Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Human Intestinal Microbiota Composition Is Associated with Local and Systemic Inflammation in Obesity

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Objective: Intestinal microbiota have been suggested to contribute to the development of obesity, but the mechanism remains elusive. The relationship between microbiota composition, intestinal permeability, and inflammation in nonobese and obese subjects was investigated.

Design and Methods: Fecal microbiota composition of 28 subjects (BMI 18.6-60.3 kg m^{-2}) was analyzed by a phylogenetic profiling microarray. Fecal calprotectin and plasma C-reactive protein levels were determined to evaluate intestinal and systemic inflammation. Furthermore, HbA_{1c}, and plasma levels of transaminases and lipids were analyzed. Gastroduodenal, small intestinal, and colonic permeability were assessed by a multisaccharide test.

Results: Based on microbiota composition, the study population segregated into two clusters with predominantly obese (15/19) or exclusively nonobese (9/9) subjects. Whereas intestinal permeability did not differ between clusters, the obese cluster showed reduced bacterial diversity, a decreased Bacteroidetes/Firmicutes ratio, and an increased abundance of potential proinflammatory Proteobacteria. Interestingly, fecal calprotectin was only detectable in subjects within the obese microbiota cluster (n = 8/19, P = 0.02). Plasma C-reactive protein was also increased in these subjects (P = 0.0005), and correlated with the Bacteroidetes/Firmicutes ratio (r_s = −0.41, P = 0.03).

Conclusions: Intestinal microbiota alterations in obese subjects are associated with local and systemic inflammation, suggesting that the obesity-related microbiota composition has a proinflammatory effect.

Obesity (2013) **21**, E607–E615. doi:10.1002/oby.20466

Introduction

The intestinal microbiota are increasingly acknowledged to be involved in the development of obesity and the metabolic syndrome (1). For instance, germ-free mice are protected from diet-induced obesity (2), while intestinal microbiota transplantation from obese mice into lean germ-free mice results in a larger fat deposition than transplantation from lean donor mice (3). Furthermore, both genetically modified (4) and diet-induced (5) obese animals display a different intestinal microbiota composition compared to lean controls. This "obese microbiota composition" is characterized by a reduction in the abundance of Bacteroidetes paralleled by an increase in Firmicutes (4,5).

Human data on gut microbiota composition in relation to obesity are however more scarce and less consistent. Increased Firmicutes and decreased Bacteroidetes have been reported (3,6,7), but a lower ratio of Firmicutes to Bacteroidetes in obesity (8) and similar microbiota composition in lean and obese subjects (9) have also been described. The mechanisms by which the intestinal microbiota affects obesity and metabolic disorders are the focus of intense research. The intestinal microbiota have been shown to influence intestinal permeability in obese mice, thereby promoting translocation of bacterial products and stimulating the low-grade inflammation characteristic of obesity and insulin resistance (10,11). Furthermore, microbiota composition alterations in obesity-prone rats have been found to coincide with intestinal inflammation (12). Finally, several studies suggest

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Disclosure: All authors hereby declare they have no competing financial interests in relation to the work described here.

Funding agencies: This work was financially supported by a Senter Novem IOP genomics grant to WAB and JWG (IGE05012A), a Transnational University Limburg grant and a Dutch Digestive Foundation project grant (WO 09-46) to SSR, and a Spinoza award to WMDV (NWO).

Author contributions: FJV, CdJ, EGZ, JWG, WAB, WMDV, and SSR conceived the study. Data was collected by FJV, SF, CdJ, EGZ, and RE, analyzed by FJV, SF, EGZ, WAB, and SSR, and interpreted by FJV, SF, CdJ, EGZ, JWG, WAB, WMDV, and SSR. Literature searches were performed by FJV, SF, EGZ, JWG, WAB, WMDV, and SSR. Figures were generated by FJV, SF, and SSR. All authors were involved in writing the article and had final approval of the submitted version.

Received: 22 December 2012; **Accepted:** 13 March 2013; **Published online 21 March 2013. doi:10.1002/oby.20466**

TABLE 1 Characteristics of the study population

	Nonobese subjects	Obese subjects	P value	Nonobese microbiota cluster[a]	Obese microbiota cluster[a]	P value
No. of patients	13	15		9	19	
Age (years)	28.2 ± 3.3	35.3 ± 2.8	<0.04	23.3± 3.3	36.2 ± 2.4	<0.0008
Sex (F : M)	8 : 5	12 : 3		6:3	14:5	
BMI (kg m^{-2})	23.4 ± 0.8 (18.6-29.6)	44.2 ± 2.3 (30.5-60.3)	<0.01	22.2 ± 0.7 (18.6-25.7)	40.4 ± 2.5 (23.7-60.3)	<0.0001
HbA_{1c} (%)	5.4 ± 0.1	6.1 ± 0.3	<0.02	5.4 ± 0.1	6.0 ± 0.3	0.07
Cholesterol (mmol L^{-1})	4.8 ± 0.4	4.6 ± 0.2	Ns	5.1 ± 0.5	4.5 ± 0.2	Ns
HDL (mmol L^{-1})	1.5 ± 0.1	1.1 ± 0.1	<0.02	1.5 ± 0.2	1.1 ± 0.1	0.05
LDL (mmol L^{-1})	2.8 ± 0.3	2.7 ± 0.3	Ns	3.0 ± 0.4	2.6 ± 0.2	Ns
TG (mmol L^{-1})	1.4 ± 0.3	1.8 ± 0.3	Ns	1.1 ± 0.3	1.8 ± 0.3	0.07
AST (IU L^{-1})	17 ± 2	19 ± 2	Ns	18 ± 3	18 ± 2	Ns
ALT (IU L^{-1})	21 ± 2	29 ± 3	Ns	21 ± 3	28 ± 3	Ns
CRP (mg L^{-1})	1.5 ± 0.2	12.4 ± 2.5	<0.01	1.5 ± 0.3	10.4 ± 2.2	<0.0005

[a]Microbiota clusters were determined by means of phylogenetic profiling (HITChip analysis).

that the intestinal microbiota influence energy extraction from nutrition and subsequent fat storage in adipose tissue (2,3,13).

In view of these data, we investigated the intestinal microbiota composition in obese and nonobese subjects by means of a phylogenetic profiling DNA microarray, and correlated these data to parameters of intestinal permeability and local and systemic inflammation. We here present the first evidence that the gut microbiota in human obesity is related to both intestinal and systemic inflammation in man.

Methods

Subjects

From May to September 2010, 28 adults (age 19-54 years, BMI 18.6-60.3 kg m^{-2} were recruited through advertising at the Atrium Medical Center Parkstad in Heerlen, the Netherlands. Thirteen subjects were nonobese, of whom nine subjects were lean (BMI 18.6-24.6 kg m^{-2} and four subjects were overweight (BMI 25.2-29.6 kg m^{-2}). Fifteen subjects were obese (BMI 30.5-60.3 kg m^{-2}), of whom nine subjects had a BMI of over 40 kg m^{-2} (range 40.4-60.3 kg m^{-2}); population characteristics are presented in Table 1. Subjects were excluded if they received antibiotic treatment in the last 6 months, used anti-inflammatory drugs, or reported alcohol consumption >63 g/week. Other exclusion criteria were acute and chronic inflammatory diseases (e.g. Crohn's disease, colitis, viral hepatitis, type 1 diabetes, auto-immune diseases, asthma, and chronic obstructive pulmonary disease). The study was approved on the 21st of December 2009 by the Medical Ethics Committee of the Atrium Medical Center and conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). Informed consent in writing was obtained from each subject individually.

Blood sampling and analysis

Venous blood samples were obtained in the outpatient clinic, collected into prechilled EDTA tubes (BD Vacutainer, Becton Dickinson Diagnostics, Erembodegem-Aalst, Belgium), and kept on ice. Parameters reflecting inflammation (high sensitivity C-Reactive Protein: CRP) and obesity comorbidity (HbA_{1c}, plasma glucose, insulin, cholesterol, HDL, LDL, free fatty acids (FFA), and liver transaminases (AST and ALT)) were assessed at the Department of Clinical Chemistry according to the protocol of the Atrium Medical Center Parkstad (Table 1).

Fecal microbiota and fecal calprotectin analysis

Subjects collected feces 24 h prior to the intestinal permeability test, and kept this refrigerated until the morning of the test, when samples were stored in aliquots at −20°C. DNA was isolated as previously described (14) and intestinal microbiota composition was assessed using the Human Intestinal Tract Chip (HITChip), a phylogenetic profiling DNA microarray containing over 4,800 probes based on 16S rRNA gene sequences of over 1,100 intestinal bacterial phylotypes. This microarray identifies both variation and relative quantity of the human intestinal tract communities (15). Hybridizations were performed in duplicate with samples labeled with Cy3 and Cy5 dyes, respectively. Slides were scanned and the data were extracted from the microarray images using the Agilent Feature Extraction software, version 10.7.3.1 (http://www.agilent.com). Array normalization was performed as previously described (15) using a set of R-based scripts (http://r-project.org) in combination with a custom designed relational database which runs under the MySQL database management system (http://www.mysql.com). This was implemented on both dyes for each sample, and duplicate hybridizations with a Pearson correlation over 0.98 were considered for further analysis. Ward's minimum variance method was used for the construction of hierarchical clusters of the total microbiota probe profiles, while the distance matrix between the samples was based on Euclidian distance. The bacterial diversity of the fecal samples was assessed by Simpson's reciprocal index of diversity (1/D) using the HITChip probe levels. Furthermore, fecal calprotectin levels reflecting intestinal inflammation were measured by ELISA (Hycult Biotech, Uden, the Netherlands) according to Van der Sluis Veer et al. to improve sensitivity (16), resulting in a detection limit of 20 μg g^{-1} feces.

Assessment of intestinal permeability

Intestinal permeability was assessed as previously described (17). In short, after at least 8 h of fasting, a multi saccharide mix was orally

administered after a double challenge with a nonsteroid anti-inflammatory drug (400 mg ibuprofen the evening prior to the test, and 400 mg the following morning) to magnify potential differences in intestinal permeability. The saccharide mix consisted of 1 g sucrose (Van Gilse, Dinteloord, the Netherlands), 1 g lactulose (Centrafarm, Etten-Leur, the Netherlands), 0.5 g L-rhamnose (Danisco, Copenhagen, Denmark), 1 g sucralose (Brenntag, Sittard, the Netherlands), and 1 g erythritol (Danisco), dissolved in 150 mL tap water. Urinary excretion of sucrose after 1 h reflects gastroduodenal permeability, the ratio of lactulose/L-rhamnose (L/R) after 5 h reflects small intestinal permeability, and large intestinal permeability is reflected by the ratio of sucralose/erythritol (S/E) after 5 h. One and 5 h after oral administration of the saccharide mix, total urine collection was recorded and sampled. Urine samples were centrifuged at 4°C for 15 min at 2,300*g*, and immediately stored in aliquots at −80°C until analysis. Urinary excretion of mono- and disaccharides was quantified by high pressure liquid chromatography and mass spectrometry (Model LTQ-XL, Thermo Electron, Breda, the Netherlands).

Statistical analysis

Multivariate statistical software Canoco 4.5 for Windows (18) (Biometrix, Plant Research International, Wageningen) was used to perform redundancy analysis (RDA) on log transformed data, and statistical significance was evaluated using a Monte Carlo Permutation Procedure (MCPP). The log transformed sum of the hybridization signals for the 131 genus-like phylogenetic groups targeted by the HITChip was used as species variables. Comparisons between groups at the genus level (subsets of phylotypes with 90% or more 16S rRNA sequence similarity) were performed using the Wilcoxon signed-rank test corrected for multiple comparisons (q value); $q < 0.05$ was considered statistically significant. Additional statistical analyses were performed using Prism 5.0 for Windows (GraphPad Software, San Diego, CA). Correlations were calculated using Spearman's rank correlation coefficient, while differences between groups were analyzed by the nonparametric Mann–Whitney test or the Chi-square test. A P value <0.05 was considered statistically significant and denoted with an asterisk in the figures. Data are presented as mean ± standard error of the mean.

Results

Obese and nonobese subjects segregate in distinct microbiota clusters with different bacterial diversity

The microbial profiles obtained from the fecal samples of all 28 subjects (13 nonobese subjects with a BMI < 30 kg m^{-2} and 15 obese subjects, BMI > 30 kg m^{-2}) were hierarchically clustered based on the signal intensity of the HITChip oligonucleotide probes. Remarkably, all obese subjects clustered separately from the nonobese subjects. Four out of the 13 nonobese subjects (two normal weight and two overweight subjects) clustered with the "obese microbiota composition" (Figure 1a). The obese microbiota cluster was characterized by a significantly lower bacterial diversity than the nonobese cluster (128.7 ± 33.2 vs. 174.6 ± 37.3, $P = 0.002$, Figure 1b), a difference which was not observed when subjects were divided based upon BMI.

Further detailed analyses of both clusters revealed significant differences in microbiota groups between the obese and non-obese clusters. The main differences were observed within the Firmicutes and the Bacteroidetes phyla (Table 2), leading to a decreased Bacteroidetes/Firmicutes ratio in the obese microbiota cluster (Figure 1c). More specifically, *Clostridium* cluster IV and XIVa of the Firmicutes phylum were more abundantly present in the obese microbiota cluster, with specific groups showing 1.8 to 2.6-fold increases. In addition, the uncultured Clostridiales I group belonging to the Firmicutes phylum was more than sixfold decreased in the obese cluster. On the other hand, Bacteroidetes were less abundantly present in the obese microbiota cluster. In particular, *Allistipes* et rel. and *Bacteroides intestinalis* et rel. showed over 3.5 fold reductions (Table 2). The relatively lower abundance of Bacteroidetes as opposed to Firmicutes in the obese cluster was confirmed by redundancy analysis (Figure 1d). Overall, bacteria associated to butyrate production accounted for 21.4% ± 7.4% of the total hybridization signal of the samples. The relative abundance of the butyrate producers was similar in the non-obese and the obese microbiota cluster (19.8% ± 7.8% vs. 22.1% ± 7.3%, respectively, $P = 0.45$).

The Bacteroidetes/Firmicutes ratio is strongly and negatively associated with BMI

Division of subjects into nonobese and obese categories according to BMI revealed similar and consistent microbiota composition differences (Table 2). The Bacteroidetes phylum was threefold less abundant in obese subjects (5.9% ± 5.8% of the total hybridization signal) compared to nonobese subjects (19.2% ± 9.2%; $P < 0.002$, Figure 2a). In contrast, Firmicutes were more numerous in obese subjects, contributing 85.8% ± 8.5% of the total hybridization signal, whereas they accounted for 74.6% ± 9.2% of the signal in nonobese subjects ($q = 0.002$, Figure 2a). As a result of these shifts in Bacteroidetes and Firmicutes abundance, the ratio of Bacteroidetes to Firmicutes was also strongly decreased in obese subjects (BMI > 30 kg m^{-2}, $P = 0.0002$, Figure 2b). In corroboration of these findings, a strong negative correlation was observed between Bacteroidetes/Firmicutes ratio and BMI ($r_s = -0.59$, $P = 0.0009$, Figure 2c).

Moreover, a positive relationship between BMI and *Roseburia intestinalis* bacteria—that are associated with butyrate producers—was found within the Firmicutes phylum (Table 3). In line with the microbiota cluster differentiation, the total signal corresponding to butyrate producers was similar in nonobese and obese subjects. Strikingly, several members of the Proteobacteria including those related *to E.aerogenes, K.pneumoniea, Vibrio, and Yersina* spp. were positively associated with BMI and more abundantly present in obese subjects (Table 3). Some of these have recently been described to be increased in mice on a high fat diet (19). In contrast, a strong negative correlation was observed between BMI and many level 2 groups belonging to the Bacteroidetes. *Allistipes* et rel. was most significantly decreased in obese subjects, by more than sixfold (Table 3).

The obese microbiota cluster is associated with intestinal and systemic inflammation

Because obesity-prone rats show intestinal inflammation in conjunction with microbiota shifts (12), we next investigated whether the obesity-associated intestinal microbiota composition changes were related to intestinal inflammation. Strikingly, the intestinal inflammation marker fecal calprotectin was only detectable in subjects within

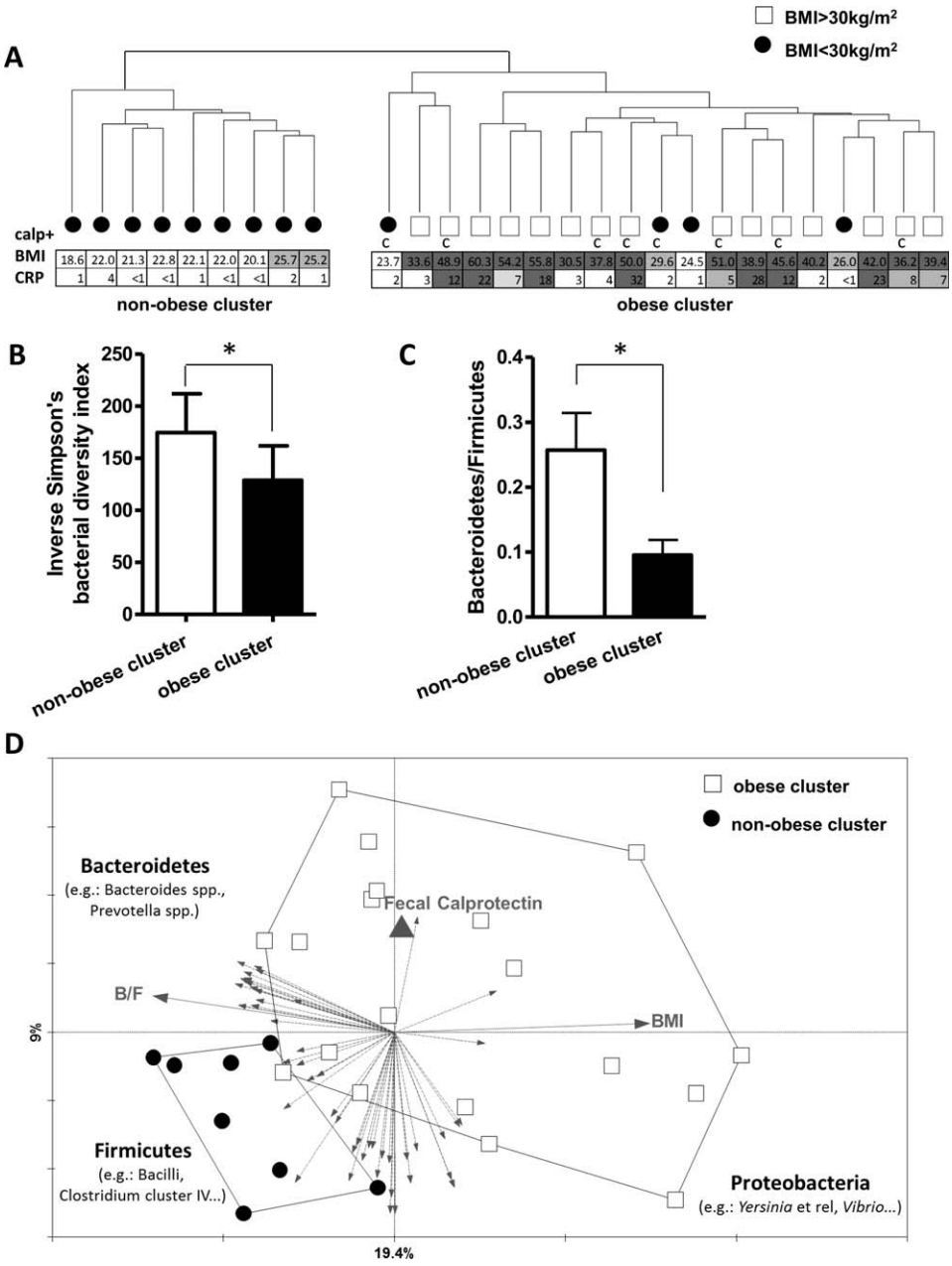

FIGURE 1 Obese and nonobese subjects segregate in distinct microbiota clusters. (**a**) Hierarchical clustering of the 28 fecal samples of obese (□) and nonobese (●) subjects as determined by the HITChip profiles. Subjects with detectable fecal calprotectin (calp+) are denoted by a "**c**." Corresponding BMI and CRP values are shown below in white for normal weight subjects (BMI < 25 kg m^{-2}; CRP < 5 mg L^{-1}), light grey for overweight subjects (25 > BMI < 30 kg m^{-2}; 5 > CRP < 10 mg L^{-1}), and dark gray for obese subjects (BMI > 30 kg m^{-2}; CRP > 10 mg L^{-1}). (**b**) The inverse bacterial diversity index according to Simpson was significantly reduced in the obese microbiota cluster (128.7 ± 33.2 vs. 174.6 ± 37.3, $P = 0.003$). (**c**) Significantly decreased bacteroidetes/firmicutes ratio in the obese microbiota cluster ($P = 0.007$). (**d**) RDA plot of subjects in the obese (□) and nonobese (●) microbiota cluster based on their microbiota composition. First and second ordination axes are plotted, explaining 19.4 and 9% of the variability in the dataset, respectively. The variation in the abundance of 48 level 2 groups (represented by the labeled arrows) belonging to the phyla Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria is explained to at least 20% by subject characteristics. Environmental variables shown in the plot (red arrows) are statistically significant (BMI $P = 0.002$, fecal calprotectin $P = 0.02$, and Bacteroidetes/Firmicutes ratio $P = 0.01$).

the obese microbiota cluster ($n = 8/19$, 42% of subjects, vs. $n = 0/9$ in the nonobese microbiota cluster, $P = 0.02$; Figures 1a and 3a). Of these subjects, two were overweight and six were obese. The mean fecal calprotectin level was 279 ± 70 $\mu g\ g^{-1}$, ranging from 80 to 570 $\mu g\ g^{-1}$. Remarkably, high sensitivity CRP plasma levels reflecting systemic inflammation correlated with the Bacteroidetes/Firmicutes

TABLE 2 Relative abundance of bacterial groups that significantly differ between the nonobese vs. the obese microbiota cluster and between nonobese vs. obese subjects

			Relative contribution (%)	
			Nonobese	Obese
Nonobese vs. obese microbiota cluster				
Bacteroidetes	Bacteroidetes	*Allistipes* et rel	3.02 ± 1.73	0.81 ± 0.80
		Bacteroides fragilis et rel	0.94 ± 0.53	0.33 ± 0.32
		Bacteroides intestinalis et rel	0.87 ± 0.48	0.25 ± 0.25
		Bacteroides splachnicus et rel	1.62 ± 1.30	0.51 ± 0.54
Firmicutes	Clostridium cluster IV	*Oscillospira guillermondii* et rel	5.56 ± 3.44	2.18 ± 2.61
	Clostridium cluster XIVa	*Clostridium colinum* et rel	0.37 ± 0.23	0.79 ± 0.34
		Clostridium symbiosum et rel	2.03 ± 0.84	3.75 ± 1.72
		Eubacterium hallii et rel	0.44 ± 0.18	0.93 ± 0.44
	Uncultured Clostridiales	Uncultured Clostridiales I	2.00 ± 3.69	0.32 ± 0.85
Nonobese vs. obese subjects				
Bacteroidetes	Bacteroidetes	*Allistipes* et rel	2.72	0.48
		Bacteroides fragilis et rel	0.84	0.25
		Bacteroides intestinalis et rel	0.72	0.21
		Bacteroides ovatus et rel	1.20	0.52
		Bacteroides plebeius et rel	1.66	0.42
		Bacteroides splachnicus et rel	1.52	0.30
		Bacteroides stercoris et rel	1.08	0.39
		Bacteroides uniformis et rel	0.91	0.26
		Parabacteroides distasonis et rel	2.11	0.60
		Prevotella oralis et rel	0.57	0.13
		Prevotella ruminicola et rel	0.50	0.16
		Prevotella tannerae et rel	1.24	0.53
		Tannerella et rel	0.83	0.33
		Uncultured *Bacteroidetes*	0.19	0.01
Firmicutes	Clostridium cluster IV	*Papillibacter cinnamivorans* et rel	0.30	0.77
	Clostridium cluster XIVa	*Clostridium symbiosum* et rel	2.54	3.76
		Dorea formicigenerans et rel	4.11	6.27

Level 2 phylogenetic groups with higher relative abundance in the obese subjects are indicated in grey. For all groups, $q < 0.05$.

ratio ($r_s = -0.41$, $P = 0.03$), implying a relationship between systemic inflammation and microbiota composition. In line with this, plasma CRP levels were also significantly higher in subjects within the obese microbiota cluster (10 ± 2.2 vs. 1.5 ± 0.31; $P < 0.0005$, Figure 3b). Moreover, both fecal calprotectin and systemic CRP levels were also related to specific groups of bacteria that were more abundant in obese subjects (Table 3). The strongest correlations were observed between fecal calprotectin and the abundance of *Clostridium nexile* et rel, and between CRP and the abundance of *Aneurinibacillus*, *Papillibacter cinnamivorans* et rel, and *Roseburia intestinalis* et rel. Conversely, plasma CRP levels showed negative correlations with seven groups belonging to the Bacteroidetes, which were all more abundant in the nonobese subjects (Table 3).

Microbiota composition and BMI are not related to intestinal permeability

Gut microbiota have been suggested to induce low-grade inflammation in obese rodents by increasing intestinal permeability (11,20). Therefore, we next studied permeability of different segments of the gastro-intestinal tract in relation to BMI and microbiota composition. Gastroduodenal permeability was almost twice as high in the obese compared to the nonobese microbiota cluster (3.6 ± 0.6 μmol vs. 2.1 ± 0.4 μmol, $P = 0.03$, Figure 4a). Obese and nonobese subjects displayed a similar difference (4.1 ± 0.7 μmol vs. 1.9 ± 0.3 μmol, $P = 0.003$, Figure 4a). However, gastroduodenal permeability was not related to the Bacteroidetes/Firmicutes ratio, BMI, CRP, or fecal calprotecin ($r_s = -0.28$, $P = 0.16$; $r_s = 0.25$, $P = 0.21$, $r_s = 0.08$, $P = 0.70$, $r_s = -0.02$, $P = 0.96$, respectively). Small intestinal permeability was also not related to the Bacteroidetes/Firmicutes ratio ($r_s = -0.01$, $P = 0.94$) or to BMI ($r_s = 0.06$, $P = 0.76$), and similar in both microbiota clusters (0.06 ± 0.02 vs. 0.06 ± 0.01; $P = 0.7$) and in nonobese and obese subjects (0.06 ± 0.02 vs. 0.05 ± 0.01; $P = 0.9$, Figure 4b).We also did not observe significant associations between small intestinal permeability and either CRP or calprotectin ($r_s = -0.03$, $P = 0.89$, $r_s = -0.44$, $P = 0.23$, respectively). Similarly, colonic permeability was not related to the

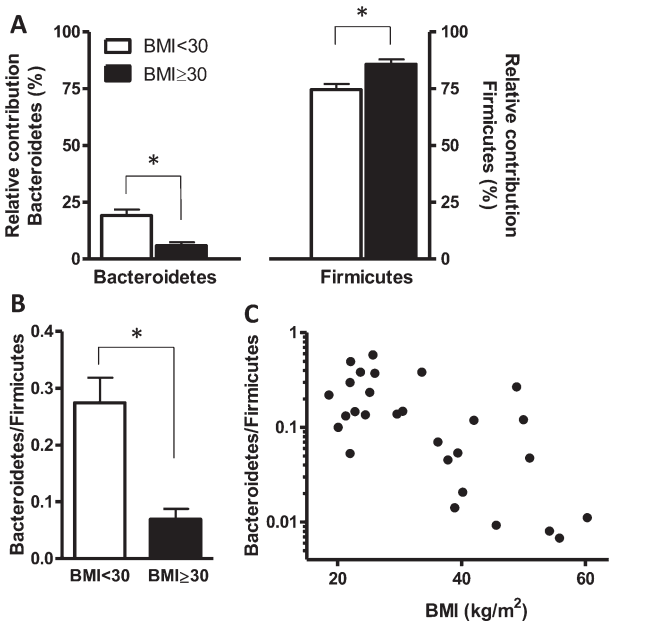

FIGURE 2 Strong relation between Bacteroidetes/Firmicutes and BMI. (**a**) Relative contribution of Bacteroidetes and Firmicutes in the samples of obese and non-obese subjects. Both phyla differed significantly between obese and nonobese populations. (**b**) The Bacteroidetes/Firmicutes ratio in obese subjects was strongly decreased ($P = 0.0002$). (c) A strong correlation between the Bacteroidetes/Firmicutes ratio and BMI was observed ($r_s = -0.59$, $P = 0.0009$).

Bacteroidetes/Firmicutes ratio or to BMI ($r_s = -0.19$, $P = 0.34$; $r_s = -0.14$, $P = 0.50$, respectively), and comparable in both the obese and nonobese groups based upon either microbiota cluster or BMI (0.04 ± 0.01 vs. 0.05 ± 0.01; $P = 0.74$; 0.03 ± 0.01 vs. 0.04 ± 0.01; $P = 0.65$, Figure 4c). Colonic permeability was not associated with plasma CRP or fecal calprotectin levels ($r_s = 0.04$, $P = 0.83$, $r_s = -0.30$, $P = 0.43$, respectively). In short, intestinal permeability was neither related to the observed differences in microbiota composition nor to BMI or inflammatory markers.

To investigate the determining and potential confounding factors in the relation between microbiota composition and inflammation, multivariate analyses were performed, taking into account BMI, age, CRP, HbA_{1c}, fecal calprotectin, intestinal permeability, and the Bacteroidetes/Firmicutes ratio. A total of 34.5% of the variation in the microbiota composition was related to these characteristics (Figure 1d). Supporting our data on the specific inflammation-associated microbiota composition in the obese population, fecal calprotectin levels were found to contribute significantly ($P = 0.004$) to the observed microbiota variations, followed by BMI ($P = 0.002$) and the Bacteroidetes/Firmicutes ratio ($P = 0.01$). Although ageing has been implied to affect gut microbiota composition later in life (>60 years) (21), age was not found to contribute to the observed variation in microbiota composition ($P = 0.74$). Collectively, our data indicate that a specific "obese" bacterial composition is related to both intestinal and systemic inflammation.

Discussion

Gut microbiota are considered to play an important role in the development of obesity and obesity-associated chronic low grade inflammation. However, the majority of microbiota studies have been performed in rodent models. Human data are more scarce and less consistent. In the present human study, we observed profound differences in fecal microbiota composition that were related to the extent of obesity. Two microbiota clusters were identified by a phylogenetic fingerprinting tool: an obese microbiota cluster on the one hand, which was characterized by diminished bacterial diversity, a decreased ratio of Bacteroidetes to Firmicutes, and associated with intestinal and systemic inflammation, and a nonobese microbiota cluster on the other hand, characterized by a higher bacterial diversity, higher Bacteroidetes/Firmicutes ratio, and a lack of inflammation. We further identified significant differences in relative abundance of specific microbiota in the obese vs. non-obese clusters and subjects. In contrast to findings in animal studies, intestinal permeability was neither altered in obesity nor related to inflammation or to microbiota composition. Our data therefore suggest that in man, the obesity-associated intestinal microbiota modulate intestinal and systemic inflammation independent of gut permeability.

Microbiota have been described to affect the intestinal barrier and promote inflammation by several mechanisms. First of all, proinflammatory bacterial compounds such as endotoxin have been shown to translocate via an increased intestinal permeability in obese rodents (10,11,20). This was also suggested to occur in obese subjects with nonalcoholic steatohepatitis (22). Furthermore, a high fat diet may enhance endotoxin absorption through chylomicron-facilitated transport (23). In addition, it was recently shown that high-fat diet-induced translocation of bacteria over the intestinal wall occurs after phagocytosis by dendritic cells, leading to systemic and adipose tissue inflammation (24). Our data are in best agreement with the last mechanism, since the observed microbiota alterations were not related to transcellular or paracellular gut permeability as probed by oligosaccharides, but nonetheless associated with local intestinal and systemic inflammation. However, we cannot rule out that the limitations of the permeability test in terms of sensitivity and/or specificity precluded the detection of potential effects of the altered microbiota on permeability. Furthermore, we did find an increased gastroduodenal permeability in obesity that could be related to potential microbiota alterations in this part of the gut, which we could not investigate.

Intestinal inflammation was only observed in subjects within the obese microbiota cluster, implying that microbiota in this cluster may have a local proinflammatory activity. Along this line, it is well known that interactions of the microbiota with the intestinal epithelium can either provoke an inflammatory response (25), or can prevent inflammation (26). Given our data, it is conceivable that the bacterial species promoting obesity-associated inflammation belong to the Firmicutes. On the other hand, bacterial species abundantly present in the nonobese microbiota composition may have a protective effect. For instance, *F. prausnitzii*, a butyrate producer from Clostridium cluster IV, was increased in the nonobese subjects. Butyrate and other short chain fatty acids are known to inhibit inflammation by limiting immune cell migration, adhesion, and cytokine production (27). In line with this, *F. prausnitzii* has been found to stimulate anti-inflammatory responses in mice (28), and its abundance was negatively correlated with inflammatory markers in obese subjects (29), suggesting that this microbe belonging to the Firmicutes may protect non-obese subjects from inflammation. Intestinal inflammation with concomitant microbiota alterations has previously been found in obese rats (12,30), which is in line with our

TABLE 3 Relative abundance of bacterial groups that correlate significantly with BMI, CRP, and/or fecal calprotectin levels in non-obese and obese subjects

		Correlation coefficient			Relative abundance	
Level 1	Level 2	BMI	CRP	Calp	Nonobese (%)	Obese (%)
Bacteroidetes						
	Allistipes et rel	−0.642[a]	−0.470[b]		2.733	0.446
	Bacteroides fragilis et rel	−0.552[a]			0.832	0.239
	Bacteroides intestinalis et rel	−0.539[a]	−0.433[b]		0.730	0.210
	Bacteroides plebeius et rel	−0.508[a]	−0.413[b]		1.680	0.413
	Bacteroides splanchnicus et rel	−0.539[a]	−0.429[b]		1.525	0.275
	Bacteroides uniformis et rel	−0.483[a]			0.921	0.264
	Bacteroides vulgatus et rel	−0.499[a]	−0.449[b]		1.663	0.599
	ParaBacteroides distasonis et rel	−0.505[a]	−0.443[b]		2.130	0.583
	Prevotella oralis et rel	−0.389[b]			0.576	0.121
	Prevotella ruminicola et rel	−0.404[b]			0.506	0.158
	Tannerella et rel	−0.511[a]	−0.440[b]		0.815	0.308
Firmicutes						
Bacilli	*Aneurinibacillus*	0.375[b]	0.458[b]		0.004	0.010
	Lactococcus			0.395[b]	0.002	0.002
C. cluster IV	*Faecalibacterium prausnitzii* et rel	−0.374[b]			9.324	6.245
	Papillibacter cinnamivorans et rel	0.522[a]	0.579[a]		0.295	0.775
	Subdoligranulum variable et rel		0.402[b]		3.669	5.377
C. cluster XIVa	*Clostridium colinum* et rel	0.409[b]			0.506	0.780
	Clostridium nexile et rel			0.437[b]	2.094	3.114
	Clostridium sphenoides et rel		0.374[b]		2.898	3.819
	Dorea formicigenerans et rel	0.487[a]			4.154	6.357
	Eubacterium rectale et rel[c]			0.378[b]	3.556	5.175
	Roseburia intestinalis et rel[c]	0.448[b]	0.479[a]		2.366	4.652
	Ruminococcus gnavus et rel	0.396[b]			1.772	2.792
C. cluster XV	*Eubacterium limosum* et rel	0.395[b]			0.001	0.003
C. cluster XVIII	*Coprobacillus catenaformis* et rel	−0.424[b]			0.059	0.026
Proteobacteria						
	Alcaligenes faecalis et rel	−0.416[b]			0.002	0.000
	Enterobacter aerogenes et rel	0.585[a]			0.006	0.018
	Klebsiella pneumoniae et rel	0.530[a]			0.004	0.008
	Vibrio	0.498[a]			0.001	0.003
	Yersinia et rel	0.562[a]			0.001	0.002
Actinobacteria						
	Bifidobacterium	0.386[b]			4.627	6.621

[a]Correlation is significant at the 0.01 level (two-tailed). Grey shading indicates groups negatively correlated to the different variables.
[b]Correlation is significant at the 0.05 level (two-tailed).
[c]Known butyrate producing bacteria.

results in man. Elevated fecal calprotectin levels have previously been reported in obese subjects (31), although microbiota composition was not analyzed. Another study did not observe enhanced fecal calprotectin levels in obese subjects, while, in support of our findings, no relation between gut permeability and obese microbiota composition was found (32). However, the subjects included in that study were less obese and a less sensitive calprotectin assay was used. This may have prevented the detection of the calprotectin levels that we observed, which are considered to be relatively low (31). These low levels might indicate that there is only a low-grade inflammation. The inflammation may be present in all parts of the intestinal tract since fecal calprotectin levels are elevated in subjects with both small intestinal and colonic inflammation (33,34).

The decreased Bacteroidetes/Firmicutes ratio that we observed in obese individuals is supported by results from several other groups (3,4,6,7), although up to now, a direct correlation between this ratio and BMI has never been shown. Contradictory results have even been

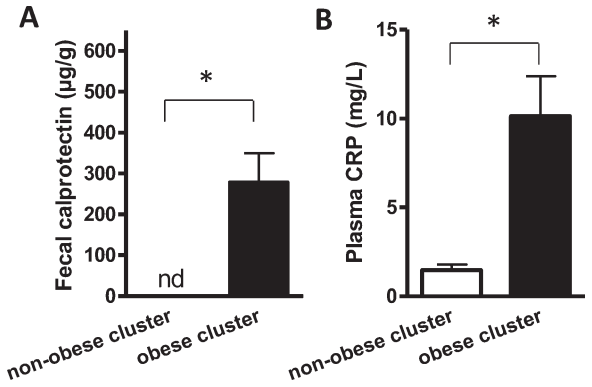

FIGURE 3 The obese microbiota cluster is associated with inflammation. (**a**) None of the subjects within the nonobese microbiota cluster had detectable fecal calprotectin levels, whereas 8 out of 19 subjects (42%) within the obese microbiota cluster showed calprotectin in their feces ($P = 0.02$). Of these subjects, two were overweight and six were obese. ND: not detectable. (**b**) Plasma CRP levels were significantly higher in subjects within the obese microbiota cluster as opposed to subjects within the nonobese microbiota cluster ($P < 0.01$).

reported, e.g., a similar microbiota composition in lean and obese subjects (9), or even an opposite change in Bacteroidetes/Firmicutes ratio in obesity (8). These conflicting data may be attributable to factors such as diet (5), recent use of antibiotics (35), host physiology (30), and the presence of obesity associated comorbidity such as insulin resistance (36). Perhaps more importantly, the subjects included in these studies were less obese than in the current study. Our data indicate that a decreased Bacteroidetes/Firmicutes ratio is particularly characteristic of severely obese individuals with a BMI > 35 kg m^{-2}.

Subjects with type 2 diabetes were recently shown to have a different microbiota profile (37). In our study, obese subjects showed a minor increase in HbA_{1c}, which was no longer significant when the population was divided into clusters according to intestinal microbiota composition. In line with this, multivariate analysis also indicated that HbA_{1c} was not related to differences in microbiota composition. Likewise, multivariate analysis did not show that age contributed to the observed microbiota composition differences. This is further supported by studies showing that gut microbiota composition of adults between the age of 20 and 50 is relatively stable (15,21,38). Nonetheless, the relationship between microbiota composition and inflammation here described needs to be confirmed in larger studies taking into account factors such as the presence of type 2 diabetes, diet, geography, and age.

The observed increase in Firmicutes and concomitant decreased Bacteroidetes/Firmicutes ratio in obese subjects could be mainly attributed to an increased abundance of *Clostridium* cluster XIVa, which contains many butyrate producing species. Interestingly, an increased synthesis of short chain fatty acids such as butyrate by obesity-associated microbiota has been suggested to contribute to increased energy harvesting in obesity (3,8). Even though it remains speculative to imply a cause and effect relationship, *Clostridium* cluster XIVa species may actively contribute to the development of obesity. More evidence for this hypothesis comes from a recent study showing that modulation of specific bacteria within *Clostridium* cluster XIVa, i.e., *Roseburia* spp, which we also identified to be related to BMI and CRP, improves body weight, insulin sensitivity, and hepatic steatosis in mice (39).

The causes of the microbiota composition changes and the associated intestinal inflammation in obesity remain speculative, though we previously found evidence for a potential involvement of Paneth cells (40). Obese subjects displayed diminished levels of Paneth cell derived

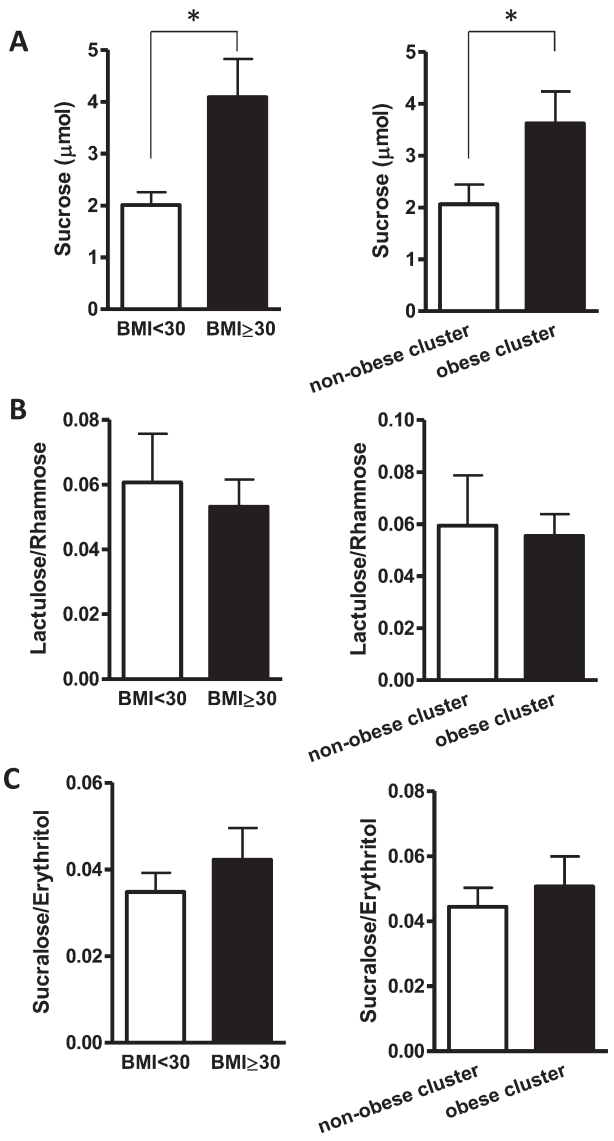

FIGURE 4 Permeability of the gastro-intestinal tract in nonobese vs. obese subjects and in obese vs. nonobese microbiota clusters. (**a**) Significantly higher gastroduodenal permeability in obese subjects and in subjects within the obese microbiota cluster, as reflected by elevated urinary sucrose levels after 1 h (4.1 ± 0.7 µmol vs. 1.9 ± 0.3 µmol, $P = 0.003$ in obese compared to nonobese subjects and 3.6 ± 0.6 µmol vs. 2.1 ± 0.4 µmol, $P = 0.03$ for the obese microbiota cluster). (**b**) A similar lactulose/rhamnose ratio was observed in both obese and nonobese subjects (0.06 ± 0.02 vs. 0.05 ± 0.01; $P = 0.9$) and obese and nonobese microbiota clusters (0.06 ± 0.02 vs. 0.06 ± 0.01; $P = 0.7$), indicating comparable small intestinal permeability. (**c**) The sucralose/erythritol ratio reflecting large intestinal permeability was not significantly different between either nonobese and obese subjects (0.03 ± 0.01 vs. 0.04 ± 0.01; $P = 0.65$), or between the non-obese and obese microbiota cluster (0.04 ± 0.01 vs. 0.05 ± 0.01; $P = 0.74$).

antimicrobial proteins. Strikingly, Paneth cells are pivotal in limiting bacterial translocation, thereby inhibiting systemic inflammation.

In conclusion, we present here the first evidence that a human obesity-associated microbiota profile is associated with both intestinal and systemic inflammation. Because no relation between the obese microbiota composition and intestinal permeability was found, our data suggest that microbiota-derived factors may directly promote inflammation in obesity. O

Acknowledgments

We thank Dr. Hans van Eijk and Babs Bessems for their contribution to the analyses of the urine samples, Annemarie van Bijnen for assistance with the calprotectin assay, and Kim van Wijck for assistance with the permeability test. We further acknowledge Wilma Akkermans-van Vliet and Ineke Heikamp-de Jong for technical assistance in performing HITChip experiments.

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