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The impact of a variety of factors on the obesity associated gut microbiota

A thesis presented to the National University of Ireland for the degree of
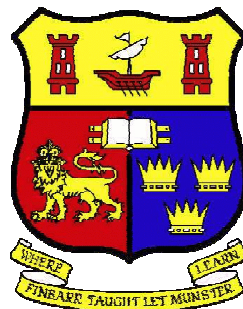
Doctor of Philosophy

by

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November 2013

DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, and has not been submitted for another degree, either at University College Cork or elsewhere.

Signed:_____

Student Number: 105447712

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ABSTRACT

The obesity pandemic has become perhaps the most prevalent health issue of our time, with more than 10% of the world's population now being obese. Obesity can be defined as abnormal or excess fat accumulation that may impair health and results from an imbalance between energy intake and energy expenditure. A decrease in physical activity due to an increase in sedentary forms of work, changing modes of transport and increasing urbanization is likely a major contributory factor. Diet is another major factor with the increased availability and intake of calorie dense, high fat foods being of global concern. Notably, with respect to this thesis, over the last decade advances in the field of next generation sequencing (NGS) have facilitated investigations to determine the relationship between the gut microbiota and obesity.

Initially, we established that a high fat diet alters microbial composition but that changes in the microbiota were dissociated from markers of energy harvest. These results highlighted that the relationship between the gut microbiota and energy harvesting capacity is more complicated than previously considered and that future studies should consider the possibility of microbial adaptation to diet and time. The objectives of this thesis were to investigate the impact of a variety of factors on the obesity-associated gut microbiota and, in turn, how these factors could affect weight gain. In order to achieve this, NGS technologies were employed to assess the impact of different interventions on the composition of the gut microbiota and, in turn, weight gain.

First, the impact of two antimicrobial strategies on metabolic abnormalities in murine diet-induced obesity was explored. Our approach involved the administration of vancomycin and a bacteriocin-producing probiotic (*Lactobacillus salivarius* UCC118 Bac⁺) to diet induced obese mice and assessing the impact on microbial composition and metabolic abnormalities associated with obesity. This study suggested that altering the gut microbiota in a specific manner can have a positive influence on

health, as vancomycin treatment resulted in a reduction in weight gain and an improved metabolic profile, but that alterations made by the bacteriocin producing probiotic provided only transient benefits. The investigation of these effects with time also highlighted the resilience of the gut microbiota and suggested that interventions may need to be monitored and continually adjusted to ensure the sustained modification of the gut microbiota.

The impact of the production of specific bile salt hydrolases on the gut microbiota and in turn weight gain was also assessed. A significant reduction is weight gain was observed in mice in receipt of an *Escherichia coli* strain producing a bile salt hydrolase (BSH) from *L. salivarius* JCM1046 (BSH1), which were fed either high or low fat diets, relative to mice fed an *E. coli* strain producing a BSH from *L. salivarius* UCC118 (BSH2). These results were accompanied by changes in microbial composition and, ultimately, highlighted the effect of diet, antibiotics and, in particular, the impact of the production of different BSHs on gut microbial communities.

Further investigations focussed on assessing the effect of diet and the microbiota on the progression of colitis and colitis-associated colorectal cancer (CAC) progression. Mice were fed a high fat or low fat diet, followed by one azoxymethane (AOM) injection and 3x dextran sodium sulphate (DSS) cycles (CAC model) or 3xDSS cycles alone (colitis model). High fat feeding protected mice from developing colitis and CAC on the basis of weight and cytokine profile, tumour incidence and number as well as colon length. NGS based analysis of the murine gut microbiota showed a reduction in gut microbial diversity in LF-colitis and LF-CAC mice relative to their HF-fed counterparts. These results provide an initial insight into the relationship between diet, inducers of colitis and colon cancer and the gut microbiota.

Finally, the degree to which exercise and diet affects the gut microbial population was examined. Since extremes of exercise often accompany extremes of diet, we addressed the issue by studying professional athletes from an international rugby union squad. Gut

microbial analysis revealed athletes had a greater microbial diversity than controls, which in turn correlated with protein consumption. These results provide evidence for a beneficial impact of exercise on the gut microbiota but also indicate that the relationship is related to accompanying dietary changes.

Overall the results presented in this thesis highlight that microbial diversity is influenced by diet, exercise, antibiotics and disease state, however it is only through further understanding of the structure and function that we can identify targets that can impact on health.

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ABBREVIATIONS

AMP	Adenosine monophoshate
AMPK	Adenosine monophoshate activated protein kinase
ANOVA	Analysis of variance
AOM	Azoxymethane
Apoa-1	Apolipoprotein A1
AST	Aspartate aminotransferase
BAC-	*Lactobacillus salivarius* UCC118 non-bacteriocin producing strain
BAC+	*Lactobacillus salivarius* UCC118 bacteriocin producing strain
BLAST	Basic local alignment search tool
BMI	Body mass index
BSH	Bile salt hydrolase
CAC	Colitis associated cancer
CBA	Conjugated bile acid
CD	Crohn's disease
CK	Creatine kinase
CRC	Colorectal cancer
DIO	Diet-induced obesity
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulphate
DXA	Dual-energy X-ray absorptiometry
EPIC	European prospective investigation into cancer
FDR	False discovery rate
FFQ	Food frequency questionnaire
FIAF	Fasting-adipose adipose factor

FISH	Fluorescence in-situ hybridisation
FNB	Food and nutrition board
FSAI	Food safety authority Ireland
GMR	Gentamicin resistance
GPR	G-protein-coupled receptor
HDL	High-density lipoprotein
HF	High fat
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
ICHEC	Irish centre for high end computing
IFN	Interferon
IGT	Impaired glucose tolerance
IL	Interleukin
IRFU	Irish rugby football union
LF	Low fat
MCP	Monocyte chemoattractant protein
MEGAN	MEtaGenome ANalyzer
MID	Molecular identifier
NCBI	National centre for biotechnology information
NMR	Nuclear magnetic resonance
NMR	Nuclear magnetic resonance
ob/ob	Genetically-determined obesity
OTU	Operational taxonomical units
PBS	Phosphate buffered saline
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction

QIIME	Quantitative Insights Into Microbial Ecology
QPCR	Quantitative real time PCR
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RDP	Ribosomal database project
rRNA	Ribosomal RNA
SCFA	Short chain fatty acids
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error
SRA	Sequence read archive
StrR	Streptomycin resistance
TLR	Toll-receptor
TNF	Tumor necrosis factor
UC	Ulcerative colitis
UCC	University College Cork
UPGMA	Unweighted pair group method
WHO	World health organisation
WISP	Weighted intake software package
γGT	Gamma Glutamyl Transferase

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Chapter 1

The gut microbiota and its relationship to diet and obesity: new insights

1.1   ABSTRACT

Obesity develops from a prolonged imbalance of energy intake and energy expenditure. However, the relatively recent discovery that the composition and function of the gut microbiota impacts on obesity has lead to an explosion of interest in what is now a distinct research field. Here, research relating to the links between the gut microbiota, diet and obesity will be reviewed under five major headings: (i) the gut microbiota of lean and obese animals, (ii) the composition of the gut microbiota of lean and obese humans, (iii) the impact of diet on the gut microbiota, (iv) manipulating the gut microbiota and (v) the mechanisms by which the gut microbiota can impact on weight gain.

1.2 INTRODUCTION

Obesity has become one of the most prevalent health issues of our time. In 2008, the World Health Organisation (WHO) estimated that there were over 1.5 billion overweight adults in the world and, of these, approximately 500 million are clinically obese. Indeed, more deaths are caused worldwide by excessive weight than those which caused by being underweight (WHO, 2013). Obesity is a multifactorial condition but it can be most simply described as being the result of a long-term imbalance between energy intake and energy expenditure. While modern eating habits and ever increasingly sedentary lifestyles are major contributory factors, researchers are gaining an ever greater appreciation of other important risk factors. One such issue that has emerged in recent years is the link between obesity and the composition and functionality of the microorganisms in the gut. Here we review the literature related to this topic under five major headings i.e. (i) the gut microbiota of lean and obese animals, (ii) the composition of the gut microbiota of lean and obese humans, (iii) the impact of diet on the gut microbiota, (iv) manipulating the gut microbiota and (v) the mechanisms by which the gut microbiota can impact on weight gain (Fig 1).

1.3 GUT MICROBIOTA OF LEAN AND OBESE ANIMALS

Mouse models are frequently employed by researchers investigating obesity and the role of the gut microbiota in obesity. The following sub-sections will focus on the various mouse models that have been employed and the outcome from studies carried out to date.

1.3.1 Microbiota of genetically obese mice

Many studies have examined the difference in the composition of the gut microbiota of lean and obese mice using a wide range of molecular methods (listed in Table 1). The possible existence of a link between obesity and the gut microbiota only became apparent upon the application of DNA sequencing on a large scale to facilitate an unbiased analysis of the entire gut microbiota (i.e. including culturable and unculturable microbes). This DNA sequencing approach initially focussed on the 16S rRNA gene which, because of the presence of highly conserved and variable regions, can be employed to classify bacteria (Fig 2). In 2005, (Ley *et al.*) first employed this approach in an obesity context to analyse 5,088 bacterial 16S rRNA sequences corresponding to the caecal microbiota of *ob*/*ob*, *ob*/+ and +/+ mice. *ob*/*ob* mice are leptin deficient, eat excessively and are obese as a consequence of this genotype. Traditional 'Sanger' sequencing was employed in this instance (Sanger *et al.*, 1977). The investigation revealed that the two most abundant bacterial divisions in mice were the phylum Firmicutes (60-80% of sequences) and the Phylum Bacteroidetes (20-40% of sequences) and it was established that the proportions of Bacteroidetes and Firmicutes were reduced and increased, respectively, in the obese animals relative to their lean counterparts. These shifts were division wide (i.e. no particular subgroup of Firmicutes and/or Bacteroidetes were lost or gained) (Ley *et al.*, 2005). (Turnbaugh *et al.*) added to our knowledge in this area in 2006 in a study which differed by virtue of being carried out on a larger scale and the use of an alternative approach, i.e. random or shotgun metagenomic sequencing of the murine (*ob*/*ob*, *ob*/+ and +/+) caecal microbial DNA (Fig 2). In this case both traditional Sanger and high throughput 454 pyrosequencing technologies were employed (Margulies *et al.*, 2005). This study again

highlighted an increased ratio of Firmicutes to Bacteroidetes in obese mice relative to their lean counterparts. It was also noted that the gut microbiota of *ob*/*ob* mice contained a higher proportion of Archaea than were present in the cecum of their lean counterparts (Turnbaugh *et al.*, 2006). Though these results are interesting, the possibility that the animal's genotype could also influence the gut microbial composition can not be excluded. Notably, since this study, high throughput DNA sequencing approaches have essentially replaced Sanger sequencing and other strategies when the objective is to assess alternations in the gut microbiota composition. The methods used to analyse the gut microbiota in the studies referred to in this review are summarised in Table 2.

1.3.2 Microbiota of diet-induced obese mice

Another murine model has been developed which focuses on obesity that arises due to consumption of a high-fat, 'western' diet (i.e. diet induced obesity or DIO), rather than genetics. In 2008, (Turnbaugh *et al.*) showed that the western diet associated caecal microbial community had a significantly lower proportion of Bacteroidetes and a specific increase in the Mollicutes subpopulation of the Firmicutes. In 2009, (Hildebrandt *et al.*) investigated the microbial communities from both wild type and resistin-like molecule (RELM) β knock-out (KO) mice fed a standard chow diet and a high fat diet. The RELMβ gene is expressed by colonic goblet cells and its expression has been shown to be dependant on the gut microbiome (He *et al.*, 2003) and can be induced by a high fat diet (Shojima *et al.*, 2005). Wildtype and RELMβ KO mice were compared in order to further investigate the relationships between diet, obesity and microbiota composition. A sequence based analysis of murine faecal samples revealed that the gut microbiota communities of 13 week old wild type and RELMβ KO mice fed a standard chow diet were very similar, with Bacteroidetes, followed by Firmicutes, being the dominant groups. The phyla Proteobacteria*,* Tenericutes and TM7 were also detected. After three months consumption of a high fat diet, the gut microbiota of both groups of animals differed from those fed the standard chow diet. More specifically, the Phylum Firmicutes class Clostridiales*,* Actinobacteria and Deltaproteobacteria increased their

respective proportions in the gut of both groups of animals fed a high fat diet which was accompanied by a reduction in the abundance of class Bacteroidales. An increase in the Mollicutes population was also noted in these animals, although this bloom was not as dramatic as had been observed by (Turnbaugh *et al.*, 2008). Despite these similarities with respect to gut microbial composition, the RELMβ KO mice consuming a high fat diet remained lean, whereas the corresponding wild type mice became obese. From this study, the authors concluded that, because the general changes in the composition of the gut microbiota were similar in the wild type and KO mice, the effect of diet was dominant i.e. the high fat diet, and not the obese state, accounted for the alteration in the gut microbial communities. We will return to the implications of this study later. A recent 16S rRNA-based study by (Murphy *et al.*, 2010) has provided further insight. Here the faecal microbiota of lean (+/+), *ob/ob* as well as +/+ mice fed a high fat (HF) diet, was investigated at 7 weeks (the time points at which the low and high fat groups were separated), 11 weeks and 15 weeks of age. It was established that, in addition to the Firmicutes and Bacteroidetes, a high proportion of Actinobacteria was present in the gut. While no significant changes in the proportions of the different microbial populations was observed in lean mice over the 8 week period, there was a progressive increase in the proportions of Firmicutes in the faecal microbiota of HF-fed and *ob/ob* mice. Bacteroidetes levels decreased overtime in all groups but this reduction reached statistical significance in *ob/ob* mice only. The levels of Actinobacteria fluctuated in all three groups with significant increases in their proportions being apparent in the *ob/ob* and HF-fed mice when samples from weeks 7 and 11 and from weeks 7 and 15 were compared. It was also noted that Proteobacteria decreased in HF-fed mice from distal weeks 11 to 15 while in *ob/ob* mice Deferribacteria and *Lactococcus* decreased overtime (Murphy *et al.*, 2010).

1.3.3 Humanized mice

Studies have revealed that although the gut of both mice and humans contains microbes from the same dominant range of bacterial phyla (i.e. Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and, to a lesser

extent, Verrucomicrobia, Cyanobacteria, TM7, Fusobacteria, and Spirochaeates), many of the bacterial genera and species present in mice are not detected in humans and vice versa (Ley *et al.*, 2005). With a view to addressing this, and to thus improve the murine model, (Turnbaugh *et al.*, 2009)used an animal model of the human gut ecosystem by transplanting human faecal microbial communities into germ-free mice. Then this demonstrated how these humanized animals can be utilized to conduct controlled proof-of-principle "clinical" metagenomic studies of host-microbiome interrelations. It was established that all bacterial phyla, 11 of 12 bacterial classes, and 88% (58 of 66) of genus-level taxa detected in the sample from the human donor were present in the recipient mice. Furthermore, the genera that were absent from the humanized mice were those present at low abundance (0.008% on average) in the donor sample. It was also established that the human gut microbiota could be successfully transferred from the humanised mice to germ-free recipient mice without a significant drop in diversity. Use of these model animals, and examination of the gut microbial community thereof, revealed that the gut microbiota in those consuming a 'western' diet contained a higher proportion of the Firmicutes classes Erysipelotrichi and a lower proportion of Bacteroidetes and *Bacillus* than did the microbiota of those in receipt of a to low fat/plant polysaccharide diet.

1.4 THE COMPOSITION OF THE GUT MICROBIOTA OF LEAN AND OBESE HUMANS

Mice are useful models in that they can be housed in controlled environments and fed specific diets. For obvious reasons, human studies lack these levels of control, and thus shifts in the associated microbiota can be considerably more variable. However, any shifts in populations deemed to be of importance with respect to weight gain ultimately need to be validated through human studies.

1.4.1 Gut microbiota of lean and obese adult humans

The gut microbiota of lean and obese individuals was compared in 2006 by (Ley *et al.*) through 16S rRNA sequencing of DNA extracted from faecal samples. It was revealed that the Bacteroidetes and Firmicutes divisions again dominated the microbiota (92.6%) but with obese individuals possessing a lower proportion of Bacteroidetes and higher levels of Firmicutes than their lean counterparts, thus resembling the patterns established in previous murine studies (Ley *et al.*, 2005). In 2009, (Turnbaugh *et al.*) characterized the gut microbiota of 154 individuals, consisting of monozygotic or dizygotic twins and their mothers. The study revealed that the composition of the gut microbiota is more similar between family members than unrelated individuals. However, it was also evident that each individual's gut microbiota was distinct and that a similar degree of co-variation existed between adult monozygotic and dizygotic twin pairs. Notably with respect to the topic of this review, it was also apparent that there was a lower proportion of Bacteroidetes in the gut of obese individuals compared to their lean counterparts, that the proportions of Actinobacteria was elevated in obese individuals and that the microbial population was in general less diverse. Notably, unlike the previous study, no significant difference in proportions of Firmicutes was apparent when the gut microbiota of lean and obese individuals was compared.

Indeed, considerable debate continues regarding the significance of the Firmicutes and Bacteroidetes proportions with respect to obesity in humans. In 2009 (Schwiertz *et al.*) studied lean and obese volunteers of both

sexes and assessed the associated faecal microbiota. Firmicutes belonging to the *Clostridium leptum* and the *Clostridium coccoides* groups as well as *Bacteroides* spp*.* (phylum Bacteroidetes) were the most abundant bacterial groups in general*.* In contrast to the studies cited above, it was noted that proportions of the genus *Bacteroides* were greater in overweight volunteers than lean and obese volunteers (p=0.002 and p=0.145, respectively) whereas the *Ruminococcus flavefaciens* subgroup, *C. leptum* group, *Methanobrevibacter* and the genus *Bifidobacterium* was less abundant in overweight and obese subjects. The significance of the proportion of Bacteroidetes and Firmicutes present was also questioned by (Duncan *et al.*, 2007) when they found that weight loss did not change the relative proportions of the *Bacteroides* spp., or the percentage of Firmicutes present, in the human gut. The composition of the gut microbiota of both African Americans and Caucasian Americans was also investigated by (Mai *et al.*, 2009). Of greatest relevance to this review is the observation by Mai *et al.,* that while a number of diet related trends were observed i.e. individuals that consumed high levels of fat had fewer Clostridia, lactic acid bacteria levels were higher in subjects that consumed fibre, and levels of *Clostridium* cluster XIVa were elevated in subjects with a higher intake of heterocyclic amines (HCAs), none of these changes were statistically significant. Similarly, no association between the body mass index (BMI) and the proportions of Bacteroidetes and *Clostridium* cluster XIVa was detected. Finally, in 2009 (Armougom *et al.*) assessed the gut microbiota of obese, lean and patients suffering from anorexia nervosa. The study revealed that the proportion of Firmicutes present in the gut of all three groups was similar, the proportion of Bacteroidetes was reduced in obese individuals and that *Methanobrevibacter smithii* was present in higher proportions in the gut of the anorexic group. A recent development in the area of human gut microbial composition is the potential discovery of distinct clusters or enterotypes in the human microbiome using sequencing data from thirty three gut microbiomes across different nationalities (French, Spanish, Italian, Danish, Japanese and American) (Arumugam *et al.*, 2011). The three enterotypes can be identified on the basis of variations in the relative levels of *Bacteroides, Prevotella* and *Ruminococcus.* Enterotype 1 is enriched in *Bacteroides* and the co-occurring

Parabacteroides. These both derive energy mainly from carbohydrates and proteins by fermentation (Martens *et al.*, 2009). Enterotype 2 is enriched in *Prevotella* which co-occurs with *Desulfovibrio.* These are known to operate in synergy to break down mucin glycoproteins (Wright *et al.*, 2000). Enterotype 3 is the most common enterotype and is distinguished on the basis of an enrichment in the levels of *Ruminococcus* and the co-occurring *Akkermansia*, both of which contain species capable of degrading mucins (Derrien *et al.*, 2004). The relationship between diet and enterotype is addressed below.

1.4.2 Impact of bariatric surgery on the gut microbiota

Bariatric surgery is increasingly employed as an anti-obesity treatment, and for a morbidly obese patient it is the only option available that can deliver substantial and sustained weight loss (Buchwald *et al.*, 2004). The surgery can be performed in a number of different ways in that, in some cases, it involves a reduction in the size of the stomach using a gastric band, in others a portion of the stomach is removed, while another option involves the creation a small stomach pouch and resecting/re-routing it to the small intestine. The impact of such surgery on the composition of the gut microbiota of patients has been investigated through a comparison of the gut microbiota of 3 obese, 3 lean and 3 post gastric bypass individuals (Zhang *et al.*, 2009b). The investigation established that the gut microbiota of individuals who had undergone gastric bypass differed from that of both the obese and lean individuals by virtue of an increase in proportions of Gammaproteobacteria (including the *Enterobacteriaceae*) and *Fusobacteriaceae* and a proportional decrease in *Clostridia*. In addition, the gut microbiota of lean individuals contained elevated proportions of sequences corresponding to the *Lachnospira* (order Clostridiales) compared to that of obese and gastric bypass individuals. It was also noted that the gut microbiota of obese individuals contained a lower proportion Verrucomicrobia and a higher proportion of Archaea relative to the other two groups. Finally, Methanobacteriales was found in all obese individuals but in only one gastric bypass patient (Zhang *et al.*, 2009b).

Another recent paper also focused on this topic but incorporated a larger number of individuals, i.e. 30 obese individuals who had undergone bariatric surgery and 13 lean volunteers, and instead relied on a qPCR-based analysis (Furet *et al.*, 2010). The investigation established that after surgery the levels of *Bacteroides*/*Prevotella* were higher than they had been prior to surgery. This increase brought the *Bacteroides*/*Prevotella* more closely in line what that observed in the lean controls. An increase in *Escherichia coli* and *Faecalibacterium prausnitzii* was also noted when post-surgical samples from individuals were compared with their pre-surgery equivalents. The relatively low levels of *F. prausnitzii* in pre-surgical samples was more apparent in the obese diabetic cohort than in their obese non diabetic counterparts. In contrast, decreases in levels of the combined *Lactobacillus*/*Leuconostoc*/*Pediococcus* group and in *Bifidobacterium* sp. were apparent in post-, relative to pre-, surgical samples. Body weight, BMI, body fat mass and leptin concentration all negatively correlated with *Bacteroides*/*Prevotella* and *E. coli* but positively correlated with *Bifidobacterium* populations. The *F. prausnitzzi* population strongly negatively correlated with changes in inflammatory markers and orosomucoid serum levels. While these results are interesting and suggest that this area requires further attention, the possibility exists that the roux-en-Y gastric bypass procedure may contribute to changes in gut microbial composition as a consequence of the associated change in pH and the downstream delivery of bile acids (Furet *et al.*, 2010).

Finally, in one case the impact of bariatric surgery on the microbial composition of wistar rats was assessed. Although differing from the previous studies by virtue of its reliance on an animal model, an increase in Gammaproteobacteria was again apparent. However, in this instance a reduction in Firmicutes and Bacteroidetes was observed, (Li *et al.*, 2011) the significance or cause of which is not known.

1.4.3 Gut microbiota of normal and overweight pregnant women

The gut microbiota of normal weight and overweight pregnant women was investigated by (Collado *et al.*, 2008). In addition to being of interest with respect to the health and weight of the mother, such investigations are also

of importance given that, in cases of natural delivery, the child is first colonized by microbiota from the mother (Langhendries, 2005). While an overall increase in the number of bacteria was observed between the first and third trimester in both groups, significant differences between the gut microbiota composition of pregnant women of different weight were noted. More specifically, higher numbers of representatives of the *Bacteroides* group and *Staphylococcus aureus* were recorded in overweight individuals. Indeed a one kilogram gain in weight correlated with a corresponding increase in *Bacteroides* numbers by 0.006 log units. The levels of the *Clostridium* group increased in overweight women from the first trimester to the third (p=0.054). *Bifidobacterium* proportions were higher in women who exhibited a relatively lower weight gain during pregnancy. Finally, it was also noted that overweight women tended to give birth to heavier infants. More recently, *(Santacruz et al., 2010)* investigated the faecal microbiota of 50 pregnant women who were assigned into one of two groups, i.e. overweight or normal weight, based on their BMI. As with the Collado study, higher numbers of *Staphylococcus* and lower numbers of *Bifidobacterium* were noted in overweight women. However, in contrast with the previous study, *Bacteroides* numbers were found to be lower in overweight women. Increased numbers of *Enterobacteriaceae* in general and of *E. coli* in particular, were also associated with overweight women. The gut microbiota of women who gained excessive weight during pregnancy underwent similar increases and decreases in microbial numbers as were associated with overweight women. *(Santacruz et al., 2010)* also investigated the relationship between gut microbiota composition and metabolomic parameters. It was found that increased *Staphylococcus* numbers corresponded with increased serum levels of cholesterol, a rise in numbers of *Enterobacteriaceae* and *E. coli* was linked with increased levels of serum ferritin, saturation transferrin index and decreased levels of transferrin, while greater numbers of *Bifidobacterium* correlated with reduced levels of ferritin, saturation transferring index and increased levels of transferrin and folic acid. Finally, increased *Bacteroides* numbers were associated with increased levels of high density lipoprotein (HDL) -cholesterol, folic acid and lower levels of triacylglycerol (TAG).

1.4.4 Gut microbiota of lean and obese children

The WHO estimated that in 2010 there were over 42 million overweight children under the age of five worldwide (WHO, 2010). The alarming increase in obesity rates in children has lead to a particular interest in investigating the gut microbiota of lean and obese children. A seven year study to investigate the composition of the faecal microbiota of children, published by (Kalliomaki *et al.*, 2008) found that normal weight development was linked to a lower number of faecal *S. aureus* number and a higher number of bifidobacteria relative to those present in the feces of overweight children. The *S. aureus* finding is doubly interesting in the context of the increase in number of *Staphylococcus* in the gut of overweight pregnant women referred to above. (Balamurugan *et al.*, 2009) noted that obese and non obese Indian children had similar dietary intakes of energy and, thus, it was apparent that other factors were at play. Thus the nature of the dominant faecal microbiota within each group was investigated. Although this revealed that there were no significant differences with respect to the levels of the *Bacteroides-Prevotella*, *Bifidobacterium*, *Eubacterium rectale* or *Lactobacillus acidophilus* groups in the gut, it did reveal that, unlike the aforementioned study by (Furet *et al.*, 2010) the obese subjects had significantly higher levels of *F. prausnitzii*, a representative of the Firmicutes which can ferment unabsorbed carbohydrate. It was thus postulated that the presence of this bacterium in greater numbers in obese children could lead to increased energy extraction from carbohydrate that would not otherwise contribute to dietary energy intake. (Luoto *et al.*, 2011) analysed the faecal microbiota of a group of children over 10 years. At 3 months of age there was no statistically significant difference in the faecal bacterial counts of the children. However children who were overweight by the time they reached age 10 years tended to have lower bifidobacterial numbers in their faeces when it were assessed at 3 months of age. Interestingly 10 year old normal weight children had significantly higher mean concentrations of serum-soluble innate microbial receptor (sCD14) than overweight children. sCD14 is involved in innate immunity and its expression is increased by the presence of LPS and fatty acids that resemble the lipid portion of LPS (Manco *et al.*,

2010). It was also noted that mothers of children who were normal weight at age 10 years had statistically significantly higher mean concentrations of adiponectin in maternal colostrum than mothers of overweight children. Adiponectin is a protein hormone secreted from adipose tissue (Gavrila *et al.*, 2003) and the placenta (Chen *et al.*, 2006) into the blood stream. It has an important role in glucose regulation and fatty acid metabolism (Okamoto *et al.*, 2006, Tsatsanis *et al.*, 2006) and provides protection against metabolic syndrome (Li *et al.*, 2009) as well as having antiatherogenic and anti-inflammatory properties (Fantuzzi, 2005). Diabetics and obese individuals have low levels of adiponectin (Gavrila *et al.*, 2003).

1.5　IMPACT OF DIET ON THE GUT MICROBIOTA

Until recently, the relationship between diet, microbes and, in turn, optimal health has remained obscure. However, a number of recent studies have investigated the primacy of diet amongst lifestyle factors that influence the composition of the gut microbiota. That which is known with respect to the impact of diet on the gut microbiota is summarised in Table 3.

1.5.1　Low carbohydrate/calorie diets

The effects of a fat restricted or carbohydrate restricted low calorie diet randomly assigned to 12 obese people has been investigated by (Ley *et al.*, 2006). While it was established that over time the relative abundance of Bacteroidetes increased and the abundance of Firmicutes decreased, these changes appeared to be irrespective of the low calorie diet consumed. (Duncan *et al.*, 2007) also studied the effect of an altered carbohydrate intake on the gut microbiota. They recruited 19 obese, but otherwise healthy individuals and allocated them to 3 different diets i.e. a maintenance diet, a high protein/medium carbohydrate diet (HPMC) and a high protein/low carbohydrate diet (HPLC). The investigation established that bacterial numbers were greatest in individuals on the maintenance diet and that the Gram negative *Bacteroides* and the Gram positive *C. coccoides* were the most abundant bacterial groups (approximately 29% and 22% of total bacteria respectively) in all cases. It was apparent that the bifidobacteria, *Roseburia* spp. and *E. rectale* from the *Clostridium* group were all negatively impacted upon by decreased carbohydrate intake. The consumption of the HPMC and HPLC diets also resulted in the lowering of the Short Chain Fatty Acid (SCFA) concentrations, with butyrate concentrations being most dramatically reduced.

The influence of an obesity treatment program on the gut microbiota and body weight of overweight adolescents has been examined by (Santacruz *et al.*, 2009). The participants in this study were subjected to a calorie restricted diet and increased physical activity programme over 10 weeks. After the treatment, a group of subjects, experiencing a >4kg weight loss and showing significant BMI reductions, was identified. The remaining

individuals lost <2.0kg in weight despite the fact that there were no significant differences in the dietary intake of the two groups. In general, the treatment led to an increase in *Bacteroides fragilis* and *Lactobacillus* groups and a decrease in the *C. coccoides*, *Bifidobacterium longum* and *Bifidobacterium adolescentis* numbers. The post intervention microbiota shifts were most significant in individuals that responded more successfully to treatment. It was also noted that *Bifidobacterium bifidum,* the *C. coccoides* group, the *Lactobacillus* group, *Bifidobacterium* and *Bifidobacterium breve* were significantly lower in the high weight loss group compared with the low weight loss group before and after the treatment. Conversely total bacteria, the *B. fragilis,* the *C. leptum*, and the *Bifidobacterium catenulatum* groups were significantly higher in the more abundant weight loss group before and after treatment (Santacruz *et al.*, 2009). In another such study by (Nadal *et al.*, 2009) the adolescent obesity treatment programmes incorporated nutritional and individual diet counselling, calorie restriction and increased physical activity over a ten week period. The maximum energy intake permitted was 1,800 kcal/d for females and 2,200 kcal/d for males. Most of the participants experienced significant weight loss ranging from 4.1 to 16.6kg after the 10 week period. Overall, the intervention programme led to reductions in the proportions of *Clostridium histolyticum* and, in line with previous investigations, *E. rectale-C. coccoides*. A correlation between *C. histolyticum* and *E. rectale-C. coccoides* proportions and BMI was also evident. *Bacteroides* proportions increased as a consequence of the intervention and almost achieved significant levels of correlation with weight loss. Although the *Lactobacillus-Enterococcus* populations also increased as weight and BMI correlations decreased, these correlations were not significant. In the group which did not experience a significant loss in weight (<2.5kg), the bacterial groups analyzed did not differ significantly as a consequence of the intervention programme. No correlations were detected between bacterial proportions and either body weight or BMI reductions in this low weight loss group. Unsurprisingly, a number of animal studies have also taken place. The caecal microbial composition of high fat fed and control mice was quantified by (Cani *et al.*, 2007). They found that, in the high fat diet mice, *Bacteroides*-like microbes were significantly reduced compared

with controls. Furthermore, although the *E. rectale-C. coccoides* group were found to be the dominant microbiota, a reduction in this group, and of *Bifidobacterium*, relative to controls was also noted.

In a study discussed briefly above (Turnbaugh *et al.*, 2008) took an alternative approach to investigate the relationship between diet and the gut microbiota. More specifically, conventionally raised (CONV-R; i.e. mice which have been allowed to acquire their microbiota naturally from birth) mice were weaned onto a 'western' or a low-fat chow diet rich in structurally complex plant polysaccharides (CHO diet) for 8-9 weeks. Mice on the 'western' diet unsurprisingly gained more weight and had a significantly larger adiposity. The researchers then carried out investigations to determine if the gut microbiota of these DIO animals possessed attributes that can more successfully increase host adiposity than the microbiota of CHO fed animals. This involved the transplantation of the caecal microbiota of the lean and obese animals to GF, CHO-fed recipients. Recipients of the DIO associated microbiota brought about a significantly greater proportional increase in body fat compared to the recipients of the CHO-associated microbiota was capable of. (Turnbaugh *et al.*, 2008) also tested the impact of defined shifts in diet on the body weight, adiposity and distal gut microbial ecology of obese mice. CONV-R mice were fed either a 'western' diet, a 'western' diet with reduced carbohydrates (CARB-R) or a 'western' diet with reduced fat (FAT-R). Mice on CARB-R or FAT-R diets had reduced Mollicutes levels and an increased abundance of Bacteroidetes. It would thus seem that both FAT-R and CARB-R diets repress the multiple effects associated with 'western' diet induced obesity.

The impact of fasting on the gut microbiota of hibernating animals has also been the subject of investigation. (Sonoyama *et al.*, 2009) compared the gut microbiota of male Syrian hamsters which were separated into a (i) fed, active, non-hibernating group, (ii) a fasted, active, non hibernating group and a (iii) hibernating group, with the latter being housed in constant darkness at 4ºC in order to bring on hibernation. It was established that the total bacterial populations were significantly reduced in fasted active hamsters when compared with fed active and hibernating hamsters, whereas there was no significant difference between the latter two groups. HPLC analysis of the

caecal contents also showed that fasted active hamsters had significantly lower concentrations of total SCFA and acetic acid than fed active or hibernating hamsters. A 16S rRNA-based investigation of caecal bacteria showed that the class Clostridia was the most abundant taxonomic group in all treatment groups but that the proportion of the Clostridia in fasted active hamsters tended to be lower than that in fed active and hibernating hamsters. Verrucomicrobia and Proteobacteria were the second and third most abundant groups. Notably, all Verrucomicrobia-associated sequences in fasted active hamsters were classified to the genus *Akkermansia*. The family *Desulfovibrionaceae* was the most common family in the phylum Proteobacteria and the proportion of this family was higher in fasted active hamsters than in the other two groups. It has been suggested that fasting stimulates the growth of sulfate-reducing bacteria such as *Desulfovibrio* spp. through increased degradation of mucins by *A. muciniphila* in the cecum of fasted active hamsters. Overall the results suggest that gut microbiota respond differently to fasting and hibernation in Syrian hamsters.

An examination of humanized mice fed a 'western' diet revealed an increased representation of the Erysipelotrichi class of bacteria or, more specifcially, *Clostridium innocuum, Eubacterium dolichum,* and *Catenibacterium mitsuokai*, in the faecal samples of these animals compared to those of mice fed a low fat/plant polysaccharide (LF/PP) diet (Turnbaugh *et al.*, 2009). A significant increase in the relative abundance of another class of Firmicutes, the Bacilli (corresponding primarily to *Enterococcus* sp.), was also associated with the 'western' diet. These increases were apparent along the entire length of the gut. A significant decrease in the representation of members of the Bacteroidetes in 'western' diet fed mice was also apparent. A parallel assessment of the gene composition of the gut microbiome revealed an obvious shift within 1 day of the switch to the 'western' diet in the form of the enrichment of ATP-binding cassette transporters and phosphotransferase systems. The microbiome associated with the LF/PP diet was enriched for pathways including N-glycan degradation, sphingolipid metabolism, and glycosaminoglycan degradation, all of which are pathways which are also enriched in Bacteroidetes.

The effects of a LF/PP and 'western' diet on two dominant phyla has been investigated by (Mahowald *et al.*, 2009). In this study the authors specifically selected *E. rectale* and *Bacteroides thetaiotaomicron* as representatives of the two dominant bacterial phyla, Firmicutes and Bacteroidetes, and investigated how these microbes were affected by changes in the host diet. Co-colonised mice were fed one of 3 diets, i.e. a standard LF/PP, a high fat, high sugar 'western-type' diet (HF/HS) or a low fat, high sugar, control diet (LF/HS). *B. thetaiotaomicron* was not affected by diet but colonisation by *E. rectale* was significantly reduced in mice fed either the LF/HS or HF/HS diets. The authors propose a number of explanations for this occurrence, i.e. (i) *E. rectale* does not possess the glycoside hydrolase and polysaccharide lyases that can process host glycans, (ii) it cannot use the sugars that are derived from mucosal polysaccharides and/or (iii) the glycobiome of the host includes enzymes that can directly process the simple sugars in these two diets. Transcriptional profiling of *B. thetaiotaomicron* revealed that it significantly up-regulated polysaccharide utilization loci (PULs) involved in breakdown of host polysaccharides and down-regulated PULs involved in breakdown of plant polysaccharides when mice were subjected to a HF/HS or LF/HS diet. *E. rectale* responded to the HF/HS and LF/HS diets by down-regulating several glycoside hydrolases and sugar transporters (Mahowald *et al.*, 2009).

The impact of a high fat diet on the gut microbiota was again examined by (Zhang *et al.*) 2009. In contrast to animals fed a normal chow diet (NC), it was noted that apolipoprotein A1 (*Apoa*-I) knockout and wild type mice fed a high fat diet (HFD) lacked *Bifidobacteriaceae* in their faeces. *Apoa*-I knockout mice were included in the study as they have been shown to have impaired glucose tolerance (IGT) and increased body fat (Han *et al.*, 2007). After 25 weeks on a HFD the WT mice also exhibited IGT. The family *Desulfovibrionaceae* was more prevalent in *Apoa*-I[-/-] on NC or HFD and WT fed a HFD than WT NC control mice. An examination of the microbiota of the mice failed to identify phylum-wide changes associated with IGT/obesity. It was apparent, however, that diet and host health can have different effects on lineages within the family more specifically, levels of *Erysipelotrichaceae* from the Class Mollicutes. The family *Erysipelotrichaceae* of the class

Mollicutes can be subdivided into four phylogenetic clusters i.e. M1, M2, M3 and M4. The M1 cluster were reduced in *Apoa*-I$^{-/-}$ and HFD mice. Levels of the M2 cluster increased in HFD mice, the M3 cluster was severely diminished in the HFD mice and the M4 cluster was only present in the HFD mice. WT mice fed a HFD were the most obese group after the 25 week trial.

Finally, it is notable that the impact of diet on a combination of ten human gut bacteria has been the subject of a recent investigation (Faith *et al.*, 2011). Strains of *Blautia hydrogenotrophica, Bacteroides ovatus, Bacteroides caccae, B. thetaiotaomicron, Clostridium symbiosum, Collinsella aerofaciens, Desulfovibrio piger, E. rectale*, *E. coli* and *Marvinbryantia formatexigens* were introduced into the gut of germ free mice which were then provided with refined diets which changed every two weeks. Each diet systematically varied the concentrations of four ingredients, i.e. casein, corn oil, cornstarch and sucrose. It was revealed that changes in diet impacted on the relative abundance of the various species. In particular, it was noted that the abundance of all ten species was significantly associated with casein, i.e. seven species showed positive correlation to increasing casein while the abundance of the others (*E. rectale, D. piger* and *M. formatexigens*) decreased with increased casein levels. A parallel increase in the expression of pathways associated with amino acid metabolism was apparent in the seven species with which a positive correlation with casein existed (Faith *et al.*, 2011).

1.5.2 Other dietary-related influences

In addition to digestible carbohydrate, the impact of other dietary components on the gut microbiota has also been investigated. In one case the impact of controlled changes in the main type of non-digestible carbohydrate components upon the microbial community of fourteen overweight humans was examined (Walker *et al.*, 2011). A significant increase in the percent of *Ruminococcus bromii*-like bacteria was apparent in individuals consuming a resistant starch (RS) diet relative to those in receipt of a non-starch polysaccharide (NSP) diet. A significant increase in *Oscillibacter valericigenes*-like bacteria was also noted in individuals on RS and a reduced carbohydrate/high protein (WL) diet compared to those fed a

maintenance or NSP diets (Walker *et al.*, 2011). Finally, *Roseburia* and *E. rectale* were also significantly increased among individuals consuming RS diets but were decreased in individuals consuming a WL diet (Walker *et al.*, 2011).

New evidence has also demonstrated a link between dietary fat and the metabolism of the intestinal microbiota with atherosclerosis (Wang *et al.*, 2011). It has been reported that the atherosclerosis-associated upregulation of two macrophage scavenger receptors, CD36 and SR-A1, occurs in mice that have choline, trimethylamine *N*-oxide (TMAO) or betaine (three metabolites of the lipid phosphatidylcholine [PC]) added to their diet (Kuchibhotla *et al.*, 2008) and that supplementation with choline and TMAO promotes the formation of atherosclerosis in mice (Wang *et al.*, 2011). The link between these phenomena and the gut microbiota was made by comparing the microbiota of mice in receipt of choline or PC with or without antibiotics. Mice fed a diet supplemented with 1% choline displayed augmented atherosclerosis but this impact was lessened in mice on the same diet but which were treated with broad spectrum antibiotics. Similarly the presence of the gut microbiota is required for the formation of TMAO. More specifically, the formation of TMAO is observed in mice in receipt of PC or choline but is suppressed in their antibiotic-treated mice equivalents (Wang *et al.*, 2011).

Using a three stage colonic model the impact of a high dietary fibre intake was examined with relation to the effect on microbial composition (Shen *et al.*, 2011). An increase in dietary fibre resulted in a significant increase in the numbers of *Bifidobacterium*, *Ruminococcus* and *Lactobacillus-Enterococcus* group in an in vitro three stage colonic model. High fibre intake in the vessel representing the proximal colon significantly increased the numbers of *F. prausnitzii* and *E. rectale – C. coccoides* groups.

The impact of diet on the microbiota is also very much evident when the microbiota of omnivores, herbivores and carnivores (thirty three mammals and eighteen humans) is compared (Muegge *et al.*, 2011). Principal coordinate analysis (PCoA) plots of both bacterial 16S rRNA and whole community gene data sets separated carnivores and omnivores from herbivores. Twelve amino acids biosynthetic enzymes were enriched in

herbivores whereas as no such enrichment was apparent among carnivores. In contrast, the enrichment of nine amino acid degradation pathways was observed in carnivores. PCoA plots of diet and the human microbiome revealed total protein intake was significantly associated with KEGG orthology (KO) data whereas, insoluble dietary fibre was significantly associated with bacterial operational taxonomic units (OTU) content (Muegge *et al.*, 2011).

Finally, in a recent study of 98 individuals, the effects of diet on the gut microbial enterotypes was examined (Wu *et al.*, 2011). It was noted that only long term diet correlated with enterotypes. Short term controlled identical feeding was shown not to affect intersubject variation. A food frequency questionnaire found that the *Bacteroides* enterotype was highly associated with animal protein. In contrast *Prevotella* enterotype was linked to high carbohydrate and simple sugars. Vegans (n=1) and vegetarians (n=11) were enriched in the *Prevotella* enterotype.

1.6 MANIPULATION OF THE GUT MICROBIOTA

In addition to the aforementioned studies, which have highlighted the impact of the overall levels of carbohydrate and/or fat on the composition of the gut microbiota, the impact of other specific components of diet, i.e. food additives, probiotics and prebiotics, have also been investigated.

Grain sorghum is an abundant source of phytochemicals that are of possible benefit to health (Carr *et al.*, 2005). (Martinez *et al.*, 2009) used the hamster model of hypercholesterolemia to investigate if changes in the gut microbiota are linked with the positive effects of grain sorghum lipid extract (GSL) on cholesterol metabolism. The hamsters' diet was supplemented with 0%, 1% or 5% GSL. Although high animal-to-animal variability at both the family and genus level was apparent, it was established that one family, the *Coriobacteriaceae*, and two genera, both of which were unclassified members of the family *Erysipelotrichaceae,* were significantly reduced in abundance as a consequence of the addition of GSL to the hamsters' diet. Furthermore, 5% GSL was shown to reduce the overall diversity of the gut microbiota. In contrast, levels of the genus *Pseudoramibacter* and *Allobaculum* increased with increased GSL levels. A significant increase in *Bifidobacterium* in hamsters fed GSL was also observed and this increase was positively linked with high-density lipoprotein (HDL) plasma cholesterol levels ($r = 0.75$, $p=0.001$). GLS feeding caused a decrease in the proportion of *Coriobacteriaceae*. This decrease in *Coriobacteriaceae* showed a strong correlation with non-HDL plasma cholesterol ($r = 36$ 0.84, $p=0.0002$). These findings suggest that GLS feeding influences the HDL/non-HDL equilibrium via a mechanism that appears to be through the alteration of the intestinal microbiota. Overall this analysis would imply that bifidobacteria are beneficial and *Coriobacteriaceae* are detrimental with respect to plasma cholesterol levels in hamsters.

The impact of probiotics on the composition of the gut microbiota has been the focus of particularly great attention in recent years. Probiotics are defined by the food and agriculture organisation of the United Nations and the WHO as "*live microorganisms which when administered in adequate amounts confer a health benefit on the host*" (WHO/FAO, 2001). Notably,

(Schiffrin *et al.*, 2009) carried out an investigation to determine if administration of a probiotic containing yoghurt can ameliorate or treat small intestinal bacterial overgrowth (SIBO) by improving gut barrier function or as a consequence of their antibacterial, immunomodulatory and/or anti inflammatory effects. SIBO most frequently occurs in older people and can lead to chronic diarrhoea, anorexia and nausea as well as malabsorption and malnutrition (Donald *et al.*, 1992, Riordan *et al.*, 1997). In the study, 23 elderly subjects with a positive glucose/H2 breath test (SPH) and 13 subjects with a negative test (SNH) were administered the probiotic yoghurt *Lactobacillus johnsonii La1* for 4 weeks. The glucose/H2 breath test measures the excretion of hydrogen in the breath which is reflective of glucose metabolism by microbes in the intestine. Patients with a positive result are suspected of having SIBO (Parlesak *et al.*, 2003, Romagnuolo *et al.*, 2002). After 4 weeks of yoghurt consumption, the authors noticed a trend towards a reduction of endotoxin concentration in the SPH group and a significant decrease in plasma endotoxin was also noted in the SNH after probiotic consumption. In conclusion, Schiffrin and colleagues suggest that an altered intestinal ecology underlies the low grade inflammatory status that favours catabolism and loss of lean body mass in the elderly and, thus, redressing such ecological imbalances could provide health benefits.

In 2009, (Hamad *et al.*) investigated the ability of milk fermented by *Lactobacillus gasseri* SBT2055 (LGSP) to impact on adipocyte size by inhibition of dietary fat absorption in Zucker rats. Obese Zucker rats have a spontaneous mutation in the leptin receptor gene causing early onset severe obesity due to over-eating (Chua *et al.*, 1996, Zucker and Zucker, 1961). Rats fed a LGSP diet had reduced total, mesenteric and subcutaneous adipose tissue masses compared with those fed a control skimmed milk diet (SM). No significant effect was observed on other white adipose tissue. The mesenteric fat mass in lean rats was reduced even more dramatically than that of obese rats fed the LGSP diet ($p<0.05$). The LGSP diet also had a significant effect on serum leptin concentrations which were decreased by 36% in lean rats but were not significantly altered in obese rats. However, the LGSP diet had no effect on the serum levels of both glucose and adiponectin in lean or obese rats. The LGSP diet caused a significant

reduction in the levels of total and HDL-cholesterol in serum and a significant increase in faecal cholesterol in both groups. Overall, the results of this study revealed that the milk fermented by *L. gasseri* reduced visceral adipose tissue mass and adipocyte hypertrophy in lean Zucker rat through a decrease in fatty acid absorption (Hamad *et al.*, 2009).

The benefits of consuming prebiotics have also been the focus of ever greater attention in recent years. Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health" (Gibson and Roberfroid, 1995). Cani and colleagues hypothesised that the control of gut permeability through the selective modulation of gut microbiota by prebiotics helps to protect *ob/ob* mice from metabolic diseases and carried out investigations which revealed that mice fed the prebiotic (oligofructose) had lower levels of the cytokines TNF-α, IL-1b, IL1-α, IL-6 and INFγ, all of which are known to promote tight-junction disruption (Cani *et al.*, 2009). This study also showed that altering the gut microbiota through the increase in abundance of *Bifidobacterium* spp*.* through the use of prebiotics is linked with a notable reduction of gut permeability. The improved gut barrier of *ob/ob* prebiotic (Ob-Pre) fed mice also correlated with lower plasma LPS levels and inflammatory tone. A decrease in markers of oxidative and inflammatory stress in liver tissue significantly correlated with the lowering of systemic inflammation by prebiotics. Overall these data suggest that prebiotics could act favourably on the gut barrier, hence improving metabolic disorders (Cani *et al.*, 2009). The authors also noted that when the gut microbiota is changed using prebiotics, an increase in endogenous production of the glucagon-like peptide-2 (GLP-2) occurs which may explain the associated improvement in intestinal barrier function. Evidence for this was provided when it was established that a GLP-2 antagonist completely blocked the positive effects of prebiotic treatment on both intestinal tight-junction proteins and proglucagon mRNA (Cani *et al.*, 2009). The effect of inulin-type fructans (ITFs) with prebiotic properties on the gut microbiota of animals fed a high fat diet was investigated by (Dewulf *et al.*, 2010). While it was established that caecal content was significantly reduced in mice fed a

high fat (HF) diet relative to that of mice fed a control (CT) diet, this effect was reversed in mice co-administered ITFs with the HF diet. Total bacterial numbers were reduced in HF fed mice relative to CT fed animals while qPCR revealed a 100 fold increase in bifidobacteria numbers and a reduction in *Roseburia* spp. and *Clostridium* cluster XIVa in ITF-HF mice compared to CT and HF fed mice. Notably, weight gain and subcutaneous adipose tissue accumulation was reduced in ITF treated HF mice even though they ingested fat to a level similar to that for non-ITF treated HF mice (Dewulf *et al.*, 2010).

The consequences of consuming different concentrations of prebiotic fibre (inulin and oligofructose), over a ten week period, on the gut microbiota of lean and obese rats was the subject of another recent investigation (Parnell and Reimer, 2011). Obese and lean rats were broken into three diet groups: controls (C), 10% prebiotic fibre (LF) and 20% prebiotic fibre (HF). In control rats the levels of *Bacteroides/Prevotella*, *C. leptum* and *Enterobacteriaceae* as well as the overall bacterial population were greater in lean than obese rats. Obese rats on the HF diet had significantly increased levels of total bacteria, *Bacteroides/Prevotella*, *C. leptum*, *Lactobacillus*, *Bifidobacterium* and *Enterobacteriaceae* when compared with obese controls. Meanwhile lean rats fed a HF diet had significantly increased *Bacteroides/Prevotella* and *Bifidobacterium* populations while a significant decrease in the *C. leptum* and *C. coccoides* populations was apparent. No statistically significant difference was seen between LF diet rats and controls. Percentage body fat, body weight, fasting insulin, insulin, incremental area under the curve (iAUC) and energy intake negatively correlated with *Bacteroides* and total bacteria. Total energy intake, glucose iAUC, body weight and fat positively correlate with *Lactobacillus* spp. The population of *Enterobacteriaceae* increases with increases in glucose iAUC and GLP-1 total area under the curve (tAUC). A positive correlation was noted between *Bacteroides* and total bacteria and ghrelin tAUC.

Despite these promising results, there has been some debate recently, prompted by a proposal by Prof Didier Raoult that a link may exist between probiotics and obesity (Raoult, 2009). Prof Raoult has suggested that there are dangers associated with promoting the consumption of products containing bacteria that have been associated with weight gain in

the animal food industry. Raoult's opinion is based on four points: 1. (Ley *et al.*, 2006) found that the gut of obese individuals contained more Firmicutes than that of lean individuals, 2. The farming industry uses probiotics which can contain Firmicutes, including *Lactobacillus* spp., *Bifidobacterium* spp. and *Enterococcus* spp. as growth promoters. 3. *L. acidophilus*, at levels equivalent to those found in functional foods, causes weight gain in piglets and, finally, 4. *Lactobacillus* species have shown to cause weight gain in children (Chouraqui and Leclaire, 2008). This view has been rejected in responses by other scientists (Delzenne and Reid, 2009, Ehrlich, 2009) and, indeed the arguments made by Dr Raoult do not accurately reflect the data present in the papers he has cited, or consider the data presented by other papers, some of which have already been discussed in this review. Most notably, lactobacilli represent just a small fraction of Firmicutes in the gut. Furthermore, Raoult's editorial refers to *Bifidobacterium* as a Firmicute, when in fact it belongs to the phylum Actinobacteria. In addition, in the studies cited by Raoult, (Chouraqui and Leclaire, 2008, Guandalini *et al.*, 2000) probiotics did not significantly impact on weight gain in children. Finally, in a recent review (Simon, 2005) it was revealed that only 3 out of 22 studies showed a significant weight gain in piglets.

Many antibiotics used to treat humans have a broad spectrum of activity which facilitates the treatment of infections of unknown aetiology. As these antibiotics are not selective in their killing, there are associated impacts on the natural biota of the human gut. In this instance the importance of the gut microbiota with respect to obesity and metabolism was investigated through disruption of the gut microbiota by treatment with ampicillin or neomycin (Cani *et al.*, 2008). WT mice were fed a control, control with antibiotics, high fat or high fat with antibiotics (carbohydrate free) diet for 4 weeks. *ob/ob* mice were also fed a control diet or a control diet with antibiotics. The 4 week antibiotic therapy had considerable impacts. The endotoxin content per gram of caecal content was considerably decreased after antibiotic treatment in both the control and high fat diet groups. Adiposity was also reduced in high fat, antibiotic-treated mice compared with high fat, untreated controls and, furthermore, antibiotic treatment significantly lowered plasma LPS levels, gut permeability. The occurrence of visceral

(mesentric) adipose tissue inflammation, oxidative stress, macrophage infiltration and metabolic disorders was also lowered. Microbiological analysis revealed that the high fat diet mice reduced *Lactobacillus* spp. and *Bifidobacterium* spp. numbers, but increased *Bacteroides-Prevotella* spp. numbers, compared to control mice. High-fat fed, antibiotic-treated mice had reduced numbers of all 3 of these groups compared with the high fat diet mice. Indeed the microbiota of the high-fat fed, antibiotic-treated mice had numbers of all 3 groups which were very similar to those present in control antibiotic mice. *ob/ob* mice had higher *Lactobacillus* spp. and *Bifidobacterium* spp. but lower *Bacteroides-Prevotella* numbers, than *ob/ob* mice. Unsurprisingly, antibiotic treatment dramatically changed the *ob/ob* mice gut microbiota, reducing *Lactobacillus* spp., *Bifidobacterium* spp. and *Bacteroides-Prevotella* spp. (Cani *et al.*, 2008).

1.7 MECHANISMS BY WHICH THE GUT MICROBIOTA MAY IMPACT ON OBESITY

Thus far this review has focussed on studies in which differences in the microbiota of lean and obese animals and humans and the impact of diet on these microbial populations were investigated. However, a key question is how these microbial populations are impacting on weight gain. In this section mechanisms *via* which these gut microbes can impact on obesity is discussed (see also Fig 3).

1.7.1 Energy extraction, leptin, *Fiaf* and AMPK

As noted briefly above, the gut microbiota is capable of the breakdown of otherwise indigestible components of the mammalian diet, thus affecting the energy balance. In 2004 (Backhed *et al.*) analysed germ free (GF), CONV-R and conventionalized (CONV-D) mice to examine the hypothesis that the microbiota acts through host signalling pathways to regulate energy storage in the host. GF mice are raised in the absence of any microbiota, while CONV-D mice are initially germ free but are then colonized with the microbiota from CONV-R donors. The study found that CONV-R animals had 42% more total body fat and 47% more epididymal fat pad weight than GF mice and that their epididymal fat pad weights were significantly greater. This was despite the fact that their consumption of chow was 29% less than their GF counterparts. CONV-D mice had 57% greater total body fat content and 61% greater epididymal fat pad weight than GF mice. Like CONV-R mice, CONV-D animals also consumed less food, in this case 27% less than GF mice. Increased energy expenditure by GF mice was excluded as an explanation for the decreased body fat content of GF mice as these animals were shown to have a metabolic rate that was 27% lower than either CONV-R or CONV-D mice. Further investigation revealed that microbial colonization caused an increase in leptin levels which was proportional to the increase in body fat. Leptin is mainly an adipocyte-derived hormone which reduces food intake and increases energy expenditure in mice and thus the impact of the microbiota on leptin levels may be of importance (Maffei *et al.*, 1995, Pelleymounter *et al.*, 1995). An increased in lipoprotein lipase (LPL) has also

been observed in the epididymal fat pads of CONV-D mice compared with GF mice. This is significant as LPL is a vital regulator in the release of fatty acids from lipoproteins in muscle, heart and fat (Preiss-Landl *et al.*, 2002). Notably, the Bäckhed study also showed that the presence of a microbial population in the gut promotes increased monosaccharide uptake (Backhed *et al.*, 2004). Analysis of the gut microbiome by (Turnbaugh *et al.*, 2006) also revealed that the gut microbiome of *ob*/*ob* mice had an increased capacity to ferment polysaccharides compared to the lean-associated equivalent. It was thus postulated that this could lead to more energy being extracted from complex carbohydrates, leading to increased energy in the host. Bomb calorimetry supported this theory, in that it was found that stool samples from obese mice contained less energy than their lean counterparts. To test this idea further, GF mice were colonised with the gut microbiota from *ob*/*ob* or +/+ donors. Noticeably the mice colonized with the *ob*/*ob* microbiota had a significantly greater percentage increase in body fat over 2 weeks than mice colonized with a +/+ microbiota. This prompted further investigation and in 2007 Bäckhed and colleagues again studied GF and CONV-R mice to establish if GF mice are resistant to DIO (Backhed *et al.*, 2007). After being fed a 'western' diet for 8 weeks it was again found that CONV-R mice had gained significantly more weight than their germ free counterparts and, as had been found previously (Backhed *et al.*, 2004), the epididymal fat pad weights were also significantly greater in conventionalised mice than GF mice. GF mice fed a 'western' diet for 8 weeks showed no significant weight gain when compared to GF mice on a low fat diet. However, unlike the previous cited investigation, it was found that the amounts of chow consumed by CONV-R and GF mice were similar. Notably, it was established that GF mice had higher levels of *Fiaf* (fasting induced adipose factor) expression in the intestine than CONV-R mice. *Fiaf* is a circulating lipoprotein lipase inhibitor whose expression is normally selectively suppressed in the gut epithelium by the microbiota (Backhed *et al.*, 2004).The relevance of *Fiaf* expression was highlighted when it was established that when GF wild type and *Fiaf*^{-/-} mice were fed a 'western diet', *Fiaf* deficient animals gained significantly more weight and had significantly greater epididymal fat pads than their wild type littermates

(Backhed *et al.*, 2007). Higher levels of LPL activity (67%) in the epididymal fat pads of *Fiaf*$^{-/--}$ mice compared to GF wild type mice was also noted (Backhed *et al.*, 2004). GF mice were also found to have increased skeletal muscle and liver levels of phosphorylated AMP activated protein kinase (AMPK). AMPK is a heterotrimeric enzyme that functions as a "fuel gauge" that monitors cellular energy status. Increased intracellular ratios of AMP to ATP results in its activation (McNeil, 1984). 40% and 50% higher levels of AMPK and AMP, respectively, were found in the gastrocnemius muscle harvested from GF mice than CONV-D mice fed a 'western diet'. This indicates that GF mice are sheltered from diet induced obesity by two mechanisms that result in increased fatty acid metabolism i.e. elevated levels of *Fiaf* and AMPK (Backhed *et al.*, 2007).

As discussed briefly above, (Hildebrandt *et al.*, 2009) investigated the effects of a high fat diet on WT and RELMβ KO mice. Higher levels of RELMβ expression were observed in high fat diet mice when compared to mice fed a standard chow diet. It was also noted that oral treatment of mice with antibiotics reduced the expression of colonic RELMβ in both mice fed a standard chow or a high fat diet. Thus it was concluded that the induction of RELMβ expression by a high fat diet is dependent upon the commensal gut microbiota. It was noted that although KO and wild type mice weighed the same at 13 weeks of age when fed a standard chow diet, following 21 weeks on a high fat diet RELMβ KO mice displayed diminished weight gain due to a decreased build up of fat mass compared with the wild type controls. This difference was not due to an alteration in food intake, fat absorption or core body temperature and it was also established that RELMβ KO mice did not exhibit any differences with respect to physical activity when compared to wild type controls during the period. However, indirect calorimetry revealed that the relative reduction in diet induced obesity in KO mice was caused by an increase in energy expenditure. Further analysis also revealed that the expression of a collection of genes encoding ABC transporters was increased in wild type mice fed the high fat diet when compared to expression of the same genes in wild type mice on a standard chow diet. The corresponding proteins are responsible for the transport of lipids, sugars and peptides as well as metals. Expression of genes for amino acid metabolism

and carbohydrate metabolism were relatively decreased. In summary, the results from this study demonstrate the importance of diet as a determinant of gut microbiome composition and suggest the need to control for dietary variation when evaluating the composition of the human gut microbiome.

The possibility that manipulation of the gut microbiota with *B. breve* might influence the fatty acid composition of host tissues has been investigated by (Wall *et al.*, 2009). In this study different animal models were fed with *B. breve* NCIMB 702258, which was selected because of its ability to synthesize bioactive isomers of conjugated linoleic acid (CLA) from free linoleic acid. The authors hypothesised that the administration of NCIMB 702258 could have an anti inflammatory impact by virtue of this metabolite. An 8 week dietary intervention study involving BALB/c mice showed that the *cis*-9, *trans*-11 CLA (c9,t11 CLA) content of the livers of mice fed the *B. breve* and a linoleic acid substrate was 2.4-fold higher than in the control mice. This CLA isomer has been previously shown to exhibit a number of beneficial activities in experimental animal models and human cell culture studies including the ability to reduce body fat and to bring about anti-diabetic effects (Terpstra, 2004, de Roos *et al.*, 2005, Moloney *et al.*, 2007). These studies should be distinguished from those involving the *cis*-12, *trans*-10 CLA isomer, which can have negative effects (Poirier *et al.*, 2005, Poirier *et al.*, 2006). The c9,t11 CLA content of the large intestine, small intestine, caecal contents and faeces of these test mice was also higher than that of controls. In the same study, the supplementation of the diet of severe combined immunodeficient (SCID; most frequently employed as animal models of inflammatory bowel disease) mice with *B. breve* and linoleic acid resulted in 4-fold higher levels of c9,t11 CLA in the liver than the group receiving linoleic acid alone. Examination of the former group also revealed 3.0- and 2.0-fold higher levels of c9,t11 CLA in the large intestines and ceca, respectively. Indeed, all tissues from SCID mice fed pure c9,t11 CLA had higher levels of this compound when compared with those fed linoleic acid alone. It was also noted that levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in adipose tissue in mice supplemented with *B. breve* alone were 3 fold higher than the corresponding tissue from control mice. EPA and DHA are also known to exert anti-inflammatory properties.

From corresponding porcine studies it was established that the livers of pigs fed the *B. breve* strain and linoleic acid had 1.5-fold greater c9,t11 CLA levels than those from unsupplemented controls. Overall this study showed that the fatty acid composition of host tissue can be positively influenced by the oral administration of a metabolically active commensal acting on a dietary substrate.

The occurrence of sexual dimorphism in the mouse model in relation to total body fat content (TBFC) was observed in a recent study (Mestdagh *et al.*, 2011). It was demonstrated that male mice had a significantly higher TBFC than female mice. Interestingly GF male and female mice did not display this sexual dimorphism. GF animals showed lower brown adipose tissue lactate levels and circulating levels of very low density lipoprotein while greater levels of (D)-3-hydroxybutyrate in liver, plasma and brown adipose tissue. These results imply that the gut microbiota adjust the lipid metabolism in brown adipose tissue as the loss of gut microbiota inhibits lipogenesis while also promoting hepatic and brown adipose tissue lipolysis.

1.7.2 Short chain fatty acids

Faecal SCFAs are predominantly produced from the fermentation of fibre in the large intestine by bacteria. In the absence of these intestinal microbes, the host would not be able to completely hydrolyse this fibre. These SCFAs, consisting mainly of acetate, propionate and butyrate, represent an additional source of energy and, indeed, it is estimated that microbially generated SCFAs provide 10% of the total dietary energy supply in humans (McNeil, 1984). SCFA levels were investigated in the previously referred to study by (Schwiertz *et al.*, 2009). In total, 98 volunteers (34 males and 64 females) were analyzed and their stool samples revealed the presence of acetate, propionate, butyrate and valerate as well as iso-valerate and iso-butyrate. It was noted that the samples from obese volunteers had 20% higher mean total SCFA concentrations than those from lean volunteers. Of the SCFA, propionate levels were most dramatically increased in this group (41%), followed by levels of butyrate (28%), valerate (21%) and acetate (18%). In contrast, the iso-SCFAs concentrations did not differ considerably. In 2007 (Duncan *et al.*) measured the changes in faecal SCFA in response to

changes in dietary intake of carbohydrates. Volunteers were given a maintenance (M) (13% protein, 52% carbohydrate and 35% fat) diet for 3 days. After this they were given a high protein (30%), low carbohydrate (4%) (HPLC) diet or a high protein (30%), moderate carbohydrate (35%) (HPMC) diet for 4 weeks. The analysis of faecal samples revealed that concentrations of SCFA were lower when the volunteers were in receipt of the HPLC and HPMC diets than when they consumed the M diet. While the concentrations of the predominant SCFAs, i.e. acetate, propionate and valerate, decreased due to the shift from the maintenance to the low carbohydrate diets (50%), butyrate levels decreased even more dramatically (75%). Notably, a linear relationship existed between carbohydrate intake and butyrate concentration. In contrast, although (Murphy *et al.*, 2010) did find that the faecal energy content of *ob/ob* mice was decreased and caecal SCFA concentrations increased at 7 weeks of age relative to lean controls, these patterns did not continue with time and were not observed in DIO mice. The (Murphy *et al.*, 2010) study also indicated that SCFA concentrations were unrelated to changes in proportions of Firmicutes, Bacteroidetes or Actinobacteria. These findings suggest that the connection between the microbial composition and energy harvest capacity is more complex than previously thought.

1.7.3 LPS-mediated impacts on obesity

Obesity and metabolic syndrome are associated with low grade inflammation, and data from several studies provides evidence that the lipopolysaccharide (LPS) endotoxin derived from certain components of the gut microbiota contributes to the increased development of adipose tissue and impaired glucose tolerance in obesity. LPS is a component of the Gram negative bacterial cell wall and is composed of lipid and a polysaccharide. The impact of LPS on the host is mediated through the toll-like receptor 4 (TLR4)/MyD88/NF-κB signalling pathway. Endogenous LPS is continuously produced in the gut as a consequence of the death of Gram-negative bacteria and is absorbed into capillaries of the intestine through a TLR4-dependant mechanism. In 2007, (Cani *et al.*) showed that LPS could be an early factor in the triggering of high-fat diet induced metabolic diseases. More specifically, their data showed that high fat feeding caused plasma LPS

concentrations to remain high throughout the whole day compared with controls which showed dinural variations in plasma LPS concentrations. Due to the fact that high fat feeding induced plasma LPS concentrations were lower than values associated with septicemia and infections, the authors defined this phenomenon as metabolic endotoxemia. To causally link high fat diet increased LPS concentrations to metabolic disease, the authors mimicked LPS concentrations of high fat feeding by implanting a subcutaneous osmotic minipump in mice and continuously infused LPS or saline for a month. It was then revealed that fasted glycemia, blood glucose, fasted insulinemia, liver triglyceride content and body weight levels were greater in mice infused with LPS than those infused with saline. Furthermore, the magnitude of weight gain and visceral and subcutaneous adipose depots in LPS infused mice was similar to that observed in mice fed a high fat diet. It was also apparent that mRNA concentrations corresponding to the genes for the main inflammatory factors involved in metabolic disease (i.e. tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and plasminogen activator inhibitor (PAI)-1) were increased in both high fat diet and LPS infused mice. In a further study, WT and CD14 mutant mice were intravenously infused with LPS for 3 hours. It was noted that, as a consequence, levels of IL-6, PAI-1, IL-1, phosphorylated nuclear factor-kB and IkappaB kinase (Claesson *et al.*, 2009) forms increased in WT mice whereas levels of the same factors decreased or were unchanged in CD14 mutant mice. In addition, the body weight, visceral and subcutaneous adipose depot weight and liver weight of WT mice were increased but were unchanged in CD14 mutant mice.

The possibility that metabolic endotoxemia could be controlled by changes of the gut microbiota has been examined by (Cani *et al.*, 2008). This involved a 4 week study during which mice were fed a control, or a high fat, carbohydrate-free diet. While mice fed a high fat diet had increased plasma LPS levels relative to controls, plasma LPS levels were not increased in high fat diet mice treated with the antibiotics ampicillin and neomycin. This study also revealed that the high fat diet significantly increased intestinal permeability through a mechanism that resulted in reduced expression of ZO-1 and occludin, i.e. tight junction proteins. Antibiotic treatment reversed this effect, suggesting that gut bacteria affected by antibiotic administration

are involved in the control of intestinal permeability and hence the occurrence of metabolic endotoxemia.

A link may also exist between serum amyloid A (SAA) proteins and LPS. SAA proteins are suspected mediators of inflammation and atherosclerosis (Lewis *et al.*, 2004, Yang *et al.*, 2006, Urieli-Shoval *et al.*, 2000). Increased serum levels of SAA proteins have been linked with obesity, chronic hyperglycemia, insulin resistance and cardiovascular disease (Andersson *et al.*, 2008, Johnson *et al.*, 2004, Lin *et al.*, 2001, Ogasawara *et al.*, 2004, Scheja *et al.*, 2008, Yang *et al.*, 2006). Four functional SAA isoforms have been identified in mice i.e. SAA1-4. SAA3 is the most abundant SAA isoform and, significantly with respect to the link between LPS and obesity, its expression is induced in adipose tissue after intraperitoneal administration of LPS (Reigstad *et al.*, 2009). The human isoform, SAA1, is most similar to the mouse SAA3 when their amino acid sequences are compared. The expression of human SAA1 has been shown to augment lipolysis in adipocytes (Yang *et al.*, 2006) and increased expression is observed in hypertrophic adipocytes of obese humans (Sjoholm *et al.*, 2005). (Scheja *et al.*, 2008) showed that SAA3 is also up-regulated in the adipose tissue of mice fed a high-fat diet and proposed that SAA3 could be a mediator of the chronic inflammation associated with insulin resistance in obesity. (Reigstad *et al.*, 2009) took these investigations a step further by comparing levels of SAA3 mRNA in adipose and intestinal tissue from GF and CONV-R mice to determine if components of the gut microbiota impacted on SAA3 levels. It was established that SAA3 mRNA levels in adipose tissue were significantly higher (9.9 fold) in CONV-R mice than in GF mice, whereas SAA1 and SAA2 levels did not increase significantly in these tissues. With respect to the gut, an analysis of cDNAs from the duodenum, jejunum, ileum and proximal colon revealed that SAA3 mRNA levels were highest in the colon of both GF and CONV-R mice and that, here too, SAA3 mRNA levels were significantly higher (7.0 fold) in CONV-R mice than GF controls. Expression of TNF-α, a commonly expressed cytokine with a likely role in chronic inflammatory disease and a known regulator of SAA3 expression, was also found to be significantly higher in the colon of CONV-R mice. Co-staining of colonic sections from CONV-R mice revealed that SAA3

is expressed by the intestinal epithelial cells and intraepithelial macrophages which may contribute to the elevated SAA3 levels identified in the colon of these animals. Indeed, the treatment of CMT-93 colonic epithelial cell lines and RAW 264.7 mouse macrophages with increasing concentrations of purified *E. coli* LPS caused a concentration-dependent increase in SAA3 mRNA levels. More specifically, a 27-fold increase was observed in the CMT-93 cells upon the addition of 1µg/ml LPS. Furthermore, SAA3 mRNA levels also increased in RAW 264.7 macrophages treated with 1µg/ml LPS, but only to a maximum of 1.6 fold. The authors speculated that mouse SAA3 might be functionally similar to human SAA1 and could represent a link between low grade microbiota-induced inflammation and obesity.

1.8 CONCLUSION

The debate regarding the significance of the Firmicutes:Bacteroidetes ratio with respect to obesity is still ongoing, and the basis for differences between the various studies investigating this phenomenon has not yet been established. While differences in host genetics represent an obvious variable, other factors such as the degree of weight change, the severity of calorie restriction and/or the duration of the study are just some of a number of other important factors. Possibly the most important of these factors relates to diet associated differences in the composition of the gut microbiota. The question as to whether specific populations are responsible for weight gain or are simply flourishing as a consequence of the diet being consumed (i.e. are these populations a 'cause' or an 'effect') remains a key one. The use of GF mice and mice conventionalized with known microbial species should help to reveal the influence of specific populations on host health and in turn reveal therapeutic targets in the fight against obesity. Further investigations, combined with the ever increasing capacity of next generation sequencing technologies, and the standardization of methods to facilitate comparisons between studies will overcome the inconsistencies that have plagued investigations into the link between human gut microbiota and obesity. Such developments are crucial as the obesity epidemic is showing no sign of abating and all possible treatments, including potentially the targeting of specific components of the gut microbiota of overweight and obese individuals, need to be considered.

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Table 1. Molecular methods used in studies discussed in this review.

Approach	Description
Sequencing strategies employed to study eubacterial (bacterial) populations	
Shotgun sequencing	Metagenomic DNA is randomly sheared into smaller fragments and sequenced at random to reveal information regarding the functional potential and, to a lesser extent, composition of a microbial population.
16S rRNA amplicon sequencing	Amplification of the 16S rRNA gene from eubacteria using degenerate PCR primers and sequencing thereof to reveal information regarding the bacterial population present in the environment.
Sequencing Platforms	
Sanger sequencing	Dideoxy chain termination method in which fluorescent labelled ddNTPs or dNTPs are incorporated into the newly synthesised DNA thereby preventing further synthesis and leaving products of different lengths. Products are separated by size by gel or capillary electrophoresis.
Pyrosequencing	Sequence by synthesis method involving the detection of pyrophosphate release upon incorporation of a known nucleotide. A number of sequencing platforms, including the Roche-454 sequencers, employ this technology.
Illumina sequencing	Sequence by synthesis method in which small DNA fragments are bound to a slide and amplified in clusters. Double stranded DNA in the clusters is separated and sequenced. Lasers detect nucleotide addition and from this the sequence of the DNA fragment is generated.
Other technologies employed to investigate microbial ecosystems	
Fluorescent *in situ* Hybridization	Fluorescent labelled oligonucleotide probes used to identify taxa *in situ* through hybridization.
Denaturing Gradient Gel Electrophoresis (DGGE)	Separation of DNA (usually 16S amplicons) based on GC content using gel electrophoresis and a denaturing agent to differentiate between microbial populations.
Terminal Restriction Fragment Length Polymorphism (T-RFLP)	Separation of terminally labelled PCR amplicons that have been enzymatically digested with restriction enzymes.
Quantitative Real Time Polymerase Chain Reaction (qPCR)	Modified form of PCR which allows for detection and quantification of fluorescent PCR amplicons or amplicons to which fluorescent probes have attached, the fluorescence of which increases in line with increasing DNA concentrations.

Table 2. Culture independent methods used by studies in this review.*

Cohort/model	Method	Reference
ob/ob, *ob*/+ and +/+ mice	16S by Sanger	(Ley *et al.*, 2005)
Obese human on low calorie diets	16S by Sanger	(Ley *et al.*, 2006)
ob/ob, *ob/+* and +/+ mice	16S by Sanger & Pyrosequencing	(Turnbaugh *et al.*, 2006)
Humans on weight loss diets	FISH	(Duncan *et al.*, 2007)
CD14 $^{-/-}$ and Wt mice	FISH	(Nadal *et al.*, 2009)
ob/ob, +/+, CD14$^{-/-}$ and *ob/ob* CD14-/- mice	DGGE and qPCR	(Cani *et al.*, 2008)
Children	FISH and qPCR	(Kalliomaki *et al.*, 2008)
DIO mice and WT mice	16S by Sanger	(Turnbaugh *et al.*, 2008)
Overweight and normal weight pregnant women	qPCR and FISH	(Collado *et al.*, 2008)
Adolescents on diets and exercise	FISH	(Santacruz *et al.*, 2009)
Monozygotic and dizygotic human twins and mothers	16S by Sanger & Pyrosequencing	(Turnbaugh *et al.*, 2009)
ob/ob mice	DGGE and qPCR	(Cani *et al.*, 2009)
Lean, obese and post gastric bypass humans	16S by Sanger & Pyrosequencing	(Zhang *et al.*, 2009b)
NMRI-KI mice on LF/HS, HF/HS or LF/PP diets	qPCR	(Zhang *et al.*, 2009a)
Adolescents on diets and exercise	qPCR	(Walker *et al.*, 2011)
Lean, overweight and obese humans	qPCR	(Schwiertz *et al.*, 2009)
Hamsters	16S by Pyrosequencing, DGGE & qPCR	(Martinez *et al.*, 2009)
Children	qPCR	(Balamurugan *et al.*, 2009)
RELMβ Knockout and Wild type mice	16S by Pyrosequencing	(Hildebrandt *et al.*, 2009)

* Table done in chronological order

Table 2. (*continued)*

Cohort/model	Method	Reference
Syrian hamsters	DGGE, 16S Sanger and qPCR	(Sonoyama *et al.*, 2009)
Obese, lean and anorexic humans	qPCR	(Armougom *et al.*, 2009)
Apoa-I$^{-/-}$ and Wt C57BL/6J mice	16S on DGGE, T-RFLP & Pyrosequencing	(Cani *et al.*, 2007)
African Americans and Caucasian Americans	DGGE, FISH and qPCR	(Mai *et al.*, 2009)
Humanized gnotobiotic mice	16S by Pyrosequencing	(Turnbaugh *et al.*, 2009)
Overweight and normal weight pregnant women	qPCR	(Santacruz *et al.*, 2010)
C57bl6/J mice	DGGE and qPCR	(Dewulf *et al.*, 2011)
Lean and obese humans	qPCR	(Li *et al.*, 2011)
HF-fed, ob/ob and Wt mice	Pyrosequencing	(Murphy *et al.*, 2010)
Overweight and normal weight children	FISH	(Luoto *et al.*, 2011)
Humans of different nationalities	Sanger & Pyrosequencing	(Arumugam *et al.*, 2011)
Gnotobiotic mice	Illumina shotgun sequencing	((Faith *et al.*, 2011)
Wistar rats	16S Pyrosequencing	(Furet *et al.*, 2010)
Mammals	Shotgun Pyrosequencing	(Muegge *et al.*, 2011)
JCR:LA-cp rats	qPCR	(Parnell and Reimer, 2011)
Healthy humans	16S by Pyrosequencing & shotgun metagenomics	(Wu *et al.*, 2011)
In vitro three stage colonic model	FISH & DGGE	(Shen *et al.*, 2011))

* Table done in chronological order

Table 3. Dietary related influences on gut microbiota.

	Human	
Diet	**Populations increasing**	**Populations decreasing**
Fat restricted	Bacteroidetes (Ley *et al.*, 2006)	Firmicutes(Ley *et al.*, 2006)
Carbohydrate restriction	Bacteroidetes (Ley *et al.*, 2006)	Firmicutes (Ley *et al.*, 2006)
Low carbohydrate/high protein	*Oscillibacter valerigens* (Walker *et al.*, 2011)	*Roseburia*, *E. rectale* (Walker *et al.*, 2011) & *Bifidobacterium* (Duncan *et al.*, 2007)
Calorie restriction & exercise	*Bacteroides fragilis*, *Lactobacillus* (Santacruz *et al.*, 2009) & *Bacteroides* (Nadal *et al.*, 2009)	*C. coccoides*, *B. longum*, *B. adolescentis* (Santacruz *et al.*, 2009), *C. histolyticum* & *E. rectale-C. coccoides* (Nadal *et al.*, 2009)
Resistant starch	*Ruminococcus bromii*, *Oscillibacter valerigens*, *Roseburia* & *E. rectale* (Walker *et al.*, 2011)	
High dietary fibre	*Bifidobacterium*, *Ruminococcus*, *Lactobacillus-Enterococcus*, *Faecalibacterium prausnitzii* & *E. rectale-C. coccoides* (Shen *et al.*, 2011)	

Table 3. *(continued)*

	Animal	
Diet	**Populations increasing**	**Populations decreasing**
High fat	Mollicutes M2 cluster (Zhang *et al.*, 2009a)	Mollicutes M1 & M3 cluster, *Bifidobacteriaceae*, (Zhang *et al.*, 2009a) *Bacteroides, E. rectale-C. coccoides* & *Bifidobacterium* (Cani *et al.*, 2007)
Western diet (high fat/high sugar)	*C. innocuum, E. dolichum, C. mitsuokai* & Bacilli (Turnbaugh *et al.*, 2009)	Bacteroidetes (Turnbaugh *et al.*, 2009) & *E. rectale* (Mahowald *et al.*, 2009)
Western diet reduced fat	Bacteroidetes (Turnbaugh *et al.*, 2008)	Mollicutes (Turnbaugh *et al.*, 2008) & *E. rectale* (Mahowald *et al.*, 2009)
Western diet reduced carbohydrate	Bacteroidetes (Turnbaugh *et al.*, 2008)	Mollicutes (Turnbaugh *et al.*, 2008)
Fasting	*Desulfovibrionaceae* (Sonoyama *et al.*, 2009)	*Clostridium* (Sonoyama *et al.*, 2009)
Increased casein		*E. rectale, D. piger & M. formatexigens* (Faith *et al.*, 2011)

Figure 1. The study of the role of the gut microbiota in obesity can be subdivided into four broad areas.
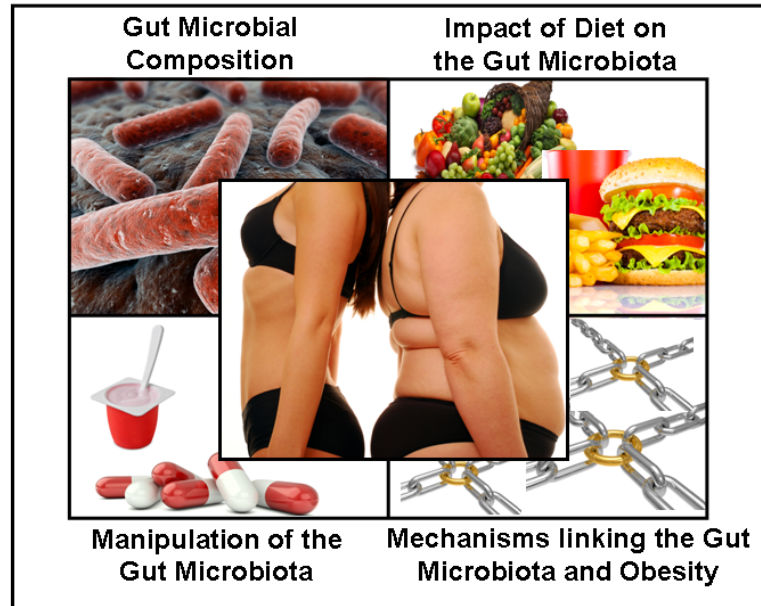
Figure 2. Next generation sequencing (NGS) – High throughput. Left – 16S rRNA gene amplification using specific PCR primers followed by sequencing to reveal eubacterial composition. Right – Random shearing of metagenomic DNA into small fragments followed by sequencing to reveal functional potential of bacterial population.
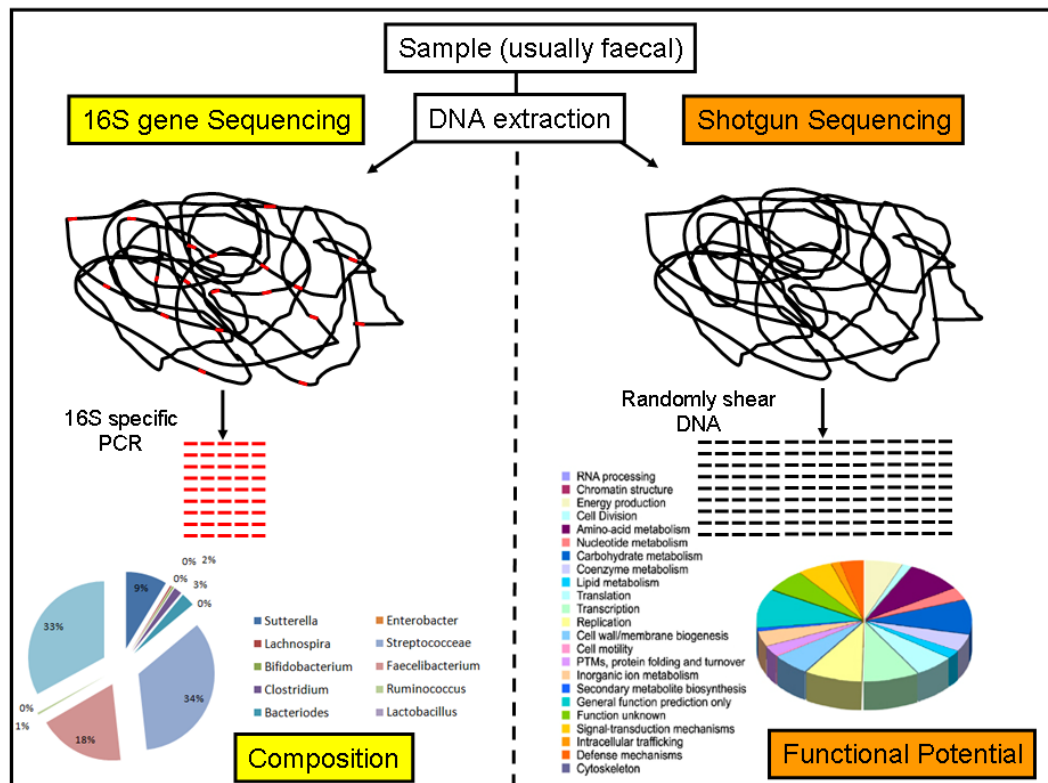
Figure 3. Comparison of germ free and Conventionally raised mice with respect to weight gain and associated biomarkers. Fasting induced adipose factor (*Fiaf*), phosphorylated AMP activated protein kinase (AMPK), lipoprotein lipase (LPL) and western diet (WD).

Germ Free	Conventionally raised
Higher *Fiaf* Higher AMPK Lower LPL activity No weight gain on WD	Lower *Fiaf* Lower AMPK Higher LPL activity Increased weight gain on WD

Chapter 2

Divergent metabolic outcomes arising from targeted manipulation of the gut microbiota in diet-induced obesity

Siobhan Clarke Chapter Contributions:

Experimental:

- Designed and performed all experiments relating to the extraction and purification of DNA from faecal pellets.
- Generated amplicons for 454 pyrosequencing.
- Designed and performed all experiments relating to the quantification of total bacterial DNA using QPCR.

Results interpretation:

- Analysed all data in relation to 16S compositional sequencing analysis with the aid of the staff bioinformatian.
- Compiled all graphical interpretations relating to 16S compositional sequencing analysis and QPCR.

Manuscript preparation:

Major contributor to manuscript preparation

2.1 ABSTRACT

Objective The gut microbiota is an environmental regulator of fat storage and adiposity. Whether the microbiota represents a realistic therapeutic target for improving metabolic health is unclear. This study explored two antimicrobial strategies for their impact on metabolic abnormalities in murine diet-induced obesity: oral vancomycin and a bacteriocin-producing probiotics (*Lactobacillus salivarius* UCC118Bac$^+$).

Design Male (7-week-old) C57BL/J6 mice (9-10/ group) were fed a low-fat (lean) or a high-fat diet for 20 weeks with/without vancomycin by gavage at 2 mg/day, or with *L. salivarius* UCC118Bac$^+$ or the bacteriocin negative derivative *L. salivarius* UCC118Bac$^-$ (each at a dose of 1×10^9 cfu/day by gavage). Compositional analysis of the microbiota was by 16S rDNA amplicon pyrosequencing.

Results Analysis of the gut microbiota showed that vancomycin treatment led to significant reductions in the proportions of Firmicutes and Bacteroidetes and a dramatic increase in Proteobacteria, with no change in Actinobacteria. Vancomycin-treated high-fat-fed mice gained less weight over the intervention period despite similar caloric intake, and had lower fasting blood glucose, plasma TNF-α and triglyceride levels compared with diet-induced obese controls. The bacteriocin producing probiotic had no significant impact on the proportions of Firmicutes but resulted in a relative increase in Bacteroidetes and Proteobacteria and a decrease in Actinobacteria compared with the non bacteriocin-producing control. No improvement in metabolic profiles was observed in probiotic-fed diet-induced obese mice.

Conclusion Both vancomycin and the bacteriocin-producing probiotic altered the gut microbiota in diet induced obese mice, but in distinct ways. Only vancomycin treatment resulted in an improvement in the metabolic abnormalities associated with obesity thereby establishing that while the gut microbiota is a realistic therapeutic target, the specificity of the antimicrobial agent employed is critical.

2.2   SIGNIFICANCE OF THIS STUDY

What is already known about this subject?
- ➢ The gut microbiota is an environmental regulator of fat storage and adiposity.
- ➢ We and others have shown the primacy of diet in influencing the microbiota in obesity.
- ➢ The composition of the gut microbiota is significantly altered in obesity and diabetes in both animal and human studies.
- ➢ It has been suggested that alteration in microbial composition increases the risk of obesity because of enhanced energy harvest from dietary intake.

What are the new findings?
- ➢ Vancomycin and the bacteriocin-producing probiotic produced distinctive modifications in the gut microbiota in diet-induced obese mice at the phylum, family and genus levels.
- ➢ To our knowledge, this is the first report to establish that a bacteriocin produced by a probiotic can substantially alter the composition of the gut microbiota in vivo.
- ➢ However, only vancomycin treatment resulted in an improvement in the metabolic abnormalities associated with obesity.
- ➢ Our findings provide further confirmation for the role of the microbiota in metabolic dysregulation and a supporting rationale for altering the microbiota as a prophylactic strategy using antimicrobial agents, including bacteriocins, but specificity of action will be crucial.

How might it impact on clinical practice in the foreseeable future?
- ➢ These findings indicate that therapeutic manipulation of the microbiota may be a useful strategy in the prevention or management of obesity and metabolic disorders.

2.3 INTRODUCTION

The basis of the modern obesity epidemic in developed societies is complex and involves a contribution from genetic susceptibility and, more importantly, changes in diet and other lifestyle elements. In addition, the molecular event underlying the pathogenesis of the metabolic abnormalities of obesity are incompletely understood and interventions to treat them are poorly developed. A body of work has recently implicated alterations of the gut microbiota as a contributory factor to obesity-related metabolic dysregulation (Ley, 2010, Tilg and Kaser, 2011).

The composition of the gut microbiota is significantly altered in obesity and diabetes in both animal and human studies and is characterised by reduced diversity (Larsen *et al.*, 2010, Ley *et al.*, 2005, Ley *et al.*, 2006, Turnbaugh *et al.*, 2008, Turnbaugh and Gordon, 2009, Turnbaugh *et al.*, 2006). It has been suggested that this alteration in microbial composition increases the risk of obesity because of enhanced energy harvest from dietary intake (Turnbaugh *et al.*, 2008, Turnbaugh and Gordon, 2009, Turnbaugh *et al.*, 2006). We and others have demonstrated that, in murine models, changes in the gut microbiota in response to diet and obesity are dissociated from markers of energy harvest over time, suggesting that mechanisms other than energy harvest may contribute to microbiota-induced susceptibility to obesity and metabolic diseases (Bajzer and Seeley, 2006, Hildebrandt *et al.*, 2009, Murphy *et al.*, 2010). In this regard, the gut microbiota and associated products such as lipopolysaccharides and short-chain fatty acids have been reported to regulate gene expression, and thereby alter energy expenditure and storage through host related mechanisms (Cani *et al.*, 2007a, Cani *et al.*, 2008, Cani *et al.*, 2007b, Samuel *et al.*, 2008). However, it is unclear if the microbiota represents a realistic therapeutic target for improving metabolic health.

The composition of the microbiome is dynamic and adaptable, and a number of strategies including antibiotics, prebiotics and probiotics have the potential to influence host metabolism favourably by targeting the gut microbiota. In this study, we explored two antimicrobial strategies for their impact on metabolic abnormalities in murine diet-induced obesity: oral

vancomycin and a bacteriocin-producing probiotics *Lactobacillus*. Vancomycin exhibits anti-Firmicutes activity and has limited systemic effects (Moellering, 2006, Pultz *et al.*, 2005, Yap *et al.*, 2008), while *Lactobacillus salivarius* UCC118 is a genetically well characterised probiotic strain that produces a broad-spectrum class II bacteriocin, Abp118, and has previously been shown to protect mice from infection by *Listeria monocytogenes* (Corr *et al.*, 2007, Claesson *et al.*, 2006, Flynn *et al.*, 2002). The availability of a bacteriocin-negative (Bac⁻) derivative of *L. salivarius* UCC118 allowed for the direct assessment of the effect of the bacteriocin on the gut microbiota and its impact on metabolic dysregulation. The results show that the antimicrobial agents modulated the gut microbiota in different ways, but only vancomycin treatment resulted in an improvement in metabolic markers in diet-induced obesity. These findings indicate that therapeutic manipulation of the microbiota may be a useful strategy in the prevention or management of obesity and metabolic disorders.

2.4   MATERIALS AND METHODS

2.4.1 Animals

C57BL/6J male mice, aged 3-4 weeks, were obtained from Harlan (Oxon, UK) and housed under barrier-maintained conditions within the biological services unit, University College Cork (UCC). Mice were allowed to acclimatise for 3-4 weeks before the start of the study. All experiments were approved by the UCC Animal Ethics Committee and experimental procedures were conducted under licence from the Irish government.

2.4.2 Experimental design

To assess the impact of the two antimicrobial strategies on metabolic abnormalities in murine diet-induced obesity, 7-week old male C57BL/J6 mice (9-10 per group) were fed either a low fat diet (control; 10% calories from fat; Research Diets, New Jersey, USA; #D12450B), a high-fat diet (diet-induced obesity; 45% calories from fat; Research Diets; #D12451) for 20 weeks or a high-fat diet for 20 weeks including an 8 week oral treatment from weeks 12 to 20 with either 2 mg/day of vancomycin (Sigma Aldrich, UK), the bacteriocin-producing probiotic *L. salivarius* UCC118Bac$^+$ at 1×10^9 cfu/day, or *L. salivarius* UCC118Bac$^-$ at 1×10^9 cfu/day. All mice were gavaged and phosphate-buffered saline was used as the vehicle control for the vancomycin-treated group. An initial study was undertaken to establish that vancomycin at a dose of 2 mg/day did not alter the health status of mice and was effective at altering the components of the gut microbiota in mice, using traditional plating methods, as outlined in the supplementary materials and methods.

2.4.3 Probiotic production

The bacteriocin-negative derivative *L. salivarius* UCC118Bac$^-$ was generated as described by Corr *et al.*, 2007. Both the *L. salivarius* UCC118Bac$^+$ and the *L. salivarius* UCC118Bac$^-$ were grown in MRS broth media for 24 h at 37°C, harvested by centrifuge and freeze dried. The powders were resuspended in water for delivery to the mice.

2.4.4 Weights and tissue sampling

Body weight and food intake were assessed weekly. At the end of the study, fat and lean body mass were measured using a Minispec mq benchtop NMR spectrometer (Bruker Instruments, Rheistetten, Germany), after which they were killed and necropsied. Internal organs (liver and spleen) and fat pads (reproductive, renal, mesenteric and inguinal) were removed, weighed and stored at -80°C.

2.4.5 DNA extractions and amplicon sequencing

Total metagenomic DNA was extracted from individual faecal samples and 16S ribosomal RNA gene tags of the V4 region were amplified, sequenced and subjected to in-silico analysis as described in the supplementary materials and methods and by Murphy *et al.*, 2010. Real-time quantitative PCR was used to determine total bacterial numbers (16S rRNA gene copies), and hierarchical clustering was used to provide an overview of the data, as outlined in the supplementary materials and methods.

2.4.6 Metabolic markers

Mice were fasted for 5-6 h and blood glucose was determined using a Coutour glucose meter (Bayer, UK) on 1 ml of blood collected from the tip of the tail vein. Blood was collected by cardiac puncture from fasted mice and plasma obtained. Tumour necrosis factor alpha (TNF-α) was measured in 25 ml of plasma using an ultrasensitive kit from MesoScale Discovery (Gaithersburg, Maryland, USA), and plasma insulin concentrations were determined in 5 ml of plasma using an ELISA kit (Mercodia, Uppsala, Sweden) as per the manufacturer's instructions. Plasma free fatty acids were determined using a Wako kit (Wako, Germany) and plasma triglyceride levels were determined on 3 ml of plasma using infinity triglyceride liquid stable reagent (Thermo Scientific, Middletown, Virginia, USA). The lipids from 50 mg of frozen liver were extracted according to the Folch method (Folch *et al.*, 1957), and triglyceride levels were determined using infinity triglyceride liquid stable reagent (Thermo Scientific).

2.4.7 Statistical analysis

Data for all variables were normally distributed and allowed for parametric tests of significance. Data are presented as mean values with their SEM. Statistical analysis was performed by analysis of variance and Student's t test (Graph-Pad Software, San Diego, CA, USA).

2.5 RESULTS

2.5.1 Diet-induced obesity alters the composition of the gut microbiota

As expected, diet-induced obese mice gained significantly more body weight compared with lean controls over the 20-week feeding period, and this increase in body weight (41.75 ± 1.12 vs 33.22 ± 0.92 g; $p<0.001$) was attributable to an increase in fat mass alone (16.03 ± 0.77 vs 6.27 ± 0.61; $p<0.001$). Diet-induced obese mice consumed significantly more calories than lean controls, as measured by the cumulative caloric intake over the 20-week period of the study (7212 ± 35 vs 6136 ± 27 kJ/mouse; $p<0.001$).

At the end of the 20-week study, the composition of the gut microbiota of individual mice was investigated by DNA sequencing (Roche-454 titanium, Roche Diagnostics Ltd, West Sussex, UK) of 16S rRNA (V4) amplicons generated from total faecal DNA. A total of 212,655 sequence reads was generated, averaging 4,340 reads per faecal sample. Species richness, coverage and diversity estimations were calculated for each dataset (Supplementary Fig 1). Rarefaction curves for each group indicated that the total bacterial diversity present was well represented. Of the reads, 173,444 (82%) were assigned at the phylum level, 100,282 (47%) at the family level and 80,899 (38%) at the genus level. In agreement with previous studies, the mouse faecal microbiota was dominated by Firmicutes and Bacteroidetes at the phylum level (Table 1). Actinobacteria, Proteobacteria and Deferribacteria were also detected but at lower proportions. Consistent with the high levels of Firmicutes and Bacteroidetes detected, the most dominant bacteria at the genus level were *Clostridium*, *Lactobacillus* and *Bacteroides* (Table 1).

A comparison of the composition of the gut microbiota of lean and diet-induced obese mice showed that high-fat feeding for 20 weeks was associated with an increase in the relative proportions of Firmicutes ($p<0.05$) and a decrease in Bacteroidetes proportions ($p<0.05$) compared with lean controls (Table 1 and Supplementary Fig 2). At the family level, diet-induced obesity in mice was associated with a relative increase in the proportions of the Firmicutes class *Lactobacillaceae* ($p<0.01$) and a decrease in the Bacteroidetes class *Bacteroidaceae* ($p<0.05$), while the abundance of

Streptococcaceae ($p<0.05$) and *Alcaligenaceae* ($p<0.05$) increased and decreased, respectively (Table 1 and Supplementary Fig 2). At the genus level, the proportions of *Lactobacillus* ($p<0.01$) and *Lactococcus* spp. ($p<0.05$) increased, while *Bacteroides* ($p<0.001$), *Odoribacter* ($p<0.05$) and *Sutterella* ($p<0.05$) decreased in abundance (Table 1 and Supplementary Fig 2).

2.5.2 Vancomycin does not alter the health status of chow-fed mice

A preliminary investigation (outlined in the supplementary materials and methods) was undertaken to determine if vancomycin treatment was associated with any adverse health effects in healthy mice. The results confirmed that vancomycin did not negatively affect body weight, appearance or consistency of the stool in chow-fed C57BL/6 male mice at a dose of 2 mg/day over 4 weeks. In addition, culture-based microbial analysis of the faeces of healthy mice confirmed that, at this dose, vancomycin impacted specific microbiota elements, with a decrease in *Enterococcus* spp. (phylum Firmicutes), an increase in total *Enterobacteriaceae* (phylum Proteobacteria) and no change in either total anaerobes or *Lactobacillus* spp. (phylum Firmicutes; see Supplementary Fig 3).

2.5.3 Vancomycin treatment alters the composition of the gut microbiota in diet-induced obese mice

DNA sequencing-based analysis of the microbiota revealed that treatment of diet-induced obese mice for 8 weeks with vancomycin at 2 mg/day resulted in a significant reduction in the relative proportions of Firmicutes ($p<0.01$), Bacteroidetes ($p<0.001$) and Deferribacteres ($p<0.05$), and a dramatic increase in the proportions of Proteobacteria ($p<0.001$) compared with diet-induced obese mice (Table 1 and Supplementary Fig 2). The increased proportion of Proteobacteria was largely accounted for by an increased percentage of *Enterobacteriaceae* ($p<0.001$), while the decrease in Firmicutes and Bacteroidetes corresponded to a decrease in the proportions of *Clostridiaceae* ($p<0.001$) and *Bacteroidaceae* ($p<0.001$), respectively, at the family level (Table 1 and Supplementary Fig 2). Vancomycin treatment of diet-induced obese mice also resulted in changes among less common

families, such as a relative decrease in the proportions of *Porphyromonadaceae* ($p<0.001$) and increases in the proportions of *Streptococcaceae* ($p<0.01$), *Desulfovibrionaceae* ($p<0.01$) and *Alcaligenaceae* ($p<0.001$). At the genus level, the proportions of *Lactococcus* ($p<0.001$), *Sutterella* ($p<0.001$) and *Desulfovibrio* spp. ($p<0.01$) increased, while *Bacteroides* ($p<0.001$), *Clostridium* ($p<0.001$) and *Odoribacter* spp. ($p<0.05$) decreased (Table 1 and Supplementary Fig 2). The overall proportions of *Lactobacillus* spp. were unchanged by vancomycin treatment of diet-induced obese mice. Clustering of the data showed that diet-induced obese mice treated with vancomycin grouped together at the phylum level and separated well from lean and diet-induced obese mice (see Supplementary Fig 4). Better separation of lean and diet-induced obese mice was observed at the family and genus level (data not shown) than at the phylum level.

2.5.4 *L. salivarius* UCC118 alters the composition of the gut microbiota in diet-induced obese mice by a mechanism involving bacteriocin production

Comparison of the *L. salivarius* UCC118Bac$^+$ with the non bacteriocin-producing strain, *L. salivarius* UCC118Bac$^-$, showed that the production of the antimicrobial agent altered the composition of the gut microbiota in diet-induced obese mice. While the proportions of Firmicutes did not change, feeding of *L. salivarius* UCC118Bac$^+$ for 8 weeks resulted in a significant increase in the relative proportions of Bacteroidetes and Proteobacteria, and a decrease in the proportions of Actinobacteria compared with the bacteriocin-negative derivative, *L. salivarius* UCC118Bac$^-$ (Table 2 and Supplementary Fig 5). Consistent with these results, the increase in Bacteroidetes corresponded to an increase in the *Bacteroidaceae* family and the genus *Bacteroides* (Table 2 and Supplementary Fig 5). The decrease in Actinobacteria corresponded to a decrease in *Bifidobacteriaceae* at the family level and *Bifidobacterium* at the genus level (Table 2 and Supplementary Fig 5). Hierarchical clustering showed that, consistent with small changes, separation was not apparent between *L. salivarius*

UCC118Bac$^+$ and the non bacteriocin-producing strain, *L. salivarius* UCC118Bac$^-$, using this technique (see Supplementary Fig 6).

2.5.5 Total bacterial counts were altered by diet-induced obesity and vancomycin treatment, but not by bacteriocin-production by *L. salivarius* UCC118, in diet-induced obese mice

Quantitative PCR analysis revealed that total bacterial counts (16S rRNA gene copies/g of stool) were significantly lower in the faeces of diet-induced obese mice compared with lean controls ($p<0.001$; Fig 1A). Treatment of diet-induced obese mice with vancomycin resulted in a further large decrease in absolute faecal bacterial numbers compared with their diet-induced obese counterparts ($p<0.001$; Fig 1A). In contrast, comparison of treatment with *L. salivarius* UCC118Bac$^+$ and its bacteriocin negative derivative showed that bacteriocin production did not alter the total bacterial numbers in the faeces of diet-induced obese mice (Fig 1B).

2.5.6 Antimicrobial strategies alter weight gain in diet-induced obese mice

Treatment of diet-induced obese mice with vancomycin resulted in a significant reduction in weight gain ($p<0.05$; Fig 2A) compared with diet-induced obese mice, despite a large increase in caecum weight ($p<0.0001$) relative to the caeca of diet induced obese controls (see Supplementary Table 1). However, a recovery in the rate of body weight gain in the vancomycin-treated diet-induced obese mice was evident after day 28 of the intervention. For probiotic-fed mice, a statistically significant reduction in weight gain was observed on treatment with *L. salivarius* UCC118Bac$^+$ in diet-induced obese mice compared with *L. salivarius* UCC118Bac$^-$ at days 14, 21 and 28 of the 8-week intervention period, but this effect did not persist over time (Fig 2B). In addition, calorie intake was not altered by treatment with vancomycin, *L. salivarius* UCC118Bac$^+$ or *L. salivarius* UCC118Bac$^-$ in diet-induced obese mice (Fig 2C).

2.5.7 Vancomycin treatment, but not bacteriocin-production by *L. salivarius* UCC118, improves metabolic markers in diet-induced obese mice

We next investigated the metabolic consequences of perturbing the gut microbiota using the two antimicrobial strategies. Comparison of lean and diet-induced obese mice after 20 weeks of feeding showed that diet-induced obese mice had elevated fasting blood glucose ($p<0.001$) and plasma insulin ($p<0.05$), while there was no change in plasma triglycerides compared with lean controls (see Supplementary Table 1). Treatment of diet-induced obese mice with vancomycin for 8 weeks resulted in an improvement in fasting blood glucose levels ($p<0.05$) and plasma triglyceride levels ($p=0.06$) compared with diet-induced obese mice, while there was no change in fasting insulin levels (see Supplementary Table 1). Diet-induced obese mice were characterised by elevated liver weight ($p<0.01$) and liver triglyceride levels ($p<0.001$) compared with lean controls. Interestingly, there was a trend towards a reduction in liver weight ($p=0.06$) in vancomycin treated diet-induced obese mice relative to diet-induced obese mice. However, liver triglyceride levels were unaltered by vancomycin in diet-induced obese mice. In contrast, comparison of *L. salivarius* UCC118Bac$^+$ with the bacteriocin-negative derivative showed that the bacteriocin-induced alterations in the gut microbiota did not alter metabolic parameters in diet induced obese mice (see Supplementary Table 2).

2.5.8 Vancomycin treatment is associated with reduced plasma TNFα levels and TNFα mRNA levels in liver and visceral adipose tissues of diet-induced obese mice

To investigate further the effect of vancomycin on metabolic health, the plasma levels of TNFα and the gene expression of TNF-α, monocyte chemoattractant protein 1 (MCP-1) and the macrophage differentiation marker (F4$^+$80) were assessed in the visceral adipose tissue and liver as markers of inflammation. Diet-induced obesity in mice was associated with an increase in plasma TNF-α ($p<0.05$) and an increase in the gene expression of F4$^+$80 ($p<0.001$), MCP-1 ($p<0.001$) and TNF-α ($p<0.05$) in

visceral adipose but not liver tissue (Fig 3A,B). Treatment of diet-induced obese mice with vancomycin resulted in a decrease in plasma TNF-α levels ($p<0.05$) compared with diet-induced obese mice (Fig 3A), and this was associated with a tendency towards a reduction in the gene expression of TNF-α in both adipose ($p=0.06$) and liver ($p=0.09$) tissue. The gene expression levels of F4[+]80 and MCP-1 in adipose or liver tissues were unchanged in vancomycin-treated diet-induced obese mice compared with diet-induced obese controls (Fig 3B).

2.6   DISCUSSION

The findings show that although vancomycin and the bacteriocin-producing probiotic, *L. salivarius* UCC118, altered the gut microbiota in diet-induced obese mice in distinct ways, only vancomycin treatment resulted in an improvement in the metabolic abnormalities associated with diet-induced obesity.

In the present study, vancomycin was chosen for its ability to target the Gram-positive component of the gut microbiota and its limited systemic impact (Moellering, 2006, Pultz *et al.*, 2005, Yap *et al.*, 2008). In addition, vancomycin exposure has been shown to alter significantly the host metabolome in healthy mice (Yap *et al.*, 2008) and the main components of the gut microbiota in a human distal colon model (Rea *et al.*, 2010). In the current study, vancomycin treatment of diet-induced obese mice resulted in a major alteration in the composition of the gut microbiota whereby the respective proportions of the three dominant phyla were altered dramatically with respect to each other, with a large reduction in Firmicutes, and in particular the Bacteroidetes, and a dramatic increase in Proteobacteria. These alterations were associated with a reduction in body weight gain and an improvement in inflammatory and metabolic health of the host. In particular, plasma TNF-α levels were reduced in vancomycin-treated diet-induced obese mice compared with diet-induced obese controls, and this corresponded to a trend towards a reduction in the gene expression of TNF-α levels in the liver and visceral adipose tissues. In both in-vitro and in animal models, an increase in TNF-α has been linked to tissue insulin resistance (Yap *et al.*, 2008, Hotamisligil *et al.*, 1995, Hotamisligil *et al.*, 1996, Hotamisligil and Spiegelman, 1994). Other studies have shown that modulation of the microbiota by broad-spectrum antibiotics results in a reduction in metabolic endotoxaemia in both high-fat-fed and *ob/ob* mice, and is associated with improvements in inflammation, glucose tolerance and hepatic steatosis, possibly through a mechanism involving Toll-like receptors (Ley *et al.*, 2005, Cani *et al.*, 2008, Membrez *et al.*, 2008). However, whether the effect on weight gain is sustained or is overcome by microbial compensatory adjustments is unclear, and more long term studies in animal

models and humans are required. These data suggest that the ability of the gut microbiota to regulate inflammatory responses in diet-induced obesity is important in the interaction between gut microbes and obesity-related metabolic dysfunction.

To our knowledge, this is the first report to establish that a bacteriocin produced by a probiotic can substantially alter the composition of the gut microbiota in vivo. We have also recently shown that the bacteriocin produced by *L. salivarius* UCC118 alters the microbiota of pigs and healthy chow-fed mice (Riboulet-Bisson *et al.*, 2012). Bacteriocin production is thought to confer a competitive advantage on the producing strain, enabling it to dominate complex microbial populations (Abee, 1995, Cotter *et al.*, 2005, Klaenhammer, 1993). In this study, the bacteriocin produced by *L. salivarius* UCC118 significantly altered the gut microbiota in diet-induced obese mice by increasing the relative proportions of Bacteroidetes and Proteobacteria and decreasing Actinobacteria compared with a bacteriocin-negative derivative. While these observations suggest that bacteriocins can play a significant role in determining the composition of gut bacterial populations in vivo, the alterations did not confer beneficial effects on metabolic health. Interestingly, the bacteriocin produced by *L. salivarius* UCC118 reduced the proportions of *Bifidobacteria* in diet-induced obese mice. A partial inhibition of *Bifidobacteria* by *L. salivarius* UCC118 has previously been observed in in-vitro studies (Dunne *et al.*, 1999). *Bifidobacteria* have been shown to be positively correlated with improved glucose tolerance and normalised inflammatory tone in high-fat-fed mice (Cani *et al.*, 2007b). These results suggest that while the gut microbiota is a realistic target for addressing obesity-related metabolic dysfunction, the specificity of the antimicrobial agent employed may be critical. Indeed, distinct clusters or enterotypes in the human microbiome have been described (Arumugam *et al.*, 2011) and support the use of targeted strategies.

While the proximate microbiota-related biomarkers of risk for obesity and metabolic dysregulation remain to be determined, recent reports have suggested an association between lactobacilli and the development of obesity (Angelakis and Raoult, 2010, Armougom *et al.*, 2009, Raoult, 2008).

Indeed, *Lactobacillus* populations have been shown to be elevated in obese subjects (Armougom *et al.*, 2009) and subjects with diabetes (Larsen *et al.*, 2010). In addition, vancomycin treatment of patients with infective endocarditis was associated with a significant increase in weight gain (Thuny *et al.*, 2010), leading the authors to speculate that this may be due to the selection of *Lactobacillus* spp. by vancomyin in the gut. However, in the present study, the introduction of *L. salivarius* UCC118 did not contribute to weight gain in diet-induced obese mice. In addition, although higher levels of *Lactobacillus* spp. were detected in diet-induced obese compared with lean mice, treatment of diet-induced obese mice with vancomycin reduced the rate of weight gain, without significantly affecting the proportions of *Lactobacillus* spp. Other studies using lactobacilli as probiotics have shown beneficial effects on metabolic health in animals (Aronsson *et al.*, 2010, Kang *et al.*, 2010, Takemura *et al.*, 2010) and humans (Kadooka *et al.*, 2010, Andreasen *et al.*, 2010). These observations suggest that lactobacilli are not related to the risk of obesity and strain-specific effects need to be taken into account.

Recent reports have shown that the relationship between obesity, gut microbiota and the risk of obesity is more complex than previously considered (Bajzer and Seeley, 2006, Murphy *et al.*, 2010, Schwiertz *et al.*, 2009, Duncan *et al.*, 2008). In the present study, in agreement with others (Ley *et al.*, 2005, Ley *et al.*, 2006, Turnbaugh *et al.*, 2006) diet-induced obesity was characterised by a relative increase in the proportions of Firmicutes and a decrease in Bacteroidetes, and was also associated with a decrease in total bacterial numbers. These results suggest that in addition to altering the composition of the gut microbiota, obesity and diet may also alter the total intestinal microbial load or 'density'. Indeed, diet has been shown to alter diversity and induce large and rapid changes in the gut microbiota (Jumpertz *et al.*, 2011, Turnbaugh *et al.*, 2009). Furthermore, vancomycin treatment of diet-induced obese mice resulted in a further large decrease in total bacterial numbers, while the bacteriocin produced by *L. salivarius* UCC118 did not alter microbial loads in diet-induced obese mice. Further work is required to understand the role of variations in the total microbial load

and diversity in obesity and related conditions and the significance of changes in phylum proportions when the total bacterial load also varies.

In conclusion, our data demonstrate that while vancomycin and the bacteriocin-producing probiotic produced distinctive modifications in the gut microbiota in diet-induced obese mice, vancomycin treatment alone resulted in an improvement in the metabolic abnormalities associated with obesity. Our findings provide further confirmation for the role of the microbiota in metabolic dysregulation, and a supporting rationale for altering the microbiota as a prophylactic strategy using antimicrobial agents, including bacteriocins, but specificity of action will be crucial.

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Table 1 Vancomycin treatment of diet-induced obese mice results in a major disturbance in the gut microbiota.

	Lean	DIO	DIO + vancomycin
Phylum‡			
Firmicutes	60.7±2.8	70.2±2.4*	53.9±0.4†††
Bacteroidetes	30.0±2.3	19.5±1.4*	3.5±1.9†††
Deferribacteria	7.1±2.2	4.7±1.6	0.7±0.3†
Proteobacteria	2.7±0.4	3.5±1.0	37.7±2.5†††
Actinobacteria	2.5±0.7	2.1±0.5	4.1±1.6
Family			
Clostridaceae	30.6±4.1	25.4±3.0	3.9±1.5†††
Bacteroidaceae	16.0±1.2	11.6±1.0*	1.5±0.6†††
Lactobacillaceae	4.9±0.9	13.6±2.7**	19.3±4.3
Bifidobacteriaceae	2.4±0.7	1.2±0.5	4.1±1.6
Porphyromonadaceae	2.3±0.5	1.4±0.2	0.1±0.1†††
Alcaligenaceae	1.0±0.4	0.0±0.0*	6.9±0.9†††
Streptococcaceae	0.4±0.1	0.6±0.1*	2.0±0.5††
Desulfovibrionaceae	0.8±0.3	0.6±0.1	1.7±0.4††
Enterobacteriaceae	0.0±0.0	0.0±0.0	28.0±0.9†††
Genus			
Clostridium	30.1±4.1	25.1±3.1	3.8±1.3†††
Bacteroides	7.4±0.7	3.3±0.5***	0.3±0.1†††
Lactobacillus	4.9±0.9	13.5±2.7**	19.6±4.2
Odoribacter	2.5±0.5	1.1±0.2*	0.0±0.0†††
Bifidobacterium	2.4±0.7	1.2±0.5	4.1±1.6
Lactococcus	0.4±0.1	0.6±0.1*	1.6±0.2†††
Desulfovibrio	0.8±0.3	0.6±0.1	1.7±0.4††
Sutterella	1.0±0.4	0.0±0.0*	6.9±0.9†††

Statistical significance was determined using a two-tailed unpaired t test; p value ≤0.05*; ≤0.01**; ≤0.001*** between lean and diet-induced obese (DIO mice and $p<0.05$†; $p<0.01$††; $p<0.001$††† between diet-induced obesity and Vancomycin-treated diet induced obese mice. ‡Values are mean percentage read number ±SEM, n=9-10.

Table 2 *L. salivarius* UCC118 alters the composition of the gut microbiota in diet-induced obese mice by a mechanism involving bacteriocin production.

	DIO + UCC118Bac[+]	DIO + UCC118Bac[-]
Phylum†		
Firmicutes	66.9±3.3	72.4±1.6
Bacteroidetes	23.9±2.1	17.6±1.9*
Deferribacteria	6.1±2.2	3.4±1.0
Proteobacteria	1.6±0.2	1.1±0.2
Actinobacteria	1.5±0.4	5.2±1.4*
Family		
Clostridaceae	15.5±2.6	22.0±2.5
Bacteroidaceae	14.4±1.3	8.0±0.7*
Lactobacillaceae	16.9±4.4	12.4±2.5
Bifidobacteriaceae	1.4±0.4	5.1±1.4*
Porphyromonadaceae	2.3±0.4	2.5±0.4
Streptococcaceae	0.8±0.1	0.7±0.1
Desulfovibrionaceae	0.7±0.2	1.2±0.7
Enterobacteriaceae	0.4±0.1	0.2±0.1
Genus		
Clostridium	16.9±4.4	11.4±2.7
Bacteroides	4.4±0.5	2.1±0.5**
Lactobacillus	16.9±4.4	11.4±2.7
Odoribacter	2.3±0.4	2.5±0.4
Bifidobacterium	1.4±0.4	5.1±1.4*
Lactococcus	0.8±0.1	0.7±0.1

Statistical significance was determined using a two-tailed unpaired t test; *p* value ≤0.05*; ≤0.01**. †Values are mean percentage read number ±SEM, n=9-10. DIO, diet-induced obese.

Figure 1 Numbers of 16S rRNA gene copies/g stool in lean, diet-induced obese (DIO) and vancomycin (vanco)- treated diet-induced obese mice (A) and in diet-induced obese mice treated with the bacteriocin-producing probiotic strain *L. salivarius* UCC118Bac[+] and a non-bacteriocin-producing derivative *L. salivarius* UCC118Bac[−] (B). Data represented as mean ±SME, n=9-10, ***p<0.001.
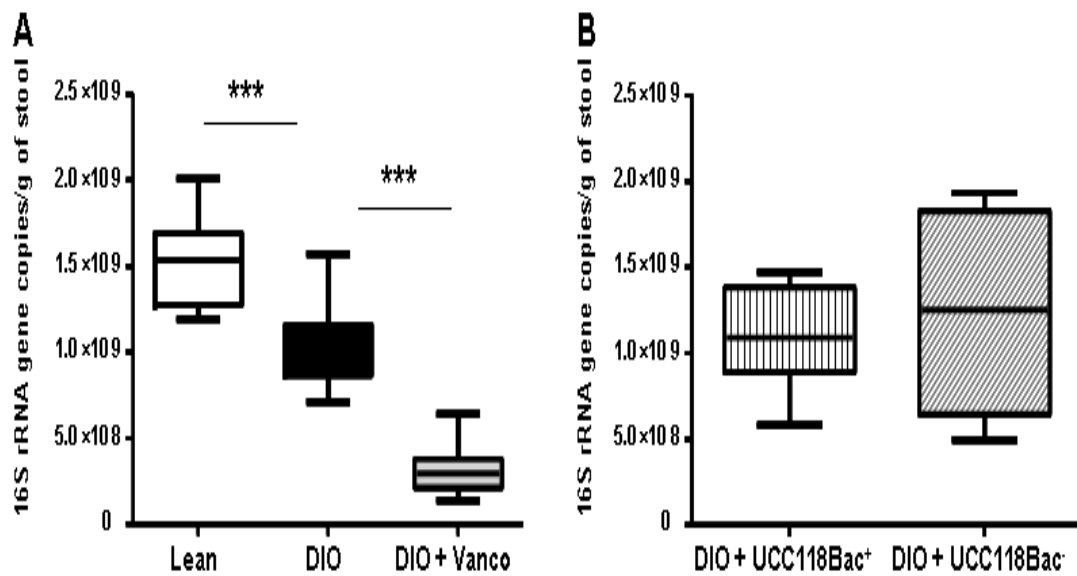
Figure 2 Weight gain (g) (A and B) and cumulative energy intake (KJ/mouse) (C) over the 8-week intervention period in lean, diet-induced obese (DIO) and vancomycin (vanco)-treated diet-induced obese mice and in diet-induced obese mice treated with the bacteriocin-producing probiotic strain *L. salivarius* UCC118Bac$^+$ (1x10^9 cfu/day) and a non-bacteriocin-producing derivative *L. salivarius* UCC118Bac$^-$ (1x10^9 cfu/day). Data represented as mean ±SEM, n=9-10. *p<0.05.
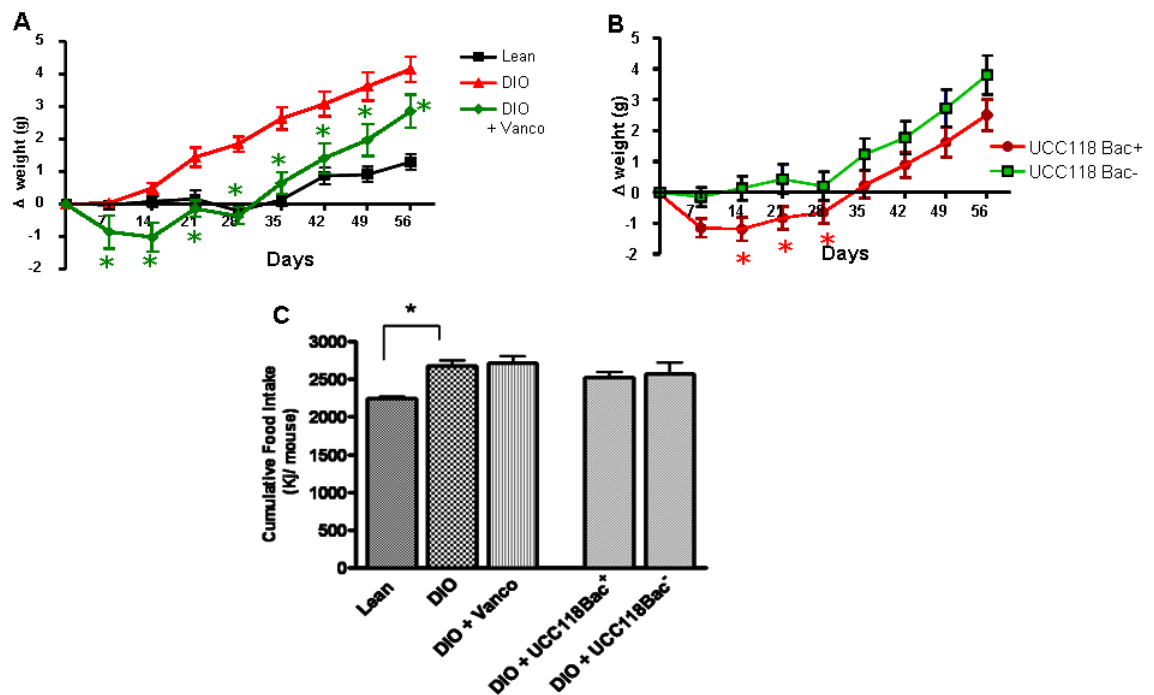
Figure 3 Vancomycin (vanco) treatment improves the inflammatory tone of diet-induced obese (DIO) mice. Plasma tumour necrosis factor alpha (TNFα) (pg/ml) (A) and gene expression levels of the macrophage differentiation marker (F4$^+$80), monocyte chemoattractant protein (MCP-1) and TNFα in visceral adipose tissue and liver (B) in lean and diet-induced obese mice (12weeeks of high-fat feeding followed followed by 8-week intervention with vancomycin (2mg/day)). Data represented as mean ±SEM, n=9-10. *$p<0.05$; ***$p<0.001$. Values for gene expression are fold change ±SEM, n=9-10. Expression is relative to β-actin.
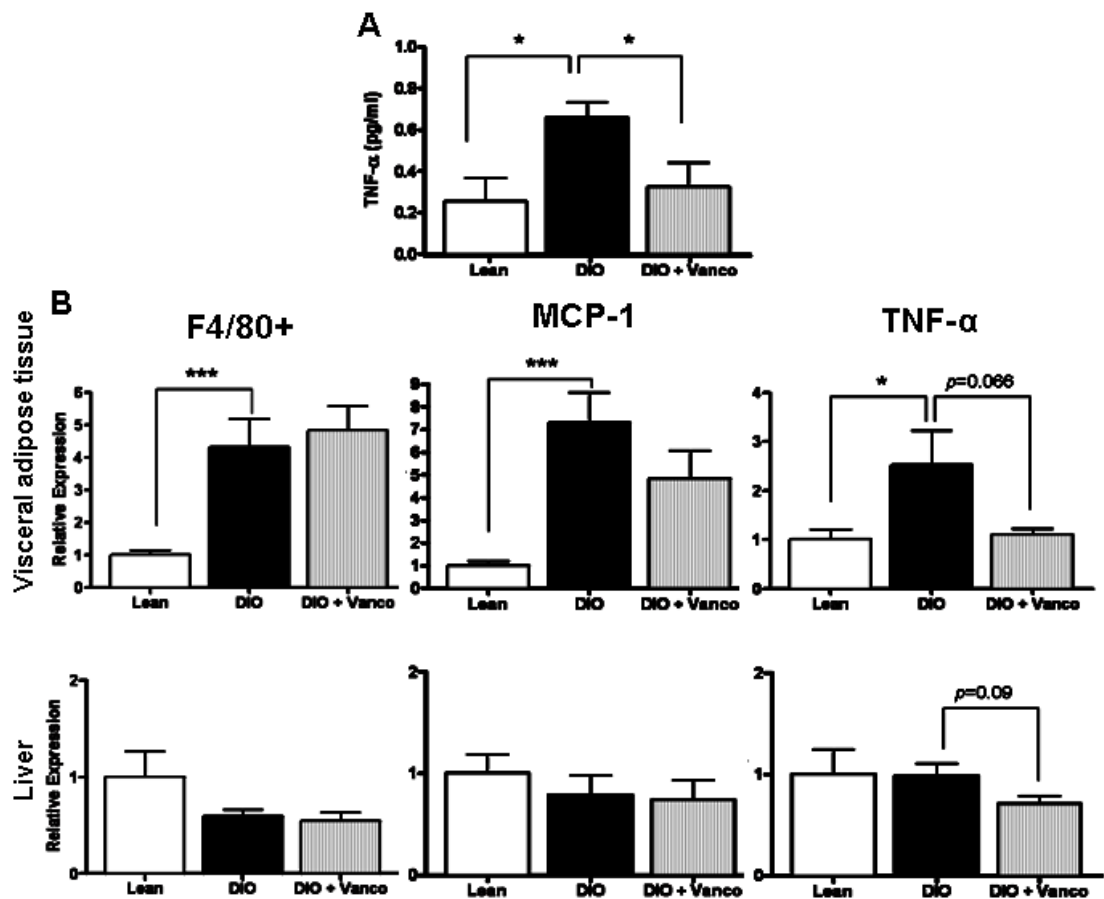
SUPPORTING INFORMATION

Supplementary Materials And Methods

The effect of vancomycin on the health status of chow-fed mice

An initial study was undertaken to establish that vancomycin at a dose of 2 mg/day does not alter the health status of mice and is effective at altering the components of the gut microbiota in mice, using traditional plating methods. Briefly, 7- week old male chow-fed C57BL/J6 mice (n=4) were gavaged with PBS or 2 mg/d of vancomycin for 4 weeks. A clinical score sheet based on weight, appearance, stool, physical signs and behaviour was kept daily. After 4 weeks of treatment, fresh faecal samples were collected under anaerobic conditions and the total numbers of culturable *Enterococcus* spp., *Enterobacteriaciae,* total anaerobes and *Lactobacillus* spp. were determined using Kenner Faecal Streptococcal (KF-Strep), Violet Red Bile Dextrose (VRBD), Wilkins-Chalgren (WC) and *Lactobacillus* selection (LBS) agar, respectively. Bacterial numbers were expressed as colony forming units (CFU)/g faecal content.

DNA extractions and amplicon sequencing

Total metagenomic DNA was extracted from individual faecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK) coupled with an initial bead-beating step. The microbial composition of these samples was determined by pyrosequencing of 16S rRNA tags (V4 region; 239 nt long) amplified using universal 16S rRNA primers predicted to bind to 94.6% of all 16S genes i.e. the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp). The primers incorporated the proprietary 19-mer sequences at the 5'-end to allow emulsion-based clonal amplification for the 454 pyrosequencing system. Unique molecular identifier (MID) tags were incorporated between the adaptamer and the target-specific primer sequence, to allow identification of individual sequences from pooled amplicons. Amplicons were cleaned using the AMPure purification system

(Agencourt) and sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, West Sussex, UK) according to 454 protocols. Sequences were trimmed and quality checked with RDP pyrosequencing pipeline. Reads with low quality scores (quality scores below 40) and short length (less than 150bp for 16S rRNA V4 region) were removed as well as reads that did not have exact matches with the primer sequence. Clustering and statistical analysis of sequence data was performed using the MOTHUR software package (Schloss and Handelsman, 2008). Trimmed fasta sequences were then BLASTed (Altschul *et al.*, 1997) against a previously published 16S rRNA-specific database (Urich *et al.*, 2008) using default parameters. The resulting BLAST output was parsed using MEGAN (Huson *et al.*, 2007). MEGAN assigns reads to NCBI taxonomies by employing the Lowest Common Ancestor algorithm which assigns each RNA-tag to the lowest common ancestor in the taxonomy from a subset of the best scoring matches in the BLAST result. Bit scores were used from within MEGAN for filtering the results prior to tree construction and summarization (absolute cutoff: BLAST bitscore 86, relative cutoff: 10% of the top hit) (Urich *et al.*, 2008). Unsupervised hierarchical cluster analyses were computed using Pearson's correlation coefficient and Ward linkage (Ward Jr, 1963) and applied to data that was dual scaled to the limits of -3 and 3. The heatplots were generated using the "made4" library in R (Culhane *et al.*, 2005).

Real-time quantitative PCR

RNA was isolated from liver tissue using the Absolute RNA Miniprep Kit from Stratagene (Texas, US) and from adipose tissue using the RNeasy Lipid Tissue Mini Kit from Qiagen (Maryland, US). Total RNA was quantified using the Nanodrop (Thermoscientific, Wilmington, Delware, US) and the quality assessed using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, California, US). Single-stranded complementary DNA (cDNA) was synthesisized from 1 µg total RNA using the reverse-transcription system from Promega (Leiden, the Netherlands) according to the manufacturer's protocol. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using 1 µl cDNA on the LightCycler 480 System using the thermal cycling conditions as per the manufacturer's instructions (Roche

Diagnostics GmbH, Germany). Polymerase chain reaction (PCR) primers and probes were designed using the Universal Probe Library Assay Design Centre (https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). All samples were analyzed in duplicate and normalized to β-actin (as a constitutively expressed control gene).

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Ward Jr, J. H. 1963. Hierarchical grouping to optimize an objective function. *Journal of the American Statistical Association,* 58**,** 236-244.

Supplementary Table 1 Tissue weights and metabolic profiles of lean, diet-induced (DIO) mice and in response to treatment of DIO mice with vancomycin.

	Lean	DIO	DIO + vancomycin
Tissue Parameters (g)			
Fat mass (NMR)	6.3±0.6	16.0±0.8*	15.0±1.0
Liver	1.3±0.1	1.7±1.3**	1.4±0.1[†]
Caecum	0.3±0.1	0.2±0.01***	0.9±0.03[†††]
Caecum contents	0.2±0.01	0.1±0.01**	0.7±0.04[†††]
Spleen	0.1±0.01	0.1±0.01	0.1±0.01
Metabolic Parameters			
Blood glucose	122.8±4.1	148.6±5.0**	116.0±13.6[†]
Insulin (µg/L)	0.5±0.01	0.6±0.02*	0.5±0.01
Plasma triglycerides (mg/dl)	29.9±2.0	31.6±3.3	22.1±2.6
Free fatty acids	10.6±1.1	14.4±2.9	14.3±2.4
Liver triglycerides (mg/dl)	101.0±12.0	192.0±20.1*	156.7±15.0

[1] Values are mean ± SEM, n= 9-10.
Statistical significance was determined using a 2-tailed unpaired t-test; p-value ≤0.05*; ≤0.01**; ≤0.001*** between lean and DIO mice and $p<0.05$[†], $p<0.01$ [††]; $p<0.001$ [†††] between DIO and vancomycin-treated DIO mice

Supplementary Table 2 Metabolic profiles of diet-induced (DIO) mice fed the bacteriocin-producing probiotic *L. salivarius* UCC118Bac[+] and a non-bacteriocin-producing derivative *L. salivarius* UCC118Bac[-].

	DIO + UCC118 Bac[+]	DIO + UCC118 Bac[-]
Metabolic Parameters		
Blood glucose	129.1±6.0	140.2±6.2
Insulin (µg/L)	0.6±0.04	0.7±0.03
Plasma triglycerides (mg/dl)	28.1±2.7	29.1±2.8
Tumor necrosis factor (pg/ml)	0.5±0.2	0.8±0.2
Liver triglycerides (mg/dl)	170.4±19.4	147.9±10.9

[1] Values are mean ± SEM, *n*= 9-10.
Statistical significance was determined using a 2-tailed unpaired *t*-test

Supplementary Figure 1 Rarefaction curves for each group at 97% similarity levels indicated that the total bacterial diversity present was well represented. Number of operational taxonomic units (OTUs) identified as a function of the number of sequence tags sampled.
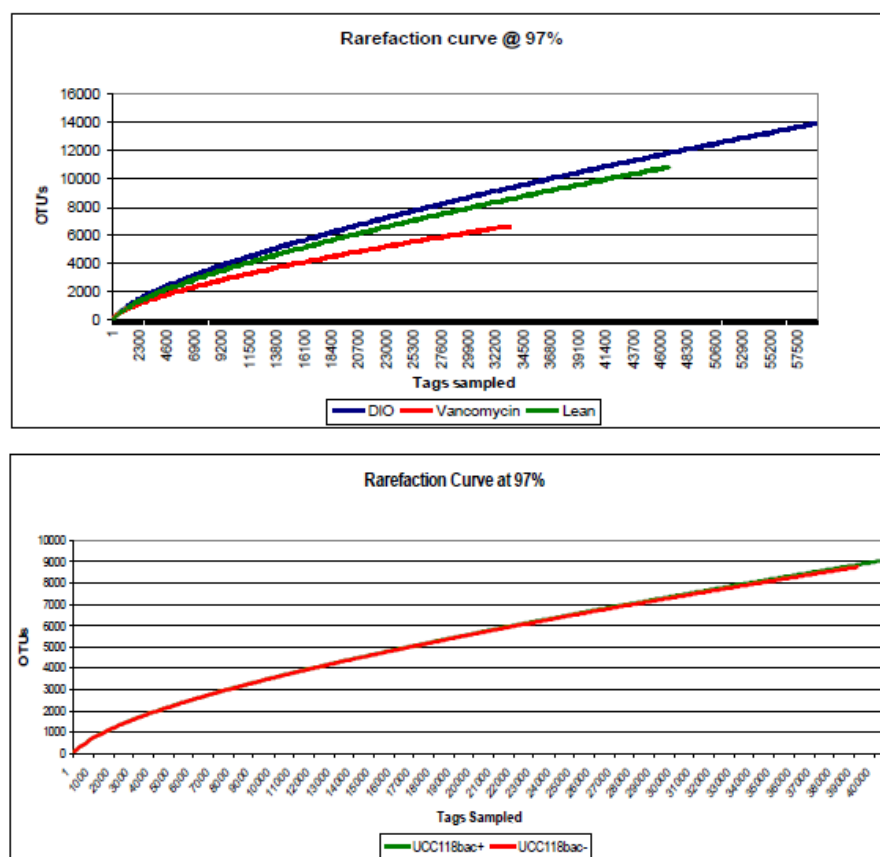
	Lean		DIO		DIO +Vancomycin		DIO +UCC118Bac$^+$		DIO +UCC118Bac$^-$	
Similarity	97%	98%	97%	98%	97%	98%	97%	98%	97%	98%
Chao1 richness estimation	36890	51096	43635	63912	18420	31026	41654	43841	27235	39871
Shannon's index for Diversity	7.7	8.2	8.0	8.5	7.1	7.7	7.7	8.3	7.7	7.3
Good's coverage	84%	78%	84%	78%	87%	81%	85%	79%	85%	79%

Supplementary Figure 2 Composition of the gut microbiota at the phylum, family and genus level as determined by pyrosequencing of 16S rRNA tags (V4 region) of lean, DIO and DIO mice treated with for 8 weeks with vancomycin (2mg/day). Data outside the pie charts represent the mean percentage read number for the corresponding colour coded family (n=9-10 per group).
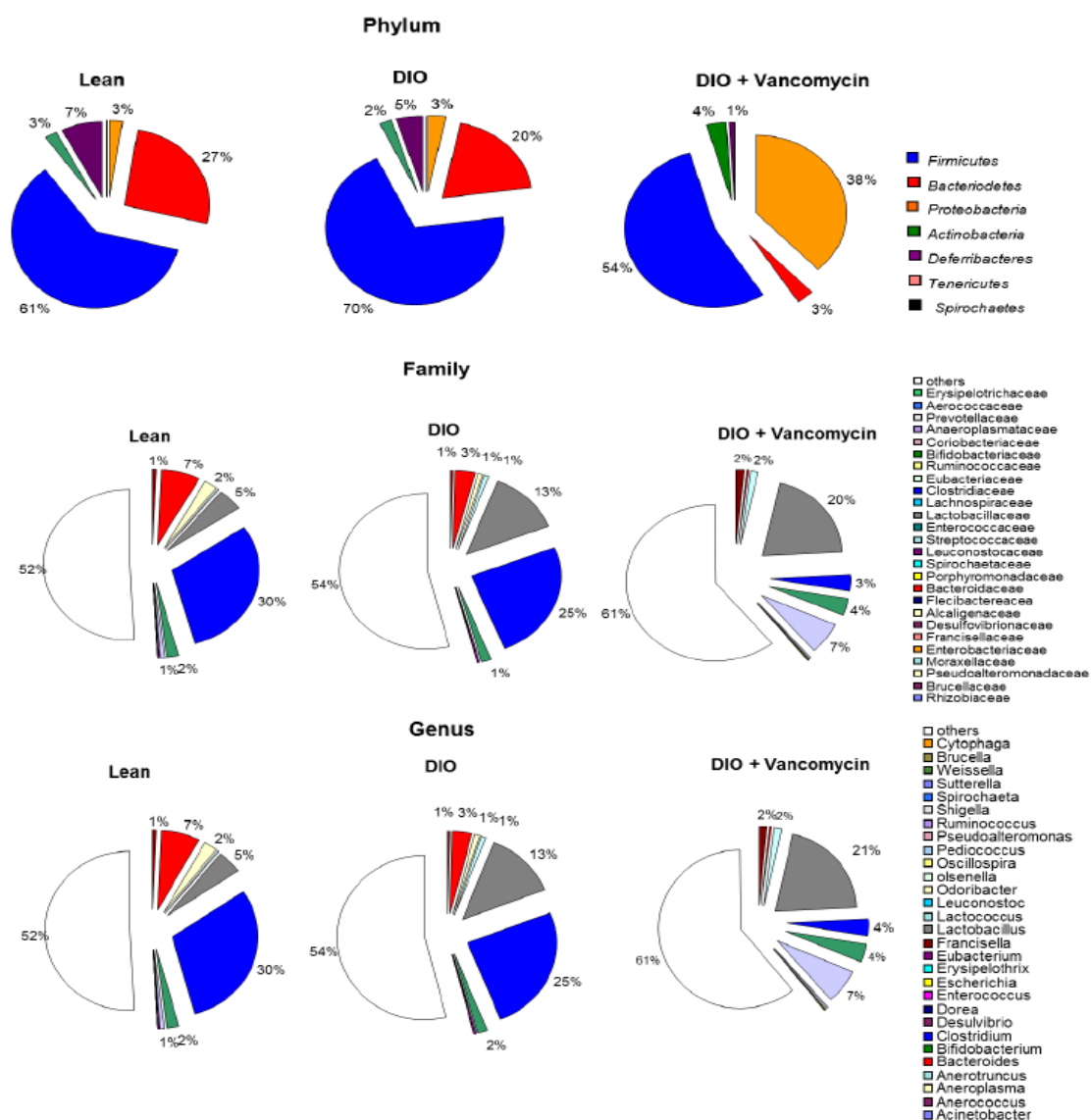
Supplementary Figure 3 Impact of the administration of 2mg/day of vancomycin (V) compared to PBS (control (C)) for 4 weeks on components of the murine faecal microbiota (n=3-4 per group) as determined using culture-based approaches. The selective media employed were FK *Streptococcus* (KF-Strep) agar for *Enterococcus* spp. (black bars), Violet Red Bile Dextrose (VRBD) agar for *Enterobacteriaciae* (white bars), Wilkins-Chalgren agar (WCA) for total anaerobes (dark grey bars) and *Lactobacillus* selection agar (LBS) for *Lactobacillus spp.* (light grey bars). Data are expressed as mean ± SD (n=3-4 per group; *p≤0.05 as determined by the Mann- Whitney non-parametric test.
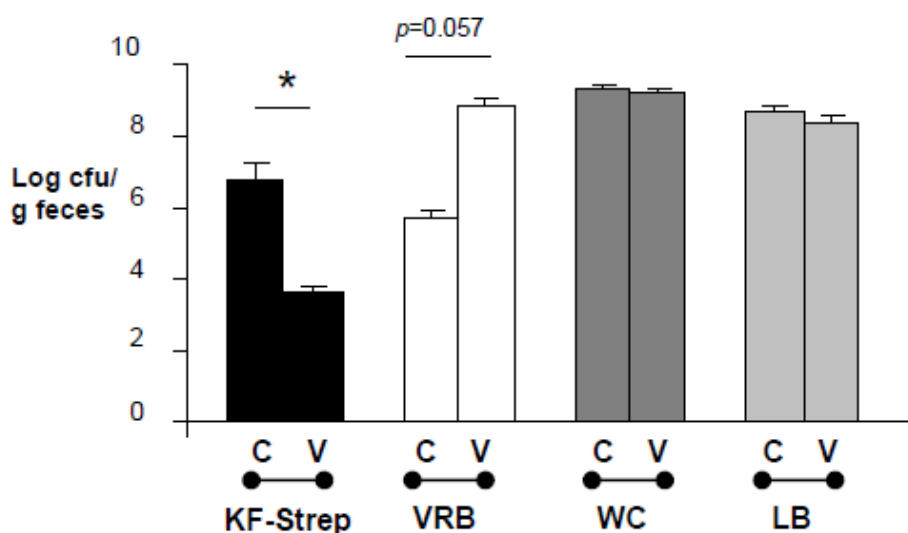
Supplementary figure 4 Clustering of microbiota composition at phylum level of lean, DIO and DIO mice treated with for 8 weeks with vancomycin (2mg/day).
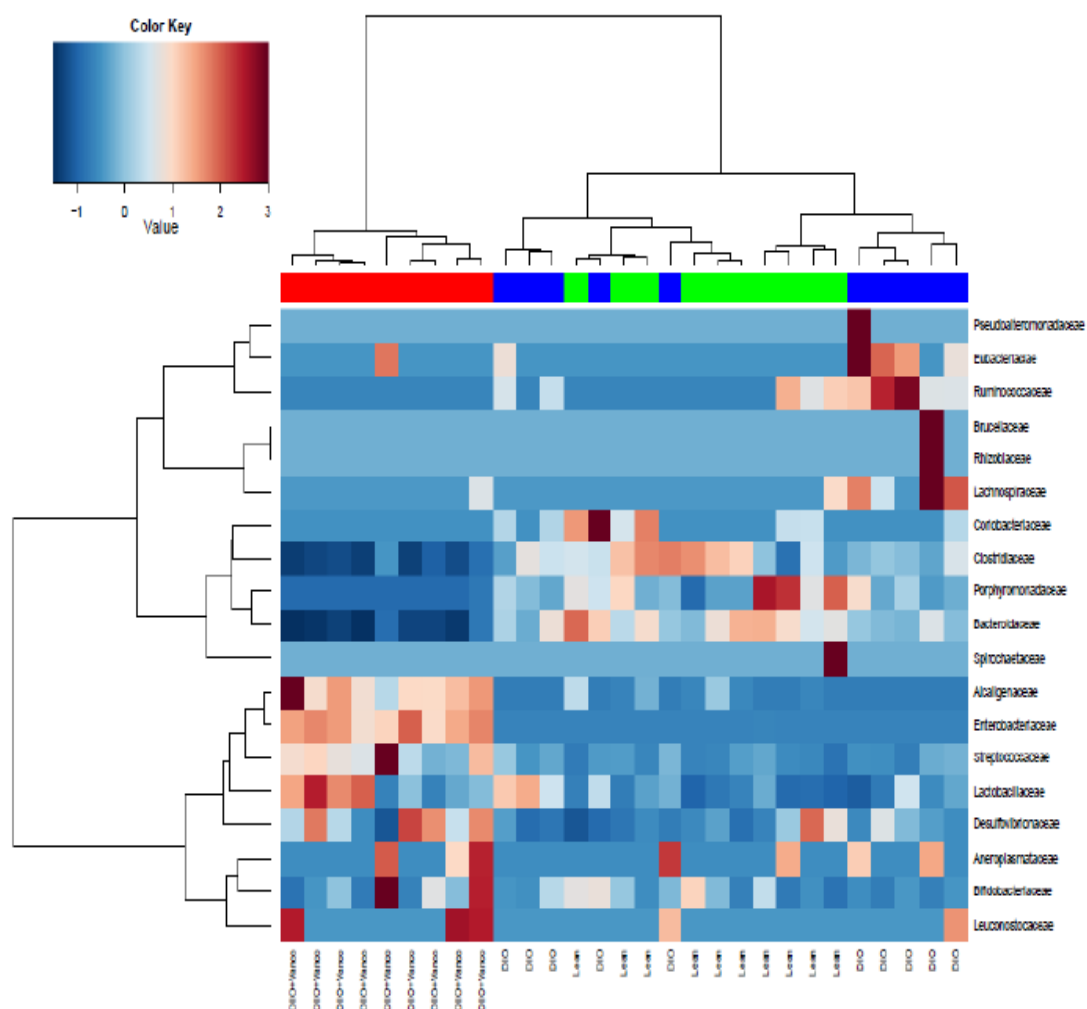
Supplementary Figure 5 Composition of the gut microbiota at the phylum, family and genus level of DIO mice treated with the bacteriocin-producing probiotic strain *L. salivarius* UCC118Bac$^+$ (1x10^9 cfu/day) compared to DIO mice treated with a non-bacteriocin-producing derivative *L. salivarius* UCC118Bac$^-$ (1x10^9 cfu/day). Data outside the pie charts represent the mean percentage read number for the corresponding colour coded family (n=10 per group).
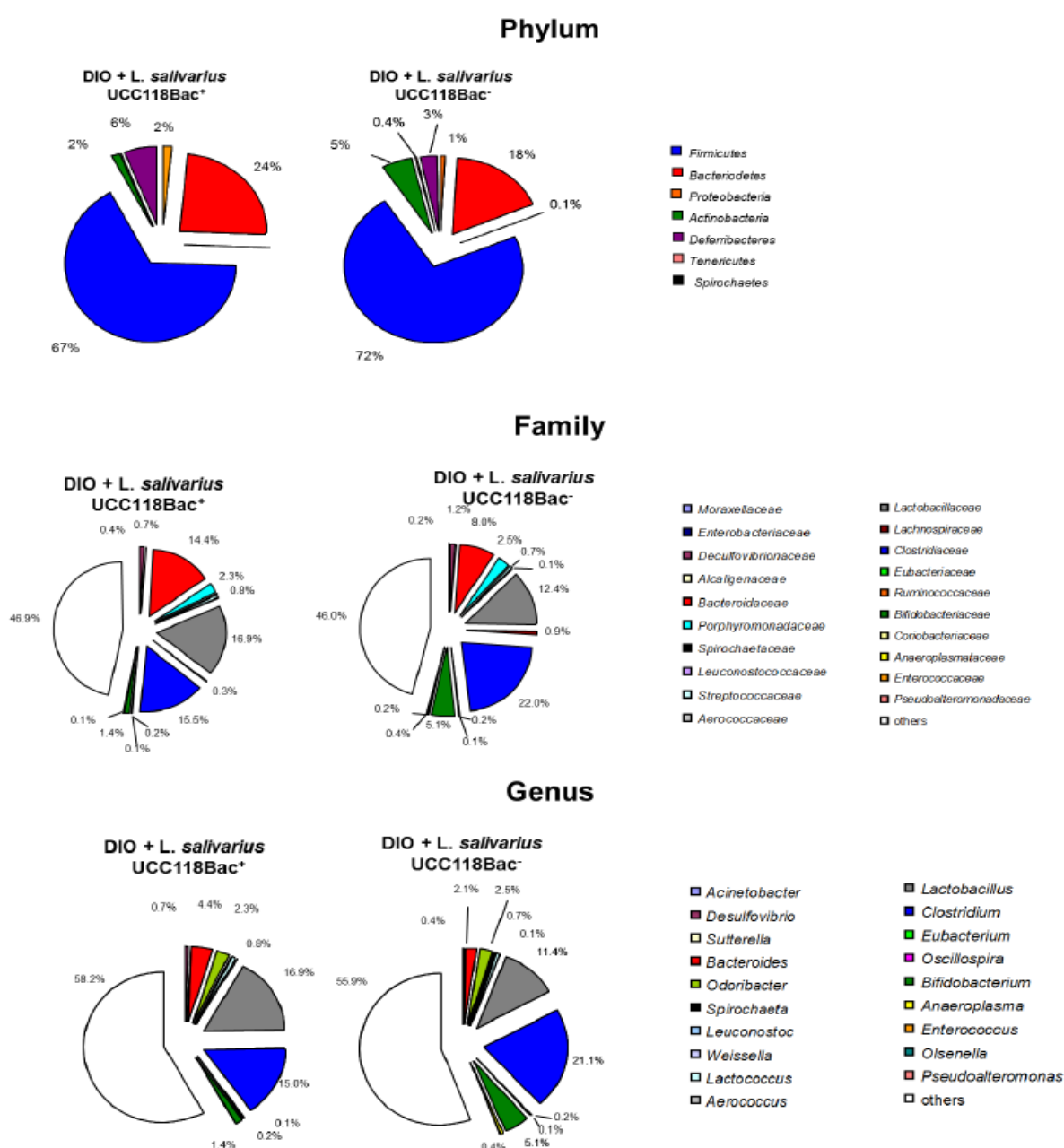
Supplementary figure 6 Clustering of microbiota composition at phylum level of DIO mice treated with the bacteriocin-producing probiotic strain *L. salivarius* UCC118Bac[+] (1x10^9 cfu/day) compared to DIO mice treated with a non-bacteriocin-producing derivative *L. salivarius* UCC118Bac[-] (1x10^9 cfu/day).
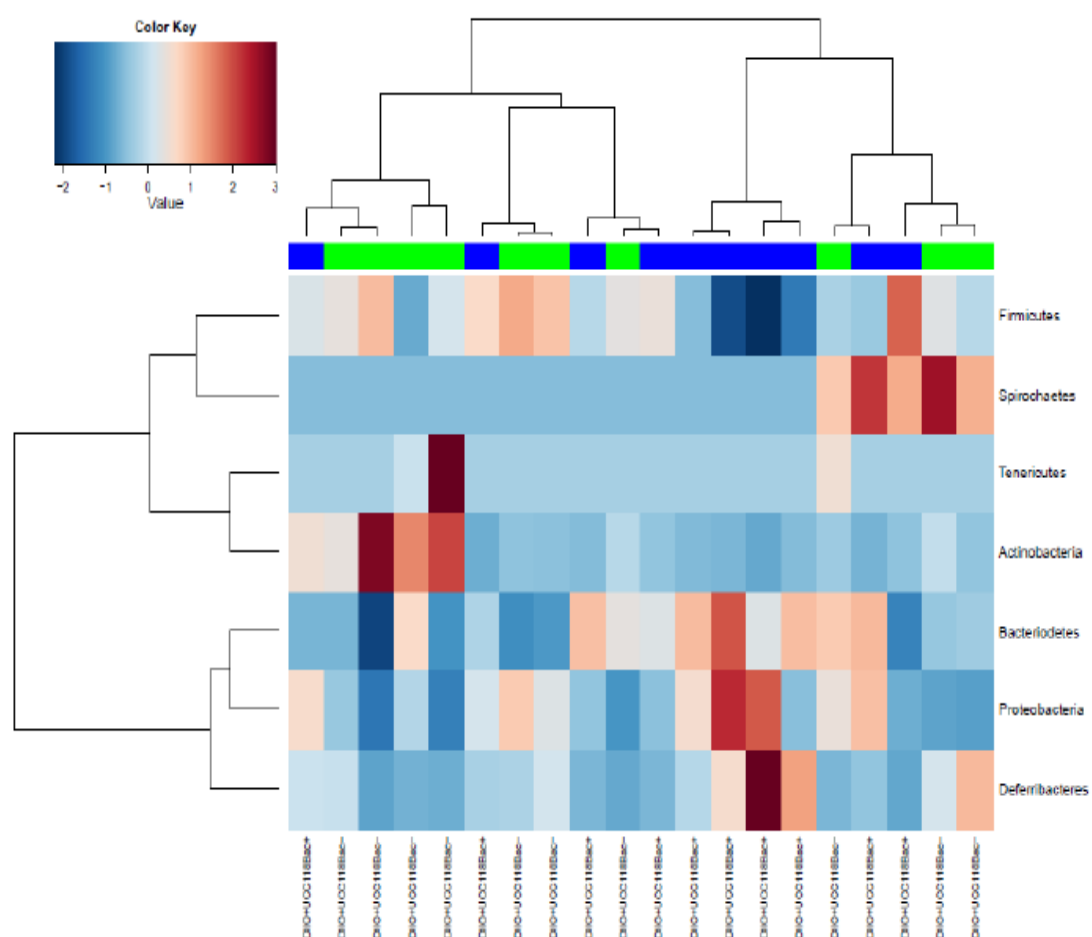
Chapter 3

Targeting the microbiota to address diet-induced obesity:
a time dependent challenge

3.1 ABSTRACT

Links between the gut microbiota and host metabolism have provided new perspectives on obesity. We previously showed that the link between the microbiota and fat deposition is age- and time-dependent subject to microbial adaptation to diet over time. We also demonstrated reduced weight gain in diet-induced obese (DIO) mice through manipulation of the gut microbiota with vancomycin or with the bacteriocin-producing probiotic *Lactobacillus salivarius* UCC118 (Bac$^+$), with metabolic improvement achieved in DIO mice in receipt of vancomycin. However, two phases of weight gain were observed with effects most marked early in the intervention phase. Here, we compare the gut microbial populations at the early relative to the late stages of intervention using a high throughput sequencing-based analysis to understand the temporal relationship between the gut microbiota and obesity. This reveals several differences in microbiota composition over the intervening period. Vancomycin dramatically altered the gut microbiota composition, relative to controls, at the early stages of intervention after which time some recovery was evident. It was also revealed that Bac$^+$ treatment initially resulted in the presence of significantly higher proportions of *Peptococcaceae* and significantly lower proportions of *Rikenellaceae* and *Porphyromonadaceae* relative to the gut microbiota of *L. salivarius* UCC118 bacteriocin negative (Bac$^-$) administered controls. These differences were no longer evident at the later time. The results highlight the resilience of the gut microbiota and suggest that interventions may need to be monitored and continually adjusted to ensure sustained modification of the gut microbiota.

3.2 INTRODUCTION

Obesity is due to a surplus of energy intake over expenditure, resulting in storage of excess energy as fat. However, this is only part of a bigger story; an emerging theme is the relationship between the composition and functionality of microorganisms in the gut with obesity (Cani and Delzenne, 2011, Clarke *et al.*, 2012, Flint, 2011, Greiner and Bäckhed, 2011, Ley, 2010). A corollary to this is the potential for manipulation of the gut microbiota in the prevention and management of obesity and associated metabolic disorders.

We previously showed that compositional changes in the faecal microbiota associated with diet-induced obesity are time-dependent and unrelated to markers of energy harvest, which change over time (Murphy *et al.*, 2010). Furthermore, we have previously investigated the impact of administering the glycopeptide antibiotic vancomycin and the bacteriocin-producing probiotic *Lactobacillus salivarius* UCC118 (Bac$^+$) to diet-induced obese (DIO) mice. Vancomycin resulted in an improvement in the metabolic abnormalities associated with obesity, including a significant reduction in weight gain, by the end of the intervention period. In contrast, when compared with an isogenic non-bacteriocin producing control (Bac$^-$), the *L. salivarius* UCC118 Bac$^+$ strain alters the gut microbiota but did not significantly alter metabolic markers or weight gain as measured at the end of the intervention period (Murphy *et al.*, 2012).

While our initial report focused on the metabolic changes evident upon completion of the intervention strategies, the temporal changes in the microbiota need to be addressed further. The impact of vancomycin intervention on weight gain was most considerable during the early stages of intervention and a significant reduction in weight gain in mice fed with the Bac$^+$ strain was apparent when compared with their Bac$^-$ fed counterparts (Murphy *et al.*, 2012). Here, we analyse and compare the gut microbial populations of these animals at the early (week 2) with the late (week 8) intervention period. The results reflect the resilience of the gut microbiota and show that therapeutic manipulation of the microbiota is

likely to be more complex than anticipated with sustained adjustment likely to require multiple interventions over time.

3.3 MATERIALS AND METHODS

3.3.1 Animals

3-4 week old male C57BL/6j mice were acquired from Harlan (oxon, UK) and housed within the biological services unit, University College Cork. UCC Animal Ethics Committee approved all experiments and experimental procedures were conducted under licence from the Irish government.

3.3.2 Experimental design

A low fat (lean) or high fat (DIO) diet was fed to male C57BL/J6 mice (aged 7 weeks) for 12 weeks followed by an intervention period during which the high fat diet was supplemented with the glycopeptide antibiotic vancomycin, the bacteriocin producing (Bac$^+$) *L. salivarius* UCC118, its bacteriocin negative derivative (Bac$^-$) or was unsupplemented (9-10 mice/cohort) for a period of 8 weeks. For full experimental design see supplementary Fig 1 and Murphy *et al.*, 2012.

3.3.3 DNA extraction and high-throughput amplicon sequencing

Individual mouse faecal samples were collected and DNA was extracted on the same day of collection from fresh samples using the QIAmp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK) combined with an additional bead-beating step (30s x 3) and stored at -20°C. The microbiota composition of the samples was established by amplicon sequencing; universal 16S rRNA primers estimated to bind to 94.6% of all 16S genes (i.e. the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP'S Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) were employed for PCR amplification. Molecular identifier tags were attached between the 454 adaptor sequence and the target-specific primer sequence, allowing for identification of individual sequences from the pooled amplicons. Ampure purification system (Beckman Coulter, Takeley, UK) was used to clean

the amplicons before being sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) in line with 454 protocols at the Teagasc high throughput sequencing centre. The amplicon sequences were deposited in the European bioinformatics institute sequence read archive (EBI-SRA) accession number ERP002448.

3.3.4 Real time quantitative PCR

Total bacterial numbers (16S rRNA gene copies per gram of wet stool (Zhang *et al.*, 2009)) were determined using real time quantitative PCR. The 16S rRNA gene sequence of *E. coli* EPI300 was amplified using the universal 16S primers 802R and 520F (Claesson *et al.*, 2009). The amplified products purified using the High Pure PCR Cleanup Micro Kit (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) were inserted into the pCR4-TOPO Vector (Invitrogen, Bio-Sciences, Dublin Ireland) and transformed into One Shot TOP10 Chemically Competent *E. coli* (Invitrogen, Bio-Sciences, Dublin, Ireland). Plasmids were extracted using the PureYield™ Plasmid Miniprep System (Promega, Madison, Wisconsin, USA) and quantified on the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Quantitative real time PCR (QPCR) was performed with SYBER-green (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) on the lightcycler 480 (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK). The standard curve was generated using dilutions of the plasmid DNA. The following program was used to quantify total bacterial numbers: 95°C for 5 min followed by 40 cycles of 95°C for 20 s, 51°C for 20s and 72°C for 20 s followed by melting curve analysis of 95°C for 5 s, 46°C for 1 min, and 97°C continuously and a final cooling at 40°C for 10 s. Samples contained 2 μl of PCR grade water, 1 μl of 520F (0.15 μM), 1 μl of the 802R (0.15 μM), 1 μl template DNA, and 5 μl of SYBR green. Samples and standards were run in triplicate. Negative controls were added to each plate with template DNA being replaced with PCR-grade water. The copy numbers of each sample were calculated from the standard curve

and copies of 16S rRNA/g wet stool was calculated using a previously outlined calculation (Zhang *et al.*, 2009).

3.3.5 Bioinformatics sequence analysis

A locally installed RDP pyrosequencing pipeline was used to quality trim the raw sequence data. Reads were removed that were shorter than the main distribution (150bp for the 16S rRNA V4 region), of low quality and not exact matches to barcoded tags and primer sequence. A locally installed version of SILVA 16S rRNA database (Pruesse *et al.*, 2007) was used to BLAST (Altschul *et al.*, 1997) the trimmed fasta sequence files using default parameters. Resulting BLAST output files were parsed through MEGAN (Huson *et al.*, 2007a) which uses a lowest common ancestor algorithm to assign reads to NCBI taxonomies. Prior to tree construction and summarization filtering was carried out within MEGAN using bit scores, similar to previous studies a bit-score cut-off of 86 was selected (Urich *et al.*, 2008, Rea *et al.*, 2011). Alpha diversity indices were generated using MOTHUR software (Schloss *et al.*, 2009). Clustering of sequence reads into operational taxonomical units (OTUs) at 97% identity was achieved using QIIME suite software tools (Caporaso *et al.*, 2010). The ChimeraSlayer program was used to remove chimeric OTUs from aligned OTUs and the FastTreeMP tool generated a phylogenetic tree (Price *et al.*, 2010, Haas *et al.*, 2011). Beta diversities were also calculated on the sequence reads based on weighted and unweighted unifrac and bray curtis distances; subsequently principal coordinate analysis (PCoA) and unweighted pair group method with arithmetic mean (UPGMA) clustering was performed on the samples. UPGMA clustering was visualised using Dendroscope software (Huson *et al.*, 2007b) while PCoA plots were viewed with KiNG viewer (Chen *et al.*, 2009). The nonparametric Kruskal-Wallis test (Kruskal and Wallis, 1952) in the Minitab statistical package was employed to establish statistical significance (significance taken to be $p \leq 0.05$).

3.4 RESULTS

3.4.1 α diversity of the murine gut microbiota increases during the intervention period

There is a significant reduction in weight gain in DIO mice at intervention weeks 2-4 (early intervention period) in the Bac[+] intervention, when compared to Bac[-] intervention, but this does not persist with time (Fig 1A) (Murphy *et al.*, 2012). Vancomycin administration results in a two phase reduction in weight gain in DIO mice. In phase one (early intervention weeks 1-4) a significant reduction in weight gain relative to the initial start weight is observed. In the second phase, DIO mice receiving vancomycin gain weight relative to the initial start weight but weight change continues to be significantly less than that in DIO controls (Fig 1B) (Murphy *et al.*, 2012). The relationship between these early intervention period-specific observations and the gut microbiota were investigated through high throughput DNA sequencing. A total of 86,103 V4 16S sequence reads, corresponding to faecal pellets from mice at intervention week 2 of the study, were generated. These corresponded to an average of 14,978 reads per group or 1,757 per mouse. These reads were analysed and compared with 212,655 16S sequence reads generated from the mice at intervention week 8 (Murphy *et al.*, 2012). Shannon diversity, simpson diversity and species richness estimations were calculated for each data set (Fig 2). The Chao1 estimator of species richness reveals significant differences in species richness with time in all populations, including lean and DIO animals not exposed to interventions. Shannon diversity data revealed a high level of biodiversity in all groups along with a significant increase in diversity with time in all cohorts except those in receipt of a lean diet only (Fig 2). The Simpson diversity index-based analysis, which also takes account the number of species present and the relative abundance of each species, particularly highlighted the significant increase in diversity in animals in receipt of vancomycin as the intervention period continued. Thus while a general increase in gut microbial diversity was apparent as the mice aged, it was apparent that exposure to vancomycin brought about an initially considerable reduction

in diversity which was diminished with time. Goods coverage ranged between 95% and 98% at week 2 and from 84% to 86% at week 8. Rarefaction curves were seen to be approaching parallel or parallel (Supplementary Fig 2) signifying that extra sampling would yield a limited increase in species richness. Of the reads at week 2, 74,686 (87%) were assigned at phylum level, 59,720 (69%) at the family level and 40,947 (48%) at genus level.

3.4.2 Taxonomical analysis highlights the temporal impact of vancomycin on specific components of the gut microbiota

Analysis of the gut microbiota composition after 2 weeks established that the major difference, at the phylum level, between the microbiota of the lean and diet induced obese (DIO) mice is the presence of relatively greater proportions of Firmicutes and relatively lower proportions of Bacteroidetes in DIO mice when compared with lean mice (p value ≤0.05). At family level, significantly greater proportions of *Rhodospirillaceae*, *Lachnospiraceae*, *Streptococcaceae*, *Lactobacillaceae*, and *Clostridiaceae* in DIO mice were apparent when compared to lean mice (p value ≤0.05), whereas significantly lower proportions of *Alcaligenaceae*, *Rikenellaceae*, *Bacteroidaceae* and *Coriobacterineae* were evident when DIO mice were compared with lean mice (p value ≤0.05). At genus level, when the microbiota of DIO mice were compared with that of lean mice, significantly more, *Thalassospira*, *Alistipes*, *Odoribacter* and *Bacteroides* and significantly less *Sutterella Lactococcus*, *Turicibacter*, *Lactobacillus*, *Clostridium* and *Anaeroplasma* were observed (p value ≤0.05; Fig 3 & Supplementary Table 1).

High-throughput DNA sequence based analysis of the gut microbiota at week 2 also revealed statistically significant differences at phylum level between DIO and DIO vancomycin treated mice. Significant decreases in proportions of Bacteroidetes and Deferibacteres (p value ≤0.05), but not Firmicutes, were noted in vancomycin treated DIO mice compared with DIO mice. An increase in proportions of Proteobacteria (p value ≤0.05) in DIO vancomycin treated mice was also evident. At family level, proportions of *Rhodospirillaceae*, *Rikenellaceae*,

Porphyromonadaceae, *Bacteroidaceae*, *Ruminococcaceae*, *Peptostrepococcaceae*, *Peptococcaceae*, *Erysipelotrichaceae*, and *Deferribacteraceae* (p value ≤0.05) were all relatively lower in the vancomycin treated mice relative to DIO controls whereas proportions of *Alcaligenaceae*, *Enterobacteriaceae, Streptococcaceae, Lactobacillaceae* and *Leuconostocaceae* (p value ≤0.05) were all relatively greater in the former group. At genus level relatively lower proportions of *Thalassospira, Alistipes, Rikenella, Parabacteroides, Odoribacter, Bacteroides, Lachnospiraceae Incertae Sedis, Coprococcus, Ruminococcaceae Incertae Sedis, Oscillibacter, Anaerotruncus, Turicibacter, Allobaculm, Mucispirillum,* uncultured *Lachnospiraceae* genus members*, Peptostreptococcaceae Incertae Sedis, Peptococcus, Clostridium and Anaeroplasma* (p value ≤0.05) were detected in DIO vancomycin treated mice compared with DIO mice. These were accompanied by the presence of relatively greater proportions of *Sutterella*, *Lactococcus*, *Lactobacillus*, *Weissella* and members of *Enterobactereaceae*-associated genera in the antibiotic treated group (p value ≤0.05) (Fig 3 & Supplementary Table 1).

The sequence data generated was investigated from a temporal perspective. As suggested from α diversity values, the six weeks which passed from intervention week 2 to 8 of the study impacted on microbiota composition even in animals where no intervention occurred, i.e. the lean and DIO controls, presumably as a consequence of the aging of the animals. At week 8, among the lean animals, it was noted that significant increases in proportions of the phyla Deferribacteres and Verrucomicrobia (p value ≤0.05) occurred and, at family level, the relative proportions of *Rhodospirillaceae, Desulfovibrionaceae, Deferribacteraceae, Lactobacillaceae, Verrucomicrobiaceae* and *Eubacteriaceae* (p value ≤0.05) were all significantly increased when compared with microbiota from the same animals at week 2. At genus level proportions of *Thalassospira, Desulfovibrio, Allobaculum, Mucispirillum, Lactobacillus, Akkermansia, Bilophila* and *Blautia* (p value ≤0.05) all increased between weeks 2 and 8. In contrast, proportions of

Bacteroidetes (phylum, *p* value ≤0.05), *Porphyromonadaceae, Bacteroidaceae, Ruminococcaceae* (family, *p* value ≤0.05) and *Alistipes, Bacteroide, Coprococcus and Ruminococcaceae Incertae Sedis* (genus, *p* value ≤0.05) all decreased between the two time points.

Among DIO mice there were relative increases in the proportions of Proteobacteria, Actinobacteria, Deferribacteres and Verrucomicrobia (*p* value ≤0.05) at the week 8, relative the week 2 time point. This corresponded to significant increases in *Rhodospirillaceae, Desulfovibrionaceae, Bifidobacteriaceae, Deferribacteraceae* and *Verrucomicrobiaceae* at the family level (*p* value ≤0.05) and *Desulfovibrio, Mucispirillum,* uncultured *Lachnospiraceae, Akkermansia, Bilophila* and *Catabacter* (*p* value ≤0.05) at the genus level. In contrast, proportions of *Ruminococcaceae, Clostridiaceae* (*p* value ≤0.05) and *Coprococcus, Ruminococcaceae Incertae Sedis, Turicibacter* and *Clostridium* decreased between the two time points (*p* value ≤0.05).

Comparison of the impacts of vancomycin treatment at week 2 relative to those at week 8 had the potential to be particularly revealing given that the relative extent to which the antibiotic impacted on weight gain was greater at the earlier time point and that α diversity also increased during the intervening period. This suggested that a compensatory effect, possibly due to the recovery of specific populations, occurred between the two time points. Analysis revealed that several taxa increased in relative proportions during this interval. These included Bacteroidetes, Actinobacteria, Defferibacteres and Verrucomicrobia (*p* value ≤0.05) at the phylum level, *Desulfovibrionaceae, Rikenellaceae, Porphyromonadaceae, Ruminococcaceae, Erysipelotrichaceae, Bifidobacteriaceae, Deferribacteraceae, Verrucomicrobiaceae* and *Enterobacteriaceae* (*p* value ≤0.05) at the family level and *Ruminococcaceae Incertae Sedis, Turicibacter, Clostridium, Akkermansia, Allobaculum, Desulfovibrio, Alistipes, Bifidobacterium, Mucispirillum, Anaeroplasma* and members of *Enterobacteriaceae*-associated genera (*p* value ≤0.05) at the genus level. Of the genus level changes, only the *Ruminococcaceae Incertae Sedis, Turicibacter,*

Clostridium and *Akkermansia* associated changes corresponded with temporal changes which were evident in the DIO control group and thus the other genera which underwent relative increases in proportions likely represent the populations which have most successfully adapted to vancomycin exposure. Within the vancomycin treatment group the only phylum to decrease significantly, from a temporal perspective, in the gut microbiota of the vancomycin administered mice was the Firmicutes (p value ≤0.05), which corresponded with a significant decrease in the relative proportions of the family *Streptococcaceae* (p value ≤0.05) and the genus *Lactococcus* (p value ≤0.05). A significant decrease over time was also apparent within the genus *Weissella* (p value ≤0.05) (Supplementary Table 1).

3.4.3 *UCC118 Bac$^+$ associated reductions in weight gain correspond to reductions in Rikenellaceae and Porphyromonadaceae and increases in Peptococcaceae populations*

While the impact of vancomycin administration on weight gain decreased from intervention week 2 to week 8 of the study, this impact continued to be significant throughout. However, the impact on weight gain of employing the bacteriocin producing probiotic relative to its isogenic non-bacteriocin producing equivalent changed from being significant to non-significant over the same duration. Comparison of the impact of the two strains at week 2 revealed no significant differences at the phylum level between the two groups. However, at family level, a relative decrease in proportions of *Rikenellaceae* and *Porphyromonadaceae* (p value ≤0.05) was noted in Bac$^+$ mice relative to their Bac$^-$ counterparts. At genus level, a relative reduction was observed in *Alistipes* (p value ≤0.05) in Bac$^+$ mice compared with Bac$^-$ mice. Furthermore, a relative increase in proportions of the family *Peptococcaceae* (p value ≤0.05) and the corresponding genus *Peptococcus* (p value ≤0.05) was noted in Bac$^+$ mice compared with Bac$^-$ mice (Fig 4 & Supplementary Table 2).

Continued exposure to the bacteriocin producing UCC118 strain brought about a variety of changes. At phylum level the relative

proportions of Firmicutes, Candidate Division TM7, Deferribacters and Verrucomicrobia (p value ≤0.05) were significantly increased by week 8. Proportions of the families *Desulfovibrionaceae, Erysipelotrichaceae, Deferribacteraceae, Verrucomicrobiaceae* and *Eubacteriaceae* (p value ≤0.05) and the genera *Desulfovibrio, Lactococcus, Akkermansia, Clostridium, Bilophila, Mucispirillum, Turicibacter* and *Catabacter* (p value ≤0.05) also increased. Over the same period relative reductions in the phylum Bacteroidetes (p value ≤0.05), the families *Rikenellaceae, Porphyromonadaceae* and *Bacteroidaceae* (p value ≤0.05) as well as the genera *Alistipes, Parabacteroides* and *Bacteroides* (p value ≤0.05) also occurred (Fig 4 & Supplementary Table 2).

The impact of administering the bacteriocin negative UCC118 strain also changed between the two time points. At phylum level relative increases in the proportions of the Firmicutes, Actinobacteria and Candidate Division TM7 (p value ≤0.05) were seen which corresponded with increases at family level in *Desulfovibrionaceae, Lachnospiraceae, Peptococcaceae, Erysipelotrichaceae, Bifidobacteriaceae, Coriobacterineae* and *Lactobacillaceae* (p value ≤0.05). At genus level increases in *Desulfovibrio, Turicibacter, Bifidobacterium, uncultured Lachnospiraceae, Peptococcus, Bilophila* and *Catabacter* (p value ≤0.05) were also noted. Relative reductions in the phylum Bacteroidetes (p value ≤0.05), families *Rikenellaceae, Porphyromonadaceae* and *Bacteroidaceae* (p value ≤0.05) as well as the genera *Alistipes, Rikenella, Parabacteroides* and *Bacteroides* (p value ≤0.05) also occurred (Fig 4 & Supplementary Table 2). It should also be noted that the proportions of *Rikenellaceae, Porphyromonadaceae and Peptococcaceae* in Bac$^+$ fed mice relative to that in Bac$^-$ fed controls did not differ significantly at the later time point (intervention week 8).

3.4.4 Vancomycin administration reduces total bacterial numbers in the gut

As high throughput sequencing reveals information with respect to relative proportions of populations rather than relative numbers, quantitative PCR was employed to determine if the antimicrobial employed, i.e. vancomcyin or the bacteriocin Abp118, impacted on the total number of gut microbes present. Analysis revealed that there were no significant differences in total bacterial numbers in the faeces of diet induced obese mice compared with lean controls (p <0.96) at week 2. While treatment of the diet-induced obese mice with vancomycin resulted in a decrease in absolute faecal bacteria compared with their diet-induced obese counterparts (p <0.006 Fig 5), bacteriocin production did not alter the total bacterial numbers in the diet induced obese mice. The significant impact of vancomycin, but not other interventions, on total bacterial counts is also apparent at week 8 (Murphy *et al.*, 2012).

3.4.5 Beta diversity highlights temporal variation in microbial populations

Principal coordinate analysis (based on unweighted unifrac distances) of the 16S rRNA sequences further highlights the temporal changes in the microbial populations from intervention week 2 to 8 with samples clearly clustering according to time point (Fig 6). In line with the α diversity and taxonomical data presented above, it is apparent that data points corresponding to DIO mice who received vancomycin (purple) cluster away from those corresponding to the other groups. The degree to which these data points are removed is more apparent at week 2, again suggesting that a recovery occurs during the subsequent weeks (Fig 6). At week 8, data points corresponding to the DIO controls (red) cluster tightly together within a larger cluster. Such tight clustering is not apparent at week 2 (Fig 6). It would also appear that the Bac$^+$ (green) and Bac$^-$ (orange) mice are more distinct at week 2 (Fig 6). Hierarchical clustering of the OTUs from each dataset also highlights the temporal instability in the mouse microbiota between the two time points. However

the unweighted pair group method with arithmetic mean (UPGMA) tree shows that, at specific time points, the microbiota of mice within each treatment group are generally more similar to each other than they are to those from the other groups (Supplementary Fig 3). Hierarchical clustering again highlights the separation of the vancomycin-exposed populations from the other groups. In addition to clustering away from the other groups, it is also clear that these populations differ at the respective time points. At week 8, the 'lean' OTUs cluster into two groups on either side of the DIO OTUs, this separation was not observed at week 2. No significant difference in weight was observed between these respective lean subgroups. At week 2 Bac$^+$ OTUs are divided into two groups on either side of DIO OTUs. This separation did not persist with time.

3.5   DISCUSSION

This analysis of the gut microbiota of animals subjected to either vancomcyin or the bacteriocin producing probiotic *L. salivarius* UCC118 intervention provides valuable information regarding the temporal nature of the resultant changes. The results reflect microbial adaptation over time and the resilience of the microbiota. Principal coordinate analysis (PCoA) and hierarchical clustering analysis reveal that clustering occurs as a feature of time and to a lesser extent, treatment groups, rather than between microbial populations from within the same animal. In agreement with previous studies (Turnbaugh *et al.*, 2006, Ley *et al.*, 2006, Ley *et al.*, 2005), we established that the diet-induced obesity-associated murine gut microbiota differed from that of lean controls. More specifically, a significant increase in the proportion of Firmicutes and a decrease in the proportion of Bacteroidetes. Vancomycin was selected because of its limited systemic impact and its apparent ability to specifically target the low GC Gram-positive organisms i.e. Firmicutes (Murphy *et al.*, 2012). Here, we establish that treatment of mice on a high fat diet with vancomycin resulted in significant alterations in the composition of the gut microbiota; including a decrease in the relative proportions of Bacteroidetes and Deferribacteres and a relative increase in the proportions of Proteobacteria relative to DIO controls. This vancomycin-induced effect on Proteobacteria populations has been noted before (Rea *et al.*, 2011, Ubeda *et al.*, 2010). In contrast to week 8, the proportion of Firmicutes was not reduced between animals receiving vancomycin and controls at intervention week 2. At this time point, the corresponding increased proportions of the families *Streptococcaceae, Lactobacillaceae* and *Leuconostocaceae* (all members of the Firmicutes phylum) might negate the detrimental impact of vancomycin on other Firmicutes members, resulting in the absence of an overall net change. The glycopeptide antibiotic vancomycin is traditionally known to be active against Gram-positive bacteria such as *Staphylococcus aureus* and *Clostridium difficile* through the inhibition of cell wall synthesis (Howden *et al.,* Rea *et al.*, 2011a, Chang *et al.*, 2003). The cell wall of Gram-negative

bacteria is protected by the presence of an outer membrane that blocks the effects of vancomycin (Barna and Williams, 1984). Nevertheless, here we found that eight genera (*Thalassospira, Alistipes, Rikenella, Parabacteroides, Odiorbacter, Bacteroides, Oscillibacter* and *Mucispirillum*) of Gram-negative bacteria were significantly reduced in vancomycin treated DIO mice at week 2. Also three Gram-positive genus (*Lactococcus, Lactobacillus* and *Weissella*) increased at week 2 in vancomycin treated mice. While the innate vancomycin resistance of many lactobacilli has been well established, there have been rare reports of resistant *Lactococcus* and *Weissella* isolates (Salimnia *et al.*, 2011, Zhang *et al.*, 2013, D'Aimmo *et al.*, 2007). Other differences between the gut microbial populations of these mice at week 2 and week 8 were also apparent. These included significant increases in the relative proportions of Bacteroidetes, Actinobacteria, Deferribacteres and Verrucomicrobia and a significant decrease in the relative proportions of Firmicutes at the later time points. The recovery of both Gram-positive (*Turicibacter, Ruminococcaceae Incertae Sedis, Clostridium, Allobaculum and Anaeroplasma*) and Gram-negative bacteria by week 8 (*Alistipes and Mucispirillum*) highlights the resilience of the gut bacteria. On the basis of PCoA and hierarchical clustering these temporal changes seems to represent a recovery of/development of resistance among the gut microbiota such that it less considerably differs from that of controls. While it is tempting to speculate that this reflects the emergence of resistant strains from among these populations, further investigations are required to definitively establish the basis for this recovery. This recovery coincides with vancomycin having a relatively less dramatic impact on weight gain by week 8 of the study but the identity of the population that may be contributing to this phenomenon is difficult to ascertain due to the numbers of different taxa which are altered, however it is also apparent that the total number of bacteria did not alter between the two time points. These results highlight the resilience of the microbiota to change, demonstrating that after the initial impact from vancomycin they start to revert back to their original profile. This highlights the challenge faced when utilising antimicrobials, prebiotics (Dewulf *et al.*, 2012), microbial

transplantation (Vrieze *et al.*, 2012) or other interventions in order to bring about long-term changes to the obesity-associated (and other) gut microbial populations. Indeed, the temporal resilience of the gut microbiota following exposure to antibiotics has been highlighted in previous studies (Cotter *et al*, 2012, Fouhy *et al.*, 2012, Lozupone *et al.*, 2012, Rea *et al.*, 2011, Tims *et al.*, 2012, Zhang *et al.*, 2012).

The comparison of the impact of Bac$^+$ and Bac$^-$ on the murine microbiota may be more revealing as a consequence of the number of changes being fewer and the fact that the impact of Bac$^+$ intervention changed from being significant to non-significant as the study continued. While previous studies with bacteriocin-producing UCC118 strain have shown it to be active against representatives of several Gram-positive taxa, including *Bacillus*, *Listeria monocytogenes*, *Enterococcus*, *Staphylococcus* and *Clostridium perfringens* (Flynn *et al.*, 2002, O'Mahony *et al.*, 2001), high throughput sequencing again provided unexpected results with respect to compositional changes, at family level, relative to Bac$^-$ controls at week 2. Specifically a relative reduction in the proportions of the Gram-negative families *Rikenellaceae* and *Porphyromonadaceae* and a relative increase in the proportions of the Gram-positive *Peptococcaceae* occurred. Interestingly, a recent study has suggested a link between the *Porphyromonadaceae* with the development of metabolic syndrome (Henao-Mejia *et al.*, 2012). While the decrease in weight gain observed in Bac$^+$, relative to Bac$^-$, mice at week 2 and the reduction in relative numbers of this family is notable, the proportions of this family decreased even further in Bac$^+$ mice by week 8 despite the fact that the impact of the probiotic with respect to weight gain was no longer significant by this time. Notably the proportions of *Rikenellaceae, Porphyromonadaceae and Peptococcaceae* in Bac$^+$ and Bac$^-$ fed mice did not differ significantly at week 8. Further studies are needed to explore the role these families play in the link between the gut microbial ecosystem and obesity.

In conclusion, the data demonstrate that though vancomycin distinctively modified the gut microbiota composition, the growth of some bacterial families over time may be responsible for this intervention

having a less dramatic impact on weight gain by the end of the 8 week intervention period. There also exist a number of changes in the microbial population of animals administered Bac$^+$ which, if successfully targeted over a longer period, could potentially extend the duration over which weight gain is significantly reduced. These results provide further rationale for altering the gut microbiota using antimicrobials but the specific identification of the populations involved and the specificity of action of the antimicrobials will be essential and may require continual modification to ensure a sustained impact and to overcome compensatory effects.

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Figure 1. Delta weight gain over the eight week intervention period. (A) Bac$^+$ intervention, when compared to Bac$^-$ intervention, causes a significant reduction in weight gain in diet induced obese mice at weeks 2-4 (early intervention period) but this does not persist with time. (B) Vancomycin treatment results in a two phase reduction in weight gain in diet induced obese mice. In phase one (early; weeks 1-4) a significant reduction in weight gain relative to the initial start weight is observed. In the second phase, diet induced obese mice receiving vancomycin gain weight relative to the initial start weight but weight change continues to be significantly less than that in diet induced obese controls. Data represented as mean ±SEM n=9-10 *p<0.05.
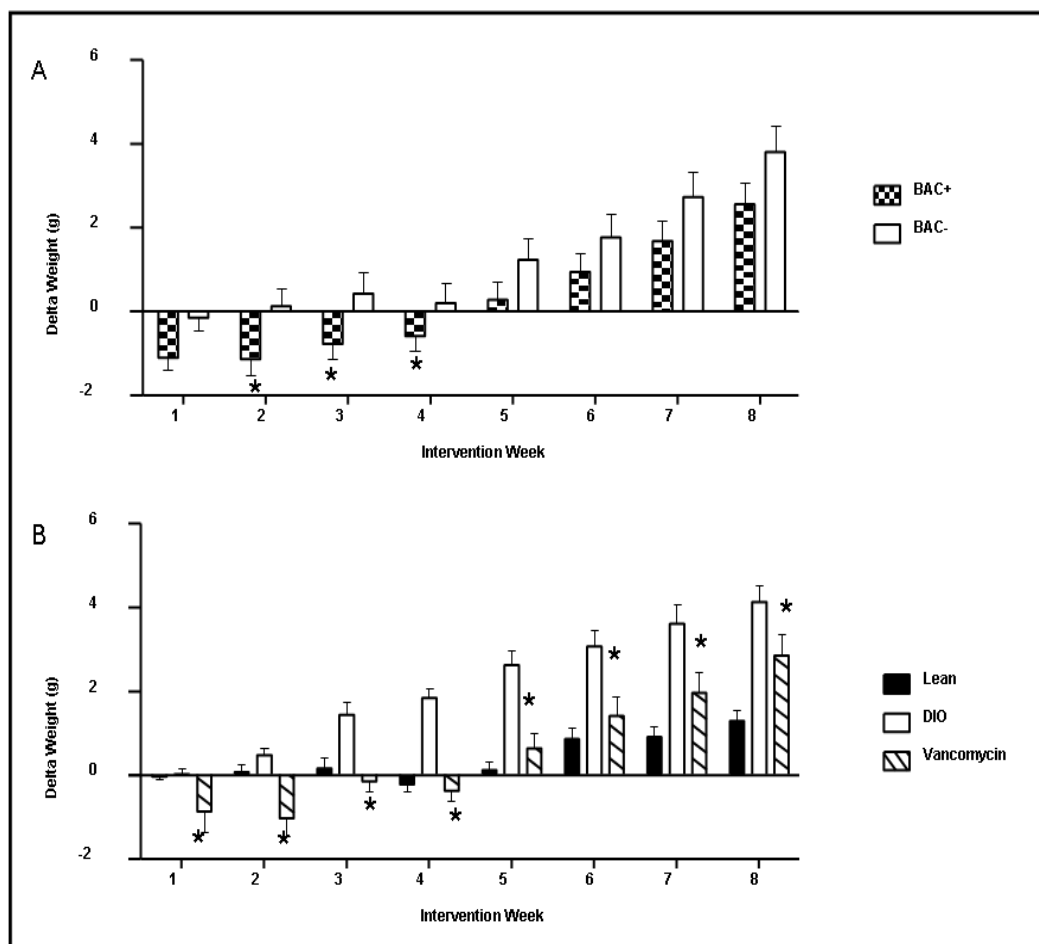
Figure 2. α diversity of the gut microbiota within each time point. In a number of instances significant increases in diversity are observed between intervention week 2 and week 8. Statistical significance was determined by Kruskal Wallis. * Statistical significant difference (*p* <0.05). Data represented as mean ±SEM (*n*=9-10).
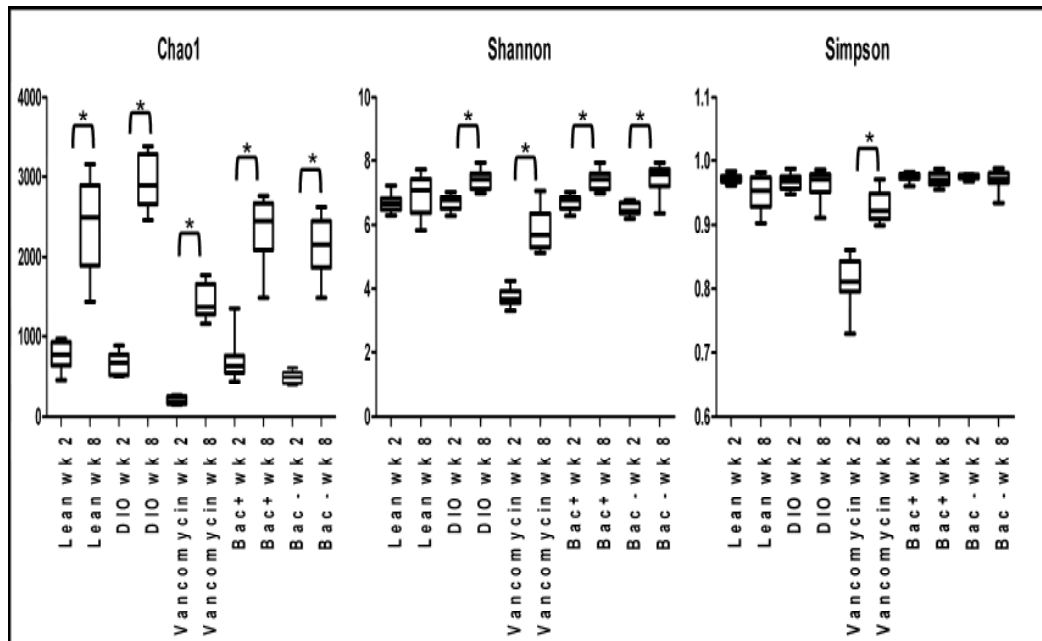
Figure 3. Microbial distribution at phylum level. Phylum level microbial distribution in all data sets at intervention week 2 and week 8. The pie charts represent total percentage read number for the corresponding colour coded phylum (*n*=9-10 per group).
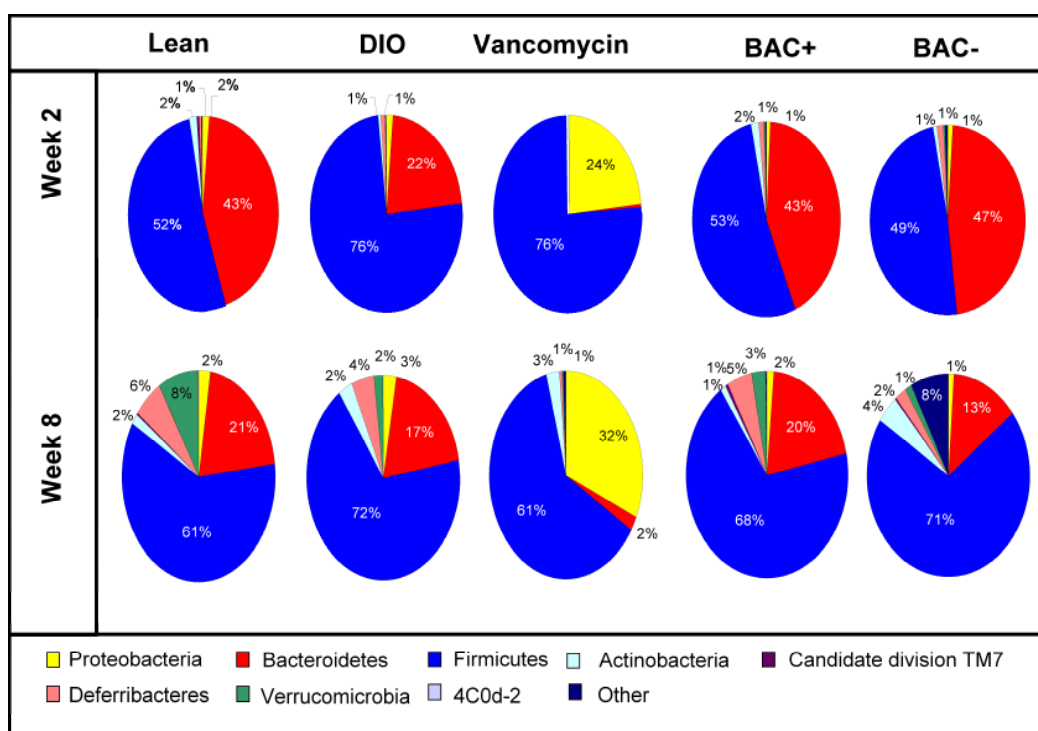
Figure 4. Microbial distribution at family level. Family level microbial distribution in all data sets at intervention week 2 and week 8. The pie charts represent total percentage read number for the corresponding colour coded family (*n*=9-10 per group).
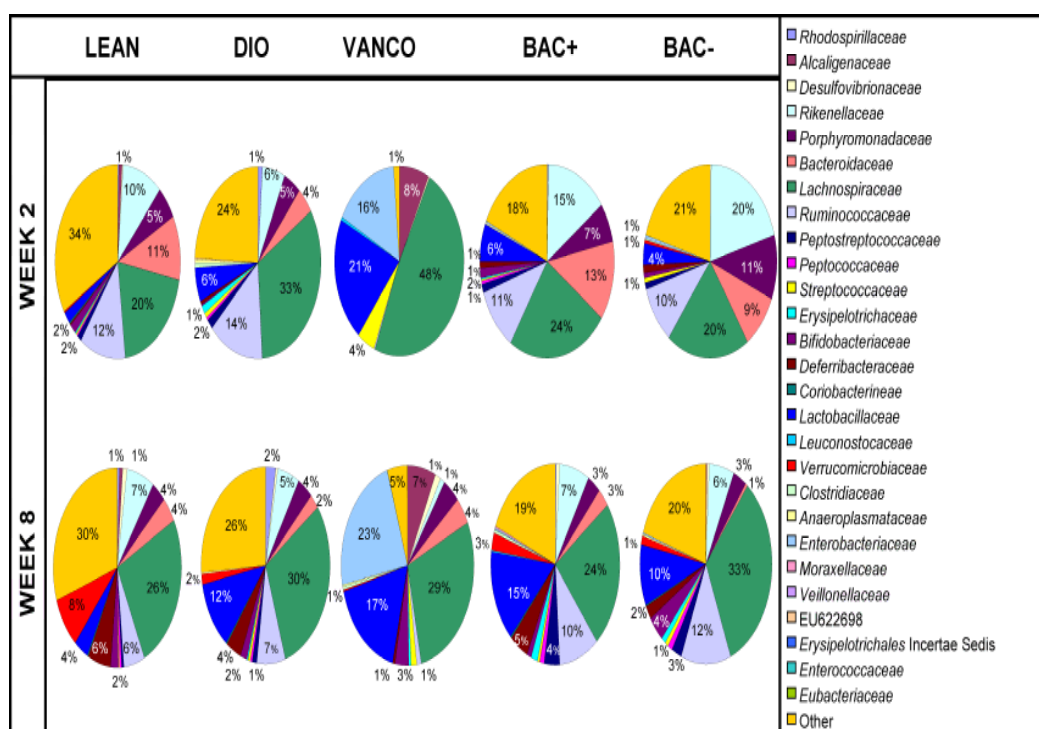
Figure 5. Total bacterial number observed in all treatment groups at both time points. Quantitative PCR reveals that the changes occurring are qualitative not quantitative as no significant difference is observed between time points. Total bacterial numbers calculated as copies of 16S rRNA/g wet stool. Statistical significant difference between treatment groups is denoted by ***. *p* value based on Kruskal Wallis analysis with statistical significant determined as *p* ≤0.05. Error bars represent the standard error of the mean.
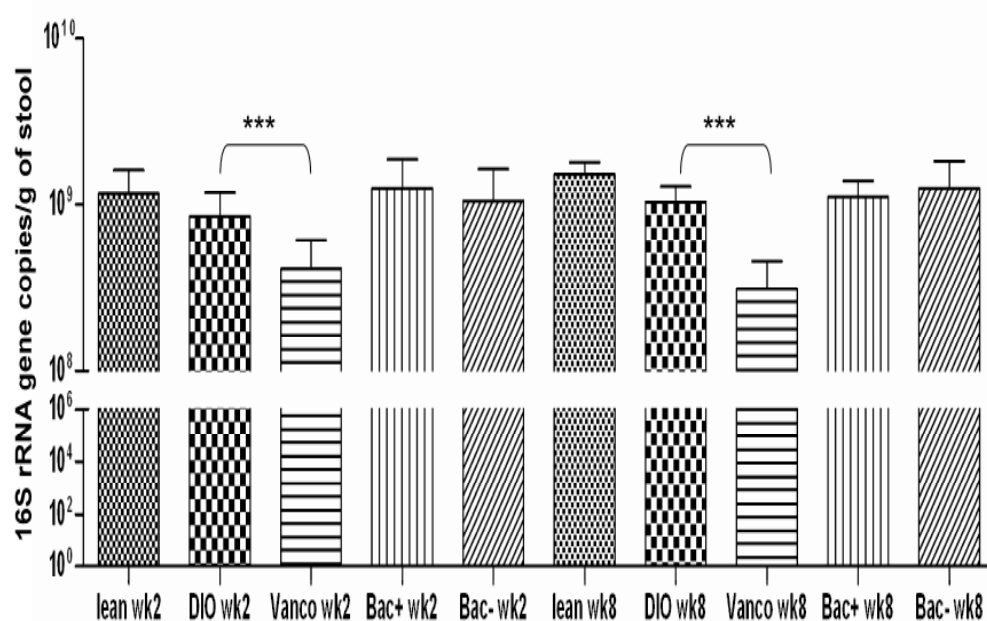
Figure 6. Principal coordinate analysis of unweighted unifrac reveals temporal shift. Vancomycin treated diet induced obese mice (purple) cluster away from other groups at both time points, however the distance between these mice and other treatment groups at week 8 is less than at week 2. Data sets: purple vancomycin, blue lean, red DIO, green Bac$^+$ and orange Bac$^-$.
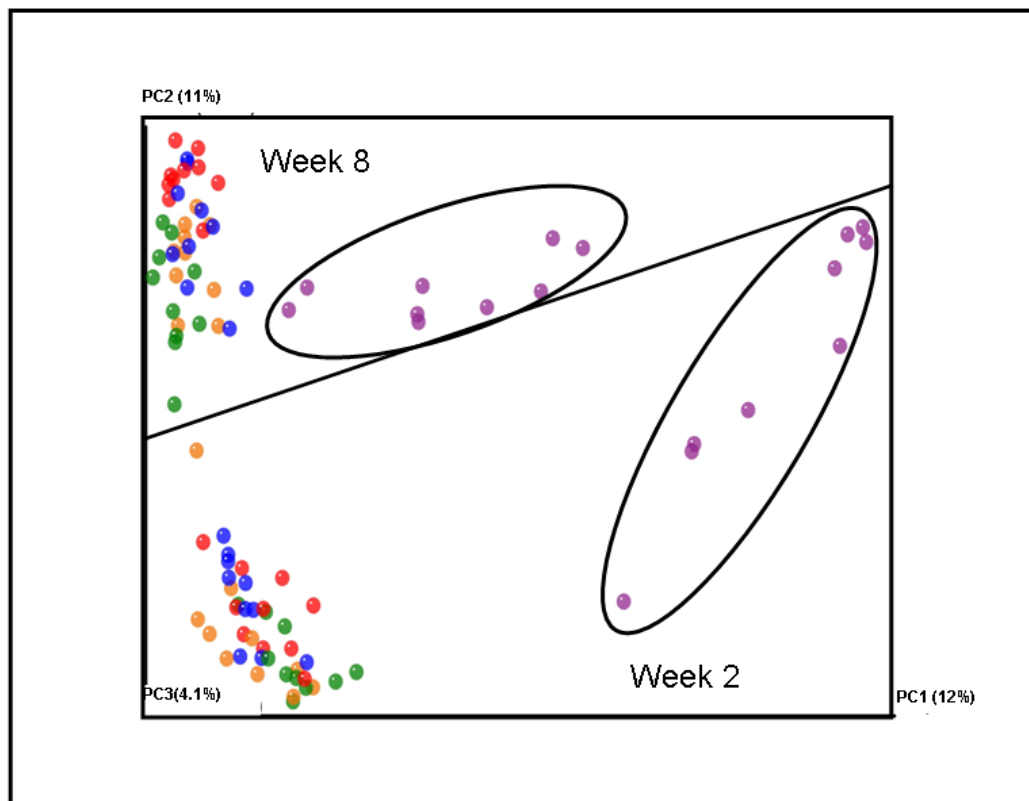
SUPPORTING INFORMATION

Table S1. Vancomycin treatment alters gut microbiota in diet induced obese mice

	Lean Wk 2	Lean Wk 8	DIO Wk 2	DIO Wk 8	Vancomycin Wk 2	Vancomycin Wk 8
Phylum						
Proteobacteria	1.51± 0.22	2.43±0.37	1.28±0.28†	3.18±0.83	24.63±6.90*	31.74±1.90
Bacteroidetes	42.89± 2.48#	21.50±2.13##	22.13±4.69	16.82±1.26	0.67±0.45*	2.24±0.76**
Firmicutes	52.92± 2.65#	60.03±2.90	74.96±4.54	72.06±2.05	74.18±7.15	61.49±2.61**
Actinobacteria	1.54± 0.66	2.20±0.61	0.58±0.22†	1.86±0.46	0.34±0.31	3.06±1.39**
Candidate Devision TM7	0.22± 0.09	0.27±0.05	0.09±0.06	0.15±0.08	0.0±0.0	0.01±0.01
Deferribacteres	0.53± 0.25	5.42±1.62##	0.61±0.18†	3.86±1.28	0.0±0.0*	0.60±0.27**
Verrucomicrobia	0.10±0.10	7.90±3.11##	0.0±0.0†	1.65±0.45	0.0±0.0	0.20±0.08**
4COd 2	0.0±0.0	0.0±0.0	0.07±0.07	0.14±0.05	0.0±0.0	0.0±0.0
Family						
Rhodospirillaceae	0.16±0.06#	0.54±.08##	0.87±0.28†	2.38±0.77	0.0±0.0*	0.01±0.01
Alcaligenaceae	0.94± 0.22#	0.99±0.42	0.0±0.0	0.03±0.02	7.74±3.09*	6.87±0.91
Desulfovibrionaceae	0.24±0.11	0.82±0.22##	0.09±0.07†	0.71±0.14	0.07±0.05	1.42±0.29**
Rikenellaceae	10.55±1.01#	7.61±1.18	5.93±1.46	5.38±0.79	0.20±0.20*	1.10±0.36**
Porphyromonadaceae	5.50±0.59	3.72±0.42##	4.71±1.44	3.92±0.54	0.0±0.0*	3.81±3.68**
Bacteroidaceae	10.40±1.65#	4.38±0.45##	4.46±2.08	2.45±0.45	0.17±0.17*	5.85±5.74
Lachnospiraceae	20.83±2.03#	24.97±2.61	32.50±4.25	29.87±2.09	48.27±11.82	27.52±5.98
Ruminococcaceae	12.13±0.97	5.62±0.99##	13.58±1.46†	7.02±1.17	0.20±0.13*	1.14±0.45**
Peptostreptococcaceae	0.96±0.23	0.51±0.24	1.67±05.6	1.19±0.41	0.0±0.0*	0.04±0.03
Peptococcaceae	0.39±0.09	0.36±0.05	0.56±0.15	0.51±0.11	0.0±0.0*	0.06±0.03
Streptococcaceae	0.37±0.09#	0.34±0.05	0.78±0.16	0.58±0.07	4.88±1.69*	1.34±0.14**
Erysipelotrichaceae	0.09±0.06	0.16±0.08	1.43±0.74	0.11±0.06	0.0±0.0*	0.63±0.29**

Statistical significance was determined using Kruskal Wallis. Values are mean percentage read number ± standard error. *p value ≤0.05 between DIO and DIO vancomycin mice at week 2; **p value ≤0.05 between DIO vancomycin mice at week 2 and DIO vancomycin mice at week 8; #p value ≤0.05 between Lean and DIO mice at week 2; ##p value ≤0.05 between Lean week 2 and Lean week 8; †p value ≤0.05 between DIO week 2 mice and DIO week 8 mice.

Table S1. (*continued*)

	Lean Wk 2	Lean Wk 8	DIO Wk 2	DIO Wk 8	Vancomycin Wk 2	Vancomycin Wk 8
Bifidobacteriaceae	1.33±0.61	2.03±0.60	0.56±0.22†	1.67±0.40	0.34±0.31	3.45±1.34**
Deferribacteraceae	0.53±0.25	5.42±1.62##	0.48±0.17†	3.86±1.28	0.0±0.0*	0.61±0.27**
Coriobacterineae	0.13±0.07#	0.14±0.04	0.0±0.0†	0.18±0.06	0.0±0.0	0.0±0.0
Lactobacillaceae	1.970±0.51#	3.88±0.69##	6.27±1.67	11.82±2.43	19.27±6.21*	16.52±3.54
Leuconostocaceae	0.05±0.05	0.04±0.02	0.0±0.0	0.04±0.02	0.40±0.13*	0.08±0.03
Verrucomicrobiaceae	0.10±0.10	7.90±3.11##	0.0±0.0†	1.65±0.45	0.0±0.0	0.20±0.08**
Clostridiaceae	0.0±0.0#	0.01±0.01	0.82±0.33†	0.0±0.0	0.13±0.09	0.73±0.31
Enterobacteriaceae	0.0±0.0	0.02±0.02	0.0±0.0	0.01±0.01	16.64±3.87*	23.32±1.20**
EU622698	0.0±0.0	0.0±0.0	0.06±0.06	0.09±0.03	0.0±0.0	0.0±0.0
Eubacteriaceae	0.0±0.0	0.06±0.03##	0.0±0.0	0.02±0.01	0.0±0.0	0.0±0.0
Genus						
Thalassospira	0.16±0.06#	0.47±0.07##	0.87±0.28	1.59±0.33	0.0±0.0*	0.01±0.01
Sutterella	0.94±0.22#	0.99±0.42	0.0±0.0	0.03±0.02	7.74±3.09*	6.87±0.91
Desulfovibrio	0.20±0.10	0.65±0.21##	0.04±0.04†	0.50±0.11	0.07±0.05	1.41±0.28**
Alistipes	7.53±0.88#	4.01±0.42##	3.52±0.98	2.43±0.25	0.13±0.13*	0.77±0.26**
Rikenella	2.06±0.32	2.82±0.76	1.71±0.67	1.89±0.63	0.0±0.0*	0.0±0.0
Parabacteroides	1.82±0.47	1.67±0.12	3.45±1.14	2.75±0.50	0.0±0.0*	0.03±0.02
Odoribacter	3.68±0.66#	2.05±0.39	1.24±0.34	1.16±0.16	0.0±0.0*	0.04±0.03
Bacteroides	10.40±1.65#	4.38±0.45##	4.46±2.08	2.45±0.45	0.17±0.17*	0.13±0.07
Lachnospiraceae Incertae Sedis	0.66±0.13	0.41±0.07	0.70±0.20	0.59±0.10	0.0±0.0*	0.10±0.06
Coprococcus	0.40±0.11	0.10±0.04##	0.58±0.14†	0.17±0.04	0.0±0.0*	0.02±0.02
Ruminococcaceae Incertae Sedis	6.66±0.62	2.12±0.36##	7.16±0.74†	3.09±0.43	0.0±0.0*	0.56±0.22**

Statistical significance was determined using Kruskal Wallis. Values are mean percentage read number ± standard error. *p value ≤0.05 between DIO and DIO vancomycin mice at week 2; **p value ≤0.05 between DIO vancomycin mice at week 2 and DIO vancomycin mice at week 8; #p value ≤0.05 between Lean and DIO mice at week 2; ##p value ≤0.05 between Lean week 2 and Lean week 8; †p value ≤0.05 between DIO week 2 mice and DIO week 8 mice.

Table S1. (*continued*)

	Lean Wk 2	Lean Wk 8	DIO Wk 2	DIO Wk 8	Vancomycin Wk 2	Vancomycin Wk 8
Oscillibacter	0.54±0.14	0.73±0.19	0.84±0.16	0.99±0.16	0.0±0.0*	0.12±0.07
Anaerotruncus	1.54±0.24	1.06±0.20	2.27±0.37	1.47±0.24	0.0±0.0*	0.12±0.06
Lactococcus	0.35±0.08#	0.33±0.05	0.74±0.15	0.56±0.07	4.76±1.67*	1.33±0.14**
Turicibacter	0.09±0.06#	0.16±0.08	1.73±0.70†	0.11±0.06	0.0±0.0*	0.63±0.29**
Allobaculum	12.77±1.61	21.18±3.34##	10.94±2.80	17.09±2.94	0.13±0.13*	2.63±1.00**
Bifidobacterium	1.36±0.61	2.03±0.60	0.56±0.22	1.46±0.42	0.34±0.31	3.45±1.34**
Mucispirillum	0.53±0.25	5.42±1.62##	0.61±0.18†	3.86±1.28	0.0±0.0*	0.61±0.27**
Lactobacillus	1.97±0.51#	3.85±0.69##	6.26±1.67	11.74±2.41	19.27±6.21*	16.49±3.53
uncultured Lachnospiraceae	0.19±0.08	0.57±0.22	0.33±0.17†	2.57±0.81	0.0±0.0*	0.22±0.10**
Weissella	0.03±0.03	0.0±0.0	0.0±0.0	0.02±0.01	0.29±0.1*	0.02±0.02**
Leuconostoc	0.02±0.02	0.0±0.0	0.0±0.0	0.0±0.0	0.04±0.04	0.03±0.02
Peptostreptococcaceae Incertae Sedis	0.11±0.06	0.20±0.16	0.52±0.22	0.33±0.11	0.0±0.0*	0.04±0.03
Peptococcus	0.09±0.10	0.0±0.0	0.34±0.15	0.21±0.10	0.0±0.0*	0.0±0.0
Akkermansia	0.10±0.10	7.90±3.11##	0.0±0.0†	1.65±0.45	0.0±0.0	0.20±0.08**
Clostridium	0.0±0.0#	0.01±0.01	0.82±0.33†	0.0±0.0	0.08±0.08*	0.73±0.13**
Anaeroplasma	0.0±0.0#	0.02±0.02	0.65±0.31	0.06±0.03	0.0±0.0*	0.07±0.04
Bilophila	0.0±0.0	0.13±0.05##	0.03±0.03†	0.21±0.04	0.0±0.0	0.0±0.0
Enterobacteriaceae genus	0.0±0.0	0.02±0.02	0.0±0.0	0.01±0.01	16.58±3.83*	23.31±1.20**
Veillonella	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.02±0.02	0.0±0.0
Streptococcus	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Catabacter	0.0±0.0	0.04±0.02	0.0±0.0†	0.05±0.02	0.0±0.0	0.0±0.0
Anaerovorax	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Acinetobacter	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Blautia	0.0±0.0	0.05±0.02##	0.0±0.0	0.05±0.02	0.0±0.0	0.0±0.0
Eubacterium	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Enterococcus	0.0±0.0	0.0±0.0	0.0±0.0	0.01±0.01	0.0±0.0	0.0±0.0

Statistical significance was determined using Kruskal Wallis. Values are mean percentage read number ± standard error. *p value ≤0.05 between DIO and DIO vancomycin mice at week 2; **p value ≤0.05 between DIO vancomycin mice at week 2 and DIO vancomycin mice at week 8; #p value ≤0.05 between Lean and DIO mice at week 2; ##p value ≤0.05 between Lean week 2 and Lean week 8; †p value ≤0.05 between DIO week 2 mice and DIO week 8 mice.

Table S2. Effects of *L salivarius* UCC118 bacteriocin production on the gut microbiota of DIO mice over time.

	BAC- wk 2	BAC- wk 8	BAC+ wk 2	BAC+ wk 8
Phylum				
Proteobacteria	0.94±0.23	1.00±0.12	0.93± 0.30	2.43±0.37
Bacteroidetes	48.66±4.0	13.4±1.6‡	41.70± 1.67†	21.50±2.13
Firmicutes	47.17±3.82	70.65±7.29‡	53.84± 1.61†	60.03±2.90
Actinobacteria	0.97±0.30	4.20±1.27‡	1.85± 0.54	2.20±0.61
Candidate Devision TM7	0.0±0.0	0.43±0.10‡	0.0±0.0†	0.27±0.05
Deferribacteres	1.23±0.56	2.40±0.72	1.25± 0.31†	5.42±1.62
Verrucomicrobia	0.49±0.29	1.17±1.02	0.16± 0.14†	7.90±3.11
4COd2	0.0±0.0	0.02±0.02	0.01±0.01†	0.0±0.0
Family				
Desulfovibrionaceae	0.10±0.07	0.58±0.10‡	0.07±0.05†	0.82±0.22
Rikenellaceae	20.99±1.88*	6.31±0.85‡	14.62±0.78†	7.61±1.18
Porphyromonadaceae	11.15±1.43*	3.14±0.57‡	6.83±0.87†	3.72±0.42
Bacteroidaceae	9.42±1.60	0.68±0.18‡	12.23±1.50†	4.38±0.45
Lachnospiraceae	19.65±2.69	33.38±3.55‡	24.54±2.20	24.97±2.61
Peptococcaceae	0.09±0.09*	1.21±0.21‡	0.69±0.14	0.36±0.05
Streptococcaceae	0.62±0.39	0.60±0.11	0.33±0.14†	0.34±0.05
Erysipelotrichaceae	0.0±0.0	1.24±0.46‡	0.09±0.06†	0.16±0.08
Bifidobacteriaceae	0.95±0.30	4.04±1.22‡	1.78±0.52	2.03±0.60
Deferribacteraceae	1.23±0.56	2.46±0.70	1.25±0.31†	5.42±1.62
Coriobacterineae	0.0±0.0	0.13±0.06‡	0.02±0.02	0.14±0.04
Lactobacillaceae	3.73±0.95	9.71±2.16‡	6.27±1.88	3.88±0.69
Leuconostocaceae	0.0±0.0	0.05±0.03	0.0±0.0†	0.04±0.02
Verrucomicrobiaceae	0.49±0.29	1.17±1.02	0.16±0.14†	7.90±3.11
Clostridiaceae	0.28±0.19	0.04±0.03	0.02±0.02†	0.01±0.01
Anaeroplasmataceae	0.17±0.12	0.29±0.22	0.03±0.03	0.02±0.02
Enterobacteriaceae	0.43±0.19	0.16±0.03	0.45±0.18	0.02±0.02
Eubacteriaceae	0.0±0.0	0.01±0.01	0.0±0.0†	0.06±0.03

Statistical significance was determined using Kruskal Wallis. Values are mean percentage read number ± standard error. *p value ≤0.05 between BAC[+] and BAC[-] at week 2; † p value ≤0.05 between BAC[+] week 2 and BAC[+] week 8; ‡p value ≤0.05 between BAC[-] week 2 and BAC[-] week 8.

Table S2. (*continued*)

	BAC- wk 2	BAC- wk 8	BAC+ wk 2	BAC+ wk 8
Genus				
Desulfovibrio	0.05±0.05	0.25±0.09‡	0.02±0.02†	0.56±0.11
Alistipes	13.92±1.18*	2.97±0.53‡	8.35±0.66†	3.60±0.39
Rikenella	3.18±0.42	2.00±0.43‡	2.64±0.48	2.55±0.67
Parabacteroides	6.80±1.11	1.22±.034‡	3.74±0.75†	1.38±0.19
Bacteroides	9.42±1.60	0.68±0.18‡	12.23±1.50†	2.87±0.54
Lactococcus	0.28±0.10	0.68±0.18	0.17±0.12†	0.62±0.09
Turicibacter	0.0±0.0	1.24±0.46‡	0.09±0.06†	1.68±0.70
Bifidobacterium	0.95±0.30	4.04±1.22‡	1.78±0.25	1.17±0.38
Mucispirillum	1.23±0.56	1.82±0.49	1.25±0.31†	4.91±1.73
uncultured Lachnospiraceae	0.32±0.16	1.46±0.55‡	0.54±0.19	1.36±0.49
Peptococcus	0.08±0.08*	0.76±0.19‡	0.47±0.13	0.51±0.14
Akkermansia	0.49±0.29	1.17±1.02	0.16±0.14†	2.85±1.05
Clostridium	0.23±0.15	0.05±0.03	0.02±0.02†	0.82±0.35
Bilophila	0.0±0.0	0.22±0.06‡	0.01±0.01†	0.36±0.11
Enterobacteriaceae genus	0.28±0.16	0.15±0.03	0.30±0.17	0.29±0.05
Catabacter	0.0±0.0	0.08±0.03‡	0.0±0.0†	0.11±0.04
Uncultured Bacteria	0.0±0.0	0.07±0.03‡	0.01±0.01†	0.21±0.05

Statistical significance was determined using Kruskal Wallis. Values are mean percentage read number ± standard error. *p value ≤0.05 between BAC[+] and BAC[-] at week 2; † p value ≤0.05 between BAC[+] week 2 and BAC[+] week 8; ‡p value ≤0.05 between BAC[-] week 2 and BAC[-] week 8.

Supplementary Figure 1. Experimental design: Seven week old C57BL/J6 mice were fed a high fat or low fat diet for 20 weeks, after 12 weeks intervention began. Sequencing was performed at intervention week 2 and week 8 of the study.
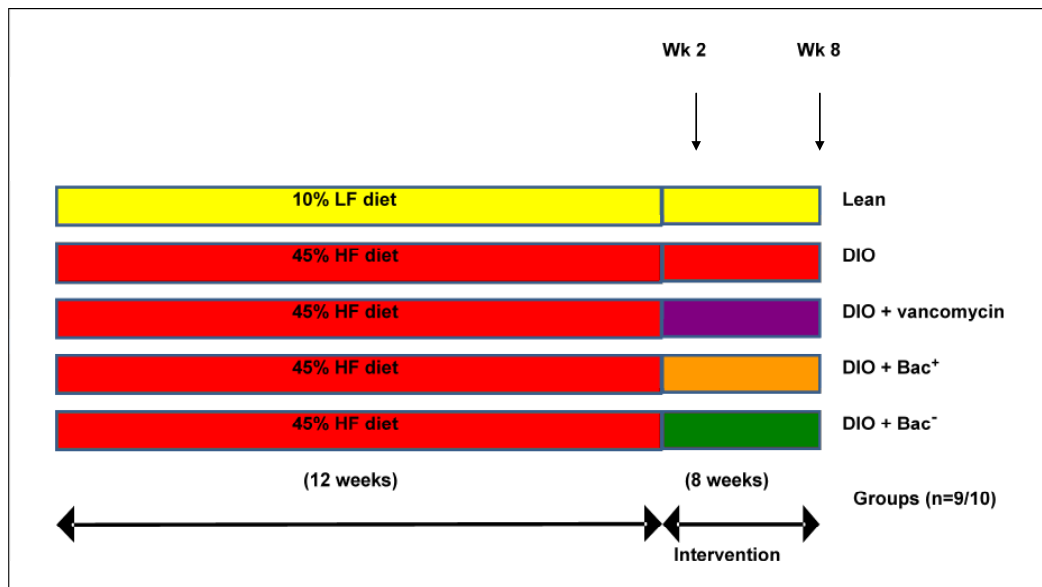
Supplementary Figure 2. Rarefaction curves for each group at 97% similarity levels for intervention week 2 (A) and week 8 (B) data sets. Amount of operational taxonomic units (OUT's) found as a function of the number of sequence tags sampled.
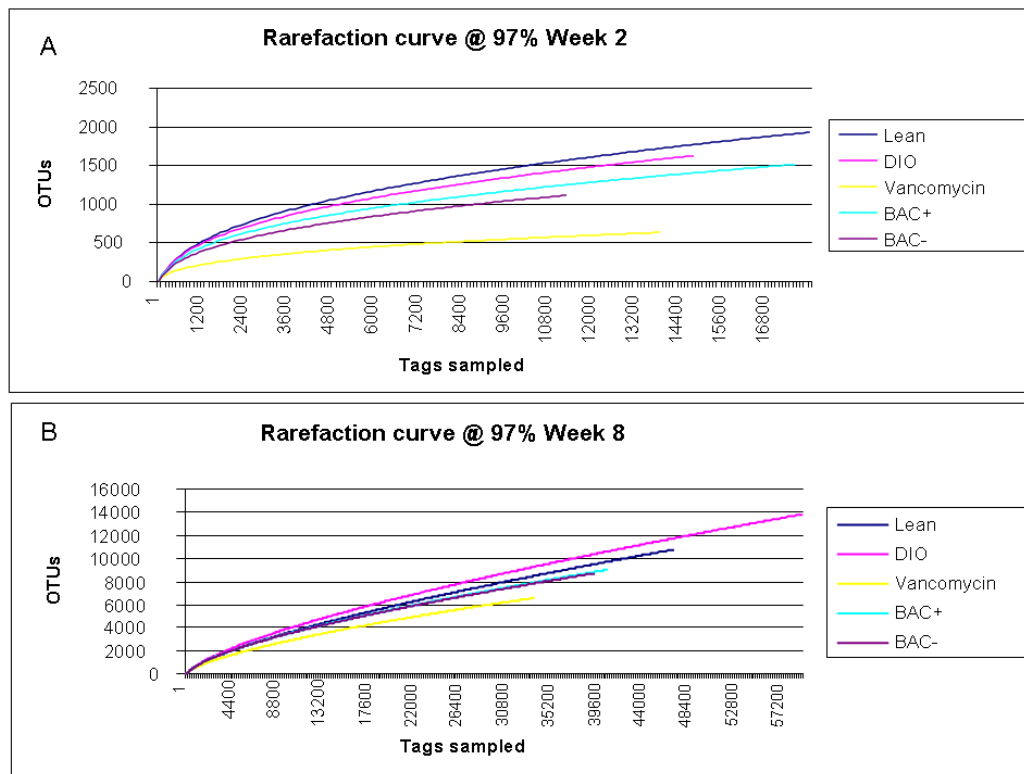
Supplementary Figure 3. Unweighted pair group method with arithmetic mean (UPGMA) tree of all samples at both time point's. Highlights temporal shift and clustering by treatment group. Vancomycin treated DIO mice present as outliers from both time points.
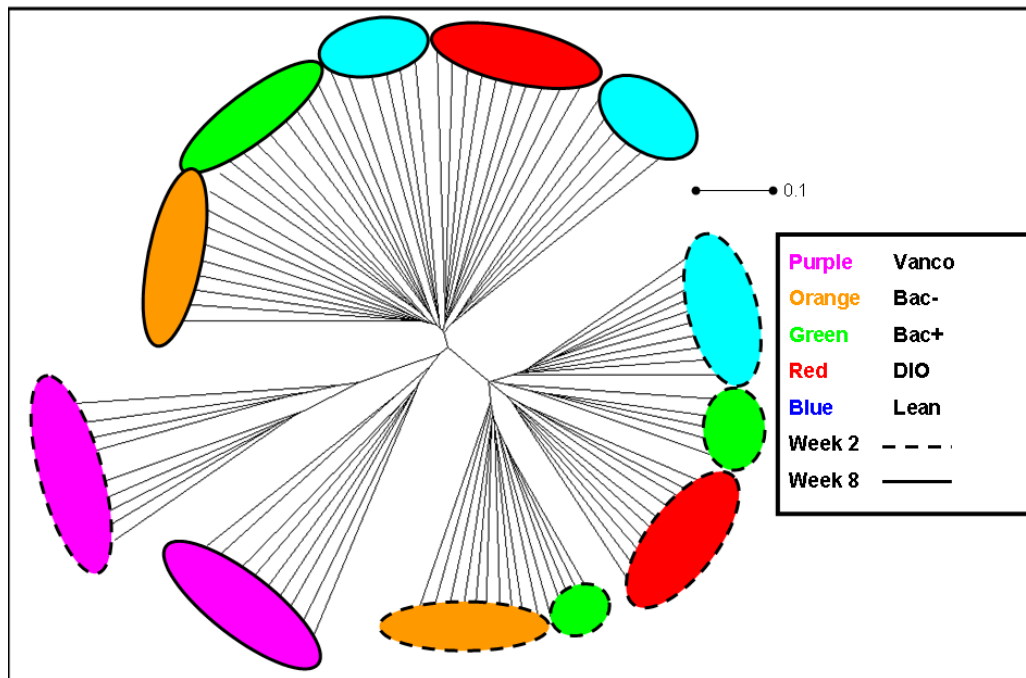
Chapter 4

Isogenic strains producing different bile salt hydrolases differ in their impact on weight gain and the composition of the gut microbiota of mice

Siobhan Clarke Chapter Contributions:

Experimental:

- Designed and performed all experiments relating to the extraction and purification of DNA from faecal pellets.
- Generated amplicons for 454 pyrosequencing.

Results interpretation:

- Analysed all data in relation to 16S compositional sequencing analysis.
- Compiled all graphical interpretations relating to 16S compositional sequencing analysis.

Manuscript preparation:

Major contributor to manuscript preparation

4.1 ABSTRACT

Conjugated bile acids (CBAs) are thought to repress gut microbiota through direct antimicrobial effects and/or up-regulation of host defences. Here we investigate the impact of the production of specific bile salt hydrolases on the gut microbiota and weight gain using a commensal *Escherichia coli* strain that produces bile salt hydrolase (BSH) from either *Lactobacillus salivarius* JCM1046 (BSH1) or UCC118 (BSH2) in the same isogenic background. To facilitate this, the streptomycin-treated mouse model was employed as it provides a less competitive niche for colonization. A significant reduction in weight gain was observed in mice administered the BSH1-producing strain fed either high and low fat diets, relative to the BSH2-producing strain. These results were accompanied by changes in microbiota composition and diversity, including an increase in the proportions of Bacteroidetes in the cecum of mice administered the BSH1-producing strain relative to those receiving the BSH2-producing strain. The study highlights the impact of the production of different BSHs on gut microbiota communities and, in turn, health.

4.2 INTRODUCTION

The human gastrointestinal tract is inhabited by a complex population of microorganisms. This community contains approximately 3 million genes (Qin *et al.*, 2010), which contrasts with the approximately 23,000 genes that comprise the human genome (Yang *et al.*, 2009). Recent advances in sequence based technologies have considerably enhanced our understanding of the composition and functional potential of the human gut microbiota and, in turn, facilitated studies to assess the role of specific microbiota populations and activities on human health, both beneficial and harmful (Qin *et al.*, 2012, Kurokawa *et al.*, 2007, Turnbaugh *et al.*, 2006).

The human liver secretes approximately one litre of bile into the intestinal tract every day (Hofmann, 1994). The main constituents of this bile include bile acids, cholesterol, phospholipids and the pigment biliverdin (Hofmann, 1999). Bile acids are synthesized *de novo* in the liver from cholesterol and are then conjugated with either glycine or taurine, which increase solubility, before secretion (Claus *et al.*, 2011). These CBAs promote the breakdown of dietary fat (Begley *et al.*, 2005b). It has been suggested that CBAs repress bacterial growth in the small intestine through direct antimicrobial effects and/or the up-regulation of host mucosal defences (Jones *et al.*, 2008). The deconjugation of CBAs by specific gut microbes is catalyzed by enzymes called bile salt hydrolases (BSHs) which hydrolyze the amide bond to liberate the glycine/taurine component from the side chain of the steroid core and release the bile acids (cholic acid or chenodeoxycholic acid and amino acids) (Begley *et al.*, 2005a). Other components of the gut microbiota can modify these bile acids through dehydroxylation, dehydrogenation and sulfation into secondary and tertiary forms (Jones *et al.*, 2008). Metagenomic analysis has identified functional BSHs from all major bacterial divisions and archaeal species in the gut, while also establishing that BSH-encoding genes are enriched within the human gut microbiome (Jones *et al.*, 2008). It has been suggested that the gut microbiota can regulate secondary bile acid metabolism and inhibit bile acid synthesis in the liver (Sayin *et al.*,

2013). BSH activity may also impact on host physiology through perturbations of bile acid controlled endocrine functions, influencing the risks of metabolic diseases such as obesity, diabetes and atherosclerosis (Jones *et al.*, 2008).

Here we investigate the respective impacts of two BSHs from different strains of *Lactobacillus salivarius* on gut microbiota composition and weight gain through two pilot studies. First, we establish a suitable experimental animal model and, second, use this model to investigate the effects of BSH-production on weight gain and the gut microbiota in a murine model of diet induced obesity.

4.3   MATERIAL AND METHODS

4.3.1 Bile salt hydrolase cloning and manipulation.

Known bile salt hydrolases from *Lactobacillus salivarius* JCM1046 (BSH1) (accession number FJ591091) and *L. salivarius* UCC118 (BSH2) (accession number FJ591081), which differ with respect to their affinity for tauro-conjugated bile acids *in vitro* (Fang *et al.*, 2009), were cloned independently into pBKminiTn7GM2 (Koch *et al.*, 2001) under the control of the constitutive P44 promoter (McGrath *et al.*, 2001) to create plasmids pTn7GMBSH1$_{UCC118}$ and pTn7GMBSH1$_{JCM1046}$. *E. coli* MG1655StrR (20 mg^{-1}) was transformed using standard methods with helper plasmid pBUX13 then conjugated with one of pBKminiTn7GM2, pTn7GMBSH1$_{UCC118}$ and pTn7GMBSH1$_{JCM1046}$. Transformants were selected on the basis of gentamicin resistance (10 mg^{-1}) and the helper plasmid was removed by sodium dodecyl sulfate (SDS) treatment. PCR and sequence analysis (GATC Biotech) confirmed the integrity of these constructs. These strains were negative for growth defects in minimal media and were named EC, ECBSH1 and ECBSH2 for those containing GM cassette, GMBSH1$_{JCM1046}$ and GMBSH1$_{UCC118}$ insertions, respectively. Primers applied are listed in supplementary information Table 1.

4.3.2 Animal studies

C57Bl/6J male mice aged 4 weeks were sourced from Harlan (Oxon, UK) and housed under barrier maintained conditions at University College Cork (UCC). Mice were allowed to acclimatise for 2 weeks prior to commencement of the study. The UCC Animal Ethics Committee approved all of the experiments performed. All procedures were conducted under license from the Irish government.

4.3.2.1   Experimental design - study 1

To reduce/remove resident Proteobacteria from the gastrointestinal (GI) tract, food was removed from 6 week old male C57Bl/6J mice for 24 hours and mice were immediately supplied with streptomycin-treated

drinking water (5 g/L final concentration) for the duration of the experiment. Water was changed every two days. After 24 hours mice were fed either a low fat diet (LFD) ((n=5) 10% calories from fat, Research Diets, New Jersey, USA D12450B) or a high fat diet (HFD) ((n=10) 45% calories from fat, Research Diets, New Jersey, USA D12451) for 10 weeks. The high fat diet group was further divided into two treatment groups (n=5 for each group) and were treated as follows (i) no treatment or (ii) gavage with 1×10^7 cfu streptomycin resistant (StrR), gentamicin resistant (GMR) *E. coli* MG1655 without a bile salt hydrolase insert (BSH) for 2 consecutive days. Faecal samples were collected, pooled and stored at $-80^{o}C$ at time points T=0, T=1, T=8 and T=10 weeks. At the end of the study, after culling of the mice, the caecum was removed and stored at $-80^{o}C$.

4.3.2.2   Experimental design - study 2

Food was removed from 6 week old male C57Bl/6J mice for 24 hours and mice were immediately supplied with streptomycin treated drinking water (5g/L final concentration) for the duration of the experiment. After this 24 hour period, mice were fed either a LFD ((n=6) 10% calories from fat, Research Diets, New Jersey, USA D12450B) or a HFD ((n=6) 45% calories from fat, Research Diets, New Jersey, USA D12451) for 8 weeks. These two diet groups were further divided into parallel treatment groups (n=3 for each group) and 1×10^7 cfu ECBSH1 or ECBSH2 were introduced by gavage on two consecutive days. Faecal samples were collected, pooled and stored at $-80^{o}C$ at time points T=0, T=1 and T=8 weeks. At the end of the study, after culling of the mice, the caecum was removed and stored at $-80^{o}C$.

4.3.3 DNA extraction and high-throughput DNA sequencing

Metagenomic DNA was extracted from murine faecal or caecal samples using the QIAmp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK) using a protocol modified to include an additional bead beating step (30 s x3) (Murphy *et al.*, 2012) and stored at $-20^{o}C$. Microbiota composition was established by 16S rRNA amplicon sequencing; PCR amplification of the

16S rRNA gene was performed using the universal primers F1 (F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAAAGNG), R2 (TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP'S Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp). Unique multiplex identifier tags were attached between the 454 adaptor sequence and the target-specific primer sequence, to facilitate the pooling and subsequent differentiation of samples. Amplicons generated from triplicate PCR reactions were pooled and cleaned using the Agencourt AMpure® purification system (Beckman Coulter, Takeley, UK) prior to being sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) in line with 454 protocols at the Teagasc high-throughput sequence centre.

4.3.4 Analysis of high-throughput DNA sequence data

A locally installed RDP pipeline for high-throughput DNA sequence data was used to quality trim and filter raw sequence data. Reads were removed that did not meet the quality criteria of a minimum quality score of 40 and sequence length shorter than 150bps for 16S amplicon reads. A locally installed version of SILVA 16S rRNA database (Pruesse *et al.*, 2007) was employed to BLAST (Altschul *et al.*, 1997) the trimmed fasta sequence files using default parameters. BLAST output files were parsed using MEGAN (Huson *et al.*, 2007) which uses a lowest common ancestor algorithm to assign reads to NCBI taxonomies. Filtering was carried out within MEGAN using bit scores of 86 similar to previous studies (Rea *et al.*, 2011, Urich *et al.*, 2008). Clustering of sequence reads into operational taxonomical units (OTUs) at 97% identity level was achieved using Qiime (Caporaso *et al.*, 2010). Chimeric OTU's were removed from the aligned OTU's using the ChimeraSlayer program (Haas *et al.*, 2011) and a phylogenetic tree was generated with the FastTreeMP tool (Price *et al.*, 2010). Alpha diversity indices and rarefaction curves were generated using Qiime. Beta diversities were also calculated on the sequence reads based on weighted and unweighted Unifrac and Bray

Curtis distance matrices; subsequently principal coordinate analysis (PCoA) plots were visualised using KiNG (Chen *et al.*, 2009).

4.3.5 Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.04 (La Jolla, California, USA) and SPSS software package version 18 (SPSS Inc., Chicago, USA). Mann–Whitney tests and unpaired Student t test were used to find significant differences. $p \leq 0.05$ was considered as statistically significant.

4.4 RESULTS

4.4.1 HFD-EC mice gain more weight than controls

Initially, the effect of continuous streptomycin treatment on weight gain and gut microbiota composition in both high and low fat fed mice over a ten week period was assessed. Streptomycin treatment was required in order to remove resident Proteobacteria from the GI tract to facilitate the subsequent establishment of the streptomycin resistant *E. coli* strain MG1655. In addition, the impact of introducing MG1655 on the gut microbiota composition of HFD mice (HFD-EC) was investigated prior to a second study, in which the effects (weight and microbiota) of utilising MG1655 derivatives that expressed heterologous BSH genes were assessed.

In the case of the first study, it was established that, as expected, HFD controls gained significantly more weight than LFD controls ($p<0.0001$). HFD mice that received 1×10^7 cfu StrR GMR *E. coli* MG1655 (HFD-EC) for two consecutive days at the start of the trial also gained significantly more body weight than the LFD controls ($p<0.0001$). HFD-EC mice gained more body weight than HFD controls and while this difference was not significant, a trend was noted ($p=0.08$) (Fig 1). HFD-EC mice consumed significantly more calories on average than both LFD and HFD controls, as measured by the average cumulative caloric intake per week (Fig 2).

4.4.2 Caecum microbiota diversity is higher in LFD mice than HFD controls

The composition of the gut microbiota of LFD, HFD and HFD-EC mice was investigated. Post quality filtering a total of 171,127 V4 16S rRNA sequence reads were generated, equating to an average of 1,321 reads per mouse for each time point and an average of 6,126 reads per cecum. Rarefaction curves were calculated at 97% similarity and were approaching parallel to the x-axis for all samples, again indicating extra sampling would yield a limited increase in species richness (Supplementary Fig 1). Shannon diversity, Simpson diversity and Chao1

values of species richness as well as Phylogenetic diversity and Observed species numbers were calculated for each sample (Supplementary Table 2). Analysis of the alpha diversity of the faecal microbiota over the course of the study revealed that treatment with streptomycin resulted in a reduction in Chao1 values in all three groups from T0 to T1. However diversity increased over time with recovery found to be greatest in the HFD-EC mice (Supplementary Fig 2). This pattern was also apparent when alpha diversity was quantified with respect to Phylogenetic diversity and Observed species, but not Simpson and Shannon diversity (Supplementary Fig 2).

Analysis of the alpha diversity of cecum microbiota revealed Observed species, Phylogenetic diversity and Chao1 values were significantly greater for the LFD caecal microbiota relative to that of HFD controls. A similar pattern was observed in the case of Shannon and Simpson values but in these instances the differences were not significant (Supplementary Fig 3). It was also noted that the number of Observed species was significantly greater in the caecum of LFD mice than in the HFD-EC controls, but that these populations did not differ significantly when other alpha diversity-related metrics were assessed (Supplementary Fig 3).

A shift in the beta diversity of the microbiota, as assessed using unweighted Unifrac, was observed in all three groups of mice following the administration of streptomycin i.e. between T0 and T1 (Fig 3). By the end of the trial, caecal microbiota and T10 faecal samples of all three groups clustered separately from each other (Fig 3)

4.4.3 Diet effects microbiota composition in streptomycin treated mice

Assignment of the 16S rRNA amplicons revealed the presence of six phyla, Bacteroidetes, Firmicutes, Actinobacteria, Deferribacteres, Proteobacteria and Verrucomicrobia, in the faecal samples of the LFD fed mice whereas just five phyla were detected in the corresponding caecal samples despite caecal samples being sequenced to a greater depth. The Proteobacterium phylum was not identified within the LFD caecal

samples and was detected in the faecal samples collected at T8 only. In both faecal and caecal samples the two dominant phyla were the Bacteroidetes and Firmicutes. Initially, the Bacteroidetes dominated but, by week eight, the Firmicutes became dominant. Bacteria corresponding to the phylum Actinobacteria were detected in low proportions in samples collected at T8 (0.5%) and in caecal samples (0.02%).

When the LFD 16S reads were assigned at family level, only fourteen families were detected in the caecal samples, whereas twenty were detected in the faecal samples. At T0, the faecal samples was dominated by a variety of families that could not be accurately identified (57%), with the most dominant 'assigned' families being *Ruminococcaceae* (22%) and *Erysipelotrichaceae* (9%) (Supplementary Table 3). After one week of intervention with streptomycin-treated water and a LFD, *Peptostreptococcaceae* (24%) and *Lactobacillaceae* (8%) became the most dominant 'assigned' families. By T10, this pattern had changed with a large reduction in unassigned families, making *Erysipelotrichaceae* (42%) the most dominant family followed by *Lactobacillaceae* (11%) and *Peptostreptococcaceae* (8%). This final pattern was also evident in the caecal samples (Supplementary Table 3). It is noteworthy that the *Enterobacteriaceae* were not detected throughout the study.

Compositional sequencing revealed the presence of four phyla in the faecal and caecal samples in the HFD fed mice. These were Bacteroidetes, Firmicutes, Deferribacteres and Verrucomicrobia. Of these, Verrucomicrobia were not identified at T1. In both faecal and caecal samples, the two most dominant phyla were the Bacteroidetes and Firmicutes. As with the LFD group, Bacteroidetes were initially the more dominant of the two phyla before a shift in favour of Firmicutes occurred. Unlike LFD group mice, reads corresponding to the phylum Actinobacteria were not detected.

The number of families detected was again lower than was the case for LFD mice i.e. only 14 families were detected in faecal samples and 13 were detected in caecal samples. After one week of intervention with streptomycin treated water and a HFD, unassigned families

dominated the composition (44%) with *Erysipelotrichaceae* (21%), *Peptostreptococcaceae* (20%), *Verrucomicrobiaceae* (3%) and *Rikenellaceae* (3%) being the most dominant 'assigned' families. By T10, a reduction in unassigned families was seen, with *Erysipelotrichaceae* (28%) and *Peptostreptococcaceae* (16%) continuing to be abundant. This pattern was also evident in the caecal samples. It is again noteworthy that *Enterobacteriaceae* were not detected throughout the study (Supplementary Table 4).

Pairwise comparison of caecal microbiota of the streptomycin-treated HFD and LFD mice using Mann-Whitney revealed that the proportions of *Bacteroidaceae* and *Peptostreptococcaceae* were significantly greater, and the proportions of *Eubacteriaceae* were significantly lower, in HFD mice.

Compositional sequencing of the microbiota of HFD mice in receipt of 1×10^7 cfu StrR GMR *E. coli* MG1655 (HFD-EC) for two consecutive days revealed the presence of seven phyla in the faecal samples and six phyla in the caecal samples. These were Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Deferribacteres, 4C0d-2 and Verrucomicrobia with reads corresponding to the 4C0d-2 being detected in T8 faecal samples only. In both faecal and caecum samples the most dominant phyla were Bacteroidetes and Firmicutes. Initially, the Bacteroidetes dominated but, by the T10, Firmicutes were the most dominant phyla. Bacteria corresponding to the phylum Proteobacteria were not initially detected but were detected in increasing proportions over subsequent time points.

As was the case for the LFD mice, there were a greater number of different families detected in the HFD-EC faecal (21) and caecal (14) samples than in HFD mice. After one week of intervention 63% of reads were 'unassigned'. Among the assigned families *Erysipelotrichaceae* (14%), *Verrucomicrobiaceae* (13%), *Rikenellaceae* (2%), *Lactobacillaceae* (2%) and *Ruminococcaceae* (2%) were the most dominant. As noted for both of the other groups, a reduction in unassigned families was observed at T10 and, as with the HFD mice, the *Erysipelotrichaceae* (51%) and *Peptostreptococcaceae* (13%) were still

the most dominant families. It is also noteworthy that *Enterobacteriaceae* were detected at T1, T8 and in the caecum (Supplementary Table 5).

Pairwise comparisons of the caecal microbiota of HFD and HFD-EC mice using Mann-Whitney revealed the presence of significantly greater proportions of *Eubacteriaceae* and significantly smaller proportions of *Rikenellaceae* and *Bacteroidaceae* in HFD-EC mice. A trend towards significantly greater Proteobacteria and associated family was seen in HFD-EC mice compared to HFD mice (p=0.054).

4.4.4 ECBSH1 mice gain less body weight than ECBSH2 mice

The streptomycin-treated DIO mouse model described above was employed to evaluate the consequences of administering *E. coli* MG1655 derivatives expressing *L. salivarius* BSH1 (accession number FJ591091) or BSH2 (accession number FJ591081) genes on weight gain and gut microbiota composition. LFD and HFD mice receiving ECBSH1 gained significantly less body weight than their ECBSH2-fed counterparts (Fig 4) despite the fact that there was no significant difference in average calorie consumption between the cohorts (Fig 5).

4.4.5 Reduced diversity in low fat diet mice in receipt of EC-BSH1

164,581 quality filtered V4 16S sequence reads were generated to investigate the gut microbiota composition of the LFD and HFD mice in receipt of either ECBSH1 or ECBSH2. This equated to an average of 2,478 reads per mouse at each time point and on average 6,919 reads per cecum. Alpha diversities were calculated in each case (Supplementary Table 6). Rarefaction curves were calculated at 97% similarity and were saturating for all samples, indicating extra sampling would yield only a limited increase in species richness (Supplementary Fig 4). Bar graph analysis of caecal alpha diversity reveals that the caecal bacteria of LFD mice receiving ECBSH1 was numerically less than that present in the other three treatment groups (Supplementary Fig 5 & 6).

4.4.6 Analysis of microbiota beta diversity highlights the dominant impact of BSH1 over diet

Principal coordinate analysis of microbial beta diversity, as determined by unweighted Unifrac, again highlights the dramatic effects that streptomycin and diet have on the microbiota population over time (Supplementary Fig 7). Tight clustering of the data points is observed prior to antibiotic treatment and dietary change but following one week of antibiotic treatment and a change in diet there is a considerable shift in the microbiota (Supplementary Fig 7). The faecal microbiota of LFD mice receiving ECBSH2 is separated from the other three treatment groups at all time points. By T8 of the trial, the faecal microbiota shifts again and clusters together with the caecal samples, with the microbiota of both LFD and HFD mice receiving ECBSH1 clustering together. Among the caecal samples, it is apparent that mice fed a LFD and receiving ECBSH2 cluster together, there is no clustering among the microbiota of HFD mice receiving ECBSH2 while all ECBSH1 receiving mice cluster together regardless of diet (Supplementary Fig S7).

4.4.7 BSH1 and 2 differ in their impacts on bacterial taxa

Although numerous changes in composition were observed following administration of the BSH1 and 2 producing strains, here we focus on family level assignments. Data with respect to the impact of introduction of these strains on phylogeny at the phylum and genus level can be found in supplementary tables 7, 8, 9 & 10. Compositional sequencing of the gut microbiota of HFD fed mice receiving ECBSH1 for two consecutive days revealed a greater number of families in faecal samples (23) than in caecal samples (16) despite greater depth of sequencing in caecal samples. Initially, faecal samples were predominantly composed of unassigned families (44%), with the most common assigned families detected similar to study 1 being *Lachnospiraceae* (17%), *Rikenellaceae* (13%), *Ruminococcaceae* (12%) and *Bacteroidaceae* (6%). However, after one week of intervention, a shift in the microbiota was evident with *Enterobacteriaceae* (13%), *Erysipelotrichaceae* (12%),

Erysipelotrichaceae Incertae Sedis (8%), *Ruminococcaceae* (3%) and *Lachnospiraceae* (3%) becoming the most dominant of the assigned families. Eight weeks of intervention further altered the microbiota composition with *Bacteroidaceae* (44% and 42%), *Erysipelotrichaceae* (15% and 16%), *Bifidobacteriaceae* (6% and 8%) and *Ruminococcaceae* (4% in both) being the most dominant families in both faecal and caecum samples, respectively. *Staphylococcaceae* were detected at a level of 16% after eight weeks but were not detected in caecum samples. *Lactobacillaceae* and *Porphyromonadaceae* were detected at 8% and 6% in caecal samples but only at 0.1% and 0.8% in T8 faecal samples (Supplementary Table 7).

Compositional sequencing of the gut microbiota of LFD fed mice receiving ECBSH1 detected twenty two families of bacteria in faecal samples whereas only fifteen were detected in caecal samples. On the basis of assigned reads, the most dominant families detected similar to study 1, were *Rikenellaceae* (17%), *Lachnospiraceae* (15%), *Ruminococcaceae* (6%) and *Bacteroidaceae* (6%) however here, *Porphyromonadaceae* (6%) were also dominant. After one week of intervention a shift in the microbiota was observed with *Lactobacillaceae* (18%), *Thermaceae* (18%), *Erysipelotrichaceae* (15%), *Enterobacteriaceae* (10%), *Ruminococcaceae* (4%) and *Lachnospiraceae* (2%) becoming the most dominant families. After eight weeks of intervention the microbiota composition shifted again, with *Bacteroidaceae* (27% and 40%) and *Erysipelotrichaceae* (23% and 17%) being the most dominant families in the faecal and caecal samples, respectively (Supplementary Table 8).

Compositional sequencing of the gut microbiota of LFD fed mice that received ECBSH2 revealed fourteen families of bacteria in caecal samples and nineteen were detected in faecal samples. As noted above, at T0, the faecal samples were predominantly composed of *Lachnospiraceae* (18%), *Ruminococcaceae* (12%) and *Rikenellaceae* (10%), however *Streptococcaceae* (31%) was also seen to be dominant in this instance. After one week of intervention, this changed such that the *Bacteroidaceae* (74%), *Enterobacteriaceae* (13%), *Rikenellaceae* (6%)

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and *Lactobacillaceae* (3%) became the most dominant families. After eight weeks of intervention *Bacteroidaceae* (54%) were still the most dominant family, followed by *Rikenellaceae* (17%), *Erysipelotrichaceae* (12%), *Bifidobacteriaceae* (5%) and *Ruminococcaceae* (4%). The caecal microbiota was again quite different, being dominated by *Lactobacillaceae* (28%), *Bacteroidaceae* (25%), *Erysipelotrichaceae* (15%) and *Rikenellaceae* (11%) (Supplementary Table 9).

Compositional sequencing of the gut microbiota of HFD mice that received ECBSH2 revealed twenty five families of bacteria in the faecal samples whereas only fifteen were detected in caecal samples. At T0 like the initial study faecal samples consisted of *Rikenellaceae* (18%), *Lachnospiraceae* (18%), *Ruminococcaceae* (11%) and *Bacteroidaceae* (6%) however unlike study 1 *Streptococcaceae* (11%) was also dominant. After one week of intervention the proportions of the different families changed with *Bacteroidaceae* (53%), *Lactobacillaceae* (22%), *Streptococcaceae* (12%) and *Erysipelotrichaceae* (8%) being the most dominant families. After eight weeks the composition changed further with *Erysipelotrichaceae* (25%), *Bacteroidaceae* (22%), *Moraxellaceae* (17%) and *Vibrionaceae* (11%) being the most dominant families. Similarly, the *Erysipelotrichaceae* (30%) and *Bacteroidaceae* (25%) were the most dominant families in the caecal samples. *Bifidobacteriaceae* were also detected at 13% (Supplementary Table 10).

4.4.8 Caecal comparisons reveal that both diet and type of BSH impact on microbiota composition

While phylogenetic data can be viewed in the context of changes within specific groups over time, it is also of value to assess differences that occur between groups. Here we will assess separately the effects both diet and the type of BSH expressed have on caecum microbiota composition. Unlike faecal samples, caecum samples were sequenced for each individual mouse which is why they were chosen for this analysis.

4.4.8.1　　　BSH activity influences microbiota composition in a strain specific manner

The impact that the two respective BSH activities had on the caecal microbiota was assessed by comparing data from animals administered identical diets but supplemented with either ECBSH1 or ECBSH2. In the case of the LFD fed animals, it is interesting to note that Bacteroidetes was the dominant phylum in mice receiving ECBSH1 whereas Firmicutes dominated in mice receiving ECBSH2 mice. Increases and decreases in the proportions of a number of families were evident throughout the study. In particular, there was a large increase in the proportions of the family *Bacteroidaceae*, and the genera *Bacteroides* and *Allobaclum*, in ECBSH1 mice. In contrast, increases in the proportions of *Lactobacillaceae* and *Streptococcaceae*, and the corresponding genera *Lactobacillus* and *Streptococcus*, were seen in ECBSH2. It is also interesting to note that representatives of the phylum Deinococcus-Thermus, and its corresponding family and genus, *Thermaceae* and *Thermus*, and the family *Erysipelotrichales Incertae Sedis* were found in ECBSH1 mice but not ECBSH2 mice. Similarly, the family *Prevotellaceae* and its corresponding genus, *Prevotella*, were detected in mice receiving ECBSH2, but not ECBSH1.

The impact of expression of the BSH genes was also assessed by comparing the caecal microbiota of HFD-fed animals that received ECBSH1 or ECBSH2. As was the case for the corresponding LFD fed animals, reads corresponding to the phylum Deinococcus-Thermus and its corresponding family and genus (*Thermaceae* and *Thermus)* were detected in mice that received ECBSH1, but not those that received ECBSH2. It was also noted yet again that Bacteroidetes were dominant in ECBSH1-administered mice whereas Firmicutes were dominant BSH2-administered mice. It was also noted that *Vibrionaceae*, and its corresponding genus *Vibrio*, were detected in mice receiving ECBSH1 only. In contrast, the families *Porphyromadaceae* and *Leuconostocaceae* and the genera *Faecalibacterium* and *Leuconostoc* were only detected in ECBSH2 mice*.* Differences in the proportions of other taxa were also

noted. In ECBSH1 mice the proportions of the families *Bacteroidaceae, Lactobacillaceae* and *Erysipelotrichales Incertae Sedis* and the genera *Bacteroides* and *Lactobacillus* were greater in ECBSH1-administered mice compared to ECBSH2 mice. In contrast, the proportions of the phylum Actinobacteia and its corresponding family and genus, *Bifidobacteriaceae* and *Bifidobacterium*, the families *Lachnospiraceae, Erysipelotrichaceae* and *Enterobacteriaceae* as well as *Lachnospiraceae Incertae Sedis* and *Enterobacteriaceae* associated genera were greater in ECBSH2-adminsitered mice.

4.5 DISCUSSION

Effective colonization of the intestine with newly introduced strains of *E. coli* can be problematic due to colonization resistance from the natural microbiota (Lawley and Walker, 2013). For this reason, the streptomycin-treated mouse model (Leatham-Jensen *et al.*, 2012, Leatham *et al.*, 2005, Cronin *et al.*, 2012) was employed in this study. Effects of streptomycin on the gut have previously been investigated using FISH, which revealed that streptomycin treatment increases the numbers of *Cytophage-Flavobacterium-Bacteroidetes*, while traditional culture methods revealed a reduction in the abundance of *Lactobacillus, Enterobacteriaceae* and enterococci/group D streptococci (Sekirov *et al.*, 2008).

On the basis of principle coordinate analysis, a clear shift in the microbiota population was apparent after one week of streptomycin treatment. While this shift could be attributed to age and diet, it is likely that antibiotic intervention plays a big part in this change. Unlike our previous study, in which a recovery of the microbiota with time was observed in HFD mice receiving the antibiotic vancomycin (Clarke *et al.*, 2013), no recovery was observed here after 10 weeks of streptomycin treatment. It is not clear why this is the case but may be due to the different antimicrobial spectra or mechanisms of action of these antibiotics. This highlights the varying and lasting effects that antibiotics can have on our gut microbiota communities. As expected, and in line with previous studies (Clarke *et al.*, 2013, Garner *et al.*, 2009, Murphy *et al.*, 2012), a reduction in microbiota diversity due to antibiotic treatment was noted in all three cohorts. Diet induced obesity in streptomycin-treated mice resulted in a gut microbiota composition, as revealed by unweighted unifrac, that was clearly distinct from that of LFD streptomycin-treated mice. Initially, and in agreement with previous studies, treatment of LFD fed animals with streptomycin for one week did not alter the proportions of Bacteroidetes. However, 10 weeks of streptomycin treatment brought about a reduction in Bacteroidetes and a bloom in Firmicutes, and of *Erysipelotrichaceae* in particular, in faecal and caecal samples. Though the blooming of Mollicutes (equivalent to

Erysipelotrichaceae in this study) has been previously associated with diet induced obesity (Turnbaugh *et al.*, 2008), this was not observed in our previous study which employed the same HFD employed here (Murphy *et al.*, 2012). Multiple antibiotics (ampicillin, gentamycin, metronidazole, neomycin and vancomycin) have been found to increase the numbers of *Erysipelotrichaceae* (Hill *et al.*, 2009*)*, with the species *Erysipelothrix rhusiopathiae* being resistant to streptomycin (Lee *et al.*, 2011). In contrast to studies investigating the impact of streptomycin on the murine microbiota (Sekirov *et al.*, 2008), but in line with diet induced obesity studies (Murphy *et al.*, 2012), a decrease in Bacteroidetes abundance and an increase in Firmicutes abundance in faecal and caecal samples was noted after 10 weeks of HFD mice receiving streptomycin. In this case, increases in the families *Erysipelotrichaceae* and *Peptostreptococcaceae* were primarily responsible for the increase in Firmicutes whereas, in our previous study, diet induced obesity did not alter the levels of *Erysipelotrichaceae* or *Peptostreptococcaceae* (Clarke *et al.*, 2013)*. This is presumably a phenomenon driven by the additional presence of streptomycin. Streptomycin also successfully reduced Proteobacteria to below detectable levels in the faecal and caecal samples of HFD and LFD mice after 10 weeks.

While the first animal trial described in this study provided information with respect to the impact of streptomycin on mice fed a HFD or LFD diet, it also revealed the impact of the colonization of the streptomycin-treated HFD fed mouse model with *E. coli* MG1655. Colonization of HFD fed animals with *E. coli* MG1655 resulted in significant changes to the microbiota from that of control HFD fed animals as revealed by unweighted Unifrac. Pairwise comparison of the caecal microbiota revealed significantly greater proportions of *Eubacteriaceae*, and significantly lower proportions of *Rikenellaceae* and *Bacteroidaceae* in HFD-EC mice compared to HFD mice. Critically, the detection of *Enterobacteriaceae* in the caecum of HFD-EC mice reflected the successful colonization of the mouse intestinal tract with *E. coli* MG1655 and suggested that this was a suitable model for investigating the influence of BSH production on the gut microbiota.

It has been noted that bile acids have a direct effect on the host with increased levels protecting against obesity in leptin deficient mice (Zhang *et al.*, 2012) and reduced levels of eight bile acids species noted in humanized obese mice (Ridaura *et al.*, 2013). Bile acid administration has also been previously noted to alter microbiota composition (Islam *et al.*, 2011). Deconjugation of bile acids by BSHs has been hypothesised to impact on gut microbiota composition as the liberated amino acids taurine and glycine could potentially be used as carbon, nitrogen and energy sources (Begley *et al.*, 2005a). Previous characterisation of BSH1$_{JCM1046}$ and BSH1$_{UCC118}$ (ECBSH1 and ECBSH2, respectively) has shown that ECBSH1 is more active than ECBSH2, detoxifying primary bile acids and producing more secondary and tertiary bile acids (Fang *et al.*, 2009). We hypothesised that ECBSH1 and ECBSH2 can modulate the gut microbiota as bacteria that are normally sensitive to bile acids can now flourish and bacteria that are sensitive to secondary and tertiary bile acids will be reduced. ECBSH1 has a greater affinity for tauro-conjugated bile (TCBA) acids than ECBSH2. As the level of TCBA is increased by dietary fat (Devkota *et al.*, 2012), we used both a LFD and HFD to determine if the effects of BSH activity on the microbiota population differed in a diet-dependent manner.

E. coli MG1655 expressing either ECBSH1 or ECBSH2 was administered to LFD or HFD fed animals for two consecutive days at the beginning of the trial. Although no significant difference in energy consumption by the respective groups was observed, significant differences in percentage weight gain were apparent. As expected, LFD mice in receipt of either ECBSH1 or ECBSH2 gained significantly less weight than their HFD counterparts. It is interesting, however, to note that ECBSH1 receiving mice gained significantly less weight than mice that received ECBSH2. This reduction could be due to increased activity of ECBSH1 altering the microbiota composition and in turn weight gain, though host related factors such as hormones and genotype could also be involved.

As with our previous trial (Clarke *et al.*, 2013) and the initial pilot study, streptomycin causes a shift in microbiota composition visualised by

PCoA. Neither diet nor BSH treatment appear to play a role in this shift with all four cohorts clustering together at each time point. However, this changed by the end of the study with caecum microbiota composition clustering by treatment group. Mice receiving ECBSH1 clustered closely together regardless of diet and LFD ECBSH2 mice clustered away from HFD ECBSH2 mice.

We speculated that comparing the gut microbiota of LFD mice receiving ECBSH1 and ECBSH2 might highlight differences that could explain the significant reduction in weight gain seen in mice receiving ECBSH1 but not in mice receiving ECBSH2. It was therefore interesting to note that the caecal samples from ECBSH1 mice had a high Bacteroidetes to Firmicutes ratio whereas the opposite was the case in mice receiving ECBSH2. A high Firmicutes to Bacteroidetes ratio has previously been associated with obesity (Murphy *et al.*, 2012, Ley *et al.*, 2005, Ley *et al.*, 2006), albeit not in all cases (Clarke *et al.*, 2013, Schwiertz *et al.*, 2009, Duncan *et al.*, 2007). Actinobacteria, specifically *Bifidobacteriaceae*, were detected in larger numbers in week eight faecal samples from LFD ECBSH1 administered mice relative to samples from LFD ECBSH2 mice. *Bifidobacterium* has previously been associated with anti-obesity effects i.e. reducing body and fat weight (An *et al.*, 2011), albeit in a strain specific manner (Yin *et al.*, 2010). A large increase in *Lactobacillus* was noted in ECBSH2 mice cecum samples. The species *Lactobacillus reuteri* has previously been found to be enriched in obese humans (Million *et al.*, 2011, Million *et al.*, 2013), but this remains highly controversial (Delzenne and Reid, 2009, Ehrlich, 2009, Raoult, 2009). As for LFD mice, HFD ECBSH1 mice gained significantly less weight than HFD ECBSH2 mice. Comparisons again reveal a high Bacteroidetes to Firmicutes ratio in HFD ECBSH1 mice (corresponding to high *Bacteroidaceae* numbers) compared to a high Firmicutes to Bacteroidetes ratio observed in ECBSH2 mice (corresponding to high *Erysipelotrichaceae* numbers). Given that a high Firmicutes to Bacteroidetes ratio and greater *Erysipelotrichi* levels have previously been observed in mice administered cholic acid (Islam *et al.*, 2011) it would seem that this phenomenon results from instances where bile salt

breakdown is greater, i.e. reflecting the greater activity of ECBSH1 over ECBSH2, or where increased substrate, i.e. cholic acid, is available for the gut microbiota to act on.

These pilot studies highlight the effect diet, antibiotics and BSH can have on gut microbiota communities. Though the reduction in weight gain associated with ECBSH1 and the composition changes are promising, further investigations using larger groups of mice and additional controls are required to determine the significance of these phenomena.

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Figure 1

HFD and HFD-EC mice gain significantly more weight than LFD mice.
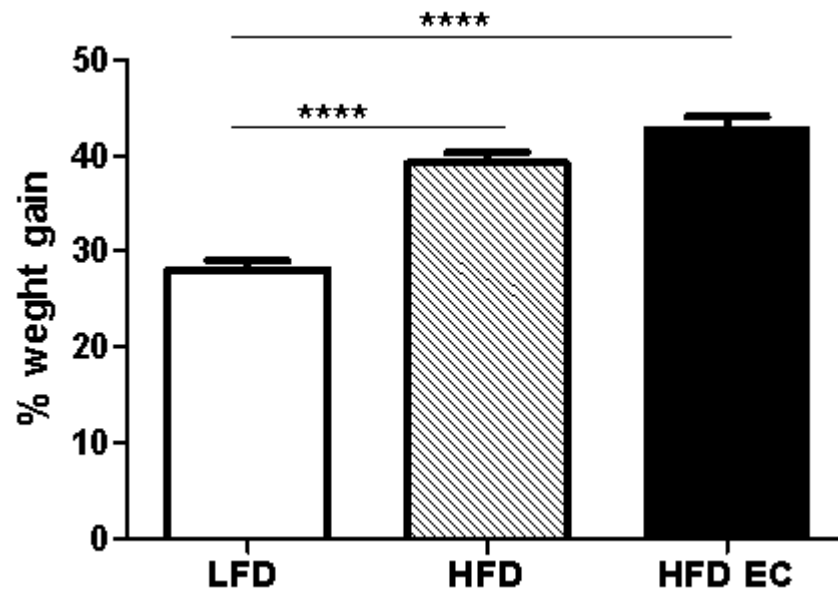
Figure 2

HFD and HFD-EC consume more calories on average than LFD mice per week.
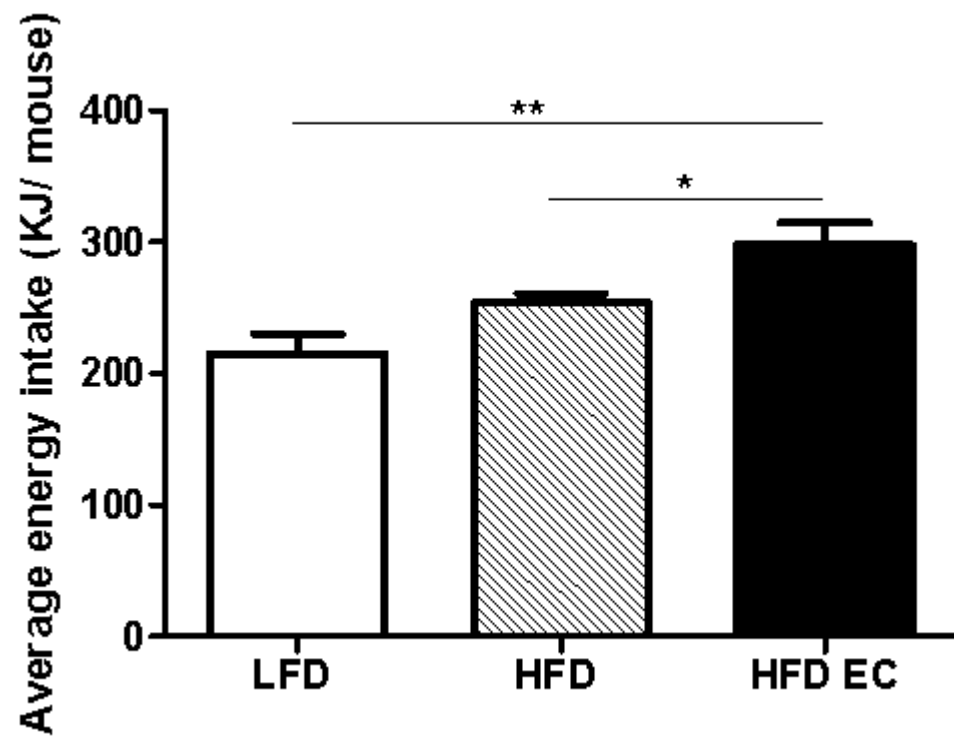
Figure 3

Principal coordinate analysis of unweighted Unifrac for each diet group.

Colour codes; low fat diet fed mice, shades of red with T1 being the darkest and getting lighter with time; high fat diet fed mice, shades of blue with T1 being the darkest and getting lighter with time and high fat diet fed mice receiving 1×10^7 cfu StrR GMR *E. coli* MG1655 with no bile salt hydrolase, shades of green with T1 being the darkest and getting lighter with time.
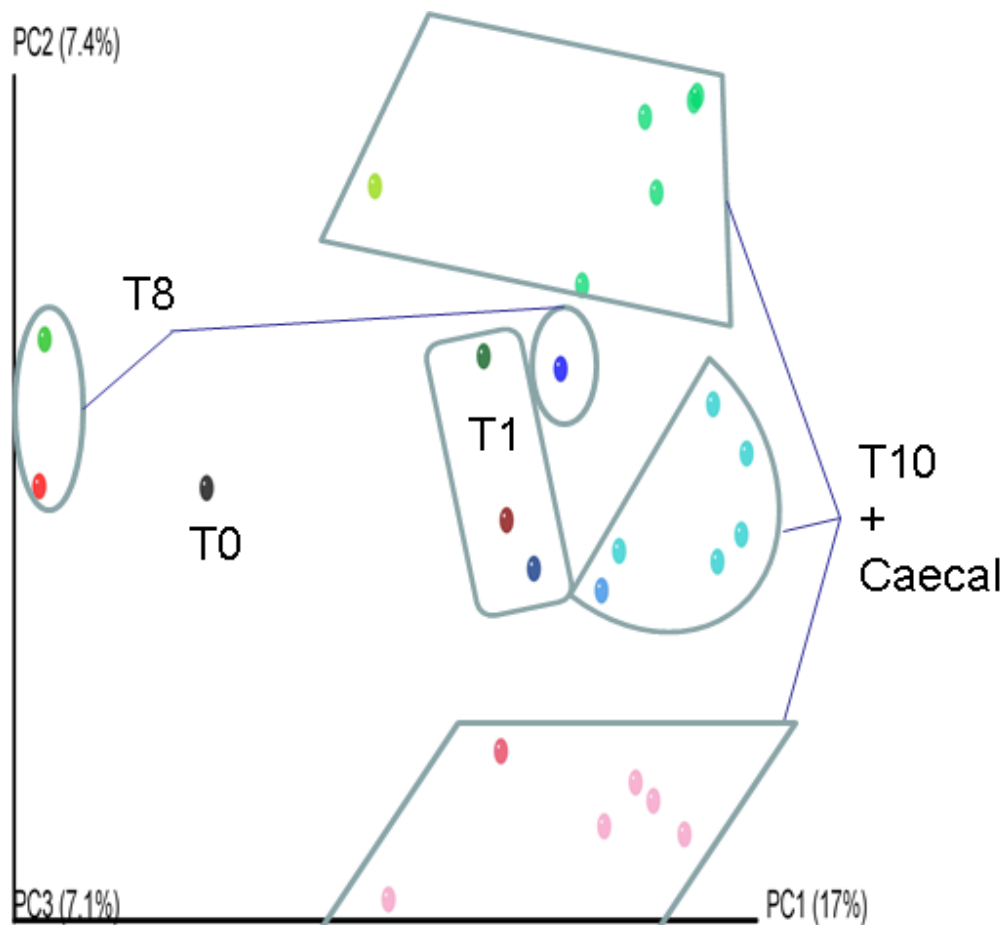
Figure 4

ECBSH1 mice gain significantly less weight than ECBSH2 mice.
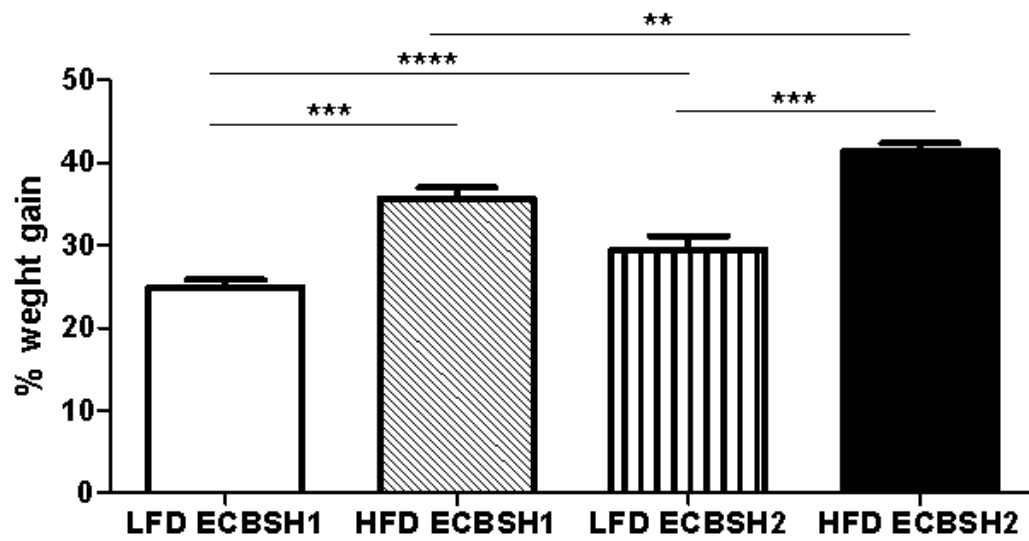
Figure 5

No significant difference in average weekly calorie intake between the cohorts.
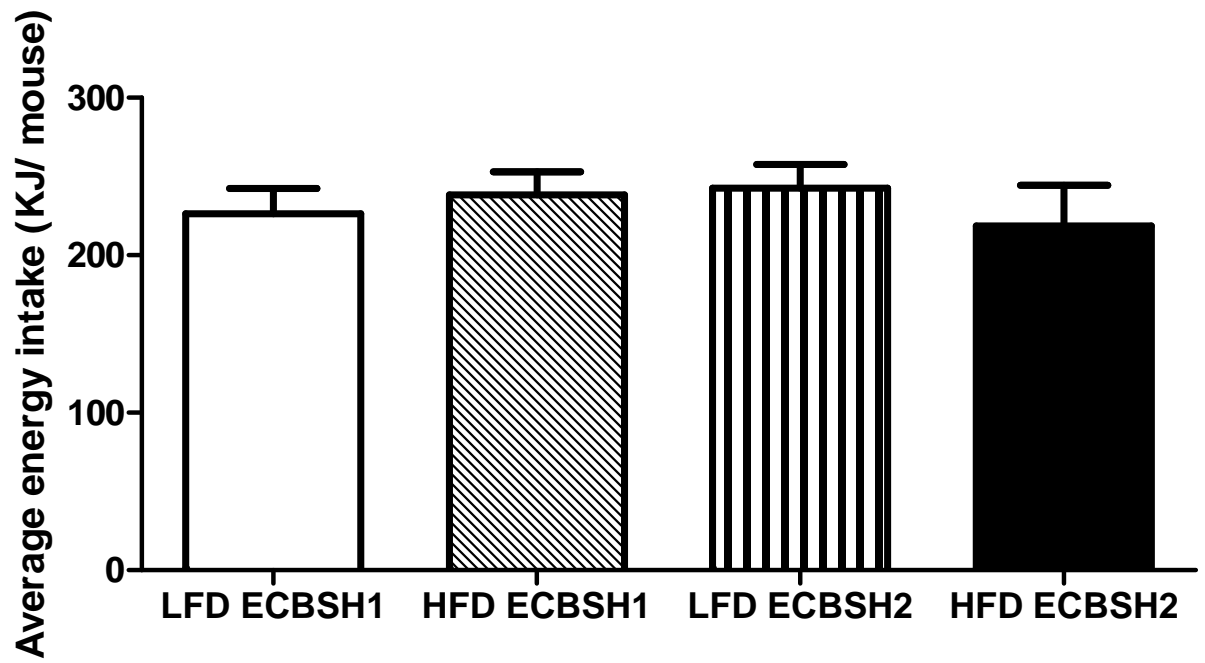
SUPPLEMENTARY INFORMATION
Supplementary Table 1

Primer	Sequence 5'-3'	Purpose
P44FA*fl*II	5'-ATAATACTTAAGACCCATCCAGGAGACGGGACGATAGC-3'	p44 Promoter amplification
p44R*Nco*I	5'-GCAGTACCCATGGTTTGGCCTCCTAAGCGCCTCCTTTCCCTCACAC ATC-3'	p44 Promoter amplification
GMF	5'-GATCCTCTAGAGTCGACCTGCAGGCATG-3'	Plasmid sequencing from GM cassette
glmSattnF	5'-GATGCTGGTGGCGAAGCTGT-3'	Chromosome integration check
glmSattnR	5'-GATGACGGTTTGTCACATGGA-3'	Chromosome integration check
p44OLRBSH1	5'-TAAAGTAATTGCTGTACACATGGTTTGGCCTCCTAAGCGCCTC CTTTCCC-3'	Amplification of p44 promoter with overlap of BSH1
BSH1OLF	5'-GGGAAAGGAGGCGCTTAGGAGGCCAAACCATGTGTACAGCAA TTACTTTA-3'	Amplification of BSH1 with overlap of p44
BSH1R	5'-ATATATCCGCGGTTAATTCAACTTATTTATTACTTGTTT-3'	Amplification of BSH1
BSH2OLF	5'-GGGAAAGGAGGCGCTTAGGAGGCCAAACCATGTGTACAGCAA TTACTTTA-3'	Amplification of BSH2 with overlap of p44
BSH2R	5'-TATATAGGTACCTTAATTTTGCATATTAATTGATTGGTGGC-3'	Amplification of BSH2

Supplementary Table 2 Alpha diversity

	Chao1	Simpson	Shannon	Phylogenetic Diversity	Observed Species
Low Fat Diet					
T0	885.5	0.953	5.589	24.147	368
T1	580.5	0.872	4.281	17.140	294
T8	845.5	0.715	4.012	30.080	443
T10	554.6	0.811	4.008	19.077	306
caecal	559.1	0.904	4.697	17.092	348
caecal	706.0	0.919	4.770	18.326	352
caecal	564.2	0.799	3.896	14.941	297
caecal	649.6	0.799	3.964	21.308	319
caecal	614.1	0.912	4.634	17.652	304
High Fat Diet					
T0	885.5	0.953	5.589	24.147	368
T1	493.0	0.877	4.220	18.211	285
T8	492.0	0.872	4.304	17.514	303
T10	536.8	0.841	4.145	16.060	290
caecal	401.6	0.926	4.793	13.949	249
caecal	487.3	0.932	4.847	14.693	270
caecal	495.1	0.916	4.696	15.097	281
caecal	464.6	0.886	4.302	15.045	284
caecal	445.7	0.896	4.517	16.243	278
High Fat Diet + MG1655					
T0	885.5	0.953	5.589	24.147	368
T1	473.9	0.889	4.373	17.658	268
T8	874.5	0.757	4.223	32.855	501
T10	700.1	0.747	3.599	21.252	338
caecal	477.1	0.913	4.738	17.044	298
caecal	541.1	0.900	4.533	15.310	296
caecal	679.3	0.888	4.391	16.858	291
caecal	614.2	0.882	4.430	14.050	282
caecal	445.7	0.915	4.663	14.719	279

Supplementary Table 3 Percentage read number Low fat diet mice

	LFD T0	LFD T1	LFD T8	LFD T10	LFD caecal
Phylum					
Proteobacteria	0.000	0.000	0.140	0.000	0.000
Bacteroidetes	52.690	53.141	12.495	22.120	35.019
Firmicutes	42.172	43.094	81.804	70.432	56.270
Actinobacteria	0.000	0.000	0.490	0.000	0.021
Deferribacteres	4.614	0.000	0.084	2.509	1.751
Candidate division TM7	0.000	0.000	0.000	0.000	0.000
4C0d-2	0.000	0.000	0.000	0.000	0.000
Verrucomicrobia	0.000	3.688	4.931	4.834	6.789
Deinococcus-Thermus	0.000	0.000	0.000	0.000	0.000
other	0.524	0.078	0.056	0.105	0.150
Family					
Rhodospirillaceae	0.000	0.000	0.000	0.000	0.000
Alcaligenaceae	0.000	0.000	0.000	0.000	0.000
Rikenellaceae	1.975	0.094	3.026	0.946	2.251
Porphyromonadaceae	0.121	0.000	0.784	0.000	0.000
Bacteroidaceae	1.350	0.375	5.379	0.171	0.141
Lachnospiraceae	1.632	3.141	1.849	4.019	1.646
Eubacteriaceae	0.222	0.125	0.616	0.355	0.563
Ruminococcaceae	22.063	1.953	4.356	2.706	5.385
Peptococcaceae	0.000	0.000	0.000	0.000	0.000
Lactobacillaceae	1.592	7.875	5.477	11.296	12.372
Erysipelotrichales Incertae Sedis	0.000	0.000	0.000	0.000	0.000
Bifidobacteriaceae	0.000	0.000	0.280	0.000	0.000
Deferribacteraceae	4.614	0.000	0.084	2.509	1.751
Erysipelotrichaceae	9.248	1.078	55.456	42.073	24.874
Coriobacterineae	0.000	0.000	0.210	0.000	0.018
Clostridiaceae	0.000	0.000	0.098	0.000	0.000
Verrucomicrobiaceae	0.000	3.688	4.917	4.834	6.789
Enterobacteriaceae	0.000	0.000	0.000	0.000	0.000
Streptococcaceae	0.181	0.359	1.961	0.328	0.545
Anaeroplasmataceae	0.000	0.000	0.000	0.000	0.000
Prevotellaceae	0.302	0.172	0.630	0.118	0.039
Moraxellaceae	0.000	0.000	0.000	0.000	0.000
Vibrionaceae	0.000	0.000	0.000	0.000	0.000
Thermaceae	0.000	0.000	0.000	0.000	0.000

	LFD T0	LFD T1	LFD T8	LFD T10	LFD caecal
Brevibacteriaceae	0.000	0.000	0.000	0.000	0.000
Staphylococcaceae	0.000	0.000	0.000	0.000	0.000
Pseudoalteromonadaceae	0.000	0.000	0.000	0.000	0.000
Leuconostocaceae	0.000	0.000	0.098	0.000	0.015
Bacillaceae	0.000	0.000	0.000	0.000	0.000
Desulfovibrionaceae	0.000	0.000	0.000	0.000	0.000
Peptostreptococcaceae	0.000	23.750	8.012	7.894	9.942
Planococcaceae	0.000	1.109	0.000	0.000	0.000
Paenibacillaceae	0.000	0.344	0.000	0.000	0.000
Veillonellaceae	0.000	0.000	0.112	0.000	0.000
Other	56.700	55.938	6.654	22.751	33.669
Genus					
Thalassospira	0.000	0.000	0.000	0.000	0.000
Sutterella	0.000	0.000	0.000	0.000	0.000
Alistipes	1.612	0.000	0.896	0.946	2.230
Rikenella	0.000	0.000	0.000	0.000	0.000
Parabacteroides	0.000	0.000	0.588	0.000	0.000
Odoribacter	0.000	0.000	0.126	0.000	0.000
Bacteroides	1.350	0.375	5.379	0.171	0.177
Lachnospiraceae Incertae Sedis	0.000	0.000	0.532	0.131	0.180
uncultured Lachnospiraceae	0.000	0.000	0.000	0.000	0.000
Ruminococcus	0.000	0.000	0.350	0.000	0.000
Ruminococcaceae Incertae Sedis	16.845	0.141	0.434	2.075	4.643
Oscillibacter	0.000	0.000	0.000	0.000	0.000
Anaerotruncus	1.229	1.344	0.196	0.066	0.186
Lactobacillus	1.592	7.875	0.546	11.296	12.372
Allobaculum	0.000	1.063	0.000	0.000	0.000
Bifidobacterium	0.000	0.000	0.280	0.000	0.000
Mucispirillum	4.614	0.000	0.084	2.509	1.751
Turicibacter	0.000	0.000	0.000	0.000	0.000
Clostridium	0.000	0.000	0.098	0.000	0.000
Coprococcus	0.000	0.000	0.000	0.000	0.000
Akkermansia	0.000	3.688	0.490	4.834	6.789
Brenneria-Yersinia	0.000	0.000	0.000	0.000	0.000
Streptococcus	0.000	0.000	0.000	0.000	0.000
Prevotella	0.302	0.172	0.630	0.118	0.039

	LFD T0	LFD T1	LFD T8	LFD T10	LFD caecal
Marvinbryantia - Bryantella	0.000	0.000	0.000	0.000	0.000
Anaeroplasma	0.000	0.000	0.000	0.000	0.000
Lactococcus	0.181	0.344	1.933	0.328	0.545
Faecalibacterium	0.101	0.000	0.798	0.000	0.000
Psychrobacter	0.000	0.000	0.000	0.000	0.000
Vibrio	0.000	0.000	0.000	0.000	0.000
Thermus	0.000	0.000	0.000	0.000	0.000
Staphylococcus	0.000	0.000	0.000	0.000	0.000
Pseudoalteromonas	0.000	0.000	0.000	0.000	0.000
Leuconostoc	0.000	0.000	0.084	0.000	0.000
Anoxybacillus	0.000	0.000	0.000	0.000	0.000
Desulfovibrio	0.000	0.000	0.000	0.000	0.000
Paenibacillus	0.000	0.344	0.000	0.000	0.000
Lysinibacillus	0.000	1.094	0.000	0.000	0.000
Peptostreptococcaceae Incertae Sedis	0.000	0.391	0.224	0.276	0.287
Anaerovorax	0.000	0.000	0.000	0.000	0.000
Catenibacterium	0.000	0.000	0.210	0.000	0.000
Barnesiella	0.000	0.000	0.000	0.000	0.000
Blautia	0.000	0.000	0.154	0.000	0.000
Subdoligranulum	0.000	0.000	0.126	0.000	0.000
uncultured Ruminococcaceae	0.000	0.000	0.084	0.000	0.000
Phascolarctobacterium	0.000	0.000	0.084	0.000	0.000
Lachnospira	0.000	0.000	0.000	0.000	0.000
Megamonas	0.000	0.000	0.000	0.000	0.000
Weissella	0.000	0.000	0.000	0.000	0.000
Uncultured bacteria	0.201	0.000	2.717	1.497	1.159
Other	71.973	83.172	82.953	75.752	69.642

Supplementary Table 4 Percentage read number high fat diet mice

	HFD T0	HFD T1	HFD T8	HFD T10	HFD caecal
Phylum					
Proteobacteria	0.000	0.000	0.000	0.000	0.000
Bacteroidetes	52.690	44.919	22.180	22.832	34.456
Firmicutes	42.172	48.591	70.171	69.128	51.592
Actinobacteria	0.000	0.000	0.000	0.000	0.000
Deferribacteres	4.614	2.981	1.480	4.145	5.097
Candidate division TM7	0.000	0.000	0.000	0.000	0.000
4C0d-2	0.000	0.000	0.000	0.000	0.000
Verrucomicrobia	0.000	3.399	6.073	3.843	8.760
Deinococcus-Thermus	0.000	0.000	0.000	0.000	0.000
other	0.524	0.109	0.096	0.053	0.095
Family					
Rhodospirillaceae	0.000	0.000	0.000	0.000	0.000
Alcaligenaceae	0.000	0.000	0.000	0.000	0.000
Rikenellaceae	1.975	3.072	1.385	3.395	2.598
Porphyromonadaceae	0.121	0.000	0.000	0.000	0.000
Bacteroidaceae	1.350	0.418	0.411	0.737	0.699
Lachnospiraceae	1.632	0.891	3.578	4.461	1.451
Eubacteriaceae	0.222	0.000	0.452	0.000	0.122
Ruminococcaceae	22.063	1.963	3.619	3.224	6.101
Peptococcaceae	0.000	0.091	0.000	0.000	0.000
Lactobacillaceae	1.592	1.545	3.578	1.500	9.285
Erysipelotrichales Incertae Sedis	0.000	0.000	0.000	0.000	0.000
Bifidobacteriaceae	0.000	0.000	0.000	0.000	0.000
Deferribacteraceae	4.614	2.981	1.480	4.145	5.097
Erysipelotrichaceae	9.248	21.196	28.430	37.163	16.814
Coriobacterineae	0.000	0.000	0.000	0.000	0.000
Clostridiaceae	0.000	0.000	0.000	0.000	0.000
Verrucomicrobiaceae	0.000	3.399	6.073	3.843	8.760
Enterobacteriaceae	0.000	0.000	0.000	0.000	0.000
Streptococcaceae	0.181	0.818	0.699	1.000	0.821
Anaeroplasmataceae	0.000	0.000	0.000	0.000	0.000
Prevotellaceae	0.302	0.345	0.000	0.000	0.024
Moraxellaceae	0.000	0.000	0.000	0.000	0.000
Vibrionaceae	0.000	0.000	0.000	0.000	0.000

	HFD T0	HFD T1	HFD T8	HFD T10	HFD caecal
Thermaceae	0.000	0.000	0.000	0.000	0.000
Brevibacteriaceae	0.000	0.000	0.000	0.000	0.000
Staphylococcaceae	0.000	0.000	0.000	0.000	0.000
Pseudoalteromonadaceae	0.000	0.000	0.000	0.000	0.000
Leuconostocaceae	0.000	0.000	0.000	0.000	0.020
Bacillaceae	0.000	0.000	0.000	0.000	0.000
Desulfovibrionaceae	0.000	0.000	0.000	0.000	0.000
Peptostreptococcaceae	0.000	19.760	24.907	15.857	15.861
Planococcaceae	0.000	0.000	0.000	0.000	0.000
Paenibacillaceae	0.000	0.000	0.000	0.000	0.000
Veillonellaceae	0.000	0.000	0.000	0.000	0.000
Other	56.700	43.519	25.387	24.674	32.346
Genus					
Thalassospira	0.000	0.000	0.000	0.000	0.000
Sutterella	0.000	0.000	0.000	0.000	0.000
Alistipes	1.612	0.491	0.795	1.908	1.882
Rikenella	0.000	0.000	0.000	0.000	0.000
Parabacteroides	0.000	0.000	0.000	0.000	0.000
Odoribacter	0.000	0.000	0.000	0.000	0.000
Bacteroides	1.350	0.418	0.411	0.737	0.699
Lachnospiraceae Incertae Sedis	0.000	0.000	0.000	0.000	0.000
uncultured Lachnospiraceae	0.000	0.000	0.000	0.000	0.000
Ruminococcus	0.000	0.000	0.000	0.000	0.000
Ruminococcaceae Incertae Sedis	16.845	1.254	1.974	2.027	3.951
Oscillibacter	0.000	0.000	0.000	0.000	0.000
Anaerotruncus	1.229	0.109	1.179	0.711	1.716
Lactobacillus	1.592	1.545	3.578	1.500	9.285
Allobaculum	0.000	0.000	0.000	0.000	0.000
Bifidobacterium	0.000	0.000	0.000	0.000	0.000
Mucispirillum	4.614	2.981	1.480	4.145	5.097
Turicibacter	0.000	0.000	0.000	0.000	0.000
Clostridium	0.000	0.000	0.000	0.000	0.000
Coprococcus	0.000	0.000	0.000	0.000	0.000
Akkermansia	0.000	3.381	6.073	3.843	8.760
Brenneria-Yersinia	0.000	0.000	0.000	0.000	0.000
Streptococcus	0.000	0.000	0.000	0.000	0.000

	HFD T0	HFD T1	HFD T8	HFD T10	HFD caecal
Prevotella	0.302	0.345	0.000	0.000	0.024
Marvinbryantia - Bryantella	0.000	0.000	0.000	0.000	0.000
Anaeroplasma	0.000	0.000	0.000	0.000	0.000
Lactococcus	0.181	0.818	0.685	0.974	0.807
Faecalibacterium	0.101	0.000	0.000	0.000	0.000
Psychrobacter	0.000	0.000	0.000	0.000	0.000
Vibrio	0.000	0.000	0.000	0.000	0.000
Thermus	0.000	0.000	0.000	0.000	0.000
Staphylococcus	0.000	0.000	0.000	0.000	0.000
Pseudoalteromonas	0.000	0.000	0.000	0.000	0.000
Leuconostoc	0.000	0.000	0.000	0.000	0.000
Anoxybacillus	0.000	0.000	0.000	0.000	0.000
Desulfovibrio	0.000	0.000	0.000	0.000	0.000
Paenibacillus	0.000	0.000	0.000	0.000	0.000
Lysinibacillus	0.000	0.000	0.000	0.000	0.000
Peptostreptococcaceae Incertae Sedis	0.000	0.491	0.315	0.355	0.424
Anaerovorax	0.000	0.000	0.000	0.000	0.000
Catenibacterium	0.000	0.000	0.000	0.000	0.000
Barnesiella	0.000	0.000	0.000	0.000	0.000
Blautia	0.000	0.000	0.000	0.000	0.000
Subdoligranulum	0.000	0.000	0.000	0.000	0.000
uncultured Ruminococcaceae	0.000	0.000	0.000	0.000	0.000
Phascolarctobacterium	0.000	0.000	0.000	0.000	0.000
Lachnospira	0.000	0.000	0.000	0.000	0.000
Megamonas	0.000	0.000	0.000	0.000	0.000
Weissella	0.000	0.000	0.000	0.000	0.000
Uncultured bacteria	0.201	0.000	1.398	2.211	0.868
Other	71.973	88.166	82.111	81.590	66.487

Supplementary Table 5 Percentage read number High fat diet mice receiving *E. coli* MG1655

	HFD-EC T0	HFD-EC T1	HFD-EC T8	HFD-EC T10	HFD-EC caecal
Phylum					
Proteobacteria	0.000	0.154	0.382	0.059	0.200
Bacteroidetes	52.690	58.773	21.229	19.841	36.000
Firmicutes	42.172	27.664	77.396	73.671	53.218
Actinobacteria	0.000	0.289	0.186	0.237	0.021
Deferribacteres	4.614	0.096	0.142	3.883	4.084
Candidate division TM7	0.000	0.000	0.000	0.000	0.000
4C0d-2	0.000	0.000	0.327	0.000	0.000
Verrucomicrobia	0.000	12.967	0.273	2.285	6.336
Deinococcus-Thermus	0.000	0.000	0.000	0.000	0.000
other	0.524	0.058	0.065	0.024	0.141
Family					
Rhodospirillaceae	0.000	0.000	0.186	0.000	0.000
Alcaligenaceae	0.000	0.000	0.000	0.000	0.000
Rikenellaceae	1.975	1.693	1.572	0.533	1.359
Porphyromonadaceae	0.121	0.000	0.830	0.000	0.000
Bacteroidaceae	1.350	0.269	7.160	0.189	0.017
Lachnospiraceae	1.632	0.539	1.790	0.675	3.449
Eubacteriaceae	0.222	0.943	0.982	0.758	0.479
Ruminococcaceae	22.063	1.501	4.224	2.084	6.243
Peptococcaceae	0.000	0.866	0.055	0.000	0.000
Lactobacillaceae	1.592	1.885	2.107	1.350	4.725
Erysipelotrichales Incertae Sedis	0.000	0.000	0.000	0.000	0.000
Bifidobacteriaceae	0.000	0.000	0.120	0.000	0.000
Deferribacteraceae	4.614	0.096	0.142	3.883	4.084
Erysipelotrichaceae	9.248	14.313	50.437	50.681	20.671
Coriobacterineae	0.000	0.289	0.065	0.225	0.017
Clostridiaceae	0.000	0.000	0.000	0.000	0.000
Verrucomicrobiaceae	0.000	12.967	0.273	2.285	6.336
Enterobacteriaceae	0.000	0.154	0.065	0.000	0.179
Streptococcaceae	0.181	1.077	2.434	2.013	0.797
Anaeroplasmataceae	0.000	0.000	0.120	0.095	0.000
Prevotellaceae	0.302	0.115	4.410	0.000	0.000
Moraxellaceae	0.000	0.000	0.000	0.000	0.000

	HFD-EC T0	HFD-EC T1	HFD-EC T8	HFD-EC T10	HFD-EC caecal
Vibrionaceae	0.000	0.000	0.000	0.000	0.000
Thermaceae	0.000	0.000	0.000	0.000	0.000
Brevibacteriaceae	0.000	0.000	0.000	0.000	0.000
Staphylococcaceae	0.000	0.000	0.000	0.000	0.000
Pseudoalteromonadaceae	0.000	0.000	0.000	0.000	0.000
Leuconostocaceae	0.000	0.000	0.000	0.000	0.038
Bacillaceae	0.000	0.000	0.000	0.000	0.000
Desulfovibrionaceae	0.000	0.000	0.055	0.000	0.000
Peptostreptococcaceae	0.000	0.289	10.194	12.490	15.290
Planococcaceae	0.000	0.000	0.000	0.000	0.000
Paenibacillaceae	0.000	0.000	0.000	0.000	0.000
Veillonellaceae	0.000	0.000	0.393	0.000	0.000
Other	56.700	63.005	12.388	22.742	36.313
Genus					
Thalassospira	0.000	0.000	0.186	0.000	0.000
Sutterella	0.000	0.000	0.000	0.000	0.000
Alistipes	1.612	1.654	1.222	0.474	1.352
Rikenella	0.000	0.000	0.000	0.000	0.000
Parabacteroides	0.000	0.000	0.458	0.000	0.000
Odoribacter	0.000	0.000	0.175	0.000	0.000
Bacteroides	1.350	0.269	7.160	0.189	0.017
Lachnospiraceae Incertae Sedis	0.000	0.000	0.502	0.000	0.252
uncultured Lachnospiraceae	0.000	0.000	0.000	0.000	0.000
Ruminococcus	0.000	0.000	0.600	0.000	0.000
Ruminococcaceae Incertae Sedis	16.845	0.750	0.666	1.705	5.050
Oscillibacter	0.000	0.000	0.000	0.000	0.000
Anaerotruncus	1.229	0.000	0.000	0.000	0.000
Lactobacillus	1.592	1.885	2.107	1.350	4.725
Allobaculum	0.000	0.000	0.000	0.000	0.000
Bifidobacterium	0.000	0.000	0.120	0.000	0.000
Mucispirillum	4.614	0.096	0.142	3.883	4.084
Turicibacter	0.000	0.000	0.000	0.000	0.000
Clostridium	0.000	0.000	0.000	0.000	0.000
Coprococcus	0.000	0.000	0.000	0.000	0.000
Akkermansia	0.000	12.967	0.273	2.285	6.336
Brenneria-Yersinia	0.000	0.154	0.000	0.000	0.179

	HFD-EC T0	HFD-EC T1	HFD-EC T8	HFD-EC T10	HFD-EC caecal
Streptococcus	0.000	0.000	0.000	0.000	0.000
Prevotella	0.302	0.115	4.388	0.000	0.000
Marvinbryantia - Bryantella	0.000	0.000	0.000	0.000	0.000
Anaeroplasma	0.000	0.000	0.120	0.095	0.000
Lactococcus	0.181	1.077	2.434	1.989	0.773
Faecalibacterium	0.101	0.000	0.775	0.000	0.000
Psychrobacter	0.000	0.000	0.000	0.000	0.000
Vibrio	0.000	0.000	0.000	0.000	0.000
Thermus	0.000	0.000	0.000	0.000	0.000
Staphylococcus	0.000	0.000	0.000	0.000	0.000
Pseudoalteromonas	0.000	0.000	0.000	0.000	0.000
Leuconostoc	0.000	0.000	0.000	0.000	0.000
Anoxybacillus	0.000	0.000	0.000	0.000	0.000
Desulfovibrio	0.000	0.000	0.000	0.000	0.000
Paenibacillus	0.000	0.000	0.000	0.000	0.000
Lysinibacillus	0.000	0.000	0.000	0.000	0.000
Peptostreptococcaceae Incertae Sedis	0.000	0.000	0.240	0.284	0.335
Anaerovorax	0.000	0.308	0.000	0.000	0.000
Catenibacterium	0.000	0.000	0.000	0.000	0.000
Barnesiella	0.000	0.000	0.196	0.000	0.000
Blautia	0.000	0.000	0.098	0.000	0.000
Subdoligranulum	0.000	0.000	0.338	0.000	0.000
uncultured Ruminococcaceae	0.000	0.000	0.076	0.000	0.000
Phascolarctobacterium	0.000	0.000	0.218	0.000	0.000
Lachnospira	0.000	0.000	0.055	0.000	0.000
Megamonas	0.000	0.000	0.098	0.000	0.000
Weissella	0.000	0.000	0.000	0.000	0.017
Uncultured bacteria	0.201	0.846	3.515	2.687	1.018
Other	71.973	79.877	73.838	85.060	75.862

Supplementary Table 6 Alpha diversities

	Chao1	Simpson	Shannon	Phylogenetic Diversity	Observed Species
Low Fat Diet + MG1655					
BSH1					
T0	2557.2	0.989	8.170	60.124	1139
T1	740.1	0.920	5.100	21.554	394
T8	1030.4	0.923	5.258	23.891	425
caecal	626.3	0.924	5.089	19.430	371
caecal	240.9	0.689	2.627	10.407	156
High Fat Diet + MG1655					
BSH1					
T0	2911.7	0.989	8.239	68.437	1277
T1	1083.4	0.916	5.127	25.681	366
T8	657.3	0.885	4.639	19.864	343
caecal	695.3	0.824	4.449	18.077	304
caecal	517.7	0.860	4.416	19.014	318
caecal	1298.4	0.935	5.497	26.521	574
Low Fat Diet + MG1655					
BSH2					
T0	2841.5	0.901	6.670	62.629	1209
T1	665.0	0.905	4.822	16.715	335
T8	510.0	0.931	5.211	14.848	263
caecal	819.5	0.894	4.841	22.899	470
caecal	871.1	0.906	4.839	21.684	437
caecal	565.1	0.772	3.804	19.164	312
High Fat Diet + MG1655					
BSH2					
T0	2594.4	0.981	7.770	62.072	1213
T1	629.7	0.822	3.813	19.675	268
T8	776.1	0.933	5.432	22.954	420
caecal	839.7	0.929	5.248	19.144	394
caecal	731.4	0.869	4.690	24.455	370
caecal	719.7	0.953	5.820	24.319	426

Supplementary Table 7 Percentage read number high fat diet mice receiving *E. coli* MG1655 BSH1

	HFD ECBSH1 T0	HFD ECBSH1 T1	HFD ECBSH1 T8	HFD ECBSH1 Caecal
Phylum				
Proteobacteria	2.333	13.441	0.650	1.235
Bacteroidetes	61.173	48.418	54.100	55.195
Firmicutes	34.279	36.031	38.493	31.975
Actinobacteria	0.798	1.791	5.895	8.608
Deferribacteres	0.222	0.221	0.749	2.774
Candidate division TM7	0.133	0.000	0.000	0.000
4C0d-2	0.089	0.000	0.000	0.000
Verrucomicrobia	0.606	0.000	0.000	0.000
Deinococcus-Thermus	0.000	0.000	0.000	0.030
Other	0.369	0.098	0.113	0.183
Family				
Rhodospirillaceae	0.798	0.000	0.000	0.000
Alcaligenaceae	1.374	0.000	0.000	0.000
Rikenellaceae	12.509	0.294	0.848	6.273
Porphyromonadaceae	1.521	0.123	0.000	0.000
Bacteroidaceae	5.716	0.883	43.780	41.709
Lachnospiraceae	17.250	2.674	0.410	0.474
Eubacteriaceae	0.177	0.000	0.000	0.809
Ruminococcaceae	11.786	2.698	4.085	4.408
Peptococcaceae	0.428	0.000	0.000	0.000
Lactobacillaceae	1.521	0.172	0.141	8.012
Erysipelotrichales Incertae Sedis	0.502	7.849	0.184	0.417
Bifidobacteriaceae	0.650	0.123	5.697	8.138
Deferribacteraceae	0.222	0.221	0.749	2.774
Erysipelotrichaceae	0.694	12.436	15.196	15.955
Coriobacterineae	0.133	1.619	0.198	0.461
Clostridiaceae	0.118	0.000	0.000	0.000
Verrucomicrobiaceae	0.606	0.000	0.000	0.000
Enterobacteriaceae	0.103	12.975	0.551	1.087
Streptococcaceae	0.000	2.821	0.947	1.213

	HFD ECBSH1 T0	HFD ECBSH1 T1	HFD ECBSH1 T8	HFD ECBSH1 Caecal
Anaeroplasmataceae	0.000	0.000	0.000	0.000
Prevotellaceae	0.000	0.172	0.000	0.000
Moraxellaceae	0.000	0.147	0.071	0.052
Vibrionaceae	0.000	0.123	0.000	0.039
Thermaceae	0.000	0.000	0.000	0.030
Brevibacteriaceae	0.000	0.000	0.000	0.000
Staphylococcaceae	0.000	0.000	16.087	0.000
Pseudoalteromonadaceae	0.000	0.000	0.000	0.000
Leuconostocaceae	0.000	0.000	0.000	0.000
Bacillaceae	0.000	0.000	0.000	0.000
Desulfovibrionaceae	0.000	0.000	0.000	0.000
Peptostreptococcaceae	0.000	0.000	0.000	0.000
Planococcaceae	0.000	0.000	0.000	0.000
Paenibacillaceae	0.000	0.000	0.000	0.000
Veillonellaceae	0.000	0.000	0.000	0.000
Other	43.893	54.673	11.055	8.147
Genus				
Thalassospira	0.738	0.000	0.000	0.000
Sutterella	1.374	0.000	0.000	0.000
Alistipes	10.767	0.196	0.848	6.247
Rikenella	1.536	0.000	0.000	0.000
Parabacteroides	0.738	0.000	0.000	0.000
Odoribacter	0.783	0.000	0.000	0.000
Bacteroides	5.716	0.883	43.780	41.709
Lachnospiraceae Incertae Sedis	3.161	0.123	0.000	0.213
uncultured Lachnospiraceae	0.192	0.000	0.000	0.000
Ruminococcus	0.340	0.123	0.000	0.000
Ruminococcaceae Incertae Sedis	5.642	1.055	3.195	3.734
Oscillibacter	0.428	0.000	0.000	0.000
Anaerotruncus	0.177	0.000	0.608	0.230
Lactobacillus	1.521	0.172	0.141	8.012
Allobaculum	0.000	0.000	0.000	0.000
Bifidobacterium	0.650	0.123	5.697	8.138
Mucispirillum	0.222	0.221	0.749	2.774
Turicibacter	0.207	0.000	0.000	0.000

	HFD ECBSH1 T0	HFD ECBSH1 T1	HFD ECBSH1 T8	HFD ECBSH1 Caecal
Clostridium	0.089	0.000	0.000	0.000
Coprococcus	0.222	0.000	0.000	0.000
Akkermansia	0.606	0.000	0.000	0.000
Brenneria-Yersinia	0.103	12.902	0.551	1.078
Streptococcus	0.000	0.000	0.254	0.713
Prevotella	0.000	0.172	0.000	0.000
Marvinbryantia - Bryantella	0.000	0.000	0.000	0.000
Anaeroplasma	0.000	0.000	0.000	0.000
Lactococcus	0.000	2.821	0.664	0.500
Faecalibacterium	0.000	0.147	0.000	0.000
Psychrobacter	0.000	0.123	0.000	0.052
Vibrio	0.000	0.123	0.000	0.035
Thermus	0.000	0.000	0.000	0.030
Staphylococcus	0.000	0.000	16.087	0.000
Pseudoalteromonas	0.000	0.000	0.000	0.000
Leuconostoc	0.000	0.000	0.000	0.000
Anoxybacillus	0.000	0.000	0.000	0.000
Desulfovibrio	0.000	0.000	0.000	0.000
Paenibacillus	0.000	0.000	0.000	0.000
Lysinibacillus	0.000	0.000	0.000	0.000
Peptostreptococcaceae Incertae Sedis	0.000	0.000	0.000	0.000
Anaerovorax	0.000	0.000	0.000	0.000
Catenibacterium	0.000	0.000	0.000	0.000
Barnesiella	0.000	0.000	0.000	0.000
Blautia	0.000	0.000	0.000	0.000
Subdoligranulum	0.000	0.000	0.000	0.000
uncultured Ruminococcaceae	0.000	0.000	0.000	0.000
Phascolarctobacterium	0.000	0.000	0.000	0.000
Lachnospira	0.000	0.000	0.000	0.000
Megamonas	0.000	0.000	0.000	0.000
Weissella	0.000	0.000	0.000	0.000
Uncultured bacteria	0.354	0.417	0.226	0.204
Other	64.437	80.402	27.198	26.328

Supplementary Table 8 Percentage read number low fat diet mice receiving *E. coli* MG1655 BSH1

	LFD ECBSH1 T0	LFD ECBSH1 T1	LFD ECBSH1 T8	LFD ECBSH1 Caecal
Phylum				
Proteobacteria	3.191	9.683	0.902	1.101
Bacteroidetes	71.011	20.663	47.350	59.945
Firmicutes	24.460	46.590	28.712	26.905
Actinobacteria	0.478	3.841	21.657	7.643
Deferribacteres	0.268	1.209	1.214	3.967
Candidate division TM7	0.000	0.000	0.000	0.000
4C0d-2	0.000	0.000	0.000	0.000
Verrucomicrobia	0.000	0.000	0.000	0.000
Deinococcus-Thermus	0.000	17.871	0.000	0.108
Other	0.592	0.142	0.164	0.331
Family				
Rhodospirillaceae	2.580	0.000	0.000	0.000
Alcaligenaceae	0.554	0.053	0.000	0.000
Rikenellaceae	17.332	0.223	7.613	12.421
Porphyromonadaceae	6.363	0.080	0.000	0.000
Bacteroidaceae	5.924	1.024	27.482	39.523
Lachnospiraceae	15.020	2.021	1.247	0.972
Eubacteriaceae	0.191	0.525	0.919	1.246
Ruminococcaceae	6.115	3.793	2.789	6.248
Peptococcaceae	0.325	0.000	0.000	0.000
Lactobacillaceae	0.936	18.378	0.000	0.341
Erysipelotrichales Incertae Sedis	0.306	0.321	0.000	0.042
Bifidobacteriaceae	0.440	0.000	21.559	7.461
Deferribacteraceae	0.268	1.211	1.214	3.980
Erysipelotrichaceae	0.325	15.493	22.888	16.658
Coriobacterineae	0.000	3.820	0.000	0.208
Clostridiaceae	0.000	0.080	0.000	0.000
Verrucomicrobiaceae	0.000	0.000	0.000	0.000
Enterobacteriaceae	0.000	9.518	0.771	1.039
Streptococcaceae	0.000	0.695	0.082	0.125
Anaeroplasmataceae	0.000	0.000	0.000	0.000
Prevotellaceae	0.000	0.000	0.000	0.000
Moraxellaceae	0.000	0.000	0.000	0.000

	LFD ECBSH1 T0	LFD ECBSH1 T1	LFD ECBSH1 T8	LFD ECBSH1 Caecal
Vibrionaceae	0.000	0.000	0.000	0.000
Thermaceae	0.000	17.897	0.000	0.108
Brevibacteriaceae	0.000	0.000	0.000	0.000
Staphylococcaceae	0.000	0.000	0.000	0.000
Pseudoalteromonadaceae	0.000	0.000	0.000	0.000
Leuconostocaceae	0.000	0.045	0.000	0.000
Bacillaceae	0.000	0.000	0.000	0.042
Desulfovibrionaceae	0.000	0.062	0.000	0.000
Peptostreptococcaceae	0.000	0.080	0.000	0.000
Planococcaceae	0.000	0.000	0.000	0.000
Paenibacillaceae	0.000	0.000	0.000	0.000
Veillonellaceae	0.000	0.000	0.000	0.000
Other	43.321	24.824	13.437	9.920
Genus				
Thalassospira	2.083	0.000	0.000	0.000
Sutterella	0.554	0.053	0.000	0.000
Alistipes	16.167	0.187	7.613	12.372
Rikenella	0.459	0.000	0.000	0.000
Parabacteroides	5.733	0.053	0.000	0.000
Odoribacter	0.631	0.000	0.000	0.000
Bacteroides	5.924	1.022	27.482	39.392
Lachnospiraceae Incertae Sedis	1.242	0.000	0.000	0.000
uncultured Lachnospiraceae	0.096	0.053	0.000	0.000
Ruminococcus	0.478	0.000	0.000	0.000
Ruminococcaceae Incertae Sedis	2.159	2.685	2.199	5.449
Oscillibacter	0.248	0.222	0.000	0.000
Anaerotruncus	0.325	0.000	0.394	0.389
Lactobacillus	0.936	18.352	0.000	0.340
Allobaculum	0.325	0.000	0.000	4.058
Bifidobacterium	0.440	0.000	21.559	7.436
Mucispirillum	0.268	1.209	1.214	3.967
Turicibacter	0.000	0.053	0.000	0.000
Clostridium	0.000	0.080	0.000	0.000
Coprococcus	0.000	0.000	0.000	0.000
Akkermansia	0.000	0.000	0.000	0.000
Brenneria-Yersinia	0.000	9.505	0.771	1.035

	LFD ECBSH1 T0	LFD ECBSH1 T1	LFD ECBSH1 T8	LFD ECBSH1 Caecal
Streptococcus	0.000	0.071	0.000	0.050
Prevotella	0.000	0.000	0.000	0.000
Marvinbryantia - Bryantella	0.000	0.000	0.000	0.000
Anaeroplasma	0.000	0.000	0.000	0.000
Lactococcus	0.000	0.622	0.082	0.050
Faecalibacterium	0.000	0.000	0.000	0.000
Psychrobacter	0.000	0.000	0.000	0.000
Vibrio	0.000	0.000	0.000	0.000
Thermus	0.000	17.871	0.000	0.108
Staphylococcus	0.000	0.000	0.000	0.000
Pseudoalteromonas	0.000	0.000	0.000	0.000
Leuconostoc	0.000	0.044	0.000	0.000
Anoxybacillus	0.000	0.000	0.000	0.041
Desulfovibrio	0.000	0.044	0.000	0.000
Paenibacillus	0.000	0.000	0.000	0.000
Lysinibacillus	0.000	0.000	0.000	0.000
Peptostreptococcaceae Incertae Sedis	0.000	0.000	0.000	0.000
Anaerovorax	0.000	0.000	0.000	0.000
Catenibacterium	0.000	0.000	0.000	0.000
Barnesiella	0.000	0.000	0.000	0.000
Blautia	0.000	0.000	0.000	0.000
Subdoligranulum	0.000	0.000	0.000	0.000
uncultured Ruminococcaceae	0.000	0.000	0.000	0.000
Phascolarctobacterium	0.000	0.000	0.000	0.000
Lachnospira	0.000	0.000	0.000	0.000
Megamonas	0.000	0.000	0.000	0.000
Weissella	0.000	0.000	0.000	0.000
Uncultured bacteria	0.248	1.574	0.870	0.480
Other	61.685	46.297	37.818	24.834

Supplementary Table 9 Percentage read number low fat diet mice
receiving *E. coli* MG1655 BSH2

	LFD ECBSH2 T0	LFD ECBSH2 T1	LFD ECBSH2 T8	LFD ECBSH2 Caecal
Phylum				
Proteobacteria	0.636	13.312	0.981	1.146
Bacteroidetes	34.969	80.143	75.234	38.750
Firmicutes	63.230	4.572	18.536	50.287
Actinobacteria	0.589	1.672	4.688	7.479
Deferribacteres	0.106	0.060	0.467	2.195
Candidate division TM7	0.000	0.000	0.000	0.000
4C0d-2	0.000	0.000	0.000	0.000
Verrucomicrobia	0.000	0.000	0.000	0.000
Deinococcus-Thermus	0.000	0.181	0.000	0.000
Other	0.471	0.060	0.093	0.142
Family				
Rhodospirillaceae	0.341	0.000	0.000	0.000
Alcaligenaceae	0.224	0.000	0.000	0.000
Rikenellaceae	10.287	5.820	16.869	11.101
Porphyromonadaceae	0.824	0.000	0.000	0.000
Bacteroidaceae	3.060	74.192	54.159	25.003
Lachnospiraceae	17.538	0.151	1.760	0.341
Eubacteriaceae	0.000	0.000	0.000	0.793
Ruminococcaceae	11.452	0.211	4.097	4.203
Peptococcaceae	0.094	0.000	0.000	0.000
Lactobacillaceae	0.577	3.414	0.000	27.596
Erysipelotrichales Incertae Sedis	0.212	0.000	0.000	0.000
Bifidobacteriaceae	0.589	0.070	4.595	7.211
Deferribacteraceae	0.106	0.060	0.467	2.195
Erysipelotrichaceae	0.400	0.081	11.682	14.955
Coriobacterineae	0.000	1.591	0.093	0.268
Clostridiaceae	0.000	0.000	0.000	0.000
Verrucomicrobiaceae	0.000	0.000	0.000	0.000
Enterobacteriaceae	0.000	12.980	0.935	1.110
Streptococcaceae	31.356	0.564	0.125	1.512
Anaeroplasmataceae	0.000	0.000	0.000	0.000
Prevotellaceae	0.000	0.000	0.000	0.024
Moraxellaceae	0.000	0.141	0.000	0.000

	LFD ECBSH2 T0	LFD ECBSH2 T1	LFD ECBSH2 T8	LFD ECBSH2 Caecal
Vibrionaceae	0.000	0.081	0.000	0.000
Thermaceae	0.000	0.181	0.000	0.000
Brevibacteriaceae	0.000	0.000	0.000	0.000
Staphylococcaceae	0.000	0.000	0.000	0.000
Pseudoalteromonadaceae	0.000	0.000	0.000	0.000
Leuconostocaceae	0.000	0.000	0.000	0.000
Bacillaceae	0.000	0.000	0.000	0.106
Desulfovibrionaceae	0.000	0.000	0.000	0.000
Peptostreptococcaceae	0.000	0.000	0.000	0.000
Planococcaceae	0.000	0.000	0.000	0.000
Paenibacillaceae	0.000	0.000	0.000	0.000
Veillonellaceae	0.000	0.000	0.000	0.000
Other	22.940	0.463	5.218	3.581
Genus				
Thalassospira	0.235	0.000	0.000	0.000
Sutterella	0.224	0.000	0.000	0.000
Alistipes	9.911	5.800	16.869	11.097
Rikenella	0.259	0.000	0.000	0.000
Parabacteroides	0.129	0.000	0.000	0.000
Odoribacter	0.694	0.000	0.000	0.000
Bacteroides	3.060	74.192	54.159	25.003
Lachnospiraceae Incertae Sedis	1.271	0.000	0.000	0.000
uncultured Lachnospiraceae	0.071	0.000	0.000	0.000
Ruminococcus	0.282	0.000	0.000	0.000
Ruminococcaceae Incertae Sedis	2.519	0.141	3.302	3.341
Oscillibacter	0.435	0.000	0.000	0.000
Anaerotruncus	0.259	0.000	0.343	0.305
Lactobacillus	0.565	3.414	0.000	27.588
Allobaculum	0.400	0.081	0.000	1.512
Bifidobacterium	0.589	0.070	4.595	7.211
Mucispirillum	0.106	0.060	0.467	2.195
Turicibacter	0.000	0.000	0.000	0.000
Clostridium	0.000	0.000	0.000	0.000
Coprococcus	0.129	0.000	0.000	0.000
Akkermansia	0.000	0.000	0.000	0.000
Brenneria-Yersinia	0.000	12.969	0.935	1.106

	LFD ECBSH2 T0	LFD ECBSH2 T1	LFD ECBSH2 T8	LFD ECBSH2 Caecal
Streptococcus	31.321	0.091	0.000	1.427
Prevotella	0.000	0.000	0.000	0.024
Marvinbryantia - Bryantella	0.000	0.000	0.000	0.000
Anaeroplasma	0.000	0.000	0.000	0.000
Lactococcus	0.000	0.443	0.125	0.041
Faecalibacterium	0.000	0.000	0.000	0.000
Psychrobacter	0.000	0.141	0.000	0.000
Vibrio	0.000	0.060	0.000	0.000
Thermus	0.000	0.181	0.000	0.000
Staphylococcus	0.000	0.000	0.000	0.000
Pseudoalteromonas	0.000	0.000	0.000	0.000
Leuconostoc	0.000	0.000	0.000	0.000
Anoxybacillus	0.000	0.000	0.000	0.081
Desulfovibrio	0.000	0.000	0.000	0.000
Paenibacillus	0.000	0.000	0.000	0.000
Lysinibacillus	0.000	0.000	0.000	0.000
Peptostreptococcaceae Incertae Sedis	0.000	0.000	0.000	0.000
Anaerovorax	0.000	0.000	0.000	0.000
Catenibacterium	0.000	0.000	0.000	0.000
Barnesiella	0.000	0.000	0.000	0.000
Blautia	0.000	0.000	0.000	0.000
Subdoligranulum	0.000	0.000	0.000	0.000
uncultured Ruminococcaceae	0.000	0.000	0.000	0.000
Phascolarctobacterium	0.000	0.000	0.000	0.000
Lachnospira	0.000	0.000	0.000	0.000
Megamonas	0.000	0.000	0.000	0.000
Weissella	0.000	0.000	0.000	0.000
Uncultured bacteria	0.494	0.000	0.109	0.183
Other	47.046	2.356	19.097	18.885

Supplementary Table 10 Percentage read number high fat diet mice receiving *E. coli* MG1655 BSH2

	HFD ECBSH2 T0	HFD ECBSH2 T1	HFD ECBSH2 T8	HFD ECBSH2 Caecal
Phylum				
Proteobacteria	2.251	2.144	31.723	2.617
Bacteroidetes	53.133	53.960	30.498	36.468
Firmicutes	43.414	43.001	31.874	41.995
Actinobacteria	0.379	0.227	4.462	13.848
Deferribacteres	0.065	0.000	0.436	4.651
Candidate division TM7	0.336	0.000	0.000	0.000
4C0d-2	0.000	0.000	0.000	0.000
Verrucomicrobia	0.065	0.000	0.000	0.000
Deinococcus-Thermus	0.000	0.580	0.000	0.000
Other	0.357	0.088	1.007	0.420
Family				
Rhodospirillaceae	1.948	0.000	0.000	0.000
Alcaligenaceae	0.206	0.303	0.000	0.000
Rikenellaceae	17.913	0.113	2.617	6.130
Porphyromonadaceae	1.764	0.000	0.000	0.037
Bacteroidaceae	6.397	53.014	21.758	25.207
Lachnospiraceae	17.772	0.315	0.503	2.386
Eubacteriaceae	0.000	0.000	0.000	0.000
Ruminococcaceae	11.311	0.517	1.258	5.740
Peptococcaceae	0.130	0.000	0.000	0.000
Lactobacillaceae	1.526	21.803	0.000	1.637
Erysipelotrichales Incertae Sedis	0.000	0.227	0.000	0.037
Bifidobacteriaceae	0.379	0.000	1.745	12.856
Deferribacteraceae	0.065	0.000	0.436	4.651
Erysipelotrichaceae	0.260	8.020	24.677	30.089
Coriobacterineae	0.000	0.139	0.000	0.980
Clostridiaceae	0.000	0.000	0.000	0.000
Verrucomicrobiaceae	0.065	0.000	0.000	0.000
Enterobacteriaceae	0.000	1.652	0.235	2.435
Streptococcaceae	10.943	11.349	3.389	0.895
Anaeroplasmataceae	0.065	0.000	0.000	0.000
Prevotellaceae	0.054	0.000	0.000	0.000
Moraxellaceae	0.000	0.088	17.245	0.067

	HFD ECBSH2 T0	HFD ECBSH2 T1	HFD ECBSH2 T8	HFD ECBSH2 Caecal
Vibrionaceae	0.000	0.063	11.206	0.000
Thermaceae	0.000	0.580	0.000	0.000
Brevibacteriaceae	0.000	0.000	0.352	0.000
Staphylococcaceae	0.000	0.000	0.000	0.000
Pseudoalteromonadaceae	0.000	0.000	2.869	0.000
Leuconostocaceae	0.000	0.000	0.252	0.134
Bacillaceae	0.000	0.000	0.000	0.000
Desulfovibrionaceae	0.000	0.000	0.000	0.000
Peptostreptococcaceae	0.000	0.000	0.000	0.000
Planococcaceae	0.000	0.000	0.000	0.000
Paenibacillaceae	0.000	0.000	0.000	0.000
Veillonellaceae	0.000	0.000	0.000	0.000
Other	29.202	1.816	11.458	6.720
Genus				
Thalassospira	1.537	0.000	0.000	0.000
Sutterella	0.206	0.303	0.000	0.000
Alistipes	17.296	0.088	2.583	6.075
Rikenella	0.487	0.000	0.000	0.000
Parabacteroides	0.216	0.000	0.000	0.000
Odoribacter	1.548	0.000	0.000	0.000
Bacteroides	6.397	53.014	21.758	25.207
Lachnospiraceae Incertae Sedis	1.223	0.000	0.151	1.017
uncultured Lachnospiraceae	0.206	0.000	0.000	0.000
Ruminococcus	0.541	0.000	0.000	0.000
Ruminococcaceae Incertae Sedis	3.561	0.277	0.000	4.937
Oscillibacter	0.303	0.000	0.000	0.000
Anaerotruncus	0.346	0.000	0.101	0.110
Lactobacillus	1.526	21.765	0.000	1.637
Allobaculum	0.260	0.000	0.000	0.000
Bifidobacterium	0.379	0.000	1.745	12.856
Mucispirillum	0.065	0.000	0.436	4.651
Turicibacter	0.000	0.000	0.000	0.000
Clostridium	0.000	0.000	0.000	0.000
Coprococcus	0.184	0.000	0.000	0.000
Akkermansia	0.065	0.000	0.000	0.000
Brenneria-Yersinia	0.000	1.652	0.201	2.423

	HFD ECBSH2 T0	HFD ECBSH2 T1	HFD ECBSH2 T8	HFD ECBSH2 Caecal
Streptococcus	10.943	10.668	0.000	0.365
Prevotella	0.054	0.000	0.000	0.000
Marvinbryantia - Bryantella	0.108	0.000	0.000	0.000
Anaeroplasma	0.065	0.000	0.000	0.000
Lactococcus	0.000	0.681	3.355	0.481
Faecalibacterium	0.000	0.000	0.000	0.037
Psychrobacter	0.000	0.088	17.245	0.055
Vibrio	0.000	0.063	10.552	0.000
Thermus	0.000	0.580	0.000	0.000
Staphylococcus	0.000	0.000	0.000	0.000
Pseudoalteromonas	0.000	0.000	2.869	0.000
Leuconostoc	0.000	0.000	0.252	0.134
Anoxybacillus	0.000	0.000	0.000	0.000
Desulfovibrio	0.000	0.000	0.000	0.000
Paenibacillus	0.000	0.000	0.000	0.000
Lysinibacillus	0.000	0.000	0.000	0.000
Peptostreptococcaceae Incertae Sedis	0.000	0.000	0.000	0.000
Anaerovorax	0.000	0.000	0.000	0.000
Catenibacterium	0.000	0.000	0.000	0.000
Barnesiella	0.000	0.000	0.000	0.000
Blautia	0.000	0.000	0.000	0.000
Subdoligranulum	0.000	0.000	0.000	0.000
uncultured Ruminococcaceae	0.000	0.000	0.000	0.000
Phascolarctobacterium	0.000	0.000	0.000	0.000
Lachnospira	0.000	0.000	0.000	0.000
Megamonas	0.000	0.000	0.000	0.000
Weissella	0.000	0.000	0.000	0.000
Uncultured bacteria	0.660	0.895	0.151	0.280
Other	51.824	9.924	38.601	39.737

Supplementary Figure 1

Rarefaction curve for each group at 97% similarity levels. Amount of operational taxonomic units (OTU's) found as a function of the number of sequence tags sampled.
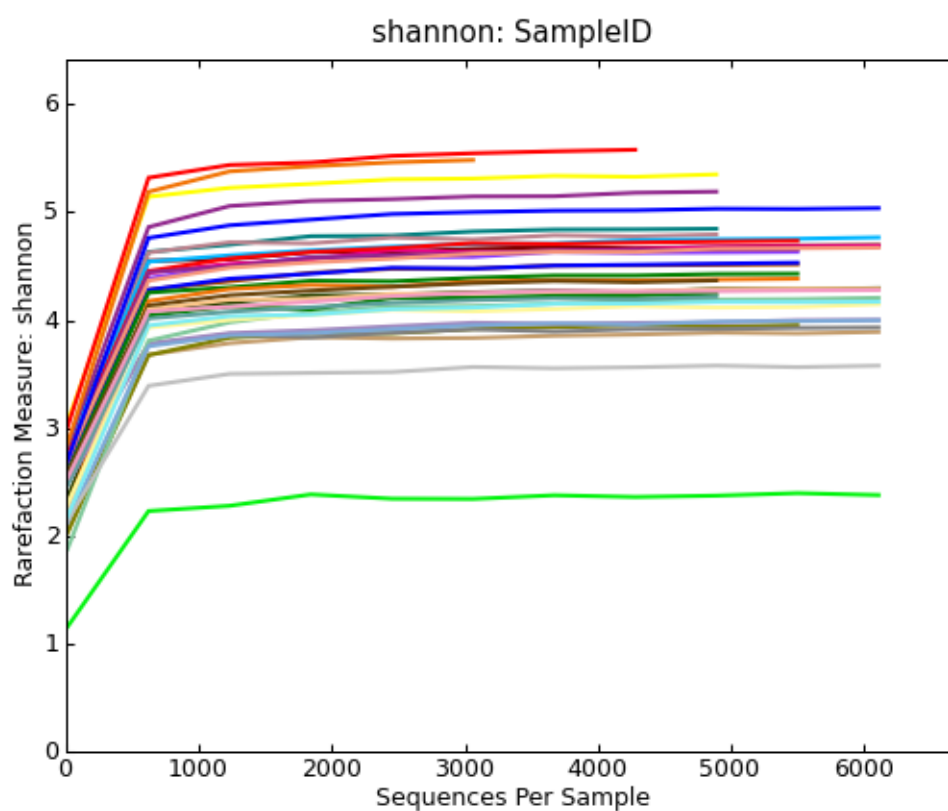
Supplementary Figure 2

Bar graph of α diversity of faecal gut microbiota within each group over time.

Colour codes blue, LFD fed mice; red, HFD fed mice; green, HFD fed mice receiving 1x10^7 cfu StrR GMR *E. coli* MG1655 with no bile salt hydrolase insert.

Supplementary Figure 3

Bar graph of α diversity of caecum microbiota within each group.

Colour codes blue, LFD fed mice; red, HFD fed mice; green, HFD fed mice receiving 1x10^7 cfu StrR GMR *E. coli* MG1655 with no bile salt hydrolase insert. Statistical significance was determined using a two-tailed unpaired t test; p value ≤0.05*; ≤0.01**.
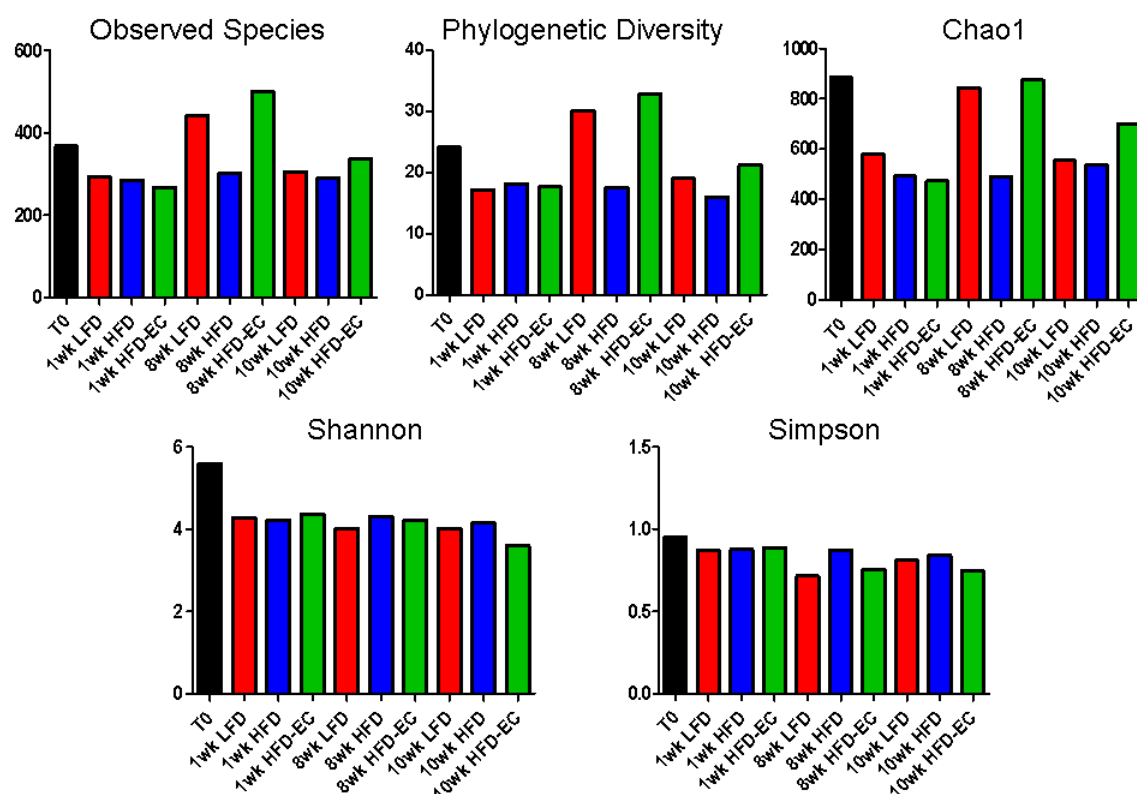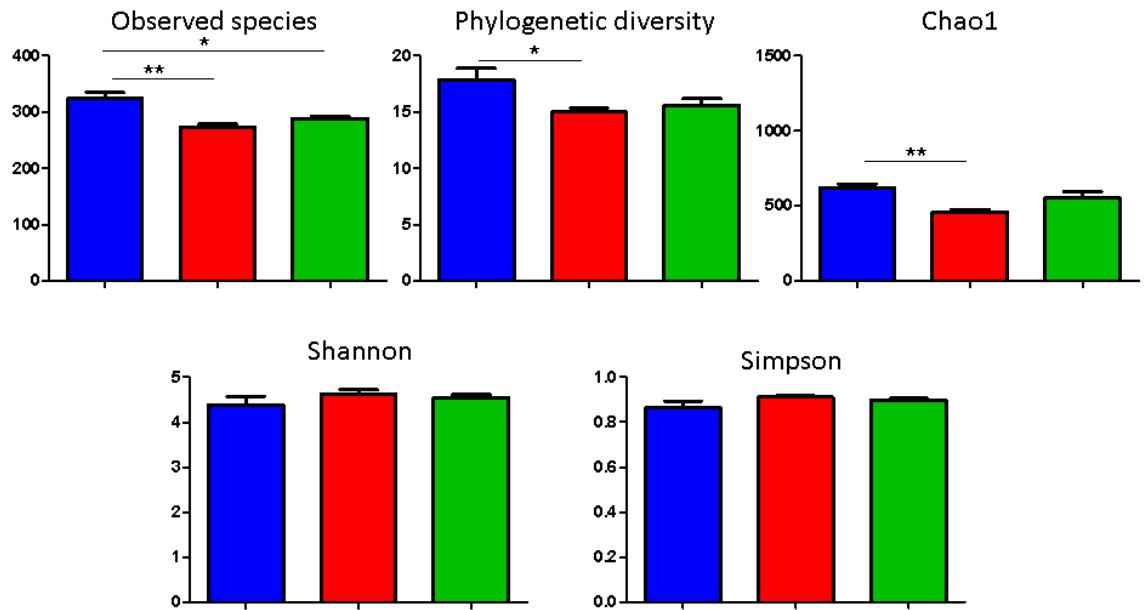
Supplementary Figure 4

Rarefaction curve for each group at 97% similarity levels. Amount of operational taxonomic units (OTU's) found as a function of the number of sequence tags sampled.
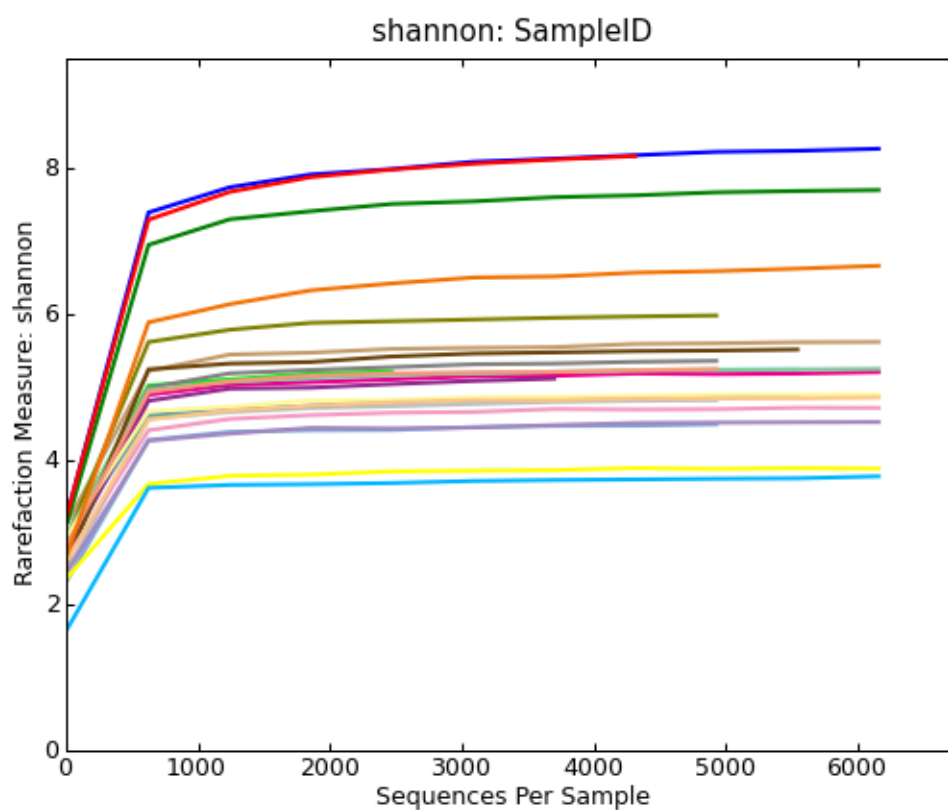
Supplementary Figure 5

Bar graph of α diversity of caecum microbiota within each diet group.

Colour codes; LFD ECBSH1, yellow; HFD ECBSH1, blue; LFD ECBSH2, red; HFD ECBSH2, green.
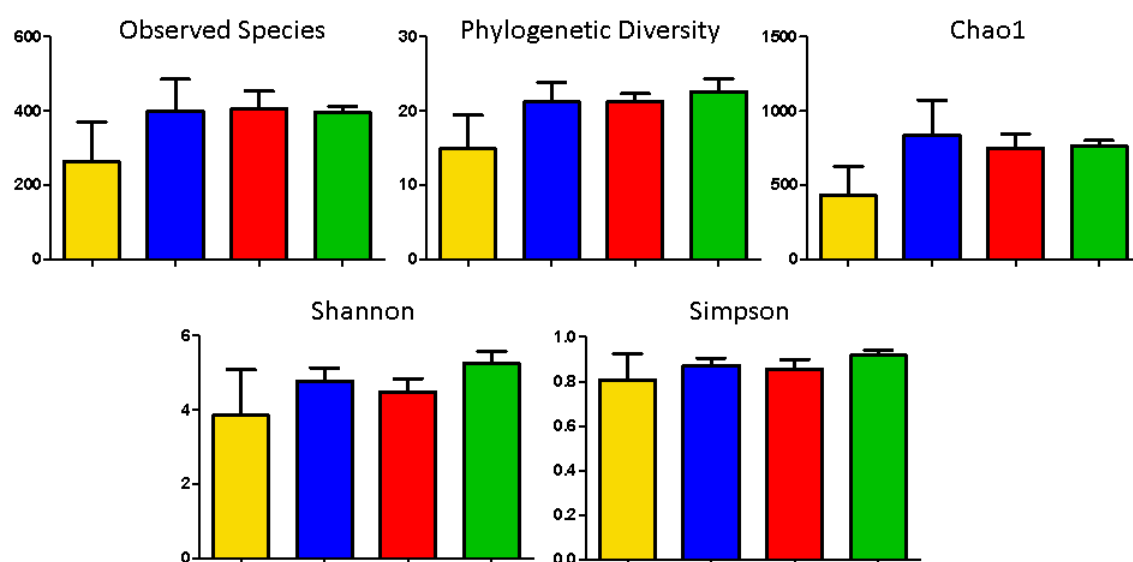
Supplementary Figure 6

Bar graph of alpha diversity from faecal gut microbiota within each group over time.

Colour codes; LFD ECBSH1, yellow; HFD ECBSH1, blue; LFD ECBSH2, red; HFD ECBSH2, green.
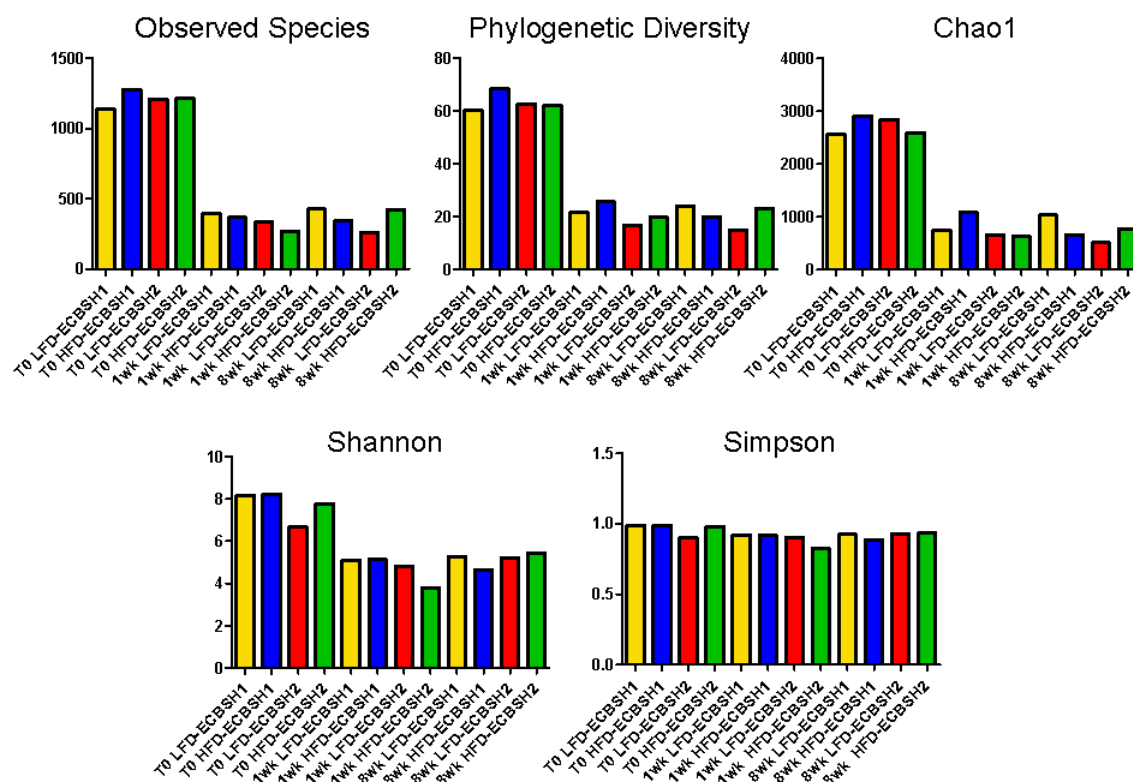
Supplementary Figure 7

Principal coordinate analysis plot of unweighted Unifrac colour coded by diet group. Colour codes, LFD ECBSH1, yellow; HFD ECBSH1, blue; LFD ECBSH2, red; HFD ECBSH2, green. Faceal time points highlighted in image. Cecum data point unlabelled.
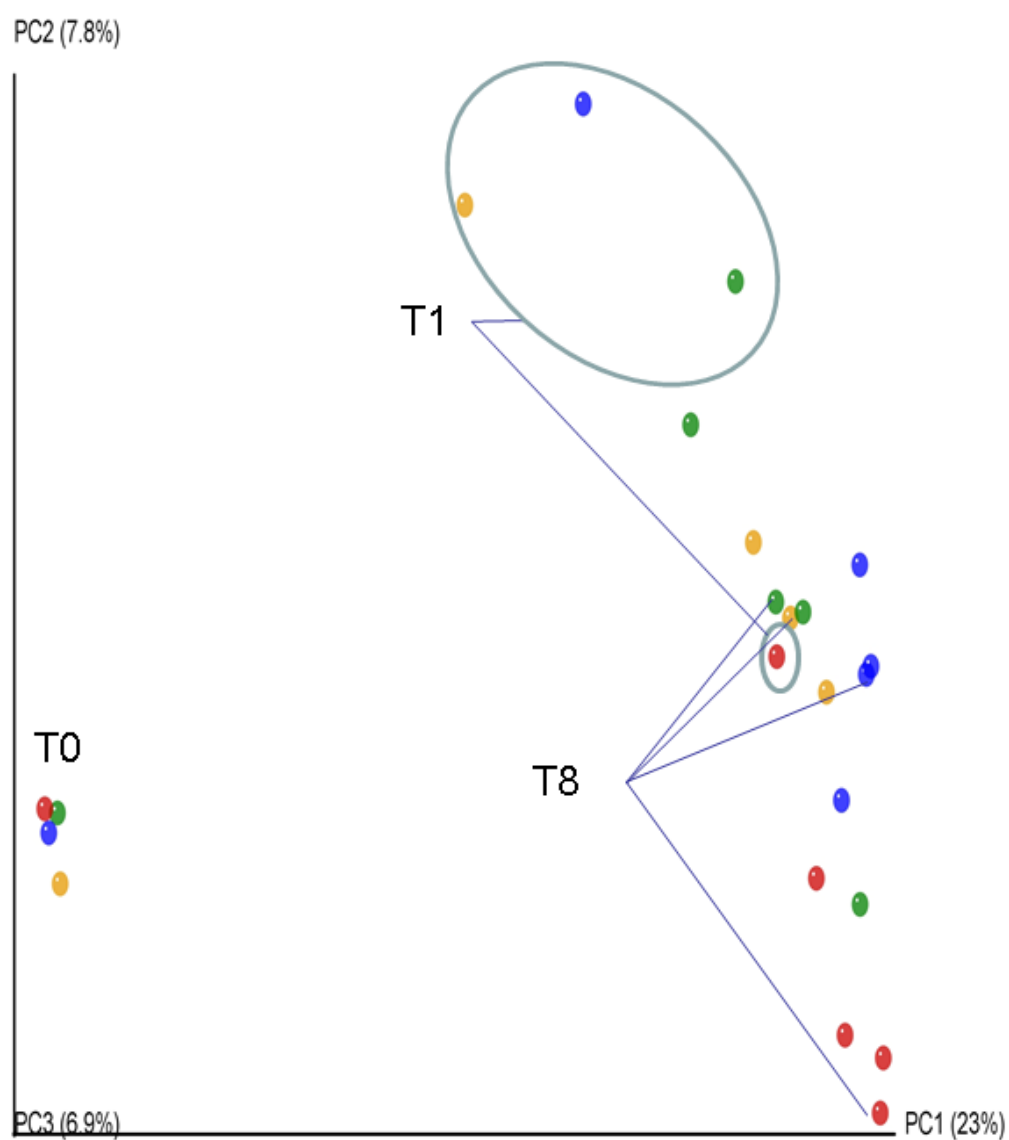
Chapter 5

High fat feeding of mice alters their gut microbiota and protects against colitis and colitis-associated colorectal cancer

Siobhan Clarke Chapter Contributions:

Experimental:

- Designed and performed all experiments relating to the extraction and purification of DNA from faecal pellets.
- Generated amplicons for 454 pyrosequencing.

Results interpretation:

- Analysed all data in relation to 16S compositional sequencing analysis.
- Compiled all graphical interpretations relating to 16S compositional sequencing analysis.

Manuscript preparation:

Major contributor to manuscript preparation.

5.1   ABSTRACT

Environmental factors such as diet and gut microbiota composition, together with immune dysregulation are thought to trigger inflammatory bowel diseases (IBD) in genetically susceptible individuals. Long term chronic inflammation increases the risk of IBD patients developing colitis-associated colorectal cancer (CAC). This study aimed to assess the effect of diet on colitis, CAC progression and microbiota composition. Mice were fed high fat (HF-45% Kcal from fat) or low fat (LF-10% Kcal from fat) diet, followed by one azoxymethane (AOM) injection and 3x Dextran Sodium Sulphate (DSS) cycles (1.5%DSS-5days & water-14days) or 3xDSS cycles alone. HF-feeding protected mice from developing colitis and CAC on the basis of weight and cytokine profile, tumour numbers and incidence and colon length and weight. Analysis of the murine gut microbiota showed a reduction in gut microbiota diversity in LF-colitis and LF-CAC mice relative to their HF-fed counterparts. This data reveals that a HF-diet provides a protective effect against colitis and CAC progression and is accompanied by alterations in gut microbiota populations. These results highlight a far more complex interaction between high fat diet on microbiota-host responses in these models warranting further investigations in patients with IBD and CAC.

5.2   INTRODUCTION

The human gut microbiota is a large and diverse population of microorganisms, the activities of which contribute to health as well as disease. Disruption of the normal gut microbiota is associated with a number of diseases including Inflammatory Bowel Diseases (IBD) (Ott *et al.*, 2004, Manichanh *et al.*, 2006), diabetes (Qin *et al.*, 2012), obesity (Flint, 2011), Irritable Bowel Syndrome (IBS) (Carroll *et al.*, 2011), autism (Kang *et al.*, 2013), Alzheimer's disease (Bhattacharjee and Lukiw, 2013) and colorectal cancer (CRC) (Sobhani *et al.*, 2011), although the quality of evidence varies considerably. Ulcerative colitis (UC) and Crohn's disease (CD) are two major phenotypes of IBD. In UC the mucosal lining of the colon and the rectum are primarily affected while in CD any part of the gastrointestinal tract can be affected (Podolsky, 2002). It has been suggested that an excessive immune response to gut microbiota-associated antigens is the main trigger of inflammation and the subsequent mucosal tissue damage in both diseases (Sartor, 2006). Furthermore, individuals with IBD have an increased risk of developing colon cancer (Jess *et al.*, 2006, Bernstein *et al.*, 2001), particularly colitis associated cancer (CAC) (Grivennikov, 2013, Danese *et al.*, 2011). CAC is a form of CRC that is preceded by IBD-like UC and CD (Grivennikov, 2013). CRC is the third most common cancer and second leading cancer killer in the United States (CDC, 2009). It has also been revealed that the risk of CRC increases by 7% as body mass index (BMI) increases by 2 (Calle and Kaaks, 2004). Diets low in fruit and vegetables and high in red meat have previously been associated with an increased risk of CRC (Mai *et al.*, 2007, Giovannucci *et al.*, 1994, Willett *et al.*, 1990, Potter, 1996), while the high fat 'western diet' has been associated with the development of CRC in mouse models (Mai *et al.*, 2007, Singh *et al.*, 1997, Newmark *et al.*, 2001). Diet-induced obesity in the mouse model of CAC (chemically induced using AOM and DSS) has also been found to increases the number colonic tumours (Flores *et al.*, 2012, Kim *et al.*, 2010, Park *et al.*, 2012). AOM is a metabolite of dimethylhydrazine (DMH) that induces colon tumours while DSS has been suggested to exert toxic

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effects on colonic epithelium creating a chronic inflammatory state (Robertis *et al.*, 2011, Thaker *et al.*, 2012). When combined, these chemicals shorten the latency time for the induction of CAC, resulting in the rapid growth of colonic tumours (Thaker *et al.*, 2012). In a recent study, feeding of a 'western style diet', where 37% of calories were from saturated milk derived fat, to IL-10$^{-/-}$ mice resulted in an increased onset and severity of colitis relative to controls fed a polyunsaturated (safflower oil) diet or low fat (5% Kcal from lard) diet (Devkota *et al.*, 2012). Examinations of the effect of high fat diets in other experimental models of colitis and CAC have provided conflicting outcomes, showing either no effect, protection or worsening of disease (Hyland *et al.*, 2009, Gäbele *et al.*, 2011, Park *et al.*, 2012)

A number of other dietary components have been shown to have an impact on the incidence of CAC. For example, high fat rice bran oil-based, as well as omega 3 fatty acids, diets have been associated with reducing the risk of CAC (Shih *et al.*, 2011, Nowak *et al.*, 2007, Jia *et al.*, 2008) while nobiletin, a polymethoxylated flavonoid in citrus fruit peels, has been shown to reduce colon tumour development (Miyamoto *et al.*, 2008). Studies into CRC have also noted that consumption of probiotics have an inhibitory effect on the development of colon cancer in animal models (Brady *et al.*, 2000, Saikali *et al.*, 2004, Appleyard *et al.*, 2011).

The role of microbiota composition in colitis has been the subject of much attention since the reports that germ free mice do not develop colitis (Sellon *et al.*, 1998). Antibiotic treatment can also alleviate colitis-associated symptoms in animal models of colitis (Hoentjen *et al.*, 2003) and in humans, especially in a sub group of patients with CD (Nomura *et al.*, 2005). With respect to CAC specifically, it has been shown that infection with *Helicobacter* spp. can result in CAC development (Chichlowski *et al.*, 2008, Fox *et al.*, 1999) and that antibiotic treatment to remove this bacterium prevented CAC development (Chichlowski *et al.*, 2008). Advances in next generation sequencing technology have allowed researchers to gain an even better insight into the altered gut microbiota populations that are associated with CD, UC and CRC. Increased proportions of Proteobacteria have consistently been noted in UC, CD

and CRC (Gophna *et al.*, 2006, Frank *et al.*, 2011, Lepage *et al.*, 2011, Arthur *et al.*, 2012, Wang *et al.*, 2012). While reductions in abundance of the bacterium *Faecalibacterium prausnitzii* (phylum Firmicutes) has been observed in patients with CD (Sokol 2008, wiling 2008) and UC (Sokol 2009), this association has been challenged by others (Hansen 2012). Similarly, although it has been reported that *Bacteroides/Prevotella* are enriched in the gut microbiota of CRC patients (Sobhani *et al.*, 2011), others have reported the opposite (Kostic *et al.*, 2012, Wang *et al.*, 2012). Genomic analysis of the microbiome of CRC patients revealed the enrichment of the bacterium *Fusobacterium (Kostic et al., 2012)* with the presence of *Fusobacterium nucleatum* accelerating the onset of colonic tumors in Apc$^{min-/-}$ mice (Kostic *et al.*, 2013). However this association was not seen in CAC models (Kostic *et al.*, 2013).

Here, we use Dextran Sodium Sulphate (DSS, a model of colitis) and Azoxymethane/DSS (AOM/DSS, a model of CAC) treated mice in receipt of a high fat (45% calories from fat) or low fat (10% of calories from fat) diets, in order to better understand the relationship between diet, the gut microbiota, colitis and CAC progression.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Male C57BL/6OlaHsD mice, 5-9 weeks old and weighing 20-22 g, were obtained from Harlan, UK. The mice were housed in a semi-conventional environment (temperature 21°C, 12h light: 12h darkness, humidity 50%) in a dedicated animal holding facility. All mice were provided with water *ad libitum* and were fed a low fat diet (LF, 10% Kcal from fat, Research Diets, New Jersey, USA; #D12450B) for 2 wk to acclimatize before entering the study. Animal husbandry and experimental procedures were approved by the UCC Animal Ethics Committee and experimental procedures were conducted under licence from the Irish government.

5.3.2 Induction of colitis and colitis-associated colorectal cancer and diet

Induction of colitis and CAC was performed as described with minor changes (Wirtz *et al.*, 2007). After acclimatization, mice were randomly divided in groups of 8-12 and received a high fat diet (HF, 45% Kcal from fat (lard), Research Diets, New Jersey, USA; #D12451) or LF for 4-5 weeks. After this period, the mice were further divided into a total of 6 groups i.e. LF-Control (Ctr), LF-colitis (3x DSS cycle), LF-CAC (AOM/3x DSS cycle), HF-Ctr, HF-colitis and HF-CAC. The CAC groups were injected intraperionatally (i.p.) once with 8mg AOM (Sigma, Aldrich, UK), and rested for 1 week after which the CAC and Colitis groups were administered 1.5% DSS (w/v, TdB Consultancy, Uppsala, Sweden) in their drinking water for 5 days, followed by 14 days of DSS-free drinking water. This DSS-cycle was repeated a total of 3 times and the mice were culled thereafter (Fig 1). Food intake, weight and general health condition was monitored once per week.

5.3.3 Weights and tissue sampling

For microbiota composition analysis, faecal samples were collected on 3 occasions; 1) immediately before DSS treatment started in the CAC and colitis groups (i.e. week 12), 2) 1 week after the first DSS treatment (i.e.

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week 14) and 3) at the end of the study (i.e. week 21). Faecal samples were collected from individual mice at the specified time point and stored at -80°C until processing. On the day on which the mice were to be sacrificed, they were first subjected to whole body composition using a Bruker MRI Body composition analyser to measure fat and lean body mass. Mice were then sedated under isoflurane anaesthesia and blood collected in EDTA-containing tubes, centrifuged, serum aliquoted, frozen and kept at -80°C until analysis. Mice were sacrificed and the cecum was dissected, weighed and the contents frozen in liquid nitrogen until processed for high-throughput sequencing. The colon was dissected and its length and weight measured. The distal colon was divided into tumour bearing (tumour+) and non-tumour bearing (tumour-) tissues. A piece (1-2cm) of distal tumour+ was rolled as "Swiss rolls" (Moolenbeek and Ruitenberg, 1981), embedded in OCT and frozen in liquid nitrogen. The rest of the colonic tissue (tumour-) was frozen in liquid nitrogen, and stored at -80°C until processing.

5.3.4 Cytokine analysis

Plasma serum and colon homogenates were assayed for the cytokines IFN-γ, TNF-α, IL-1β, IL-6, IL-10, IL-12p70, keratinocyte chemoattractant [KC] using the 7-proinflammatory-plex kit (MesoScale Discovery, USA) and IL-17 were examined using ELISA (R&D Systems, Minnesota, USA). Colons were homogenised as described previously (Murphy *et al.*, 2010). All assays were performed according to the manufacturers' instructions. Colonic cytokine levels are expressed as pg cytokine/100mg colonic tissue.

5.3.5 DNA extraction and 16S rRNA gene amplicon sequencing

DNA was extracted from faecal and cecum samples using the QIAmp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK) combined with an additional bead-beating step (30s x 3) and stored at -20°C. The microbiota composition of the samples was established by high-throughput DNA sequencing of 16S rRNA amplicons generated using the universal 16S rRNA primers (i.e. the forward primer F1 (5'-

AYTGGGYDTAAAGNG) and reverse primer V5 (5'-CCGTCAATTYYTTTRAGTTT) (RDP'S Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp). Molecular identifier tags were attached between the 454 adaptor sequence and the target-specific primer sequence, allowing for identification of individual sequences from the pooled amplicons. Ampure purification system (Beckman Coulter, Takeley, UK) was used to clean the amplicons before being sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) in line with 454 protocols at the Teagasc high throughput sequencing centre.

5.3.6 Bioinformatic analysis of 16s rRNA sequence data

A locally installed RDP pyrosequencing pipeline was used to quality trim the raw sequence data. Reads were removed that were shorter than the main distribution (150bp for the 16S rRNA V4 region), of low quality or not exact matches to barcoded tags and primer sequence. A locally installed version of SILVA 16S rRNA database (Pruesse *et al.*, 2007) was used to BLAST (Altschul *et al.*, 1997) the trimmed fasta sequence files using default parameters. Resulting BLAST output files were parsed through MEGAN (Huson *et al.*, 2007) which uses a lowest common ancestor algorithm to assign reads to NCBI taxonomies. Prior to tree construction and summarization filtering was carried out within MEGAN using bit scores, similar to previous studies a bit-score cut-off of 86 was selected (Urich *et al.*, 2008, Rea *et al.*, 2011). Alpha diversity indices were generated using MOTHUR software (Schloss *et al.*, 2009). Clustering of sequence reads into operational taxonomical units (OTUs) was achieved using QIIME suite software tools (Caporaso *et al.*, 2010). The ChimeraSlayer program was used to remove chimeric OTUs from aligned OTUs (Haas *et al.*, 2011) and the FastTreeMP tool generated a phylogenetic tree (Price *et al.*, 2010). Beta diversities were also determined based on weighted and unweighted Unifrac and bray curtis distances. Principal coordinate analysis (PCoA) was performed on the samples. PCoA plots were visualised with KiNG viewer (Chen *et al.*, 2009). The nonparametric Kruskal-Wallis test (Kruskal and Wallis, 1952)

in the Minitab statistical package was employed to establish statistical significance (significance taken to be $p \leq 0.05$).

5.3.7 Statistics

All statistical tests were performed using commercially available statistic software (GraphPad Software, CA, USA) unless otherwise stated. Statistical significance was determined with one-way analysis of variance (ANOVA) with post-hoc analysis and the unpaired Student's t-test. Data are presented as Mean ±SEM. A p value of <0.05 was considered significant.

5.4 RESULTS

5.4.1 High fat feeding protects mice from inflammation of tumour development

Mice were randomised and fed LF and HF diets for 4-5 weeks, with mice that consumed a HF diet gaining more weight than LF-mice i.e. 45 to 51% body weight increase from start of feeding in HF-groups compared 20 to 25% increase in body weight in LF-groups (Fig 2). Injection of AOM did not affect weight gain in either LF or HF-fed mice. Mice in the LF-CAC group (treated with AOM and one DSS cycle and thus predicted to develop CAC) lost 14.5% body weight and mice with LF-colitis group (treated with one DSS cycle and thus predicted to develop colitis) lost 17.8% body weight in line with previous reports (Thaker *et al.*, 2012). In contrast, mice fed a HF diet lost significantly less weight ($p<0.05$; Fig 2), i.e. 3.9% lost in HF-CAC mice and 4.6% lost in HF-colitis animals. Similar body weight changes were observed after the 2nd and 3rd cycle of DSS-treatment with a significant difference in the final body weight at W21 in the HF-CAC and HF-colitis groups compared to the LF-CAC and LF-colitis equivalents (Fig 2). Reductions in colon length and increased colon weight are indicative of inflammation and were apparent in LF-colitis ($p<0.05$) and LF-CAC ($p<0.05$ and $p<0.001$ respectively), when compared to LF-Ctr, mice (Fig 3). HF-CAC mice had significantly longer intestines ($p<0.01$) and lower colon weights ($p<0.01$) than LF-CAC mice (Fig 3). A trend was observed in the intestinal length and a significant difference in colon weight ($p<0.05$) of the HF-colitis group when compared with those of LF-colitis mice. As noted above, combined AOM/DSS treatment is known to result in accelerated tumour development (Robertis *et al.*, 2011, Rosenberg *et al.*, 2009). Here, we observed that mice fed LF and treated with AOM/DSS (LF-CAC) presented on average with 6.75 tumours per mouse. The corresponding HF diet fed mice developed significantly fewer tumours ($p<0.01$), i.e. on average 2.7 per mouse (Fig 4). In addition to the reduced number of

tumours, the size of the tumours was, on the basis of visual observation (Fig.4), smaller in the HF-CAC mice.

5.4.2 High fat diet reduces plasma and colonic cytokines in colitis and CAC model animals

We also examined the effect of the LF- and HF-diet on the local and mucosal inflammatory response in mice with colitis and CAC. IL-1β ($p<0.05$), IFNγ ($p<0.05$) and IL-12 ($p<0.001$) levels were significantly higher in LF-colitis, compared to LF-Ctr, mice. The increase in IL-1β ($p<0.001$) was sustained in LF-CAC relative to LF-Ctr mice and a significant increase in IL-4 ($p<0.001$) levels was also apparent (Fig 5). IL-1β ($p<0.001$) was also significantly increased in HF-CAC, relative to HF-Ctr mice, with significantly greater levels of IL-2 ($p<0.05$) and IL-10 ($p<0.05$) also being apparent in the former group. In contrast to the LF groups, no significant differences in plasma cytokines were detected when HF-colitis and HF-Ctr groups were compared. Comparisons between HF and LF colitis and CAC data revealed that plasma IL-1β ($p<0.05$), IL-12 ($p<0.001$) and IFNγ ($p<0.05$) levels were significantly reduced in HF-colitis relative to LF-colitis animals and IL-4 ($p<0.01$) was also significantly reduced in HF-CAC relative to LF-CAC animals. No other significant differences were observed between the HF-CAC and LF-CAC groups (Fig 5). No difference in plasma levels of IL-5, IL-17, TNFα and mKC were detected between any of the groups. Thus, at the systemic level, the HF diet reduces the inflammatory tone in animals with DSS-induced colitis relative to their LF diet fed counterparts.

We next examined the effect of diet on cytokine levels in colon tissue devoid of tumours. A significant increase in the levels of colonic IL-1β ($p<0.001$), IFNγ ($p<0.05$) and mKC ($p<0.05$) was found in LF-colitis, compared to LF-Ctr mice (Fig 6). IL-1β and mKC, in addition to IL-6 ($p<0.05$), were significantly greater in LF-CAC relative to LF-Ctr mice. HF-colitis mice also had significantly greater levels of colonic IFNγ ($p<0.05$), IL-6 ($p<0.05$) and mKC ($p<0.05$) compared to HF-Ctr mice. HF-CAC mice had significantly higher levels of mKC ($p<0.05$) compared to HF-Ctr mice. HF-CAC mice had significantly less IL-6 ($p<0.05$) than HF-colitis mice.

There were no significant differences in the levels of colonic cytokines between HF-colitis and LF-colitis mice or HF-CAC and LF-CAC mice, although the cytokine levels were in general lower in HF-group compared to their LF fed counterparts (Fig 5). Overall, a lower inflammatory tone was found in HF-diet mice with colitis and CAC relative to their LF-fed counterparts.

5.4.3 Microbiota diversity is reduced in LF-colitis and LF-CAC mice

The composition of the gut microbiota in these mice was investigated. Faecal samples were collected at 3 different time points i.e. 1 week after CAC mice were injected with AOM (week 12 – W12), after the first DSS cycle (week 14, W14) and immediately prior to culling (week 21, W21) to explore the changes that diet and the different onslaughts exerts on the microbiota. A total of 477,108 V4 16S rRNA sequence reads were generated. These corresponded to an average of 17,211 reads per group or 1,912 per mouse. Rarefaction curve analysis established that extra sampling would yield only a limited increase in species richness (Supplementary Fig 1). α diversity metrics were calculated for each data set (Supplementary Table 1). In almost all cases diet did not affect the diversity in the control LF and HF mice. One cycle of DSS (W14) significantly reduced α diversity in LF-colitis mice relative to HF-colitis mice. While this difference was not apparent in subsequent faecal samples, lower Simpson (p=0.0015) and Shannon (p=0.0026) values were attributed to cecum samples of LF-colitis mice relative to HF-colitis mice. No significant difference in α diversity was found between the faecal samples from HF-CAC and LF-CAC mice faecal samples but a significantly greater (p=0.035) Shannon diversity was noted in HF-CAC cecum samples relative to those of LF-CAC mice.

Principal coordinate analysis (PCoA) based on unweighted Unifrac distances highlights the clear separation of the microbiota populations in a treatment-specific manner (Fig 10A). It can be clearly seen that data points cluster according to treatment groups and away from the HF and LF controls. Data points corresponding to the LF-colitis and LF-CAC microbiota cluster together and, in general, diverged considerably from

the data points corresponding to controls. The data points corresponding to the HF-colitis and HF-CAC microbiota are generally located between those corresponding to the controls and the LF-colitis/CAC populations (Fig 10A). When the PCoA is visualised as a function of time, regardless of treatment, clustering of the data points at week 12 and in the cecum is most evident (Fig 10B).

5.4.4 Altered gut microbiota composition in animals fed a high fat, rather than a low fat, diet.

Of the reads, 413,069 (87%) were assigned at phylum level, 365,218 (76%) at family level and 281,785 (59%) at genus level. Comparisons between the gut microbiota compositions of many of the different groups of mice were possible. First, we compared the microbiota composition of control animals fed a LF or HF diet. At 12 weeks of age, i.e. 6 weeks after being divided into HF and LF diet groups, significantly lower proportions of the phyla Actinobacteria and Verrucomicrobia, the families *Alcaligenaceae*, *Bifidobacteriaceae* and *Verrucomicrobiaceae* and the genera *Sutterella*, *Allobaculum*, *Bifidobacterium* and *Akkermansia* were observed in HF-fed compared to LF-fed control mice (Fig 7). Significantly greater proportions of the families *Lachnospiraceae*, *Ruminococcaceae* and *Erysipelotrichaceae* and genera *Lachnospiraceae Incertae Sedis*, uncultured *Lachnospiraceae*, *Ruminococcaceae Incertae Sedis*, *Oscillibacter* and *Turicibacter* were also observed among HF-fed compared to LF-fed control mice (Supplementary Fig 2).

When the microbiota was assessed two weeks later (W14), significantly higher proportions of reads corresponding to the phylum Firmicutes, the family *Ruminococcaceae* and the genus uncultured *Lachnospiraceae*, *Ruminococcaceae Incertae Sedis* and *Oscillibacter* were detected among HF-fed relative to LF-fed animals. Significantly lower proportions of the phylum Proteobacteria, the families *Alcaligenaceae* and *Rikenellaceae* and the genera *Sutterella* and *Alistipes* were also evident in the HF-fed group.

At 21 weeks of age (W21), no significant differences were seen between HF-fed and LF-fed mice microbiota at phylum level (Fig 7).

However, significantly greater proportions of, at family level, *Streptococcaceae*, and, at genus level, *Oscillibacter*, *Turicibacter* and *Lactococcus* were observed in HF-fed animals. Among the same animals, significantly lower proportions of the genera *Alistipes* and *Parabacteroides* were evident in HF-fed, relative to LF-fed, animals.

Analysis of cecal samples revealed the presence of significantly greater proportions of, at phylum level, Deferribacteres, at family level, *Lachnospiraceae, Deferribacteraceae* and *Desulfovibrionaceae* and, at genus level, *Mucispirillum* and *Desulfovibrio* in HF-fed compared to LF-fed mice. In the same samples, it was noted that proportions of Bacteroidetes (phylum). *Rikenellaceae* (family) and *Alistipes* and *Thalassospira* (genus) were lower in HF-fed mice than in the LF-fed equivalents (Fig 7; Supplementary Fig 2 & 3 and Supplementary Tables 2 & 3).

5.4.5 The abundance of Proteobacteria in the colitis mouse model is lower in animals fed a high fat diet

At W14, when the mice present the highest degree of acute inflammation, analysis of the gut microbiota composition revealed specific DSS effects (Fig 8) (i.e. significant changes not previously found in our control mice at W14). Significantly lower proportions of *Bacteroidaceae, Ruminococcaceae* (family) and *Bacteroides* (genus) were detected in the HF-colitis mice relative to LF-colitis mice (Supplementary Fig 4 and 5). In contrast, significantly greater proportions of the phylum Deferribacteres and the genera *Anaerotruncus* and *Odoribacter* were seen in the HF-colitis mice relative to LF-colitis mice (Fig 8, Supplementary Fig 4 and 5).

Analysis of the gut microbiota composition at W21, revealed there was significantly lower proportions of the phylum Proteobacteria, the family *Alcaligenaceae* and in the corresponding genus *Sutterella* in HF-colitis mice compared to LF-colitis counterparts (Fig 8). Significantly greater proportions of the phylum Verrucomicrobia, the families *Bifidobacteriaceae*, *Verrucomicrobiaceae*, *Peptostreptococcaceae* and the genera *Lachnospiraceae Incertae Sedis*, *Akkermansia* and

Peptostreptococcaceae Incertae Sedis were noted in the HF-colitis mice compared to the LF-colitis mice.

Analysis of the microbiota composition of the murine cecal samples revealed that, at phylum level, as for the corresponding faecal samples, proportions of Proteobacteria were also greater in LF-fed mice, but in this instance not to a significant degree (p=0.2). At family level, significantly greater proportions of *Rikenellaceae*, *Ruminococcaceae*, *Peptococcaceae*, *Veillonellaceae* and of the genera *Alistipes, Ruminococcaceae Incertae Sedis* and *Oscillibacter* were apparent in HF-colitis mice, while significantly reduced proportions of the family *Porphyromonadaceae* and the genus *Parabacteroides*, were noted in HF-colitis mice compared to their LF-colitis equivalents (Fig 8; Supplementary Fig 4 & 5 and Supplementary Tables 3 & 4).

5.4.6 Proportions of Proteobacteria are also lower in the colitis associated cancer mouse model fed a high fat diet

Analysis of the gut microbiota composition after six weeks of diet (W12) and one week post AOM injection (but prior to DSS exposure) between the HF-CAC mice and the LF-CAC mice revealed the presence of relatively lower proportions of the phyla Proteobacteria and Bacteroidetes, the family *Prophyromonadaceae* and the genera *Sutterella* and *Rikenella* in HF-CAC mice compared to LF-CAC mice. Significantly greater proportions of the phylum Firmicutes, the family *Lactobacillaceae* and the genus *Lactobacillus* were apparent in these HF-CAC animals.

After eight weeks of dietary intervention (W14 i.e. three weeks after AOM injection and 5 days post DSS cycle one), the proportions of the phylum Bacteroidetes, the families *Bacteroidaceae*, *Peptostreptococcaceae* and *Prophyromonadaceae* were significantly lower in HF-CAC mice compared to LF-CAC controls. Only the family *Erysipelotrichaceae* were present in significantly greater proportions within the gut microbiota of the HF-CAC, relative to LF-CAC mice.

After 15 weeks of dietary intervention (W21 i.e. 10 weeks post AOM and two weeks post the third DSS cycle), a significantly lower proportion of the phylum Proteobacteria and the corresponding family

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Alcaligenaceae and genus *Sutterella* was found in HF-fed, relative to LF-fed mice*.*

Analysis of the cecal microbiota composition established that at phylum level a significantly lower proportion of Proteobacteria were observed in HF-CAC mice compared to their LF-fed equivalents. Significant reductions at family level in *Alcaligenaceae*, *Peptostreptococcaceae, Clostridiaceae* and at genus level in *Sutterella* and *Clostridium* were noted in HF-CAC mice relative to LF-CAC mice (Fig 9; Supplementary Fig 6 & 7 Supplementary Tables 3 & 5).

5.5   DISCUSSION

The 'western diet', characterised by high fat and calorie rich content, and its contribution to colorectal cancer, has been the focus of much attention. Previous studies examining 'western diet' in experimental models of colitis and colitis associated cancer (CAC) have been inconclusive. Here we assessed the relative effects of high and low fat diets on the development of colitis and CAC in the widely used DSS and AOM/DSS murine models (Rosenberg *et al.*, 2009, Thaker *et al.*, 2012, Robertis *et al.*, 2011). AOM induces colon tumours while DSS has been suggested to exert toxic effects on colonic epithelium creating a chronic inflammatory state (Robertis *et al.*, 2011, Thaker *et al.*, 2012) and, when combined, they result in rapid growth of colon tumours shortening the latency time for induction of CAC (Thaker *et al.*, 2012). Here we found that, in contrast to previous studies in CRC mice fed a high fat diet (Singh *et al.*, 1997, Newmark *et al.*, 2001) a high lard based fat diet protected mice from colitis and colitis associated cancer when compared to a low fat diet. Though there are differences seen between this study and those cited, this study does follow a similar design to that employed by Flores *et al.* (2012). These results are in line with reports by Hyland and colleagues in the TNBS-model of colitis and Gäbele and colleagues in chronic DSS-colitis, both studies using similar lard-containing HF-diet (Hyland *et al.*, 2009, Gäbele *et al.*, 2011). In addition, two other studies reported HF diet reduction in a colon carcinogenesis but in these cases the high fat diets were rice bran oil-based or were olive oil-based and supplemented with fruit and vegetable extract (Shih *et al.*, 2011, Mai *et al.*, 2007). Major differences between this study and previous studies reporting that HF diets induced colitis and CAC are the strain used (BALB/c, A/J (Kim *et al.*, 2010, Park *et al.*, 2012)), the concentration of AOM (10-12.5mg/kg (Flores *et al.*, 2012, Park *et al.*, 2012, Kim *et al.*, 2010)), the DSS-provider (Laroui *et al.*, 2012, Park *et al.*, 2012, Kim *et al.*, 2010), percentage (Laroui *et al.*, 2012, Flores *et al.*, 2012, Park *et al.*, 2012, Kim *et al.*, 2010) and time of exposure of DSS (Laroui *et al.*, 2012, Park *et al.*, 2012, Kim *et al.*, 2010), the concentration of HF diet (Flores *et al.*, 2012, Kim *et al.*,

2010, Laroui *et al.*, 2012) and the provider of the mice (Flores *et al.*, 2012, Kim *et al.*, 2010, Park *et al.*, 2012).

Similar to other conditions, including obesity and diabetes, IBD has an increasing incidence worldwide suggesting that environmental factors such as diet might be important. There are currently no studies that correlate obesity with the increased incidence of IBD and one recent study did not find a correlation between BMI and incidence for CD or UC (Chan *et al.*, 2013). Obesity is associated with an increased pro-inflammatory cytokine production and infiltration of macrophages into the adipose tissue suggesting an interaction between the adipose tissue and the immune system. In the current study, the protective effect of high fat diet was already noticed after the first cycle of DSS (based on body weight loss), suggesting that diet positively regulates responses from other non-mucosal organ such as the adipose tissue as well as the microbiota. Recent reports have suggested that the crosstalk between mesenteric adipose tissue and immune cells is of a protective nature with the adipose tissue being a barrier for microbiota translocation (Kredel *et al.*, 2013, Batra *et al.*, 2012). We are currently examining the response of the adipose tissue in these models with a view to identifying novel mechanisms associated with the protective effect.

It is well accepted that diet regulates the microbiota. Analysis of the murine gut microbiota composition was particularly revealing in this current study, since no such analysis were performed in the previous reports. Principal coordinate analysis revealed a shift in the microbiota composition in colitis and CAC mice which was influenced by diet. High gut microbiota diversity is considered desirable with low diversity associated with UC and CD (Ott *et al.*, 2004). The current study revealed a reduction in gut microbiota diversity of LF-colitis and LF-CAC mice relative to their HF counterparts. It was revealed that in both controls and experimental groups, HF-fed mice had significantly lower proportions of the phylum Proteobacteria relative to LF-fed comparators. The phylum Proteobacteria, as previously stated, has been found to be associated with CD, UC and CRC patients (Lepage *et al.*, 2011, Gophna *et al.*, 2006, Frank *et al.*, 2011). Phylogenetic analysis of the faecal microbiota of

colitis model mice revealed numerous differences between HF-fed and LF-fed mice. Of particular interest was the increase in proportions of the genus *Ordoribacter* in HF-fed mice. *Ordoribacter* was found previously to be reduced in CD and DSS colitis mice (Samanta *et al.*, 2012, Morgan *et al.*, 2012). Notably, the genus *Akkermansia* was increased in the "HF-fed colitis mice" given that a member of this genus, *Akkermansia muciniphila*, has been previously reported to be reduced in CD and UC patients (Png *et al.*, 2010). A recent report also highlighted the regulatory role of *A. muciniphila* in a model of diet-induced obesity whereby administration of *A. muciniphila* reduced the metabolic profile of the disease by improving gut barrier function (Everard *et al.*, 2013)**.** In cecum samples, a reduction in *Prophyromonadaceae* and increase in *Ruminococcaceae* was noted. These two families have previously been shown to be increased and decreased, respectively, in CRC and UC patients (Wang *et al.*, 2012, Lepage *et al.*, 2011, Kostic *et al.*, 2012, Chen *et al.*, 2012, Bibiloni *et al.*, 2006). Analysis of the gut microbiota composition of CAC mice revealed, like colitis mice, numerous differences between HF-fed and LF-fed animals. Of particular interest is the greater proportion of the phylum Firmicutes in HF-fed CAC mice, as others have reported the presence of relatively low proportions of Firmicutes in CRC patients (Kostic *et al.*, 2012). It is interesting that reductions in the families *Porphyromonadaceae* and *Peptostreptococcaceae* were noted in HF-fed CAC mice given that two corresponding genera, *Porphyromonas* and *Peptostreptococcus*, have previously been found to be increased in CRC patients (Wang *et al.*, 2012, Chen *et al.*, 2012) while an unclassified member of the family *Porphyromonadaceae* was detected in UC patients, but not healthy controls (Bibiloni *et al.*, 2006).

Ultimately, this data reveals that a high fat diet protected against the progression of colitis and CAC and highlights changes to the gut microbiota that accompany this phenomenon. The data also highlight a far more complex outcome of high fat diet on microbiota-host responses warranting further investigations on these factors in patients with IBD and CAC.

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Figure 1. Schematic of experimental design. Male C57BL/6 mice were fed a low fat (LF, 10% fat) or high fat (HF, 45% fat) diet for 15 weeks (Control group). Male C57BL/6 mice were fed with LF or HF diet for 15 weeks including 3 cycles of DSS, 5 days 1.5% DSS and 14 days of water (Colitis group). Male C57BL/6 mice were fed a LF or HF diet for 15 weeks, after 4 weeks injected intraperitoneally with axozymethane (AOM, 8mg) and 1 week later supplied with 3 cycles of DSS, 5 days 1.5% DSS and 14 days of water (Colitis-associated cancer (CAC)-group).
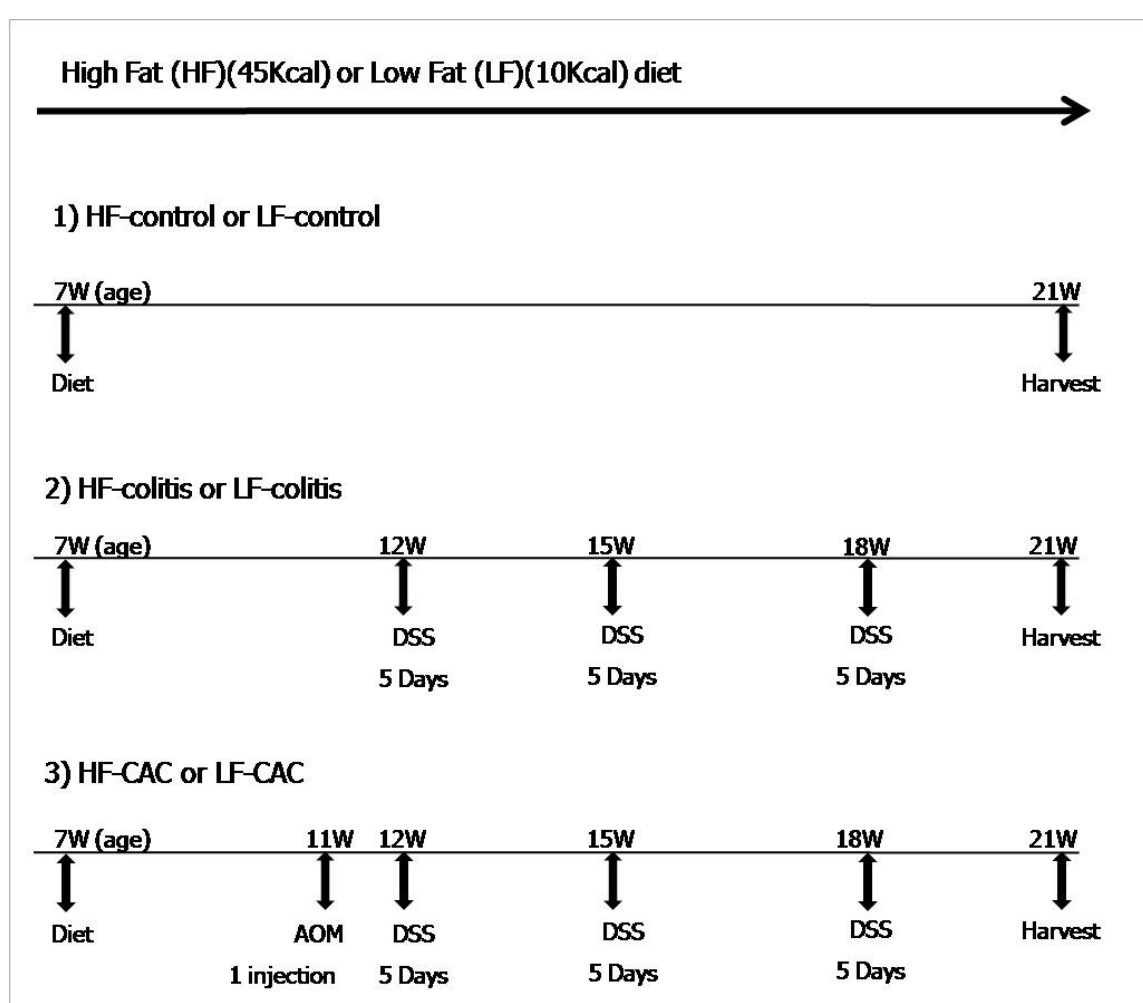
Figure 2. Reduced body weight loss in HF diet colitis and CAC model mice relative to LF diet counterparts. Body weight change was monitored twice a week and the relative change is depicted as percentage of starting day zero. Significance differences were determined using ANOVA with post-hoc corrections unless otherwise stated: %%% $p<0.001$ for differences between HF-CAC vs HF-Ctr; \$\$\$ $p<0.001$ for differences between HF-colitis vs LF-Colitis; &&& $p<0.001$ for differences between HF-Ctr vs LF-Ctr. Data are mean ±SEM. n=8–12 mice per group, representative of 2 experiments.
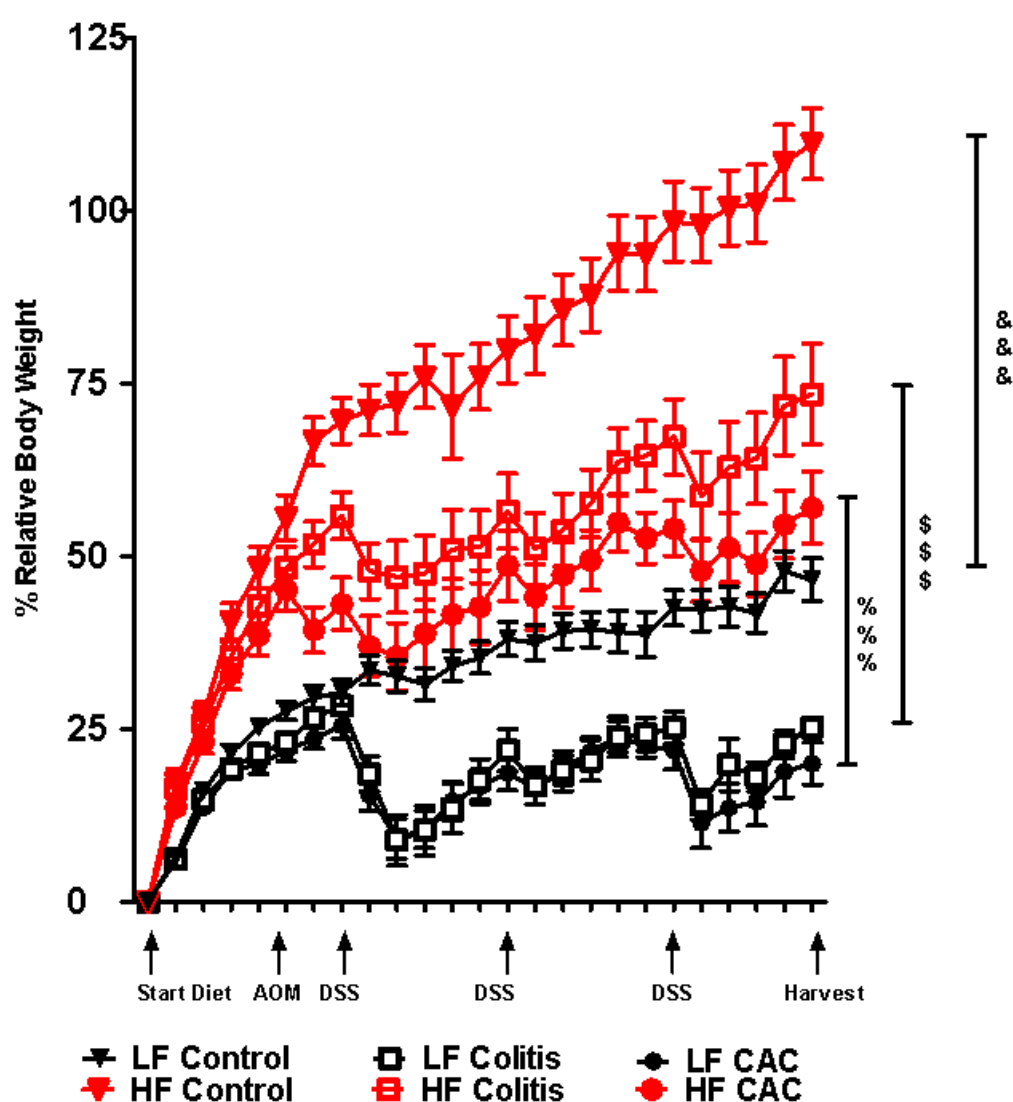
Figure 3. Colonic macroscopic signs of disease. Colon Length and weight were measured at time of necropsy. Significance differences were determined using ANOVA with post-hoc corrections unless otherwise stated: **$p<0.01$ for differences between HF-CAC vs LF-CAC; # $p< 0.05$ for differences between LF-colitis vs LF-Ctr; ## $p< 0.05$ ($p<0.001$ relative colon weight) for differences between LF-CAC and LF-Ctr; ### $p<0.05$ for differences between LF-colitis vs LF-CAC; $ $p<0.05$ for differences between LF-colitis vs HF-colitis and % $p<0.05$ for differences between HF-CAC vs HF-Ctr. Data are mean ±SEM. n=8–12 mice per group, representative of 2 experiments.

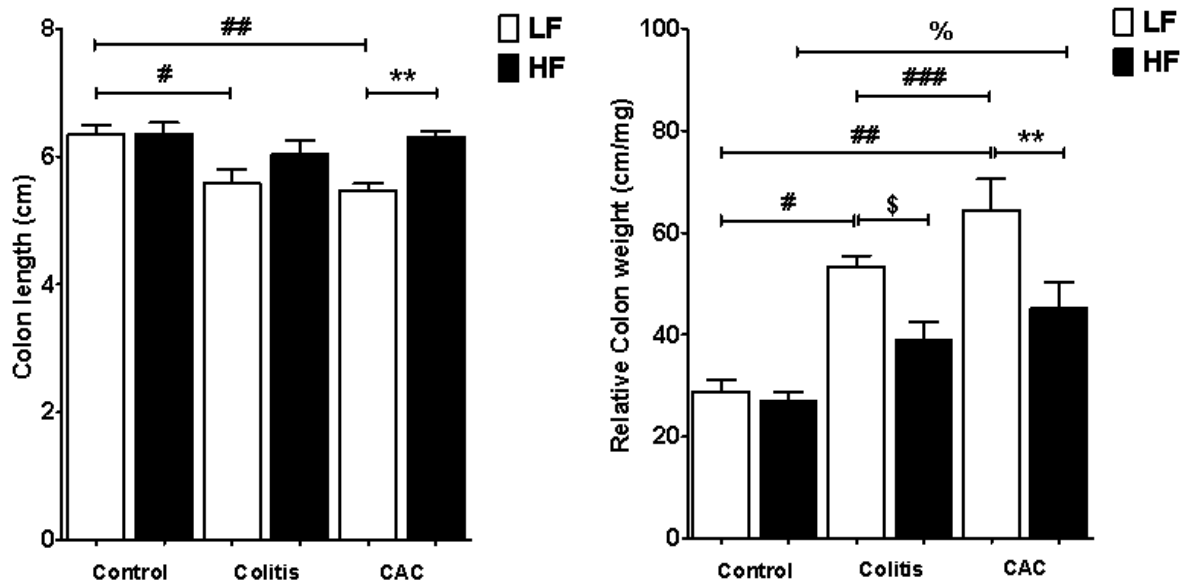**Figure 4. Tumor number and incidence.** Representative images of intestine with tumours from mice with LF-CAC and HF-CAC at 20x and a close up of tumours at 40x. Number of tumours per mouse fed LF and HF diet. Data are mean ±SEM. n = 9–12 mice per group, representative of two experiments. Significance differences were determined using ANOVA with post-hoc corrections - **p<0.01 for differences between HF-CAC vs LF-CAC.
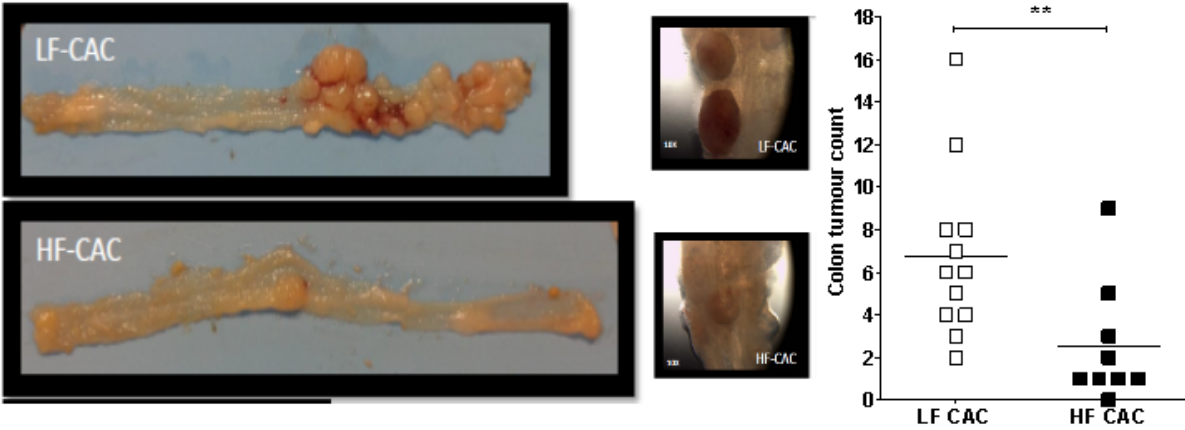
Figure 5. Reduced plasma cytokines in HF fed colitis and CAC model animals relative to LF diet counterparts. Plasma was collected at the end of the study from mice exposed to LF and HF-diet (LF/HF-Ctr), diets and DSS (LF/HF-colitis) and diets and AOM/DSS (LF/HF-CAC). Plasma levels of IL-1β, IFN-γ, IL-12, IL-2, IL-10 and IL-4 were measured using MSD-technology. Data are mean ±SEM. n=8–12 mice per group, representative of 2 experiments. Significance differences were determined using ANOVA with post-hoc corrections - ***p<0.001 for differences between HF-CAC vs LF-CAC; # p<0.05 and ### p<0.001 for differences between LF-colitis or LF-CAC vs LF-Ctr; ## p<0.05 for differences between LF-colitis and LF-CAC; % p<0.05 and %%% p<0.001 for differences between HF-CAC or HF-colitis vs HF-Ctr; %% p<0.05 between HF-CAC and HF-Colitis; \$\$ p<0.05 and \$\$\$ p<0.001 for differences between HF-colitis vs LF-colitis.
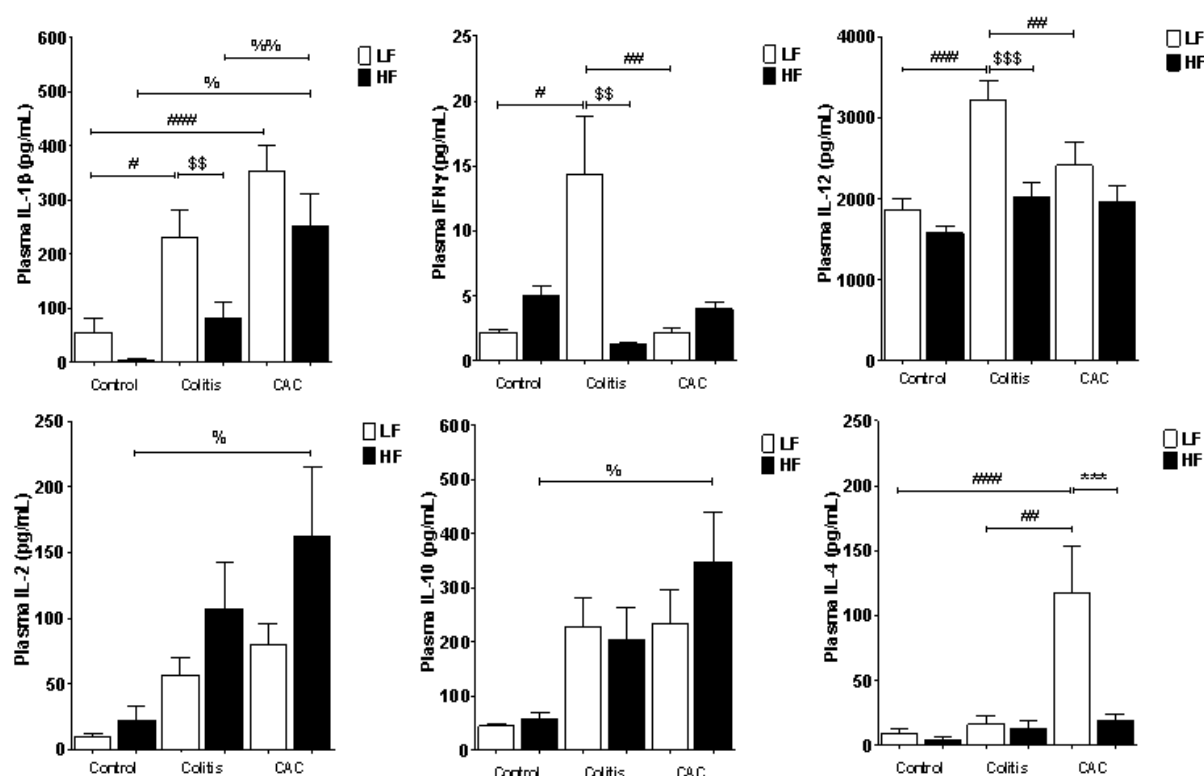
Figure 6. Reduced colon cytokines in HF fed colitis and CAC model animals relative to LF diet counterparts. Proximal colon was collected at necropsy from mice exposed to LF and HF-diet (LF/HF-Ctr), and DSS (LF/HF-colitis) and AOM/DSS (LF/HF-CAC). Colon homogenates were measured for IL-1β, IFN-γ, IL-6 and mKC using MSD technology. Data are mean ±SEM. *n*=8–12 mice per group. Significance differences were determined using ANOVA with post-hoc corrections unless otherwise stated - # *p*<0.05 and ### *p*<0.001 for differences between LF-colitis or LF-CAC vs LF-Ctr; ## *p*<0.05 for differences between LF-colitis and LF-CAC; % *p*<0.05 for differences between HF-CAC or HF-colitis vs HF-Ctr; %% *p*<0.05 between HF-CAC and HF-colitis.
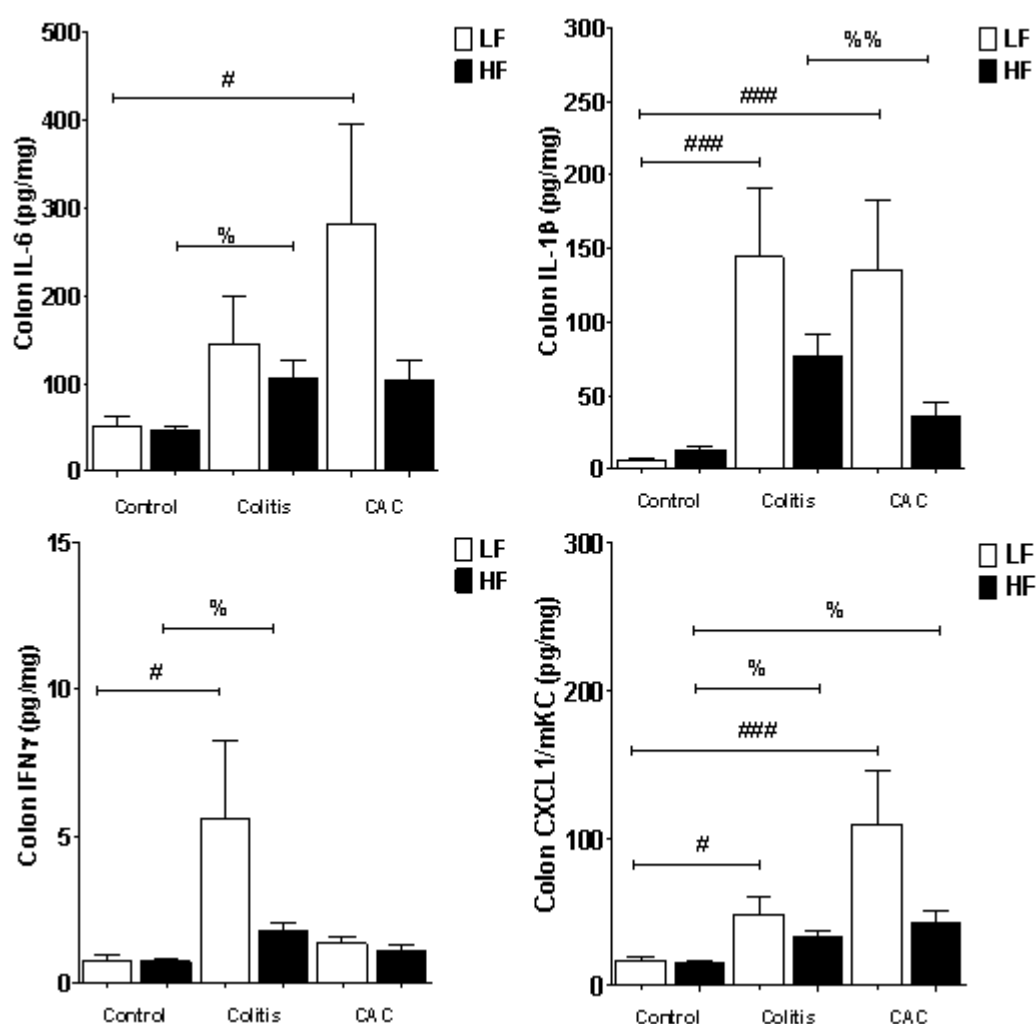
Figure 7. Faecal microbiota composition at phylum level in Control mice. Faecal samples were collected at week 12 (W12, before DSS start), W14 (after 5 days DSS and 1 week of water) and W21 (at time of necropsy). DNA extracted and microbiota composition determined. (a) Microbiota distribution at phylum level in LF- and HF-diet control mice as determined by pyrosequencing of 16rRNA tags (V4) region. Data outside the pie charts represent the mean percentage read number for the corresponding colour coded family (*n*=8 mice/group).
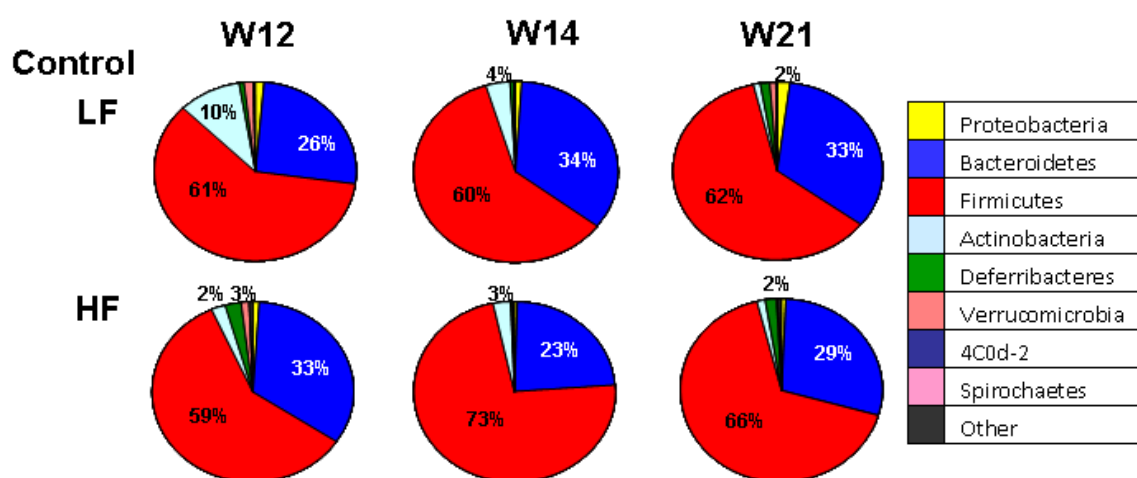
Figure 8. Faecal microbiota composition at phylum level in mice with colitis. The gut microbiota composition at phylum level of mice fed a HF diet is altered in colitis conditions compared to mice on a LF diet as determined by pyrosequencing of 16rRNA tags (V4) region. Data outside the pie charts represent the mean percentage read number for the corresponding colour coded family (*n*=7-11 mice/group).
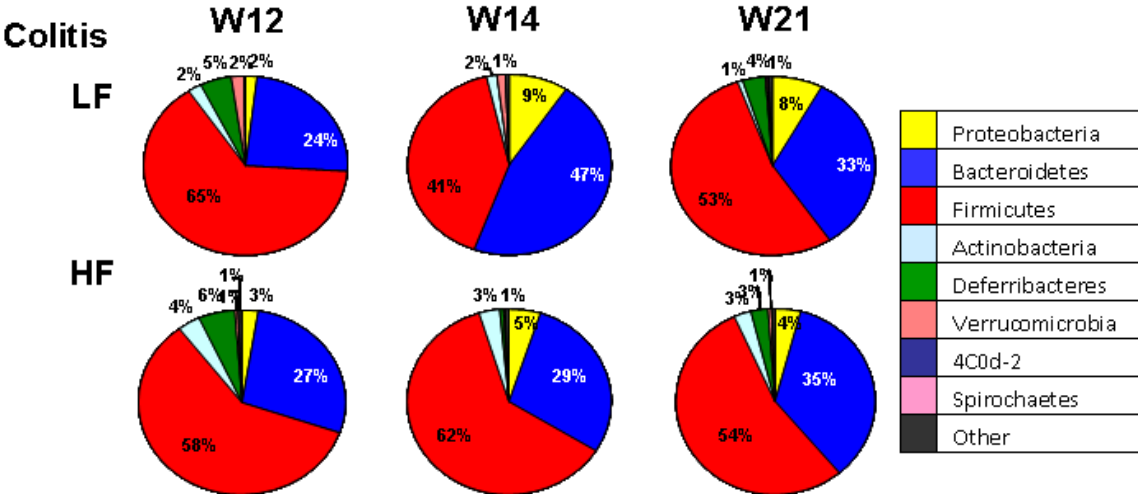
Figure 9. Faecal microbiota composition at phylum level in mice with colitis-associated colorectal cancer (CAC). The gut microbiota composition at phylum level of mice fed a HF diet is altered in CAC compared to mice on a LF diet as determined by pyrosequencing of 16rRNA tags (V4) region. Data outside the pie charts represent the mean percentage read number for the corresponding colour coded family (*n*=7-10 mice/group)
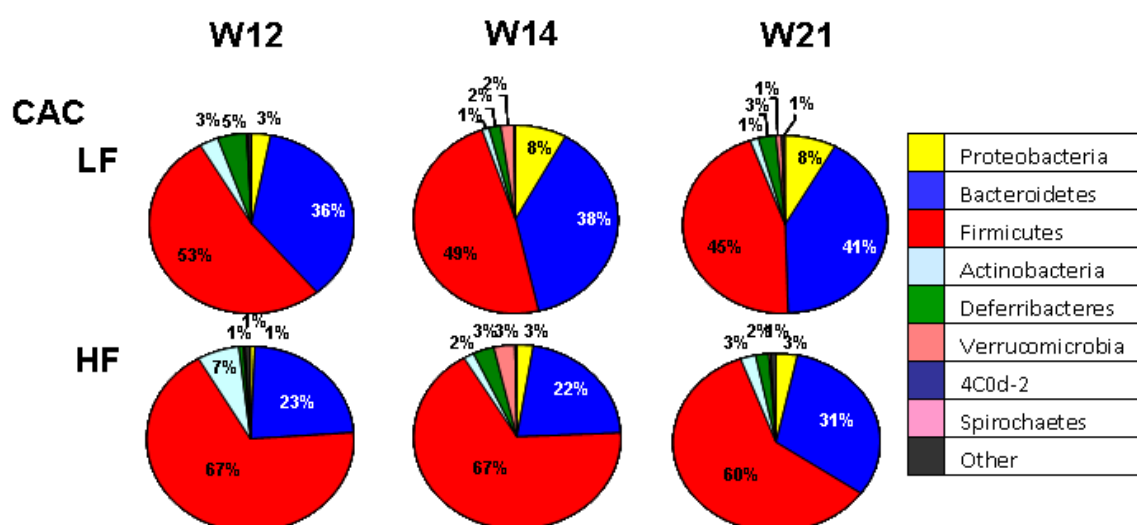
Figure 10. Microbiota composition. Principal coordinate analysis of unweighted Unifrac reveals separation by treatment group. Data sets A: Yellow - low fat diet control, Purple - high fat diet control, Red - CAC high fat diet, Blue - CAC low fat diet, Orange - Colitis high fat diet and Green - Colitis low fat diet. Data sets B: Blue - week 12, Orange - week 14, Green - week 21 and Red - cecum.

SUPPLEMENTARY INFORMATION

Supplementary Table 1 - α Diversity of the gut microbiota in each group within each time point

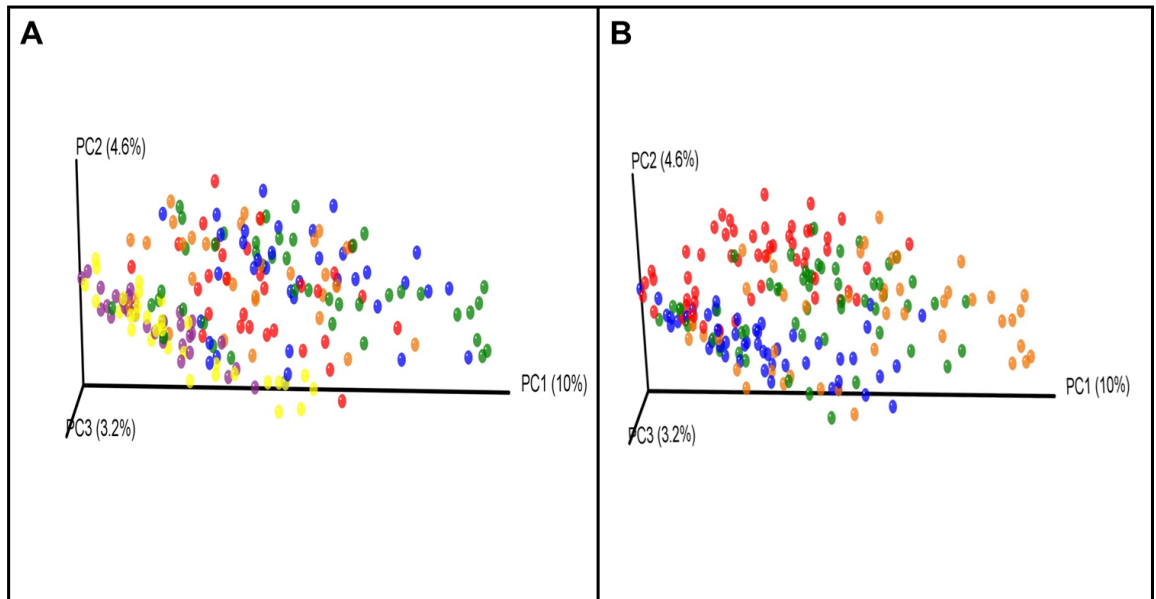|	Group	AMODSSHF	AMODSSLF	DSSHF	DSSLF	HF	LF
Week 12	Simpson	0.78	0.85	0.87	0.86	0.93	0.79**
	CHao1	239.58	306.33	395.67	465.49	589.53	389.53
	Shannon	3.96	4.41	4.66	4.79	5.52	4.15
	Observed Species	121.86	138	159.88	219.55	257.5	193.13
	Phylogenetic Diversity	14.21	15.46	16.60	20.10	21.98	18.48
Week 14	Simpson	0.75	0.84	0.85	0.82	0.75	0.77
	CHao1	302.93	209.28	405.21	172.85**	303.48	364.02
	Shannon	3.70	3.97	4.42	3.60**	3.86	4.04
	Observed Species	144.2	104.25	175.5	81.73***	163.67	170.4
	Phylogenetic Diversity	15.03	12.10	17.65	10.44***	17.14	17.40
Week 21	Simpson	0.77	0.87	0.83	0.85	0.83	0.81
	CHao1	285.60	344.08	319.23	237.65	468.31	408.20
	Shannon	3.91	4.50	4.14	4.33	4.50	4.40
	Observed Species	143.22	163.4	149.63	123.30	217.63	187.25
	Phylogenetic Diversity	15.70	16.98	15.34	14.10	20.04	18.35
Cecum	Simpson	0.94	0.93	0.93	0.88**	0.93	0.93
	CHao1	529.30	371.40	554.60	451.43	721.32	804.34
	Shannon	5.46	5.14*	5.40	4.65**	5.55	5.58
	Observed Species	203.22	178.4	228	189.73	271.5	310.38
	Phylogenetic Diversity	19.70	18.21	20.78	18.67	22.60	25.87

Statistical significance was determined using Mann Whitney. *p value ≤0.05 ** p value <0.005 and *** p value <0.0005 between HF-fed and LF-fed mice within each treatment group.

Supplementary Table 2. Gut microbiota changes in the control mouse model over time

	WK 12 HF	WK 12 LF	WK 14 HF	WK 14 LF	WK 21 HF	WK 21 LF
Phylum						
Proteobacteria	1.116	1.487	0.617	1.098*	0.91	1.95
Firmicutes	58.931	60.205	72.764	60.128*	66.51	60.87
Actinobacteria	2.243	10.146*	2.806	3.901	1.32	1.33
Verrucomicrobia	1.417	1.497*	0.172	0.067	0.16	0.65
Family						
Alcaligenaceae	0.000	0.995*	0.043	0.652*	0.00	0.90
Rikenellaceae	9.196	8.721	3.954	14.338*	5.46	8.06
Lachnospiraceae	21.500	8.536*	8.957	8.520	14.73	11.29
Ruminococcaceae	13.004	4.696*	11.389	5.139*	6.54	7.97
Bifidobacteriaceae	2.205	10.095*	2.641	3.761	1.26	1.24
Verrucomicrobiaceae	1.417	1.497*	0.172	0.067	0.16	0.65
Erysipelotrichaceae	17.791	39.81*	45.737	41.856	36.304	37.631
Streptococcaceae	0.224	0.000	0.000	0.000	0.10	0.00*

Note only significant changes in % read number shown. Statistical significance was determined using Kruskal Wallis. *Pvalue ≤0.05 between high fat and low fat within each time point.

Supplementary Table 2 (continued)

| | WK 12 | WK 12 | WK 14 | WK 14 | WK 21 | WK 21 |
	HF	LF	HF	LF	HF	LF
Genus						
Sutterella	0.000	0.995*	0.043	0.652*	0.00	0.90
Alistipes	6.631	6.942	2.727	12.294*	3.37	6.39*
Lachnospiraceae Incertae Sedis	1.198	0.441*	0.581	0.459	0.68	0.76
uncultured Lachnospiraceae	0.624	0.077*	0.746	0.160*	0.19	0.07
Ruminococcaceae Incertae Sedis	6.078	1.933*	6.301	2.689*	3.25	4.26
Oscillibacter	1.346	0.272*	1.593	0.379*	0.90	0.53*
Allobaculum	16.905	39.810*	45.464	41.856	34.85	37.63
Bifidobacterium	2.205	10.095*	2.641	3.761	1.26	1.24
Akkermansia	1.417	1.405*	0.172	0.067	0.16	0.62
Turicibacter	0.886	0.000*	0.273	0.000	1.45	0.00*
Lactococcus	0.148	0.000	0.050	0.000	0.09	0.00*
Other	38.137	24.076*	20.906	24.256	33.56	30.74

Note only significant changes in % read number shown. Statistical significance was determined using Kruskal Wallis. *Pvalue ≤0.05 between high fat and low fat within each time point.

Supplementary Table 3. Cecum composition within each cohort at W21

	Cecum HF	Cecum LF	Cecum DSS HF	Cecum DSS LF	Cecum AMO DSS HF	Cecum AMO DSS LF
Phylum						
Proteobacteria	4.110	3.890	4.990	7.420	4.460	8.250*
Bacteroidetes	24.530	33.620*	23.690	29.030	34.170	36.500
Deferribacteres	7.500	3.880*	7.440	3.050*	5.130	3.630
Family						
Alcaligenaceae	0.100	1.460	2.390	6.020	2.280	6.010*
Rikenellaceae	5.710	10.200*	5.260	2.200*	6.580	4.510
Porphyromonadaceae	4.220	4.450*	2.130	3.940*	2.920	4.270
Lachnospiraceae	31.350	17.240*	24.350	20.470	20.060	17.290
Ruminococcaceae	15.010	16.860	15.600	10.130*	16.470	10.630
Deferribacteraceae	7.500	3.880*	7.440	3.050*	5.130	3.630
Desulfovibrionaceae	3.700	1.670*	2.000	0.910*	1.730	1.550
Peptostreptococcaceae	0.050	0.110	0.320	0.150	0.000	0.260*
Peptococcaceae	0.720	0.510	0.700	0.250*	0.560	0.690
Clostridiaceae	0.000	0.000	0.000	0.020	0.000	0.150*
Veillonellaceae	0.000	0.000	0.090	0.00*	0.040	0.000

Note only significant changes in % read number shown. Statistical significance was determined using Kruskal Wallis. *Pvalue ≤0.05 between high fat and low fat within group

Supplementary Table 3 (continued)

	Cecum HF	Cecum LF	Cecum DSS HF	Cecum DSS LF	Cecum AMO DSS HF	Cecum AMO DSS LF
Genus						
Sutterella	0.100	1.460	2.390	6.020	2.280	6.010*
Alistipes	4.050	8.550*	3.900	1.550*	5.240	3.540
Parabacteroides	1.990	3.010	0.950	2.830*	1.850	3.610
Ruminococcaceae Incertae Sedis	8.450	10.060	8.020	4.890*	9.930	5.610
Oscillibacter	2.770	1.470	1.730	1.020*	1.370	1.300
Mucispirillum	7.500	3.880*	7.440	3.050*	5.130	3.630
Desulfovibrio	3.430	1.530*	1.580	0.770*	1.410	1.410
Thalassospira	0.210	0.520*	0.370	0.420	0.230	0.310
Clostridium	0.000	0.000	0.000	0.020	0.000	0.150*

Note only significant changes in % read number shown. Statistical significance was determined using Kruskal Wallis. *Pvalue ≤0.05 between high fat and low fat within each group.

Supplementary Table 4. Gut microbiota changes in the colitis mouse model overtime

	WK12 colitis HF	WK12 colitis LF	WK14 colitis HF	WK14 colitis LF	WK21 colitis HF	WK21 colitis LF
Phylum						
Proteobacteria	2.519	1.913	5.033	9.476*	4.19	7.96*
Firmicutes	59.266	64.608	61.548	40.730*	54.49	53.79
Deferribacteres	5.568	5.247	0.566	0.091*	2.97	3.85
Verrucomicrobia	0.592	1.777	0.364	1.385	0.52	0.24*
Family						
Alcaligenaceae	0.318	1.053	4.528	8.608	2.63	6.07*
Rikenellaceae	7.097	5.501	1.280	3.602*	4.91	2.35
Bacteroidaceae	11.012	4.921	14.230	27.494*	12.03	10.15
Lachnospiraceae	16.792	19.514	12.700	2.181*	9.38	14.64
Ruminococcaceae	8.988	8.668	8.779	1.531*	4.30	5.94
Bifidobacteriaceae	3.535	1.898	2.062	0.231	3.05	0.54*
Verrucomicrobiaceae	0.592	1.777	0.364	1.385	0.52	0.24*
Peptostreptococcaceae	0.106	0.178	0.640	1.841	0.65	0.00*
Peptococcaceae	0.186	0.246	0.371	0.061	0.35	0.00*

Note only significant changes in % read number shown. Statistical significance was determined using Kruskal Wallis. *Pvalue ≤0.05 between high fat and low fat within each time point.

Supplementary Table 4 (continued)

	WK12 colitis HF	WK12 colitis LF	WK14 colitis HF	WK14 colitis LF	WK21 colitis HF	WK21 colitis LF
Genus						
Sutterella	0.318	1.053	4.528	8.608	2.63	7.97*
Parabacteroides	0.097	1.879*	1.348	2.570	0.60	2.60
Bacteroides	11.012	4.921	14.230	27.494*	12.03	10.15
Lachnospiraceae Incertae Sedis	1.149	1.315	1.961	0.383*	1.67	0.22*
uncultured Lachnospiraceae	0.000	0.269*	0.155	0.000	0.00	0.32
Ruminococcaceae Incertae Sedis	4.490	4.262	5.195	0.589*	1.71	1.91
Oscillibacter	0.946	0.754	0.445	0.140*	0.37	0.28
Anaerotruncus	0.928	1.489	0.364	0.000*	0.68	1.37
Akkermansia	0.592	1.777	2.244	4.459	2.43	0.51*
Odoribacter	1.918	1.424	0.404	1.233*	0.45	0.00
Peptostreptococcaceae Incertae Sedis	0.000	0.045	0.061	0.000	0.37	0.00*
uncultured bacteria	0.362	0.000	0.418	0.000*	0.00	0.00
Other	30.040	34.039	31.573	23.545*	26.66	35.54

Note only significant changes in % read number shown. Statistical significance was determined using Kruskal Wallis. *Pvalue ≤0.05 between high fat and low fat within each time point.

Supplementary Table 5 Gut microbiota changes in the colitis associated cancer mouse model (CAC) overtime

|	WK 12	WK 12	WK 14	WK 14	WK 21	WK 21
	CAC HF	CAC LF	CAC HF	CAC LF	CAC HF	CAC LF
Phylum						
Proteobacteria	0.855	3.014*	2.586	8.112*	3.430	8.130*
Bacteroidetes	23.055	35.969*	21.959	38.381*	31.330	41.200
Firmicutes	67.640	52.702*	66.839	48.117*	59.610	45.200
Family						
Alcaligenaceae	0.342	1.587*	2.255	6.898*	2.270	6.710*
Porphyromonadaceae	1.539	3.382*	0.952	2.686*	2.190	3.570
Bacteroidaceae	8.218	12.358	10.074	25.219*	12.620	20.290
Lactobacillaceae	8.475	2.513*	1.634	3.766	2.640	1.800
Peptostreptococcaceae	0.000	0.000	0.217	2.588*	0.030	0.170
Erysipelotrichaceae	42.569	33.759	41.963	23.898*	44.133	24.980

Note only significant changes in % read number shown. Statistical significance was determined using Kruskal Wallis. *Pvalue ≤0.05 between high fat and low fat within each time point.

Supplementary Table 5 (continued)

	WK 12 CAC HF	WK 12 CAC LF	WK 14 CAC HF	WK 14 CAC LF	WK 21 CAC HF	WK 21 CAC LF
Genus						
Sutterella	0.342	1.587*	2.255	6.898*	2.270	6.710*
Parabacteroides	0.550	1.663	0.652	2.650*	1.470	3.120
Bacteroides	8.218	12.358	10.074	25.219*	12.620	20.290
uncultured Lachnospiraceae	0.220	0.047	0.434	0.080*	0.200	0.230
Allobaculum	42.569	33.692	41.963	23.737*	44.130	24.980
Lactobacillus	8.426	2.513*	1.634	3.757	2.060	1.790
Peptostreptococcaceae Incertae Sedis	0.000	0.000	0.134	1.303*	0.000	0.060
Rikenella	0.000	0.595*	0.000	0.000	0.310	0.060

Note only significant changes in % read number shown. Statistical significance was determined using Kruskal Wallis. *Pvalue ≤0.05 between high fat and low fat within each time point.

Supplementary Figure 1. Rarefaction curves for each group at 97% similarity levels for all twenty four data sets. Amount of operational taxonomic units (OTU's) found as a function of the number of sequence tags sampled.
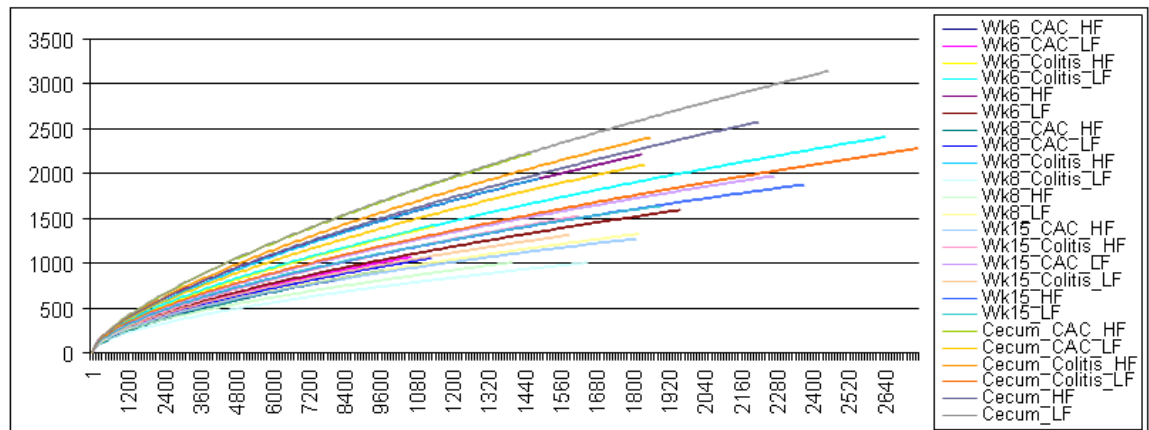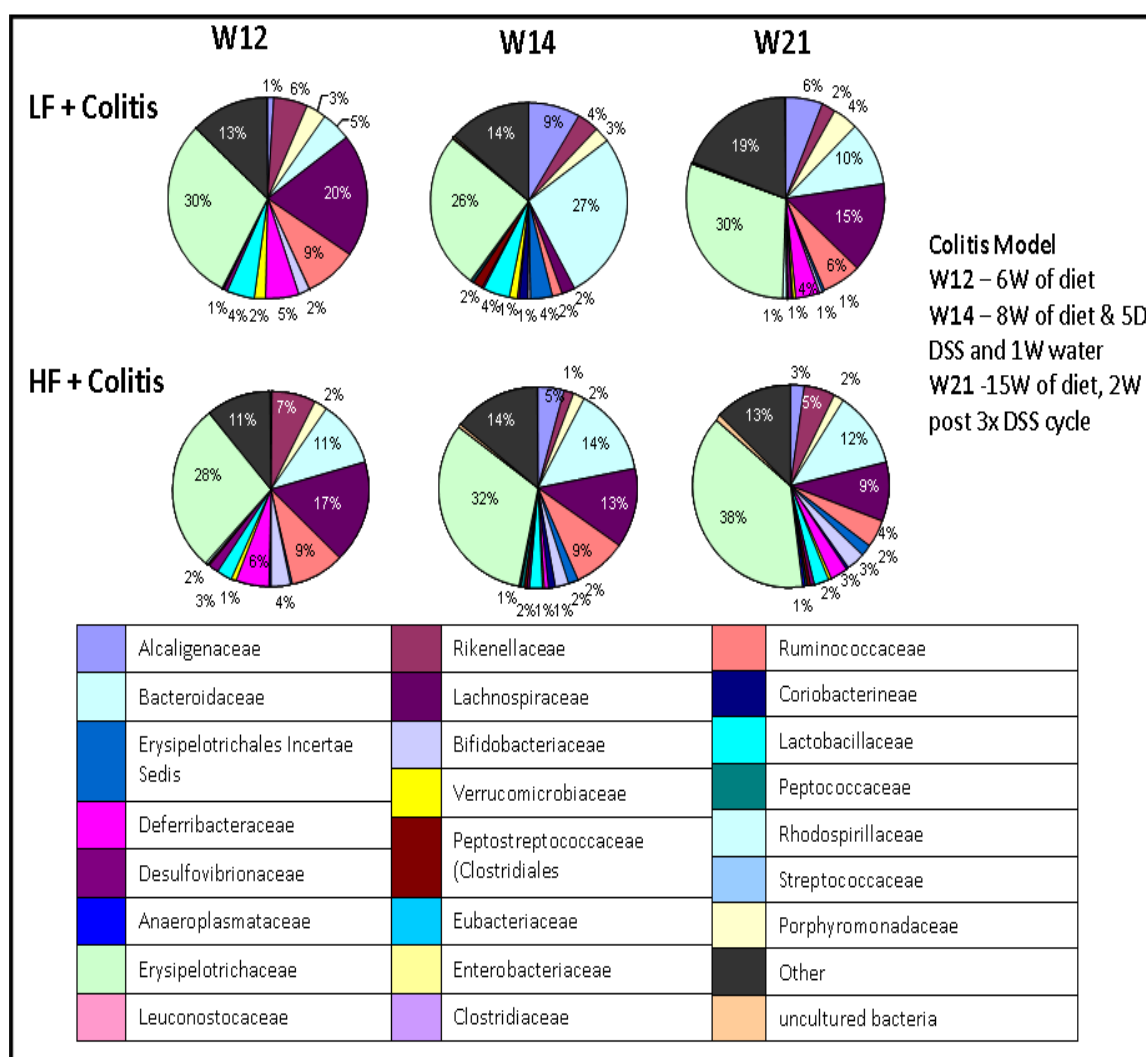
Supplementary Figure 2. Faecal microbiota composition at family level in control mice. Microbiota distribution at family level in high fat diet and low fat diet mice as determined by pyrosequencing of 16rRNA tags (V4) region. Data outside the pie charts represent the mean percentage read number for the corresponding colour coded family (n=8 mice/group).
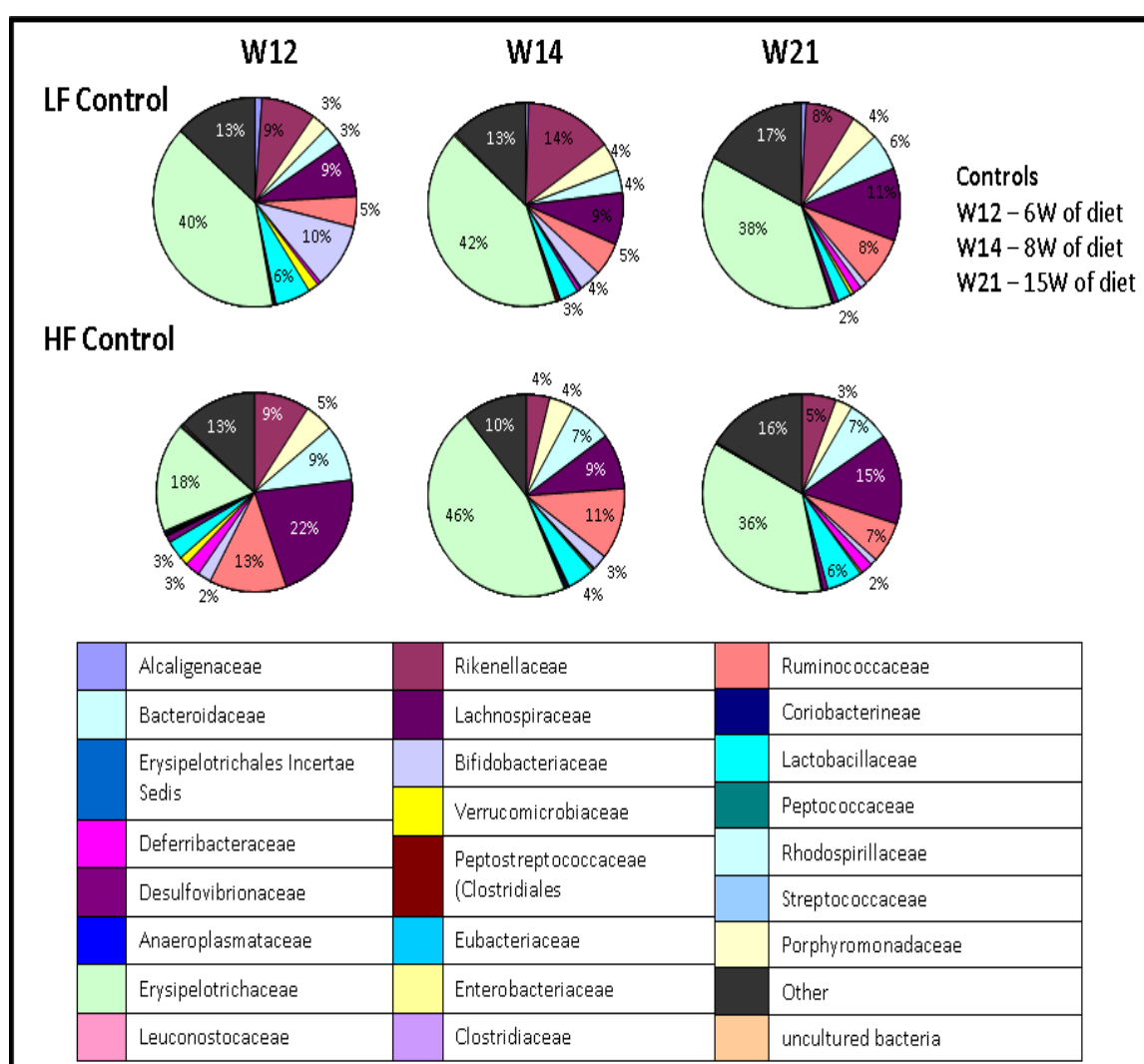
Supplementary Figure 3. Faecal microbiota composition at genus level in control mice. Microbiota distribution at genus level in high fat diet and low fat diet mice as determined by pyrosequencing of 16rRNA tags (V4) region. Data outside the pie charts represent the mean percentage read number for the corresponding colour coded family (n=8 mice/group).
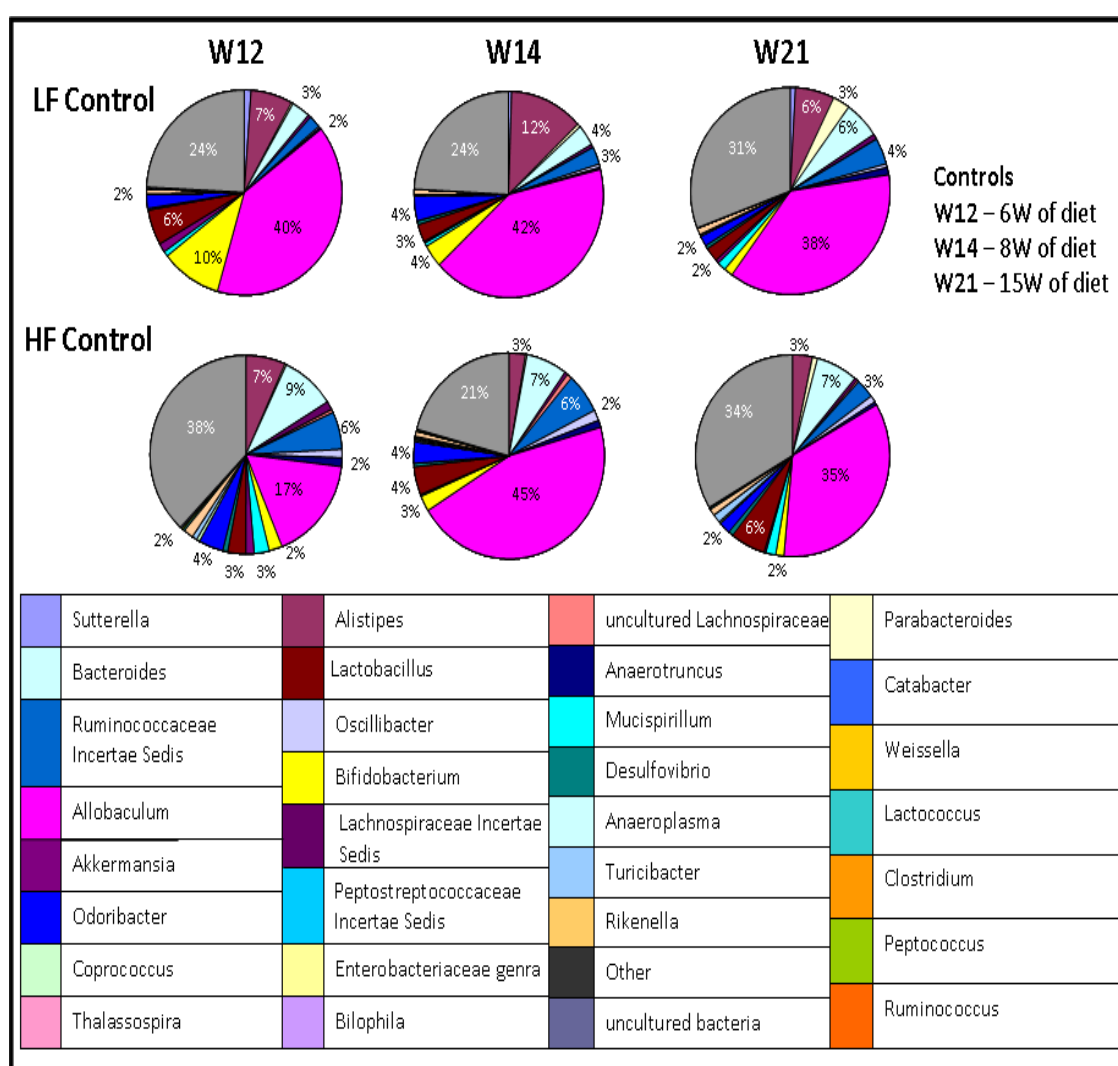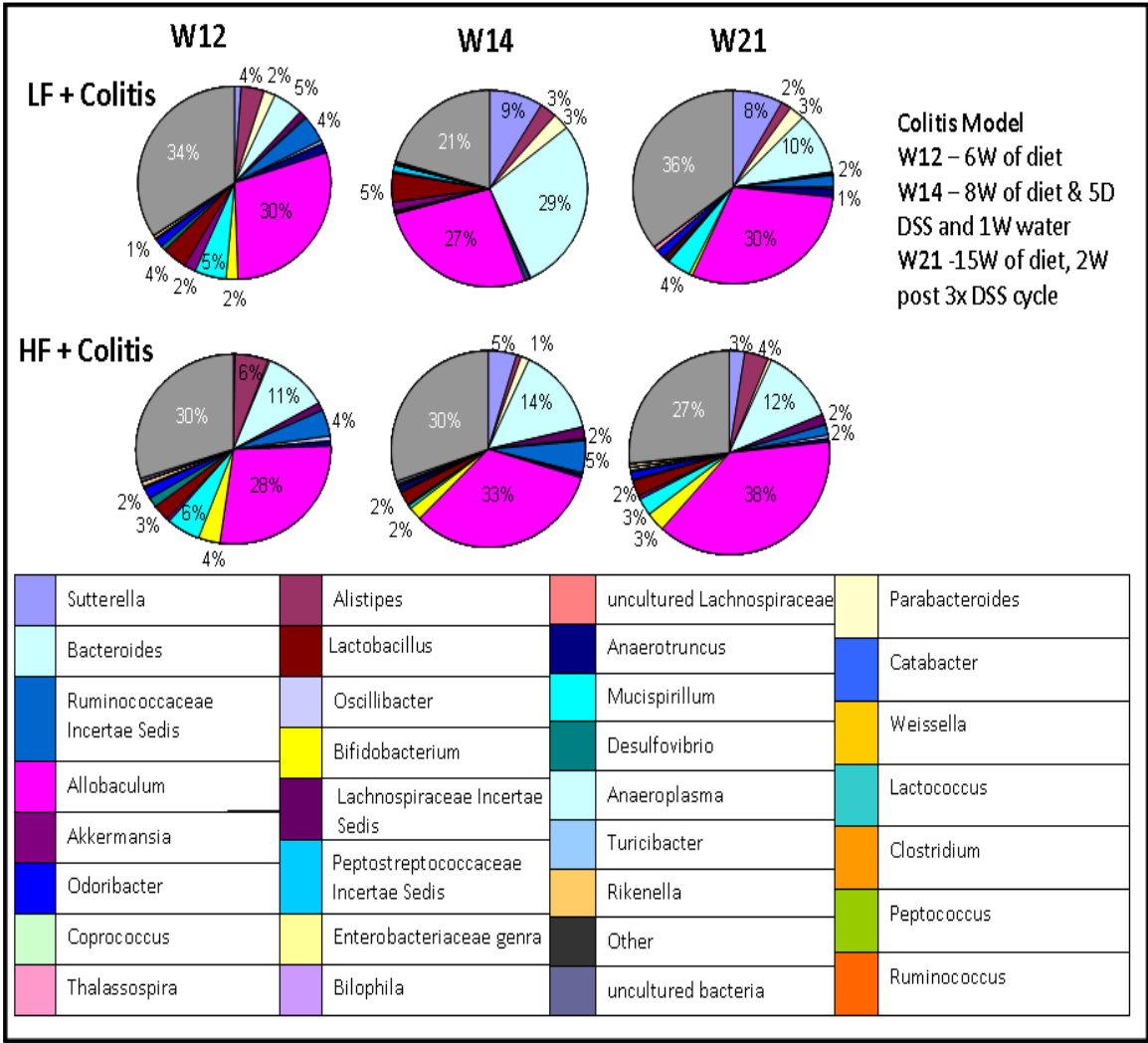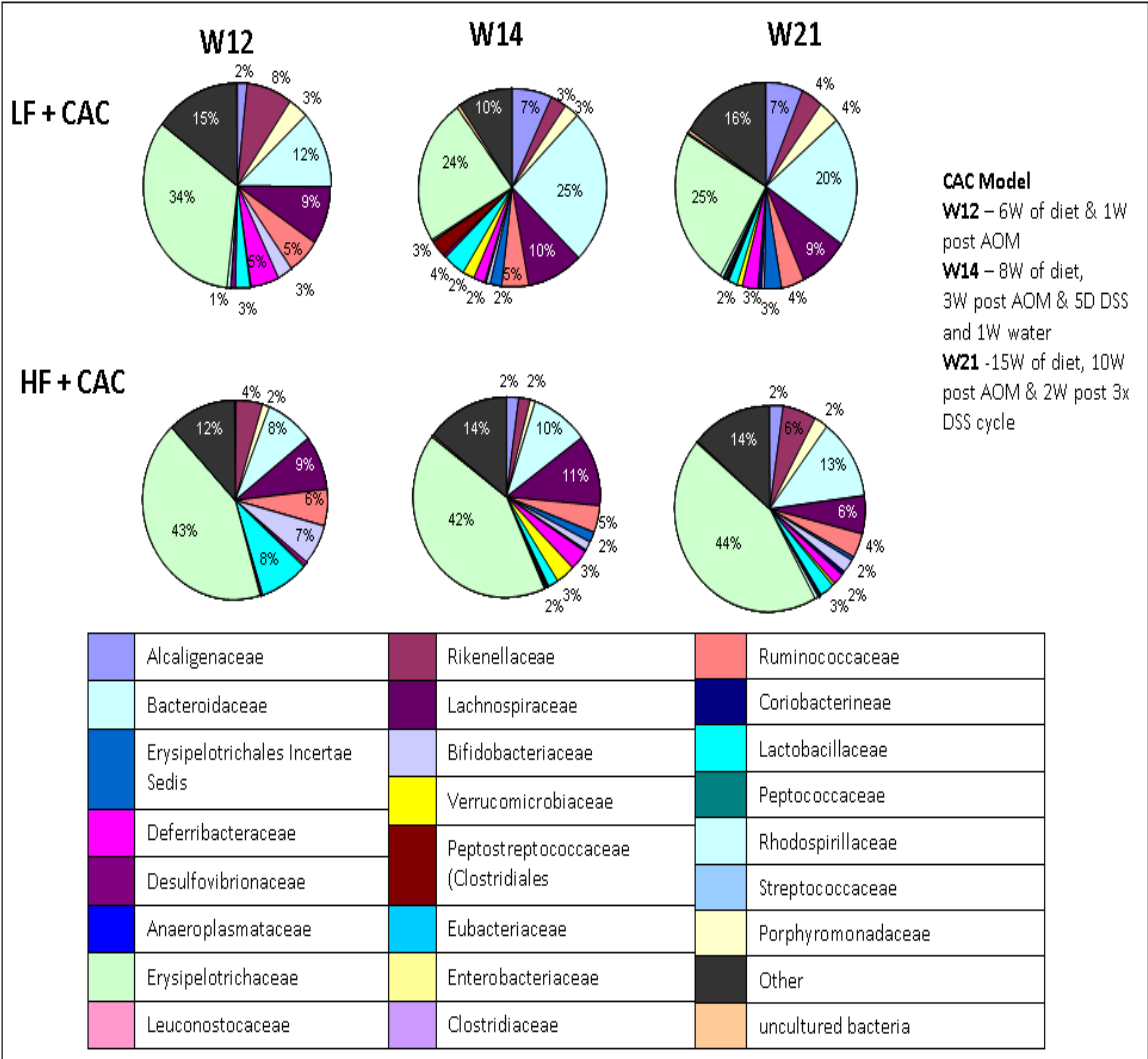
Supplementary Figure 4. Faecal microbiota composition at family level in colitis mice. The gut microbiota composition at family level of mice fed a HF diet is altered in colitis conditions compared to mice on a LF diet as determined by pyrosequencing of 16rRNA tags (V4) region. Data outside the pie charts represent the mean percentage read number for the corresponding colour coded family (n=7-11 mice/group).

Supplementary Figure 5. Faecal microbiota composition at genus level in colitis mice. The gut microbiota composition at genus level of mice fed a HF diet is altered in colitis conditions compared to mice on a LF diet as determined by pyrosequencing of 16rRNA tags (V4) region. Data outside the pie charts represent the mean percentage read number for the corresponding colour coded family (n=7-11 mice/group).

Supplementary Figure 6. Faecal microbiota composition at family level in mice with colitis-associated colorectal cancer (CAC). The gut microbiota composition at family level of mice fed a HF diet is altered in CAC conditions compared to mice on a LF diet as determined by pyrosequencing of 16rRNA tags (V4) region. Data outside the pie charts represent the mean percentage read number for the corresponding colour coded family (n=7-10 mice/group).

Supplementary Figure 7. Faecal microbiota composition at genus level in mice with colitis-associated colorectal cancer (CAC). The gut microbiota composition at genus level of mice fed a HF diet is altered in CAC compared to mice on a LF diet as determined by pyrosequencing of 16rRNA tags (V4) region. Data outside the pie charts represent the mean percentage read number for the corresponding colour coded family (n=7-10 mice/group).
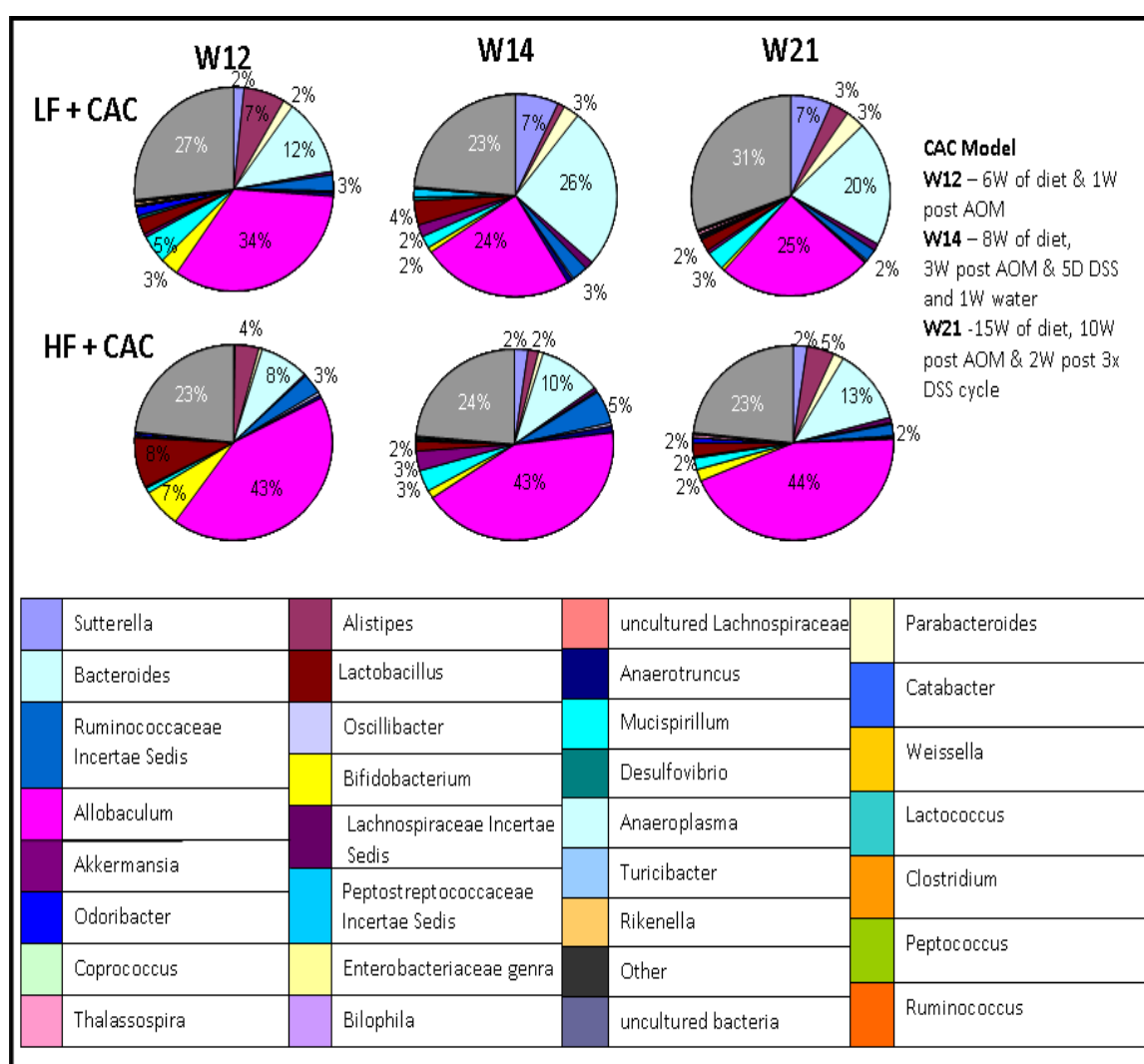
Chapter 6

Exercise and associated dietary extremes impact on gut microbial diversity

Siobhan Clarke Chapter Contributions:

Experimental:

- Designed and performed all experiments relating to the extraction and purification of DNA from faecal pellets.
- Generated amplicons for 454 pyrosequencing.

Results interpretation:

- Analysed all data in relation to 16S compositional sequencing analysis.
- Compiled all graphical interpretations relating to 16S compositional sequencing analysis.

Manuscript preparation:

Major contributor to manuscript preparation

6.1   ABSTRACT

The commensal microbiota, host immunity and metabolism participate in a signalling network, with diet influencing each component of this triad. In addition to diet, many elements of a modern lifestyle influence the gut microbiota but the degree to which exercise affects this population is unclear. Since extremes of exercise often accompany extremes of diet, we addressed the issue by studying professional athletes from an international rugby union squad. Two groups were included to control for physical size, age and gender. As expected, athletes and controls differed significantly with respect to plasma creatine kinase (a marker of extreme exercise), inflammatory and metabolic markers. More importantly, athletes had a higher diversity of gut microorganisms, which in turn positively correlated with protein consumption. The results provide evidence for a beneficial impact of exercise on the microbiota but also indicate that the relationship is complex and related to accompanying dietary extremes. The results provide rationale for a prospective controlled study.

6.2 INTRODUCTION

The commensal human gut microbiota has become the focus of converging interest from diverse disciplines, primarily because of its contribution to health and risk of disease throughout life (Sommer and Bäckhed, 2013, Sekirov *et al.*, 2010). The changing composition of the human microbiota is linked with changes in human behaviour (Shanahan, 2012). Many of the elements of a modern lifestyle, particularly in early life, influence the composition of the microbiota (O'Toole and Claesson, 2010). Disturbances of the microbiota at various ages may confer disease risk. In the elderly, the diversity and composition of the microbiota has been linked with various health parameters, including levels of inflammatory cytokines (Claesson *et al.*, 2012). The microbiota may also be a risk or protective factor in relation to immunoallergic and metabolic disorders (Le Chatelier *et al.*, 2013, Larmonier *et al.*, 2013, Bisgaard *et al.*, 2011, Sha *et al.*, 2012). Thus, a signalling network among the microbiota, host immunity and host metabolism has become evident, with diet influencing each component of this triad (Cotillard *et al.*, 2013, Jeffery and O'Toole, 2013).

Although obesity-related disorders have been linked with alterations in the microbiota (Qin *et al.*, 2012), the relationship between the microbiota and exercise or a sedentary lifestyle has received less attention. Some evidence suggests that exercise may modify the microbiota (Queipo-Ortuño *et al.*, 2013); therefore, we predicted that athletes should have a more diverse microbiota than their sedentary counterparts. To address this, we studied a professional rugby team while in the regulated environment of pre-season camp and performed a high-throughput DNA sequencing-based analysis of faecal microbiota with contemporaneous measurements of inflammatory cytokines and metabolic health. Since extremes of exercise are often associated with dietary extremes, the findings were correlated with diet. Because of the physical size of modern rugby players, two control groups were assessed; one matched for athlete size with a comparable body mass index (BMI) and another reflecting the background age- and gender-matched population. The results confirm differences in composition and diversity of the microbiota of athletes when compared to both control groups which correlate

with dietary differences, and which are linked with a more favourable metabolic and inflammatory profile.

6.3 RESULTS

6.3.1 Athletes have lower inflammatory and improved metabolic markers relative to controls

Subject characteristics are shown in Table 1. Despite increased levels of creatine kinase (CK) (Fig. 1), the athletes studied had a lower inflammatory status than controls (Supplementary Figs S1 and S2). Metabolic markers in athletes and low BMI controls were improved relative to the high BMI cohort (Supplementary Table S1 and Supplementary Figs S3 and S4).

6.3.2 The gut microbiota of athletes is more diverse than that of controls

A total of 1,217,954 (1.2 million) 16S rRNA reads were generated from faecal samples provided by elite athletes and controls, with an average of 14,736 (±6,234 s.d.) reads per athlete and 11,941 (±4,515 s.d.) reads per control. In both cases rarefaction curves established that extra sampling would be of limited benefit (Supplementary Fig. S5). Analysis of this data revealed that the alpha diversity of the elite athlete microbiota was significantly higher than that of the high BMI (Shannon index, Simpson) or both control groups (Phylogenetic diversity, Chao1, Observed species) (Fig. 2). The alpha diversity of the two control groups did not differ significantly from each other. Principal coordinate analysis based on unweighted Unifrac distances of the 16S rRNA sequences highlighted a clear clustering of the microbial populations of athletes away from that of controls (Fig. 3). This was further confirmed using hierarchical clustering (Supplementary Fig. S6). No separation was observed between control groups. None of the previously reported enterotypes were identified.

6.3.3 Proportions of several taxa are significantly higher in the gut microbiota of elite athletes relative to controls

Reads corresponding to 22 phyla, 68 families and 113 genera were detected in athlete faecal samples. In contrast, just 11 phyla, 33 families and 65 genera were detected in low BMI samples and 9 phyla, 33 families and 61 genera in high BMI samples. Pairwise comparisons of the elite athlete gut microbiota

and the high BMI controls revealed that athletes had significantly greater proportions of 48 taxa than high BMI controls while only one taxon, Bacteroidetes was significantly (P=0.022) less abundant in athletes (Supplementary Fig.S7). The top six flux changes in relative abundance were in the Firmicutes, *Ruminococcaceae, S24-7, Succinivibrionaceae, RC9 gut group* and *Succinivibrio*. Notably, there were significantly higher proportions of *Akkermansiaceae* (family; P=0.049) and *Akkermansia* (genus; P=0.035) in elite athletes compared to the high BMI controls. A comparison of the elite athlete gut microbiota with that of low BMI controls revealed significantly higher proportions of 40 taxa and lower proportions of only three taxa, *Lactobacillaceae* (P=0.001) *Bacteroides* (P=0.035) and *Lactobacillus* (P=0.001), in the former (Supplementary Fig. S8). The top six flux changes in relative abundance were noted among the *Prevotellaceae, Erysipelotrichaceae*, *S24-7, Succinivibrionaceae, Prevotella* and *Succinivibrio*. Pairwise comparisons of the microbiota of the control groups revealed differences in the proportions of seven taxa (Supplementary Fig. S9). At genus level, significantly greater proportions of *Dorea* (P=0.026) and *Pseudobutyrivibrio* (P=0.022) and significantly lower proportions of *Ruminococcaceae Incertae Sedis* (P=0.021) and *Akkermansia* (P=0.006) were observed in the high BMI, relative to low BMI, controls*.*

6.3.4 Protein accounted for considerably more of the total energy intake of athletes than of controls

As expected, total energy intake was significantly higher in athletes than either control group (Table 2). Athletes consumed significantly higher quantities of calories, protein, fat, carbohydrates, sugar and saturated fat per day than either of the control groups and consumed significantly higher quantities of fibre, monounsaturated fat and polyunsaturated fat than the high BMI control group (Table 2). Protein accounted for considerably more (22%) of the total energy intake of athletes, than of the low BMI (16%) and high BMI (15%) control groups (Table 2). While meat/meat products were the top contributors of dietary protein across all groups, supplements were the second highest (15%) contributor to protein in athletes and did not contribute considerably to protein consumption in controls (Supplementary Fig. S10).

Visualisation of dietary data with correspondence analysis highlighted a clear separation between the types of foods consumed by athletes and controls (Fig. 4a). The most discriminating food types were fruit, vegetables (athletes) and snacks (controls), whose consumption changes in a gradual manner along the y axis (Fig. 4b).

6.3.5 Exercise and protein intake as drivers of increased gut microbiota diversity in athletes

Correlations between health parameters (BMI, waist hip ratio, metabolic and inflammatory markers) or diet with the respective taxa or microbial diversity were examined using Pearson correlations. Significantly positive correlations were revealed between leptin levels and BMI, body fat percentage and waist hip measurement, while significant negative correlations between leptin and lean body mass (Supplementary Fig. S11), as well as between adiponectin levels and both BMI and lean body mass (Supplementary Fig. S2), were observed. Significantly positive associations was noted between microbial diversity and protein intake (Fig. 5), CK levels (Fig. 6) and urea (Supplementary Fig. S13). No correlations were observed after adjustment for multiple testing using FDR values between microbial taxa and health parameters.

6.4   DISCUSSION

Although the relationship between diet, the gut microbiota, host immunity and host metabolism is becoming more evident (Cotillard *et al.*, 2013, Jeffery and O'Toole, 2013), the relationship between the microbiota and exercise has not been fully explored. Our findings show that a combination of exercise and diet impact on gut microbial diversity. In particular, the enhanced diversity of the microbiota correlates with exercise and dietary protein consumption in the athlete group.

Diversity is important in all ecosystems to promote stability and performance. Microbiota diversity may become a new biomarker or indicator of health (Shanahan, 2010). Loss of biodiversity within the gut has been linked to an increasing number of conditions such as autism, gastrointestinal diseases and obesity associated inflammatory characteristics (Claesson *et al.*, 2012, Kang *et al.*, 2013, Ott *et al.*, 2004, Le Chatelier *et al.*, 2013, Chang *et al.*, 2008). In this study, the diversity of the athlete gut microbiota was significantly higher than both control groups matched for physical size, age and gender. The proportions of several gut microbial taxa were also altered in athletes relative to controls. However, few differences were seen between the two control cohorts. Of note, the athletes and low BMI group had significantly higher proportions of the genus *Akkermansia* levels than the high BMI group. *Akkermansia muciniphilla* has been identified as a mucin-degrading bacteria that resides in the mucus layer and its abundance has been shown to inversely correlate with obesity and associated metabolic disorders in both mice and humans (Everard *et al.*, 2013, Karlsson *et al.*, 2012). Everard *et al.,* 2013 recently showed that feeding *Akkermansia*, or restoration of *Akkermansia* levels by prebiotic treatment in diet-induced obese mice, correlated with an improved metabolic profile possibly due to enhanced barrier function.   Interestingly, the athletes had lower inflammatory and improved metabolic markers relative to controls, and in particular the high BMI controls, demonstrating the enhanced health profile of this group. While the microbiota diversity of the two control groups did not differ significantly, it was noted that alpha diversity in the high BMI controls was numerically, although not significantly, lower than that of their low BMI counterparts. Reductions in

the diversity of microbial populations in high BMI individuals has been reported in a recent study with a larger cohort size (Le Chatelier *et al.*, 2013) which also showed that dietary intervention can increase microbiota diversity in an obese cohort (Cotillard *et al.*, 2013).

Rugby is a vigorous contact sport requiring considerable fitness and increased dietary requirements (Lundy *et al.*, 2006). The levels of plasma CK (a marker of extreme exercise) and creatinine were significantly elevated in the elite athlete group consistent with the high exercise loads (Brancaccio *et al.*, 2007, Lazarim *et al.*, 2009, McLellan *et al.*, 2010). Furthermore, diet was significantly different from that of controls, with increased intake of calories, protein, fat and carbohydrate. Diversity in the diet has been linked to microbiota diversity (Claesson *et al.*, 2012). In our study, microbiota diversity indices positively correlated with protein intake and CK suggesting that both diet and exercise are drivers of biodiversity in the gut. The protein and microbiota diversity relationship is further supported by a positive correlation between urea levels, a by-product of diets that are rich in protein, and microbiota diversity. Long-term diets have been linked to clusters in the gut microbiota with protein and animal fat associated with *Bacteroides* and simple carbohydrates with *Prevotella* (Wu *et al.*, 2011). Fermentation of protein has also been suggested to result in the production of various potentially toxic products, such as amines and NH_3, and in one report, with growth of potential pathogens (Rist *et al.*, 2013). In contrast, feeding of whey protein to mice mediates against the negative effects of a high fat diet (McAllana *et al.*, submitted, Tranberg *et al.*, 2013). Indeed, in athletes, whey protein supplements represented a significant component of the protein intake in athletes but not controls. Whey protein has been associated with reductions in body weight and increased insulin sensitivity in the past and is frequently a major component of the athlete diet (Tipton and Wolfe, 2004, Belobrajdic *et al.*, 2004). Taken together, our results suggest that the relationship between exercise, diet and the gut microbiota warrants further investigation.

In conclusion, exercise seems to be another important factor in the relationship between the microbiota, host immunity and host metabolism, with diet playing an important role. Further, intervention-based studies to tease apart this relationship will be important and provide further insights into

optimal therapies to influence the gut microbiota and its relationship to health and disease.

6.5 MATERIALS AND METHODS

6.5.1 Subjects characteristics

Male elite professional rugby players (n=40) were recruited for this study; the mean age of the athletes was 29 (±4) years and they had a mean BMI of 29.1 (±2.9). Healthy male controls were recruited from the Cork city and county region of Ireland; the mean age of controls was 29 (±6) years. Two groups of control were specifically recruited based on their physical size (BMI) relative to the athletes, with group 1 (n=23) having a BMI of less than or equal to 25 and group 2 (n=23) having a BMI of greater than 28 (Supplementary Table 2). All subjects except one (Indian ethnicity) were of Irish ethnicity and all subjects gave written informed consent prior to the beginning of the study. This study was approved by the Cork Clinical Research Ethics Committee. Exclusion criteria included having a BMI between 25 and 28, antibiotic treatment within the previous 2 months or suffering from any acute or chronic cardiovascular, gastrointestinal or immunological condition.

6.5.2 Experimental design

Faecal and blood samples were collected from all participants. DNA was extracted from fresh stool samples which were stored on ice prior to use. Each participant was interviewed by a nutritionist and completed a detailed food frequency questionnaire (FFQ). Body composition analysis data from dual-energy X-ray absorptiometry (DXA) scans were received from the Irish Rugby Football Union (IRFU) for all athletes, DXA scans for controls were performed in University Hospital Cork, waist:hip measurements were taken for both athletes and controls.

6.5.3 Nutritional and clinical data collection

Dietary data were collected by means of a FFQ which was administered by a research nutritionist. The FFQ was an adapted version of that used in the UK arm of the European Prospective Investigation into Cancer (EPIC) study (Bingham *et al.*, 1997) which in turn, is based on the original Willett FFQ (Willett *et al.*, 1988, Willett *et al.*, 1985). To more comprehensively reflect the Irish diet, the 130-food item EPIC FFQ was extended to include an additional

57 food items. Participants were asked to recall dietary intakes over the previous 4-weeks. A photographic food atlas was used to pictorially quantify foods and beverages (Nelson *et al.*, 1997). Manufacturer's weights on packaging and household measures were also used to quantify foods. Intakes of nutritional supplements were recorded. Completed FFQ's were coded and quantified by researchers and entered in the Weighed Intake Software Package (WISP©) (Tinuviel Software, Anglesey, UK), which uses McCance and Widdowson's *The Composition of Foods*, sixth edition plus all supplemental volumes to generate nutrient intake data (Roe *et al.*, 2002). Data were subsequently imported into SPSS© version 18 (SPSS Inc., Chicago, USA) for analysis. Dietary data was visualised with correspondence analysis (R statistical package version 2.13.1) (RDevelopmentCoreTeam, 2010). Fasting blood samples were collected and analysed at the Cork Mercy University Hospital clinical laboratories. Commercial multi-spot microplates (Meso Scale Diagnostics) were used to measure cytokines.

6.5.4 Control physical activity levels

As the athletes were involved in a rigorous training camp we needed to assess the physical activity levels of both control groups. To determine this we used an adapted version of the EPIC-Norfolk questionnaire (Wareham *et al.*, 2002). T-tests were carried out to compare high BMI and low BMI controls.

6.5.5 DNA extraction and high-throughput amplicon sequencing

Stool samples were stored on ice until processed. DNA was purified from fresh stool samples using the QIAmp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK) according to manufacture's instructions with addition of a bead-beating step (30s x 3) and stored at -20°C. The microbiota composition of the samples was established by amplicon sequencing of the 16S rRNA gene V4; universal 16S rRNA primers estimated to bind to 94.6% of all 16S genes (i.e. the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAAAGNG), R2 (TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP'S Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) were employed for PCR amplification.

Molecular identifier tags were attached between the 454 adaptor sequence and the target-specific primer sequence, allowing for identification of individual sequences from the pooled amplicons. Ampure purification system (Beckman Coulter, Takeley, UK) was used to clean the amplicons before being sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) in line with 454 protocols at the Teagasc high throughput sequencing centre. DNA sequence reads from this study are available from the Sequence Read Archive (accession numbers PRJEB4609)

6.5.6 Bioinformatic analysis

The Stoney supercomputer at the Irish Centre for High End Computing (ICHEC) was used for the following analysis. Raw sequences were quality trimmed using the Qiime Suite of programmes (Caporaso *et al.*, 2010), any reads not meeting the quality criteria of a minimum quality score of 25 and sequence length shorter than 150bps for 16S amplicon reads. The SILVA 16S rRNA (version 106) database was employed to BLAST the trimmed fasta sequence files using default parameters (Altschul *et al.*, 1997, Pruesse *et al.*, 2007). Parsing of the resulting BLAST output files was achieved through Megan which uses a lowest common ancestor algorithm to assign reads to NCBI taxonomies (Huson *et al.*, 2007). Filtering was carried out within MEGAN using bit scores prior to tree construction and summarization, similar to previous studies a bit-score cut-off of 86 was selected (Urich *et al.*, 2008). Clustering of sequence reads into operational taxonomical units (OTUs) at 97% identity level was achieved using Qiime. The ChimeraSlayer program was used to remove chimeras from aligned OTU and the FastTreeMP tool generated a phylogenetic tree (Haas *et al.*, 2011, Price *et al.*, 2010). Alpha diversity indices and rarefaction curves were generated using Qiime. Beta diversities were also calculated on the sequence reads based on weighted and unweighted Unifrac and bray curtis distance matrices; subsequently principal coordinate analysis (PCoA) and unweighted pair group method with arithmetic mean (UPGMA) clustering was performed on the samples. KiNG viewer and Dendroscope software were used to visualise PCoA plots and UPGMA clustering respectively (Chen *et al.*, 2009). Enterotype clustering was

carried out according to the approach previously described (Arumugam *et al.*, 2011).

6.5.7 Statistical methods

Statistical analysis was carried out using GraphPad Prism version 5.04 (La Jolla, California, USA) R statistical package (version 2.13.1), and SPSS software package version 18 (SPSS Inc., Chicago, USA)(RDevelopmentCoreTeam, 2010). Kruskal–Wallis and Mann–Whitney tests were used to find significant differences in microbial taxa, alpha diversity, clinical and biochemical measures. Adjustment for multiple testing was estimated using the FDR functions (phylum and family level) in the R statistical package (version 2.13.1) using the Benjamini & Hochberg method (Benjamini and Hochberg, 1995).

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Table 1 Subject Characteristics

	Elite Athletes (n = 40)	Low BMI Controls (n = 23)	High BMI Controls (n = 23)
Age (years)	28.8±3.8	28.1± 5.1	30.8±5.6
BMI (kg/m2)	29.1±3.0$^+$	22.7±1.8**	31.2±3.0¥
Body mass (Kg)	101.3±13.8	74.3±6.3	103.1±13.8
Body Fat % (Kg)	16.9±6.1^{++}	15±4.6*	33.9±8.8¥
Lean body mass (Kg)	80±8.9^{++}	55.4±5.6**	65±8¥
Waist/Hip Ratio	0.8±0.04^{++}	0.8±0.05	0.9±0.07¥

Data shown as mean ± SD, *$P<0.01$ or **$P<0.0005$ athletes versus low BMI controls $^+P<0.01$ or $^{++}P<0.0005$ athletes versus high BMI controls ¥$P<0.0005$ high BMI versus low BMI controls. Note only data for 39 athletes was available for waist hip ratio while only data for 22 controls was assessed for fat (Kg) and lean (Kg).

Table 2 Macronutrient intake in study participants

Macronutrients	Recommended daily intakes	Athletes (n=40)		Control BMI <25 (n=23)		Control BMI >28 (n=23)	
		Median	IQR	Median	IQR	Median	IQR
Energy (Kcal)	2400-2800(FSAI, 2011)	4449**	3610-5656	2937	2354-3917	2801	2358-3257
Protein (g)		248**	192-305	117	83-144	105	88-131
Protein (g/kg bw)		2.36**	0.99-4.42	1.55†	0.88-2.82	1.1	0.55-1.66
Fat (g)		131**	113-186	100	80-152	101	78-127
Saturated fat (g)		44**	35-55	37	28-45	33	30-49
Monounsaturated fat (g)		41*	32-57	35	25-48	32	25-42
Polyunsaturated fat (g)		18*	16-31	17	12-29	15	11-22
Carbohydrate (g)		572**	442-875	375	288-529	316	266-423
Sugars (g)	≤10% TE (FSAI, 2011)	330**	250-569	163	131-256	159	102-247
Fibre (g)	≥25g (FSAI, 2011)	39*	32-51	30	25-36	25	19-33
% Total energy from protein	10-35% TE (FNB, 2005)	22	17-27	16	11-20	15	13-19
% Total energy from total fat	20-35% TE (FSAI, 2011)	27	23-37	31	25-47	32	25-41
% Total energy from saturated fat	≤10% TE (FSAI, 2011)	9	7-11	11	9-14	11	10-16
% Total energy from carbohydrate	45-65% TE (FNB, 2005)	49	39-79	51	40-72	45	38-60

** P value ≤ 0.05 between athletes and both control groups. * P value ≤ 0.05 between athletes and >28 controls. † P value ≤ 0.05 between control groups.

Figure 1. Creatine kinase levels elevated in athletes compared to controls. Plasma creatine kinase levels are significantly elevated in athletes (n=39) compared to low (n=23) and high (n=23) BMI controls. Results are expressed as mean value ± SEM. Statistical significance was determined by Mann-Whitney for each pair-wise comparison.
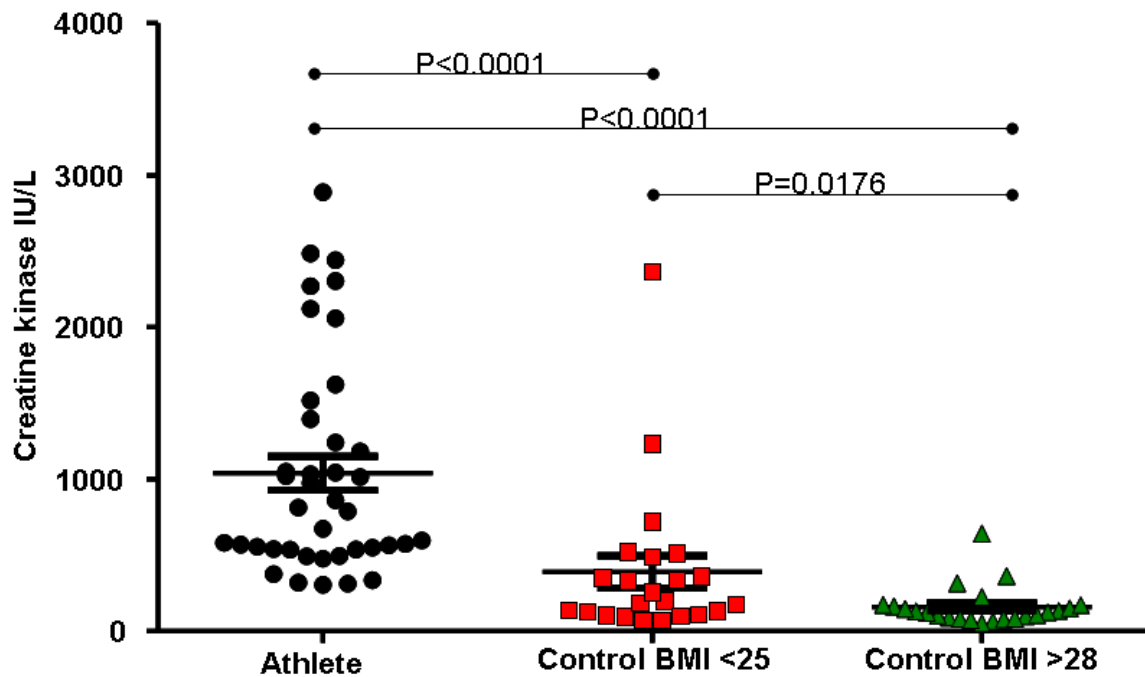
Figure 2. Increased α diversity in athletes compared to controls.
Comparison of microbiota indices across the three cohorts. a, Phylogenetic diversity; b, Shannon index; c, Simpson; d, Chao1 and e, Observed species. Mann-Whitney tests were performed for each pair-wise comparison. * $p<0.05$ ** $p<0.009$ *** $p<0.009$. Kruskal Wallis p values refer to tests performed across all three groups.
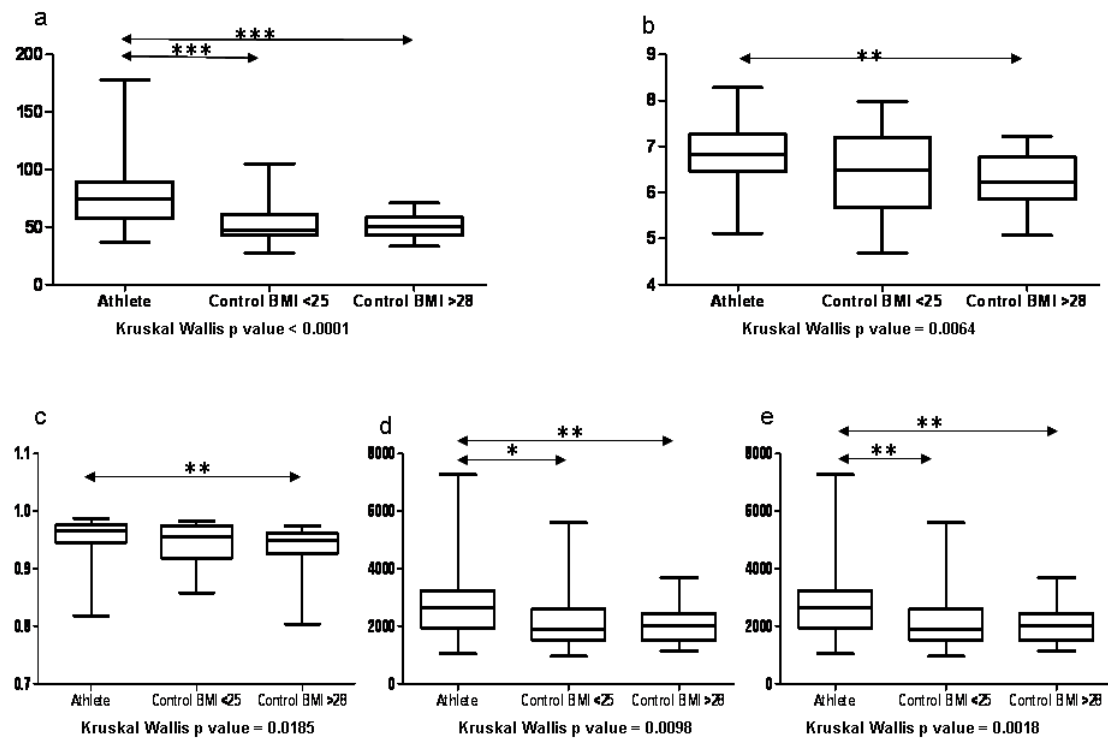
Figure 3. Unweighted UniFrac separates the athlete and control microbiota. Unweighted UniFrac PCoA of faecal microbiota from 86 subjects. Subject colour coding: black, elite athletes; green, high BMI controls; and red, low BMI controls.
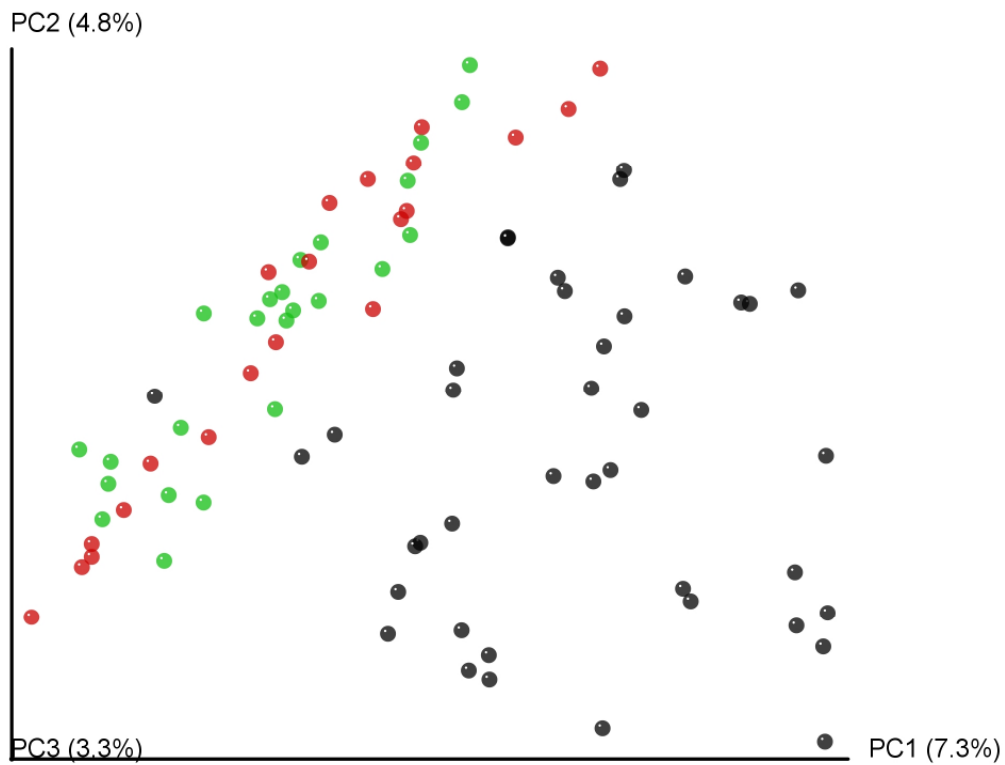
Figure 4. Dietary patterns separate elite athletes from controls. Food correspondence analysis, A, Food frequency data visualised by correspondence analysis. By describing subjects' responses according to their dominant sources of variation (food types), correspondence analysis can produce a single score for a subject from multiple measurements. Colour codes green-high BMI controls, red-low BMI controls and black-elite athletes. B, Driving food types. Using the same scoring system, food can be evaluated and plotted on the same axis, providing a visual aid to get an overall view of how food drives the clustering. Colour codes green-meat, red-fish, blue-bread and cereal, brown-eggs, yellow-carbohydrates, cyan-dairy, maroon-spreads and sauces, orange-fruit and vegetables, black-snacks, violet-non alcoholic beverages and dark grey-alcohol.
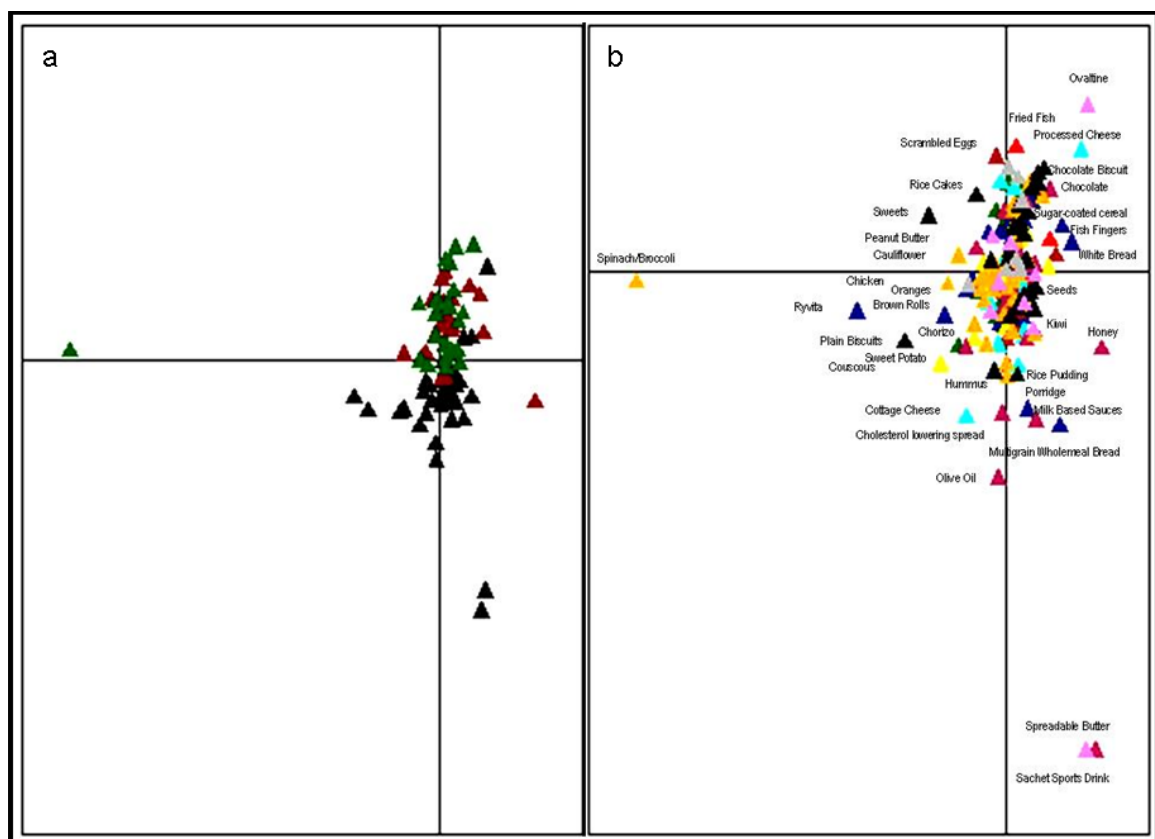
Figure 5. Protein intake positively correlates with α diversity. Subject colour coding: black, elite athletes; green, high BMI controls; and red, low BMI controls.
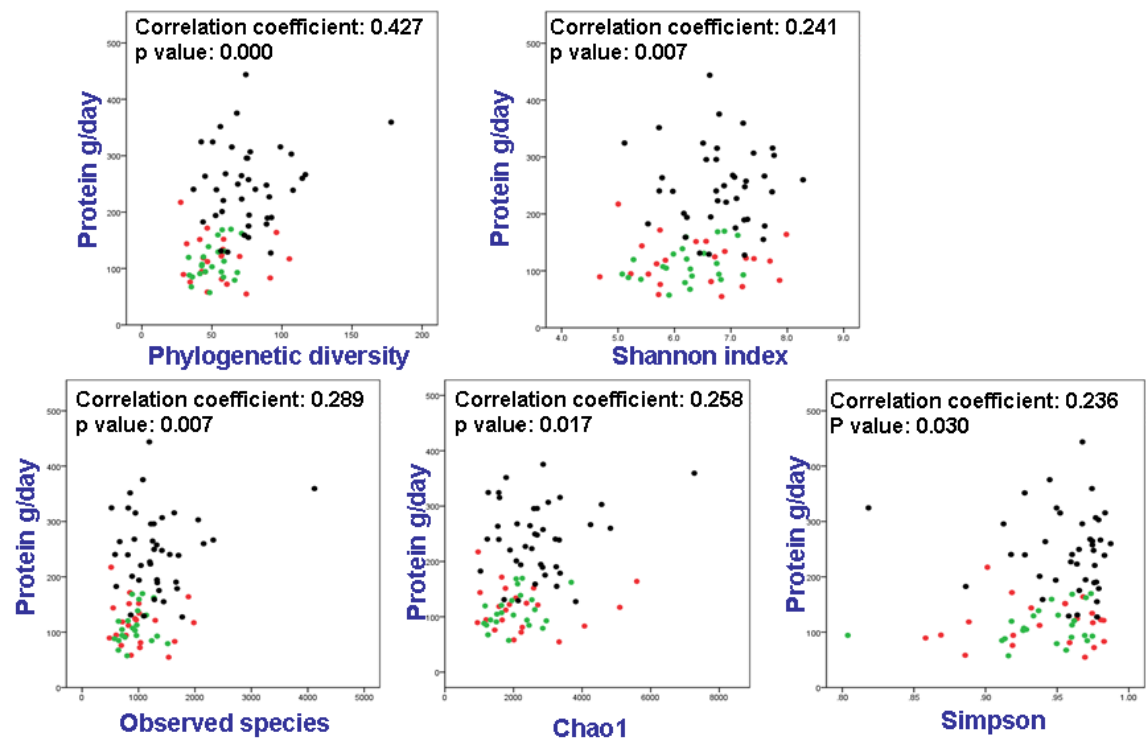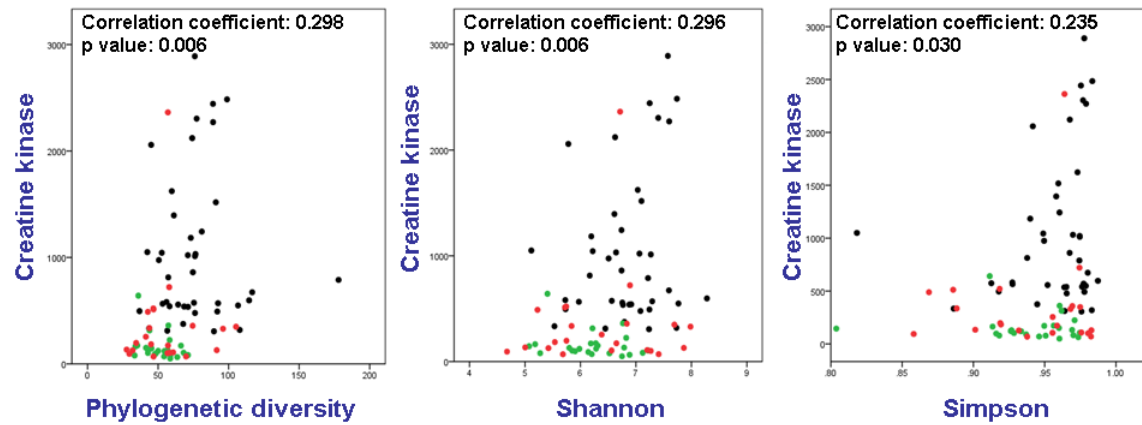
Figure 6. Creatine kinase positively correlates with α diversity. Subject colour coding: black, elite athletes; green, high BMI controls; and red, low BMI controls.

SUPPLEMENTARY INFORMATION

Supplementary Table S1 Metabolic, inflammatory and other markers

|	Athletes n=40	Control BMI >28 n=23*	Controls BMI <25 n=23*
Glucose (mmol/L)	4.94±0.1	4.95±0.1*	4.69±0.1*
CRP (mg/dL)	1.12±0.1	1.94±0.5	2.18±0.7
Adiponectin (ug/ml)	7.02±0.39	8.5±0.6	9.8±0.6#
Insulin (pg/ml)	227.88±24.6▲	469.1±84.9	490.3±123.04#
Leptin (pg/ml)	1411.01±234.5▲	10382.5±2117.9†	4237.1±1937.6
IFN-γ (pg/ml)	5.2±2.6	1.88±0.2	1.7±0.2
IL-10 (pg/ml)	8.78±3.8	2.67±0.3	63.5±53.4
IL-12p70 (pg/ml)	7.2±3.1	1.97±0.2	11.47±5.9
IL-1B (pg/ml)	0.5±0.05▲	1.56±0.4	5.6±4.8
IL-6 (pg/ml)	0.79±0.19▲	0.82±0.1	4.2±3.5
IL-8 (pg/ml)	10.45±5.8▲	2.79±0.4	3.2±0.3#
TNF-α (pg/ml)	4.86±0.2▲	6.68±0.4	32.7±27.6
Sodium (mmol/L)	139±0.2▲	139.2±0.7	139.52±0.6
Potassium (mmol/L)	5.89±0.1▲	4.2±0.1†	4.62±0.3#
Urea (mmol/L)	8.57±0.2▲	5.2±0.2†	6.07±0.2#
Creatine (μmol/L)	97.42±2▲	82.44±2	88.48±3.9#
CK (IU/L)	1038.17±112▲	159.44±26.9†	389.52±105.7#
AST (IU/L)	45.65±2.4▲	23.95±3.2	26.57±2.3#
YGT (IU/L)	27.90±3.2	32.0±5.2†	23.44±4.8#
Alkaline Phosphatase (IU/L)	68.15±2.2	64.7±2.7	64.22±4.2
Total Bilirubin (μmol/L)	15.97±0.8	14.44±1.2	16.74±2.57
Total Cholesterol (mmol/L)	4.91±0.2	7.11±2.3	4.5±0.2
HDL (mmol/L)	1.44±0.04▲	1.2±0.4†	1.39±0.05
LDL (mmol/L)	3.07±0.1	2.94±0.1	2.73±0.2
Triglycerides (mmol/L)	0.86±0.05▲	7.6±5.9†	0.84±0.1
Total Protein (g/L)	71.03±0.5▲	73.95±0.9	73.74±0.8#
Albumin (g/L)	41.45±0.3▲	43.8±0.6	44.44±0.4#
Globulin (g/L)	29.58±0.43	30.2±0.7	29.3±0.7
Calcium (mmol/L)	2.43±0.01	2.41±0.2	2.34±0.1

Data is expressed as mean value ± standard error of mean. All intergroup comparisons were made using the Mann Whitney U test. *n=22, # $P<0.05$ between athletes and low BMI controls. ▲ $P<0.05$ between athletes and high BMI controls. † $P<0.05$ between high BMI and low BMI controls.

**Supplementary Table S2** Control physical activity assessment

	BMI<25	BMI >28
Walking		
winter (hours per week)	6.26	4.30
summer (hours per week)	7.04	5.07
Cycling		
winter (hours per week)	3.00	0.39
summer (hours per week)	3.61	0.98
Gardening		
winter (hours per week)	0.28	0.21
summer (hours per week)	0.98	0.53
Physical exercise e.g. aerobics, dancing swimming etc		
winter (hours per week)	1.16	0.61
summer (hours per week)	3.74	2.61
DIY (hours per week)	4.22	3.41
Housework (hours per week)	4.41	4.13
How many hours weekly of vigorous activity (sweating/ ↑ heart rate)	7.21	3.85
No. flights of stairs per day	7.17	9.09

Values shown are expressed as mean. t-test comparisons between low BMI and high BMI controls was undertaken.

Supplementary Figure S1. Cytokine levels vary between cohorts. Scatter plot of A, Interlukin-1β; B, Interlukin-8; C, Interlukin-6; and D, TNF-α. (n=40 Athletes; n=23 low BMI control and n=23 high BMI control). Data expressed as mean value ± SEM. Statistical significance was determined by Mann-Whitney for each pair-wise comparison. Note one low BMI control was removed from these graphs due to elevated cytokine values associated with completion of a marathon prior to blood collection. Removal of this control changes the p value between athletes and low BMI controls to p=0.0051 for IL-8. Two new significant results also appear between athletes and low BMI controls in IL-6 p=0.0373 and low BMI controls and high BMI controls in TNF-α p=0.0121.
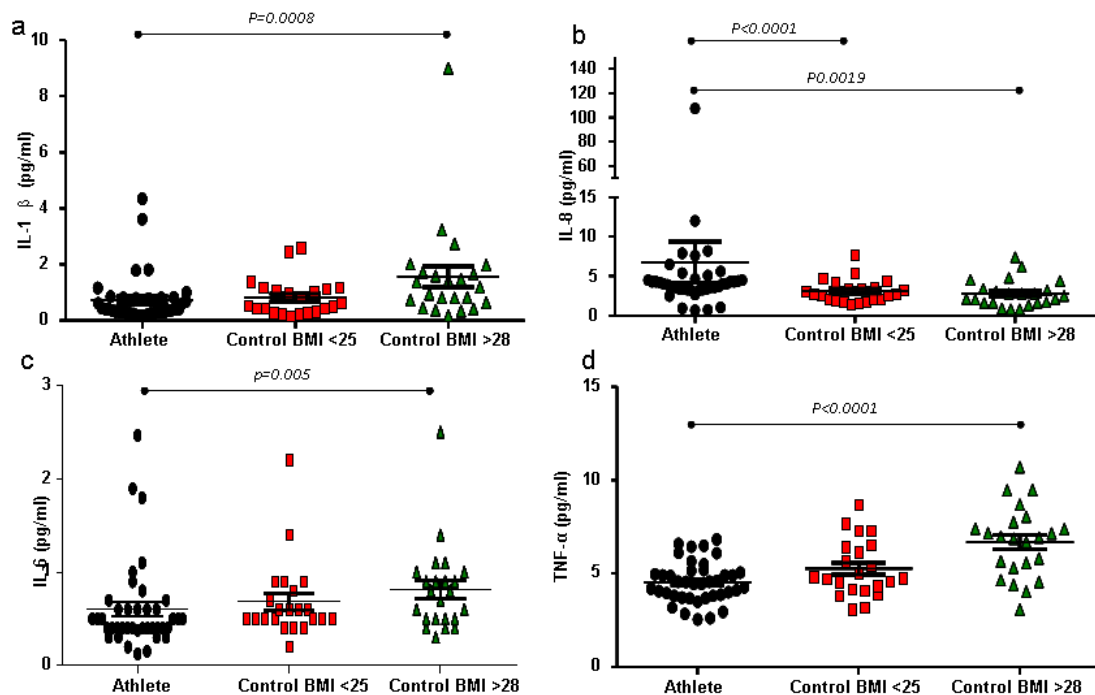
Supplementary Figure S2. Differences in the blood biochemistry of athletes and controls. Scatter plot of A, creatinine; B, sodium; C, potassium; D, urea; E, AST; and F, YGT. Mann- (n=40 Athletes; n=23 low BMI control and n=23 high BMI control). Data expressed as mean value ± SEM. Statistical significance was determined by Mann-Whitney for each pair-wise comparison.
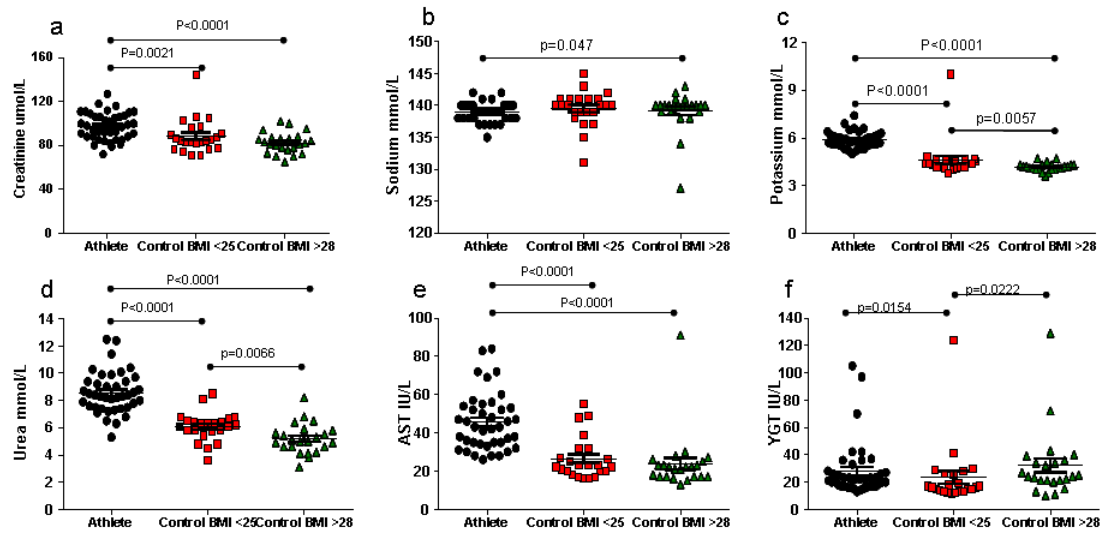
Supplementary Figure S3. Study participants blood biochemistry. Scatter plot of A, HDL; B, triglycerides; C, total protein; and D, albumin. (n=40 Athletes; n=23 low BMI control and n=23 high BMI control). Data expressed as mean value ± SEM. Statistical significance was determined by Mann-Whitney for each pair-wise comparison.
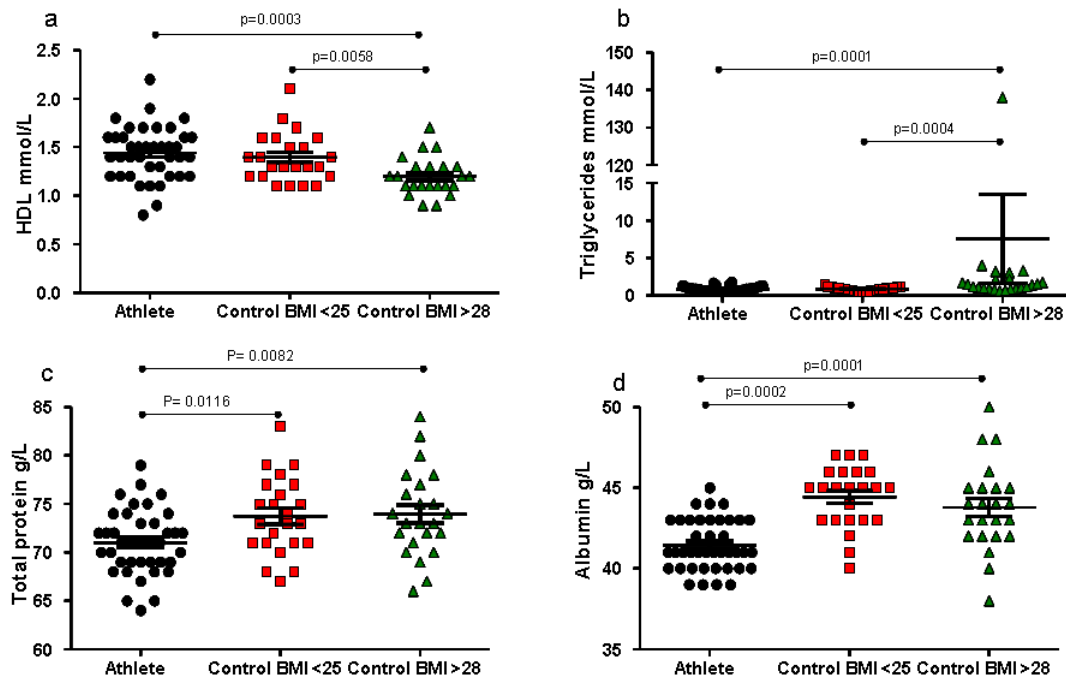
Supplementary Figure S4. Significantly lower leptin and insulin levels seen in elite athletes. Scatter plot of A, insulin; B, adiponectin and C, leptin across the three cohorts. (n=40 Athletes; n=23 low BMI control and n=23 high BMI control). Data expressed as mean value ± SEM. Statistical significance was determined by Mann-Whitney for each pair-wise comparison.
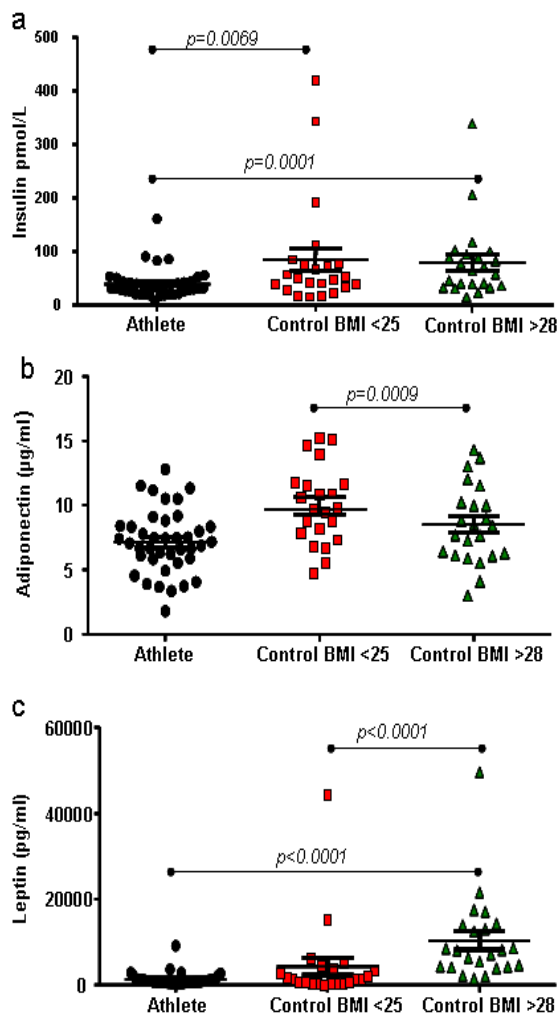
Supplementary Figure S5. Rarefaction curves for a, controls and b, athletes at 97% similarity levels. Amount of operational taxonomic units (OUT's) found as a function of the number of sequence tags sampled.
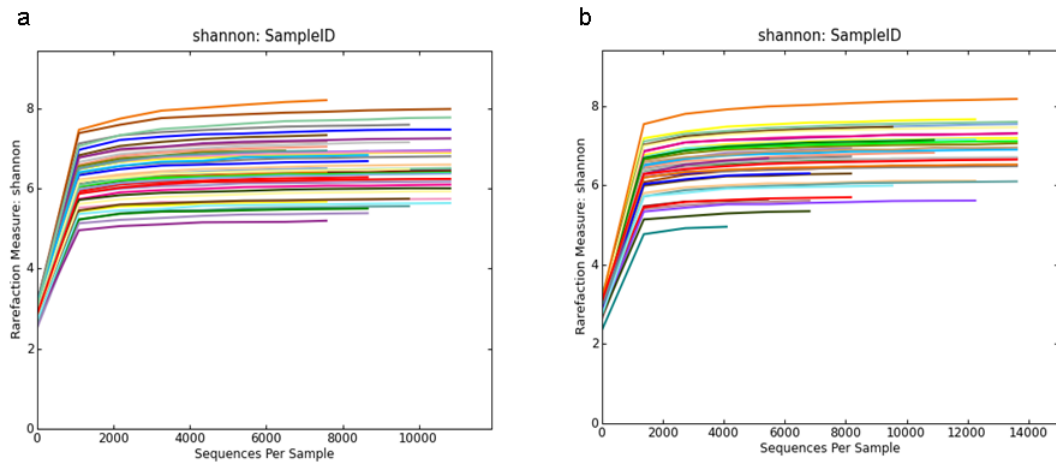
Supplementary Figure S6. Unweighted pair group method with arithmetric mean (UPGMA) tree of all subjects. Subject colour coding: black, elite athletes; green, high BMI controls; and red, low BMI controls.
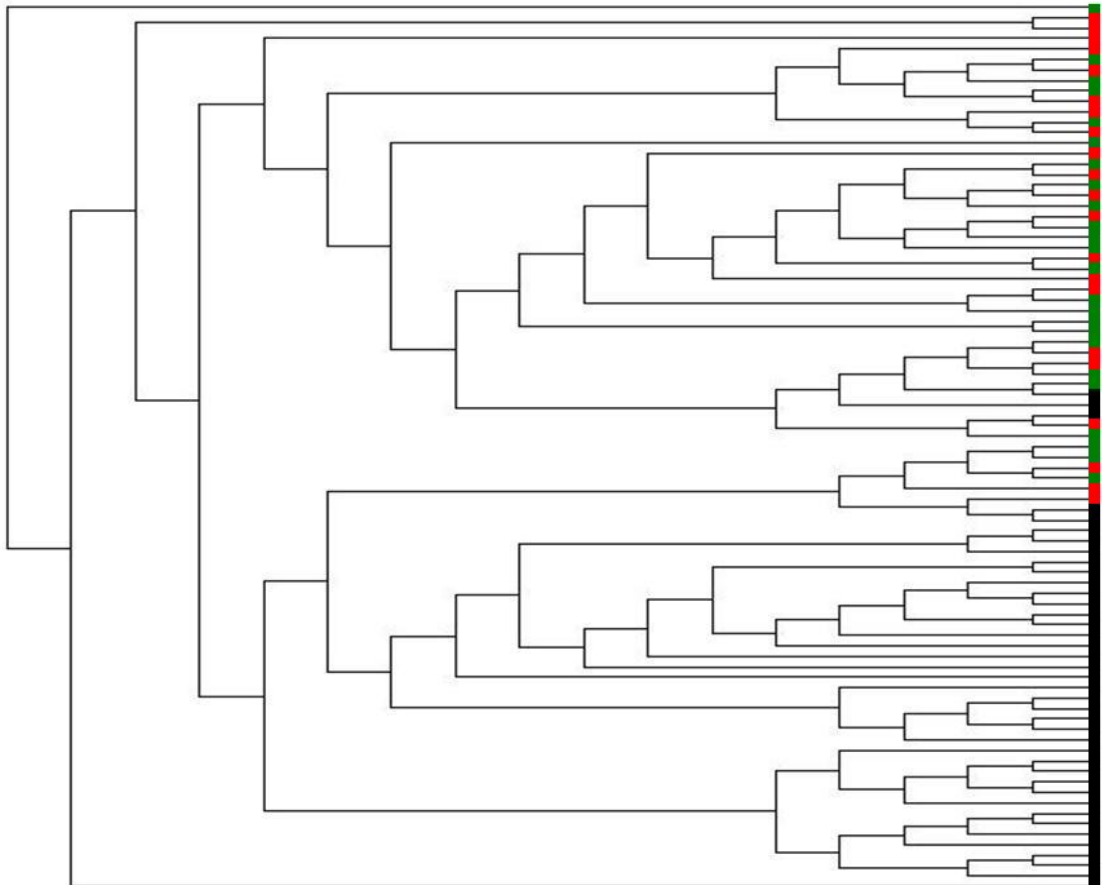
Supplementary Figure S7. Relative change in the abundance of selected taxa in athletes relative to high BMI controls. Only taxa with significant differences in population numbers are illustrated.
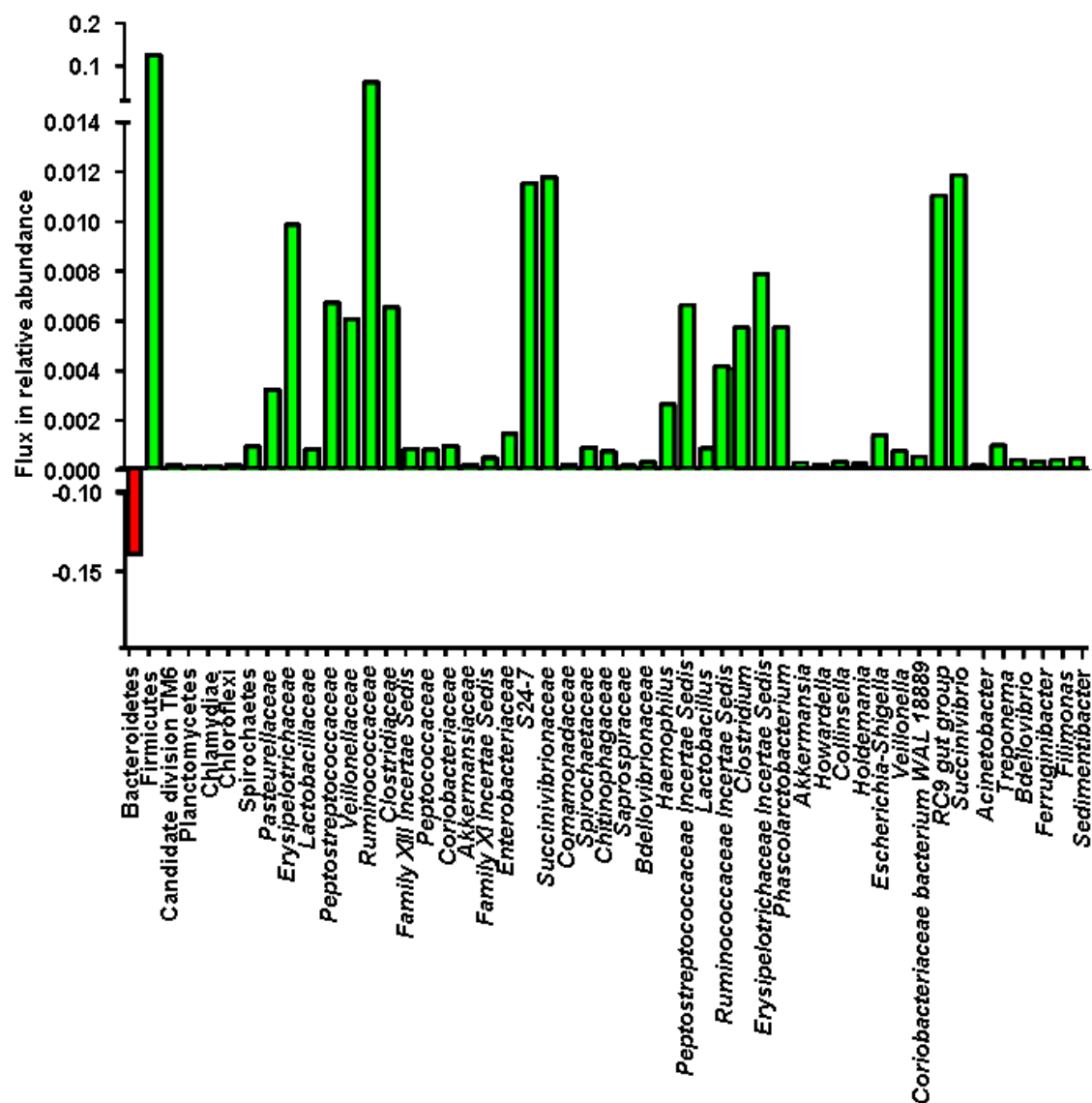
Supplementary Figure S8. Relative change in the abundance of selected taxa in athletes relative to low BMI controls. Only taxa with significant differences in population numbers are illustrated.
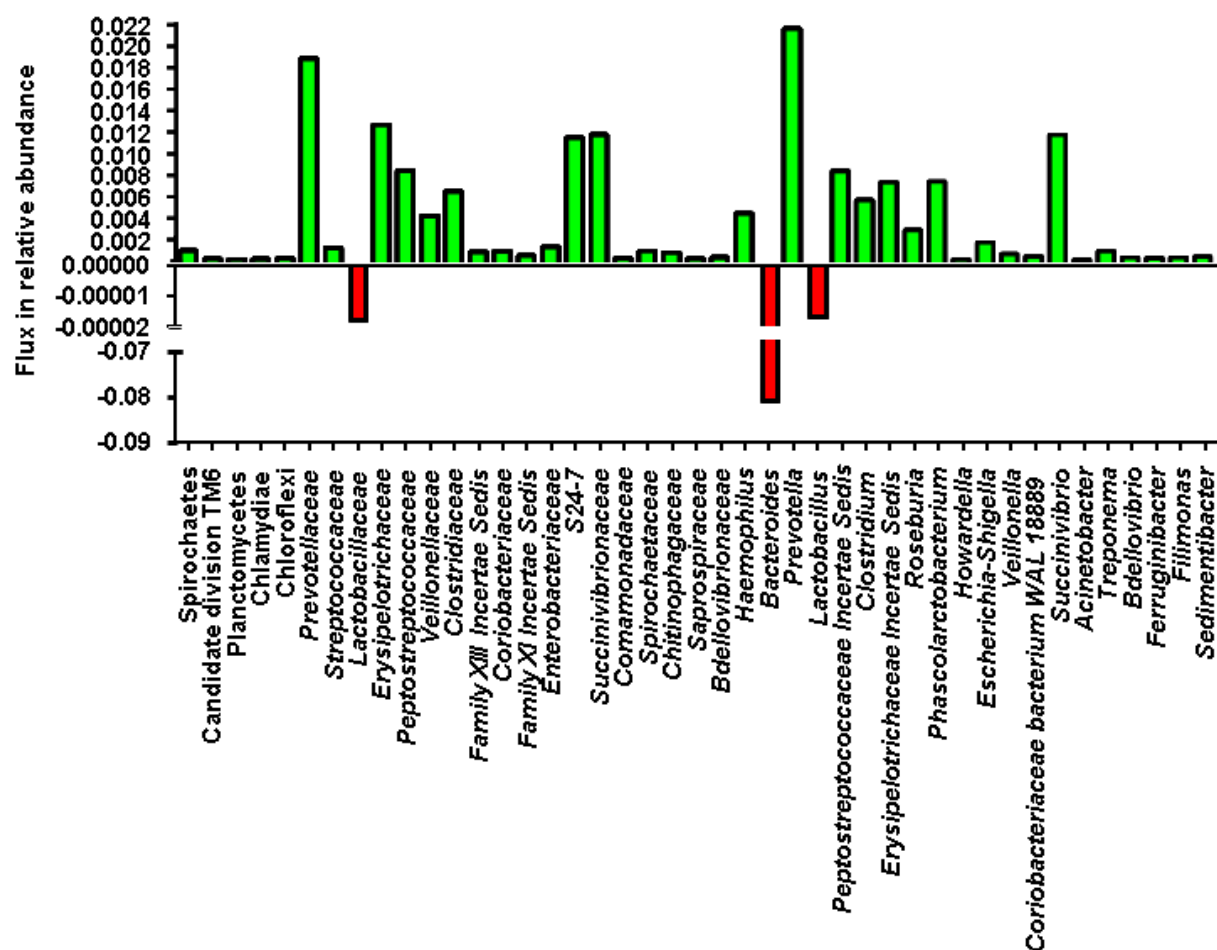
Supplementary Figure S9. Relative change in the abundance of selected taxa in low BMI controls relative to high BMI controls. Only taxa with significant differences in population numbers are illustrated.
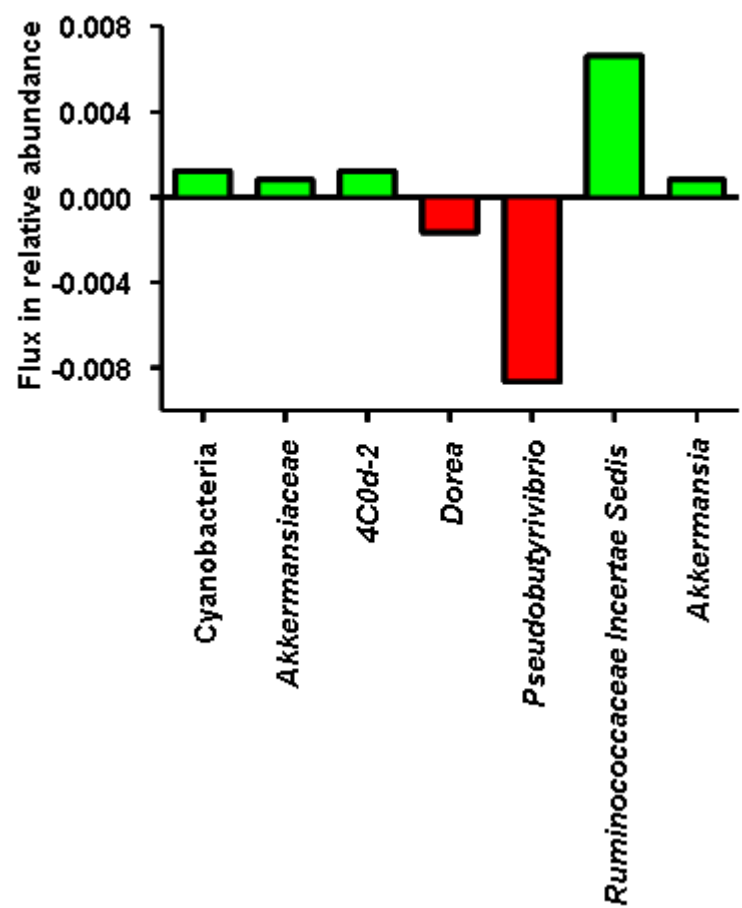
Supplementary Figure S10. Top 5 food groups contributing to protein in all subjects. A, athletes: b, low BMI controls and c, high BMI controls.
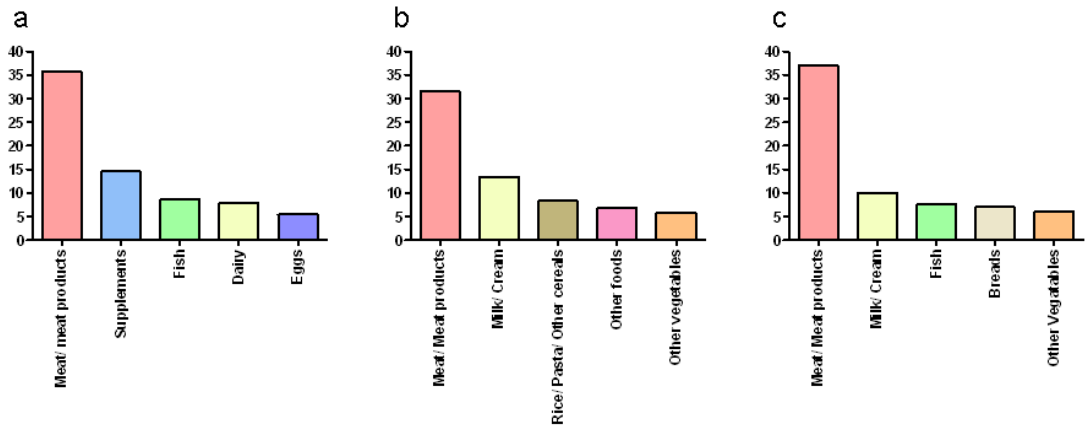
Supplementary Figure S11. Leptin levels positively correlate with percentage body fat, BMI, waist:hip ratio and negatively correlate with lean body mass. Subject colour coding: black, elite athletes; green, high BMI controls; and red, low BMI controls.
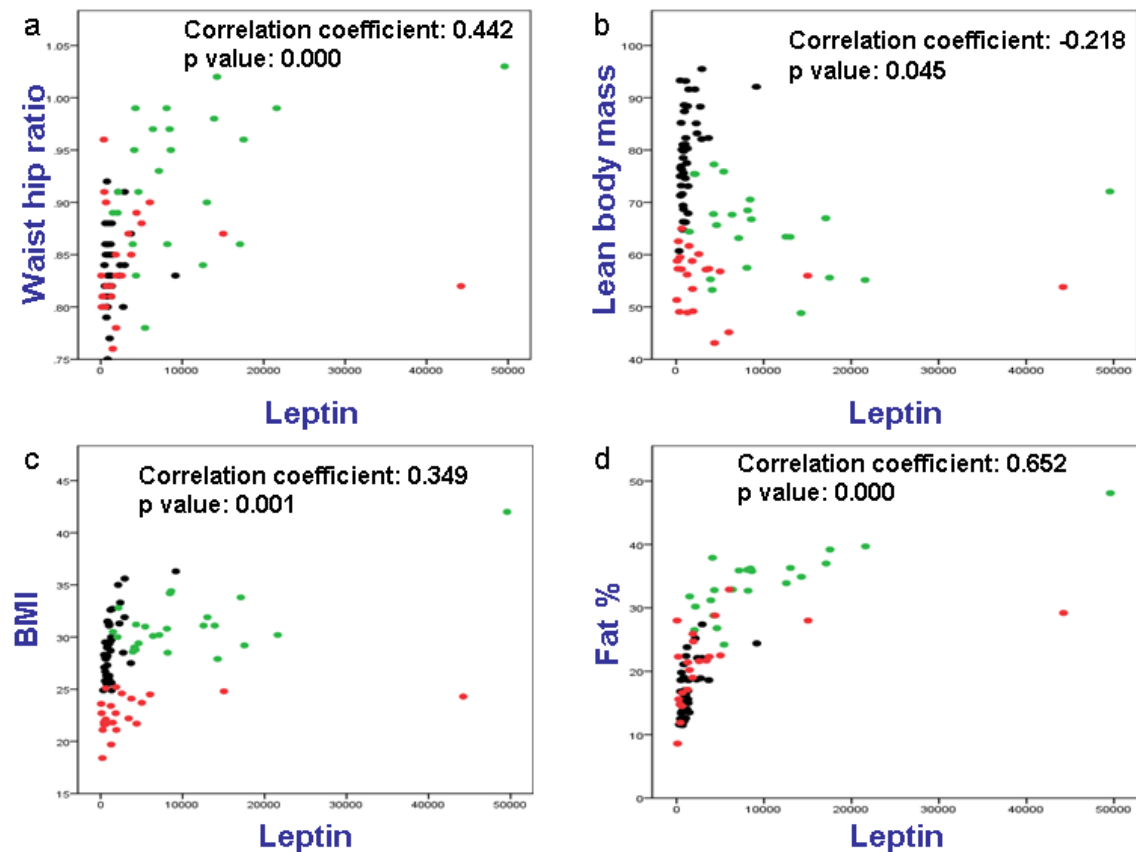
Supplementary Figure S12. Adiponectin levels negatively correlate with BMI and lean body mass. Subject colour coding: black, elite athletes; green, high BMI controls; and red, low BMI controls.
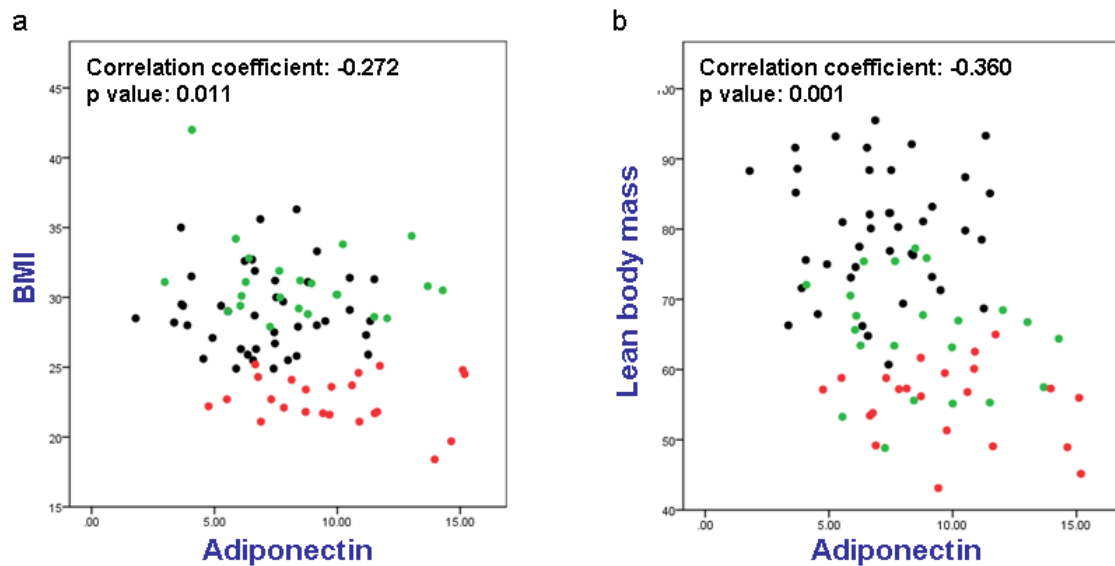
Supplementary Figure S13. Urea levels positively correlates with α diversity. Subject colour coding: black, elite athletes; green, high BMI controls; and red, low BMI controls.
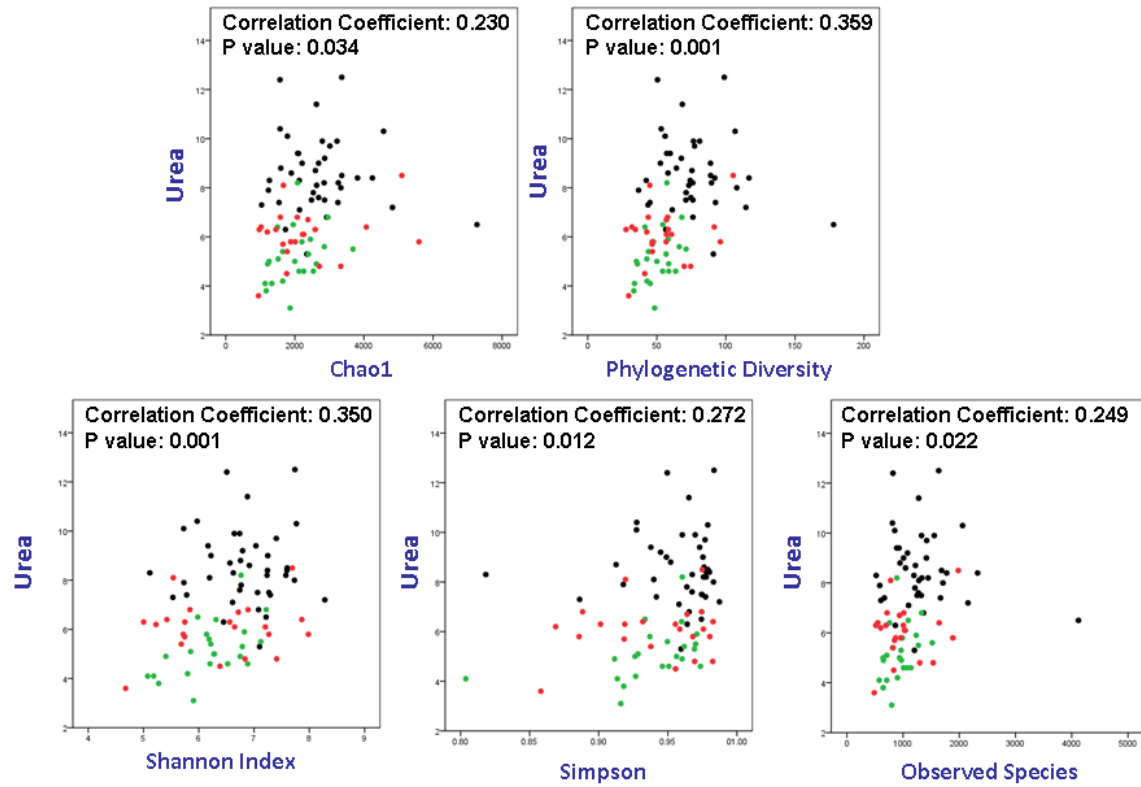
APPENDIX I

Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models

Siobhan Clarke Chapter Contributions:

Experimental:

- Generated amplicons for 454 pyrosequencing.

Results interpretation:

- Analysed all data in relation to 16S compositional sequencing analysis.

7.1 ABSTRACT

Background and Aims Increased efficiency of energy harvest, due to alterations in the gut microbiota (increased Firmicutes and decreased Bacteroidetes), has been implicated in obesity in mice and humans. However, a causal relationship is unproven and contributory variables include diet, genetics and age. Therefore, we explored the effect of a high-fat (HF) diet and genetically determined obesity (*ob/ob*) for changes in microbiota and energy harvesting capacity over time.

Methods Seven-week-old male *ob/ob* mice were fed a low-fat (LF) diet and wild-type mice were fed either a LF-diet or a HF-diet for 8 weeks (*n*=8/group). They were assessed at 7, 11 and 15 weeks of age for: fat and lean body mass (by NMR); faecal and caecal short-chain fatty acids (SCFA, by gas chromatography); faecal energy content (by bomb calorimetry) and microbial composition (by metagenomic pyrosequencing).

Results A progressive increase in Firmicutes was confirmed in both HF-fed and *ob/ob* mice reaching statistical significance in the former, but this phylum was unchanged over time in the lean controls. Reductions in Bacteroidetes were also found in *ob/ob* mice. However, changes in the microbiota were dissociated from markers of energy harvest. Thus, although the faecal energy in the *ob/ob* mice was significantly decreased at 7 weeks, and caecal SCFA increased, these did not persist and faecal acetate diminished over time in both *ob/ob* and HF-fed mice, but not in lean controls. Furthermore, the proportion of the major phyla did not correlate with energy harvest markers.

Conclusion The relationship between the microbial composition and energy harvesting capacity is more complex than previously considered. While compositional changes in the faecal microbiota were confirmed, this was primarily a feature of high fat feeding rather than genetically induced obesity. In addition, changes in the proportions of the major phyla were unrelated to markers of energy harvest which changed over time. The possibility of microbial adaptation to diet and time should be considered in future studies.

7.2 SIGNIFICANCE OF THE STUDY

What is already known about this subject?

➢ Increased efficiency of energy harvest, due to alterations in the gut microbiota (increased Firmicutes and decreased Bacteroidetes) has been implicated in obesity in mice and humans.

➢ Studies in humans have provided variable finding with respect to changes in the gut microbiota in obese individuals and placed less emphasis on changes in the proportions of Firmicutes and Bacteroidetes in obesity

➢ Recent studies in mice suggest that diet plays an important role in modulating the gut microbiota.

➢ Further mouse studies have highlighted the role of the gut microbiota in the regulation of energy homeostasis, in the pathogenesis of insulin resistance, in fatty liver, in lipid and amino acid metabolism and as a modulator of host fatty acid composition.

What are the new findings?

➢ While compositional changes in the faecal microbiota were confirmed, this was primarily a feature of high-fat feeding rather than genetically-induced obesity.

➢ The relationship between the microbial composition and energy harvesting capacity is more complex than previously considered.

➢ The changes in proportions of Firmicutes and Bacteroidetes were unrelated to markers of energy harvest which changed over time.

➢ The mouse gut microbiota was dominated not only by Firmicutes and Bacteroidetes, but also by a third major phylum, the Actinobacteria.

How might it impact on clinical practice in the foreseeable future?

➢ Our findings suggest that microbial adaptation to diet over time, and perhaps with age, is an important variable in the complex relationship between the composition of the microbiota, energy harvesting capacity and obesity and should be taken into account in the design and interpretation of future clinical studies.

7.3 INTRODUCTION

Obesity, one of the great pandemics of our time, is a major threat to public health and to healthcare resources. This complex syndrome is influenced by host susceptibility and by environmental or lifestyle factors, such as diet and sedentary behaviour. At one level, obesity is frequently thought as the outcome of a relative imbalance in energy intake versus energy expenditure. Recently, enhanced energy harvest from dietary intake, due to an alteration in microbial composition, has been highlighted as a potential contributor to the pathogenesis of obesity (Ley *et al.*, 2005, Ley *et al.*, 2006, Turnbaugh *et al.*, 2008, Turnbaugh *et al.*, 2006, Ley, 2010).

Sequencing of the gut microbiota of the caecum of genetically obese *ob/ob* mice revealed a 50% lower relative abundance of Bacteroidetes, whereas the Firmicutes were correspondingly higher (Ley *et al.*, 2005). Similarly, the provision of a high-calorie, high-fat/simple carbohydrate, obesity-inducing 'Western' diet to wild-type mice brought about an overall decrease in the diversity of the gut microbiota, a decrease in Bacteroidetes and a bloom of a single class of Firmicutes - the *Mollicutes* (Turnbaugh *et al.*, 2008). On the basis of these investigations it was suggested that the obese microbiome possesses metabolic pathways that are highly efficient at extracting energy from food (Turnbaugh *et al.*, 2008, Turnbaugh *et al.*, 2006). This has been supported by the observation that transplantation of the microbiota of chow-fed *ob/ob* or 'Western' diet-fed wild-type mice into germ-free wild-type mouse recipients resulted in mice receiving an 'obese' microbiota gaining more fat than recipients of a 'lean' microbiota (Turnbaugh *et al.*, 2006, Turnbaugh *et al.*, 2008). Furthermore, *Ley et al., 2006* showed a decrease in the Firmicutes/Bacteroidetes ratio in obese human subjects in response to weight loss following dietary intervention. In a larger follow up study in obese and lean twins, Turnbaugh and colleagues reported that while the faecal microbiome of obese subjects had significantly lower proportions of Bacteroidetes and higher levels of Actinobacteria

compared to lean individuals, there was no change in the proportion of Firmicutes (Turnbaugh *et al.*, 2009).

Further mouse studies have highlighted the role of the gut microbiota in the regulation of energy homeostasis (Backhed *et al.*, 2004, Backhed *et al.*, 2007), in the pathogenesis of insulin resistance (Cani *et al.*, 2007a, Cani *et al.*, 2008a, Cani *et al.*, 2008b, Cani *et al.*, 2007b, Membrez *et al.*, 2008), in fatty liver (Dumas *et al.*, 2006), in lipid and amino acid metabolism (Martin *et al.*, 2007) and as a modulator of host fatty acid composition (Wall *et al.*, 2009). These studies suggest that interventions which target the metabolic activity of the gut microbiota may have efficacy in obesity and associated disorders. However, a variety of intrinsic and environmental factors, such as age, diet and host physiology and genotype are considered to significantly affect the structure and functionality capabilities of gut microbial communities (Hopkins *et al.*, 2001, Kurokawa *et al.*, 2007, McGarr *et al.*, 2005, Stewart *et al.*, 2005). Indeed, it has been suggested that the Firmicutes/Bacteroidetes ratio of the human microbiota changes with age (Mariat *et al.*, 2009).

A recent study in mice has shown that a high-fat diet determines the composition of the gut microbiota independent of obesity (Hildebrandt *et al.*, 2009). Furthermore, switching from a low-fat to a high-fat diet resulted in a rapid and dramatic shift in the structure of the gut microbiota in mice in a single day (Turnbaugh and Gordon, 2009). These studies suggest that diet plays an important role in modulating the gut microbiota. In addition, a number of follow-up studies in humans have provided variable findings with respect to changes in the gut microbiota in obese individuals and placed less emphasis on changes in the proportions of Firmicutes and Bacteroidetes in obesity (Ley, 2010, Turnbaugh *et al.*, 2009, Schwiertz *et al.*, 2009, Duncan *et al.*, 2008, Santacruz *et al.*, 2009, Zhang *et al.*, 2009, Collado *et al.*, 2008). Moreover, few reports have examined the composition of the gut microbiota over time. Therefore, we examined the energy harvesting capacity and composition of the gut microbiota over time in murine models in response to high fat (HF) feeding and genetically determined obesity. The results confirm the compositional changes in the faecal microbiota, particularly in response

to diet rather than genetically determined obesity, but these are unrelated to markers of energy harvest, which change over time.

7.4 MATERIALS AND METHODS

7.4.1 Animals and diets

ob/ob (leptin deficient) and wild-type C57BL/6J mice were obtained from Charles River (L'arbresle, France) and housed under barrier-maintained conditions within the biological services unit, University College Cork (UCC). Mice were received at 5 weeks of age and allowed to acclimatise for 2 weeks on a low-fat diet (10% calories from fat; Research Diets, New Brunswick, New Jersey, USA; #D12450B) to 'normalise' the gut microbiota. Seven-week-old male *ob/ob* mice, wild-type controls fed a low-fat diet and wild-type mice fed a HF-diet (45% calories from fat; Research Diets; #D12451) were housed individually for 8 weeks (*n*=8 per group). Body weight was assessed weekly. Precautions were taken to ensure that food intake and faecal collections were performed as accurately as possible. Food intake was measured weekly taking account of residual spillage. Faecal output per mouse was measured over 48 h at age 7, 11 and 15 weeks and weights recorded. Fat and lean body mass were measured at age 15 weeks using a Minispec mq benchtop NMR spectrometer (Bruker Instruments, Rheinstetten, Germany).

7.4.2 Experimental design

To examine the relationship between the composition of the gut microbiota and the efficiency of energy harvest, the effects of diet and obesity on the composition and the energy harvesting capacity of the gut microbiota were determined over time (age 7, 11 and 15 weeks). The levels of short-chain fatty acids (SCFA), the major fermentation end-products and source of energy for the host, and the energy content of the faeces were used as markers of energy harvesting.

7.4.3 Analysis of the composition of the gut microbiota in response to diet and obesity over time

For these studies, fresh faecal pellets (*n*=8 per group) were collected at 7, 11 and 15 weeks of age from lean, HF-fed and *ob/ob* mice. As a consequence of the number of samples that required processing, the samples were stored at -80°C prior to DNA extraction. As it has been suggested that freezing may adversely affect the levels of Bacteroidetes in stored human faecal samples (Duncan *et al.*, 2008), an initial study was undertaken to determine the impact of freezing on the microbial populations of mouse faeces. Faecal pellets were collected from C57BL/J6 mice on the same day (*n*=20) and divided into two groups. Faecal samples in the first group were stored on ice and DNA was extracted from these fresh samples on the day of collection (*n*=10) while in the second group, faecal samples were stored at -80°C and DNA was extracted (*n*=10) after 1 month of freezing.

7.4.4 DNA extractions and pyrosequencing

Total metagenomic DNA was extracted from individual faecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK) coupled with an initial bead-beating step. The microbial composition of these samples was determined by pyrosequencing of 16S rRNA tags (V4 region; 239 nt long) amplified using universal 16S primers predicted to bind to 94.6% of all 16S genes; that is, the forward primer F1 (59-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (59-TACCRGGGTHTCTAATCC), R2 (59-TACCAGAGTATCTAATTC), R3 (59-CTACDSRGGTMTCTAATC) and R4 (59-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp). The primers incorporated the proprietary 19-mer sequences at the 59-end to allow emulsion-based clonal amplification for the 454 pyrosequencing system. Unique molecular identifier (MID) tags were incorporated between the adaptamer and the target-specific primer sequence, to allow identification of individual sequences from pooled amplicons. Amplicons were cleaned using the

AMPure purification system (Beckman Coulter, Takeley, United Kingdom) and sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) according to 454 protocols. De-noising was performed using traditional techniques implemented in the RDP pyrosequencing pipeline. Reads with low quality scores (quality scores below 40) and short length (less than 150 bp for the 16S rRNA V4 region) were removed as well as reads that did not have exact matches with the primer sequence. Clustering and statistical analysis of sequence data were performed using the MOTHUR software package (Schloss and Handelsman, 2008). Trimmed fasta sequences were then BLASTed (Altschul *et al.*, 1997) against a previously published 16S-specific database (Urich *et al.*, 2008) using default parameters. The resulting BLAST output was parsed using MEGAN (Huson *et al.*, 2007). MEGAN assigns reads to NCBI taxonomies by employing the lowest common ancestor algorithm which assigns each RNA-tag to the lowest common ancestor in the taxonomy from a subset of the best scoring matches in the BLAST result. Bit scores were used from within MEGAN for filtering the results prior to tree construction and summarisation (absolute cut-off: BLAST bitscore 86, relative cut-off: 10% of the top hit) (Urich *et al.*, 2008).

7.4.5 Bomb calorimetry

Faecal samples were collected over 48 h from individual mice at 7, 11 and 15 weeks of age and used for bomb calorimetry and SCFA analysis. For bomb calorimetry analysis, the samples were weighed and oven-dried at 60°C for 48 h. The energy content of the faeces 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 analysed in triplicate.

7.4.6 SCFA analysis

SCFA analysis was performed according to previously published methods (Bakker-Zierikzee *et al.*, 2005, Costabile *et al.*, 2008, Pereira *et al.*, 2003).

Mice were killed at 7, 11 and 15 weeks of age and the contents of the caecum from individual mice were collected for SCFA analysis. To have sufficient quantity for gas chromatography, the caecum contents were pooled with an n=3-4 per pool. SCFA were extracted from the caecal and faecal contents using 2.0 ml Milli-Q water per 0.1 g fresh weight. The solution was centrifuged for 5 min at 10 000 g to pellet bacteria and other solids. The supernatant was collected and added to 3.0 mM 2-ethylbutyric acid in formic acid, used as the internal standard. Calibration was done using standard solutions containing 10.0 mM, 8.0 mM, 6.0 mM, 4.0 mM, 2.0 mM, 1.0 mM and 0.5 mM of acetic acid, propionic acid and butyric acid. The concentration of SCFA was determined by gas chromatography using a Varian 3500 GC system, fitted with a TRB-FFAP column (30 m x 0.32 mm x 0.50 mm; Teknokroma, Barcelona, Spain) and a flame ionisation detector. Helium was supplied as the carrier gas at an initial flow rate of 1.3 ml/min. The initial oven temperature was 100°C, maintained for 0.5 min, raised to 180°C at 8°C/min and held for 1.0 min, then increased to 200°C at 20°C/min, and finally held at 200°C for 5.0 min. The temperatures of the detector and the injection port were set at 250°C and 240°C, respectively. The injected sample volume was 0.5 mL. Peaks were integrated using Varian Star Chromatography Workstation version 6.0 software. Additional phials containing standards were included in each run to maintain calibration and a cleaning injection of 1.2% formic acid was used before each analysis.

7.4.7 Statistical analysis

Data for all variables were normally distributed and allowed for parametric tests of significance. Data are presented as mean values with their standard errors (SEM). Statistical analysis was performed by ANOVA and the Student t test (Graph-Pad Software, San Diego, CA, USA). Correlations analysis was performed using Pearson's (r) correlation test. p<0.05 was considered as statistically significant.

7.5 RESULTS

7.5.1 *ob/ob* and HF-fed mice gain more weight than lean mice and vary in body composition

ob/ob mice gained significantly more body weight compared to lean controls ($p<0.001$) while a modest although statistically significant increase in body weight was observed for mice fed a high-fat diet compared to lean mice ($p<0.05$) over 8 weeks (Fig 1A,B). The increase in body weight was associated with an increase in fat mass and a decrease in lean mass in *ob/ob* mice compared to lean mice, while the increase in body weight in HF-fed mice was attributable to an increase in fat mass alone (Fig 1C). In agreement with previous studies, *ob/ob* mice consumed significantly more calories than lean controls, as measured by the cumulative caloric intake over the 8 week period of the study (Fig 1D). There were no differences in cumulative caloric intake between lean and HF-fed mice.

7.5.2 Freezing of murine faecal samples for 1 month does not significantly alter compositional analysis

It has been suggested that freezing may reduce the levels of Bacteroidetes in stored human faecal samples (Duncan *et al.*, 2008). High throughput sequencing and phylogenetic assignment of the resultant amplicons revealed that, at both the phylum and genus levels, there was no significant alteration in the composition of the gut microbiota between fresh and frozen samples (Supplementary Fig 1). Therefore, for processing large numbers of samples, frozen storage was deemed acceptable prior to DNA isolation for the purposes of the present study.

7.5.3 The mouse gut microbiota is dominated by Firmicutes, Bacteroidetes and Actinobacteria

A total of 249,409 V4 16S sequence reads were generated, corresponding to an average of 31,176 reads per group or 3,897 per mouse. Species richness, coverage and diversity estimations were calculated for each data set (Supplementary Table 1 and Supplementary

Fig 2). Rarefaction curves for each group indicated that the total bacterial diversity present was well represented. Of the reads, 208,202 (83%) were assigned at the phylum level, 148,363 (59.5%) at the family level and 130,950 (52.5%) at the genus level. Taxonomy-based analysis of the assigned sequences showed that, at the phylum level, the mouse gut microbiota was dominated by Firmicutes, Bacteroidetes and Actinobacteria (Fig 2 and Table 1). The high proportion of Actinobacteria detected may reflect differences in the DNA extraction protocol employed or advances in primer design (primers employed match perfectly to 94.6% of sequences within RDP release 9.53 [http://pyro.cme.msu.edu/pyro/help.jsp]). Proteobacteria, Tenericutes, Spirochaetes and Deferribacteria were detected but at considerably lower levels. Consistent with the high levels of Firmicutes and Actinobacteria detected, the most dominant bacteria at the genus level were *Clostridium* and *Bifidobacterium* (Table 1).

7.5.4 The composition of the murine gut microbiota changes over time in response to diet and obesity

In lean mice, from age 7 through 11 to 15 weeks, there were no significant changes in the proportions of bacteria at the level of phylum (Fig 2A and Table 1) or genus (Table 1). In contrast, there was a progressive increase in the proportions of Firmicutes in both HF-fed and *ob/ob* mice, reaching statistical significance in the former ($p<0.05$). Reductions in Bacteroidetes over time were evident in all three groups of mice but reached statistical significance only in the *ob/ob* mice ($p<0.001$). The levels of Actinobacteria, and of the associated genus *Bifidobacterium*, fluctuated in all three groups with significant increases observed in *ob/ob* and HF-fed mice from age 7 to 11 weeks ($p<0.05$) but not between 7-15 or 11-15 weeks of age.

While the proportions of other phyla and genera were very low, changes were detectable. Proteobacteria were found to decrease in abundance over time in HF-fed mice ($p<0.001$); Deferribacteria and *lactococci* increased and decreased, respectively, from age 7 to 11 ($p<0.001$) and from 7 to 15 ($p<0.001$) weeks in ob/ob mice (Table 1).

7.5.5 Energy harvesting changes in response to diet and obesity over time and does not correlate with the proportions of Firmicutes, Bacteroidetes and Actinobacteria

To examine the relationship between the composition of the gut microbiota and the efficiency of energy harvest, the levels of SCFA, the major fermentation end-products and source of energy for the host, and the energy content of the faeces were used as markers of energy harvesting. Faecal SCFA and energy content were then correlated with the levels of Firmicutes, Bacteroidetes and Actinobacteria in lean, *ob/ob* and HF-fed mice.

As reported by others (Turnbaugh *et al.*, 2006), the energy content of faeces from *ob/ob* mice, as assessed by bomb calorimetry, was modestly but significantly lower at 7 weeks than that of lean controls (Table 2 and Fig 3A). However, this relationship did not persist at ages 11 and 15 weeks. In contrast, in mice on a high-fat diet, the faecal energy content increased from age 7 to 11 weeks without any further increase at 15 weeks (Table 2 and Fig 3A). Faecal output was similar in lean and HF-fed mice over time and there was no significant changes in faecal output between lean and HF-fed mice at age 7 (0.31 vs 0.32 g/day), 11 (0.30±0.01 vs 0.34±0.01 g/day) and 15 (0.29±0.01 vs 0.32±0.01 g/day) weeks. *ob/ob* mice produced significantly more faeces (0.47±0.02 vs 0.31±0.01 g/day; $p<0.001$) at age 7 weeks compared to lean controls. However, faecal production decreased over time in *ob/ob* mice from age 7 to 11 (0.47±0.02 to 0.34±0.02 g/ day; $p<0.001$) and from 7 to 15 (0.47±0.02 to 0.30±0.01; $p<0.001$) weeks of age. Calculation of energy assimilation (data not shown) suggests that the energy excreted is a function of food intake and the contribution of the gut microbiota to energy extraction is very small as suggested by Bajzer and Seeley, 2006.

Faecal and caecal SCFA analysis showed that while acetate, propionate and butyrate were detected in the caecum, only acetate was detected in the faeces of the lean, HF-fed and *ob/ob* groups (Table 2 and Fig 3B). Faecal acetate concentration was higher in *ob/ob* compared to lean mice at age 7 and 11 weeks. However, faecal acetate levels

decreased progressively over time in both the *ob/ob* and HF-fed mice. Analysis of caecal SCFA showed that, at 7 weeks, caecal acetate and propionate levels were significantly higher in *ob/ob* mice compared to lean controls (Table 2). However, this observation did not persist with time and no further pattern in caecal SCFA levels was observed in lean, HF-fed and *ob/ob* groups.

Correlation analysis revealed that the proportions of Firmicutes, Bacteroidetes and Actinobacteria did not correlate with energy harvesting markers (Table 3). Furthermore, while a positive correlation between faecal energy content and acetate levels in lean mice was observed (r=0.50; *p*<0.01), there was no relationship between these parameters in *ob/ob* and HF-fed mice suggesting a more complex relationship between faecal energy and faecal SCFA levels in obesity (Fig 3C).

7.6   DISCUSSION

The results confirmed the compositional changes in the microbiota previously linked with obesity, particularly in response to a high-fat diet, but these were unrelated to energy harvesting capacity, which changed over time. Furthermore, markers of energy harvesting were not correlated with the proportions of the major phyla. The study also showed that the mouse gut microbiota was dominated not only by Firmicutes and Bacteroidetes, but also by a third major phylum, the Actinobacteria.

This is the first report to detect high levels of the phylum Actinobacteria and the associated genus *Bifidobacterium* in the mouse gut microbiota using a pyrosequencing approach. There appears to be discordance in detection rates and levels of *Bifidobacterium* between pyrosequencing (Ley *et al.*, 2005, Ley, 2010, Hildebrandt *et al.*, 2009, Eckburg *et al.*, 2005, Palmer *et al.*, 2007, Suau *et al.*, 1999, Wang *et al.*, 2003) and other techniques such as fluorescence in-situ hybridisation (FISH) and traditional culture-based methods (Rajilić-Stojanović *et al.*, 2007, Vrieze *et al.*, 2010, Zoetendal *et al.*, 2006). The low percentage of Actinobacteria in some studies has been attributed to difficulties associated with extracting the associated DNA, a difference in the GC

content of the template or the accuracy of PCR primers (Turroni *et al.*, 2008). In this study, the particular DNA extraction protocol employed and/or the use of primers predicted to bind to 94.6% of all 16S genes, may account for the greater proportion of Actinobacteria reads.

Another potential pitfall in studies of this type is the impact of freeze storage prior to DNA extraction (Duncan *et al.*, 2008). In our hands, a preliminary assessment revealed that storage of frozen samples over 1 month had no major influence on the DNA-based compositional analysis. It is noteworthy that a recent large metagenomics study in Europe (MetaHIT) also used frozen stool samples (Qin *et al.*, 2010).

The evidence supporting the concept that the gut microbiota in obesity facilitates the extraction of additional calories from ingested food has been reviewed elsewhere (DiBaise *et al.*, 2008, Tilg *et al.*, 2009, Tilg, 2010). Although we were able to confirm that the energy content of faeces from *ob/ob* mice was reduced and that caecal SCFA were increased at 7 weeks (consistent with increased harvest), as reported by others (Turnbaugh *et al.*, 2006), this did not persist with time and was not found in HF-fed mice. It is noteworthy that faecal acetate levels decreased in both *ob/ob* and HF-fed mice over time but not in lean controls. The decrease in faecal acetate may be due to the observed alterations in the gut microbiota or an increase in its uptake/absorption in response to diet and obesity. Interestingly, it has been reported that, while obese individuals have higher faecal SCFA levels, these changes were not associated with a higher proportion of Firmicutes leading the authors to speculate that the amount of SCFA produced and not the ratio of Firmicutes and Bacteroidetes is important in obesity (Schwiertz *et al.*, 2009). It is unlikely that the Firmicutes/Bacteroidetes ratio is solely responsible for obesogenic shifts in the microbiota as indicated by Turnbaugh and colleagues (Turnbaugh *et al.*, 2009) suggesting that altered proportions of other phyla including the Actinobacteria should also be further investigated. Indeed, the critical biomarker of obesity is uncertain (Firmicutes, Bacteroidetes, Firmicutes/Bacteroidetes ratio thereof and other phyla) and remains to be determined.

The primacy of diet in determining the composition of the gut microbiota independent of obesity has been elegantly demonstrated by Hildebrandt *et al.*, 2009. Moreover, Turnbaugh and Gordon, 2009 demonstrated that the structure of the microbiota changes in a rapid and dramatic manner after switching from a low-fat polysaccharide-rich diet to a high-fat, high-sugar 'Western' diet. In our study, while time-dependent decreases in Bacteroidetes in *ob/ob* mice were observed, the levels of Firmicutes significantly increased in HF-fed but not *ob/ob* mice over time. This observation suggests that a high-fat diet and not the obese genotype of *ob/ob* mice exerts the greater influence on the composition of the gut microbiota, in agreement with the results by Hildebrandt *et al.*, 2009. Furthermore, Duncan *et al.*, 2008 demonstrated a significant diet-dependent reduction in Firmicutes levels in faecal samples from obese individuals on a low-carbohydrate diet. Taken together, these observations suggest that diet plays an important role in modulating the gut microbiota and suggests that dietary variations must be taken into account in human studies.

In the present study, there was no significant change in the composition and energy harvesting of the gut microbiota of lean mice over the duration of the study suggesting that most of the changes observed in the HF-fed mice were probably attributable to change in diet. However, some temporal variations in the microbiota were apparent in the *ob/ob* mice over this period including a decrease over time in Bacteroidetes and Deferribacteria, at the phylum level, and *Lactococcus* at the genus level. While there has been an increasing appreciation of the impact of age on the gut microbial composition for example the Firmicutes/Bacteroidetes ratio of the human gut microbiota changes with age, changing from 0.4 to 10.9 to 0.6 as one moves from infants to adults and onto elderly individuals, respectively (Mariat *et al.*, 2009), we did not address age-associated alterations. However, our findings suggest that a comprehensive assessment of the role of gut microbiota in obesity should take account of temporal variation and adaptation.

Although Proteobacteria decreased in response to a high-fat diet, these bacteria were only present in low proportions in the murine gut

microbiota. Interestingly, increases in the associated classes (Gamma-Proteobacteria) and family (*Enterobacteriaceae*) have been associated with weight loss in adolescents (Santacruz *et al.*, 2009) and gastric bypass in obese individuals (Zhang *et al.*, 2009), respectively.

Our data demonstrate that changes in the microbiota were dissociated from markers of energy harvest, suggesting that mechanisms other than energy harvesting may contribute to microbiota-induced alterations in obesity and metabolic diseases. In this regard, other work has suggested that the gut microbiota and its products (eg, LPS and SCFA) regulate host gene expression and, thereby, affect host energy expenditure and storage acting through mechanisms involving fasting-adipose adipose factor (FIAF) (Backhed *et al.*, 2004), adenosine monophoshate (AMP)-activated protein kinase (AMPK) (Backhed *et al.*, 2007), G-protein-coupled receptor (GPR)41 (Samuel *et al.*, 2008) and CD14/Toll-like-receptor (TLR)4 (Cani *et al.*, 2007a). Indeed, germ-free mice are protected from the development of diet-induced obesity (Backhed *et al.*, 2007). Moreover, a recent study in the TLR5-deficient mice suggests that malfunction of the innate immune system may promote the development of metabolic syndrome through a mechanism involving the gut microbiota (Vijay-Kumar *et al.*, 2010).

In conclusion, while compositional changes in the faecal microbiota were confirmed, this was primarily a feature of high fat feeding rather than genetically determined obesity. In addition, changes in the proportions of the major phyla of the gut microbiota were unrelated to markers of energy harvest which changed over time. These findings suggest that microbial adaptation to diet over time, and perhaps with age, is an important variable in the complex relationship between the composition of the microbiota, energy harvesting capacity and obesity and should be considered in future studies.

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Table 1 Obesity and diet alter the composition of the gut microbiota in high-fat (HF)-fed and *ob/ob* mice over time.

	Lean			HF-fed		*ob/ob*		
Weeks of age	**7**	**11**	**15**	**11**	**15**	**7**	**11**	**15**
Phylum*:								
Firmicutes	56.2±3.5	45.4±2.4	56.6±5.3	65.2±3.4 †	70.5±4.3 ¶	56.2±2.0	57.6±2.4 †	63.5±4.0
Bacteroidetes	24.4±4.7	19.0±5.6	16.9±3.9	19.3±3.3	15.2±3.4	25.1±3.7	11.2±1.3 ‡	12.2±1.4 ¶
Actinobacteria	15.5±5.1	32.8±6.6	24.5±8.0	14.6±2.5	13.1±3.7	15.9±3.2	29.7±3.0 ‡	22.9±4.3
Proteobacteria	3.0±0.4	2.2±0.8	1.3±0.2	0.6±0.4 †‡	0.2±0.2 †¶	0.8±0.2	0.6±0.1 †	0.6±0.2 †
Deferribacteria	1.0±0.3	0.7±0.3	0.6±0.2	0.3±0.1	1.0±0.6	1.6±0.3	0.4±0.1 ‡	0.3±0.1 ¶
Genus:								
Bacteroides	2.7±0.5	2.3±0.6	1.4±0.3	2.9±0.5	1.7±0.6	2.7±0.6	1.7±0.3	1.9±0.3
Clostridium	23.2±5.1	25.1±2.9	27.7±4.5	35.2±5.1	29.5±6.7	19.6±3.2	31.8±2.6	32.7±5.7
Lactococcus	2.5±0.5	2.1±0.4	3.4±1.0	2.8±0.6	4.6±0.9	3.5±0.5	1.6±0.2 ‡	1.5±0.2 ¶
Lactobacillus	6.0±2.0	1.6±0.6	3.2±2.0	1.8±0.5	1.9±0.4	7.1±2.1	3.7±1.3	3.0±1.1
Bifidobacterium	14.2±5.3	32.5±6.5	24.1±8.0	12.0±2.9 †	12.8±3.8	15.4±3.2	29.5±3.0 ‡	22.8±4.9

*Values are mean percentage read number ±SEM, *n*=8. † Indicated significant differences ($p<0.05$, two-tailed Student t test) in bacterial populations relative to the corresponding lean control time-point (age 7 weeks in lean controls are used as baseline before introduction of high-fat feeding). ‡, ¶ indicates significant differences ($p<0.05$, one-way ANOVA using the Tukey post-hoc t-test) in the composition of the gut microbiota between ages 7, 11 and 15 weeks within lean and ob/ob groups separately and in response to high-fat feeding (‡ between 7 and 11 weeks, § between ages 11 and 15 weeks and ¶ between ages 7 and 15 weeks).

Table 2 Energy harvesting capacity of the gut microbiota is altered in lean, high-fat (HF)-fed and ob/ob mice over time.

Weeks of age	Lean			HF-fed			*ob/ob*		
	7	11	15	7	11	15	7	11	15
Faecal *:									
Energy (kJ/g)	13.5±0.4	13.6±0.3	13.5±0.3	13.6±0.3	14.6±0.8 †‡	14.3±0.6 §	13.1±0.4 †	13.5±0.4	13.5±0.2
Acetate (μmol/g)	13.9±9.6	15.0±6.3	10.6±6.3	11.8±3.1	9.7±4.4 †	4.1±2.7 §¶	36.9±20.6 †	29.9±10.4 †	14.4±3.8 §¶
Caecal (μmol/g):									
Acetate	51.6±2.2	67.1±17.8	37.3±2.0 §	49.8±6.8	54.5±11.6	37.8±1.9	68.1±5.4 †	48.8±5.35	40.8±13.0 ¶
Propionate	5.7±1.2	8.7±3.4	ND § ¶	6.6±1.6	1.9±3.3 †‡	ND ¶	9.2±1.6 †	6.7±1.6	1.0±2.0 ¶
Butyrate	1.9±1.7	4.25±1.5	2.1±1.9	1.7±1.3	1.6±1.8	2.6±2.6	3.7±3.1	3.9±3.2	1.2±1.9

*Values are mean ±SEM, n=8. For caecal analysis, samples were pooled; n=3-4 per group. Propionate and butyrate were not detected (ND) in faeces. † Indicated significant differences (p<0.05, two-tailed Student t test) in energy harvesting markers relative to the corresponding lean control time-point. ‡, §, ¶ indicates significant differences (p<0.05, one-way ANOVA using the Tukey post-hoc t-test) in energy harvesting markers between ages 7, 11 and 15 weeks within lean and *ob/ob* groups separately and in response to high-fat feeding (‡ between 7 and 11 weeks, § between ages 11 and 15 weeks and ¶ between ages 7 and 15 weeks).

Table 3 The proportion of Firmicutes, Bacteroidetes and Actinobacteria do not correlate with energy harvesting markers.

Comparisons*:	Lean		High-fat-fed		*ob/ob*	
	r	p-value	r	p-value	r	p-value
Firmicutes versus faecal energy	-0.33	0.11	-0.38	0.14	-0.16	0.46
Firmicutes versus faecal acetate	-0.56	0.005	-0.16	0.55	-0.01	0.98
Bacteroidetes versus faecal energy	-0.26	0.22	0.06	0.82	0.23	0.27
Bacteroidetes versus faecal acetate	-0.25	0.24	-0.29	0.28	-0.35	0.09
Actinobacteria versus faecal energy	0.33	0.12	0.28	0.29	0.17	0.43
Actinobacteria versus faecal acetate	0.61	0.002	0.33	0.21	0.09	0.66

*Values refer to Pearson's r correlation and corresponding p-value.

Figure 1 Phenotype of mouse models of obesity. Body weight (A) and appearance (B) over the 8 week study period. Body composition (g) for lean controls, high-fat (HF) fed and *ob/ob* mice at age 15 weeks (C) and cumulative energy intake per mouse (kj/mouse) (D). Data presented as mean ±SEM, (*n*= 8 per group). ****p*<0.001.
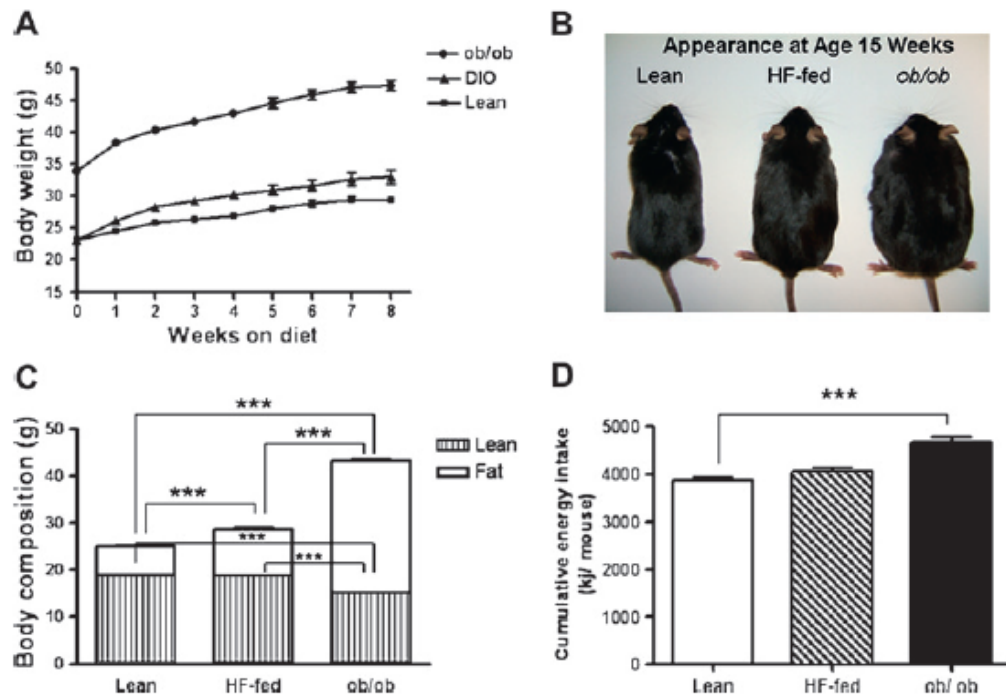
Figure 2 The gut microbiota composition is altered in *ob/ob* mice (C) at age 7, 11 and 15 weeks and in response to high-fat (HF) feeding (4 and 8 weeks) (B) but not lean mice (A) as determined by pyrosequencing of 16S rRNA tags (V4 region). Data outside the pie charts represent the mean percentage read number for the corresponding colour coded phylum (*n*=8 per group). Note: Microbial composition of faeces at age 7 weeks in lean mice is used as the baseline prior to introduction of HF-feeding. Thus, the pie charts for the gut microbiota composition of lean and HF-fed mice at age 7 weeks are the same only at this time-point.

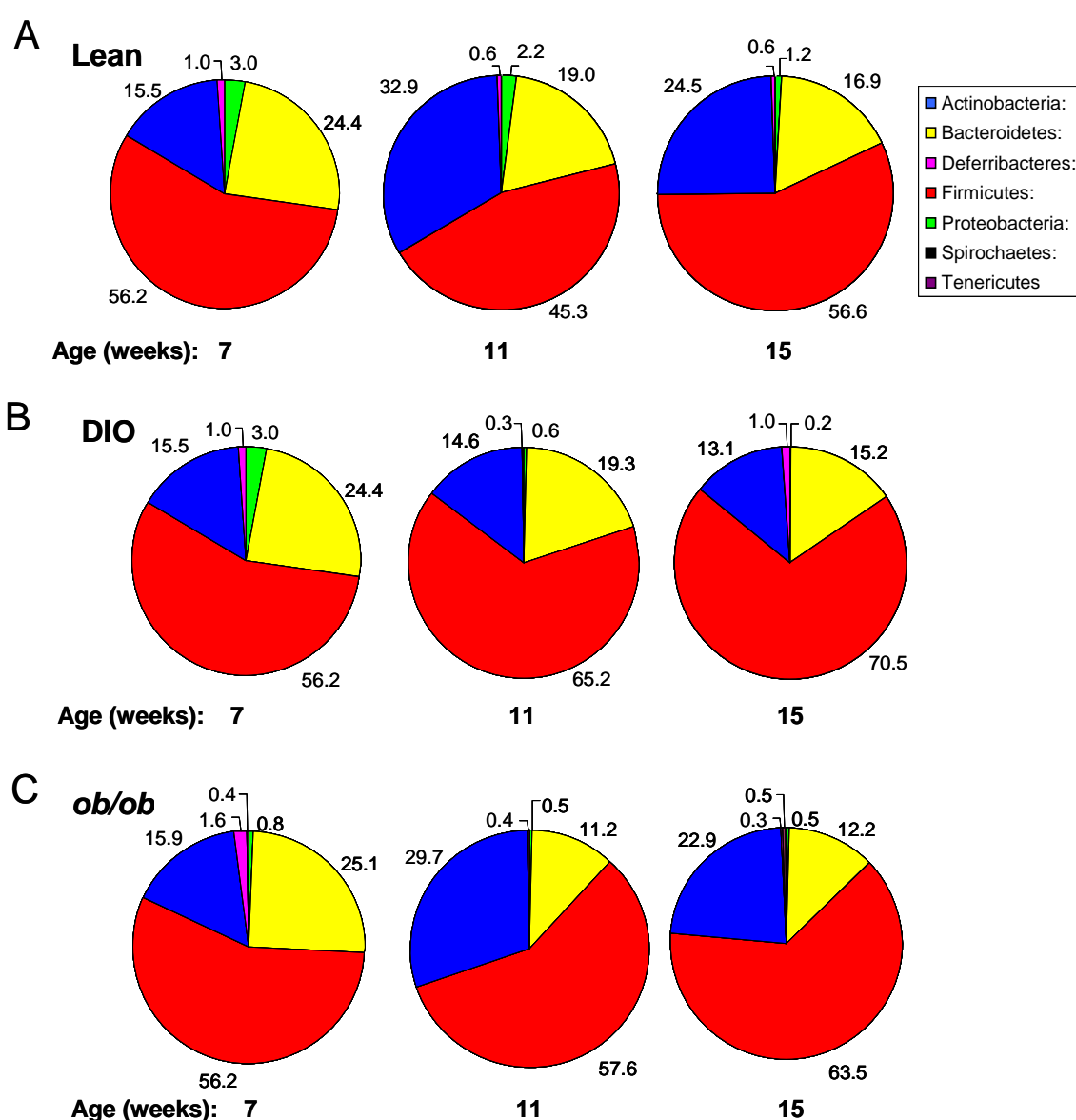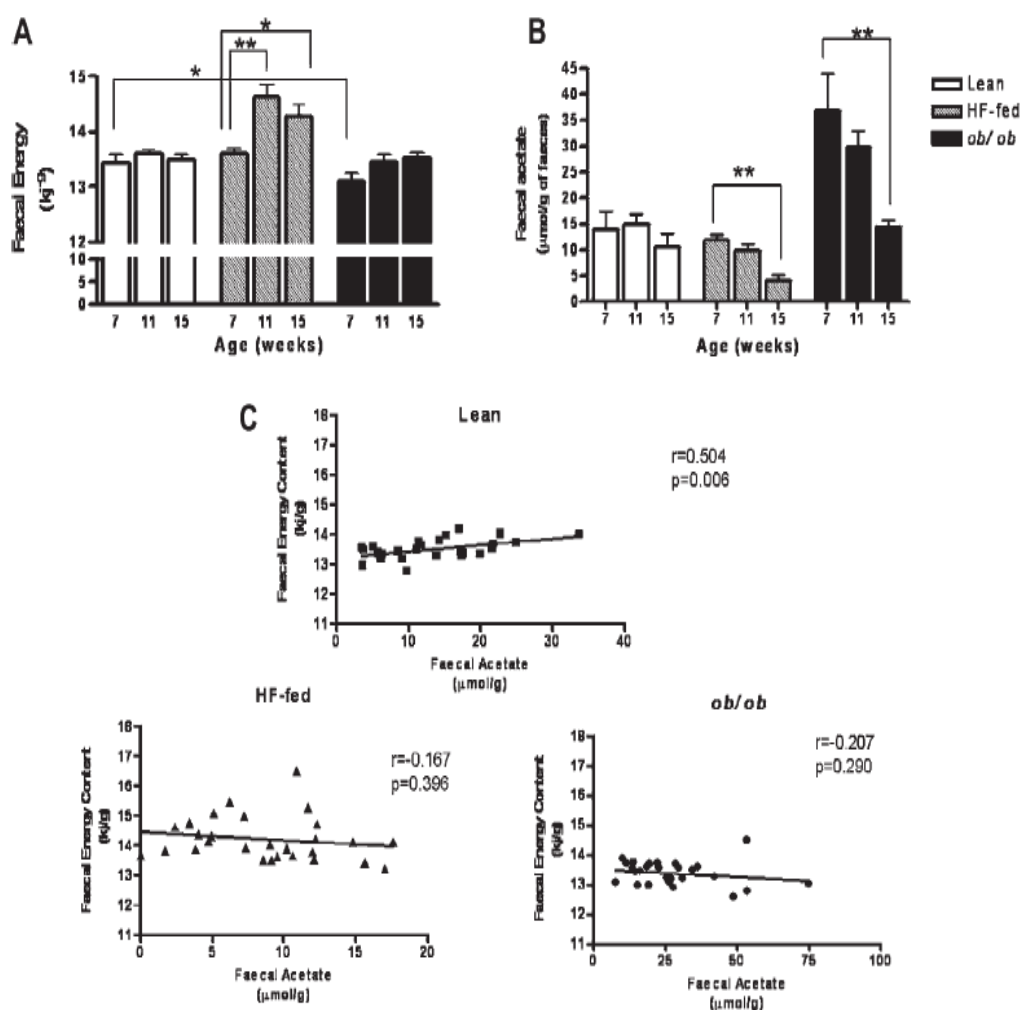**Figure 3** Markers of energy harvest are altered in mouse models of obesity. Faecal energy (A) and acetate (B) levels in lean and *ob/ob* mice at age 7, 11 and 15 weeks and in response to high-fat (HF) feeding (0, 4 and 8 weeks). Correlation of faecal energy content and faecal acetate levels in lean, HF-fed and *ob/ob* mice (C). Values refer to Pearson's r correlation and corresponding p value.

SUPPLEMENTARY INFORMATION

Supplementary Table 1. Diversity estimates for lean, DIO and *ob/ob* mice at age 7, 11 and 15 weeks.

	Lean						DIO				*ob/ob*					
Weeks of age	7		11		15		11		15		7		11		15	
Similarity (%)	97	98	97	98	97	98	97	98	97	98	97	98	97	98	97	98
Chao1 richness estimation	13987	19323	10376	13899	26874	40796	9678	13870	25655	39014	11622	15784	24958	37679	12089	17108
Shannon's index for Diversity	7.0	7.6	6.7	7.2	7.4	8.0	6.7	7.3	7.3	7.9	7.2	7.8	7.4	7.9	6.9	7.4
Good's coverage (%)	83.6	77.6	83.3	77.7	89.4	84.9	84.9	79.1	89.5	84.9	86.7	81.9	88.4	83.6	85.3	79.6

Supplementary Figure 1. Freezing (1 month at -80 ^{o}C) does not significantly alter the composition of the gut microbiota at the phylum level in murine fecal samples as determined by pyrosequencing of 16S rRNA tags (V4 region). Data outside the pie charts represent the mean percentage read number ±SEM of corresponding color-coded phylum.
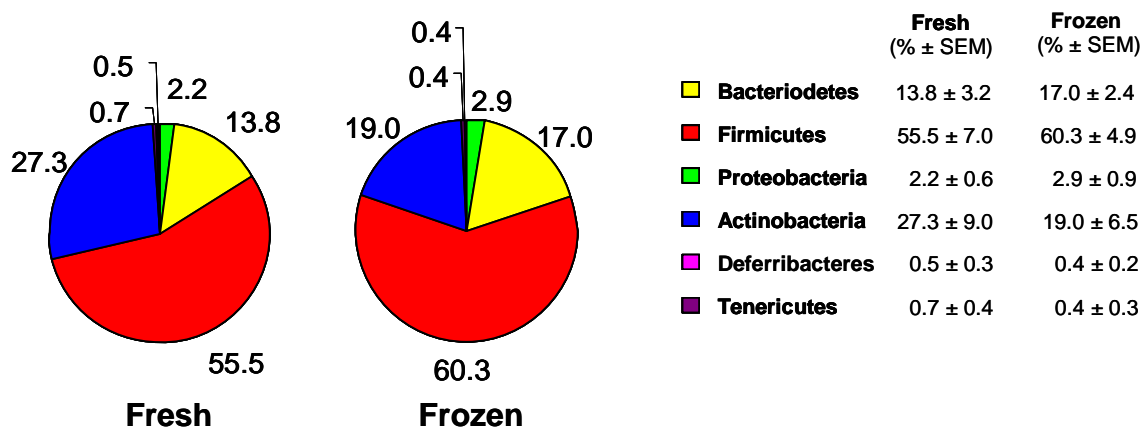
	Fresh (% ± SEM)	Frozen (% ± SEM)
☐ Bacteriodetes	13.8 ± 3.2	17.0 ± 2.4
☐ Firmicutes	55.5 ± 7.0	60.3 ± 4.9
☐ Proteobacteria	2.2 ± 0.6	2.9 ± 0.9
☐ Actinobacteria	27.3 ± 9.0	19.0 ± 6.5
☐ Deferribacteres	0.5 ± 0.3	0.4 ± 0.2
☐ Tenericutes	0.7 ± 0.4	0.4 ± 0.3

Supplementary Figure 2. Rarefaction curves for each group at 97% similarity levels indicated that the total bacterial diversity present was well represented. Number of operational taxonomic units (OTUs) identified as a function of the number of sequence tags sampled.
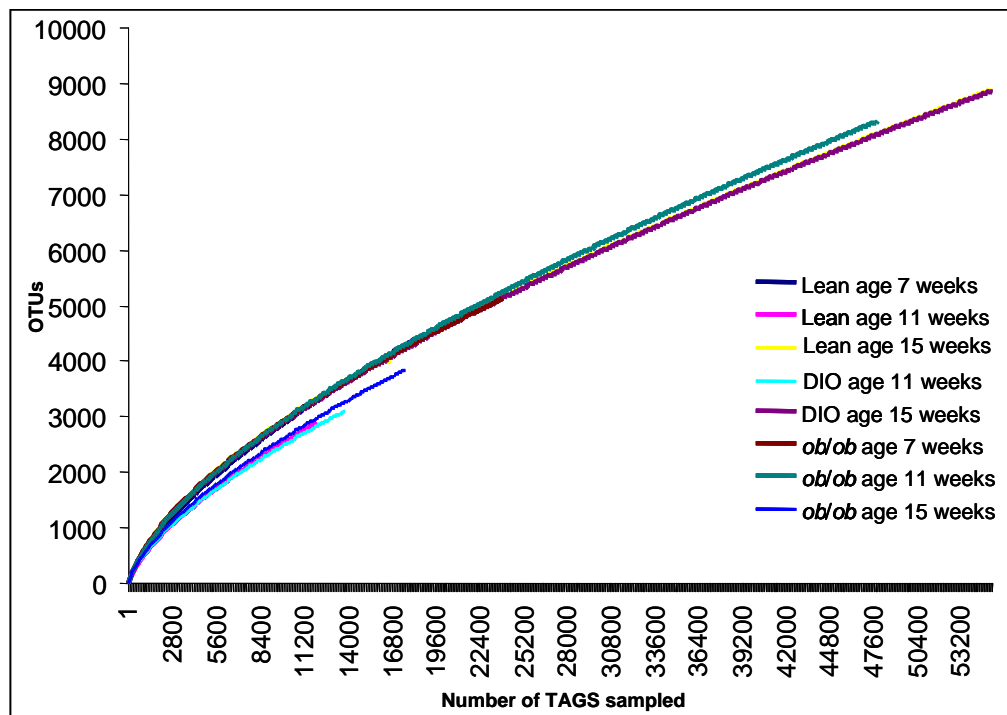
APPENDIX II

Antimicrobials: Strategies for targeting obesity and metabolic health?

Siobhan Clarke Chapter Contributions:

Major contributor to manuscript preparation

8.1 ABSTRACT

Obesity is associated with a number of serious health consequences, including type 2 diabetes, cardiovascular disease and a variety of cancers among others and has been repeatedly shown to be associated with a higher risk of mortality. The relatively recent discovery that the composition and metabolic activity of the gut microbiota may affect the risk of developing obesity and related disorders has led to an explosion of interest in this distinct research field. A corollary of these findings would suggest that modulation of gut microbial populations can have beneficial effects with respect to controlling obesity. In this addendum, we summarize our recent data, showing that therapeutic manipulation of the microbiota using different antimicrobial strategies may be a useful approach for the management of obesity and metabolic conditions. In addition, we will explore some of the mechanisms that may contribute to microbiota-induced susceptibility to obesity and metabolic diseases.

8.2 INTRODUCTION

Obesity, the great pandemic of our time, is a major threat to public health and challenge to healthcare resources. This complex syndrome is influenced by host susceptibility and by environmental or lifestyle factors, such as diet and physical activity. Obesity is associated with a number of serious health consequences, including type 2 diabetes (T2DM), cardiovascular disease and a variety of cancers amongst others (Hensrud and Klein, 2006) and has been repeatedly shown to be associated with a higher risk of mortality (Ogden *et al.*, 2007). The relatively recent discovery that the composition and metabolic activity of the gut microbiota may affect the risk of developing obesity and related disorders has led to an explosion of interest in this distinct research field (for review see refs. (Clarke *et al.*, 2012, Ley, 2010)). A corollary of these findings would suggest that modulation of gut microbial populations can have beneficial effects with respect to controlling obesity. A number of strategies including specific functional foods, probiotics, prebiotics and/or antimicrobials antibiotics have the potential to favourably influence host metabolism by targeting the gut microbiota. In this addendum, we

summarize our recent data, showing that therapeutic manipulation of the microbiota using different antimicrobial strategies may be a useful approach for the management of obesity and metabolic conditions. In addition, we will explore some of the mechanisms that may contribute to microbiota-induced susceptibility to obesity and metabolic diseases.

8.3   OBESITY AND THE GUT MICROBIOTA

Both animal and human studies have shown that the composition of the gut microbiota is significantly altered in obesity and diabetes and characterized by reduced diversity (Larsen *et al.*, 2010, Ley *et al.*, 2005, Ley *et al.*, 2006, Turnbaugh *et al.*, 2008, Turnbaugh and Gordon, 2009, Turnbaugh *et al.*, 2006). We and others have demonstrated the primacy of diet in influencing the microbiota in obesity (Murphy *et al.*, 2010, Wu *et al.*, 2011). Further mouse studies have highlighted the role of the gut microbiota in the regulation of energy homeostasis (Backhed *et al.*, 2004a, Backhed *et al.*, 2007), in the pathogenesis of insulin resistance (Cani *et al.*, 2007a, Cani *et al.*, 2008a, Cani *et al.*, 2008b, Cani *et al.*, 2007b, Membrez *et al.*, 2008), in fatty liver (Dumas *et al.*, 2006), in lipid and amino acid metabolism (Martin *et al.*, 2007) and as a modulator of host fatty acid composition (Wall *et al.*, 2009). These studies suggest that interventions which target the metabolic activity of the gut microbiota may have efficacy in obesity and associated disorders.

A number of mechanisms have been proposed to contribute to microbiota-induced susceptibility to obesity and metabolic diseases. Enhanced energy harvest from dietary intake, due to an alteration in microbial composition, has been highlighted as a potential contributor to the pathogenesis of obesity. Other work has suggested that the gut microbiota and its products affect host energy regulation acting through mechanisms involving fasting-adipose adipose factor (FIAF) (Backhed *et al.*, 2004b), adenosine monophoshate (AMP)-activated protein kinase (AMPK) (Backhed *et al.*, 2007), and G-protein-coupled receptor (GPR)41 (Samuel *et al.*, 2008, Cani *et al.*, 2007a). Reciprocal signalling between the immune system and the microbiota, partially via the interaction between LPS and CD14/Toll-

receptor (TLR)4, appears to also play a pivotal role in linking alterations in gut microbiota and chronic low-grade inflammation with risk of metabolic disease in the host (Cani *et al.*, 2008a). Indeed, germ-free mice are protected from the development of diet-induced obesity (Backhed *et al.*, 2007). However, whether the gut microbiota represents a realistic target is unclear.

8.4 A MICROBIAL INTERVENTION

The gut microbiota contains a large, and relatively uncharted, repository of molecules and metabolites that can be deployed in a variety of settings. One common feature among gut microbes is the ability to produce bacteriocins. Bacteriocins are bacterially produced, ribosomally synthesized, small, heat-stable antimicrobial peptides that can have broad or narrow spectrum activity against other bacteria and to which the producer has a specific immunity mechanism (Cotter *et al.*, 2005). Although bacteriocin production by probiotics has been regarded as a beneficial trait for some time (Dunne *et al.*, 1999), the full extent of the benefits of bacteriocin production in the gut is only beginning to be appreciated. The ability of some bacteriocins to modulate specific undesirable components of the gut microbiota, without causing major collateral damage to the remainder of the population, is a very attractive trait (Rea *et al.*, 2011, Cotter, 2011). While the majority of studies to date have focussed on the ability of bacteriocin-producing probiotics to target and control well established gut pathogens, it is to be expected that as high-throughput sequencing analyses of human microbial populations evolves, new targets will emerge. Indeed, distinct clusters or enterotypes in the human microbiome have been described (Arumugam *et al.*, 2011) and provide further support for the use of targeted strategies. With respect to obesity, specific populations that merit targeting have yet to be clearly defined. Although increases in the Firmicutes to Bacteroidetes ratio have been observed in the gut of obese animals (Ley *et al.*, 2005, Turnbaugh *et al.*, 2006), the subject remains controversial (Clarke *et al.*, 2012, Ley, 2010), and it is anticipated that other, more specific, targets will emerge (Pennisi, 2011).

In our study published in *Gut* earlier this year (Murphy *et al.*, 2012) we explored the concept of targeting the gut microbiota using antimicrobials to impact on metabolic abnormalities in murine diet-induced obesity (DIO). Two antimicrobial strategies were used a bacteriocin-producing probiotic *Lactobacillus* and oral vancomycin. Vancomycin is a well known clinical antibiotic that demonstrates anti-Firmicutes activity and has limited systemic effects (Moellering, 2006, Pultz *et al.*, 2005, Yap *et al.*, 2008) while *Lactobacillus salivarius* UCC118 is a genetically well characterized probiotic strain (Claesson *et al.*, 2006) that produces a broad-spectrum class II two peptide bacteriocin, Abp118, which is active against Firmicutes (Flynn *et al.*, 2002). To differentiate between the influence of the bacteriocin and that of the probiotic *per se* on metabolic dysregulation, a bacteriocin-negative (Bac⁻) isogenic derivative of *L. salivarius* UCC118 was used (Corr *et al.*, 2007). This approach was used previously to establish the critical importance of Abp118 production with respect to the ability of *L. salivarius* UCC118 to control *Listeria monocytogenes* infection in the murine gut.

The experimental design involved the feeding of a high fat (45%; HF) diet to 4 groups of mice over a 12 week period following by an 8-week intervention period where mice continued to receive a 1) HF diet, 2) HF diet in addition to oral vancomycin, 3) HF diet in addition to *L. salivarius* UCC118 and 4) HF diet with the Bac⁻ strain, respectively. A fifth control group received a low fat (10%) diet throughout the 20 week study. A number of analyses took place during and/or at the end of the intervention period. A high throughput DNA sequencing based analyses of the gut microbiota of faecal pellets collected at the end of the intervention period showed that both vancomycin and the bacteriocin-producing probiotic significantly altered the gut microbiota in diet-induced obese mice, but in distinct ways (Fig 1). From a bacteriocin perspective, the latter observation is particularly notable as, to our knowledge, it is the first occasion upon which the full extent of the impact of a bacteriocin producing probiotic on the gut microbiota has been assessed *in vivo.* Bioinformatic analysis of sequence data showed that vancomycin dramatically impacted upon the overall composition of the gut microbiota whereas the bacteriocin had a more subtle impact. More specifically, at the family level, vancomycin brought about a significant decrease in the

proportions of the *Clostridiaceae*, *Bacteroidaceae* and *Porphyromonadaceae* and a significant increase in the *Enterobacteriaceae* (from levels below the detection threshold to 28% of the population), *Streptococcaceae*, *Desulfovibrionaceae* and *Alcaligenaceae* relative to the HF only alone controls. Comparison of the *L. salivarius* UCC118 Bac$^+$ with the non-bacteriocin-producing strain, *L. salivarius* UCC118 Bac$^-$, showed that the production of the antimicrobial resulted in an increase in *Bacteroidaceae* and a reduction in the proportions of *Bifidobactereaceae* in the gut microbiota of DIO mice.

Notably, some of the populations inhibited were not previously known to be targeted by the antimicrobials employed, highlighting the value of culture-independent analyses. It should also be noted that in recent work involving mice and pigs fed a standard diet, high throughput sequencing again revealed that the production of Abp118 by UCC118 had a significant impact on the gut microbiota (Riboulet-Bisson *et al.*, 2012). Furthermore, traditionally vancomycin is directed against members of the Firmicutes and is reserved as a drug of "last resort" treatment of infections caused by Gram-positive bacteria. However, studies using high throughput sequencing have shown that vancomycin appears to reduce Bacteroidetes and other taxa *in vivo (Rea et al., 2011, Robinson and Young, 2010).* These obeservations highlight the advantages of using global profiling of the gut microbiota and emphasise the risks associated with relying solely on *in vitro* approaches to assess the impact of an antimicrobial (or other bioactive) on the gut microbiota.

In the obese mouse model both vancomycin and bacteriocin derived from *L. salivarius* UCC118 impacted on weight gain over the 8 week intervention period. However, a recovery in the rate of body weight gain for both strategies was observed, suggesting that compensatory microbial adjustments and/or host physiologic adaptations such as changes in energy expenditure, satiety, and food intake (Cani and Delzenne, 2009, Cani *et al.*, 2009, Murphy *et al.*, 2010) (perhaps triggered by changes in the microbiota), may be at play. Of the interventions, only vancomycin treatment resulted in an improvement in the metabolic abnormalities. While these changes are desirable, the negative consequences of such a dramatic alteration of the gut

microbiota, increasing *Enterobacteriaceae* populations and enhancing the risk of antibiotic resistance, ensure that a vancomycin-based weight management programme will remain of theoretical interest and a proof of concept. It is noteworthy that the study shows the potential utility of bacteriocin-producing bacteria to favourably modify the gut microbiota but further work is required to identify bacteriocin-producing probiotics that can have a prolonged effect on energy, metabolism, and weight control.

8.5 MECHANISMS CONTRIBUTING TO MICROBIOTA-INDUCED SUSCEPTIBILITY TO OBESITY AND METABOLIC DISEASES.

The gut microbiota has the potential to influence weight gain and fat deposition through a variety of mechanisms. Changes in gut microbiota composition have been shown to influence energy expenditure, satiety, and food intake (Cani and Delzenne, 2009, Cani *et al.*, 2009, Murphy *et al.*, 2010). There is increasing evidence that the gut microbiota and their metabolic products can influence gut hormones, inflammation, and gut motility (Cani *et al.*, 2012, Lin *et al.*, 2012, Quigley, 2011). Another factor is the ability of the gut microbiota to extract energy by fermenting otherwise indigestible components of the diet ("energy harvest"). We have performed additional unpublished work to examine the effect of manipulating the gut microbiota using vancomycin and the bacteriocin-producing probiotic, *Lactobacillus salivarius* UCC118, on the efficiency of energy harvest, using the levels of short chain fatty acids (SCFA), the major fermentation end-products, and on the energy content of the feces. No difference was found in acetate and propionate production between lean and DIO mice over the 8 week feeding period. Only vancomycin treatment resulted in a decrease in faecal acetate (Fig 2), while neither antimicrobial strategy altered fecal propionate levels or energy content. Although precise energy balance studies were not performed, when combined with our previous work where the energy content of the diet, food intake and faecal output were measured, these data further support our observations that changes in the microbiota are dissociated from markers of energy harvest (Murphy *et al.*, 2010). Indeed, our work suggested that the improvement in metabolic abnormalities

observed with vancomycin treatment of DIO mice may be due to alterations in the inflammatory tone. Of note, a recent study by Cho *et al.*, 2012 showed that subtherapeutic antibiotic therapy increased adiposity, altered bone development and increased hormone levels related to metabolism in young mice suggesting that exposure of the infant gut microbiota to antibiotics may have long-term metabolic consequences. This study highlights the important of age and lifestage may be important factors to consider in the complex relationship between the gut microbiota and obesity.

A recent study in TLR5-deficient mice suggests that malfunction of the innate immune system may promote the development of metabolic syndrome through a mechanism involving the gut microbiota (Vijay-Kumar *et al.*, 2010). Our work showed that vancomycin treatment of DIO mice resulted in an improvement in the inflammatory and metabolic health of the host. In particular, plasma TNF-α levels were reduced in vancomycin-treated DIO mice compared to DIO controls and this corresponded with a trend towards a reduction in the gene expression of TNF-α levels in the liver and visceral adipose tissues. The fact that vancomycin treatment was associated with a decrease in inflammatory tone in DIO mice, despite an increase in the relative levels of *Enterobactericeae*, again highlights the complexities of the host/microbiota relationship. Furthemore, studies by others have suggested that the gut microbiota may contribute to the onset of insulin resistance and the low-grade inflammatory tone characterizing obesity through a mechanism involved in high-fat induced metabolic endotoxemia and toll-like receptors but the specific components of the gut microbiota responsible for the interaction remain to be identified (Cani *et al.*, 2008a, Hotamisligil and Spiegelman, 1994, Ley *et al.*, 2005). Interestingly, *Serino et al., 2011* recently identified a gut microbial profile specific to the diabetes-sensitive and diabetes-resistant metabolic phenotypes and found an increased Bacteroidetes to Firmicutes ratio and a reduction in *Lachnospiraceae* family and *Oscillibacter* genus associated with the diabetic phenotype.

In addition to SCFA production and energy extraction from the diet, a possible link between diet, gastrointestinal bacterial metabolism, and immune and inflammatory responses seems likely. The G protein-coupled receptors, GPR43 and GPR41 have been identified as endogenous

receptors for SCFAs. Maslowski *et al.*, 2009 showed that stimulation of GPR43 by SCFAs was necessary for the resolution of inflammatory responses, in models of colitis, arthritis and asthma through a mechanism involving GPR43. In addition, new research has highlighted SCFAs and their receptors as potential targets for the treatment of obesity and diabetes. A recent study showed that chronic treatment of butyrate and propionate to DIO mice suppressed food intake, protected against high-fat diet-induced weight gain and glucose intolerance, and stimulated gut hormone secretion (Lin *et al.*, 2012). Indeed, it has been suggested that the amount of SCFA produced by the gut microbes, more than the composition of the microbiota could impact on the host's weight balance (Schwiertz *et al.*, 2009). However, deciphering the role of SCFA in obesity, diabetes and inflammatory conditions will depend on identifying the components of the obese gut microbiota actively involved in the production of SCFAs and interactions amongst them, in addition to, an improved understanding of how diet and age relate to SCFA levels.

8.6   CONCLUSION

There is increasing evidence to suggest that gut microbiota and their metabolic products can influence obesity and metabolic health. Harnessing the bacteriocin-producing capacity of the gut and identifying selective pharmabiotics which can alter the development of obesity and associated conditions as a consequence of changing the gut microbiota represents a realistic therapeutic strategy for future development.

8.6 REFERENCES

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Figure 1 Schematic overview of the effect of bacteriocin-producing probiotic *Lactobacillus salivarius* UCC118 and oral vancomycin on the composition of the gut microbiota at family level in diet-induced obese mice.
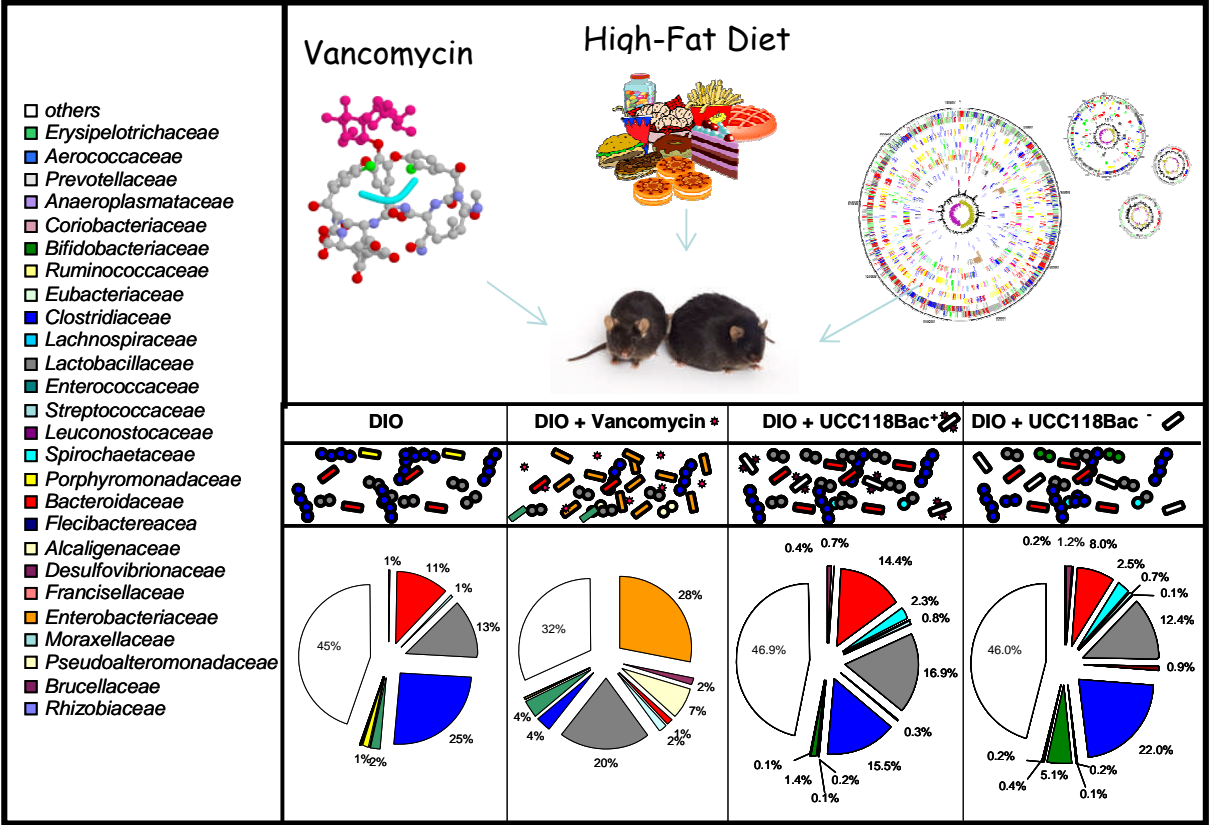
Figure 2 Acetate and propionate production (µmol/g) over the 8 week intervention period in (i) lean, DIO and vancomycin-treated DIO mice and (ii) DIO mice treated with the bacteriocin-producing probiotic strain *L. salivarius* UCC118 Bac$^+$ (1x10^9 cfu/day) compared to DIO mice treated with a non-bacteriocin-producing derivative *L. salivarius* UCC118Bac$^-$ (1x10^9 cfu/day). Data represented as mean ±SEM, *n*=9-10. *p<0.05
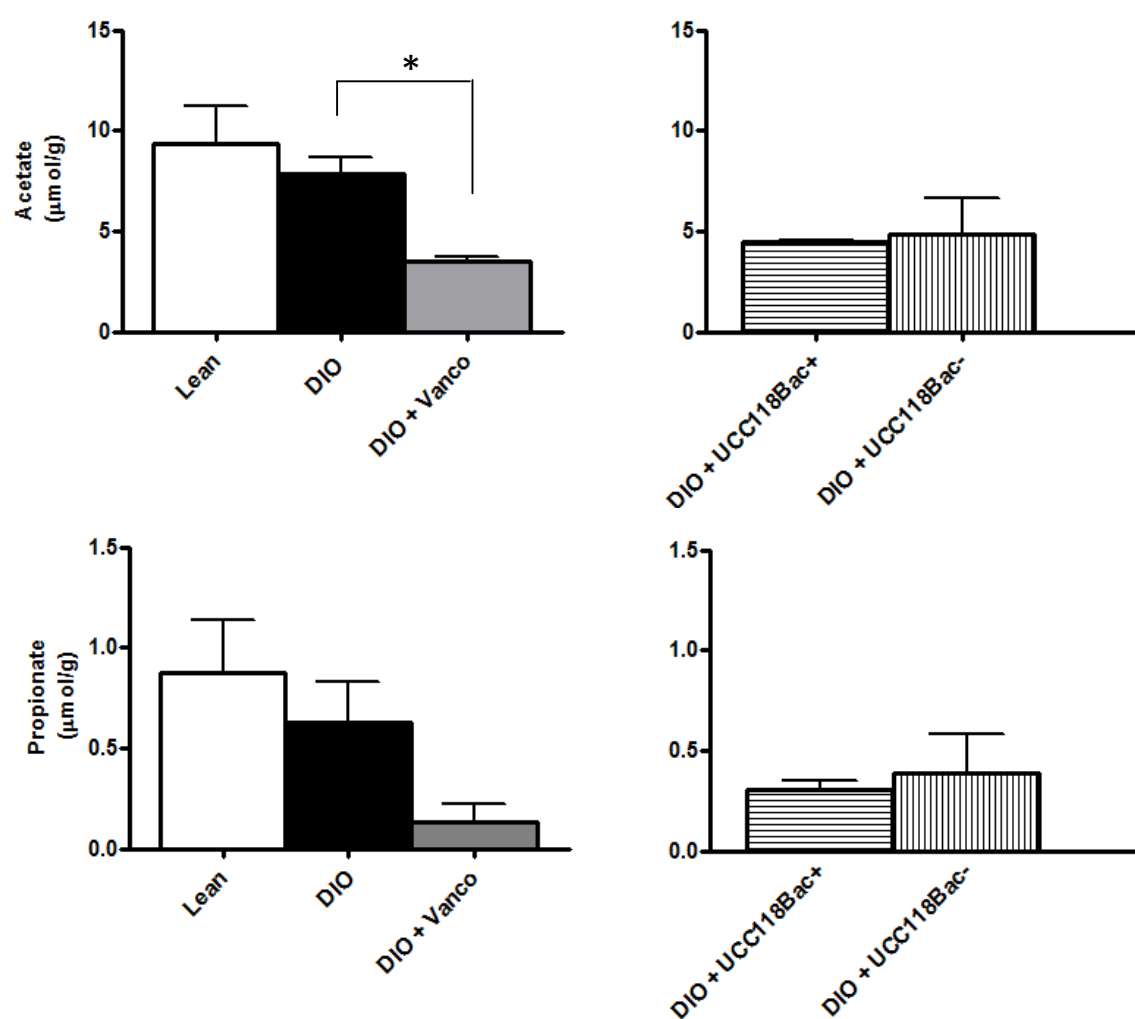
CONCLUSION

Obesity has become one of the greatest health concerns of the twenty first century, with worldwide obesity numbers having nearly doubled since 1980 (WHO, 2013). This multifactorial condition is most simply described as a prolonged imbalance between energy intake and energy expenditure resulting in storage of excess energy as fat. While modern eating habits and decreased physical activity are major contributory factors, research has pointed towards a link between obesity and the composition and functionality of the gut microbiota (Cani and Delzenne, 2011, Flint, 2011, Greiner and Bäckhed, 2011). More specifically, the gut microbiota composition is significantly altered in obesity and diabetes in both animal and human studies and is characterised by reduced diversity (Larsen *et al.*, 2010, Ley *et al.*, 2005, Ley *et al.*, 2006, Turnbaugh *et al.*, 2008, Turnbaugh *et al.*, 2006, Turnbaugh and Gordon, 2009). Therefore the aim of the work reported in this thesis was to investigate the impact of a variety of factors on the obesity-associated gut microbiota and, in turn, how these factors could impact on weight gain.

The gut microbiota is dynamic and adaptable, and a number of treatments including antibiotics, prebiotics and probiotics could hypothetically influence host metabolism favourably by targeting the gut microbiota. In the initial study reported here (Chapter 2), disruption of the gut microbiota with the glycopeptide antibiotic vancomycin and/or the bacteriocin-producing probiotic *L. salivarius* UCC118 was shown to reduce weight gain in diet-induced obese mice (transiently in the latter case), with vancomycin also improving the metabolic abnormalities associated with obesity. To our knowledge, this is the first evidence that a bacteriocin producing probiotic can significantly alter the gut microbiota *in vivo*. Bacteriocin production increased the relative proportions of Bacteroidetes and Proteobacteria and decreased Actinobacteria compared with a bacteriocin-negative derivative. The use of the antibiotic vancomycin was prompted by the fact that the antibiotic had been reported to specifically target Firmicutes (Yap *et al.*, 2008), members of which had previously been found to be increased in the obesity associated gut microbiota (Ley *et al.*, 2005, Ley *et al.*, 2006, Turnbaugh *et al.*, 2006). However, culture independent analysis revealed that

vancomycin treatment had a more global impact, with a large reduction in Firmicutes and Bacteroidetes and a dramatic increase in Proteobacteria being observed. Our findings provide further evidence for the role of the microbiota in metabolic dysregulation, and highlight the merits of altering the microbiota with antimicrobial agents, such as bacteriocins, to address such issues.

The subsequent chapter (Chapter 3), represents a further extension of that described in Chapter 2. Here, the effects of vancomycin and the bacteriocin-producing probiotic on the gut microbiota were further investigated by assessing their impact in a time-dependent manner. This investigation highlighted the resilience of the gut microbiota to change as, after the initially considerable vancomycin-induced changes, the gut microbiota began to revert back to a profile more comparable to that present in controls. The temporal resilience of the gut microbiota following exposure to antibiotics has also been highlighted in previous studies (Cotter PD, 2012, Fouhy *et al.*, 2012, Lozupone *et al.*, 2012, Rea *et al.*, 2011, Tims *et al.*, 2012, Zhang *et al.*, 2012a). These findings demonstrate that though manipulation of the gut microbiota has the potential to favourably influence host health, challenges remain when employing antimicrobials, prebiotics (Dewulf *et al.*, 2012), microbial transplantation (Vrieze *et al.*, 2012) or other interventions in order to bring about long-term modifications to the obesity-associated (and other) gut microbial populations..

Conjugated bile acids (CBAs) are thought to impact on the gut microbiota through direct antimicrobial effects and/or up-regulation of host defences (Jones *et al.*, 2008, Kumar *et al.*, 2006). Indeed, it has previously been observed that bile acids have a direct effect on the host with increased levels protecting against obesity in leptin deficient mice (Zhang *et al.*, 2012b) and reduced levels of eight bile acids species noted in humanized obese mice (Ridaura *et al.*, 2013). Bile salt hydrolase (BSH) enzymes are produced by gut microbiota to catalyse the breakdown of conjugated bile salts and neutralise this activity (Kumar *et al.*, 2006). In Chapter 4, isogenic strains producing different bile salt hydrolases were shown to vary in the extent to which they alter the gut

microbiota and reduced weight gain in mice. This was established through administration of *E. coli* strains containing aBSH from *L. salivarius* JCM1046 (BSH1) (accession number FJ591091) or from *L. salivarius* UCC118 (accession number FJ591081). These results suggest that the application of different BSH-producing strains as a means of modulating the gut microbiota merits further attention.

Colorectal cancer (CRC) is the third most common cancer and second leading cancer killer in the United States (CDC, 2009). Obese individuals are at an increased risk of developing colorectal cancer (Calle and Kaaks, 2004). Colitis associated cancer is a form of CRC that is preceded by the Inflammatory Bowel Diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD) (Grivennikov, 2013). In Chapter 5 of this thesis the effect of diet and the microbiota on the progression of colitis and colitis-associated colorectal cancer (CAC) progression was investigated. In contrast to previous reports (Singh *et al.*, 1997, Newmark *et al.*, 2001), high fat lard based feeding was shown to protect mice from developing colitis and CAC on the basis of weight and cytokine profile, tumour incidence and number, as well as colon length. High gut microbiota diversity is considered desirable with low diversity reported to be associated with UC and CD (Ott *et al.*, 2004). Analysis of the murine gut microbiota showed a reduction in gut microbial diversity in low fat colitis and low fat CAC mice relative to their high fat fed counterparts. Additionally, it was revealed that high fat colitis and high fat CAC mice had significantly lower proportions of the phylum Proteobacteria relative to low fat fed comparators. The phylum Proteobacteria has been found to be associated with CD, UC and CRC patients (Lepage *et al.*, 2011, Gophna *et al.*, 2006, Frank *et al.*, 2011). These results provide a further insight into the relationship between diet, inducers of colitis and colon cancer and the gut microbiota, and suggest that the impact of high fat diets on microbiota-host responses, inflammation and CAC are more complex than previously anticipated.

The degree to which exercise and diet influences the gut microbiota was examined in Chapter 6. Diet and gut microbiota composition was assessed in professional athletes from an international

rugby union squad while in the regulated environment of pre-season camp. Two control groups, one matched for athlete size with a comparable body mass index (BMI) and another reflecting the background age- and gender-matched population, were also investigated. Gut microbial analysis revealed that exercise and diet, and protein in particular, impact on gut microbial diversity. Microbiota diversity may become a new biomarker or indicator of health (Shanahan, 2010) as loss of biodiversity within the gut has been connected to an increasing number of disorders such as autism, gastrointestinal diseases and obesity associated inflammatory characteristics (Claesson *et al.*, 2012, Kang *et al.*, 2013, Ott *et al.*, 2004, Le Chatelier *et al.*, 2013, Chang *et al.*, 2008). Notably, athletes and low BMI controls had significantly higher proportions of the genus *Akkermansia* than the high BMI control group. *Akkermansia muciniphilla* abundance has been shown to inversely correlate with obesity and associated metabolic disorders in both mice and humans (Everard *et al.*, 2013, Karlsson *et al.*, 2012). Despite these observations, prospective studies will be required in order to better determine the specific impact of protein consumption and exercise, respectively, on the gut microbiota.

Overall the studies in this thesis highlight that microbial diversity is influenced by diet, exercise, antibiotics and disease state, and, in the process, highlights a number of interventions that merit consideration with respect to altering the microbiota with a view to improving host health.

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