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**Effects on weight gain and gut microbiota in rats given bacterial
supplements and a high-energy-dense diet from fetal life through to 6
months of age**

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Running title: Bacteria affect obesity and gut microbiota

Abbreviations

ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
cfu	colony forming unit
HEDD	high-energy dense diet
LPS	lipopolysaccharides
OPLS-DA	orthogonal partial least-squares to latent structures discriminate analysis
T-RFLP	terminal restriction fragment length polymorphism
T-RF	terminal restriction fragment
VRBD	violet red bile dextrose

Abstract

The aim of this study was to assess the long-term effects of a high-energy dense diet, supplemented with *Lactobacillus plantarum* (Lp) or *Escherichia coli* (Ec) on weight gain, fattening and the gut microbiota in rats. Since the mother's dietary habits can influence offspring physiology, the dietary regimes started with the dams at pregnancy and through lactation, and continued with the offspring for six months. The weight gain of group Lp was lower than for groups C (control) and Ec ($P=0.086$). More retroperitoneal adipose tissue ($P=0.030$) and higher plasma leptin ($P=0.035$) were seen in group Ec compared to group Lp. The viable count of *Enterobacteriaceae* was higher in group Ec than in group Lp ($P=0.019$) and when all animals were compared, *Enterobacteriaceae* correlated positively with body weight ($r=0.428$, $P=0.029$). Bacterial diversity was lower in group Ec than in groups C ($P<0.05$) and Lp ($P<0.05$). *Firmicutes*, *Bacteroidetes* and *Verrucomicrobia* dominated in all groups, but *Bacteroidetes* were more prevalent in group C than in groups Lp ($P=0.036$) and Ec ($P=0.056$). The same five bacterial families dominated the microbiota of groups Ec and C, and four of these were also present in group Lp. The other five families dominating in group Lp were not found in any of the other groups. Multivariate data analysis pointed in the same directions as the univariate statistics. Our results suggest that supplementation of *L. plantarum* or *E. coli* can have long-term effects on the composition of the intestinal microbiota, as well as on weight gain and fattening.

Keywords: body weight; probiotics; biochemical markers; gut microbiota; high-energy dense diet

Introduction

Although the primary reason for obesity is energy intake exceeding energy expenditure, it is becoming increasingly clear that overweight can also be associated with individual differences in energy extraction capacity, genetic background and gut microbiota⁽¹⁻⁶⁾. While intestinal microbes contribute to colonisation resistance, nutrition, and the development and tuning of the immune system⁽⁷⁾, studies in both human and animals have also shown a connection between the gut microbiota and obesity⁽¹⁻⁵⁾.

Antibiotic treatment is known to manipulate the intestinal microbiota. In experimental studies it has been postulated that antibiotic treatment during pregnancy impairs the physiology and intestinal microbiota of rat pups⁽⁸⁾, and antibiotic modulation of mice gut microbiota has been shown to change the metabolic status of the host⁽⁵⁾.

Since the sterile foetus is colonised by microorganisms from the mother and the surrounding environment at birth, it can be hypothesised that the microbial heritage from the mother can be important for the offspring's development and health. Furthermore, the microbiota of the mother can be affected by dietary factors. For example, a high intake of fat can affect the gut microbiota⁽⁵⁾.

Constant exposure to bacteria through diet may well have effects in the long run. In this study, the two non-pathogenic bacteria *Lactobacillus plantarum* DSM 15313, designated in the division *Firmicutes* (family *Lactobacillaceae*), and *Escherichia coli* CCUG 29300^T (T = type strain), designated in the division *Proteobacteria* (family *Enterobacteriaceae*), were given to rat dams and their offspring. *L. plantarum* frequently occurs spontaneously and in high numbers in most lactic acid fermented foods, especially those based on plant material, for example, brined olives, capers, sauerkraut, salted gherkins, cassava, and in grape juice and

wine⁽⁹⁾. However, *L. plantarum* is also present on human intestinal mucosa⁽¹⁰⁾ and probiotic effects of certain strains of this species have been documented in humans, e.g. reduction in abdominal bloating and pain in patients with irritable bowel syndrome⁽¹¹⁾ and reduction in cardiovascular disease risk factors⁽¹²⁾. Furthermore, administration of *L. plantarum* to pregnant dams has been shown to stimulate gut growth and development in their suckling offspring⁽¹³⁾. The particular strain, *L. plantarum* DSM 15313, used in this study has been shown to mitigate colitis, translocation and liver injury in rats⁽¹⁴⁻¹⁵⁾. Refrigerated foods such as milk, meat and vegetables in an early phase of deterioration, before loss of apparent edibility, expose the consumer to different types of bacteria. Those bacteria are typically Gram-negative *Proteobacteria* such as *Pseudomonas* and *Enterobacteriaceae* that have pro-inflammatory lipopolysaccharides (LPS) associated to their cell membranes⁽¹⁶⁾. *E. coli* is a Gram-negative commensal in the gastrointestinal tract. Non-pathogenic strains of *E. coli* can also exert inflammatory stimuli by its LPS, and changed caecal microbiota, as well as increased inflammatory level, has been observed in pups to rats receiving *E. coli* during pregnancy and lactation⁽⁸⁾. It has been shown in mice that LPS in the blood (endotoxemia) is a starting point for insulin resistance and obesity⁽¹⁷⁾. Moreover, it has been depicted that a high-fat diet increased LPS-containing organisms in the gut and increased transfer of LPS into the blood, which resulted in increased systemic inflammation⁽⁵⁾.

High biological diversity normally indicates an ecosystem in healthy balance, while a low bacterial diversity in the gastrointestinal tract has been observed in patients with Crohn's disease, ulcerative colitis and atopic eczema⁽¹⁸⁻²⁰⁾. Recently, obese twins were reported to have lower intestinal diversity compared to lean twins⁽⁴⁾. Interestingly, the probiotic strain *Lactobacillus plantarum* DSM9843 has been shown to improve colonic bacterial diversity in patients with incipient arteriosclerosis⁽²¹⁾. However, there is a lack of knowledge of the long-

term effect of probiotic administration regarding both gut microbiota and body weight regulation.

The present study was designed to evaluate the long-term effects on microbiota, weight gain and fattening of regular intake of two fundamentally different non-pathogenic commensal bacteria in combination with a challenging energy-rich diet. To further stress the system, by exposure from foetal life to adulthood, rat dams of the outbreed Sprague-Dawley stock were mated and fed with high-energy dense diet (HEDD) and bacterial supplement during pregnancy and lactation, and then their offspring received the same treatment for six months.

Experimental Methods

Study design and animals

For each study group, three adult female Sprague-Dawley rats (Taconic, Ry, Denmark) of similar weight and age were mated with different males. From two weeks before parturition, dams were housed individually and were at that time assigned diet and bacterial supplements as below. At weaning, about four weeks of age, nine of the female offspring from each study group were randomly mixed into cages with three animals in each cage. They continued in the experiment for six months. Throughout the experiment, animals were housed in polycarbonate cages on chopped aspen wood bedding (Beekay bedding; Scanbur BK AB, Sollentuna, Sweden) and the experiment was run in a controlled environment ($21 \pm 1^\circ\text{C}$, $50 \pm 10\%$ relative humidity, 12:12-h light-dark cycle). The experiment was conducted in compliance with all relevant Swedish laws and institutional guidelines and Lund Regional Ethical Review Board approved the study.

Diets, bacterial supplement and body weight recordings

The purified HEDD with 41 energy percent (E%) fat and 41E% carbohydrates, of which half was sucrose, and 18E% protein, was produced by Research Diets (New Brunswick, NJ, USA) (Table S1, available online). Feed was administered *ad libitum* and the consumption per cage was recorded once a week. In addition to the HEDD, one group received *L. plantarum* DSM 15313 suspended in freezing medium (4.28 mM- K_2HPO_4 , 1.31 mM- KH_2PO_4 , 1.82 mM-Na-Citrate, 0.87 mM- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.48 mM-98 % glycerol) (Lp) while another group was given *E. coli* CCUG 29300^T suspended in freezing medium (Ec) in their drinking water. The control (C) group received a corresponding amount of freezing medium in their water. The treatment to dams began during the last two-thirds of pregnancy and continued during lactation. The offspring were then given the same treatment as their mothers until six months of age.

A frozen high-density preparation of *L. plantarum* DSM 15313, originating from the mouth of a healthy human individual, was provided by Probi AB, Lund, Sweden. *E. coli* CCUG 29300^T, a non-pathogenic strain of human origin, was grown in brain heart infusion medium (Oxoid, Basingstoke, Hampshire, England) in a shaking water bath at 36°C for 16 hours. The cells were stored in freezing medium after harvesting. All bacteria were kept at -80°C until feeding. The bacterial suspension was poured into the drinking water on the day of administration. Water was changed, and consumption was recorded daily. Confirmed by culturing, viable counts of *L. plantarum* and *E. coli* were stable during 24 hours in tap water at room temperature. Each cage was given 10⁹ colony forming units (cfu) per day of *L. plantarum* or *E. coli* in 200 ml water. Every rat consumed approximately 10⁸ cfu per day of *L. plantarum* or *E. coli*. The birth weight of the litter (all pups taken together) was recorded the day after delivery and a mean birth weight of each animal was assigned. Body weight was measured weekly from weaning until sacrifice.

Procedure at animal sacrifice and blood and organ sampling

At six months of age, non-fasted animals were anaesthetised by subcutaneous injection of 0.375 mg fluanisone and 0.012 mg fentanyl citrate (Hypnorm[®] VetPharma, Leeds, UK)/100 g body weight and 0.188 mg midazolam (Dormicum[®] Roche, Basel, Switzerland)/100 g body weight. Under aseptic conditions a laparotomy was performed through a midline incision. Blood from the aorta was sampled into ice-chilled endotoxin-free polystyrene tubes (Lonza, Copenhagen, Denmark) containing EDTA as an anticoagulant to obtain plasma. Blood was also taken in BD Vacutainer tubes (Becton Dickinson, Plymouth, UK) to obtain serum. Plasma and serum were stored at -80°C until further analyses. Plasma for determination of endotoxin was stored in endotoxin-free tubes (Biopur 1.5 ml tubes, Eppendorf, Hamburg, Germany). Left retroperitoneal and periovarial adipose tissue, spleen and adrenals were

carefully dissected and weighed. Caecum and rectal content and liver tissue were frozen, in freezing medium for culturing, in liquid nitrogen, and stored at -80°C until analyses.

Biochemical analyses

Plasma leptin was analysed with ELISA (Crystal Chem, IL, USA), with detection limit 0.2 ng/ml. Plasma haptoglobin was analysed colorimetrically, with an assay sensitivity of 0.05 mg/ml (Phase™ Range Haptoglobin Assay; Tridelata Development Ltd, Ireland), according to the manufacturer's instructions. Plasma endotoxin was determined using an accredited chromogenic endpoint method based on limulus amebocyte extract (Charles River Laboratories, Charleston, SC, USA). Samples were diluted 1:10 and heated at 75°C for 10 minutes; detection limit was 0.03 EU/ml. Each sample was spiked with endotoxin and 50% recovery was ensured in order to analyse lack of inhibitors and verify reliability. Serum bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were analysed colorimetrically and rectal content of calprotectin was analysed with ELISA (Immundiagnostic AG, Bensheim, Germany).

Liver histology

Liver tissue was fixed in 4% buffered formalin (Histolab, Göteborg, Sweden), rinsed in water and dehydrated in graded ethanol concentrations, followed by xylen and embedded in paraffin. Sections (5 µm) were mounted on slides, deparaffinised, and stained with hematoxylin and eosin and the prevalence of steatosis was scored as follows: 0 = no visible steatosis, 1 = steatosis in <1/3 of the hepatocytes; 2 = steatosis in 1/3 to 2/3 of the hepatocytes; 3 = steatosis in >2/3 of the hepatocytes. The histological evaluation was performed by a blinded scientist.

Microbial analyses

In order to analyse caecal amount of viable lactobacilli and *Enterobacteriaceae*, conventional dilution series were performed and samples from appropriate dilutions were plated on Rogosa agar (Oxoid) and violet red bile dextrose agar (VRBD; Oxoid) and incubated at 37°C for 72 hours anaerobically and 24 hours aerobically, respectively. Translocation of viable bacteria was evaluated by anaerobic and aerobic cultivation of liver tissue on brain heart infusion agar (Oxoid) at 37°C for 72 hours. Microbial diversity was assessed with the Terminal Restriction Fragment Length Polymorphism (T-RFLP) method, and cloning and sequencing were used to imply phylogenetic affiliations of the intestinal microbiota. The procedures are described in the supplementar material available online.

Calculations

The relative abundance of each T-RF within a given T-RFLP pattern was calculated as the peak area of the respective T-RF divided by the total peak area of all T-RFs, in the given T-RFLP pattern, detected within a fragment length of 20 to 600 base pairs (bp). Relative abundance was used for calculations of diversity index. Shannon and Weaner (Shannon, H') and Simpson (D) indices were calculated for all individuals by using the equations: $H' = -\sum p_i \ln p_i$ and $1-D$ where $D = \sum p_i^2$, where p_i is the relative abundance of i th peak in the community⁽²²⁾. T-RF incidences were calculated with Fisher's Exact test and two-tailed P -values were reported.

Statistical evaluation was performed using Kruskal-Wallis followed by Nemenyi-Damico-Wolfe-Dunn test⁽²³⁾ using the package coin⁽²⁴⁾ in the software R 2.9.1. Spearman rank order correlation was calculated by SigmaStat 3.1 (Systat Software, Point Richmond, USA) and Fischer's Exact Test was calculated with Statistics Online Computational Resource (<http://www.socr.ucla.edu/SOCR.html>). A P -value of <0.05 was considered significant.

Multivariate data analysis with orthogonal partial least-squares to latent structures discriminate analysis (OPLS-DA) (SIMCA-P+12.0.1, Umetrics, Sweden) were performed to reveal differences between the treatment groups⁽²⁵⁻²⁶⁾.

Results

Animal health and performance

All animals (n=9/group) appeared healthy throughout the study, with the exception of one rat in group Ec that was sacrificed after 12 weeks after veterinarian consultation and recommendation, due to the finding of a subcutaneous tumour. The data from this rat was excluded. One of the remaining Ec rats was perceptually leaner than the others, as confirmed at dissection at the end of the study. No signs of disease could be diagnosed, and this rat was included in the data evaluation. No difference in feed and water consumption was observed among the groups.

Effects on body and organ weights

Pups in group Lp had the lowest mean birth weight (7.47 g) and group Ec had the highest (9.65 g) while group C had intermediate birth weight (8.15 g). However, because of the limited numbers of litters, statistical analysis could not be performed. Group Lp had lower body weight throughout the study compared to groups C and Ec, and at six months the difference approached statistical significance ($P=0.086$; Fig. 1, Table 1). Significantly more retroperitoneal fat was observed in group Ec compared to group Lp ($P=0.03$; Table 1). The weight of retroperitoneal fat correlated positively to body weight after six months ($r=0.454$, $P=0.02$). Group Ec had a higher median weight of the periovarial fat deposit compared to the other groups but the difference was not significant (Table 1). The weight of spleen was higher in group Ec than in group Lp ($P=0.006$; Table 1). Group Lp had heavier adrenals than group C ($P=0.022$; Table 1).

Effects on liver histology

The incidence of liver steatosis was significantly lower in group Ec compared to group C ($P=0.008$; Table 1).

Effects on biochemical markers

Group Ec showed significantly higher plasma leptin concentrations than group Lp ($P=0.035$; Table 2). Haptoglobin was significantly lower in group Ec compared to group C ($P=0.003$; Table 2), but no significant differences were observed in the blood levels of endotoxin, bilirubin or the liver enzymes AST, ALT and ALP, nor intestinal calprotectin (Table 2). However, group C had the highest AST concentration, which approached statistical significance ($P=0.087$).

Effects on microbiota

Significantly lower bacterial diversity was seen in the caecum of group Ec compared to that of group C when diversity indices were calculated for the T-RFLP profiles (Table 3). When *MspI* was used for DNA digestion, group Ec had significantly lower diversity than group Lp (Table 3).

Clone libraries from the different groups showed that divisions *Firmicutes*, *Bacteroidetes* and *Verrucomicrobia* were represented in all three groups at a level ranging 65-77%, 4-26% and 5-15%, respectively. At the hierarchical level of family, the same five taxa dominated the microbiota in animals with the most diverse microbiota (according to T-RFLP profiles) in groups C and Ec, while nine taxa were seen in group Lp (Fig. 2). As regards proportions of the microbiota, significant differences were observed between the treatment groups at division level. Members of the *Bacteroidetes* were less prevalent in the Ec library than in the C library (LibCompare in Ribosomal Database Project, $P=0.036$) with close to significance for less prevalence in the Lp library compared to the C library ($P=0.056$). No significance in proportions of sequences belonging to *Firmicutes* was observed. All sequences of *Verrucomicrobia*, independent of treatment group, corresponded to *Akkermansia muciniphila*.

Insertae Sedis XIII included sequences most similar to those of *Anaerovorax odorimutans* but with a low similarity score (0.94). Incidence of T-RF differed significantly between groups Lp and C, and between group Ec and C (Table S2, available online). The gut microbiota was also significantly different in groups Lp and Ec since group Ec, with *AluI* digestion, had the highest incidence of T-RF at 77bp and the lowest incidence of 203bp. With *MspI* digestion, the T-RF at 257bp was higher and T-RFs of 80bp, 258bp and 516bp were found at significantly lower incidence in group Ec than in group Lp.

The viable count of *Enterobacteriaceae* in caecal content was significantly higher in group Ec compared to that in group Lp ($P=0.019$; Table 3), and correlated positively with body weight after six months ($r=0.428$, $P=0.029$). No significant differences were seen in lactobacilli count of caecal content or translocation of viable bacteria to the liver.

Multivariate analysis

Results of multivariate data analysis with OPLS-DA depict substantial difference between the different groups. Important for the separation of group Lp and Ec were fat deposition, viable count of *Enterobacteriaceae* and microbial diversity (Fig. 3). Significant for the separation of the groups Ec and C were fat deposition and diversity indices while separation of the groups Lp and C were based on body weight and weight of adrenals, Q2 (cum) 0.79 and 0.58, respectively.

Discussion

The feeding with HEDD and the two bacterial supplements was initiated during pregnancy because it is known that metabolic mal-programming in the offspring can occur when the dietary intake of the pregnant dam is manipulated⁽²⁷⁻²⁸⁾. In the present study, *E. coli* was chosen as one supplement since it has been shown that this Gram-negative bacterium, with pro-inflammatory LPS associated to its cell wall, can occasionally dominate the intestinal microbiota in human infants⁽²⁹⁾. *E. coli* was also seen to dominate on the sigmoidal mucosa in one out of nine 60-year old volunteers without clinical symptoms or medication when sequence analysis of cloned 16S rRNA genes, as enriched by PCR from biopsies, was applied (91% of the clone library were related to *E. coli*; G.M., unpublished results). In contrast, *L. plantarum* was selected since it is a Gram-positive commensal of the gastrointestinal tract and can confer health benefits as probiotics⁽¹⁰⁻¹¹⁾.

In this six-month study, long-term bacterial supplementation seemed to affect the weight development of the rats, without having any effect on the feed consumption. Furthermore, when repeated measures analysis of variance was calculated, group Lp had significantly lower body weight compare to group C (data not shown). Importantly, *L. plantarum* decreased body weight gain, and *E. coli* increased body fat, the latter consistent with the hypothesis of Cani *et al.*⁽⁵⁾, which suggests increased body weight gain by high fat feeding and increased load of Gram-negative bacteria. To our knowledge, effects of probiotics on weight gain have not previously been experimentally evaluated from foetal life to adulthood. However, *Lactobacillus gasseri* SBT2055 has been shown to significantly reduce abdominal visceral and subcutaneous fat⁽³⁰⁾ and lower body mass index has been suggested in children after *Lactobacillus rhamnosus* GG administration⁽³¹⁾.

Intestinal diversity has previously been shown to increase in rodents receiving probiotics⁽³²⁻³³⁾. However, these findings were from short-term studies in which no physiological parameters were evaluated. Thus, there is a general lack of knowledge regarding the long-term effects of bacterial supplements on intestinal microbial diversity in combination with changed homeostasis. Calculation of diversity index is a common way to estimate microbial diversity^(18, 20-22), and low diversity has been associated with various diseases⁽¹⁸⁻²⁰⁾.

In the present study, reduced bacterial diversity indices were observed in group Ec compared to group C. Group Ec had significantly increased viable count of *Enterobacteriaceae* in the caecum compared to that of group Lp, as well as lower diversity indices, thereby indicating that *E. coli* consumption might associate with a tendency to overgrowth of unfavourable bacterial groups. Furthermore, the amount of *Enterobacteriaceae* in the caecum content was positively correlated with body weight. The incidence of specific T-RFs varied among the groups, which further supports the observation of different intestinal microbiota according to treatment.

Previous reports suggest a reduction in the abundance of *Bacteroidetes* and a proportional increase of *Firmicutes* in obese individuals⁽²⁻³⁾. However, this was not the case in the present study as both the group of the heavier Ec animals and the leaner Lp animals had lower proportions of *Bacteroidetes* compared to group C. Furthermore, the proportion of *Firmicutes* did not vary between the groups. This suggests that the microbiota must be dealt with on lower hierarchical levels than that of division, preferably on the species level but at least at family and genus levels. It also seems important to look into different experimental models with different strains and species of animals, and also different categories of humans. Hence, more extensive studies are required to fully explain the connection between gut microbiota and obesity.

In an attempt to evaluate the correlation of intestinal microbiota and changed homeostasis, we measured various biochemical markers. Previously, adult offspring to LPS-exposed dams were reported to have higher body weight, heavier fat deposits and higher serum leptin⁽³⁴⁾. The same scenario was true in this study for the group where the mothers and the offspring received *E. coli*. Interestingly, the opposite effect was seen in the group receiving *L. plantarum*: lower leptin levels were observed in combination with lower body weight.

The acute-phase protein haptoglobin, mainly produced by the liver⁽³⁵⁾, is known to increase during inflammation, but contradictory results have also been reported⁽³⁶⁻³⁷⁾. Since obesity is characterised by a low-grade inflammation^(5, 38) and since group Ec in our study had more body fat and higher leptin levels, the animals in this group were assumed to have a higher inflammatory tone. In spite of this, group Ec had significantly lower haptoglobin concentrations compared to group C. Interestingly, one of the primary functions of haptoglobin is to restrict iron availability to pathogenic bacteria by formation of an irreversible complex with haemoglobin released from damaged erythrocytes⁽³⁹⁻⁴⁰⁾. The decreased haptoglobin concentration found in group Ec might be a result of even non-pathogenic *E. coli* strains being equipped with efficient means (siderophores) to absorb iron⁽⁴¹⁾. In the present study, significantly heavier spleen was found in group Ec compare to group Lp indicating systemic inflammation in group Ec⁽⁴²⁾. Furthermore, intravascular haemolysis is associated with splenomegaly and decreased haptoglobin levels⁽⁴³⁾.

Esposito *et al.* reported reduced liver damage and inflammation in young rats on high-fat diet when this was combined with the probiotic mixture VSL#3 containing different strains of *Lactobacillus* and *Bifidobacterium*, but no microbial evaluation was performed⁽⁴⁴⁾. In the present study, no statistically significant changes in liver enzymes were observed. However,

AST and haptoglobin concentrations were highest in group C, which is in accordance with the higher occurrence of liver steatosis in this group. Non-pathogenic strains of *E. coli* indicate improvement of liver function while other *Enterobacteriaceae* showed the opposite⁽⁴⁵⁾. This highlights the potential to influence the level of steatosis and liver function by modulating the intestinal microbiota. Notably, in the present study, eating behaviour was not controlled prior to blood sampling at sacrifice and, for example, plasma LPS is enhanced after a meal⁽⁴⁶⁾, so some measurements may be on the small side.

It should be stressed that in this study calprotectin in the intestinal content did not vary between groups, suggesting absence of intestinal inflammation. Hence, although systemic effects were observed, neither *E. coli* nor *L. plantarum* seemed to have injured the gastrointestinal tract.

The weight of adrenals was significantly higher in group Lp than in group C, but it was not possible to distinguish the weights of the medulla and cortex, making it hard to evaluate the consequences and underlying relevance of the difference in the weight of this organ.

Although not totally conclusive, the results of this study indicate that it may be possible to affect host homeostasis by bacterial supplementation to rats consuming a high-energy dense diet. In future studies, larger study populations should be used and physiological and microbial characterisation of rat dams should be addressed more extensively. Furthermore, extensive evaluation of the offspring early in life should be performed to distinguish the effect obtained on the foetus *in utero*, during the neonatal and weaning period. Litter sizes and social behaviour, such as cage hierarchy, should also be considered.

In summary, the long-term effects of bacterial supplements to HEDD-fed rats were studied from foetal life to adulthood in outbreed rats. Animals receiving *L. plantarum* had lower body weight, less retroperitoneal adipose tissue and lower plasma leptin compared to those that were given *E. coli*. The diversity of the intestinal microbiota was reduced in rats consuming *E. coli*, which highlights the potential of modulating intestinal microbiota and homeostasis. As shown by the multivariate analysis, fat deposition and weight gain were affected by the gut microbiota, and manipulated by the supplementation of bacteria to an HEDD.

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1 Table 1. Body weights, weights of fat deposits, spleen weight, adrenals weights and liver steatosis in rats fed a high-energy dense diet (HEDD) with
 2 bacterial supplements for six months.

	group C [*] (n=9)		group Lp [*] (n=9)		group Ec [*] (n=8)	
Body weight (g)	340·6 ^a	(313·8 – 373·4)	303·8 ^a	(298·3 – 306·6)	351·8 ^a	(300·1 – 401·7)
Left retroperitoneal fat deposit (mg)	1674 ^{ab}	(1547 – 1824)	1308 ^a	(1103 – 1496)	2903 ^b	(1532 – 4115)
Left periovarial fat deposit (mg)	5777 ^a	(5040 – 5969)	4689 ^a	(4584 – 5161)	8548 ^a	(4451 – 12152)
Spleen (mg)	674 ^{ab}	(564 – 700)	620 ^a	(598 – 626)	735 ^b	(680 – 839)
Adrenals (mg)	61 ^a	(60 – 63)	70 ^b	(65 – 72)	65 ^{ab}	(64 – 72)
Liver steatosis scoring	2 ^a	(1 – 2)	1 ^{ab}	(1 – 1)	0 ^b	(0 – 1)

3 ^{*}C, control; Lp, *L. plantarum* DSM 15313; Ec, *E. coli* CCUG29300^T.

4 Values are expressed as group medians (interquartile range) and values within a row with unlike superscript letters were significantly different
 5 ($P \leq 0.05$).

6

7 Table 2. Concentrations of biochemical markers in rats fed a high-energy dense diet (HEDD) with bacterial supplements for six months.

	group C [*] (n=9)		group Lp [*] (n=9)		group Ec [*] (n=8)	
Leptin in blood (ng/ml)	6.20 ^{ab}	(5.24 – 7.60)	4.47 ^a	(3.73 – 5.75)	9.89 ^b	(7.04 – 12.53)
Haptoglobin in blood (mg/ml)	0.58 ^a	(0.43 – 0.82)	0.35 ^{ab}	(0.33 – 0.49)	0.22 ^b	(0.10- 0.38)
Endotoxin in blood (EU/ml)	0.031 ^a	(<0.030 – 0.035)	<0.030 ^a	(<0.030 – 0.036)	0.035 ^a	(0.033 – 0.045)
Bilirubin in blood (μmol/L)	2.0 ^a	(1.8 – 2.2)	1.6 ^a	(1.5 – 1.7)	1.9 ^a	(1.8 – 1.95)
AST in blood (μkat/L)†	1.80 ^a	(1.74 – 1.95)	1.33 ^a	(1.21 – 1.54)	1.27 ^a	(1.26 – 1.30)
ALT in blood (μkat/L)	0.49 ^a	(0.41 – 0.54)	0.40 ^a	(0.34 – 0.47)	0.44 ^a	(0.39 – 0.46)
ALP in blood (μkat/L)	2 ^a	(1.7 – 2.1)	1.7 ^a	(1.7 – 1.9)	1.5 ^a	(1.3 – 2.1)
Calprotectin in intestine (ng/ml)	1.91 ^a	(1.76 – 1.99)	1.96 ^a	(1.92 – 2.02)	1.89 ^a	(1.84 – 1.94)

8 ^{*}C, control; Lp, *L. plantarum* DSM 15313; Ec, *E. coli* CCUG29300^T.

9 † denotes $P=0.087$.

10 Values are expressed as group medians (interquartile range) and values within a row with unlike superscript letters were significantly different
 11 ($P \leq 0.05$).

12

13 Table 3. Microbial diversity in caecal content, calculated with Shannon and Simpson diversity indices, and viable counts of *Enterobacteriaceae* and
 14 lactobacilli.

	group C* (n=9)		group Lp* (n=9)		group Ec* (n=8)	
Diversity index,						
restriction endonuclease						
Shannon, <i>AluI</i>	2.85 ^a	(2.69 – 2.89)	2.74 ^{ab}	(2.66 – 2.90)	2.50 ^b	(2.32 – 2.63)
Shannon, <i>MspI</i>	2.87 ^a	(2.65 – 2.92)	2.82 ^a	(2.78 – 2.95)	2.48 ^b	(2.23 – 2.60)
Simpson, <i>AluI</i>	0.91 ^a	(0.89 – 0.92)	0.89 ^{ab}	(0.86 – 0.91)	0.86 ^b	(0.82 – 0.88)
Simpson, <i>MspI</i>	0.91 ^a	(0.90 – 0.93)	0.90 ^a	(0.84 – 0.93)	0.88 ^a	(0.83 – 0.93)
Viable count						
<i>Enterobacteriaceae</i>	5.57 ^{ab}	(5.28 – 6.29)	4.91 ^a	(4.63 – 5.13)	5.94 ^b	(5.50 – 6.63)
(log cfu/g caecum content)						
lactobacilli	6.57 ^a	(6.50 – 7.38)	7.15 ^a	(6.98 – 7.74)	6.56 ^a	(6.37 – 6.72)
(log cfu/g caecum content)						

15 *C, control; Lp, *L. plantarum* DSM 15313; Ec, *E. coli* CCUG29300^T.

- 16 Values are expressed as group medians (interquartile range) and values within a row with unlike superscript letters were significantly different
- 17 ($P \leq 0.05$).

18 **Figure legends**

19 Fig. 1. Body weight development

20 Group means \pm SE of body weights from weaning until six months of age. At four weeks,
 21 group Ec had significantly higher body weight than group C (* $P=0.002$) and at five weeks
 22 group Ec were heavier than groups C and Lp ($\dagger P=0.003$ and $\ddagger P<0.001$ respectively). After
 23 six months, group Lp tended to have significantly lower body weight ($P=0.086$). ■, control; ●,
 24 *L. plantarum* DSM 15313; ▲, *E. coli* CCUG29300^T.

25

26 Fig. 2. Identification of sequences in clone libraries to the level of family

27 The figure shows the families to which clones in each library are related. Results are given as
 28 percentage of total number of clones for each specific library. In group C 41 clones were
 29 sequenced and in groups Lp and Ec 44 and 84 clones were sequenced, respectively. C, control
 30 (black bars); Lp, *L. plantarum* DSM 15313 (white bars); Ec, *E. coli* CCUG 29300^T (grey
 31 bars).

32

33 Fig. 3. Multivariate analysis with OPLS-DA

34 OPLS-DA score and loading column plots (a and b respectively) to discriminate the
 35 correlation between groups Lp and Ec. Fat deposition, viable count of *Enterobacteriaceae* and
 36 diversity of the intestinal microbiota were of importance for separation of groups Lp and Ec.
 37 Q2 (cum) was 0.55. Lp, *L. plantarum* DSM 15313; Ec, *E. coli* CCUG 29300^T.

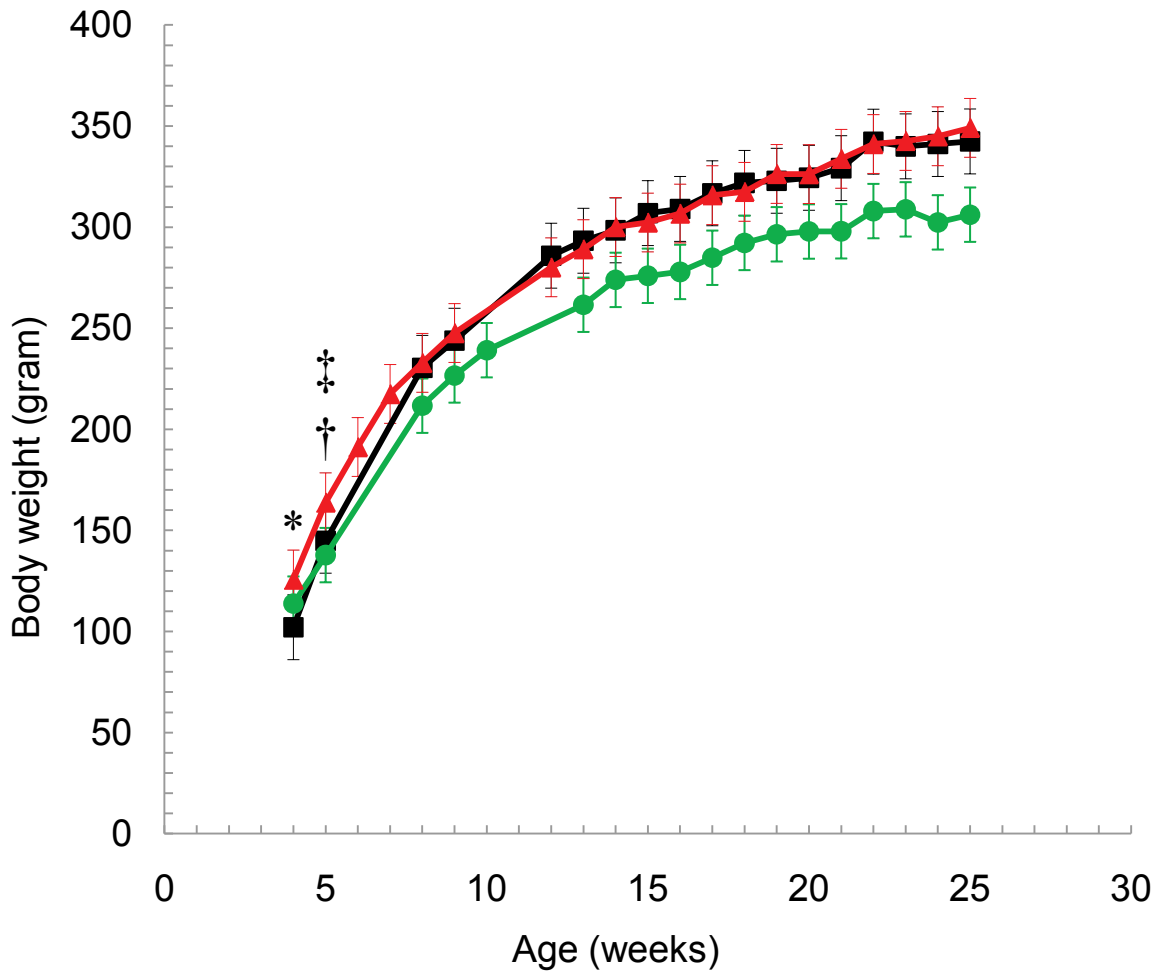


Fig. 1.

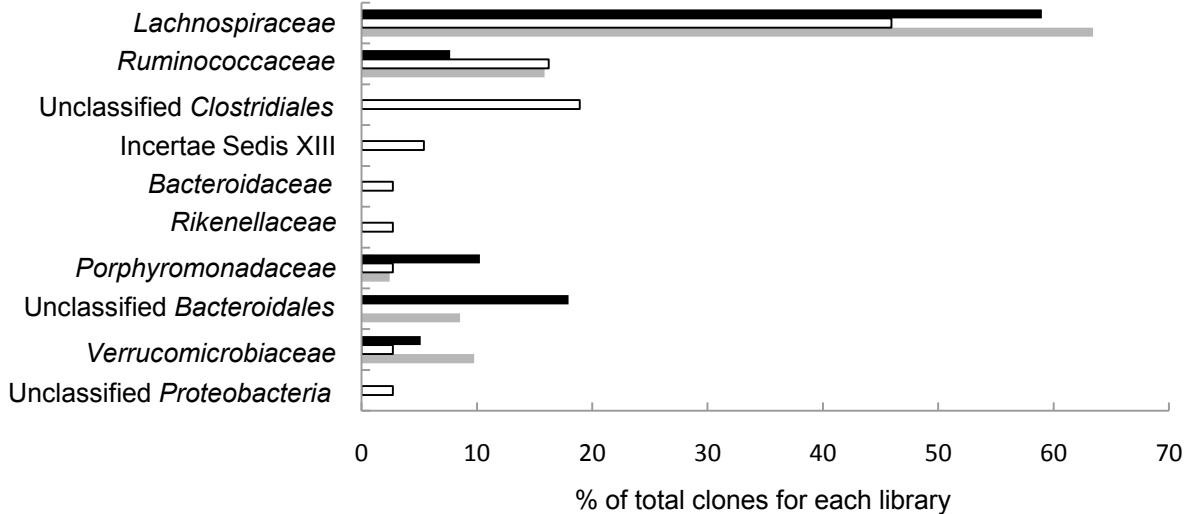


Fig. 2.

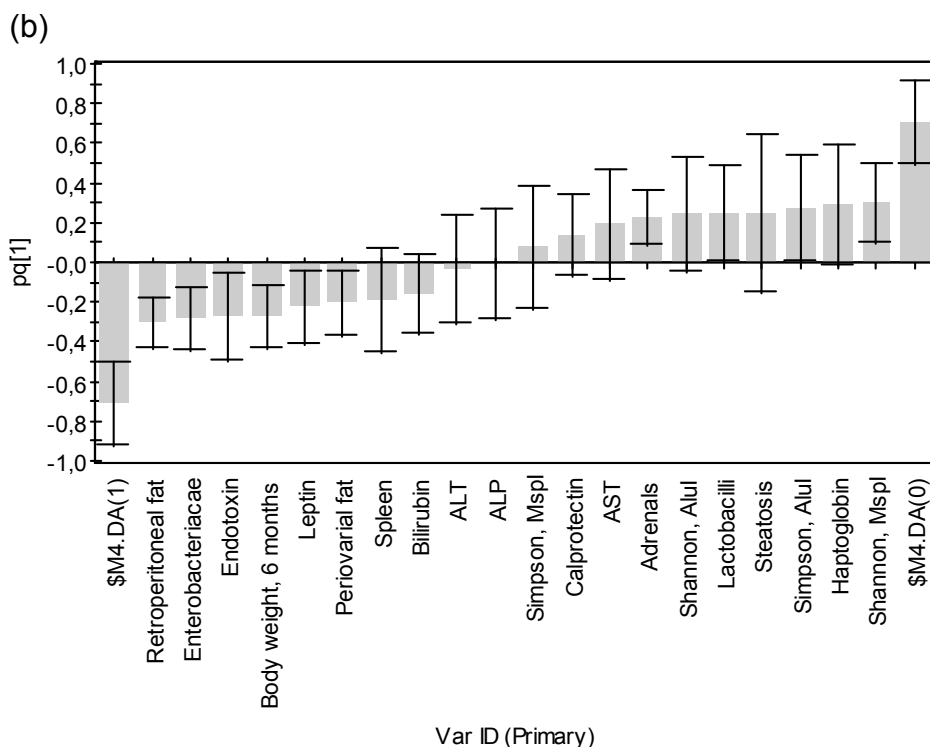
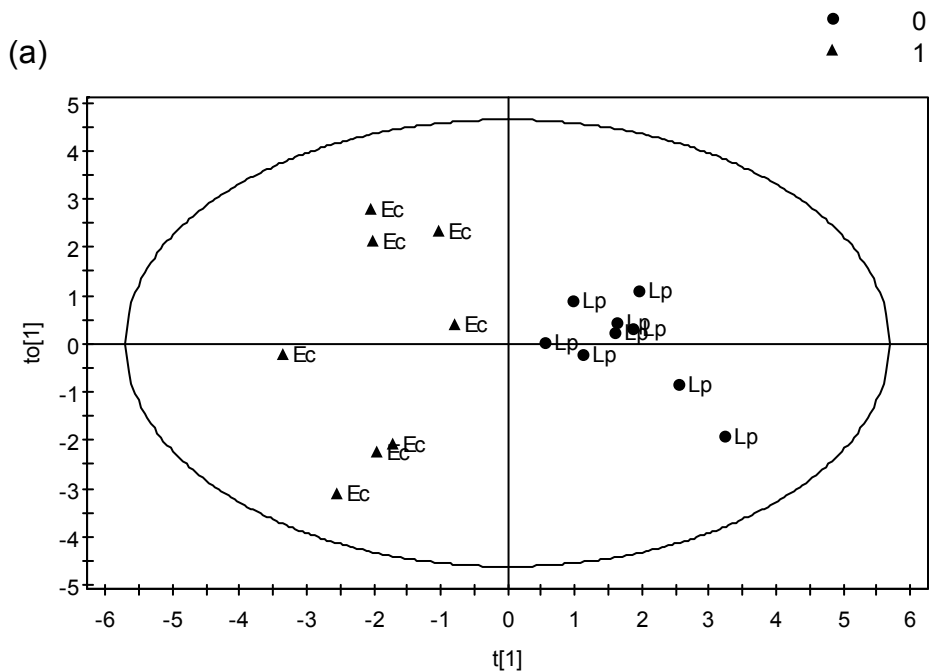


Fig. 3.