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Investigating fecal microbiota transplants from individuals with anorexia nervosa in antibiotic-treated mice using a cross-over study design

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Abstract

Anorexia nervosa (AN) is a complex and serious mental disorder, which may affect individuals of all ages and sex, but primarily affecting young women. The disease is characterized by a disturbed body image, restrictive eating behavior, and a lack of acknowledgment of low body weight. The underlying causes of AN remain largely unknown, and current treatment options are limited to psychotherapy and nutritional support. This paper investigates the impact of Fecal Microbiota Transplants (FMT) from patients with AN on food intake, body weight, behavior, and gut microbiota into antibiotic-treated mice. Two rounds of FMT were performed using AN and control (CO) donors. During the second round of FMT, a subset of mice received gut microbiota (GM) from a different donor type. This split-group cross-over design was chosen to demonstrate any recovery effect of FMT from a non-eating disorder state donor. The first FMT, from donors with AN, resulted in lower food intake in mice without affecting body weight. Analysis of GM showed significant differences between AN and CO mice after FMT1, before cross-over. Specific bacterial genera and families *Ruminococcaceae*, *Lachnospiraceae*, and *Faecalibacterium* showed different abundances in AN and CO receiving mice. Behavioral tests showed decreased locomotor activity in AN mice after FMT1. After FMT2, serum analysis revealed higher levels of appetite-influencing hormones (PYY and leptin) in mice receiving AN-GM. Overall, the results suggest that AN-GM may contribute to altered food intake and appetite regulation, which can be ameliorated with FMT from a non-eating disorder state donor potentially offering FMT as a supportive treatment for AN.

Plain English summary

In our study, we explored whether characteristics associated with Anorexia Nervosa (AN), such as low body weight and altered appetite, could be transferred to mice pretreated with antibiotics through fecal transplants from individuals with AN. We used mice with antibiotic-depleted gut bacteria and humanized them by transplanting human donor gut microbiota. After observing reduced food intake in mice receiving AN gut microbiota, we conducted a second transplant with non-eating disorder donor microbiota to counteract these effects. We found

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that the food intake patterns of mice could be influenced by the type of microbiota they received after the first rounds of transplants, suggesting a potential for microbiota transplants to modify AN-related behaviors. Biomarker analysis indicated changes in appetite-regulating hormones in mice receiving AN microbiota, further supporting the role of gut microbiota in influencing eating behaviors. Our findings highlight the potential of microbiota transplants as a therapeutic approach for treating AN.

Keywords Microbiota, Anorexia nervosa, Split-group cross-over design, Appetite, Gut-brain axis, Mice

Introduction

Anorexia nervosa (AN) is a complex and serious mental disorder, which may affect individuals of all ages and sex with the highest prevalence in young women, with a lifetime prevalence rate up to 4% in females and 0.3% in males [1–3]. Restrictive AN is characterized by a disturbed body image or lack of acknowledgement of low body weight and behavior that interferes with weight gain [4]. AN is in some cases preceded by depression, trauma, gastrointestinal symptoms, an infection, or a combination hereof, however, the etiology is largely unknown [5]. Treatment options for AN are still limited to mainly psychotherapy and nutritional support and new avenues of approach to therapy are warranted.

Growing evidence suggests that the human gut microbiota (GM) plays a crucial role in health, weight regulation, and metabolic function, and, therefore, prompting a hypothesis of its involvement of the gut-brain axis as interaction between GM influence on neuropsychiatric diseases and possibly the etiology of AN [6, 7]. Several studies have highlighted significant differences in the GM of patients with AN compared to non-eating disorder individuals, termed a dysbiosis [8–14]. A microbiota dysbiosis is characterized by either loss of commensals, expansion of pathobionts, loss of microbial diversity, or combinations hereof [15]. It has also been demonstrated that interventions aimed at weight restoration, regular diets and therapy do not rectify the dysbiosis of AN-GM [16–18]. Currently, two single case studies, and one protocol, have explored fecal matter transplantation (FMT) as a potential therapeutic approach to address dysbiosis by transferring GM from non-eating disorder donors to patients with AN [19–21].

Studies in mice investigating the transfer of AN phenotypical and behavioral traits via fecal microbiota transplantation (FMT) from patients with AN to germ-free (GF) mice remain limited. Fan et al. (2023) showed a larger initial decrease in body weight and a slower weight gain over time in mice receiving AN microbiota compared to those receiving non-eating disorder state, control FMT. Hata et al. (2019) reported reduced body weights and behavioral changes, such as anxiety-like and compulsive behaviors, in offspring from GF mice that received FMT from patients with AN [22]. Conversely, Glenny et al. (2021) reported no relationship between AN-associated GM and changes in body weight gain in adult recipient GF female and male mice [23]. All three studies employed GF mice but differ with respect to inoculums, mouse strain and other methodologies.

Humanizing mouse models with human-to-mice FMT is an emerging concept with pros and cons that provides an ecologically relevant context to study the role of GM in health and disease. However, the major obstacles are low colonization rates from donor GM and immunological abnormalities observed in these mice [24]. GF mice are typically preferred for FMT studies due to their immunological naivety and absence of microbial competition, facilitating accurate assessment of transferred phenotypes. However, the option to use antibiotics to deplete native GM is important to consider when studying phenotype transfers linked to GM alterations. This approach enables the examination of GM disruptions at specific life stages and in immune-competent mice, providing clearer insights into the GM´s role in these processes [25].

In this study, our approach is to use human-to-mouse FMT to transfer AN phenotypic traits to adolescent, antibiotic-treated, immunocompetent female mice. We monitor food intake per cage, body weight gain, and behavioral characteristics over time. We apply a split-group cross-over design, as we hypothesize that FMT from a non-eating disorder state, control donor could partially remedy an AN-GM induced phenotype and vice versa. We correlate this to appetite-related biomarkers in mouse serum at the point termination.

Results

Study design

Briefly, 48 mice were first treated with ampicillin for two weeks. The mice were then split into two groups and subjected to FMT with GM from either a patient with AN or a control (CO) person. The mice received one FMT per week for 5 weeks (FMT1). After, all the mice received 3 weeks of AB treatment and then went through a second bout of FMT for 4 weeks (FMT2). Here, the mice received either the same FMT as before (ANAN and COCO) or the opposite FMT (ANCO and COAN). The whole experiment was run in two series (ExpA and ExpB) staggered one week. During both FMT rounds food intake and body weight was measured twice a week. We collected five fecal samples (FS1-FS5) from the mice over the 14 weeks. For further details, see the Material & Methods section.

Fecal microbiota transplants from patients with anorexia nervosa lowers food intake without impacting body weights

Body weights: No body weight differences were observed in mice receiving fecal microbiota transplants (FMT) from donors with anorexia (AN mice) compared to those receiving FMT from control donors (CO mice) following the first round (five weeks) of fecal microbiota transplants (FMT1) (Fig. 1, left). During the three weeks of AB treatment, the weight gain of both AN and CO mice were identical at an average of 0.9 g. Also, no distinction in body weights were observed after the split cross-over and four weeks of the second round of FMT (FMT2). This finding remained consistent regardless of whether the analysis considered only the last received donor type (AN or CO) (data not presented) or the combined donors from both FMT rounds (ANAN, COAN, ANCO, COCO) (Fig. 1, right).

Food intake: Food was weighed per cage twice a week. Overall, AN cage (after FMT1) and ANAN cages (after FMT2) exhibited lower food intake ranging between 1.9% and 6.9% over the period and all time points, compared to other donor combinations (see Supplementary Table 1). As shown in Fig. 2, food intake during FMT1 was significantly lower ($^*p = 0.020$) in AN cages ($n = 6$) compared to CO cages ($n = 6$). There were no statistical differences between our two consecutive experiments, ExpA and ExpB, after FMT1 and hence analyzed together. ExpA and ExpB ran under identical conditions, except 1 week staggered (See Methods).

However, after FMT2, ExpA and ExpB showed factorial differences ($^*p = 0.013$) and were analyzed separately (Fig. 3A and B). Following FMT2, ANAN cages exhibited lower accumulated food intake than COCO cages (31.9 g/6.7% in ExpA and 32,0 g/7,0% in ExpB) or the cross-over groups COAN and ANCO. Both cross-over groups showed intermediate food intake levels. Although a trend was observed, the results from the separate ExpA or ExpB did not reach statistical significance.

Satiety-inducing hormones are increased in AN mice

At the end of the FMT2, the animals were sacrificed, and serum concentrations of six satiety and appetite-related hormones were measured. As depicted in Fig. 4, ANAN mice exhibited significantly higher concentrations of PYY ($^*p = 0.012$) and leptin ($^*p = 0.019$), than COCO mice. Insulin and glucagon showed an average elevation in AN mice but did not reach statistical significance. Additionally, CO mice showed on average higher serum concentrations ghrelin but did not reach statistical significance. There was no difference in active GLP-1 serum levels between groups.

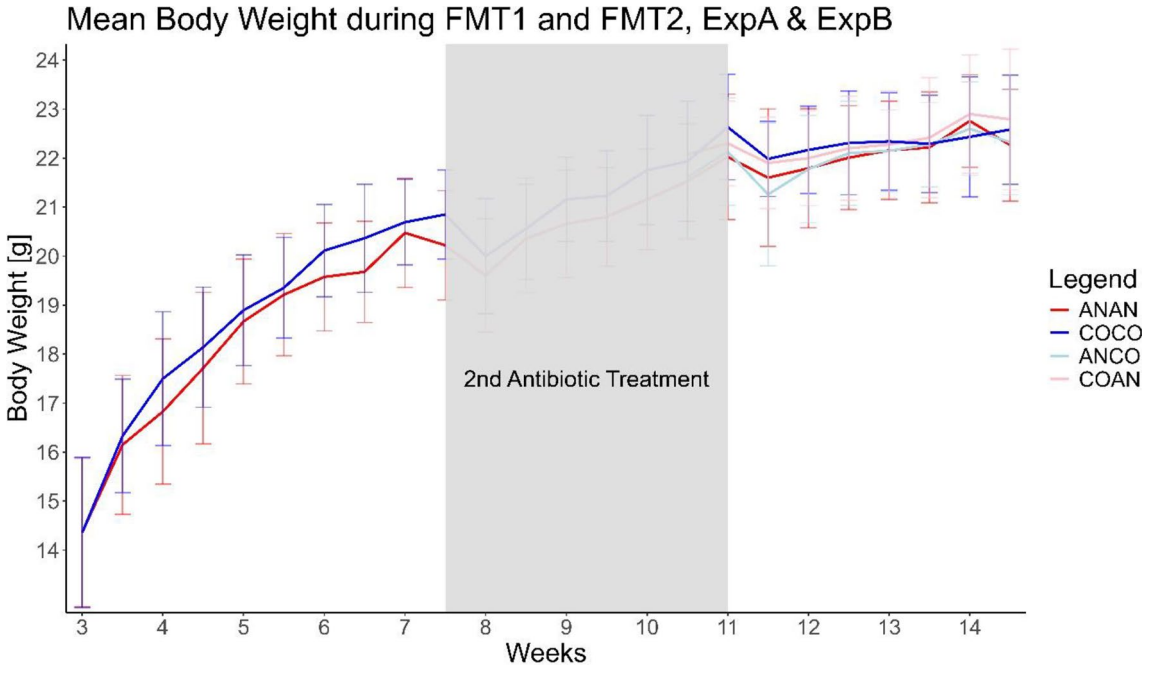

Fig. 1 Effects of FMT from AN and CO donors on body weight development. **Left)** FMT1 for five weeks. Mice received either FMT from patients with AN or from a CO donor (AN mice, $n = 24$, CO mice, $n = 24$, (6 + 6 cages). **Middle)** three weeks of antibiotics. **Right)** FMT2 shows the body weight of mice based on combined donor for FMT1 and FMT2. ANAN mice received GM from patients with AN in both rounds, while COCO mice received GM from control donors in both rounds. ANCO and COAN mice were given GM from patients with AN first and control donors second, or vice versa. All mice were weighed twice a week and Areas under the Curve (AUCs) were calculated and evaluated by 2-way ANOVA with no statistical differences based on donor type

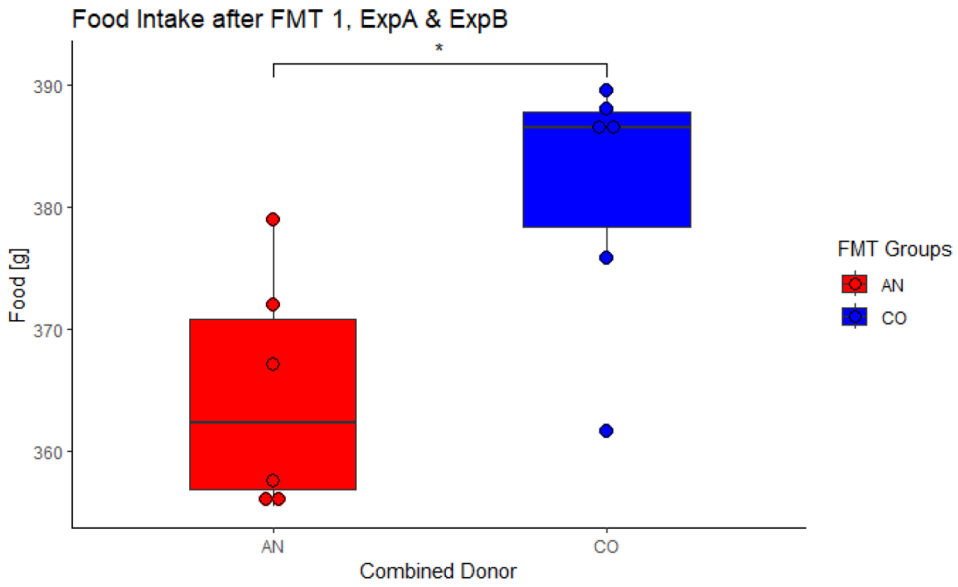

Fig. 2 Effect of FMT1 on accumulated food intake over five weeks per cage (4 mice/cage). Statistical differences between AN cages ($n=6$, (mice $n=24$)) and CO cages ($n=6$, (mice $n=24$)) were evaluated by 2-way ANOVA (AN/CO: $*p<0.020$)

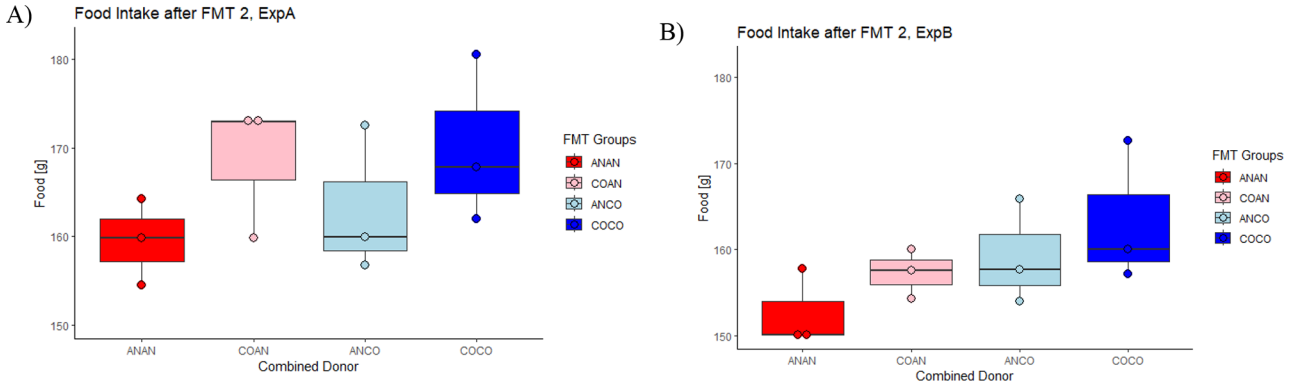

Fig. 3 Effect after FMT2 on accumulated food intake per cage, over four weeks in a split cross-over design. Combined donor explanation: ANAN = same AN donor at FMT1 and FMT2, COAN = CO donor at FMT1 and AN donor at FMT2, ANCO = AN donor at FMT1 and CO donor at FMT2, COCO = same CO donor at FMT1 and FMT2. Statistical differences between combined donor groups and ExpA vs. ExpB were evaluated by 2-way ANOVA. Since there was a significant difference between ExpA and ExpB ($*p=0.013$), the two experiments were analyzed separately. Accumulated food intakes were then calculated and evaluated by 1-way ANOVA. (A) shows ExpA (Cages $n=12$, (mice $n=24$)) ($p=$ ns). (B) shows ExpB (Cages $n=12$, (mice $n=24$)) ($p=$ ns)

Humanization of gut microbiota in mice after antibiotics treatment

We manipulated the GM of the mice with AB and humanized them during FMT1 and FMT2 (Fig. 5, p < **0.01). Based on the similarity coefficient Jaccard index, which relies on the presence and absence of ASVs in fecal samples (FS), we successfully depleted the GM from the original mouse GM (FS1 = red) with antibiotics, as illustrated by the shift in clustering from red to blue (FS2 = blue). After five weeks of FMT1 the humanized mouse GMs, both donor with AN and CO donors (FS3 = orange) are shifted (from blue) towards the human donors (AN = pink and CO = black) while remaining separate from the original mouse GM (FS1 = red). Following a second round of AB, split cross-over, and four weeks of FMT2, the humanized mouse GM shifted once again (FS5 = green).

After FMT1, our two consecutive experimental runs, ExpA and ExpB, were factorially different ($**p<0.01$, Supplementary Fig. 1) and thus analyzed separately. In both ExpA and ExpB, the GM of the AN group was significantly different from the CO group ($*p<0.02$, $**p<0.01$, Fig. 6). After FMT2, the two experimental runs remained significantly different ($*p<0.02$, Supplementary Fig. 2). However, after FMT2, there was no longer a significant difference between the now four subgroups in either experimental run (Supplementary Fig. 3). There was also no statistical difference when comparing either the four subgroups regarding their last donor or when comparing only the ANAN and COCO groups (data not shown).

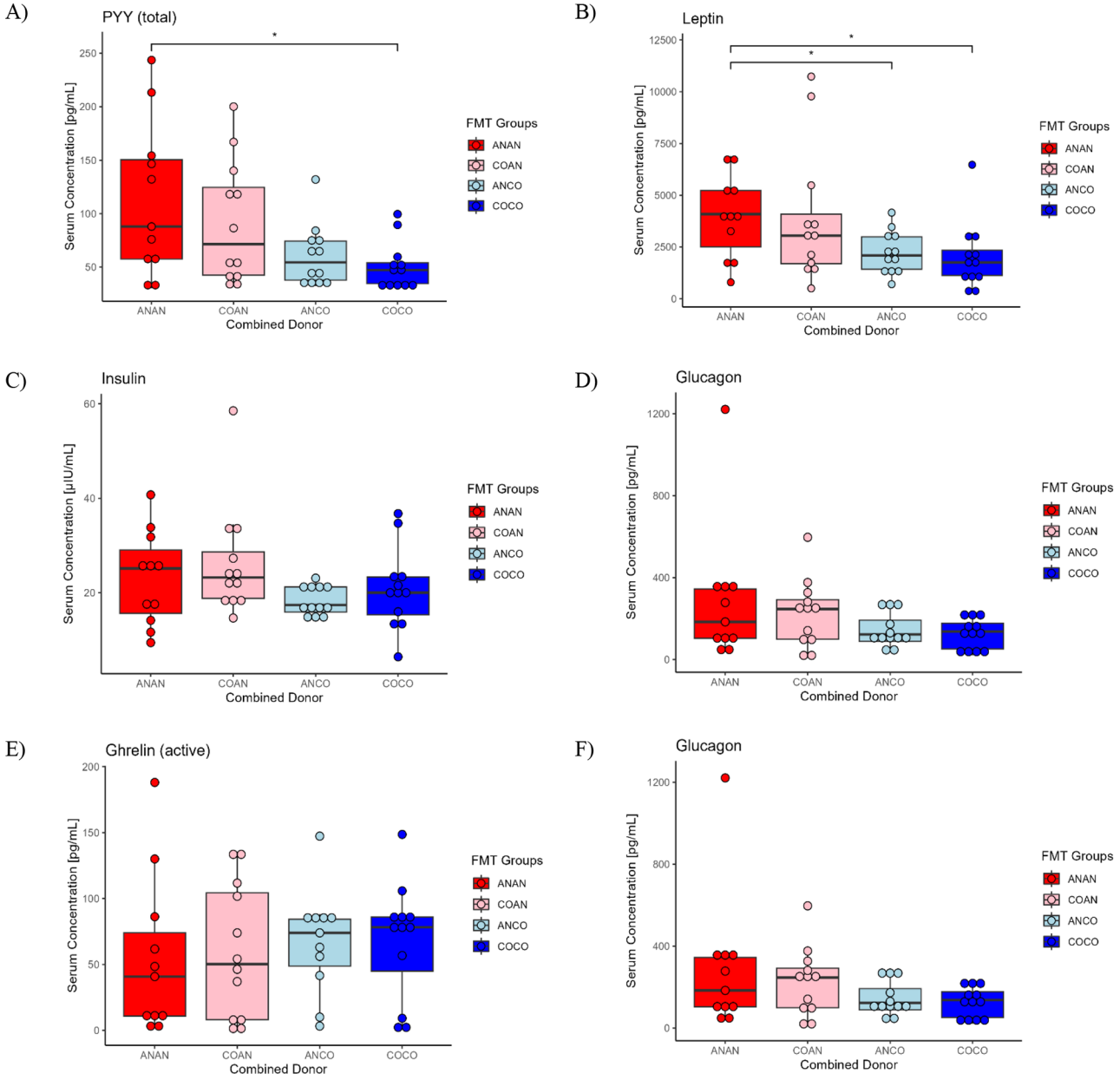

Fig. 4 Effects of FMTs (Combined donor) on serum concentrations (pg/ml or µIU/mL) on appetite related molecules in serum. (1) PYY, (2) leptin, (3) insulin, (4) glucagon, (5) ghrelin and (6) GLP-1 (active), were measured and evaluated by 2-way ANOVA. There were significant differences in PYY and leptin serum concentrations (*$p = 0.012$, *$p = 0.012$ respectively) and no significant differences in insulin, glucagon, ghrelin and GLP-1 (active). Both measurements from ExpA and ExpB are shown together

We also sequenced the GM from the six human donors. The relative abundances can be seen in Fig. 7 and Supplementary Fig. 5. With six different donors attributed to two categories (AN or CO), there are no meaningful statistical comparisons to examine their differences, but noteworthy GM families are *Ruminococcaceae* and *Lachnospiraceae*, as well as the genera *Faecalibacterium*, *Bifidobacterium* and *Ruminococcus*.

When analyzing the GM of all the mice after five weeks of FMT1, the ExpA and ExpB were factorial different (*$p = 0.007$) (Supplementary Fig. 1), and the two experiments were analyzed separately. Examining the bacterial difference in the GM, there is a significant difference in *Clostridium* abundance (Fig. 8).

After FMT1, in ExpA, the GM of the AN mice was significantly different from that of CO mice (Jaccard, ***$p < 0.001$) (Fig. 6A1). In ExpA, AN mice had a higher abundance in *Bilophila* (Fig. 6A2). In ExpB (After FMT1), the GM of the AN mice was also significantly different from that of CO mice (*$p = 0.004$) (Fig. 6B). However,

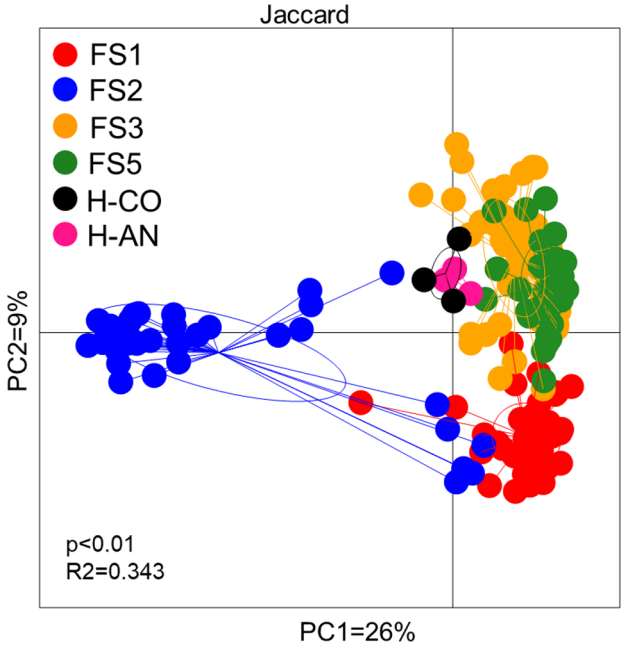

Fig. 5 Gut microbiota of human and mice samples shown by the Jaccard index. The effect of the antibiotic treatment can be seen in the first shift in microbiota from the non-manipulated mouse microbiota (FS1, red) to the mouse GM which was taken after AB treatment (blue, FS2). After five weeks of FMT1 the humanized mouse GM (orange, FS3) shifted from blue towards the human donor (AN, pink and CO, black) while still separated from the original mouse GM (FS1, red). After the second round of AB (not shown, FS4) and four weeks of FMT2, the humanized mouse GM is shifted again (green, FS5). Measurements from both ExpA and ExpB are shown together

there were no statistical significances when analyzed at lower taxonomic levels.

When analyzing the GM of all the mice after four weeks of FMT2, there were no significant differences when analyzed either based on last donor type (data not shown) or the combined donor after FMT2 (Supplementary Fig. 3). There were, however, significant differences between the two consecutive experiments ExpA and ExpB in the abundance level of *Adlercreutzia*,*S24-7*, *Lachnospiraceae*, *Ruminococcaceae*, *Ruminococcus*, *Rikenellaceae* and *Streptophyta* (Fig. 9).

AN mice show less active behavior during open field tests

To assess any effect of FMTs on general locomotor activity levels, the mice underwent open field (OF) tests. Baseline measurements were conducted after the initial two weeks of AB treatment but before the first FMT. Baseline data did not reveal any differences in general locomotor activity between groups (data not shown).

After five weeks of FMT1, the OF data demonstrated a factorial difference between our two consecutive experiments, ExpA and ExpB ($^{***}p < 0.001$), leading to separate analyses (Fig. 10). In ExpA, there were no differences in either the general locomotor activity level (total distance moved) or the total frequency of crossing the fields' subdivisions (Fig. 10A). In ExpB, both the total distance ($^{*}p = 0.024$) and frequency ($^{*}p = 0.024$) were significantly lower in AN mice than CO mice (Fig. 10B). After cross-over and FMT2, there were no differences in general locomotor activity and frequency in either ExpA or ExpB when analyzed by last donor type or combined donor (data not shown).

Over time and regardless of FMT donors, we observed a significant decrease in general locomotor activity ($^{***}p < 0.001$) and total frequency ($^{*}p = 0.012$) for both ExpA and ExpB between baseline and FMT1. Additionally, we observed a significant decrease general locomotor activity ($^{***}p < 0.001$) and total frequency ($^{***}p < 0.001$) between FMT1 and FMT2 (Supplementary Fig. 4).

Discussion

We investigated if phenotypes related to Anorexia Nervosa (AN), such as lower body weight and appetite, and altered behavior, could be transferred by fecal microbiota transplants (FMT) from patients with AN to antibiotic (AB) GM depleted mice. AB was used first to decimate the mouse GM and enable humanization by FMT1. Using a split-group cross-over design, we hypothesized that, after 3 weeks washout with AB to prevent or minimize carry-over effects, a second round of FMT with GM from a non-eating disorder state donor might counteract the effects of the initial FMT of AN gut microbiota (AN-GM), aiming to address AN-GM dysbiosis in humans. Two single case studies indicated promising outcomes for FMT as an additional treatment modality for AN. Prochazkova et al. (2019) reported a substantial increase in bacterial diversity in a patient persisting for one year [19]. The second study demonstrated weight gain induced by FMT treatment in a patient with recurrent AN, suggesting the potential of FMT to facilitate nutritional rehabilitation [20].

We effectively depleted the indigenous mouse gut microbiotas with AB and humanized the model by transplanting the human donor GM to Specific Pathogen Free (SPF) mice as before [26]. The choice between using AB-depleted SPF mice instead of germ-free (GF) mice depends on the specific research questions and the human scenario being mimicked [25]. Fan et al. (2023) used FMTs on GF mice that received a calorie-restricted diet [14], while Hata et al. (2019) inoculated pregnant GF mice to explore AN characteristics in the offspring [22]. Glenny et al. (2021) transferred AN-GM to adolescent GF mice, mimicking induced changes in GM on a naïve immunological and physiological background [27]. In our study, we opted for AB-depleted SPF mice to investigate GM shifts in an adolescent, immune-competent background, resembling potential human scenarios where GM transitions from a previously non-eating disorder

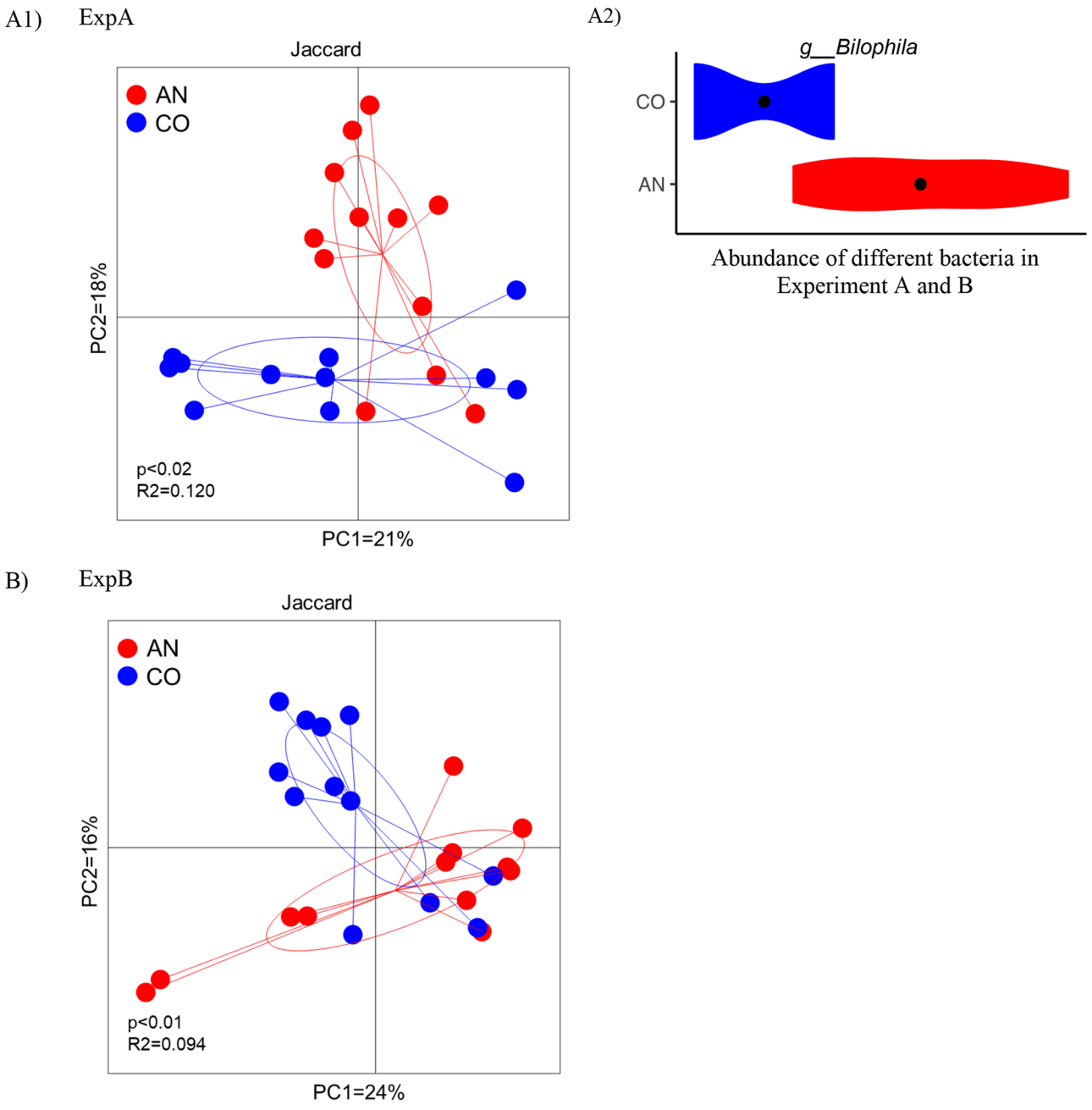

Fig. 6 Effects of FMT1 on the mice GM in ExpA (**A**) and ExpB (**B**). The microbiota is compared by the Jaccard index. After FMT1 the mice receiving AN microbiota (red) were significantly different from CO receiving mice (blue) in both experimental runs (ExpA: $*p < 0.02$, ExpB: $**p < 0.01$). There is a significant difference in the abundance of *Bilophila*, which is higher in AN mice (A2)

state to a dysbiotic state due to altered patient behaviors. It should be noted as a limitation that we did not determine the extent of engraftment of human derived GM. But, based on previous studies by others, and us, engraftment is expected to be similar to engraftment using GF mice between 40 and 60%, depending on methodology [25, 28–30].

Following FMT1, we observed a significant decrease in accumulated food intake in AN-inoculated cages compared to CO cages (ages six to eleven weeks), ranging from 1.9 to 6.9% less food. If directly translatable to humans, a cumulative 1.9-6.9% reduction in food intake due to GM dysbiosis in AN would be significant, as even small decreases can exacerbate malnutrition and weight loss over time. The reduction is low compared to established forced starvation mouse models of AN, which require up to 60% restriction and remains within a range (20–30% restriction) considered non-detrimental to mice

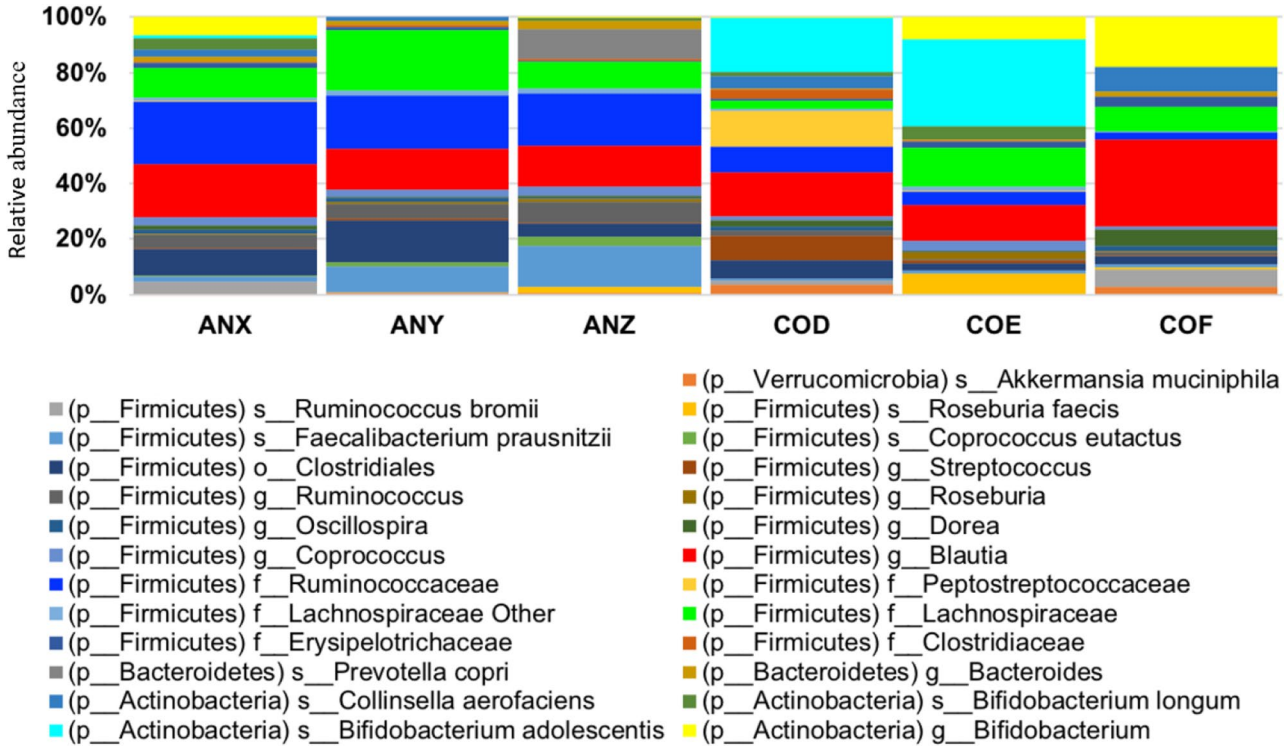

Fig. 7 Composition of the GM of the six human donors. ANX, ANY and ANZ are the three donors with AN and COD, COE and COF are the control donors. The bacterial compositions are presented in a relative correlation to each other. In the donors with AN the abundances of *Faecalibacterium* (light blue), *Rumicnococcaceae* (blue) and *Lachnospiraceae* (green) are higher compared to CO donors. In two of the CO donors the abundance of *Bifidobacterium* (yellow and bright blue) is higher

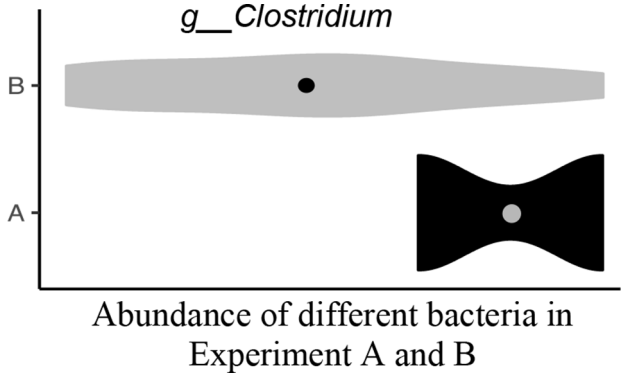

Fig. 8 After FMT1 the abundance of *Clostridium* is significantly different between the two experimental runs ExpA and ExpB. Only taxa that reached significance, defined as having a pseudo p-value < 0.05 are reported

health [31, 32]. Our food reduction data does align with findings from Hata et al. (2019), reporting approximately a 4% reduction in food intake between ages four to ten weeks. Conversely, Glenny et al. (2021) found no changes in average daily food intake between experimental groups using different (C57BL/6) mice of both sexes aged nine to thirteen weeks. Discrepancies in food intake results may stem from differences in mouse strain, potential sex differences, and the age range. Despite reduced food intake, our study did not result in lower body weights, consistent with Glenny et al. (2021). However, Fan et al. (2023) observed decreased weight gain in offspring of AN-inoculated mice under calorie restrictions, while Hata et al. (2019) found decreased weight gain under *ad libitum* food conditions [14, 22, 23]. Various explanations exist for why reduced food intake does not directly lead to weight loss, including potential differences in energy expenditure [33], or efficiency of energy extraction facilitated through the gut microbiota [34, 35].

Following our 3 weeks AB washout period, split cross-over, and FMT2, there was decreased food intake in the pure ANAN cages compared to pure COCO, which received GM from a single donor through both FMT1 and FMT2. As food intake is measured per cage each group only consist of 3 cages/per experiment/group, the result did not reach statistical significance. In the cross-over groups (ANCO or COAN) with different donors in FMT1 and FMT2, intermediate food intake levels were observed, primarily influenced by the last donor type. We report data post-FTM2 results as "combined donor (e.g. COAN)" since we anticipated the second AB treatment would not fully erase the FMT1 effect, which is confirmed by the trends in both cross-over groups. Considering food intake, we can influence CO mice (FMT1) to eat less by inoculating them with AN-GM in FMT2. That also seems to be the case in the opposite situation,

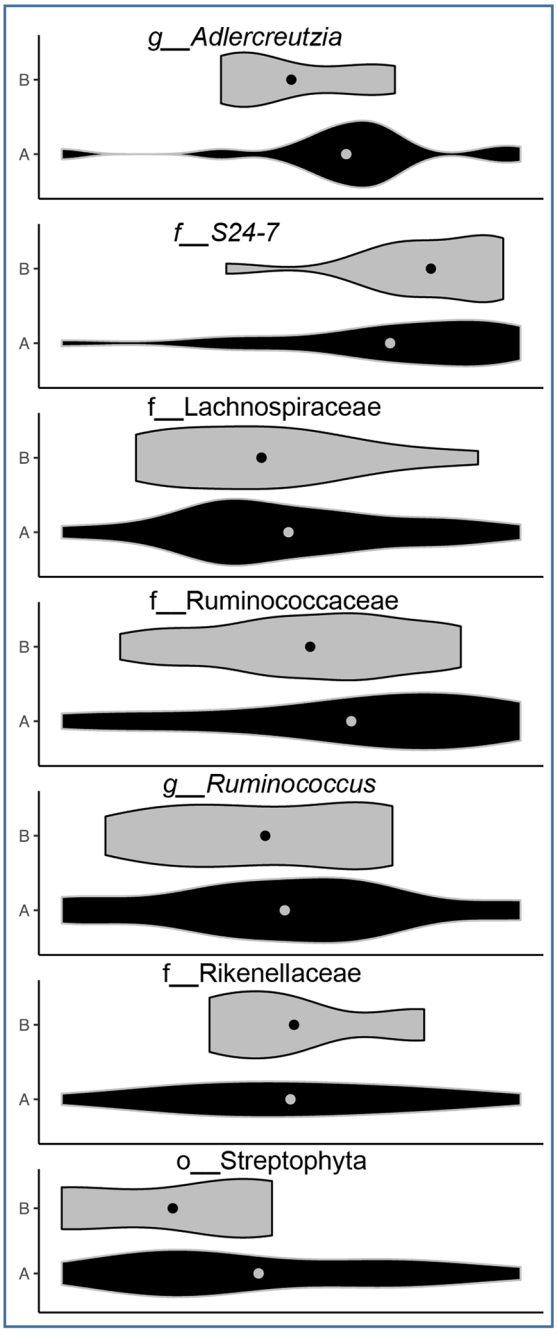

Fig. 9 Statistically significant bacteria when comparing ExpA and ExpB after FMT2. The genera *Adlercreutzia*, *Ruminococcus*, and the families *Lachnospiraceae*, *Ruminococcaeae*, *Rikenellaceae* and the order *Streptophyta* have a higher abundance in ExpA. While *S24-7* has a higher abundance in ExpB. Only taxa that reached significance, defined as having a pseudo p-value < 0.05 are reported. The figure shows violin plots with a boxplot, where the width of each violin indicates the distribution and density of the data for each category. The central boxplot highlights the median, interquartile range, and data spread

in which we influence AN mice (FMT1) to eat more by inoculating them with a CO-GM in FMT2. This suggests potential amelioration of food intake, by FMT from a non-eating disorder state donor.

To explore whether the correlation between FMT donors and food intake could be explained by changed appetite and satiety signals in the blood, we measured six relevant biomarkers in the serum of the mice at termination after FMT2. Although it would have been informative to assess these biomarkers after FMT1, the limited serum volume available prevented pre-termination measurements. Hata et al. (2019) suggests that endocrine biomarkers like PYY (peptide tyrosine tyrosine), ghrelin and leptin are indicative of chronic food restriction [36]. Our results reveal significantly higher serum concentrations of PYY and leptin in ANAN mice than COCO after FMT2. PYY functions as an appetite-suppressing gut hormone increasing satiety and reducing food intake [37]. Similarly, leptin mediates long-term energy regulation balance, suppressing food intake [38]. While insulin was elevated in ANAN and COAN mice, the differences were not statistically significant. In the cross-over groups (ANCO or COAN) intermediate biomarker levels were observed, mostly influenced by the last donor type. Although we did not reach statistically significance on ghrelin or GLP-1, the mean levels of ghrelin, known to increase appetite [36], were higher in both CO groups (ANCO and COCO) compared to both AN groups (ANAN and COAN). Elevated serum PYY levels in our AN align with reports of PYY and leptin contributing to lower food intake and increased satiety in patients with AN [39]. PYY acts on the brain by inhibiting the orexigenic neuropeptide Y (NPY) [40], indicating that the AN-GM activity transferred to our mice affects the gut-microbiota-brain axis by enhancing the PYY signal.

In our study, we observed increased leptin levels in ANAN and COAN mice compared to COCO and ANCO mice, suggesting heightened satiety in these mice. However, it has been reported that the serum leptin concentrations are significantly lower in fasting patients with AN, other types of malnutrition and starvation, than normal individuals [41–44]. This discrepancy may stem from differences in adipose tissue composition, as leptin is primarily secreted from subcutaneous fat [45], which is notably reduced in adult patients with AN [46]. Although the body weight of our mice was similar between AN and CO mice and adipose tissue levels may vary, our study only investigated rather short-term effect from altered GM and not a comparable severe disease state of individuals with AN. However, Fan et al. (2023) did find that FMT from human donors with AN resulted in increased expression of specific genes in inguinal fat of AN-transplanted mice, indicating enhanced adipose tissue thermogenesis. Serum insulin levels were also

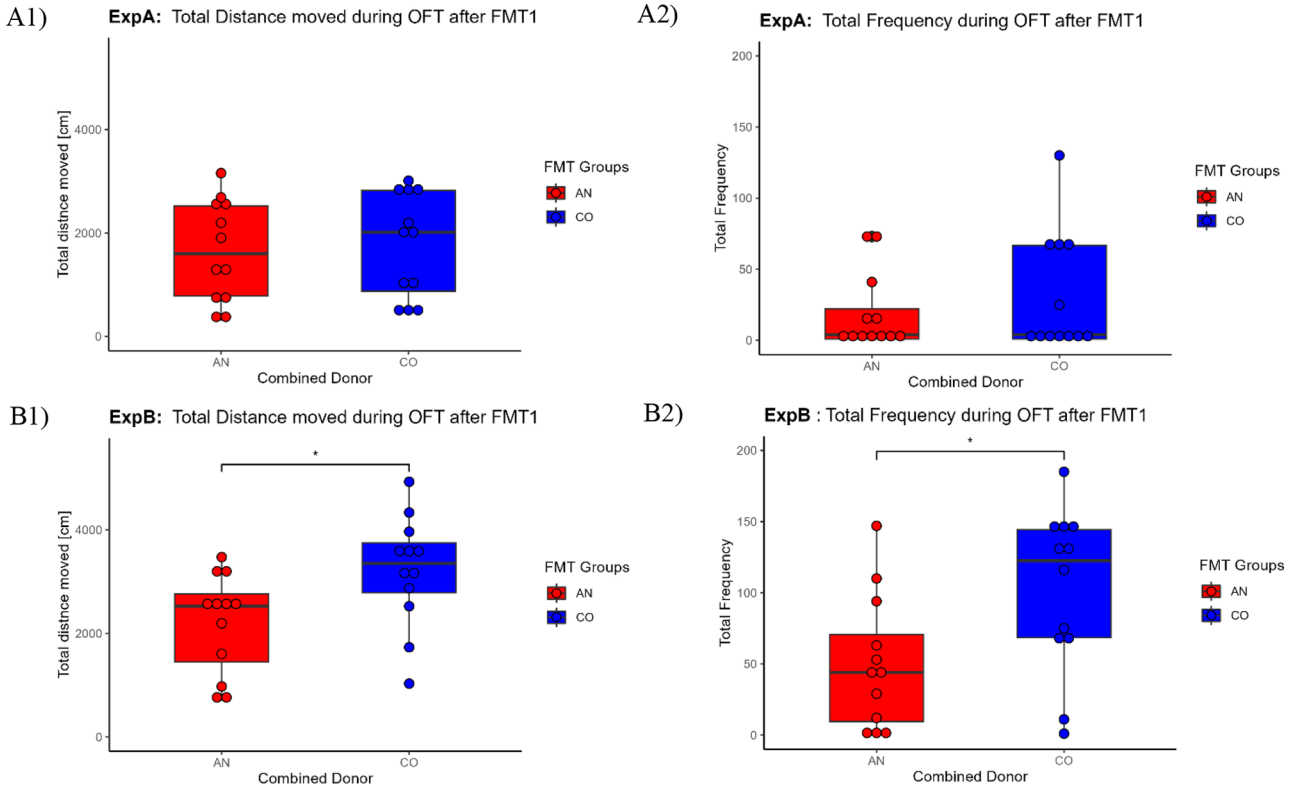

Fig. 10 General locomotor activity. Effect on total distance moved (cm) in the open field and total frequency of crossing between the three open field subdivisions (outer, middle and center subdivision) from FMT1 compared between AN versus CO donor. In ExpA there were no differences observed. **A1)** Total distance moved in ExpA, **A2)** Total frequency in ExpA (AN, $n = 12$) (CO, $n = 12$). In ExpB, we observed a significant decrease in the AN mice compared to CO mice in both total distance moves and crossing frequency. **B1)** Total distance moved in ExpB, **B2) T**otal frequency in ExpB (AN, $n = 12$) (CO, $n = 12$) (*$p < 0.010$)

elevated in ANAN and COAN mice, however, fasting the mice before blood sampling should be considered in future studies, as insulin rapidly increases in response to glucose levels [47].

To comprehensively evaluate the impact of the FMTs on the microbiota of humanized mice, we sequenced fecal samples collected at different time points. We analyzed the microbiota using the Jaccard index, which considers the presence and absence of ASVs, and subsequently, we assessed statistical differences in bacterial genera at lowest possible taxonomical level.

The primary observation was the successful shift in the GM of naïve mouse through the antibiotic treatment. After five weeks of FMT1, the humanized mouse GM transitioned towards that of the human donors while maintaining distinctiveness from the original mouse GM. Following the second AB treatment and four weeks of FMT2, the humanized GM underwent another shift.

After FMT1, the two experimental runs, ExpA and ExpB, were significantly different and were therefore analyzed separately. In both experimental runs, we observed significant differences between AN and CO mice based on the Jaccard Index. However, after FMT2, no statistically significant differences in GM were observed when comparing the combined donor types (ANAN, COCO, ANCO, COAN). Additionally, no significant differences were found when comparing groups based on their last donor types (ANAN and COAN vs. COCO and ANCO) or when comparing groups that received only one donor type during both FMTs (ANAN vs. COCO). This may be attributed to the lower group sizes after cross-over. Furthermore, FMT2 is one week shorter than FMT1, and given an expected GM establishment period.

A recent meta-analysis of 14 AN-GM studies, including 476 AN patients and 554 controls, revealed that AN patients had significantly lower levels of the genera *Faecalibacterium* and *Roseburia*, and higher levels of *Methanobrevibacter* compared to non-eating disorder state controls [48]. Among our six human donors, we noted differences in the abundance of *Ruminococcaceae*, *Lachnospiraceae* and *Faecalibacterium*, which were higher in donors with AN compared to CO donors. *Lachnospiraceae* has been associated with weight gain following AB treatment and increased blood glucose levels, suggesting a link to energy utilization and rapid weight gain [49–51]. Higher *Lachnospiraceae* abundance at hospital admission has also been indicated as a predictor of shorter inpatient treatment duration in a human study of anorexia

[18]. This study also shows that *Faecalibacterium* abundance is lower in patients with AN at hospital admission, with level increasing significantly with weight gain and AN improvement [18]. This aligns with Fan et al. (2023) reporting lower *Faecalibacterium* abundance in patients with AN compared to non-eating disorder controls. A limitation of our study is the higher abundance of *Faecalibacterium* in the human donors used, which differs from reports in the literature. *Ruminococcaceae* has also been described to be altered in human patients with AN and influenced by weight gain [18].

The humanized mice GM after FMT1 in ExpA and ExpB showed significant differences, notably in the abundance of *Clostridium*, which have been implicated in the regulation of eating behavior and neuropsychiatric symptoms [52]. This could explain the variability observed between ExpA and ExpB.

Moreover, when comparing the GM of AN and CO mice, we found higher levels of *Bilophila* in AN mice of ExpA after FMT1. In a weight-loss surgery study, *Bilophila* has been associated with weight loss and a reduction in body fat [53].

Following FMT2, there were no statistically significant bacterial differences when analyzing combined donor types. However, significant differences between ExpA and ExpB were identified. Considering that some of them, like *Lachnospiraceae*, *Ruminococcaceae* and *Ruminococcus* have been previously shown to be altered, depending on the study, in human patients with AN and influenced by body weight [18, 48, 54], these varying concentrations of bacteria could contribute to the differences between the two experimental runs.

To assess any effect of FMTs on general locomotor activity (GMA) levels, the mice underwent open field (OF) tests. We did observe a statistically significant decrease in AN-mouse activity and frequency of crossing the subdivisions of the OF after FMT1 in ExpB (ten to eleven weeks old). Hata et al. (2019) found no significant differences in GMA [22]. In our study, any behavioral changes due to FMT leading to decreased activity level and energy expenditure might explain the maintained body weight in the AN mice despite their decreased food intake. Continuously measuring GMA in the home cages or combing FMT with AN mouse model of exercise [55] could reveal any accelerated weight loss. After FMT2, there were no differences in general locomotor activity (GMA), or frequency based on FMT donors. However, a significant decrease in GMA and frequency in crossing subdivisions was observed over time, potentially masking subtle GM-induced behavioral changes. The exact cause of these decreases remains unclear, whether it is due to FMT procedures, repeated exposures, antibiotic treatment, or age-related factors. To mitigate repeated exposures, non-interfering systems like those developed by Poffé et al. (2018) could be employed for measuring spontaneous physical activity [56].

In conclusion, our study suggests that gut microbiota transplants from patients with AN may contribute to the pathological characteristics of AN by altering food intake through the gut-brain axis in immune competent AB-treated mice. The decreased food intake after FMT1 and appetite biomarkers can be ameliorated by a second FMT with gut microbiota from a non-eating disorder state donor. These results may ultimately contribute to the development of supportive treatment with FMT in anorexia nervosa.

Table 1 Characteristics of patients with anorexia nervosa and non-eating disorder controls for feces collection

Donor	Age	BMI, kg/m^2
ANX	18	16.90
ANY	21	15.42
ANZ	22	16.89
Mean ± SE	**20.33 ± 1.20**	**16.43 ± 0.49**
COD	25	19.70
COE	30	23.60
COF	27	19.83
Mean ± SE	**27.33 ± 1.45**	**21.04 ± 1.28**

Methods

Subject recruitment and sample collection

We recruited three female patients diagnosed with restricting-type anorexia nervosa (AN), admitted to Mental Health Centre Ballerup, along with three non-eating disorder state female controls (CO) with no history of any eating disorders or digestive diseases such as IBD or IBS (Table 1). None of the donors had used antibiotics three months prior to study participation. The study protocol was approved by the local ethics board (id: H-15012537; addendum 77106) under the PROspective Longitudinal all-comer Eating Disorder study (PROLED) study. Written informed consent was obtained from all participants.

A fresh fecal sample was collected from the three donors with anorexia (ANX, ANY, and ANZ) and from the three control (CO) donors (COD, COE, and COF). Body mass index (BMI) and age of the participants are shown in Table 1. A better age matching would have been optimal. At the time of sample collection, the fresh stools were frozen at − 80 ℃ until further processed for animal experiments.

Animals and housing

48 specific-pathogen free (SPF) female BALB/cJTac mice (Taconic Biosciences, Ejby, Denmark), aged three to four weeks at arrival, were randomly distributed to cages and housed at the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited

barrier protected facility of the Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark. The sample size was founded on a power analysis, which revealed a sample size of ten per donor group with 90% power and 5% significance level based on comparable results from Hata et al. (2019) [22].

The facility maintained a temperature of 22 ℃ (+/- 2 ℃), a humidity of 55% (+/- 10%), 12/12-hour light/dark cycle (lights on from 7 a.m., summertime), and 15–20 air changes per hour. Upon arrival, all mice were randomized blindly, earmarked and then housed four per open cage (Techniplast, 1290D Eurostandard type III, Scanbur A/S, Karlslunde, Denmark), with Tapvei® aspen bedding, nesting material Enviro-dri®, a cardboard Smart Home shelter, a fresh 1 × 1 × 5 cm Tapvei® aspen chewing block, fresh cotton Nestlets, a cardboard Mini Fun Tunnel with changing of bedding, and a clear plastic tunnel (Brogaarden, Lynge, Denmark) with water and feed *ad libitum*. The bedding was changed at the end of each AB treatment for maximum bacterial exposure. The mice received the maintenance diet Altromin 1324 feed (Brogaarden, Lynge, Denmark) consisting of 11% fat, 24% protein, and 65% carbohydrates. According to Federation of European Laboratory Animal Science Associations (FELASA) guidelines [57] routine health monitoring of the mice revealed no listed pathogens.

Animal ethics

This study was conducted according to the Danish Act on Animal Experimentation (BEK nr 2028 af 14/12/2020) and the EU directive "on the protection of animals used for scientific purposes" (2010/63/EU). The Animal Experimentation Inspectorate, Ministry of Food, Fisheries, and Agriculture, Denmark approved this study (license no. 2017-15-0201-01262). The study was planned according to PREPARE guidelines [58], and it will be reported according to ARRIVE guidelines [59, 60].

Body weights

For the entire experimental period, all mice were weighed twice a week. For body weights, AUCs were calculated and evaluated by 2-way ANOVA. When significant we applied Tukey's HSD.

Antibiotic treatment

All mice were treated with Ampicillin (Sigma-Aldrich, St. Louis, MO, USA) in the drinking water (1 g/L) in two bouts of treatment, first for two and later for three weeks, as previously described [26]. The second bout of antibiotics serves as the washout period for the split-group cross over design. The mice received the water with ampicillin *ad libitum* fresh every three days starting on the third day of arrival. The antibiotic and dose previously showed to consistently reduce gut bacterial density and facilitate transfer of donor GM [61, 62].

Fecal matter transplant (FMT)

Before FMT, the fecal samples from patients and controls were thawed on ice and subsequently suspended 1:5 in 50% glycerol. 300 µL of the suspension was divided into 2 mL Biosphere® SafeSeal tubes, one tube per FMT. The samples were stored at -80 ℃ until thawing on ice for administration. FMT was performed once a week on all mice, where 50 µL of suspension was applied at room temperature to the mouth of the mice with a sterile 1 mL disposable syringe.

A "split-group cross-over" study design (FMT1 and FMT2)

There were two consecutive FMT phases (FMT1 and FMT2) in this study performed on the same mice ($n = 48$), with a partial cross-over midway using the 3-week AB treatment as the washout period [63]. All mice were weighed twice a week and food intake per cage was calculated twice a week. We performed this as two identical series (A and B) in parallel staggered one week with 24 different mice in each experiment (ExpA, $n = 24$ and ExpB, $n = 24$). Food Intake was analyzed with one or two-way ANOVA with experimental run (ExpA vs. ExpB) and FMT donor type or combined donor type as factors. For an overview see Table 2.

After two weeks of antibiotic treatment all mice were subjected to FMT1: For five consecutive weeks mice were given a FMT from one of six human donors, from either three AN and three CO. After a second antibiotic treatment (three weeks) the mice were subjected to the second phase. In FMT2, we moved two mice randomly per cage to new cages, effectively doubling our number of cages, with now two mice/cage. During FMT2 mice received either the same human donor as during FMT1 (ANAN or COCO cages) or they received a cross-over donor (COAN or ANCO).

Sampling and behavioral recording

We collected mouse fecal pellets from all mice before and after the first AB treatment and after both FMT1 and FMT2 phases. Figure 11 summarizes all experimental procedures performed in both ExpA and ExpB, data collection and sampling in a timeline. Open field data collection was done before and after FMT1 and again after FMT2. At termination we collected serum.

Open field behavioral test

To assess general locomotor activity level the mice were tested in an open field arena. The mice were habituated to the test room for maximum 30 min between 13.00 and 16.30, then in random order placed individually in the arena (48 × 48 × 28 cm) under low illumination (< 5 lx). A

Table 2 A "split-group cross-over" study design (FMT1 and FMT2). After two weeks of antibiotic treatment cages were randomly distributed to either experiment A (ExpA) or repeat experiment B (ExpB). **ExpA**: the six cages received one of each of the six human donors (three AN or three CO) (four mice/cage, total 24). After five weeks of FMT1, all mice were again subjected to antibiotic treatment (three weeks). At the "split-group cross-over" all cages were subdivided into two new cages (all cages, two mice/cage) either receiving GM from the exact same donor as during FMT1 (ANAN and COCO) or from the cross-over donor (ANCO and COAN) at random. **ExpB**: exactly, repeating ExpA, but a week delayed for practical reasons

	FMT1					FMT2 / Cross-over					
Experiment	AB treatment	Donor Type	FMT	#mice	#cages	AB treatment	Donor Treatment	Comb. Donor	FMT	#cages	#mice
ExpA	2 weeks	AN	5x	12	3	3 weeks	AN	ANAN	4x	3	6
							CO	ANCO	4x	3	6
ExpA	2 weeks	CO	5x	12	3	3 weeks	CO	COCO	4x	3	6
							AN	COAN	4x	3	6
ExpB	2 weeks	AN	5x	12	3	3 weeks	AN	ANAN	4x	3	6
							CO	ANCO	4x	3	6
ExpB	2 weeks	CO	5x	12	3	3 weeks	CO	COCO	4x	3	6
							AN	COAN	4x	3	6

camera placed above the arena recorded the mice activity for ten minutes. The software Ethovision XT version 13 (Noldus Information Technologies, The Netherlands) was used to sub-divide the open field arena virtually into a center zone, eight middle zones, and 16 outer zones as previously described [64] and tracking the animals' movements in the arena. The 25 total zones are merged into three subdivisions, an outer, middle and center subdivision, on which the analyses were done. Variables used for analysis were the total distance moved (cm), mean velocity (m/s) and time and number of entries in the three different subdivisions (frequency) of the arena. ***General locomotor activity and frequency evaluated by paired t-test (2-tailed).*** Before each trial, the arena was disinfected with 70% ethanol.

Appetite-related biomarkers in serum

Serums were diluted 1:2 in metabolic assay working solution (MSD®, Rockville, MD, USA) and GLP-1, glucagon, insulin, leptin, ghrelin, and PYY levels were analyzed using the U-PLEX® Mouse Metabolic Group 1 multiplex immunoassay (MSD®), following the manufacturer's protocol. Serum biomarkers were analyzed with one or two-way ANOVA with experimental run (ExpA vs. ExpB) and FMT donor type or combined donor type as factors.

Gut microbiota analysis

The DNA was extracted from fecal pellets using the Bead-Beat Micro AX Gravity kit (A&A Biotechnology, Gdynia, Poland, Cat. # 106-100-M1), following the manufacturer's protocol. The isolated DNA was stored at -80℃ until DNA library construction.

16S gene amplicon sequencing and data processing

The 16 S rRNA gene amplicon sequencing and data processing were performed as described in Arildsen et al. (2021) (57). Gut microbial composition was examined by sequencing near full-length 16 S rRNA gene amplicon using GridION (Oxford Nanopore Technologies, Oxford, UK) as previously described (59). Overview of primers used to target the hypervariable region (V1-V8) is found in Supplementary Table 2. The data generated using GridION was collected using MinKNOW software v22.10.7 (Oxford Nanopore Technologies, Oxford, UK). The Guppy v6.2.8 basecalling toolkit was used to base call raw FAST5 to FASTQ (Oxford Nanopore Technologies, Oxford, UK). The abundance table was generated from raw FASTQ files using the Long Amplicon Consensus Analysis pipeline (LACA, GitHub: https://github.com/yanhui09/laca). Taxonomy assignment of quality-corrected reads was performed against the SILVA database [65].

The subsequent analysis was conducted using the Qiime2 (v2020.8.0) bioinformatic platform (Bolyen et al., 2019). Alpha diversity and beta diversity were calculated through rarefaction to 3,000 reads per sample. In total, 19 samples collected fewer reads than 3,000 and were thus excluded from the analysis. Permutational multivariate analysis of variance (PERMANOVA) was used to test differences between the three groups based on Bray-Curtis and Jaccard distance matrices. Analysis of Composition of Microbiomes (ANCOM) was used to identify taxonomic groups that are differentially abundant across tested categories.

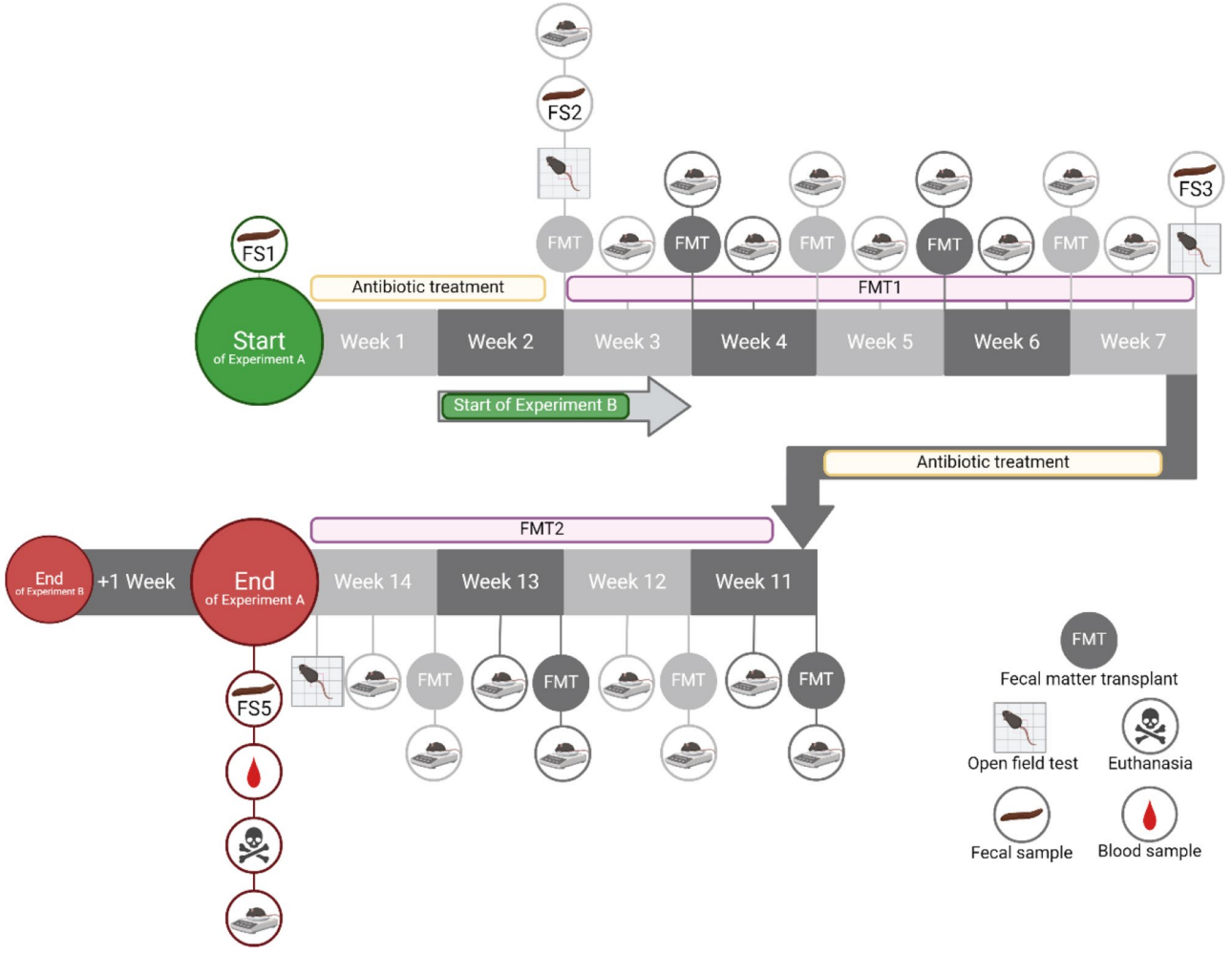

Fig. 11 Sampling and behavioral recording in a timeline. **Start**: FMT1: Before AB treatment we collected fecal samples from all mice (FS1). All mice were subjected to two weeks of AB treatment and FS2 was collected, and the first open field behavior recorded pre-FMT1. After five weeks of FMT1, with either AN- or CO-GM, FS3 was collected and repeated open field data was recorded. After three weeks of AB treatment and split-group cross-over, mice were subjected to FMT2 for four weeks. The final open field data was collected at the end of FMT2. At the **End**: The final FS5 was taken, and mouse blood was collected. The experiment was run as two identical series (A and B) in parallel staggered one week with 24 different mice in each experiment (ExpA, $n = 24$ and ExpB, $n = 24$). *Created with BioRender.com. Publication code: HM25XADTL4*

Supplementary Information

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Supplementary Material 1

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Author contributions
SM wrote and revised the manuscript. THØ conducted the experiments and handled data collection and processing. LK and CMJM performed bioinformatics and data analysis and interpreted the results. LFZ, AKH, DBS, and JMS made intellectual and specialist contributions. KKB conceived and designed the analysis, detailed the protocol, and supervised all phases of the study, including paper writing. All authors contributed to the review and editing of the paper.

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Data availability
All data is published on zenodo: https://zenodo.org/uploads/8410580?token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6ImM1OWEwMzg3LWQ0MGQtNGZhYy05ZDA2LTMwMzk0NmE1NzU0YyIsImRhdGEiOnt9LCJyYW5kb20iOiI3NGE2MWRmMGMzMWM4ZmNjZWIxNjMzYjRkN2Q5NDZkOSJ9.oy1ODNK-toEAT8×58Erl53ZCoRvhjXwaa6LzD0Y3GyWRSGs-Ip6Q5-N47MbIg1-rrQhHaeLDGA3kV3hXDo09Nw.

Declarations

Competing interests

DBS declares that he has collaborated with pharmaceutical industry and received funding from this source as described on https://research.ku.dk/search/result/?pure=en/persons/114880AKH declares that he has collaborated with pharmaceutical industry and received funding from this source, as well as he is the owner of a diabetes related patent as described on https://ivh.ku.dk/english/employees/?pure=en/persons/107126.

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