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Metagenomic analysis of salivary microbiota in patients with anorexia nervosa and association with functional digestive disorders (ORMICAN pilot study)

Luc Vignal ,[1] Raynald de Lahondès ,[2] André Gillibert,[3] Marie-Pierre Tavolacci,[4] Edi Prifiti,[5,6] Etienne Formstecher,[2] David Ribet,[7,8] Muriel Quillard,[9] Moïse Coeffier,[4] Pierre Déchelotte[4,10]

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For numbered affiliations see end of article.

Correspondence to
Dr Luc Vignal;
luc.vignal@icloud.com

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ABSTRACT

Background Patients with anorexia nervosa (AN) have intestinal dysbiosis and are frequently affected by oral and upper gastrointestinal disorders. Until now, no metagenomic sequencing data were available on oral microbiota in AN.

Design This observational study enrolled 46 patients with restrictive/purging AN and 20 controls. Salivary samples were performed after fasting. DNA of oral microbiota from salivary samples was analysed by whole genome shotgun deep sequencing. The primary objective was to compare the diversity of oral microbiota between patients with AN and healthy individuals. Secondary endpoints were to assess the associations between the diversity of oral microbiota and the severity of functional digestive disorders, between patients with a restrictive type of AN and patients with a mixed/purging type and between the diversity of oral microbiota and the severity of AN.

Results We observed not only a significant decrease in the alpha diversity of oral microbiota in AN patients (4.47 (4.05; 4.75)) versus controls (4.81 (4.68; 5.04)) (p=0.001) but also in gene richness (p=0.00023). There was no significant correlation (95% CI) between oral microbiota diversity and functional digestive disorders nor between patients with a restrictive type of AN and patients with a mixed/purging type of AN, nor between the diversity of oral microbiota and the severity of AN. In addition, we observed four bacterial taxa that were decreased in AN patients.

Conclusion Our study highlights a decreased diversity of oral microbiota in AN patients. Future larger studies may help identify the prognostic and therapeutic value of oral microbiota in AN.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Patients with anorexia nervosa (AN) have intestinal dysbiosis and are frequently affected by oral and upper gastrointestinal disorders. Until now, no metagenomic sequencing data were available on oral microbiota in AN.

WHAT THIS STUDY ADDS

⇒ This study is the first to highlight a disruption in the oral microbiota of patients with AN in comparison with healthy individuals, including a decreased diversity of oral microbiota, decreased gene and species richness and a specific bacterial signature.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study paves the way for future research focusing on prognostic or treatment strategies targeting oral microbiota in AN.

INTRODUCTION

Anorexia nervosa (AN), a typical restrictive eating disorder (ED), affects nearly 1.4% of women and 0.2% of men in developed countries,[1] with a high mortality rate.[2] Incidence is increasing around the world with a sharp rise since the COVID-19 pandemic.[3] AN is characterised by restriction of energy intake in relation to nutritional needs leading to undernutrition associated with an intense fear of gaining weight and an alteration of body image perception.[4] Two subtypes of AN are described: the purely restrictive type and the mixed/purging type, where food restriction is associated with purging behaviours (ie, vomiting, laxative abuse), preceded or not by bulimic episodes. The current conception of AN and other EDs is based on a multifactorial metabo-psychiatric model involving genetic risk factors and environmental triggering and perpetuation factors, with an implication of microbiota-gut-brain axis dysfunction.[5] The diagnosis of AN is clinical, with no specific biomarkers. In the absence of specific drug therapy, AN care remains based on symptoms improvement. More research is needed to improve

our understanding of underlying neurobiological mechanisms and develop more specific treatments for better patient outcome.

Digestive disorders are common in patients with AN. Symptoms of functional dyspepsia (FD) such as gastric fullness, abdominal distension, nausea or early satiety, also known as post prandial distress syndrome, can affect 23 to 45% of patients with ED.[6–8] Santonicola *et al* reported a prevalence of 90% of postprandial distress syndrome in AN patients.[9] Irritable bowel syndrome can affect 41%–52% of patients with ED, and constipation can affect 67% to 83% of patients with AN.[7]

Oral manifestations are frequently found in patients with AN, as a consequence of vomiting or undernutrition. The most frequently found conditions are dental erosions, xerostomia and salivary gland hypertrophy; sialadenosis is more frequent in patients with bulimic episodes.[10 11]

Diet, one of the main factors shaping the intestinal microbiota, is profoundly disturbed in patients with AN. Several studies have highlighted gut dysbiosis in patients with AN.[12 13] Despite some heterogeneity between studies, some taxa show reproducible altered levels in AN patients such as a decrease in butyrate-producing species and an increase in mucin-degrading species.[12 13]

Oral microbiota (OM) is the second most diverse microbiota after intestinal microbiota, with more than 700 species and densities reaching 2×10^9 bacteria/mL of saliva.[14] Studies in patients with FD reported a decrease in microbial diversity in the saliva of dyspeptic patients vs controls and an increase in *Veillonella*.[15] *Veillonella* spp. is a commensal bacteria in the oral cavity but can be associated with a periodontal infection. An imbalance in the OM could lead to systemic inflammation, particularly if pathobionts gain access to the bloodstream.[16] Other studies highlighted that the severity of gastric symptoms was strongly related to higher levels of the genus *Streptococcus* in oral, oesophageal, gastric and duodenal mucosa-associated microbiota.[17]

In contrast to gut microbiota, data on OM in AN are sparse. Some early studies in the 1990s reported some pH and bacteria species modifications, for instance, an increase of *Streptococcus sobrinus* and *Streptococcus mutans*, related to bulimic episodes. However, these studies based solely on aerobic culture methods[18 19] were not able to detect many non-cultivable species. To our knowledge, no recent study has attempted to describe OM in AN using modern comprehensive metagenomic techniques.

To fill this gap, we explored OM in patients with AN and healthy controls (HC), using metagenomic sequencing. Our primary objective was to compare the diversity of OM between patients with AN and HC. Secondary endpoints were to assess the associations between the diversity of OM and the severity of functional digestive disorders, between patients with a restrictive type of AN and patients with a mixed/purging type of AN and between the diversity of OM and the severity of AN.

PATIENTS AND METHODS

Patients

This single-centre observational study enrolled patients with AN and HC. Patients with AN were recruited during external consultations for AN follow-up at the department of nutrition of Rouen University Hospital (France). HC were recruited via the healthy volunteer registry of the clinical investigation centre (CIC) of Rouen University Hospital. Inclusion criteria for AN patients were: female sex, aged over 18 years with a diagnosis of restrictive or purging AN according to The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5). Inclusion criteria for HC were: female sex, a body mass index (BMI) between 18.5 and 24.9, negative screening for ED according to the SCOFF questionnaire and irritable bowel syndrome according to ROME IV criteria. Patients filled in questionnaires (online supplemental table S1) on lifestyle and medical history. AN was evaluated using the Eating Disorder Inventory (EDI-2) questionnaire, and digestive symptoms were evaluated using the Francis score for irritable bowel syndrome and the FSSG score (Frequency Scale for the Symptoms of Gastro-oesophageal reflux disease (GERD)) for upper gastrointestinal (GI) disorders. Patients or controls who had taken antibiotics or probiotics during the last 3 months were identified but kept in the study and analysed separately.

Patient and public involvement statement

Patients or the public were not involved in the design, conduct, reporting or dissemination plans of our research.

Saliva sampling

Patients and controls were given the questionnaires and a tube for saliva sampling with DNA shield (DNA/RNA Shield Collection Tube w/ Swab ZymoBIOMICS Cat. No. R1211-E). Saliva was sampled at home at wake-up, prior to any food intake or drink, smoking, chewed gum or teeth brushing. The tube containing the saliva sample and the questionnaires was sent by post directly to the CIC, and aliquots of saliva were taken and stored at −80°C on reception. The frozen aliquots were later sent to the CeGaT sequencing platform (Tübingen, Germany) for shotgun deep sequencing. Metagenomic sequencing results were analysed by the GMT Science bioinformatics team and the statistics department of Rouen University Hospital.

DNA isolation and sequencing

Please see online supplemental appendix 5.

Metagenomic sequencing

Please see online supplemental appendix 6.

Metagenomic data processing

Please see online supplemental appendix 7.

Planned sample size

Please see online supplemental appendix 8.

Statistics

Primary and sensitivity analyses

Bacterial alpha-diversity could be defined by three statistics (Shannon index, Simpson and richness) on three taxonomies (bacterial species, genes, and Kyoto Encyclopedia of Genes and Genomes (KEGG) modules) leading to nine alpha-diversity metrics. The three statistics are defined in online supplemental appendix 2. The primary outcome was the Shannon index of bacterial species, while bacterial species Simpson index and gene richness were used in sensitivity analyses. Alpha-diversity indexes were compared between the control and AN group by Mann-Whitney tests without multiple testing procedure. The area under receiver operating characteristic (ROC) curve between the bacterial diversity (quantitative) and group (binary variable) was estimated by DeLong's method.

The mean Shannon index was compared between the control and AN groups without and with adjustments on age (linear effect), active tobacco consumption (yes vs no), number of tooth brushing per day (linear effect), daily mouth washing (yes vs no) and antibiotics use in the last 3 months (yes vs no) in post hoc linear models.

Beta-diversity

Average beta-diversity was computed in each group (AN or control group), by the mean Bray-Curtis distance between all possible pairs of two distinct patients of the group. Three means were calculated: mean Bray-Curtis between two controls, between two patients and between a control and a patient (planned secondary analyses). Mean beta-diversities were compared by bias-correlated accelerated (BC_a) bootstrap.

Secondary analysis: correlations between diversity and disease severity

Spearman's correlation coefficients between Shannon index and EDI-2, FSSG GERD, FSSG dyspepsia and Francis score were estimated by BC_a bootstrap in the AN group. In a post hoc analysis, Spearman's correlation between bacterial species abundances and BMI was estimated.

Secondary analysis: subgroup analysis

Shannon index was compared between patients with pure restrictive AN and patients with purging AN using a Mann-Whitney test.

General characteristics

For general clinical features, percentages were compared by Fisher's exact tests and means by Student's t tests, without multiple testing procedure.

Post hoc comparison by bacterial species

A post hoc comparison of the relative abundance of bacterial species, genera, families or orders between AN and control groups was performed using Mann-Whitney tests (see online supplemental appendix 4).

Software

All statistical analyses were performed and figures were drawn with R (V.4.2, The R Foundation for Statistical Computing, Vienna, Austria) statistical software. Medians and quartiles were calculated with the default method (type 7 according to 'Hyndman & Fan').

RESULTS

Flow chart

Initially, 50 patients and 21 HC were included. Four patients who did not send saliva samples were secondarily excluded. One control was secondarily excluded because she had a positive SCOFF score. Final statistical analysis included 20 HC and 46 patients with AN (29 restrictive type, 17 mixed/purging type).

Baseline characteristics of patients and controls

As expected, BMI was significantly lower and digestive disorders significantly more frequent in patients than in controls (43.5% vs 10%, p<0.0001). GERD or dyspepsia was more severe in patients (21.7%) than in controls (0%) (p=0.026). Smoking, medication use, especially antidepressants and anxiolytics were also more prevalent in AN patients than controls. AN patients tended to have an increased number of oral diseases. (online supplemental table S1)

OM alpha diversity

The median (IQR) of the Shannon index in the control group (n=20), estimated at 4.81 (4.68; 5.04), is significantly higher (p=0.001) than in the total AN group, estimated at 4.47 (4.05; 4.75) (n=46); the area under ROC curve (AUC) separating AN from control patients was estimated at 0.75 (95% CI 0.62 to 0.87) (figure 1). Seven AN patients who had taken antibiotics or probiotics in the last 3 months were kept in the study and analysed separately. Without these seven patients, the median (IQR) of the Shannon index in the control group (n=20) was estimated at 4.81 (4.68; 5.04) compared with 4.47 (4.1; 4.71) for the AN group (n=39) with an AUC at 0.76 (IC95: 0.63; 0.88, p=0.001). Moreover, within the AN patients, the difference was not significant between patients with (n=7) and without (n=39) antibiotics/probiotics according to a Mann-Whitney test (p=0.93) (sensitivity analysis, online supplemental appendix 1 figure S2). Thus, these seven patients were kept in the AN group.

Sensitivity analysis

The median (IQR) Simpson index of bacterial species was 1.59% (1.39; 1.94%) in the control group (n=20) vs 2.45% (1.86; 3.32%) in the AN group (n=46) with an area under ROC curve at 0.73 (95% CI 0.60 to 0.87,

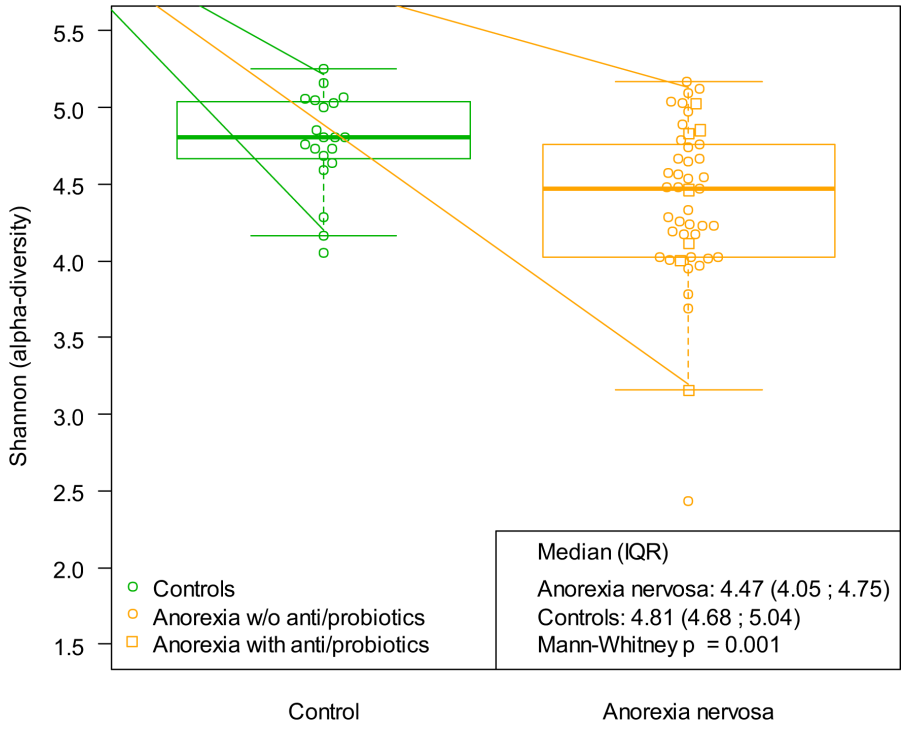

Figure 1 : Comparison of the alpha diversity of the oral samples between the control and anorexic groups by the Shannon index represented as beeswarm plots. Green plots represent controls samples whereas orange plots are AN patients' samples. Rectangles represent patients who took antibiotics or probiotics in the last 3 months.

p=0.002). The median (IQR) bacterial species richness (ie, number of different bacterial species found in the samples) was 280 (253.5; 323.25) in the control group versus 201.5 (164; 267) in the AN group with an area under ROC curve at 0.78 (95% CI 0.67 to 0.89, p=0.0003). These sensitivity analyses are better interpreted knowing the correlations between alpha-diversity indices described in online supplemental appendix 3 figure S2 and table S1.

The mean±SD Shannon index of bacterial species was 4.78±0.32 in the control group (n=20) vs 4.38±0.52 in the AN group (n=46) with a mean unadjusted difference at −0.39 (95% CI −0.65 to −0.14, p=0.003). After adjustment (post hoc analysis) on age, tobacco consumption, tooth brushing, mouth washing, antibiotics use in the last 3 months, the mean difference between the AN and control groups was −0.46 (95% CI −0.74 to −0.19, p=0.002). Adjustment on BMI was statistically invalid due to quasi-complete disjunction of the BMI between AN and control groups.

OM gene richness

Gene richness provided by shotgun analysis correlated well with species richness (Spearman test rho=0.93) (figure 2A). Gene richness was significantly decreased in AN patients compared with controls (p=0.00023) (figure 2B). According to the distribution of gene richness across samples, gene richness separated controls from patients (figure 2C).

OM beta-diversity

The difference in oral microbial population between two patients was greater than between two controls (figure 3). Indeed, the mean±SD bacterial species Bray–Curtis (distance of microbiota) among all pairs of women was estimated at 0.57±0.11 in AN versus 0.48±0.11 in pairs of controls. For a pair of women, one with AN and the other being a control, it was estimated at 0.55±0.12. The difference between the mean Bray–Curtis of two women in the anorexia group (0.57) and the mean Bray–Curtis of two women in the control group (0.48) was estimated to be +0.093 (95% CI 0.014 to 0.148, p=0.02). The difference between the mean Bray–Curtis of one woman in the anorexia group and one woman in the control group (0.55) and the mean Bray–Curtis of two women in the anorexia group (0.57) was estimated to be −0.017 (95% CI −0.050 to −0.001, p=0.04); this can be explained by the larger heterogeneity of AN patients compared with control patients. A post hoc analysis was performed to graphically assess beta-diversity based on projection of patients and controls on the first two components of a principal coordinate analysis (PCoA) based on the Bray–Curtis distance of bacterial species frequencies, without scaling (figure 3).

Subgroup and correlation analyses

Patients with purging AN (n=17) had a median (IQR) bacterial species Shannon index of 4.48 (4.19; 4.66) compared with 4.33 (4.03; 4.79) for restrictive AN anorexia (n=29), with an estimated AUC of 0.48 (95%

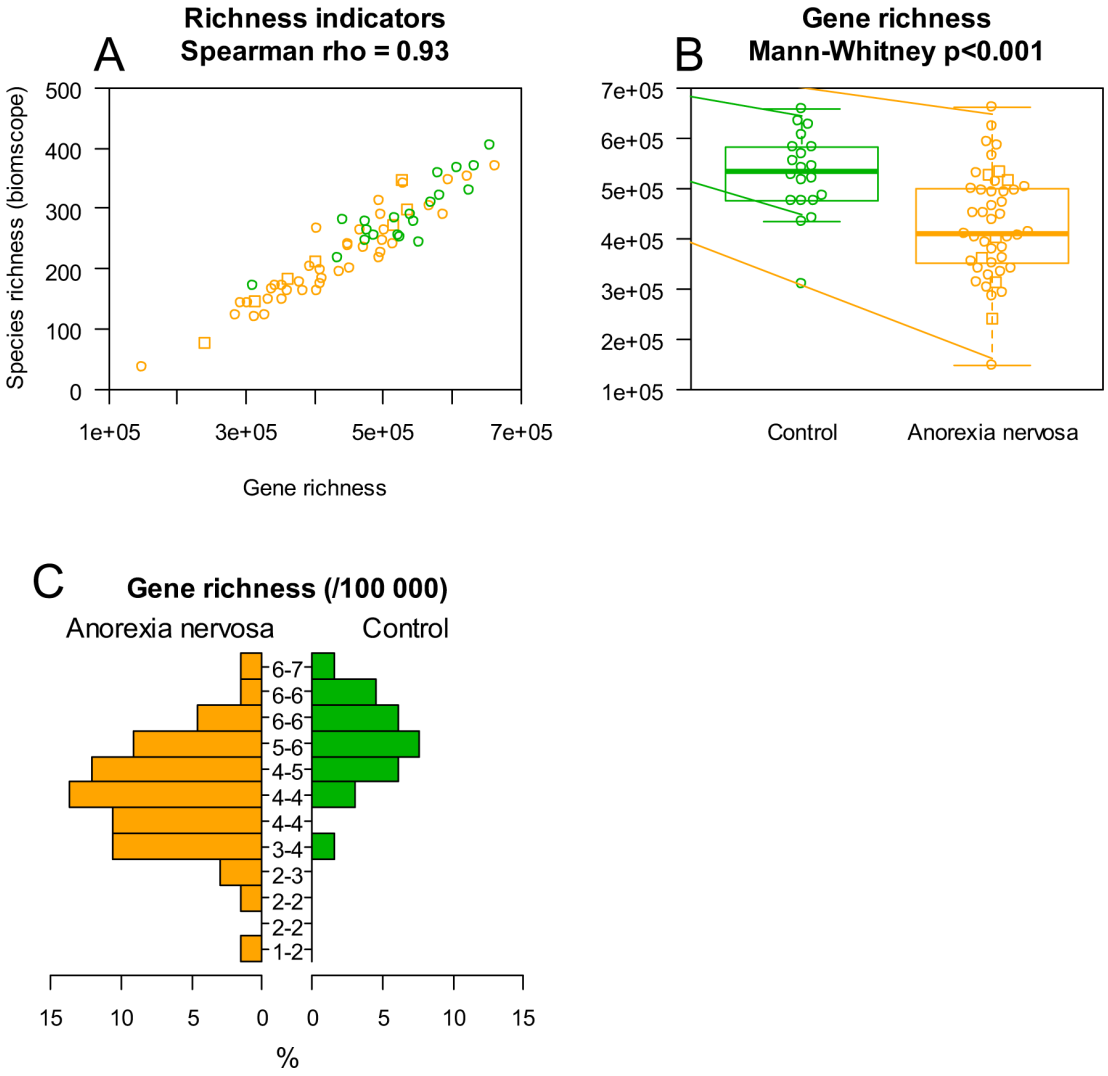

Figure 2 Comparison of gene richness of oral microbiota between control (green) and anorexia (orange) groups; squares represent patient who took antibiotics/probiotics within the last 3 months. (A) Association between gene richness and species richness assessed by Spearman index. (B) Comparison of gene richness between the two groups. (C) Distribution of participants (x-axis: % of all participants) according to gene richness (y-axis) of salivary samples.

CI 0.31 to 0.66, p=0.87). Thus, no significant difference for alpha-diversity was observed between purging versus restrictive AN patients.

In AN patients (n=46), we did not find any significant correlation (table 1) between the Shannon index and the EDI-2, Francis, FSSG GERD and FSSG dyspepsia scores. There was a non-significant statistical trend (R=0.30, 95% CI −0.02 to +0.57, p=0.06) for a positive correlation between EDI-2 and Shannon index.

Comparisons by bacterial species

Differences were computed between the mean relative abundance of bacterial species isolated or automatically grouped in genus, family and order if their relative abundance was too low (at least 10 patients having at least 0.1% of relative abundance for this species). From 700 bacterial species originally identified, after grouping low-abundance species, 421 isolated species, 58 genera, 23 families and seven orders were compared between AN patients and controls, for a total of 509 statistical tests (online supplemental tables S2 and S3). All results significant for an FDR at 20% are shown, in increasing P value order, in table 2. Benjamini-Yekutieli P values shown in table 2 can be interpreted as the lowest FDR that would have to be defined to accept the difference as statistically significant. At 5% FDR, four bacteria were significantly under-represented in AN patients: s__F0428 sp003043955, msp_2412 (*Bergeyella_A*), msp_2628 (*Mogibacterium*) and s__JABZIP01 sp015258265. At 20% FDR, there was a significant increase in the *Veillonella* genus and a decrease in the *Alloprevotella* genus in the AN group compared with the control group (table 2).

DISCUSSION

To the best of our knowledge, this study presents the first data on oral bacterial microbiota in AN patients using deep sequencing shotgun metagenomics and bioinformatic

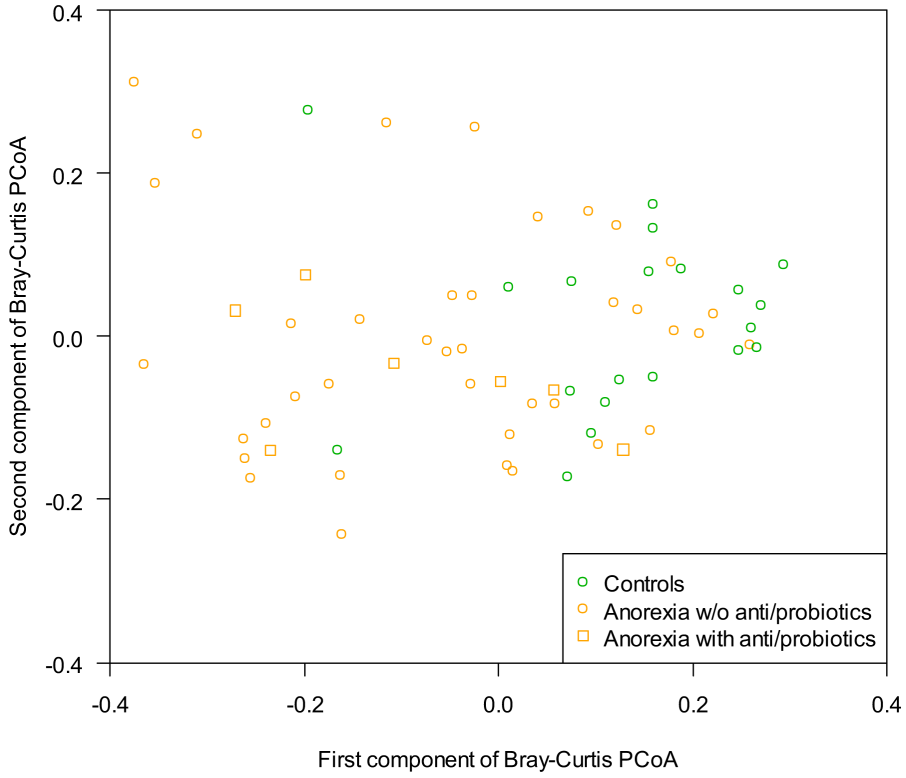

Figure 3 Comparison of Beta-diversity between the AN and control groups by graphical presentation of the first two principal components of PCoA with corrected "explained variances" (relative eigenvalues with Lingoes correction) equal to 18.9% and 8.7%.

analysis based on recently updated databases.[19 20] Our main results show that the OM of AN patients was significantly less diverse than that of controls. Secondary analyses did not show significant associations between the presence of digestive disorders and alpha-diversity, nor between the severity of AN and alpha-diversity. We did not find a significant difference in terms of alpha-diversity between the subgroups of AN (purging or pure restrictive), in contrast to some studies of gut microbiota in AN patients.[21]

We observed that the Bray–Curtis distance (beta-diversity) between patients was greater than between controls, suggesting that the composition of the OM in patients is more heterogenous than in controls. The high beta-diversity between patients could also be related to heterogeneity of severity and risk factors. The variance was higher in AN patients than controls, but the barycentres of groups are close to each other, suggesting that PCoA cannot distinguish anorexia patients from control.

Nevertheless, non-identified bacteria s__F0428 sp003043955, msp_2412 (*Bergeyella_A*), msp_2628 (*Mogibacterium*) and s__JABZIP01 sp015258265 were found more abundantly in controls than in patients with a false discovery rate at 5%. Despite what has been described in the literature in patients with AN,[11] we found a non-significant increase in *Streptococcus mutans* and *Lactobacillus* spp. We can explain this result by the metagenomic approach, which is more precise and exhaustive than previous studies that force us to correct the multiplicity of tests, which results in a loss of power for each individual germ. The *Veillonella* genus was more abundantly found in patients, and, on the contrary, *Alloprevotella* was less frequent in patients. In the literature, patients with squamous cell carcinoma exhibit significantly lower levels of *Veillonella* than HC.[22] *Bergeyella* was found to be enriched from superficial gastritis to gastric cancer.[23] This genus was found highly abundant in Chinese children without caries,[24] although this strain exhibits circadian fluctuations.[25] The *Veillonella* genus has been found to be in higher abundance in the OM of smokers, which is depleted as a whole, compared with a non-smoking group.[26] *Veillonella* spp. is involved in lactate metabolism from pyruvate in the propionate production pathway.[27]

Table 1 Correlation between Francis, EDI-2, FSSG and BMI scores and bacterial diversity with Shannon index

n=46 AN patients	Correlation with Shannon estimate (95% CI)	P value
FSSG GERD	−0.04 (−0.35; 0.29)	0.83
FSSG dyspepsia	−0.25 (−0.52; 0.07)	0.12
Francis score	−0.26 (−0.55; 0.10)	0.14
EDI-2	0.30 (−0.02; 0.57)	0.06
BMI (post hoc)	0.23 (−0.07; 0.49)	0.13

BMI, body mass index; EDI-2, Eating Disorder Inventory; FSSG, Frequency Scale for the Symptoms of GERD; GERD, gastro-oesophageal reflux disease.

Table 2 Main differences in bacterial population between AN and control groups corrected by the Benjamini Yekuteli (BY) method (FDR 20%)

Family	Genus	Species	MSP	Control group n=20 Mean±SD (% of alignments)	AN group n=46 Mean±SD (% of alignments)	Crude P val	BY P val
Lachnospiraceae	F0428	sp003043955	2255	0,175±0170	0.083±0202	3.4E-05	0.048
Weeksellaceae	Bergeyella_A	sp916710115	2412	0.177±0333	0.036±0119	3.3E-05	0.048
Anaerovoracaceae	*Mogibacterium*	sp916438645	2628	0.081±0108	0.012±0041	5.6E-05	0.048
Anaerovoracaceae	JABZIP01	sp015258265	2501	0.065±0099	0.009±0029	4.4E-05	0.048
Bacteroidaceae	*Alloprevotella*	Any		5.47±4.70	2.37±2.17	1.4E-04	0.054
Bacteroidaceae	*Bacteroidales*	Bacterium	2212	2.07±3.75	0.59±1.30	8.9E-05	0.054
X112	HOT-345	Any		1.381±0911	0.551±0668	1.4E-04	0.054
Neisseriaceae	*Neisseria*	*cinerea*	2327	0.297±0348	0.090±0177	1.3E-04	0.054
Filifactoraceae	*Peptoanaerobacter*	Unknown	2423	0.029±0029	0.006±0020	1.1E-04	0.054
UBA660	Any	Any		0.318±0349	0.095±0153	1.7E-04	0.057
Streptococcaceae	*Streptococcus*	Unknown	2589	0.210±0158	0.066±0123	1.9E-04	0.057
Weeksellaceae	Bergeyella_A	Any		0.178±0337	0.038±0119	2.0E-04	0.057
Veillonellaceae	*Veillonella*	Any		6.73±2.64	11.40±5.57	2.5E-04	0.065
Bacteroidaceae	*Alloprevotella*	sp900095835	2283	1.085±1405	0.253±0592	2.7E-04	0.065
UBA660	CAJPPJ01	Any		0.213±0301	0.058±0115	2.9E-04	0.065
Treponemataceae	*Treponema*	Unknown	2267	0.112±0110	0.035±0073	3.0E-04	0.065
Pasteurellaceae	*Mesocricetibacter*	sp905373425	2304	0.020±0019	0.006±0016	3.2E-04	0.066
Actinomycetaceae	*Pauljensenia*	Unknown	2350	0.327±0274	0.688±0496	3.5E-04	0.066
Filifactoraceae	*Peptoanaerobacter*	Unknown	2446	0.101±0092	0.044±0094	4.5E-04	0.082
Veillonellaceae	*Veillonella*	parvula_A	0313	1.23±1.63	2.23±2.01	5.4E-04	0.093
Streptococcaceae	*Streptococcus*	*parasanguinis*	0742	0.64±0.57	2.10±4.42	7.1E-04	0.104
Fusobacteriaceae	*Fusobacterium*	*polymorphum*	1790	0.218±0095	0.341±0180	6.8E-04	0.104
Selenomonadaceae	*Selenomonas*	Any		0.186±0179	0.093±0086	7.8E-04	0.104
Unknown	Unknown	Unknown	2709	0.174±0350	0.015±0052	7.4E-04	0.104
Bacilli RF39	Bacilli RF39 UBA660	Unknown	2581	0.084±0093	0.028±0067	7.6E-04	0.104
Bacilli RF39	Bacilli RF39 UBA660	CAJPPJ01 sp905372515	2640	0.054±0035	0.023±0037	6.9E-04	0.104
Absconditicoccaceae	Absconditicoccaceae HOT-345	sp013333295	2386	0.300±0253	0.110±0198	8.3E-04	0.107
Micrococcaceae	*Rothia*	mucilaginosa_A	2250	0.392±0435	1.129±1191	9.9E-04	0.112
Gemellaceae	*Gemella*	Unknown	2719	0.568±1208	0.110±0184	1.1E-03	0.112
Neisseriaceae	*Neisseria*	*gonorrhoeae*	2906	0.202±0222	0.057±0121	1.0E-03	0.112

Continued

Table 2 Continued

Family	Genus	Species	MSP	Control group n=20 Mean±SD (% of alignments)	AN group n=46 Mean±SD (% of alignments)	Crude P val	BY P val
Porphyromonadaceae	*Porphyromonas*	sp003640335	2306	0.113±0158	0.031±0100	1.1E-03	0.112
Selenomonadaceae	*Selenomonas*	sp916438625	2456	0.074±0185	0.011±0027	9.3E-04	0.112
Bacilli RF39	Bacilli RF39 UBA660	Unknown	2584	0.055±0072	0.016±0052	1.1E-03	0.112
Nanosyncoccaceae	*Nanosyncoccus*	Unknown	2656	0.059±0079	0.011±0038	9.8E-04	0.112
Neisseriaceae	*Wielerella*	Unknown	2457	0.044±0047	0.013±0023	1.1E-03	0.112
Aerococcaceae	*Granulicatella*	Unknown	2937	0.202±0280	0.060±0123	1.4E-03	0.134
Porphyromonadaceae	*Porphyromonas*	Any		1.98±1.08	1.09±1.06	1.5E-03	0.137
Bacteroidaceae	*Prevotella*	*aurantiaca*	2813	0.206±0396	0.022±0092	1.7E-03	0.158
Actinomycetaceae	*Actinomyces*	*oris*	0437c	0.319±0346	0.592±0666	1.9E-03	0.161
Nanosyncoccaceae	*Nanosyncoccus*	Any		0.511±0348	0.269±0345	1.9E-03	0.161
Gemellaceae	*Gemella*	Unknown	2926	0.374±0203	0.204±0211	1.9E-03	0.161
Pasteurellaceae	*Haemophilus*	*seminalis*	1785	0.591±0637	0.182±0198	2.0E-03	0.164
Actinomycetaceae	*Actinomyces*	*naeslundii*	2219	0.435±0266	0.236±0321	2.0E-03	0.164
Anaerovoracaceae	Any	Any		1.32±0.53	0.89±0.49	2.3E-03	0.169
Nanogingivalaceae	*Nanogingivalis*	sp900556165	2671	0.238±0293	0.120±0283	2.3E-03	0.169
Bacteroidaceae	*Prevotella*	sp000599605	2377	0.082±0064	0.043±0086	2.2E-03	0.169
Anaerovoracaceae	Unknown	Unknown	2547	0.021±0019	0.011±0036	2.2E-03	0.169
CAJPTX01	Bacteroides_D	sp013333195	2353	0.178±0205	0.054±0088	2.4E-03	0.170
Lachnospiraceae	*Catonella*	Unknown	2515	0.026±0035	0.005±0013	2.7E-03	0.189
Fusobacteriaceae	*Fusobacterium*	sp000235465	2636	0.492±0321	0.250±0301	2.8E-03	0.191
Streptococcaceae	*Streptococcus*	*timonensis*	2938	0.505±0417	0.252±0489	2.9E-03	0.196
Actinomycetaceae	*Mobiluncus*	sp916438445	2492	0.019±0017	0.009±0020	3.0E-03	0.199

Green items differ significantly between AN and control groups at false discovery rate (FDR) at 5%, orange items differ significantly at FDR 20% but not 5% and black items do not differ significantly. MSP describes the species number in the 8.4M catalogue of genes and species of the human oral microbiota.[20]
AN, anorexia nervosa; MSP, metagenomic species pan-genomes.

The level of bacteria of the *Alloprevotella* genus and in particular the *Alloprevotella rava* species has recently been inversely associated with the risk of suicidal ideation in the OM of adolescents,[28] and it is well established that patients with AN have a high risk of self-injury and suicide.[29] Furthermore, oral *Alloprevotella rava* was found highly abundant in consumers of high-sugar beverages[30] and in patients with IBS-diarrhoea.[31]

Like other studies before us, we did not find any association between poor OM and upper GI disorders in AN patients. This may be due to changes in specific bacterial populations (such as *Bergeyella*) or to the causal plurality of functional intestinal disorders.[15]

The main strength of this study is to use shotgun sequencing and recently updated bacterial DNA sequence databases for the comparison of carefully phenotyped patients and controls. Limitations include the relatively small number of participants and the lack of identification of certain bacterial sequences, which prevented us from accurately and fully describing the OM of patients with AN. This shortcoming is explained by the current performance limits of taxonomic profilers, which open possibilities for future re-analysis of this study. Finally, with respect to patient characteristics, the proportion of smokers and the frequency of tooth brushing and mouthwashing were higher in patients than in controls, which could constitute a confounding factor. Data should be stratified on these characteristics in larger future studies to explore the influence of these factors alone or in combination. Similarly, the frequency of antidepressant use was much higher in the patients. This could be the cause of dry mouth, itself responsible for a change in oral flora.[32] Data should be stratified on these characteristics in larger future studies to reduce bias. Although we had initially decided to exclude patients who had taken antibiotics or probiotics over the last 3 months, we did not exclude patients who had been included by protocol amendment. However, the sensitivity analysis excluding these patients did not change the results. Finally, we also decided not to present a metagenomic functional study with KEGG modules that we could not interpret clearly without a metabolomic analysis of saliva samples.

From a pathophysiological point of view, it is conceivable that the reduced oral bacterial diversity in patients with AN could interfere with taste or feelings of hunger and satiety or food palatability.[33 34] It has been reported that the composition of microbiota in contact with gustatory papillae might affect the orosensory perception of lipids in obese subjects.[35] Some taste alterations have been reported in AN patients with inconsistent findings, and microbiota data were rarely available.[34] Whether taste and oral microbial alterations could contribute to the onset or perpetuation of the disease remains to be determined. Another hypothesis is that oral dysbiosis could activate the immune system and cause systemic inflammation, which could maintain the disease.[36] More and more links are being established between the OM and neurological diseases such as autism, schizophrenia, depression and Alzheimer's disease. The effect of the OM on these pathologies may be via the destruction of the blood-brain barrier or indirectly via the gut-brain axis.[37] This study opens up a new link between OM and another neuro-psychiatric disease such as AN.

In conclusion, our study brings new data that may help to understand the physiopathology of AN. Although oral dysbiosis may be more a consequence than a cause of AN, it may at least contribute to the perpetuation of the disease via orosensorial changes. Future beneficial interventions might target OM, such as the use of oral probiotics, improved oral hygiene or specific dietary supplements, for example, to restore lipid intake. Further longitudinal studies are needed to investigate whether the analysis of OM will lead to the identification of prognostic biomarkers or help in the prediction of response to treatment.

Author affiliations
[1]Nutrition, Rouen Institut hospitalo-universitaire de recherche biomédicale et d'innovation, Rouen, France
[2]GMT science, Paris, France
[3]Statistics, Rouen Institut hospitalo-universitaire de recherche biomédicale et d'innovation, Rouen, France
[4]INSERM unit 1073, Rouen, France
[5]Integromics team, Institute of Cardiometabolism and Nutrition, ICAN, Paris, France
[6]unité de modélisation mathématique et informatique des systèmes complexes (UMMISCO), Sorbonne Universités, UPMC Univ Paris06, Bondy, France
[7]INSERM UMR 1073 "Nutrition inflammation and gut-brain axis", Rouen, France
[8]UNIROUEN, Rouen, France
[9]Rouen Institut hospitalo-universitaire de recherche biomédicale et d'innovation, Rouen, France
[10]Nutrition Unit, Rouen Unversity Hospital, Rouen, France

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Contributors PD planned the study; he is the principal investigator. He contributed to the writing of the paper. He is the guarantor of the paper. LV conducted the study and wrote the paper. AG planned and conducted the statistical analyses. He contributed to the writing of the paper. RdL conducted metagenomic analysis, and M-PT was responsible for recruitment of controls and the storage of saliva samples. EP participated in metagenomic analysis and formatting the metagenomic data. EF was responsible for organising the shipment of samples and sequencing. DR participated in the proofreading of the paper. MQ was responsible for the collection of saliva samples. MC helped order the supplies and participated in the proofreading of the paper.

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ORCID iDs
Luc Vignal https://orcid.org/0009-0004-4871-8262
Raynald de Lahondès https://orcid.org/0009-0000-2862-9589

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