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

Calum J. Walsh, Selena Healy, Paul W. O'Toole, Eileen F. Murphy, and Paul D. Cotter

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.

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 the likely role of the gut microbiota in the development of obesity is becoming better understood. The contribution of the gut microbiota to obesity is multifactorial and involves processes such as enhanced energy harvest and fat storage, altered metabolic pathways and bacterial translocation leading to chronic low-grade inflammation. The manipulation of the gut microbiota by probiotics is a potential therapeutic approach to help ameliorate obesity and associated metabolic disorders. *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. Obesity is characterized *inter alia* by a chronic low-grade inflammation 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. We also investigate...
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 (*).](image-url)
The alpha-diversity of the fecal microbiota of the LFD and HFD control groups did not differ significantly, 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,
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,
TG, HDL-c lipid and random blood glucose levels when compared to HFD group. PERMANOVA reported that \textit{L. casei} LC-XCAL supplementation ($p = .003$), but not \textit{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 \textit{L. casei} LC-XCAL-fed group compared to the \textit{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 \textit{L. casei} LC-XCAL-fed group compared to either HFD or LFD groups. Alpha-diversity was reduced in the \textit{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 \textit{L. plantarum} AH0315-fed group was significantly different from the HFD group ($p = .004$) while the \textit{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 \textit{L. casei} LC-XCAL supplementation, notably increased relative abundance of \textit{Acinetobacter}, \textit{Oscillibacter}, \textit{Lactococcus}, and \textit{Elizabethkinga} (Table 1, Table S2).

PERMANOVA revealed that the overall function of the fecal microbiota was significantly altered by supplementation by \textit{L. plantarum} AH0315 ($p = .006$) but not by \textit{L. casei} LC-XCAL ($p = .106$). As \textit{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 \textit{L. casei LC-
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 over-abundance 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.
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 \textit{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 \textit{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 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 ($R^2 = 0.08669, p = .002157831$) of the host.

**Discussion**

The anti-inflammatory effects of selected EPS-producing bacteria in gastrointestinal inflammatory conditions have previously been reported.\textsuperscript{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,\textsuperscript{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.\textsuperscript{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,\textsuperscript{26–32} conjugated linoleic acid production,\textsuperscript{33,34} improvement of gut barrier function,\textsuperscript{35} and bile salt hydrolase activity.\textsuperscript{36,37} There have been many animals and human studies investigating the potential of strains of lactobacilli and bifidobacteria in improving lipid profiles,\textsuperscript{38–44} however relatively few have demonstrated the translation of the beneficial effects for some lactobacilli strains from animal studies to humans.\textsuperscript{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

**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.
humans 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 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 knockout 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 re-suspended 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 x 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 10^6 cells/ml (25: 1 Bacteria:PBMC), 1 x 10^7 cells/ml (50:1 Bacteria:PBMC) and 2 x 10^7 cells/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 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 x 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...
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 \((TT-TC)/TC \times 100\) 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: \(\text{VLDL} = \frac{\text{Triglycerides (mg/dl)}}{5}\). 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

### 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 10^9 cfu/dose/day in drinking water, daily       |
| Group 4 (HFD + L. plantarum AH0315) | 12 | 45% calories from fat | 1 x 10^9 cfu/dose/day in drinking water, daily       |
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.\textsuperscript{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 pre-weighed 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, \textit{L. casei} LC-XCAL\textsuperscript{™} and \textit{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 QIAamp 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’TCGTCGACGACGCTATGATGTAATACGACAGGCTACAGGGGNGGC WGCAG; reverse primer 5’GTCTCGTGGGCTCGGAGATGTGTATAAGACAGGACTACHVGGGT 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 equimolar concentration and the pool underwent a final cleaning step. The quality of the pool was determined using the Agilent Bioanalyzer 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). PyNAST62 was used to align OTUs and taxonomy was assigned using BLAST against the SILVA SSURef database release 123.63,64

The R package compareGroups (v. 3.1)65 and LEFSe66 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 ≤ 0.05 after FDR multiple correction.

PICRUSt67 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) 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.

ORCID

Calum J. Walsh http://orcid.org/0000-0003-3113-9534
Paul W. O’Toole http://orcid.org/0000-0001-5377-0824

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