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ARTICLE

Low-gainer diet-induced obese microbiota transplanted mice exhibit increased fighting

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Abstract

Weight gain variation is a great challenge in diet-induced obesity studies since low-gainer animals are of limited experimental value. The inbred C57BL/6 (B6) mice are frequently used models due to their genetic homogeneity and susceptibility to diet-induced obesity (DIO). The aim of this study is to investigate if the gut microbiota (GM) influences the fraction of low weight gainers in DIO studies. A total of 100 male B6 mice (donor population) were fed a high-fat diet for 14 weeks and divided into the study groups high gainer (HG) and low gainer (LG) based on their weight gain. Subsequently, fecal matter transplantation (FMT) was done on germ-free B6 mice with GM from HG and LG donors (FMT population). LG (13.35 ± 2.5 g) and HG (25.52 ± 2.0 g) animals were identified by the weight gain from week 1 to week 12. Interestingly, the start weight of the LG (20.36 ± 1.4 g) and HG (21.59 ± 0.7 g) groups differed significantly. Transplanting LG or HG fecal matter to germ-free mice resulted in significant differences in weight gain between HG and LG, as well as differences in serum leptin levels and epididymal fat pad weight. A clear LG-specific GM composition could not be distinguished by 16S rRNA gene amplicon sequencing. Surprisingly, significantly more fighting was recorded in LG groups of both donor populations and when transplanted to germ-free mice. The HG and LG phenotypes could be transferred to germ-free mice. The increased fighting in the LG group in both studies suggests not only that the tendency to fight can be transferred by FMT in these mice, but also that fighting should be prevented in DIO studies to minimize the number of LG animals.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Diet-induced obesity studies in mice suffer from variance in weight gain where low weight gainers are of limited value for weight loss intervention studies.

Both human and mouse studies have shown that obesity is affected by the gut microbiota.

WHAT QUESTION DID THIS STUDY ADDRESS?

If gut microbiota variation is contributing to low weight gainers in diet-induced obesity studies in B6 mice.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

The low- and high- gainer phenotypes can be transferred by fecal microbiota transplant; however, a clear microbial signature could not be distinguished in this study. There was significantly more fighting in the low-gainer groups indicating that this behavior should be prevented in diet-induced obesity studies for the benefit of animal welfare as well as study outcome.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Reflecting on how the gut microbiota can affect the study outcomes and animal welfare in preclinical studies may lead to lower variance in weight gain in diet-induced obesity studies. This could limit the number of animals needed for obtaining translatable results.

INTRODUCTION

The inbred C57BL/6 (B6) mouse is commonly used for induction of the diet-induced obesity (DIO) model, as they become obese when fed a high-fat diet (HFD), whereas they maintain normal body weight (BW) when fed a low-fat diet (LFD).[1,2] Furthermore, secondary complications in human obesity, including hyperglycemia, insulin resistance, and atherosclerosis can be mimicked in B6 mice.[3–5] Nevertheless, for an inbred mouse which is highly genetically homogeneous,[6] B6 has a large variation in BW after HFD feeding and up to 20% of the B6 mice fed a HFD will maintain a lower BW, often referred to as low gainers (LG).[7] For pharmaceutical weight loss intervention studies, such LG mice will be excluded, and this is an ethical issue since being fed an HFD is categorized as a mild-to-moderate severity grade according to the EU directive on the protection of animals used for scientific purposes (2010/63/EU). Efforts to identify LG animals before initiation of the HFD feeding have previously been made. Koza et al.[8] studied the variability of BW in B6 mice as a DIO model and found a coherence of being the smallest mouse (age 6 weeks) and becoming an LG after receiving HFD, but no effects on litter size or the BW at weaning. The BW differences between LG and high gainers (HG) were significantly different after the first week on HFD.[8] Zhang et al.[9] investigated the variability of BW in B6 mice and found that initial body composition measured by DEXA scan as well as physical activity was predictive of weight gain during HFD feeding and that these features were influenced by the lactation period in the mice.[9]

Differences in gut microbiota (GM) composition are also known to have a strong impact on weight gain in B6 mice. Germ-free B6 mice are resistant to diet-induced obesity,[10,11] but Ridaura et al.[12] and Turnbaugh et al.[13] found that GM transferred from obese mice or humans into germ-free mice resulted in obesity, even on LFD. Generally, the GM is necessary for host's energy utilization of the diet and indirectly influences the host's regulation of energy expenditure and storage. It ferments indigestible fibers and carbohydrates into short-chain fatty acids.[14] The GM itself is changed by stress in the mice,[15] and therefore, it may be hypothesized that the LG mice are more stressed than the HG mice. The overall purpose of the study was to decrease the number of mice to be induced without effect in future obesity studies. Therefore, we investigated whether GM differs between HG and LG mice and if the LG and HG phenotypes can be transferred to germ-free mice by fecal matter transplantation (FMT). Fighting among the mice, a common stress factor in group-housed males, was recorded during the studies, and it is related to a low weight gain was investigated.

MATERIALS AND METHODS

Ethics

These experiments were conducted in accordance with the Danish Animal Experimentation Act (LBK nr 474 of 15/05/2014), which implements the directive 2010/63/EU on the protection of animals used for scientific purposes. The study was furthermore approved by the

Animal Experimentation Inspectorate, Ministry of Food, Fisheries and Agriculture, Denmark (Donor population study: License No. 2014-15-0201-00412, FMT study: 2017-15-0201-01262), and the health monitoring was performed according to FELASA guidelines.[16]

Donor population

A total of 100 inbred male C57BL/6JCrl mice (4–5 weeks at arrival) completed the 14-week DIO study. The mice were randomly assorted in cages (3 mice per cage) stored in three ventilated NOVOtainers (custom-made by Scanbur, Karlslunde, Denmark). The mice were acclimatized to chow diet (1324 Altromin, Brogaarden, Lynge, Denmark) the first week of arrival, hereafter HFD (D12451, 45% of energy from fat, Research Diets, Brogaarden, Lynge, Denmark) was provided. Diet and water were provided ad libitum.

BW was measured weekly, and fights were registered on cage level simultaneously with the weighing procedure. Fights were defined as situations when two mice fought, or one mouse chased another and wounds were noted. The mice had their cages changed once a week, on the same day they were weighed. If notable aggression was present (wounds), the animals were separated.

Feces were collected in week 0, week 1 (before HFD), week 8, and week 14. After 12 weeks, 36 mice were nominated into three study groups: low gainer (LG, $n = 12$, range: 28.4–32.3 g), mean gainers (MG, $n = 12$, range: 35.9–36.9 g), and high gainer (HG, $n = 12$, range: 41.4–48.2 g) based on the BW in week 12, that is, the mice with the lowest and highest weight were categorized as LG and HG, respectively, whereas mice with a terminal weight closest to the total mean of the mice's BW were denoted MG. The remaining mice were termed in-between-gainers (IBG) and excluded from the study (see Figure S1 for an overview).

Blood samples for acute blood glucose (BG) and long-term blood glucose (HbA1c) measurements were drawn by tail vein puncture before the initiation of HFD for the total population and at study week 14 for the study population. BG was analyzed by the immobilized glucose oxidase method using a BIOSEN 5040S line autoanalyzer (Eppendorf, Germany) following manufacturer's instructions. HbA1c was analyzed on the COBAS 900 autoanalyzer (Roche Diagnostic Systems, Basel, Switzerland). And 2 weeks after study completion the mice were terminated by cervical dislocation, and epididymal adipose tissue (EWAT) was weighed.

FMT study

Fecal pellets from the top 3 HG donors and top 3 LG donors sampled at week 0 and week 14 in the donor population were dissolved in 10 mL PBS by vortexing and mixing using a 2.5 mL syringe. Donor feces suspension was stored at −80°C and thawed immediately before inoculation.

Germ-free adult C57BL/6NTac mice (Taconic, Germantown, NY) were housed in two separate isolators (PFI Systems, Milton Keynes, U.K.) (pressure, 110 Pa; 23°C) in the AAALAC-accredited germ-free facility (Faculty of Health and Medical Sciences, University of Copenhagen) with unlimited access to irradiated Altromin 1324 diet (Brogaarden) and sterile water. After a week of acclimatization, the female mice were inoculated with LG ($n = 5$) and HG donor feces ($n = 6$) (FMT) in separate isolators before mating and 1 week after by oral inoculation using a syringe without a needle to gently drip the inoculum into the mouth. Weaned and inoculated pups (FMT inoculated at week 1 + 3) were used to breed the study population inside the isolators (ISO) and in a conventional (CONV) animal facility. Time mating was set up in the isolators and in the conventional animal facility and pups for the study were born within the same week. ISO pups were FMT inoculated at week 1 and week 3, while CONV pups were not.

ISO pups were transferred to the CONV facility and the male and female pups were separated, earmarked, and housed with littermates ($n = 2$–4) in open-lid cages and fed ad libitum on chow diet (1314 Altromin, Brogaarden). The animals were mainly handled using a mouse handling tunnel (Datesan, Stockport, UK) to minimize anxiety[17] and their cages were enriched with a mini fun tunnel, aspen bedding, aspen chew block, nesting material, and a cardboard shelter (Brogaarden, Lynge, Denmark).

The FMT study population ($N = 60$, $n = 31$ HG, $n = 29$ LG, $n = 34$ males, $n = 26$ females, $n = 30$ ISO, $n = 30$ CONV) were fed HFD (Research Diets D12492 60% energy from fat, Brogaarden, Karlslunde, Denmark) from 6 weeks of age for 13 weeks. In week 12 study, glycosylated hemoglobin (HbA1c) was measured using tail vein puncture on a DCA Vantage Analyzer (Siemens Healthcare Diagnostics, Cat# 6651932), and an oral glucose tolerance test (OGTT) was performed after 6 h fast. Animals were orally dosed with 0.15 mL glucosemonohydrate 500 mg/mL (SAD, 823122, Sygehus Apotekerne Danmark, Denmark) and BG was measured by tail vein puncture using a Freestyle Mini Glucometer (Hermedico, Copenhagen, Denmark) at times 0, 15, 30, 45, 60, 90 and 120 min. Fights were recorded by examining wounds on the animals and fights when handling the animals. Mice were separated into new cages if they were engaged in extensive fighting ($n = 7$ mice, all males from the LG and ISO group).

The study was terminated in study week 13 where the animals were anesthetized using a Hypnorm (BN: P736/005, VetaPharma Ltd, Leeds, UK)/Midazolam (BN: 3530418, Braun, Melsungen, Germany) mixture in sterile water in the ratio of 1:1. Blood was sampled retro-orbitally

and animals were euthanized by cervical dislocation whereafter ileum tissue was sampled and the gonadal fat pad and the liver were weighed. Serum leptin and insulin were measured using the Mouse Metabolic Kit (K15124C-1, Meso Scale Discovery, Rockville, Maryland, USA). Ileum Cytokines were measured using the Proinflammatory Panel 1 (mouse) kit V-PLEX (K15048D-2, Meso Scale Discovery, Maryland, USA) following manufacturer's instructions. The design of the study was summarized in Figure 1.

Fecal DNA extraction and 16S rRNA gene amplicon sequencing

DNA extraction was conducted with Bead-beat Micro AX Gravity mod 1 (106-100-M1, A&A Biotechnology, Gdansk, PL) according to the user manual. The bead beating step was performed using Fast Prep® -24 (MPBiomedicals, Strasbourg, France) following manufacturer's instructions. The V3 region (~190 bp) of the 16S rRNA gene was amplified using primers NXt_338_F: (5′-TCGTCGGCAG CGTCAGATGT GTATAAGAGA CAGACWCCTA CGGGWGGCAGCAG-3′) and NXt_518_R: (5′-GTCTC GTGGG CTCGGAGATG TGTATAAGAG ACAGATTACC GCGGCTGCTGG-3′) compatible with Nextera Index Kit (Illumina, CA, USA). The amplicon library followed a two-step PCR strategy as previously described and was sequenced using NextSeq 500/550 Mid Output Kit v2.5 (300 Cycles, Illumina, CA, USA).[18]

16S rRNA gene amplicon sequencing data analysis

The USEARCH pipeline was used to generate the feature table from fastq data as previously described.[18] Briefly, the raw fastq files containing pair-ended reads with corresponding quality scores were merged and trimmed, eliminating chimeric reads, and zero radius Operational Taxonomic Units (zOTU) were constructed. Taxonomy assignment was conducted against Greengenes database (13.8). The generated feature table was further analyzed in R (version 4.0.5) using the PhyloSeq package (version 1.34.0) for data transformation, low count zOTU filtering, and calculating alpha-diversity measures (rarefied zOTU counts) and beta-diversity measures (CSS transformed zOTU counts).[19] Permutational multivariate analysis of variance (PERMANOVA) from the Adonis2 function

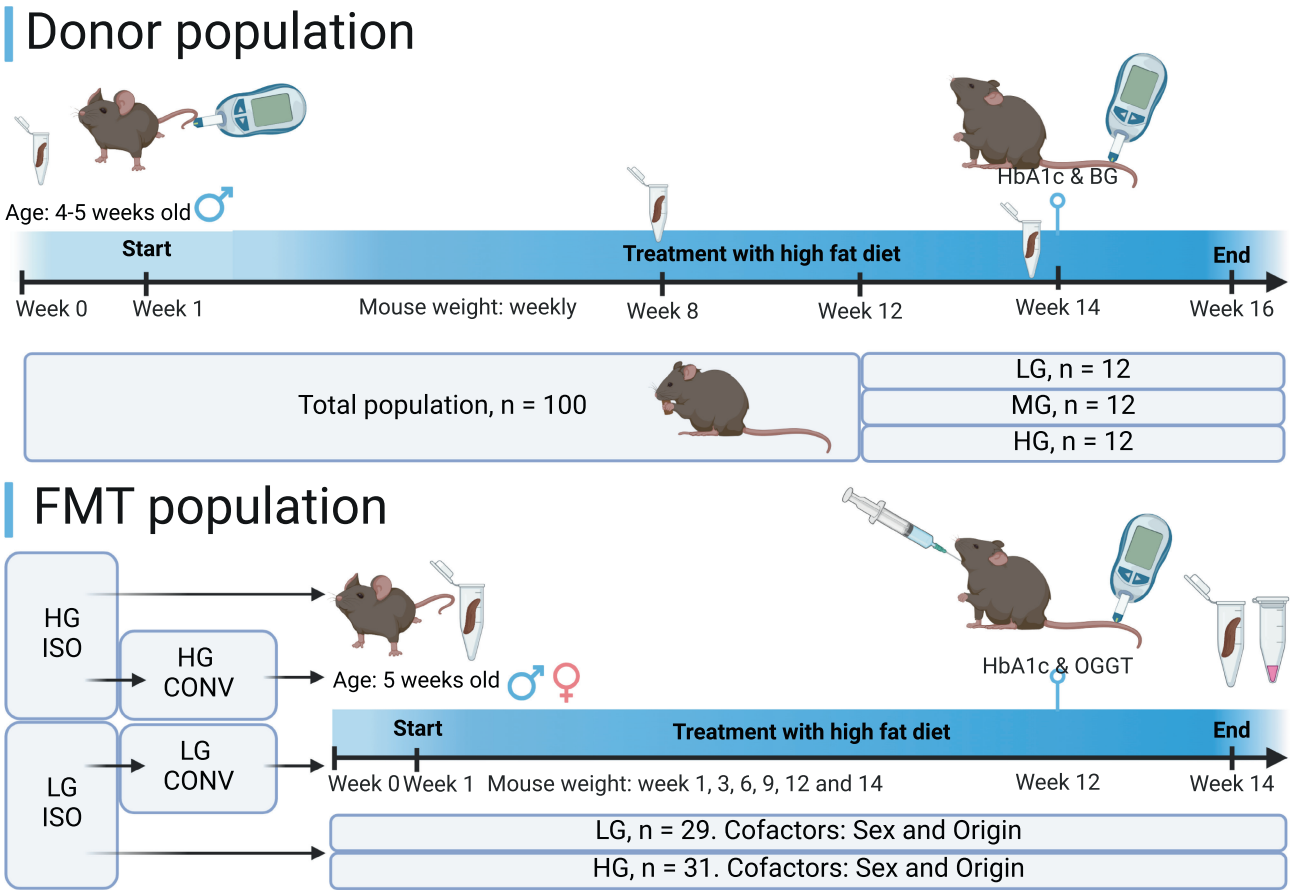

FIGURE 1 Overview of the study design and timeline of Donor population and FMT studies.

in Vegan package (version 2.5-7) was used to evaluate group differences based on weighted and unweighted UniFrac distance metrics.[20] Differential abundance was analyzed using DESeq2 (version 1.30.1)[21] Ampvis2 (version 2.7.13) was used to visualize differentially abundant taxonomic features identified by DESeq2.[22]

Statistics on in vivo data

All in vivo data were analyzed in R (R version 4.0.5). For the donor population, a one-way ANOVA with Tukey's post hoc test was applied, if data were following a normal distribution, and a Kruskall–Wallis with Dunns post-test if they did not. In the FMT study, a Student's *t*-test was applied when analyzing two groups, while a linear model ($y \sim \text{Group(HG/LG)} + \text{Origin(ISO/CONV)} + \text{Sex(M/F)}$) was applied due to unbalanced group sizes and multiple cofactors in the setup. Data with non-normally distributed residuals were log2 transformed before analysis. Contingency tables of fighting versus non-fighting were analyzed using the Fisher's exact test and adjusted for multiple testing by the Holm correction for multiple testing when applied to the three groups of the donor population. Student's *t*-test was used for weight gain comparisons between two groups and between fighters and non-fighters.

RESULTS

High-, middle-, and low-gainer mice could be identified after high-fat feeding

Following 12 weeks on HFD, 36 of the 100 animals in the total donor population were allocated to the LG, MG, and HG study populations (each $n = 12$) based on BW. As expected, the donor population showed a normally distributed weight gain (Figure 2a) and there were significant differences in weight gain in the selected study population of the donor population due to the inclusion criteria: LG (13.35 ± 2.5 g) and HG group (25.52 ± 2.0 g, $p < 0.001$), but also between the LG and MG (19.40 ± 1.9 g) ($p < 0.001$) and MG and HG ($p < 0.001$) (Figure 2b). The initial BW of the mice was different between the LG (18.85 ± 1.2 g) and the HG (20.3 ± 0.8 g) groups ($p < 0.05$), but not between the MG (19.6 ± 1.0 g) (Figure 2c). However, the initial BW did not correlate significantly to the total weight gain (Figures S2 and S3). The weight of the EWAT was significantly lower in the LG (1328 ± 462 mg) animals compared with both the MG (2323 ± 350 mg, $p < 0.001$) and HG (2455 ± 428 mg, $p < 0.001$) groups (Figure 2d). In addition, acute BG measured in the study population was higher in the HG (10.67 ± 1.10 mmol/L) compared with the LG (8.74 ± 0.86 mmol/L, $p < 0.001$) and to the MG animals (9.48 ± 0.83 mmol/L, $p < 0.05$) (Figure 2e).

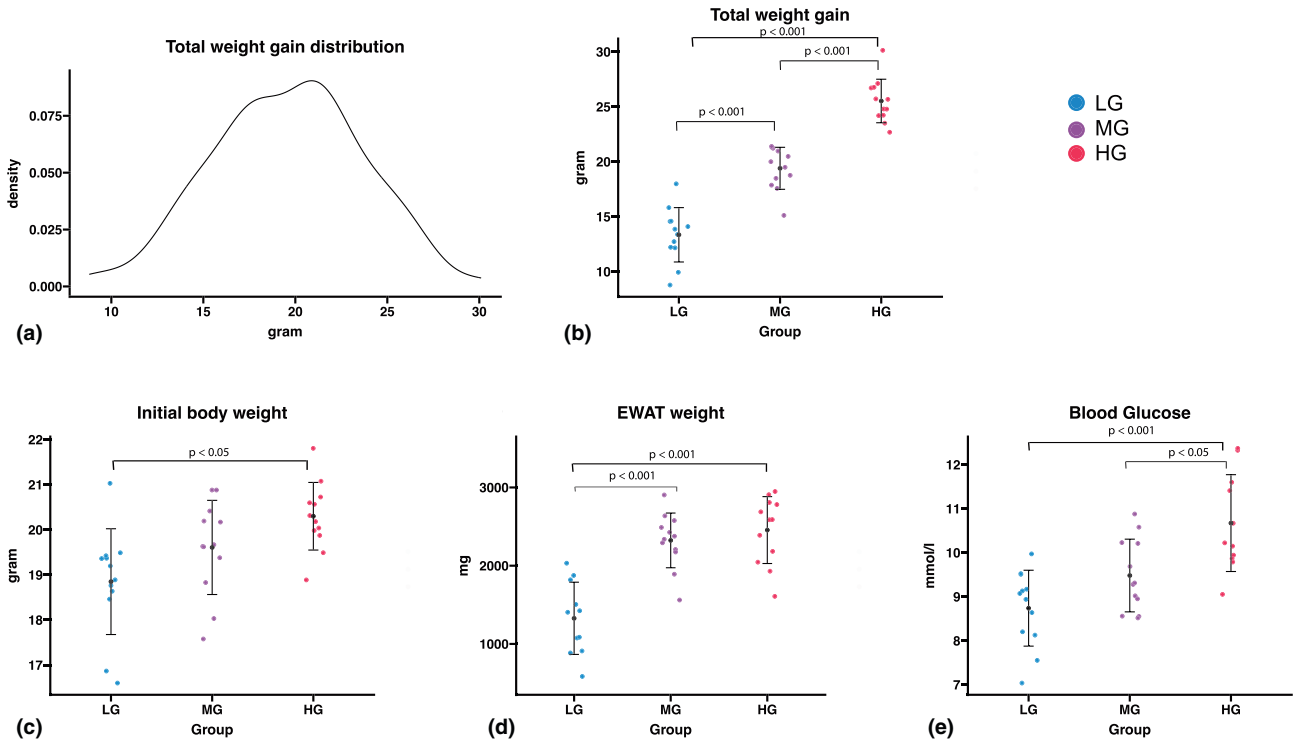

FIGURE 2 The male donor population. (a) Weight gain distribution in the total donor population $n = 100$. (b) Total weight gain in the selected donor population study groups: High gainer (HG, $n = 12$), middle gainer (MG, $n = 12$) and low gainer (LG, $n = 12$). (c) Initial BW upon arrival from a breeder in the three study groups. (d) Weight of epididymal adipose tissue (EWAT) in the three study groups at study termination (16 weeks HFD). (e) Blood glucose measured at study week 14 in the three study groups.

The high- and low-gainer phenotype was transferable with gut microbiota to germ-free mice

To establish if the LG and HG phenotypes were transferable by the GM. The top three donors from the LG and the HG groups of the donor population were selected, pooled, and transferred to germ-free animals by FMT. The total FMT population showed a normal distribution with a lower mean due to the inclusion of female mice in this study (Figure 3a). The weight gain distribution for males however shows a comparable mean to the donor population (Figure S4). After 13 weeks of HFD, the total weight gain throughout the study of the FMT population inoculated with HG (16.6 ± 6 g) microbiota was significantly 2.2 g higher than the LG (14.4 ± 5 g) when adjusted for sex and origin (ISO vs. CONV, $p < 0.05$) (Figure 3b). The start weight of the FMT population correlated significantly with their total weight gain, but there were no significant differences between the start weight of the animals in the HG and LG groups (Figures S5 and S6). The weight gain was mainly driven by the males (18.9 ± 3.6 g) that gained 7.9 g more than the females (11.01 ± 4.1 g) ($p < 0.001$) (Figure 3b). The that EWAT weight analyzed for male mice was higher in the HG (1039 ± 174 mg) animals than in the LG (882 ± 262 mg) animals ($p < 0.05$) (Figure 3c). The serum leptin analyzed for males was higher in HG (49,753 ± 14,328 pg/mL) animals compared with the LG (36,377 ± 27,017 pg/mL) animals ($p < 0.05$) (Figure 3d). Of the cytokines measured in the ileum, IFNg was the only one significantly higher in the HG group (0.086 ± 0.066 pg/mL) compared with the LG group (0.053 ± 0.024 pg/mL) (Figure 3e). To detect hyperglycemia, HbA1c, OGTT, fasting BG, and insulin were also measured in this population but no significant differences between LG and HG animals were found (data not shown).

Only microbiota transplanted mice exhibited gut microbiota differences distinguishable by 16S rRNA gene amplicon sequencing

Fecal DNA from weeks 0, 1, 8, and 12 of the study from the 36 selected animals in the donor population was purified and subjected to 16S rRNA gene amplicon sequencing to evaluate GM compositional alterations. Figures S7 and S8 show that a distinct GM signature for the LG and HG

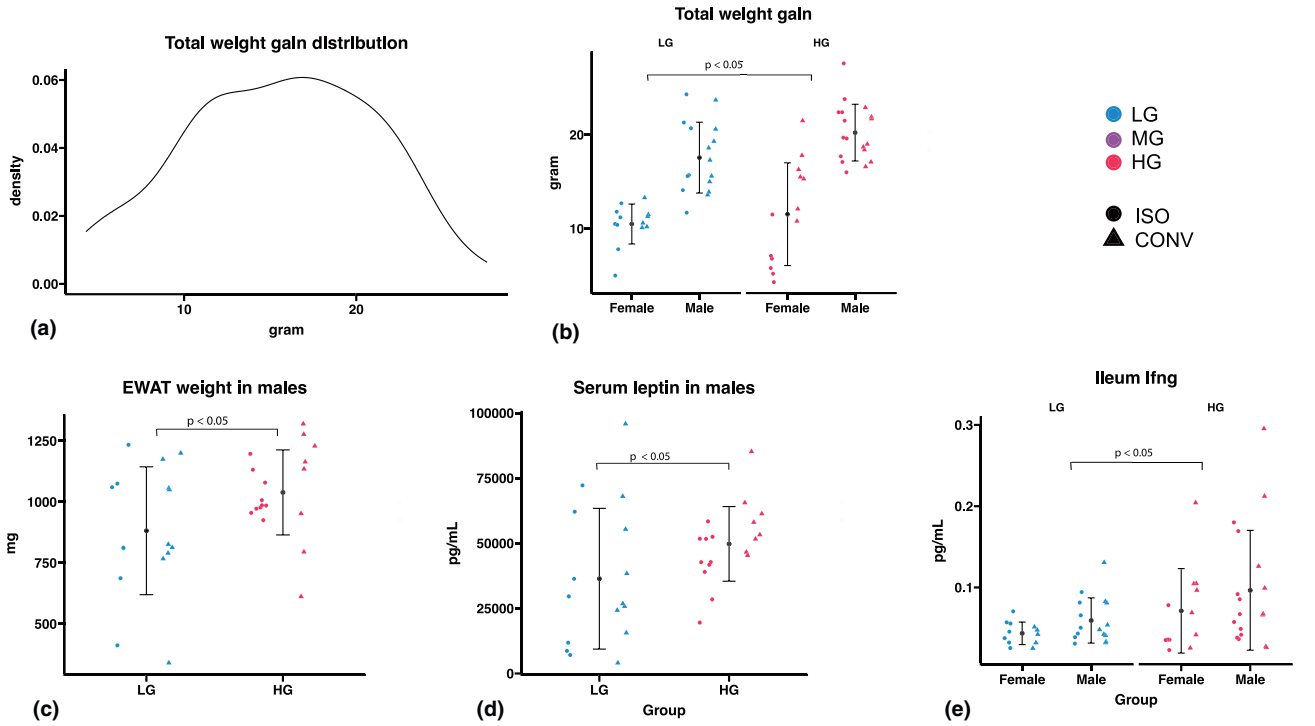

FIGURE 3 The FMT population ($N = 60$) with the groups receiving GM from HG donors ($n = 31$) and LG donors ($n = 29$). Cofactors include sex ($n = 34$ males, $n = 26$ females) and Origin (born in isolators or in conventional housing unit ($n = 30$ ISO and $n = 30$ CONV). (a) Weight gain distribution in the FMT population $n = 60$. (b) Total mouse weight gain after 13 weeks of HFD. (c) Weight of epididymal adipose tissue (EWAT) in the males at study termination. (d) Serum leptin levels in the males at study termination. (e) Ileum IFNG at study termination.

animals could not be observed, and that time is the main driver of microbial changes.

Fecal DNA from weeks 1 and 13 of the FMT study was also purified and subjected to 16S rRNA gene amplicon sequencing. At the beginning of the study (week 1) the LG and HG animals differed significantly in their GM composition (PERMANOVA on weighted and unweighted UniFrac distance matrices) (Figure 4a,b). Differential abundance analysis (Figure 4c) show that this difference is mainly driven by different Lacnospiracea genera, including *Blautia* spp., which are more abundant in the LG group, and *Adlercreutzia* spp. and *Prevotella* spp., which have a higher abundance in the HG group. At the end of the study, the difference between the LG and the HG groups is reduced as seen in the beta-diversity plots (Figure 4b and Figure S9) and only a few low abundant

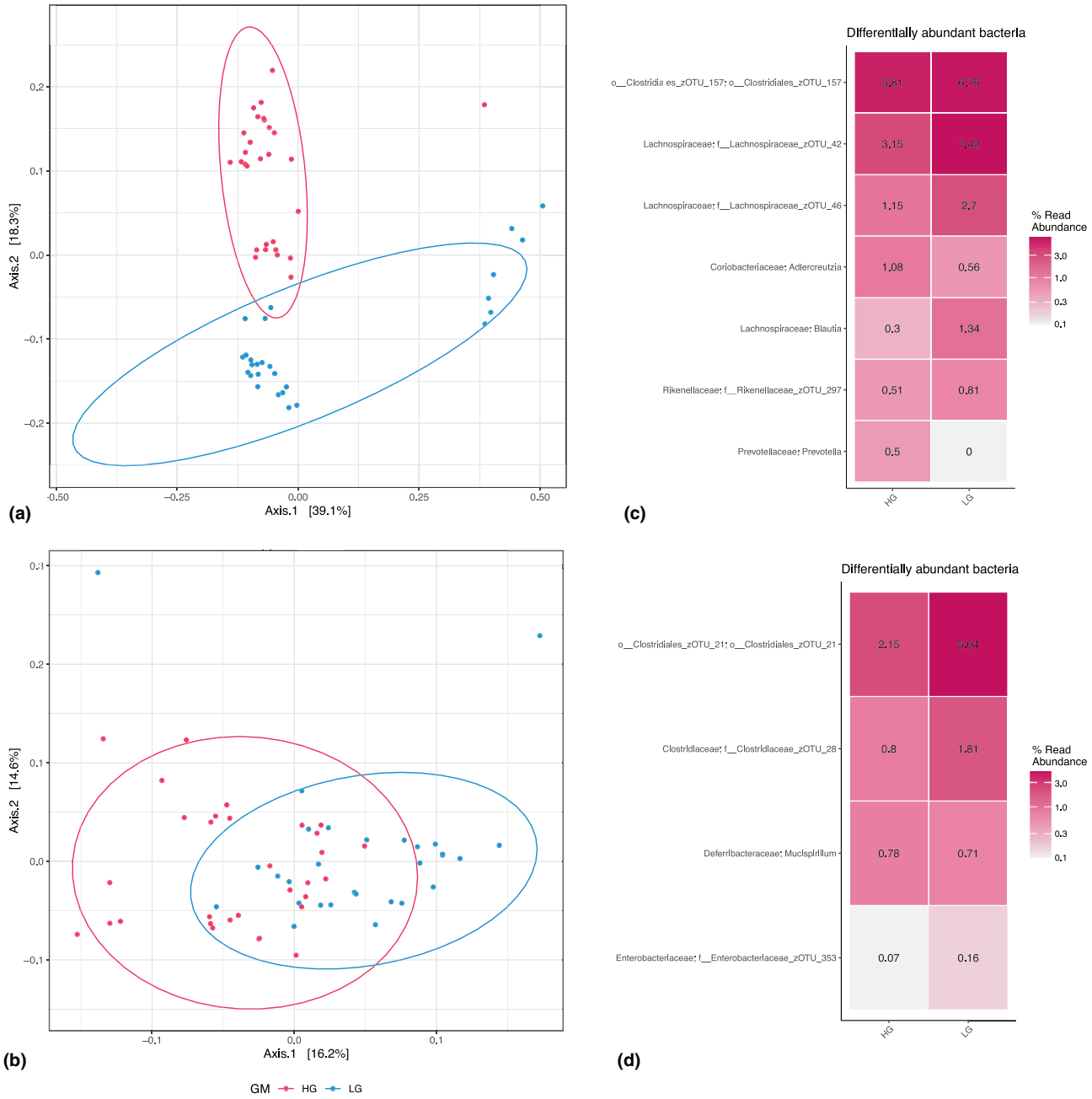

FIGURE 4 Gut microbiota analysis in the FMT study, consisting of both males and females. (a) Beta-diversity PCoA plot based on the unweighted UniFrac distance matrix presenting groups LG and HG at the beginning of the study before HFD (week 1). (b) Beta-diversity PCoA plot based on the unweighted UniFrac distance matrix presenting groups LG and HG at the end of the study after 13 weeks of HFD. (c) Heatmap presenting significantly differentially abundant bacteria between HG and LG at study start before HFD. (d) Heatmap presenting significantly differentially abundant bacteria between HG and LG at the following 13 weeks of HFD.

bacteria are driving the difference where Clostridiales are even more different between the groups at study end that at study start (Figure 4d). Notably, no significant differences in alpha-diversity indices (Observed species, Shannon, and phylogenetic diversity) between HG and LG were observed before or after HFD (Figure S10).

LG groups recorded significantly more fighting events

In both the donor population and the FMT study, fighting among the animals were detected, with no fights observed in the HG groups. The distribution of fights (YES, NO) in the donor population is given in Figure 5a. The significant differences in the weight gain were observed when animals were grouped into fighters (YES, 15.8 g) or non-fighters (NO, 21.25 g, $p < 0.01$) (Figure 5b). In the FMT study, no fights were reported in the HG group and no significant difference in the total weight gain was found (Figure 5c,d).

DISCUSSION

As expected, the total donor population could be divided into LG, MG, and HG animals that had significant differences in weight gain even though the total donor population was subjected to identical treatment and the B6 is an inbred mouse line. This variation in weight gain is in line with other studies on B6 weight development during high-fat diet feeding.[2,7–9,16,23] The HG and LG phenotypes were transferable to germ-free mice through FMT and to the best of our knowledge, this is the first study that aims

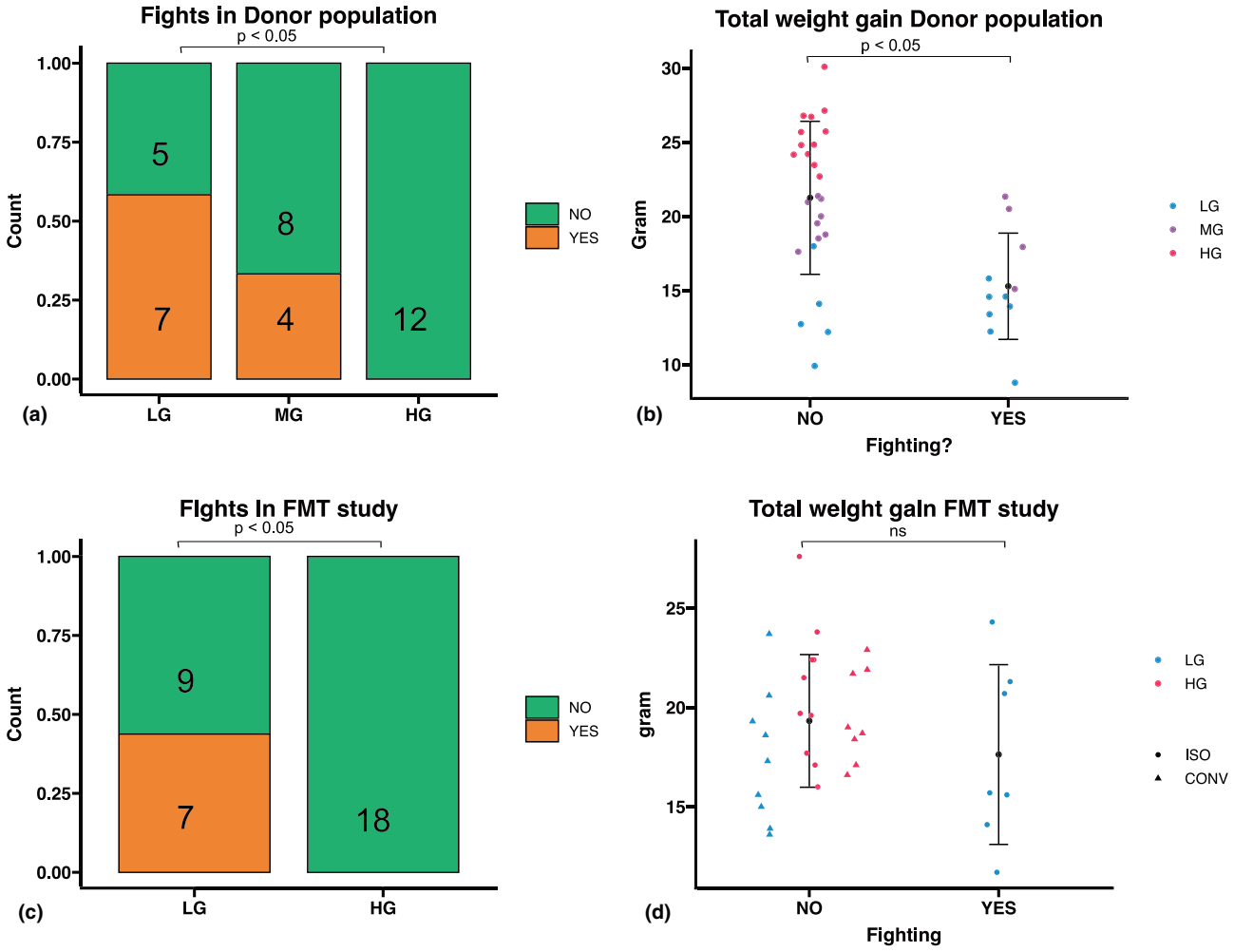

FIGURE 5 Fighting recorded in the donor population and the FMT study and its effect on weight gain. (a) Frequency of fighting (YES or NO) in the three study groups in the donor population ($n = 12$). Counts from the contingency table are reported on the bars. (b) Total weight gain of the animals in selected study groups in the donor population when divided into fighters (YES, $n = 11$) and non-fighters (NO, $n = 25$). (c) Frequency of fighting in males from the FMT study. Counts from the contingency table is reported on the bars. (d) Total weight gain of the male FMT animals when divided into fighters (YES, $n = 7$) and non-fighters (NO, $n = 27$).

to utilize GM characterization to discover LG animals during HFD studies to dismiss the LG animals before weight loss intervention to decrease variation and promote the use of fewer animals. There was no significant correlation between the start weight of the mice and weight gain in the total donor population or in the selected 36 mice (Figures S2 and S3). However, the initial bodyweight was significantly different between the selected LG and HG group (Figure 2c). In the FMT study, there was an overall significant correlation between the weight of the animals before high-fat feeding and the weight gain at study termination, but no significant difference between the HG and LG groups (Figures S5 and S6). This indicates that LG animals to some extent can be identified by a low BW before an HFD study as previously shown.[9]

There were no significant differences in the common measures of glucose metabolism (fasting BG, HbA1c, OGTT) between HG and LG animals in either study as expected, as the wild-type B6 mouse does not develop type-2 diabetes[24]; however, acute BG was measured in the donor study and the MG and HG groups had higher BG levels compared with the LG (Figure 2e). In both donor and FMT studies, the EWAT weight was higher in the HG animals (Figures 2d and 3d) which is also reflected by the serum leptin levels measured in the FMT study (Figure 3d) supporting increased adiposity in the HG group. Cytokines measured in the ileum of the FMT study showed an upregulation of IFNG in the high gainer group in both males and females. IFN-gamma upregulation is also seen in human obesity.[25]

Albeit the clear differences in weight gain and other adiposity measures, there were no significant differences in the microbiota composition of the selected study groups in the donor study population measured by 16S rRNA gene amplicon sequencing. However, the HG-LG phenotype was transferable to the germ-free mice in the FMT study, and there was a significant separation of the groups according to the beta-diversity (Figure 4a,b), indicating that the bacterial communities of the HG and LG groups were distinct. Differential abundance analysis before high-fat diet feeding (Figure 4c) revealed differences between zOTUs belonging to the members of the *Lachnospiracae* family that were downregulated in the HG mice. *Lachnospiracae* abundance is also lower in human studies of obesity.[26,27] *Lachnospiracae Blautia* was among the lower abundant *Lachnospiracae* in the FMT HG group and a member of the *Blautia* genus has previously been shown to ameliorate obesity and type 2 diabetes.[28] Of the lower abundant bacteria, *Prevotalla* was significantly higher abundant in the HG mice and a zOTU belonging to the *Rikenellaceae* family was less abundant in the HG mice, a pattern also seen in a human study.[29] zOTU belonging to the order *Clostridiales* was less abundant both before and after the HF-diet feeding (Figure 4c,d) and *Clostridiales* is also decreased in overweight humans.[30] In general, the differentially abundant bacteria after HF-diet feeding was of very low abundance and probably not of high biological impact in the gut (Figure 4d). Diet is one of the key contributors to GM alterations. Some unknown interactions between the host and GM could be hidden under the same feeding background, especially when a phenotypic co-variate is indirectly linked to GM and HF-diet feeding affects the GM to an extent where it is difficult to see differences between groups both fed HF-diet.[31]

An interesting observation was that in both donor and FMT studies, there were increased incidences of fighting between the males in the LG groups. Group housing is the common practice in mouse studies, although mice, in spite of being social animals in nature, do not share territories with males.[32] A study on group-housed vs. single-housed B6 mice showed that single-housing decreases variance slightly, but the single-housed animals have lower BW when fed a chow diet.[33] However, it has been reported that B6 males show a very docile behavior toward each other, compared with other strains when fed a standard chow diet.[34] In a study of single and group-housed males on HFD, the single-housed males gained more BW after weeks on HFD.[35] HFD feeding itself is a risk factor for increased aggression in mice, so single housing may be the best option in DIO studies.[36] The aggression is mainly an issue when using male mice; however, as also shown in the FMT study where females were included, male mice have a much higher weight gain on the HFD. Female mice are to a large extent protected against DIO and associated comorbidities.[37–39] B6 mice exposed to a fighting male induced minor changes in microbial abundances in the colon, mainly visible by altered beta-diversity and a decline in *Lactobacillius* spp. abundance.[40] When the donor and the FMT population were divided into fighters or non-fighers there was no significant difference in the GM and the fact that we were not able to see a clear difference in the GM in the donor population and nevertheless transferred the LG phenotype to the germ-free mice in the FMT study suggests that it may be a difference not driven by specific prokaryotes. To follow-up on these results it could be relevant to investigate the microbial metabolites as this might differ in LG and HG animals, or the differences in actual encoded functional genes could be assessed by shotgun sequencing. The differences in phenotype may also be driven by the virome (bacteriophages and eukaryotic viruses), in the mice which is not observable by 16S rRNA gene amplicon sequencing.[41,42] We have previously shown that bacteriophages can modulate the obese phenotype in B6 mice.[43]

While this study provides valuable insights into the variability in weight gain among C57BL/6 mice and its potential association with GM, it is essential to acknowledge certain limitations. Firstly, the use of a single inbred strain may limit the applicability of the findings to other mouse strains with different genetic backgrounds. The C57BL/6 mouse was chosen as a model in this study as it is widely used in DIO studies and the results obtained in this study is relevant for anyone performing diet- induced obesity studies in C57BL/6 mice.[1–5] This study does not provide additional information on the translation of data obtained in rodents to humans; however, it does add information on the path to decrease the high variation seen in DIO using C57BL/6 mice.[7] Additionally, the absence of a detailed investigation into the functional aspects of the gut microbiota (GM) and its metabolites leaves open the possibility of undiscovered factors influencing the observed phenotypic differences. The increased fighting in the FMT LG group may be a coincidence, but could also be a transferred component of the GM from the donor LG mice that was not discovered by the methods utilized in this study.

Despite these limitations, the B6 mouse remains a relevant model for weight loss studies due to their fast weight gain when fed an HF diet and their highly translational response to the increasingly popular GLP-1 analogs for weight loss.[6,44]

In summary, this study shows that there is no clear difference in the GM of HG and LG animals in DIO studies that can be used to identify potential LG animals; however, as the phenotype could be transferred to germ-free mice a GM component responsible for the HG and LG phenotypes cannot be ruled out. A more notifiable object to modify in a DIO study is to prevent the fighting of the mice, as there was significantly more fighting in the LG groups of both studies. Also as there were differences in the initial BW of LG and HG animals, the smallest mice could be abolished from DIO studies to lower weight gain variance.

AUTHOR CONTRIBUTIONS

CMJM, LK, and AKH wrote the manuscript. AKH, LK, DSN, and MK designed the research. CMJM, TMSH, MK-D, LFZ, AA, and YL performed the research. CMJM, LK, LFZ, and YH analyzed the data. DSN, LHH, and LK contributed analytical tools.

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CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

The raw sequencing data is available via Short Read Archive (https://www.ncbi.nlm.nih.gov/sra) under BioProject ID: PRJNA1023381.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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