Supplemental Material

Gram staining

The gram staining technique is used to differentiate prokaryotes into two groups based on differences in staining and form (i.e., rods and cocci) (1) . Gram-positive prokaryotes stain purple because their relatively impermeable cell wall contains a thick layer of peptidoglycan that retains crystal violet. In contrast, gram-negative prokaryotes have a thin peptidoglycan layer that does not retain crystal violet during decolorization; therefore, the cells are stained red (2). The archaeal cell wall does not contain peptidoglycan, and its envelope structure is very different from that of the eubacterial envelope (3). Gram stain results for archaeal cells are variable: some *Archaea,* such as *Methanospirillum bungatei*, stain gram-negative (3), whereas *Methanobrevibacter smithii*, *Methanosphaerae stadtmanae* and *Methanomassiliicoccus lumyniensis,* the 3 archaeal species that have been isolated from the human gut microbiota to date (4,5), stain gram-positive (6).

Quantification by flow cytometry

For flow cytometric analyses, the BD LSR Fortessa™ Cell Analyzer (Becton Dickinson, Franklin Lakes, NJ, USA) was used, equipped with 3 lasers (a 405 nm purple laser, a 488 nm blue laser and a 633 nm red laser). We quantified bacteria using a suspension of fluorescent microspheres (Cytocount™, DakoCytomation, Glostrup, Denmark) as a reference population.

Transmission electron microscopy

The samples were washed with PBS and fixed overnight in 2% glutaraldehyde in 0.1 M cacodylate buffer. After washing 3 times in 0.1 M cacodylate buffer, the specimens were post-fixed in 1% OsO2 in potassium ferricyanide (0.1 M) (7) for one hour, washed 3 times with a water solution and dehydrated in an ascending series of ethanol dilutions ranging from 30% to 100%. Finally, the samples were submerged in Epon 815 resin (8). After

polymerization for 3 days, the blocks of resin containing the samples were cut using a microtome (a transversal cut was made, which is why differentiating between cocci and rods is not possible). Sections with a thickness of 70 nm were stained with 3.5% uranyl acetate and lead citrate before examination in a TEM Philips Morgagni™ 268(D) (FEI, Hillsboro, Oregon) at 60 kV.

16S rDNA pyrosequencing

In the first case, the forward and reverse primers were designated 454A_917F (CGTATCGCCTCCCTCGCGCCATCAGGAATTGACGGGGRCCC) and 454B_1391R (CTATGCGCCTTGCCAGCCCGCTCAGGACGGGCGGTGWGTRCA). The 13 others samples were amplified to allow unidirectional sequencing using the following forward and reverse primers:

ShotA_917F (CCATCTCATCCCTGCGTGTCTCCGACTCAGGAATTGACGGGGRCCC) and

ShotB_1391R (CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGACGGGCGGTGWGTRCA), respectively. For both approaches, the amplicon library consisted of a simple PCR reaction using 1 µl of the DNA template extracted as described above and a pair of special fusion primers consisting of two parts. The PCR reactions were performed in 20 µL reaction volumes and used 30 cycle reactions with Taq Phusion (Finnzymes, Thermo Scientific, Waltham, MA, USA). The PCR reagents and thermocycling parameters were those suggested in the protocol suggested for the Taq Phusion enzyme (optimal annealing temperature of 60°C, an elongation time of 30 sec at 72°C for 20 cycles and a final elongation time of 10 min at 72°C). The amplicon lengths (544 bp) were visualized using the BioAnalyzer DNA labchip 7500. The amplicons were purified as recommended using Ampure beads (Agencourt) and were quantified using a Tecan Genios fluorometer according to the manufacturer's instructions (procedure 454_Roche, "The Amplicon Library Preparation Method Manual").

Three bidirectional libraries were clone-amplified with 1 cpb in a 1 cup emPCR reaction using the GS Titanium LV emPCR Kit (Lib-A) v2 for each sample, and the 13 unidirectional libraries were amplified with 1.5 cpb using the GS Titanium LV emPCR Kit (Lib-L). Each project was loaded onto one half of a GS Titanium PicoTiterPlate PTP Kit 70x75 and sequenced with the GS Titanium Sequencing Kit XLR70. The sequence (355 Mb) was generated as it passed the filter sequences with an average read length of 283 bp.

16S ribosomal RNA copy number correction

The number of 16S rDNA gene copies is used as a measure of microbial population abundance and diversity (9), varying from one to 15 copies in different bacteria (10). To compare the employed pyrosequencing technique with the employed morphological tools (gram staining and TEM) without introducing molecular bias, we corrected the number of reads obtained from the major phyla (*Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria* and *Verrucomicrobia*) by pyrosequencing using the mean number of 16S rDNA gene copies for these phyla. We selected bacterial species composing the human gut microbiota from the Human Microbiome Project catalogue (http://www.hmpdacc.org) based only on previous studies (11, 12) in which the entire genome had been sequenced. We used the Gold database (13) and the NCBI genome and gene databases (http://www.ncbi.nlm.nih.gov/genome) to analyze the complete sequenced genome and assess the 16S rDNA copy number for each species selected. We then divided the concentration for each phylum, as indicated by the number of reads obtained by pyrosequencing, by the mean number of 16S rDNA gene copies of each phylum and compared the results with quantifications obtained using gram staining and TEM. Unidentified reads were divided by the mean number of 16S rDNA gene copies from the *Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria* and *Verrucomicrobia* phyla.

Quantification of Gram-negative and gram-positive prokaryotes per gram of feces

For each sample, the quantity of gram-negative and gram-positive prokaryotes per gram of feces was calculated by multiplying the percentage of gram-negative or gram-positive prokaryotes obtained using each technique (i.e., the number of cells counted using gram staining and TEM and the number of reads assigned to each phylum using pyrosequencing) by the total prokaryote concentration obtained using flow cytometry. For quantitative PCR, the concentrations of *Bacteroidetes*, *Firmicutes* and *Methanobrevibacter smithii* were calculated using the concentration of chimeric plasmid. In addition, the quantifications of gram-positive bacteria that were obtained by ram staining and TEM were corrected using *M. smithii* concentrations obtained using quantitative PCR. The quantifications of bacteria in the *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia* phyla that were obtained by pyrosequencing were normalized to the mean 16S rDNA copy number to compare with TEM, while no correction was made for the quantifications of bacteria in the *Bacteroidetes* and *Firmicutes* phyla that were obtained by comparing pyrosequencing data with the qPCR results.

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Supplementary Figures and Tables

TABLE S1. Patient origins and characteristics.

Sample number	SRA number	Sex	Age	Geographical origin	Weight (kg)	BMI (kg/m2)	Diseases	Antibiotics
1	SRS385004	M	49	French Polynesia = Raiatea	84	27,4	Overweight	No
2	SRA049748	F	26	France	120	48,2	Morbid obesity	No
3	SRA062687	F	20	France	27,7	10,4	Anorexia nervosa	No
4	SRA058885	F	47	France	NA	NA	ICU 3 months	piperacillin tazobactam, cefotaxime, ceftazidime, amoxicillin, clavulanic acid, ertapenem, caspofungin, voriconazole
5	SRA058885	M	62	France	NA	NA	ICU 10 days	imipenem
6	SRA062846	M	32	France	80	25,2	HIV Type 1	No
7	SRS387496	M	44	French Polynesia	103,0	33,0	Obesity	No
8	SRS387497	M	46	French Polynesia	111,0	31,0	Obesity	No
9	SRS387498	M	65	French Polynesia	65,0	24,0	Overweight	No
10	SRS387488	F	72	France	NA	NA	HIV Type 1	No
11	SRS387489	M	57	France	NA	NA	HIV Type 2	No
12	SRS387490	M	42	France	NA	NA	HIV Type 1	No
13	SRS387491	M	32	France	NA	NA	HIV Type 1	No
14	SRS385014	M	46	France	NA	NA	HIV Type 1	No
15	SRS387493	M	44	France	NA	NA	HIV Type 1	No
16	SRS387495	F	23	France	NA	NA	Elite Controller	No

TABLE S2. Primers and probes used for quantitative real-time PCR.
>f: Forward primer
>r: Reverse primer

Phylum	Target gene	Amplicon length (bp)	Primer	Probe
Firmicutes	16S rRNA	179	>**f** GTCAGCTCGTGTCGTGA >**r** CCATTGTAKYACGTGTGT	*GTCAANTCATCATGCC*
Bacteroidetes	16S rRNA	184	>**f** AGCAGCCGCGGTAAT **>r** CTAHGCATTTCACCGCTA	*GGGTTTAAAGGG*
Methanobrevibacter smithii	16S rRNA	123	>**f** CCGGGTATCTAATCCGGTTC **>r** CTCCCAGGGTAGAGGTGAAA	*CCGTCAGAATCGTTCCAGTCAG*

TABLE S3. Quantification of prokaryotes obtained using the Kovaslide and flow cytometry methods for 16 samples at a dilution of 10^{-4} (**A**). Quantification of prokaryotes obtained from 10 control stool samples at 3 different dilutions (10^{-4} to 10^{-6}) (**B**).

A

	Count/g wet weight (Kovaslide)	Count/g wet weight (Cytometry)
1	3,10E+10	1,49E+11
2	1,80E+10	3,21E+10
3	1,40E+10	2,83E+10
4	1,40E+10	2,61E+09
5	1,40E+10	6,12E+09
6	2,00E+10	4,60E+10
7	2,20E+10	3,28E+10
8	9,60E+10	1,50E+10
9	1,50E+10	3,10E+10
10	2,00E+10	8,18E+09
11	2,20E+10	1,90E+10
12	1,50E+10	9,04E+09
13	9,90E+09	1,52E+10
14	2,00E+10	1,04E+10
15	6,30E+09	7,33E+09
16	9,90E+09	2,99E+10
Mean	2,17E+10	2,76E+10
SD	2,07E+10	3,47E+10

B

	1/10000		*1/100000*		*1/1000000*	
	KovaS	Cyto	KovaS	Cyto	KovaS	Cyto
A	6,39E+10	8,90E+10	8,10E+10	1,17E+11	9,00E+10	2,28E+11
B	8,10E+10	5,01E+10	9,90E+10	5,16E+10	9,00E+10	1,17E+11
C	6,39E+10	5,62E+09	4,50E+10	1,22E+10	9,00E+10	3,63E+10
D	6,93E+10	2,03E+10	8,10E+10	3,11E+10	9,00E+10	4,66E+10
E	3,15E+10	1,48E+10	7,20E+10	5,67E+10	9,00E+10	1,72E+11
F	8,55E+10	9,11E+10	9,00E+10	1,87E+11	9,00E+10	3,58E+10
G	4,77E+10	6,61E+10	6,30E+10	8,90E+10	9,00E+10	3,67E+11
H	6,75E+10	4,12E+10	5,40E+10	7,63E+10	9,00E+10	3,94E+11
I	3,87E+10	2,36E+10	8,10E+10	6,18E+10	9,00E+10	5,68E+11
J	3,87E+10	3,76E+10	7,20E+10	2,70E+11	9,00E+10	1,02E+12
Mean	5,88E+10	4,39E+10	7,38E+10	9,52E+10	9,00E+10	2,98E+11
SD	1,86E+10	3,00E+10	1,63E+10	7,84E+10	0,00E+00	3,08E+11

TABLE S4. The 16S rDNA copy numbers for *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* (**A**). The 16S rDNA copy numbers for *Methanobrevibacter smithii* obtained using quantitative PCR (**B**).

A

	16S rDNA copy number
Firmicutes	
Eubacterium rectale 33656	5
Enterococcus faecalis	4
E. faecium	6
Lactobacillus reuteri	6
L. rhamnosus	5
Clostridium perfringens	8
C.difficile CD196	10
Veillonella parvula	4
Ruminococcus albus	4
E. eligens	5
Bacteroidetes	
Bacteroides vulgatus	7
B. thetaiotaomicron VPI-5482	5
Parabacteroides distasonis ATCC 8503	7
B. fragilis 638R	6
Alistipes finegoldii	2
Prevotella melaninogenica	4
Odoribacter splanchnicus DSM 20712	4
Porphyromonas gingivalis ATCC 33277	4
Actinobacteria	
Bifidobacterium adolescentis ATCC 15703	5
B. bifidum	3
B. breve ACS-071-V-Sch8b	3
B. longum DJO10A	4
B. animalis subsp. Lactis AD011	2
Eggerthella lenta	3
Rothia mucilaginosa	3
Propionibacterium acnes	2
Atopobium parvulum	1
Clavibacter michiganensis subsp. michiganensis NCPPB 382	2
Verrucomicrobia	
Akkermansia muciniphila ATCC BAA-835	3
Proteobacteria	
Escherichia Coli	7
Proteus mirabilis	7
Klebsiella pneumoniae	8
Helicobacter pylori	2
Enterobacter cloacae	8
Pseudomonas aeruginosa	4
Citrobacter koseri ATCC BAA-895	7
Haemophilus parainfluenzae	6
Edwardsiella tarda	8
Aeromonas veronii	10

B

	M. smithii	
	Ct	Quantification
1	30,0	1,05E+06
2	25,8	7,75E+06
3	22,6	1,33E+09
4	20,5	3,60E+08
5	24,8	8,88E+08
6	27,3	3,19E+06
7	22,3	1,48E+08
8	22,4	2,96E+08
9	24,5	2,21E+07
10	25,7	8,32E+07
11	19,4	3,96E+08
12	25,6	2,34E+06
13	25,5	9,82E+06
14	17,2	2,28E+09
15	27,6	1,57E+07
16	19,6	1,85E+09

TABLE S5. The number of reads obtained for the 16 samples by pyrosequencing. (X) indicates the percentage of each phylum within a sample. The two most important phyla in each sample are indicated by bold typeface. Blue font indicates gram-positive phyla, and red font indicates gram-negative phyla.

Firmicutes	*Bacteroidetes*	*Actinobacteria*	*Proteobacteria*	*Fusobacteria*	*Deinococcus Thermus*	*Cyanobacteria Chloroplast*	*Verrucomicrobia*	*Synergistetes*	*Lentisphaerae*	*TM7*	*Others*	TOTAL
2364 (1,66)	**74813 (52,64)**	872 (0,61)	**64082 (45,09)**	1 (0)	0	0	0	0	0	0	0	142132
83261 (91,12)	14 (0,02)	931 (1,02)	**739 (0,81)**	0	0	2 (0)	0	0	0	4	**6421 (7,03)**	91372
64216 (76,49)	4983 (5,94)	**8439 (10,05)**	2434 (2,90)	1 (0)	0	25 (0,03)	2 (0)	17 (0,02)	0	0	3834 (4,57)	83951
47052 (78,54)	**5230 (8,73)**	1851 (3,09)	57 (0,10)	9 (0,02)	0	0	1 (0)	772 (1,29)	0	0	4936 (8,24)	59908
968 (7,39)	580 (4,43)	1 (0,01)	230 (1,76)	0	0	0	**11280 (86,09)**	0	0	0	43 (0,33)	13102
58791 (49,07)	**56107 (46,84)**	2165 (1,81)	1750 (1,46)	0	0	1 (0)	39 (0,03)	0	0	0	938 (0,78)	119781
113331 (50,15)	432 (0,19)	42020(18,6)	**69880 (30,93)**	2 (0)	0,0	2 (0)	0	3 (0)	0	103 (0,05)	192 (0,08)	225965
76403 (72,57)	7749 (7,36)	**12448 (11,82)**	1235 (1,17)	0	0,0	0,00	5780 (5,49)	2 (0)	0	0	1661 (1,58)	105278
63867 (40,75)	1290 (0,82)	4687 (2,99)	**86868 (55,42)**	0	0,0	1 (0)	7 (0)	1 (0)	0	6 (0)	11 (0,01)	156738
35474 (76,92)	**5739 (12,44)**	207 (0,45)	1134 (2,46)	0	0	0	2762 (5,99)	0	4 (0,01)	0	800 (1,73)	46120
32989 (80,68)	1774 (4,30)	441 (1,08)	**5217 (12,8)**	0	0	0	0	0	0	1	467 (1,14)	40889
59476 (71,35)	1867 (2,24)	**16962 (20,35)**	3879 (4,65)	0	0	0	0	0	0	7 (0,01)	1162 (1,39)	83353
188372 (87,15)	4443 (2,06)	**11377 (5,26)**	662 (0,31)	1 (0)	0	1 (0)	0	0	0	8 (0)	11258 (5,21)	216145
37427 (70,28)	1294 (2,43)	**4165 (7,82)**	1448 (2,72)	0	0	0	0	79 (0,15)	8 (0,02)	1 (0)	8830 (16,58)	53252
121974 (83,06)	**23925 (16,29)**	195 (0,13)	713 (0,49)	2 (0)	0	4 (0)	0	0	0	0	36 (0,02)	146849
9281 (80,49)	84 (0,73)	474 (4,11)	**1640 (14,22)**	0	0	0	16 (0,14)	0	0	0	36 (0,31)	11531

TABLE S6. Concentration of gram-positive (**A**) and gram-negative (**B**) prokaryotes obtained with each method.
A

	qPCR (*Firmicutes*)	Pyro (*Firmicutes*)[2]	Pyro (*Firmicutes*)[1]	Pyro (all gram +)[1]	Gram staining	TEM
1	1,37E+08	2,48E+09	4,35E+08	7,60E+08	6,15E+10	7,61E+10
2	2,26E+12	2,92E+10	5,13E+09	5,24E+09	1,55E+10	1,41E+10
3	7,34E+11	2,17E+10	3,80E+09	4,82E+09	1,42E+10	1,83E+10
4	1,02E+11	2,05E+09	3,60E+08	3,89E+08	1,64E+09	1,72E+09
5	1,11E+08	4,52E+08	7,93E+07	7,95E+07	2,82E+09	4,99E+09
6	4,18E+11	2,26E+10	3,96E+09	4,26E+09	3,46E+10	2,90E+10
7	4,35E+10	1,65E+10	2,89E+09	5,07E+09	9,07E+09	2,41E+10
8	6,50E+10	1,09E+10	1,91E+09	2,54E+09	3,65E+09	1,17E+10
9	1,78E+10	1,26E+10	2,21E+09	2,54E+09	1,24E+10	2,20E+10
10	7,92E+11	6,29E+09	1,10E+09	1,12E+09	1,68E+09	5,40E+09
11	3,55E+11	1,53E+10	2,69E+09	2,76E+09	5,37E+09	1,41E+10
12	1,49E+11	6,45E+09	1,13E+09	1,79E+09	1,51E+09	6,87E+09
13	3,74E+11	1,33E+10	2,33E+09	2,62E+09	2,51E+09	1,21E+10
14	4,85E+11	7,34E+09	1,29E+09	1,58E+09	4,11E+09	1,05E+10
15	1,25E+10	6,08E+09	1,07E+09	1,07E+09	5,66E+09	4,92E+09
16	5,10E+10	2,41E+10	4,22E+09	4,66E+09	5,89E+09	2,37E+10
Median	1,26E+11	1,17E+10	2,06E+09	2,54E+09	5,51E+09	1,31E+10
SD	5,68E+11	8,65E+09	1,52E+09	1,75E+09	1,58E+10	1,76E+10

[1]correction using 16S rDNA copy number

[2] no correction using 16S rDNA copy number

B

	qPCR (*Bacteroidetes*)	Pyro (*Bacteroidetes*)[2]	Pyro (*Bacteroidetes*)[1]	Pyro (all gram -)[1]	Gram staining	TEM
1	2,13E+08	7,85E+10	1,60E+10	2,61E+10	8,78E+10	7,31E+10
2	3,09E+09	6,41E+06	1,31E+06	4,01E+07	1,66E+10	1,80E+10
3	1,74E+09	1,68E+09	3,43E+08	4,80E+08	1,54E+10	1,13E+10
4	1,48E+09	2,28E+08	4,66E+07	8,12E+07	1,34E+09	1,25E+09
5	1,68E+08	2,71E+08	5,53E+07	1,83E+09	4,18E+09	2,02E+09
6	1,80E+10	2,15E+10	4,40E+09	4,50E+09	1,14E+10	1,75E+10
7	1,48E+08	6,23E+07	1,27E+07	1,54E+09	2,39E+10	8,86E+09
8	4,27E+08	1,10E+09	2,25E+08	5,26E+08	1,16E+10	3,60E+09
9	7,02E+07	2,54E+08	5,18E+07	2,61E+09	1,86E+10	8,98E+09
10	1,92E+09	1,02E+09	2,08E+08	4,02E+08	6,58E+09	2,86E+09
11	5,67E+08	8,24E+08	1,68E+08	5,31E+08	1,40E+10	5,31E+09
12	7,31E+08	2,02E+08	4,13E+07	1,05E+08	7,53E+09	2,17E+09
13	3,37E+08	3,14E+08	6,41E+07	7,12E+07	1,27E+10	3,20E+09
14	4,95E+09	2,54E+08	5,18E+07	1,12E+08	8,62E+09	2,19E+09
15	1,85E+09	1,19E+09	2,44E+08	2,49E+08	1,68E+09	2,42E+09
16	4,42E+09	2,18E+08	4,46E+07	6,94E+08	2,59E+10	8,08E+09
Median	1,10E+09	2,93E+08	5,97E+07	5,03E+08	1,22E+10	4,46E+09
SD	4,40E+09	1,99E+10	4,05E+09	6,40E+09	2,02E+10	1,75E+10

[1]correction using 16S rDNA copy number

[2] no correction using 16S rDNA copy number

Fig. S1. Observation of prokaryotes by TEM: Gram-negative prokaryotes (**A**); Gram-positive prokaryotes (**B**); *Mycobacterium szulgai* (14) (**C**); bacteria with unique cell envelopes (15) (**D**).

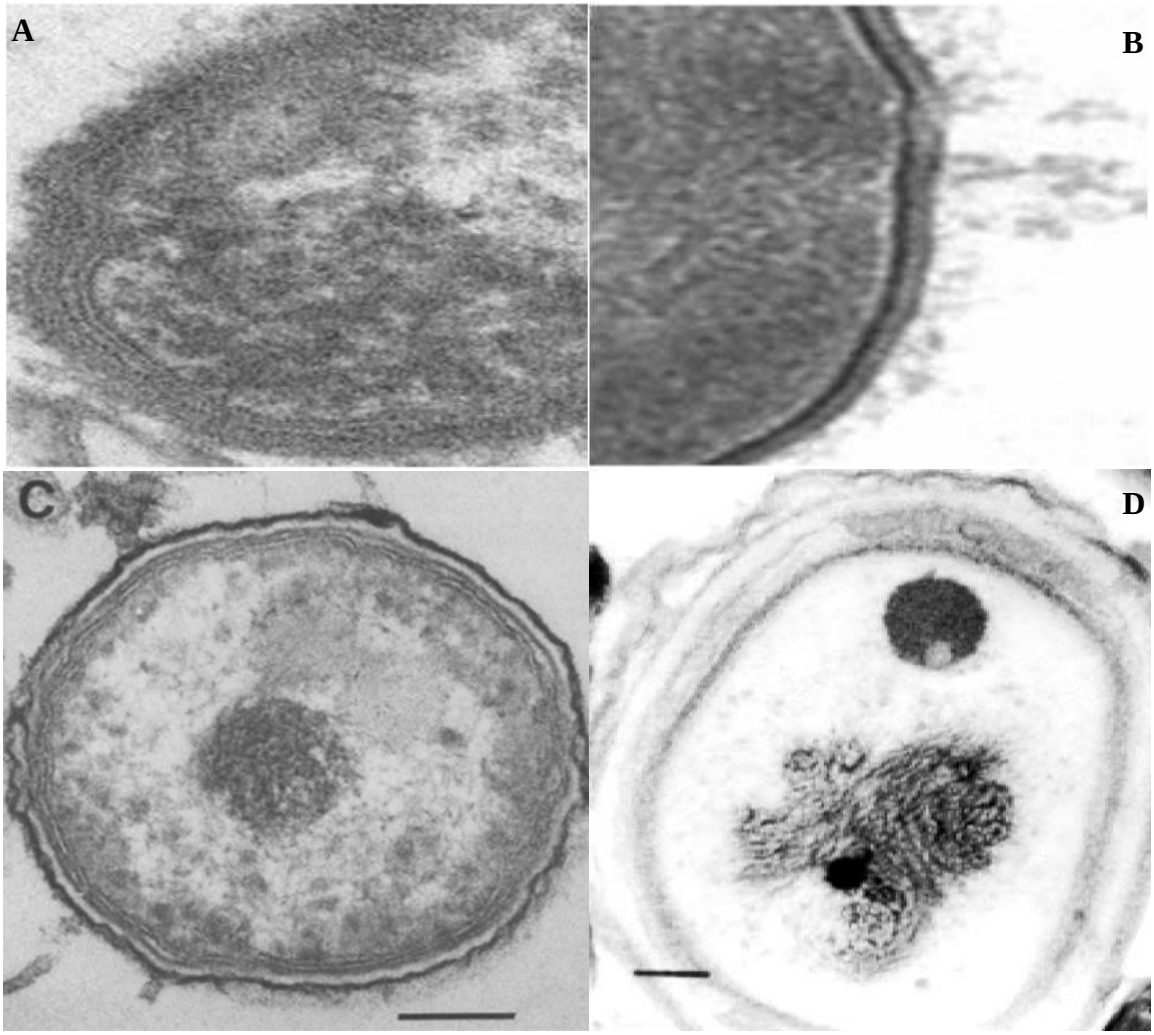
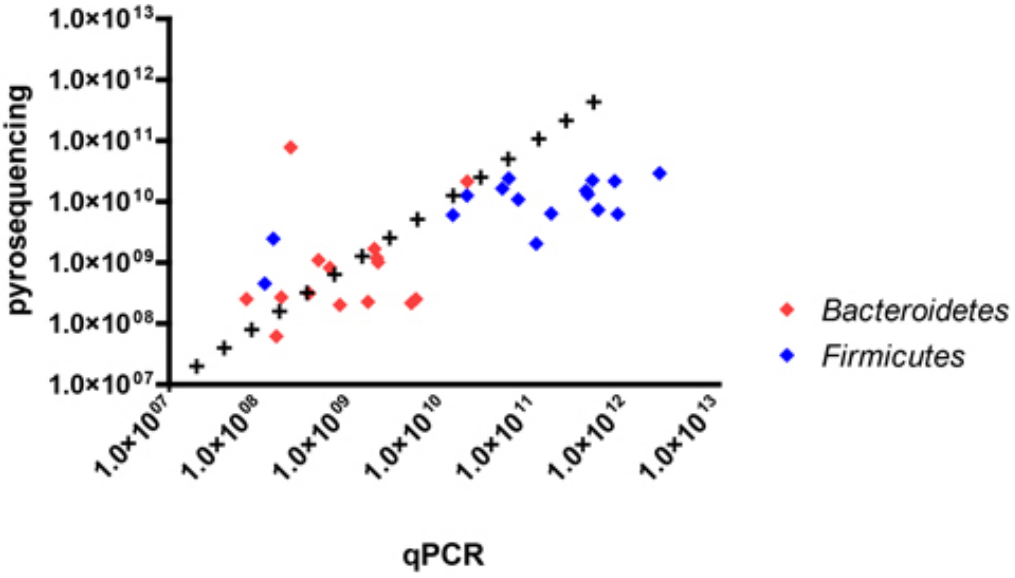
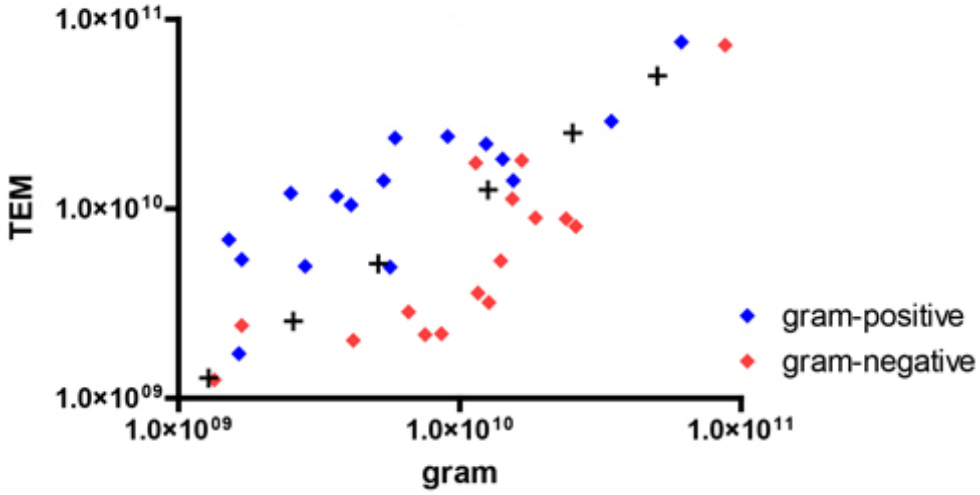


Fig. S2. Correlational analysis of the *Firmicutes* and *Bacteroidetes* concentrations obtained using qPCR and 454 FLX pyrosequencing (**A**). Correlational analysis of the prokaryote concentrations obtained using gram staining and TEM (**B**).



Spearman ρ = 0.4912, p= 0.0534 (*Firmicutes* concentration)
Spearman ρ = 0.05887, p= 0.8286 (*Bacteroidetes* concentration)



Spearman ρ = 0.8035, p=0.0002 (gram-positive prokaryotes)
Spearman ρ = 0.8176, p=0.0001 (gram-negative prokaryotes)