Clinical intervention using Bifidobacterium strains in celiac disease children reveals novel microbial modulators of TNF-α and short-chain fatty acids

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Original article

Clinical intervention using *Bifidobacterium* strains in celiac disease children reveals novel microbial modulators of TNF-α and short-chain fatty acids

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SUMMARY

Background & aims

Celiac disease (CD) is an immune-mediated systemic disease, caused by ingestion of gluten in genetically predisposed individuals. Gut microbiota dysbiosis might play a significant role in pathogenesis of chronic enteropathies and its modulation can be used as an intervention strategy in CD as well. In this study, we aimed to identify correlations between fecal microbiota, serum tumor necrosis factor alpha (TNF-α) and fecal short-chain fatty acids (SCFAs) in healthy children and children with CD after administration of probiotic *Bifidobacterium breve* BR03 and B632.

Methods

A double-blind placebo-controlled study enrolled 40 children with CD (CD) and 16 healthy children (HC). CD children were randomly allocated into two groups, of which 20 belonged to the placebo (PL) group and 20 to the Probiotic (PR) group. The PR group received a probiotic formulation containing a mixture of 2 strains, *B. breve* BR03 (DSM 16604) and *B. breve* B632 (DSM 24706) in 1:1 ratio for 3 months. Subsequently, for statistical analysis, blood and fecal samples from CD children (on enrolment - T0 and after 3 months, at the end of intervention with probiotic/placebo - T1) and HC children were used. The HC group was sampled only once (T0).

Results

Verrucomicrobia, *Parcubacteria* and some yet unknown phyla of *Bacteria* and *Archaea* may be involved in the disease, indicated by a strong correlation to TNF-α. Likewise, *Proteobacteria* strongly correlated with fecal SCFAs concentration. The effect of probiotic administration has disclosed a negative correlation between *Verrucomicrobia*, some unknown phyla of *Bacteria*, *Synergistetes*, *Euryarchaeota* and some SCFAs, turning them into an important target in microbiome restoration process. *Synergistetes* and *Euryarchaeota* may have a role in the anti-inflammatory process in healthy human gut.

Conclusions

Our results highlight new phyla, which may have an important relation to disease-related parameters, CD itself and health.

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1. Introduction

Diagnostic rates of celiac disease (CD) are rising, leading towards an estimated global prevalence of about 1% [1]. Ingestion of wheat and other gluten-containing cereals causes a specific damage to the small intestinal mucosa, a typical pathology of CD, which is considered an autoimmune enteropathy [2].

The role of susceptibility genes in the pathogenesis of CD has been described [3,4]. However, additional environmental factors are involved, as only 2–5% of CD-related gene carriers eventually develop the disease [5]. Epidemiological and clinical data suggest a role of various environmental factors in the pathogenesis of CD, such as infections, early feeding practices [6], antibiotic administration, mode of delivery and breastfeeding [7]. In addition, alteration of gut microbiota may also play an important role in the disease development. Whether it is a cause or a consequence of the disease, remains unclear [8–10]. Due to inconsistent findings concerning both active and non-active disease phase [11–15], CD still lacks a distinctive 'microbial footprint', although some bacterial species may associate with the disease [16]. Moreover, microbial metabolites such as short-chain fatty acids (SCFAs) play an important role in trigger-response relationship between host diet, microbiota and homeostasis in many pathological conditions, also in CD [17,18]. However, changes in the fecal SCFAs pattern are a reflection of complex mechanisms [19] and studies about their relationship and effects on CD are scarce [20].

Application of probiotics in clinical practice has been frequently used due to their immunomodulatory [21,22] and microbiota modulation effects [23–26], demonstrated in several inflammatory and autoimmune diseases. Moreover, the effect of *Bifidobacterium* strains on gut microbiota composition and their applications as probiotics in infants have been reviewed [27] and their administration in one *in-vivo* study [25] has revealed an impact on *Firmicutes* abundance, resulting in an increase of *Firmicutes/Bacteroidetes* ratio. Furthermore, its immunomodulatory characteristics have been described *in-vitro* [28] and *in-vivo* [29–32]. Several *in-vitro* studies have shown that *Bifidobacterium* strains decrease levels of pro-inflammatory cytokines, such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and interleukin 2 (IL-2) [33–36]. Moreover, a decrease in TNF-α level after administration of *Bifidobacterium* strains has been reported *in-vivo* [30,31]. In fact, TNF-α secretion, triggered by an increased production of IFN-γ in CD, plays an essential role in inducing damage and inflammation of intestinal mucosa [26,37].

Our aim was to study the effects of *Bifidobacterium breve* BR03 and B632 administration on children with CD and to determine statistically significant correlations between fecal microbiota composition analyzed by next generation sequencing, serum TNF-α and fecal SCFAs levels. To our knowledge, this is the first study on correlations between these parameters with the aim to evaluate their potential significance in CD pathogenesis.

2. Material and methods

2.1. Study design and sample collection

The research study was a double-blind placebo-controlled intervention involving 40 children with CD (CD) and 16 healthy children (HC), who were enrolled at the Department of Pediatrics, University Clinical Center Maribor in a period from October 2013 to June 2014. The research was registered at https://www.clinicaltrials.gov (registration number: NCT02244047).

A selection of HC as control group was based on a clinical examination, excluding any clinical disorder or any acute and chronic illness status. None of HC was on medication or antibiotic therapy for at least one month preceding the research study. HC were children, matching on age and gender and consuming a regular (gluten containing) diet.

All invited CD children, aged from 1 till 19 years, were previously diagnosed with positive serologic markers for CD and had positive small bowel biopsy. Their CD diagnosis were established on ESPGHAN criteria for CD [38,39]. The children were consuming gluten-free diet (GFD) (different time periods - half a year to 15 years). Children with acute or chronic illness and children on permanent medication or antibiotics for at least one month preceding the research study were excluded. CD children were randomly allocated into two groups, of which 20 belonged to the placebo (PL) group and 20 to the Probiotic (PR) group. The PR group received a probiotic formulation containing a mixture of 2 strains, *B. breve* BR03 (DSM 16604) and *B. breve* B632 (DSM 24706) in 1:1 ratio for 3 months. Probiotic and placebo packages contained 2 g of probiotic culture or placebo in a powder form. A daily dosage of each probiotic strain was 10^9 Colony Forming Unit (CFU)/g of powder. In both groups, cytokine analysis, analysis for CD serological markers (EMA, tTG) and clinical examination were performed (on enrolment (T0), at the end of intervention with probiotic/placebo (T1) and on follow up – 3 months after intervention period (T2)). A more detailed information about probiotic administration and inclusion/exclusion criteria of participating children has been described before [30].

Blood samples of CD children were collected 3 times (at T0, T1 and T2). The HC group was sampled only once (T0). Please refer to the article of Klemenak et al. [30] for more details. However, for statistical analysis, samples from periods T0 and T1 were collected (see section *2.7 Statistical analysis of NGS*, *SCFAs and TNF-α results*).

Fecal sample of CD children were collected twice, on T0 and T1. The HC group was sampled only once (T0). Please refer to the detailed description of fecal collection in Primec et al. [20].

Researchers carrying out DNA extraction, molecular (NGS) and HPLC analysis of fecal samples were blind to the children group identity (HC, PR and PL).

2.2. DNA extraction

DNA extraction from 200 mg of feces, which was preserved at −80 °C, was accomplished with the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK), according to manufacturer's instructions. A slight modification was performed, in order to improve the bacterial cell rupture [40]. A detailed protocol was described in Quagliariello et al. [25].

2.3. Preparation of DNA libraries for next-generation sequencing (NGS; Illumina MiSeq sequencing)

Samples of the following 5 groups of children were subjected to sequencing: 20 PR group T0 and 20 PR group T1, 20 PL group T0 and 20 PL group T1 and 16 HC group T0. Libraries were prepared for amplification of V3—V4 region of the 16S rRNA gene, using forward and reverse primers [41], respectively: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3′, and 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC 3′. Their approximate length was 460 bp. A detailed NGS protocol was described in Quagliariello et al. [25].

2.4. SCFA analysis

SCFAs were derivatized and analyzed by reverse-phase HPLC. Acetic, propionic and butyric acid were quantified and results were expressed in μmol/g of wet weight feces. For derivatization procedure and HPLC analysis of SCFAs please refer to the article of Primec et al. [20].

2.5. TNF-α detection

After centrifugation, serum samples for TNF-α detection were collected and stored at −80 °C until analysis. Quantification was performed using a solid-phase enzyme-labeled chemiluminescent immunometric assay, according to the manufacturer's instructions (Immulite One, Siemens Healthcare Diagnostics). A more detailed information about the blood sampling procedure and further TNF-α detection is described in the article of Klemenak et al. [30].

2.6. Bioinformatics and statistical analyses of NGS experiment

In bioinformatics and statistical analyses, the generated raw data has been checked for quality levels, length and elimination of chimeric sequences in order to obtain reliable double-stranded reads for the 16S reference sequence database alignment at the Ribosomal Data Project (RDP). Finally, RDP outputs were processed and further statistically analyzed [25,42–44].

2.7. Statistical analysis of NGS, SCFAs and TNF-α results

Patients were grouped according to the treatment and disease, i.e. probiotic group (PR; PR group T0, PR group T1), placebo group (PL; PL group T0, PL group T1), healthy controls (HC group T0) and CD patients (CD group T0). The CD group T0 consisted of all CD patients at T0 (PR and PL group, both T0). Results from SCFAs (i.e. acetic, propionic, butyric acid and total SCFAs), microbial phylum abundance and TNF-α analysis from 6 groups were statistically correlated for placebo and probiotic groups at the beginning and at the end of probiotic intervention.

Obtained data were analyzed using IBM SPSS Statistics 22.0 software (IBM Inc., Armonk, New York). Age differences between study groups were analyzed using non-parametric Kruskal–Wallis H test. Correlations between two continuous variables were determined using non-parametrical Spearman correlation after Shapiro–Wilk test of data distribution normality. Where indicated, p value of ≤ 0.05 or ≤ 0.01 was considered statistically significant.

3. Results and discussion

3.1. NGS analysis

DNA was extracted out of 96 fecal samples and was sequenced using the Illumina MiSeq apparatus. Sequencing runs generated 4,348,432 joint reads with high quality pass filter with average of 46,259 sequence reads per sample with quality scores between 30 and 35. Two samples were excluded from further analysis due to low quality reads [25]. A detailed microbial profile of each group is shown in Supplementary Tables 1a–f.

3.2. Characteristics of study groups used for statistical analysis of correlations

Basic characteristics of children whose parameters were used for statistical analysis of correlations are summarized in Table 1. Three samples were excluded due to low quality reads in NGS and insufficient data.

3.3. Statistical analysis of correlations

3.3.1. Correlation values at T0 in CD patients

The CD patient group was analyzed at the enrollment day (T0). Figure 1 shows the results of statistically significant correlation values. TNF-α had a positive correlation to *Verrucomicrobia* ($\rho = 0.404$, $p = 0.013$) and a negative one to *Parcubacteria* ($\rho = 0.396$, $p = 0.015$). Moreover, a strong positive association and a high statistical significance ($\rho = 0.532$, $p = 0.001$) between TNF-α and unclassified *Bacteria* group and a positive correlation ($\rho = 0.396$, $p = 0.003$) between TNF-α and unclassified *Archaea* group was found, indicating that *Verrucomicrobia* and some yet unknown phyla, belonging to *Bacteria* and *Archaea*, may be involved in an increased production of TNF-α in CD patients, while *Parcubacteria* indicated a negative association with TNF-α. *Verrucomicrobi*a is commonly encountered in the colonic microbiota [7], but is relatively less than 10% abundant [45]. *Parcubacteria* is a largely unknown phylum, with representatives found in anoxic environments [46]. Indicated correlations could play an important role in the pathogenesis of the disease.

Proteobacteria correlated positively with acetic and propionic acid ($\rho = 0.452$, $p = 0.004$ and $\rho = 0.331$, $p = 0.045$, respectively), which resulted in a positive correlation between *Proteobacteria* and total SCFAs ($\rho = 0.380$, $p = 0.017$). *Proteobacteria* is the major gut-resident phylum of Gram-negative bacteria and includes a wide variety of pathogens, including members of Enterobacteriaceae family. Furthermore, the phyla has been found characteristically increased in duodenal and fecal microbiota of CD patients [16,26,47–50]. Increased values of acetic, propionic acid and total SCFAs in CD have been suggested before [20,51–53], describing them as a consequence of microbiota dysbiosis in the disease. Tjellström et al. [53] even described acetic acid as a potential pro-inflammatory agent. Positive correlation data obtained in our studies indeed indicate that *Proteobacteria* may be responsible for an increased acetic and propionic acid production in CD. *Proteobacteria*, *Bacteroides-Prevotella* group (*Bacteroidetes*) and *Bifidobacterium* spp. have been already described as acetate and propionate producers [54]. In contrast, butyric acid has been proposed to originate mostly from the metabolism of *Firmicutes* [18]. From the *Archaea* group, *Euryarchaeota* phylum also had a positive correlation ($\rho = 0.351$, $p = 0.029$) to acetic acid. *Euryarchaeota* phylum is the most commonly found *Archaea* in the human ecosystem, contributing to less than 10% of the total microbiota population [55]. *Euryarchaeota* is known to metabolize nutrients and other microbial metabolites to end products such as acetate. This results in an increase of total SCFAs concentration and energy

Table 1
Cohort used for the correlation assessment.

	PR group (n = 20)	PL group (n = 19)	HC group (n = 14)	*P* value
Age,Years	9.15 ± 4.35	10.53 ± 5.05	10.14 ± 6.01	0.709
Sex, M/F	4/16	6/13	5/9	/

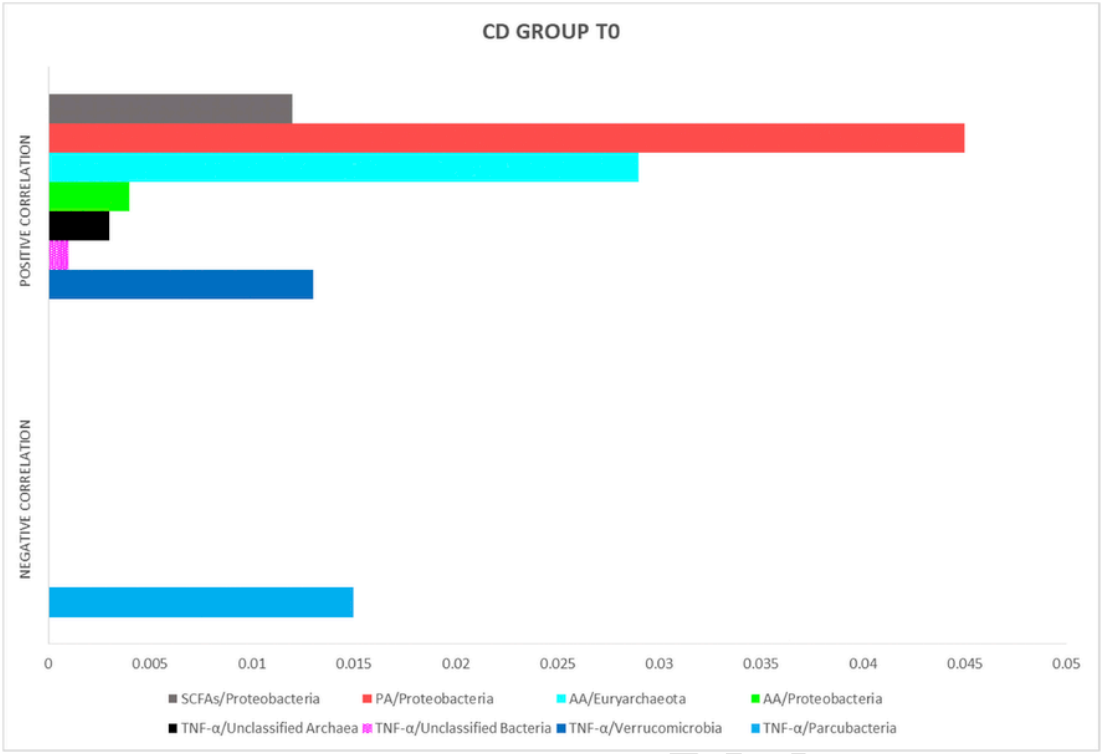

Fig. 1. Statistically significant correlations in the CD group at T0. CD: celiac disease; T0: enrolment day; AA: acetic acid; PA: propionic acid; SCFAs: short-chain fatty acids; TNF-α: tumor necrosis factor alpha.

harvesting [56]. Till now, the potential relationship between *Euryarchaeota* and acetic acid has not been linked to CD.

3.3.2. Correlation values at T0 in healthy children (HC)

Figure 2 shows the results of statistically significant correlation values in the HC group at T0. Members of the *Firmicutes* phylum are mostly Gram-positive bacteria, an abundant group comprising 80% of the intestinal microbiota [45] in healthy subjects and tend to decrease in number in CD patients [16]. Not surprisingly, proinflammatory TNF-α had a strong negative association and a high statistical significance ($\rho = 0.660$, $p = 0.010$) to *Firmicutes* and a negative correlation to *Euryarchaeota* ($\rho = 0.654$, $p = 0.011$). Apparently, the later relationship may play an important role only in the HC population, as it was not identified in the CD group T0. *Synergistetes* is evidently a minority phylum in human feces with an abundance of 0.01% [57]. Regardless of the low quantity, the phylum appears to be relevant for human health [58] and its negative correlation ($\rho = 0.658$, $p = 0.011$) to TNF-α may indicate an important anti-inflammatory factor in healthy population.

Furthermore, acetic acid had a positive correlation ($\rho = 0.569$, $p = 0.034$) to *Candidatus Saccharibacteria*, a group of *Bacteria* still under investigation for its potential role in human health [59]. *Lentisphaerae* negatively correlated ($\rho = 0.556$, $p = 0.039$ and $\rho = 0.584$, $p = 0.028$, respectively) with butyric acid and total SCFAs. This phylum of *Bacteria* is closely related to *Verrucomicrobia*, but its activity and role in host microbiota still needs to be determined [58].

3.3.3. Effect of probiotics on correlation values in the PR group at T1

Significant correlations after probiotic administration are presented in Fig. 3. No significant correlations have been found in the PR group on the enrolment day (T0). However, several significant correlations emerged in the same group after 3-month treatment with the probiotic (PR group T1).

TNF-α is so far known for its important role in pro-inflammatory conditions. In fact, its appearance as an inflammatory mediator in CD patients have been already described [37,60]. However, in the work of Klemenak et al. (2015), the administration of both probiotic strains (*B. breve* BR03 (DSM 16604) and *B. breve* B632 (DSM 24706)) revealed a decrease in TNF-α in PR group after 3 months compared to PL group. The baseline TNF-α levels in both groups were similar to the ones in HC group. As both groups (placebo and probiotic, respectively) had a compliance to GFD of 81% and 91%, the researchers concluded that the reduction in TNF-α occurred because of the combination of *B. breve* strains and GFD. Moreover, Quagliariello et al. (2016) reported that the 3-months probiotic administration in PR group affected the abundance of *Firmicutes phylum* by increasing their percentage, while keeping similar percentage of *Bacteroidetes*, thus resulting in an increase of *Firmicutes/Bacteroidetes* ratio. The ratio in CD subjects is normally lower, usually because of lower percentage of *Firmicutes* or higher percentage of *Bacteroidetes.* In relation to both parameters, an interesting observation has been found by evaluating the correlation results between TNF-α and *phylum Firmicutes*. After 3 months of probiotic administration, TNF-α had a negative correlation ($\rho = 0.468$, $p = 0.038$) to *Firmicutes*, which is in concordance with the article of Klemenak et al. (2015) and Quagliariello et al. (2016), revealing a decrease in TNF-α and re-establishment of the *Firmicutes/Bacteroidetes* ratio, respectively, upon probiotic treatment. Acetic acid correlated negatively ($\rho = 0.502$, $p = 0.024$; $\rho = 0.498$, $p = 0.026$ and $\rho = 524$, $p = 0.018$) with *Verrucomicrobia*, unclassified group of *Bacteria* and *Euryarchaeota*, respectively. Moreover, acetic acid had a negative strong association and a high statistical significance to *Synergistetes* ($\rho = 0.587$, $p = 0.006$). The *Synergistetes phylum* clearly confirmed a negative association with

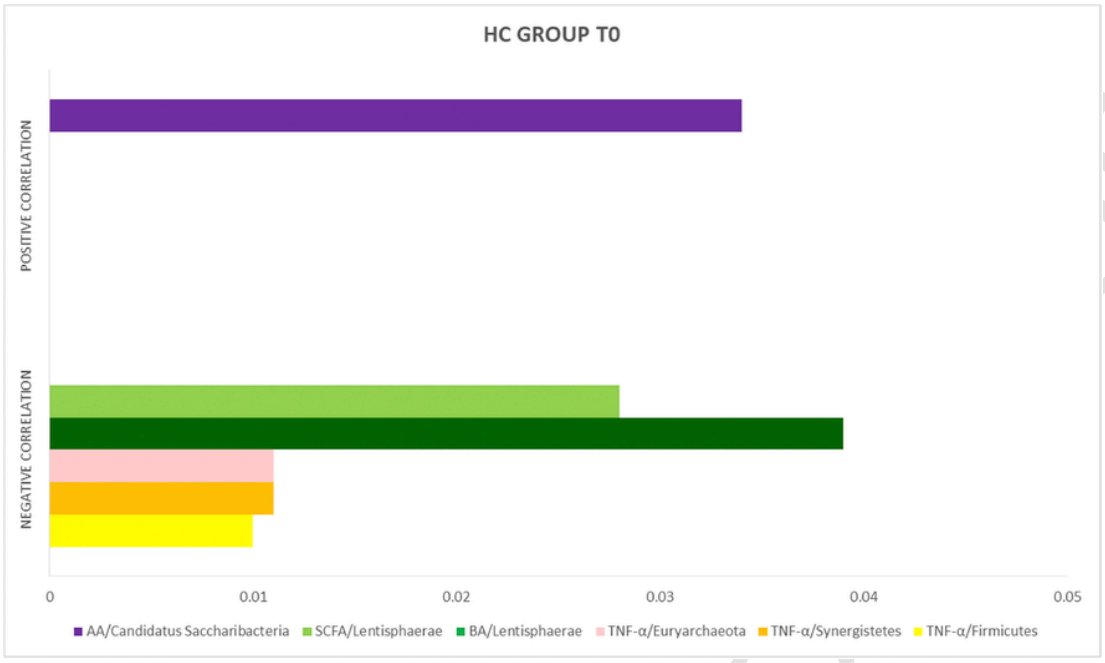

Fig. 2. Statistically significant correlations in the HC group at T0. HC: healthy children; T0: enrolment day; AA: acetic acid; BA: butyric acid; SCFAs: short-chain fatty acids; TNF-α: tumor necrosis factor alpha.

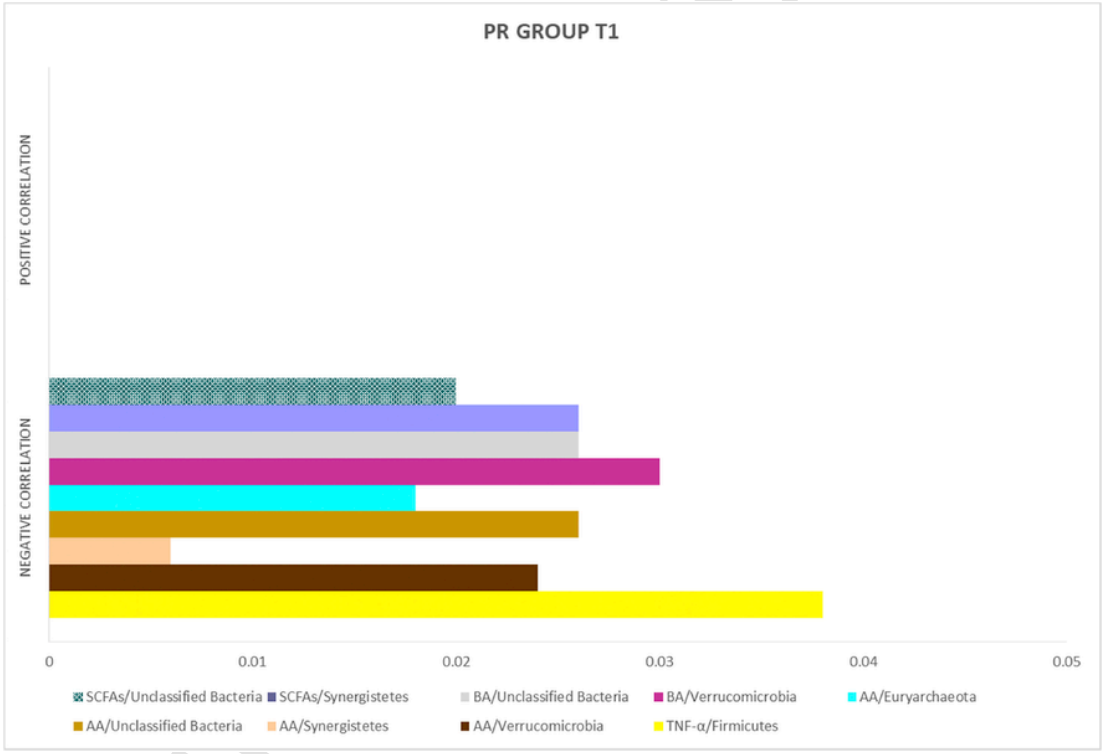

Fig. 3. Statistically significant correlations in the PR group at T1 as a result of probiotic administration. PR: probiotic group; T1: after 3-month treatment; AA: acetic acid; BA: butyric acid; SCFAs: short-chain fatty acids; TNF-α: tumor necrosis factor alpha.

pro-inflammatory acetic acid, previously seen in healthy subjects and may play an important role in anti-inflammatory process too, however in this case as a consequence of a probiotic administration. *Verrucomicrobia* had also a negative correlation ($\rho = 0.486$, $p = 0.030$) to butyric acid, but the later negatively correlated to unclassified *Bacteria* ($\rho = 0.498$, $p = 0.026$). SCFAs had a negative correlation to *Synergistetes* and unclassified group of *Bacteria* ($\rho = 0.496$, $p = 0.026$ and $\rho = 0.517$, $p = 0.020$, respectively). Identified correlations between the mentioned SCFAs and phyla are largely unknown, but they may

play a role in the microbiome restoration as a result of probiotic administration.

3.3.4. Effect of placebo on correlation values in the PL group at T1

When comparing PL group at T0 and T1 (Figs. 4 and 5, respectively), the phylum *Proteobacteria* and *Verrucomicrobia* confirmed again its important role in CD, already observed in CD group T0. In both PL groups (T0 and T1), *Proteobacteria* positively correlated to acetic, propionic acid and total SCFAs (between $\rho = 0.574$, $p = 0.010$ and $\rho = 0.505$, $p = 0.027$). Furthermore, *Verrucomicrobia* had a strong positive association and a high statistical significance to TNF-α in PL group T0 ($\rho = 0.780$, $p = 0.000135$) and continued to positively correlate to TNF-α in PL group T1 ($\rho = 0.495$, $p = 0.037$). However, *Parcubacteria* again confirmed its important role in CD, while revealing a strong negative association and a high statistical significance ($\rho = 0.590$, $p = 0.010$) to TNF-α in PL group T0. Surprisingly, no statistically significant correlation between the two parameters has been found in PL group T1. Moreover, although not expecting any particular differences between the PL group T0 and the PL group T1, the results in PL group after 3-month placebo treatment revealed some new statistically significant correlations. TNF-α had a negative correlation ($\rho = 0.507$, $p = 0.032$) to *Bacteroidetes* and a positive correlation ($\rho = 0.507$, $p = 0.032$) to *Deinococcus-Thermus*. Furthermore, acetic acid negatively correlated ($\rho = 0.521$, $p = 0.022$) to the group of unclassified *Bacteria*. Propionic acid had a negative ($\rho = 0.471$, $p = 0.042$) correlation to *Synergistetes* and butyric acid had a positive correlation ($\rho = 0.498$, $p = 0.030$) to *Proteobacteria*. Microbiota composition is continuously changing as a result of the complex interplay between environmental factors, such as diet, psychological factors and the host itself. Since there has been a 3-month difference between T0 and T1, microbiota shift was likely to occur even in the PL group.

4. Conclusions

Many physiological changes related to CD have been already described (Fig. 6). However, the complexity of the disease is puzzling with many questions still open. Besides classically documented microbiota changes in the *Firmicutes* phylum, our results have shown that additional phyla such as *Verrucomicrobia*, *Parcubacteria* and some yet unknown phyla belonging to *Bacteria* and *Archaea Kingdom*, may also play an important role in CD-related pathology. Moreover, *Proteobacteria* seems to be responsible for the increase of fecal SCFAs in the disease. In healthy subjects, *Synergistetes* and *Euryarchaeota* are present in a minor relative abundance in the human gut system, but they may be additional phyla next to *Firmicutes* contributing to anti-inflammation. Probiotic administration has clearly revealed a negative relationship between *Firmicutes* and pro-inflammatory TNF-α. Moreover, probiotic effect has exposed some new phyla, particularly *Synergistetes*, which negatively correlated to acetic acid and total SCFAs, suggesting a potential role in microbiome restoration. Nevertheless, alterations of microbiota in CD subjects may not be considered exclusively as a consequence of the disease itself, but rather as a part of a complex relationship between many causative factors, including those of diet and psychological nature.

Statement of authorship

Designed research: DMT, DG and TL; conducted research; MK, MP, IA, NBC, AQ; analyzed data: MP, MG and TL; wrote paper: MP and TL; made final approval of the version to be submitted: TL, DMT and DG; had primary responsibility for final content: MP, MK, DG, IA, NBC, AQ, MG, DMT and TL.

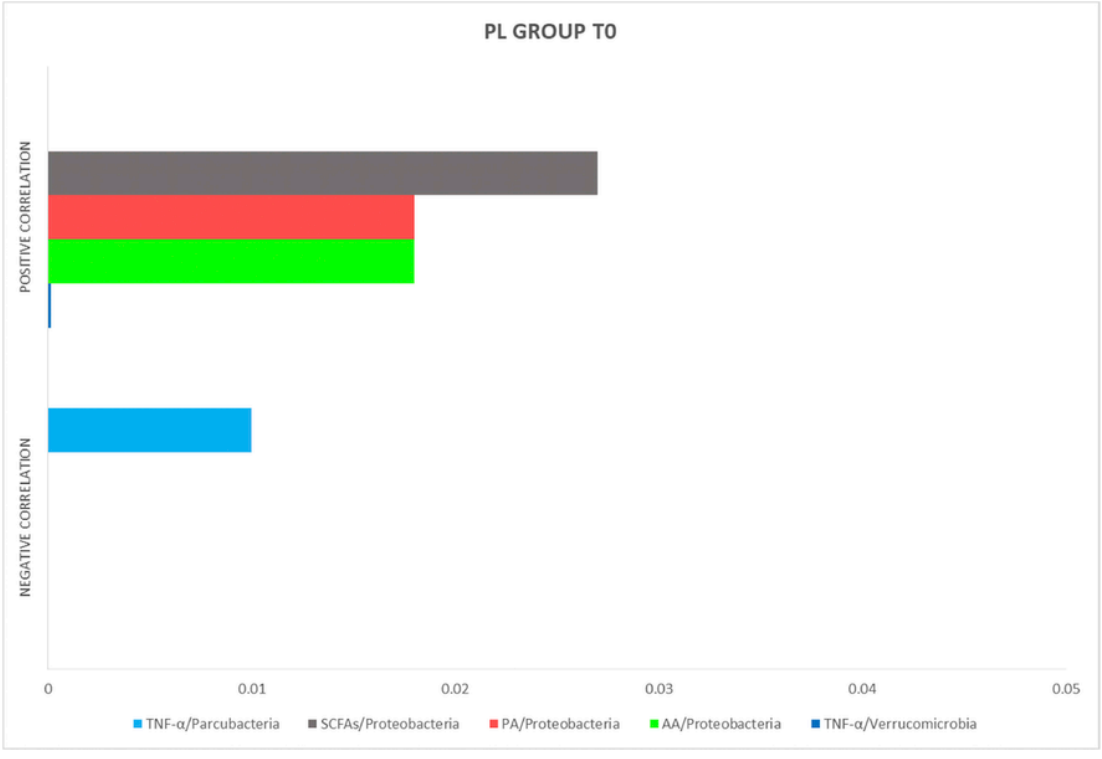

Fig. 4. Statistically significant correlations in PL group at T0. PL: placebo group; T0: enrolment day; AA: acetic acid; PA: propionic acid; SCFAs: short-chain fatty acids; TNF-α: tumor necrosis factor alpha.

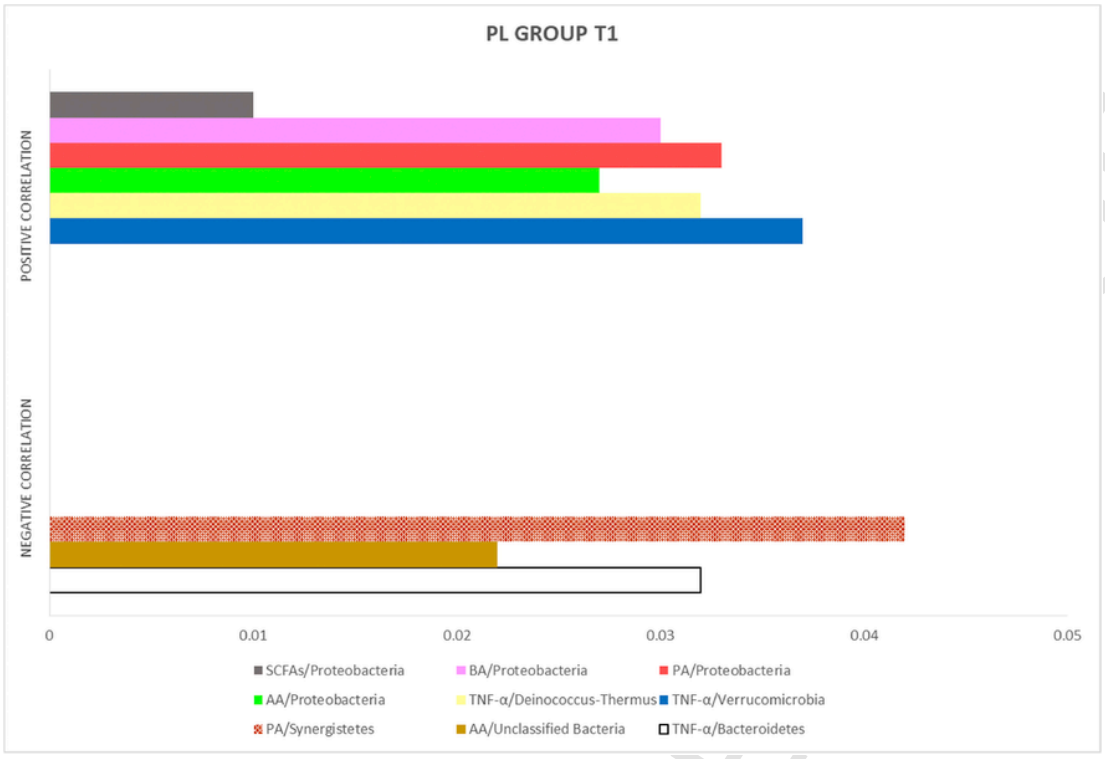

Fig. 5. Statistically significant correlations in PL group at T1 as a result of placebo administration. PL: placebo group; T1: after 3-month treatment; AA: acetic acid; BA: butyric acid; PA: propionic acid; SCFAs: short-chain fatty acids; TNF-α: tumor necrosis factor alpha.

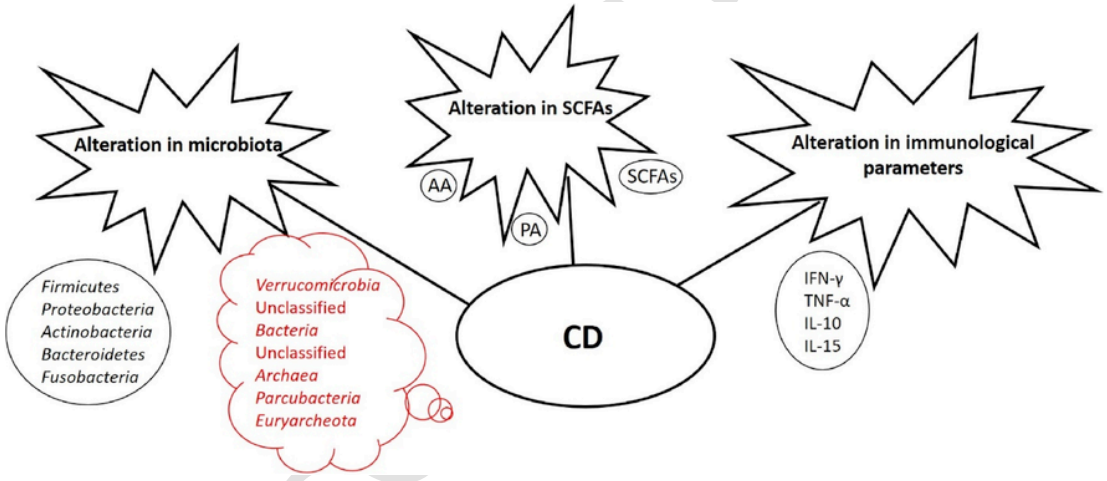

Fig. 6. Schematic representation of the main physiological changes related to CD (shown in black). Potential alterations in microbiota related to CD are shown in red. AA: acetic acid; PA: propionic acid; SCFAs: short-chain fatty acids; IFN-γ: interferon gamma; TNF-α: tumor necrosis factor alpha; IL-10: interleukin 10; IL-15: interleukin 15. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.06.931.

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