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ORIGINAL ARTICLE

Gut microbiome composition is linked to whole grain-induced immunological improvements

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The involvement of the gut microbiota in metabolic disorders, and the ability of whole grains to affect both host metabolism and gut microbial ecology, suggest that some benefits of whole grains are mediated through their effects on the gut microbiome. Nutritional studies that assess the effect of whole grains on both the gut microbiome and human physiology are needed. We conducted a randomized cross-over trial with four-week treatments in which 28 healthy humans consumed a daily dose of 60 g of whole-grain barley (WGB), brown rice (BR), or an equal mixture of the two (BR + WGB), and characterized their impact on fecal microbial ecology and blood markers of inflammation, glucose and lipid metabolism. All treatments increased microbial diversity, the Firmicutes/Bacteroidetes ratio, and the abundance of the genus *Blautia* in fecal samples. The inclusion of WGB enriched the genera *Roseburia*, *Bifidobacterium* and *Dialister*, and the species *Eubacterium rectale*, *Roseburia faecis* and *Roseburia intestinalis*. Whole grains, and especially the BR + WGB treatment, reduced plasma interleukin-6 (IL-6) and peak postprandial glucose. Shifts in the abundance of *Eubacterium rectale* were associated with changes in the glucose and insulin postprandial response. Interestingly, subjects with greater improvements in IL-6 levels harbored significantly higher proportions of *Dialister* and lower abundance of Coriobacteriaceae. In conclusion, this study revealed that a short-term intake of whole grains induced compositional alterations of the gut microbiota that coincided with improvements in host physiological measures related to metabolic dysfunctions in humans.
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Introduction

Obesity is associated with an increased risk in cardiovascular disease, type-2 diabetes, non-alcoholic fatty-liver disease and some cancers, and constitutes a major health concern worldwide (Cornier *et al.*, 2008; Hu, 2011). A diet high in whole grains and dietary fibers has been shown to improve metabolic parameters related to these metabolic disorders (Liu *et al.*, 1999; Fung *et al.*, 2002; Liu *et al.*, 2003; Murtaugh *et al.*, 2003; Jensen *et al.*, 2004; Nettleton *et al.*, 2008). The mechanisms responsible for the benefits of whole grains are not completely understood. It has been proposed that the dietary fiber present in whole grains increases

the viscosity of the digesta and binds to bile acids in the small intestine, thus contributing to decreased sugar and lipid (cholesterol) absorption (Behall *et al.*, 2004; Alminger and Eklund-Jonsson, 2008). In addition, phytochemicals and other bioactive compounds in whole grains might provide metabolic benefits (Adom and Liu, 2002; Nilsson *et al.*, 2006; Harris and Kris-Etherton, 2010). Furthermore, the metabolic inflammation associated with obesity and related diseases is now considered to trigger metabolic dysfunctions (Gregor and Hotamisligil, 2011), and the benefits of whole grains might be due to an anti-inflammatory action (Nilsson *et al.*, 2008b; Rosén *et al.*, 2011). In this respect, bacterial fermentation of undigestible constituents of whole grains in the gastrointestinal tract has been suggested to be partly responsible for the benefits of whole grains (Nilsson *et al.*, 2008a; North *et al.*, 2009; Harris and Kris-Etherton, 2010).

A consideration of the gut microbiome in the context of the health effects of whole grains has

become especially relevant in light of recent research that indicated an etiological role of gut bacteria in metabolic disorders. Obesity and type 2 diabetes have been linked to alterations in the intestinal microbiota in both the humans and animal models (Ley et al., 2006; Turnbaugh et al., 2006; Cani et al., 2007; Larsen et al., 2010; Vijay-Kumar et al., 2010). If these aberrations contribute to human disease is still unclear, but pathophysiological indicators are reduced in animal models when animals are kept germ-free or when treated with antibiotics, and manifestations of disease can be transmitted through the gut microbiota (Ley et al., 2005; Cani et al., 2008; Vijay-Kumar et al., 2010; Henao-Mejia et al., 2012). Proposed mechanisms by which microbiota contribute to metabolic aberrations are the induction of lipolysis leading to increased fat storage (Bäckhed et al., 2007), hepatic de-novo synthesis of triglycerides (Bäckhed et al., 2004) and the alteration of bile acid metabolites with consequences to lipid metabolism in the host (Claus et al., 2011). Furthermore, the gut microbiome might exacerbate the systemic inflammation associated with obesity and related metabolic disorders (Hotamisligil, 2006; Ding et al., 2010), possibly through the induction of endotoxemia driven by lipopolysaccharide translocation through the intestinal epithelium (Cani et al., 2007; Amar et al., 2008; Cani et al., 2008; Li and Hotamisligil, 2010).

The interplay between the gut microbiota and host metabolism and the ability of whole grains to affect both of these aspects suggest that one mechanism by which whole grains confer their benefits might be through a modulation of the gut microbiome. Recent research has revealed that the composition and metabolism of the gut microbiota can be modulated through prebiotics and fiber (Flint et al., 2007; Louis et al., 2007), and these carbohydrates have been shown to improve metabolic markers in experimental models (Cani et al., 2007; Neyrinck et al., 2011). Despite these encouraging findings, human studies that investigate the effects of whole grains and cereal fibers on host metabolism have neglected, until now, to characterize the gut microbiome and explore its potential contribution to health improvements (Tilg and Kaser, 2011). In addition, although the effect of fiber on the gut microbiota has been recently studied in experimental animals (Neyrinck et al., 2011; Van den Abbeele et al., 2011), information on how whole grains impact human gut microbiome composition is lacking.

The aims of this study were to characterize the impact of the incorporation of whole grains to an otherwise unrestricted diet on gut microbial ecology in healthy human subjects, and to investigate whether a connection with metabolic and immunological improvements exists. For this purpose, we performed a human crossover study with three four-week whole grain treatments, and collected fecal and blood samples at baseline and at the end of each treatment. The effect of whole grains on fecal microbiota composition was characterized by pyrosequencing of 16S rRNA gene tags, and inflammatory and metabolic markers related to metabolic dysfunctions in humans were measured in blood samples. The molecular characterization of fecal microbiota in parallel to host phenotyping allowed an investigation of associations between diet-induced metabolic changes and shifts in the gut microbiome.

Materials and methods

Subjects
The human trial was approved by the Institutional Review Board of the Kansas State University (IRB Approval Number: 5298), and written informed consent was obtained from all subjects. Healthy participants (see Supplementary materials for inclusion/exclusion criteria) were recruited through leaflets distributed on-campus by the College of Human Nutrition at the Kansas State University, Manhattan, KS. Twenty-eight participants, 17 females and 11 males (age 25.9 ± 5.5 years), took part in the study.

Test meals
Whole grain Prowashonupana Barley (Sustagrain Barley Quick Flakes, ConAgra Mills, Omaha, NE, USA) and whole-grain brown rice (Insta Grains Brown Rice Flakes, Briess, Chilton, WI, USA) flakes were used in this study. Three test meals with different amounts of total dietary fiber were included: a barley treatment (WGB), consisting of 60 g of barley (18.7 g total dietary fiber); a brown rice and barley treatment (BR + WGB), consisting of 30 g each barley and BR (11.5 g total dietary fiber); and a BR treatment, consisting of 60 g of BR (4.4 g total dietary fiber). Subjects were provided with individual bags containing a daily dose of the corresponding treatment (60 g of flakes). Nutritional information of the whole-grain flakes used in the study is available in the Supplementary materials and Supplementary Table S1.

Study design
The study was conducted as a randomized crossover trial over 17 weeks (Figure 1). The first week served as a baseline period, after which each subject underwent three four-week dietary treatments (BR, BR + WGB, WGB) in random order, and interspaced
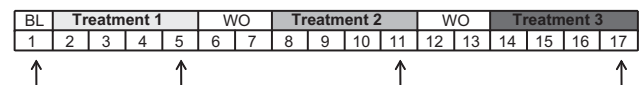

Figure 1 Experimental design. Time line of the randomized crossover trial. Three four-week dietary treatments were assessed in succession. The treatments were interspaced by two-week washout (WO) periods. Blood and stool samples (indicated by arrows) were collected during the baseline (BL) and at the end of each treatment period.

by two-week washout periods. The study was conducted under free-living conditions, and no dietary restrictions were imposed except that subjects were expected to be non-vegetarian. Subjects were instructed to consume the 60 g of flakes daily either plain, with yogurt or with milk, without time restrictions. Weekly symptom diaries were completed by the subjects in which they self-reported bowel movement, discomfort, flatulence, bloating, stool consistency and general well-being on a scale from 1 to 5 (1 being optimal/normal and 5 worst/abnormal).

Subject parameters and determination of metabolic and immunological markers

Subject parameters were measured at the Human Metabolism Laboratory at Kansas State University. Total body composition was assessed at baseline with dual-energy X-ray absorptiometry (Prodigy GE-Lunar, GE, Waukesha, WI, USA). Blood samples were drawn at baseline and at the end of each dietary treatment after a 12 h overnight fast. An initial blood sample was drawn (time 0). A standard drink containing 75 g of glucose (Fisher Scientific, Pittsburg, PA, USA) was consumed within 10 min, and blood samples were collected at 15, 30, 45, 60, 90 and 120 min for the determination of postprandial glucose and insulin responses. Blood was immediately placed in tubes containing K_2-EDTA (Vacutainer, BD, Franklin Lakes, NJ, USA) and centrifuged at 1000–1500$x g$ for 13 min at 5–10 °C. Aliquots of plasma were transferred into tubes for storage at -80 °C until further testing.

Glucose and insulin were measured in plasma samples in duplicate using an automated analyzer (YSI 2300, YSI Life Sciences, Yellow Springs, OH, USA) and the Human Gut Hormone Immunoassay kit (Milliplex, EMD Millipore, Billerica, MA, USA) with a dual laser flow cytometer (Luminex, EMD Millipore, Billerica, MA, USA), respectively. A lipid profile, consisting of total cholesterol, high-density lipoprotein (HDL) and non-HDL cholesterol was performed on the preprandial samples (time 0) using the Cholestech LDX System (Alere, Waltham, MA, USA). Three markers of inflammation were measured in plasma samples by enzyme-linked immunosorbent assays (in duplicate): lipopolysaccharide-binding protein (LBP) (USCN Life Science and Technology, Huston, TX, USA), high-sensitive C-reactive protein (hs-CRP) (Symansis, Timaru, New Zealand), and interleukin 6 (IL-6) (R&D Systems, Minneapolis, MN, USA).

Short-chain fatty acids were quantified in fecal samples by gas chromatography as described in the Supplementary materials.

Compositional analysis of the fecal microbiota by pyrosequencing

Despite the fact that fecal samples represent microbial communities that are shed from the gut and not resident, they provide a good overview over the microbiota present in the distal colon, and are the most practical samples that can be obtained from subjects participating in nutritional trials. Subjects provided fecal samples within 24 h of blood sampling and 2 h of defecation. Fecal material and 1:10 fecal homogenates in phosphate-buffered saline (pH = 7) were immediately frozen (-80 °C) and stored until further processing. Bacterial DNA was extracted from fecal homogenates as described by Martínez et al. (2010), using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) in combination with enzymatic and mechanical cell lysis. Pyrosequencing of amplicons obtained by PCR with universal primers targeting the V1–V3 region of the 16 S rRNA gene was performed as previously described (Martínez et al., 2010), using the 454 Genome Sequencer FLX with GS FLX Titanium series reagents at the Core for Applied Genomics and Ecology (University of Nebraska). Sequences obtained during this study are deposited in the MG-RAST server under the accession numbers 4498555.3, 4498556.3 and 4498557.3.

Sequence processing was performed combining features of QIIME (Caporaso et al., 2010) and the Ribosomal Database Project pipeline (Cole et al., 2009). Three-thousand quality-controlled sequences per sample were randomly selected and used for taxonomic classification. Sequences were assigned to a bacterial phylum, family and genus using the Classifier tool of the Ribosomal Database Project (Wang et al., 2007). In addition, sequences were assigned to operational taxonomic units with 97% sequence homology as described in the Supplementary materials. Chao1 species richness estimator, and Shannon's and Simpson's (defined as 1-Dominance) diversity indices were computed with QIIME.

Query for genes encoding β-glucanases in genomes of human gut microbes

Bacterial genomes available in the Joint Genome Institute database were used to identify large-bowel associated bacteria with β-glucanase encoding activity. The integrated microbial genomes platform of Joint Genome Institute was used to conduct this survey. A list of the strains included in the survey is presented in the Supplementary materials. For the species identified to contain β-glucanase genes, their abundance in the fecal microbiota of our subjects was quantified by BLASTn.

Statistics

Results are presented as means ± s.d. Differences in bacterial taxa and host phenotypes among treatments were determined by one-way analysis of variance with repeated measures in combination with Tukey's post-hoc tests, and $P < 0.05$ was considered statistically significant. If the data were not normally distributed, values were subjected to transformations such as square root or logarithm with base 10 to

achieve normality. If normality could not be achieved through transformations, the non-parametric Kruskal-Wallis test was performed. When only two groups of data were compared, Student's *t*-tests were performed. Correlations between host parameters and bacterial populations were assessed by Pearson's correlation tests using GraphPad Prism version 5.00 (GraphPad Software, La Joya, CA, USA). Associations between inflammatory markers and gut microbiome composition were also analyzed through linear models using SAS (SAS Institute Inc., Cary, NC, USA). Additional information on the statistical methods can be found in the Supplementary materials.

Results

Physiologic, metabolic and microbiome characteristics of the study population
Twenty-eight volunteers, 11 males and 17 females, participated in the nutritional trial, and subjects' parameters are presented in Table 1. Based on percent body fat, 13 subjects were classified as overweight, using as cutoff values >31% body fat for women and >25% for men. This Metabolic and immunological markers included in the study were plasma fasting glucose and insulin levels, glycemic and insulin postprandial response, a lipid panel (total cholesterol, HDL, non-HDL), and inflammatory markers (hs-CRP, IL-6 and LBP). The rationale for the inclusion of these markers is their suitability in determining the progression of metabolic aberrancies and the risk of cardiovascular disease (Schumann *et al.*, 1990; Spranger *et al.*, 2003; Cardellini *et al.*, 2005; Ridker, 2009), and their association with obesity (Sun *et al.*, 2010). Accordingly, positive correlations between body fat and all three inflammatory markers were observed (Figures 2a–c). LBP and hs-CRP were highly correlated ($r = 0.90$, $P < 0.0001$) (Figure 2d). The linear model identified body fat as a significant factor affecting IL-6 ($P < 0.01$), hs-CRP ($P < 0.0001$) and LBP ($P < 0.0001$). Furthermore, significant positive correlations existed between IL-6 and postprandial glucose response (Figure 2a). Together, these associations substantiate the link between adiposity, a low-grade systemic inflammation, and glucose metabolism (Hotamisligil, 2006).

Pyrosequencing revealed that the baseline fecal microbiota of the participants was dominated by the phyla Firmicutes and Bacteroidetes, with lower proportions of Verrucomicrobia and Actinobacteria, in agreement with previous molecular characterizations of the human fecal microbiota (Ley *et al.*, 2006; Martínez *et al.*, 2010). We investigated whether associations between host phenotypes and microbial populations existed (Supplementary Figure S1). No significant correlation was observed between any bacterial group and body fat or BMI, although overweight subjects harbored significantly lower abundances of Ruminococcaceae ($10.8 \pm 5.4\%$ versus $17.9 \pm 9.9\%$, $P < 0.05$) and *Faecalibacterium* ($1.8 \pm 1.8\%$ versus $3.7 \pm 2.5\%$, $P < 0.05$). The analysis revealed negative correlations between the family Ruminococcaceae and all the three inflammatory markers at baseline (Figures 2e and f, and Supplementary Figure S1). Within this family, the genera *Faecalibacterium* and *Ruminococcus* displayed negative correlations with hs-CRP ($r = -0.48$, $P < 0.05$, and $r = -0.60$, $P < 0.01$, respectively). The analysis also revealed a negative association between *Oscillibacter* and postprandial glucose area under the curve (Figure 2f). Regarding the markers of lipid metabolism, proportions of Bacteroidetes, Bacteroidaceae and *Bacteroides* were positively correlated to plasma HDL values ($r = 0.54$, $P < 0.05$; $r = 0.56$, $P < 0.05$; $r = 0.56$, $P < 0.05$; respectively) (Supplementary Figure S2).

Effects of whole grains on fecal microbial communities
Sequence data obtained by pyrosequencing were used to establish the effects of whole grains on the gut microbiota composition. This analysis revealed that whole grains had a measurable effect on gut microbiota composition. All three treatments significantly increased the bacterial diversity measured by Shannon's and Simpson's indices but not by Chao1 (Supplementary Figure S3). These results indicated an increase in community evenness (Shannon's and Simpson's), but not in total species richness (Chao1).

In accordance to previous studies that assessed the effect of diet on the gut microbiome (Martínez *et al.*, 2010; Davis *et al.*, 2011), substantial inter-individual variation was observed in response to whole grains (Supplementary Table 2). Despite this variability, several diet-induced shifts reached statistical significance in the entire study population. The proportion of the phylum Firmicutes increased, while Bacteroidetes were reduced (Table 2). The decrease in Bacteroidetes was largely caused by a reduction of the genus *Bacteroides* (Table 2).

The increase in Firmicutes was more comprehensive and shifts in the abundance of several taxa were detected. All three dietary treatments increased the abundance of the genus *Blautia* and two operational taxonomic units within this genus (Table 2), although significance was only achieved when WGB was included in the treatment. Several compositional shifts were strictly associated with the consumption of WGB, namely the genera *Roseburia*, *Bifidobacterium* and *Dialister* and the species *E. rectale*, *R. faecis* and *R. intestinalis* (Table 2), and many of these taxa increased gradually with WGB intake. The linear regression model confirmed all of these significant changes except for the species *Bifidobacterium,* and *Dialister*. Other taxa clearly responded to WGB, but because of inter-individual variation, these shifts did not reach statistical significance. For example, *Bacteroides*

Table 1 Baseline characteristics of the 28 subjects, and differentiated by gender and percent body fat (values are presented as mean ± s.d.)

	Overall	Gender			Body fat[a]		
	All subjects (n = 28)	Male (n = 11)	Female (n = 17)	P-value	Overweight (n = 13)	Normoweight (n = 15)	P-value
Age	25.9 ± 5.4	26.7 ± 5.4	25.4 ± 5.8	NS	28.6 ± 6.6	23.6 ± 3.0	< 0.05
Weight (kg)	72.3 ± 18.3	87.7 ± 17.1	62.3 ± 10.5	< 0.001	79.7 ± 19.5	65.9 ± 14.9	< 0.05
BMI (kg m^{-2})	25.1 ± 4.5	27.4 ± 4.5	23.6 ± 3.7	< 0.05	27.9 ± 4.4	22.7 ± 3.0	< 0.001
Body fat mass (kg)	20.8 ± 10.3	20.2 ± 11.6	21.2 ± 9.8	NS	29.7 ± 8.1	13.1 ± 3.6	< 0.001
Body fat (%)	29.6 ± 11.0	22.8 ± 8.5	34.0 ± 10.8	< 0.01	39.2 ± 7.4	21.3 ± 6.3	< 0.001
Cholesterol (mmol l^{-1})							
Total cholesterol	4.86 ± 1.12	4.07 ± 0.69	5.22 ± 1.67	< 0.01	4.76 ± 1.26	4.75 ± 1.07	NS
Non-high-density lipoprotein	3.13 ± 1.03	2.78 ± 0.74	3.29 ± 1.38	NS	3.23 ± 1.21	2.94 ± 0.85	NS
High-density lipoprotein	1.65 ± 0.42	1.30 ± 0.28	1.84 ± 0.57	< 0.001	1.53 ± 0.45	1.65 ± 0.42	NS
Fasting plasma glucose (mmol l^{-1})	5.17 ± 0.74	5.14 ± 0.72	5.15 ± 1.42	NS	5.44 ± 0.93	4.94 ± 0.40	NS
Fasting plasma insulin (μIU ml^{-1})	43.44 ± 18.86	42.76 ± 20.55	44.93 ± 21.14	NS	49.77 ± 19.92	40.34 ± 18.38	NS
Inflammatory markers							
IL-6 (pg ml^{-1}) (min–max)	1.75 ± 1.43 (0.06–5.17)	1.18 ± 0.81 (0.33–2.59)	2.01 ± 1.60 (0.06–5.17)	NS	2.29 ± 1.34 (0.65–4.89)	1.28 ± 1.33 (0.06–5.17)	NS
Hs-CRP (mg l^{-1}) (min–max)	1.69 ± 2.24 (0.002–7.039)	0.32 ± 0.22 (0.052–0.805)	2.38 ± 2.46 (0.002–7.039)	< 0.01	2.47 ± 2.36 (0.115–6.696)	0.97 ± 1.87 (0.002–7.039)	NS
LBP (μg ml^{-1}) (min–max)	15.09 ± 19.85 (0.12–88.05)	4.42 ± 3.03 (1.00–9.61)	20.91 ± 22.41 (0.12–88.05)	< 0.01	23.98 ± 24.90 (2.12–88.05)	7.48 ± 8.60 (0.12–28.51)	NS

Abbreviations: hs-CRP, high-sensitive C-reactive protein; IL, interleukin; LBP, lipopolysaccharide-binding protein; NS, not significant.
[a]Women with over 31% body fat, and men with over 25% body fat were considered as overweight individuals. All others were considered lean.
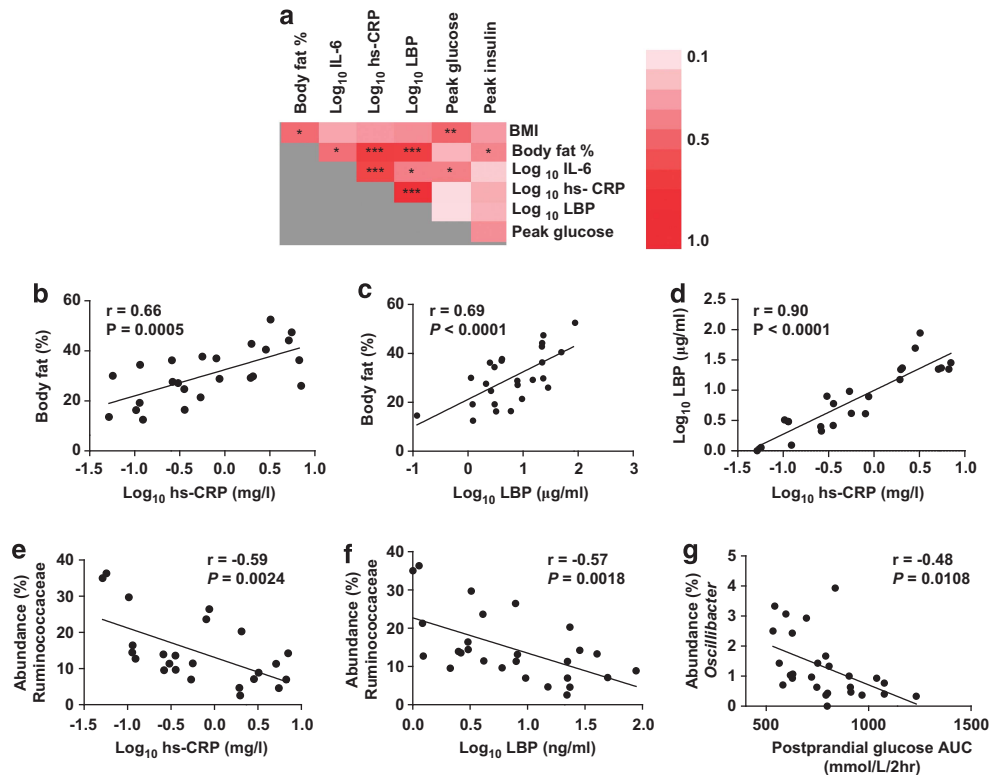

Figure 2 Associations among host physiological characteristics and their correlation with bacterial populations in fecal samples at baseline. Heatmap displaying correlation coefficients between metabolic and physiological parameters of the study population at baseline (**a**). Correlations between hs-CRP with body fat (**b**), LBP with body fat (**c**), hs-CRP and LBP (**d**), hs-CRP and Ruminococcaceae (**e**), LBP with Ruminococcaceae (**f**) and *Oscillibacter* with postprandial AUC glucose (**g**). Pearson's correlation (*r*) and the corresponding *P*-values are presented.

Table 2 Abundance of dominant bacterial taxa (% of total microbiota) in fecal samples as determined by 454 pyrosequencing (values are presented as mean ± s.d.)

	Baseline	BR	BR + WGB	WGB	P-value	Confirmation by linear model
Phylum						
Firmicutes	57.30 ± 14.13	65.06 ± 11.40^a	65.53 ± 10.64^a	65.42 ± 12.05^a	0.003	Yes
Bacteroidetes	37.99 ± 14.35	30.74 ± 11.62^a	29.85 ± 11.93^a	30.32 ± 12.22^a	0.01	Yes
Verrucomicrobia	1.82 ± 1.98	1.34 ± 1.53	0.68 ± 0.80	0.59 ± 0.80	NS	Yes
Actinobacteria	1.24 ± 0.97	1.42 ± 1.78	2.23 ± 3.32	2.05 ± 2.73	NS	Yes
Family						
Bacteroidaceae	28.55 ± 15.73	22.89 ± 10.37	21.19 ± 11.87^a	23.48 ± 12.62	0.013	Yes
Lachnospiraceae	22.21 ± 7.90	22.62 ± 7.91	23.11 ± 6.56	22.65 ± 7.63	NS	Yes
Ruminococcaceae	14.64 ± 8.76	17.32 ± 8.90	16.53 ± 8.06	15.82 ± 8.32	NS	Yes
Incertae Sedis XIV	5.79 ± 3.15	7.63 ± 4.47	8.16 ± 3.97^b	8.62 ± 4.32^b	0.001	Yes
Porphyromonadaceae	3.40 ± 3.07	2.69 ± 3.42	2.76 ± 3.10	1.95 ± 1.55^a	0.022	No
Prevotellaceae	2.97 ± 9.24	2.34 ± 6.56	3.59 ± 10.10	2.39 ± 6.50	NS	Yes
Verrucromicrobiaceae	1.85 ± 4.58	0.77 ± 1.53	0.68 ± 1.28	0.59 ± 0.80	NS	Yes
Rikenellaceae	1.77 ± 2.09	1.68 ± 1.85	1.12 ± 1.06	1.35 ± 1.68	NS	Yes
Veillonellaceae	1.59 ± 1.13	1.52 ± 1.19	1.86 ± 1.19	1.97 ± 1.60	NS	Yes
Genus						
Bacteroides	28.55 ± 15.73	22.89 ± 10.37	21.19 ± 11.87^a	23.48 ± 12.62	0.022	Yes
Blautia	5.68 ± 3.15	7.61 ± 4.47	8.14 ± 3.97^b	8.61 ± 4.32^b	0.001	Yes
Ruminococcus	4.20 ± 4.91	5.35 ± 5.05	4.171 ± 5.75	3.46 ± 4.32	NS	Yes
Faecealibacterium	2.82 ± 2.38	3.06 ± 2.29	3.86 ± 3.22	3.86 ± 3.19	NS	Yes
Prevotella	2.79 ± 8.89	1.99 ± 6.24	3.34 ± 9.84	2.02 ± 6.30	NS	Yes
Dorea	2.59 ± 2.01	3.12 ± 2.22	3.08 ± 1.80	2.75 ± 1.86	NS	Yes
Parabacteroides	2.58 ± 3.05	2.06 ± 3.23	2.10 ± 3.14	1.59 ± 1.44	NS	Yes
Roseburia	1.98 ± 1.35	1.70 ± 1.25	2.42 ± 1.58	3.06 ± 2.91^e	0.01	Yes
Akkermansia	1.85 ± 4.58	0.77 ± 1.53	0.68 ± 1.28	0.59 ± 0.80	NS	Yes
Coprococcus	1.82 ± 2.09	1.91 ± 2.08	1.47 ± 2.22	1.35 ± 1.78	NS	Yes
Alistipes	1.76 ± 2.08	1.67 ± 1.85	1.11 ± 1.05	1.34 ± 1.67	NS	Yes
Oscillibacter	1.27 ± 1.04	1.24 ± 1.00	1.08 ± 0.83	0.96 ± 0.61	NS	Yes
Bifidobacterium	0.99 ± 1.88	1.02 ± 1.64	1.95 ± 3.16	1.84 ± 2.54^d	0.011	No
Subdoligranulum	0.94 ± 1.03	1.17 ± 1.43	1.42 ± 1.73	1.09 ± 1.02	NS	Yes
Dialister	0.75 ± 1.17	0.60 ± 0.89	0.94 ± 1.21	1.14 ± 1.69^d	0.027	No
Odoribacter	0.26 ± 0.24	0.28 ± 0.35	0.28 ± 0.41	0.15 ± 0.18^b	0.002	No
Operational Taxonomic Units (OTU number, closest hit in database, % identity with 16S rRNA gene)						
1737, *Odoribacter splanchnicus*, 99%	0.15 ± 0.14	0.13 ± 0.18	0.15 ± 0.24	0.07 ± 0.10^b	0.001	No
679, Eubacterium rectale, 94%	0.25 ± 0.32	0.31 ± 0.42	0.43 ± 0.57	$0.57 \pm 0.63^{b,e}$	< 0.0001	Yes
956, *Roseburia faecis*, 99%	0.12 ± 0.17	0.06 ± 0.07	0.26 ± 0.31	$0.53 \pm 0.92^{b,f}$	< 0.0001	Yes
770, *Roseburia intestinalis*, 100%	0.09 ± 0.12	0.04 ± 0.05	0.17 ± 0.18^d	$0.30 \pm 0.42^{a,f}$	< 0.0001	Yes
3, *Blautia wexlerae*, 100%	1.07 ± 0.78	1.58 ± 1.11	1.49 ± 0.98	$1.82 \pm 1.14^{c,g}$	< 0.0001	Yes
179-188, *Blautia spp.*	1.81 ± 1.13	2.38 ± 1.69	2.75 ± 1.75^a	2.80 ± 2.04^b	0.006	Yes
44-19-1999-93, *Eubacterium rectale*, 98%	2.48 ± 2.67	2.75 ± 3.27	3.65 ± 3.45	$4.83 \pm 3.98^{a,e,g}$	0.001	Yes

Abbreviations: BR, brown rice; NS, not significant; OUT, operational taxonomic unit; WGB, whole grain barley.
Significantly different to baseline: $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.
Significantly different to BR: $^dP < 0.05$, $^eP < 0.01$, $^fP < 0.001$.
Significantly different to BR + WGB: $^gP < 0.05$.

coprocola, which was only detected in three subjects, showed a 10-fold increase with WGB consumption in only two of the subjects (Supplementary Table S2). Although both whole grains led to an equivalent increase in the Firmicutes/Bacteroides ratio, no family or genus showed a significant increase for BR, suggesting that this test meal induced diverse alterations in the gut microbiome that are not consistent among subjects.

No significant differences were detected in the amounts of short-chain fatty acids for any of the treatments. It is possible that an increase in short-chain fatty acids could not be detected in fecal samples they are for the most part absorbed in the gastrointestinal tract (Millet *et al.*, 2010).

Distribution of β-glucanase genes in human gut microbes

WGB contains a high amount of β-glucans (14.1%), while none were detected in BR (Supplementary Table S1). In order to test if the ability to hydrolyze β-glucans could explain the specific shifts in the fecal microbiota induced through WGB, we investigated distribution of β-glucanase genes in 112 strains originating from the human gut. This

analysis revealed that β-glucanase genes are present in a variety of gut bacterial species from a broad taxonomic range, including ten *Bacteroides,* four *Bifidobacterium*, three *Collinsella*, two *Clostridium*, two *Coproccus*, two *Eubacterium*, one *Ruminococcus*, two *Roseburia*, and one *Akkermansia* species (Supplementary Table S2). Of these species, only *E. rectale*, *R. faecis* and *R. intestinalis* were significantly increased through WGB, indicating that the mere presence of β-glucanase encoding genes does not predict the changes in community composition in response to the diet.

Whole grain-induced metabolic and immunological changes
The daily consumption of 60 g of whole grains for 4 weeks improved immunological and metabolic markers in the human subjects. The findings for the entire study population are shown in Supplementary Table S3, and differentiated by gender and body fat in Supplementary Tables S4 and S5. A significant decrease in plasma IL-6 levels for the BR + WGB treatment versus baseline values was detected (Figure 3a). Quantitatively, this reduction was highest in overweight subjects (Figure 3b). In women, all three treatments significantly reduced IL-6 (Figure 3c). The linear model analysis confirmed the anti-inflammatory effect of whole grains and revealed a significant reduction of IL-6 for BR + WGB and WGB treatments ($P < 0.01$, $P < 0.05$). Despite not achieving statistical significance due to high inter-individual variation, hs-CRP plasma levels were halved during the BR + WGB period compared with the baseline (Supplementary Tables S3-S5).

Whole-grain consumption significantly improved glucose and lipid metabolism. Postprandial peak glucose levels were significantly lowered in overweight subjects during the BR + WGB period ($P < 0.05$), and the reduction approached significance in the entire study population ($P < 0.1$) (Figures 3d and e). Fasting glucose levels were significantly decreased in women and overweight subjects, and in females, total cholesterol was significantly reduced (Supplementary Tables S4 and S5).

Links between whole grain-induced metabolic improvements and fecal microbial community structure
To determine whether effects of whole grains were related to the gut microbiome, a correlation analysis was performed between bacterial shifts and changes in the metabolic markers that occurred during the BR + WGB period. We focused the analysis on the BR + WGB treatment as it induced the most significant metabolic improvements (Figure 3). This analysis revealed that increases in the abundance of *E. rectale* were associated with improvements in the postprandial glucose and insulin response (Supplementary Figures S4A and SB). The association between *E. rectale* and maximum postprandial
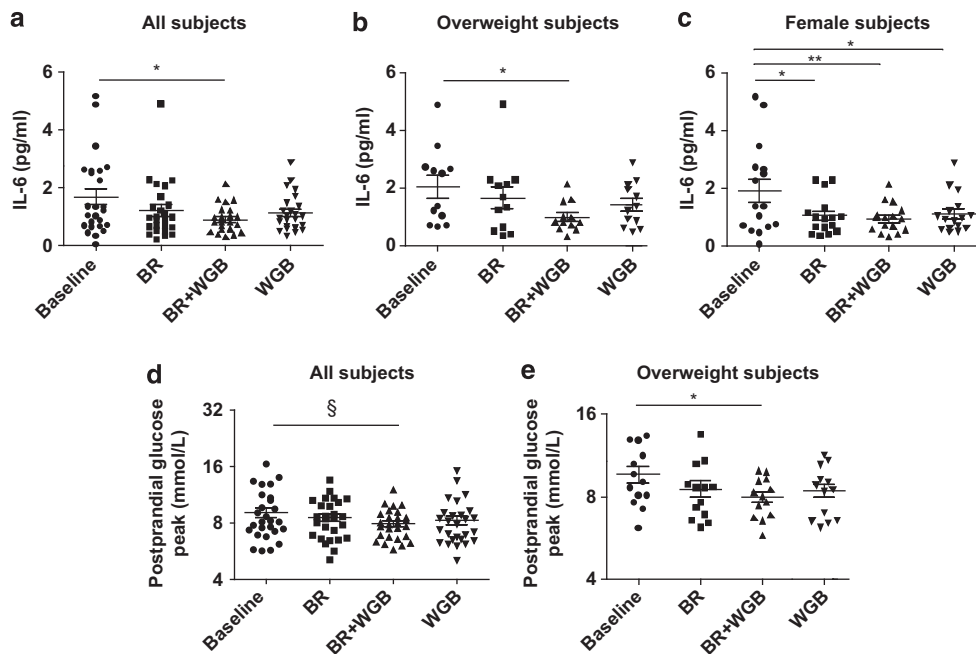

Figure 3 Immunological and metabolic improvements induced through whole-grain consumption. Plasma IL-6 levels in the entire subject population (**a**), in overweight participants (**b**), and in females (**c**). Maximum postprandial glucose levels in the entire subject population (**d**) and overweight subjects (**e**) during the three treatments (BR, BR + WGB, WGB) and at baseline. *$P < 0.05$, **$P < 0.01$, §$P < 0.1$.
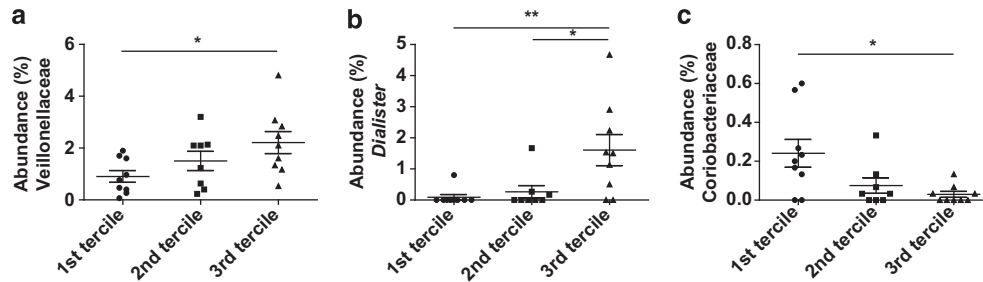
Figure 4 Abundance of specific taxa in subjects that showed differences in their IL-6 response to whole grains. Subjects were classified into terciles according to the magnitude of the change in plasma IL-6 levels induced by whole-grain consumption (BR + B treatment versus baseline). The proportions of bacterial taxa in fecal samples during the baseline were compared in the three terciles and significant differences existed in the proportions of Veillonellaceae (**a**), Dialister (**b**) and Coriobacteriaceae (**c**) in fecal samples during baseline. $*P < 0.05$, $**P < 0.01$.

glucose levels approached significance (Supplementary Figure S4C).

In addition, we categorized subjects into the three groups (terciles) according to the magnitude of the improvements in IL-6, hs-CRP, fasting glucose and glucose peak through BR + WGB. The baseline proportions of the bacterial groups between the three groups were compared. This analysis revealed that the gut microbiota of subjects with the highest improvement in IL-6 (3rd tercile) contained significantly higher percentages of Veillonellaceae (Figure 4a), and within this family, the genus *Dialister* (Figure 4b). Conversely, Coriobacteriaceae were significantly decreased in subjects with the highest improvement in IL-6 (Figure 4c). No significant differences in microbiome composition were detected between the terciles generated for hs-CRP, fasting glucose and postprandial glucose peak.

Gastrointestinal symptoms
Self-reported symptoms diaries revealed that 60 g of WGB significantly increased all the gastrointestinal symptoms surveyed, especially flatulence, while 30 g caused only a slight increase in flatulence (Supplementary Table S6). The addition of BR to the diet did not result in any reported changes in symptoms.

Discussion

The metabolic and immunological benefits of whole grains have been shown in various studies (Fung *et al.*, 2002; Behall *et al.*, 2004; Jensen *et al.*, 2004; Nilsson *et al.*, 2006, 2008b), and a contribution of the gut microbiome to these effects has been suggested (North *et al.*, 2009). However, the assessment of bacterial participation in these processes has been limited to hydrogen breath measurements, and the effects of whole grains on the gut microbiome structure have not been investigated. In this

study, we showed that whole grains have a significant effect on the composition of the fecal microbiota that coincided with metabolic and immunological improvements in healthy human individuals.

All whole-grain test meals caused an increase in community diversity within the subjects, driven by an increase in evenness of bacterial species. Therefore, WGB and BR seem to differ in their effects on the gut microbiota when compared with prebiotics and dietary fibers, which have not been shown to increase community diversity (Martínez *et al.*, 2010; Davis *et al.*, 2011; Van den Abbeele *et al.*, 2011). These differences might be due to compositional complexity of whole grains, which contain a variety of carbohydrates, potentially affecting a wider scope of bacterial taxa. Interestingly, a higher microbial diversity in fecal samples was also observed in children from Burkina Faso, who consumed a diet high in whole grains, legumes and vegetables, when compared with Europeans (De Filippo *et al.*, 2010). In addition, weaning in human infants leads to a drastic increase in diversity likely caused by the incorporation of more diverse arrays of dietary carbohydrates (reviewed in Koropatkin *et al.*, 2012). Therefore, it appears that bacterial diversity in the gut can be increased by providing a broader range of undigestible substrates, and our findings showed that this can be achieved by intake of whole grains.

This study revealed shifts in the fecal microbiota that were induced by both BR and WGB, while others were specific to WGB intake. Both whole grains increased the Firmicutes/Bacteroidetes ratio and the abundance of the genus *Blautia*. The overall shift in microbiota structure in favor of an expansion of Firmicutes could be the result of an increased carbohydrate intake (Duncan *et al.*, 2008). However, in a previous study, we did not observe an increase in the Firmicutes/Bacteroidetes ratio with the consumption of crackers containing resistant starches (Martínez *et al.*, 2010), although the dose of carbohydrates and fiber in these crackers exceeded

that of the whole-grain test meals. Interestingly, a decrease of *Bacteroides* was also shown to be associated with a long-term consumption of diets rich in whole grains, dietary fibers and vegetables in African children and US individuals (De Filippo *et al.*, 2010; Wu *et al.*, 2011). These and our findings suggest that other components included in whole grains and other plant-derived food products might influence community structure at the phylum level, specifically decreasing Bacteroidetes. The reason for the increase in the genus *Blautia* through whole grains might be due to a syntrophic effect. *Blautia* species are acetogenic and might benefit from the production of hydrogen, which is a product of glycan fermentation, and, therefore, likely induced by whole grain consumption (Nakamura *et al.*, 2010; Koropatkin *et al.*, 2012).

We detected several bacterial taxa that displayed a specific increase with WGB, several with a clear dose response. This is likely due to its high content of β-glucans. Accordingly, the bacteria that specifically responded to WGB harbor genes encoding for β-glucanases and utilize the substrate *in vitro* (Hughes *et al.*, 2008; Tasse *et al.*, 2010). However, the *in vivo* findings cannot solely be explained based on functional and genomic attributes of community members, as *Bacteroides* species decreased during WGB consumption, but possess β-glucanase genes and can utilize β-glucans *in vitro* (Crittenden *et al.*, 2002; Tasse *et al.*, 2010; Zhao and Cheung, 2011). A possible explanation for the *in vivo* findings could entail preferences towards distinct β-glucan structures and molecular weights. The β(1–4) to β(1–3) linkage ratio in barley is 2.3-3, while *Bacteroides* species have been shown to especially possess β(1–3)-glucanase activity (Salyers *et al.*, 1977). Moreover, barley-derived β-glucan fractions of high molecular weight have also been shown to be poorly fermented by *Bacteroides* (Hughes *et al.*, 2008). However, previous human trials with prebiotics and resistant starches have also revealed that the ability of a species to utilize substrates *in vitro* does not predict population shifts *in vivo* (Martínez *et al.*, 2010; Davis *et al.*, 2011; Koropatkin *et al.*, 2012). Therefore, although the findings obtained suggest that β-glucans are the main cause for the shifts in composition induced by WGB, the exact mechanisms by which these changes are restricted to only a small number of taxa are likely to be due to competitive interactions.

A main objective of this study was to determine whether the effects of whole grains on the gut microbiome are associated with physiological benefits. The whole grains used in our study led to immunological and metabolic improvements, especially when BR + WGB was consumed. Plasma IL-6 was reduced, and a tendency for a decrease in plasma hs-CRP was detected. In addition to this anti-inflammatory effect, an improvement in the glycemic response during BR + WGB treatment was detected. Our findings are in agreement with previous research that established the immunological and metabolic benefits of whole grains (Casiraghi *et al.*, 2006; Kallio *et al.*, 2008; Nilsson *et al.*, 2008b; Rosén *et al.*, 2011). Most importantly, inflammation has been identified as a main cause of metabolic disorders (Hotamisligil, 2006), and the anti-inflammatory effect could provide a mechanism by which whole grains improve glucose metabolism.

The anti-inflammatory effect of whole grains might be mediated through its effect on the gut microbiota. A remarkable positive correlation between LBP and hs-CRP was identified in our study population, supporting a link between bacterial lipopolysaccharide and systemic inflammation. The associations of these markers with body-fat support the hypothesis that endotoxemia could contribute to obesity (Cani *et al.*, 2007; Delzenne and Cani, 2011). WGB led to an increase of bacterial taxa such as bifidobacteria and *Roseburia*, which have been suggested to affect immune/inflammatory and metabolic functions in animal models (Cani *et al.*, 2008; Neyrinck *et al.*, 2011). Although one could envision that these shifts might underlie the anti-inflammatory effect of whole grains, no significant correlations between these taxa and inflammatory markers were observed. However, shifts in the abundance of *E. rectale* induced through the BR + WGB diet correlated with decreased postprandial glucose and insulin responses. This organism produces butyrate, which might contribute to the immunological benefits of whole grain consumption through its anti-inflammatory effects.

Interestingly, compositional differences at baseline were detected in the gut microbiome of subjects that differed in the magnitude of their anti-inflammatory response to whole grains. Subjects with the greatest reduction in plasma IL-6 concentration had significantly higher proportions of *Dialister* and a lower abundance of Coriobacteriaceae. These bacterial groups have been linked to chronic inflammation in previous studies. *D. invisus* and Coriobacteriaceae have been shown to be reduced and increased in patients with Crohn's disease and colitic mice, respectively (Clavel *et al.*, 2009; Würdemann *et al.*, 2009; Willing *et al.*, 2010; Joossens *et al.*, 2011). The association of *Dialister* and Coriobacteriaceae with IL-6 response suggests that these taxa may condition the capability of an individual to be immunologically responsive to whole grains.

Before the start of the treatments, associations between bacterial groups, inflammatory state and host metabolism were observed (Figure 3). Ruminococcaceae negatively correlated with markers of inflammation and were more dominant in normoweight individuals. In addition, Bacteroidetes positively correlated with HDL cholesterol. These observations could result from an impact of these taxa on host physiology, and these associations provide a rationale to develop dietary strategies that target Ruminococcaceae and Bacteroidetes to improve human metabolic and immunological

functions. However, host physiology (inflammatory state, cholesterol/bile acid metabolism) might also shape the microbiome composition. If systemic inflammation and cholesterol metabolism impact levels of Ruminococcaceae and Bacteroidetes, respectively, then these interactions could explain the discrepancies related to an altered microbiome in obese versus normoweight individuals (Ley et al., 2006; Duncan et al., 2008; Schwiertz et al., 2010). Not obesity per se, but the associated inflammatory and metabolic aberrations could shape microbiome composition and might cause variable and more complex patterns of dysbiosis.

This study has provided novel information about the relationship between whole grains, the gut microbiota and host metabolism. Whole grain-induced alterations in the characteristics and composition of the fecal microbiota coincided with immunological and metabolic benefits, and the clear associations between the reduction of IL-6 and the presence of certain bacterial taxa (*Dialister*, Coriobacteriaceae) indicate an important functional role of gut bacteria in the physiologic effects of whole grains.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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