Dietary modulation of clostridial cluster XIVa gut bacteria (*Roseburia* spp.) by chitin–glucan fiber improves host metabolic alterations induced by high-fat diet in mice ☆

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Abstract

Recent studies have provided new evidence that alterations in the composition of the gut microbiota — known as dysbiosis — participate in the development of obesity. The aim of the present study was to investigate the ability of chitin–glucan (CG) from a fungal source to modulate both the gut microbiota and glucose and lipid metabolism in high-fat (HF) diet-induced obese mice. Supplementation of the HF diet with fungal CG (10% w/w) induced caecal enlargement with prominent changes in gut microbiota: it restored the number of bacteria from clostridial cluster XIVa including *Roseburia* spp., which were decreased due to HF feeding. Furthermore, CG treatment significantly decreased HF-induced body weight gain, fat mass development, fasting hyperglycemia, glucose intolerance, hepatic triglyceride accumulation and hypercholesterolemia, independently of the caloric intake. All those parameters were negatively correlated with specific bacteria of clostridial cluster XIVa, i.e., *Roseburia* spp. (Pearson's correlations analysis). In contrast to prebiotics that more specifically target the bifidobacteria species, CG effects on obesity appear to be independent of the incretin glucagon-like peptide 1 (GLP-1) production, since portal GLP-1 and proglucagon (its precursor) expression were not modified by the dietary intervention. In conclusion, our findings support the view that chronic consumption of CG has potential beneficial effects with respect to the development of obesity and associated metabolic diabetes and hepatic steatosis, through a mechanism related to the restoration of the composition and/or the activity of gut bacteria, namely, bacteria from clostridial cluster XIVa.
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1. Introduction

Obesity is typically associated with a cluster of several metabolic disorders such as type 2 diabetes and insulin resistance. In the liver, insulin resistance is reflected by impaired suppression of glucose production — which largely accounts for fasting hyperglycemia and glucose intolerance — as well as an increased lipid synthesis and storage [1]. These events link obesity and metabolic syndrome to hepatic steatosis or non-alcoholic fatty-liver disease [1-3].

The literature provides new evidence that gut microbiota dysbiosis — meaning changes in the composition and/or the activity of gut bacteria (at the phyla, genus or species level) — affects host metabolism and energy storage [4]. Several mechanisms are proposed which link events occurring in the colon with the regulation of energy metabolism, such as energy harvest from the diet, the synthesis of gut peptides involved in energy homeostasis (i.e., GLP-1) and the regulation of lipid storage [5]. We have previously reported that high-fat (HF) feeding was associated with changes in gut microbiota in mice, leading among others to a drop in bifidobacteria counts [6,7]. Another study demonstrated that diet-induced obesity was linked to changes in the gut microbial ecology, resulting in an increased capacity of the distal gut microbiota to promote host adiposity [8]. Furthermore, our recent studies have demonstrated that fructans obtained from chicory root inulin, a non-digestible carbohydrate which selectively increases bifidobacteria numbers in the caeco-colon (therefore called "prebiotic"), may be promising nutrients in the control of metabolic alterations associated with obesity, including steatosis and dyslipidemia [6,7,9-12]. Indeed, our results demonstrate that the selective increase of bifidobacteria in the gut microbiota upon fructan supplementation improves HF diet-induced

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diabetes in mice. An additional mechanism involves the promotion of proglucagon expression and GLP-1 production in the proximal colon, thereby allowing this hormone to play its functional role on glucose homeostasis [7,13,14]. In addition, fructans are also able to improve gut barrier functioning during obesity and diabetes through a mechanism which involves glucagon GLP-2, another proglucagon-derived peptide [9]. Taken together, these data suggest that non-digestible carbohydrates, when selectively fermented by gut microbes, exert a beneficial effect on obesity and associated metabolic disturbances.

The cell wall of fungi is composed of a polysaccharide-based three-dimensional network in which the central core contains branched chitin–β-1,3 glucan [15]. We have previously shown that a deacety-lated form of chitin — i.e., chitosan — extracted from the fungal exoskeleton (*Agaricus bisporus*) counteracts some metabolic altera-tions occurring in diet-induced obese mice, such as increased fat mass, plasma adipokine levels and ectopic fat deposition in the liver [16]. Furthermore, fungal chitosan shares characteristics with dietary fibre, since (i) it modulates bacterial population isolated from the human colon; (ii) it induces caecal enlargement in mice; and (iii) it increases fecal short-chain fatty acid concentrations [16-18]. The experimental material used in the present study was derived from the cell walls of the mycelium of *Aspergillus niger*, in which chitin and β glucan are combined. Although only a few reports on metabolic effects of such chitin–glucan (CG) formulations exist, the presence of the two biologically active polysaccharides support the potential of enhanced biological activity of the complex. Therefore, the aim of the present study was to investigate the ability of fungal CG to modulate (i) the gut microbial community and (ii) glucose and lipid metabolism in mice with HF diet-induced obesity.

2. Materials and methods

2.1. Animals and diet

Twenty-four male C57bl6/J mice (9 weeks old at the beginning of the experiment; Charles River Laboratories, France) were housed in groups of four per cage in a controlled environment (12-h daylight cycle, lights off at 6 p.m.) with free access to food and water. After 1 week of acclimatisation, the mice were divided into three groups (n=8/group): a control group (CT) fed with a control diet (AO4, SAFE, Villemoison-sur-Orge, France), a group fed with a HF diet and a group fed the same HF diet, supplemented with CG [90% HF (w/w)+10% KiOnutrime-CG from KitoZyme, Belgium; HF-CG group]. The HF diet (D12492, Research Diets) contained 35% fat — soybean oil and lard (g/100 g of total diet) — 9% sucrose, 16% maltodextrin, 26% protein and 6.5% cellulose. The energy content of the HF diet was 60% of fat, 20% carbohydrate and 20% protein. CG used in the study was derived from the cell walls of the mycelium of *A. niger*, in which two types of polysaccharide chains, i.e., chitin (poly *N*-acetyl-D-glucosamine) and β(1,3)-D-glucan, are associated. The CG ratio (w/w) was 35:65 as determined by ^{13}C-solid state NMR (data not shown). Food intake was recorded taking into account spillage twice a week during 4 weeks. The feed efficiency was calculated as the weight gain divided by calories consumed during the whole treatment. After 4 weeks and a 6-h period of fasting, mice were anaesthetised (ketamine/xylazine intraperitoneally, 100 and 10 mg/kg, respectively) and blood samples were harvested for further analysis. Liver, adipose tissues and intestinal tissues (jejunum, ileon, proximal colon and caecum) were carefully dissected and immersed in liquid nitrogen before storage at −80°C. The animal experiments were approved by the local ethics committee and housing conditions were as specified by the Belgian Law of 14 November 1993 on the protection of laboratory animals (agreement no. LA 1230314).

2.2. Oral glucose tolerance test

After 3 weeks of treatment, an oral glucose tolerance test (OGTT) was performed on 6-h fasted mice. Glucose was administered orally (3 g/kg glucose, 66% glucose solution) and blood glucose levels were determined using a glucose meter (Roche Diagnostics) on 3.5 μl of blood collected from the tip of the tail vein both before (−30 min and 0 min) and after glucose administration (15, 30, 60, 90 and 120 min). Twenty microliters of blood was sampled 30 min before and 15 min after the glucose load to assess plasma insulin concentrations. The insulino-resistance index (HOMA-IR) was calculated by multiplying fasting glycemia (mM) and fasting insulinemia (pM) divided by 22.5 [19].

2.3. Microbial analysis of the caecal contents

After killing of the animals, the total caecum content was collected and weighed before storage at −80°C. For analysis of the microbial content, metagenomic DNA was extracted from the caecal content of all mice, using the QIAamp DNA stool mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Denaturing gradient gel electrophoresis (DGGE) on total bacteria, bifidobacteria and lactobacilli was performed to study the qualitative effect of the treatment on the structure and composition of the intestinal microbial community [20]. DGGE with a 45–60% denaturant gradient was used to separate the polymerase chain reaction (PCR) products obtained with a nested approach for the 16S rRNA genes of bifidobacteria (primers BIF164f-BIF662r) and lactobacilli (SGLAB0158f-SGLAB0667). The first PCR round was followed by a second amplification with primers 338F-GC and 518R. The latter primers were also used to amplify the 16S rDNA of all bacteria on total extracted DNA. The DGGE patterns obtained were subsequently analyzed using the Bionumerics software version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). In brief, the calculation of the similarities was based on the Pearson (product moment) correlation coefficient. Clustering analysis was performed using the unweighted pair group method with arithmetic mean clustering algorithm (UPGMA) to calculate the dendrograms of each DGGE gel and a combination of all gels. The latter was performed on a created composite dataset. The composite dataset of the three DGGE patterns was also used to perform principal component analysis (PCA). PCA ordinations were calculated using the Pearson product-moment correlation coefficient. Within each character set, this coefficient subtracts each character from the average value and divides it by the variance of the character set.

Quantitative PCR (qPCR) was performed to study the quantitative effect of the treatment on the composition of the intestinal microbial community. The qPCR for total bacteria (using primers PRBA338f and P518r) and in particular for bifidobacteria, lactobacilli or the *Eubacterium rectal–Clostridium coccoides* cluster was performed as reported by Possemiers et al. [21]. The qPCR for *Roseburia* spp. was performed as described before [22], using the primers Ros-F1 and Ros-R1, and the Power SYBR Green PCR Master kit (Applied Biosystems, Foster City, CA, USA). The qPCR for *Bacteroides-Prevotella* spp. was performed as described by Rinttilä et al. [23], using the qPCR Core kit for SYBR Green I (Eurogentec, Seraing, Belgium) and primers Bacter140f and Bacter140r. All qPCR was performed with an ABI PRISM SDS 7000 Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d Ijssel, the Netherlands).

2.4. Expression of selected genes in tissues

Total RNA was isolated using the TriPure isolation reagent kit (Roche Diagnostics Belgium, Vilvoorde, Belgium). cDNA was prepared by reverse transcription of 1 μg total RNA using the kit Reverse Transcription System (Promega, Leiden, The Netherlands). Real-time PCRs were performed with the StepOnePlus real-time PCR system and software (Applied Biosystems, Den Ijssel, The Netherlands) using Mesa Fast qPCR (Eurogentec) for detection according to the manufacturer's instructions. RPL19 RNA was chosen as the housekeeping gene. Primer sequences for the targeted mouse genes are available on request (audrey.neyrinck@uclouvain.be). All samples were run in duplicate in a single 96-well reaction plate and data were analysed according to the $2^{-\Delta C_T}$ method [6]. The identity and purity of the amplified product were checked through analysis of the melting curve carried out at the end of amplification.

2.5. Blood parameters

Plasma insulin concentrations were determined using an ELISA kit (Mercodia, Uppsala, Sweden) on 5 μl of plasma collected from tail blood during OGTT. Upon killing of the animals, portal vein blood samples were collected in EDTA tubes (Sarstedt, Nümbrecht, Germany) containing dipeptidyl peptidase IV inhibitor (Linco Research, St. Charles, MO, USA) and plasma was stored at −80°C. GLP-1 (7–36) amide concentrations were measured using an ELISA kit (GLP-1 active ELISA kit; Linco Research). Plasma triglycerides, cholesterol and non-esterified fatty acid concentra-tions were measured using kits coupling enzymatic reaction and spectrophotometric detection of reaction end products (Diasys Diagnostic and Systems, Holzheim, Germany). High-density lipoprotein cholesterol (HDL-cholesterol) concentration was measured enzymatically after very low density lipoprotein, chylomicrons and low-density lipoprotein cholesterol (LDL-cholesterol) antibody precipitation (Diasys Diagnostic and Systems). LDL was estimated by the Friedwald formula [24].

2.6. Lipid analysis in the liver

Triglycerides and cholesterol were measured in the liver tissue after extraction with chloroform–methanol according to Folch et al. [25]. Briefly, 100 mg of liver tissue was homogenized in 1 ml of phosphate buffer (pH 7.4). Lipids were extracted by mixing 125 μl of the homogenate with 1 ml of chloroform/methanol (2:1). The chloroform phase was evaporated under nitrogen flux, and the dried residue was solubilized in 100 μl of isopropanol. Triglycerides and total cholesterol were measured as previously described for plasma samples. Free and esterified cholesterol were also determined using a kit coupling enzymatic reaction and spectrophotometric detection of reaction end products (Diasys Diagnostic and Systems). Protein concentrations were measured by the Bradford method using bovine serum albumin as standard [26].

2.7. Fat histochemical detection

A fraction of the main liver lobe was fixed frozen in Tissue-tek in liquid nitrogen–cold isopentane. For the detection of neutral lipids, frozen sections were sliced and stained for 10 min at 60°C with oil red O, using 0.5% oil red O dissolved in propylene glycol. The sliced sections were then counterstained.

2.8. Statistical analysis

Results are presented as mean±S.E.M. Statistical analysis was performed by ANOVA followed by *post hoc* Tukey's multiple comparison test (GraphPad Software, San Diego, CA, USA). Correlations between parameters were assessed by Pearson's correlation test. $P < .05$ was considered as statistically significant.

3. Results

3.1. Supplementation of the HF diet with CG induces caecal enlargement with changes in the gut microbiota

HF feeding induced important changes in the microbial community structure, as shown in Fig. 1. Clustering of the DGGE fingerprints for total bacteria indicated a separate cluster of the HF fed mice, and PCA analysis of combined DGGE fingerprints of total bacteria, bifidobacteria and lactobacilli led to a distinct clustering of the fingerprint profiles for the HF group as compared to the CT mice. This was further confirmed by the changes in quantitative numbers of different bacterial groups due to the HF administration (Fig. 2). HF diet decreased caecum volume (Fig. 2G,H) and induced a drop in the number of all analyzed bacterial groups, except bifidobacteria (Fig. 2 and Supplemental Fig. 1).

CG supplementation to the HF diet induced caecal enlargement together with a profound shift in the microbial community in comparison with the HF group, as shown by the distinct clustering in the DGGE profile and PCA analysis (Fig. 1). Analysis of the quantitative values of the analyzed microbial groups (Fig. 2) showed that CG supplementation modestly increased the quantities of *Bacteroides-Prevotella* spp., whereas the *Clostridium coccoides–Eubacter-*

ium rectale cluster group and *Roseburia* spp. were completely restored after CG treatment. Bifidobacteria in the HF-CG fed mice were higher than in the HF fed mice or control mice only when considering their caecal pool (Supplemental Fig. 1) and not their quantity per gram of caecal content (Fig. 2).

3.2. Supplementation of the HF diet with CG decreases body weight gain and fat mass development

The total caloric intake was obtained by multiplying total food intake (g) for four mice per cage by the caloric value of the diets, i.e., 3.10, 5.24 and 4.72 kcal/g for the CT, HF and HF-CG diets, respectively; the total energy intake for four mice was then 1250±31, 1579±30 and 1627±160 kcal for the CT, HF and HF+CG groups, respectively. Moreover, it is important to note that the total fat consumption during the 4-week period of treatment was not affected by the supplementation of the HF diet with CG (12.1±0.3, 105.2±2.0 and 108.4±10.6 g total fat for the CT, HF and HF+CG groups, respectively). The HF diet significantly increased body weight gain in combination with a higher development of epididymal, visceral and subcutaneous fat, as compared to the CT group (Fig. 3), whereas CG decreased body weight gain by about 28% as compared to HF (Fig. 3B). This effect was accompanied by lower fat mass development (Fig. 3D–F). The feed efficiency (weight gain divided by calories consumed during the whole treatment) was significantly lower in the HF-CG-treated mice (Fig. 3C).

3.3. Supplementation with CG improves glycemia and glucose tolerance upon HF feeding

After 3 weeks of dietary treatment, HF diet induced glucose homeostasis disorders, as evidenced by fasting hyperglycemia, fasting hyperinsulinemia, a higher glucose-induced insulin secretion following OGTT and a higher index of insulino-resistance (HOMA-IR), as compared to mice fed normal chow diet (Fig. 4). Most of these
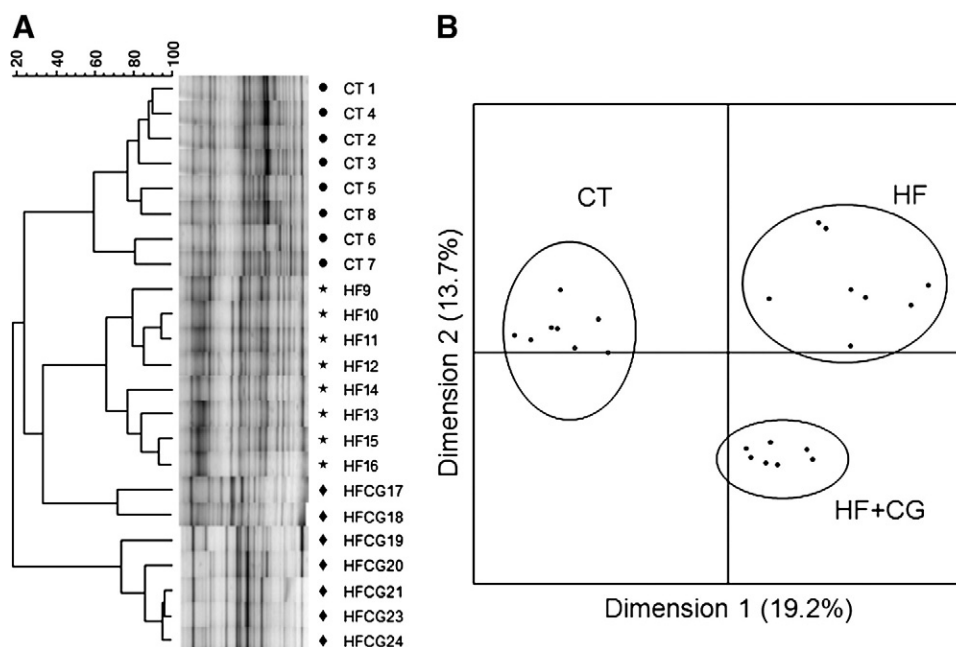

Fig. 1. Denaturing gradient gel electrophoresis (DGGE) fingerprint patterns of the caecal microbial community of mice fed a standard (CT), high fat (HF) or HF diet supplemented with 10% fungal CG (HF-CG) for 4 weeks (A). The DGGE profiles were constructed using primers for total bacteria. Principal component analysis (PCA) was used to explore the similarity within a composite dataset consisting of DGGE fingerprints of total bacteria, bifidobacteria, and lactobacilli (B).

parameters were improved in HF-CG-treated mice (Fig. 4A, D and E), except for insulinemia, HOMA-IR and insulin response after the glucose load.

3.4. Supplementation with CG changes the liver and plasma lipid profiles and prevents HF-induced hepatic steatosis

The HF diet induced triglyceride accumulation (by about 28%) in the liver as shown in Table 1 and Fig. 5. Fat staining of the tissue showed that lipid accumulation in the liver due to the HF diet was reduced by the CG treatment (Fig. 5). Indeed, triglyceride content was lower in the liver of HF-CG mice, as compared to the HF group (Table 1). The expression of two genes in the liver that regulate lipid metabolism was examined (Table 1): the sterol regulatory element-binding protein-1c (SREBP-1c) — emerging as a major mediator of insulin action on glycolytic and lipogenic gene expression — and fatty acid synthase (FAS). CG did not significantly counteract HF-induced SREBP1c and FAS expression. Concerning the serum lipid profile, no modification in triglyceridemia or non-esterified fatty acids was observed (Table 1). The HF diet induced significant increases in total, LDL- and HDL-cholesterol. CG supplementation decreased plasma cholesterol (total cholesterol and HDL-cholesterol) concentration, without significant modification of the liver cholesterol

content, whatever the form of cholesterol (esterified- and free cholesterol) (Table 1).

3.5. Supplementation with CG does not change portal GLP-1 and proglucagon expression in the gut

The HF diet alone, or supplemented with CG, did not modify the portal levels of GLP-1 (7.48 ± 0.71, 5.15 ± 0.75 and 5.60 ± 0.54 pM for the CT, HF and HF-CG groups, respectively; $P > .05$ ANOVA). Furthermore, although proglucagon mRNA content increased in different intestinal segments due to HF treatment, its expression was unchanged by the addition of CG in the HF diet vs. the HF group, whatever the part of the gut (jejunum, ileon and proximal colon) (Table 1).

3.6. Improvement of HF-induced metabolic alterations due to CG supplementation is correlated to higher number of gram-positive bacteria from the clostridial cluster XIVa

In the present study, only *Roseburia* spp. and the *Clostridium coccoides–Eubacterium rectale* cluster were decreased through HF feeding and were restored by CG supplementation. Therefore, we performed correlation analysis taking into account those bacteria and
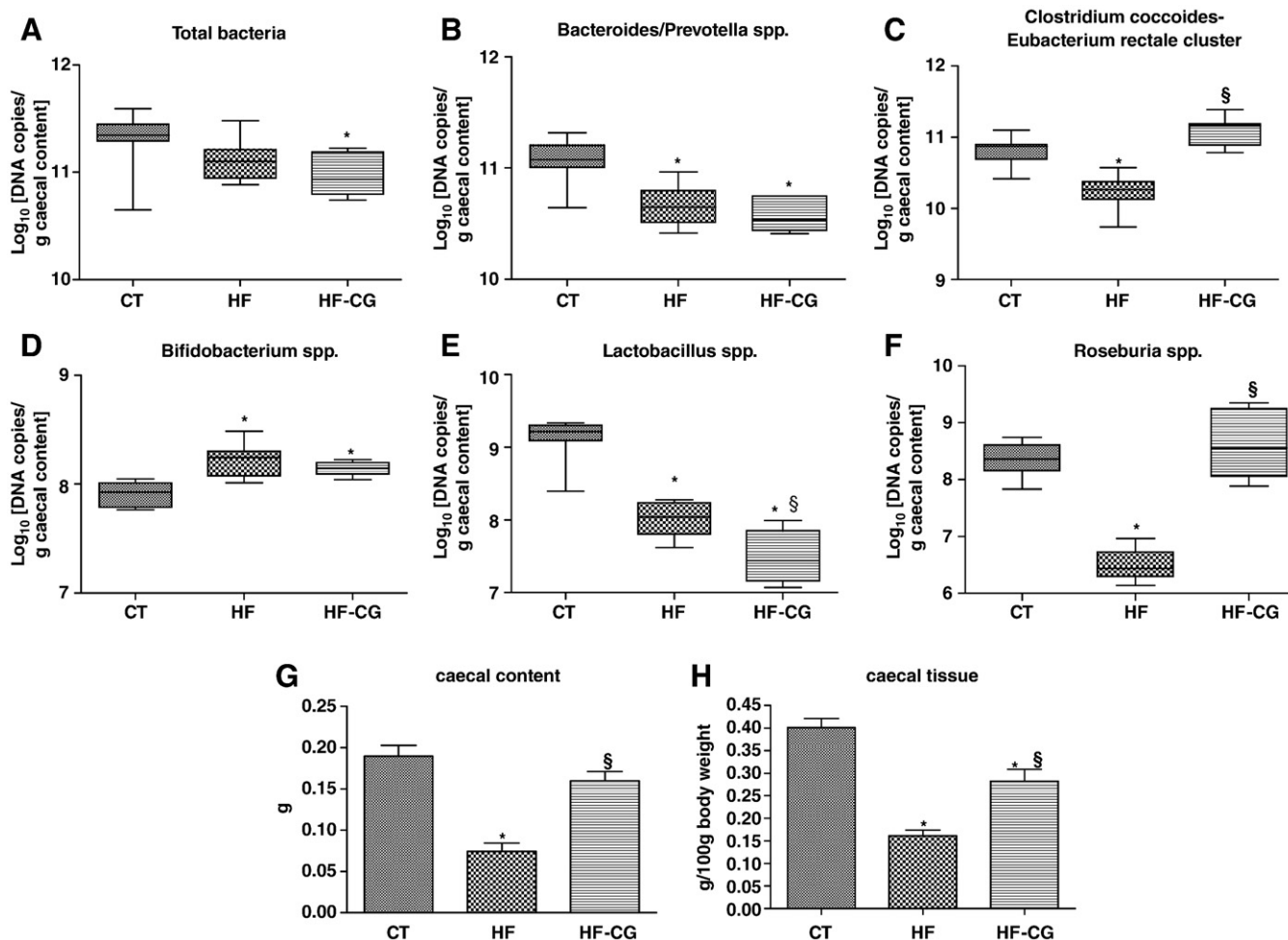

Fig. 2. Caecal bacterial content of total bacteria (A), *Bacteroides-Prevotella* spp. (B), the *Eubacterium rectale–Clostridium coccoides* group (C), *Bifidobacterium* spp. (D), *Lactobacillus* spp. (E), *Roseburia* spp. (F). Caecal content (G) and caecal tissue (H) weight are also presented. Bacterial quantities are expressed as log10 (bacterial cells/gram of caecal content wet weight). Mice were fed a CT, HF or HF diet supplemented with 10% fungal CG (HF-CG) for 4 weeks. *$P < .05$ vs. CT and §$P < .05$ vs. HF (ANOVA).
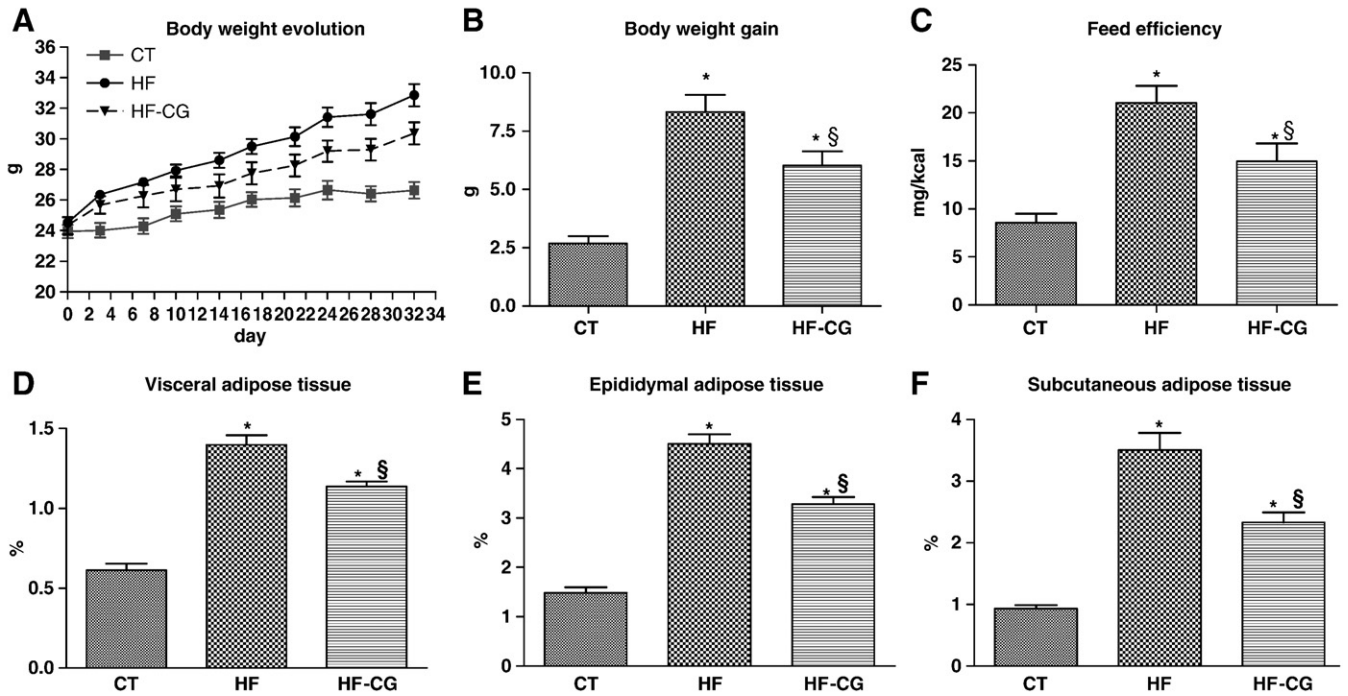
Fig. 3. Body weight evolution (A), body weight gain (B), feed efficiency (C) and visceral (D) epididymal (E) and subcutaneous (F) adipose tissue weight of mice fed a CT, HF or HF diet supplemented with 10% fungal CG (HF-CG) for 4 weeks. *$P<.05$ vs. CT and §$P<.05$ vs. HF (ANOVA).

related their level with the metabolic parameters which were improved through CG supplementation. Significant negative correlations were observed between *Roseburia* spp. and all parameters that were both altered by HF diet and improved by CG (Fig. 6). Interestingly, we observed two distinct clusters: cluster for HF-treated mice and a cluster for both CT- and HF-CG mice, whatever the parameters correlated with *Roseburia* spp. Similar results were obtained with the *Clostridium coccoides–Eubacterium rectale* cluster (data not shown).

4. Discussion

Recently, it has been proposed that alterations in the composition of the gut microbiota — known as dysbiosis — participate in the
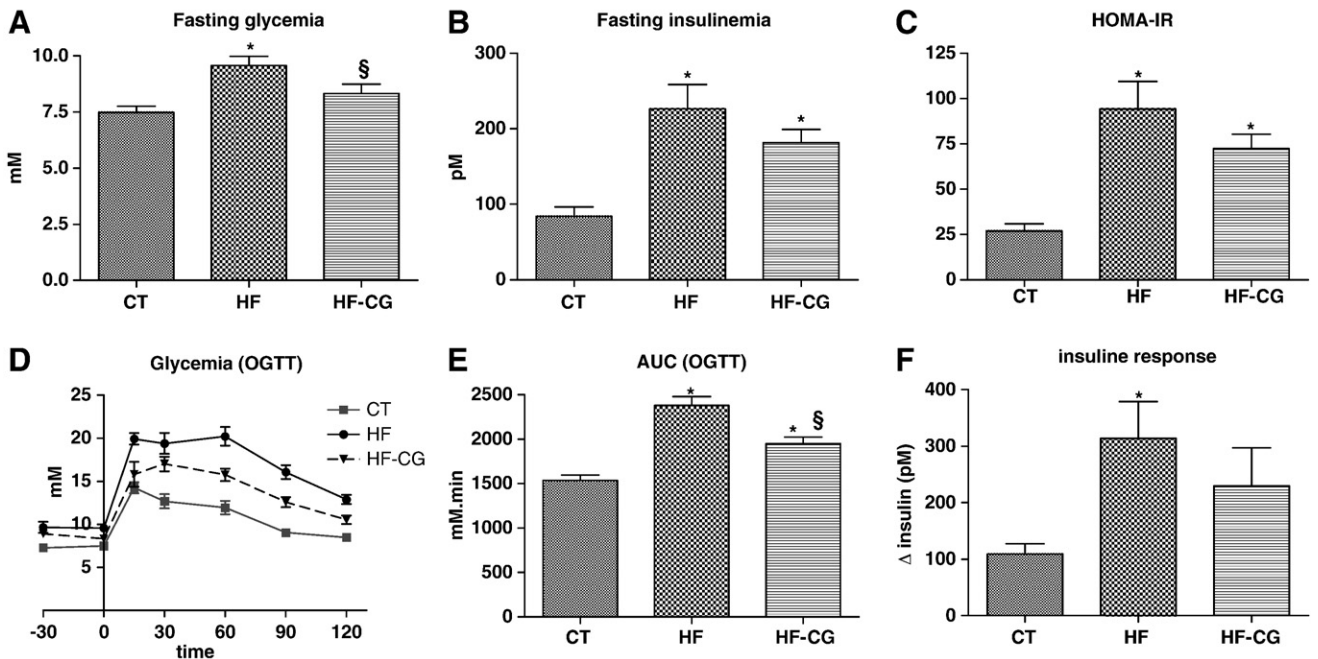

Fig. 4. Fasting glycemia (A), fasting insulinemia (B), insulino-resistance index (HOMA-IR) (C), plasma glucose levels after the oral glucose load (D), area under the curve (AUC) of the glucose excursion (E) and insulin response (F) after the oral glucose load (Δ insulin concentrations 30 min before and 15 min after the oral glucose load) in mice fed a CT, HF or HF diet supplemented with 10% fungal CG (HF-CG) for 3 weeks. *$P<.05$ vs. CT and §$P<.05$ vs. HF (ANOVA).

Table 1
Serum lipids, liver lipids and gene expression of mice fed a CT, HF or a HF diet supplemented with 10% fungal CG (HF-CG) for 4 weeks

	CT	HF	HF-CG
Serum lipids, mM			
Triglycerides	1.47±0.11	1.71±0.13	1.68±0.11
Non-esterified fatty acids	0.27±0.03	0.31±0.03	0.28±0.02
Total cholesterol	1.75±0.06	3.00±0.03 [*]	2.67±0.12 [*,§]
LDL-Cholesterol	0.31±0.05	1.22±0.08 [*]	1.02±0.13 [*]
HDL-Cholesterol	0.78±0.02	1.00±0.03 [*]	0.89±0.03 [*,§]
Liver lipid content, nmol/mg protein			
Triglycerides	60.9±3.04	77.8±5.26 [*]	61.1±3.42 [§]
Total cholesterol	41.1±4.05	74.2±11.0 [*]	74.4±6.84 [*]
Free cholesterol	27.0±2.16	40.3±4.62 [*]	34.7±2.13
Esterified cholesterol	14.1±3.21	33.9±6.59 [*]	39.7±4.97 [*]
Hepatic gene expression			
SREBP1c mRNA	1.00±0.10	2.14±0.25 [*]	1.73±0.16
FAS mRNA	1.00±0.07	1.34±0.13	1.47±0.10
Intestinal proglucagon mRNA			
Jejunum	1.00±0.11	1.29±0.11	1.22±0.15
Ileon	1.00±0.04	1.80±0.27 [*]	1.83±0.18 [*]
Proximal colon	1.00±0.06	1.47±0.15 [*]	1.30±0.14

[*] $P<.05$ vs. CT (ANOVA).

[§] $P<.05$ vs. HF (ANOVA).

development of obesity [4]. In our previous studies, we demonstrated that the development of obesity and type 2 diabetes following a HF diet feeding is characterized by specific changes of the bacterial populations, which are predominant in the gut microbiota. Indeed, we found that diet-induced obesity markedly reduced the number of *Bifidobacterium* spp., *Lactobacillus* spp., Bacteroides-related bacteria and the *Eubacterium rectale–Clostridium coccoides* cluster in the caecal content of mice [7,27]. In the present study, we have confirmed that HF feeding decreased the dominant members of the mouse intestinal microbiota — *Bacteroides-Prevotella* spp. and *Clostridium coccoides–Eubacterium rectale* cluster — together with the specific populations of Lactobacilli and *Roseburia* spp. However, we did not observe any drop in bifidobacteria and even a higher number of this specific bacterial group was detected when the results were expressed per gram of caecal content. The discrepancy between this last observation and our previous studies could be the result of dietary composition of the HF diet (HF-free carbohydrate vs. HF–high-carbohydrate diet). In the present study, we have demonstrated for the first time that fungal CG modulates bacterial populations in the caecal content, as characterised by a marked increase in gram-positive bacteria from clostridial cluster XIVa including *Roseburia* spp. Both *in vitro* analysis of bacterial growth and short-chain fatty acid production in the presence of CG and *in vivo* mechanistic study of bacterial enzyme involved in CG breakdown would constitute interesting perspectives in order to elucidate the mechanisms involved in the prebiotic effect of CG. Among them, we can cite, e.g., increased/decreased transit time as a result of fiber intake, or direct bactericidal effects, which have been observed for the related oligomers, chitosan [28]. To date, only one study has been published having the aim to investigate whether consumption of CG from *A. niger* had potential beneficial effects in a HF model (a diet-induced atherosclerosis hamster model) [29]. In that study, it was reported that CG did not affect plasma cholesterol but lowered triglycerides after 12 weeks of treatment. Importantly, CG resulted in the almost complete prevention in the development of fatty aortic streaks, via a mechanism linked to an improvement of the antioxidant status. The authors indicated that further investigations are needed to define the exact mechanisms by which CG provided protection. In the present study, in parallel to changes in gut bacterial population, we demonstrated the beneficial effects of CG on several metabolic parameters which are classically altered by a HF diet. CG significantly decreased HF-induced body weight gain, fat mass development, fasting hyperglycemia, glucose intolerance, hepatic

triglyceride accumulation and hypercholesterolemia, independently of the caloric intake. Although chitosan — derived from chitin — has the capacity to bind fatty acids and to increase the lipid content in the caecum [16,30], this mechanism does not seem to be involved in the improvement of HF-induced metabolic alterations due to CG. Indeed,
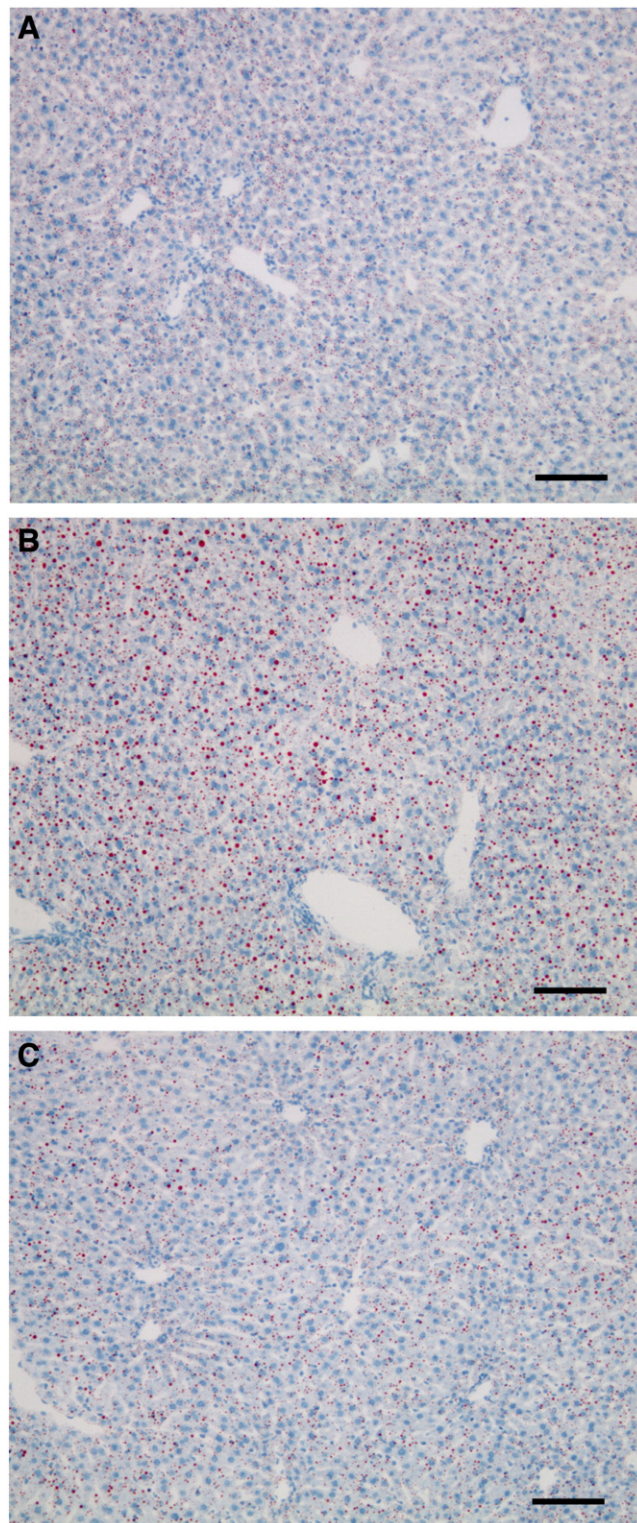

Fig. 5. Histological analysis of liver cryostat sections stained with oil red O for lipid detection of mice fed a CT (A), HF (B) or HF diet supplemented with 10% fungal CG (C) for 4 weeks (bar=100 μm).
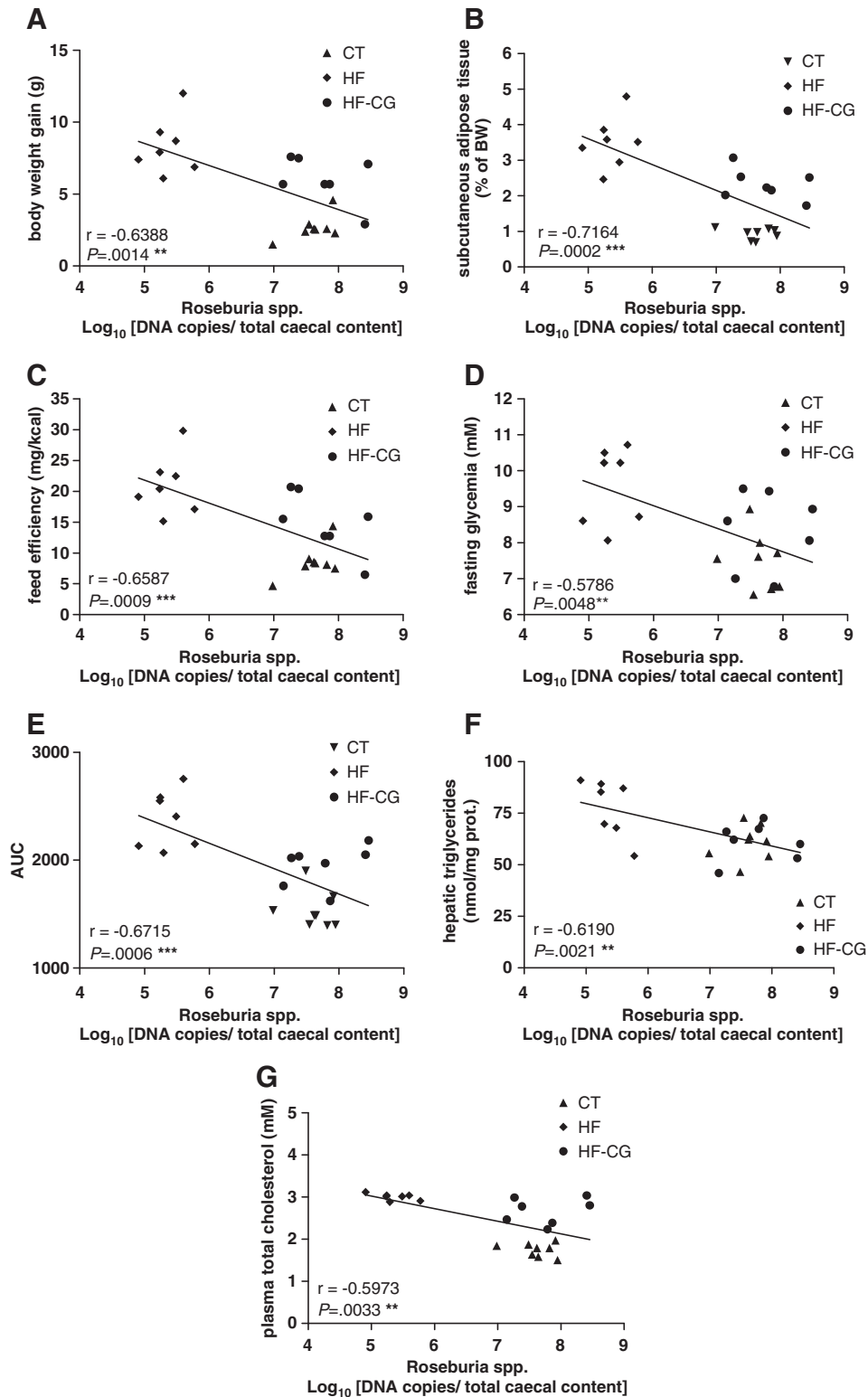
Fig. 6. Correlation between the number of *Roseburia* spp. in the caecal content (expressed as log10 − bacterial cells/total caecal content wet weight) and body weight gain (A), subcutaneous adipose tissue weight (B), feed efficiency (C), fasting glycemia (D), area under the curve (AUC) of the glucose excursion after the oral glucose load (E), hepatic triglycerides (F) and plasma cholesterol (G). Mice were fed a CT, HF or a HF diet supplemented with 10% fungal CG (HF-CG). Insert corresponds to Pearson's *r* correlation and the *P* value.

the proportion of lipid accumulated in the caecum vs. the lipid ingested during a 12-h feeding period was very modestly increased due to CG supplementation in the HF diet, whereas this ratio was increased by twofold with chitosan (Supplemental Fig. 2A). Further-

more, in this additional control experiment, CG supplementation had no effect on the fatty acid content in the caecum of mice fed a HF diet, whereas chitosan induced a huge increase of this parameter (Supplemental Fig. 2B).

We have previously shown that feeding mice with fructans (oligofructose) restored the number of intestinal bifidobacteria and reduced the impact of HF diet-induced metabolic disorders through endogenous production of the intestinotrophic peptide GLP-2 and the incretin GLP-1 [7,9,14]. In the present study, although CG increased the number of bifidobacteria in the caecum of mice, metabolic alterations could not be related to a drop in bifidobacteria. Furthermore, portal GLP-1 and intestinal precursor of GLP-1 and GLP-2 (proglucagon mRNA) were not modified due to dietary CG treatment, whatever the intestinal segments analyzed. Therefore, in contrast to oligofructose, the mechanism of CG seems to be independent of GLP-1 production and bifidobacteria changes.

In view of our correlation analysis, we postulate that CG affected host lipid metabolism through the modulation of gut microbiota. The gut microbiota may affect host lipid metabolism through several mechanisms since it is able (1) to regulate chylomicron formation and lipid uptake by affecting gut transit time and bile salt metabolism (modulation of the enterohepatic circulation of bile acids) [31,32]; (2) to ferment complex polysaccharides into short-chain fatty acids that may act either as a lipogenic substrates in the liver or as an inhibitor of cholesterologenesis and lipogenesis [33]; (3) to suppress expression of fasting-induced adipose factor in the intestinal mucosa, which increases lipoprotein lipase-dependent triglyceride storage in adipose tissue and reduces serum triglyceride level [31,34].

Importantly, multiple Pearson's correlations analysis indicated that body weight gain, fat mass development, fasting glycemia, glucose tolerance, feed efficiency, hepatic triglycerides and cholesterolemia were negatively correlated with specific bacteria of clostridial cluster XIVa, as shown by quantitative analysis of *Roseburia* spp. and the *Clostridium coccoides–Eubacterium rectale* cluster. It is known that the anaerobic microbial communities of the mammalian large intestine produce short-chain fatty acids — acetate, propionate and butyrate — as their main non-gaseous fermentation end products. In particular, clostridial cluster XIVa in general and *Roseburia* spp. in particular are important butyrate-producing bacteria. We thus asked the following question: Can we attribute the effect of CG to butyrate produced by the bacteria of clostridial cluster XIVa? A recent study demonstrated that dietary supplementation of butyrate can prevent and treat diet-induced insulin resistance in mouse [35]. However, butyrate is the preferred energy source for colonocytes and concentrations in blood are usually undetectable as a result of rapid utilisation [36]. Moreover, butyrate, together with the other short-chain fatty acids, is considered as a driver of energy sparing and is presented as a potential mechanism involved in the increase of fat mass in microbiota-bearing mice [36–38]. *Roseburia* spp. has another specific characteristic: its high capacity to form conjugated-linoleic acid (CLA) from linoleic acid [39]. Since CLA appears to exhibit a number of health benefits related to anti-obesitic and antidiabetogenic effects [40], we postulate that CLA producers such as *Roseburia* spp. could be an important actor of the interesting metabolic effects observed in mice fed with CG. Further experimental investigations are needed to distinguish between these potential mechanisms.

In conclusion, our findings support the view that chronic consumption of CG has potential beneficial effects with respect to the development of obesity and associated metabolic disorders such as diabetes and hepatic steatosis. The selective modulation of the gut microbiota due to the dietary treatment with CG is different from changes observed with fructans. CG has metabolic interest that could be dependent on specific changes in microbiota such as the increase in clostridial cluster XIVa bacteria. The possibility to treat animals with selected *Roseburia* as oral probiotics could constitute one interesting perspective to investigate further on the role of this specific gut bacteria in HF diet-induced obesity. Interestingly, the CG is unable to mimic some microbial changes occurring upon fructan administration (namely, the increase in bifidobacteria per gram of caecal content) and which are implicated in the production of gastrointestinal peptides (GLP-1, GLP-2) driving the metabolic effects. Therefore, we can conclude that all fermentable carbohydrates that differently modify the gut microbiota can exert interesting physiological effects, by various mechanisms.

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