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Comparative analysis of the duodenojejunal microbiome with the oral and fecal microbiomes reveals its stronger association with obesity and nutrition

Emilie Steinbach[a#], Eugeni Belda[a,b#], Rohia Alili[a], Solia Adriouch[a], Charlène J. G. Dauriat[c,d], Gianfranco Donatelli[a,e], Jean-Loup Dumont[e], Filippo Pacini[e], Thierry Tuszynski[e], Véronique Pelloux[a], Flavien Jacques[a], Laura Creusot[f,g], Emavieve Coles[a], Paul Taillandier[a], Marta Vazquez Gomez[a], Davide Masi[a,h], Véronique Mateo[a], Sébastien André[a], Melissa Kordahi[c,d], Christine Rouault[a], Jean-Daniel Zucker[a,b], Harry Sokol[f,g,i], Laurent Genser[a,j], Benoit Chassaing[c,d], Tiphaine Le Roy[a*], and Karine Clément[a,k*]

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ABSTRACT

The intestinal microbiota is increasingly recognized as a crucial player in the development and maintenance of various chronic conditions, including obesity and associated metabolic diseases. While most research focuses on the fecal microbiota due to its easier accessibility, the small intestine, as a major site for nutrient sensing and absorption, warrants further investigation to determine its microbiota composition and functions. Here, we conducted a clinical research project in 30 age- and sex-matched participants with ($n = 15$) and without ($n = 15$) obesity. Duodenojejunal fluid was obtained by aspiration during endoscopy. Phenotyping included clinical variables related to metabolic status, lifestyle, and psychosocial factors using validated questionnaires. We performed metagenomic analyses of the oral, duodenojejunal, and fecal microbiome, alongside metabolomic data from duodenojejunal fluid and feces, integrating these data with clinical and lifestyle information. Our results highlight significant associations between duodenojejunal microbiota composition and usual dietary intake, as well as clinical phenotypes, with larger effect sizes than the associations between these variables and fecal microbiota. Notably, we found that the duodenojejunal microbiota of patients with obesity exhibited higher diversity and showed distinct differences in the abundance of several duodenojejunal microbiota species compared with individuals without obesity. Our findings support the relevance of studying the role of the small intestinal microbiota in the pathogenesis of nutrition-related diseases.

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Introduction

Obesity is a cornerstone of non-communicable diseases and has become a critical global health issue. Indeed, recent estimates indicate that 1 billion people worldwide are living with obesity.[1] Obesity accounts for 2 to 8% of the total health-related costs across different countries, due to its numerous complications, including cardiovascular diseases, type 2 diabetes, and cancers. The growing burden of these conditions underscores the urgent need for preventive actions and the development of new therapeutic approaches to mitigate the impact of obesity on global health. The composition of the gut microbiota varies between individuals and interacts with the host to exert positive or negative effects on metabolism. Through these effects, the

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gut microbiome plays a role in the regulation of body weight, blood glucose, blood lipids, and the development and/or progression of the aforementioned cardiometabolic diseases.[2] Yet, previous research has primarily focused on the fecal microbiome, which is representative of the distal colonic microbiota. While these studies have provided valuable insights into the compositional and functional changes associated with obesity,[3] the fecal microbiota represents only a fraction of the gastrointestinal tract microbiome.

The microbiota of the proximal small intestine is thus crucial to study, as this segment of the digestive tract has critical functions in food digestion, nutrient sensing, absorption, enterohormone production, and metabolic homeostasis.[4,5] Indeed, alterations in these functions of the proximal small intestinal epithelium have been described in obesity and metabolic diseases and are thought to contribute to the development of these pathologies.[6] Conversely, fatty acid malabsorption in the upper small intestine, together with duodenal inflammation, is a cornerstone of childhood stunting.[7,8] It has been observed that the size of the mucosa is increased in people with obesity,[9] an observation that has been recapitulated in rodents fed a high-fat diet in several studies.[10] The barrier function of the small intestine has also been shown to be altered in people with obesity and associated cardiometabolic disease.[11] In addition, the mucus layer in mice is disrupted by a Western diet not only in the colon but also in the small intestine.[12] Obesity is also associated with a reduced stimulation of postprandial insulin secretion by gut hormones,[13] which is linked to reduced sensing of the nutrients and to epithelial insulin resistance in the jejunum.[9,14]

While nutrients overload is a major driver of the metabolic alterations observed in cardiometabolic diseases, evidence points toward a role for the intestinal microbiota as a mediator between the diet and the host metabolism. Indeed, germ-free animals fed the same diet but colonized with the microbiota from healthy or diseased individuals exhibit different degrees of obesity and development of metabolic alterations.[15,16] Studies in rodents fed a high-fat diet have shown that alterations in small intestine microbiota are concomitant to the alteration of epithelial functions within the same gut segment. However, few of them have demonstrated the causal role of the microbiota alterations in these pathophysiological processes. Notably, Bauer et al. elegantly demonstrated that ACSL3-fatty acids sensing is abolished by a high-fat diet and can be restored by increasing the abundance of *Lactobacillus gasseri* in the small intestine lumen, which subsequently reduces whole-body gluconeogenesis.[17] Similarly, the altered jejunal microbiota derived from animals fed a high-fat diet has been shown to enhance triglyceride and cholesterol absorption in mice fed a low-fat diet.[18] Conversely, Vonaesch and colleagues showed that Streptococci isolated from the duodenal fluid of children with enteropathy are able to reduce fatty acids absorption by mICcl2 murine fetal intestinal epithelial cell line.[7] The small intestine microbiota is not only able to modulate the absorption of fatty acids but also of cholesterol. Indeed, increased or decreased intestinal cholesterol absorption in the upper small intestine is transmissible from humans to mice by fecal microbiota transfer.[19] Nonetheless, it should be noted that most of these studies were conducted on rodent models that are coprophagous. Coprophagy exposes the small intestine epithelium to fecal microbes and fecal metabolites. Consequently, knowledge about the role of the small intestine microbiota, particularly in its proximal parts, drawn from rodent models cannot be fully translated to human physiopathology. This underscores the need for focused research on the human duodenal and jejunal microbiome, as it may provide insights into the role of this microbiota in nutrient absorption and metabolic regulation in humans.

Distinct physicochemical conditions exist in each segment of the GIT, shaping the microbial ecosystems in each region.[20] Recently, major efforts based on metagenomic sequencing have been made to describe the composition of the upper small intestine microbiota under physiological conditions,[21] showing that the upper small intestine microbiota is dominated by the genera *Neisseria*, *Lactobacillus*, *Haemophilus*, *Streptococcus*, *Veillonella*, and *Actinomyces*.[22] While studies on rodents have demonstrated the causal influence of the microbiome residing in the proximal small intestine on metabolic regulation, clinical investigations of this microbiome in human obesity, remain limited and conflicting.[5]

In particular, Nardelli and colleagues constituted a population of 16 lean participants and 19 patients with obesity and observed, using 16S rRNA gene profiling, that obesity is associated with a decrease in the abundance of the Firmicutes phylum and an increase in the abundance of the Proteobacteria phylum in the duodenum.[23] This observation has not been confirmed in a recent larger cohort, where obesity status was not associated with a decrease of the Firmicutes phylum but with an increase in alpha diversity and of the abundance of several *Lactobacillus* species.[24] There is thus a pressing need for further research to elucidate the intricate interplay between the upper small intestine microbiota and metabolic health in humans,[4,5] as well as to better understand the effect of lifestyle on the host-microbiota dialogue.

In the present study, we investigated the proximal small intestine microbiome and the associated metabolome in thoroughly characterized participants with ($n = 15$, OB) or without ($n = 15$, NOB) obesity matched for age and sex in a population named JeMiMe (Jejunal microbiota in metabolic diseases). We compared the duodenojejunal fluid (DJF) microbiome obtained by aspiration during endoscopy at the Treitz Angle with the oral and fecal microbiome. Additionally, we conducted statistical analyses to explore potential associations between these microbiomes, the duodenojejunal metabolome, and participants' lifestyles and clinical phenotypes.

Material and methods

JeMiMe population

The study, conducted at Hôpital-Privé des Peupliers, Ramsay-Santé, Paris, France, is an observational study. Prior to inclusion, informed written consent was obtained from participants. The study adhered to the Helsinki Declaration and obtained an agreement from the ethics committee (IRB) "Comité de protection des personnes Ile de France VIII" (CPP Ile de France 8; approval number: 210648). The « Institut National de la Santé Et de la Recherche Médicale (INSERM) » is promoter of this clinical study.

The studied population comprised 30 participants categorized into two groups: the Non-Obese Group (NOB; $n = 15$): composed of individuals without obesity or known metabolic disorders for which endoscopy was scheduled due to minor epigastralgia that did not necessitate medication; the Obesity Group (OB; $n = 15$): composed of candidates for bariatric surgery for which gastroscopy was a prerequisite procedure.

The study employed specific inclusion and exclusion criteria to select eligible participants, these criteria are listed on the Clinical Trial.gov website (NCT05186389). Notably, patients with prior use of prebiotics, probiotics, and antibiotics within the 3 months before their inclusion in the study were excluded.

Clinical and lifestyle data

Clinical and lifestyle data were managed and/or collected using REDCap electronic data capture tools hosted at Sorbonne Université.[25,26] In addition to a general medical questionnaire, various standardized questionnaires were used to evaluate dietary intakes (previously validated food frequency questionnaire[27] (FFQ)), alcohol consumption (Alcohol Use Disorders Identification Test), nicotine dependence (Fagerström Questionnaire), perceived stress (Perceived Stress Scale-10), anxiety (Hospital Anxiety Depression Scale, only anxiety-items), depression (Beck Depression Inventory), circadian rhythm (Horne and Ostberg Questionnaire), and eating behavior (Dutch Eating Behavior Questionnaire (DEBQ)).[28–33]

Anthropometric measurements and body composition were measured (MC-780 MA P, Tanita, Amsterdam, the Netherlands). To avoid redundancy, the results only display a subset of body composition and corpulence variables (fat mass %, visceral fat rating, and BMI).

Except for stools, samples were collected at overnight fasted state before the endoscopy, early in the morning. Participants had been fasting overnight for at least 12 hours.

Circulating markers related to glucose metabolism (fasting glycemia, insulin, Hba1c), lipid profile (total cholesterol, LDL, HDL, and triglycerides), liver function (aspartate transaminase – ASAT, alanine transaminase – ALAT, gamma-glutamyl transferase (GGT), and alkaline phosphatase), and thyroid function (ultra-sensitive measurement of

thyroid-stimulating hormone) were measured using biochemical assays as part of routine medical care (Alinity-Abbott; Cerballiance, Paris).

Quantification of circulating levels of Amylin, C-Peptide, Ghrelin, gastric-inhibitory-polypeptide, Glucagon-like Peptide-1, Glucagon, Interleukin-6 (IL-6), Insulin, Leptin, Monocyte-chemoattractant protein-1 (MCP-1), Pancreatic Polypeptide, Peptide-YY, Secretin, and tumor necrosis factor-alpha (TNFα) was performed on serum treated with dipeptidyl peptidase-4 inhibitors and protease inhibitors using a multiplex immunoassay kit (MILLIPLEX® Human Metabolic Hormone Panel V3, Millipore).

Quantification of inflammatory cytokines IL-6/8, C-reactive protein, serum-amyloid A, MCP-1, and TNFα were measured on serum using a multiplex immunoassay kit (Meso-Scale Discovery's ultra-sensitive assay). Subsequently, a cumulative score of low-grade inflammation (Z-score) was calculated following the previously described methodology.[34]

Quantitative determination of human High Molecular Weight Adiponectin (Human HMW Adiponectin/Acrp30 Immunoassay), human Growth Differentiation Factor-15, and human Fibroblast growth factor-21 were also performed on serum (QuantikineTM, ELISA). Tryptophan metabolites were quantified through liquid chromatography coupled with high-resolution mass spectrometry from serum, as previously described.[35]

Stools sampling

Total fresh stools were collected in a hermetic container at the patient's home during the two weeks preceding the endoscopy. When the sample was collected, participants placed an anaerocult (bioMérieux, Paris, France) on the stools and hermetically closed the box. The sample was transported and aliquoted on ice in an anaerobic hood within two hours for different analyses and stored at −80°C.

Saliva sampling

Participants were asked not to brush their teeth in the morning before saliva sampling, and they had been fasting for at least 8 hours. Saliva was collected by spitting into a 50 mL tube. After collection, saliva was transported to the laboratory on ice within two hours and aliquoted and stored at −80°C.

Duodenojejunal fluid sampling

After saliva sampling, participants thoroughly brushed their mouth and teeth to prevent duodenojejunal fluid contamination from the oral cavity.[36] Then, endoscopy was performed. The endoscope was thoroughly washed 3 times in the stomach with sterile saline then DJF was aspirated at the Treitz Angle and collected in a sterile tube. DJF was immediately aliquoted and placed within five minutes after sampling on dry ice, then stored at −80°C.

Metabolome analysis

Untargeted metabolomics was performed using Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy on duodenojejunal fluid and stool (Metabolon®, Durham, North Carolina, United States).

Metagenomic analysis

Bacterial DNA extraction from saliva, DJF, and homogenized feces was performed using NucleoMag DNA Microbiome kit (Macherey-Nagel, Vertrieb GmbH & Co.Kg). Two cycles of chemical- and mechanical-lysis were performed (Precellys®, Bertin Technologies, Montigny-le-Bretonneux, France). We used an automated robot for DNA extraction and purification using paramagnetic beads (Auto-Pure96, Nucleic Acid Purification System Hangzhou Allsheng Instruments CO., Ltd. Hangzhou, Zhejiang, China). Purity ratio and DNA quantity were controlled (NanoDrop and Qubit, ThermoFisher).

DNA was physically sheared to approximately 250–550 bp then purified (QIAquick Purification kit, Qiagen, Hilden, Germany). Library preparation for sequencing was performed using the Invitrogen ColibriTM PS DNA Library Prep Kit for IlluminaTM (ThermoFisher Scientific, Waltham, Massachusetts, United States). PCR amplification of the purified adaptor-ligated DNA library

was performed, followed by a third purification of the amplified DNA library using reagents included in the Colibri kit. One fecal sample was excluded due to library preparation failure. Sequencing was performed with NextSeq 2000 (P2 300 cycles: 2 × 150 bp). 5.89 M (±2.45 M), 4.95 (±1.06 M), and 5.07 (±1.39 M) read pairs per sample were generated for fecal, DJF, and saliva samples, respectively (Supplemental Table S1).

Metagenomic analyses were performed using the bioBakery tools.[37] Read-level quality control was performed using KneadData with default settings, including quality filtering with Trimmomatic (SLIDINGWINDOW:4:20 minLEN:75) and removal of human contaminant sequences with bowtie2 in very-sensitive mode vs. hg37dec_v0.1 assembly reference. 5.04 M (±2.34 M), 1.29 M (±1.108 M), and 2.14 M (±1.13 M) high-quality human-decontaminated read pairs per sample were retained for fecal, DJF, and saliva samples, respectively (Supplemental Table S1). Taxonomic profiling was performed using MetaPhlAn4-catalog[38] vs mpa_vJan21_CHOCOPhlAnSGB_202103 reference database (21 978 species-level genome bins (SGB) derived from a reference gene catalog of 5.1 million taxonomic markers). To correct for variations in sequencing depth, Metaphlan4 normalized marker gene abundances (reads per kilobase, RPK) were divided by metagenome size (quality-filtered non-human read pairs) before robust average calculation of SGB (0.2 default quantile value).

Integration of metagenomic profiles from Shalon et al. study

Raw fastq files from the study of Shalon et al.[21] corresponding to saliva ($n = 29$ samples), small intestine device 1 ($n = 53$ samples) and stools ($n = 58$ samples) were downloaded from the ENA project PRJNA822660. Sequence files were processed with the same bioinformatic workflow as the present study samples for quality control, host contaminant removal, and generation of quantitative metagenomic profiles with Metaphlan4.

Statistical and ecological analyses

Statistical analyses were performed with R (version 4.2.2) (https://www.R-project.org/). For clinical and anthropometric data, the normality of the data distribution was evaluated by a Shapiro–Wilk test, followed by either a Student t-test or a Wilcoxon rank-sum test depending on the distribution of the data.

Vegan v2.6.4 R package was used for ecological analyses of metagenomic profiles. Alpha diversity (Metaphlan4 gene marker richness, SGB-based richness and Shannon diversity) was determined from a random sampling of 350K read pairs per sample with seqtk to account for variations in sequencing depth across samples. Species-level genome bins profiles derived from normalized marker gene abundances (RPK + corrected by metagenome size) were used for Principal Coordinate Analyses (PCoA) based on Bray-Curtis (quantitative profiles) and Jaccard (binary presence/absence data) distances computed with *vegdist* and *cmdscale* vegan functions. Permutational multivariate analyses of variance (PERMANOVA) with *adonis2* function were used to evaluate the impact of different clinical covariates on microbiome and metabolome composition based on Bray-Curtis and Euclidean distances, respectively. P-values of PERMANOVA tests were False Discovery Rate (FDR) corrected with Benjamini–Hochberg method by block of clinical/nutritional variables tested. Associations between metagenomic gene richness (Metaphlan4 marker genes) and clinical/nutritional variables were evaluated with Spearman correlations (for numerical variables) and Wilcoxon rank-sum test (for discrete variables at two levels). P-values were FDR corrected with Benjamini–Hochberg method by block of clinical/nutritional variables tested and effect sizes (spearman rho's for numerical variables, Cliff's Delta for discrete variables at two levels) were retained for visualization.

Non-parametric Kruskal–Wallis tests followed by post-hoc pairwise Dunn tests were used to identify taxonomic (species-level Metaphlan4 relative abundance profiles followed with centered log-ratio (CLR) transformation to account for compositionality of the data) and metabolomic features associated with different ecosystems. Only the

features present in > 20% of the samples were retained for analyses. P-values derived from Kruskal–Wallis tests were corrected for multiple testing using the Benjamini – Hochberg method (Padj), only Padj < 0.05 were reported as significant. Over the same data, Wilcoxon rank-sum tests were used to identify taxonomic and metabolomic features associated to clinical study group. P-values were corrected for multiple testing using the Benjamini – Hochberg method. Cliff's delta was used as an indicator of the effect size of feature change across pairwise comparisons. Linear regression analyses were used to evaluate the association of taxonomic and metabolomic markers with clinical covariates unadjusted and adjusted by alcohol intake. In order to reinforce the normality of metagenomic variables, quantile normal transformation was applied as previously described.[39,40]

Results

Microbiome patterns across three ecosystems of the human digestive tract

Metagenomic profiles of oral (salivary), duodenojejunal, and fecal samples from all participants were analyzed by Illumina sequencing, which showed that the duodenojejunal microbiota exhibited a lower alpha diversity than the oral microbiota and the fecal microbiota, as evidenced by a significantly lower number of marker genes and species genome bins and a significantly lower Shannon index in the duodenojejunal fluid (Figure 1(a)). Principal coordinate analysis (PCoA) of the samples based on Bray-Curtis distances derived from the coverage of species genomes bins highlighted significant segregation of stool samples from oral and duodenojejunal samples (Figure 1(b)). Notably, the duodenojejunal microbiota was highly similar to the oral microbiota (Figure 1(b)). This similarity was confirmed by analyzing the distribution of the Bray-Curtis distances between ecosystems, with dissimilarities between duodenojejunal microbiota and oral microbiota being significantly lower than the dissimilarities of both ecosystems compared to fecal samples ($p < 0.05$, Figure 1(c)). Similar analyses on intra-ecosystems' dissimilarities showed similar levels of compositional variation in the stools and DJF, with oral microbiome showing a lower degree of compositional variation between samples ($p < 0.05$, Figure 1(c)). The major compositional differences between the fecal microbiome and the microbiome of the duodenojejunal fluid (DJF) and saliva were further confirmed by univariate statistical analyses (Cliff's Delta) at the species level, as 56 species were significantly altered between the oral and duodenojejunal microbiota. In contrast, 169 species were significantly altered between the fecal and oral microbiota, and 182 species were significantly altered between the fecal and DFJ microbiota (Figure 1(d), supplemental Table S2). Finally, the prevalence analysis of the species altered between the ecosystems showed the presence of aerobic species belonging to the *Streptococcaceae*, *Veillonellaceae*, and *Prevotellaceae* families in the oral microbiota and the duodenojejunal microbiota, while strict anaerobes belonging to the *Lachnospiraceae* and *Ruminococcaceae* families were prevalent in the fecal samples (Supplementary Figure S1).

Pairwise comparisons of microbiome and metabolome dissimilarities showed a strong significant positive correlation in stools (Spearman Rho = 0.5, p-value <2.2e-16), whereas no significant correlation was observed in the duodenojejunal fluid (Spearman Rho = 1.1e-03, p-value = 0.98), suggesting that the microbiota is a strong contributor to the luminal metabolome in the distal colon but not in the proximal small intestine (Supplemental Figure S2a). Stratified analyses on subjects without obesity and with obesity showed that the positive correlation at fecal level is reproduced with the same strength in both groups (Spearman Rho = 0.51, p-value = 4.9e-07 in controls; Spearman Rho = 0.56, p-value = 5.5e-10 in the group with obesity; Supplemental Figure S2b). In contrast, we observed positive association between duodenojejunal microbiota and metabolome composition in control participants (Spearman Rho = 0.3, p-value = 1.7e-03) but not in the context of obesity (Spearman Rho = 0.14, p-value = 0.14, Supplemental Figure S2b), suggesting potential altered interactions between microbial and metabolome composition in the duodenojejunal ecosystem in obesity.

Recently, Shalon *et al.* reported that the small intestine microbiota shares more compositional similarity with stools than with saliva.[21] This result, derived from the analysis of digestive contents obtained from ingestible devices and later recovered stools, differs significantly from

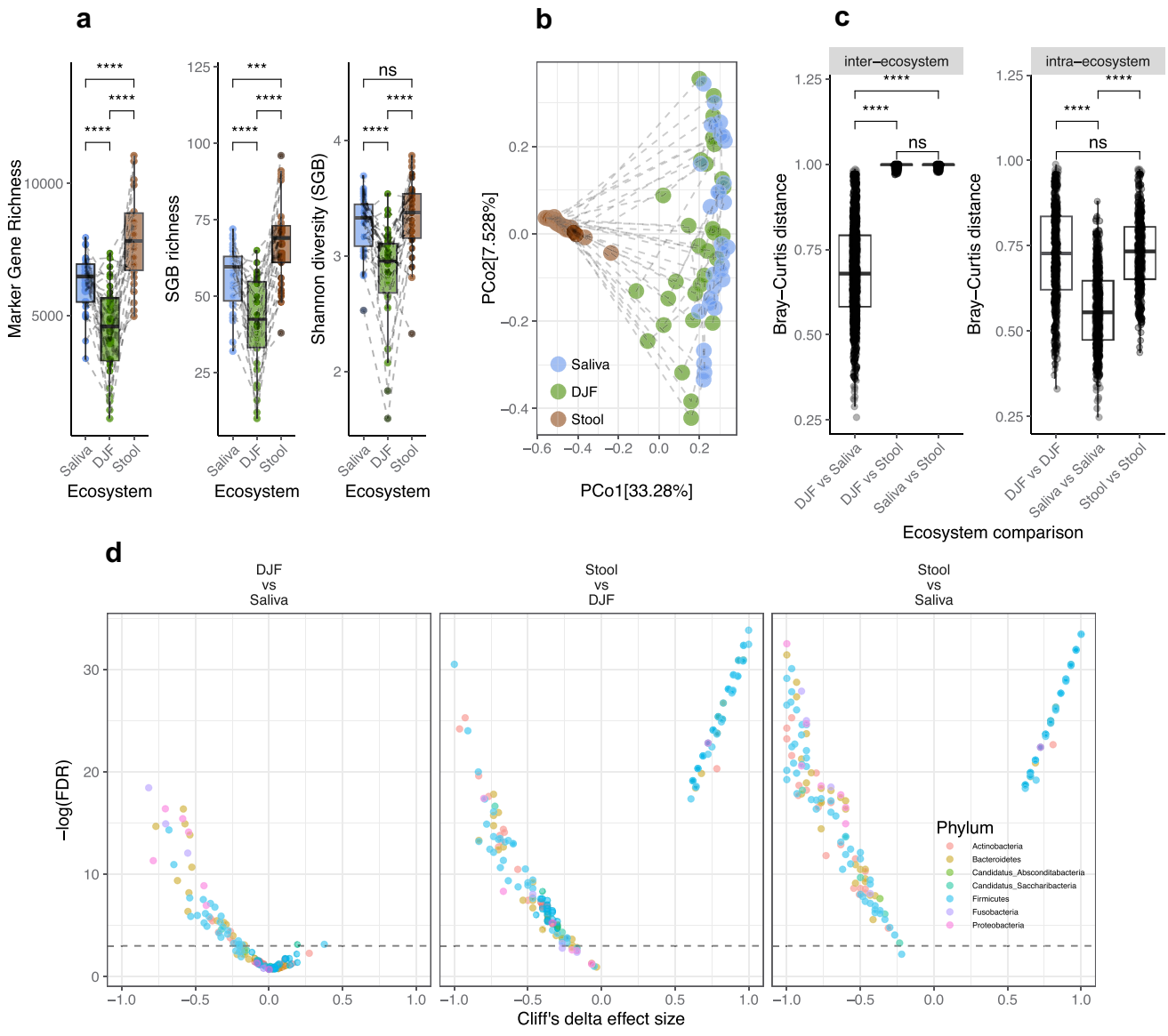

Figure 1. Microbiome patterns across three ecosystems of the digestive tract. (a) Metagenomic richness across the three ecosystems. Boxplots of the metagenomic richness at the marker gene level (left panel), the species genome bin level (center panel), and Shannon diversity computed from the species genome bins abundances (right panel; y-axis) of each ecosystem (x-axis): saliva (blue), duodenojejunal fluid (DFJ, green), and stool (brown). P-values result from Wilcoxon tests. Legend: ***$p < 0.001$; ****$p < 0.0001$. (b) Principal Coordinate Analysis of the samples from the three ecosystems based on Bray-Curtis distance derived from SGB abundance: saliva (in blue), duodenojejunal fluid (in green), and stool (in brown). Samples from the same patient are connected with a dotted line. (c) Comparison of Bray-Curtis distances between samples from different ecosystems (left panel) and within the same ecosystem (right panel). ***$p < 0.001$; ****$p < 0.0001$; ns = p > .5; Wilcoxon rank-sum test. (d) Volcano plots of SGB abundances between microbial ecosystems. Univariate tests between each pair of ecosystems were performed on clr-transformed SGB abundances (Kruskal–Wallist test followed by pairwise Dunn's comparison between ecosystems). X-axis represents the Cliff's delta effect sizes (>0 indicates higher abundance in the reference ecosystem of the comparison and <0 indicates a higher abundance in the compared ecosystem; saliva, DJF, and saliva respectively). Y-axis represents the -log-transformed FDR values. Dashed line indicates FDR = 0.05. Detailed results available in Supplemental Table 2.

our observations, that are derived from the analysis of duodenojejunal aspirates obtained during endoscopy. Sampling by aspiration presents a risk of contamination with saliva, which we mitigated as much as possible by repeated thorough rinsing of the endoscope with sterile saline in the stomach before reaching the sampling site, i.e., the transition between the duodenum and the jejunum (Treitz angle). We retrieved the metagenomic sequences from the

Shalon *et al.* study, corresponding to the ecological niches in our study ($n = 29$ saliva samples, $n = 53$ samples of the small intestine device 1, $n = 58$ fecal samples) from ENA repository and subjected them to the same bioinformatic pipeline as the samples from the present study and compared the microbiome composition.

First, we computed a principal coordinates analysis based on the Bray-Curtis distances between the samples from Shalon and colleagues[21] samples and the samples from our study. Saliva samples from both studies clustered together, as did stool samples from both studies (Figure 2(a), Supplemental Figure S3), highlighting the similarities between the populations and metagenomic sequencing pipelines of the two studies. The duodenojejunal fluid samples collected by aspiration clustered closely with saliva samples from both studies. In contrast, samples of proximal small intestine lumen content collected by the ingestible device 1 (designed to open and collect fluid in the duodenum) clustered with the stools. All the small intestine samples from the ingestible device 1 showed higher similarity to stools from both studies than to the duodenojejunal fluids collected by aspiration (Figure 2(a), Supplemental Figure S3). Quantitatively, this translated into a greater dissimilarity between ingestible device 1 samples and all saliva samples than between duodenojejunal fluid samples collected by aspiration and saliva samples (FDR < 0.05, Figure 2(b)). Similar conclusions were drawn using the Jaccard distances (based on the presence and absence of SGB, Supplemental Figure S3b). These observations reflect a greater abundance of families considered typical of fecal microbiota, such as *Ruminococcaceae* and *Lachnospiraceae*, in the samples from the ingestible device 1 than in aspirates. Concurrently, there was a lower abundance of *Streptococcaceae* and *Actinomycetaceae* in these samples (Supplemental Figure S3c).

We then sought to investigate the association between the microbiota composition of the

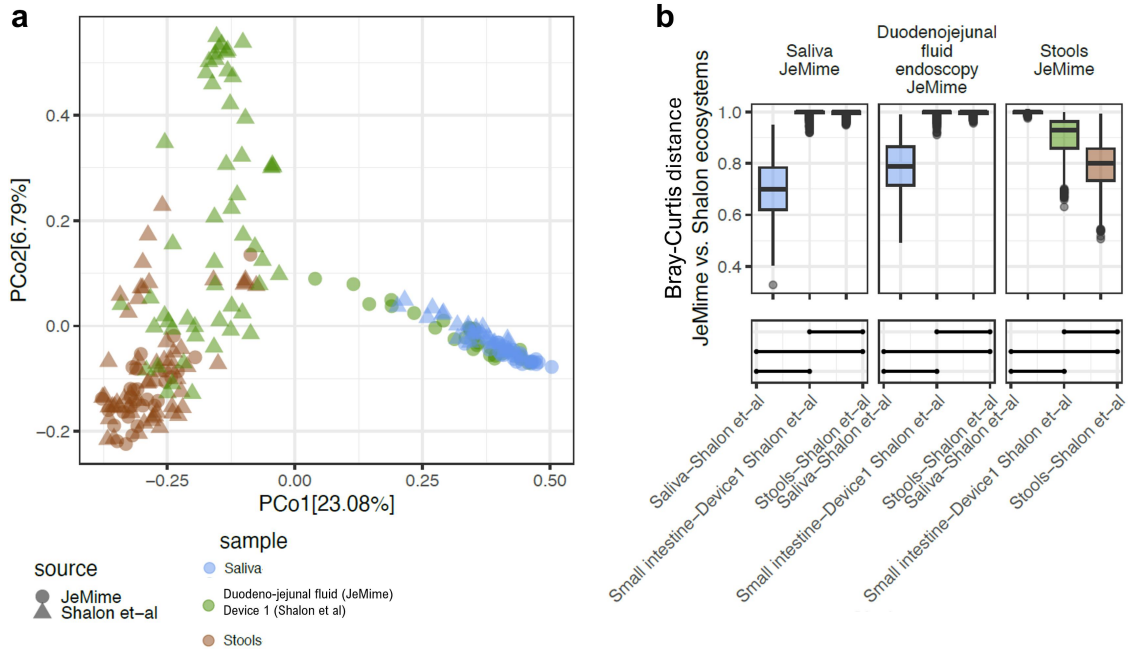

Figure 2. Integrated compositional landscape of human gut ecosystems from JeMiMe and Shalon et al. studies. (a) Principal Coordinates Analyses (PCoA) from Bray-Curtis distances derived from Metaphlan4 abundance profiles (Species-level Genome Bins; SGB) of 229 samples ($n = 89$ JeMiMe samples; $n = 140$ Shalon et-al samples). (b) Boxplots representing the dissimilarities (Bray-Curtis distance) between JeMiMe samples of different ecosystems (Saliva, Duodenojejunal fluid, Stools; facets in plots) vs. samples from different ecosystems/devices from the Shalon et al. cohort (x-axis). Bottom panels connect pairs of devices/ecosystems of the Shalon et al. study for which the distances vs. the corresponding JeMiMe samples are significantly different (FDR < 0.05; post-hoc Dunn's test).

three digestive ecosystems, the metabolic phenotype, and the lifestyle, including dietary information, of the participants.

Characteristics of the participants with and without obesity

There were no significant differences in age and sex ratio between the groups with and without obesity. As expected, considering the inclusion criteria for each group, the group with obesity (Ob group) had a significantly higher body mass index and body fat proportion (%) than the control group (Table 1). This was associated with significantly higher glycemia, insulin levels, and insulin resistance index, although without reaching the thresholds defining type 2 diabetes.[41] The elevated insulin levels in participants with obesity but without type 2 diabetes resulted from higher insulin production, as evidenced by 2.5-fold higher levels of c-peptide in participants with obesity than in the control group (Supplemental table 3). Additionally, the participants with obesity had a comparatively altered blood lipid profile, characterized by higher triglyceride levels and lower proportions of HDL to LDL-cholesterol. This overall altered metabolic profile in the participants with obesity was further confirmed by metabolic hormone assay, which showed a moderate decrease in ghrelin and a dramatic increase in leptin levels (Supplementary Table 3). Although still within the normal range, C-reactive protein levels were also higher in the group with obesity than in the control group (Supplementary Table 4). This was associated with non-significant increases in pro-inflammatory cytokines and chemokines IL-6, TNFα, and MCP-1 circulating levels (Supplementary Table 4). However, combining inflammatory cytokine levels in a cumulative Z-score revealed a significantly increased low-grade inflammation in people with obesity (Table 1).[34]

The characterization of the participants also included multiple questionnaires to assess the patients' lifestyle, digestive symptoms, sleep, psycho-emotional health, and eating behaviors

Table 1. Characteristics of the study participants. Results are expressed as mean (SD) or median [min;max] for continuous data and n (%) for categorical data. *p* values result from the Student's t-test or from Mann-Whitney-Wilcoxon depending on data distributions for each continuous variable and from the Chi-square test for categorical variable (sex).

	Control Group	Obesity Group	*p-value*
Age (years)	29.4 [26.5;53.2]	29.6 [18.5;59.5]	*ns*
Sex (male), n (%)	3 (20.0)	1 (6.6)	*ns*
Adiposity markers			
Weight (kg)	61.6 [46.4;88.7]	111 [84.7;132]	****
BMI (kg/m^2)	21.5 [18.6;28.2]	39.4 [32.5;50.6]	****
Fat mass (%)	21.3 [14.5;37.0]	45.4 [36.5;53.6]	****
Visceral fat rating	3.0 [1.0;5.0]	12.0 [9.0;18.0]	****
BMR (Kcal/day)	1447 (237.8)	1894 (290.9)	****
Plasma Glucose homeostasis			
Glycemia (mmol/L)	4.60 (0.34)	5.21 (0.46)	***
Insulinemia (mIU/L)	3.80 [1.80;10.2]	15.6 [6.90;41.8]	****
HOMA-IR	0.80 [0.30;2.10]	3.50 [1.50;9.10]	****
HbA1c (%)	5.06 (0.26)	5.58 (0.31)	****
Plasma lipid homeostasis			
Total cholesterol (mmol/L)	4.48 (0.85)	4.80 (0.75)	*ns*
Total triglycerides (mmol/L)	0.70 (0.29)	1.14 (0.27)	****
HDL cholesterol (mmol/L)	1.59 [0.97;2.25]	1.11 [0.86;1.60]	***
LDL cholesterol (mmol/L)	2.52 (0.66)	3.16 (0.70)	*
Liver Enzymes			
ASAT (IU/L)	27.7 (6.86)	23.9 (5.13)	*ns*
ALAT (IU/L)	25.0 [11.0;64.0]	30.0 [15.0;101]	*ns*
ASAT/ALAT ratio	1.06 (0,22)	0.82 (0,26)	**
GGT (IU/L)	15.0 [9.00;35.0]	33.0 [15.0;106]	***
Inflammation			
Z-score	−3.03 (3.25)	3.02 (2.66)	****

Legend: BMI: Body Mass Index; BMR; calculated basal metabolic rate, HOMA-IR: homeostatic Model assessment of insulin resistance. ASAT: aspartate transaminase; ALAT: alanine transaminase; GGT: gamma glutamyl-transpeptidase; ns P-value > 0.05; *P-value ≤ 0.05; **P-value ≤ 0.01; ***P-value ≤ 0.001; ****P-value ≤ 0.0001.

(Supplementary Table S5). The scores obtained did not differ significantly between groups.

Participants with obesity have a lower dietary quality

Diet is a major determinant of the composition of the fecal microbiota and is thought to have an even greater effect on the composition of the small intestine microbiota.[42] We therefore studied the dietary intake of the participants using food frequency questionnaires (FFQ) and found that participants with obesity consumed significantly more red meat, processed meat and soft drinks, together with less legumes, vegetables, nuts, and oily fish (Table 2). The control participants, on their end, consumed significantly more coffee, beer, and wine. As a consequence, protein and lipid dietary intakes were similar in the two groups, while alcohol intake was higher in the participants without obesity and carbohydrates intake was higher in individuals with obesity. Taken together, these food consumption data resulted in a higher food quality,[43] measured by aHEI or DASH indices, in the control participants than in the participants with obesity.

Obesity is associated with alterations in the small intestine microbiota and metabolome composition

We performed a beta-diversity analysis (PERMANOVA analyses on Bray-Curtis distances derived from SGB abundances) to compare the metagenome composition between participants with and without obesity and observed no clustering of the samples according to the obesity status in the oral ecosystem (Figure 3(a)). However, the duodenojejunal fluid metagenome clustered separately, with a higher and more significant impact of obesity status ($p = 0.01$), indicating that the duodenojejunal ecosystem is significantly affected by obesity status. The impact of obesity on the fecal ecosystem approached, but did not reach, statistical significance.

Similarly, PERMANOVA analyses based on Euclidean distances computed from non-targeted metabolomic profiles also showed a statistically significant effect of the obesity status in the duodenojejunal fluid, but only a trend in the stools ($p = 0.047$ and $p = 0.064$, respectively, Figure 3(b)). Consistently, PERMANOVA analyses with clinical, dietary, and lifestyle variables showed that body composition variables significantly explained the compositional variance of the duodenojejunal fluid microbiota, with a higher effect size than that observed for the oral and fecal microbiota (FDR < 0.05, Figure 3(c)). In particular, there was a statistically significant association between the duodenojejunal fluid microbiota composition and blood HDL-cholesterol level, which was not observed for oral or fecal microbiota. Similarly, significant associations were found between the duodenojejunal microbiota compositional variance

Table 2. Dietary intakes of the study participants. Results are expressed as median [min;max]. *p* values result from the Mann-Whitney-Wilcoxon non-parametric test (W test).

	Control Group	Obesity Group	*p-value*
Repartition of macronutrients and alcohol on total caloric intake			
Alcohol (%)	4.46 [2.53;5.38]	0.11 [0.01;0.98]	***
Carbohydrates (%)	39.9 [36.1;43.0]	44.3 [41.6;46.8]	*
Proteins (%)	17.2 [16.5;19.3]	17.6 [15.0;19.8]	*ns*
Lipids (%)	37.2 [34.9;41.4]	38.1 [35.0;41.5]	*ns*
Food groups (Daily intake)			
Coffee (mL)	180 [90.0;225]	0.00 [0.00;38.7]	***
Beer (mL)	35.0 [8.75;108]	0.00 [0.00;0.00]	***
Wine (mL)	51.6 [34.2;73.2]	0.00 [0.00;8.40]	***
Red meat (g)	17.0 [3.69;27.6]	48.9 [39.2;56.0]	**
Nuts (g)	3.24 [0.81;9.94]	0.00 [0.00;1.62]	**
Pulses (g)	21.4 [16.1;65.9]	10.7 [0.00;21.4]	**
Sweetened beverages (mL)	0.00 [0.00;21.0]	71.0 [0.00;176]	**
Processed meat (g)	3.89 [0.00;6.29]	19.1 [7.13;41.6]	*
Vegetables (g)	271 [243;424]	202 [135;276]	*
Oily fish (g)	8.83 [4.41;18.0]	4.41 [3.31;5.52]	*ns*
Diet quality scores			
aHEI %	52.4 [43.5;62.8]	31.0 [20.9;37.4]	***
DASH %	58.5 [51.0;64.8]	48.4 [41.8;56.3]	**

Legend: aHEI (Alternate Healthy Eating Index) and the DASH (dietary approaches to stop hypertension) scores. Ns P-value >0.05; * P-value ≤0.05; ** P-value ≤0.01; *** P-value ≤0.001; **** P-value ≤0.0001.

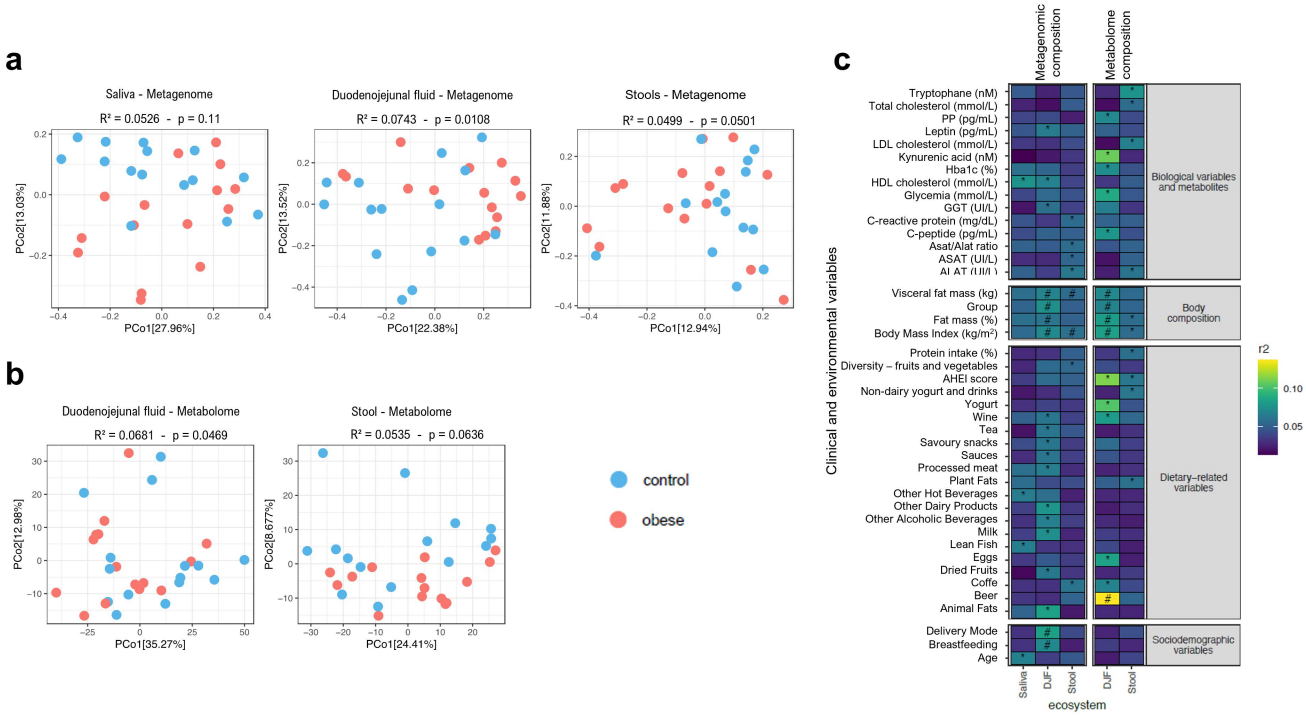

Figure 3. Oral, duodenojejunal microbiome and metabolome structure in obesity and their association with lifestyle and clinical phenotype. (a) Principal Coordinates Analyses (PCoA) from Bray-Curtis distances derived from Metaphlan4 abundance profiles (Species-level Genome Bins; SGB) in saliva, duodenojejunal fluid, and stools and (b) Principal component analyses (PCA) based on the Euclidean distances derived from the metabolome in the duodenojejunal fluid and stools of control (blue) and with obesity (red) participants. R^2 and p-values of PERMANOVA test to evaluate the impact of obesity status on microbiome composition are shown on top of each panel. (c) Proportion of compositional variance explained by different clinical or environmental variables. The Permutational Multivariate Analysis of Variance using the Adonis function was computed from the Bray-Curtis dissimilarity matrix for metagenomic data and the Euclidean distances for metabolome data. Legend: *: $p < 0.05$ and #: FDR < 0.1.

and body composition of the participants (visceral and overall fat mass and body mass index), while the associations with the oral and fecal microbiota were weaker or absent. Among other sociodemographic and dietary-related variables, we observed a significant impact of delivery mode (FDR = 0.02) and being breastfed during infancy (FDR = 0.04) on the DJF microbiome composition (Figure 3(c)). Similarly, the associations between the duodenojejunal fluid metabolome and body composition were statistically stronger than those with the fecal metabolome. Taken together, these results suggest a possibly stronger association between the host metabolic profile and the duodenojejunal microbiota than with the oral or fecal microbiota.

We also observed significant associations between the intake of several FFQ-derived dietary items and metagenome compositional variance in the three ecosystems (Figure 3(c)). The strength of these associations was comparable in duodenojejunal fluid and stools, but weaker in the oral microbiota. A higher number of dietary intake variables had a significant association with DJF metagenome and metabolome ($n = 2$, 10 and 2 for Saliva, DJF, and Stool, respectively; Figure 3(c) and Supplemental Figure S4). Notably, the duodenojejunal microbiota composition was associated with consumption of various food items such as alcohol/wine, processed meat, pastries, and dairy products.

We observed relationships between dietary quality (as measured by the aHEI index) and duodenojejunal metabolome. A strong association of the duodenojejunal metabolome with dietary quality and beer consumption was also observed.

Further analysis of the differences in microbiomes characteristics in participants either lean or with obesity showed that obesity was associated with an increase in the gene richness exclusively in the duodenojejunal microbiota (Figure 4(a)). Other markers of microbial diversity like the number of species genome bins (SGB) and the Shannon index were also increased in the duodenojejunal

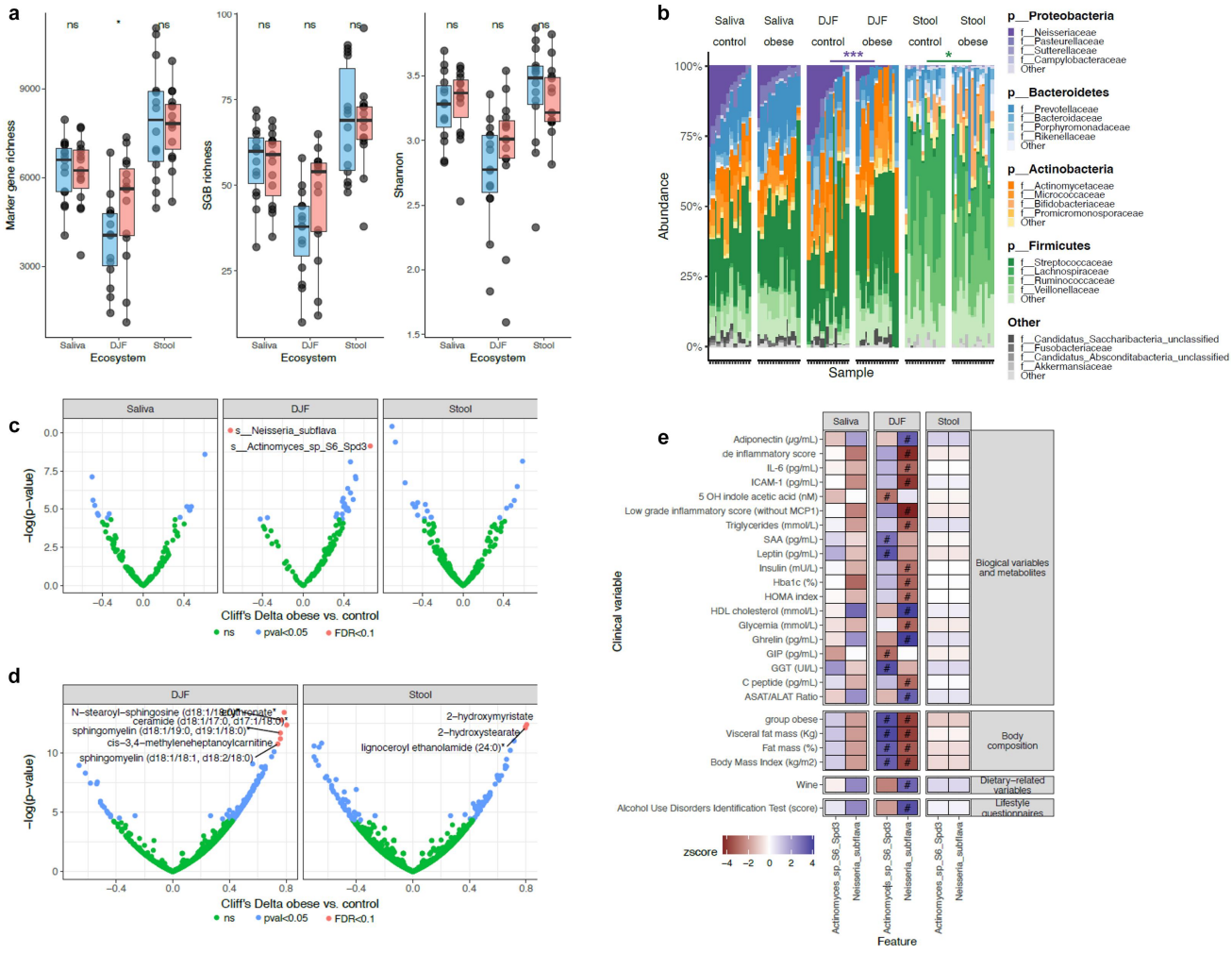

Figure 4. Alterations of the oral, duodenojejunal microbiome and metabolome composition in obesity and their association with lifestyle and clinical phenotype. (a) Metagenomic richness at the marker gene level (left panel), the species genome bin level (center), and the Shannon index (right panel; y-axis) in each ecosystem (x-axis), stratified per group (control group: blue; with obesity group: red). P-values result from between-group comparisons using the Wilcoxon test. Legend: *p < 0.05. (b) Relative abundance (%, y-axis) of bacterial families per ecosystem. The different Phyla are listed in bold and color-coded, and bacterial families are colored in different shades within each Phyla. Each column represents a participant. P-values result from between-group comparisons using the Wilcoxon test. (c) and (d) Volcano plots representing the Cliff's Delta effect size analysis between bacterial species and metabolites and the participant's group within each ecosystem. Log-transformed *p* values and FDR are represented on the y-axis, and associations with the cohort group on the x-axis (>0: higher abundance in obese group; <0: higher abundance in the control group). Each dot represents a bacterial species or a metabolite and is color-coded with regard to the level of significance of the association (green: not significant – ns; blue: p < 0.05; red: FDR < 0.1). (e) Associations between Actinomyces sp S6-Spd3 and Neisseria subflava relative abundances and clinical and lifestyle variables in the whole cohort of 30 participants derived from linear regression analysis (red: negative association; blue: positive association). Effect sizes are represented as Z-scores. * p < 0.05 and #: FDR < 0.1.

microbiota of subjects with obesity although without reaching statistical significance (Figure 4(a)). The α-diversity in the other ecosystems was similar in both groups.

Comparing the groups with or without obesity within each ecosystem, we found that the relative abundance of the *Neisseriaceae* family was lower in the duodenojejunal microbiota of the group with obesity (Figure 4(b)). At the species level, 19 species were significantly more abundant in the duodenojejunal microbiota of the participants with obesity (Cliff's delta effect size obese vs. control > 0; $p < 0.05$, Supplemental Table 6), while only three species were more abundant in the control group (Cliff's delta effect size obese vs. control < 0; $p < 0.05$, Figure 4(c)). Notably, none of the species' abundances differed significantly in participants with and without obesity in

saliva after correction for multiple comparisons, while two species still differed significantly between participants with and without obesity in the duodenojejunal fluid (Supplementary table 6). In the duodenojejunal microbiota, *Actinomyces sp* S6-Spd3 species was more abundant in participants with obesity, while *Neisseria subflava* species was more abundant in the control group. These two taxa were also detected in the oral microbiota of the study participants. Notably, *Actinomyces sp* S6 Spd3 abundance was similar in the saliva of both groups of participants. *Neisseria subflava* relative abundance was reduced both in the saliva and duodenojejunal fluid of participants with obesity although the magnitude of the effect was larger in duodenojejunal fluid ($p < 0.05$ and FDR > 0.1, Cliff's delta effect size obese vs. control = −0.44 vs. −0.77 in saliva and DJF, respectively, Supplemental Table 6). In stools, *Ruminococcus lactaris* species was significantly more abundant in the control group, with effect sizes similar to those of *Neisseria subflava* in duodenojejunal fluid. Consistent with the proportion of the compositional variance analysis (Figure 3(c)), a greater number of species were altered at nominal p-value level by the obesity status in the duodenojejunal fluid (22 out of 311 detected species, i.e. 6.6%) than in the stools (19 of detected 863 species, i.e. 2.2%).

To determine whether changes in the duodenojejunal microbiota in obesity are associated with changes in the metabolome, we performed similar univariate analyses (Cliff's delta analysis) of the duodenojejunal and fecal metabolomes, which showed that several lipids, and in particular sphingolipids, were enriched in the duodenojejunal fluid of the participants with obesity (Figure 4(d), Supplemental Table S7). Similar to the previous observation made on the microbiome composition and coherently with the higher proportion of metabolome variance explained by anthropometric data in the duodenojejunal fluid (Figure 3(c)), a higher absolute number and fraction of metabolites had significantly different levels between the participants with and without obesity in the duodenojejunal fluid than in the stools. Indeed, 16.1% (142 out of the 822 metabolites detected) of the metabolites were altered at nominal p-value level in the duodenojejunal fluid, versus only 10.0% (102 out of 1,019 metabolites detected) in the stool.

Linear regression analyses on quantile-normal transformed clr abundances of *Actinomyces sp* S6-Spd3 in the duodenojejunal fluid showed that this bacterium was positively associated with BMI, body fat percentage, visceral fat rating, along with higher circulating levels of leptin, γ-glutamyl Transferase, and Serum-Amyloid A (FDR < 0.1, Figure 4(e)). The relative abundance of this species in the duodenojejunal fluid also displayed a negative association with the circulating levels of gastric-inhibitory-polypeptide. Conversely, the relative abundance of *N. subflava* in the duodenojejunal fluid showed negative association with corpulence and body composition variables and circulating markers such as glycemia, HbA1c, triglycerides, and inflammatory markers (also combined in the Z-score). Additionally, it showed positive associations with circulating levels of active ghrelin, HDL-cholesterol, adiponectin, and the ASAT/ALAT ratio, as well as lifestyle factors such as wine consumption (FDR < 0.1, Figure 4(e)). Further adjusted association analysis highlighted the association of *N. subflava*'s relative abundance with BMI even after accounting for alcohol consumption (Supplemental Figure S5), suggesting a relationship between *N. subflava* and leanness while taking into account potential confounding factors.

Discussion

The intestinal microbiota is recognized as an important player in the metabolic regulation of the host. Most studies on this topic are based on the analysis of fecal microbiota, which is only representative of the microbiota of a small part of the digestive tract. This is obviously due to the differences in the ease with which stool and small intestine content samples can be collected. Yet, the paucity of studies focusing on the small intestinal microbiota hinders our understanding of how the microbiota regulates host metabolism, particularly because the small intestine is central in host metabolism compared to the more distal parts of the digestive tract. Here, we investigated the relationship between the composition of the oral, duodenojejunal and fecal microbiota, the duodenojejunal

and fecal metabolomes, lifestyle, and anthropometric and clinical variables in 30 participants, who were either lean or with obesity.

As expected, the oral and duodenojejunal microbiomes differed significantly from fecal microbiota. A large part of the species we detected with metagenomic sequencing were previously identified as members of the proximal small intestine microbiota previously found using 16S rRNA gene sequencing.[22,44] Moreover, we report a high similarity between the oral and duodenojejunal microbiomes, which supports several previous studies, also based on 16S rRNA gene sequencing.[36,45] This is in contrast to a recent metagenomic analysis across the entire digestive tract,[21] which reported that the small intestine microbiota, collected using ingestible capsules, is more akin to the fecal microbiome.[4] These discrepancies may be attributed to several factors, in particular differences in sampling location and variations in collection techniques between studies. All studies reporting a high similarity between the oral and proximal small intestine microbiomes are based on endoscopic aspiration, which carries a significant risk of sample contamination by saliva. Nonetheless, it should be noted that the total daily flow of saliva in adults is comprised between 1 and 1.5 liters.[46] As saliva represents a significant proportion of the content of the proximal small intestine, a high degree of compositional similarity between oral and duodenojejunal ecosystems would be expected and should not be significantly affected by minimal contamination of duodenojejunal samples by saliva during sampling. Another factor that may explain discrepancies between studies using different sampling methods is variation in sampling site. Endoscopic sampling inherently limits the collection of small intestine fluid to the very proximal part of the digestive tract, that is to say the duodenum and the duodenum-jejunum junction at the Treitz angle. Here, we sampled duodenojejunal fluid precisely at the Treitz angle, whereas Shalon and colleagues used capsules that may have collected luminal fluid at a more variable and possibly slightly more distal location, which may also explain the discrepant results. Another factor influencing the compositional results depending on the sampling method is the length of the time period between the sampling and the freezing of the samples. In our study, the duodenojejunal fluid was snap frozen with dry ice within minutes of sampling, whereas the luminal fluid in the ingestible devices underwent an incubation period at body temperature of approximately 7 to 66 hours (approximately one hour less than the transit time) between the opening of the ingestible devices and their recollection after defecation. We cannot exclude that the duodenal or jejunal content enclosed in an ingestible device without preservative medium undergoes compositional changes during this incubation period at body temperature. Finally, another significant difference between the studies is the sampling in the postprandial state using ingestible devices, whereas endoscopic aspiration obligatory requires a fasting state. The amount of microbes present in the food items as well as the transient multiplication of endogenous microbes due to the abundance of nutrients is likely to influence the composition of the proximal small intestine microbiome in the post-prandial state, whereas during fasting the comparatively higher concentration of digestive fluids secreted by the host, such as saliva, bile, intestinal mucus, and pancreatic secretion, will modulate the microbiota, although it is not yet known exactly how and to what extent. Ultimately, the two sampling methods provide different but complementary results.

Previous research has repeatedly shown that the diversity of the fecal microbiota is reduced in individuals with obesity.[47,48] However, our study shows the opposite trend in the proximal small intestine microbiota, suggesting that increased richness is associated with obesity at this location. Other reports show an elevated bacterial count in the duodenal mucosa-associated microbiome of hyperglycemic compared to normoglycaemic individuals and in overweight versus lean individuals.[24,49] These observations suggest that the increased diversity of the small intestinal microbiota in individuals with metabolic disorders is a confirmed phenomenon and may be a marker of metabolic deterioration. The determinants of the richness of the small intestine microbiota remain largely unknown, but the influence of the diet, the immune system, and variations in the environment provided by the host to the microbes are strongly suspected to play significant roles. In a population of 51 patients with cirrhosis, Hussain and

colleagues reported that duodenal microbiota richness is increased by protein intake and decreased with coffee consumption,[50] which is in contrast to our observation, as we did not observe a difference in protein consumption between lean and with obesity participants and a greater coffee consumption in controlled lean participants. Our study demonstrates significant associations between the microbiome and obesity-related metrics (e.g., BMI, percent fat mass, and android fat); nevertheless, it is important to note that dietary habits and alcohol consumption, which were significantly different between the subjects' groups with or without obesity, may influence the microbiome, as seen in many studies on fecal microbiota. These factors represent potential confounders that could contribute to the observed associations, independently of obesity. In the present study, associations between clinical variables and duodenojejunal microbiota composition remained in large part statistically significant after statistical adjustment on alcohol intake. Future studies with controlled dietary intake or stratified analyses in larger populations are warranted to disentangle these effects and provide more precise insights into the role of the microbiome of each part of the digestive tract in obesity.

A limitation of dietary assessment in such studies is the reliance on a Food Frequency Questionnaire (FFQ), which, while useful for capturing long-term dietary patterns, do not reflect short-term dietary variations that could influence microbiota composition, especially in the proximal small intestine. Future studies utilizing 24-hour dietary recalls or 3-day food records, especially the days preceding duodenojejunal fluid sampling, would provide more precise data on the relationship between recent dietary intake and microbial community structure. Ultimately, intervention studies in humans and in animal models in order to decipher the factors determining the richness of the proximal small intestine microbiota.

Supporting the hypothesis that the host, independently of the diet, directly regulates the small intestine microbiota composition and richness through a modulation of the environment provided to the microbes, Leite and colleagues showed that chronological age, and more importantly, aging process (exemplified by the number of age-related pathologies and systemic inflammation) decreases duodenal microbiome richness.[51] An increased diversity of the small intestine microbiome in obesity could also, at least partially, result from decreased control of the endogenous microbes by the host immune system as studies performed in rodent models show that the depletion of specific populations of immune cells (macrophages, B cells, T cells, and dendritic cells) systematically result in an increase in the duodenal microbiome diversity.[52] As such, our team previously demonstrated major alterations of immune cell profiles in the proximal intestine in people with severe obesity.[9] We hypothesize that the higher microbial diversity in the small intestine of individuals with obesity partially results from alterations in immune system fitness. However, this hypothesis requires deeper exploration of the small intestine barrier and mechanistic studies for validation. Notably, we found modifications in duodenojejunal fluid metabolites, which are rarely explored at this location, and potential interactions of these molecules with immune cells could be of significant interest for further investigation.

Notably, we observed a decreased relative abundance of the species *Neisseria subflava* and an increase in the species *Actinomyces sp* S6-Spd3 in the duodenojejunal fluid of the patients with obesity, suggesting a link between the abundance of these species in the proximal small intestine, metabolic health, and lifestyle factors. Of note, after adjusting for potential confounders (including alcohol consumption), the association analysis still demonstrated a link between the relative abundance of *N. subflava*, *Actinomyces sp* S6-Spd3, and corpulence traits. These two species belong to two genera, each comprising more than 30 species with names with Standing in Nomenclature, of which a significant proportion is classified as opportunistic pathogens. Species of both genera are known members of the oral and fecal microbiota in humans.[53,54] In the present study, only one species of each genus was associated with metabolic health despite the detection of 16 *Actinomyces* and 7 *Neisseria* species in the samples, suggesting that the associations observed between these taxa and metabolism only concern these species and not the genera as a whole. To our knowledge, the abundance of these taxa in the proximal small intestine has not yet been

associated with obesity and metabolic diseases, and with dietary intake features. Nevertheless, it has been observed that the abundance in the stomach of *Neisseria* and *Actinomyces* genera is decreased by *Helicobacter pylori* infection.[55] The role of *Helicobacter pylori* in obesity, either directly or mediated by bacteria whose abundance is affected by *H. pylori* colonization, is still debated as studies report a positive association between *H. pylori* prevalence and BMI, while others report negative associations.[56,57] Of note, the systematic search for *Helicobacter pylori* showed that all the participants in this study did not carry *H. pylori*. It has also been shown that species belonging to the *Actinomyces* genus are enriched in the ileal content of patients with inflammatory bowel disease, suggesting a connection between *Actinomyces* and inflammation in the small intestine.[58] Whether these two species are causally involved in the regulation of the host metabolism or affected by the diet or by the differences of environment provided to the microbes by the host depending on the obesity status cannot be determined in the present study. To this end, experiments using preclinical models would be necessary.

An additional limitation of our study is the potentially low coverage of the microbial ecosystem in certain duodenojejunal samples, which is attributable to high levels of human DNA contamination (ranging from 0.25% to 89.34% of quality-trimmed reads, compared to just 0.02% to 0.14% in fecal samples). Moreover, the use of a reference-based quantitative metagenomics approach (MetaPhlAn4),[38] while highly comprehensive for known microbial diversity, may fail to detect specific microbial species unique to understudied ecosystems like the duodenojejunal microbiome. To uncover potentially hidden microbial diversity, future studies employing de novo metagenomic assembly with larger sample sizes and deeper sequencing will be necessary.

By leveraging the metagenome characterization in three segments of the digestive system (mouth, proximal small intestine and stools) combined to an in-depth metabolic phenotyping of participants with and without obesity, we demonstrated that the duodenojejunal microbiota composition exhibits the most significant associations with the metabolic health and the diet of the participants, without excluding the contribution of lifestyle and dietary factors. This observation needs to be replicated in larger studies, as the small number of participants (30) in our study is a limitation. Nonetheless, this finding strongly supports the hypothesis that the duodenojejunal microbiome may exert a stronger influence on host metabolic health than the oral and fecal microbiomes, in connection with the crucial functions of the small intestine in host metabolism and its large interface with luminal content.

In conclusion, our study underscores the relevance of investigating the connections between the small intestinal microbiota, the diet and metabolic health in order to better understand human physiology. Developing preventive and therapeutic strategies based on the modulation of the proximal small intestine composition or activity will nonetheless require intervention studies in humans as well as preclinical and in vitro experiments.

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Availability of data and materials

All data produced in the present study are available upon reasonable request to the corresponding authors. Metagenomics sequencing reads are available on the European Nucleotide Archive under Project PRJEB69217.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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