Correlation between body mass index and faecal microbiota from children

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Abstract

Childhood obesity is an increasing problem at the global level and considered as a risk factor for obesity development and the associated co-morbidities in adult life. In this study, the occurrence of *Bacteroides fragilis* group, *Clostridium* spp., *Bifidobacterium* spp. and *Escherichia coli* in 84 faecal samples from 30 obese, 24 overweight and 30 lean children was verified by culture technique and quantitative determination by quantitative PCR. In addition, *Lactobacillus* spp. and *Methanobrevibacter smithii* were also analysed. A correlation between the body mass index (BMI) and these bacteria was sought. *Bacteroides vulgatus*, *Clostridium perfringens* and *Bifidobacterium adolescentis* were most prevalent in all samples evaluated by culture-method. The *B. fragilis* group were found at high concentrations in obese and overweight children when compared with the lean ones (p 0.015). The obese and overweight children harboured higher numbers of *Lactobacillus* spp. than lean children (p 0.022). The faecal concentrations of the *B. fragilis* group (r = 0.24; p 0.026) and *Lactobacillus* spp. (r = 0.44; p 0.002) were positively correlated with BMI. *Bifidobacterium* spp. were found in higher numbers in the lean group than the overweight and obese ones (p 0.042). Furthermore, a negative correlation between BMI and *Bifidobacterium* spp. copy number (r = −0.22; p 0.039) was observed. Our findings show some difference in the intestinal microbial ecosystem of obese children compared with the lean ones and a significant association between number of *Lactobacillus* spp. and *B. fragilis* group and BMI.

Keywords: Anaerobic bacteria, body mass index, childhood obesity, *Escherichia coli*, faecal microbiota, quantitative PCR
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Introduction

Obesity is a consequence of the massive fat mass expansion corresponding to the body mass index (BMI) >30 kg/m^2 [1]. The high incidence rate of childhood obesity or overweight is a risk factor for obesity in adult life and it has been found to be associated with co-morbidities such as diabetes mellitus, coronary diseases, respiratory disorders and cancer [2,3].

According to the World Health Organization, obesity prevalence is increasing among adults, adolescents and children worldwide, and it is being considered as a public health problem [3]. The aetiology of obesity is complex and involves environmental, genetic, endocrine and neural factors [4,5]; and recently, many studies have associated obesity development with a specific profile of gut microbiota [6–8].

The gut microbiota enables enzymatic digestion of non-digestible polysaccharides producing absorbable monosaccharides; and it activates lipoprotein lipase on intestinal epithelium, which causes rapid absorption of glucose and fatty acids, contributing to the fat mass expansion and weight gain [9,10].

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Reports based on quantitative methods and pyrosequencing show differences in the faecal microbial composition of obese individuals displaying high levels of *Firmicutes* and a lower proportion of the phyla *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Archaea* [11–13].

Species of *Lactobacillus*, *Escherichia coli* and *Staphylococcus aureus* are found to be associated with obesity, whereas *Bifidobacterium* spp. and *Methanobrevibacter smithii* are found in high concentrations in lean individuals [14–16]. Intestinal microbiota composition in adults and infants has been extensively evaluated; however, there are few studies analysing intestinal microbiota composition in individuals older than 2 years [10,17]; and the obtained reports are controversial [4,5].

In this study, the occurrence of the *Bacteroides fragilis* group, *Clostridium* spp., *Bifidobacterium* spp. and *E. coli* in obese, overweight and lean children was verified using a culture-based technique. These bacterial species are representative of resident members of the intestinal microbiota and their viability is of interest to determine their presence in this ecosystem. In addition, a determination by real-time PCR was also performed to quantify these bacteria, and also *Lactobacillus* spp. and *M. smithii* because of their frequent association with weight gain. Finally, a correlation between bacterial quantification and BMI was established.

Materials and Methods

Children and sample collection

Faecal samples were obtained from healthy children at the Institute of Children (Hospital das Clinicas) and private and municipal schools of Sao Paulo city, SP, Brazil. Demographic and clinical data (date of birth, weight, height, gender, length/weight of birth, birth delivery and clinical history) were recorded using a standardized questionnaire. Children were grouped based on Z-score as follows: lean children (≥ -2 and $< +1$), overweight children ($\geq +1$ and $< +2$) and obese children ($\geq +2$). The Z-score was calculated using WHO Anthro Plus Software, taking gender, age, weight and height into consideration [18,19]. All children were 3–11 years old without diarrhoea, and none of them had undertaken antibiotic therapy in at least the 3 months prior to the sample collection. Faeces were collected in sterile universal collecting vials and immediately stored at −80°C until use.

Analyses of the gut microbiota

Bacterial isolation. Fresh faeces were streaked onto selective media *Bacteroides* Bile Esculin agar (BBE) for the *B. fragilis* group, Cycloserin Cefoxitin Fructose agar (CCFA) supplemented with 5% blood for *Clostridium* spp., *Bifidobacterium* modified agar (using as base Reinforced *Clostridium* Medium, dextrose, and L-cysteine) for *Bifidobacterium* spp. and MacConkey agar for *E. coli*. The streaked plates (BBE, CCFA and *Bifidobacterium* agar) were incubated in anaerobic conditions for 5 days and MacConkey agar was incubated in aerobic conditions for 24 h at 37°C. To assure proper bacterial identification, four characteristic colonies for each microorganism were subcultured on blood or Luria–Bertani agar. Bacterial DNA from the *B. fragilis* group, *Bifidobacterium* spp. and *E. coli* was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The *Bacteroides fragilis* group was identified by multiplex-PCR assays [20]; and *Bifidobacterium* spp. [21] and *E. coli* [22] by conventional PCR using 16S rRNA primers. Species of *Clostridium* were identified using an API 20A kit (BioMérieux, Rio de Janeiro, RJ, Brazil).

Faecal DNA extraction. Total DNA was obtained from the collected faeces by using a QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions. DNA concentrations were determined by spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, USA), and 10 µL of each DNA sample was checked for integrity on 1% agarose gel. DNA samples were stored at −80°C until use.

Bacterial quantification by real-time PCR

The PCR assays were performed using 16S rRNA genes species-specific sequences (Table 1). DNA amplifications were performed in final volumes of 20 µL containing: 2× SYBR® Green PCR Master Mix (GoTaq qPCR Master Mix, Promega Corporation, Sao Paulo, Brazil), 5 µM of each primer and 2 ng of faecal DNA. Amplification reactions were performed in a Rotor Gene 6000 (Corbett Life Science, Mort lake, NSW, Australia) programmed as follows: initial denaturation at 95°C for 10 min; 40 cycles of 95°C for 15 s and annealing temperature suitable for each primer pair for 60 s.

Statistical analyses

The clinical parameters were analysed by Kruskal–Wallis (Dunn), analysis of variance (Tukey) and chi-square tests. A comparison of quantitative bacterial detection among lean, overweight and obese children was performed with Kruskal–Wallis test. Possible correlations between BMI and quantitative bacterial detection were evaluated by Spearman's test (r). Taking into account possible confounders like age and gender, a model to identify bacteria whose presence was closely related with BMI was obtained using multiple linear regressions. Variables included in the model were: concentrations of all bacteria (values in $\log_{10}$), age and gender. A further logistic regression method based on the three weight categories (lean = 1; overweight = 2; obese = 3) was performed. Overweight and obese

TABLE 1. 16S rRNA oligonucleotides used to detected bacterial groups or species, and real-time PCR conditions

Microorganisms	Oligonucle0tides 5′ → 3′	Tm (°C)	Amplicon (bp)	Strain[c]	References
Bacteroides fragilis group[a]	F: GAG GAA GGT CCC CCA CAT TG R: TCC TTC ACG CTA CTT GGC TG	60	113	*B. fragilis* ATCC 25285	This study
Clostridium Cluster I[b]	F: ATG CAA GTC GAG CGA KG R: TAT GCG GTA TTA ATC TYC CTT T	60	120	*C. perfringens* ATCC 13124	[34]
Lactobacillus spp.	F: AGC AGT AGG GAA TCT TCC A R: ATT YCA CCG CTA CAC ATG	60	380	*L. acidophilus* ATCC 4356	[35]
Bifidobacterium spp.	F: GCG TGC TTA ACA CAT GCA AGT C R: CAC CCG TTT CCA GGA GCT ATT	60	125	*B. bifidum* ATCC 15696	[35]
Escherichia coli	F: AGA AGC TTG CTC TTT GCT GA R: CTT TGG TCT TGC GAC GTT AT	60	120	*E. coli* ATCC 25922	[36]
Methanobrevibacter smithii	F: AGG TAC TCC CAG GGT AGA GG R: TCC CTC ACC GTC AGA ATC G	59	92	*M. smithii* ATCC 35061	This study

[a]*Bacteroides fragilis* group: *B. fragilis*, *B. vulgatus*, *B. uniformis*, *B. eggerthii*, *B. thetaiotaomicron*, *B. ovatus* and *B. caccae*.
[b]*Clostridium* cluster I: *C. perfringens*, *C. homopropionicum*, *C. cadaveris*, *C. intestinalis*, *C. putrificum*, *C. botulinum*, *C. novyi*, *C. sporogenes*, *tyrobutyricum*, *C. kluyveri*, *C. ljungdahlii*, *C. scatologenes*, *C. acetireducens*, *C. subterminale*, *C. estertheticum*, *C. argentinense*, *C. sardiniensis*, *C. paraputrificum*, *C. longisporum*, *C. septicum*, *C. cellulovorans*, *C. baratti*, *C. absonum*, *C. chauvoei*, *C. carnis*, *C. butyricum*, *C. beijerinckii*, *C. kainantoi*, *C. corinoforum*, *C. puniceum*, *C. histolyticum*, *C. proteolyticum*, *C. limosum*.
[c]Strains used to the standard curve construction.

groups were compared with the lean group, which was used as control. For all statistic analyses, significance levels of 5% were obtained with the GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA, USA), and BioEstat 2009 version 5.3.5 as statistical packages.

Results

Of the 84 children enrolled in this study, 30 were obese, 24 were overweight and 30 were lean. No statistically significant differences were observed regarding gender (p 0.27), weight at birth (p 0.63), length at birth (p 0.25) and birth-delivery mode (p 0.8). Significant differences for age (p 0.008) and BMI ($p \leq 0.001$) were observed (Table 2).

The children harboured at least one species of the *Bacteroides fragilis* group, *Clostridium* spp., *Bifidobacterium* spp. or *E. coli* by culture-method. All obese children harboured species of the *Bacteroides fragilis* group and *E. coli*, 22 (77.3%) *Clostridium* spp. and 23 (76.6%) *Bifidobacterium* spp. Among the 24 overweight children, 20 (83.3%) harboured *B. fragilis* group, 17 (70.8%) *Clostridium* spp., 15 (62.5%) *Bifidobacterium* spp. and 21 (87.5%) *E. coli*. Among the 30 lean children, 21 (70%) harboured species of the *B. fragilis* group, 16 (48%) *Clostridium* spp., 18 (60%) *Bifidobacterium* spp. and 28 (93.3%) *E. coli*. *Bacteroides vulgatus*, *Clostridium perfringens*, *Bifidobacterium adolescentis* and *E. coli* were prevalent in all three groups (Table 3).

TABLE 2. Demographic parameters obtained from the evaluated children

Parameters	Obese ($n = 30$)	Overweight ($n = 24$)	Lean ($n = 30$)	p value
Age (years), mean ± SD[a]	8.5 ± 2.6[a]	8 ± 2[a]	6.1 ± 2.4	0.008[c]
Gender (male:female)	19:11	14:10	13:17	0.273[d]
BMI[b] (kg/m^2), mean ± SD	27.12 ± 5.9[a,b]	19.67 ± 1.62[a]	16.06 ± 1.18	<0.001[c]
BMI (z-score), mean ± SD	3.5 ± 1.6[a,b]	1.68 ± 0.33[a]	0.19 ± 0.72	<0.0001[c]
Birth weight (kg), mean ± SD	3.3 ± 0.7	3.15 ± 0.56	3.14 ± 0.68	0.632[c]
Birth length (cm), mean ± SD	48.3 ± 3.4	47.3 ± 3.2	46.2 ± 5.8	0.253[c]
Birth-delivery mode, (caesarean:vaginal delivery)	20:10	14:10	18:12	0.792[d]

Abbreviations: BMI, body mass index; SD, standard deviation.
[a]Overweight and obese ≠ lean.
[b]Obese ≠ overweight;
[c]Analysis of variance and Tukey's test.
[d]Chi-square test.

Bacterial quantitative analyses by real-time PCR revealed the occurrence of the *B. fragilis* group and *Lactobacillus* spp. in the children and it was higher in obese and overweight children than in lean ones (p <0.05) (Table 4).

Lower quantitative values (number of copies) for *Bifidobacterium* spp. were observed in obese and overweight children compared with the lean ones (p 0.042) by real-time PCR. The PCR quantification of *Clostridium* Cluster I, *M. smithii* and *E. coli* in the three groups of children did not show significant differences (Table 4).

By using Spearman's test a positive and significant correlation between the high concentration of *Lactobacillus* spp. ($r = 0.44$; p 0.002) or *B. fragilis* group ($r = 0.24$; p 0.026) with BMI was observed. The concentration of *Bifidobacterium* spp. was high in the lean group; a negative correlation between this microorganism and BMI ($r = -0.22$; p 0.039) was verified. For Cluster I, *E. coli* and *M. smithii* showed no association with BMI (Fig. 1).

A significant correlation between the increased BMI values associated with quantification of *Lactobacillus* spp. ($r = 0.51$; p 0.006) and *M. smithii* ($r = 0.38$; p 0.042) in obese children was found. In overweight and lean groups no significant correlation between evaluated microorganisms with BMI was observed (see Supplementary material, Figs S1, S2 and S3,).

Differences in gender were observed in *Bifidobacterium* spp. copy number in obese children. Specifically, obese girls had

TABLE 3. Bacterial species identified in feces from 30 obese, 24 overweight and 30 lean children by culture method

Microorganisms	Obese (n = 30) Prevalence[a] n (%)	Overweight (n = 24) Prevalence n (%)	Lean (n = 30) Prevalence n (%)	p value[b]
Bacteroides fragilis group				
B. vulgatus	24 (80)	10 (41.6)	16 (53.3)	0.011
B. fragilis	3 (10)	2 (8.3)	3 (10)	0.055
B. ovatus	3 (10)	5 (20.8)	1 (3.3)	0.116
B. caccae	0	2 (8.3)	0	ND
B. uniformis	0	2 (8.3)	0	ND
B. stercoris	0	0	2 (6.6)	ND
P. distasonis	11 (36.6)	8 (33.3)	6 (20)	0.333
P merdae	2 (6.6)	1 (4.1)	2 (6.6)	0.908
Clostridium spp.				
C. perfringens	11 (36.6)	8 (33.3)	8 (26.6)	0.701
C. clostridioforme	4 (13.3)	1 (4.1)	1 (3.3)	0.257
C. glycolicum	0	1 (4.1)	1 (3.3)	ND
C. innocuum	4 (13.3)	5 (20.8)	2 (6.6)	0.308
C. sordelli	0	0	1 (3.3)	ND
C. paraputrificum	2 (6.6)	1 (4.1)	2 (6.6)	0.908
C. baratii	2 (6.6)	1 (4.1)	0	ND
C. difficile	1 (3.3)	1 (4.1)	0	ND
C. sporogenes	0	1 (4.1)	1 (3.3)	ND
C. septicum	1 (3.3)	0	0	ND
C. tertium	1 (3.3)	0	0	ND
Clostridium sp.	14 (46.6)	10 (41.6)	5 (16.6)	0.034
Bifidobacterium spp.				
B. adolescentis	19 (63.3)	13 (54.1)	15 (50)	0.569
B. infantis	15 (50)	5 (20.8)	8 (26.6)	0.048
Escherichia coli	30 (100)	21 (70)	28 (93.3)	0.152

ND, without sufficient positive samples to perform the chi-square test.
[a]Prevalence reflects the number of positive samples by culture-based technique.
[b]Chi-square test was applied.

significantly higher *Bifidobacterium* spp. levels than obese boys (p <0.05). Further stratification of bacterial copy number by gender revealed significantly higher *Lactobacillus* spp. levels in obese and overweight girls compared with the lean girls (p 0.001); there was no difference in *Lactobacillus* spp. levels between obese, overweight and lean boys. For the others microorganisms evaluated no statistically significant differences were observed (Table 5).

By linear regression analysis, bacterial concentrations (quantitative PCR) were correlated with BMI and a significant association between *Lactobacillus* spp. (coefficient 0.718; 95% CI 0.024–1.412; p 0.043), *B. fragilis* group (coefficient 3.731; 95% CI 0.765–6.758; p 0.015) and age (coefficient 1.109; 95% CI 0.680–1.537; p <0.001) was observed (see Supplementary material, Table S1). In a logistic regression the variables eligible for the final model were *Bifidobacterium* spp., *Lactobacillus* spp., *B. fragilis* group and age. The logistic regression analysis showed that *Lactobacillus* spp. and age are significantly associated with overweight; *B. fragilis* group and age are associated with obese children; and *Bifidobacterium* spp. were associated with lean children (Table 6). These results confirm the findings obtained by univariate analysis.

Discussion

Studies have shown that the gut microbiota composition is associated with diet, host genetic, socio-economic status, lifestyle and others diseases, including allergy, obesity and type 2 diabetes mellitus [23–25]. Exogenous factors such as vaginal or caesarean delivery, breastfeeding and administration of antibiotics in infants can affect the intestinal microbial diversity; however, it is not clear if these factors might have an obesogenic effect [26]. In this study, no differences were found between delivery mode, weight and length at birth and BMI, indicating no association between these factors and weight gain in the evaluated children.

Qualitative culturing was used to identify and characterize strains with specific traits, and to determine their viability in the intestinal ecosystem. Culture-base technique is a 'gold standard' for isolation of selected bacterial group and it can help to

TABLE 4. Bacterial prevalence and quantification verified in faeces of obese, overweight and lean children by quantitative PCR

	Obese (n = 30)	Overweight (n = 24)	Lean (n = 30)	Total (n = 84)	p value
Presence of genus or species[a]					
Bacteroides fragilis group	30 (100%)	24 (100%)	30 (100%)	84 (100%)	ND
Clostridium Cluster I	29 (96.6%)	24 (100%)	30 (100%)	83 (98.8%)	ND
Bifidobacterium spp.	28 (93.3%)	24 (100%)	30 (100%)	82 (97.6%)	ND
Lactobacillus spp.	29 (96.6%)	24 (100%)	27 (90%)	80 (95.2%)	ND
Escherichia coli	30 (100%)	23 (95.8%)	30 (100%)	83 (98.8%)	ND
Methanobrevibacter smithii	20 (66.6%)	21 (87.5%)	27 (90%)	68 (80.9%)	0.044
Quantitative determination (log_{10} copies/g faeces)[b]					
Bacteroides fragilis group	9.2 (9–9.6)[c]	9.1 (8.9–9.5)[c]	8.9 (8.7–9.7)	9.1 (8.9–9.7)	0.015
Clostridium Cluster I	4 (2.8–6.3)	4 (3–6.2)	4.4 (3.7–6.2)	4.1 (3.1–6.3)	0.702
Bifidobacterium spp.	7 (6.6–8.6)	6.8 (6.2–9.8)	7.6 (7.2–8.9)[d]	7.3 (6.6–9.8)	0.042
Lactobacillus spp.	5.7 (5.2–7.8)[c]	5.5 (5.3–7.5)[c]	5.2 (4.8–6.2)	5.5 (5–7.8)	0.022
Escherichia coli	7.3 (6.8–8.5)	7.7 (6.6–9.2)	7.5 (6.9–9.4)	7.5 (6.9–9.4)	0.672
Methanobrevibacter smithii	4.1 (0–8.4)	4.5 (3.9–8.6)	4.5 (3.8–8.8)	4.4 (3.7–8.8)	0.262

ND, without sufficient positive samples to perform the chi-square test.
[a]Values noted as number (percentage), chi-square test.
[b]Data were presented as median (interquartile range; IQR); differences among three groups were compared using Kruskal–Wallis test (Dunn post-test).
[c]p < 0.05 indicated significant differences as compared with the lean group.
[d]p <0.05 indicated significant differences compared with the obese and overweight groups.

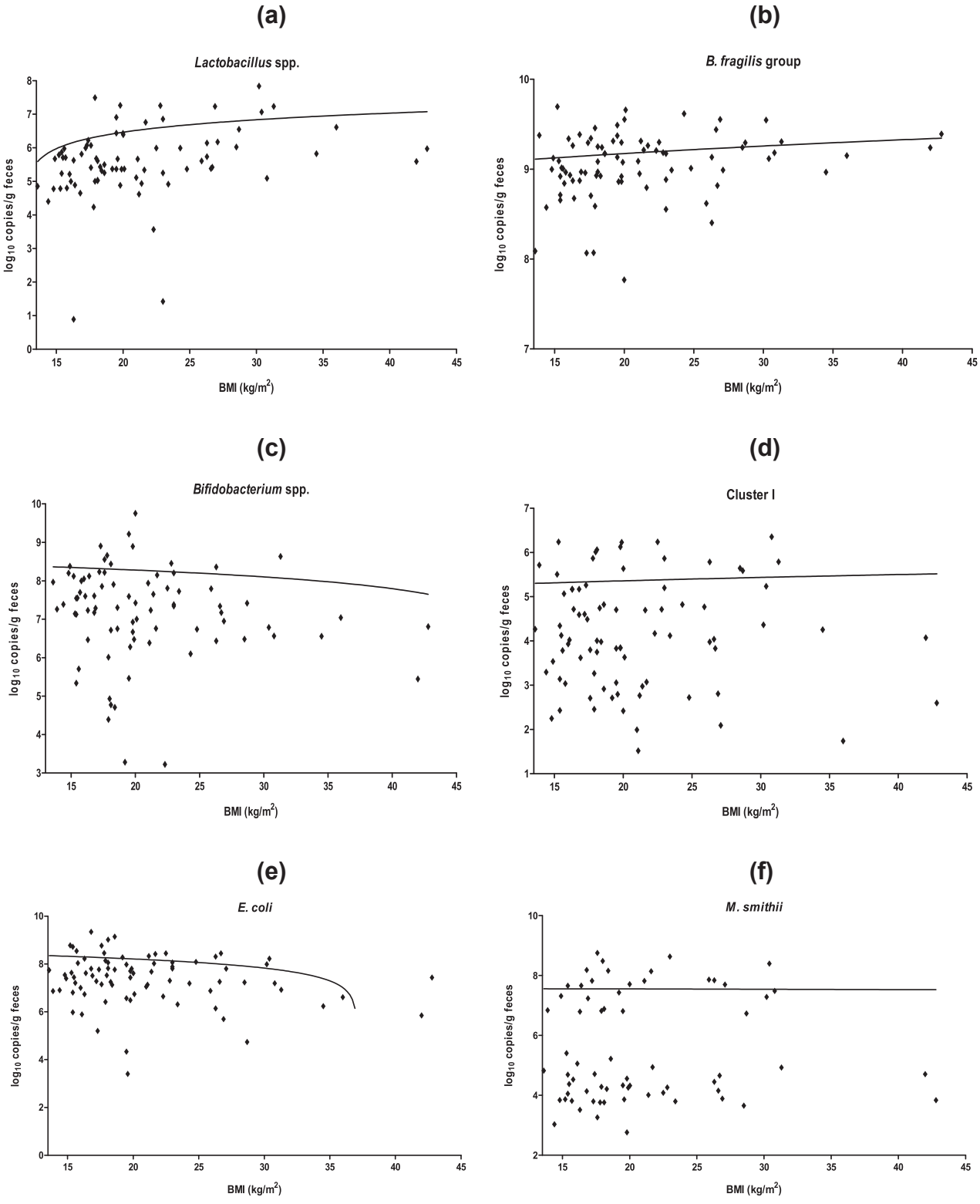

FIG. 1. Correlation between bacterial copies detected by quantitative PCR and body mass index (BMI) in total children (n = 84). Spearman correlation test (r): (a) *Lactobacillus* spp. (r = 0.44; p 0.002), (b) *Bacteroides fragilis* group (r = 0.24; p 0.026), (c) *Bifidobacterium* spp. (r = −0.22; p 0.039), (d) Cluster I (r = 0.00; p 0.966), (e) *Escherichia coli* (r = −0.12; p 0.254) and (f) *Methanobrevibacter smithii* (r = −0.05; p 0.599).

TABLE 5. Univariate analysis of the bacterial association with body mass index levels by gender

Variables	Obese children (n = 30)		Overweight children (n = 24)		Lean children (n = 30)		Total (n = 84)		
	Pr[a]	Median (IQR)	Pr[a]	Median (IQR)	Pr[a]	Median (IQR)	Pr[a]	Median,(IQR)	p value
Girls		(n = 11)		(n = 10)		(n = 17)		(n = 38)	
Bacteroides fragilis group	11	9.1 (9–9.6)	10	9.1 (8.9–9.4)	17	8.9 (8.6–9.4)	40	9.0 (8.7–9.6)	0.190
Clostridium Cluster I	11	4.8 (2.9–5.9)	10	3.6 (2.8–6.2)	17	4.6 (3.8–6.2)	40	4.2 (3.1–6.2)	0.280
Bifidobacterium spp.	11	7.4 (7–8.4)[b]	10	7.5 (6.7–9.8)	17	8 (7.3–8.9)	40	7.7 (7.1–9.8)[b]	0.339
Lactobacillus spp.	11	6.1 (5.3–7.2)[c]	10	5.9 (5.4–7.5)[c]	14	4.8 (4.2–6.1)	37	5.5 (4.9–7.5)	0.001
Escherichia coli	11	7.1 (6.7–8.4)	9	7.8 (6.7–8.8)	17	7.3 (6.9–8.6)	39	7.2 (6.8–8.8)	0.708
Methanobrevibacter smithii	7	4.5 (0–8.4)	9	6.5 (3.6–8.6)	16	4.8 (3.8–8.8)	33	4.7 (3.8–8.8)	0.731
Boys		(n = 19)		(n = 14)		(n = 13)		(n = 46)	
Bacteroides fragilis group	19	9.2 (9–9.6)	14	9.1 (8.9–9.5)	13	8.9 (8.8–9.7)	44	9.1 (8.9–9.7)	0.159
Clostridium Cluster I	18	4 (2.9–6.3)	14	4.7 (3.9–6.2)	13	4.1 (3.6–6.1)	43	4.1 (3.5–6.3)	0.384
Bifidobacterium spp.	17	5.1 (6.3–8.6)	14	6.6 (5.1–9.2)	13	7.5 (7.2–8.4)	42	6.9 (6.2–9.2)	0.209
Lactobacillus spp.	18	5.6 (5–7.8)	14	5.4 (5.3–7.3)	13	5.6 (5.2–6.2)	43	5.5 (5.2–7.8)	0.955
Escherichia coli	19	7.3 (7.1–8.5)	14	7.6 (6.8–9.2)	13	7.8 (7.4–9.4)	44	7.5 (7.2–9.4)	0.414
Methanobrevibacter smithii	13	4 (0–7.8)	12	4.5 (4–8.5)	11	4.3 (3.9–7.3)	35	4.3 (3.6–8.5)	0.332

Data were presented as $\log_{10}$ median (interquartile range; IQR); differences among three groups were compared using Kruskal–Wallis test (Dunn post-test). $p < 0.05$.
[a]Prevalence (Pr) reflects the number of positive samples by quantitative PCR assay.
[b]$p < 0.05$, indicates significant differences between girls and boys in obese and total group. No significant differences between girls and boys were found in either the overweight group or the lean group.
[c]$p < 0.05$ indicates significant differences compared with the lean group.

TABLE 6. Factors associated with body mass index based on multiple logistic regression (logistic regression analysis using quantitative PCR results; n = 84 children)

	Overweight[a]		Obese[a]	
	OR (95% CI)	p value	OR (95% CI)	p value
Bifidobacterium spp.	0.632 (0.389–1.024)	0.062	0.600 (0.369–0.977)	0.040
Lactobacillus spp.	1.781 (1.034–3.068)	0.037	1.321 (0.913–1.913)	0.140
Bacteroides fragilis group	4.206 (0.662–26.738)	0.128	15.863 (1.997–126.016)	0.009
Age	1.382 (1.053–1.814)	0.020	1.612 (1.225–2.120)	0.001

[a]Overweight and obese groups were compared with the lean group (used as control).

elucidate the host–microbiota interaction in obesity development [7,15].

In addition to qualitative culturing, real-time PCR was also used for a better determination of bacterial number in each group of children. These results were used to determine a possible association of bacterial number with BMI for each group. Species-specific primers used in quantitative PCR have shown good reproducibility, sensitivity and specificity; however, significant differences between culture and PCR have also been observed for *Lactobacillus* spp. by Million *et al.*, [15]. In this study, PCR showed much more sensitivity than culture to detect selected species of *B. fragilis* group, *Bifidobacterium*, *Clostridium* and *E. coli*.

The obese children showed high numbers of viable bacteria compared with the overweight and lean children. However, the obese children showed low bacterial diversity when compared with the lean and overweight children, similar to that observed by Karlsson *et al.* [5]. Interestingly, overweight and lean children harboured similar numbers of bacterial isolates, and these results might be explained because of the transition stage from lean to overweight.

The *B. fragilis* group were detected by quantitative PCR in higher concentrations in obese and overweight children than in lean children, and it showed a significant correlation with BMI and weight gain. It is known that the *B. fragilis* group is predominant in the intestinal resident microbiota. Obese or overweight children eat a diet rich in carbohydrates, which can be used by the host to store as fat and by intestinal *Bacteroides* to produce short-chain fatty acids, increasing a risk for development of obesity [12,35]. Previous studies on children found no positive association between the *Bacteroides* level and BMI [16,17,24,27]. These differences in the results can be ascribed to the different methodologies used in the studies.

In accordance with previous findings [16,28], in this study, *Lactobacillus* spp. were present in higher concentrations in the obese and overweight subjects; and a positive correlation between this microorganism and BMI was observed. Some studies correlated the specie *Lactobacillus reuteri* with BMI in adults [8,15], though we have not evaluated this species, the continual findings of *Lactobacillus* spp. indicate a strong association between this genus and obesity, not only in adulthood but also during infancy.

Moreover, gender differences in the levels of *Lactobacillus* spp. were observed in the obese and overweight children but not in the lean group (Table 5), which is in disagreement with Xu *et al.* [27], and Mueller *et al.* [29], whereas the cause of this difference is unclear. Differences observed in girls and boys regarding the qualitative intestinal microbiota, may be due to hormonal, endocrine, behavioural and socio-economic factors; whichever, this has not been, evaluated herein.

Species of *Bifidobacterium*, especially *B. adolescentis*, were recovered more from obese and overweight children compared with lean but showed inverse numbers in real-time PCR analysis. In addition, a negative and significant correlation between *Bifidobacterium* spp. levels and BMI was observed; suggesting that this genus may not be associated with weight gain.

Bifidobacterium spp. and *Lactobacillus* spp. are considered benefical bacteria in the human intestinal microbiota, but gut colonization by these lactic acid bacteria are easily influenced by diet. Probiotics have been used to increase the weight gain in animals for decades, therefore the oral administration of specific probiotics might be a possible factor involved in obesity development in humans [15].

Clostridium cluster I was observed in children but with no statistical difference in the number of copies. Zuo *et al*. [30], by using bacterial cultivation and counting technique, found lower numbers of *C. perfringens* in obese adults. In our study, similar percentages for *C. perfringens* and *Clostridium* cluster I were found in lean, overweight and obese children. Certainly, the role of these microorganisms in the intestinal microbiota of obese subjects needs further investigation.

Enterobacteriaceae is a family of gram-negative commensal bacteria; mainly *E. coli* is predominant in the intestinal ecosystem during childhood [31]. As expected, most of the children showed a high prevalence of *E. coli* as determined by culture and molecular techniques. In contrast to a previous study by Karlsson *et al*. [5], our study found an inverse correlation with BMI, suggesting no association with increased BMI. Considering the mean age of the obese children, our results are in accordance with the data reported by Million *et al*. [8], who also detected lower bacterial loads in obese adults.

Methanogenic archaea are important because they are involved in the removal of excess H_2 from the mammalian gut. *Methanobrevibacter smithii* is the most common archaea found in the human intestinal microbiota [32,33]. As the lean children showed high numbers of *M. smithii* compared with overweight and obese children, the presence of this microorganism might not be associated with obesity, in accordance with other reports [8,12].

In addition, our results suggest that the quantitative changes of species of the *B. fragilis* group and *Lactobacillus* spp. are associated with obesity; although no association with birth delivery mode or weight at birth was observed. The better knowledge of the intestinal microbiota composition in obese, overweight and lean individuals and its interaction with host endocrine factors could help elucidate obesity development.

Transparency declaration

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2015.10.031.

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