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BRIEF REPORT

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The probiotic *L. casei* LC-XCAL™ improves metabolic health in a diet-induced obesity mouse model without altering the microbiome

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ABSTRACT

Chronic low-grade inflammation associated with obesity may be a target for improvement of metabolic health. Some exopolysaccharide (EPS)-producing bacteria have been shown to have anti-inflammatory effects in gastrointestinal inflammatory conditions. However, evidence for the role of EPS-producing probiotics in the management of obesity and associated conditions is scarce and the role of the microbiota is unclear. In this study, two probiotic candidates were screened for their effects on metabolic health using the diet-induced obesity (DIO) mouse model.

Mice fed a high-fat diet supplemented with the anti-inflammatory, EPS-producing strain *L. casei* **LC-XCAL**™ showed significantly reduced hepatic triglycerides, hepatic total cholesterol, and fat pad weight compared to those fed a high-fat diet alone, likely as a result of reduced energy absorption from food. 16-S rRNA amplicon analysis of the fecal microbiota of these mice indicated that the altered metabolic phenotype as a result of the *L. casei* LC-XCAL strain administration was not associated with an overall change in the composition or inferred functional capacity of the fecal microbiota despite some abundance changes in individual taxa and functions.

These findings provide evidence that specific microbial strategies can improve metabolic health independent of the microbiome and reinforce the importance of carefully selecting the most appropriate strain for specific indications by thorough screening programmes.

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Lactobacillus casei; fecal microbiota; metabolic health; obesity; probiotic; exopolysaccharide; anti-inflammatory

Introduction

Obesity is a multifactorial disorder which is the result of a long-term imbalance between energy intake and expenditure and is influenced by genetic and environmental factors. Since the observation that germ-free mice were leaner than their conventionally raised counterparts[1] the likely role of the gut microbiota in the development of obesity is becoming better understood.[2–9] The contribution of the gut microbiota to obesity is multifactorial and involves processes such as enhanced energy harvest and fat storage, [10,11] altered metabolic pathways[12] and bacterial translocation leading to chronic low-grade inflammation.[2,13] The manipulation of the gut microbiota by probiotics is a potential therapeutic approach to help ameliorate obesity and associated metabolic disorders.[14,15] *Lactobacillus* and *Bifidobacterium* strains are commonly used as probiotics and a number of studies have described the beneficial effects of some strains on the characteristics of the metabolic syndrome and obesity. However, in the majority of cases, the mechanisms involved are still unclear.

Some bacterial exopolysaccharides (EPS) have been shown to have anti-inflammatory effects in gastrointestinal inflammatory conditions.[16,17] Obesity is characterized *inter alia* by a chronic low-grade inflammation[18] and this is one characteristic of the condition that may be targeted by administration of probiotics. Here, we describe an anti-inflammatory profile for *Lactobacillus casei* **LC-XCAL**™, a copious EPS-producer, in a peripheral blood monocyte cytokine (PBMC) induction assay, similar to the anti-inflammatory prototype strain *Bifidobacterium longum* NCIMB 41003; which has been demonstrated to mediate such effects in the human gastrointestinal tract as well as extra-intestinally.[19–21] We also investigate

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the impact of LC-XCAL on weight gain and metabolic health in a diet-induced obesity (DIO) mouse model.

Results

PBMC cytokine induction

L. casei LC-XCAL and *B. longum* NCIMB 41003, both prolific EPS-producers, induced broadly similar anti-inflammatory immune profiles in a PBMC cytokine induction assay, although *L. casei* LC-XCAL induced significantly less IL-10 production at lower concentrations (50:1 p = .042, 25:1 p = .014) (Figure 1). *L. plantarum* AH0315, a low-EPS producing strain, significantly increased TNF-α induction (p = 3.46e-06) and induced significantly less IL-10 production (p = 5.92e-05) compared to *B. longum* NCIMB 41003.

Estimation of energy and fat excretion

A slight increase of fecal GCV was observed in *L. casei* LC-XCAL-fed mice compared to those on the high-fat control diet at all three timepoints tested (Figure 2) despite no significant change in food intake (Figure 2). Extrapolation of these results over the length of the trial, combined with measurements of fecal fat content, suggest that *L. casei* LC-XCAL supplementation is increasing the amount of energy excreted and/or reducing lipid absorption in the gut (Figure 2).

Diet-induced obesity alters metabolic phenotype of the host and overall composition, but not function, of the fecal microbiota

Feeding of a high-fat diet for 16 weeks resulted in a significant increase in body weight (Figure S1) and fat mass (Figure 3) in C57BL/6 J mice compared to low-fat diet controls. These increases were accompanied by significant increases in the weights of subcutaneous fat, brown adipose tissue, epididymal fat, retroperitoneal fat, mesenteric fat, and liver. No significant difference was found in skeletal muscle, spleen, cecum, brain, intestine weights, or lean mass between the two control groups. The HFD group exhibited elevated hepatic TC, hepatic TG, random blood glucose levels, terminal blood glucose and plasma TC, TG, HDL-c, LDL-c, and VLDL-c levels compared to the LFD group. Permutation multivariate analysis of variance (PERMANOVA) revealed that overall murine metabolic phenotype was significantly altered by diet (p = .001)

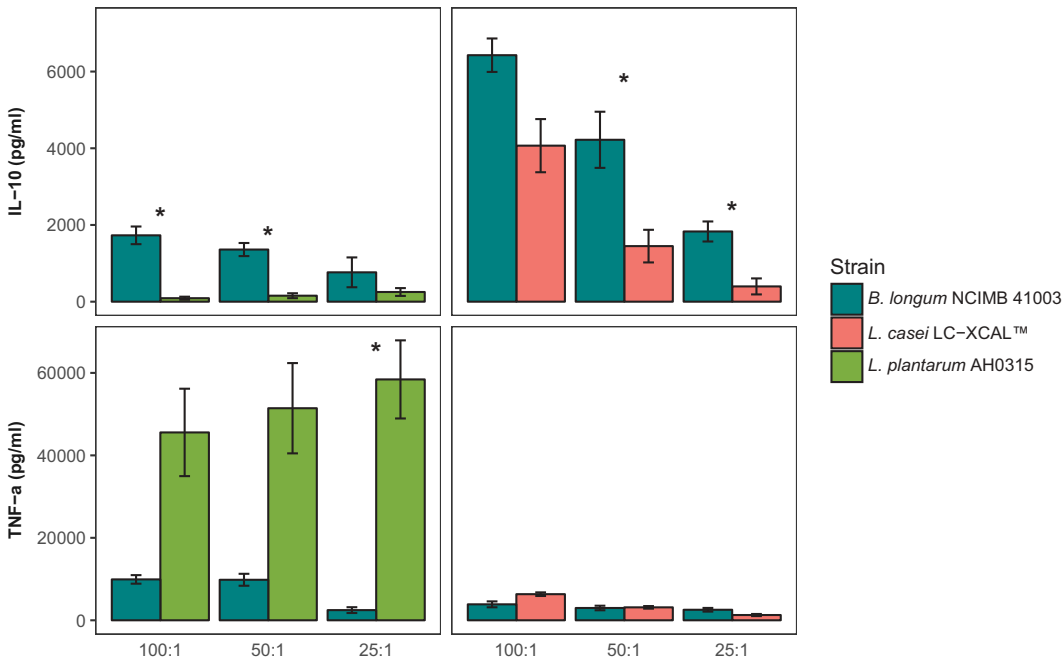

Figure 1. PBMC anti-inflammatory profiles of strains used in this study. IL-10 and TNF-α induction in the PBMC cytokine induction assay following 48 h stimulation with *B. longum* NCIMB 41003, *L. plantarum* AH0315 and *L. casei* LC-XCAL (n = 3). Significant differences between strains are denoted by (*).

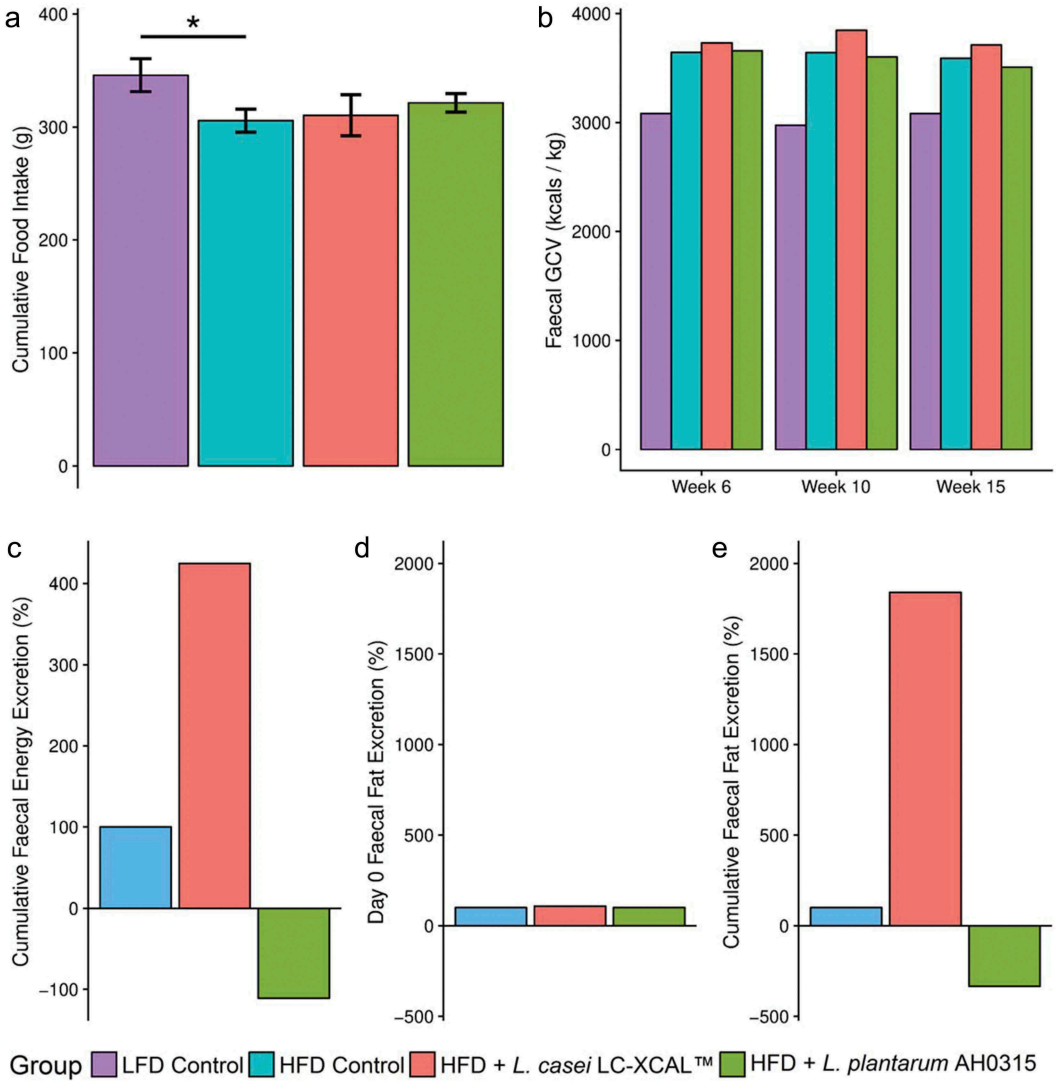

Figure 2. Effect of diet and probiotic supplementation on food intake and energy and fat excretion. Effect of *L. casei* LC-XCAL and *L. plantarum* AH0315 on (A) cumulative food intake over the duration of the DIO mouse trial, (B) fecal gross calorific value (GCV), (C) estimation of % cumulative energy excretion over the duration of the DIO mouse trial, (D) day 0 fecal fat excretion before commencement of probiotic supplementation, and (E) estimation of % cumulative fecal fat excretion over the duration of the DIO mouse trial. A) LFD v HFD were statistically compared by unpaired t-test; probiotic groups were compared relative to HFD by one-way ANOVA followed by Tukey's multiple comparison test; *p < .05; **p < .01; ***p < .001. (B-E) No statistical analysis was performed for this data as they are based on a single value per group.

The alpha-diversity of the fecal microbiota of the LFD and HFD control groups did not differ significantly, other indicating that the diversity of the mouse fecal microbiota in this study was not affected by high-fat feeding (Figure 4). However, PERMANOVA highlighted a significant overall alteration of fecal microbiota composition based on diet (p = .04) (Figure 4). The functional capacity of the fecal microbiota, as inferred by PICRUSt, was not significantly impacted by diet (p = .143). High-fat diet feeding caused a significant decrease in the abundance of genes involved in glycerophospholipid metabolism (p = .008), fatty acid elongation in mitochondria (p = .009), steroid biosynthesis (p = .009), arachidonic acid metabolism (p = .013), biosynthesis of unsaturated fatty acids (p = .033), and fatty acid metabolism (p = .05) compared to low-fat diet-fed controls.

Probiotic treatment significantly impacts host metabolic health and the composition and function of the fecal microbiota in a strain-specific manner

When compared to the HFD control group, the probiotic intervention groups did not show any significant reduction in body weight gain,

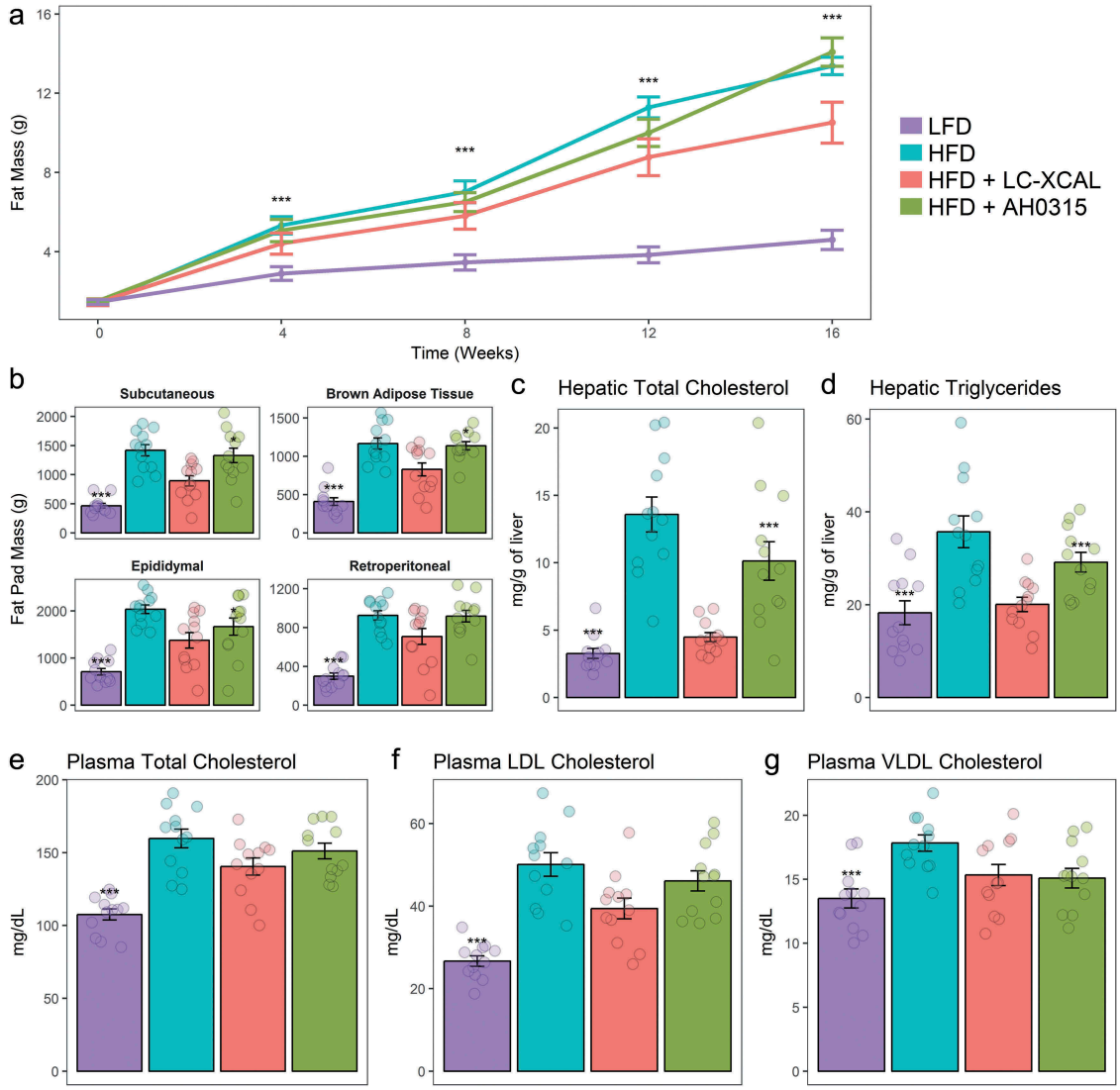

Figure 3. Effect of diet and probiotic supplementation on metabolic phenotype. (A) Effect of diet, *L. casei* LC-XCAL, and *L. plantarum* AH0315 on fat mass (Weeks 0, 4, 8, 12 and 16). LFD v HFD were statistically compared by unpaired t-test *p < .05; **p < .01; ***p < .001. Probiotic groups were compared relative to HFD by one-way ANOVA (no significance found). (B-G) Effect of diet, *L. casei* LC-XCAL and *L. plantarum* AH0315 on fat pad weight, hepatic total cholesterol, hepatic triglycerides, 6 h fasted terminal total cholesterol, 6 h fasted terminal plasma low-density lipoprotein (LDL) cholesterol, and 6 h fasted terminal plasma very low-density (VLDL) cholesterol levels. LFD v HFD were statistically compared by unpaired t-test; probiotic groups were compared relative to HFD by one-way ANOVA followed by Tukey's multiple comparison test; *p < .05; **p < .01; ***p < .001.

although the *L. casei* LC-XCAL-treated group showed a trend toward a reduction (Figure S1). When compared to the HFD control group, *L. casei* LC-XCAL supplemented mice did not show any significant difference in fat mass throughout the study (Figure 3). However, a trend toward a reduction of fat mass gain was observed and there were significant reductions in subcutaneous fat, brown adipose tissue, and epididymal fat. The *L. plantarum* AH0315-treated group did not show any significant change in fat mass or fat pad weights. When compared to the HFD control group, neither probiotic intervention groups showed any significant change in lean mass.

When compared to the HFD control group, the *L. casei* LC-XCAL-treated group showed statistically significant reductions in hepatic TC (Figure 3) and TG levels which were not detected in the *L. plantarum* AH0315-treated group. Neither *L. casei* LC-XCAL nor *L. plantarum* AH0315 supplementation significantly altered terminal plasma TC,

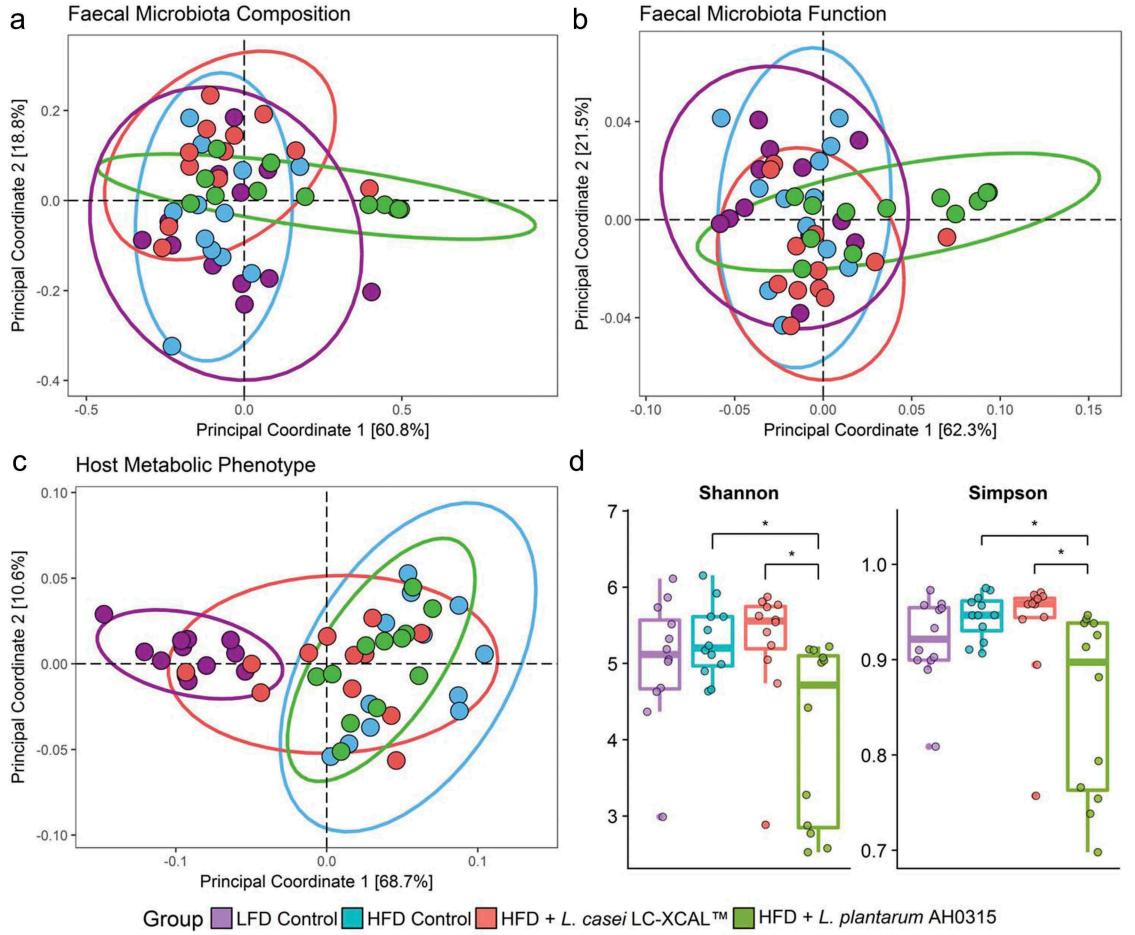

Figure 4. Community-level impact of diet and probiotic supplementation on the fecal microbiota. (A-C) Principal coordinate analysis of fecal microbiota composition, fecal microbiota function, and host metabolic phenotype based on Bray–Curtis dissimilarity. Ellipses represent 95% confidence intervals. (D) Boxplots showing alpha diversity (Shannon and Simpson indices) of fecal microbiota composition. Significant differences ($p < .05$) are shown (*).

TG, HDL-c lipid and random blood glucose levels when compared to HFD group. PERMANOVA reported that *L. casei* LC-XCAL supplementation ($p = .003$), but not L. *plantarum* AH0315 supplementation ($p = .221$), significantly altered the overall metabolic phenotype of the high-fat diet-fed host (Figure 4).

Alpha-diversity of the fecal microbiota was significantly higher in the *L. casei* LC-XCAL-fed group compared to the *L. plantarum* AH0315-fed group, as determined by Shannon ($p = .005$) and Simpson ($p = .008$) diversity indices. However, there was no significant difference in the alpha-diversity of the *L. casei* LC-XCAL-fed group compared to either HFD or LFD groups. Alpha-diversity was reduced in the *L. plantarum* AH0315-fed group relative to the HFD group (Shannon $p = .03$; Simpson $p = .012$). PERMANOVA revealed that the overall composition of the fecal microbiota of the *L. plantarum* AH0315-fed group was significantly different from the HFD group ($p = .004$) while the *L. casei* LC-XCAL-fed group showed no significant difference from this control group ($p = .057$). There were, however, several genera which were significantly altered by *L. casei* LC-XCAL supplementation, notably increased relative abundance of *Acinetobacter, Oscillibacter, Lactococcus*, and *Elizabethkinga* (Table 1, Table S2).

PERMANOVA revealed that the overall function of the fecal microbiota was significantly altered by supplementation by *L. plantarum* AH0315 ($p = .006$) but not by *L. casei* LC-XCAL ($p = .106$). As *L. casei* LC-XCAL supplementation was shown to increase fecal fat excretion, lipid metabolism was chosen as the primary focus for in-depth functional analysis of the fecal microbiota. The *L. casei* LC-

Table 1. Genera whose significant enrichment (*) or reduction ($) by *L. casei* LC-XCAL feeding were either reversed or not significant (n.s.) in the *L. plantarum* AH0315-fed group.

	P value	
Group	HFD + LC-XCAL	HFD + AH0315
Genus		
Acidovorax	0.015*	n.s.
Acinetobacter	<0.001*	n.s.
Anaeroplasma	0.01*	n.s.
Burkholderia	<0.001*	n.s.
Chryseobacterium	0.006*	n.s.
Clostridiaceae | Candidatus Arthromitus	0.021*	n.s.
Comamonas	0.033*	n.s.
Elizabethkingia	<0.001*	n.s.
Erysipelotrichaceae | Incertae Sedis	<0.001*	n.s.
Oscillibacter	0.008*	n.s.
Pseudobutyrivibrio	0.025*	n.s.
Pseudomonas	0.002*	n.s.
Sphingobacterium	0.003*	n.s.
Sphingomonas	0.001*	n.s.
Stenotrophomonas	0.003*	n.s.
Cyanobacteria | *4C0d2* | Uncultured Prokaryote	<0.001$	n.s.

XCAL-fed group exhibited a microbiota with a predicted increase in genes involved in ether lipid metabolism ($p < .001$) and glycerolipid metabolism ($p = .028$), accompanied by decreased steroid hormone biosynthesis ($p = .006$) compared to the HFD group. The *L. plantarum* AH0315-fed group also showed predicted increased levels of pathways involved in ether lipid metabolism ($p = .001$) and glycerolipid metabolism ($p = .007$), as well as linoleic acid metabolism ($p = .018$), and synthesis and degradation of ketone bodies ($p = .05$), along with decreased levels of steroid hormone biosynthesis ($p = .001$), sphingolipid metabolism ($p = .002$), and alpha linoleic acid metabolism ($p = .006$), compared to the HFD group (Table 2).

Identification of group-specific microbial biomarkers

Analysis of filtered, genus-level relative abundance data by LEFSe identified 11 genera whose overabundance discriminated one group from the remaining three (Figure S2). Increased levels of *Akkermansia, Turicibacter*, and an uncultured member of the *Christensenellaceae* family were characteristic of the LFD group, while the HFD group was distinguished by an overabundance of a single genus, an uncultured member of the *Peptostreptococcaceae* family; a bloom that was partly reduced by *L. casei* LC-XCAL feeding (Table S1). The *L. casei* LC-XCAL-fed group was characterized by increased populations of *Elizabethkingia, Acinetobacter, Prevotella, Oscillibacter*, and *Enterococcus* and differentiation of the *L. plantarum* AH0315-fed group was based on an overabundance of *Lactobacillus* and *Bifidobacterium*.

Machine learning can accurately predict diet and probiotic supplementation status based on murine fecal microbiota composition

Random forests (RF) were used to build a predictive model based on genus-level relative abundance data to assess the predictive power of the fecal microbiota (Figure S3). Based on 500 repeat tests, RF achieved a mean classification error of 0.103, compared to 0.86 on the same data when the group labels were randomized. The Boruta feature selection algorithm was used to select 18 genera with significant predictive power and the analysis was repeated using only the abundance data of these genera. This resulted in an even lower mean classification error of 0.085, meaning that this model correctly classified, on average, 91.5% of samples into their treatment group using these selected genera, compared to 89.7% using all genera and 14% by random chance.

Reanalysis of the overall profile of the microbiota using only these 18 genera provided some interesting results (Figure S4). PERMANOVA reported that there were significant differences in microbiota composition as a result of both diet (LFD vs HFD, $p = .006$) and probiotic supplementation of the high-fat diet with both *L. casei* LC-XCAL ($p = .006$) and *L. plantarum* AH0315 ($p = .001$). The two probiotic supplemented groups also significantly differed from each other ($p = .001$).

Examination of the relationship between fecal microbiota composition and function and host metabolic phenotype

The composition of the fecal microbiota was examined for correlations between microbial relative abundances and the physiological measurements

Table 2. Microbial-encoded functions whose significant enrichment (*) or reduction ($) by *L. casei* LC-XCAL feeding were either reversed or not significant (n.s.) in the *L. plantarum* AH0315-fed group.

Function	*p* value	
Group	HFD + LC-XCAL	HFD + AH0315
Cellular Processes | Transport and Catabolism | Endocytosis	<0.001*	n.s.
Genetic Information Processing | Transcription | Transcription factors	0.024*	n.s.
Human Diseases | Cancers | Bladder cancer	0.013*	n.s.
Human Diseases | Cardiovascular Diseases | Hypertrophic cardiomyopathy (HCM)	0.031*	n.s.
Human Diseases | Infectious Diseases | Bacterial invasion of epithelial cells	0.003*	n.s.
Human Diseases | Infectious Diseases | Vibrio cholerae infection	0.031*	n.s.
Human Diseases | Neurodegenerative Diseases | Prion diseases	0.005*	n.s.
Metabolism | Amino Acid Metabolism | Lysine biosynthesis	0.038*	n.s.
Metabolism | Amino Acid Metabolism | Phenylalanine, tyrosine and tryptophan biosynthesis	0.024*	n.s.
Metabolism | Biosynthesis of Other Secondary Metabolites | Isoflavonoid biosynthesis	0.006*	n.s.
Metabolism | Metabolism of Terpenoids and Polyketides | Tetracycline biosynthesis	0.05*	n.s.
Metabolism | Xenobiotics Biodegradation and Metabolism | Nitrotoluene degradation	0.05*	n.s.
Organismal Systems | Digestive System | Bile secretion	0.02*	n.s.
Organismal Systems | Endocrine System | GnRH signaling pathway	<0.001*	n.s.
Organismal Systems | Endocrine System | Renin angiotensin system	0.002*	n.s.
Organismal Systems | Immune System | Fc gamma R mediated phagocytosis	<0.001*	n.s.
Unclassified | Cellular Processes and Signaling | Electron transfer carriers	0.05*	n.s.
Unclassified | Cellular Processes and Signaling | Germination	0.043*	n.s.
Unclassified | Cellular Processes and Signaling | Sporulation	0.021*	n.s.
Unclassified | Metabolism | Carbohydrate metabolism	0.05*	n.s.
Human Diseases | Infectious Diseases | Vibrio cholerae pathogenic cycle	0.004*	0.004$
Metabolism | Carbohydrate Metabolism | Ascorbate and aldarate metabolism	<0.001*	0.002$
Metabolism | Carbohydrate Metabolism | Pentose and glucuronate interconversions	0.009*	0.011$
Metabolism | Energy Metabolism | Nitrogen metabolism	0.009*	0.033$
Metabolism | Glycan Biosynthesis and Metabolism | Glycosphingolipid biosynthesis (lacto and neolacto series)	0.001*	0.043$
Organismal Systems | Endocrine System | Insulin signaling pathway	0.038*	0.018$
Cellular Processes | Cell Growth and Death | Apoptosis	0.038$	n.s.
Environmental Information Processing | Signal Transduction | Phosphatidylinositol signaling system	0.003$	n.s.
Environmental Information Processing | Signaling Molecules and Interaction | Cellular antigens	0.05$	n.s.
Genetic Information Processing | Folding, Sorting and Degradation | Proteasome	0.05$	n.s.
Metabolism | Amino Acid Metabolism | Amino acid related enzymes	0.038$	n.s.
Metabolism | Biosynthesis of Other Secondary Metabolites | Flavonoid biosynthesis	0.038$	n.s.
Metabolism | Energy Metabolism | Sulfur metabolism	0.002$	n.s.
Metabolism | Glycan Biosynthesis and Metabolism | Glycosyltransferases	0.013$	n.s.
Metabolism | Metabolism of Cofactors and Vitamins | Riboflavin metabolism	0.043$	n.s.
Metabolism | Metabolism of Other Amino Acids | Selenocompound metabolism	0.021$	n.s.
Metabolism | Metabolism of Other Amino Acids | Taurine and hypotaurine metabolism	0.05$	n.s.
Metabolism | Metabolism of Terpenoids and Polyketides | Prenyltransferases	0.043$	n.s.
Metabolism | Xenobiotics Biodegradation and Metabolism | Aminobenzoate degradation	0.002$	n.s.
Organismal Systems | Nervous System | Glutamatergic synapse	0.028$	n.s.

recorded. A total of 2483 correlations were performed; resulting in 25 significant associations (FDR corrected $p < .1$). Only four of the physiological measurements were significantly associated with microbiota composition but, notably, these were plasma HDL cholesterol, plasma total cholesterol, fat mass, and body weight. All significant correlations are detailed in Table S3. The *Akkermansia* genus showed that the some of the strongest correlations in the dataset, exhibiting negative relationships with body weight (−0.53, $p = .029775172$), fat mass (−0.6, $p = .004922817$), plasma HDL (−0.48, $p = .078125976$), and plasma total cholesterol (−0.47, $p = .078125976$). An uncultured member of the *Prevotellaceae* family also showed a strong negative relationship with fat mass (−0.48325828, $p = .078125976$). ANOVA reported a significant inverse relationship between alpha-diversity and fat mass (Shannon: $R^2 = 0.1484$, $p = .00686$; Simpson: $R^2 = 0.1544$, $p = .00574$), meaning that mice with low fat mass possessed a more diverse microbiota.

Similar analysis of the PICRUSt-predicted microbiota functions and physiological measurements recorded identified 519 significant associations (FDR corrected $p < .1$) from 4264 performed correlations. It is again notable that the greatest proportion of these associations involved fat mass (23.1%), followed by plasma LDL cholesterol

(14.8%), plasma total cholesterol (14.3%), plasma HDL (13.9%), and body weight (13.5%). Lean mass was the only physiological measurement not associated with any microbiota-encoded functions. All significant correlations are detailed in Table S4. Four of the strongest correlations in the data were between microbiota-encoded lipid metabolism pathways and the fat mass of the host, namely biosynthesis of unsaturated fatty acids (−0.61, p = .002157831), fatty acid elongation in mitochondria (−0.59, p = 002157831), steroid biosynthesis (−0.59, p = .002157831) and steroid hormone biosynthesis (−0.59, p = .002157831). ANOVA of the first principal coordinate generated from Bray–Curtis dissimilarities showed a significant relationship between overall microbiota function and the fat mass (R^2 = 0.1945, p = .001705) and plasma HDL cholesterol (R^2 = 0.08669, p = .04222) of the host.

Examination of the relationship between the fecal microbiota and host hepatic total cholesterol and hepatic triglyceride levels

Hepatic total cholesterol (mg/g of liver) and hepatic triglyceride (mg/g of liver) levels were selected for further investigation as both were significantly decreased by *L. casei* LC-XCAL treatment compared to the HFD group. Regression analysis reported no significant relationship between genus-level relative abundances and either hepatic total cholesterol (adjusted R^2 = 0.2074, p = .3267) or hepatic triglycerides (adjusted R^2 = 0.0311, p = .5077). There was also no significant relationship between lipid metabolism and hepatic triglycerides (adjusted R^2 = 0.08075, p = .2928). There was, however, a significant relationship between lipid metabolism and hepatic total cholesterol (adjusted R^2 = 0.2718, p = .04358). All-subset regression to select a subset of variables from the lipid metabolism dataset that would more accurately model hepatic total cholesterol resulted in a new model composed of eight predictor variables (adjusted R^2 = 0.397, p = .000325), meaning that approximately 40% of the variation in the host's hepatic total cholesterol level can be explained by these eight microbial functions.

Discussion

The anti-inflammatory effects of selected EPS-producing bacteria in gastrointestinal inflammatory conditions have previously been reported.[16,17] The effectiveness of EPS-producing bacteria in the management of obesity, which is characterized by chronic low-grade inflammation, was investigated in this study by inclusion of one EPS-producing and one non-EPS producing strain in the diet-induced obesity (DIO) mouse model. The EPS-producing strain *L. casei* LC-XCAL had a positive impact on metabolic outcomes in the DIO mouse model while the non-EPS-producing *L. plantarum* AH0315 did not. This work further suggests that probiotics may be useful as a tool to manage obesity and metabolic health. However, the effects are strain specific and further work is required to better understand the relevant probiotic properties of the strain which showed a benefit.

Due to the chronic inflammation associated with obesity, the production of EPS was chosen as the criterion of inclusion for *L. casei* LC-XCAL in this study. Unsurprisingly, as EPS molecules differ in composition, charge and molecular structure, [22–25] *L casei* LC-XCAL displayed a similar, but not identical, anti-inflammatory cytokine induction profile in a PBMC assay to that of *B. longum* NCIMB 41003, a strain whose anti-inflammatory effect in gastrointestinal disease is well characterized.[19,21]

The mechanisms of action involved in the improvement of metabolic health are still very unclear and would appear to be related to the individual properties of the strain rather than a general probiotic response. Myriad suggested mechanisms have been reported including modulation of genes linked with energy metabolism and inflammation, [26–32] conjugated linoleic acid production, [33,34] improvement of gut barrier function, [35] and bile salt hydrolase activity.[36,37] There have been many animals and human studies investigating the potential of strains of lactobacilli and bifidobacteria in improving lipid profiles, [38–44] however relatively few have demonstrated the translation of the beneficial effects for some lactobacilli strains from animal studies to humans.[17,28,40,45-47] It should be noted that a number of *Lactobacillus* strains were also reported to elicit weight loss in animals and, more recently, in

humans[29,48-51] while a study has demonstrated the protective effects of VSL#3 (a multi-strain probiotic consisting of an *S. thermophilus* strain and a number of *Lactobacillus* and *Bifidobacterium* strains) against increases in body mass and fat mass in healthy young adults consuming a high-fat diet.[52]

Changes in the gut microbiota in response to high-fat feeding have been shown previously[53,54] with resulting alterations in energy harvest, production of LPS and production of short-chain fatty acids, for example, reported to regulate gene expression, and, thereby, alter energy expenditure and storage through host-related mechanisms.[2,13,55] However, it remains unclear whether modulation of the gut microbiota following probiotic administration is a cause contributing to the mechanism of action or a consequence of the improved metabolic health status. Here, *L. casei* LC-XCAL feeding resulted in a more compositionally diverse fecal microbiota than that conferred by *L. plantarum* AH0315, although neither was significantly more diverse than the HFD group. Although *L. plantarum* AH0315 appeared to result in a clustering of the fecal microbiota in a manner more similar to that of the LFD control compared to *L. casei* LC-XCAL, only *L. casei* LC-XCAL administration resulted in an improvement in the metabolic abnormalities associated with diet-induced obesity. Interestingly, despite feeding *Lactobacillus* for 16-weeks, an increase in *Lactobacillus* in the fecal microbiota of the mice was only observed for the *L. plantarum* AH0315-fed group, suggesting the probiotic may be colonizing the host and driving the overall shift in the microbiota composition of this group. However, as there was no change in any physiological measurements taken, we are led to infer that either the mechanism improving metabolic disease in the *L. casei* LC-XCAL-fed group is independent of an extensive overall change in the microbiota or the responsible taxon/functional pathway is impacted by *L. casei* LC-XCAL but not by *L. plantarum* AH0315. The former is supported by the failure of PERMANOVA to identify any observable shift in the overall microbiota composition and function in the LC-XCAL-fed group despite improvement of the host's metabolic health. This is also supported by the observation that *L. casei* LC-XCAL supplementation increased the amount of energy excreted and/or reduced lipid absorption in the gut as evidenced by increased fecal caloric and fat content. Should the effect be caused by a more specific alteration than can be detected by PERMANOVA, it will most likely be listed in Tables 1 and 2, which contain a list of genera and inferred functions, respectively, upon whom a significant impact by *L. casei* LC-XCAL feeding was either not significant or reversed in the *L. plantarum* AH0315-fed group. Interestingly, a subset of the microbiota identified by machine learning feature selection was significantly altered by diet and probiotic supplementation. Suggesting that, while the overall fecal microbiota was unaltered by *L. casei* LC-XCAL supplementation, a core or important community was impacted and may have played a role in influencing the metabolic benefits observed in the host.

Additionally, the *Akkermansia* genus repeatedly appeared here to be associated with improved metabolic health, further highlighting its potential as a probiotic for the improvement of metabolic health.[6,56] The taxon was significantly enriched in the low fat diet-fed group, correlated with lower fat mass, body weight, and blood glucose, was identified by LEFSe as a biomarker for low-fat diet feeding, and was recognized to hold predictive power by Boruta.

Multiple mechanisms may be associated with the effects of probiotics on metabolic health that are linked with the intrinsic properties of the strain itself. The findings presented to highlight the strain specific effects of probiotics on diet-induced obesity, reinforcing the importance of identifying the correct strain for the right indication through thorough screening programmes. Further work to elucidate the mechanisms of action and host response involved as well as clinical trials to confirm these effects in humans are needed. Furthermore, inclusion of an EPS knock-out mutant of *L. casei* LC-XCAL would confirm the specific involvement of the EPS in the probiotic effects observed for this strain.

Materials and Methods

Bacterial strains

The strains employed in this study were selected by PrecisionBiotics Group Ltd., (the industry sponsor) and provided in freeze-dried powder format under a Material Transfer Agreement (MTA).

The LC-XCAL™ strain is a rifampicin resistant variant derived from the parent strain *Lactobacillus casei* AH0099, which was originally isolated from unpasteurized milk. *Lactobacillus plantarum* AH0315 was isolated from a human adult fecal sample. *L. casei* LC-XCAL is an EPS-producing strain, while *L. plantarum* AH0315 is not.

Peripheral blood mononuclear cell (PBMC) cytokine induction assay

Blood was obtained from three healthy volunteers under approval of the Clinical Research Ethics Committee of the Cork Teaching Hospitals. Subjects had all abstained from probiotic, antibiotic, or anti-inflammatory medication usage for one month or longer prior to blood donation. PBMCs were extracted from whole blood by density gradient separation using histopaque (Sigma-Aldrich), a hydrophilic polysaccharide that separates layers of blood, with a 'buffy coat' forming under a layer of plasma which contains the PBMCs. For each strain, 100 mg of freeze-dried powder was weighed out and resuspended in sterile Dulbecos PBS (Sigma-Aldrich). The bacterial cells were washed twice by centrifugation (4000rpm/10 min/4°C/Brake 0) and re-suspended in sterile PBS. Direct microscopic counts were performed and the cell preparations were diluted to the appropriate concentrations to give ratios of 100:1; 50:1; 25:1 total bacteria:PBMC cells. Technical replicates were performed in triplicate. PBMCs were then incubated at a concentration of 2×10^5 cells/ml for 48 h at 37°C (in the presence of penicillin and streptomycin (Sigma-Aldrich)) with control media, or with increasing concentrations of the bacterial strains: 1 x 10^6cells/ml (25: 1 Bacteria: PBMC), 1 x 10^7cells/ml (50:1 Bacteria:PBMC) and 2 x 10^7cells/mL (100:1 Bacteria:PBMC). Supernatants were assayed for the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokine TNF-α which were measured using the MesoScale Discovery (MSD) multiplex platform tissue culture kits (Meso Scale Diagnostics, Maryland, USA). *B. longum* NCIMB 41003, which has previously been shown to have anti-inflammatory activity[20] was used as a positive control to validate the accuracy of the assay.

Statistical analysis was performed using two-way ANOVA followed by an unpaired t-test to identify differences between strains at each cell ratio when suitable. Data were analyzed in R and the results were considered statistically significant when $p < .05$.

Diet-induced obesity (DIO) mouse model

Seven-week old male C57BL/6 J mice (Harlan Laboratories, Netherlands) (48 mice, n = 12 per group), randomized based on body weight, were maintained in a controlled environment at 22 ± 3° C temperature, 50 ± 2% humidity, a light/dark cycle of 12 h each and 15–20 fresh air changes per hour. Male mice were chosen for this model because of their higher susceptibility than female mice in gaining weight and as male mice do not have hormonal changes related to estrous stages, which may affect parameters analyzed. Mice were housed group wise (4 mice per cage) and autoclaved corncob were used as bedding material. Mice were received at 5-weeks of age and were quarantined for one week followed by acclimatization for a further week prior to commencement of the study.

Experimental design

From day 0, mice were fed *ad libitum*; group 1 were fed a low-fat diet (LFD) (10% calories from fat, gamma irradiated; Research Diets Inc, USA) and the other three groups were fed a high-fat diet (HFD) (45% calories from fat) for a period of 16 weeks. The LFD and HFD control groups were provided with plain sterile drinking water via polycarbonate bottles fitted with stainless steel sipper tubes while the two probiotic-supplemented groups were provided with drinking water containing 1×10^9 CFU/dose/day of the appropriate strain (Table 3). General health observation was performed on a daily basis at the same time of the day and involved checking alertness, hair texture, cage movement and presence of any discharge from nose, eyes, mouth and ears. Pre-measured feed was kept in each cage and the left over feed was measured and recorded on every third day to access the amount of food consumed by the mice. Water consumption by the animals was measured on a daily basis starting

Table 3. Experimental DIO mouse groups and associated diet and treatment regimens. LFD = Low-fat diet control; HFD = high-fat diet control.

Groups	Number of mice/group	Diet regimen	Treatment regimen
Group 1 (LFD control)	12	10% calories from fat	Plain sterile drinking water, daily
Group 2 (HFD control)	12	45% calories from fat	Plain sterile drinking water, daily
Group 3 (HFD + *L. casei* LC-XCAL)	12	45% calories from fat	1 x 10^9cfu/dose/day in drinking water, daily
Group 4 (HFD + *L. plantarum* AH0315)	12	45% calories from fat	1 x 10^9cfu/dose/day in drinking water, daily

from the first dosing day. Mice were provided with 50 ml of water daily and the water remaining in each cage was measured every 24 h.

Weight determination and tissue sampling

Body weights were recorded individually for all animals at receipt, day of randomization, prior to treatment, and every three days thereafter. The percent change in bodyweight was calculated according to the formula (TT-TC)/TC * 100 where TT is the test day treated and TC is the test day control. Mice, placed in a plastic holder without sedation or anesthesia, were subjected to Echo Magnetic Resonance Imaging (EchoMRI) using an Echo MRI (EchoMRI-700™) on day −1 and on weeks 4, 8, 12 and 16 to assess body fat and lean mass composition. Plastic holders were sanitized between animals from different groups to avoid cross-contamination. Aseptic technique was followed while handling animals from different groups. At the end of week 16, the animals were sacrificed by CO_2 asphyxiation. Liver, skeletal muscle, visceral fat (epididymal, renal and mesenteric), subcutaneous fat, spleen, cecum, brown adipose fat, brain and intestine were collected, weighed and stored at −80°C for future biochemical and genetic analysis.

Measurement of metabolic markers

Blood samples were collected at 9am by the tail-nipping method (non-anesthetic mode of blood collection) on weeks 0, 4, 8, 12 and 16 for random blood glucose, starting/including the first dosing day. Blood glucose analysis was done using a Johnson and Johnson glucometer (One touch Ultra 2). Aseptic technique was followed while handling animals from different groups. At the end of 16 weeks, mice were fasted for 6 h and blood glucose was estimated as above. Estimation of total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and non-esterified fatty acids (NEFA) was performed using a fully automated random access clinical chemistry analyzer (EM-360, Erba Mannheim, Germany) on plasma from blood, collected by retro-orbital puncture under light isoflurane anesthesia. Plasma very low-density lipoprotein (VLDL) cholesterol levels were obtained by the calculation method: VLDL = Triglycerides (mg/dl)/5. For hepatic TC and TG estimation, liver was homogenized in isopropanol (1 ml/50 mg tissue) and incubated at 4°C for 1 h. The samples were centrifuged at 4°C for 5 min at 2,500 rpm. Cholesterol and triglyceride concentrations in the supernatants were measured by a fully automated random access clinical chemistry analyzer (EM-360, Erba Mannheim).

Two fecal pellets were collected from each mouse once every 2 weeks (weeks 0, 2, 4, 6, 8, 10, 12, 14 and 15) and these samples were immediately stored at −80°C. Aseptic technique was followed while handling animals from different groups. Fecal samples have taken on weeks 6, 10 and 15 were estimated for their gross calorific value (GCV) by bomb calorimetry. For this analysis, the samples were weighed and oven-dried at 60°C for 48 h. The energy content of the feces was assessed with a Parr 6100 calorimeter using an 1109 semi-micro bomb (Parr Instruments & Co., Moline, Illinois, USA). The calorimeter energy equivalent factor was determined using benzoic acid standards and each sample (100 mg) was analyzed in triplicate.

Estimation of energy excretion

Two fecal pellets were collected from each mouse at Weeks 6, 10 and 15 and analyzed for their gross calorific value by bomb calorimetry. For bomb

calorimetry analysis, the samples were weighed and oven-dried at 60°C for 48 h. The energy content of the feces was assessed with a Parr 6100 calorimeter using an 1109 semi-micro bomb (Parr Instruments & Co., Moline, Illinois, USA). The calorimeter energy equivalent factor was determined using benzoic acid standards and each sample (100 mg) was analyzed in triplicate. Cumulative energy excretion of probiotic fed mice over the course of the study was estimated as a percentage relative to energy excreted by mice from the high-fat diet control group. No statistical analysis was performed for this data as they are based on a single value per group.

Estimation of fat excretion

Two fecal pellets were collected from each mouse at Weeks 0, 6, 10 and 14 and stored at −80°C until further analysis. Fecal fat content was determined according to a modified method of Folch *et al* .[57,58] Fecal samples were weighed in 15 ml conical polypropylene tubes (Sarstedt) and deionized water (10x v/w) was added. Samples were vortexed for 60 seconds at high speed at soaked overnight at room temperature. To extract lipids 4x volume of chloroform and methanol mixture (2:1, v:v) to deionized water was added and vortexed for 60 seconds at high speed. The mixture was then centrifuged at 2000 g for 10 min. The bottom lipophilic layer from the extraction was collected by insertion of a 22 G 1½ hypodermic needle (BD) through the tube wall and drained into preweighed tubes. The collected lipophilic layer was allowed to dry overnight. Total fat content was weighed using an analytical laboratory balance (Sartorius). Cumulative fat excretion of probiotic fed mice over the course of the study was estimated as a percentage relative to fat excreted by mice from the high-fat diet control group. No statistical analysis was performed for this data as they are based on a single value per group.

Statistical analysis for DIO study

Statistical analysis was performed using unpaired t-test for differences between two groups. One-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test was used when more than two groups were assessed. Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software). The results were considered statistically significant when $p < .05$.

Fecal microbiota compositional analysis

Total DNA extraction

The microbial composition of fecal pellets from LFD control, HFD control, *L. casei* LC-XCAL™ and *L. plantarum* AH0315 groups was determined by 16 S rRNA sequencing (performed in Teagasc Moorepark, Cork). Total metagenomic DNA was extracted from fresh pellets with the QIamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) coupled with an initial bead-beating step. DNA was quantified using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Ireland).

16S rRNA amplicon sequencing

The V3-V4 variable region of the 16S rRNA gene was amplified from each extracted DNA sample according to the 16S metagenomic sequencing library protocol (Illumina, Sweden). Initially, the template DNA was amplified using primers specific to the V3-V4 region of the 16S rRNA gene, which also incorporates the Illumina overhang adaptor (Forward primer 5'TCGTCGGCAGCGTCAGATGTGTATAAGAG-ACAGCCTACGGGNGGC WGCAG; reverse primer 5'GTCTCGTGGGCTCGGAGATGTGTATAAGA-GACAGGACTACHVGGGT ATCTAATCC). Each PCR reaction contained 2.5 µl DNA template, 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM) (Sigma, Ireland) and 12.5 µl Kapa HiFi Hotstart Readymix (2X) (Kapa Biosystems, London, United Kingdom). The template DNA was amplified under the following PCR conditions: 95°C for 3 min (initialization); followed by 25 cycles of 95 °C for 30 sec (denaturation), 55°C for 30 sec (annealing), 72°C for 30 sec (elongation); followed by a final elongation period of 5 minutes. A negative control reaction whereby the DNA template was replaced with PCR grade water was employed to confirm lack of contamination and PCR products were visualized using gel electrophoresis (1X TAE buffer, 1.5% agarose gel, 100 V) post PCR reaction. Successful amplicons were then cleaned using the AMpure XP purification system (Labplan, Dublin, Ireland). A second PCR reaction was then performed using the previously

amplified and purified DNA as the template. Two indexing primers (Illumina Nextera XT indexing primers, Illumina) were used per sample to allow all samples to be pooled, sequenced and subsequently identified. Each reaction contained 25 µl Kapa HiFI HotStart ReadyMix (2X), 5 µl template DNA, 5 µl index 1 primer (N7xx), 5 µl index 2 primer (S5xx) and 10 µl PCR grade water. PCR conditions were the same as previously described with the samples undergoing just eight cycles instead of 25. PCR products then underwent the same electrophoresis and cleaning protocols as described above. Samples were then quantified using the Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) in conjunction with the broad range DNA quantification assay kit (Biosciences, Dublin, Ireland). All samples were then pooled to an eqimolar concentration and the pool underwent a final cleaning step. The quality of the pool was determined using the Agilent Bioanalyser prior to sequencing. The sample pool was then denatured with 0.2 M NaOH, diluted to 4pM and combined with 10% (v/v) denatured 4pM PhiX. Samples were then sequenced in-house (Teagasc Moorepark, Fermoy, Co. Cork) on the MiSeq sequencing platform using a 2.300 cycle V3 Kit following protocols outlined by Illumina.

Bioinformatic and statistical analysis

Two-hundred and fifty base pair paired-end reads were assembled using FLASH.[59] Reads were further processed with the inclusion of quality filtering, based on a quality score of >25, followed by subsequent removal of mismatched barcodes and sequences below length threshold using QIIME.[60] USEARCH v7 (64-bit)[61] was used for noise removal and chimera detection as well as clustering into operational taxonomic units (OTUs). PyNAST[62] was used to align OTUs and taxonomy was assigned using BLAST against the SILVA SSURef database release 123.[63,64]

The R package comapreGroups (v. 3.1)[65] and LEFSe[66] were employed to detect and visualize statistically significant differences in abundances of individual taxa between groups using the Wilcoxon rank-sum test with multiple corrections. Statistical significance was accepted as $p \leq 0.05$ after FDR multiple correction.

PICRUSt[67] was used to investigate the abundances of gene families based on the 16 S-data available and, from this data, infer functional alterations in the microbiota. For this, the pick OTUs module was performed at 97% identity in a closed reference way using the Greengenes database (13_8)[68] in QIIME. Data were normalized for 16S rRNA gene copy numbers and the metagenomes were predicted. KEGG Orthologs (KO) were identified from the inferred metagenomes and the R package compareGroups (v. 3.1) was used to identify differentially expressed functions between groups.

The remaining statistical analysis was all performed in R (v. 3.2.3) .[69] The phyloseq package (v. 1.10)[70] was used to calculate Alpha diversities and compareGroups was used to test for significant difference. The vegan package (v. 2.3–1)[71] was used to calculate Beta diversities based on Bray-Curtis distance matrices and principal coordinate analysis (PCoA) plots were then visualized using the ggplot2 (v. 2.1.0) package for R .[72] Permutational multivariate analysis of variance (PERMANOVA) was used to test for differences in overall host physiology, microbiota composition, and microbiota function between groups using the vegan package's 'adonis' function.

Identification of group-specific microbial biomarkers

Linear discriminant analysis effect size (LEFSe) was used with default parameters on genus-level relative abundance data to identify genera whose overabundance differentiates one group from the remaining three. Before analysis, genera with a mean relative abundance <0.1% were removed from the data to simplify visualization of results.

Predictive modeling

Random Forests (RF) was used to predict to which group a sample belonged based on its microbiota profile (genus-level relative abundance data) using the default parameters of the 'randomForest' package in R (v. 4.6–12)[73] with "ntree" set to 2000. Repeated tests (n = 500) were used to assess the classification accuracy. The classification performance was evaluated by analyzing the same data with randomized group labels. The Boruta package for R (v. 5.1)[74] was used to identify genera with predictive power. The Boruta package iteratively performs random forests classification and removes genera whose ability to differentiate

between groups are not significantly greater than random chance. The 'leaps' package for R (v. 2.9)[75] was used to perform all-subset regression.

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Data availability

Sequence data have been deposited in the European Nucleotide Archive (ENA) under the project accession number PRJEB35063.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

1. Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A. 2004;101:15718–15723. doi:10.1073/pnas.0407076101.
2. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes. 2007;56:1761–1772. doi:10.2337/db06-1491.
3. Cani PD, Delzenne NM, Amar J, Burcelin R. Role of gut microflora in the development of obesity and insulin resistance following high-fat diet feeding. Pathol Biol (Paris). 2008;56:305–309. doi:10.1016/j.patbio.2007.09.008.
4. Cani PD, Osto M, Geurts L, Everard A. Involvement of gut microbiota in the development of low-grade inflammation and type 2 diabetes associated with obesity. Gut Microbes. 2012;3:279–288. doi:10.4161/gmic.19625.
5. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck A, Lambert DM, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 2009;58:1091–1103. doi:10.1136/gut.2008.165886.
6. Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, Guiot Y, Derrien M, Muccioli GG, Delzenne NM, et al. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci U S A. 2013;110:9066–9071. doi:10.1073/pnas.1219451110.
7. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, Lombard V, Henrissat B, Bain JR, et al. Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science. 2013;341:1241214. doi:10.1126/science.1241214.
8. Vrieze A, Van Nood E, Holleman F, Salojarvi J, Kootte RS, Bartelsman JF, Dallinga-Thie GM, Ackermans MT, Serlie MJ, Oozeer R, et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology. 2012;143:913–6 e7. doi:10.1053/j.gastro.2012.06.031.
9. Backhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A. 2007;104:979–984. doi:10.1073/pnas.0605374104.
10. Schwiertz A, Taras D, Schafer K, Beijer S, Bos NA, Donus C, Hardt PD. Microbiota and SCFA in lean and overweight healthy subjects. Obesity (Silver Spring). 2010;18:190–195. doi:10.1038/oby.2009.167.
11. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444:1027–1031. doi:10.1038/nature05414.
12. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, et al. A core gut microbiome in obese and lean twins. Nature. 2009;457:480–484. doi:10.1038/nature07540.
13. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes. 2008;57:1470–1481. doi:10.2337/db07-1403.

14. Cani PD, Van Hul M. Novel opportunities for next-generation probiotics targeting metabolic syndrome. Curr Opin Biotechnol. 2015;32:21–27. doi:10.1016/j.copbio.2014.10.006.
15. Delzenne NM, Neyrinck AM, Backhed F, Cani PD. Targeting gut microbiota in obesity: effects of prebiotics and probiotics. Nat Rev Endocrinol. 2011;7:639–646. doi:10.1038/nrendo.2011.126.
16. Fanning S, Hall LJ, Cronin M, Zomer A, MacSharry J, Goulding D, Motherway MO, Shanahan F, Nally K, Dougan G, et al. Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. Proc Natl Acad Sci U S A. 2012;109:2108–2113. doi:10.1073/pnas.1115621109.
17. Jones ML, Martoni CJ, Prakash S. Cholesterol lowering and inhibition of sterol absorption by Lactobacillus reuteri NCIMB 30242: a randomized controlled trial. Eur J Clin Nutr. 2012;66:1234–1241. doi:10.1038/ejcn.2012.126.
18. Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. Annu Rev Immunol. 2011;29:415–445. doi:10.1146/annurev-immunol-031210-101322.
19. O'Mahony L, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, et al. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. Gastroenterology. 2005;128:541–551. doi:10.1053/j.gastro.2004.11.050.
20. Groeger D, O'Mahony L, Murphy EF, Bourke JF, Dinan TG, Kiely B, Shanahan F, Quigley EM. Bifidobacterium infantis 35624 modulates host inflammatory processes beyond the gut. Gut Microbes. 2013;4:325–339. doi:10.4161/gmic.25487.
21. Whorwell PJ, Altringer L, Morel J, Bond Y, Charbonneau D, O'Mahony L, Kiely B, Shanahan F, Quigley EM. Efficacy of an encapsulated probiotic Bifidobacterium infantis 35624 in women with irritable bowel syndrome. Am J Gastroenterol. 2006;101:1581–1590. doi:10.1111/j.1572-0241.2006.00734.x.
22. Adams EL, Rice PJ, Graves B, Ensley HE, Yu H, Brown GD, Gordon S, Monteiro MA, Papp-Szabo E, Lowman DW, et al. Differential high-affinity interaction of dectin-1 with natural or synthetic glucans is dependent upon primary structure and is influenced by polymer chain length and side-chain branching. J Pharmacol Exp Ther. 2008;325:115–123. doi:10.1124/jpet.107.133124.
23. Bland EJ, Keshavarz T, Bucke C. The influence of small oligosaccharides on the immune system. Carbohydr Res. 2004;339:1673–1678. doi:10.1016/j.carres.2004.05.009.
24. Hidalgo-Cantabrana C, Lopez P, Gueimonde M, de Los Reyes-gavilan CG, Suarez A, Margolles A, Ruas-Madiedo P. Immune modulation capability of exopolysaccharides synthesised by lactic acid bacteria and bifidobacteria. Probiotics Antimicrob Proteins. 2012;4:227–237. doi:10.1007/s12602-012-9110-2.
25. Kankainen M, Paulin L, Tynkkynen S, von Ossowski I, Reunanen J, Partanen P, Satokari R, Vesterlund S, Hendrickx AP, Lebeer S, et al. Comparative genomic analysis of Lactobacillus rhamnosus GG reveals pili containing a human- mucus binding protein. Proc Natl Acad Sci U S A. 2009;106:17193–17198. doi:10.1073/pnas.0908876106.
26. Kondo S, Kamei A, Xiao JZ, Iwatsuki K, Abe K. Bifidobacterium breve B-3 exerts metabolic syndrome-suppressing effects in the liver of diet-induced obese mice: a DNA microarray analysis. Benef Microbes. 2013;4:247–251. doi:10.3920/BM2012.0019.
27. Zhong Z, Zhang WY, Du RT, Meng H, Zhang HP. Lactobacillus casei Zhang stimulates lipid metabolism in hypercholesterolemic rats by affecting gene expression in the liver. Eur J Lipid Sci Technol. 2012;114:244–252. doi:10.1002/ejlt.201100118.
28. Park DY, Ahn YT, Park SH, Huh CS, Yoo SR, Yu R, Sung MK, McGregor RA, Choi MS. Supplementation of Lactobacillus curvatus HY7601 and Lactobacillus plantarum KY1032 in diet-induced obese mice is associated with gut microbial changes and reduction in obesity. PLoS One. 2013;8(3):e59470.
29. Kang JH, Yun SI, Park MH, Park JH, Jeong SY, Park HO. Anti-obesity effect of Lactobacillus gasseri BNR17 in high-sucrose diet-induced obese mice. PLoS One. 2013;8(1):e54617.
30. Fak F, Backhed F. Lactobacillus reuteri prevents diet-induced obesity, but not atherosclerosis, in a strain dependent fashion in Apoe-/- Mice. PLoS One. 2012;7(10):e46837.
31. McNulty NP, Yatsunenko T, Hsiao A, Faith JJ, Muegge BD, Goodman AL, Henrissat B, Oozeer R, Cools-Portier S, Gobert G, et al. The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. Sci Transl Med. 2011;3:106ra106.
32. Aronsson L, Huang Y, Parini P, Korach-Andre M, Hakansson J, Gustafsson JA, Pettersson S, Arulampalam V, Rafter J. Decreased fat storage by lactobacillus paracasei is associated with increased levels of angiopoietin-like 4 protein (ANGPTL4). PLoS One. 2010;5(9):e13087.
33. Lee HY, Park JH, Seok SH, Baek MW, Kim DJ, Lee KE, Paek KS, Lee Y, Park JH. Human originated bacteria, Lactobacillus rhamnosus PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice. Biochimica Et Biophysica Acta-Mol Cell Biol Lipid. 2006;1761:736–744. doi:10.1016/j.bbalip.2006.05.007.
34. Lee K, Paek K, Lee HY, Park JH, Lee Y. Antiobesity effect of trans-10,cis-12-conjugated linoleic acid-producing Lactobacillus plantarum PL62 on diet-induced obese mice. J Appl Microbiol. 2007;103:1140–1146. doi:10.1111/j.1365-2672.2007.03336.x.

35. Naito E, Yoshida Y, Makino K, Kounoshi Y, Kunihiro S, Takahashi R, Matsuzaki T, Miyazaki K, Ishikawa F. Beneficial effect of oral administration of Lactobacillus casei strain Shirota on insulin resistance in diet-induced obesity mice. J Appl Microbiol. 2011;110:650–657. doi:10.1111/j.1365-2672.2010.04922.x.
36. Joyce SA, MacSharry J, Casey PG, Kinsella M, Murphy EF, Shanahan F, Hill C, Gahan CGM. Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut. Proc Natl Acad Sci U S A. 2014;111:7421–7426. doi:10.1073/pnas.1323599111.
37. Joyce SA, Shanahan F, Hill C, Gahan CG. Bacterial bile salt hydrolase in host metabolism: potential for influencing gastrointestinal microbe-host crosstalk. Gut Microbes. 2014;5:669–674. doi:10.4161/19490976.2014.969986.
38. Andrade S, Borges N. Effect of fermented milk containing Lactobacillus acidophilus and Bifidobacterium longum on plasma lipids of women with normal or moderately elevated cholesterol. J Dairy Res. 2009;76:469–474. doi:10.1017/S0022029909990173.
39. Ataie-Jafari A, Larijani B, Majd HA, Tahbaz F. Cholesterol-lowering effect of probiotic yogurt in comparison with ordinary yogurt in mildly to moderately hypercholesterolemic subjects. Annal Nutr Metab. 2009;54:22–27. doi:10.1159/000203284.
40. Fuentes MC, Lajo T, Carrion JM, Cune J. Cholesterol-lowering efficacy of Lactobacillus plantarum CECT 7527, 7528 and 7529 in hypercholesterolaemic adults. Br J Nutr. 2013;109:1866–1872. doi:10.1017/S000711451200373X.
41. Greany KA, Bonorden MJL, Hamilton-Reeves JM, McMullen MH, Wangen KE, Phipps WR, Feirtag J, Thomas W, Kurzer MS. Probiotic capsules do not lower plasma lipids in young women and men. Eur J Clin Nutr. 2008;62:232–237. doi:10.1038/sj.ejcn.1602719.
42. Lewis SJ, Burmeister S. A double-blind placebo-controlled study of the effects of Lactobacillus acidophilus on plasma lipids. Eur J Clin Nutr. 2005;59:776–780. doi:10.1038/sj.ejcn.1602139.
43. Miremadi F, Ayyash M, Sherkat F, Stojanovska L. Cholesterol reduction mechanisms and fatty acid composition of cellular membranes of probiotic Lactobacilli and Bifidobacteria. J Funct Foods. 2014;9:295–305. doi:10.1016/j.jff.2014.05.002.
44. Moroti C, Magri LFS, Costa MD, Cavallini DCU, Sivieri K. Effect of the consumption of a new symbiotic shake on glycemia and cholesterol levels in elderly people with type 2 diabetes mellitus. Lipids Health Dis. 2012;11:29.
45. Ahn HY, Kim M, Ahn YT, Sim JH, Choi ID, Lee SH, Lee JH. The triglyceride-lowering effect of supplementation with dual probiotic strains, Lactobacillus curvatus HY7601 and Lactobacillus plantarum KY1032: reduction of fasting plasma lysophosphatidylcholines in nondiabetic and hypertriglyceridemic subjects. Nutr Metab Cardiovasc Dis. 2015;25:724–733. doi:10.1016/j.numecd.2015.05.002.
46. Ahn HY, Kim M, Chae JS, Ahn YT, Sim JH, Choi ID, Lee SH, Lee JH. Supplementation with two probiotic strains, Lactobacillus curvatus HY7601 and Lactobacillus plantarum KY1032, reduces fasting triglycerides and enhances apolipoprotein A-V levels in non-diabetic subjects with hypertriglyceridemia. Atherosclerosis. 2015;241:649–656. doi:10.1016/j.atherosclerosis.2015.06.030.
47. Yoo SR, Kim YJ, Park DY, Jung UJ, Jeon SM, Ahn YT, Huh CS, McGregor R, Choi MS. Probiotics L. plantarum and L. curvatus in combination alter hepatic lipid metabolism and suppress diet-induced obesity. Obesity. 2013;21:2571–2578. doi:10.1002/oby.20428.
48. Hamad EM, Sato M, Uzu K, Yoshida T, Higashi S, Kawakami H, Kadooka Y, Matsuyama H, El-Gawad IAA, Imaizumi K. Milk fermented by Lactobacillus gasseri SBT2055 influences adipocyte size via inhibition of dietary fat absorption in Zucker rats. Br J Nutr. 2009;101:716–724. doi:10.1017/S0007114508043808.
49. Kadooka Y, Sato M, Imaizumi K, Ogawa A, Ikuyama K, Akai Y, Okano M, Kagoshima M, Tsuchida T. Regulation of abdominal adiposity by probiotics (Lactobacillus gasseri SBT2055) in adults with obese tendencies in a randomized controlled trial. Eur J Clin Nutr. 2010;64:636–643. doi:10.1038/ejcn.2010.19.
50. Kadooka Y, Sato M, Ogawa A, Miyoshi M, Uenishi H, Ogawa H, Ikuyama K, Kagoshima M, Tsuchida T. Effect of Lactobacillus gasseri SBT2055 in fermented milk on abdominal adiposity in adults in a randomised controlled trial. Br J Nutr. 2013;110:1696–1703. doi:10.1017/S0007114513001037.
51. Kang JH, Yun SI, Park HO. Effects of Lactobacillus gasseri BNR17 on body weight and adipose tissue mass in diet-induced overweight rats. J Microbiology. 2010;48:712–714. doi:10.1007/s12275-010-0363-8.
52. Osterberg KL, Boutagy NE, McMillan RP, Stevens JR, Frisard MI, Kavanaugh JW, Davy BM, Davy KP, Hulver MW. Probiotic supplementation attenuates increases in body mass and fat mass during high-fat diet in healthy young adults. Obesity. 2015;23:2364–2370. doi:10.1002/oby.21230.
53. Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, Knight R, Ahima RS, Bushman F, Wu GD. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology. 2009;137:1716–1724. doi:10.1053/j.gastro.2009.08.042.
54. Murphy EF, Cotter PD, Healy S, Marques TM, O'Sullivan O, Fouhy F, Clarke SF, O'Toole PW, Quigley EM, Stanton C, et al. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. Gut. 2010;59:1635–1642. doi:10.1136/gut.2010.215665.
55. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, Gibson GR, Delzenne NM. Selective

increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia. 2007;50:2374–2383. doi:10.1007/s00125-007-0791-0.
56. Schneeberger M, Everard A, Gomez-Valades AG, Matamoros S, Ramirez S, Delzenne NM, Gomis R, Claret M, Cani PD. Akkermansia muciniphila inversely correlates with the onset of inflammation, altered adipose tissue metabolism and metabolic disorders during obesity in mice. Sci Rep. 2015;5:16643.
57. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957;226:497–509.
58. Kraus D, Yang Q, Kahn BB. Lipid extraction from mouse feces. Bio-protocol. 2015;5:e1375. doi:10.21769/BioProtoc.1375.
59. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 2011;27:2957–2963. doi:10.1093/bioinformatics/btr507.
60. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7:335–336. doi:10.1038/nmeth.f.303.
61. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26:2460–2461. doi:10.1093/bioinformatics/btq461.
62. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics. 2010;26:266–267. doi:10.1093/bioinformatics/btp636.
63. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590–6. doi:10.1093/nar/gks1219.
64. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–410. doi:10.1016/S0022-2836(05)80360-2.
65. Subirana I, Sanz H, Vila J. Building bivariate tables: the comparegroups package for R. J Stat Softw. 2014;57(12):1–16.
66. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12;R60.
67. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Thurber RLV, Knight R, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol. 2013;31:814-+. doi:10.1038/nbt.2676.
68. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006;72:5069–5072. doi:10.1128/AEM.03006-05.
69. R Core Team. 2016. R: A language and environment for statistical computing.
70. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One. 2013;8:e61217. doi:10.1371/journal.pone.0061217.
71. Jari Oksanen FGB, Michael F, Roeland K, Pierre L, Dan McGlinn PR, Minchin RB, O'Hara GL, Simpson PS, Henry M, Stevens H, et al. Vegan: community ecology package. R package version 2.3-1. 2015.
72. Wickham H. ggplot2: elegant graphics for data analysis. Ggplot2: elegant graphics for data analysis.2009. p. 1–212. doi:10.1007/978-0-387-98141-3:.
73. Wiener A. Classification and regression by Random forest. R News 2; 2002
74. Kursa MB, Rudnicki WR. Feature selection with the boruta package. J Stat Softw. 2010;36:1–13. doi:10.18637/jss.v036.i11.
75. Lumley T 2017. Leaps: regression subset selection.