completed a detailed questionnaire covering demographic, lifestyle, and physiological factors, ensuring that we minimized potential sources of variability. Detailed statistical data regarding the cohort's characteristics are provided in Dataset S1. The participants' ages ranged from 83 to 102 years, with a mean age of 88.9 ± 4.5 years. The cohort displayed a relatively low average BMI of 19.0 ± 3.0. Among the participants, 21 individuals were current areca nut chewers, while the remaining 59, comprising former and never-chewers, served as the non-chewer group.

Comparative analysis revealed no statistically significant differences between current and non-current areca nut chewers in terms of age ($P = 0.694$; Student's t-test), BMI ($P = 0.925$; Student's t-test), ethnicity ($P = 0.801$; Chi-squared test), gender (P = 0.138; Chi-squared test), alcohol consumption ($P = 0.832$; Chi-squared test), or tea consumption ($P = 0.801$; Chi-squared test). Saliva samples were collected from each participant, followed by DNA extraction and shotgun metagenomic sequencing to assess the composition and function of the oral microbiome (Figure 1B). To evaluate the impact of major metadata variables on the microbial profiles across these dimensions, Permutational multivariate analysis of variance analysis (PERMANOVA) was employed (Figure 1C). The results indicated that ethnicity had the most significant effect, explaining the largest proportion of variance across species (R = 0.24), pathways (R = 0.30), genes (R = 0.32), and SNVs (R = 0.33). Interestingly, while the effects of areca nut chewing on species (R = 0.09) and pathway (R = 0.16) levels were slightly lower than those associated with sampling location (R = 0.24 for species and R = 0.30 for pathways), areca nut chewing exerted a more pronounced influence at the gene (R = 0.17) and SNV (R = 0.19) levels. This finding suggests that areca nut chewing significantly impacts oral microbiome functionality, even if it exerts less influence on species composition. In contrast, gender, smoking, and tea consumption had minimal effects on microbial profiles (R < 0.15), with smoking and tea consumption showing no significant impact across any data layers ($P > 0.05$).

3.2 Species-level profiling revealed changes in Streptococcus in areca nut chewers

We first analyzed the species-level microbial composition among all participants. Consistent with previous studies [46–48], we found that the five most predominant phyla were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Fusobacteria*, collectively accounting for 98.5% of the total sequences (Figure S1). *Prevotella*, *Neisseria*, *Streptococcus*, and *Porphyromonas* were the most abundant genera. Notably, we observed an elevated prevalence of *Methyloversatilis* across all samples, a genus broadly distributed in the natural environment with a capability of methylotroph, and potentially associated with poor prognosis in head and neck squamous cell carcinoma patients [49].

To further explore microbial diversity, we compared alpha and beta diversity measures at the species level. No significant variations in Shannon index were observed between current and non-current areca chewers ($P = 0.34$, Figure 2A). Similarly, there were no significant differences based on ethnicity ($P = 0.06$), gender ($P = 0.49$), or tea consumption ($P = 0.83$). However, current smokers exhibited a significantly higher Shannon index ($P = 0.019$). For beta diversity, Bray-Curtis dissimilarity analysis revealed a clear distinction between individuals based on areca nut chewing status ($P = 0.016$, Figure 2B), whereas no separation was observed between current and non-current smokers ($P = 0.5$, Figure 2C). Additionally, we performed an exploratory analysis by stratifying the cohort into three distinct groups based on exposure status: current, former, and never chewers. PERMANOVA on species-level β-diversity showed that current chewers were significantly different from both former chewers ($P = 0.003$) and never chewers ($P = 0.003$), while the latter two groups were statistically indistinguishable from each other ($P = 0.484$) (Figure S2).

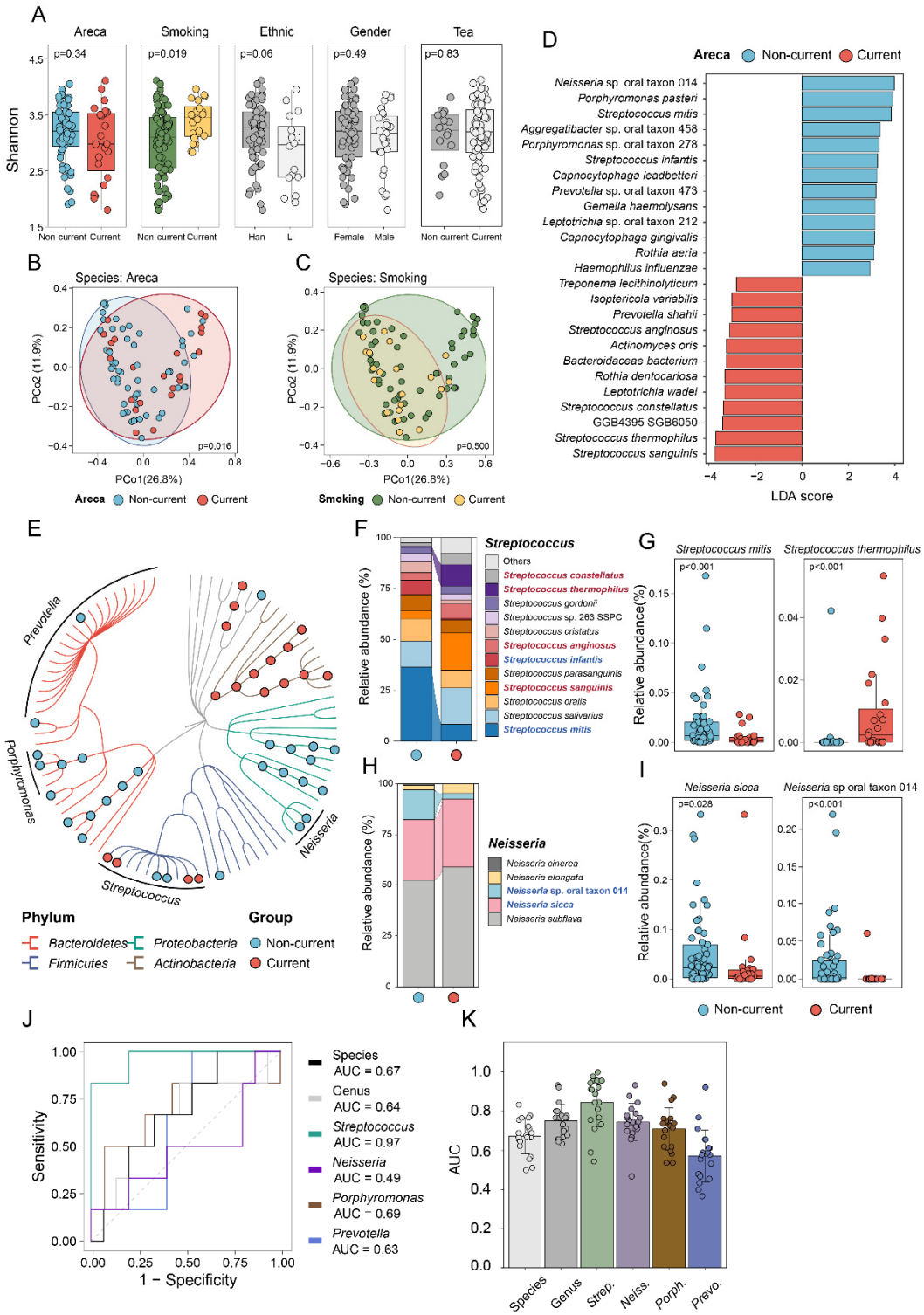

Figure 2 Species-level profiling revealed changes in *Streptococcus* in areca nut chewers. (A) Boxplot of the microbiome α-diversity across areca nut chewing status, smoking, ethnicity, gender, and tea consumption (ANOVA). **(B-C)** Principal Coordinates Analysis (PCoA) of Bray–Curtis distances based on species-level composition between areca nut chewing groups (B) and smoking groups (C). The P-values were calculated using ANOSIM. **(D)** Linear Discriminant Analysis Effect Size (LEfSe) was used to calculate the species difference between areca nut chewers and controls, with P-values were corrected using Benjamini-Hochberg procedure. **(E)** Phylogenetic trees showing the differential bacterial taxonomic units. The circles are marked for significant differential species ($P < 0.05$) identified via LEfSe. **(F-I)** Species-level composition of *Streptococcus* (F) and *Neisseria* (H). Relative abundances of *Streptococcus infantis* and *Streptococcus mitis* (G), and *Neisseria sicca* and *Neisseria* sp. oral taxon 014 (I) were compared between chewers and controls (Wilcoxon test). **(J)** A random forest model was trained to predict areca nut chewing status using feature tables of all species, all genera, and species-level compositions of *Streptococcus*, *Neisseria*, *Porphyromonas*, and *Prevotella*. The ROC curve was computed to evaluate performance. **(H)** Random under-sampling (n = 20) was used to fit additional random forest models, and the AUC was calculated to evaluate predictive performance.

Given the observed differences in microbial diversity and composition, we employed LEfSe to identify areca-associated taxonomic signatures (Figure 2D-E, Dataset S2). The analysis identified 13 species with decreased abundance in areca chewers, including *Porphyromonas pasteri*, *Haemophilus influenzae*, *Capnocytophaga leadbetteri*, and *Capnocytophaga gingivalis*. Interestingly, *Porphyromonas pasteri* has been implicated in the progression of periodontitis and identified as a bacterial marker for oral squamous cell carcinoma diagnosis, underscoring its clinical relevance [50]. Meanwhile, *Haemophilus influenzae*, *Capnocytophaga leadbetteri*, and *Capnocytophaga gingivalis* have been linked to oral and respiratory infections under specific conditions, and all have previously been associated with oral squamous cell carcinoma development [51]. Conversely, 12 species were enriched among current chewers, with a marked increase in the oral pathogen *Treponema lecithinolyticum*, which is associated with chronic periodontitis [52]. Intriguingly, we also observed an enrichment of the probiotic *Streptococcus thermophilus* in areca chewers [53]. In the oral cavity, certain *S. thermophilus* strains are known to produce folic acid, tyramine, and histamine, which have demonstrated cytotoxic effects against cancer cells, and have potential for both oral health protection and anticancer properties [54]. The increase in probiotic species such as *S. thermophilus* in the oral microbiota of the elderly areca chewers reflects the complex relationship between areca nut chewing and shifts in oral microbial ecology.

Remarkably, significant alterations in species composition were observed within the genera *Streptococcus* and *Neisseria*. In areca nut chewers, *Streptococcus constellatus*, *Streptococcus thermophilus*, *Streptococcus anginosus*, and *Streptococcus sanguinis* were significantly enriched, whereas *Streptococcus infantis* and *Streptococcus mitis* were found at lower proportions (Figures 2F-G, Figure S3). Additionally, we consistently observed a significant reduction in *Streptococcus mitis*, an increase in *Streptococcus thermophilus* in the “current” group. In contrast, “former” and “never” chewers exhibited a high degree of similarity in the species composition. These results suggest that the community structure of former chewers is statistically comparable to that of never chewers (Figure S2). Similarly, a decline was observed in *Neisseria sicca* and *Neisseria* sp. oral taxon 014, both of which are typically asymptomatic colonizers of the human oral cavity (Figures 2H-I) [50]. These changes in microbial composition are likely driven by the alterations in the oral environment induced by areca nut chewing, including shifts in chemical composition, physical stimulation, changes in the salivary environment, and potentially other underlying mechanisms. These findings prompted us to explore whether the dynamic characteristics of these microbiomes could indicate the areca nut chewing status. We assessed the effectiveness of microbiome composition, including relative abundance for all species, all genera, and selected genera (*Streptococcus*, *Neisseria*, *Porphyromonas* and *Prevotella*) in distinguishing areca nut chewers from all individuals using the random-forest classification model. The initial results showed that classifiers based on species (AUC = 0.67) and genus (AUC = 0.64) were insufficient for accurate identification. However, the *Streptococcus* composition achieved a significantly higher AUC of up to 0.97, compared to the *Neisseria* (AUC=0.49), *Porphyromonas* (AUC = 0.69), and *Prevotella* (AUC = 0.63) (Figure 2J). To validate these findings, we performed random under-sampling and

fitted additional random-forest models (n = 20), recalculating the average AUC to confirm predictive accuracy (Figure 2K). Consistently, the *Streptococcus* composition achieved the highest AUC, underscoring its robustness to predict areca nut chewing status. Our findings emphasize that the microbial composition, particularly within the *Streptococcus* genus, is highly effective in distinguishing areca nut chewers from non-chewers, highlighting its potential utility as a biomarker.

3.3 Areca nut chewing induces alterations in oral thiamine metabolism

Gene functional analysis of the microbiome provides crucial insights into how microbial communities impact health, disease, and environmental processes by revealing the specific roles and biochemical functions of microbial genes. In this study, we investigated whether oral microbial functional profiles were associated with areca nut chewing. Beta diversity analysis, based on Bray–Curtis dissimilarity, showed that microbial pathways differed between current and non-current areca chewers (P = 0.002, Figure 3A), whereas no significant differences were observed between current and non-current smokers (P = 0.072, Figure 3B). Using MaAsLin2, we identified four differential MetaCyc pathways associated with areca nut chewing (P < 0.05, coefficient > 1.5), with the most notable being a community-level shift in microbial function related to thiamine metabolism (Figure 3C). This shift encompassed intertwined salvage and synthesis pathways of thiamine diphosphate, including salvage pathways involving pyrithiamine and oxythiamine (Figure 3D).

To further explore the effects of areca nut chewing on thiamine metabolism, we identified genes involved in both thiamine synthesis and salvage pathways (Figure 3E). Using HUMANN3, we traced the species origin of these differential genes, revealing significant reductions in thiamine salvage genes among areca nut chewers. This reduction correlated with decreases in species such as *Streptococcus mitis* and *Neisseria* sp. oral taxon 014. Additionally, complex changes were observed in genes within the 4-amino-2-methyl-5-diphosphomethylpyrimidine biosynthesis I pathway, essential for pyrimidine heterocycle formation in thiamine diphosphate biosynthesis. Specifically, genes responsible for pyridoxal 5'-phosphate oxidation and phosphoribosylamine formation, *pdxH* and *purF* [55], were underrepresented in areca nut chewers. Conversely, genes *pdxK* and *pdxL*, encoding pyridoxal kinase and pyridoxine 4-dehydrogenase [56], respectively, were enriched, correlating with increased proportions of *S. thermophilus* and *Saccharomyces cerevisiae*. These findings indicate a complex reorganization of thiamine-related metabolic pathways in response to areca nut chewing.

To visualize these relationships, we constructed a network of microbial features, showing associations between thiamine metabolism—including curated MetaCyc pathways, enzymes, and species encoding these microbial functions (Figure 3F). We hypothesize that the reduction of species such as *S. mitis* contributes to an overall downregulation of the microbial community's thiamine salvage pathway, while the increased presence of *S. thermophilus* may support pyrimidine heterocycle synthesis for thiamine diphosphate biosynthesis using pyridoxine as a precursor. These results suggest that alterations in the oral microbiota associated with areca nut chewing induce a substantial shift in the community's thiamine metabolic pathway. Several studies have highlighted a significant association between thiamine levels and cancer development. Notably, low thiamine

concentrations have been linked to an increased risk of certain cancers[57–59]. This suggests that the enhanced de novo synthesis of thiamine within the oral microbiome, induced by areca nut chewing, may pose health risks, particularly regarding oral health. These findings underscore the need for further exploration of how thiamine metabolism alterations in the oral microbiota might contribute to the onset of oral diseases and cancer.

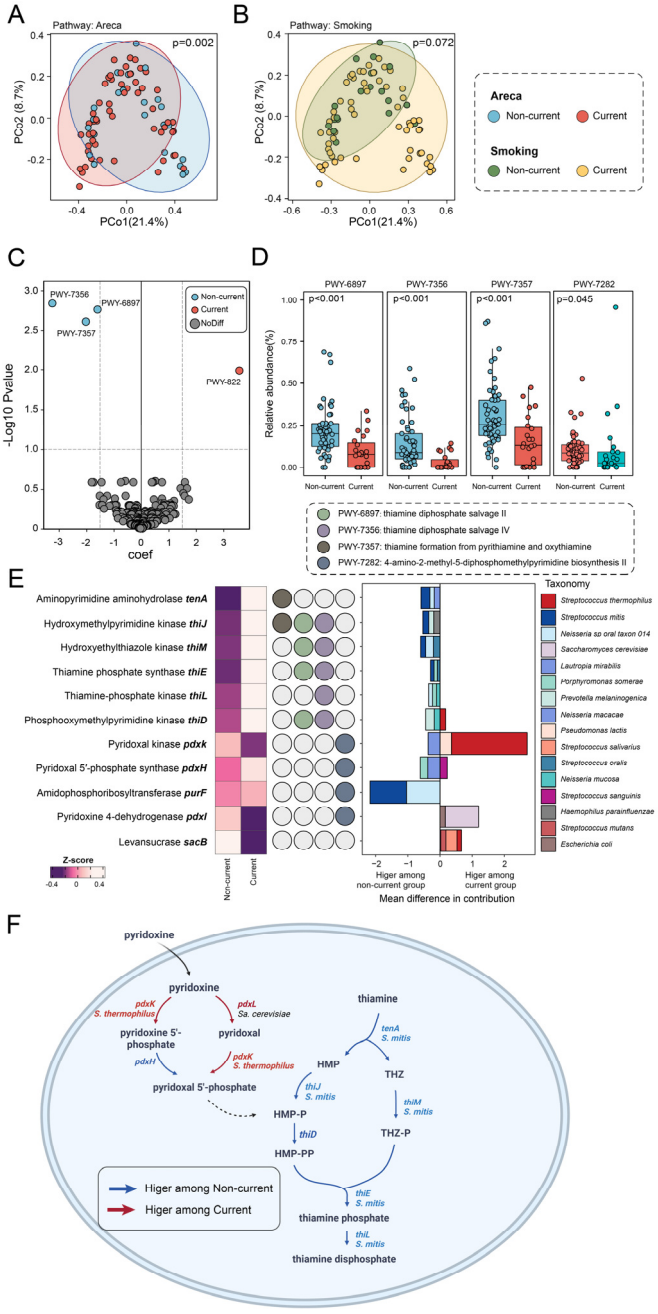

Figure 3 Alterations in thiamine metabolism were observed in areca nut chewers. (A-B) Principal Coordinates Analysis (PCoA) of of Bray–Curtis distances based on pathway-level composition by areca nut chewing (A) and smoking (B) status. The P-values were calculated using ANOSIM. **(C)** Volcano plot showing the differential MetaCyc pathways in all samples identified via MaAsLin2. **(D)** Relative abundance of differential pathways across areca nut chewers and controls (Wilcoxon test). **(E)** Metagenomic analysis associations of microbial functions (as MetaCyc pathways) involved in thiamine metabolism with areca nut chewing. Heatmap showing normalized relative abundance of thiamine metabolism genes in different groups. Different colored circles indicate genes were classified into different thiamine metabolic pathways. **(F)** A network of microbial features showing the intertwined relationships between thiamine metabolic pathways. The network includes curated MetaCyc enzymes significantly associated with areca nut chewing and species encoding these enzymes are marked in the network.

3.4 Areca nut chewing-associated microbial SNV signatures

Microbial SNVs act as crucial molecular markers, capturing strain-level genetic variations that reveal functional shifts often overlooked in species-based studies. We examined SNV signatures in areca nut chewers, focusing on 90 commonly detected microbial species. Initial analyses assessed differences in the proportion of SNV sites across all profiled coding regions based on areca nut chewing status (Figure 4A). This analysis revealed differences in SNV site proportions for only three species: *Methyloversatilis discipulorum* A (P = 0.032), *Fusobacterium polymorphum* (P = 0.041), and *Bulleidia moorei* (P = 0.042). To further investigate the distribution of SNVs across samples from areca nut chewers, we calculated Bray-Curtis distances between all sample pairs and performed ANOSIM (Figure 4B). The analysis identified 12 species with significant differences in SNV composition ($P < 0.05$), primarily within the *Neisseria* and *Streptococcus* genera. These findings suggest that microbial strains within these genera may undergo adaptive changes in response to long-term alterations in the oral environment associated with areca nut chewing.

Given the marked differences and the number of SNVs, we selected *Neisseria elongata* and *Neisseria subflava* for further analysis. Using the Wilcoxon test, we examined allele frequencies in the coding regions of these species, identifying 442 differential SNV loci in *N. elongata* (Figure 4C) and 113 in *N. subflava* (Figure 4D) within the areca nut chewing group. No specific biases in the genome positions of these differential SNVs were observed. We then identified coding genes with notable variation in SNV composition between groups (ANOSIM, $P < 0.05$; Dataset S3). Many of these genes play roles in key metabolic functions, including glycolysis, fatty acid degradation, and tyrosine metabolism. Interestingly, three genes involved in transmembrane transport displayed distinctive SNV frequency patterns (Figures 4E-G). Further annotation revealed that these genes encode ATPase components of the ABC transport systems in Gram-negative bacteria, specifically facilitating the uptake of exogenous corrinoids, such as cobalamin (Figure 4H). As part of the cobalamin salvage pathway, these ATPase components hydrolyze ATP to power the transport of corrinoids across the inner membrane via the BtuCD complex. Efficient corrinoid import through this energy-dependent mechanism allows bacteria to sustain essential cobalamin-dependent metabolic processes. We hypothesize that *Neisseria* species, represented by *N. elongata* and *N. subflava*, may have adapted to shifts in the oral vitamin environment induced by areca nut chewing, potentially by modifying their cobalamin transport mechanisms through SNV-based alterations.

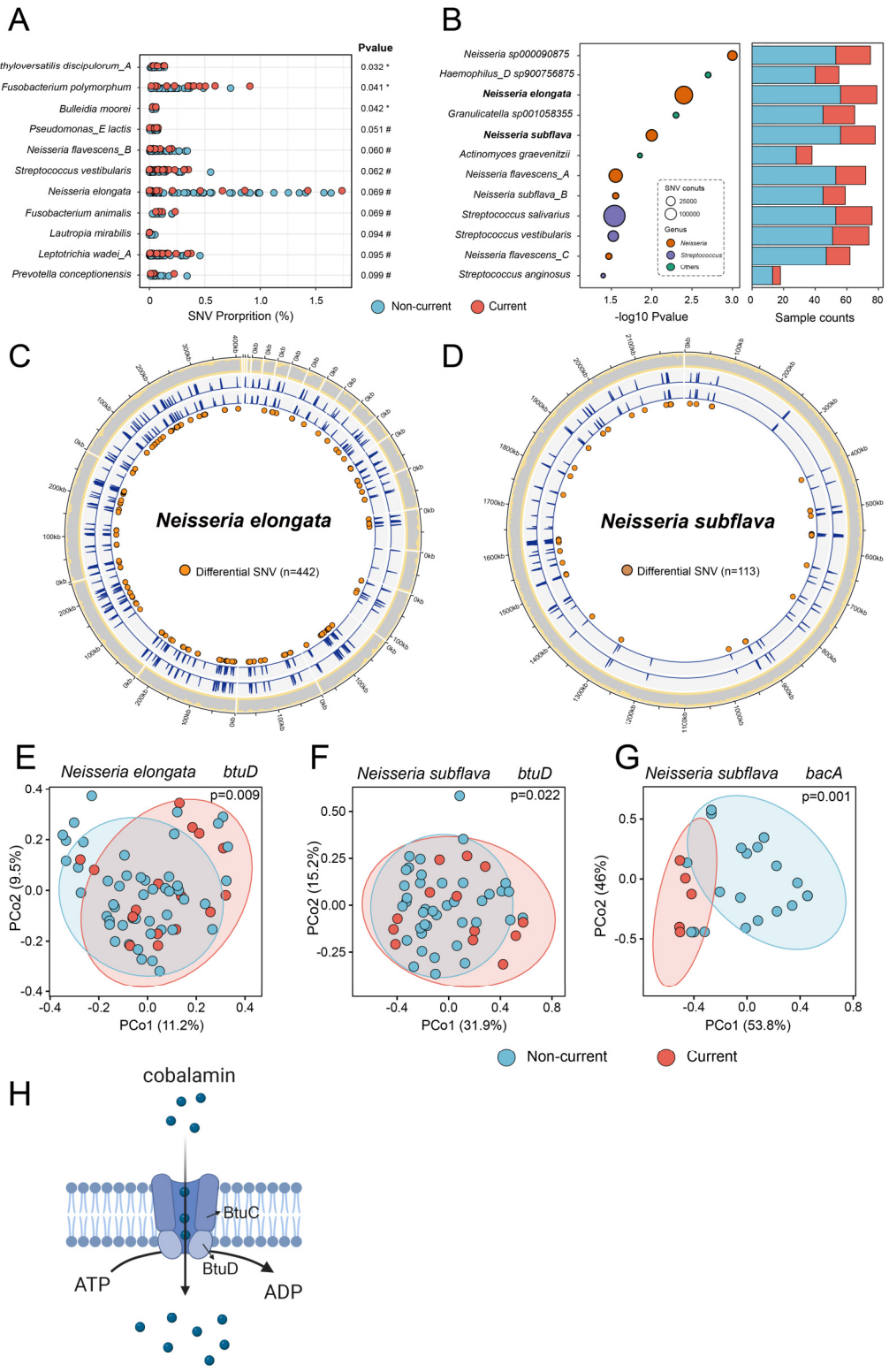

Figure 4 Identification of SNV signatures associated with areca nut chewing. (A) Fraction of single-nucleotide variants (SNVs) within core genes in each sample for different species. **(B)** SNV compositions differing by areca nut chewing status were assessed using ANOSIM. The size of each circle represents the number of SNVs detected in the corresponding species. The bar plot on the right shows the number of samples in which SNVs were detected. **(C-D)** Genomic locations of SNVs in strains of *Neisseria elongata* (C) and *Neisseria subflava* (D). The yellow outer rings represent the GC content of the reference genomes for the annotated strains. SNV frequencies in areca nut chewers and controls are indicated by blue lines in the second (chewers) and third (controls) rings, respectively. The fourth ring marks the locations of identified differential SNVs (yellow circles). (E-G) Principal Coordinates Analysis (PCoA) of Bray-Curtis distances based on SNV-level composition of *btuD* from *Neisseria elongata* (E), and *btuD* and *bacA* from *Neisseria subflava*, across different areca nut chewing conditions (P-values from ANOSIM). **(H)** Schematic representation of the role of BtuD in the transmembrane transport of vitamin B12 (cobalamin) in bacteria.

Since we detected SNVs in the coding region of the ATPase associated with cobalamin salvage—significantly influenced by areca nut chewing—we speculate that these SNVs could affect protein stability and thereby impact functionality. We first modeled the protein structures using the SWISS-MODEL web server. The resulting GMQE scores were 0.79, 0.91, and 0.90 for BtuD_1, BtuD_2, and BacA, respectively, indicating that the predicted models are of high quality. Specifically, mutations were identified at the 21st, 120th, and 264th nucleotide positions within *btuD*_1 of *Neisseria elongata*, resulting in non-synonymous amino acid substitutions (Figure 5A). Subsequently, the structural models and the amino acid changes were submitted to DDMut to assess the potential structural impact of the mutations. Structural predictions of BtuD_1, based on the AlphaFold Protein Structure Database entry for the ATP-binding cassette transporter from *Neisseria bacilliformis* ATCC BAA-1200 (F2BBG8) (Figure 5C) [43], indicated that two out of three SNVs may significantly impact protein stability, with predicted Gibbs Free Energy (ΔΔG) changes of -0.48 kcal/mol and -0.84 kcal/mol, respectively [45] (Figure 5B). Thus, the mutations in *btuD*_2 likely alter protein structure and function, potentially linked to areca nut chewing. Similarly, we identified mutations in *btuD*_2 and *bacA* from *Neisseria subflava* that may impact protein stability, such as V32I in *btuD*_2 (ΔΔG = -0.26 kcal/mol) and Q17R in *bacA* (ΔΔG = -0.17 kcal/mol) (Figure 5C-F).

3.5 Single nucleotide variants in the cobalamin transporter are associated with oral squamous cell carcinoma

We observed variations in SNV composition associated with areca nut chewing in the coding region of the vitamin B12 ABC transporter, indicating possible disruptions in oral cobalamin levels. Cobalamin, acting as a free radical scavenger, plays a key role in immune activation, and enabling immune cells to target and eliminate mutated cells, and it is essential for innate immunity. Previous studies have shown a strong association between reduced serum cobalamin levels and oral cancer, with cobalamin deficiency notably more common in advanced-stage oral cancer patients [60]. This suggests that altered cobalamin metabolism could contribute to oral cancer pathogenesis and progression. Therefore, we hypothesize that similar SNV composition changes driven by cobalamin dysregulation may be detectable in patients with oral cancer.

To assess the SNV signal observed in our main analysis, we further utilized two independent validation cohorts: one with adenoid cystic carcinoma (Chongqing Cohort) [24] and another with squamous cell carcinoma (Shanghai Cohort) [25]. To ensure subtype-specificity and minimize potential confounders, we adopted an analytical strategy wherein each cancer cohort was compared exclusively against its own internal healthy controls. Using custom scripts, we identified SNV locations and frequencies within three targeted genes by directly comparing shotgun metagenomic reads with reference sequences across samples. Interestingly, a higher frequency and diversity of SNVs were observed in the oral microbiomes of cancer patients in both cohorts (Figure S4), particularly in *btuD_2* in squamous cell carcinoma patients from Shanghai cohort, where these mutations were entirely absent in the healthy controls (Figure 5G-H). We calculated Bray-Curtis distances of SNV composition for three genes across different groups within each cohort and performed PCoA analysis. Results revealed distinct SNV composition differences (P = 0.001 for

Chongqing cohort and P = 0.004 for Shanghai cohort) between cancer patients and healthy controls, suggesting that cancer status significantly affects the SNV landscape of these cobalamin transporter genes (Figure 5I-J).

In summary, our findings indicate that areca nut chewing induces notable SNV changes in specific cobalamin transporter genes in some oral *Neisseria* species. These alterations could serve as indicators of oral cancer and may be closely related to the onset and progression of the disease.

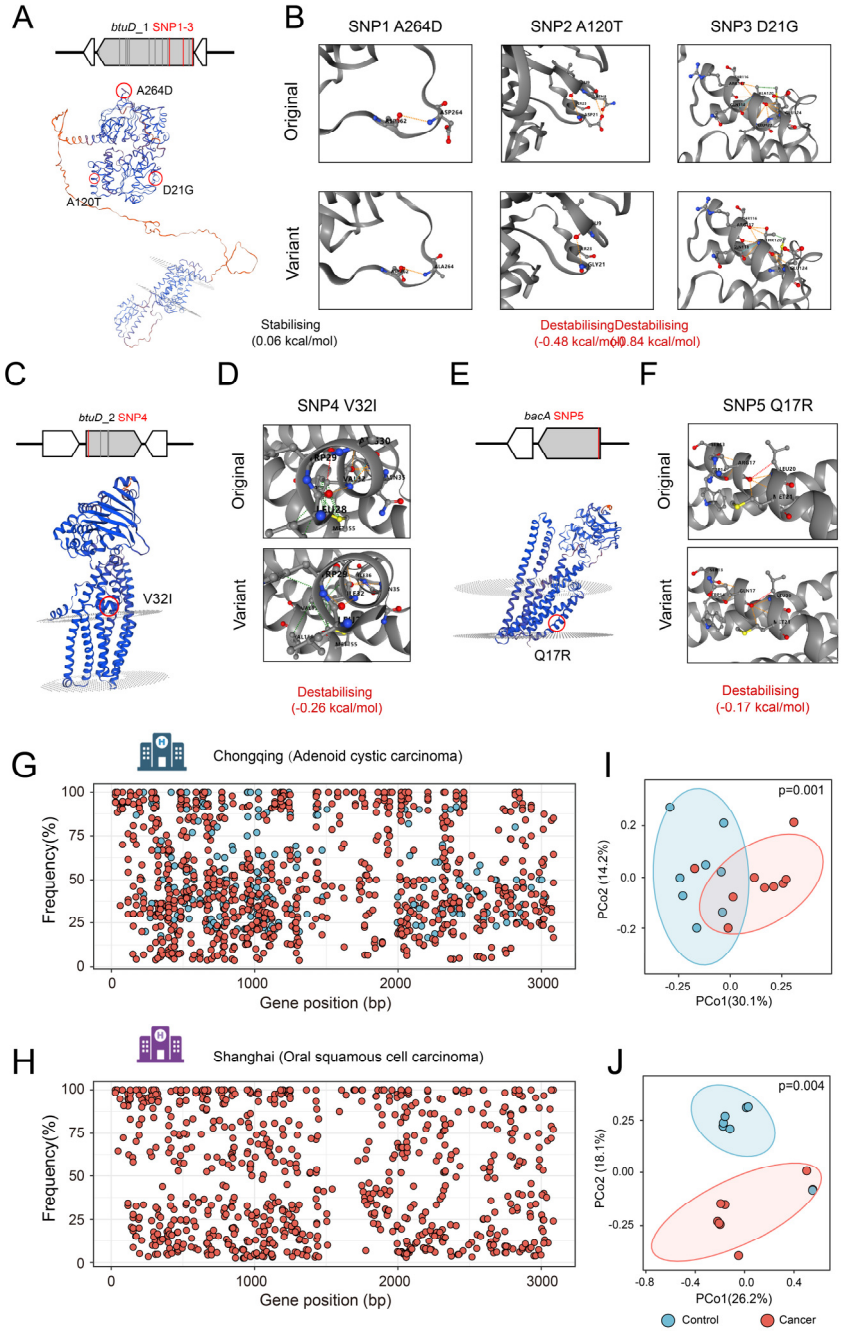

Figure 5 Single nucleotide variants (SNVs) in the cobalamin transporter. (A-F) The positions of detectable SNVs of *btuD*_1 from *Neisseria elongata* (A), *btuD*_2 from *Neisseria subflava* (C), and *bacA* from *Neisseria subflava* (E). Structures were predicted based on AlphaFold models F2BBG8_9NEIS, Q5F634_NEIG1, and A0A1F1GMW2, respectively. DDMut predicted the structural impact of variants in BtuD_1 (B), BtuD_2 (D), and BacA (F). **(G-H)** The positions and frequencies of the detected SNVs of *btuD*_1 in salivary adenoid cystic carcinoma patients (Chongqing Cohort) (G) and oral squamous cell carcinoma patients (Shanghai Cohort) (H). **(I-J)** Principal Coordinates Analysis (PCoA) of Bray–Curtis distances based on SNV-level composition of *btuD*_1, *btuD*_2, and *bacA* across different patients and controls from Chongqing Cohort (I) and Shanghai Cohort (J). P-values calculated using ANOSIM.

3.6 Combinatorial effects of smoking and areca nut chewing on oral microbiome

To explore the potential overlapping effects of smoking and areca nut chewing on the oral salivary microbiome, we divided participants into four groups based on their smoking and chewing habits: current smoking only (Smoking), current areca nut chewing only (Areca), both smoking and areca nut chewing (Areca-Smoking), and a control group (Control). A comparison of the Shannon index indicated that areca nut chewing significantly reduced species-level alpha diversity in the oral microbiota (Figure 6A, $P < 0.05$). In contrast, no significant differences were observed between the other groups and the control group. Additionally, individuals in the Areca group exhibited significantly lower alpha diversity compared to those in the Areca-Smoking and Smoking groups ($P < 0.05$), suggesting a potential interaction effect between areca nut chewing and smoking on the oral microbiota.

To further disentangle the effects of areca nut chewing and smoking on the oral microbiome, we performed distance-based redundancy analysis (db-RDA) at both the species and pathway levels (Figures 6B and 6C). The ordination plots revealed clear separation patterns associated with areca nut use and smoking, with the combined exposure group (Areca & Smoking) showing a distinct shift along the primary axes in both analyses. These patterns suggest a potential interactive effect between areca nut chewing and smoking, jointly shaping the taxonomic structure and functional capacities of the oral microbial community. Notably, several taxa and metabolic pathways appeared to be differentially associated with this combined exposure, indicating that dual lifestyle factors may amplify microbial alterations beyond their individual effects. Further investigation into the species and functional characteristics of the salivary microbiota associated with areca nut chewing and smoking was conducted using shotgun metagenomics sequencing data (Figure 6D, Dataset S4). LEfSe analysis revealed no microbial signatures in the Smoking group, indicating that smoking alone did not significantly alter microbial composition or functional pathways. In contrast, some differential species, such as *Porphyromonas pasteri*, *Rothia dentocariosa*, *Streptococcus sanguinis*, and *Haemophilus influenzae*, were uniquely associated with areca nut chewers, reinforcing the notion that smoking and areca nut chewing have distinct impacts on microbial species composition.

In addition, *S. thermophilus* and three thiamine metabolic pathways were consistently enriched in areca nut chewers irrespective of smoking status, indicating a strong association between areca nut exposure and shifts in microbial thiamine metabolism. Moreover, compared to the Control group samples, several microbial functional pathways were significantly altered exclusively in the Areca-Smoking group, suggesting a combined effect of smoking and areca nut chewing. Notable changes in the Areca-Smoking group included a reduction in fructan biosynthesis (PWY-822) and the assimilatory sulfate reduction pathway (SO4ASSIM-PWY), along with a decrease in the superpathway of pyrimidine deoxyribonucleoside degradation (PWY0-1298), which may be associated with disruptions in microbial nitrogen cycling. Increased activity in pathways associated with the biosynthesis of phosphatidylglycerol (PWY4FS-7), a primary lipid component of bacterial membranes, was also observed [61]. Taken together, these findings suggest that the combined exposure to areca nut chewing and smoking may have a greater impact on the structure and

metabolic functions of the oral microbiome than either factor alone, particularly through pathways related to nutrient cycling and membrane biosynthesis. Although causality cannot be firmly established, the observed alterations likely reflect a compounding effect, which may contribute to oral ecological imbalance and associated health risks.

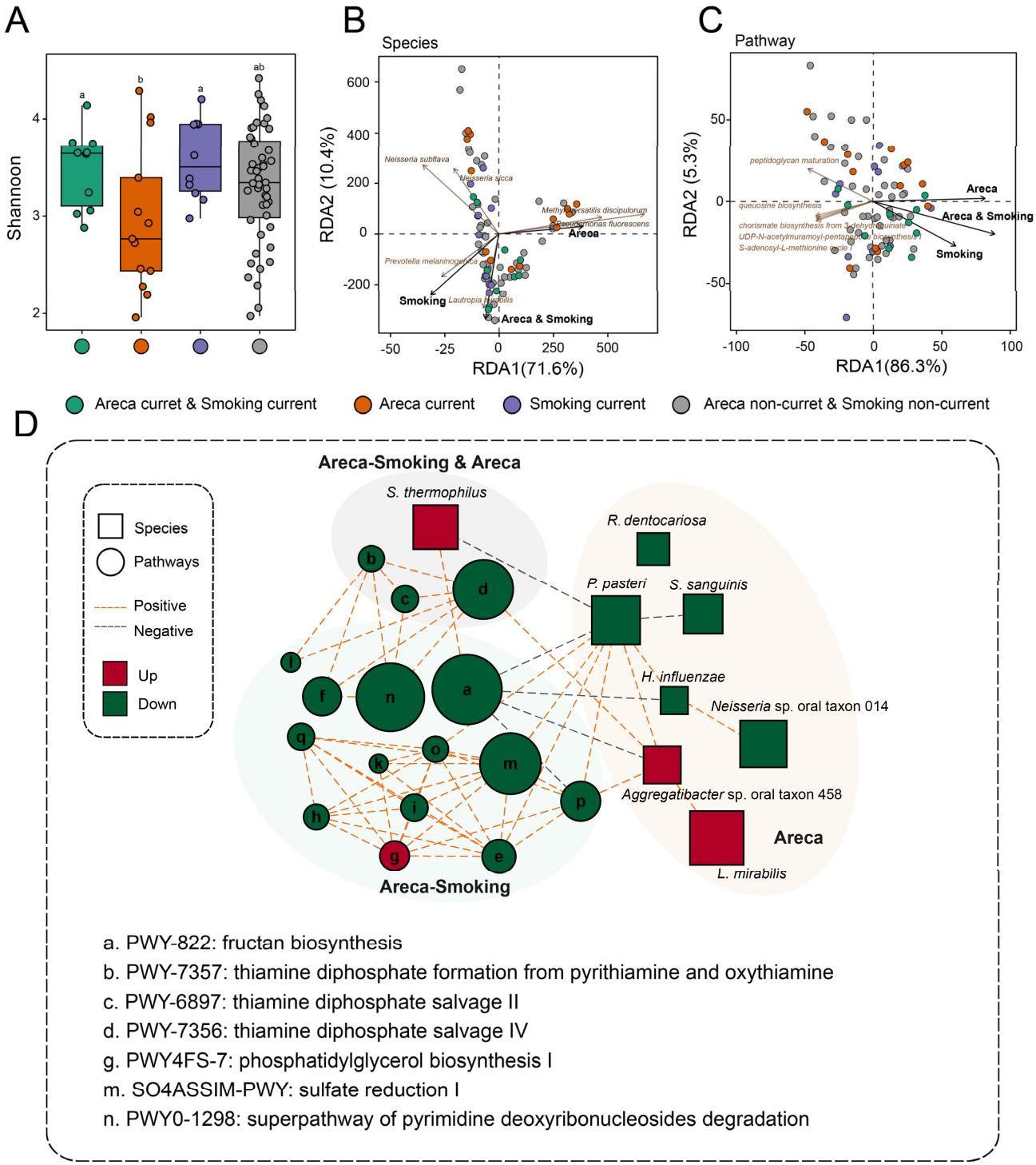

Figure 6 The potential overlapping effects of smoking and areca nut chewing on the oral microbiome. (A) Boxplot of microbiome α-diversity across groups with only areca nut chewing (Areca), only smoking (Smoking), areca nut chewing and smoking (Areca-Smoking) and controls (Wilcoxon test). **(B-C)** Plots from distance-based redundancy analysis (db-RDA) of the microbiota composition at the species level (B) and pathway level (C), relative to areca nut chewing and smoking status. **(D)** The Spearman interaction network of differential pathways and differential species among the indicated groups was constructed. Differential features were identified using Linear Discriminant Analysis Effect Size (LEfSe), comparing each group against the controls.

4. Discussion

In this study, we established a well-defined cohort of long-lived elderly individuals to explore the impact of prolonged areca nut chewing on the oral microbiome. Our aim was to focus on the impact of prolonged chronic areca nut chewing on the oral microbiota of long-lived elderly individuals, as this demographic provides a unique opportunity to study the long-term effects of areca nut consumption on microbial communities in the oral cavity. Unlike studies with shorter exposure durations, which may capture only transient microbial alterations, our research highlights how chronic exposure over several decades can lead to more substantial and enduring shifts in microbial composition and function. This extended exposure could offer deeper insights into the potential long-term health consequences of areca nut chewing, particularly regarding oral diseases and microbial resilience in aging populations. Using shotgun metagenomic sequencing, we performed a comprehensive analysis of the bacterial species composition, functional pathway enrichment, and SNV profiles in the saliva microbiome of these individuals, with a specific focus on the influence of areca nut chewing. Our findings demonstrate that areca nut chewing not only alters the species composition within the *Streptococcus* and the strain-level diversity of *Neisseria*, but also disrupts the homeostasis of essential vitamins such as thiamine (vitamin B1) and cobalamin (vitamin B12). Such changes may induce a functional reorganization of the oral microbiome within the context of host-microbe interactions, potentially influencing host health.

The substantial shifts in the oral microbiota observed in areca nut chewers likely reflect pronounced environmental changes that selectively favor the growth of specific microbial species, thereby reshaping the overall community structure. Previous studies have demonstrated the antimicrobial properties of both water and methanol extracts of areca nut *in vitro*, with tannic acid shown to inhibit the growth of *Streptococcus* species [62,63]. These effects may partially explain the observed reductions in specific bacterial taxa. The increasing use of plant-derived substances as alternatives to synthetic chemical additives in pharmaceuticals and foods has attracted considerable attention, due to the diverse bioactive properties of herbal extracts and flavoring agents [64]. Our findings highlight the need for a more rigorous assessment of the impact of areca nut extracts on the oral microbiome during their application, to minimize or avoid disruptions to normal microbial functions.

A significant shift in the species composition of *Streptococcus* was associated with alterations in thiamine metabolism, which may serve as potential biomarkers. Thiamine, or vitamin B1, is a vital coenzyme in multiple essential metabolic pathways, including glycolysis, the pentose phosphate pathway, and the citric acid cycle [65]. A deficiency in thiamine disrupts these pathways and can lead to impaired glucose metabolism and compromised cellular bioenergetics [66]. As humans cannot synthesize thiamine, it must be obtained from dietary intake or through microbial synthesis in the microbiome [66]. In microbial communities, thiamine is commonly cross-fed and actively shared, facilitating nutrient balance within the oral environment [67]. While some bacterial taxa are capable of *de novo* thiamine synthesis, this process requires substantial energy and resources, leading many bacteria to rely on exogenous sources for efficiency in normal

conditions [68]. In this study, a significant reduction in *Streptococcus* species among areca nut chewers was associated with a decrease in genes and pathways related to the exogenous absorption of thiamine in the oral microbiome. Conversely, the increased abundance of *Streptococcus thermophilus* and *Saccharomyces cerevisiae* predominantly contributed to genes involved in the synthesis of the thiamine pyrimidine pathway. This indicates an unusual shift in the oral microbiome, from reliance on environmental thiamine absorption to autonomous thiamine biosynthesis. Indeed, research has highlighted a strong association between thiamine levels and cancer progression, likely mediated through pathways involving the solute carrier transporter gene and transketolase [65,69]. Thiamine supplementation has also been observed to influence tumor cell survival, proliferation, and chemotherapy resistance[70], suggesting the critical role in cancer biology. Considering the limited and persistent sources of vitamins in the oral cavity, we hypothesize that significant alterations in microbiome-related metabolism may lead to changes in oral thiamine levels, potentially increasing the associated risks of cancer.

In addition to the functional study of the oral microbiome, we also focused on strain-level changes within the oral microbiota, as microorganisms may alter their functional characteristics through genomic variations to adapt to the altered host environment [71]. Interestingly, our findings also revealed substantial SNV variations related to cobalamin (vitamin B12) transmembrane transport in *Neisseria* species, particularly in *N. elongata* and *N. subflava*. These SNV differences suggest that the microbial community in areca nut chewers may adapt to the altered oral environment by selectively promoting strains that better accommodate shifts in nutrient and vitamin availability, thereby affecting overall microbial function and stability. Cobalamin, a crucial coenzyme synthesized exclusively by certain bacteria and archaea, plays key roles in nerve health, brain function, and red blood cell production in humans [72]. Elevated levels of cobalamin have been reported in several types of cancer, which may indicate a connection between cobalamin dysregulation and cancer development or progression [73,74]. For bacteria capable of de novo cobalamin synthesis, the pathway involves approximately 30 enzymatic steps, making it an energetically intensive and time-consuming process [58]. To reduce this metabolic load, many bacteria instead salvage cobalamin intermediates or import the vitamin through transmembrane transporters, such as the BtuCD complex, which is a primary uptake system in gram-negative bacteria [23]. The BtuCD complex, which consists of two ATP-binding proteins (BtuD) and two transmembrane proteins (BtuC), utilizes ATP hydrolysis to drive cobalamin transport into the cell. BtuD, the ATPase component, is particularly critical as it provides the energy required for the active transport of cobalamin across the cell membrane [75]. In our study, strain-level variations in *Neisseria* species were linked to significant SNV differences in the *btuD* gene, which may alter the protein's structure and function. This, in turn, could modify the efficiency of cobalamin uptake from the surrounding oral environment, potentially impacting overall cobalamin availability within the host's oral cavity. To explore the potential link between *btuD* and *bacA* SNVs and cancer status, we extended our analysis to two small cancer-associated cohorts, each compared independently to its own healthy control group. Despite limitations, including small control groups and heterogeneity in geography, age, and cancer pathology, we found significant and consistent differences in

SNV profiles of *btuD* and *bacA* between cancer patients and their respective healthy controls in each cohort. This reproducible pattern across both cohorts suggests a potential generalizable biological signature associated with oral cancer, meriting further study in larger, well-controlled cohorts.

Tobacco smoking is a well-known risk factor for oral cancer and is believed to act synergistically with areca nut chewing in promoting oral precancerous conditions such as oral submucous fibrosis [76]. In this study, we further investigated the combined effects of smoking and areca nut chewing on microbial functional pathways and uncovered complex interactions that may destabilize the oral microbial ecosystem and contribute to disease progression. One of the most notable changes was the downregulation of the assimilatory sulfate reduction pathway in the Areca-Smoking group. This pathway is essential for converting sulfate into hydrogen sulfide (H_2S), a key intermediate in the biosynthesis of sulfur-containing amino acids. Although H_2S plays physiological roles for some oral bacteria, its accumulation in the periodontal environment is toxic, promoting inflammation, epithelial damage, and contributing to halitosis, periodontitis, and oral squamous cell carcinoma [26,77]. Thus, while reduced pathway activity may transiently limit H_2S production, such disruption may also reflect a broader imbalance in sulfur metabolism that compromises the functional capacity and homeostasis of the microbial community. In parallel, we observed a significant downregulation in the superpathway of pyrimidine deoxyribonucleoside degradation, a key contributor to amino nitrogen cycling through cytidine breakdown. This pathway supports microbial nitrogen assimilation and sustains bacterial growth under nutrient-limited conditions [78]. Impairment of this route could restrict nitrogen availability, a critical resource that shapes microbial competition, community structure, and resilience. Together, these findings suggest that the combined exposure to areca nut and tobacco smoking perturbs both the sulfur and nitrogen metabolic networks in the oral microbiome, potentially influencing oral ecological stability.

Collectively, our results highlight the complex, multifaceted effects of prolonged areca nut use and smoking on the functional potential of the oral microbiota. By modulating microbial community structure and functional pathways, these habits may influence the stability and adaptability of the oral microbiome, potentially altering host-microbiota interactions and potentially increasing the risk for oral diseases, including cancer. These findings suggest that interventions aimed at modulating vitamin levels in the oral cavity of areca nut chewers could be a promising approach to mitigate health risks. However, our analysis is based on shotgun metagenomic data and descriptive analyses of the oral microbiome. We still lack direct experimental evidence to establish a causal relationship between microbiome functional shifts, host oral vitamin status, and clinical outcomes. Further experimental validation is necessary, including host transcriptomics and metabolomics under controlled conditions, to clarify the precise role of microbial communities in host-microbe interactions and to elucidate the mechanisms underlying oral disease risk.

In addition, our current cohort has limitations. The sample size is relatively small, and all participants were recruited from a single geographic region, which may restrict the generalizability of our findings. Moreover, due to the cross-sectional study design and limited metadata, we were unable to perform stratified analyses based on the duration of areca nut chewing, and potential heterogeneity in chewing history may have

introduced additional variability. Future large-scale longitudinal or stratified studies examining the influence of chewing duration on microbial community dynamics could provide valuable insights into how long-term areca nut use progressively drives microbial dysbiosis. Such studies may offer translational potential for understanding and mitigating the detrimental ecological impact of areca nut chewing on the oral microbiome.

5. Conclusions

In this study, we performed a metagenomic analysis of a cohort of older adults to assess the effects of prolonged areca nut chewing on oral microbial composition, function, and strain-level diversity. Our results revealed significant changes in the microbial community associated with areca nut chewing. Specifically, shifts in the abundance of *Streptococcus* strains affected thiamine salvage functions within the microbial community, while variations in *Neisseria* strains influenced vitamin B12 uptake and utilization, potentially elevating cancer risk. In addition, the interaction between smoking and areca nut chewing may shape complex microbial dynamics that result in broad alterations in sulfur and nitrogen metabolic functions within the oral microbiome, with potential implications for oral health. Overall, our study provides a more comprehensive understanding of the relationship between areca nut chewing and the oral microbiota, emphasizing significant alterations in vitamin metabolism. The functional shifts in the microbial community that arise from areca nut chewing may impact host health, and interventions aimed at regulating oral vitamin levels could be helpful for maintaining overall health in individuals who chew areca nuts.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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