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Gut microbiota alteration in a mouse model of Anorexia Nervosa

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SUMMARY

Background & aims: Anorexia Nervosa is a severe disease depending on both biological, psychological and environmental factors. The gut microbiota has recently been proposed as one of the biological factors potentially involved in the onset or maintenance of Anorexia Nervosa. To unravel the potential role of the gut microbiota in this disease, we characterized the dysbiosis occurring in a mouse model of Anorexia and correlated bacteria level changes with different physiological parameters such as body weight, food intake or levels of hypothalamic neuropeptides.

Methods: We used the Activity-Based Anorexia (ABA) mouse model, which combines food restriction and physical activity, and which mimics core features of Anorexia Nervosa. We characterized the gut microbiota alteration in ABA mice by combining 16S rRNA gene sequencing and quantitative PCR analyses of targeted genera or species.

Results: We identified 68 amplicon sequence variants (ASVs) with decreased levels and 8 ASVs with increased levels in the cecal content of ABA mice compared to control mice. We observed in particular in ABA mice increases in the abundance of *Clostridium cocleatum* and several *Lactobacillus* species and a decrease in the abundance of *Burkholderiales* compared to control mice. Interestingly, we show that most of the observed gut microbiota alterations are due to food restriction and are not affected by physical activity. In addition, we identified several bacterial groups that correlate with mice body weight, food intake, lean and fat masses as well as with hypothalamic mRNA levels of NPY (Neuropeptide Y) and POMC (Pro-opiomelanocortin).

Conclusions: Our study provides a comprehensive characterization of the gut microbiota dysbiosis occurring in the Activity-Based Anorexia mouse model. These data constitute a valuable resource to further decipher the role of the gut microbiota in the different facets of anorexia pathophysiology, such as functional gastrointestinal disorders, appetite regulation and mood disorders.

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1. Introduction

Anorexia Nervosa (AN) is a highly morbid eating disorder characterized by underweight (Body Mass Index (BMI) < 18.5 kg/m²), achieved by addictive food restriction and increased physical activity, a fear of gaining weight, and a disturbed self-body image

(DSM-V criteria) [1]. The prevalence of AN is increasing in most countries and is estimated to be 1.4% for women and 0.2% for men [2]. AN is usually considered as the psychiatric illness with the highest mortality rate (standardized mortality ratio > 5) [3] and thus constitutes a serious public health issue. The etiology and pathophysiology of AN remains poorly understood and treatments targeting the causal factors of AN are still lacking [4]. As a consequence, current treatments, that mainly focus on both psychological and nutritional approaches, have only limited efficacy with a relapse rate of ~40% within 18 months [5,6].

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AN is a multifactorial disease depending on biological, psychological and environmental factors. The gut microbiota has recently been proposed as one of the biological factors potentially involved in the onset and/or maintenance of Anorexia Nervosa [7–14]. Indeed, several studies have now established that anorectic patients have an intestinal dysbiosis (i.e. an alteration of the composition and/or activity of the gut microbiota in comparison to healthy individuals) [15–23]. However, the consequences of gut microbiota alterations in AN still remain hypothetical. As the gut microbiota has been involved in weight regulation, energy harvest from diet, eating behavior, as well as anxiety and depression (which are frequent comorbidities of AN), its potential role in AN may be highly versatile [24–27].

Animal models constitute key experimental approaches to decipher the potential role of the gut microbiota in human diseases. Among the various animal models of Anorexia Nervosa described in the literature, the Activity-Based Anorexia (ABA) model is one of the most studied ones [28,29]. In this model, rodents are isolated in cages equipped with an activity wheel and have a progressive time restricted food access, combined to a voluntary physical activity. This model leads to body-weight loss (between 10 and 25% depending of animal species, strains or sex) and was shown to mimic core features of AN. It has been instrumental to document brain alterations, gastrointestinal functional disorders and hormonal changes characteristic of AN [28–35]. The ABA model thus constitutes an interesting animal model to study the role of the gut microbiota in AN pathophysiology. Here, by combining 16S rRNA gene sequencing and quantitative PCR analysis of targeted genera or species, we provide a comprehensive characterization of the gut microbiota dysbiosis occurring in murine model of Anorexia and demonstrate that most of the identified gut microbiota alterations are due to food restriction. We identified in addition several bacterial groups which levels correlate with body weight, food intake, lean and fat masses as well as with hypothalamic levels of NPY (Neuropeptide Y) and POMC (Pro-opiomelanocortin) neuropeptides.

2. Materials and methods

2.1. Animals

Animal care and experimentation were approved by a regional Animal Experimentation Ethics Committee (authorization N/05-11-12/28/11-15) and complied with the guidelines of the European Commission for the handling of laboratory animals (Directive 2010/63/EU). All efforts were made to minimize suffering of animals. All animals were fed with a standard diet (3430PMS10; Serlab, France). The ABA protocol was performed as previously described [30]. Eight-week-old C57Bl/6JrJ male mice (Janvier Labs, Le-Genest-Saint-Isle, France) were housed individually at 23 °C with a reversed 12-h light-dark cycle (dark phase from 10:00 am to 10:00 pm) and split in three groups: a control group, with food *ad libitum* (CTRL), a group with restricted access to food (Limited Food Access; LFA) and a group with restricted access to food combined with a free access to a running wheel (Activity-Based Anorexia group; ABA). Food access was progressively limited for LFA and ABA mice from 6h at day 6 to 3h at day 9, and until the end of the experiment at day 17. Food was given at the beginning of the dark phase. All mice had free access to water. For each independent experiment, 6–8 animals were used per group. CTRL and LFA mice were housed in standard cages whereas ABA mice were housed in cages equipped with an activity wheel connected to the Running Wheel® software (Intellibio, Seichamps, France), that recorded ABA mice physical activity during the whole protocol. Body weight and food intake were monitored each day at the end of the light phase. In

accordance with ethical procedures, mice showing excessive weight loss (>20%) over 3 consecutive days were euthanized. Whole body composition was assessed on vigil animals at day 16 using fast nuclear magnetic resonance (Minispec LF110, Bruker, Wissembourg, France). At the end of the protocol (day 17), all animals were euthanized. Cecal contents and hypothalamus were removed, frozen in liquid nitrogen and stored at –80 °C.

2.2. Gut microbiota analyses

DNA from mouse cecal contents were extracted using the QIAamp DNA Stool Mini Kit (QIAGEN), including a bead-beating step (0.1 mm zirconia silica beads, BioSpec products, Bartlesville, USA) [36].

For Illumina sequencing, DNA samples from 2 independent animal experiments were PCR-enriched for the V5–V6 region of the 16S rRNA gene and then underwent a library tailing PCR as previously described [37]. The amplicons were purified, quantified and sequenced using an Illumina MiSeq to produce 2 × 300 bp sequencing products. Initial quality-filtering of the reads was conducted with the Illumina Software, yielding an average of 121 594 pass-filter reads per sample. Quality scores were visualized and reads were trimmed to 220 bp (R1) and 200 bp (R2). The reads were merged with the merge-Illumina-pairs application [38]. For all samples but two, a subset of 48 000 reads was randomly selected using Mothur 1.32.1 [39]. The UPARSE pipeline implemented in USEARCH was used to further process the sequences. Amplicon sequence variants (ASVs) were identified using UNOISE3 [59]. Taxonomic prediction was performed using the *nbc_tax* function [60], an implementation of the RDP Naive Bayesian Classifier algorithm [40]. The phylotypes were computed as percent proportions based on the total number of sequences in each sample. Indexes of alpha diversity were computed using QIIME [41]. Raw data generated during the analysis of the gut microbiota composition can be accessed on SRA (SRA accession: PRJNA565878).

Quantitative real-time polymerase chain reaction (qPCR) was performed on DNA samples from 3 independent animal experiments (including the 2 experiments used for Illumina sequencing). qPCR signals were detected on a Mastercycler *ep* Realplex system (Eppendorf, Hamburg, Germany) using Itaq Universal SYBR Green Supermix (BioRad). Primer sequences are detailed in Table S3. Primers specificity was checked using Ribosomal Database Project tools [42]. All samples were run in duplicate in 96-well reaction plates. Final concentrations were as follow: DNA 0.1 ng/μL (excepted for *Akkermansia muciniphila* 1 ng/μL), primers 0.5 μM, and SYBR Green Supermix 1X. Thermocycling conditions were as follow: initiation step at 95 °C 5 min; cycling stage at 95 °C 5 s, 60 °C 30 s (unless a different annealing temperature is indicated in Table S3), 40 cycles; melt curve stage at 95 °C 15 s, 65 °C 15 s, increment of 1 °C every 10 s until reaching 95 °C. The purity of the amplified product was verified by analyzing the melt curve performed at the end of amplification. At least 80% of the duplicates show a variation lower than 0.5 Cq units. Serial dilution of DNA from cecal content was included on each plate to generate a relative curve and to integrate primer efficiency in the calculations. Analyses were considered as acceptable when amplification efficiencies reached values between 70% and 110%. For detection of total Eubacteria, Cq of each sample were compared with a standard curve made by diluting genomic DNA extracted from a pure culture of *Escherichia coli*, for which cell counts were determined prior to DNA isolation. Non-template controls were included on each plate. A qPCR is considered valid if the Cq of the non-template control is at least 3 units higher than the Cq of the templates or, for targets with low expression levels, if the melt curve of the non-template control was different from the templates.

2.3. Quantification of neuropeptide expression

Extraction of total RNAs from CTRL, LFA and ABA mice hypothalamus and quantification of NPY and POMC mRNA levels were performed as described in [31]. Rps18 gene was used as an internal reference for normalization. Primer sequences are detailed in Table S3.

2.4. Statistical analyses

Comparison of body weight at day 17 and lean and fat masses at day 16 were performed using one-way ANOVA with Holm-Sidak's multiple comparison test. Comparison of body weight, food intake and physical activity during the protocol were performed using two-way ANOVA with Bonferroni's multiple comparison test. Comparison of bacterial taxa levels quantified by qPCR were performed using one-way ANOVA with Holm-Sidak's multiple comparison test. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, San Diego, USA) except for the sequencing results. Significant ASVs and taxa were selected using a Kruskal-Wallis test, with Benjamini-Hochberg correction for multiple testing (q-value) [43], followed by Dunn's post-tests. The significance threshold was placed at a q-value < 0.05. Comparison of alpha diversity indexes were performed using Kruskal-Wallis test followed by Dunn's post-test. Correlations were computed using Spearman correlations with Benjamini-Hochberg correction for multiple testing. Correlograms were performed with RStudio 1.1.383.

3. Results

We compared in this study three groups of mice: a control group with food *ad libitum* (CTRL), a group with restricted access to food (Limited Food Access; LFA) and a group with restricted access to food combined with a free access to a running wheel (Activity-Based Anorexia group; ABA). Body composition was determined at day 17 for CTRL, LFA and ABA mice. As previously reported in this animal model, we observed a significant decrease in body weight for LFA and ABA mice compared to CTRL mice, which correlates with a decrease in food intake [30] (Fig. 1A and B). At the end of the protocol (day 17), body weight loss was more important in ABA mice than LFA mice (Fig. 1C). The lean mass of both LFA and ABA mice was significantly decreased compared to CTRL mice (Fig. 1D). Physical activity pattern of ABA mice was significantly modified during the restriction period (day 6 to day 17) compared to the acclimatization period (day 1 to day 5), with a progressive increase in wheel activity during light phase and a decrease in wheel activity during dark phase (Fig. 1F).

In order to characterize the potential gut microbiota alteration occurring in the ABA mouse model, we extracted DNA from the cecal content of CTRL, LFA and ABA mice at day 17. Using 16S rDNA-targeting qPCR analysis, we monitored changes in the levels of Eubacteria, Archaea, Firmicutes and Bacteroidetes in these samples to detect potential broad alterations in the ABA mice gut ecosystem. We did not observe any significant differences in the abundance of Eubacteria, Archaea, Firmicutes and Bacteroidetes between CTRL, LFA and ABA mice (Fig. 2 and Table S1). As no difference in bacterial taxa at high taxonomic levels were observed between mice groups, we performed Illumina sequencing of 16S rDNA on mice cecal contents to obtain an accurate characterization of the gut microbiota composition from CTRL, LFA and ABA mice.

We did not observe any significant differences in alpha diversity indexes of richness (observed species) or richness and evenness (Shannon index) between CTRL, LFA and ABA mice (Fig. 2E and F). Using non-taxonomy based analysis of our sequencing data, we

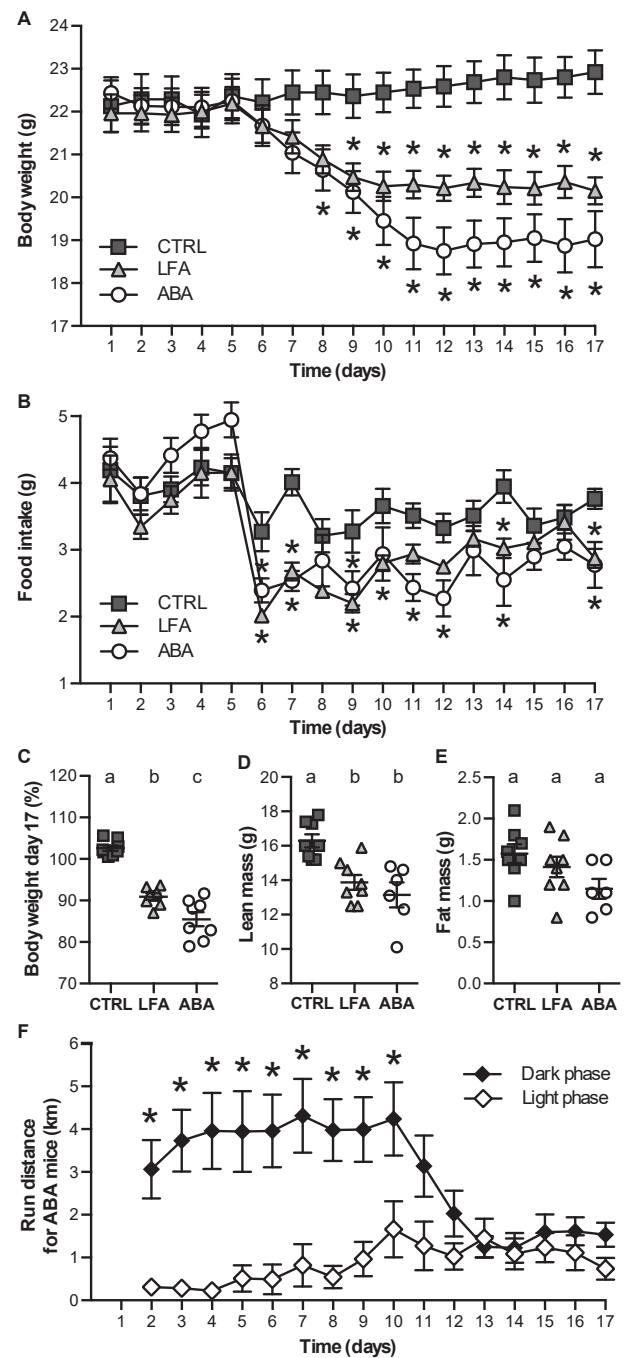


Fig. 1. Body weight loss and changes in physical activity in ABA mice. A, Changes in body weight during the protocol (values are means $\pm$ SEMs, $n = 8$; *, Different from control, $P < 0.05$). B, Food intake (values are means $\pm$ SEMs, $n = 8$; *, Different from control, $P < 0.05$). C, Body weight at day 17 (individual values corresponding to percentage of body weight at day 5 are represented with means $\pm$ SEMs, $n = 8$; Labeled means without a common letter differ, $P < 0.05$). D-E, Lean and fat masses at day 16 (individual values are represented with means $\pm$ SEMs, $n = 6-8$; Labeled means without a common letter differ, $P < 0.05$). F, Physical activity of ABA mice (values are means of run distance $\pm$ SEMs; $n = 8$; *, Different from physical activity during light phase, $P < 0.05$).

identified 85 Amplicon Sequence Variants (ASVs) with significant altered levels between mice groups (among the 1466 ASVs identified in this analysis; Tables S2 and S4). Eight ASVs show increased levels in ABA versus CTRL mice (including two ASVs attributed to

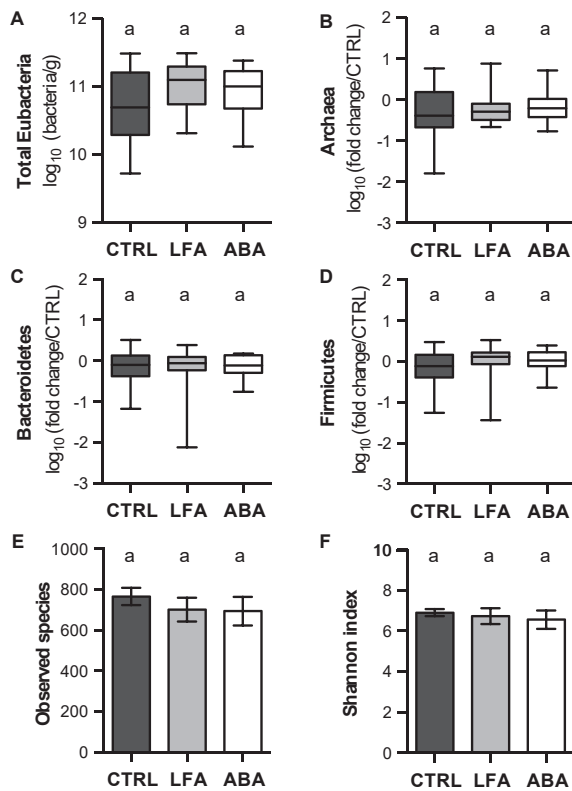


Fig. 2. Bacteria levels and alpha diversity indexes in CTRL, LFA and ABA mice gut microbiota. A–D, Relative quantification of total Eubacteria, Archaea, Bacteroidetes and Firmicutes in cecal contents from CTRL, LFA and ABA mice, as determined by qPCR analysis (values are represented as whisker plots with minimum and maximum values, $n = 20–23$; Labeled plots without a common letter differ, $P < 0.05$). E–F, Alpha diversity indexes in CTRL, LFA and ABA mice (values are means $\pm$ SEMs; $n = 13–15$; Labeled means without a common letter differ, $P < 0.05$).

Lactobacillus spp. and one attributed to *Clostridium* cluster XVIII) whereas 68 ASV show decreased levels in ABA versus CTRL mice (mainly belonging to the *Lachnospiraceae* and family and the *Bacteroidales* order). Interestingly, among the 76 ASVs with altered levels in ABA versus CTRL mice, 75 (99%) show similar levels between the two groups of food deprived mice (i.e. LFA and ABA mice). These data suggest that the main alterations that we observed in ABA mice cecal microbiota compared to CTRL mice are due to food restriction and are neither restored nor further impaired by physical activity. Only one ASV, corresponding to a yet unclassified bacterium, shows significant increased levels in ABA mice compared to both CTRL and LFA mice (ASV1396; CTRL: $0.0013 \pm 0.0009\%$; LFA: $0.0027 \pm 0.0012\%$; ABA: $0.0112 \pm 0.0039\%$; Tables S2 and S4).

In line with these analyses, using taxonomy-based analysis of our sequencing data, we identified 3 taxa with significant altered levels between CTRL, LFA and ABA mice (with significant differences also observed in parent taxa): the *Burkholderiales* order, the *Clostridium* cluster XVIII genus and the *Lactobacillus* genus (Fig. 3 and Fig. 4). Again, the abundance of these taxa differs between CTRL mice and food deprived mice (LFA and ABA), with no significant differences observed between LFA and ABA mice.

To confirm these results and to narrow down the species from *Clostridium* cluster XVIII showing increased levels in ABA mice, we focused on the only identified ASV belonging to *Clostridium* cluster XVIII in our dataset (ASV153; Table S4). This ASV is highly homologous to the 16S rRNA gene of *Clostridium cocleatum* (one of the four bacterial species constituting *Clostridium* cluster XVIII) [44]. We

thus used a couple of primers specific for *C. cocleatum* 16S rRNA gene [45] to monitor its abundance by qPCR in the cecal bacterial community from CTRL, LFA and ABA mice. We observed that *C. cocleatum* is significantly increased in the cecum of both LFA and ABA mice compared to control mice (Fig. 4D) (14.5 fold-change increase for LFA vs CTRL and 11.9 fold-change increase for ABA vs CTRL). This result confirms our 16S rRNA sequencing data and suggests that food restriction increases the level of *C. cocleatum* in mice gut microbiota.

In addition to *C. cocleatum*, our sequencing data indicate that *Lactobacillus* species are significantly increased in both LFA and ABA mice (Fig. 4B). To confirm this result, we monitor by qPCR changes in *Lactobacillus* spp. levels. We observed that *Lactobacillus* spp. levels are significantly increased in both LFA and ABA mice compared to control mice (5.1 fold-change increase for LFA vs CTRL and 5.7 fold-change increase for ABA vs CTRL) (Fig. 4E). To better characterize the changes in the *Lactobacillus* genus in ABA mice, we quantified the levels of the most abundant *Lactobacillus* species in C57Bl/6 mice gut microbiota (i.e. *Lactobacillus reuteri*, *Lactobacillus murinus/animalis* and *Lactobacillus johnsonii/gasseri*) using specific primers [46,47]. We observed a significant increase in the abundance of all tested *Lactobacillus* species in ABA mice compared to CTRL mice (Fig. 4). This result confirms again our 16S rRNA sequencing data and suggests that the dominant *Lactobacillus* species are all increased in ABA mice as a result of food restriction.

To complete our characterization of the gut microbiota in ABA mice, we quantified by qPCR the levels of specific bacterial species that were not detected by 16S rRNA Illumina sequencing but which represent interesting candidates to understand the putative link between anorexia-associated functional gastrointestinal disorders and gut microbiota dysbiosis. These species include (i) *Methanobrevibacter smithii*, the predominant human gut methanogen archaeon, which was found overrepresented in the microbiota of anorectic patients from two independent cohorts [15,20], (ii) *Roseburia* spp, which are butyrate producers shown as decreased in the microbiota of anorectic patients from three independent cohorts [19,20,23], (iii) *Faecalibacterium prausnitzii*, a Firmicutes exhibiting anti-inflammatory effects and shown in several studies as decreased in patients with Inflammatory Bowel Diseases [48], and (iv) *A. muciniphila*, a mucin-utilizing specialist showing decreased abundance in several pathological conditions such as obesity or type 2 diabetes [49]. We did not observe any significant differences in the levels of *M. smithii*, *Roseburia* spp and *F. prausnitzii* between CTRL, LFA and ABA mice (Table S1). We observed a significant 2.1-fold decrease in the level of *A. muciniphila* in LFA mice compared to CTRL mice (Fig. 4I). A similar pattern was observed in ABA mice for *A. muciniphila*, but difference did not reach significance. This result suggests that food restriction leads to a decrease in the cecal abundance of *A. muciniphila* in C57Bl/6 mice.

By correlating the levels of the different identified bacterial taxa together, we identified several clusters of bacteria with positively correlated levels (corresponding to bacteria frequently observed together in a given mice) and, in contrast, bacterial taxa with negatively correlated levels (corresponding to mutually exclusive bacteria) (Fig. 5A). For example, we could observe that the genera *Clostridium* cluster XIVa, *Clostridium* cluster XI, *Escherichia/Shigella* and *Enterococcus* form a cluster of positively correlated bacteria, and that this cluster is negatively correlated with bacteria belonging to the *Prevotella* and *Acetatifactor* genera (Fig. 5A).

Finally, in order to highlight potential links between the gut microbiota dysbiosis observed in ABA mice and animal physiology, we correlated the levels of the identified bacterial taxa and ASVs with biological parameters such as body weight, food intake, lean and fat masses or the mRNA levels of two neuropeptides, NPY and POMC, that we quantified in the hypothalamus of CTRL, LFA and

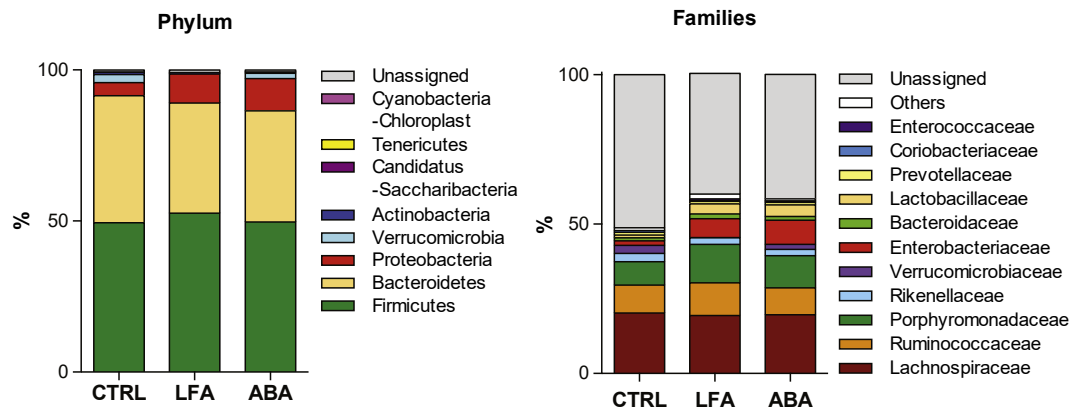


Fig. 3. Relative abundance of bacterial taxa in CTRL, LFA and ABA mice gut microbiota. Relative abundance of bacterial phyla (left) and families (right) identified in cecal contents from CTRL, LFA and ABA mice (families with relative abundance below 0.5% in all mice groups are classified as “others”).

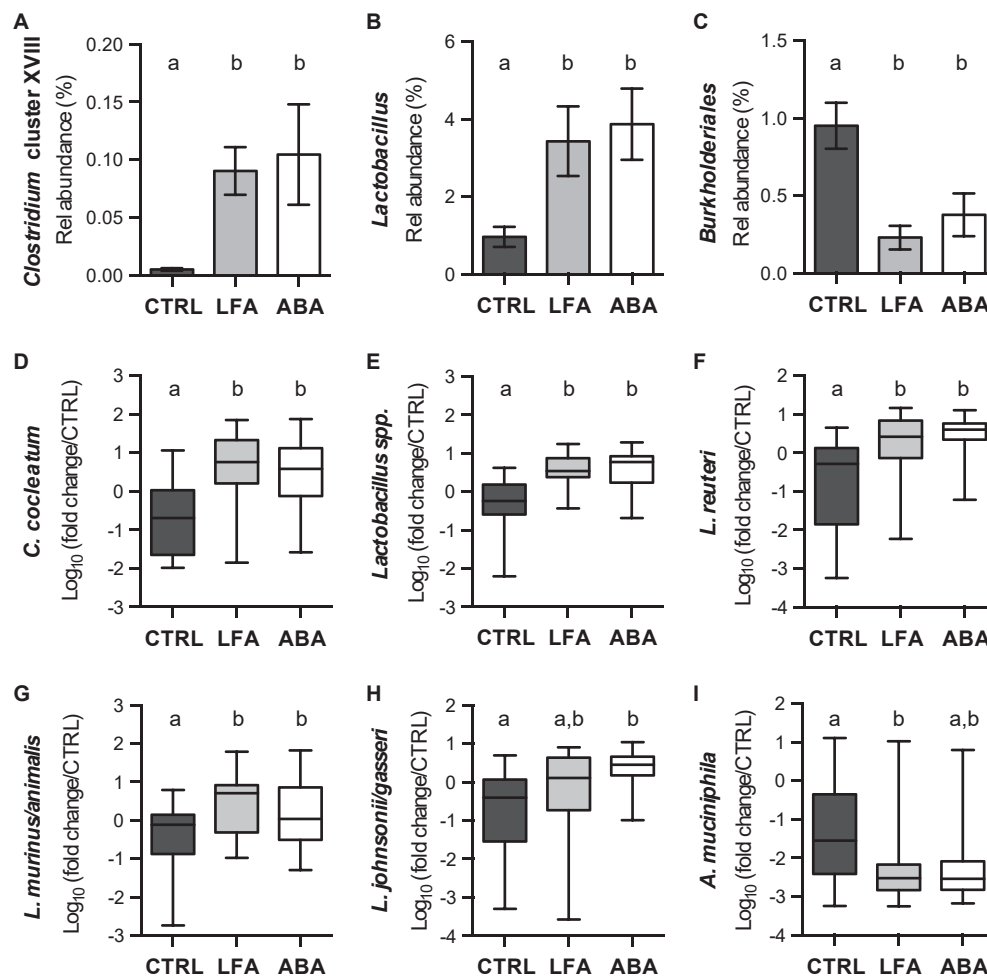


Fig. 4. Bacteria levels alteration in CTRL, LFA and ABA mice gut microbiota. A-C, Relative abundance of bacteria belonging to *Clostridium* cluster XVIII, *Lactobacillus* and *Burkholderiales* taxa in cecal contents from CTRL, LFA and ABA mice, as determined by V5–V6 16S rRNA gene sequencing (values are means $\pm$ SEMs; $n = 14–15$; Labeled means without a common letter differ, $P < 0.05$). D-I, Relative quantification of different bacterial species in cecal contents from CTRL, LFA and ABA mice, as determined by qPCR analysis (values are represented as whisker plots with minimum and maximum values, $n = 19–23$; Labeled plots without a common letter differ, $P < 0.05$).

ABA mice. These two neuropeptides play opposite roles in appetite regulation: NPY is a potent orexigenic neuropeptide whereas POMC is the precursor of the α -melanocyte-stimulating hormone (α MSH), a potent inducer of satiety pathways [27]. We identified several

bacterial taxa and ASVs that correlate positively or negatively with body weight, food intake, lean mass and fat mass (Fig. 5B). In agreement with our previous results, we observed for example that *Burkholderiales* are positively correlated with body weight, food

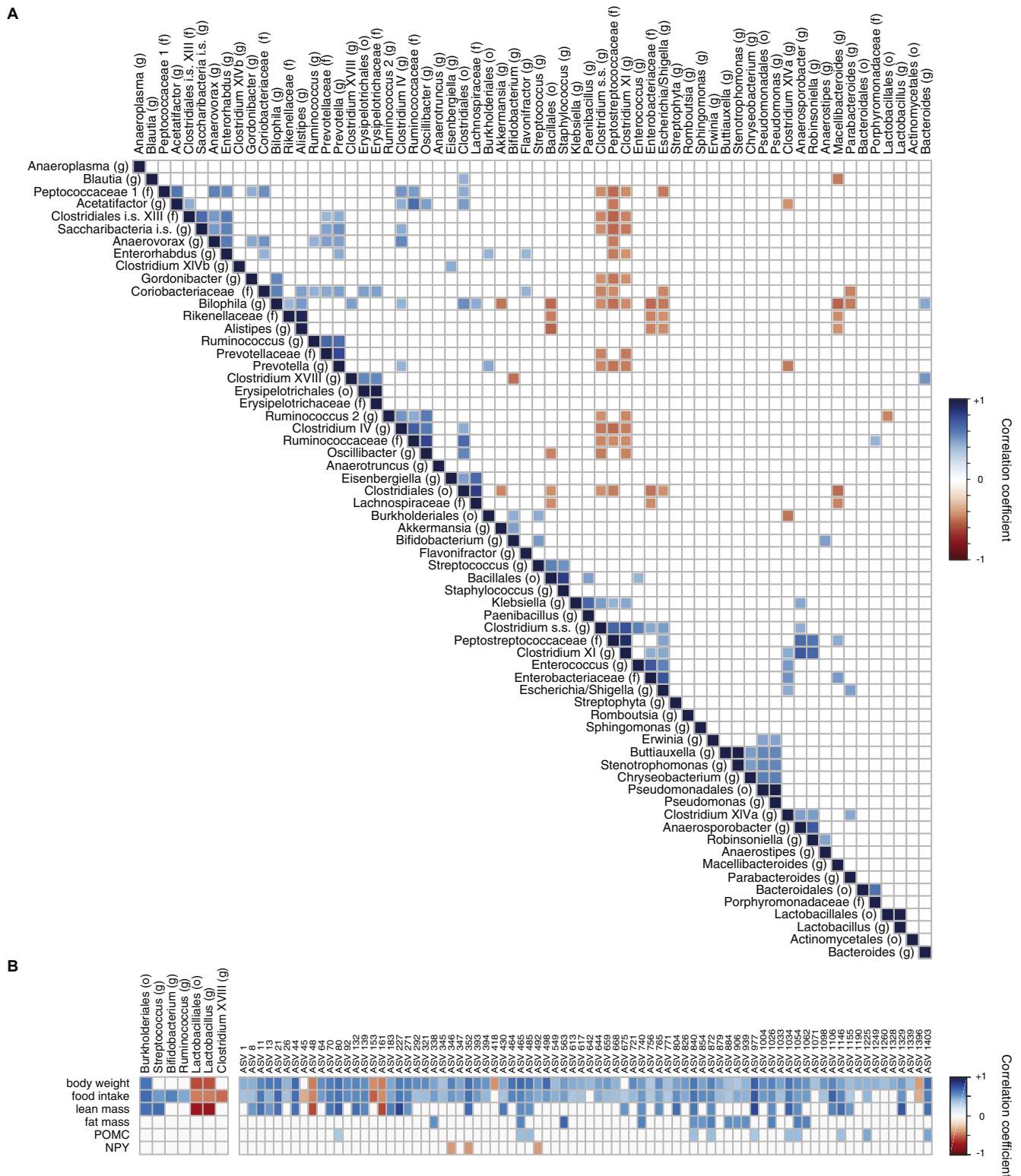


Fig. 5. Correlations between gut microbiota alterations and biological parameters variations in CTRL, LFA and ABA mice. Correlations between the different bacteria taxa quantified in mouse cecum (A) or between bacteria levels and mouse biological parameters (B) (heatmaps of Spearman correlation coefficients; only significant correlations are displayed; adjusted P-value<0.05). The blue color indicates a positive correlation and the red color a negative correlation. Color intensity is proportional to the correlation coefficients. Taxonomy-based analyses were restricted to bacterial orders, families and genera (o, order; f, family; g, genus; i.s., incertae sedis; s.s., sensus stricto).

intake and lean mass (i.e. increased in mice with high body weight, high food intake and high lean mass) in contrast to *Lactobacillales* which are negatively correlated with these three parameters. Interestingly, we also identified 11 bacterial units that are positively correlated with POMC hypothalamic levels and 3 bacterial units that are negatively correlated with NPY hypothalamic levels (all of them being positively correlated with body weight and food intake, as expected) (Fig. 5B).

4. Discussion

The Activity-Based Anorexia model mimics core features of AN in humans [28,29]. This animal model has been instrumental to decipher pathophysiological mechanisms occurring during AN. In order to unravel the potential role of the gut microbiota during AN, we performed a detailed characterization of the gut dysbiosis occurring in ABA mice after 12 days of food restriction. We identified 8 bacterial units showing increased levels and 68 bacterial units showing decreased levels in ABA mice compared to CTRL mice.

Almost all bacteria showing increased or decreased levels in ABA mice compared to CTRL mice are similarly altered in ABA and LFA mice, which suggests that the main driver for the gut microbiota dysbiosis observed at the end of our protocol is food restriction and that the effect of physical activity is only marginal. Of note, we analyzed gut microbiota composition in ABA mice at day 17, where mice exhibit decreased physical activity compared to the day 1-day 10 period (Fig. 1F). We thus cannot exclude that physical activity has a more pronounced effect on gut microbiota composition at the beginning of the food restriction period in ABA mice.

Alteration of gut microbiota in a rat ABA model has been partially described in a previous study using PCR-denaturing gradient gel electrophoresis and qPCR approaches [50]. In agreement with our observations, most of the detected alterations in bacteria levels in ABA rats were similarly observed in food restricted rats that had not access to a running wheel. This again suggests a major impact of food restriction on gut microbiota alteration in a context of anorexia. In contrast to our findings, ABA rats showed decreased levels in *Firmicutes*, *Bacteroidetes* and *Lactobacillus* and increased levels in *M. smithii* in comparison to rats fed *ad libitum* [50]. These differences in gut microbiota alterations between mouse and rat ABA models might be due either to rodent-specific differences, to differences in the type of samples used for 16S rRNA gene sequencing (feces for rats versus caecal contents for mice) or to divergences in the ABA protocol, such as the daily duration for food access (1h for rats versus 3h for mice) or the total length of the restriction period (6 days for rats versus 12 days for mice) [50].

It was recently shown that major metabolic perturbations are induced in the Activity-Based Anorexia model, which was proposed to reflect both host and microbiome metabolic pathways adaptation to food restriction [51]. It was shown in particular that undernutrition was the major driver for urinary and blood metabolite variations in ABA mice whereas physical activity had no significant impact [51]. This nicely echoes our own observation of a major role of food restriction on gut microbiota alteration. As gut bacteria are important providers of metabolites for the host, we can thus propose that, in this animal model of Anorexia, food restriction alters gut microbiota composition and metabolic capacities, which in turn has an impact on host circulating and urinary excreted metabolites.

We identified in this study several bacterial groups correlating with biological parameters such as body weight, food intake and

lean/fat masses. Interestingly, we identified bacterial units positively correlating with POMC hypothalamic levels or negatively correlating with NPY hypothalamic levels. These bacteria constitute interesting candidates potentially involved in gut-brain axis communication. One interesting hypothesis is that the dysbiosis observed in ABA mice alters the communication along the gut-brain axis and has an impact on animal behavior and, in particular, on appetite regulation [27]. Determining whether the gut microbiota changes observed in anorectic patients facilitates or perpetuates eating behaviors dysregulations is an essential objective that may have repercussions on the clinical management of AN [14].

Our data reveal that the abundance of *C. cocleatum*, which belongs to *Clostridium* cluster XVIII, is strongly increased in response to food restriction (>10-fold increase) in ABA mice. *C. cocleatum* is a mucin degrader that possesses numerous glucosidase activities involved in the degradation of mucin oligosaccharide chains in the digestive tract [52]. Interestingly, a decrease in enteral nutrition was reported to stimulate the growth of mucin degrading bacteria, probably because these micro-organisms present a competitive advantage in the context of food nutrient deprivation [53]. *C. cocleatum* may thus possess a competitive advantage in the digestive tract of ABA mice, which may explain its observed high levels in these animals. Of note, the *Clostridium* cluster XVIII taxon was observed as being increased in a cohort of anorectic patients compared to healthy individuals [19]. A refined characterization of the species belonging to *Clostridium* cluster XVIII increased in anorectic patients gut microbiota and their potential consequences on both animal and human physiology would deserve further investigations.

In addition to *C. cocleatum*, we observed an increase in the abundance of *Lactobacillus* in response to food restriction in C57BL/6 mice. Our data are consistent with several studies that linked increases in *Lactobacillus* levels with calorie-restricted diets. It was shown for example that a 14 days-long calorie restriction diet in C57BL/6 mice (leading to ~20% of weight loss) shifts the gut microbiota and create a *Lactobacillus*-predominated gut ecosystem, which was associated with decreased markers of systemic inflammation [54,55]. In humans, a calorie-restricted diet associated with increased physical activity over 10 weeks increased the abundance of gut *Lactobacillus* [56]. We observed that all major *Lactobacillus* species were increased in ABA mice. This suggests that the relative proportions of *Lactobacillus* species in ABA mice remain probably unchanged, in contrast for example to cancer-induced anorexia mouse models, where *L. murinus/animalis* outcompete *L. reuteri* and *L. johnsonii/gasseri* [47].

Finally, we identified a significant decrease in the level of *Burkholderiales* in food-restricted mice and observed that this bacterial order is positively correlated with mice body weight. These results are consistent with other reports showing a positive correlation between *Burkholderiales* and body weight in a model of mice fed with a Western-style diet [57], or with reports showing a potential role of *Burkholderiales* in BMI gain in risperidone-treated psychiatric patients [58].

Although several studies have described the gut dysbiosis associated with Anorexia Nervosa in humans, no clear consensus have emerged yet [14]. Only very few bacterial species were repeatedly described as being increased or decreased in independent human studies [14,15,19,20,23]. This heterogeneity in the observed dysbiosis associated with human AN may be due to methodological differences (sample collection, bacterial DNA extraction, data analysis), to variations in the design of the clinical

study (timepoint for sample collection, criteria for the selection of reference individuals) or to anorectic patients' heterogeneity (variations in BMI, in levels of calorie intake by patients or in patients' medical history). Not surprisingly, we observed differences in the dysbiosis of ABA mice compared to the reported dysbiosis in humans. Indeed, several studies observed no significant differences in the abundance of *Lactobacillus* species between patients and healthy controls, and we did not detect significant differences in *M. smithii* or *Roseburia* abundances in ABA mice [15–17,19]. These discrepancies may reflect human versus rodent-specific differences, and in particular differences between the laboratory feeds given to mice and human diets. They may also reveal the involvement of additional factors in the human disease that, for example, limit the expansion of *Lactobacillus* despite food restriction or promotes alterations in *M. smithii* and *Roseburia* levels independently of changes in food intake. In contrast to human Anorexia Nervosa, which is a multifactorial disease, the ABA animal model constitutes a simplified system in which genetic and environmental variables are controlled. This model thus constitutes an interesting tool to disentangle microbiota changes induced by food restriction and physical activity from changes induced by the other factors involved in this complex disease in humans. In addition, this model can be used to study the interactions between bacterial species in the gut ecosystem in response to an environmental disruption (food restriction in our case; Fig. 5A).

In conclusion, the potential role of the gut microbiota in AN has recently emerged as a promising field of research. We anticipate that our results will pave the way for future studies aiming at manipulating mouse gut microbiota in order to determine the role of this ecosystem in the different facets of anorexia pathophysiology, such as functional gastrointestinal disorders, appetite regulation or mood disorders.

Statement of authorship

Jonathan Breton: Conceptualization, Formal analysis, Investigation, Writing- Original Draft, Visualization. **Pauline Tirelle:** Investigation. **Soujoud Hasanat:** Investigation. **Arthur Pernot:** Investigation. **Clément L'Huillier:** Investigation. **Jean-Claude do Rego:** Resources. **Pierre Déchelotte:** Conceptualization, Writing-Original Draft. **Moïse Coëffier:** Conceptualization, Writing-Original Draft. **Laure Bindels:** Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing- Original Draft, Visualization. **David Ribet:** Conceptualization, Validation, Investigation, Data curation, Writing- Original Draft, Writing- Review & Editing, Visualization, Supervision, Funding acquisition.

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Conflicts of interest

PD is a co-founder of the TargEDys company; JB, PT, SH, AP, CL, JCDR, MC, LB, DR, no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clnu.2020.05.002>.

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