Shifts in clostridia, bacteroides and immunoglobulin-coating faecal bacteria associated with weight loss in obese adolescents

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Running title: Gut microbiota associated with weight loss.

Abstract

Objective: To evaluate the effects of a multidisciplinary obesity treatment programme on faecal microbiota composition and immunoglobulin-coating bacteria in overweight and obese adolescents and their relationship to weight loss.

Design: Longitudinal intervention study based on both a calorie-restricted diet (calorie reduction= 10-40%) and increased physical activity (calorie expenditure=15-23 kcal/kg body weight/wk) for 10 weeks.

Subjects: Thirty-nine overweight and obese adolescents (BMI mean 33.1 range 23.7-50.4; age mean14.8; range 13.0-16.0)

Measurements: BMI[1], BMI z-scores[2] and plasma biochemical parameters were measured before and after the intervention. Faecal microbiota was analyzed by fluorescent *in situ* hybridization. Immunoglobulin-coating bacteria were detected using fluorescent-labelled F(ab´)2 antihuman IgA, IgG and IgM.

Results: Reductions in *C. histolyticum* and *E. rectale-C. coccoides* proportions significantly correlated with weight and BMI z-score reductions in the whole adolescent population. Proportions of *C. histolyticum, C. lituseburense* and *E. rectale-C. coccoides* dropped significantly while those of the *Bacteroides-Prevotella* group increased after the intervention in those adolescents that lost more than 4 kg. Total faecal energy was almost significantly reduced in the same group of adolescents but not in the group that lost less than 2.5 kg. IgA-coating bacterial proportions also decreased significantly in subjects that lost more than 6 kg after the intervention, paralleled to reductions in *C. histolyticum* and *E. rectale-C. coccoides* populations. *E. rectale-C. coccoides* proportions also correlated with weight loss and BMI z-score reduction in subjects whose weight loss exceeded 4 kg.

Conclusions: Specific gut bacteria and an associated IgA response were related to body weight changes in adolescents under lifestyle intervention. These results suggest interactions between diet, gut microbiota and host metabolism and immunity in obesity.

Keywords: obesity, overweight, microbiota, weight management, IgA

INTRODUCTION

Obesity and the associated metabolic disorders, such as diabetes and metabolic syndrome, have become major public-health issues in adult and paediatric populations worldwide.[3] Obesity results from a positive energy balance and is characterized by a state of chronic, low-grade inflammation with abnormal cytokine and acute-phase inflammatory protein production.[4] Treatments based on calorie restriction, exercise and behavioural changes have succeeded to some extent to control obesity, but usually yield limited and transient weight loss.[5] More efficient strategies to control obesity and tackle its metabolic consequences are, therefore, urgently needed. In this context, it is essential to identify interactions between the environmental factors and host mechanisms involved in energy regulation with a view to developing additional intervention strategies. [3,6]

The environmental factors accounting for the dramatic rise in obesity in recent decades are not fully understood. Breastfeeding seems to be a protective factor against obesity later in life, while increased energy intake in formula or mixed-fed infants seems to be detrimental.[7] In recent studies, lack of breastfeeding, high early energy intake and high intake of sugar-sweetened beverages have also been shown to contribute to obesity in adolescents.[8] In addition, shifts in the composition of gut microbiota in response to dietary factors, such as total quantity and quality of carbohydrate and fat intake, have been reported.[9] In fact, the microbes populating the gut are currently being investigated as potential environmental factors involved in obesity.[6,9,10] Gut microbiota is viewed as a metabolic organ that plays a pivotal role in the physiology of energy homeostasis.[11] Commensal bacteria contribute to the digestion of nutrients otherwise inaccessible to humans, such as complex polysaccharides. The microbial fermentation of undigested dietary compounds in the large intestine can provide up to 10-15% of human daily energy supply.[11] Gut microbes are known to be involved in the absorption of monosaccharides and short-chain fatty acids, as well as in their

conversion to complex lipids in the liver and their storage in adipocytes. [6] In addition, commensal bacteria colonizing the gut or in transit may also regulate the signalling pathways that link obesity with inflammation by interacting with the epithelium and host immune system.[12] So far, some studies associated obesity with an increase in the proportion of *Firmicutes* and a reduction in *Bacteroidetes*, in mice obesity models and adult human subjects through small-scale intervention studies[10] but other results were controversial.[13] In mice fed on a high-fat diet, increases in *Bifidobacterium* levels achieved by intake of prebiotics were correlated with normalization of inflammatory status and endotoxaemia.[12] Notable differences in microbiota composition have also been shown between exercised and sedentary rats.[14] Nevertheless, the associations between specific gut bacteria and human host metabolism and immunity in relation to obesity remain largely uncharacterized.

The objective of this study was to evaluate the effects of a multidisciplinary obesity treatment programme (including energy-restricted diet and increased physical activity) on faecal microbial composition and immunoglobulin-coating bacteria in overweight or obese adolescents and assess their relationship to biochemical parameters and weight loss. Thus, sounder links between gut microbes and human obesity can be established.

METHODS

Subjects and experimental design

Subjects for the study were selected according to their body mass index (BMI) [weight (kg)/[height $(m)^2$] and classified as overweight or obese according to the International Obesity Task Force criteria defined by Cole et al.[1] during the course of the EVASYON study. The current study was designed to develop a multidisciplinary obesity-treatment programme adapted to Spanish primary health care centres and was assessed by Paediatric services in five cities around Spain. The treatment program included nutritional and individual diet

counselling, including calorie restriction and increased physical activity, as well as group therapies aim at changing behaviour, providing support and encouraging adolescents to change lifestyle and follow treatment recommendations. A total of 39 overweight or obese Spanish adolescents (20 females and 19 males; mean age 14.8 years) were included in this study, and their characteristics are shown in Table 1. BMI z-scores were calculated as a function of the subject's obesity degree when compared with BMI local reference standards.[2] Over a 10-week period, the participants followed an energy-restricted diet (a 10-40% reduction) established according to both obesity degree and regular physical activity determined by accelerometry.[15] The maximum energy intake was 1800 kcal/day for females and 2200 kcal/day for males. The physical activity program was established to increase energy expenditure by 15-23 kcal/kg body weight per week. None of the volunteers were treated with antibiotics during the study.

Energy food intake

To determine the intake of energy food diary records were kept for 72h (2 weekdays and 1 weekend day) both before the start of the study (baseline intakes) and after the intervention (week 10). Detailed information on how to record food and drink consumed using common household measures was provided. Food diary records were returned to their dietician, and analyzed for energy contents based on the CESNID food-composition database of Spanish foods.[16]

Biochemical analyses

Fasting plasma glucose, total cholesterol, triglycerides, and HDL cholesterol were measured by enzyme-colorimetric automated methods (Roche®, Neuilly sur Seine Cedex, France). LDL cholesterol was calculated by the Friedwald equation. Fasting plasma insulin was measured

by the LINCOplex KIT Human Gut Hormone Panel (CAT-HGT-68K, Linco Research-St Charles, Missouri - USA).

Faecal sample collection and preparation for microbiological analyses

Faecal samples were collected at baseline and after 10 weeks of the intervention, frozen immediately after collection at -20 ºC, and stored until analysed. Faeces were diluted 1:10 (w/v) in PBS (pH 7.2) and homogenized in a Lab Blender 400 stomacher (Seward Medical London, UK) for 5 min. After low-speed centrifugation (2, 000 *g*, 2 min), the supernatant was collected. For bacterial quantification, cells were fixed by adding 4% paraformaldehyde solution (Sigma, St Louis, MO) and incubated overnight at 4 ºC. After fixation, bacteria were washed twice in PBS by centrifugation (12, 000 *g* for 5 min). Finally, cell pellets were suspended in a PBS/ethanol mixture (1:1) and stored at −80 °C until analyzed as previously described.[17]

Fluorescent *in situ* hybridisation for microbiological analysis

The bacterial groups present in faeces were quantified by fluorescent in *situ* hybridization (FISH) using group-specific probes (MOLBIOL, Berlin, Germany). The specific probes and controls used in this study, as well as the hybridization conditions, are shown in Table 2. The EUB 338 probe, targeting a conserved region within the bacterial domain, was used as a positive control,[16] and the NON 338 probe was used as a negative control to eliminate background fluorescence.[17] Control probes were covalently linked at their 5´end either to indocyanine dye Cy3 or to fluorescein isothiocyanate (FITC). Specific cell enumeration was performed by combining each of the group-specific FITC-probes with the EUB 338-Cy3 probe as previously described.[17] Briefly, fixed cell suspensions were incubated in the hybridization solution (10 mM Tris-HCl, 0.9 M NaCl, pH 8.0 and 10% SDS) containing 4

ng/µl of each fluorescent probe at appropriate temperatures, overnight. Then, hybridised cells were pelleted by centrifugation (10,000 *g* for 5 min) and resuspended in 500µl PBS solution for flow-cytometry analysis. The proportion of each bacterial group was expressed as a ratio of cells hybridising with the FITC-labelled specific probe to cells hybridising with the EUB 338-Cy3 probe.[17]

Immunoglobulin-coating bacterial analysis

Bacterial cells from 20 µl of the supernatant obtained after low-speed centrifugation were collected (10,000 *g* for 5 min). The pellet was resuspended in 60 µl 1% (w/v) BSA/PBS, containing 1% (v/v) FITC-labelled F(ab´)2 antihuman IgA, IgG or IgM (CALTAG Laboratories, Burlingame, CA). Another aliquot of each sample was pelleted and resuspended in 60 µl 1% (w/v) BSA/PBS and used as control. After 30 min incubation, suspensions were washed twice with PBS. Bacterial pellet was finally resuspended in 500 µl PBS and mixed with 20 µl propidium iodine (100mg/l) to label total bacteria before flow cytometry detection.[28]

Flow cytometry

Flow cytometry detections were performed using an EPICS® XL-MCL flow cytometer (Beckman Coulter, Florida, USA) as previously described.[17] This instrument is equipped with two light scatter detectors that measure forward (FSC) and side scatter (SSC) and fluorescence detectors that detect appropriately filtered light at green (FL1, 525 nm) and red-orange (FL3, 620 nm) wavelengths. The event rate was kept at the lowest setting (200-300 events per second) to avoid cell coincidence. A total of 15,000 events were recorded in a list mode file and analyzed with the System II V.3 software (Beckman Coulter). The proportion of each bacterial group was expressed as a ratio of cells hybridising with the FITC-labelled

specific probe to cells hybridising with the universal EUB 338-Cy3 probe.[17] Immunoglobulin coating of faecal bacteria was expressed as a ratio of bacterial cells labelled with FITC-labelled F(ab´)2 antihuman IgA, IgG or IgM to the total cell population hybridising with propidium iodine.[28]

Faecal energy determination

Energy content of faeces was determined by calorimetry as described elsewhere[29] using an Automatic Adiabatic Bomb Calorimeter (Gallenkamp, Leicestershire, UK). Faecal samples were dried by lyophilisation and samples of 1.5 g dry weight were analysed in duplicated. Gross energy content of faecal samples was defined as the amount of heat developed by the total combustion of a unit of dry weight sample.

Statistical analyses

Statistical analyses were done using the SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). Results are expressed as median values and ranges of the proportions of each bacterial group determined in duplicate. Total bacteria, Gram-positive and Gram-negative bacteria were calculated by adding the proportions of the corresponding groups detected by specific probes, which do not overlap. Thus, total Gram-positive bacteria was calculated by adding the proportions obtained with the probes Chis150, Erec0482, Bif164, Clit135 and Lab158 and total Gram-negative was calculated by adding the proportions obtained with the probes Bac303, Ent1432 and SBR687. Differences in bacterial populations and immunoglobulin-coating bacteria detected before and after the intervention programme were determined using the Mann–Whitney *U* test of non-normal data distribution. The Pearson correlation test was used to calculate the correlations between bacterial count changes and weight loss as a result of the intervention. In every case, a *P*-value <0.05 was considered statistically significant.

Statement of Ethics

We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research. Informed consent was obtained from all adolescents and their parents, and the study was approved by the local Ethics Committees.

RESULTS

Subjects

Clinical characteristics did not differ significantly between the two groups of adolescents (A and B) at recruitment time (Table 1). Base line values of BMI and BMI z-score of group A were 30.7 (26.4-36.3) and 2.95 (1.6-4.03), respectively and those of group B were 33.1 (30.0-35.0) and 3.22 (2.57-4.16), respectively. The subjects, 51% female (20/39) and 49% male (19/39), were 14.4 years old (13.0-16.0 years), maintained an apparently good health status and did not consume antibiotics during the study (Table 1). Most of the subjects (n = 26) experienced significant ($P = 0.050$) weight loss from 4.1 to 16.6 kg (mean decrease of 7.6 kg) after 10 weeks of following the intervention programme. Some of them (n =13) did not experience remarkable weight loss (mean decrease of 1.1 kg; range 0.8-2.4 P=0.798). These two groups showed significant differences in their weight loss (P<0.001) and BMI z-score reduction (P<0.001) and, accordingly, were sub-divided for comparisons of their faecal bacterial populations into groups A (> 4 kg, mean 7.6 kg weight loss) and B (<2.5 kg, mean 1.1 kg weight loss). BMI, and, BMI z-score (P=0.033 and P=0.039, respectively) detected before and after the dietary intervention was also significantly different in group A but not in group B. Group A was further subdivided into other two groups that also displayed significant differences in weight loss (P<0.001), one (group A1) integrated by subjects with a weight loss of 4-6 kg (corresponding to a 5.5 % decrease in body weight) and the other (group A2)

integrated by those subjects with a weight loss exceeding 6 kg (corresponding to 9.4 % decrease in body weight) for comparisons of faecal immunoglobulin coating bacteria as indicated below.

The dietary intervention resulted in a significant reduction ($P< 0.050$) in total energy intake in both adolescent groups from 2284 (2739-1549) to 1429.4 (1049-1782) kcal/day in group A and from 2159 (1926-2414) to 1416 (1296-1508) kcal/day in group B. No significant differences in dietary energy intake were found between both adolescent groups before and after the intervention program.

Microbiota composition and energy of faeces from adolescents

A follow-up study was made of the shifts in composition of faecal microbiota of the subjects under study during the weight-loss intervention programme. The results of the faecal microbiota analyses before and after intervention by FCM-FISH techniques are shown in Fig 1 and 2, and Table 3. In the whole adolescent population, the intervention programme led to reductions in *C. histolyticum* proportions, which correlated with weight loss (Fig. 1A; r=0.43; P =0.009), as did reductions in *E. rectale-C. coccoides* proportions (Fig 1B; r=0.50, P=0.001). Similar correlations were found between *C. histolyticum* and *E. rectale-C. coccoides* proportions and BMI z-scores (r= 0.41; P=0.012 and r=0.39; P=0.014, respectively). *Bacteroides* proportions increased as a result of the intervention and almost reached significant levels of correlation with weight loss (Fig 1C. r=-0.28; P =0.083). Although increases in *Lactobacillus-Enterococcus* proportions were also parallel to reductions in weight (Fig 1D. r =-0.15; P =0.361) and BMI z-scores (r = -0.29; P=0.074), correlation was not significant. Shifts in *Bifidobacterium, C. lituseburense*, *Enterobacteriaceae*, *E. coli*, *Roseburia* and sulphate-reducing bacterial groups were neither significantly correlated with weight loss nor with BMI z-score reductions.

The composition of the faecal microbiota of two groups of adolescents A and B, which displayed significant differences in weight loss (> 4 kg in group A versus < 2.5 kg in group B) and BMI z-score reduction after intervention, was compared at base line, revealing the presence of significantly higher levels (P=0.008) of *Lactobacillus* proportions in the group A than in group B (Table 3). In group A, Gram-positive bacterial populations, estimated by adding the proportions of corresponding groups targeted by the probes, were significantly lower (P=0.046) after the intervention, while significant differences in Gram-negative bacteria and total bacteria were not detected (Fig 2A). By contrast, in group B no differences were detected in Gram-positive, Gram-negative or total bacteria proportions present in faeces before and after the intervention (Fig 2B).

In group A, which experienced important weight loss (> 4 kg) and BMI z-score reductions (mean decrease 1.05; range 1.86-0.38), *C. histolyticum, C. lituseburense* and *E. rectale-C. coccoides* proportions decreased significantly after the intervention programme (P=0.011, P=0.049, and P=0.033, respectively), while those of *Bacteroides-Prevotella* group were significantly increased (P=0.047). *Lactobacillus-Enterococcus*, *Enterobacteriaceae*, *E. coli,* and *Roseburia* groups showed slight increases while *Bifidobacterium* and sulphate-reducing bacteria tended to decrease but these changes were not statistically significant. Reduced *C. histolyticum,* and *E. rectale-C. coccoides* proportions significantly correlated with percentage of body weight loss (r= 0.48; P=0.020 and r= 0.41; P=0.036, respectively) and those of *E. rectale-C. coccoides* with BMI z-score reductions (r= 0.36; P=0.020 and r= 0.41; P=0.036, respectively). In the adolescents group B, who did not experience a significant weight loss, none of the analyzed bacterial groups showed statistically significant differences before and after the intervention programme (Table 3). No correlations were detected between bacterial proportions and either body weight or BMI z-score reductions.

Faecal energy content before (5.43 [5.11-5.90] kcal/g) and after (5.16 [4.94-5.28] kcal/g) the intervention was almost significantly reduced (*P*=0.055) in group A of adolescents. In contrast, faecal energy content before (5.42 [5.04-5.66] kcal/g) and after (5.33 [5.2-5.42] kcal/g) the intervention was not significantly different (*P*=0.513) in group B of adolescents.

Biochemical parameters and correlations with faecal microbiota

Biochemical parameters in both groups of adolescents before and after the intervention are shown in Table 4. No significant differences were found in the analyzed biochemical parameters between both adolescent groups A and B before the intervention (base line) except for LDL levels, which were higher in group B of adolescents (P=0.034). Serum HDL-cholesterol values were significantly higher (*P*=0.031) before than after the intervention in group B of adolescents but did not correlate with any bacterial group changes. Slight changes in glucose concentration correlated with slight changes in *E. rectale-C. coccoides* as a result of the intervention in group B of adolescents (*r*=0.683, *P*=0.030). Serum glucose (*P*=0.029) and total cholesterol (*P*=0.012) concentration significantly dropped in group A of adolescent after the intervention. Changes in glucose and cholesterol significantly correlated with changes in the enteric group proportions (*r*=-0.547, *P*=0.006 and *r*=-0.462, *P*=0.035, respectively). In addition, changes in glucose significantly correlated with changes in total Gram-negative bacteria (*r*=-0.538, *P*=0.012). LDL-cholesterol was reduced after the intervention although not significantly and correlated to changes in *C. lituseburense* proportions (*r*=-0.508, *P*=0.019).

Immunoglobulin-coating bacteria in faeces from adolescents

Immunoglobulin-coating bacteria were detected in faeces of adolescents that experienced the greatest loss (> 4 kg; group A) in body weight (Fig 3). Overall, higher percentages of IgA,

IgM and IgG-coating bacteria were detected in faeces of adolescents before the intervention than after it. The proportions of IgA-coating bacteria were significantly reduced (P=0.034) after the intervention programme in group A1 of subjects, who lost over 6 kg, while these differences were not significant in group A2 of subjects with weight reductions between 4-6 kg (Fig 3). Group A1 also revealed significant reductions in proportions of *C. histolyticum,* and *E. rectale-C. coccoides* groups *(*P=0.046 and P=0.044, respectively*)* while changes in group A2 did not reached statistical significance (P<0.050), indicating that these bacteria exerted the greatest influence on IgA-coating bacterial changes. In addition, reduced *E. rectale-C. coccoides* proportions in group A1 significantly correlated with percentage of body weight loss and BMI z-score reduction (r= 0.56; P=0.023 and r= 0.53; P=0.035, respectively). Group A1 also showed significantly lower proportions of total Gram-positive bacterial populations (P=0.034) after the intervention programme, while no significant differences in Gram-negative bacteria and total bacteria were detected. Therefore, changes in IgA secretion against the gut microbiota could be explained by specific changes in its composition associated with weight loss.

Discussion

Diet is considered to be one of the main environmental factors shaping the composition of the gut microbiota within a host and affecting their functional relationships. This study has demonstrated that a weight-loss intervention programme, based on a calorie restricted diet and increased physical activity, induced changes in the gut microbiota structure of obese adolescents and that some of these changes correlated with weight loss and BMI z-score reductions. Therefore, the relative abundance of specific gut bacteria seems to be susceptible to lifestyle intervention and may be an additional element for consideration in weight management strategies.

Reduced *E. rectale-C. coccoides* and *C. histolyticum* proportions were significantly correlated with weight loss and BMI z-score reduction in the total population. By contrast, *Bacteroides* proportions increased as a result of the intervention programme, although their correlations with weight loss and BMI z-scores did not reach statistical significance. In previous human studies in adults, decreases in *Firmicutes* division, which include *Clostridium* clusters, as well as increases in *Bacteroidetes* division have also been correlated with the percentage of body weight loss; however, a very limited number of subjects were included in the corresponding study.[10] Therefore, the present report has confirmed that the abundance of these two bacterial groups in the distal gut could be linked to human weight loss although controversial results have also been reported recently.[13]

Specific faecal bacterial proportions differed significantly in group A of adolescents, who experienced a remarkable weight loss (> 4 kg) representing on average 8.1 % of their body weight, as a result of the intervention. Accordingly, previous studies have indicated that *Firmicutes* and *Bacteroides* changes are associated with weight-loss percentage. These changes were only evident when the individuals had lost at least between 2 and 6% of their body weight, without finding a relationship to the type of diet (either fat or carbohydrate

restricted).[10] Overall, total Gram-positive bacterial populations were significantly reduced and Gram-negative bacteria slightly increased after the intervention programme in group A of adolescents. *C. histolyticum, E. rectale-C. coccoides* and *C. lituseburense* were identified as the main contributors to the overall reduction in Gram-positive bacteria, whereas *Bacteroides* group contributed to the increase in total Gram-negative bacteria.

These gut microbes could play a role in obesity together with diet by affecting either host metabolism,[6] or the signalling pathways that link inflammation with obesity.[12] *E. rectale-C. coccoides* group includes clostridia cluster XIV, which integrates the main butyrate-producing bacteria in the distal colon.[29] These bacteria are responsible for generating butyric acid from carbohydrate fermentation, which fuels epithelial cells covering up to 70% of their energy needs.[30] The reduction of these bacterial groups by intervention in obese adolescents could contribute to reducing the overall ability of the gut microbiota to harvest energy from the diet, which could account for up to 10-15% of our daily caloric supply.[31] Nevertheless, butyrate generation by gut microbes has generally been associated with beneficial effects, including satiety promotion, rather than with obesogenic features,[32] indicating that more complex mechanisms related to fatty-acid metabolism could link *Firmicutes* and *Clostridium* clusters with obesity.

Clostridium histolyticum proportions were also reduced after the intervention programme in adolescents and these shifts were correlated with weight loss. This group belongs to clostridia cluster II, which are highly proteolytic and produce acetate as the main end-product of metabolism.[33] Increased levels in colonic and serum acetate, which may stimulate lipid synthesis, have also been associated with microbial gut colonization.[34,35] In addition, *Clostridium histolyticum* produces proteases that are cytotoxic for cells and tissues and could be pathogenic factors in the gut environment. The prevalence of this clostridial group could also increase protein fermentation in the colon with the subsequent generation of toxic

compounds such as sulphur-containing metabolites.[11] *Clostridium lituseburense* group, which is included in clostridia cluster XI, was also decreased after the intervention in group A of adolescents. This is a heterogeneous phylogenetic cluster but comprises opportunistic pathogens, such as *Clostridium difficile*, and its abundance together with that of *C. histolyticum* group could modify the potential virulence of the gut microbiota in obese patients, which in turn was improved in adolescents showing the highest weight loss.[33] In contrast to *Clostridium* groups, *Bacteroides* were increased in individuals showing a significant weight loss (< 4 kg) after the dietary intervention, which may be related to changes in the type of short-chain fatty acid generated and colonic pH increases.[36] Increased *Bacteroides* populations could contribute to generating propionate, which has been shown to inhibit lipid synthesis from acetate and may favour a lean phenotype.[35,37]

The present study has also showed almost significant changes in total faecal energy in group A of adolescents after the intervention while not in group B. Although the faecal energy only reflect part of the energy supply that could be due to the colonic microbiota, the obtained results could partly explain the detected differences in weight loss between the two adolescent groups (A and B) paralleled to microbiota changes. In fact, differences in total faecal energy between both adolescent groups could not be related to differences in total dietary energy intake. Therefore, these and previous results point for a role of gut bacteria other than common probiotic genera in weight management and, therefore, the current dietary strategies used to modulate the gut microbiota based on the administration of lactobacilli, bifidobacteria and prebiotics that favour their predominance could be questioned in obesity control.

Nevertheless, a recent study has not found correlations between *Bacteroides* populations and obesity by comparing obese and non-obese subjects and there was not significant relationship between changes in the percentage of *Bacteroides* in faeces and weight loss under reduced calorie diets.[13] Significant shifts detected in *Roseburia-E. rectale* groups detected with the

probe Rrec482 and *Bifidobacterium* were related to reductions in carbohydrate intake but regardless weight loss.[13] By contrast, the present study has not shown significant reductions in these bacterial groups in faeces of subjects submitted to energy intake reduction, including carbohydrate restrictions (approximately 28% reduction), but confirmed previous relations between *Clostridium* and *Bacteroides* groups with weight loss under dietary intervention.[10] Therefore, the possibility that weight loss depends on both the diet and its interactions with gut microbiota could not be completely disregarded.

Glucose and LDL-cholesterol reductions detected in group A of adolescents were also correlated with shifts in total Gram-negative bacteria and *C. lituseburense*. Although further studies should be carried out to confirm these trends, the results also suggest interactions between diet, gut bacteria and host's metabolism as previously proposed.[10]

Obesity and related disorders, such as the metabolic syndrome, are also associated with a chronic low-grade inflammation even at early ages, exemplifying the link between metabolism and immunity.[4] In this study, gut microbiota has been identified as a factor stimulating host immunity to different extents depending on weight loss. Elevation of IgA coating bacteria before the intervention programme could be an indication of low-grade inflammation triggered by the gut microbiota before intervention, since this microbiota is characterized by increased levels of opportunistic pathogens when compared with that detected after the diet in group A1 of adolescents.[38] The decrease in IgA-coating bacteria detected after the intervention was particularly associated with reductions in *C. histolyticum* and *E. rectale-C. coccoides* proportions. The reductions in butyrate-producing bacteria of *E. rectale-C. coccoides* group, as a result of the intervention programme, could also be responsible for reducing energy availability for immune cells leading to reducing IgA-producing cells and mucosal IgA concentrations.[39] Our results suggest that the increased host immune response trigger by the gut microbiota can be modified in overweight adolescents by

a lifestyle intervention, confirming that there is a relationship between gut microbiota and host immunity in obese human subjects

The limitations of the study include the relative small sample size of subgroups due to differences in weight loss responses of the whole population group and the short duration of the intervention, which could reduce the significance of the detected changes. However, this short-term study confirmed and complemented the results of a previous long-term study (1 year) by using different molecular techniques that target specific bacterial groups.

All in all, the present study has provided sounder links between specific bacterial groups and body weight in adolescents under a lifestyle intervention. This study also suggest a role for the gut microbiota in this disorder related to both host metabolism and immunity, evidenced by shifts in the bacteria driving the main metabolic pathways in the colon and showing different pathogenic features, although direct evidence should still be provided.

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FIGURE LEGENDS

Figure 1. Correlation between changes in different bacterial groups (% before - % after intervention) and weight loss (kg before - kg after diet). Pearson's correlations: $r = 0.43$, $P = 0.009$ (A); $r = 0.50$, $P = 0.001$ (B);$r = -0.28$, $P = 0.083$ (C); $r = -0.15$, $P = 0.365$ (D).

Figure 2. Overall bacterial composition in two adolescents groups: group A with >4 kg weight loss (A); group B with <2.5 kg weight loss (B). total Gram-positive bacteria was calculated by adding the proportions obtained with the probes Chis150, Erec0482, Bif164, Clit135 and Lab158 and total Gram-negative was calculated by adding the proportions obtained with the probes Bac303, Ent1432 and SBR687. *Significant differences ($P < 0.05$) between median values before (grey) and after (white) intervention programme by using the Mann-Whitney *U* test.

Figure 3. Mean percentage of faecal bacteria coated with IgA. *Significant differences ($P < 0.05$) between median values of different groups before (black) and after (white) intervention programme by using the Mann-Whitney *U* test.

Table 1. Clinical characteristics of the studied subjects

Characteristics*	Total subjects n=39	
Age (years)	14.4 (13.0-16.0)	
Diet (kcal/day)	1762 (1300-2200)	
Weight loss (kg)	4.3 (0.8-16.7)	
Energetic expenditure/wk	15-23 kcal/kg body weight	
	Before intervention	After intervention
†BW (kg)	91.7 (62.0-145.0)	87.4 (62.8-131.2)
‡BMI (kg/m^2)	33.1 (24.8-50.7)	31.5(23.7-50.4)
BMI z-score	3.4 (0.9-9.5)	2.9 (0.7-9.4)

†BW, body weight; ‡BMI, body mass index.
*Data are expressed as mean value (range)

Table 2. Oligonucleotide probes and hybridization conditions used in the analysis of intestinal bacterial by fluorescent in *situ* hybridization.

Probe	Target bacterial group	Sequence (5'–3')	Hybridization Conditions (ºC)	References
EUB338	Domain bacteria	GCT GCC TCC CGT AGG AGT	50	18
NON338	Negative control	ACA TCC TAC GGG AGG C	50	19
Bif164	*Bifidobacterium*	CAT CCG GCA TTA CCA CCC	50	20
Chis150	*Clostridium histolyticum*	TTA TGC GGT ATT AAT CT(C/T) CCT TT	50	21
Clit135	*Clostridium lituseburense*	GTT ATC CGT GTG TAC AGG G	50	21
Erec0482	*Eubacterium rectale/Clostridium coccoides*	GCT TCT TAG TCA GGTACCG	50	21
Lab158	*Lactobacillus/Enterococcus*	GGT ATT AGC A(C/T)C TGT TTC CA	45	22
Bac303	*Bacteroides/Prevotella*	CCA ATG TGG GGG ACC TT	45	23
Enter1432	*Enterobacteriaceae* group	CTTTTGCAACCCACT	50	24
Ecol1513	*Escherichia coli*	CAC CGT AGT GCC TCG TCA TCA	50	25
Rrec584	*Roseburia* subcluster	GGGACGTTGTTTCTGAGT	50	26
SRB687	Sulphate-reducing bacteria	TAC GGA TTT CAC TCC T	50	27

Table 3. Bacterial composition[1] of faecal samples as assessed by fluorescence in situ hybridization.

Bacterial group	Group A Adolescents with >4.0 kg weight loss (n=26)		P-value	Group B Adolescents with <2.5 kg weight loss (n=13)		*P*-value
	Before intervention	After intervention		Before intervention	After intervention	
Bifidobacterium	8.31 (20.48-0.51)	7.85 (19.58-1.2)	0.898	8.72 (25.48-1.49)	7.14 (15.67-4.23)	0.497
C. histolyticum	5.38 (13.04-2.02)	2.95 (13.12-0.47)	0.011*	6.04 (13.34-1.44)	7.31 (12.52-4.14)	0.573
C. lituseburense	2.53 (17.3-0.33)	1.45 (17-0.16)	0.049*	2.73 (28.04-0.42)	1.98 (18.61-0.52)	0.538
E.rectale/C.coccoides	7.51 (19.4-1.53)	4.55 (20.57-0.51)	0.033*	5.98 (14.23-1.37)	8.02 (21.33-0.76)	0.978
Lactobacillus/Enteroccoccus	1.01 (3.15-0.16)	1.31 (5.64-0.08)	0.604	0.57 (2.88-0.24)	0.65 (2.79-0.1)	0.663
Bacteroides/Prevotella	2.51 (6.92-1.13)	3.09 (16.14-0.93)	0.047*	1.83 (4.30-0.22)	1.77 (7.10-0.34)	0.681
Enteric group	7.27 (22.17-0.43)	7.96 (23.21-0.74)	0.833	6.44 (14.58-2.60)	6.78 (17.59-1.64)	0.682
E. coli	4.48 (17.96-0.11)	5.12 (26-0.1)	0.503	2.30 (11.57-0.20)	1.97 (7.76-0.56)	0.457
Roseburia	6.01 (15.75-2.8)	8.36 (19.49-2.02)	0.304	4.78 (11.55-1.56)	5.34 (12.67-2.97)	0.383
Sulphate-reducing bacteria	7.41 (20.61-0.15)	6.76 (22.45-0.3)	0.749	4.79 (17.07-0.87)	5.62 (15.12-1.46)	0.758

[1] Data were expressed as median proportions of bacterial cells hybridizing with specific-group probes to total bacteria hybridizing with EUB probe 338 and ranges.

*Significant differences ($P < 0.050$) between median values of different bacterial group proportions before and after diet were established by using Mann-Whitney *U* test.

Table 4. Biochemical parameters determined in plasma of adolescents before and after the intervention.

Parameters	Group B Adolescents with <2.5 kg weight loss (n=13)					Group A Adolescents with >4.0 kg weight loss (n=26)				
	Before intervention		After intervention			Before intervention		After intervention		
	Median	Range	Median	Range	*P*	Median	Range	Median	Range	*P*
Glucose (mg/dl)	85.5	83.5-90.0	83.0	78.0-87.0	0.309	87.5	83.0-99.7	83.0	76.7-90.2	0.029*
Total cholesterol (mg/dl)	141.0	121.0-158.0	141.0	129.0-152.0	0.977	152.5	133.0-163.0	132.5	123.7-147.0	0.012*
Triglycerides (mg/dl)	70.0	54.5-88.0	71.0	59.0-100.0	0.562	84.0	57.5-121.5	73.5	50.0-106.5	0.527
HDL cholesterol (mg/dl)	48.0	40.0-82.0	45.0	36.0-49.0	0.236	46.0	40.5-50.0	45.0	39.0-63.0	0.836
LDL cholesterol (mg/dl)	73.0	70.5-101.0	49.0	40.5-75.5	0.031*	79.0	52.2-98.7	75.5	65.0-86.2	0.391
Insulin (pg/ml)	344.2	529.0-664.0	504.6	349.0-760.0	0.555	471.6	417.1-772.0	421.5	263.0-632.0	0.139

* Statistical differences before and after the intervention were calculated using the Mann-Whitney *U*-test and established at $P < 0.050$.

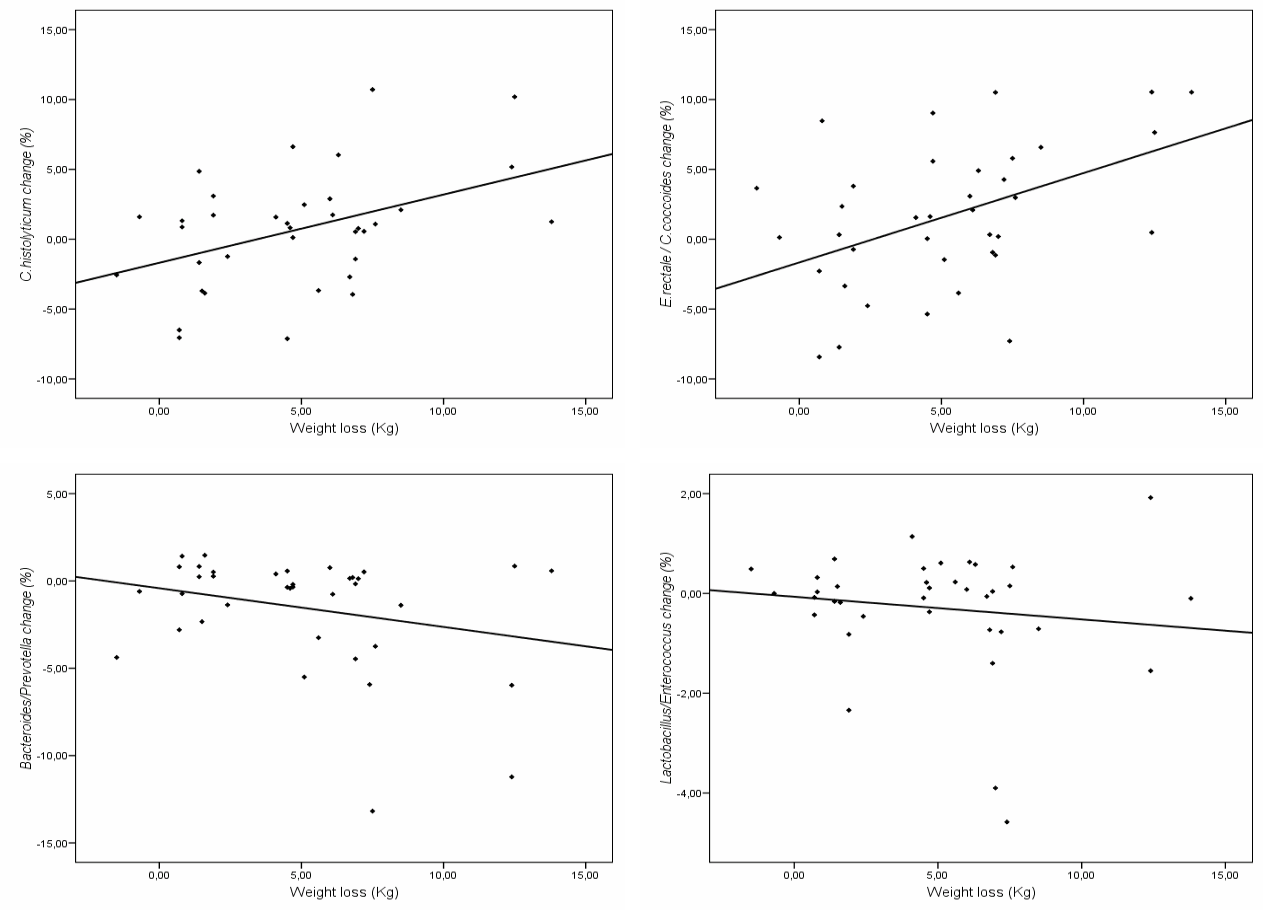

Fig 1. Nadal et al.

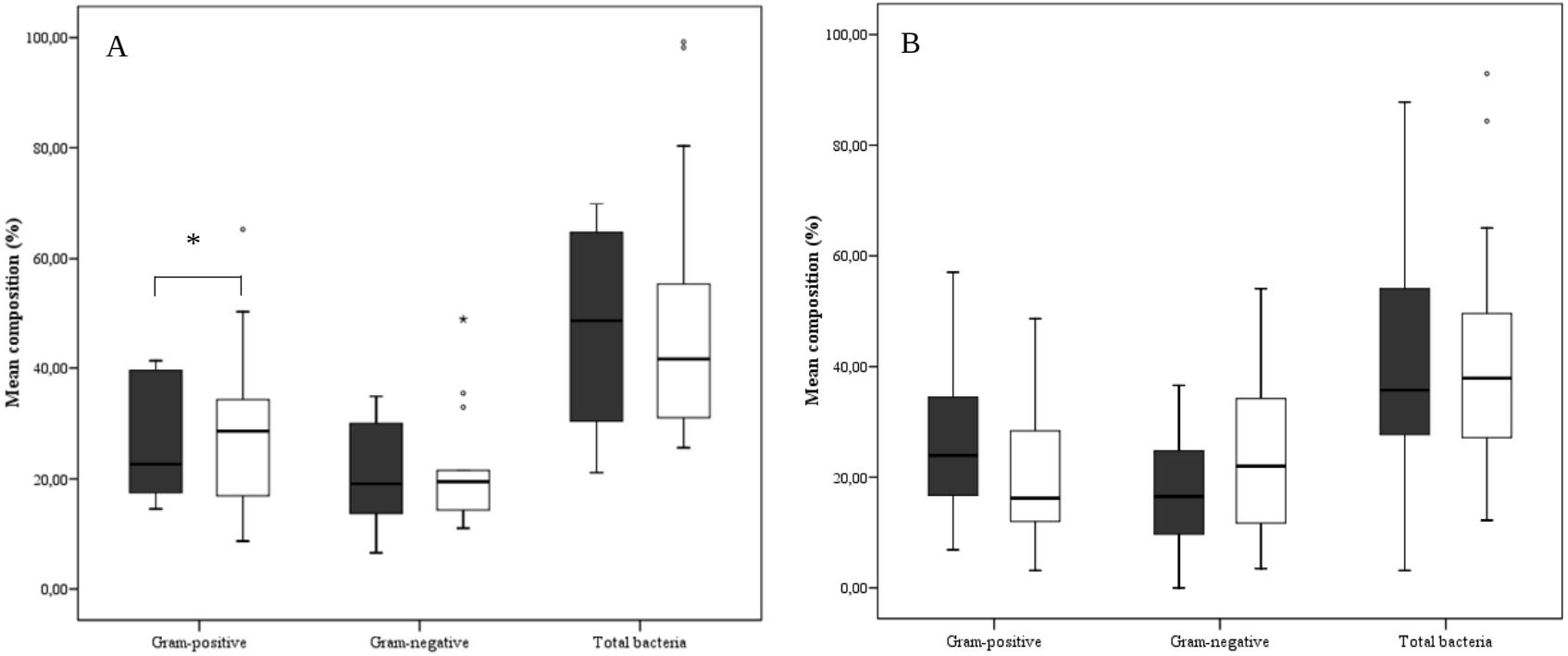

Fig 2. Nadal et al.

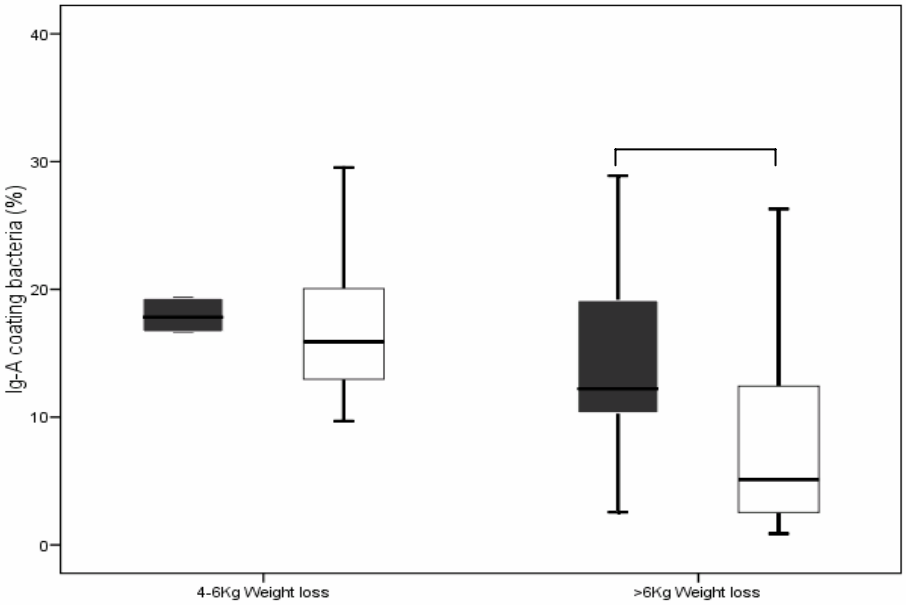

Fig 3. Nadal et al.