

## Changes in gut microbiota due to supplemented fatty acids in diet-induced obese mice

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### Abstract

Consumption of a high-fat diet (HFD), which is associated with chronic ‘low-grade’ systemic inflammation, alters the gut microbiota (GM). The aim of the present study was to investigate the ability of an oleic acid-derived compound (S1) and a combination of *n*-3 fatty acids (EPA and DHA, S2) to modulate both body weight and the GM in HFD-induced obese mice. A total of eighty mice were fed either a control diet or a HFD, non-supplemented or supplemented with S1 or S2. At week 19, faeces were collected in order to analyse the GM. Group-specific primers for accurate quantification of several major bacterial groups from faecal samples were assayed using quantitative PCR. The HFD induced an increase in body weight, which was reduced by supplementation with S1. Furthermore, S1 supplementation markedly increased total bacterial density and restored the proportions of bacteria that were increased (i.e. clostridial cluster XIVa and Enterobacteriales) or decreased (i.e. *Bifidobacterium* spp.) during HFD feeding. S2 supplementation significantly increased the quantities of Firmicutes (especially the *Lactobacillus* group). Correlation analysis revealed that body weight correlated positively with the phylum Firmicutes and clostridial cluster XIVa, and negatively with the phylum Bacteroidetes. In conclusion, the consumption of a HFD induced changes in the faecal microbiota, which were associated with the appearance of an obese phenotype. Supplementation of the HFD with S1 counteracted HFD-induced gut dysbiosis, together with an improvement in body weight. These data support a role for certain fatty acids as interesting nutrients related to obesity prevention.

**Key words:** Gut microbiota; Fatty acids; Obese mice; Quantitative PCR

The worldwide prevalence of overweight and obesity has increased dramatically in the past decades, reaching epidemic levels<sup>(1)</sup>. It is well known that a high-fat diet (HFD) leads, especially in genetically predisposed individuals, to an accumulation of adipose tissue<sup>(2)</sup> and to the development of a cluster of metabolic and cardiovascular disorders such as type 2 diabetes, atherosclerosis, hypertension and stroke<sup>(3)</sup>.

The gut microbiota (GM) has an important role in supplying nutrients and vitamins, giving colonisation resistance against pathogenic bacteria and interacting with the host immune system and intestinal epithelium<sup>(4)</sup>. The possible involvement of the host genotype, particularly as it relates to immunophenotype, has been frequently postulated as a major influence on GM composition and stability. Other variables known to broadly influence the composition of the GM include the type of delivery (vaginal or caesarean), age, the use of antibiotics and other drugs, and ‘lifestyle’ (such as diet, physical activity and stress)<sup>(5–9)</sup>. The significance of the protective role of the GM has been highlighted by the profound impact

seen when the GM is absent or disrupted. Germ-free mice have poorly developed mucosal architecture and rudimentary development of the mucosa-associated lymphoid tissue, being generally small and underweight, and also highly susceptible to intestinal infection<sup>(10)</sup>.

In obese patients, there is a significant change in the composition of the GM compared with lean controls<sup>(11,12)</sup>, and, in rats, these modifications can be induced by the ingestion of a HFD<sup>(13)</sup>. Furthermore, host nutritional status may be markedly influenced by the composition and activities of the GM since the Bacteroidetes:Firmicutes ratio seems to be decreased in obese individuals and genetically obese mice harbour an ‘obese microbiome’, with a transferable elevated capacity for energy sequestration<sup>(14)</sup>.

Given the important role of the GM in association with obesity, several studies have focused on the hypothesis that the onset of obesity may be influenced by targeted modification of the GM by specific nutrients. Indeed, the decrease in bifidobacteria occurring in obese mice fed with

**Abbreviations:** CD, control diet; GM, gut microbiota; HFD, high-fat diet; HFD-S1, high-fat diet supplemented with an oleic acid-derived compound; HFD-S2, high-fat diet in combination with *n*-3 fatty acids; qPCR, quantitative PCR.

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a HFD<sup>(15,16)</sup> was counteracted through the administration of non-digestible oligosaccharides such as inulin-type fructans<sup>(17)</sup>.

The Mediterranean diet has been related to lower rates of obesity<sup>(18,19)</sup>. In this diet, there is a high consumption of olive oil, which contains the MUFA oleic acid, and fish, a rich source (especially oily fish) of *n*-3 PUFA. Some authors have described the effects of oleic acid and others unsaturated fatty acids on body weight in human and experimental models. Vögler *et al.*<sup>(20)</sup> evaluated the actions of C18 fatty acids on the body weight of rats and found that oleic acid could lead to a reduction in adipose tissue mass. In the same study, the authors showed that 2-hydroxyoleic acid, a synthetic derivative of oleic acid, promoted a drastic decrease in the body weight of the treated rats<sup>(20)</sup>. Moreover, supplementation with long-chain PUFA, notably EPA and DHA, can attenuate weight gain and reduce body fat in rodents, particularly epididymal (visceral) fat<sup>(21)</sup>. It has been hypothesised that these fatty acids could exert their effects by promoting changes in GM.

The aim of the present study was to evaluate the effect of a HFD, supplemented or not with fatty acids frequently found in the Mediterranean diet (such as oleic acid and *n*-3 fatty acids), on body weight in a mouse model, and to relate that effect to the changes in the faecal microbiota composition.

## Materials and methods

### Animals

A total of eighty female ICR (CD-1<sup>®</sup>) outbred mice (8 weeks old at the beginning of the study; Harlan Laboratories) were housed in groups of 6 ( $\pm$  2) animals per cage (polyurethane boxes) and maintained at a constant temperature ( $22 \pm 2^\circ\text{C}$ ) in a controlled environment (12 h light–12 h dark cycle, lights off at 18.00 hours), with free access to food and water. Mice were specifically pathogen-free as tested by Harlan and according to the Federation of European Laboratory Science Associations recommendations. Mice were treated according to the guidelines of the European Community Council Directives (86/6091 EEC) as well as to the Spanish laws about protection of animals. The experiments were performed according to the institutional guidelines and were approved by the Complutense University Ethical Committee for Animal Experimentation.

### Diet intervention

During the acclimatisation period (for their adaptation to their new location), all mice were fed with a maintenance diet (Teklad Global 14% Protein Rodent Maintenance Diet, reference 2014; Harlan Laboratories). At week 4, mice were separated into four groups (three cages per group: cage A, *n* 8; cage B, *n* 6; cage C, *n* 6) as follows:

(1) The control diet (CD) group continued with the maintenance diet until the end of the study.

- (2) The HFD group received a HFD (Adjusted Calories Diet 60/Fat, reference TD.06414; Harlan Laboratories) until the end of the study.
- (3) The HFD-supplementation 1 (S1) group was fed for 8 weeks with the HFD, and for another 7 weeks with the same HFD supplemented with an oleic acid-derived compound (1500 mg/kg per d; BTSA-Biotecnologías Aplicadas S.L.).
- (4) The HFD-S2 group was fed for 8 weeks with the HFD, and for another 7 weeks with the same HFD supplemented with a combination of *n*-3 fatty acids (EPA and DHA, 3000 mg/kg per d; BTSA-Biotecnologías Aplicadas S.L.).

The full composition of both the maintenance diet and the HFD is given in Table 1. The energy content of the maintenance diet consisted of 67.3% carbohydrate, 20.1% protein, 12.6% fat and 22.1% fibre; meanwhile the HFD consisted of 21.3% carbohydrate, 18.4% protein, 60.3% fat and 6.5% fibre. Body weight and food intake (monitored by weighing the food provided and left uneaten, and taking into account spillage) were recorded twice per week.

### Faecal microbiota analysis

**Extraction and purification of DNA from faecal samples.** At the end of the study (week 19), faeces from every cage were collected and stored at  $-80^\circ\text{C}$ . For analysis of the microbial community composition, DNA was extracted from the frozen faecal samples (cages B and C), using the QIAamp<sup>®</sup> DNA Stool Mini Kit (QIAGEN GmbH), according to the manufacturer's instructions, using the optional high-temperature step (at  $95^\circ\text{C}$ ). The faecal samples were thawed on ice; for each extraction, 471–955 mg of faeces were weighed and diluted to 100 mg/ml with stool lysis buffer. At the end, DNA was eluted in 200  $\mu\text{l}$  of elution buffer and stored at  $-20^\circ\text{C}$  until use.

The concentrations of the extracted DNA were determined by a fluorometric method based on the fluorochrome Hoechst 33258 (Fluorescent DNA Quantification Kit; Bio-Rad), which is fluorescent when bound to double-stranded DNA. The measurements were performed using the Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (Biotek Instruments) with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Data analysis

**Table 1.** Composition of the diets

	Maintenance diet	High-fat diet
Carbohydrate (%)	48.0	27.3
Protein (%)	14.3	23.5
Fat (%)	4.0	34.3
Saturated (%)	0.7	12.7
Monounsaturated (%)	0.8	16.1
Polyunsaturated (%)	2.5	5.5
Fibre (%)	22.1	6.5
Soluble (%)	4.1	–
Insoluble (%)	18.0	6.5
Energy density (kJ/g)	12.1	21.4
Energy from carbohydrate (%)	67.3	21.3
Energy from protein (%)	20.1	18.4
Energy from fat (%)	12.6	60.3



was performed using Gen5 Software (version 1.10.8), also supplied by Biotek Instruments.

**Primers.** The abundance of specific intestinal bacterial groups was measured by quantitative PCR (qPCR) with group-specific 16S rRNA gene primers (Isogen; Table 2). A short segment of the 16S rRNA gene (200 bp) was specifically amplified by qPCR, using a conserved 16S rRNA-specific primer pair (Table 2), to determine the total amount of commensal bacteria in the faeces (the so-called 'Eubacteria/Pan-bacteria' group). Using the same genomic DNA from each sample, qPCR were completed using group-specific primers to determine the amount of bacteria in each of the following major groups: (1) the phylum Firmicutes, the first quantitatively dominant phylum in mouse faeces, which contains the cell wall-less mycoplasmas (class Mollicutes) and the low G + C Gram-positive bacteria (classes Clostridia and Bacilli); (2) clostridial cluster XIVa (the largest group of the class Clostridia); (3) the group *Lactobacillus* (the so-called lactic acid bacteria, the largest group of the order Lactobacillales, from the class Bacilli); (4) the Gram-negative phylum Bacteroidetes, the second quantitatively dominant phylum in mouse faeces; (5) the order Enterobacteriales (from the Gram-negative phylum Proteobacteria); (6) *Bifidobacterium* spp. (from the high G + C Gram-positive phylum Actinobacteria), one of the major genera of bacteria that make up the colon microbiota in mice.

**Quantitative PCR amplification of 16S rRNA gene sequences.** Faecal DNA samples from the four treatment groups (*n* 2 animals per group) were subjected to qPCR, which were performed to study the effect of the HFD (alone or in combination with supplementations S1 and S2) on the composition of the intestinal microbial community. qPCR experiments were carried out with a Stratagene Mx3000P (Agilent Technologies), using Mx3000P<sup>®</sup> ninety-six-well non-skirted PCR plates (Agilent Technologies) covered with PCR adhesive films (Eppendorf AG). Analyses were performed in duplicate and mean values were calculated. Each qPCR, with a final volume of 20 µl, was composed of 10 µl of 2 × Brilliant III Ultra-Fast SYBR<sup>®</sup> Green QPCR Master Mix (Agilent Technologies), 2 µl of 2 µM forward primer, 2 µl of 2 µM reverse primer, 0.3 µl of the diluted reference dye ROX (30 nM final

concentration), 3.7 µl of nuclease-free PCR-grade water and 2 µl of either an optimised dilution of 1:500 of extracted template DNA for specimen analysis or a serial dilution series of bacterial reference genomic DNA for standard curves. All reactions were paralleled by a non-template control analysis. The amplification programme started with an initial step of 3 min at 95°C, followed by thirty cycles of 15 s at 95°C and 20 s at 60°C. Data were acquired in the final step at 60°C.

Melt curve analysis was carried out after each qPCR assay to distinguish the fluorescence signal obtained from the specific amplification product from artifacts such as primer-dimers. Melting curves were obtained by slow heating from 55 to 95°C, with fluorescence measurements taken at every 1°C increase in temperature.

Data analysis was performed using MxPro–Mx3000P software (version 4.10; Agilent Technologies). In 'analysis term settings' amplification-based threshold fluorescence and adaptive baseline correction were selected.

**Preparation of PCR standards and quantification of target bacterial DNA in faecal samples by quantitative PCR.** Standard curves for individual qPCR assays were used for the quantification of target bacterial DNA (in relative units) from faecal DNA preparations. The standard curves were generated using duplicate 2-fold dilutions of the DNA extracted from the faeces of one of the cages of the CD group (cage A, week 6). At least five non-zero standard dilutions were used in each assay. The same amount of all faecal DNA samples (1000 or 100 µg) was amplified by the qPCR assays, so that the threshold cycle (*C<sub>T</sub>*) values were always inside the range of the standard curves. No positive signals were generated within the standard curves in these assays by non-template control.

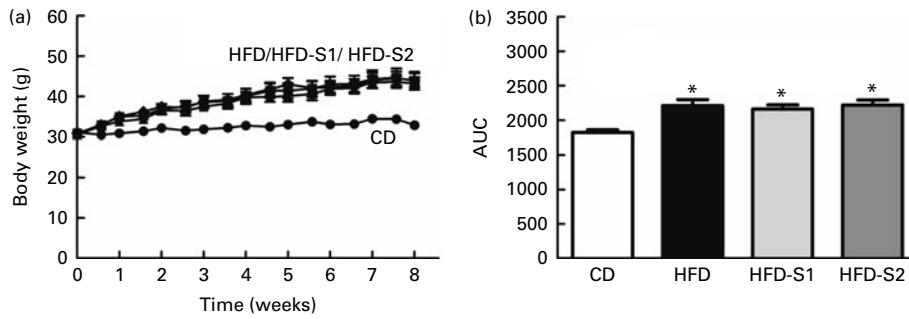
**Statistical analysis**

Statistical analysis was performed using GraphPad Prism Software for Windows OS (version 5.04; Graphpad Software). Statistically significant differences in body weight and DNA content between the four treatment groups were determined by the non-parametric Kruskal–Wallis test, with Dunn's multiple comparison post-test. Correlations between body weight and DNA

**Table 2.** Primers used for bacterial quantification by quantitative PCR

Bacterial group	Oligonucleotide sequence	Amplicon size (bp)	Reference
Total bacteria	F: 5'-ACTCCTACGGGAGGCAGCAG-3' R: 5'-ATTACCGCGGCTGCTGG-3'	200	36
Firmicutes phylum	F: 5'-GGAGYATGTGGTTTAATTGCAAGCA-3' R: 5'-AGCTGACGACAACCATGCAC-3'	126	36
<i>Lactobacillus</i> group	F: 5'-AGCAGTAGGGAATCTTCCA-3' R: 5'-CACCGCTACACATGGAG-3'	341	14
Clostridial cluster XIVa	F: 5'-GCGGTRCGGCAAGTCTGA-3' R: 5'-CCTCGACACTCTAGTMCGAC-3'	81	37
Enterobacteriales order	F: 5'-ATGGCTGTCGTCAGCTCGT-3' R: 5'-CCTACTTCTTTGCAACCCACTC-3'	177	38
<i>Bifidobacterium</i> spp.	F: 5'-TCGCGTCYGGTGTGAAAG-3' R: 5'-RCCACATCCAGCRTCCAC-3'	243	14
Bacteroidetes phylum	F: 5'-GGARCATGTGGTTAATTCGATGAT-3' R: 5'-AGCTGACGACAACCATGCAG-3'	126	36

F, forward; R, reverse.



**Fig. 1.** (a) Effect of a high-fat diet (HFD) on body weight. Mice were fed for 8 weeks with either a control diet (CD) or a HFD (non-supplemented (HFD), supplemented with an oleic acid-derived compound (HFD-S1) and a combination of *n*-3 fatty acids (HFD-S2)). Body weight was determined twice per week. Values are means (cages B and C, *n* 12), with standard errors represented by vertical bars. (b) The groups' AUC were compared by the non-parametric Kruskal–Wallis test, with Dunn's multiple comparison post-test. \* Mean value was significantly different from that of the CD group ( $P < 0.05$ ).

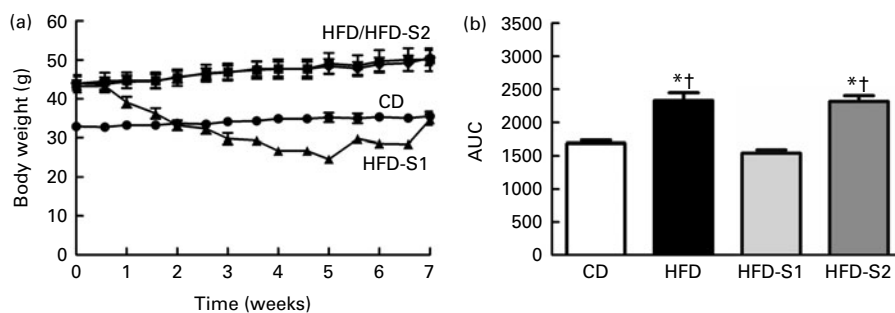
content were assessed by the non-parametric Spearman's correlation test.  $P < 0.05$  was considered as statistically significant.

**Results**

*Effect of the high-fat diet on mice weight*

After an acclimatisation period of 4 weeks, mice were divided into four groups: one control group fed with a maintenance diet (CD group) and three groups fed with a HFD for 8 weeks (HFD, HFD-S1 and HFD-S2 groups). Body weight increased progressively only in the three groups of mice that consumed the HFD (Fig. 1(a)). The evolution of weight gain was statistically different between the control group (CD, mice fed with the maintenance diet) and the other three groups (HFD, HFD-S1 and HFD-S2, mice fed with the HFD) (Fig. 1(b)).

After diet-induced obesity, we evaluated the effect of two different supplementations on weight gain in obese mice. We found that S1 supplementation (an oleic acid-derived compound) led to a progressive decrease in body weight (Fig. 2(a)). This effect could not be explained by changes in food consumption, since the total food intake was not different between the HFD and HFD-S1 groups (data not shown). S2 supplementation (a combination of *n*-3 fatty acids) did not show any effect on mouse body weight or weight gain (Fig. 2(a) and (b)).



**Fig. 2.** (a) Effect of supplementation with different fatty acids on body weight. Diet-induced obese mice continued with the high-fat diet (HFD) for another 7 weeks, non-supplemented (HFD) or supplemented with an oleic acid-derived compound (HFD-S1) or a combination of *n*-3 fatty acids (HFD-S2). Meanwhile, the control diet (CD) group continued with the maintenance diet. Body weight was determined twice per week. Values are means (cages B and C, *n* 12), with standard errors represented by vertical bars. (b) The groups' AUC were compared by the non-parametric Kruskal–Wallis test, with Dunn's multiple comparison post-test. \* Mean value was significantly different from that of the CD group ( $P < 0.05$ ). † Mean value was significantly different from that of the HFD-S1 group ( $P < 0.5$ ).

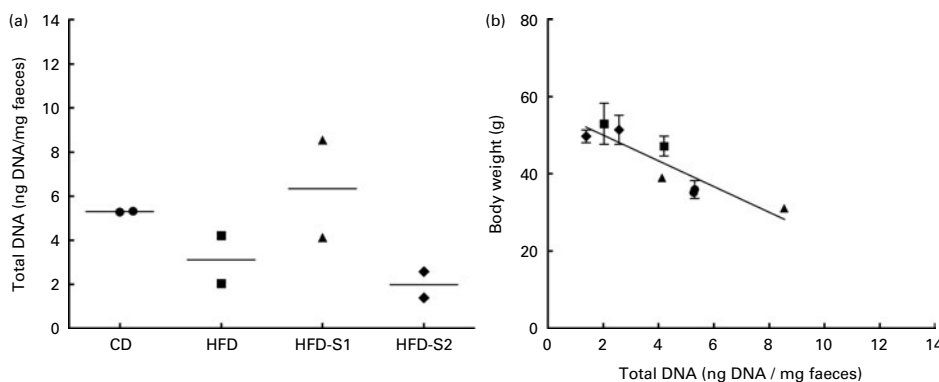
*Effect of the high-fat diet on total DNA content in the faeces*

Compared with the control group, the HFD (non-supplemented and supplemented with S2) decreased the total DNA content in the faeces (Fig. 3(a)). However, S1 supplementation was capable of increasing the total DNA content up to a similar level to that of the control group (Fig. 3(a)). Although these differences were not statistically significant ( $P > 0.1$ ), when the total DNA content was plotted against body weight, a significant negative correlation was observed (Fig. 3(b)).

*Effect of the high-fat diet on faecal microbiota composition*

The standard curves had correlation coefficient values ranging from 0.990 to 0.999 (Table 3). The amplification efficiencies for the SYBR Green I assays were obtained by plotting the  $C_T$  values against the target DNA starting quantity. Using the formula  $E = (10^{(-1/\text{slope})} - 1)$ , the efficiencies for the individual assays ranged from 86.0 to 101.4% (Table 3). The fluorescence signal was not detected in the non-template control during amplification, which indicated that the primer-dimers were denatured at this temperature and could not contribute to the fluorescence.

At the end of the study (week 19), the faecal levels of total bacteria, Firmicutes phylum, *Lactobacillus* group, clostridial cluster XIVa, Enterobacteriales order, *Bifidobacterium* spp.



**Fig. 3.** Total DNA content and its correlation with body weight. Mice were fed with either a control diet (CD, ●) or a high-fat diet (HFD, ■) or supplemented with an oleic acid-derived compound (HFD-S1, ▲) or a combination of *n*-3 fatty acids (HFD-S2, ◆). Total DNA content is expressed as ng DNA/mg faeces. Body weight values (g) are presented as means (cages B and C, *n* 6 animals per cage), with standard errors represented by vertical bars. (b) Spearman's correlation  $r = -0.8810$ ,  $P = 0.0072$ ,  $R^2 = 0.8108$ .

and Bacteroidetes phylum were determined by qPCR (Fig. 4). The correlations between these levels and body weight were also calculated (Fig. 5).

**Total bacteria.** Although not statistically significant, the administration of the HFD induced an increase in total bacteria when compared with control mice, an effect that was potentiated by the co-administration of an oleic acid-derived compound (HFD-S1), which significantly increased the total bacteria ( $P < 0.1$ ; Fig. 4(a)). No correlation was found between total bacteria and body weight ( $r = 0.0$ ,  $P = 1.0232$ ; Fig. 5(a)). Nevertheless, when considering DNA content per mg of faeces, total bacteria in HFD and HFD-S2 mice were lower than that in the control mice (data not shown), and a negative correlation was established between total bacteria and body weight ( $r = -0.7143$ ,  $P = 0.0576$ ).

**Bacterial groups.** When compared with the CD group (fed with the maintenance diet), the administration of the HFD induced an increase in all the groups of Firmicutes (the phylum Firmicutes, the *Lactobacillus* group and clostridial cluster XIVa), as well as the order Enterobacteriales (Fig. 4(b)–(e)), a difference that was statistically significant only in the last group ( $P < 0.1$ ). Conversely, and although not statistically significant, *Bifidobacterium* spp. and the phylum Bacteroidetes were decreased after the ingestion of the HFD (Fig. 4(f) and (g)).

HFD-induced microbial community changes were (partially or completely) counteracted by the supplementation of the

HFD with an oleic acid-derived compound (HFD-S1), as shown by the modest decrease in the phylum Firmicutes and the group *Lactobacillus* (Fig. 4(b) and (c)), the significant decrease in clostridial cluster XIVa and the order Enterobacteriales (Fig. 4(d) and (e)) and the significant increase in *Bifidobacterium* spp. and the phylum Bacteroidetes (Fig. 4(f) and (g)), up to a level even higher than the CD group in the last group of bacteria.

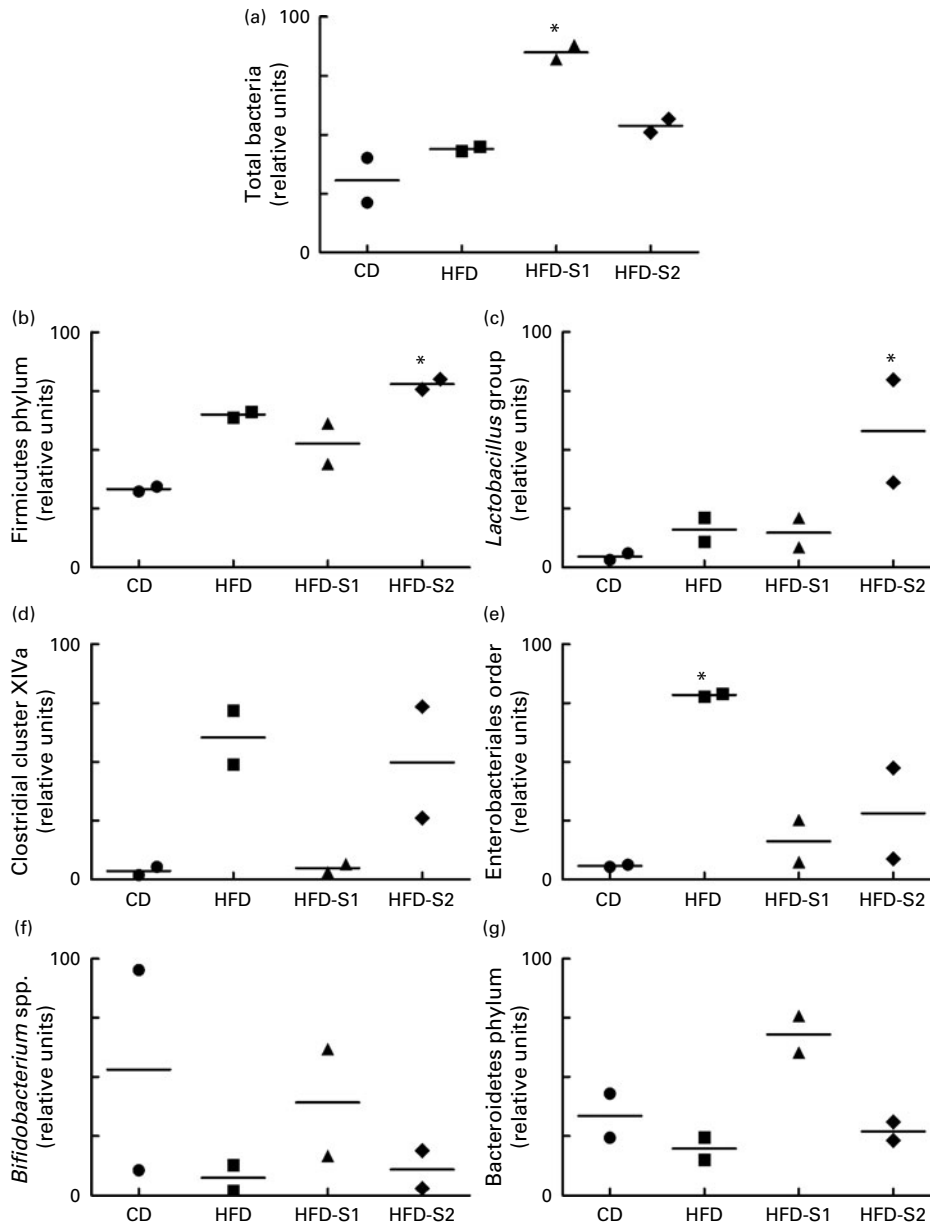
When compared with the HFD group, supplementation with a combination of *n*-3 fatty acids (HFD-S2) did not change the levels of clostridial cluster XIVa, *Bifidobacterium* spp. and the phylum Bacteroidetes (Fig. 4(d), (f) and (g)). However, S2 supplementation was capable of increasing the phylum Firmicutes and the group *Lactobacillus*, compared with the HFD group (NS) and the CD group ( $P < 0.1$ ; Fig. 4(b) and (c)). Similarly to S1 supplementation, the increase in the order Enterobacteriales induced by the HFD was partially restored by the S2 supplementation (Fig. 4(e)).

Although many of the mentioned differences were not statistically significant ( $P > 0.1$ ), when the quantitative values of the analysed microbial groups were plotted against body weight, significant positive correlations were observed with the phylum Firmicutes (Fig. 5(b)) and clostridial cluster XIVa (Fig. 5(d)) and a significant negative correlation with the phylum Bacteroidetes (Fig. 5(g)). No correlations were found for the *Lactobacillus* group, the order Enterobacteriales and *Bifidobacterium* spp. (Fig. 5(c), (e) and (f)).

**Table 3.** Amplification slopes, efficiencies and correlation coefficients for individual quantitative PCR assays

PCR assay	Slope	PCR efficiency (%)	Correlation coefficient
Total bacteria	-3.288	101.4	0.999
Firmicutes phylum	-3.382	97.6	0.999
<i>Lactobacillus</i> group	-3.709	86.0	0.999
Clostridial cluster XIVa	-3.439	95.3	0.999
Enterobacteriales order	-3.379	97.7	0.990
<i>Bifidobacterium</i> spp.	-3.613	90.2	0.997
Bacteroidetes phylum	-3.401	96.9	0.999





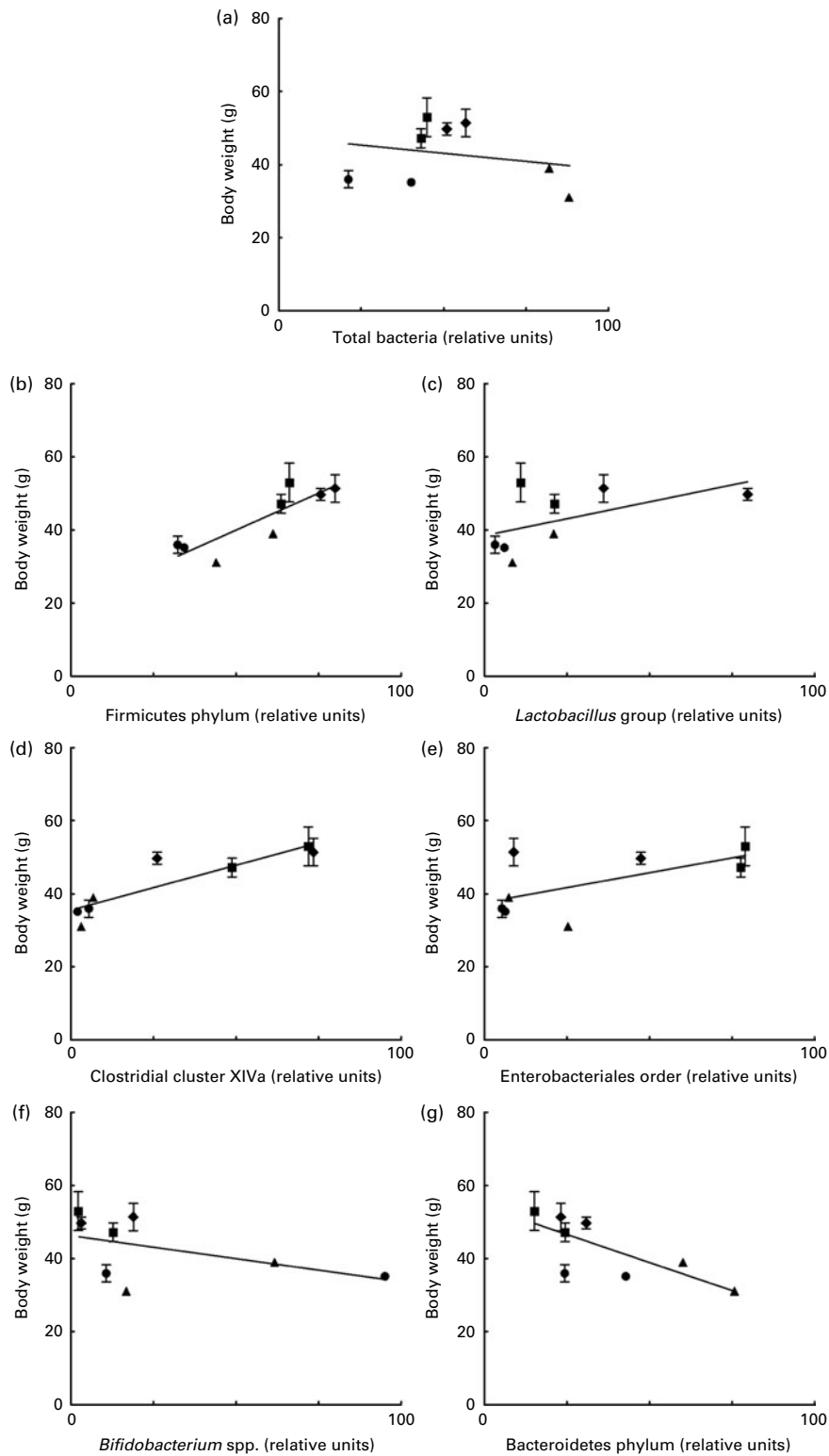
**Fig. 4.** Faecal bacterial content of (a) total bacteria, (b) Firmicutes phylum, (c) *Lactobacillus* group, (d) clostridial cluster XIVa, (e) Enterobacteriales order, (f) *Bifidobacterium* spp. and (g) Bacteroidetes phylum. Bacterial quantities are expressed as relative units. Mice were fed with either a control diet (CD, ●) or a HFD, non-supplemented (HFD, ■) or supplemented with an oleic acid-derived compound (HFD-S1, ▲) or a combination of *n*-3 fatty acids (HFD-S2, ◆). \* Mean value was significantly different from that of the CD group ( $P < 0.1$ ; Kruskal–Wallis).

## Discussion

The HFD used in the present study induced an increase of body weight and a decrease of total DNA content in the faeces. Supplementation of the HFD with an oleic acid-derived compound (HFD-S1) restored both parameters up to similar levels to that of the control group (Figs. 2(b) and 3(a)).

The GM is a complex ecosystem made up of 500–1000 different bacterial species<sup>(22)</sup>, the majority of which are obligate anaerobes whose investigation by culture-dependent techniques is laborious and prone to misinterpretation<sup>(23)</sup>. Recent advances in molecular approaches to the identification and quantification of bacteria using 16S ribosomal sequences

have significantly advanced our understanding of the GM<sup>(24)</sup>. qPCR is a powerful advancement of the basic PCR technique, which has been successfully applied for the quantification of bacterial DNA in various environments such as faeces, colonic tissue, rumen, gastric tissue and periodontal samples<sup>(25)</sup>. In the present study, selected predominant bacteria from mouse faecal samples were quantified by qPCR, using SYBR Green I chemistry (Table 2). The DNA isolation and purification protocol yielded high-quality DNA, which was established by the fact that increasing the total DNA template amount subjected to qPCR did not have any inhibitory effect. This facilitates the detection and quantification of minor subpopulations in the faeces.



**Fig. 5.** Correlation between body weight and the faecal bacterial content of (a) total bacteria (Spearman's  $r = 0.0$ ,  $P = 1.0232$  (NS),  $R^2 = 0.0329$ ), (b) Firmicutes phylum (Spearman's  $r = 0.8333$ ,  $P = 0.0154$ ,  $R^2 = 0.7424$ ), (c) *Lactobacillus* group (Spearman's  $r = 0.6429$ ,  $P = 0.0962$  (NS),  $R^2 = 0.1897$ ), (d) clostridial cluster XIVa (Spearman's  $r = 0.9286$ ,  $P = 0.0022$ ,  $R^2 = 0.5072$ ), (e) Enterobacteriales order (Spearman's  $r = 0.5952$ ,  $P = 0.1323$  (NS),  $R^2 = 0.3643$ ), (f) *Bifidobacterium* spp. (Spearman's  $r = -0.5538$ ,  $P = 0.1966$  (NS),  $R^2 = 0.2453$ ) and (g) Bacteroidetes phylum (Spearman's  $r = -0.7857$ ,  $P = 0.0279$ ,  $R^2 = 0.5678$ ). Mice were fed with either a control diet (CD, ●) or a HFD, non-supplemented (HFD, ■) or supplemented with an oleic acid-derived compound (HFD-S1, ▲) or a combination of *n*-3 fatty acids (HFD-S2, ◆). Bacterial quantities are expressed as relative units. Body weight values (g) are presented as means (cages B and C,  $n = 6$  animals per cage), with standard errors represented by vertical bars.

Animal and human studies have revealed a remarkable microbial influence on host metabolism, energy utilisation and storage, and metabolic diseases<sup>(26–29)</sup>. However, this complex ecosystem remains poorly studied; therefore, more precise monitoring of the gut bacteria is vital for better understanding of their contribution to health and disease.

Recently, it has been proposed that alterations in the composition of the GM (known as dysbiosis) participate in the development of obesity<sup>(30)</sup>. In the present study, administration of a HFD induced a significant increase in the Enterobacteriales order ( $P < 0.1$ ; Fig. 4(e)). A bloom in the  $\gamma$ -Proteobacteria class, to which belongs the Enterobacteriales order, was also observed in the study of Hildebrandt *et al.*<sup>(31)</sup>. An increase in the Enterobacteriaceae family within this order has been associated with gut inflammation; induction of experimental colitis in rodents was followed by an increase in this family, suggesting that it may be a consequence of gut inflammation rather than a cause<sup>(32)</sup>.

More than 90% of the distal GM of mice and humans is composed of the Bacteroidetes and Firmicutes phyla<sup>(31)</sup>, while other members, such as lactic acid bacteria and Proteobacteria, are present in much lower amounts<sup>(33)</sup>. Similar to previously reported well-defined experiments using in part even genetically modified animals<sup>(34)</sup> or strictly controlled human studies<sup>(14,35)</sup>, administration of a HFD induced an increase in all the groups of Firmicutes (the phylum Firmicutes, the group *Lactobacillus* and clostridial cluster XIVa; Fig. 4(b)–(d)) and a decrease in the phylum Bacteroidetes (Fig. 4(g)). However, further reports have linked an increased proportion of Bacteroidetes in obese rats<sup>(13)</sup> and human subjects<sup>(9,36,37)</sup>. The most probable explanation for this discrepancy could be that some of these studies did not control for other possible confounding variables of obesity, such as intensity, regularity of exercise as well as total daily energy intake<sup>(37)</sup>. Methodological differences in DNA extraction protocols, primer design and qPCR techniques may also have caused additional variation. Either way, obesity seems to be related to changes in the Firmicutes:Bacteroidetes ratio that could be involved in a greater capacity to extract energy from nutrients<sup>(38,39)</sup>.

The compositions of the diets employed in the present study are probably responsible for some changes related to the GM composition. According to the manufacturer, the CD contains cereal products that contribute to 18.0% of insoluble fibre (including cellulose, hemicellulose and lignin) and 4.1% of soluble fibre (including fermentable carbohydrates). In contrast, the HFD has much less fibre (6.5%), which is exclusively obtained from cellulose, a poorly fermentable insoluble fibre. The absence of soluble fibre in the HFD could explain the lower levels of *Bifidobacterium* spp. (Fig. 4(f)).

Supplementation of the HFD with S1 markedly increased total bacterial density and restored the proportions of bacteria that were increased (i.e. clostridial cluster XIVa and Enterobacteriales) or decreased (i.e. *Bifidobacterium* spp.) during HFD feeding (Fig. 4(d)–(f)). The decrease in body weight only in the HFD group supplemented with an oleic acid-derived compound (S1) could be explained by the decrease in the Firmicutes:Bacteroidetes ratio up to a level

even lower than the CD group, but a combination of *n*-3 fatty acids (S2) did not.

The HFD-induced levels of clostridial cluster XIVa, *Bifidobacterium* spp. and the phylum Bacteroidetes were not restored by supplementation with a combination of *n*-3 fatty acids (HFS-S2; Fig. 4(d), (f), and (g)). However, the elevated number of Enterobacteriales induced by the HFD, and probably associated with gut inflammation, was partially restored by S2 supplementation (Fig. 4(e)), which are well-known modulators of the inflammatory process<sup>(40)</sup>.

*n*-3 PUFA influence the gastrointestinal microbiota and specifically the population level of lactic acid bacteria (mainly bifidobacteria and lactobacilli), which are generally considered to be beneficial. A study carried out in fish has reported an increase in the content of lactobacilli by *n*-3 PUFA consumption<sup>(41)</sup>. According to Kankaanpaa *et al.*<sup>(42)</sup>, low concentrations of *n*-3 fatty acids (5  $\mu$ g/ml) promoted growth and mucus adhesion of *Lactobacillus casei* Shirota. In gnotobiotic piglets, the oral administration of an oil containing PUFA significantly increased the number of *Lactobacillus paracasei* adhering to the jejunal mucosa compared with the control group<sup>(43)</sup>. Moreover, mice fed with a diet depleted in *n*-3 PUFA for two generations exhibited a huge decrease in lactobacilli and, unexpectedly, an increase in bifidobacteria in the caecal content when compared with mice fed a diet with an adequate content in *n*-3 PUFA<sup>(44)</sup>. Coherently, we show here in mice that S2 supplementation increased the quantities of Firmicutes (especially the *Lactobacillus* group), when compared with the HFD (NS) and CD groups ( $P < 0.1$ ; Fig. 4(b) and (c)).

The adhesion to mucosal surfaces is pivotal in health-promoting effects by probiotics. Although further studies are needed to confirm this hypothesis *in vivo*, evidence suggests that some physiological effects of probiotics could be associated with the interactions between probiotics and dietary PUFA. It has been suggested that dietary PUFA affect the attachment sites for the gastrointestinal microbiota, possibly by modifying the fatty acid composition of the intestinal wall<sup>(41–43)</sup>. The stimulatory effect of PUFA upon adhesion of lactobacilli could be used for enhancing the effectiveness of probiotics in inhibiting digestive tract pathogens.

## Conclusions

Consumption of a HFD induced changes in the faecal microbiota (an increase in all the tested groups of Firmicutes, as well as the order Enterobacteriales, and a decrease in *Bifidobacterium* spp. and the phylum Bacteroidetes), which were associated with the appearance of an obese phenotype. Correlation analysis revealed that body weight correlated positively with the phylum Firmicutes and clostridial cluster XIVa, and negatively with the phylum Bacteroidetes. Supplementation of the HFD with S1 counteracted HFD-induced gut dysbiosis, together with an improvement in body weight.

These data support a role for certain fatty acids as interesting nutrients related to obesity prevention. Even if a direct extrapolation of the present study to humans is still questionable due to differences in digestive tract structure and in GM,





the present findings support the view that chronic consumption of an oleic acid-derived compound (S1) could confer potential beneficial effects with respect to the development of obesity, through a mechanism related to the restoration of the composition of gut bacteria. The possibility to treat animals with selected bacteria as oral probiotics could also constitute one interesting perspective to investigate further on the role of these specific gut bacteria in HFD-induced obesity. However, and until these issues are clarified, the best way to prevent obesity in humans is by promoting a healthy diet and lifestyle.

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### References

- WHO (2011) Obesity and overweight – Fact sheet No. 311. Geneva: WHO.
- Dewulf EM, Cani PD, Neyrinck AM, *et al.* (2011) Inulin-type fructans with prebiotic properties counteract GPR43 overexpression and PPARgamma-related adipogenesis in the white adipose tissue of high-fat diet-fed mice. *J Nutr Biochem* **22**, 712–722.
- Hotamisligil GS (2006) Inflammation and metabolic disorders. *Nature* **444**, 860–867.
- Hooper LV, Wong MH, Thelin A, *et al.* (2001) Molecular analysis of commensal host–microbial relationships in the intestine. *Science* **291**, 881–884.
- Sekirov I, Russell SL, Antunes LC, *et al.* (2010) Gut microbiota in health and disease. *Physiol Rev* **90**, 859–904.
- Kau AL, Ahern PP, Griffin NW, *et al.* (2011) Human nutrition, the gut microbiome and the immune system. *Nature* **474**, 327–336.
- Guarino A, Wudy A, Basile F, *et al.* (2012) Composition and roles of intestinal microbiota in children. *J Matern Fetal Neonatal Med* **25**, Suppl. 1, 63–66.
- Shanahan F (2009) Therapeutic implications of manipulating and mining the microbiota. *J Physiol* **587**, 4175–4179.
- Wu GD, Chen J, Hoffmann C, *et al.* (2011) Linking long-term dietary patterns with gut microbial enterotypes. *Science* **334**, 105–108.
- Wostmann BS (1981) The germfree animal in nutritional studies. *Ann Rev Nutr* **1**, 257–279.
- Nadal I, Santacruz A, Marcos A, *et al.* (2009) Shifts in clostridia, bacteroides and immunoglobulin-coating fecal bacteria associated with weight loss in obese adolescents. *Int J Obes (Lond)* **33**, 758–767.
- Santacruz A, Marcos A, Warnberg J, *et al.* (2009) Interplay between weight loss and gut microbiota composition in overweight adolescents. *Obesity (Silver Spring)* **17**, 1906–1915.
- de La Serre CB, Ellis CL, Lee J, *et al.* (2010) Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. *Am J Physiol Gastrointest Liver Physiol* **299**, G440–G448.
- Turnbaugh PJ, Ley RE, Mahowald MA, *et al.* (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027–1031.
- Cani PD, Amar J, Iglesias MA, *et al.* (2007) Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* **56**, 1761–1772.
- Cani PD, Neyrinck AM, Fava F, *et al.* (2007) Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia* **50**, 2374–2383.
- Roberfroid M (2007) Prebiotics: the concept revisited. *J Nutr* **137**, 3 Suppl. 2, 830S–837S.
- Schroder H, Marrugat J, Vila J, *et al.* (2004) Adherence to the traditional Mediterranean diet is inversely associated with body mass index and obesity in a Spanish population. *J Nutr* **134**, 3355–3361.
- Razquin C, Martinez JA, Martinez-Gonzalez MA, *et al.* (2009) A 3 years follow-up of a Mediterranean diet rich in virgin olive oil is associated with high plasma antioxidant capacity and reduced body weight gain. *Eur J Clin Nutr* **63**, 1387–1393.
- Vögler O, Lopez-Bellan A, Alemany R, *et al.* (2008) Structure–effect relation of C18 long-chain fatty acids in the reduction of body weight in rats. *Int J Obes (Lond)* **32**, 464–473.
- Buckley JD & Howe PR (2010) Long-chain omega-3 polyunsaturated fatty acids may be beneficial for reducing obesity – a review. *Nutrients* **2**, 1212–1230.
- Eckburg PB, Bik EM, Bernstein CN, *et al.* (2005) Diversity of the human intestinal microbial flora. *Science* **308**, 1635–1638.
- Vaughan EE, Schut F, Heilig HG, *et al.* (2000) A molecular view of the intestinal ecosystem. *Curr Issues Intest Microbiol* **1**, 1–12.
- Backhed F, Ley RE, Sonnenburg JL, *et al.* (2005) Host–bacterial mutualism in the human intestine. *Science* **307**, 1915–1920.
- Rinttila T, Kassinen A, Malinen E, *et al.* (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* **97**, 1166–1177.
- Backhed F & Crawford PA (2010) Coordinated regulation of the metabolome and lipidome at the host–microbial interface. *Biochim Biophys Acta* **1801**, 240–245.
- Cani PD & Delzenne NM (2009) The role of the gut microbiota in energy metabolism and metabolic disease. *Curr Pharm Des* **15**, 1546–1558.
- Larsen N, Vogensen FK, van den Berg FW, *et al.* (2010) Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PLoS One* **5**, e9085.
- Armougom F, Henry M, Vialettes B, *et al.* (2009) Monitoring bacterial community of human gut microbiota reveals an

- increase in *Lactobacillus* in obese patients and methanogens in anorexic patients. *PLoS One* **4**, e7125.
30. Cani PD & Delzenne NM (2009) Interplay between obesity and associated metabolic disorders: new insights into the gut microbiota. *Curr Opin Pharmacol* **9**, 737–743.
  31. Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, *et al.* (2009) High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology* **137**, 1716–1724.e1-2.
  32. Lupp C, Robertson ML, Wickham ME, *et al.* (2007) Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* **2**, 204.
  33. Flint H, Louis P, Scott K, *et al.* (2007) Commensal bacteria in health and disease. In *Virulence Mechanisms of Bacterial Pathogens*, pp. 101–114 [KA Brogden and JM Guthmiller, editors]. Washington, DC: ASM Press.
  34. Ley RE, Backhed F, Turnbaugh P, *et al.* (2005) Obesity alters gut microbial ecology. *Proc Natl Acad Sci U S A* **102**, 11070–11075.
  35. Ley RE, Turnbaugh PJ, Klein S, *et al.* (2006) Microbial ecology: human gut microbes associated with obesity. *Nature* **444**, 1022–1023.
  36. Collado MC, Isolauri E, Laitinen K, *et al.* (2008) Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. *Am J Clin Nutr* **88**, 894–899.
  37. Schwartz A, Taras D, Schafer K, *et al.* (2010) Microbiota and SCFA in lean and overweight healthy subjects. *Obesity (Silver Spring)* **18**, 190–195.
  38. Backhed F, Ding H, Wang T, *et al.* (2004) The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A* **101**, 15718–15723.
  39. Musso G, Gambino R & Cassader M (2010) Gut microbiota as a regulator of energy homeostasis and ectopic fat deposition: mechanisms and implications for metabolic disorders. *Curr Opin Lipidol* **21**, 76–83.
  40. Calder PC (2006) *n*-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr* **83**, 6 Suppl., 1505S–1519S.
  41. Ringo E, Bendiksen HR, Gausen SJ, *et al.* (1998) The effect of dietary fatty acids on lactic acid bacteria associated with the epithelial mucosa and from faecalia of Arctic charr, *Salvelinus alpinus* (L.). *J Appl Microbiol* **85**, 855–864.
  42. Kankaanpaa PE, Salminen SJ, Isolauri E, *et al.* (2001) The influence of polyunsaturated fatty acids on probiotic growth and adhesion. *FEMS Microbiol Lett* **194**, 149–153.
  43. Bomba A, Nemcova R, Gancarcikova S, *et al.* (2002) Improvement of the probiotic effect of micro-organisms by their combination with maltodextrins, fructo-oligosaccharides and polyunsaturated fatty acids. *Br J Nutr* **88**, Suppl. 1, S95–S99.
  44. Pachikian BD, Neyrinck AM, Portois L, *et al.* (2011) Involvement of gut microbial fermentation in the metabolic alterations occurring in *n*-3 polyunsaturated fatty acids-depleted mice. *Nutr Metab* **8**, 44.