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ORIGINAL ARTICLE

Associations between common intestinal parasites and bacteria in humans as revealed by qPCR

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Abstract Several studies have shown associations between groups of intestinal bacterial or specific ratios between bacterial groups and various disease traits. Meanwhile, little is known about interactions and associations between eukaryotic and prokaryotic microorganisms in the human gut. In this work, we set out to investigate potential associations between common single-celled parasites such as *Blastocystis* spp. and *Dientamoeba fragilis* and intestinal bacteria. Stool DNA from patients with intestinal symptoms were selected based on being *Blastocystis* spp.-positive (B+)/negative (B−) and *D. fragilis*-positive (D+)/negative (D−), and split into four groups of 21 samples (B+ D+, B+ D−, B− D+, and B− D−). Quantitative PCR targeting the six bacterial taxa *Bacteroides*, *Prevotella*, the butyrate-producing clostridial clusters IV and XIVa, the mucin-degrading *Akkermansia muciniphila*, and the indigenous group of *Bifidobacterium* was subsequently performed, and the relative abundance of these bacteria across the four groups was compared. The relative abundance of *Bacteroides* in B– D– samples was significantly higher compared with B+ D− and B+ D+ samples ($P < 0.05$ and $P < 0.01$, respectively), and this association was even more significant when comparing all parasite-positive samples with parasite-negative samples ($P < 0.001$). Additionally, our data revealed that a low abundance of *Prevotella* and a higher abundance of Clostridial cluster XIVa was associated with parasite-negative samples ($P < 0.05$ and $P < 0.01$, respectively). Our data support the theory that *Blastocystis* alone or combined with *D. fragilis* is associated with gut microbiota characterized by low relative abundances of *Bacteroides* and Clostridial cluster XIVa and high levels of *Prevotella*.

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Introduction

A variety of microbial species inhabit the human gut, and every person has a unique gut microbiota consisting of about 170 microbial species, mainly reflecting different species of bacteria [1, 2]. Billions of bacteria colonize the adult gastrointestinal tract (GIT), and colonization potentially begins already at the fetal stage and develops throughout our lives [3, 4]. Sequencing of bacterial DNA to determine the intestinal microbiome has identified Firmicutes, Bacteroidetes, and Actinobacteria as the dominant bacterial phyla in the adult human intestine [5]. Firmicutes comprise butyrate-producing bacterial groups such as Clostridial cluster XIV and IV, while Bacteroidetes primarily comprise *Bacteroides*, *Prevotella*, and *Porphyromonas* [6, 7].

Arumugam et al. demonstrated that humans could be stratified into enterotypes, the three most common ones being defined by a high abundance of *Bacteroides*, *Prevotella*, and *Ruminococcus*, respectively [8]. Until now, most comprehensive studies of human gut microbiota, i.e., gut microbiome and metagenomics studies, have focused entirely on bacteria; hence, little is known about other types of intestinal organisms such as parasites, fungi and viruses in a microbiome context [9, 10].

Blastocystis spp. and *D. fragilis* represent two common, single-celled intestinal parasites. While the role of these parasites in human health and disease remains unsettled, recent data suggest that these parasites are more common in healthy individuals than in patients with functional and inflammatory bowel diseases [11–13]. Differences in interactions between

parasites and bacteria may reflect differences in the clinical and public health significance of common intestinal parasites. The impact of bacterial communities on the pathogenicity and growth of parasites has been documented to some extent; for instance, the presence of *Lactobacillus* may reduce the growth of *Giardia* [14]. Additionally, a recent study analyzing metagenomics data showed a negative association between *Blastocystis* spp. and the *Bacteroides* enterotype in human fecal samples [15], suggesting that parasite colonization might be a biomarker of gut microbiota structure. However, the original material used to generate the metagenomics data was not available for studies that could corroborate these finding by other methods.

The aim of the present study was to validate previous data suggesting a clear negative association between *Blastocystis* spp. and the *Bacteroides* enterotype, and to identify associations between common intestinal parasites with intestinal bacterial groups using qPCR. This is the first time that bacterial DNA from clinical stool samples is investigated by targeted qPCR to reveal associations between two common intestinal parasitic protists, *Blastocystis* spp. and *D. fragilis*, and selected groups of intestinal bacteria with potential relevance for human health.

Materials and methods

Sample inclusion/exclusion criteria

Since the purpose of this study was to test for associations between common intestinal parasites and bacteria, 750 clinical fecal samples submitted by unique patients (in the period from 28th of March to 25th of April 2013) were considered as candidates. DNA from stool samples was extracted using the NucliSENS® easyMag® protocol (bioMérieux, Denmark) according to the manufacturer's recommendations (Protocol B, 200 mg stool, 60 μL silica). All 750 samples were screened for *Blastocystis* spp., *Dientamoeba fragilis*, *Giardia intestinalis*, *Entamoeba dispar*, *Entamoeba histolytica*, and *Cryptosporidium* spp. by PCR using methods previously described [16, 17]. Exclusion criteria were as follows: (1) Samples positive for *G. intestinalis*, *E. histolytica*, *E. dispar*, and *Cryptosporidium* spp. as determined by qPCR, (2) patients who had been travelling abroad, and (3) patients less than 6 years of age since the intestinal microbiota is not yet completely stable in this age group [18]. All patients were referred from hospitals, specialists, or general practitioners. No information was available regarding potential consumption of antibiotics. A total of 84 samples were selected from eligible samples with a view to obtaining four groups with similar age and gender distribution and evenly distributed in terms of being *Blastocystis* spp.- and *D. fragilis*-positive/-negative, with 21 patients in each group, as follows: *Blastocystis* spp.- and *D. fragilis*-positive patients (B+ D+ group; age range, 9–70 years; mean age, 31 years [SD, 19.1]); *Blastocystis* spp.-positive and *D. fragilis*-negative patients (B+ D− group; age range, 6–69 years; mean age, 34 years [SD, 17.2]); *Blastocystis* spp.-negative and *D. fragilis*-positive patients (B– D+ group; age range, 9–69 years; mean age, 29 years [SD, 18.4]); and *Blastocystis* spp.- and *D. fragilis*-negative patients (B– D– group; age range, 17–69 years; mean age, 38 years [SD, 15.9]).

Detection of bacteria

The abundance of *Bacteroides*, *Prevotella*, Clostridial cluster IV, Clostridial cluster XIVa, *Akkermansia muciniphilia*, and *Bifidobacterium* was determined in quadruplets by qPCR on a LightCycler® 480 II System (Roche applied Systems, Penzberg, Germany). The relative abundance of each taxon was calculated as the ratio of the mean abundance of the specific taxon and the mean abundance of the total bacteria targeted by the primers HDA1 and HDA2 [19]. The LinRegPCR software program (The Heart Failure Research Center [HFRC], Amsterdam, Netherlands) was used to calculate abundance data. Amplification by qPCR, applied primers, and the calculation of relative abundance was performed as previously described [20]. One sample in the B+ D− group failed in qPCR, and this group therefore contained only 20 samples.

Data analysis

For statistical testing of differences between the four individual groups, a one-way ANOVA test was applied with the post-hoc Tukey's range test [21]. To test the 0-hypothesis that there was no statistical difference in mean values between parasite-positive samples (B+ D+, B+ D− and B− D+ combined) and parasite-negative samples (B– D–), the Wilcoxon rank-sum test was applied. This variant was chosen since the number of samples differed in the two groups ($N=62$ and $N=21$ for parasite-positive and parasite-negative samples, respectively) [22].

Results

Analyzing for differences between the four individual groups, the relative abundance of *Bacteroides* was found to be significantly higher in the B− D− group compared with the B+ D+ and B+ D− groups ($P<0.01$ and $P<0.05$, respectively) (Fig. 1). No statistically significant difference was observed between the B-D+ group and any of the other groups with respect to the abundance of *Bacteroides*.

The relative abundance of Clostridial cluster IV was significantly lower in the B+ D− group compared with the B− D+

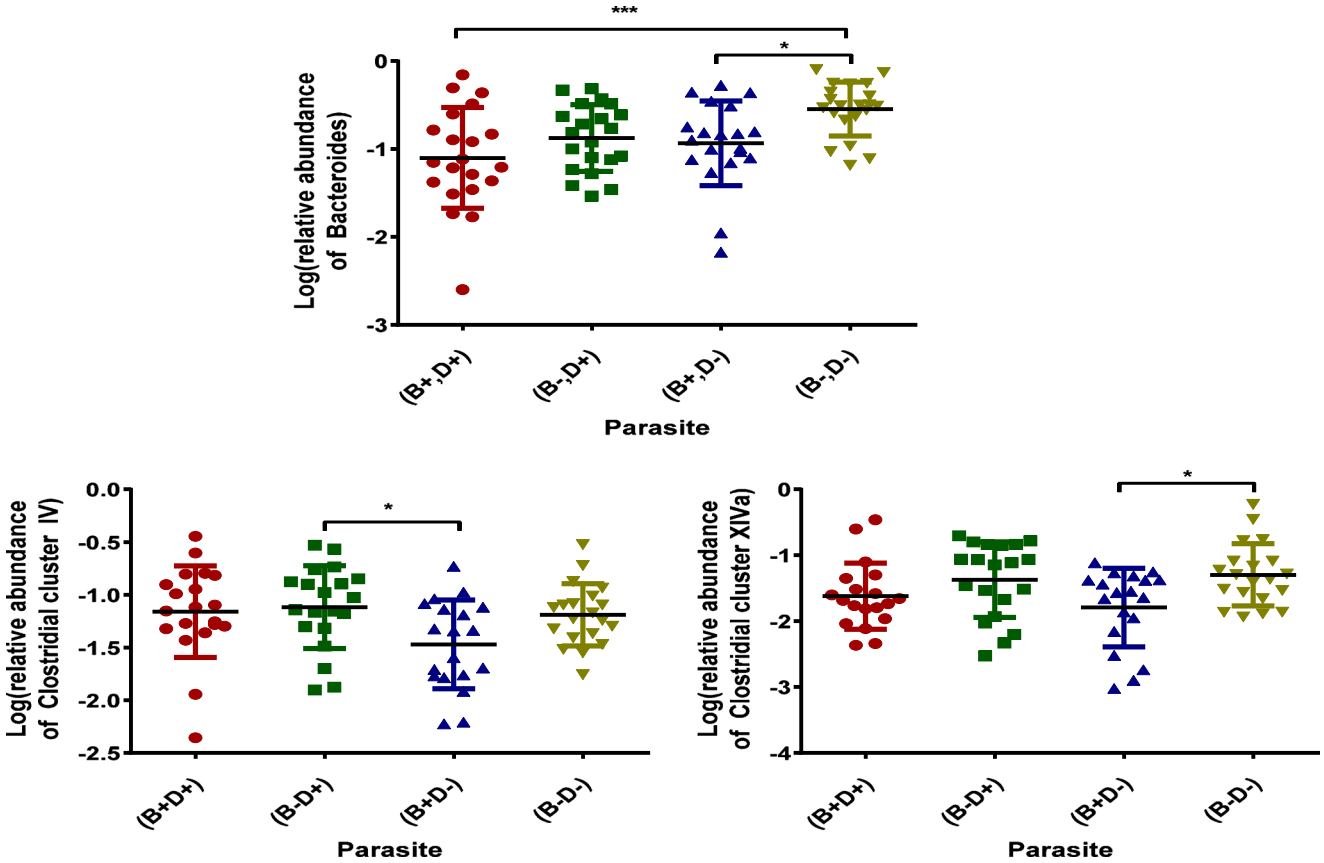

Fig. 1 The log-normalized relative abundance of *Bacteroides*, Clostridial cluster IV, and Clostridial cluster XIVa in each of the four groups (B+ D+, B− D+, B+ D−, and B− D−). The *middle bar* indicates the mean value, and *whiskers* indicate the standard deviation. * $P<0.05$, *** $P<0.001$. (+/−) denotes whether the group is positive and/or negative for *Blastocystis* spp. and *Dientamoeba fragilis* (B/D)

group ($P<0.05$; Fig. 1). Furthermore, the relative abundance of Clostridial cluster XIVa was found in higher levels in the B– D– group compared with the B+ D− group ($P<0.05$; Fig. 1). The abundance of *Akkermansia muciniphilia* and *Bifidobacterium* did not differ among the four groups.

Combining the two *Blastocystis* spp.-positive groups and the two *Blastocystis* spp.-negative groups, the relative abundance of *Bacteroides* was significantly lower in *Blastocystis* spp.-positive patients ($P=0.006$). A comparison of all parasite-positive samples and all parasite-negative samples showed an even more pronounced difference in *Bacteroides* abundance ($P<0.001$), and a borderline significant difference in the relative abundance of Clostridial cluster XIVa (Fig. 2). When grouping individuals based on age and disregarding parasite presence, the relative abundance of Clostridial cluster XIVa was higher in individuals aged 41–60 years than in those aged 61–80 years ($P<0.05$).

The relative abundance of *Prevotella* did not differ significantly between the four groups. A bimodal distribution of the *Prevotella*-to-*Bacteroides* ratio was found (Fig. 3). Of note, most individuals in the B– D– group were found to have a low P/B ratio (Fig. 3), which was due to higher levels of *Bacteroides* in the B– D– group (Fig. 1). The other three groups were more similar in terms of the distribution of low and high P/B ratios.

Discussion

A negative association between *Blastocystis* spp. and *Bacteroides* in stool samples has previously been reported in healthy subjects [15]. In concordance with this, we found a higher relative abundance of *Bacteroides* in B– D– individuals than in *Blastocystis*-positive individuals, suggesting a negative association between *Bacteroides* and *Blastocystis*. The previous study [15] described a negative association between the *Bacteroides* enterotype and the presence of *Blastocystis*, but not in a quantitative manner; meanwhile, the present study sought to focus on the quantitative relationships between the studied taxa. When taking the relative abundance of *Bacteroides* of every single study individual into account—not only that of the *Bacteroides*-dominated individuals—a quantitative relationship between *Bacteroides* and *Blastocystis* spp. was revealed. Our data also revealed that the lower abundance of *Bacteroides* in *Blastocystis* spp.-positive individuals is not only observed in healthy subjects [15],

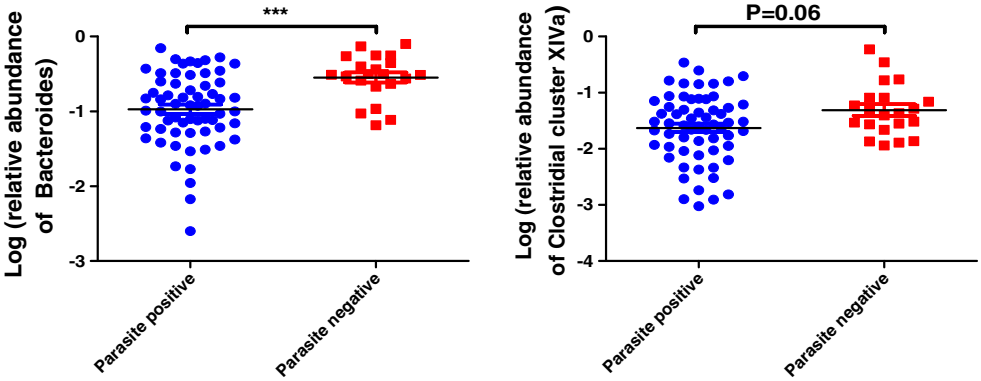

Fig. 2 Comparison of the log-normalized relative abundance of *Bacteroides* and Clostridial cluster XIVa in the combined parasite-positive groups (B+ D+, B− D+, B+ D−) versus the parasite-negative group (B− D−). *** $P < 0.001$. The *middle bar* indicates the mean value, and *whiskers* indicate the standard deviation

but also in patients with intestinal symptoms. This suggests that the association between *Bacteroides* and *Blastocystis* is independent of health status.

The B+ D− group showed a significantly lower abundance of *Bacteroides* than the B− D− group ($P < 0.05$), and the two *Blastocystis* spp.-positive groups exhibited an even higher negative association to *Bacteroides* compared with the two *Blastocystis* spp.-negative groups ($P = 0.006$). Eventually, when combining all parasite-positive individuals, a strong negative association between *Bacteroides* and parasites was observed ($P < 0.001$). Our data indicate that *Blastocystis* spp. drive the difference in *Bacteroides* abundance observed across study individuals but also appear to indicate that *Blastocystis* spp. and *D. fragilis* in conjunction have an even more pronounced influence on the abundance of *Bacteroides*. It could be speculated that the presence of another organism such as *D. fragilis* could increase this association; however, further analysis of healthy individuals should also be performed in order to confirm this. Additionally, it would be interesting to explore whether the negative correlation existing between these common intestinal parasites and *Bacteroides* also parallels an increase in bacterial richness in the presence of these parasites, as indicated in a previous study [15]. If high bacterial richness reflects a healthy gut ecology, the influence of these common parasites on bacterial richness warrants further investigation. If colonization by *Blastocystis* is linked to high gut microbiota diversity, the use of in vitro and in vivo models may help clarify whether *Blastocystis* selects for high gut microbiota diversity or whether it is the other way around. For instance, experiments involving the introduction of *Blastocystis* in a *Blastocystis*-naïve bacterial ecosystem could focus on potential changes in bacterial structure over time.

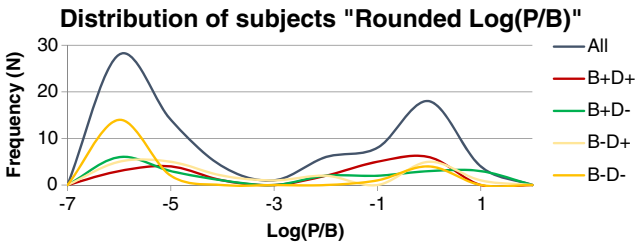

Fig. 3 Bimodal distribution of individuals according to *Prevotella* to *Bacteroides* (*P*/*B*) ratio. (+/−) denotes whether the group is positive or negative for *Blastocystis* spp. and/or *Dientamoeba fragilis* (B/D)

The abundance of Clostridial cluster XIVa was lower in parasite-positive individuals. As there was no statistical difference in age distribution between the four groups, the establishment of cluster XIVa also appears to be influenced by co-colonization with parasites and not just determined by the individual's age as described by Claesson et al. [23].

The bimodal distribution of *Prevotella*/*Bacteroides* ratio as detected by qPCR has previously been reported in children [24, 25]. Our data indicate that the absence of *Blastocystis* spp. and *D. fragilis* is associated with a low *Prevotella*/*Bacteroides* ratio, which may correspond to the *Bacteroides*-driven enterotype as defined by Arumugam et al. [8]. However, the ecology underlying this association remains to be elucidated.

In the present study, no information was available regarding potential consumption of antibiotics. Of course, previous antibiotic use in some of the patients could have resulted in perturbation of the microbiota in certain directions, including clearance of *Blastocystis* and selection for a certain microbiota structure. However, if any drug would be used to treat patients suspected of intestinal parasitosis, this would be metronidazole or mebendazole, both of which are quite inefficient in terms of eradicating *Blastocystis* [26]. Meanwhile, metronidazole is a broad-spectrum antibiotic potentially resulting in shifts in gut microbiota; for example, extensive metronidazole use may lead to *Clostridium difficile* infections, which reflect gut microbiota perturbation. Therefore, any metronidazole use leading to gut microbiota perturbation may indirectly result in eradication of *Blastocystis*. This situation, however, does not affect the hypothesis that *Blastocystis* is a biomarker of gut microbiota diversity and/or intestinal homeostasis.

Conclusion

Our data corroborate the theory that common intestinal parasitic micro-eukaryotes can be viewed as markers of gut microbiota structure. Further studies are needed to elaborate on the

associations between common intestinal parasitic microeukaryotes and the composition of the intestinal microbiota in relation to health and disease.

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Study conception and design: CRS, TRL, KAK, ABK and LOA. Acquisition of data: ABK, HMR, LKV, TRL, CRS and LOA. Analysis and interpretation of data: LOA, ABK, HMR, CRS, KAK and TRL. Drafting of manuscript: LOA and CRS. Critical revision: All

Compliance with ethical standards

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Conflict of interest All authors report of no conflict of interests.

Informed consent Formal consent was not required as this was a retrospective observational study.

References

1. Zoetendal EG, Akkermans AD, De Vos WM (1998) Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl Environ Microbiol 64:3854–3859
2. Qin J, Li R, Raes J et al (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464:59–65
3. Jiménez E, Fernández L, Marín ML, Martín R, Odriozola JM, Nueno-Palop C, Narbad A, Olivares M, Xaus J, Rodríguez JM (2005) Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. Curr Microbiol 51: 270–274
4. Ottman N, Smidt H, de Vos WM, Belzer C (2012) The function of our microbiota: who is out there and what do they do? Front Cell Infect Microbiol 2:104
5. Aziz Q, Doré J, Emmanuel A, Guarner F, Quigley EM (2013) Gut microbiota and gastrointestinal health: current concepts and future directions. Neurogastroenterol Motil 25:4–15
6. Rajilić-Stojanović M, de Vos WM (2014) The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiol Rev 38:996–1047
7. Gerritsen J, Smidt H, Rijkers GT, de Vos WM (2011) Intestinal microbiota in human health and disease: the impact of probiotics. Genes Nutr 6:209–240
8. Arumugam M, Raes J, Pelletier E et al (2011) Enterotypes of the human gut microbiome. Nature 473:174–180
9. Andersen LO, Vedel Nielsen H, Stensvold CR (2013) Waiting for the human intestinal Eukaryotome. ISME J 7:1253–1255
10. Lukeš J, Stensvold CR, Jirků-Pomajbíková K, Wegener Parfrey L (2015) Are human intestinal eukaryotes beneficial or commensals? PLoS Pathog 11:e1005039
11. Krogsgaard LR, Engsbro AL, Stensvold CR, Nielsen HV, Bytzer P (2015) The prevalence of intestinal parasites is not greater among individuals with irritable bowel syndrome: a population-based case–control study. Clin Gastroenterol Hepatol 13:507.e502–513.e502
12. Rossen NG, Bart A, Verhaar N, van Nood E, Kootte R, de Groot PF, D'Haens GR, Ponsioen CY, van Gool T (2015) Low prevalence of *Blastocystis* sp. in active ulcerative colitis patients. Eur J Clin Microbiol Infect Dis 34:1039–1044
13. Petersen AM, Stensvold CR, Mirsepasi H, Engberg J, Friis-Møller A, Porsbo LJ, Hammerum AM, Nordgaard-Lassen I, Nielsen HV, Krogfelt KA (2013) Active ulcerative colitis associated with low prevalence of *Blastocystis* and *Dientamoeba fragilis* infection. Scand J Gastroenterol 48:638–639
14. Clemente JC, Ursell LK, Parfrey LW, Knight R (2012) The impact of the gut microbiota on human health: an integrative view. Cell 148:1258–1270
15. Andersen LO, Bonde I, Nielsen HB, Stensvold CR (2015) A retrospective metagenomics approach to studying *Blastocystis*. FEMS Microbiol Ecol 91. doi:10.1093/femsec/fiv072
16. Stensvold CR, Ahmed UN, Andersen LO, Nielsen HV (2012) Development and evaluation of a genus-specific, probe-based, internal-process-controlled real-time PCR assay for sensitive and specific detection of *Blastocystis* spp. J Clin Microbiol 50:1847–1851
17. Verweij JJ, Mulder B, Poell B, van Middelkoop D, Brienen EA, van Lieshout L (2007) Real-time PCR for the detection of *Dientamoeba fragilis* in fecal samples. Mol Cell Probes 21:400–404
18. Mackie RI, Sghir A, Gaskins HR (1999) Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr 69: 1035S–1045S
19. Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Ng J, Munro K, Alatossava T (1999) Identification of *Lactobacillus* isolates from the gastrointestinal tract, silage, and yoghurt by 16S-23S rRNA gene intergenic spacer region sequence comparisons. Appl Environ Microbiol 65:4264–4267
20. Bergström A, Licht TR, Wilcks A, Andersen JB, Schmidt LR, Grønlund HA, Vigsnaes LK, Michaelsen KF, Bahl MI (2012) Introducing GUt low-density array (GULDA): a validated approach for qPCR-based intestinal microbial community analysis. FEMS Microbiol Lett 337:38–47
21. Tukey JW (1949) Comparing individual means in the analysis of variance. Biometrics 5:99–114
22. Blair RC, Higgins JJ (1980) A comparison of the power of Wilcoxon's rank-sum statistic to that of Student's t statistic under various nonnormal distributions. J Educ Stat 5:24
23. Claesson MJ, Cusack S, O'Sullivan O et al (2011) Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci USA 108(Suppl 1):4586–4591
24. Roager HM, Licht TR, Poulsen SK, Larsen TM, Bahl MI (2014) Microbial enterotypes, inferred by the prevotella-to-bacteroides ratio, remained stable during a 6-month randomized controlled diet intervention with the new nordic diet. Appl Environ Microbiol 80: 1142–1149
25. Bergström A, Skov TH, Bahl MI, Roager HM, Christensen LB, Ejlerskov KT, Mølgaard C, Michaelsen KF, Licht TR (2014) Establishment of intestinal microbiota during early life: a longitudinal, explorative study of a large cohort of Danish infants. Appl Environ Microbiol 80:2889–2900
26. Stensvold CR, Smith HV, Nagel R, Olsen KE, Traub RJ (2010) Eradication of *Blastocystis* carriage with antimicrobials: reality or delusion? J Clin Gastroenterol 44:85–90