Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant woman

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Running title: Pregnant gut microbiota and overweight

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Abstract

Obesity is associated with complications during pregnancy and increased health risks in the newborn. The objective of this study was to establish possible relationships between gut microbiota, body weight, weight gain, and biochemical parameters in pregnant woman. Fifty pregnant women were classified according to their body mass index (BMI) in normal weight (n=34) and overweight (n=16) groups. Gut microbiota composition was analyzed by quantitative real-time PCR in faeces and biochemical parameters in plasma at 24 weeks of pregnancy. Reduced numbers of *Bifidobacterium* and *Bacteroides* and increased numbers of *Staphylococcus, Enterobacteriaceae* and *E. coli* were detected in overweight compared to normal weight pregnant women. *E. coli* numbers were higher in women with excessive weight gain than in woman with normal weight gain during pregnancy, while *Bifidobacterium* and *Akkermansia muciniphila* showed an opposite trend. In the whole population, increased total bacteria and *Staphylococcus* numbers were related to increased plasma cholesterol levels. Increased *Bacteroides* numbers were related to increased HDL cholesterol and folic acid levels, and reduced triglyceride levels. Increased *Bifidobacterium* numbers were related to increased folic acid levels. Increased *Enterobacteriaceae* and *E. coli* numbers were related to increased ferritin and reduced transferrin, while *Bifidobacterium* levels showed the opposite trend. Therefore, gut microbiota composition is related to body weight, weight-gain and metabolic biomarkers during pregnancy, which might be of relevance to the management of woman and infant's health.

Key words: pregnancy, gut microbiota, obesity, cholesterol, triglycerides, folic acid, ferritin.

Introduction

The prevalence of obesity is rapidly increasing worldwide, constituting an important health issue. Obesity is the result of a positive imbalance between energy intake and energy expenditure over a long period and is related to the development of other disorders such as diabetes, dyslipemia and cardiovascular diseases. Obesity is also associated with complications during pregnancy and at the delivery for women and with increased health risks in newborn [1-3].

There are several genetic and environmental factors such as diet, cultural behaviour, and socioeconomic status, which influence obesity [4,5]. In addition, recent reports suggest that the nature and composition of the intestinal microbiota are altered in obesity [6,7]. Lean individuals have more *Bacteroidetes*, while obese individuals have more *Firmicutes*, including *Clostridium* clusters, in their intestinal microbiota [6,7]. It has been proposed that such bacterial composition improved the ability of the host to extract energy from the diet and to store this energy in the adipose tissue [7]. Gut microbiota has also been related to body weight and body weigh loss under a lifestyle intervention in humans [8,9]. Although obesity is an important health issue during pregnancy, the relationships between the gut microbiota composition and obesity has been scarcely studied in pregnant women [10].

The aim of the present study was to analyse the microbiota composition of pregnant women and establish its possible relationships with body weight, weight gain and biochemical parameters to progress in the understanding of the role of the microbiota in the health status of pregnant woman.

Experimental methods

Study participants

The pregnant women were recruited at 20 weeks of pregnancy at the Clinical University Hospital "San Cecilio" de Granada, Spain. Women were classified according to their pre-pregnancy Body Mass Index (BMI) into two groups, overweight women (n=16) with BMI>25 and normal weight women (n=34) with BMI<25 (Table 1). Signed informed consent was obtained from the studied women after a full explanation of the study was given by a member of the team at the first visit. Participants were assured of anonymity and confidentiality. After the visit at the first trimester, women were examined by the obstetrician again at 24 (2^{nd} trimester) and 34 weeks (3^{rd} trimester) and clinical parameters were recorded. At 24 weeks of pregnancy, faecal and blood samples were obtained for microbiological and biochemical analysis. Data on weight before pregnancy was used to calculate weight gain during pregnancy. Normal weight gain ranges were from 11.5 to 16.0 kg for normal weight women (BMI 19.8–25.0) and from 7.0 to 11.5 kg for overweight women (BMI >25), respectively, over pregnancy according to the Institute of Medicine (IOM) criteria [(11)]. Total weight gains above these values, >16 kg for normal-weight women and >11.5 kg for overweight women, were considered excessive weight. Data on gestation time and birth weights of the newborns were also collected. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethics committee of the Hospital involved in the study. Written informed consent was obtained from all subjects before their inclusion in the study.

Dietary assessment

Food diary records of pregnant women were kept for 72h (2 weekdays and 1 weekend day) at 24 weeks of pregnancy. 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, water and nutrient contents based on the CESNID food-composition database of Spanish foods[12].

Biochemical parameters

Fasting plasma glucose, total cholesterol, HDL cholesterol, triglycerides, urea, creatinine, uric acid, bilirubin and iron were measured by enzyme-colorimetric automated methods for clinical chemistry (Modular analytics EVO, Roche, Neuilly sur Seine Cedex, France). LDL cholesterol was calculated using the Friedwald's formula [13]. Ferritin, transferrin, folate and thyroid - stimulating hormone (TSH) levels were measured by using the automatic analyser Elecsys 2010 with modular analytics E170 (Roche, Neuilly sur Seine Cedex, France). The transferrin saturation index was calculated using the following formula: TSI (%) = (ferritin (ug/ml).100)/(transferrin (mg/dl)x1.24).

Sample preparation and DNA extraction

Faecal samples were frozen immediately at -20ºC and kept until processing. Faeces (1g) were diluted 1: 10 (w/v) in PBS (pH 7.2), homogenized and used for DNA extraction. DNA from pure cultures of reference bacterial strains and faecal samples were extracted using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration of DNA was determined with a Nanodrop-1000 spectrophotometer (Nanodrop, Wilmington, DE).

Analysis of faecal microbiota composition

Quantitative real time PCR (qPCR) was used to characterize the microbiota by using of specific primers targeting different bacterial groups and the SYBR® Green PCR Master Mix (SuperArray Bioscience Corporation, Frederick, MD, USA), as previously described [9,10]. PCR amplification and detection were performed with an ABI PRISM 7000-PCR sequence detection system (Applied Biosystems, Warrington, UK). Bacterial concentration from each sample was calculated by comparing the Ct values obtained from standard curves. Standard curves were created using serial 10-fold dilution of pure culture DNA corresponding to 10^2 to 10^9 cell equivalents/ml (genome equivalents/ml). Conversion of the amount of bacteria DNA in samples determined by qPCR to theoretical genome equivalents required the assumption that the genome size and 16S rRNA gene copy number for each bacterial group analyzed was similar. The following genome sizes were used in the study: 2.3 Mb for *Bifidobacterium* (using *B. longum* as standard), 2.9 Mb for *Lactobacillus* (*L. casei*), 5.2 Mb for *Bacteroides* (*B. fragilis*), 4 Mb for *C. coccoides* group, 3.3 Mb for *C. leptum* group, 4.6 Mb for *Enterobacteriaceae* and *E. coli*, 2.8 Mb for *Staphylococcus* (*St. aureus*) and 2.7 Mb for *Akkermansia muciniphila*. Genome sizes were obtained from NCBI data base (Genome project). Standard curves were created using the following reference strains: *Bifidobacterium longum* subsp. *longum* CECT 4503, *Bacteroides fragilis* DSMZ 2451; *Clostridium coccoides* DSMZ 933; *C. leptum* DSMZ 935; *Staphylococcus aureus* CECT 86; *Lactobacillus casei* ATCC 393; *E. coli* CECT 45 and *Akkermansia muciniphila* strain Muc^T (ATCC BAA-835^T).

Statistical analyses

Statistical analyses were done using the SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). Data distribution was analysed by applying the Kolmogorov-Smirnov test and creating a Gaussian. Due to non-normal distribution, microbial data are expressed as medians with interquartile ranges (IQR). The Mann-Whitney U-test was applied for comparisons between bacterial numbers of normal and overweight women and between women with excessive and normal weight gain over pregnancy. Differences in prevalence of bacterial groups were established by applying the Chi-square test. Correlations between variables were determined by applying the Spearman's rank correlation. A P <.050 was considered statistically significant for all tests.

RESULTS

Body weight, body mass index, and weight gain over pregnancy

Clinical characteristics of the studied women at recruitment time were similar in both groups (Table 1) except for BMI and body weight. The body weight of the overweight women was significantly higher than that of normal weight women during pregnancy, although no significantly differences (P= .120) in weight gain were detected between the groups over time. BMI was significantly different (P <.050) between normal weight and overweight women and increased in both groups over pregnancy. The infants were born at term and the infant's birth weight of the overweight women were higher than those of normal weight women (P= .028).

Dietary intakes

Dietary data of normal weight and overweigh pregnant women at 24 weeks of pregnancy are shown in Table 2. No significant differences in dietary intake of energy, macronutrients or on food group level were found between both groups of women. Only

the intake of fiber was slightly higher (*P*= .057) in normal weight than in overweight woman. When women were grouped according to the total weight gain over pregnancy into two groups (excessive and normal weight gain), no significant differences in dietary intake of energy, macronutrients or on food group level were found between the two groups. No correlations were found between dietary intakes, body weight and body weight gain.

Biochemical parameters

Biochemical parameters of pregnant women at 24 weeks subdivided according to their BMI in normal and overweight women are shown in Table 3. Bilirubin, iron and folic acid levels were significantly higher in normal than in overweight women (*P*=.021, *P*= .021 and *P*= .042, respectively). HDL cholesterol was higher (*P*= .050) in normal than in overweight women, whereas total cholesterol and triglycerides levels were significantly higher in overweight than in normal weight women (*P*= .019 and *P*= .034, respectively). Moreover, increased levels of triglycerides (R=0.30, *P* =.033) and total cholesterol (R=0.43, *P* =.002) and reduced levels of bilirubin (R=-0.36, *P* = .019) and iron (R=-0.33, *P* = .019) correlated to overweight women.

When women were grouped according to the total weight gain over pregnancy into two groups (excessive and normal weight gain), correlations with some biochemical parameters were also detected. Increased levels of total cholesterol (R=0.33, *P* = .020) and ferritin (R=0.45, *P* = .001) correlated with women with excessive weight gain over pregnancy.

Microbiota composition in normal and overweight women

The bacterial numbers detected in faecal samples of normal- and overweight women are shown in Table 4. *Bifidobacterium* and *Bacteroides* numbers were significantly higher ($P > .001$ and $P = .035$, respectively) in normal weight women than in overweight women, whereas *Enterobacteriaceae* ($P = .001$), *E. coli* ($P = .005$) and *Staphylococcus* ($P = .006$) numbers were lower in normal weight than in overweight women. *C. coccoides* group numbers were slightly higher in overweight women than in normal weight woman, but not significantly ($P = .088$).

The ratio of *Bifidobacterium* to *C. coccoides* group was significantly higher ($P < .001$) in normal weight than in overweight women. The ratio of *Bifidobacterium* to both *Clostridium* groups (*C. coccoides* plus *C. leptum*) was also significantly higher ($P < .001$) in normal weight than in overweight women.

Increased numbers of *Bifidobacterium* (R=- 0.56, $P < .001$) and *Bacteroides* (R= -0.34, $P = .020$) correlated to normal weight women, while a different trend was found for *Staphylococcus* (R=0.67, $P = .003$), *Enterobacteriaceae* (R=0.46, $P < .001$) and *E. coli* (R=0.40, $P = .004$) (Fig 1). An increased ratio of *Bifidobacterium* to *C. coccoides* correlated to lower BMI (R=-0.60, $P < .001$). Similarly, an increased *Bifidobacterium* to *C. coccoides* plus *C. leptum* ratio was positively related to normal weight women (R=-0.54, $P < .001$).

Microbiota composition according to weight gain over pregnancy

Faecal microbiota composition of woman showing normal or excessive weight gain over pregnancy is shown in Table 5. *E. coli* numbers were significantly higher ($P= .045$) in women with excessive weight gain than in women with normal weight gain over pregnancy. A similar trend was found for *Enterobacteriaceae* numbers although the differences were not significant ($P = .142$). Contrary to this tendency, *Akkermansia muciniphila* and *Bifidobacterium* numbers were higher ($P = .020$ and $P = .078$,

respectively) in women with normal weight gain than in those with excessive weight gain.

The prevalence of *C. leptum* group and *Staphylococcus* was higher in women with excessive weight-gain than in women with normal weight gain over pregnancy (P = .545 and P = .124).

Increased numbers of *Bifidobacterium* (R=-0.31, P = .029), *Bacteroides* (R=-0.36, P= .019) and *A. muciniphila* (R= -0.34, P = .017) correlated significantly to normal weight gain over pregnancy (Fig 2). Opposite, increased numbers of *Enterobacteriaceae* (R=0.28, P = .050) and *E. coli* (R=0.42, P = .002) correlated with excessive weight gain over pregnancy (Fig. 2)

Relationships between microbiota composition and dietary intakes

In the whole women population, only increased numbers of total bacteria correlated to reduced energy (R= -0.71 P< .001), animal protein (R= -0.66, P= .001), cholesterol (R= -0.57, P= .007) and PUFA (R= -0.52 P< .015) intakes. The same trend was detected between total bacteria and energy (R=-0.78 P< .001 and R=-0.07 P= .002), animal protein (R= -0.61 P< .015 and R= -0.75 P= .001), and cholesterol (R= -0.52, P< .043 and R=- 0.58 P= .018) intakes in the normal weight group and in the normal weight gain group.

Relationships between microbiota composition and biochemical parameters

In the whole women population, total bacterial positively correlated to cholesterol (R=0.350, P =.013, respectively). Increased numbers of *Staphylococcus* were related to increased levels of cholesterol (R=0.68, P = .003). Increased numbers of *Enterobacteriaceae* and *E. coli* counts were significantly correlated to increased levels

of ferritin (R=0.324, *P*= .023 and R=0.425, *P* = .002) and saturation transferrin index (R=0.302, *P* = .035 and R=0.439, *P* = .002) and reduced levels of transferrin (R=-0.353, *P* = .013 and R=-0.341, *P*= .017). In contrast, increased numbers of *Bifidobacterium* were related to reduced levels of ferritin (R=-0.420, *P* = .003) and saturation transferrin index (R=-0.388, *P*= .006) and to increased levels of transferrin (R=0.348, *P*= .014). In addition, increased numbers of *Bifidobacterium* were related to increased levels of folic acid (R=0.308, *P*= .032). Increased numbers of *Bacteroides* were related to increased levels of HDL cholesterol (R=0.518, *P* < .001) and folic acid (R=0.333, *P* = .020) and to reduced levels of triglycerides (R=-0.371, *P* = .009).

In normal weight women, increased numbers of total bacteria correlated to increased levels of cholesterol (R=0.383, *P*=.025), while in overweight women the correlations were not significant.

In normal weight gain women, increased levels of total bacteria were related to increased levels of total cholesterol (R=0.390, *P* = .019), HDL cholesterol (R=0.335, *P* = .046) and folic acid (R=0.338, *P* = .044). Increased numbers of *Staphylococcus* correlated with increased levels of total cholesterol (R=0.881, *P* < .001). Moreover, increased numbers of *Bacteroides* correlated with higher levels of HDL cholesterol (R=0.620, *P* = .002). In women with excessive weight gain over pregnancy, increased numbers of *Bifidobacterium* were related to increased levels of HDL cholesterol (R=0.572, *P* = .042) and reduced levels of total triglycerides (R=-0.682, *P* = .010). Increased *Bacteroides* numbers were related to reduced levels of triglycerides (R=-0.809, *P* = .001).

Relationships between maternal microbiota composition and infant's birth weight

In the whole women population, significant positive correlations were found between *E. coli* (R=0.331, *P* = .039) and *C. coccoides* (R=0.323, *P* = .045) numbers and infant's birth weight were found. In overweight women, positive correlation were also found between *E. coli numbers* and infant's birth weight (R=0.673, *P* = .035). In excessive weight gain women, significant negative correlations were found between numbers of *Lactobacillus* group and infant's birth weight (R=-0.917, *P* = .001).

Discussion

This study reports differences in the intestinal microbiota of normal weight and overweight pregnant women, associated with body weight and weight gain over pregnancy, suggesting that the intestinal microbiota is a relevant target to weight management in pregnancy. Moreover, newborns form overweight pregnant woman had higher birth weight than those from normal weight pregnant women, suggesting the transference of the mother's features to their newborns. In this context, the results can also be of relevance to the transference of the aberrant microbiota to the newborns, which use the mother's microbiota as inoculums for microbiota development [(14)]. In this context, a positive relationship between the maternal intestinal *E. coli* numbers and infant's birth weight was demonstrated, which could be related to infant's body weight regulation. In contrast, in excessive weight gain women increased *Lactobacillus* numbers were related to reduced infant's birth weight, suggesting a positive role of this bacterial group in infant's body weight regulation.

In the present study, increased numbers of *Bacteroides*, which belong to *Bacteroidetes* phylum, were detected in normal weight compared to overweight women. In previous studies, the faecal microbiota of lean human subjects was characterized by having increased numbers of *Bacteroidetes* compared to that of obese subjects. Moreover,

weight loss under dietary intervention was associated with increases in *Bacteroidetes* and *Bacteroides fragilis* group numbers in adults and adolescents [6,8,9]. Therefore, the association of *Bacteroidetes* with a lean phenotype established in previous studies has also been confirmed in pregnant woman included in this study. Nevertheless, *Bacteroides* numbers were significantly higher in overweight than in normal weight women and associated with excessive weight gain over pregnancy in the only previous study carried out in pregnant women[10]. These results contradict all previous findings on the role of *Bacteroides* in obesity and highlight the importance of the new evidence provided by this study in this regard.

Increased numbers of *Bifidobacterium* were also related to normal weight women compared to overweight women, and a similar trend was detected in women with normal weight gain compared to those with excessive weight gain over pregnancy. This is in agreement with recent studies, which showed that levels of *Bifidobacterium* were reduced in infants who developed overweight at 7 years old, compared to normal weight children[14]; however, this association was not established in the previous study conducted in pregnant women[10]. In animal models, a role has also been attributed to *Bifidobacterium* in obesity. Obese Zucker rats (*fa*/*fa*) and mice fed a high fat diet showed reduced *Bifidobacterium* counts [15, 16]. Moreover, the administration of prebiotics to mice fed a high fat diet increased the intestinal *Bifidobacterium* numbers, which positively correlated with improved glucose tolerance and glucose-induced insulin secretion and with the normalization of the inflammatory tone [16].

In addition, the ratio of *Bifidobacterium* to either *C. coccoides* or to *C. coccoides* plus *C. leptum* group numbers was also significantly higher in normal weight than in overweight women, suggesting a negative role of *Clostridium* in obesity. In agreement, obese human subjects were shown to have increased numbers of *Firmicutes* in their faecal microbiota

as compared to lean subjects [6]. Moreover, weight loss under dietary intervention has also been associated with reduction in *Firmicutes* or *C. coccoides* and *C. histolyticum* group proportions [6, 8, 9]. Altogether, these results confirm that increases in the relative abundance of members of *Firmicutes* and, in particular, of some *Clostridium* clusters is associated with excessive body weight.

Staphylococcus numbers were also increased in overweight compared to normal weight women in agreement with a previous study conducted in pregnant woman [10]. Moreover, children becoming overweight at 7 years old showed a greater number of *Staphylococcus aureus* in faeces during infancy [14]. In addition, *Enterobacteriaceae* and *E. coli* were significantly higher in overweight than in normal weight women and also in women with excessive weight gain over pregnancy. Increased levels of Gram-negative bacteria, which could include *Enterobacteriaceae* and *E. coli*, could be related to the endotoxaemia and inflammatory tone associated with obesity as evidenced in animal models [16].

Total cholesterol and triglycerides levels were significantly higher in overweight than in normal weight women and increased cholesterol levels correlated with excessive weight gain over pregnancy, as expected. In addition, folic acid was significantly lower in overweight than in normal weight women, which is a nutrient involved in the correct differentiation of the neural tube during foetal organogenesis. In fact, obesity is a risk factor for neural tube defects [17]. Moreover, iron levels were also lower in overweight than in normal weight women and increased levels of ferritin correlated to higher weight gain in the whole population and in the excessive weight gain group. It has been described a relationship between obesity and iron deficiency, which can be reflected in reduced plasma levels of iron and transferrin and increased plasma levels of ferritin and saturation transferrin index [18, 19, 21, 22]. The iron deficiency associated with obesity has a

multifactorial aetiology and could be due to impairment of intestinal iron uptake and iron release from stores, and to inadequate iron bioavailability because of inflammation. In particular, abnormal ferritin concentrations have been explained by the chronic low-grade inflammation associated with obesity, metabolic syndrome and gestational and type 2 diabetes [20, 21, 22]. Increases in serum ferritin concentrations early in gestation also constitute a risk of gestational diabetes, partly mediated by the maternal fat mass and obesity [22].

This study also reports interesting relationships between biochemical parameters and specific intestinal bacterial groups in pregnant women. While *Bacteroides* numbers seemed to have a positive effect on plasma biomarkers of lipid metabolism, *Staphylococcus* numbers seemed to have a negative effect particularly on plasma cholesterol. Cholesterol and other sterols have been shown to stimulate the growth of at least *S. aureus*[23]; however, in this study no correlation was found between cholesterol intake and *Staphylococcus* numbers, which could explain a link with plasma cholesterol levels. Other mechanisms have been proposed to justify the influence of the intestinal microbiota on lipid metabolism, including generation of different short-chain fatty acids and regulation of the host gene expression [6, 7, 24, 25] but the specific relationships found in the present study remain to be elucidated.

Bifidobacterium numbers were positively related to plasma folic acid levels in the whole population, which may be due to the ability of some strains of this genus to synthesise and secrete folates in the human intestinal environment, providing a complementary endogenous source of this vitamin [26]. This metabolic trait of *Bifidobacterium* strains could contribute to improving the nutritional status of the pregnant woman and the foetus.

Enterobacteriaceae/E. coli and *Bifidobacterium* showed inverse relationships with transferrin, saturation transferrin index and ferritin, as well as with body weight in the whole population. Increases in serum transferrin saturation index, because of a transferrin decrease and ferritin increase, have been associated with a decrease of antibacterial activity of serum against enterobacteria, such as *Salmonella enterica*, which could contribute to favouring the survival of this bacterial group [(27)]. In fact, infections are one of the conditions that can depress transferrin levels. The possibility that the overgrowth of *Enterobacteriaceae* in the gut environment might favour their translocation to some extent and cause a similar effect could not be disregarded. By contrast, the administration of inulin to pigs led to increased *Lactobacillus* and *Bifidobacterium* numbers and to up-regulating the expression of genes encoding for iron transporters in the enterocytes, which suggest a connexion between these bacterial groups and/or the prebiotic, and improved iron absorption [(28)]. Therefore, the relative abundance of *Bifidobacterium* and *Enterobacteriaceae* may differently influence iron metabolism and, in turn, exert opposite effects on the nutritional status of pregnant woman. Unlikely the present study, a previous report on pregnant woman microbiota did not provide any data on biochemical parameters and their possible associations with the microbiota[(10)].

In summary, specific bacterial groups are oppositely related to overweight and weight gain during pregnancy, pointing for a beneficial role of *Bacteroides* and *Bifidobacterium* in body weight regulation. In addition, novel associations between these bacterial groups and beneficial changes in metabolic biomarkers are provided, suggesting a connexion between the gut microbiota and the host metabolism. Altogether, these findings open new possibilities for the management of body weight and of the nutritional status of

pregnant women through modulation of the intestinal microbiota, which may have consequences on later infant's health and deserve further investigations.

Acknowledgements

This work was supported by grants AGL2008-01440/ALI, Consolider Fun-*C*-Food CSD2007-00063 from the Spanish Ministry of Science and Innovation, AP-124/09 from Consellería de Sanitat (Valencia, Spain) and P06-CTS-02341 (Excellence Project PREOBE) from Consejería de Innovación, Ciencia y Empresa de la Junta de Andalucía. (Spain). M.C Collado was recipients of I3P fellowships from CSIC (Spain). *Akkermansia muciniphila* strain Muc^T (=ATCC BAA-835^T=CIP 107961^T) was kindly provided by Molecular Microbial Ecology Group, Laboratory of Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, Wageningen, The Netherlands.

Y. Sanz conceived and coordinated the microbiological study, and draft the manuscript. A. Santacruz and M.C. Collado carried out the microbiological and statistical analyses. C. Campoy coordinate the clinical follow-up of pregnant woman. L García-Valdés, M.T. Segura, J.A. Martín-Lagos and T. Anjos collected clinical and biochemical data. M. Martí-Romero, R.M. Lopez and J. Florido recruited and followed-up pregnant woman. All authors have read, reviewed and approved the final version of the manuscript. The authors do not have any conflict of interest.

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FIGURE LEGENDS

Figure 1. Relations between numbers of faecal bacterial groups and weight. Data represent the positive samples. The line in the box is the median (50% percentile), with the lower line the lower 25% border (25% percentile) and the upper line the 75% (75% percentile) border. The end of the upper vertical line is the maximum data value, outliers not considered. The end of the lower vertical line is the lowest value, outliers not considered. The separate dots or asterisks indicate outliers. Lines showed the Spearman correlation (linear adjustment).

Figure 2. Relations between numbers of faecal bacterial groups and weight gain over pregnancy. Data represent the positive samples. The line in the box is the median (50% percentile), with the lower line the lower 25% border (25% percentile) and the upper line the 75% (75% percentile) border. The end of the upper vertical line is the maximum data value, outliers not considered. The end of the lower vertical line is the lowest value, outliers not considered. The separate dots or asterisks indicate outliers. Lines showed the Spearman correlation (linear adjustment).

Table 1. Clinical characteristics of the studied subjects

Characteristics[1]	Women		*P value
	Normal weight (n=34)	Overweight (n=16)	
Women			
Age (years)	31.0 (27.7-34.2)	29.0 (28.0-33.5)	.646
Height (cm) prior pregnancy	163.0 (158.0-167.0)	162.0 (158.0-166.0)	.967
Weight (kg)			
prior pregnancy	58.0 (53.2-64.5)	73.0 (70.0-84.0)	> .001
1st trimester	62.5 (58.1-66.2)	75.4 (71.4-86.4)	> .001
2nd trimester (24 wk)	66.4 (60.7-72.3)	77.3 (73.5-88.6)	> .001
3rd trimester (34 wk)	70.4 (66.0-75.5)	81.2 (78.0-100.0)	> .001
Weight (kg) gain over pregnancy	11.7 (8.8-14.3)	10.0 (6.2-11.4)	.120
Body mass index (BMI)			
prior pregnancy	23.0 (20.8-24.3)	28.7 (26.3-31.2)	> .001
1st trimester	23.3 (21.0-25.0)	29.0 (27.8-32.7)	> .001
2nd trimester (24 wk)	24.0 (22.7-25.7)	30.0 (26.8-33.2)	> .001
3rd trimester (34 wk)	26.6 (25.1-28.2)	30.8 (28.9-35.5)	> .001
Newborns			
Duration of gestation (weeks)	39.0 (38.5-40.0)	39.5 (39.0-41.0)	.165
Birth weight (kg)	3.20 (3.1-3.4)	3.50 (3.2-4.0)	.028

[1]Data are presented as medians (interquartile range).

*Significant differences were calculated using Mann-Whitney U-test at $P < .050$.

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Table 2. Daily energy and nutrient intake in normal and overweight women at 24 weeks of pregnancy.

	Normal weight group (18<BMI<25) (n=34)		Overweight group (BMI>25) (n=16)		Mann-Whitney U-test
	Median	IQR	Median	IQR	*P*-value
Energy (kJ)	8.86	7.85 – 10.16	8.06	6.68 – 9.74	0.430
Water (g)	8.40	6.47 – 11.17	8.02	7.27 – 12.31	1.000
Protein (g)	82.14	73.0 - 99.0	86.54	75.0 - 103.0	0.610
Protein (%)	16.31	14.7 - 17 7	17.53	15.2 - 20.1	0.265
Plant protein (g)	25.04	22.7 - 28. 7	23.22	16.4 - 26.5	0.265
Plant protein (%)	4.73	4.4 - 7.2	4.90	3.5 - 5.1	0.458
Animal protein (g)	56.85	48.3 - 71.7	61.25	50.6 - 74.8	0.546
Animal protein (%)	11.77	9.9 - 12.7	13.15	10.3 - 15.7	0.063
Fat (g)	91.35	75.4 - 105.0	85.00	62.5 - 105.0	0.458
Energy from fat (%)	40.28	35.8 - 44.7	40.31	33.5 - 44.3	0.926
Saturated fat (g)	30.36	27.1 - 40.4	26.71	20.1 - 42.0	0.577
Energy from saturated fat (%)	13.77	12.2 - 14.9	13.33	10.0 - 17.6	0.963
MUFA (g)	37.20	30.0 - 48.5	34.34	27.4 - 41.0	0.577
Energy from MUFA (%)	16.23	14.0 - 20.0	16.00	15.1 - 17.7	0.889
PUFA (g)	13.88	11.2 - 16.4	12.00	9.2 - 16.7	0.458
Energy from PUFA (%)	5.6	4.8 - 7.3	5.56	4.4 - 6.5	0.642
Cholesterol (mg)	279.70	228.5 - 414.8	352.46	222.1 - 478.5	0.458
CH (g)	227.70	200 - 262.0	197.00	171.8 - 262.7	0.330
Energy from CH (%)	43.06	38.0 - 48.3	42.43	38.0 - 45.1	0.610
Simple CH (g)	125.20	111.8 - 153.3	102.12	79.0 - 139.5	0.150
Energy from simple CH (%)	22.02	19.5 - 27.0	21.23	18.6 - 23.2	0.280
Complex CH (g)	101.51	87.5 - 110.8	98.00	74.8 - 120.2	0.889
Energy from complex CH (%)	18.60	17.0 - 24.1	20.45	17.0 - 23.4	0.816
Dietary fiber (g)	19.81	16.2 - 24.3	16.75	13.6 - 19.0	0.057

Abbreviations: PUFA = Polyunsaturated fatty acids, MUFA = Monounsaturated fatty acids, CH = Carbohydrates
*Statistical significant differences were calculated by using the Mann-Whitney *U* test and established at $P < 0.050$

Table 3. Biochemical parameters recorded at 24 weeks of pregnancy of normal and overweight women.

Biochemical parameter	Units	Reference values	Values at 24 weeks of pregnancy		Mann- Whitney Test
			BMI<25 (n=34) [a]	BMI>25(n=16) [a]	*P value
Glucose	mg/dl	65-110	76.5 (69.7-81.0)	77.0 (63.5-90.0)	.840
Urea	mg/dl	10-50	20.0 (17.5-25.6)	19.7 (14.7-22.7)	.288
Creatinine	mg/dl	0.5-1.2	0.6 (0.5-0.7)	0.5 (0.5-0.6)	.072
Uric acid	mg/dl	2.4-7.0	3.1 (2.7-3.7)	3.3 (2.9-3.7)	.493
Bilirubin	mg/dl	0-1	0.2 (0.2-0.3)	0.1 (0.1-0.3)	.021
Cholesterol	mg/dl	120-220	233.0 (206.7-256.0)	259.0 (230.0-281.0)	.019
Triglycerides	mg/dl	50-170	148.0 (119.0-186.0)	192.0 (163.0-225.0)	.034
HDL cholesterol	mg/dl	45-65	77.0 (69.7-95.5)	66.0 (58.0-83.0)	.050
LDL cholesterol	mg/dl	50-150	135.0 (99.7-150.0)	130.0 (99.0-138.0)	.580
Total protein	g/dl	6.5-8.7	7.0 (6.6-7.1)	7.0 (6.7-7.1)	.502
Albumin	g/dl	3.5-5.0	4.0 (3.7-4.1)	3.8 (3.6-4.0)	.395
Iron	μg/dl	45-150	79.5 (63.7-105.7)	60.0 (53.0-95.0)	.021
Ferritin	ng/ml	30-400	19.0 (10.5-31.2)	20.0 (16.3-33.7)	.356
Transferrin	mg/dl	212-360	358.5 (320.7-413.0)	350.0 (305.0-397.0)	.288
Saturation transferrin index	%	17.1-30.6	18.7 (14.0-25.0)	16.5 (11.6-23.0)	.362
Folic acid	ng/ml	3.1-17.5	15.3 (10.6-18.5)	10.5 (7.3-17.0)	.042
TSH	μUI/ml	0.3-4.2	1.4 (0.9-1.7)	1.6 (1.0-1.6)	.368

[a] Data are shown as medians and interquartile range (IQR)

* Statistical differences were calculated by using the Mann-Whitney *U* test. Significantly difference was considered at $P < .050$.

Table 4. Bacterial numbers in faecal samples analyzed by qPCR at 24 weeks of pregnancy.

Microbial groups	Normal weight women (n=34)		Overweight women (n=16)		Mann-Whitney test* *P* value
	Pr[a]	Log genome equivalent/g [b]	Pr[a]	Log genome equivalent/g [b]	
Total cell counts	34/34	9.85 (9.40-10.24)	16/16	9.89 (9.40-10.02)	.630
Bifidobacterium	34/34	9.10 (8.53-9.52)	16/16	8.36 (7.74-8.57)	>.001
Lactobacillus group	34/34	7.48 (7.35-7.60)	16/16	7.70 (7.40-7.78)	.053
Clostridium coccoides group	34/34	8.52 (7.78-8.87)	16/16	8.75 (8.29-9.12)	.088
Clostridium leptum group	30/34	8.40 (8.04-8.78)	14/16	8.35 (7.37-8.66)	.313
Bacteroides	34/34	6.88 (6.21-7.23)	16/16	6.20 (6.00-6.66)	.035
Enterobacteriaceae	34/34	6.37 (6.10-6.76)	16/16	7.23 (6.65-7.90)	.001
E. coli	34/34	5.17 (4.68-5.70)	16/16	6.20 (5.50-7.14)	.005
Staphylococcus	8/34	4.40 (3.94-4.74)	9/16	5.78 (4.83-6.37)	.006
Akkermansia muciniphila	34/34	8.35 (7.56-9.00)	16/16	8.50 (7.10-9.45)	.763

[a] Prevalence (Pr) reflects the number of positive amplifications from total samples analysed by PCR (n=number of samples analysed)
[b] Data are shown as medians and interquartile range (IQR) of cell equivalents (genome equivalent) per gram of faeces.
* Statistical differences were calculated by using Mann-Whitney *U* test. Significantly difference between groups was considered at $P < .050$.

Table 5. Bacterial numbers in faecal samples analyzed by qPCR according to recommend weight gain over pregnancy.

Microbial groups	Normal weight gain (n=36)		Excessive weight gain (n=14)		Mann-Whitney test* *P* value
	Pr[a]	Log genome equivalent/g [b]	Pr[a]	Log genome equivalent/g [b]	
Total cell counts	36/36	9.90 (9.51-10.25)	14/14	9.73 (9.18-10.00)	.218
Bifidobacterium	36/36	8.92 (8.27-9.44)	14/14	8.46 (8.13-8.22)	.078
Lactobacillus group	36/36	7.48 (7.39-7.64)	14/14	7.56 (7.35-7.76)	.449
Clostridium coccoides group	36/36	8.71 (8.07-8.97)	14/14	8.35 (8.15-8.67)	.315
Clostridium leptum group	32/36	8.42 (8.16-8.78)	12/14	8.17 (7.20-8.68)	.268
Bacteroides	36/36	6.42 (6.06-7.03)	14/14	6.64 (6.20-7.36)	.331
Enterobacteriaceae	36/36	6.55 (6.21-6.86)	14/14	6.84 (6.16-8.04)	.142
E. coli	36/36	5.26 (4.70-5.94)	14/14	6.25 (5.06-8.08)	.045
Staphylococcus	10/36	4.50 (4.33-5.74)	7/14	4.46 (4.08-5.62)	.527
Akkermansia muciniphila	36/36	8.54 (7.90-9.50)	14/14	8.12 (6.52-8.50)	.020

[a] Prevalence (Pr) reflects the number of positive amplifications from total samples analysed by PCR (n=number of samples analysed)
[b] Data are shown as medians and interquartile range (IQR) of cell equivalents (genome equivalents) per gram of faeces.
*Statistical differences were calculated by using Mann-Whitney *U* test. Significantly difference between groups was considered at $P < .050$.
Normal weight gains over pregnancy according to IOM were < 16.0 Kg (BMI<25) and < 11.5 Kg (BMI>25)

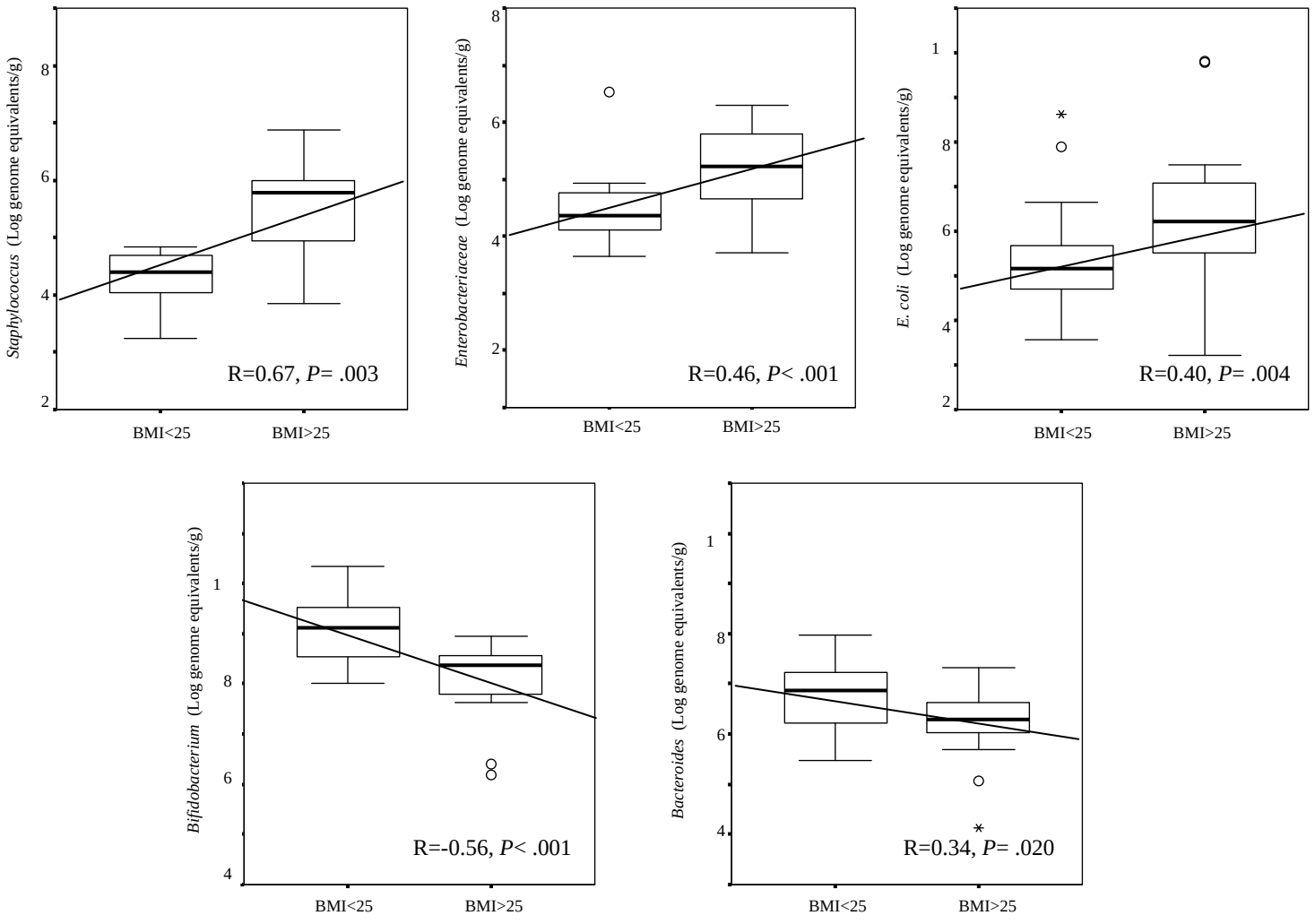
Staphylococcus (Log genome equivalents/g)
R=0.67, P= .003
BMI<25
BMI>25
Enterobacteriaceae (Log genome equivalents/g)
R=0.46, P< .001
BMI<25
BMI>25
E. coli (Log genome equivalents/g)
R=0.40, P= .004
BMI<25
BMI>25
Bifidobacterium (Log genome equivalents/g)
R=-0.56, P< .001
BMI<25
BMI>25
Bacteroides (Log genome equivalents/g)
R=0.34, P= .020
BMI<25
BMI>25

Figure 1.

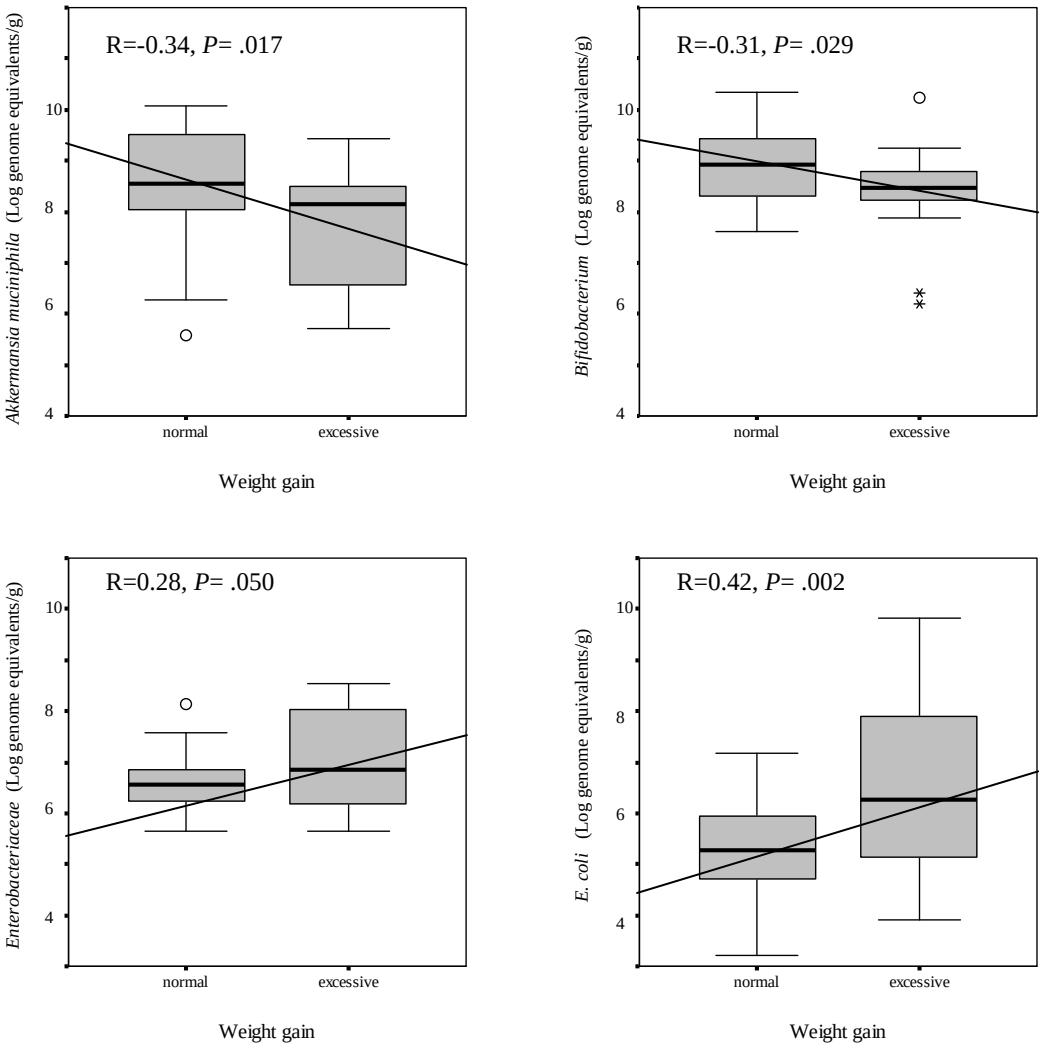
R=-0.34, P= .017
Akkermansia muciniphila (Log genome equivalents/g)
10
8
6
4
normal
excessive
Weight gain
R=-0.31, P= .029
Bifidobacterium (Log genome equivalents/g)
10
8
6
4
normal
excessive
Weight gain
R=0.28, P= .050
Enterobacteriaceae (Log genome equivalents/g)
10
8
6
4
normal
excessive
Weight gain
R=0.42, P= .002
E. coli (Log genome equivalents/g)
10
8
6
4
normal
excessive
Weight gain

Figure 2.