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1 **Gut microbiota alteration in a mouse model of Anorexia Nervosa**

2

3 Jonathan Breton^{a,b,d}, Pauline Tirelle^{a,b}, Soujoud Hasanat^{a,b}, Arthur Pernot^{a,b}, Clément
4 L’Huillier^{a,b}, Jean-Claude do Rego^{b,c}, Pierre Déchelotte^{a,b,d}, Moïse Coëffier^{a,b,d}, Laure B.
5 Bindels^c and David Ribet^{a,b,*}

6

7

8 a. Normandie Univ, UNIROUEN, INSERM UMR 1073, Nutrition, Inflammation et
9 dysfonction de l'axe intestin-cerveau, Rouen, France.

10 b. Normandie Univ, UNIROUEN, Institute for Research and Innovation in Biomedicine
11 (IRIB), Rouen, France.

12 c. Normandie Univ, UNIROUEN, Animal Behaviour Platform (SCAC), Rouen, France.

13 d. Rouen University Hospital, Nutrition Department, Rouen, France.

14 e. Metabolism and Nutrition Research Group, Louvain Drug Research Institute, Université
15 catholique de Louvain, Brussels, Belgium.

16

17 * Corresponding author: david.ribet@inserm.fr

18 INSERM UMR1073 – Université de Rouen

19 UFR Santé - 22 Boulevard Gambetta

20 76183 ROUEN CEDEX

21 Tel : 33 (0) 2 35 14 82 40

22 **ABSTRACT**

23

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

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

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

44 *Conclusions.* Our study provides a comprehensive characterization of the gut microbiota
45 dysbiosis occurring in the Activity-Based Anorexia mouse model. These data constitute a
46 valuable resource to further decipher the role of the gut microbiota in the different facets of

47 anorexia pathophysiology, such as functional gastrointestinal disorders, appetite regulation
48 and mood disorders.

49

50 **KEYWORDS:** Anorexia Nervosa; Eating disorders; Gut microbiota; Activity-Based
51 Anorexia; Food restriction; physical activity.

52 INTRODUCTION

53

54 Anorexia Nervosa (AN) is a highly morbid eating disorder characterized by underweight
55 (Body Mass Index (BMI) <18.5 kg/m²), achieved by addictive food restriction and increased
56 physical activity, a fear of gaining weight, and a disturbed self-body image (DSM-V criteria)
57 (1). The prevalence of AN is increasing in most countries and is estimated to be 1.4% for
58 women and 0.2% for men (2). AN is usually considered as the psychiatric illness with the
59 highest mortality rate (standardized mortality ratio >5) (3) and thus constitutes a serious public
60 health issue. The etiology and pathophysiology of AN remains poorly understood and
61 treatments targeting the causal factors of AN are still lacking (4). As a consequence, current
62 treatments, that mainly focus on both psychological and nutritional approaches, have only
63 limited efficacy with a relapse rate of ~40% within 18 months (5,6).

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

73 Animal models constitute key experimental approaches to decipher the potential role of the
74 gut microbiota in human diseases. Among the various animal models of Anorexia Nervosa
75 described in the literature, the Activity-Based Anorexia (ABA) model is one of the most
76 studied ones (28,29). In this model, rodents are isolated in cages equipped with an activity

77 wheel and have a progressive time restricted food access, combined to a voluntary physical
78 activity. This model leads to body-weight loss (between 10 and 25% depending of animal
79 species, strains or sex) and was shown to mimic core features of AN. It has been instrumental
80 to document brain alterations, gastrointestinal functional disorders and hormonal changes
81 characteristic of AN (28-35). The ABA model thus constitutes an interesting animal model to
82 study the role of the gut microbiota in AN pathophysiology. Here, by combining 16S rRNA
83 gene sequencing and quantitative PCR analysis of targeted genera or species, we provide a
84 comprehensive characterization of the gut microbiota dysbiosis occurring in murine model of
85 Anorexia and demonstrate that most of the identified gut microbiota alterations are due to
86 food restriction. We identified in addition several bacterial groups which levels correlate with
87 body weight, food intake, lean and fat masses as well as with hypothalamic levels of NPY
88 (Neuropeptide Y) and POMC (Pro-opiomelanocortin) neuropeptides.

89 **MATERIALS AND METHODS**

90

91 **Animals**

92 Animal care and experimentation were approved by a regional Animal Experimentation
93 Ethics Committee (authorization N/05-11-12/28/11-15) and complied with the guidelines of
94 the European Commission for the handling of laboratory animals (Directive 2010/63/EU). All
95 efforts were made to minimize suffering of animals. All animals were fed with a standard diet
96 (3430PMS10; Serlab, France). The ABA protocol was performed as previously described
97 (30). Eight-weeks-old C57Bl/6JRj male mice (Janvier Labs, Le-Genest-Saint-Isle, France)
98 were housed individually at 23 °C with a reversed 12-h light-dark cycle (dark phase from
99 10:00 am to 10:00 pm) and split in three groups: a control group, with food *ad libitum*
100 (CTRL), a group with restricted access to food (Limited Food Access; LFA) and a group with
101 restricted access to food combined with a free access to a running wheel (Activity-Based
102 Anorexia group; ABA). Food access was progressively limited for LFA and ABA mice from
103 6h at day 6 to 3h at day 9, and until the end of the experiment at day 17. Food was given at
104 the beginning of the dark phase. All mice had free access to water. For each independent
105 experiment, 6-8 animals were used per group. CTRL and LFA mice were housed in standard
106 cages whereas ABA mice were housed in cages equipped with an activity wheel connected to
107 the Running Wheel® software (Intellibio, Seichamps, France), that recorded ABA mice
108 physical activity during the whole protocol. Body weight and food intake were monitored
109 each day at the end of the light phase. In accordance with ethical procedures, mice showing
110 excessive weight loss (>20%) over 3 consecutive days were euthanized. Whole body
111 composition was assessed on vigil animals at day 16 using fast nuclear magnetic resonance
112 (Minispec LF110, Bruker, Wissembourg, France). At the end of the protocol (day 17), all

113 animals were euthanized. Cecal contents and hypothalamus were removed, frozen in liquid
114 nitrogen and stored at -80°C.

115

116 **Gut microbiota analyses**

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

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

135 Quantitative real-time polymerase chain reaction (qPCR) was performed on DNA samples
136 from 3 independent animal experiments (including the 2 experiments used for Illumina

137 sequencing). qPCR signals were detected on a Mastercycler *ep* Realplex system (Eppendorf,
138 Hamburg, Germany) using Itaq Universal SYBR Green Supermix (BioRad). Primer
139 sequences are detailed in Table S3. Primers specificity was checked using Ribosomal
140 Database Project tools (42). All samples were run in duplicate in 96-well reaction plates.
141 Final concentrations were as follow: DNA 0.1 ng/μL (excepted for *A. muciniphila* 1 ng/μL),
142 primers 0.5 μM, and SYBR Green Supermix 1X. Thermocycling conditions were as follow:
143 initiation step at 95°C 5 min; cycling stage at 95°C 5 s, 60°C 30 s (unless a different
144 annealing temperature is indicated in Table S3), 95°C 15 s, 40 cycles; melt curve stage at
145 95°C 15 s, 65°C 15 s, increment of 1°C every 10 s until reaching 95°C. The purity of the
146 amplified product was verified by analyzing the melt curve performed at the end of
147 amplification. At least 80% of the duplicates show a variation lower than 0.5 Cq units. Serial
148 dilution of DNA from cecal content was included on each plate to generate a relative curve
149 and to integrate primer efficiency in the calculations. Analyses were considered as acceptable
150 when amplification efficiencies reached values between 70% and 110%. For detection of total
151 Eubacteria, Cq of each sample were compared with a standard curve made by diluting
152 genomic DNA extracted from a pure culture of *E. coli*, for which cell counts were determined
153 prior to DNA isolation. Non-template controls were included on each plate. A qPCR is
154 considered valid if the Cq of the non-template control is at least 3 units higher than the Cq of
155 the templates or, for targets with low expression levels, if the melt curve of the non-template
156 control was different from the templates.

157 **Quantification of neuropeptide expression**

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

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164 **Statistical analyses**

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

176 **RESULTS**

177

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

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

201 We did not observe any significant differences in alpha diversity indexes of richness
202 (observed species) or richness and evenness (Shannon index) between CTRL, LFA and ABA
203 mice (Fig. 2E and 2F). Using non-taxonomy based analysis of our sequencing data, we
204 identified 85 Amplicon Sequence Variants (ASVs) with significant altered levels between
205 mice groups (among the 1466 ASVs identified in this analysis; Table S2 and S4). Eight ASVs
206 show increased levels in ABA versus CTRL mice (including two ASVs attributed to
207 *Lactobacillus* spp. and one attributed to *Clostridium* cluster XVIII) whereas 68 ASV show
208 decreased levels in ABA versus CTRL mice (mainly belonging to the *Lachnospiraceae* and
209 *Ruminococcaceae* families and the *Bacteroidales* order). Interestingly, among the 76 ASVs
210 with altered levels in ABA versus CTRL mice, 72 (95%) show similar levels between the two
211 groups of food deprived mice (*i.e.* LFA and ABA mice). These data suggest that the main
212 alterations that we observed in ABA mice cecal microbiota compared to CTRL mice are due
213 to food restriction and are neither restored nor further impaired by physical activity. Only one
214 ASV, corresponding to a yet unclassified bacterium, shows significant increased levels in
215 ABA mice compared to both CTRL and LFA mice (ASV1396; CTRL: 0.0013 ± 0.0009 %;
216 LFA: 0.0027 ± 0.0012 %; ABA: 0.0112 ± 0.0039 %; Table S2 and S4).

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

223 To confirm these results and to narrow down the species from *Clostridium* cluster XVIII
224 showing increased levels in ABA mice, we focused on the only identified ASV belonging to
225 *Clostridium* cluster XVIII in our dataset (ASV153; Table S4). This ASV is highly homolog to

226 the 16S rRNA gene of *C. cocleatum* (one of the four bacterial species constituting
227 *Clostridium* cluster XVIII) (44). We thus used a couple of primers specific for the rRNA 16S
228 gene from *C. cocleatum* (45) to monitor its abundance by qPCR in the cecal bacterial
229 community from CTRL, LFA and ABA mice. We observed that *C. cocleatum* is significantly
230 increased in the cecum of both LFA and ABA mice compared to control mice (Fig. 4D) (14.5
231 fold-change increase for LFA vs CTRL and 11.9 fold-change increase for ABA vs CTRL).
232 This result confirms our 16S rRNA sequencing data and suggests that food restriction
233 increases the level of *C. cocleatum* in mice gut microbiota.

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

246 To complete our characterization of the gut microbiota in ABA mice, we quantified by qPCR
247 the levels of specific bacterial species that were not detected by 16S rRNA Illumina
248 sequencing but which represent interesting candidates to understand the putative link between
249 anorexia-associated functional gastrointestinal disorders and gut microbiota dysbiosis. These
250 species include (i) *Methanobrevibacter smithii*, the predominant human gut methanogen

251 archaeon, which was found overrepresented in the microbiota of anorectic patients from two
252 independent cohorts (15,20), (ii) *Roseburia* spp, which are butyrate producers shown as
253 decreased in the microbiota of anorectic patients from three independent cohorts (19,20,23),
254 (iii) *Faecalibacterium prausnitzii*, a Firmicutes exhibiting anti-inflammatory effects and
255 shown in several studies as decreased in patients with Inflammatory Bowel Diseases (48), and
256 (iv) *Akkermansia muciniphila*, a mucin-utilizing specialist showing decreased abundance in
257 several pathological conditions such as obesity or type 2 diabetes (49). We did not observe
258 any significant differences in the levels of *M. smithii*, *Roseburia* spp and *F. prausnitzii*
259 between CTRL, LFA and ABA mice (Table S1). We observed a significant 2.1-fold decrease
260 in the level of *A. muciniphila* in LFA mice compared to CTRL mice (Fig. 4I). A similar
261 pattern was observed in ABA mice for *A. muciniphila*, but difference did not reach
262 significance. This result suggests that food restriction leads to a decrease in the cecal
263 abundance of *A. muciniphila* in C57Bl/6 mice.

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

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

276 of CTRL, LFA and ABA mice. These two neuropeptides play opposite roles in appetite
277 regulation: NPY is a potent orexigenic neuropeptide whereas POMC is the precursor of the α -
278 melanocyte-stimulating hormone (α MSH), a potent inducer of satiety pathways (27). We
279 identified several bacterial taxa and ASVs that correlate positively or negatively with body
280 weight, food intake, lean mass and fat mass (Fig. 5B). In agreement with our previous results,
281 we observed for example that *Burkholderiales* are positively correlated with body weight,
282 food intake and lean mass (*i.e.* increased in mice with high body weight, high food intake and
283 high lean mass) in contrast to *Lactobacillales* which are negatively correlated with these three
284 parameters. Interestingly, we also identified 11 bacterial units that are positively correlated
285 with POMC hypothalamic levels and 3 bacterial units that are negatively correlated with NPY
286 hypothalamic levels (all of them being positively correlated with body weight and food
287 intake, as expected) (Fig. 5B).

288

289 **DISCUSSION**

290

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

297 Almost all bacteria showing increased or decreased levels in ABA mice compared to CTRL
298 mice are similarly altered in ABA and LFA mice, which suggests that the main driver for the
299 gut microbiota dysbiosis observed at the end of our protocol is food restriction and that the
300 effect of physical activity is only marginal. Of note, we analyzed gut microbiota composition

301 in ABA mice at day 17, where mice exhibit decreased physical activity compared to the day
302 1-day 10 period (Fig. 1F). We thus cannot exclude that physical activity has a more
303 pronounced effect on gut microbiota composition at the beginning of the food restriction
304 period in ABA mice.

305 Alteration of gut microbiota in a rat ABA model has been partially described in a previous
306 study using PCR-denaturing gradient gel electrophoresis and qPCR approaches (50). In
307 agreement with our observations, most of the detected alterations in bacteria levels in ABA
308 rats were similarly observed in food restricted rats that had not access to a running wheel.
309 This again suggests a major impact of food restriction on gut microbiota alteration in a
310 context of anorexia. In contrast to our findings, ABA rats showed decreased levels in
311 *Firmicutes*, *Bacteroidetes* and *Lactobacillus* and increased levels in *M. smithii* in comparison
312 to rats fed *ad libitum* (50). These differences in gut microbiota alterations between mice and
313 rat ABA models might be due either to rodent-specific differences, to differences in the type
314 of samples used for 16S rRNA gene sequencing (feces for rats versus caecal contents for
315 mice) or to divergences in the ABA protocol, such as the daily duration for food access (1h
316 for rats versus 3h for mice) or the total length of the restriction period (6 days for rats versus
317 12 days for mice) (50).

318 It was recently shown that major metabolic perturbations are induced in the Activity-based
319 anorexia model, which was proposed to reflect both host and microbiome metabolic pathways
320 adaptation to food restriction (51). It was shown in particular that undernutrition was the
321 major driver for urinary and blood metabolite variations in ABA mice whereas physical
322 activity had no significant impact (51). This nicely echoes our own observation of a major
323 role of food restriction on gut microbiota alteration. As gut bacteria are important providers of
324 metabolites for the host, we can thus propose that, in this animal model of Anorexia, food

325 restriction alters gut microbiota composition and metabolic capacities, which in turn has an
326 impact on host circulating and urinary excreted metabolites.

327 We identified in this study several bacterial groups correlating with biological parameters
328 such as body weight, food intake and lean/fat masses. Interestingly, we identified bacterial
329 units positively correlating with POMC hypothalamic levels or negatively correlating with
330 NPY hypothalamic levels. These bacteria constitute interesting candidates potentially
331 involved in gut-brain axis communication. One interesting hypothesis is that the dysbiosis
332 observed in ABA mice alters the communication along the gut-brain axis and has an impact
333 on animal behavior and, in particular, on appetite regulation (27). Determining whether the
334 gut microbiota changes observed in anorectic patients facilitates or perpetuates eating
335 behaviors dysregulations is an essential objective that may have repercussions on the clinical
336 management of AN (14).

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

350 In addition to *C. cocleatum*, we observed an increase in the abundance of *Lactobacillus* in
351 response to food restriction in C57Bl/6 mice. Our data are consistent with several studies that
352 linked increases in *Lactobacillus* levels with calorie-restricted diets. It was shown for example
353 that a 14 days-long calorie restriction diet in C57Bl/6 mice (leading to ~20% of weight loss)
354 shifts the gut microbiota and create a *Lactobacillus*-predominated gut ecosystem, which was
355 associated with decreased markers of systemic inflammation (54,55). In humans, a calorie-
356 restricted diet associated with increased physical activity over 10 weeks increased the
357 abundance of gut *Lactobacillus* (56). We observed that all major *Lactobacillus* species were
358 increased in ABA mice. This suggests that the relative proportions of *Lactobacillus* species in
359 ABA mice remain probably unchanged, in contrast for example to cancer-induced anorexia
360 mouse models, where *L. murinus/animalis* outcompete *L. reuteri* and *L. johnsonii/gasseri*
361 (47).

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

368 Although several studies have described the gut dysbiosis associated with Anorexia Nervosa
369 in humans, no clear consensus have emerged yet (14). Only very few bacterial species were
370 repeatedly described as being increased or decreased in independent human studies (14,15,
371 19,20,23). This heterogeneity in the observed dysbiosis associated with human AN may be
372 due to methodological differences (sample collection, bacterial DNA extraction, data
373 analysis), to variations in the design of the clinical study (timepoint for sample collection,
374 criteria for the selection of reference individuals) or to anorectic patients' heterogeneity

375 (variations in BMI, in levels of calorie intake by patients or in patients' medical history). Not
376 surprisingly, we observed differences in the dysbiosis of ABA mice compared to the reported
377 dysbiosis in humans. Indeed, several studies observed no significant differences in the
378 abundance of *Lactobacillus* species between patients and healthy controls, and we did not
379 detect significant differences in *M. smithii* or *Roseburia* abundances in ABA mice
380 (15,16,17,19). These discrepancies may reflect human versus rodent-specific differences, and
381 in particular differences between the laboratory feeds given to mice and human diets. They
382 may also reveal the involvement of additional factors in the human disease that, for example,
383 limit the expansion of *Lactobacillus* despite food restriction or promotes alterations in *M.*
384 *smithii* and *Roseburia* levels independently of changes in food intake. In contrast to human
385 Anorexia Nervosa, which is a multifactorial disease, the ABA animal model constitutes a
386 simplified system in which genetic and environmental variables are controlled. This model
387 thus constitutes an interesting tool to disentangle microbiota changes induced by food
388 restriction and physical activity from changes induced by the other factors involved in this
389 complex disease in humans. In addition, this model can be used to study the interactions
390 between bacterial species in the gut ecosystem in response to an environmental disruption
391 (food restriction in our case; Fig. 5A).

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

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399

400 **STATEMENT OF AUTHORSHIP**

401 **Jonathan Breton:** Conceptualization, Formal analysis, Investigation, Writing- Original Draft,

402 Visualization. **Pauline Tirelle:** Investigation. **Soujoud Hasanat:** Investigation. **Arthur**

403 **Pernot:** Investigation. **Clément L’Huillier:** Investigation. **Jean-Claude do Rego:** Resources.

404 **Pierre Déchelotte:** Conceptualization, Writing- Original Draft. **Moïse Coëffier:**

405 Conceptualization, Writing- Original Draft. **Laure Bindels:** Conceptualization, Validation,

406 Formal analysis, Investigation, Data curation, Writing- Original Draft, Visualization. **David**

407 **Ribet :** Conceptualization, Validation, Investigation, Data curation, Writing- Original Draft,

408 Writing- Review & Editing, Visualization, Supervision, Funding acquisition.

409

410 **CONFLICT OF INTEREST STATEMENT**

411 PD is a co-founder of the TargEDys company; JB, PT, SH, AP, CL, JC DR, MC, LB, DR, no

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413

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419

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583 **FIGURE LEGENDS**

584

585 **Figure 1 : Body weight loss and changes in physical activity in ABA mice.**

586 A, Changes in body weight during the protocol (values are means \pm SEMs, $n=6-8$; *,
587 Different from control, $P<0.05$). B, Food intake (values are means \pm SEMs, $n=6-8$; *,
588 Different from control, $P<0.05$). C, Body weight at day 17 (individual values correspond to
589 percentage of body weight at day 5 with means \pm SEMs, $n=6-8$; Labeled means without a
590 common letter differ, $P<0.05$). D-E, Lean and fat masses at day 16 (individual values are
591 represented with means \pm SEMs, $n=6-8$; Labeled means without a common letter differ,
592 $P<0.05$). F, Physical activity of ABA mice (values are means of run distance \pm SEMs; $n=6-8$;
593 *, Different from physical activity during light phase, $P<0.05$).

594

595 **Figure 2 : Bacteria levels and alpha diversity indexes in CTRL, LFA and ABA mice gut**
596 **microbiota.**

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

603

604 **Figure 3 : Relative abundance of bacterial taxa in CTRL, LFA and ABA mice gut**
605 **microbiota.**

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

609

610 **Figure 4 : Alteration in bacteria levels in CTRL, LFA and ABA mice gut microbiota.**

611 A-C, Relative abundance of bacteria belonging to *Clostridium* cluster XVIII, *Lactobacillus*
612 and *Burkholderiales* taxa in cecal contents from CTRL, LFA and ABA mice, as determined
613 by V5-V6 16S rRNA gene sequencing (values are means \pm SEMs; $n=13-15$; Labeled means
614 without a common letter differ, $P<0.05$). D-I, Relative quantification of different bacterial
615 species in cecal contents from CTRL, LFA and ABA mice, as determined by qPCR analysis
616 (values are represented as whisker plots with minimum and maximum values, $n=20-23$;
617 Labeled plots without a common letter differ, $P<0.05$).

618

619 **Figure 5 : Correlations between gut microbiota alterations and biological parameters**
620 **variations in CTRL, LFA and ABA mice.**

621 Correlations between the different bacteria taxa quantified in mouse caecum (A) or between
622 bacteria levels and mouse biological parameters (B) (heatmaps of Spearman correlation
623 coefficients; only significant correlations are displayed; adjusted P-value <0.05). The blue
624 color indicates a positive correlation and the red color a negative correlation. Color intensity
625 is proportional to the correlation coefficients. Taxonomy-based analyses were restricted to
626 bacterial orders, families and genera (o, order; f, family; g, genus; i.s., incertae sedis; s.s.,
627 sensus stricto).

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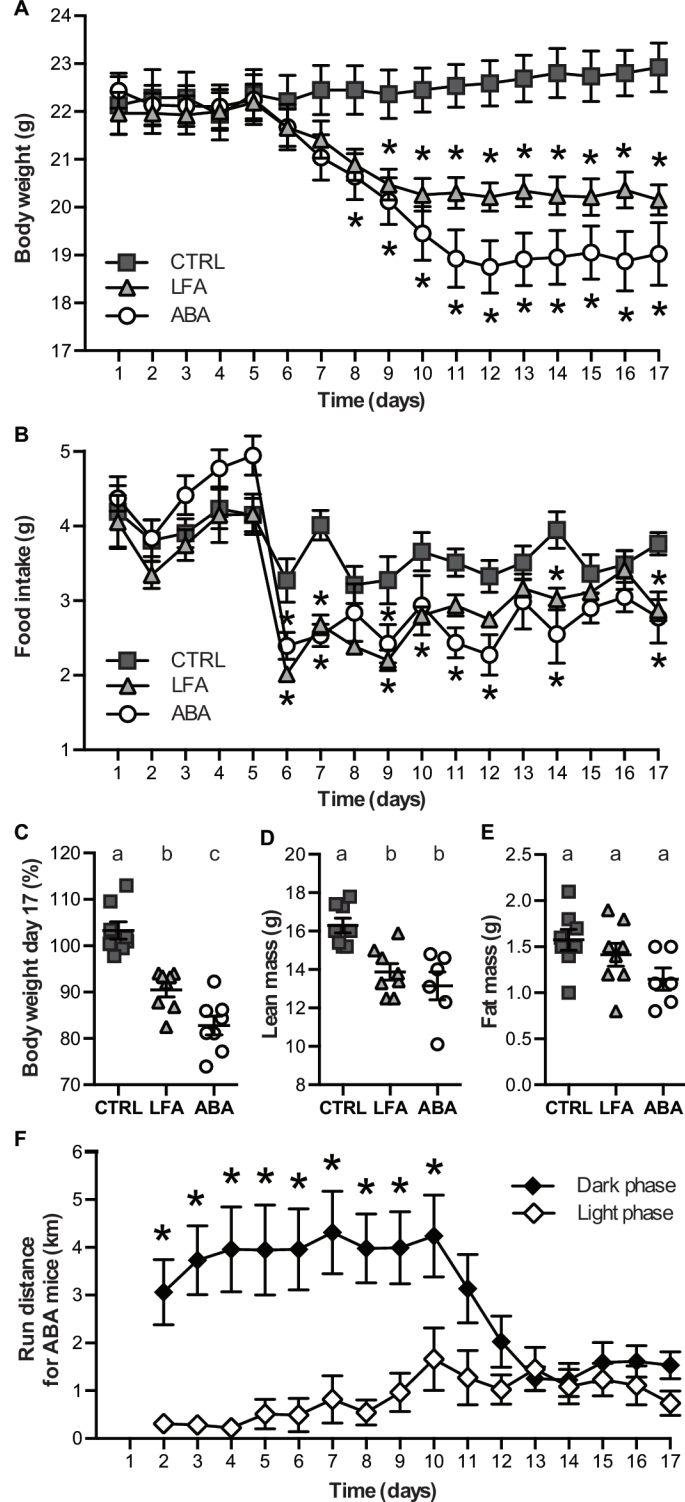


Figure 1 : Body weight loss and changes in physical activity in ABA mice.

A, Changes in body weight during the protocol (values are means \pm SEMs, $n=6-8$; *, Different from control, $P<0.05$). B, Food intake (values are means \pm SEMs, $n=6-8$; *, Different from control, $P<0.05$). C, Body weight at day 17 (individual values correspond to percentage of body weight at day 5 with means \pm SEMs, $n=6-8$; 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; $n=6-8$; *, Different from physical activity during light phase, $P<0.05$).

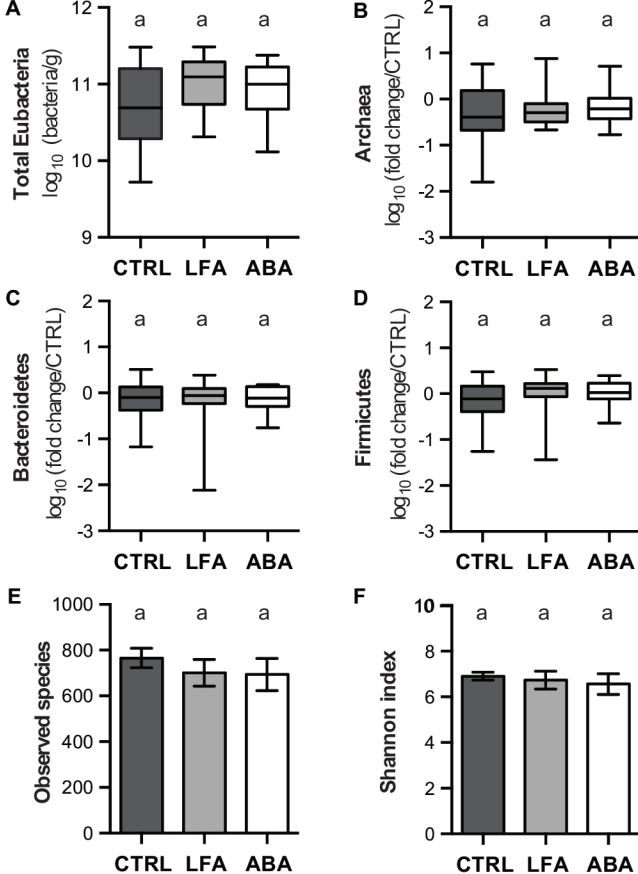


Figure 2 : Bacteria levels and alpha diversity indexes in CTRL, LFA and ABA mice gut microbiota.

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).

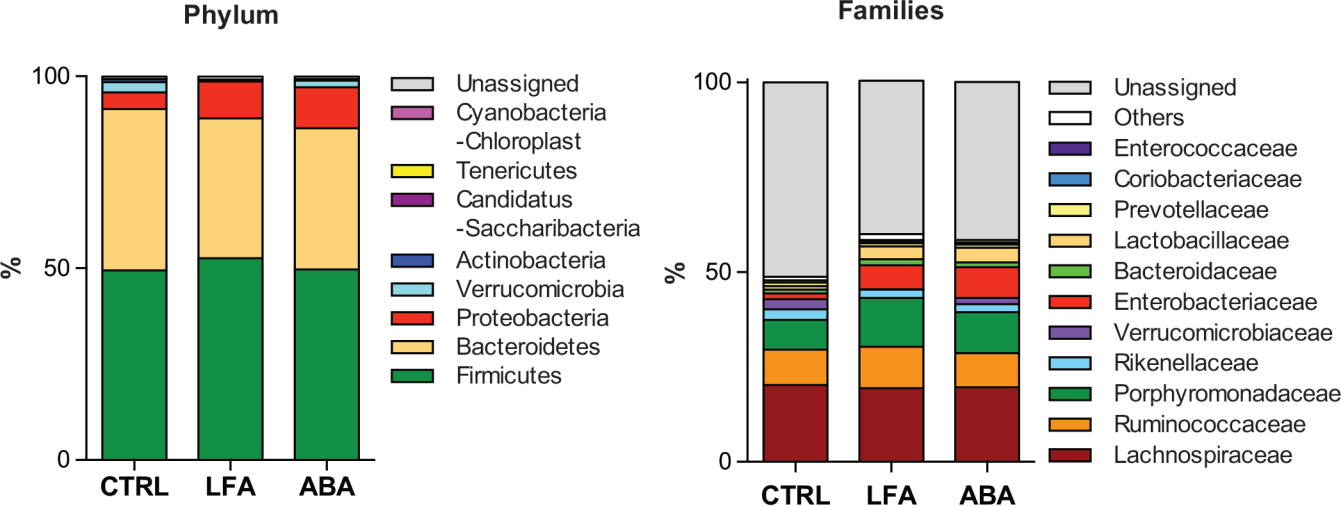


Figure 3 : Relative abundance of bacterial taxa in CTRL, LFA and ABA mice gut microbiota.

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”).

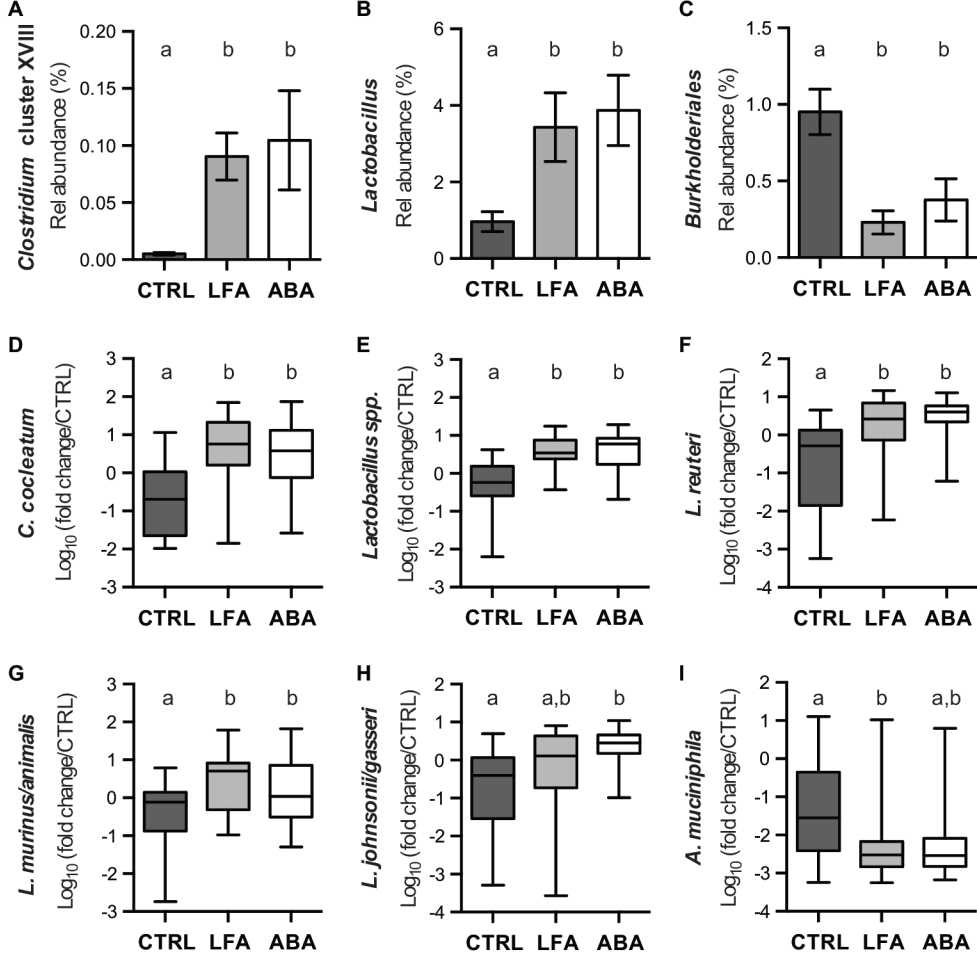


Figure 4 : Alteration in bacteria levels in CTRL, LFA and ABA mice gut microbiota.

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=13-15; 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=20-23; Labeled plots without a common letter differ, P<0.05).

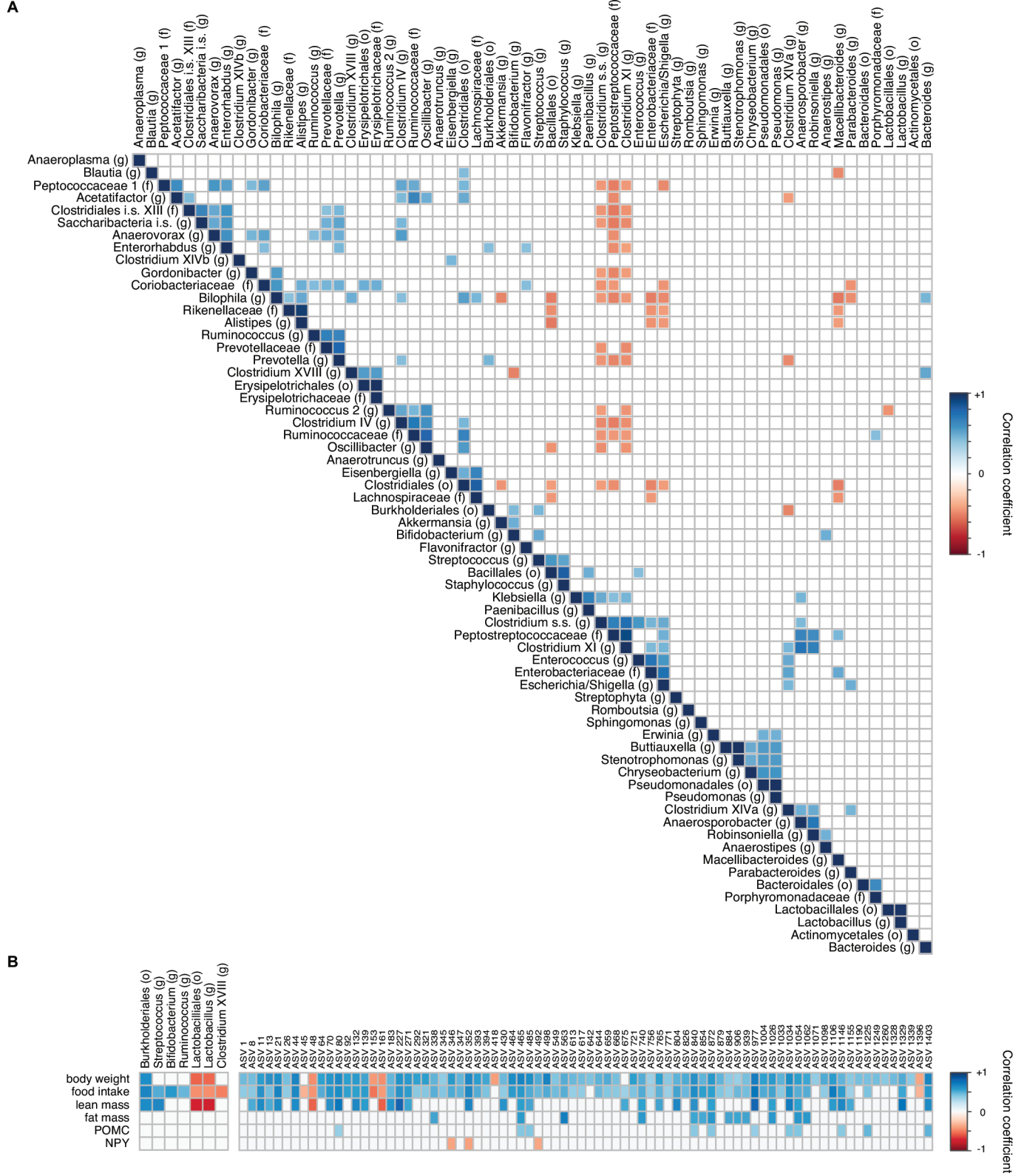


Figure 5 : Correlations between gut microbiota alterations and biological parameters variations in CTRL, LFA and ABA mice.

Correlations between the different bacteria taxa quantified in mouse caecum (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., sensu stricto).