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## **Proteome modifications of gut microbiota in mice with activity-based anorexia and starvation – role in ATP production**

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**Abstract (<250 words)**

**Objective:** Activity-based anorexia (ABA) in rodents is a behavioral model of anorexia nervosa, both characterized by negative energy balance, hyperactivity and dysbiosis of gut microbiota. Gut bacteria are known to produce energy substrates including adenosine triphosphate (ATP) and acetate. Here we studied whether ABA may alter the proteome of gut microbiota relevant to ATP and acetate productions.

**Methods:** The ABA was developed in male mice and compared with food-restricted and *ad libitum*-fed conditions. Proteomic analysis of faeces was performed using the 2-dimensional gel electrophoresis and mass spectrometry. The *in vitro* ATP-producing capacity of proteins extracted from faeces was assayed.

**Results:** Increased level of the phosphoglycerate kinase, an ATP-producing glycolytic enzyme was detected in faeces of food-restricted mice and this enzyme was further increased in the ABA group. Starvation also upregulated several other proteins synthesized by order Clostridiales including *Clostridiaceae* and *Lachnospiraceae* families. No significant differences in the *in vitro* ATP producing capacity by bacterial proteins from ABA, food-restricted and *ad libitum*-fed control mice were found. However, plasma levels of acetate strongly tended to be increased in the activity groups including ABA mice.

**Conclusion:** The data reveal that starvation in food-restricted and ABA mice induces proteome modification in gut bacteria favoring ATP production mainly by the order *Clostridiales*. However, this does not result in increased total ATP-production capacity by gut microbiota. These changes can be interpreted as an adaption of specific gut bacteria to the host malnutrition beneficial for host survival.

**Key words:** gut-brain axis; microbiota; ATP, acetate; eating disorders; anorexia nervosa, activity-based anorexia.

## Introduction

Anorexia nervosa (AN) is a form of eating disorders (ED) characterized by low food intake and for a subtype of patients, by hyperactivity leading to the negative energy balance and progressive loss of body weight [1]. Although the exact pathophysiological mechanisms of AN and ED remain unknown, gut microbiota appeared recently as an important participant in the mechanisms regulating host appetite and feeding behavior as well as other functions involving the gut-brain axis (reviewed in [2-4]). Several studies in patients with AN reported the presence of gut microbiota dysbioses [5-10]. However, the significance of the reported modifications of gut bacterial composition to the pathophysiology of AN remains obscure (reviewed in: [11-15] and may also non-specifically reflect the host starvation [16]. Host starvation and negative energy balance imposed to microbiota may impact on the energy metabolism of gut bacteria, which are actively involved in both energy consumption, necessary for bacterial multiplication, as well as in energy production used by both bacteria and the host (reviewed in: [2, 17]). In fact, gut bacteria are a major source of several energy substrates in the body including adenosine triphosphate (ATP), short chain fatty acids (SCFA), such as acetate, butyrate etc (reviewed in [18, 19]). Moreover, we have previously shown that proteins extracted from *E.coli* dose-dependently catalyzed ATP production from various nutritional sources and influenced feeding behavior in rats and mice [20].

Activity-based anorexia (ABA) in rodents is a behavioral model of AN sharing the features of negative energy balance and hyperactivity. The relationship between the running wheel and the progressive limitation of food (from 6h of free access at day 6 to 3h at day 9; Figure 1A) leads to the ABA phenotype. In fact, under the imposed restricted feeding schedule, rats and mice engage themselves in physical activity instead of feeding resulting in a “voluntary” decrease in food intake and progressive loss of body weight [21, 22]. Besides

this central aspect in AN diagnosis, ABA rodents also share many similarities with patients, both physiologically (hypothermia [23-25], anhedonia [26, 27], increased susceptibility in younger rodents [28, 29]) and behaviorally (females exercise more than males [30-32]). However, despite these well described features, a complete neurocognitive assessment is needed to better characterize the model [33].

Dysbiosis of gut microbiota has been reported in ABA rats, which may share some modifications of gut microbiota found in patients with AN [34]. It is presently unclear whether the observed changes of gut bacterial composition in AN and ABA may alter energy substrate production capacity by gut bacteria towards catabolic or anabolic processes, which may indicate a pathological or physiological impact of gut microbiota on the host energy balance, respectively.

In the present study, we used the ABA model in mice to analyze the proteome of fecal microbiota for its relevance to ATP production. For this purpose we compared fecal-extracted proteins between ABA, food restricted and *ad libitum*-fed groups of mice using 2D gel electrophoresis followed by mass spectrometry and by their *in vitro* capacity to catalyze ATP production from a mixed nutrient source of the Mueller-Hinton (MH) medium.

## **Material and methods**

### ***Animals***

Animal care and experimentation complied with both French and European Community regulations (Official Journal of the European Community L 358, December 18, 1986), and M.C. was authorized by the French government to use animal models (authorization no. 76–107). Male C57Bl/6 mice (Elevage Janvier, Le Genest St Isle, France) were acclimatized in individual cages at 23 °C during 7 days. During this period and all the experiment, the 12-h light-dark cycle was inverted (dark phase: 9:30 AM - 9:30 PM). At day (D) 1 of the

experiment, mice were randomized into 4 groups depending on the body weight : ABA (n=8), Feeding-time restriction (FTR; n=8), ad libitum fed controls with monitored activity (CTPA; n=8) and without (CT; n=8) as previously described [35]. ABA and CTPA mice were placed in individual cages with an activity wheel with RunningWheel software (Intellibio, France). Wheel activity was continuously recorded. FTR and control mice were placed in individual cages without activity wheel. From D1 to D5, mice had free access to water and standard diet. Food access was progressively limited in ABA and FTR groups from 6 h at D6 to 3 h at D9 until the end of experiment. Food was given at the beginning of the dark phase (9:30 AM). Food consumption was measured when food was removed. Body weight, water and food consumption were measured daily at 9:00 AM. In the ABA and FTR groups, water and food intake were also monitored when food was removed. Experiments were carried out until d17. Mice showing excessive weight loss (>20%) over 3 consecutive days were euthanized for ethical reasons. At d17, mice were euthanized and pellets were collected directly in colon and immediately frozen at - 80°C for further proteomic analysis of gut microbiota. Trunk blood was collected after decapitation and plasma was separated by centrifugation.

### ***Body composition***

Total, lean and fat mass were measured in vigil mice on the last day of the experimental protocol (D17) using EchoMRI EMR-185 (EchoMRI, Houston, TX), a nuclear magnetic resonance instrument.

### ***Proteins extraction***

Proteins extractions from faeces were performed as described by Flores et al, with slight modifications [36]. Briefly, faeces were thawed in phosphate buffered saline then transferred to a proteins extraction buffer [urea 7mol/L, thiourea 2mol/L, dithiothreitol 20 mmol/L, CHAPS and tributylphosphate]. The fecal content was homogenized by vortexing for 2 min. Then, bacterial cells were lysed by sonication during 90 s (Bioblock Scientific, France) before centrifugation 22.000g during 30 min at 4°C. Supernatants were sampled to perform ultracentrifugation at 100.000 rpm during 1 hour at 4°C to isolate proteins from residual debris.

### ***Two-dimensional PAGE***

For two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE), 300 µg of fecal protein extract was used to rehydrate immobilized pH gradient (IPG) strips (pH 4–7; 18 cm; BIO-RAD, Hercules, CA). Proteins were separated in the first dimension by isoelectric focusing for a total of 85,000 V-h by using the IPGphor isoelectric focusing system (GE Healthcare). After focusing, IPG strips were incubated for 15 min in the equilibration buffer [urea 6 mol/L, 30% (vol:vol) glycerol, 2% (wt:vol) sodium dodecyl sulfate (SDS), Tris-HCl 50 mmol/L pH 8.8, and 0.25% (wt:vol) bromophenol blue containing 2% (wt:vol) dithiothreitol] and then alkylated for 15 min in the equilibration buffer containing 4% (wt:vol) iodoacetamide. IPG strips were subsequently fixed onto 10% polyacrylamide gradient gels (20 cm · 18 cm · 1 mm) for SDS-PAGE. Separation of proteins in second dimension according to molecular weight was performed overnight in the Ettan Daltsix vertical electrophoresis system (GE Healthcare) with 12 mA/gel at 25°C. After SDS-PAGE, the 2D gels were fixed for 2 h in 2% (vol:vol) orthophosphoric acid and in 50% (vol:vol) methanol at RT. Gels were then rinsed with water, and the protein spots were visualized by CBB G-250 (BIO-RAD) staining [34% (vol:vol)

methanol, 17% (wt:vol) ammonium sulfate, 2% (vol:vol) orthophosphoric acid, and 0.66 g CBB G-250/L].

### ***Analysis of differential protein expression***

Images of stained 2D gels were scanned by an ImageScanner II (GE Healthcare) calibrated with a grey scale marker (Kodak, Rochester, NY) and digitalized with Labscan 6.0 software (GE Healthcare). Analysis of differential protein expression including spot detection, quantification, matching, and comparative analysis was performed using Progenesis sameSpot software (GE Healthcare). Each protein sample was subjected to 2D-PAGE at least 3 times to minimize run-to-run variation, and each set of 3 gels was compared using Progenesis to confirm the non-appearance of statistically differential spots within the set of gels. The expression level was determined by the relative volume of each spot in the gel and expressed as %volume, calculated as spot volume/ $\Sigma$ volumes of all spots resolved in the gel. This normalized spot volume takes into account variations due to protein loading and staining by considering the total volume over all the spots present in the gel. Variations in abundance were calculated as the ratio of average values of % volume for a group of spots between the 2 phases. Only spots with a % volume variation ratio >1.5 were considered relevant. The absence of a spot within a gel indicated that no detectable expression could be reported for the relative protein under the selected experimental condition. The corresponding *p* values were determined by Student's t-test (significance level *p*<0.05) after spot % volume log-transformation.

### ***Protein identification by liquid chromatography-electrospray ionization MS/MS***



171 The protein spots of interest were excised from CBB G-250-stained 2D gels using the Ettan  
172 Spot Picker (GE Healthcare), and automated in-gel digestion of proteins was performed on  
173 the Ettan Digester (GE Healthcare). Protein extracts were then resuspended in 10  $\mu$ L of 5%  
174 (vol:vol) acetonitrile/0.1% (vol:vol) formic acid and then analyzed with a nano-LC1200  
175 system coupled to a 6340 Ion Trap mass spectrometer equipped with a nanospray source  
176 and an HPLC-chip cube interface (Agilent Technologies, Les Ulis, France). Briefly, peptides  
177 were enriched and desalted on a 40 nL RP-C18 trap column and separated on a Zorbax (30-  
178 nm pore size, 5- $\mu$ m particle size) C18 column (43 mm long x 75  $\mu$ m inner diameter; Agilent  
179 Technologies). A 9-min linear gradient (3%-80% acetonitrile in 0.1% formic acid) at a flow  
180 rate of 400 nL/min was used and the eluent was analyzed with an Ion Trap mass  
181 spectrometer.

182 For protein identification, MS/MS peak lists were extracted and compared with the  
183 protein databases by using the MASCOT Daemon version 2.2.2 (Matrix Science) search  
184 engine. The searches were performed with the following specific parameters: enzyme  
185 specificity, trypsin; one missed cleavage permitted; no fixed modifications; variable  
186 modifications, methionine oxidation, cysteine carbamidomethylation, serine, tyrosine and  
187 threonine phosphorylation; monoisotopic; peptide charge, 2+ and 3+; mass tolerance for  
188 precursor ions, 1.5 Da; mass tolerance for fragment ions, 0.6 Da; ESI-TRAP as instrument;  
189 taxonomy, *Bacteria*; National Center for Biotechnology Information (NCBI) database [NCBI nr  
190 20120531 (18280215 sequences, 6265275233 residues)] (Bethesda, MD). Protein hits were  
191 automatically validated if they satisfied one of the following criteria: identification with at  
192 least two top ranking peptides (bold and red) each with a MASCOT score of more than 54 ( $p$   
193  $< 0.01$ ), or at least two top ranking peptides (bold and red) each with a MASCOT score of  
194 more than 47 ( $p < 0.05$ ). To evaluate false-positive rates, all the initial database searches

were performed using the “decoy” option of MASCOT. Results were considered relevant if the false-positive rate never exceeded 1%.

### ***ATP assay***

In vitro ATP production was measured using ATP colorimetric / fluorometric assay kit according to the manufacturer’s instructions (BioVision, CA). Briefly, bacterial proteins from faeces of ABA, CT, FTR and CT-PA mice were put in duplicates into the wells at 1 µg/µL concentration in ATP assay buffer and adjusted to 40 µL/well with ATP assay buffer. Then, 10 µL of nutrients solution Mueller-Hinton (MH) culture medium were added; MH medium contains 30% beef infusion, 1.75% casein hydrolysate and 0.15% starch (Becton, Dickinson, MD). The plate was incubated for 2 h at 37°C. After the incubation, 50 µL of ATP reaction mix (containing ATP assay buffer, ATP probes, ATP converter and developer mix) were added in each wells. Optical density was measured at 570 nm after 30 min of incubation at room temperature (RT), protected from day light.

### ***Acetate assay***

In vitro acetate production was measured using an Acetate Colorimetric Assay kit according to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, USA). Briefly, plasma from ABA, CT, FTR and CT-PA was placed into the plate with 50 µL per well. After the incubation, 50 µL of acetate reaction mix (containing acetate assay buffer, probes, ATP, acetate enzyme and acetate substrate mix) were added to each well. OD was measured at 450 nm after 40 min of incubation at RT, protected from day light.

### ***Statistical analysis***

Data were analyzed and graphs were plotted using the GraphPad Prism 5.02 (GraphPad Software Inc., San Diego, CA, USA). Normality was evaluated by the Kolmogorov-Smirnov test. Group differences were analyzed by the analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis (K-W) test with the Tukey's or Dunn's post-tests, according to the normality results. Where appropriate, individual groups were compared using the Student's *t*-test or the Mann-Whitney (M-W) test according to the normality results. Data are shown as means  $\pm$  standard error of means (s.e.m), and for all test,  $p < 0.05$  was considered statistically significant.

## Results

### *Body weight and body composition*

In response to food restriction, both FTR and ABA mice showed a significant decrease of body weight as compared to the sedentary control group (CT) and control mice with free access to a running wheel (CT-PA; control with physical activity). At the beginning of the feeding time restriction (D6 to D12) body weight loss was more pronounced in ABA than in FTR mice, but the final weight loss was similar at the end of the experiment (**Figure 1B,C**). Analysis of body composition revealed a decrease of total, lean and fat mass in both groups of food-restricted mice (**Figure 1D-F**). However, the decrease of fat mass in ABA mice was more pronounced than in the FTR group (**Figure 1F**).

### *Food and water intakes*

As expected, during the food restriction period, food intake in both ABA and FTR mice was lower than in *ad libitum*-fed groups but it was not significantly different between the ABA and FTR groups (**Figure 2A,B**). Water intake was not significantly different in food restricted

vs. sedentary controls, while the CT-PA group displayed higher water intake (**Figure 2C,D**). Both ABA and FTR mice showed a decrease in food intake/water intake ratios during the food restriction period as compared to CT and CT-PA groups (**Figure 1E,F**).

#### *Physical activity*

ABA mice showed a transitory increase of physical activity during food restriction from D7 to D9 followed by a progressive decline of total activity towards the end of the protocol (**Figure 3A**). In CT-PA mice physical activity occurred only in the dark phase while in ABA mice it was mainly in the dark phase but some activity was also present in the light phase, which was due to feeding anticipatory activity (**Figure 3A vs. B**). Indeed, the analysis of physical activity (from D8 to D17) during 3h preceding food provision showed a significant increase in ABA mice (**Figure 3C**). During feeding periods, ABA mice progressively decreased their physical activity showing an adaptation to the feeding time restriction schedule (**Figure 3D**).

#### *ATP-production by bacterial proteins from faeces*

To analyze whether the capacity of bacterial proteins to produce ATP is altered in starved mice, we incubated *in vitro* for 2h total proteins from feces of CT, CT-PA, FTR and ABA mice with MH culture medium (as a source of nutrient) and measured ATP concentration in the incubation medium. No significant difference of *in vitro* ATP production by bacterial proteins was found between the groups (**Figure 4A**). Adjustment of ATP production by body weight of mice did not result in significant differences between the groups either (**Figure 4B**).

#### *Plasma acetate and correlations between biological parameters*

Acetate is one of the end products of carbohydrate fermentation by colonic microbiota and is an alternative source of energy for the brain [37]. Since acetate has also been shown to induce anorexigenic effect in the brain [38], we measured acetate plasma concentrations in mice on D17. We found that plasma acetate was higher in the group of mice with physical activity (CT-PA) and tended to be higher in ABA mice ( $t$ -test two-tailed  $p = 0.094$ ) as compared to CT mice (**Figure 4C**).

To gain further insight into the possible links between the biological parameters assayed in the present study, they were compared by correlation analysis. As illustrated by the correlograms no significant correlations were found between ATP-production capacity or plasma acetate with behavioral parameters either in all groups (**Figure 4D**) or in groups of mice with physical activity (**Figure 4E**). Significant negative correlations were found between the feeding anticipatory activity and food intake and body weight, indicating that such physical activity was a contributor to the weight loss in the ABA group. Conversely, as expected, food and water intakes correlated positively with body weight (**Figure 4D,E**)

#### *Proteomic analysis of faeces*

2D-electrophoresis gels were run using total proteins samples extracted from feces collected from the colon of CT, FTR and ABA groups on D17 (**Figure 5**). After the comparison of proteome profiles we found that 20 protein spots were differentially expressed between CT and FTR groups. Among them, 16 proteins were overexpressed in CT group whereas only 4 proteins were overexpressed in FTR group (**Figure 5A**). Moreover, 26 protein spots were found to be differentially expressed between FTR and ABA groups. Among them, 14 proteins were overexpressed in FTR group and 12 were overexpressed in ABA group (**Figure 5B**). Some, but not all differentially expressed proteins have been identified by mass

spectrometry. The results of the identification are presented in **Table 1**. Of note, increased levels of the phosphoglycerate kinase, an ATP-producing glycolytic enzyme belonging to the *Clostridium* sp. ASF502, were detected in feces of FTR mice and this enzyme was further increased in the ABA group. Starvation also upregulated several other proteins from the *Clostridium* sp. ASF502 and from *Lachnospiraceae* family, both belonging to the *Clostridiales* order.

## Discussion

In the present study, we analyzed gut bacterial proteome in chronically starved mice using behavioral models with limited access to food with or without physical activity in a running wheel – i.e. in the ABA and FTR groups, respectively. The validity of the experimental approach was confirmed by significant loss of body weight and decrease of food and water intake in both ABA and FTR groups. The limitation of this model is that such “anorexia” will disappear as soon as the animals will have *ad libitum* access to food. In the present experiment, mice of the ABA group did not, for the majority, display lower food intake than the FTR group, apart from few sporadic decreases (Fig. 2A). Nevertheless, lower levels of fat mass and a more significant loss of total body mass than in FTR group show a greater degree of starvation in the ABA group. This starvation was due to both a decrease of food intake and increased anticipatory physical activity supported by significant correlations with body weight of both parameters. Weight loss was not related to dehydration since no decrease in water intake was observed resulting in lower ratios of food to water intakes in both ABA and FTR groups. We also observed that during starvation, mice adapted to the restricted feeding mainly by reducing physical activity than by increasing food intake [30]. Since we used male mice, which we previously found to be more susceptible to develop ABA, these data are in

agreement with results showing reduced plasticity of hippocampal GABA-ergic neurons in male mice contributing to the development of ABA [39]. Although AN is predominantly a female pathology, the sex ratio tends to evolve a bit over the time, suggesting that AN pathology could be also a male concern [40].

Starvation creates an energy deficit for the body including the brain, which consumes about 20% of oxygen and 25% of glucose. It has been estimated that glutamate-mediated neurotransmission is responsible for most (~80%) of the energy expenditure in the gray matter [41]. Neurons receive their energy substrates mainly in the form of lactate from astrocytes, which are highly glycolytic [42]. This is of relevance to AN because reduced astrocyte density was observed in animals with ABA [43] possibly underlying the loss of brain volume in patients with AN [44]. Also, ABA mice exhibit brain modifications of hypothalamic proteins mainly involved in energy metabolism such as glycolysis and TCA cycle [45]. To meet the demands of the host, gut bacteria could represent an alternative source of energy substrates for the body and the brain due to the capacity of bacterial enzymes to catalyze production of ATP, lactate and SCFA such as acetate, butyrate and propionate, which pass from the gut to the systemic circulation [38]. The present results revealed modifications of the bacterial proteome in faeces of starved mice by showing an upregulation of several glycolytic enzymes including phosphoglycerate kinase (PGK), a major ATP-producing glycolytic enzyme [46].

In our study, the proteomic investigations of the fecal/colonic microbiota could represent a limit because the main place of the fermentation is located in the caecum. However, the specific fecal/colonic microbiota may be of interest since increased colonic permeability characterized the ABA mice [47] which could lead to gut microbiota modifications. Also a specific fecal microbiota profile has been found to be significantly

different from the cecal one with biological and functional relevance regarding the bacterial catabolism [48]. It is also worth noting that the proteins identified in this study (and assigned to *Clostridium* sp ASF502) could match with other bacteria either from the *Lachnospiraceae* family or from the *Clostridiaceae* family both belonging to the order *Clostridiales*. Of notice, the PGK belonged to the order *Clostridiales* and it was further up-regulated in ABA as compared to FTR mice suggesting that PGK levels can be linked to the starvation degree. This finding supports an idea that starvation-induced increase of the PGK expression by selective gut bacteria may provide an additional tool for the ATP supply for the body to combat energy deficit. Another glycolytic enzyme, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) produced by *Clostridium* was found to be up-regulated in the fecal proteome of ABA mice. GAPDH precedes PGK in the glycolysis metabolic pathway and generates energy by producing nicotinamide adenine dinucleotide. The fecal microbiota of ABA mice seems to produce energy substrates with a greatest efficacy than the fecal microbiota of CT mice. Also, an upregulation of glycolytic enzymes (such as phosphoglycerate kinase) can be the result of utilization of substrates from lipolysis and glycerol metabolism for the host.

In spite of increased levels of the *Clostridium* PGK detected by proteomics, our *in vitro* assay of gut bacterial enzyme activity did not show differences in ATP production between starved and *ad libitum*-fed mice. These results suggest that the capacities of bacterial ATP production are limited to the maintenance of normal levels of ATP synthesis during starvation, which may partly compensate for eventual losses of ATP and other energy substrate production following modification of the microbiome composition. In fact, several studies revealed low levels of SCFA in faeces of patients with AN [6, 8, 9]. This may reflect a decrease of SCFA-producing bacterial species in AN such as *Roseburia* (family *Lachnospiraceae*, order *Clostridiales*), which abundance correlates with butyrate [8, 9]. A



decrease of SCFA production by gut bacteria is of functional relevance as SCFA may account for up to 10% of energy substrate supply [49]. In the present study we measured plasma levels of acetate, which tended to be higher in ABA vs. control mice. However, it was not increased in FTR mice suggesting that it was not primarily linked to starvation, but rather to physical activity because *ad libitum*-fed running mice also displayed significant elevated plasma acetate.

It is of interest that all the identified proteins, which were upregulated in both ABA and FTR mice belonged to the order *Clostridiales* (Table 1). In fact, beside the glycolytic enzymes, increased expression of uridine diphosphate (UDP) kinase and of the elongation factor Tu both produced of *Lachnospiraceae* bacterium were detected in ABA and FTR mice, respectively. Flagellin, a structural protein from a butyrate-producing bacterium *Butyrivibrio proteoclasticus* (family *Lachnospiraceae*, order *Clostridiales*) was also increased in FTR mice. Taken together, these data point to increased metabolic activities of *Clostridiales* during starvation. Increased abundance of *Clostridiales* has been also found in ABA model in rats correlating negatively with plasma leptin [34]. Thus, although the ABA model in rodents can only partly reflect chronic starvation in patients with AN, the present data support a role of the order *Clostridiales* in the adaptation of gut microbiota to host starvation via increased capacity in energy harvesting to produce energy substrates and maintain ATP synthesis.

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## Author contributions

SOF conceived and designed the study and wrote the manuscript together with JB, who contributed to the study design, performed the experiments, analyzed the data and made the figures. RL, NA helped with experiments. PC performed the mass spectrometry analysis. MC & PD contributed to the study design.

## Conflicts of interest

SOF is a co-founder and consultant of TargEDys, SA. PD is a co-founder of TargEDys, SA. Other co-authors declare no conflict of interest.

## Figure legends

### Figure 1. Body weight, and body composition.

**A**, Protocol of daily food access in ABA and FTR mice. **B**, Daily dynamics of body weight changes. **C**, Area under curve (AUC) of body weight from day 6. **D**, Total tissue mass **E**, Lean tissue mass and **F**, Fat tissue mass measured by echoMRI at day 17. **C**, ANOVA  $p < 0.0001$ , Bonferroni's post-test CT-PA. and CT vs. ABA.,  $***p < 0.0001$ , CT-PA. and CT. vs. FTR.,  $**p < 0.001$  **D**, ANOVA  $p = 0.0017$ , Dunns post-test CT. vs. FTR.,  $*p < 0.05$  and CT. vs. ABA., ANOVA  $**p < 0.01$ . **E**, ANOVA  $p = 0.0019$ , Dunns post-test CT. vs. FTR.,  $*p < 0.05$  and CT. vs. ABA., ANOVA  $**p < 0.01$ . **F**, ANOVA  $p = 0.0035$ , Dunns post-test CT. vs. ABA.  $**p < 0.01$ .  $*p < 0.05$  Student's  $t$ -test CT. vs. FTR.  $^{\$}p = 0.032$ ,  $t$ -test two-tailed FTR. vs. ABA.,  $p = 0.0952$ . CT, Control; FTR, feeding-time restriction; ABA, activity-based anorexia; CT-PA, control with physical activity.

**Figure 2. Food and water intakes.**

**A**, Dynamics of daily food intake (FI) **B**, Area under curve (AUC) from day 6 of food intake. **C**, Dynamics of daily water intake (WI) **D**, AUC from day 6 of water intake. **E**, Dynamics of daily ratios of food and water intakes **F**, AUC from day 6 of FI/WI ratios. **B**, ANOVA  $p < 0.0001$ , Bonferroni's post-test CT-PA. and CT vs. ABA. and FTR,  $***p < 0.0001$ . **D**, ANOVA  $p = 0.0214$ , Bonferroni's post-test CT. vs. CT-PA.,  $*p < 0.05$ . **F**, ANOVA  $p < 0.0001$ , Bonferroni's post-test CT-PA. and CT vs. FTR., CT. vs. ABA.  $***p < 0.0001$ , CT-PA. vs. ABA.  $**p < 0.01$ . CT, Control; FTR, feeding-time restriction; ABA, activity-based anorexia; CT-PA, control with physical activity.

**Figure 3. Physical activity.**

**A**. Daily physical activity (running distance in Km) in ABA mice and in CT-PA mice (**B**). **C**. Feeding anticipatory activity 3h before food access and physical activity during 3h of feeding period (**D**) in ABA and CT-PA mice. **C**, 2 way-ANOVA, Bonferroni's post-test ABA. vs. CT-PA., day 8, day 13, day 15  $***p < 0.0001$  ; day 12, day 14  $**p < 0.01$  ; day 9  $*p < 0.05$  **D**, 2 way-ANOVA, Bonferroni's post-test ABA. vs. CT-PA., from day 9 to day 16,  $***p < 0.0001$ . ABA, activity-based anorexia; CT-PA, control with physical activity.

**Figure 4. ATP-production by bacterial proteins from faeces**

**A**, *In vitro* assay of ATP production by gut bacterial proteins extracted from faeces of ABA, FTR, CT and CT-PA mice. **B**, ATP production relative to body weight of mice. **C**, Acetate plasma concentration in ABA, FTR, CT and CT-PA mice. **D**, Correlations of study parameters in all groups. **E**, Correlations of study parameters including physical activity in ABA and CT-PA groups. Circles denote significant ( $p < 0.05$ ) negative (blue) and positive (red) Spearman's

correlations. **C**, \*  $p < 0.05$  Student's  $t$ -test CT. vs. CT-PA.  $^{\S}p=0.032$ ,  $t$ -test two-tailed CT. vs. ABA.,  $^{\#}p=0.0923$ . CT, Control; FTR, feeding-time restriction; ABA, activity-based anorexia; CT-PA, control with physical activity.

**Figure 5. Representative images of Coomassie-stained 2-D gels of fecal proteins.** A. CT vs FTR and B FTR vs ABA. Differentially expressed proteins (i.e., with at least 1.5 fold change, Student's  $t$ -test,  $p < 0.05$ ) are shown as encircled spots. Red-increase, blue-decrease. CT, Control; FTR, feeding-time restriction; ABA, activity-based anorexia.

**Table 1. Identified bacterial proteins in faeces of mice.** Differentially expressed proteins (i.e., with at least 1.5 fold change, Student's  $t$ -test,  $p < 0.05$ ), which were identified by liquid chromatography-tandem mass spectrometry in CT, FTR and ABA groups. CT, Control; FTR, feeding-time restriction; ABA, activity-based anorexia.

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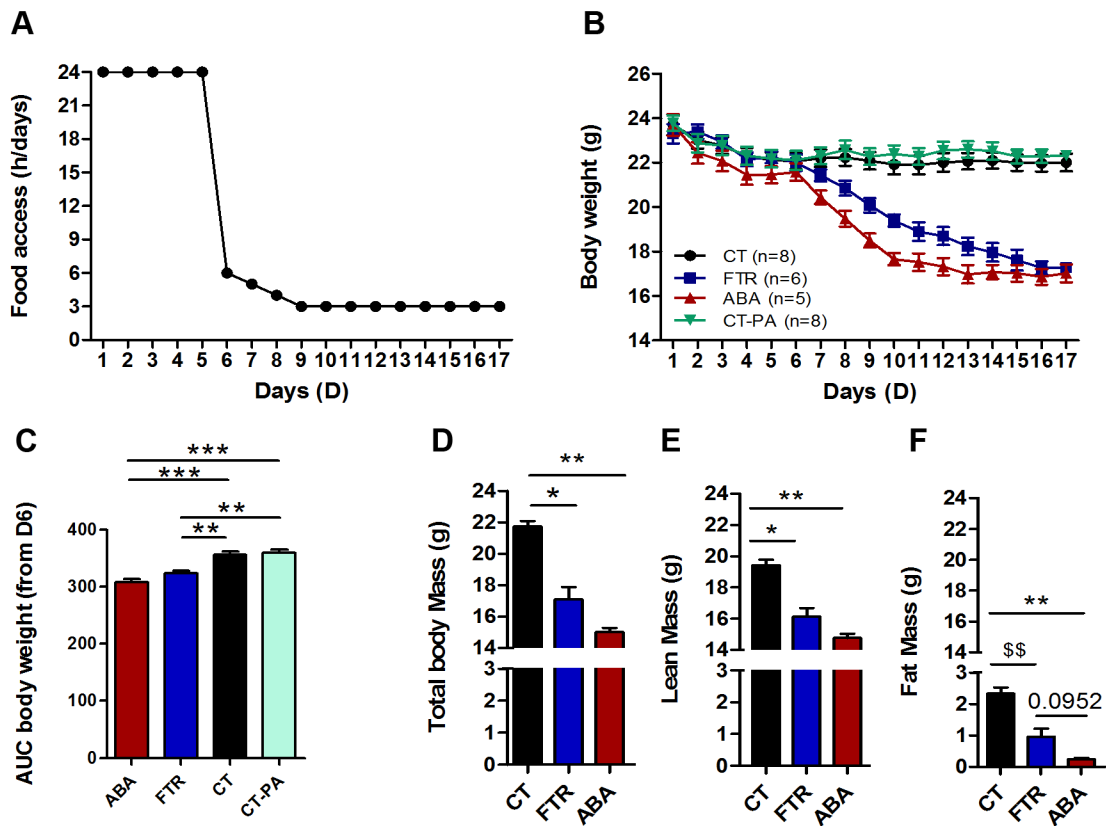
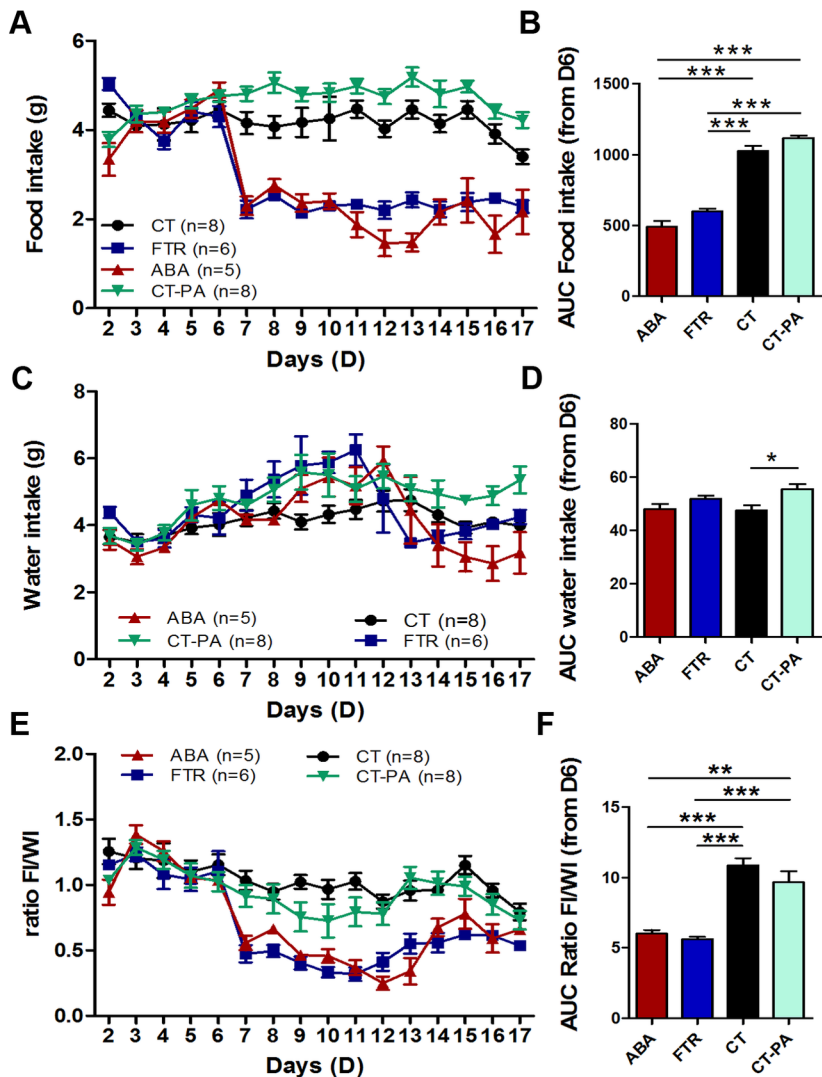


Figure 1





**Figure 2**

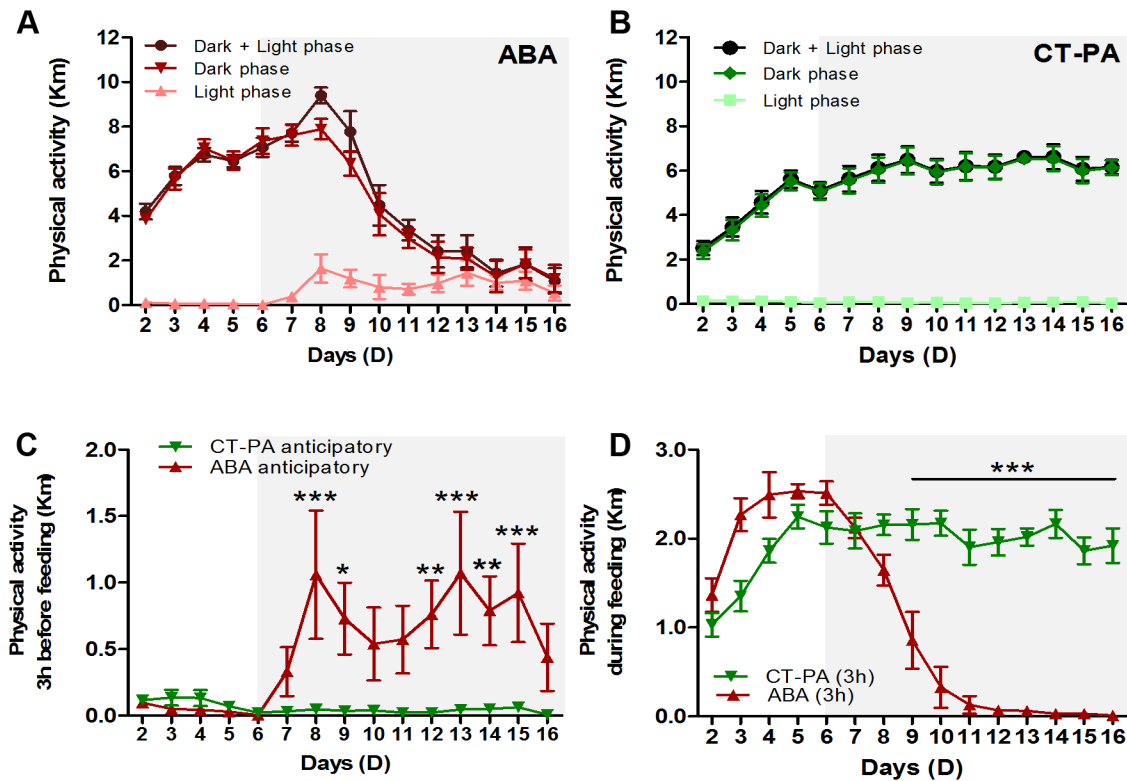
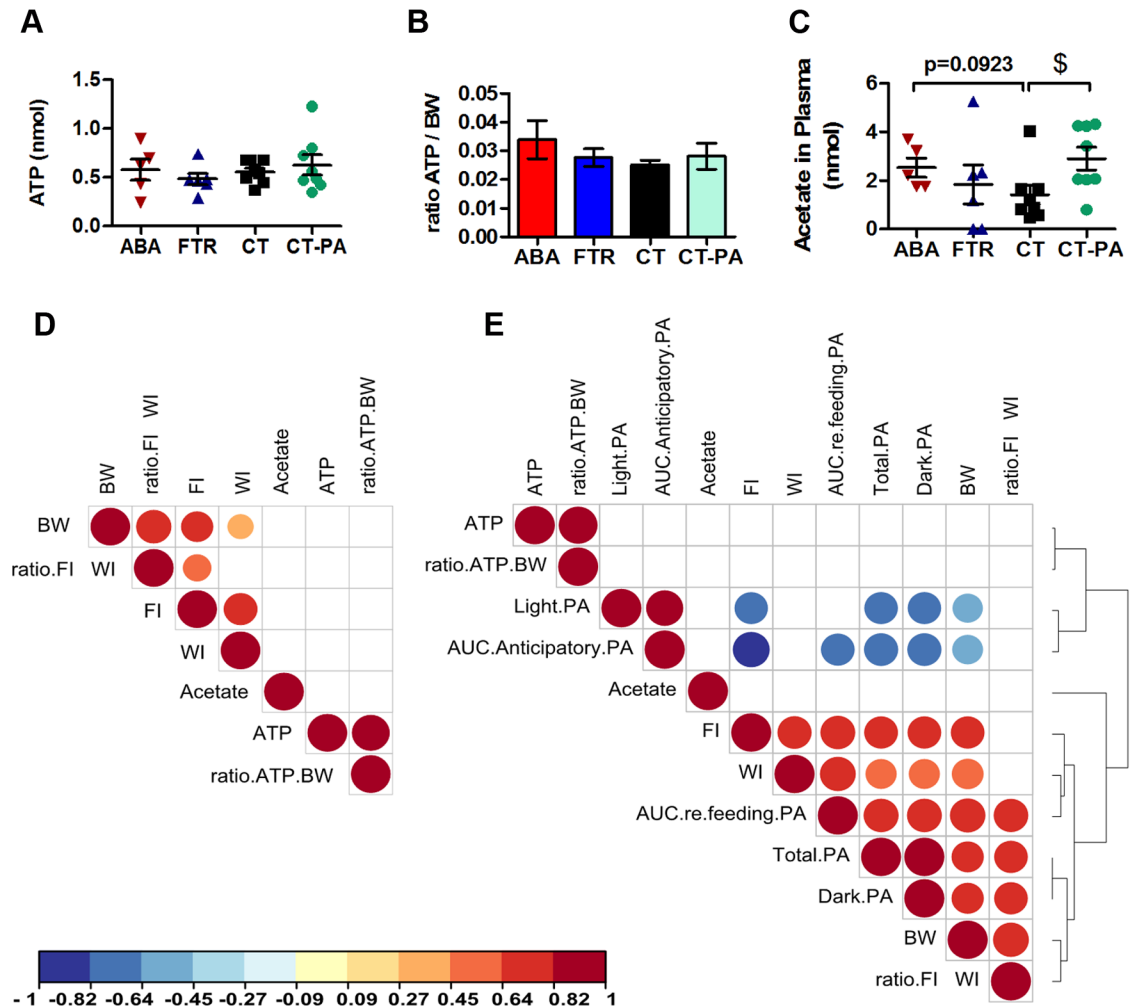
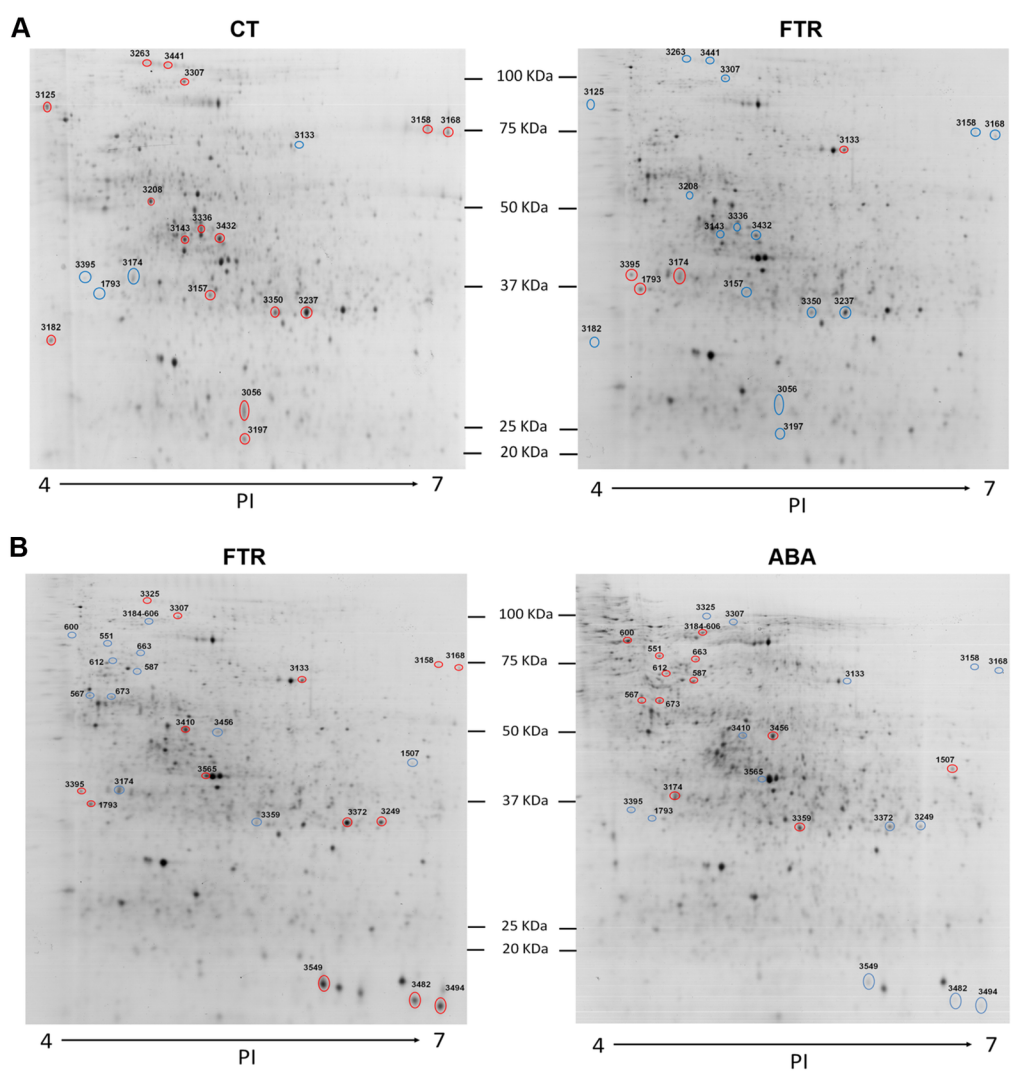


Figure 3



**Figure 4**



**Figure 5**

Table 1. Identified bacterial proteins in faeces of mice. Differentially expressed proteins (i.e., with at least 1.5 fold change, Student's t-test, p<0.05), which were identified by liquid chromatography-tandem mass spectrometry in CT, FTR and ABA groups. CT, Control; FTR, feeding-time restriction; ABA, activity-based anorexia.

Spot	NCBI nr accession number	Protein name	Bacterial clades	IP	MW (Da)	Cover (%)	Fold changes
CT group							
3056	gi 393783424	Hypothetical protein HMPREF1071_02465	<i>Bacteroidales</i> ( <i>Bacteroidaceae</i> , <i>Bacteroidetes solyersiae</i> CT02712C01)	5.44	72169	7	2,8 vs FTR
3125	gi 46909475	Flagellin (uncultured bacterium)	<i>Unknown</i>	5.05	48935	9	4,8 vs FTR
3143	gi 255283697	Translation elongation factor Tu	<i>Clostridiales</i> ( <i>Clostridiaceae</i> , <i>Bryantella formateigen</i> DSM 14469)	5.04	43815	32	3,6 vs FTR
FTR group							
1793	gi 302669857	Flagellin Flic1	<i>Clostridiales</i> ( <i>Lochnospiraceae</i> , <i>Butyrivibrio proteoclasticus</i> B316)	5.3	31337	14	2,4 vs CT/ABA
3174	gi 476628170	Phosphoglycerate kinase	<i>Clostridiales</i> ( <i>Clostridiaceae</i> , <i>Clostridium</i> sp ASF502)	4.93	41780	22	1,8 vs CT
3410	gi 336430440	Elongation factor Tu	<i>Clostridiales</i> ( <i>Lochnospiraceae</i> bacterium 3157FAA CT1)	5.05	43928	6	3,6 vs ABA
ABA group							
663	gi 490181412	Hypothetical protein	<i>Clostridiales</i> ( <i>Clostridiaceae</i> , <i>Clostridium</i> sp ASF502)	4.92	89955	27	2,9 vs FTR
673	gi 496554534	UDP kinase	<i>Clostridiales</i> ( <i>Lochnospiraceae</i> bacterium 7_1_58FAA)	5.42	42664	14	2,6 vs FTR
1507	gi 490173254	Hypothetical protein	<i>Clostridiales</i> ( <i>Clostridiaceae</i> , <i>Clostridium</i> sp ASF502)	7.03	29270	29	2,5 vs FTR
3174	gi 490169794	Phosphoglycerate kinase	<i>Clostridiales</i> ( <i>Clostridiaceae</i> , <i>Clostridium</i> sp ASF502)	4.93	41780	25	1,8 vs FTR
3359	gi 490169796	Gyceraldehyde3Phosphate dehydrogenase	<i>Clostridiales</i> ( <i>Clostridiaceae</i> , <i>Clostridium</i> sp ASF502)	5.1	35913	29	2,63 vs FTR