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Evolution of oral microbiota during adolescence – a longitudinal study from Finland

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Abstract

Background: Obesity is increasing globally among children and adolescents. Saliva microbiota is being studied as an alternative to gut microbiota due to its links to diseases, including obesity, though findings are inconsistent. We conducted a follow-up study to examine changes in saliva microbiota composition among adolescents with stable weight compared to those with increased weight.

Methods: We analyzed saliva samples from 440 adolescents in the Finnish Health in Teens cohort at ages 11.7 and 14.2, using 16S rRNA gene sequencing. Adolescents were grouped by BMI based on International Obesity Task Force criteria. We examined differences in alpha diversity, beta diversity, and composition between children who stayed in the same BMI category and those who increased their BMI.

Results: The core microbiota composition varied between two timepoints, with significant alterations in adolescents with persistent overweight or obesity. Specifically, the genera *Haemophilus* and *Neisseria*increased in these individuals. The microbiota remained more stable in adolescents who were previously normal weight, even if they gained weight.

Conclusions: Our findings suggest that saliva microbiota evolves during adolescence. The changes in saliva microbiome with time were more pronounced in those with persistent overweight/obesity, pointing towards chronic, low-grade inflammation and metabolic dysregulation.

Introduction

The oral cavity hosts one of the richest microbial ecosystems in the human body, which is acquired during childhood and influenced by oral diseases, oral hygiene habits, and various lifestyle choices [1]. However, the development and changes over time within this ecosystem are not well understood, despite their potential to provide insights into human health and well-being [2]. Current evidence is based mainly on cross-sectional studies comparing the oral microbiome in children of different ages suggesting that the microbiome matures with age [3,4]. During the puberty, oral microbiome composition is distinct from that of adults, and recent reviews suggest that the primary succession of the oral microbiome continues until young adulthood [5]. This age dependency likely results from changes in dentition and the development of both immune and endocrine systems.

Some studies indicate that the oral microbiome remains relatively stable over time, with core microbiota preserved despite events such as tooth eruption, travel, and illness [3,4]. A longitudinal study of only five individuals showed changes in microbial profiles over time [6], with intra-individual variation at the strain level generally being smaller than differences between individuals. Another study found that while alpha diversity varied between months, beta diversity remained stable in 10 individuals throughout a one-year period [7]. The only exception was bacterial load, indicated by 16S rRNA gene copy numbers, peaked at the end of the winter months [7]. However, weight changes or metabolic alterations among participants were not monitored in previous studies, even though these could interact the oral microbiome.

Understanding short-term changes in the oral microbiome can provide valuable insights into pathways leading to microbiome-related conditions like obesity [8]. Our study aimed to describe longitudinal changes in saliva microbiota composition between two timepoints in a population-based sample of over 400 Finnish adolescents. We investigated whether the bacterial microbiota from the first timepoint could predict short-term weight changes; and explored intra-individual alterations of salivary microbiota in adolescents with a stable weight status, and in adolescents whose weight category changed during the follow-up time.

Results

Background characteristics

The mean (SD) age for the participants at baseline was 11.6 (0.3), and at follow-up was 14.2 (0.3) years. The mean follow-up time was between 2.3 (0.8) years. Majority of participants had a normal weight, while 19–20% lived with overweight/obesity at both time point. The frequency of central obesity was 10%. All children with central obesity also belonged to the overweight/obesity BMI category at both timepoints. The background characteristics of the participants are shown in Table 1.

Table 1
Background characteristics of the participants.

Sex, n (%)	Baseline (n = 440)	Follow-up (n = 473)
Girls	244 (55.5)	259 (54.8)
Boys	196 (44.5)	214 (45.2)
Age, years[1]	11.6 (0.3)	14.2 (0.3)
Follow up time, years[2]		1.69–2.96
BMI categories (%)		
Underweight (UW)	57 (12.9)	38 (8)
Normal-weight (NW)	300 (68.1)	340 (71.8)
Overweight/Obesity (OW)	83 (18.8)	95 (20)
Central Obesity (%)		
Without central obesity (nonCO)	391 (88.8)	423 (89.4)
With central obesity (CO)	45 (10.2)	50 (10.5)
[1] mean (SD)		
[2] range		

Alpha-diversity

Alpha diversity – Shannon index and Inverse Simpson index – did not differ between genders (Wilcoxon test; Shannon index p = 0.97; Inverse Simpson p = 0.73) suggesting that the genders could be analyzed together.

Overview of baseline and follow-up microbiota abundance

The baseline core saliva microbiota at the phylum level consisted of Firmicutes (51.7%), Bacteroidetes (18%), Proteobacteria (15.5%), and Actinobacteria (8.1%), as previously reported [9]. Corresponding microbiota composition at the follow-up was Bacteroidetes (28.7%), Firmicutes (23.9%), Proteobacteria (20.7%), and Actinobacteria (18.1%).

Figure 2 illustrates the distribution of microbiota at the genus level with relative abundance across different BMI categories (UW, NW, OW) and central obesity status (CO vs nonCO) at both baseline and follow-up.

Compositional differences in follow-up microbiota by obesity measures

The baseline differences in microbiota composition based on BMI are previously reported [9]. Here, we observed no differences in microbiota composition between central obesity categories at baseline (p_{corr} > 0.9). We investigated compositional differences between BMI/central obesity categories at the follow-up timepoint.

Composition analysis suggested the relative abundance of *Prevotella 7*, *Rothia*, and *Veillonella* differed between the BMI categories (Fig. 2), with *Veillonella* showing the largest difference between the categories (1.9% at UW, 3.9% at NW, 16.5% at OW).

Post hoc, group-wise comparisons confirmed the *Veillonella* composition to be higher in OW category than in UW category (p_{corr} = 0.056, fold change = 0.36). Additionally, the relative abundance of Actinobacteriota at the phylum and class levels was lower in

OW category (p_{corr} = 0.077, fold change = -2.54) compared with UW (Table 2).

Table 2
Differences in microbiota between UW (n = 38) vs OW (n = 95) groups at follow-up.

Taxon	Fold Change	P	P_{corr} (FDR)
Bacteria_Firmicutes_Negativicutes_Veillonellales.Selenomonadales_Veillonellaceae_Veillonella	0.36	0.005	0.056
Bacteria_Firmicutes_Negativicutes_Veillonellales.Selenomonadales_Veillonellaceae	0.36	0.006	0.056
Bacteria_Firmicutes_Negativicutes	0.40	0.008	0.056
Bacteria_Firmicutes_Negativicutes_Veillonellales.Selenomonadales	0.40	0.008	0.056
Bacteria_Actinobacteriota	-2.54	0.01	0.077
Bacteria_Actinobacteriota_Actinobacteria	-2.55	0.01	0.077

When comparing the differences between central obesity groups, the abundance of *Veillonella* was noted to be twice as high in CO compared to non-CO (11.8% vs. 5.6%). In group-wise comparisons the relative abundance of *Rothia* (p = 0.04; fold change = −1.88) was lower in the CO category compared to nonCO category at all taxonomic levels. However, these differences were not retained after FDR correction (p_{corr} > 0.1).

Alterations in the composition of the microbiota during the follow-up period according to changes in obesity metrics

We investigated whether the microbiota composition changed within the same individuals over time. This analysis was segmented by changes in BMI/central obesity categories.

Firstly, we compared the microbiota of the participants who remained in the NW category from baseline until follow-up. They presented no changes in microbiota composition between the two timepoints. Initially, we observed an increase in Firmicutes, at the phylum and class level, where bacterial class of *Lactobacillales* appeared increased, but not after applying FDR correction (p_{corr} > 0.19). Participants who experienced weight gain from normal weight to overweight/obesity showed no change in microbiota between the two timepoints either (p_{corr} > 0.7).

Next, the microbiota of the participants with stable OW were compared at two timepoints. These participants experienced an increase in the abundance of *Haemophilus* (p_{corr} = 0.052; fold change = 1.75) at all taxonomic levels. Correspondingly, *Neisseria* increased during the follow-up, but only at the family level (p_{corr} = 0.1, fold change = 1.62) (Table 3).

Table 3
Alterations in microbiota with time in participants with stable overweight/obesity

BL OW vs FU OW (n = 59)			
Taxon	**Fold Change**	**P**	**P_{corr} (FDR)**
Bacteria_Proteobacteria_Gammaproteobacteria_Enterobacterales_Pasteurellaceae_Haemophilus	1.75	0.004	0.052
Bacteria_Proteobacteria_Gammaproteobacteria_Enterobacterales_Pasteurellaceae	1.78	0.007	0.052
Bacteria_Proteobacteria_Gammaproteobacteria_Enterobacterales	1.78	0.007	0.052
Bacteria_Proteobacteria_Gammaproteobacteria	1.78	0.01	0.052
Bacteria_Proteobacteria	1.78	0.01	0.052
Bacteria_Proteobacteria_Gammaproteobacteria_Burkholderiales_Neisseriaceae	1.62	0.02	0.10

The category with persistent central obesity (CO) showed a decrease in *Neisseriaceae* at family level (p_{corr} = 0.04, fold change = -1.84) between the two timepoints (Table 4). Interestingly, participants who recovered from central obesity during follow-up time also showed an increase in Actinobacteriota at phylum (p_{corr} = 0.05, fold change = 1.57) and class level (p_{corr} = 0.005, fold change = 4.80) (Table 4).

Table 4
Alterations in microbiota with time in participants with a) stable central obesity and b) decreased central obesity.

a) BL CO vs FU CO (n = 21)			
Taxon	**Fold Change**	**P**	**P_{corr} (FDR)**
Bacteria_Proteobacteria_Gammaproteobacteria_Burkholderiales_Neisseriaceae	-1.84	0.003	0.048
Bacteria_Proteobacteria_Gammaproteobacteria_Burkholderiales	-1.81	0.004	0.048
b) **BL CO vs FU nonCO (n = 24)**			
Taxon	**Fold Change**	**P**	**P_{corr} (FDR)**
Bacteria_Actinobacteriota_Actinobacteria	4.80	0.0002	0.005
Bacteria_Actinobacteriota	1.57	0.006	0.05

Baseline microbiota composition was not related to short-term weight changes (**Supplementary material**).

Discussion

The key findings of our longitudinal study in 440 adolescents, conducted approximately two years apart, revealed significant compositional alterations saliva microbiota in individuals with persistent overweight status. Specifically, the abundance of *Haemophilus* and *Neisseria* increased during the follow-up time. In contrast, the microbiota composition remained stable in adolescents who were previously normal-weight, even if they experienced weight gain. As changes in microbiota were observed also in participants with persistent central obesity, we assume that prolonged inflammation may drive these alterations.

Initially, among the 11-year-old participants, the core microbiota was predominated by six genera (in descending order): *Veillonella, Prevotella 7, Streptococcus, Neisseria, Rothia*, and *Haemophilus* that together accounted for 80% of the total abundance. After two years, the order of these genera was altered: *Prevotella 7*, *Rothia*, *Haemophilus*, *Neisseria*, *Veillonella*, and *Streptococcus*, and together they accounted for 60% of the overall abundance, implying that some evolution concurred with time in the group level. This is a unique finding, as there are only a few longitudinal studies describing temporal changes in saliva microbiota composition in adults [6, 7], but none concerning this age group. However, the evolution of saliva microbiota is assumed to continue until the permanent dentation [5] which is implied by cross-sectional studies as well [3, 4].

Surprisingly, none of the genera were consistently linked with overweight or obesity at either timepoint, but the genus *Veillonella* caught our attention. Previously, *Veillonella* was reported to be less abundant in 11-year-old participants with obesity compared to those with normal-weight [9]. However, in the same individuals at 14 years, a higher abundance of *Veillonella* was noted in the OW category compared to the UW category. Others have demonstrated *Veillonella* spp. (especially *V. parvula*) to associate with caries among children [27]. Further evidence shows that *V. denticariosi* occurs only in diseased sites, whereas *V. rogosae* is found only in healthy plaque [28, 29]. Here, we did not include data on oral health, but in our previous study, we have reported an association between caries experience and development of overweight [30] suggesting that caries experiences occur prior to weight development, which could explain the higher *Veillonella* abundance observed here.

Additionally, Actinobacteriota showed a lower abundance at both the phylum and class levels in the OW category compared to the UW category in adolescence. Of the phylum Actinobacteriota, genera *Actinomyces, Rothia*, and *Bifidobacterium* are commonly observed in the oral cavity, and their lower abundance has been associated with obesity in previous studies in children [31–33]. In

fact, in a study by Coker et al., *A. odontilyculus* was inversely associated with various obesity outcomes i.e., higher fat mass, BMI z-score and presence of overweight. Similarly, the abundance of *Rothia* is reported to be lower in individuals living with obesity [34]. Moreover, *R. mucilaginosa* and *R. dentocariosa* were inversely associated with several obesity outcomes in children [33].

Previous studies have identified several unique associations between oral microbiota and weight outcomes in children [33, 35, 36]. However, these results have been rarely replicated in other studies, likely due to variations in geographical location, participant age, microbiota analysis methods, obesity measures, among other things. We were unable to replicate the previously reported differences in microbiota composition between weight categories, as reported [9] among the same individuals after two years. Additionally, we could not predict short-term weight changes with initial microbiota composition (**Supplementary material**). Therefore, we believe the microbiota signals related to excess weight are not robust as suggested before [37]. On the other hand, multiple obesity phenotypes with varying pathophysiology [38] could explain the varying microbiota signals. A follow-up study involving the same individuals enables the tracking of intra-individual patterns over time. Since changes occurred only among those with persistent overweight, this suggests that metabolic abnormalities affect the oral microbiota evolution.

An increase in the abundance of *Haemophilus* and *Neisseria* was observed among adolescents with persistent overweight in a longitudinal setting. While *Haemophilus* is typically regarded as commensal, meaning it coexists with the host without causing harm, certain *Haemophilus* species can also serve as opportunistic pathogens, promoting infections beyond the oral cavity. In our pilot study of 50 adolecents using shotgun metagenomic sequencing, we found *Haemophilus* genus, particularly *H. parainfluenzae*, was the second most common species in the saliva of adolecents, and positively associated with sugar intake [39]. Correspondingly, the genus *Neisseria* is a commensal inhabitant in the mouth, where certain species are thought to play a role in maintaining oral health, potentially by preventing the colonization of harmful bacteria [40–42]. As we do not have species level information, we are not able to evaporate the clinical relevance of our findings.

Interestingly, a reduction in the abundance of the *Neisseria* family was observed among participants with persistent central obesity, which may contrast with our findings based on BMI categories. The groups with persistent BMI-based overweight and persistent central obesity overlapped, and the number of participants was halved for the central obesity group. These groups, representing distinct obesity phenotypes as previously discussed, might account for the observed decrease in family-level abundance while permitting an increase in certain genera of *Neisseria*.

Finally, we marked a higher abundance of Actinobacteriota at the phylum and class level in participants who recovered from the central obesity during the follow-up. In the gut, the genus *Actinobacteria* has been associated with leanness in children [43], and may have a similar association in the mouth as well. The finding implies that the microbiota alterations may be more pronounced when based on waist circumference than BMI. Waist-to-height is considered a superior indicator of obesity-related health risks than BMI [44–46], which may partly explain the different outcomes observed here. Still, this finding supports the role of low-grade inflammation driving microbiota changes, as we can assume that the inflammatory status was alleviated when the waist circumference normalized. More in-depth sequencing and additional biomarkers could reveal the precise mechanism involved in microbiota modification.

A key strength of our study is the longitudinal study design with large sample size, using the same sequencing protocol. However, the study is limited by the ability to measure microbiota only at the genus level. More comprehensive sequencing, such as shotgun sequencing, would have enabled species-level identification and functional annotation of the microbiota. We addressed technical variation in our sequencing by incorporating it as a confounding factor in our analysis, along with age, which can be seen as a strength. We relied on self-reported anthropometric data at follow-up; while self-reports are recognized as a valid method in epidemiological studies [47], some may view this as a limitation. Factors such as puberty, lifestyle choices like physical activity, and diet were not addressed, as we focused to reveal among who and how oral microbiota evolved during the follow-up period. The relative nature of our microbiota data poses another limitation, particularly in a longitudinal context.

Conclusion

We analyzed the saliva microbiota composition in over 400 Finnish adolescents at the ages of 11 and 14. Overall, minor alterations were detected in the microbiota composition with time. The most notable changes were observed in adolescents with persistent overweight or central obesity, suggesting that inflammation might be influencing alterations in microbiota composition.

However, these changes were not fully consistent, indicating variability in obesity-related signals within the saliva microbiota. Further research employing advanced sequencing techniques and additional biomarkers is needed to comprehend the underlying mechanisms.

Methods

Data source

This study utilized material from the Finnish Health in Teens (Fin-HIT) study, a prospective cohort initially consisting of over 11,000 Finnish children. The cohort is described in detail elsewhere [10,11]. The baseline data collection, which included self-reported health behaviors, saliva sampling, and anthropometric measurements, was conducted in 2011–2014 in schools under supervision. In 2015–2016, 53% of the participants took part in the follow-up at their homes.

Saliva samples

Children provided unstimulated saliva samples using the Oragene® DNA Self-Collection Kit (DNA Genotek Inc., Ottawa, ON, Canada) [12]. The saliva samples were mixed with a stabilizing reagent within the collection tube and stored at room temperature until the analysis [13].

Anthropometry

Anthropometric measures, including height, weight, and waist circumference, were obtained as previously described [10]. For this study, we used two obesity measures: BMI (kg/m^2) and waist-to-height ratio. Initially, children were categorized into four BMI categories: underweight, normal-weight, overweight, and obesity based on the International Obesity Task Force cut-offs [14]. To ensure adequate sample size, the overweight and obesity categories were combined, leaving us with three categories: underweight (UW), normal-weight (NW), and overweight/obesity (OW).

Waist-to-height ratio (WHtR) was used to define central obesity. The participants were divided into two categories: with central obesity (WHtR > = 0.5; CO), and without central obesity (WHtR < 0.5; nonCO).

We monitored the changes in BMI categories between the baseline and the follow-up. Consequently, participants were classified as having increased, decreased, or stable weight status between the two time points (**Figure 1a-c**). A similar classification was applied for central obesity as well (**Figure 1d-e**). At baseline, no participants moved from UW to OW category at follow-up or vice-versa.

DNA Extraction and Library Preparation

The baseline saliva samples were sequenced in 2015, and the follow-up saliva samples in 2018. The procedure for 16S rRNA gene sequencing was the same both times and is detailed elsewhere [9]. In brief, intensive lysis and mechanical disruption protocol of microbial cells were performed, and genomic DNA was extracted using a CMG-1035 saliva kit and Chemagic MSM1 nucleic acid extraction robot (PerkinElmer) [9]. The V3–V4 variable regions of the 16S rRNA gene were amplified with primers [S-D-Bact-0341-b-S-17 (5' CCTACGGGNGGCWGCAG 3') and S-D-Bact-0785-a-A-21 (5' GACTACHVGGGTATCTAATCC 3')] [15]. The Truseq (TS)-tailed 1-step amplification protocol was used to amplify the 16S rRNA gene [16]. The 2 × 270 bp paired-end sequencing of the PCR amplicons was carried out on the Illumina HiSeq1500 platform (Illumina Inc., San Diego, CA, United States).

Analysis of Sequencing Data

We had repeated saliva measures from both baseline and follow-up from 486 participants, which were processed together (total n = 972) using the CLC Genomics Workbench (Version 20.0.4) (https://digitalinsights.qiagen.com). To ensure high-quality data for analysis, reads containing ambiguous bases, more than one mismatch in the primer sequence, fewer than 40 base pair assembly overlaps, and over 5 unaligned mismatched ends under the default parameters in CLC were removed. Assembled reads shorter than 100 bp and over 470 bp in length were excluded from the analysis. The high-quality assembled reads were aligned to the SILVA 16S rRNA database (Version v138.1) [17], clustered into Operational Taxonomic Units (OTUs) and assigned taxonomy with

a cut-off value of > 99% similarity levels among sequences. Due to low sequencing-depth (< 10,000 reads) 59 samples were omitted during the analysis, leaving the total count of 913 samples (440 baseline samples and 473 follow-up samples).

The sequencing generated ~49 million reads from 913 samples. The reads were categorized based on sequence similarities into 4790 Operational Taxonomic Units (OTUs), divided into 26 phyla, 41 classes, 85 orders, 124 families, and 241 genera.

Statistical Analysis

To illustrate the differences within the sample, the saliva microbiota alpha-diversity indices (Shannon and inverse Simpson) were tested with Wilcoxon test. The overall microbiota composition was calculated up to genus level with R packages vegan [18] and microbiome [19]. Phyloseq objects were created from both the baseline and follow-up data together for processing [20]. Generalized linear models with negative binomial distribution (glm.nb) from the MASS package [21] and Generalized Least Squares (gls) from the nlme package [22] within the mare package [23] were used to analyze differences in the microbiota composition between the weight categories. The *p*-values for taxa-specific differences were corrected using false discovery rate (FDR; Benjamini–Hochberg method) [24]. In our study, FDR *p-value* ≤ 0.1 was considered significant [25,26]. Sequencing batch (technical variation) and age were considered as confounders in all our analyses.

Declarations

Ethics declarations

The Fin-HIT study protocol was approved by the Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa (decision number 169/13/03/00/10). The study protocol was conducted according to the Declaration of Helsinki. Children and their guardians have given written informed consent to combine the national health register data as part of the research material.

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Data availability

The datasets generated and analysed during the current study are available in the EGA repository, https://ega-archive.org/studies/EGAS00001003039.

Author contributions

NA, RVH, and HV have contributed to the conception of the study. All authors have assisted with data acquisition, analysis, and interpretation of data. All authors have drafted the work, critically revised and approve the current presentation of it.

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Additional Information

The authors declare no competing interests.

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Figures

Figure 1.

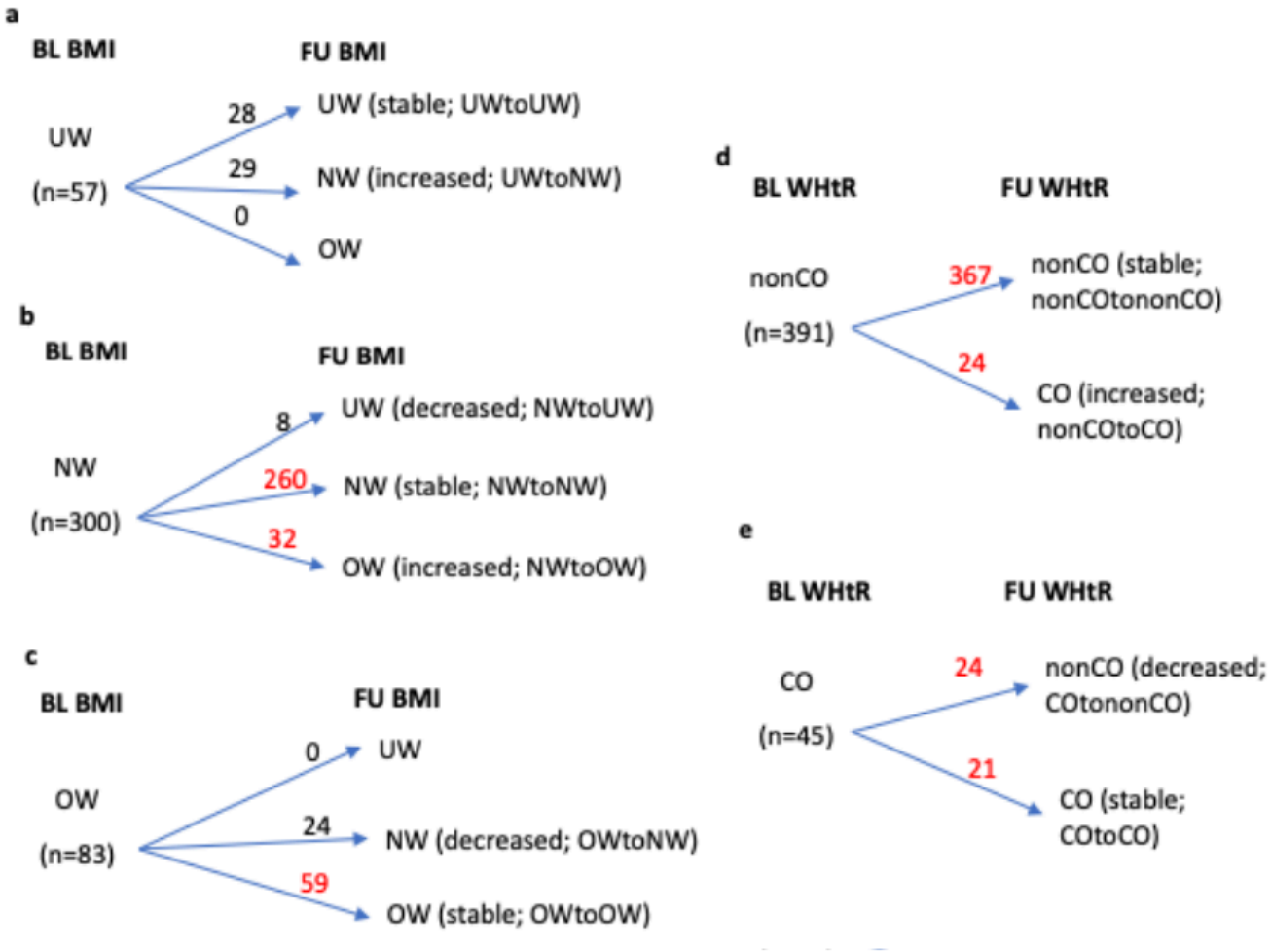


Figure 1

Flow chart of participants between BMI categories (a-c) and central obesity groups (d-e) from baseline (BL) to follow-up (FU). With the red font we have indicated the groups whose microbiota composition was focused in this study.

Figure 2.

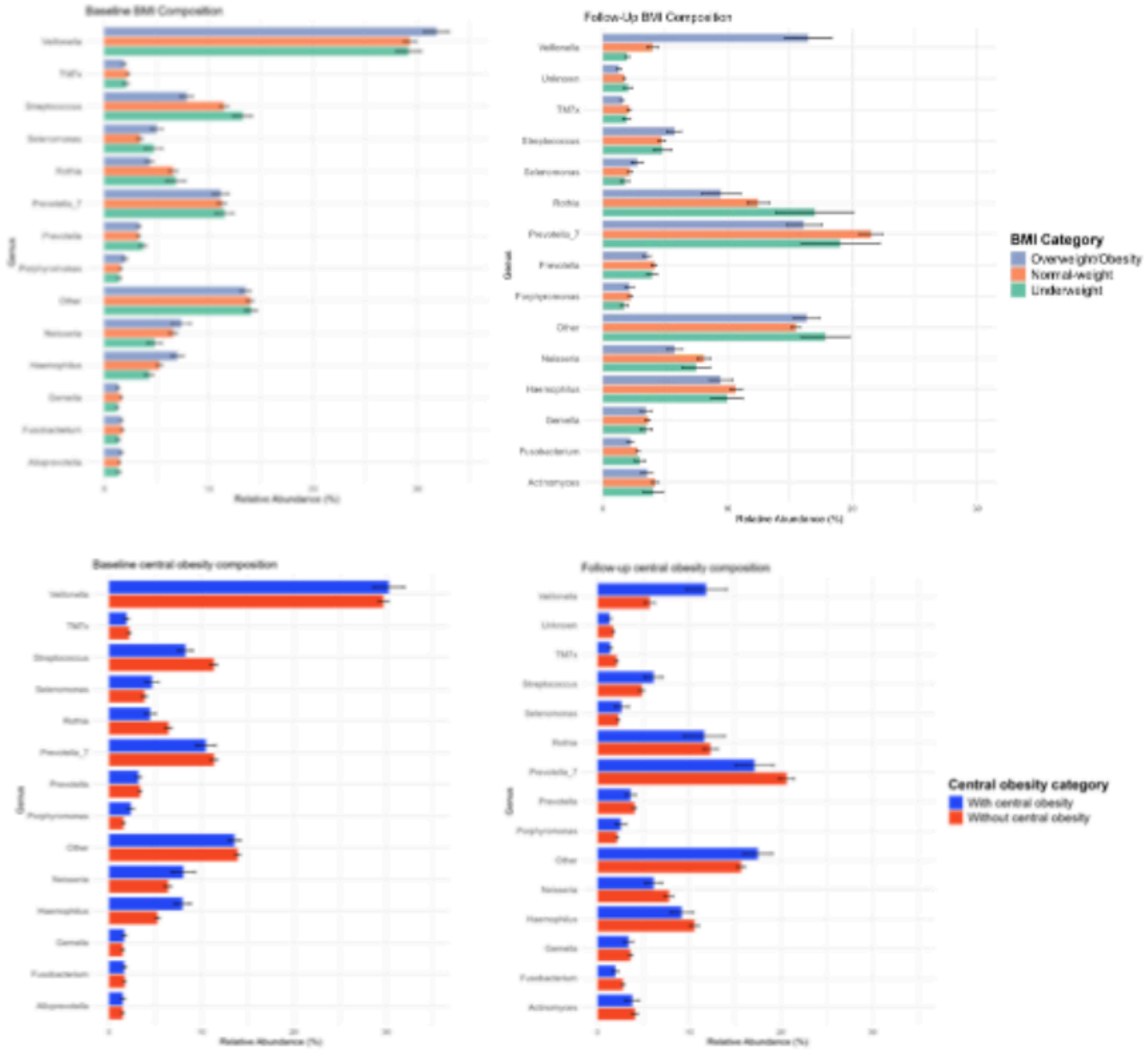


Figure 2

The relative abundance distribution of microbiota between a) BMI categories (UW, NW, OW) and b) central obesity categories (CO vs nonCO) at baseline and follow-up. The error bars represent SD.

Supplementary Files

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- 20260108Supplementarymaterial.docx