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Influence of betel nut chewing on oral microbiome in Papua New Guinea

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ABSTRACT

Background and objectives

For thousands of years, betel nut has been used as a psychoactive agent in Asian and Oceanian populations. Betel nut chewing was associated with the alteration of human oral microbiome and with diseases such as oral cancer and periodontitis, but only in populations of Asian cultural background. We studied the influence of betel nut chewing on the oral microbiome in Papua New Guinea, where half of the population uses betel nut and the prevalence of these diseases is one of the highest in the world.

Methodology

We characterised the oral microbiomes of 100 Papua New Guineans. We defined two cohorts of betel chewers (n=50) and non-chewers (n=50) based on a genetic approach to identify the presence of betel nut in saliva. We statistically compared the alpha and beta microbial diversities between the two cohorts. We performed linear discriminant analyses to identify bacterial species more prevalent in each cohort.

Results

We found that oral microbial diversity is significantly different between betel chewers and non-chewers. The dysbiosis observed in betel chewers, led to an increase of pathogenic bacterial species including *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*, known to be in the aetiology of periodontal diseases.

Conclusions and implications

Our study strongly supports the alteration of human oral microbiome by betel nut use, potentially leading to periodontal diseases. It also shows the need to consider local specificities (e.g. different habits, betel nut types, and oral microbial diversities) to better characterise the impact of betel nut chewing on health.

LAY SUMMARY

Betel nut is a widely used psychoactive agent, especially in Papua New Guinea, with strong deleterious impact on health. We found that betel nut chewing significantly alters the oral microbiome diversity, increasing the prevalence of bacteria associated to periodontal diseases. This effect is potentially region-specific.

KEYWORDS: Microbiome, *Areca catechu*, Oceania, Periodontal diseases, Metagenomics

INTRODUCTION

Betel nut is the fourth most common psychoactive agent worldwide after caffeine, alcohol, and nicotine [1]. This fruit of areca palm trees (*Areca* spp., *Arecaceae*) contains several alkaloids that can trigger euphoria, relaxation and improved concentration [2]. Often chewed in combination with tobacco (*Nicotiana tabacum*) and the inflorescence of betel piper vine (*Piper betle*), betel nut is used by over 600 million people, especially in South Asia and Oceania where it has a strong significance in the expression of cultural and social identities [3]. Its most ancient use is archaeologically documented in Thailand around 8,000 years ago [4]. Betel nut has long been used in traditional medicine for its potential anthelmintic effect and antibacterial activity [5]. Such a long-term use of betel nut could have influenced the evolutionary history of human populations, ultimately leading to specific biological diversities and adaptations [6,7]. However, several studies have shown its deleterious impact on human health [8]. Betel nut chewing has negative physiological effects across multiple organ systems, including the nervous, cardiovascular, gastrointestinal, respiratory, endocrine, and reproductive systems [1]. It has been associated with several pathologies such as anaemia, oral cancer and periodontal diseases [9–11].

In Papua New Guinea, betel nut chewing concerns almost half of the population, equally prevalent among men and women [12]. Locally known as 'buai' in *Tok Pisin*, betel nut (mainly *Areca catechu*) is usually chewed in combination with a vine fruit (mainly *Piper betle*, known as 'daka') and lime (known as 'kambang'), usually without tobacco [13]. While the origins of the betel nut cultivation in Papua New Guinea remains unclear, there are archaeological and genetic evidences that *Areca catechu* has been spread in Papua New Guinea over the last three millennia, probably contemporaneously to the Austronesian dispersal [14–16]. Yet, wild local substitutes, such as *Areca macrocalyx* and *Piper gibbilimum*, may have been used before [14]. Widely used in the coastal regions during the 1960s, areca palm trees are now frequently cultivated in the highlands as well [12]. It is currently grown in the rural locations and traded across the country, representing a major source of income for communities. Apart from being perceived as "green gold for grassroots" [13], betel nut is also regarded as a symbol of peace, traditionally used in peace ceremonies, bride price exchanges, and rituals to ward off evil spirits. However, betel nut chewing in Papua New Guinea has also been linked to several deleterious impact on health, such as anemia [17], reduced child birth weight [18] and the spread of tuberculosis [19]. Papua New Guinean population has currently one of the highest prevalence of oral cancer and periodontal diseases of the world [8,20].

Statistical analyses

The alpha diversity within each sample, based on the relative abundance of all genera, was estimated by the Shannon and Inverse Simpson indices using the R package *vegan* v.2-6-4 [35]. T-tests were performed to evaluate the difference of alpha diversities between Betel chewers and Non-chewers. All statistical tests are corrected for multiple testing using the Bonferroni correction (p_c).

The beta diversity between samples, based on the relative abundance of all genera, was estimated by the Bray-Curtis index using the R package *vegan* v.2-6-4. Principal coordinates analyses (PCoA) and non-metric multidimensional scaling analyses (NMDS) were computed to plot the beta diversities. PERMANOVA was performed to estimate the difference of beta diversities between the two groups (10,000 permutations). We identified microbial genera significantly more present in one of the two groups using LEfSE [36]. For each genus giving a significant result in the Linear Discriminant Analysis, t-tests were performed at the species-level between the two groups. A cladogram was computed, based on the phylogeny at the Domain, Phylum, Class, Order, Family, Genus levels from eHOMD (https://www.homd.org/ftp//phylogenetic_trees/genome).

RESULTS

Genetic identification of betel nut chewing

Among the 262 shotgun sequencing data available, we detected at least one of the plant genera characterizing betel nut chewing (*Areca* and *Piper*) for 125 individuals. It results in a ratio of 48%, close to the reported use of betel nut in the general Papua New Guinean population (around 50%) [12]. *Cannabis* (*Cannabis*) was detected in 5% of individuals which is lower than the estimation (around 29%), considering that this plant is mainly smoked (i.e. probably less DNA in saliva) and that no accurate survey was available for Papua New Guinea [12]. Tobacco (*Nicotiana*) was detected in 27% of individuals which corresponds to the expected ratio in Papua New Guinea (around 30%) [37]. Overall, our method revealed proportions of the use of these plants close to the surveys.

In order to be conservative, we defined individuals as “Betel chewers” if at least 10 fastq reads mapped to both *Areca* and *Piper*, or as “Non-chewers” if no read can be mapped to *Areca* or to *Piper*. We identified 50 individuals as “Betel chewers” out of 262. This group included 41 men and 9 women, with an average age of 36.7 years, representing all four sampling places (National Capital District, East Sepik Province, Western Province, Simbu Province). Based on these characteristics and the absence of any read mapping to either *Areca* or *Piper*, we defined a group of 50 “Non-chewers” composed by 38 men and 12 women, with an average age of 26.2 years from the four sampling places, in order to have a relative balanced

aggregation and metabolic interdependency among these three bacterial species related to the aetiology of periodontal diseases. A recent paleogenetic analysis in Europe found that *Tannerella forsythia* was drastically impacted by changes in dietary habits over the last millenium [41]. These changes accelerated with industrialisation leading to the modification of the virulence repertoire in *Tannerella forsythia* genome, especially in genes coding S-layer proteins that are key factors in the interaction between the host and the pathogen [41]. The habit of betel nut chewing in Papua New Guinea has been present since at least 3,000 years and recently increased in the last century [12]. Such a long-term and widespread use of betel nut could have modified the original diversity of the red complex as well as the evolution of its virulence repertoire, ultimately impacting the immune response of chewers. A direct impact of betel nut biochemical components on bacteria was reported [42], but we cannot discard the possibility that our results also depends on other factors linked to the habit. The hunger suppressor effect of betel nut chewing could indirectly lead to oral dysbiosis [43]. Tobacco, which was detected in some betel chewers (Table S3), could also impact the oral microbiome [44]. Our results may also have been influenced by the use of genomic datasets not primarily designed for microbiome analysis (e.g. DNA extraction was performed without bacterial cell lysis buffer), favouring the detection of Gram-negative bacterial species, which are commonly found in dysbiosis [45]. While considering these possible cofactors, our results strongly suggested that the habit of betel nut chewing causes a significant dysbiosis of the oral microbiome which could favour the proliferation of important species involved in periodontitis, a major health issue in Papua New Guinea [20].

The association between betel nut chewing and periodontal diseases has been consistently documented in many parts of the world [1,10,46,47]. However, the influence of betel nut chewing on oral microbiome is more complex to interpret globally [11]. While we observed a significantly higher alpha diversity of the oral microbiome in betel chewers, as found in Sri Lanka [23], the opposite was found in India, Pakistan and Guam [24–26]. Moreover, the bacterial taxa influenced by betel nut chewing are not similar across studies [11]. Even when comparing our results to those from Sri Lanka [23], a higher abundance of bacterial taxa involved in periodontal diseases is found but the species are not fully concordant (*Porphyromonas*, *Treponema* and *Tannerella* in Papua New Guinea vs. *Actinomyces*, *Tannerella*, and *Prevotella* in Sri Lanka)[23]. Many factors could explain these discrepancies from technical differences (ex: 16S sequencing or shotgun sequencing) to the type of sampling (ex: buccal swab or saliva sampling). Future meta-analyses would benefit from common study designs. In that respect we believe that our approach to genetically identify the plants used in betel nut chewing could be a powerful complementary tool to questionnaires to improve the cohort definition. We could not compare our method to

questionnaires on betel nut use, leading to possible pitfalls regarding the detection threshold of plant genetic markers. As it is, our method is likely very conservative, discarding several individual data. The combination of both methods could greatly improve the characterisation of betel chewers, and establish the link between the number of betel nut genetic reads in saliva and the amount of used plants. It would also be informative to characterise the correlation between the number of betel nut genetic reads in saliva and the time of the last consumption, and potentially differentiate between occasional and regular consumers. A significant improvement of our method would be to capture and to quantify the genetic signature of all plants in order to obtain a proportion of each species and to explore the quantitative nature of the effect of betel nut chewing on oral microbiome [33]. But outside this global perspective, all of these studies highlight probable regional specificities of betel nut chewing and their impact on the oral microbiome. They could be the reflect of specific human oral microbial diversities [7], different sociocultural behaviour related to betel nut chewing [1], and even different biomolecular compositions of the *Areca* nut itself [48]. Specificities within a region should also be considered. For example, in Papua New Guinea, inter-population oral microbial differences were suggested [27], different uses of betel nut were documented across cultures [12] and two types of *Areca* nuts and *Piper* fruits are used, with distinct origins [12,14] and potentially biochemical properties [49]. This complexity could lead to diverse influences of betel nut chewing on human oral microbiome within Papua New Guinea. The biocultural context thus needs to be considered to characterize the interaction between human biology and plants. Our study strongly suggested that betel nut chewing in Papua New Guinea has a detrimental impact on human oral microbiome homeostasis, which could ultimately lead to periodontal diseases. Our study has some limitations such as the absence of questionnaires on betel nut chewing habit, the moderate number of participants, and the use of a publicly available dataset not optimised for microbiome analysis. However, it paved the way towards a better understanding of the impact of betel nut on Papua New Guinean biology, and it presented a novel and complementary approach to characterise betel nut chewing that can be extended to other regions of the world. Given the difficulty to control the population's habits of betel nut chewing and the current increase of oral diseases in Papua New Guinea [50], our study advocates to limit its use and supports more education and awareness programs about the risks of betel nut consumption.

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AUTHOR CONTRIBUTIONS

N.B. and F.-X.R. designed the study. N.B. and V.A. performed the data analyses. N.B. wrote the manuscript with the contribution of all the co-authors.

CONFLICT OF INTEREST

The authors declare no competing interest.

DATA AVAILABILITY

All data used in this study have been deposited in the European Genome-phenome Archive under accession codes EGAD00001010142 (<https://ega-archive.org/datasets/EGAD00001010142>), EGAD00001010143 (<https://ega-archive.org/datasets/EGAD00001010143>), EGAD500000000050 (<https://ega-archive.org/datasets/EGAD500000000050>) and EGAD00001007783 (<https://ega-archive.org/datasets/EGAD00001007783>). All codes used in this study are available at: <https://github.com/nbrucato/Microbiome/tree/main>.

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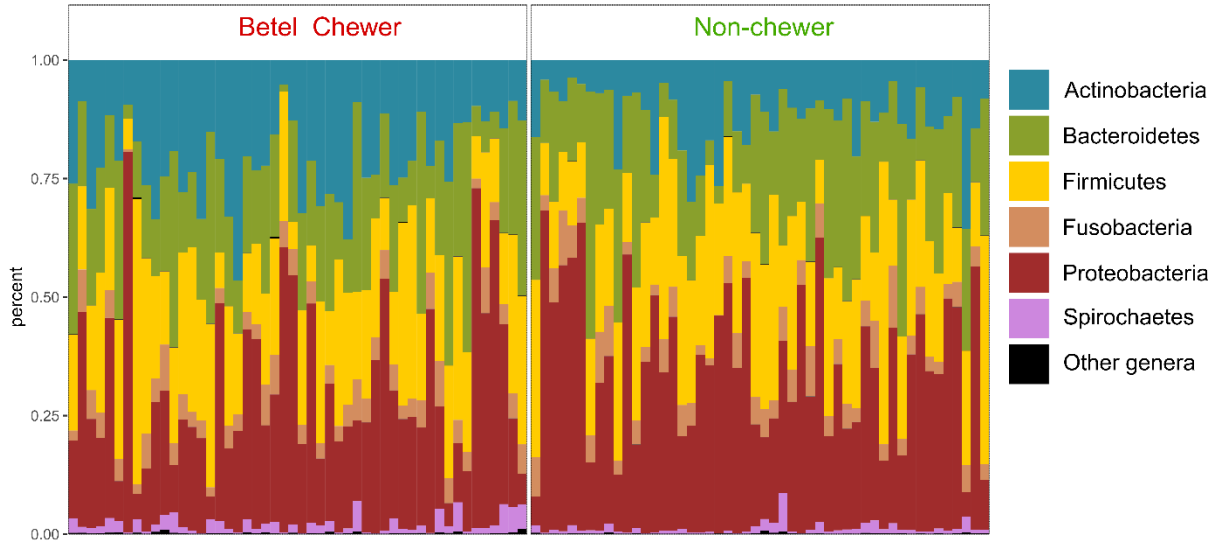
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Figure 1. Relative abundances of the six main oral microbial phyla in saliva of Betel chewers and Non-chewers from Papua New Guinea. Any other phyla are pulled together in 'Other phyla' category. Each bar corresponds to one individual.

Figure 2. Alpha and beta diversities of the oral microbiome in relation to betel chewing in Papua New Guinea. (A) Violin plot of the Inverse Simpson alpha diversity in the two groups. Stars indicate the level of significance of the t-tests (** $p > 0.01$). (B) PCoA representing the Bray-Curtis beta diversity of the cohort (principal components 1 and 3).

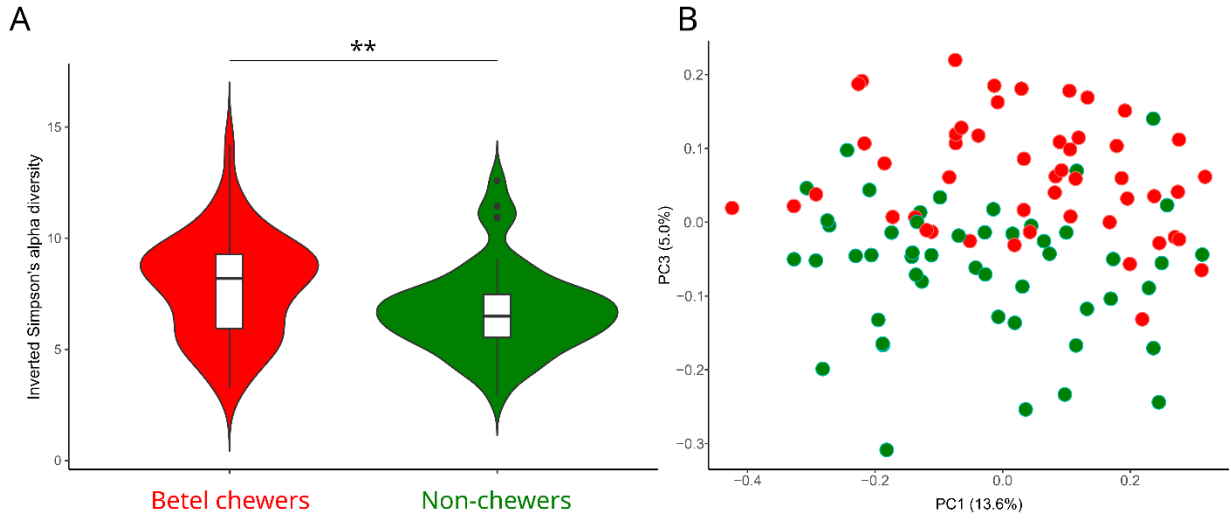
Figure 3. Influence of betel nut chewing in Papua New Guinea on specific bacterial taxa. (A) Cladogram from LEfSe analysis highlighted taxa that were significantly discriminant (LDA score >2) between the Betel chewers group, in red, and the Non-chewers group, in green. The hierarchical tree represented the taxonomy, from phyla in the innermost part of the plot to genera in the outermost part. Each circle is a member of the taxonomic level, which size was proportional to its abundance. Shading represents multiple taxa with LDA score >2 belonging to the same phylotype. a: Actinomycetaceae; b: Actinomycetales; c: Micrococcaceae; d: Micrococcales; e: Actinomycetia; f: Porphyromonadaceae; g: Flavobacteriaceae; h: Flavobacteriales; i: Flavobacteriia; j: Aerococcaceae; k: Bacillales; l: Bacilli; m: Selenomonadaceae; n: Selenomonadales; o: Veillonellaceae; p: Veillonellales; q: Negativicutes; r: Fusobacteriaceae; s: Leptotrichiaceae; t: Neisseriaceae; u: Neisseriales; v: Betaproteobacteria; w: Campylobacteraceae; x: Campylobacterales; y: Epsilonproteobacteria; z: Enterobacteriaceae; a0: Enterobacteriales; a1: Spirochaetaceae; a2: Spirochaetales; a3: Spirochaetia (B) Violin plot of the relative abundance of *Neisseria mucosa*. (C) Violin plot of the relative abundance of *Porphyromonas gingivalis*. Stars indicate the level of significance of the t-tests: **** $p = 0.0001$ and ***** $p = 0.00001$.

Figure 1



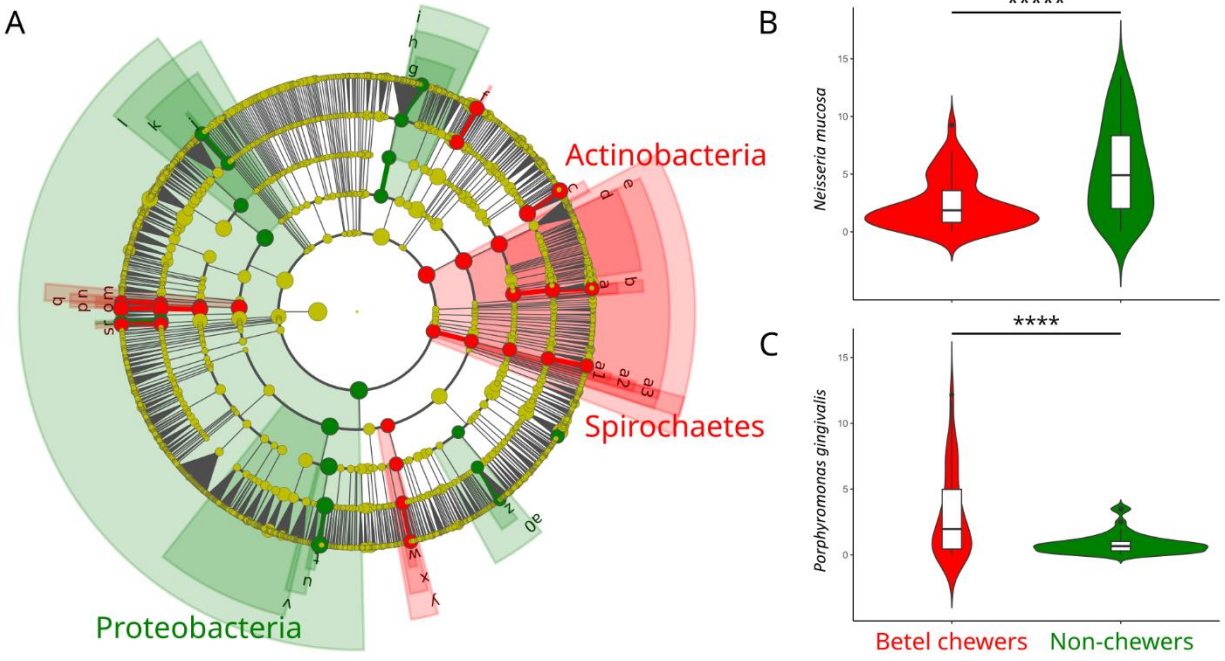
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Figure 2



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Figure 3



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