Dental calculus microbiome correlates with dietary intake

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Summary

The occurrence of dental calculus is widespread among pediatric and adult patients. It is originated by oral bacterial biofilm, but the microbial variability embedded within and whether it is influenced by host diet remain questioned.

We highlighted the presence of a significant microbiome variability, with clusters of patients characterized by specific bacterial taxonomic composition and functional capabilities, such as virulence factors. Moreover, the observed differences among groups of patients were strongly related to specific dietary intake.

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This evidence suggests that diet may influence the presence of pro-inflammatory microbiome adhered to the tooth surface, and that dental calculus microbiome may be tested to inspect oral health conditions.

Abstract

Background: Dental calculus is the result of dental plaque mineralization, originating by tooth associated bacterial biofilm. Recent evidence revealed that the dental calculus microbiome has a more complex composition than previously considered, including an unstructured mix of both aerobes and anaerobes bacteria. Actually, we lack information about the influence of host lifestyle factors, such as diet and health on this highly biodiverse ecosystem. Here, we provide a pilot study investigating dental calculus microbial biodiversity and its relation with host diet.

Methods: We collected 40 dental calculus samples during routine dental inspection; DNA was extracted and analysed through 16S amplicon sequencing, while dietary information were retrieved through a questionnaire. Associations between diet and oral bacteria taxonomy and functional pathways were statistically tested.

Results: Overall, microbiome composition was dominated by 10 phyla and 39 bacterial genera, which were differently distributed among samples. Cluster analysis revealed four main groups based on the taxonomic profile, and two groups based on functional pathways. Each taxonomic cluster was dominated by different microbial biomarkers: *Streptococcus, Rothia, Tannerella, Lautropia* and *Fusobacterium*. Bacteria genera and pathways were also associated to specific dietary element, especially vegetable and fruit intake suggesting an overall effect of diet on dental calculus microbiome.

Conclusions: The present study demonstrate that it exists an inter-variability in the microbial composition of dental calculus among individuals of a rather homogenous population. Furthermore, the observed biodiversity and microbial functions can find an association to specific dietary habits, such as a high fiber diet or a protein rich diet.

Introduction

The interaction between humans and their microbiomes has become one of the main growing interests in human health in the last few decades(Ursell, Metcalf, Parfrey, & Knight, 2012). The recent breakthrough in personalized medicine has made it possible to integrate additional data beyond host genetics or microbiome composition, such as human lifestyle data such as diet, drug use and sport activity(Flores-Pérez, de la Rosa Oliva, Argenes, & Meneses-Garcia, 2019; Malm, Jakobsson, & Isaksson, 2019). In particular, diet is known to be one of the main factors associated with microbiome compositional shifts, explaining 60% of such variations(Zhang et al., 2010). Oral tract microbiome is gaining attention as a new target of omics studies, as it can capture humans' dietary habits, immune responses, and environmental factors(Millen et al., 2022; Willis et al., 2022). Actually, the oral cavity represents one of the richest substrates for microorganisms to grow and establish complex microbial communities(Wade, 2013), directly affecting oral health state. Previous research comparing changes in microbial communities driven by diet (i.e., Mediterranean, vegan) has focused mainly on gut microbiota(Bailey & Holscher, 2018; Tomova et al., 2019). As a consequence, the influence of dietary regimen on the human oral microbiome remains largely elusive.

Recently, molecular anthropology and archaeology have greatly improved our knowledge regarding the evolution of human–microbe symbiosis during human history(Fellows Yates et al., 2021). The ability to retrieve conserved DNA from ancient samples has resulted from the analysis of tooth-associated dental calculus, a mineralized derivation of the commonly known dental plaque. Dental calculus has been demonstrated to be an extremely rich resource, harboring a variety of biological molecules including host and microbial DNA, proteins, and metabolic compounds(Radini & Nikita, 2022), which has allowed us to obtain important information regarding past populations life conditions. Recent evidence made possible to propose a new model of plaque microbiome formation revealing that there are no early and later colonizer as previously hypothesized(Li et al., 2004), but multiple bacterial species that are found concomitantly in polymicrobial aggregates harboring complex communities which forms the human plaque. Once bound to the teeth, this microbial consortia starts biofilm formation, which then engulfs the bacterial single static cells found in saliva(Simon-Soro et al., 2022). However, to date, we lack information regarding the microbial biodiversity harbored in the dental calculus, and regarding whether it presents a certain level of inter-individual variability or, conversely, a flattened microbial biodiversity. Moreover, we currently have no evidence to disclose whether lifestyle and health variables are able to shape its composition, as observed in microbiomes belonging to other body niches.

In this study, we analyzed the microbiome composition of modern dental calculus in order to define (i) the level of inter-individual variability and (ii) the role of the diet in driving its structure. To this end, taxonomic diversity and microbial community functional profiles were

investigated by applying 16S amplicon sequencing methodology to 40 omnivorous female and male subjects, ranging from 20 to 77 years of age, following a Western lifestyle. This work can be considered a pilot study testing how the dental calculus microbiome could be exploited to obtain relevant clinical information on diet effects and patient health status.

Materials and methods

Sample collection and surveys

This study was approved by the human subjects ethics board of the Hospital of Padova (Italy), with Prot. N°AOP2258, and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. Dental calculus samples were collected at the Unit of Dentistry of the Hospital of Padova (Italy) during routine inspections. Patients were informed about the study aims and provided their informed consent. A questionnaire was submitted to each patient, requesting anthropometric, lifestyle, health status, and dietary information. Regarding the clinic procedure to collect the calculus, one expert operator used a standard 5/6 stainless steel periodontal curettes with an octagonal handle (Hu-Friedy, Chicago, IL, USA). We report below the inclusion criteria applied for samples collection: (1) For all patients we collected samples always from the same location and type, which were the supragingival mineralized plaque of lower anterior teeth. These criteria were applied both for the highest presence of mineralized plaque on lower anterior teeth, and to minimize possible bias related to the oral geography(Fagernäs et al., 2022; Mark Welch, Rossetti, Rieken, Dewhirst, & Borisy, 2016). (2) Only patients who did not present any sign of current gingival inflammation, bleeding, and caries were selected. (3) All participants were evaluated during the scheduled periodontal maintenance (~ every 6 months) to assure a similar time of dental calculus formation. (4) Finally, before collecting the mineralized plaque, the patients underwent 30 seconds of mouth washing (especially in the sampling area) by using a solution of 50% water and chlorhexidine (0.2%, Meridol, Colgate Palmolive company) in order to remove bacteria adhered on calculus surface.

A published dietary survey was applied in order to collect specific data about subject diet and adherence to the Mediterranean diet(Sofi, Macchi, Abbate, Gensini, & Casini, 2014). Samples were collected in 1.5ml Eppendorf tubes and directly transferred to the biomolecular laboratory at the Department of Comparative Biomedicine and Food Science of the University of Padova (Italy).

DNA extraction, sequencing and taxonomic analysis

Genomic DNA was extracted using a Qiagen PowerSoil kit, following the manufacturer's instructions (Qiagen, Hilden, Germany). PowerSoil kit was recommended by the Human Microbiome Project (HMP) for DNA extraction in microbiome studies(Aagaard et al., 2013; Hagan et al., 2020). This kit ensures a mechanical, chemical and enzymatic cell lysis which is needed to break the mineralized matrix of dental calculus and assure a highest DNA yield. Taq Platinum HiFi (Thermofisher, USA) was used for 16S amplicon sequencing on the V3–V4 regions(Takahashi, Tomita, Nishioka, Hisada, & Nishijima, 2014) using MiSeq System (Illumina, Inc., USA). Raw reads were deposited in the BioProject database under accession number PRJNA828039.

The produced sequences were analyzed according to Callahan et al. 2016(Callahan, Sankaran, Fukuyama, McMurdie, & Holmes, 2016). Paired-end sequenced reads were filtered on the basis of their quality using the package *dada2* (v. 1.14.1) on R version 3.6.3, and then organized using Amplicon Sequencing Variants (ASV) classification approach. Taxonomy was assigned until genus level using SILVA 16S Database (NR98 version 138.1).

Analysis of microbial variability was performed using the *microbiome* (v 1.8.0) and *phyloseq* (v 1.30.0) packages. Alpha-diversity difference among clusters was assessed through ANOVA test. Finally, Kruskal–Wallis test and LDA effect size with LEfSe(Segata et al., 2011) were computed to determine significant difference at genus level among clusters.

The association between diet type and microbiome composition was checked through a redundancy analysis (RDA). A permutation analysis was computed to assess the significance of dietary variables through the *vegan* (v. 2.4-2) package in R. Finally, a Spearman test was performed to determine which and to what extent each genus-level taxon correlated with Mediterranean scores.

Additional information is reported in Supplementary Materials.

Functional analysis

To assess each sample's gene content, Picrust2 software(Douglas et al., 2020) was applied to the pre-filtered ASV sequences. Gene families and relative abundances were computed using the KEGG Ontology database(Kanehisa, Sato, Kawashima, Furumichi, & Tanabe, 2016), enabling the collapse of these functions to the pathway levels 2 and 3 (L2, L3) based on the KEGG mapping tree. Hierarchical clustering was computed and compared to the

taxonomy-based clusters using *dendextend* package v1.15.2, with the aim to understand which taxonomic clusters fell into diverse functional groups and to derive the relations between microbiome activity, microbial composition, and sample dietary information.

Additional information is reported in Supplementary Materials.

Results

Patient metadata reveal a diverse range of food consumption

Our dataset was composed of 40 samples comprising 13 male and 27 female participants of varying ages, ranging from 20 to 77 years old, with an overall mean of 48.1 (male mean age = 47.69, female mean age = 48.34) and a median of 50. The age distributions of males and females were similar to ensure that every age range and sex was well represented (Figure 1A). The majority of patients (39 out 40) were Italians, mainly from Northern Italian regions (37 out of 40, corresponding to 92.5%), with 2 of them originating from South Italy (Sicily), while the remaining participant was Spanish (Table 1).

Anthropometric information was used to obtain the body mass index (Table 1). The mean for the overall values of BMI was 23.85, which falls in the normal weight score range according to the World Health Organization (WHO) Europe standard (https://www.euro.who.int/en/). Specifically, the mean BMI of males was 25.63, which is considered slightly above the pre-obesity threshold of 25, while for females, the average BMI was 22.95, corresponding to a normal weight. Within the dataset, only two males (ID2 and ID31) showed obesity conditions, surpassing the value of 30 in the BMI index scale, and also recognizing their obesity in the survey (Table 1 and Figure 1B). There were also two cases of underweight (ID27 and ID32), both in females, presenting a BMI score lower than 19 (Table 1 and Figure 1B). Despite these specific cases, no significant difference was detected in sex distribution within the binned age groups and BMI (chi-squared test $p>0.05$).

Data regarding dietary habits showed a very homogenous profile, considering that 39 out 40 individuals were omnivores, but still presented a diverse range of food preferences and daily consumption (Supplementary Table 1). For this purpose, the Mediterranean score assignment strategy was used to define the level of each individual's closeness to the Mediterranean diet, showing different diet styles (Supplementary Figure 1).

Dental calculus microbiome composition and diversity

A total of 39 samples were selected for the microbiome composition analysis. One sample (ID40) was discarded because of a poor read count, probably due to the low quantity of extracted DNA (Supplementary Table 2). Focusing on the relative abundance of each taxon, we found 10 phyla, among which Firmicutes was found to be dominant in ~75% of the samples, followed by Actinobacteria, Bacteroidetes, and Proteobacteria (Figure 2A). Specifically, dental calculus microbiome was populated by a total of 39 genera, mainly represented by *Streptococcus*, *Tannerella*, *Fusobacterium*, *Freticobacterium*, *Porphyromonas*, *Bacteroidetes oral taxon sp.*, and *Prevotella* (Figure 2B). The core microbiota comprised 11 taxa, including *Streptococcus*, *Rothia*, *Fusobacterium*, *Campylobacter*, *Tannerella*, *Treponema*, *Porphyromonas,* and *Actinomyces*, among others (Supplementary Figure 2). In particular, *Streptococcus* showed the highest prevalence in our dataset. Furthermore, the core microbiota showed the presence of all three taxa comprising the red complex, a group of bacteria formed by *Treponema*, *Tannerella*, and *Porphyromonas*. At the same time, the accessory microbiome comprised bacteria commonly found in the oral cavity, such as *Freticobacterium*, *Abiotrophia*, and *Aggregatibacter.*

Taxonomic differences among samples are associated with different dietary habits

Intra-sample variability was compared across samples through principal coordinate analysis, showing that the microbial composition slightly changed across individuals. Four main clusters were identified through hierarchical clustering, based both on taxonomic differences and on differential abundances (Figure 3A) (C1 = 16 samples, C2 = 7 samples, C3 = 7 samples, C4 = 9 samples). The analysis of alpha-diversity revealed a high degree of similarity and higher variability of C1 and C2 compared to C3 and C4, which were also similar in composition to each other (Supplementary Figure 3). The prevalence of *Fusobacterium*, *Tannerella*, *Rothia*, *Streptococcus*, and *Lautropia* spp. significantly varied across the clusters. These genera fell into five of the main phyla composing the oral microbiome (Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, and Actinobacteria), accounting for most of the diversity of whole microbial communities. In particular, *Fusobacterium* and *Tannerella* were both significantly enriched in C4, while *Streptococcus*, *Rothia*, and *Lautropia* were respectively enriched in C1, C2, and C3 (Figure 3B and Supplementary Figure 4).

According to RDA, we found that dietary variables (type of food consumed) were significantly associated with the taxonomic composition of our dataset. Specifically, legumes,

fruit, vegetables, and fish were associated with C1; cereals pointed to C3; milk, meat, and their derivatives had a stronger association with C4 (Figure 3C). Furthermore, groups related with higher consumption of fibers (vegetables, fruit) were also enriched with the *Streptococcus* genus, while C3 and C4, which were mainly associated with *Tannerella* and *Porphyromonas*, were characterized by diet rich in animal proteins (e.g., milk and meat) and higher cereal consumption.

Finally, we did not detect any relevant associations between the Mediterranean score (based on the overall food consumption) and the taxonomical and clustering data (Supplementary Figure 5). However, we checked for correlations between the Mediterranean score and each bacterial genus, and we found that Mediterranean values negatively correlated with *Corynebacterium* and *Conchiformibius*, while they positively correlated with *Eikenella*, *Prevotella*, and *Lentimicrobium* (Figure 3D).

Functional analysis and microbiota pathways

Cluster analysis obtained on the KEGG profile revealed potential associations between diet type and bacterial functions. We observed two functional clusters (FCs) according to gap statistics: FC1 was composed of 20 individuals, while FC2 included 19 individuals. Notably, FC1 was mostly composed of the taxonomy-based C1, while FC2 mainly included components belonging to C2, C3, and C4 (Figure 4A and B). The two functional clusters were differentially enriched by important pathways involved both in catabolic and anabolic processes, allowing a possible reconstruction of the different functions of microbes ascribed to the two groups. Specifically, glycan, carbohydrate, and nucleotide metabolism were enriched in FC1, while FC2 was mainly characterized by amino acid, lipid, and transport metabolism (Figure 4C). In addition, level 3 KEGG categories allowed us to gain further details on the differential enrichment of many pathways (Supplementary Figure 6). For instance, FC1 was enriched in starch and sucrose metabolism, as well as amino sugars, nucleotide sugars, galactose, fructose, and mannose metabolism, which may suggest a more carbohydrate- and fiber-oriented diet, while FC2 was mostly characterized by metabolism of amino acids such as histidine, alanine, aspartate, and glutamate, pointing to a high-protein diet, and further supporting the information retrieved from the taxonomic analysis.

Discussion

The present study shows that the microbiome embedded in the dental calculus falls within the known variability for the oral microbiota. Indeed, the taxonomic composition comprised 10 out of the 13 phyla commonly belonging to the oral microbiome signature(Dewhirst et al., 2010) as well as a conspicuous number of representative genera (39). Among these, it is interesting to observe the concomitant presence of typical commensals, mainly associated with a healthy core microbiota (e.g., *Streptococcus*, *Fusobacterium*, *Rothia*, and *Actinomyces*) and of taxa associated with a higher risk of developing periodontal diseases, such as *Treponema*, *Tannarella*, and *Porphyromonas*, being flagged as microorganisms of the red complex(Socransky, Haffajee, Cugini, Smith, & Kent, 1998). Intriguingly, all these pathogens were found to be highly represented across our samples, making them relevant members of the overall core dental calculus microbiome. The first aim of this study was to investigate the level of inter-variability in the microbial composition of dental calculus among individuals of a rather homogeneous population. The identification of four taxonomy-based clusters was indicative of a diverse microbiota profile among samples. The second element we wished to address in this study was whether the observed inter-variability had any association with specific dietary habits. Indeed, we found that a diet rich in viscous fibers, such as those in fruit, vegetables, and legumes, was significantly associated with C1, which was mainly represented by the *Streptococcus* genus. These specific types of fiber constitute part of the healthy Mediterranean diet, and are characterized by a low saturated fatty acid content, low glycemic indices(Jenkins et al., 1981), and a high concentration of polyunsaturated fatty acids such as omega-3 (PUFA). Furthermore, their composition includes vegetal proteins and a wide range of functional metabolites able to reduce the postprandial increase in blood glucose and to reduce LDL cholesterol(Brown, Rosner, Willett, & Sacks, 1999; Jenkins et al., 1978). The high presence of *Streptococcus* among the subjects belonging to the enriched vegetable and legume consumption group (i.e., C1), might be associated with the ability of different *Streptococcus* species to express amylase-binding proteins, which would bind to host salivary α-amylases and thus exploit dietary starch for their nutrition(Haase et al., 2017). It has been widely demonstrated that a high-fiber diet benefits the proliferation of many species of microorganisms, resulting in high microbial diversity(Makki, Deehan, Walter, & Bäckhed, 2018). Conversely, high consumption of meats, cured meats, and dairy products, which are considered signatures of a Western-based diet, is associated with higher caloric content, increased amino acid intake, and a reduction in microbial biodiversity(Statovci, Aguilera, MacSharry, & Melgar, 2017). In this study, such nutrients were mostly associated with C4 and, to a lesser extent, with C3, which were characterized by reduced biodiversity and a moderate association with some opportunistic pathogens, such as *Fusobacterium*, *Porphyromonas*, and *Treponema*(Han, 2015; Tan et al., 2014). Remarkably, the microbiota diversity of C1 and C2 was significantly higher compared to that of C3 and C4. These data seem to fit the cited association model of higher fiber intake with increased microbiota richness and higher meat consumption with decreased biodiversity(Wastyk et al., 2021). As for C2, this category can be considered an admixture of the characteristics of C1 and the other groups, as it does not show signs of strong associations with a particular diet.

Interestingly, the functional analysis showed a significant association of FC1 with carbohydrate metabolism, terpenoid metabolism, and glycan pathways; thus, it is in accordance with the collected dietary information. Conversely, FC2 was characterized by enriched amino acid metabolism, energy-related pathways, and lipid metabolism. Such characteristics reflect and partially corroborate the statements made for the taxonomy-based clusters. Indeed, considering that FC1 mainly overlaps with C1, these data support the effects observed on the taxonomic level by diet: the higher carbohydrate, glycan, and terpenoid metabolism could be explained by the association with a vegetable- and fruit-rich diet, which characterized ~65% of FC1. In particular, fibers and glycans, highly present in a vegetable-based diet, are considered to play an important role in shaping oral microbiota metabolism(Koropatkin, Cameron, & Martens, 2012), producing an increase in all the carbohydrate processing pathways, such as fructose, galactose, and mannose metabolism, all of which were enriched in FC1. However, FC2, showing a heterogeneous sample composition comprising ~55% of both C3 and C4, reflected amino acid and fatty acid pathway enrichment. By way of a deeper assessment, regarding L3-associated pathways, it was possible to observe an enrichment in FC2 of several pro-inflammatory pathways. Indeed, a number of virulence factors, such as bacterial motility proteins, cell motility and secretion, and bacterial chemotaxis(Josenhans & Suerbaum, 2002), were increased within this group, along with antibiotic biosynthesis (i.e., Novobiocin), xenobiotics metabolism(Johnson, Patterson, Idle, & Gonzalez, 2012), and glutathione metabolism(Smirnova & Oktyabrsky, 2005), which help in adaptation to stress. Other signals of possible dietary connection with the dental calculus microbiome composition came from the correlation of the value of adherence to the Mediterranean diet with some of the genera found in our cohort. Particularly, we suggested that *Prevotella*, *Eikenella*, and *Lentimicrobium* seem to be positively associated with greater adherence to a Mediterranean diet. This result is in accordance with what has already been observed in other studies in the positive selection exerted by high-fiber and -vegetable diets on *Prevotella* spp. and *Eikenella*(Hansen et al., 2018; Kovatcheva-Datchary et al., 2015).

In conclusion, this pilot study shows that the dental calculus microbiome accounts for a very biodiverse environment and that it is specific of the oral cavity. Moreover, our results suggest that its composition and gene contents may be driven by specific dietary elements in the studied population. This is, to our knowledge, the first evidence of an association between the dental calculus microbiome and food consumption. However, further analyses are needed to corroborate this association. Specifically, our study is the first to consider dental calculus variability in relation with specific dietary information, thus we were not able to compare our results with external cohorts. Moreover, future analyses should consider the application of longitudinal protocols assessing the association between different dietary regimens (e.g., vegan or vegetarian) and calculus microbial composition across time and seasons, as well as focus on the influence of specific dietary macronutrients (e.g., carbohydrates) on bacterial composition (Millen et al., 2022). Finally, the results presented here suggest the possibility for a future adoption of dental calculus microbiome as signature of individual nutrition, even on ancient populations.

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Authors Contribution

G. I., performed the bioinformatics analysis and drafted and critically revised the manuscript. MEM and ES, contributed to results discussion and critically revised the manuscript. AdF, contributed to design the project, supervised sample and questionnaire collection, and critically revised the manuscript. AQ, conceived the study, performed DNA extraction, supervised the statistical analysis and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

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Conflicts of interest

The authors declare no conflict of interest.

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Figure and Table caption

Table 1. Dataset information about sample age, sex, weight (kg), height (cm), body mass index (BMI), place of birth, and geographical location.

Sample-ID	Age	Sex	Weight (cm)	Height (cm)	BMI	Location
ID1	77	f	78	170	27	North Italy
ID2	67	m	95	170	33	North Italy
ID3	63	m	66	170	23	North Italy
ID15	39	m	83	182	25	North Italy
ID16	50	f	68	155	28	North Italy
ID17	39	f	67	173	22	North Italy
ID18	53	f	75	160	29	North Italy
ID19	50	m	75	174	25	North Italy
ID20	34	f	55	170	19	North Italy
ID21	44	f	53	163	20	North Italy
ID22	50	f	57	166	21	Spain
ID23	58	f	57	160	22	North Italy
ID24	34	f	59	166	21	North Italy
ID25	54	f	63	158	25	North Italy
ID26	21	m	80	175	26	North Italy
ID27	25	f	49	167	18	North Italy
ID28	29	f	55	160	21	North Italy
ID29	57	f	68	168	24	North Italy
ID30	45	f	67	161	26	North Italy
ID31	62	m	110	180	34	North Italy

ID32	60	f	50	168	18	North Italy
ID33	63	m	65	173	22	North Italy
ID34	38	m	87	187	25	North Italy
ID35	39	f	72	162	27	North Italy
ID36	50	m	85	185	25	North Italy
ID37	56	f	58	160	23	North Italy
ID38	62	f	48	150	21	North Italy
ID39	20	m	62	180	19	North Italy
ID40	29	f	56	175	18	North Italy
ID41	62	f	62	164	23	North Italy
ID42	46	f	58	160	23	North Italy
ID43	54	f	70	160	27	North Italy
ID44	70	m	80	168	28	North Italy
ID45	45	f	60	168	21	North Italy
ID46	63	m	63	170	22	North Italy
ID47	54	f	65	172	22	North Italy
ID48	40	m	80	170	28	South Italy
ID49	28	f	57	170	20	South Italy
ID50	50	f	58	158	23	North Italy
ID51	37	m	70	178	22	North Italy
ID52	52	f	59	157	24	North Italy

Figure 1. A. Pie charts showing the dataset composition in terms of age and sex variables. Age was binned using 10-year ranges. **B.** Body mass index distribution. Dashed lines indicate BMI ranges according to WHO Europe: the green line indicates the lower limit for a normal-weight BMI, while the orange line represents the upper limit of the same; the red line indicates the limit between overweight and concrete obesity.

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ID21	44	f	53	163	20	North Italy
ID22	50	f	57	166	21	Spain
ID23	58	f	57	160	22	North Italy
ID24	34	f	59	166	21	North Italy
ID25	54	f	63	158	25	North Italy
ID26	21	m	80	175	26	North Italy
ID27	25	f	49	167	18	North Italy
ID28	29	f	55	160	21	North Italy
ID29	57	f	68	168	24	North Italy
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ID51	37	m	70	178	22	North Italy
ID52	52	f	59	157	24	North Italy

Figure 2. Stacked barplots showing phylum (**A**) and genus (**B**) distribution.

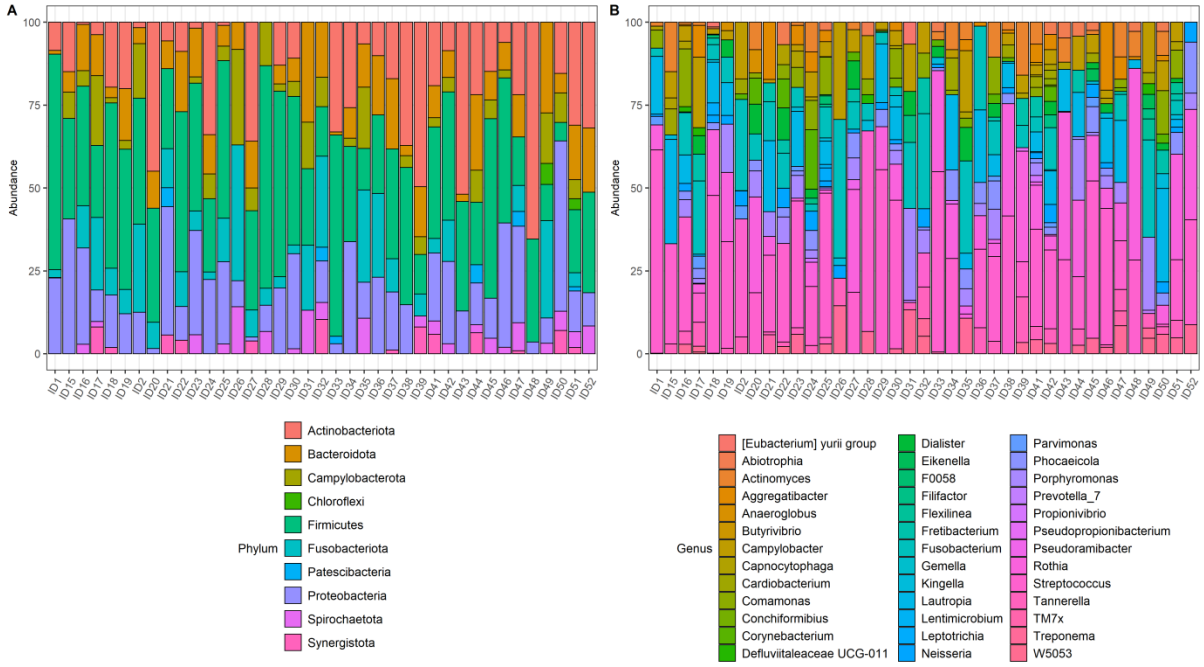

Figure 3. A. Hierarchical tree based on the Bray–Curtis distance matrix. Clusters were calculated by 500 bootstraps. **B.** Linear discriminant analysis (LEfSe) results showing significant bacterial genera for each cluster. **C.** Redundancy analysis (RDA) triplot showing relationships between sample taxonomical distances coloured by cluster, genera relative abundances, and food metadata categorical variables. **D.** Spearman correlation between all genus-level taxa and the Mediterranean score.

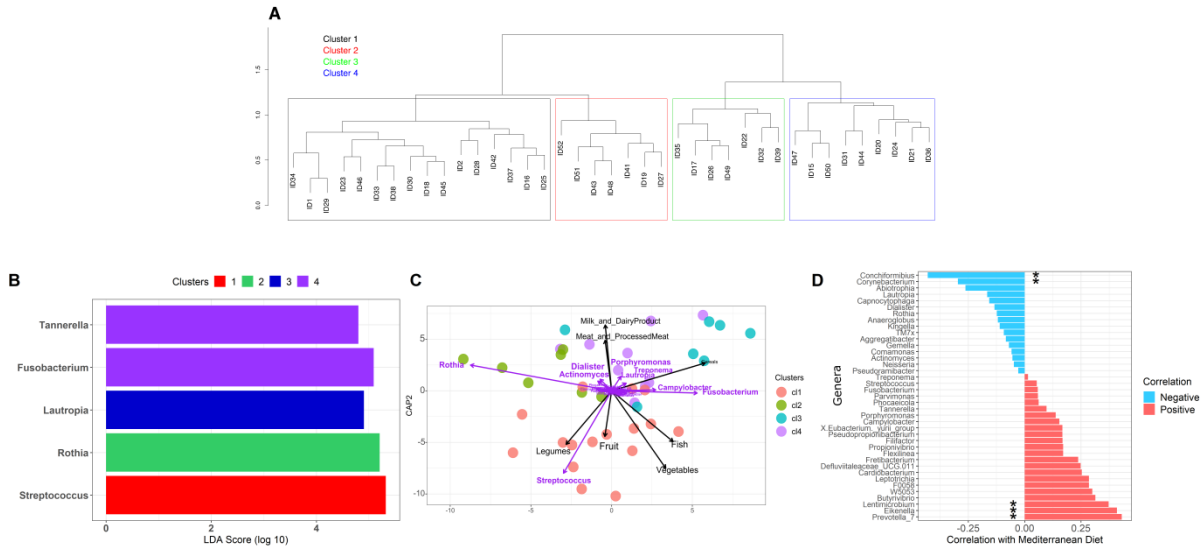

Figure 4. A. Comparison between taxonomy-based (on the left) and functional-based (on the right) dendrograms. **B.** Pie charts showing the distribution of each taxonomy-based

clusters (C1–4) inside the two functional-based clusters (FC1,2). **C.** Linear discriminant analysis using L2 KEGG ontology categories between the two functional clusters.