Child's Nervous System (2023) 39:633–645
https://doi.org/10.1007/s00381-022-05688-0

ORIGINAL ARTICLE

Gut microbiota and pediatric patients with spina bifida and neurogenic bowel dysfunction

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Received: 8 August 2022 / Accepted: 20 September 2022 / Published online: 30 September 2022

Abstract

Purpose Gut microbiota has recently been recognized to be influenced by a broad range of pathologies. Alterations of gut microbiota are known as dysbiosis and have found to be related to chronic constipation, a condition which affects also pediatric patients with spina bifida (SB).

Methods In this study, gut microbiota richness and composition were investigated by 16S rRNA sequencing and bioinformatic analysis in 48 SB patients (mean age, 11.9 ± 4.8 years) with secondary neurogenic constipation and 32 healthy controls (mean age, 18.0 ± 9.6 years). The study also aimed at exploring eventual effects of laxatives and transanal irrigation (TAI) adopted by SB subjects to get relief from the symptoms of neurogenic constipation.

Results Collected data demonstrated that the microbiota richness of SB patients was significantly increased compared to healthy controls, with a higher number of dominant bacteria rather than rare species. The absence of SB condition was associated with taxa *Coprococcus 2*, with the species *C. eutactus* and *Roseburia*, *Dialister*, and the *[Eubacterium] coprostanoligenes* group. On the other hand, the SB patients displayed a different group of positively associated taxa, namely, *Blautia*, *Collinsella*, *Intestinibacter*, and *Romboutsia* genera, the *[Clostridium] innocuum* group, and *Clostridium sensu stricto 1*. *Bifidobacterium* and the *[Eubacterium] hallii* group were also found to be positively associated with SB gut microbiome.

Conclusions Among SB patients, the administration of laxatives and TAI did not negatively affect gut microbiota diversity and composition, even considering long-term use (up to 5 years) of TAI device.

Keywords Gut microbiota · Spina bifida · Neurogenic constipation · Trans anal irrigation

Introduction

Gut microbiota is defined as a population of microorganisms living in the human gastrointestinal tract; it is one of the most complex ecosystems of the planet, for abundance, biodiversity, and interaction with the host organism [1]. The set of genes encoded by all microbes populating the intestine is referred to as gut microbiome and is known to regulate both the maintenance of health and the pathogenesis of disease in the host [2].

The knowledge about the microbiota and microbiome is gaining high clinical value [3], as recognized by the Human

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Microbiome Project launched in the USA in 2007 [4] and the European Union Project on Metagenomics of the Human Intestinal Tract (MetaHIT) launched in 2008 [5], with the goal of understanding the impact of human microbial components on normal physiology and predisposition to disease. Combined data from the MetaHit and the Human Microbiome Project have provided the most comprehensive view of the human-associated microbial repertoire to date, creating the Integrated Gene Catalog (IGC) [6] which has been applied to the study of microbiome composition in different clinical contexts (i.e., type 2 diabetes, obesity) [7].

The human intestinal microbiota includes more than 10^{14} bacterial cells, mainly in the final part of the gut (the colon), where 10^{12} cells per gram of feces are found. Most human gut microorganisms are strictly anaerobic, and they belong to phyla Bacteroidetes, Firmicutes, and Proteobacteria. Less represented gut bacteria (usually below 1% of the whole microbiota) belong to phyla Actinobacteria, Verrucomicrobia, Acidobacteria, or Fusobacteria [8]. Mucosa-associated bacteria isolated from gut biopsies showed an enrichment of *Lactobacillus*, *Veillonella* (Firmicutes), and *Helicobacter* (Proteobacteria) in proximal gut, whereas *Bacilli*, *Streptococcaceae* (Firmicutes), *Actinomycinaeae*, and *Corynebacteriaceae* (both Actinobacteria) are abundant in the duodenum, jejunum, or ileum, and increased concentrations of *Lachnospiraceae* (Firmicutes) and *Bacteroidetes* are found in the colon [8].

Owing to its large genomic content and metabolic complement, the intestinal microbiota promotes the maintenance of the mucosal barrier integrity, the provision of nutrients (essential amino acids, vitamins, short-chain fatty acids), or protection against pathogens. Additionally, the interaction between commensal microbiota and the mucosal immune system is fundamental for proper immune function [1].

Specific alterations in the composition of the gut microbiota that cause a drastic imbalance between the beneficial and potentially pathogenic bacteria are known as dysbiosis. It has been associated with a vast number of diseases whose incidence is rapidly growing (i.e., obesity, diabetes, inflammatory bowel diseases, colorectal cancer, diverticulitis, irritable bowel syndrome) [9]. Thus, maintaining a healthy profile (e.g., correct intake of dietary fibers, reduction of fat and sugar in food, active lifestyle) is essential to preserve the physiological and metabolic homeostasis of the host and correct bowel function [10].

Recent clinical evidence seems to demonstrate that chronic constipation is also characterized by intestinal dysbiosis. Since the 50% of the fecal volume consists of bacteria, prolonged stasis in the colon can alter the saprophytic microbial pattern [11]. Most patients with chronic constipation have a shortage of *Bifidobacterium* and *Lactobacilli* with an increase in *Bacteroides* and *Enterobacteriaceae* [11, 12]. On the other hand, poor analysis of the microbiome in children suffering from chronic constipation has been reported, and the few available results do not lead to statistically significant values [12]. Furthermore, studies in pediatric age analyzing the microbiome in functional constipation linked to impaired motility of the gastrointestinal tract are completely absent.

Neurogenic constipation represents one of the functional impairments which affect pediatric patients suffering from spina bifida (SB) [13]. SB is the most common central nervous system birth defect, and myelomeningocele is the most common form of SB, but other forms of open and closed lesions exist. The incidence is estimated around 0.1–0.3%. The etiology is multifactorial and involves both genetic and environmental factors. It is defined by characteristic development abnormalities of the vertebrae and spinal cord and associated changes in the cerebrum, brainstem, and peripheral nerves. As a result of denervation, many are the consequences that can affect bladder and bowel function [14].

To date, the medical treatment of neurogenic bowel dysfunction (NBD) has been largely empirical and mainly based on therapeutic solutions designed for the single patient. Clinical data collected from SB patients aged 8–17 years showed that transanal irrigation (TAI) provides relief from the symptoms of neurogenic constipation in the majority (60%) of treated subjects [15]. More recently, the effects of TAI on gut microbiota were clinically demonstrated on SB patients treated for 3 months and reporting significant improvement in constipation, with increased abundance in intestinal bacteria which play a regulatory role in the intestinal motility and host immune system. This resulted in reduction of urinary infections, despite persistent fecal incontinence [13].

For the treatment of intractable neurogenic constipation, the Peristeen transanal irrigation system (Coloplast A/S, Humlebaek, Denmark) proved to reduce symptoms of constipation and fecal incontinence compared with conservative bowel management in patients with spinal injury and SB, significantly improving symptom-related quality of life [16]. Specifically, after changing from conservative bowel management to Peristeen, children and youths with SB and NBD experienced significantly reduced symptoms of bowel dysfunction, including fecal incontinence, and achieved greater partial or total independence, reducing the need for assistance with bowel evacuation [17].

Based on the above considerations, the characterization of the gut microbiota in SB patients can be a fundamental tool (a) to understand and treat some functional impairments related to this condition, including neurogenic constipation and (b) to clarify the effects that therapeutic and nutritional approaches or clinical procedures have on gut microbial population. Thus, the primary objective of this study is to investigate the microbiota profile in pediatric patients with SB (compared to healthy patients); furtherly, the secondary objective is to evaluate the effect of TAI on the intestinal microbiota of these patients.

Methods

Study design and study population

This study was an open-label, interventional, prospective, cross-sectional, and multicenter clinical trial which covered 11 different national hospitals and medical centers treating SB pediatric patients, namely, (1) Fondazione Policlinico Universitario A. Gemelli-IRCCS, Roma; (2) Ospedale Regina Margherita, Torino; (3) Ospedale Casa del Sollievo della Sofferenza, San Giovanni Rotondo (Foggia); (4) Azienda Provinciale Sanitaria, Caltanissetta; (5) Azienda Ospedaliera – Universitaria, Centro Spina Bifida, Parma; (6) Azienda Ospedaliera – Università degli Studi della Campania Luigi Vanvitelli, Napoli; (7) Ospedale San Bortolo, Vicenza; (8) ASST Papa Giovanni XXIII, Bergamo; (9) Fondazione IRCCS Policlinico San Matteo, Pavia; (10) Presidio Ospedaliero Santo Spirito, Pescara; and (11) Azienda Ospedaliera Brotzu, Cagliari.

The study protocol was approved by the ethical committees of the participating hospitals.

In each center, SB patients were enrolled from March to December 2020, in a day hospital setting, in a non-competitive way, after having obtained signed informed consent from participants or their parents. In parallel, a dataset of thirty-two healthy volunteers whose gut microbiota samples were obtained from the NCBI Short Read Archive (SRA) under the project IDs PRJNA355083 [18], SRP073251 [19], and PRJNA401981 [20] (average age, 18 ± 9.6 years; sex, female; 68.8% (22/32)) was introduced in this study as a control healthy group. We kept the healthy controls from peer-reviewed literature if they met the following inclusion criteria: less than 45 years of age, Italian origin, and at least 3000 sequencing reads after QC filtering.

Patients were considered eligible for enrolment if they met the following criteria: male and female children/youths aged between 6 and 18 years, suffering from neurogenic bowel, and not subjected to surgical interventions or hospitalizations in the last 6 months.

Exclusion criteria were the following: presence of pre-existing intestinal diseases such as inflammatory bowel diseases, chronic hepatitis, celiac disease, neoplasms, previous extensive intestinal resections, diarrhea of any origin in progress (intended as more than 6 evacuations per day of watery stools and/or fecal volume in 24 h greater than 250 ml), severe septic state in progress, state of pregnancy, and use of antibiotics in the last 2 months.

Among the SB population, the effect of TAI on gut microbiota was assessed by comparing the following four SB patient groups: group 1 (GR1), including the patients who had been using Peristeen transanal irrigation system (Coloplast A/S, Humlebaek, Denmark) and laxatives; group 2 (GR2), including the patients who had been using laxatives only; group 3 (GR3), including patients who had been using Peristeen device only; and group 4 (GR4), in which the patients who had been using neither of the mentioned treatments were included.

Furtherly, any effect of the long-term use of Peristeen on the gut microbial composition of the SB patients was also investigated by collecting information regarding the years of use of the device. Within GR3, four subgroups could be identified of at least 3 patients each, where the subjects had been using the device for 2 ($n = 4$ people), 3 ($n = 6$ people), 4 ($n = 6$ people), or 5 years ($n = 3$ people); thus, this information could be retrieved from a total of 19 subjects who participated in the study.

Study objectives

The primary objective of the study was the qualitative and quantitative determination of the gut microbiota composition of subjects with secondary neurogenic constipation and SB. To this end, the microbiome was analyzed on a fecal sample of enrolled patients, in comparison with the already known profiles of the healthy Italian pediatric population.

The secondary objective of the study was to investigate eventual effects of TAI on gut microbiota in constipated SB patients who have been using the Peristeen device for different periods of time.

Fecal sample collection and methodology

The intervention consisted in collecting fecal samples from enrolled patients to analyze the composition of their microbiome through biomolecular methods at the Wellmicro S.r.l. Laboratory (Bologna).

Fecal sampling occurred at any time following a spontaneous or scheduled evacuation, as per standard procedure in clinical practice.

Gut microbiota characterization

The characterization of the gut microbiota of SB patients was carried out by using next-generation sequencing (NGS) techniques, a methodological approach which allows to perform a comprehensive analysis of patients' microbial ecosystem.

Total microbial DNA extraction

Bacterial DNA was extracted from fecal samples according to a specific protocol developed and validated by the research group of the Microbial Ecology of Health Unit, Department of Pharmacy and Biotechnology—University of Bologna [21]. Briefly, 250 mg of feces were resuspended in a lysis buffer and homogenized in a FastPrep (MP Biomedicals,

Santa Ana, CA, USA) in the presence of glass and zirconium beads. After several incubation and precipitation steps, the DNA was purified using the QIAamp Mini Spin columns (Qiagen, Hilden, Germany). The concentration and quality of the extracted DNA were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Qubit™ 4 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA).

16S rRNA gene sequencing

After DNA quantification, V3 to V4 region of the 16S rRNA gene was amplified using the primer set S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 [22]. PCR products were purified with a magnetic bead-based clean-up system (Agencourt AMPure XP; Beckman Coulter, Brea, CA, USA). Indexed libraries were prepared by limited-cycle PCR using Nextera technology and further cleaned up with AMPure XP magnetic beads (Beckman Coulter). Libraries were pooled at equimolar concentrations (4 nM), denatured, and diluted to 5 pM before loading onto the MiSeq flow cell. Sequencing on Illumina MiSeq platform was performed by using a 2×250 bp paired-end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

Bioinformatic analysis and determination of the gut microbial ecosystem composition

Raw 16S rRNA gene sequences obtained from the sequencing platforms were processed using the Quantitative Insights Into Microbial Ecology (QIIME) open-source software pipeline. After appropriate filtering by length and quality, the reads were grouped into operational taxonomic units (OTUs) with an identity threshold of 97% using the UCLUST algorithm. The taxonomic assignment was performed using the RDP classifier against the latest version of Greengenes database.

About 20,000 reads for each sample were provided, identifying and quantifying, in terms of relative abundance, all the bacterial genera present in the sample.

The sequencing and bioinformatics analysis pipelines were developed by the research group of the Health Microbial Ecology Unit of the Department of Pharmacy and Biotechnology, University of Bologna, according to previously published methodology [21].

Microbiota fingerprint

In order to analyze the gut microbiota of SB patients in terms of "health-promoting" or dysbiotic potential, the following key parameters were considered:

1. *Ecosystem diversity*, expressed as a numerical index compared to the values detected in the healthy population.
2. *Microbial dysbiosis index*, expressed as a single value, which is the index of the degree of dysbiosis of the microbiota.
3. *Description of the ecosystem* in terms of relative abundance of the most relevant bacterial taxa at different phylogenetic levels, which cover over 90% of the ecosystem. For each taxon, patient values will be compared with those of the healthy population as control.
4. *Description of the functionality of the ecosystem*, in terms of relative abundance of key bacterial groups, selected on the basis of their metabolic capacities and, consequently, of the benefits or disadvantages they exert to the host health (i.e., production of acetate, butyrate, propionate, and lactate; proteolysis, mucolysis, hydrogen sulfide production, endo/exotoxigenic potential). For each functional class, patient values were compared with known mean values in the healthy population.

Data analysis

Paired-end sequenced reads of forty-eight SB patients gut microbiota were analyzed using QIIME2 (version 2020.6). The DADA2 (Divisive Amplicon Denoising Algorithm 2) plugin was used to remove noise and chimeras and to generate ASVs (amplicon sequence variants). Quality filtering and clustering were performed using VSEARCH. High-quality reads were classified taxonomically using the SILVA reference database, version 132 with a similarity threshold of 99%. Samples that had less than 10,000 reads after Illumina MiSeq sequencing were excluded. The bacterial abundance data were imported into R (version 4.0.3) on Rstudio v1.4.1103 where all statistical analysis were performed using R package *phyloseq*. Environmental microbial contaminants were excluded from the present analysis by filtering out ASVs that were specifically present in the negative controls (water) using the *decontam* R package at 5% stringency. Normalization by rarefaction to the number of sequences in the sample with the least coverage was performed to correct for different sequencing depth of each sample (3,402 reads). After filtering and performing rarefaction, 11,114 taxa were present across the samples and were used in the downstream analysis. The overall gut microbiota richness and diversity among the study groups were evaluated by calculating the alpha-diversity indices. The differences in alpha-diversity were then assessed, based on the data distribution of metrics, using ANOVA and Tukey's HSD (honestly significant difference) tests for normally distributed data or Wilcoxon–Mann–Whitney with Holm–Bonferroni correction method for non-normally distributed data.

To evaluate the similarity of gut microbial communities among the study groups, the beta-diversity characteristics

were analyzed by performing principal coordinate analysis (PCoA) based on unweighted UniFrac measures. PCoA was applied on the distance matrices to generate bi-dimensional plots in R. Dispersion of the PCoA clusters was compared using the *betadisper* function in R *vegan* package. The permutational analysis of variance (PERMANOVA) test, calculated using the function *adonis* in the *vegan* package, was performed to determine whether there was a significant separation between different sample groups. The plots were graphed using *ggplot2* R packages.

To determine the potential bacterial biomarkers that drove the differentiation of the microbiota among the patient groups, the linear discriminant analysis (LDA) effect size (LEfSE) algorithm [23] at the genus level was performed. This tool is hosted on the Galaxy web application at https://huttenhower.sph.harvard.edu/galaxy/. LEfSe uses the two-tailed nonparametric Kruskal–Wallis test to evaluate the significance of differences in ASVs in two groups. Using the unpaired Wilcoxon test, a set of pairwise tests was performed. Ultimately, LDA was performed to estimate the effect size of each differentially abundant ASV at the genus level. A strength of the LEfSe method compared with standard statistical approaches is that it provides *p* values along with an estimation of the magnitude of the association between each ASV and the categories under study. For stringency, the samples were considered significantly different if their differences had a p value < 0.05 and an LDA score (log10) > 3, which is one order of magnitude greater than the default of the LEfSe method.

Results

Characteristics of study participants

For this study, 48 children (average age, 11.9 ± 4.8 years; sex, female; 52.1% (25/48)) of Italian origin were enrolled. The healthy controls were sex-matched, with a similar median age (11 years in the healthy controls and 13 years of age in the SB subjects) but with a significantly higher average age than the SB patients (Table 1). More of the SB patients were underweight when compared with the control group (35.4%, 17 out of 48, p value = 0.0007) (Table 1).

Among SB patients enrolled by the study, the majority resulted to suffer from open (47.9%) or closed (6.3%) myelomeningocele. Regarding concomitant NBD, 39.6% of SB patients reported neurogenic constipation, whereas the use of transanal irrigation system (Peristeen) and the use of laxatives were reported by the 47.9% and 31.3% of SB subjects, respectively. Finally, a minority of patients (18.8%) resulted to also suffer from urinary tract infection (UTI) (Table 2).

Table 2 Characteristics of SB patients included in the study

Characteristics of SB patients		
Type of lesion	Open myelomeningocele	47.9% (23/48)
	Closed myelomeningocele	6.3% (3/48)
	Lipomyelomeningocele	18.8% (9/48)
	Others	27.1% (13/48)
Alvus characteristics	Constipation	39.6% (19/48)
	Hard stools	6.3% (3/48)
	Loose stools	2.1% (1/48)
	Normal stools	6.3% (3/48)
	Data not available	45.8% (22/48)
Urinary tract infection	Yes	18.8% (9/48)
	No	81.2% (39/48)
Use of Peristeen transanal irrigation system	Yes	47.9% (23/48)
	No	52.1% (25/48)
Use of laxatives	Yes	31.3% (15/48)
	No	68.7% (33/48)

Differences in gut microbial diversity between spina bifida patients and healthy controls

The rarefaction curve approached an asymptote as the number of sequences increased until the read number which was present in the less rich sample of the dataset after quality filtering (3,402 high-quality reads), indicating that most of

Table 1 Anthropological data of the study population (SB patients) and healthy control subjects

Variable		Spina bifida ($n = 48$)	Healthy controls ($n = 32$)	p value*
Average age	years (± SD); median of age	11.9 (± 4.8); 13	18.0 (± 9.6); 11	0.041
Sex	F	52.1% (25/48)	68.8% (22/32)	0.17
	M	47.9% (23/48)	31.2% (10/32)	
BMI	Underweight BMI ≤ 18.5	35.4% (17/48)	3.1% (1/32)	0.0007
	Overweight 25 ≤ BMI < 30	14.6% (7/48)	3.1% (1/32)	0.14
	Obese BMI ≥ 30	10.4% (5/48)	0.0% (0/32)	0.080

*Fischer's exact test; Mann–Whitney *U* test for continuous data

the gut microbiota species were captured at this level of rarefaction (Online Resource 1).

According to the alpha-diversity measures, the microbiota richness of SB patients was significantly increased compared to that of the healthy controls (FDR-corrected *p* value of observed species index = 0.049) (Fig. 1). We therefore applied the Shannon–Wiener, as well as the inverse Simpson's indexes, which evaluate both richness and evenness; nonetheless, the Shannon–Wiener index places more emphasis on rare species, and the inverse Simpson's index gives more weight to dominant species [24], but both values are linked to diversity with higher values indicating more existing species and higher evenness of their distribution in the microbial ecosystem. Based on these findings, the SB patients have a higher number of dominant bacteria rather than rare species as indicated by the significant difference found between the inverse Simpson's index values of the two study groups (FDR-corrected *p* value = 0.0043] (Fig. 1). Regarding phylogenetic relationships among taxa in each subject of the two study groups, the healthy controls and patients had a similar phylogenetic diversity (FDR-corrected *p* value = n.s.).

Principal coordinate analysis revealed that the gut microbiota of SB patients was distinct from that of the healthy controls (*p* value = 0.001, PERMANOVA, with beta-dispersion *p* value = 0.001), and the fecal microbiota composition among the patients was more similar within one another than it was in the control subjects (Fig. 2). The weighted UniFrac measures display a distinct, but not significant clusterization between SB and control samples (*p* value = n.s.).

Differences in microbiota composition between spina bifida patients and healthy controls

Significant microbial taxa differences between the SB patients and healthy subjects (LDA score > 3, $p < 0.05$) are reported in Online Resource 2, showing the results of LEfSe analysis. Bacterial taxa positively associated with the absence of SB condition included, for example, *Coprococcus 2*, with the species *C. eutactus* and *Roseburia*, *Dialister*, and the *[Eubacterium] coprostanoligenes* group. On the other hand, the SB patients displayed a different group of positively associated taxa, namely, *Blautia*, *Collinsella*, *Intestinibacter*, and *Romboutsia* genera, the *[Clostridium] innocuum* group, and *Clostridium sensu stricto 1*. *Bifidobacterium* and the *[Eubacterium] hallii* group were also found to be positively associated with SB gut microbiome.

Transanal irrigation (TAI) or laxative use alone do not increase disease-associated bacterial groups

Comparing the gut microbiota of SB patients who used both Peristeen device and laxatives (GR1) or only one of the two

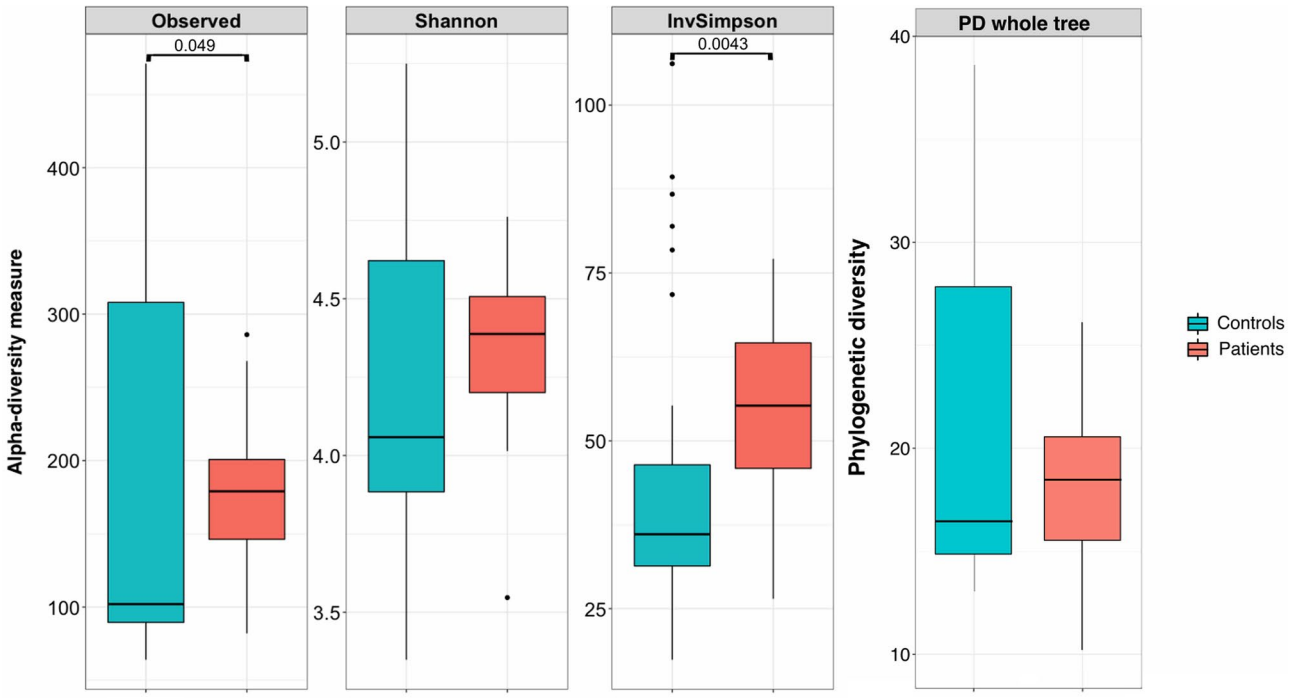

Fig. 1 Alpha-diversity indexes of gut bacterial microbiomes. Boxplots with whiskers showing the comparison of alpha-diversity measures between SB patients ($n = 48$) and healthy controls ($n = 32$). Median, first and third quartile, and *p* values with FDR correction and outliers are shown

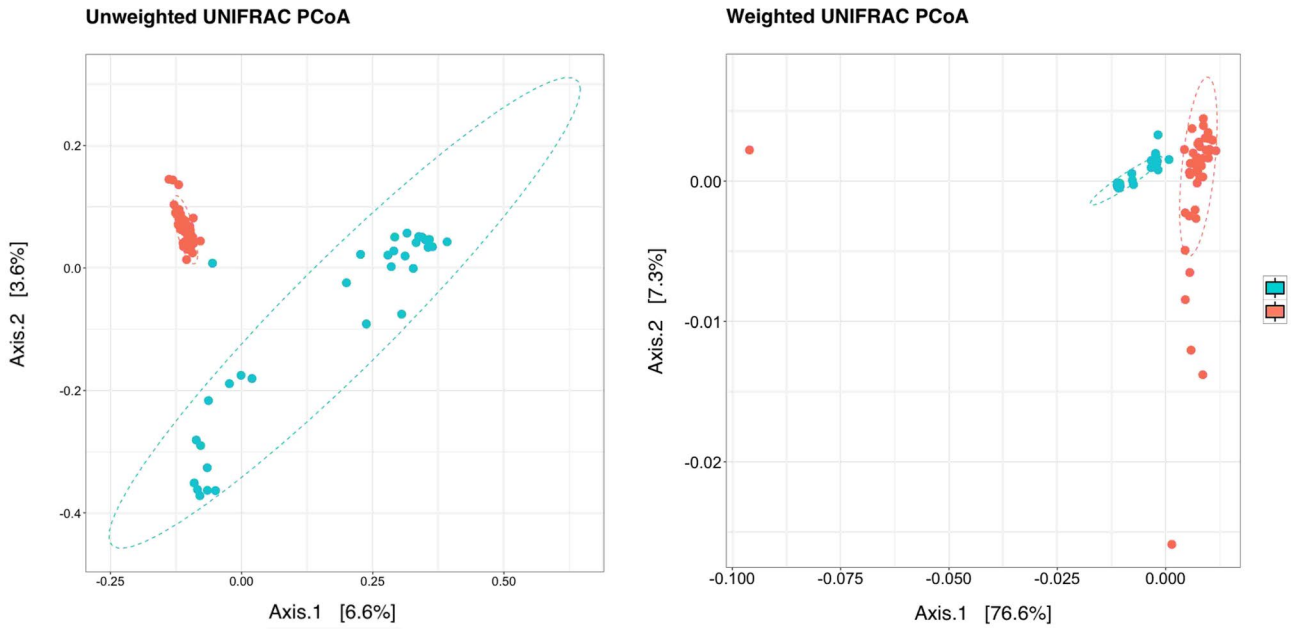

Fig. 2 Principal coordinate analysis (PCoA) on unweighted and weighted UniFrac distance metric at the ASV level calculated on SB patients ($n=48$, salmon pink dots) and healthy controls ($n=32$, light blue dots). Each sample is represented by a dot. Axis 1 explained 6.6% and 76.6% of the variation observed, in the left and right graph, respectively, and Axis 2 explained 3.6% and 7.3% of the variation, in the left and right graph, respectively

treatments (GR2, laxatives; GR3, Peristeen) or none of that (GR4), no significant differences were detected in the alpha-diversity or beta-diversity measures among studied groups (Fig. 3).

Conversely, the results of LDA LEfSE analysis comparing the gut microbial communities of GR1, GR2, GR3, and GR4 revealed some differences among the groups (Fig. 4).

GR1 was positively associated with the *Ruminococcaceae UBA1819* group and with *Anaerotruncus*. GR2 was positively associated with *Ruminococcus gnavus* group, but this association was mainly driven by some outliers, so we showed the result but dismissed this finding. GR3 was associated with *Family XIII UCG001* unknown genus, while GR4 was positively associated with *Flavonifractor* genus and *[Clostridium] innocuum* group.

Long-term use of Peristeen does not negative change gut microbiome composition

The gut bacterial microbiota of SB patients using Peristeen device was finally compared according to the period of use (i.e., 2, 3, 4, or 5 years). No significant differences were detected among subgroups in the alpha- or beta-diversity or LDA LEfSE analysis. Thus, it was demonstrated that the gut microbiota of the patients using the Peristeen® device from 2 to 5 years did not show any significant negative changes (Fig. 5).

Discussion

Gastrointestinal dysfunction is recognized to be an important issue affecting physical and mental health of patients with spinal cord lesions and defects [25], including spina bifida [13]. Gut microbiota has recently become a research hotspot, with growing awareness of the importance to understand its association with the pathophysiological changes and chronic consequences of neurogenic bowel impairments, like constipation in SB patients. Nevertheless, there are scant clinical evidence on SB patients that focus on gut microbiota, and substantial efforts are needed to develop research on this topic. Considering that, this work aimed at characterizing the gut microbiota diversity and composition in SB patients compared to healthy subjects, also considering the effect of TAI treatment of neurogenic constipation associated with the spinal cord defect. Collected data demonstrated that, although the healthy controls and SB patients showed a similar Phylogenetic diversity, a higher number of bacterial species were found in the pathological group, with a prevalence of dominant bacteria, rather than rare species. This suggests that the gut microbiota of SB patients is characterized by an increase in the number of dominant taxa, which however share a similar ecological differentiation (phylogenetic diversity) with the healthy controls.

Fecal microbiota richness is widely considered as a marker of gut health, stability, and resilience to perturbation

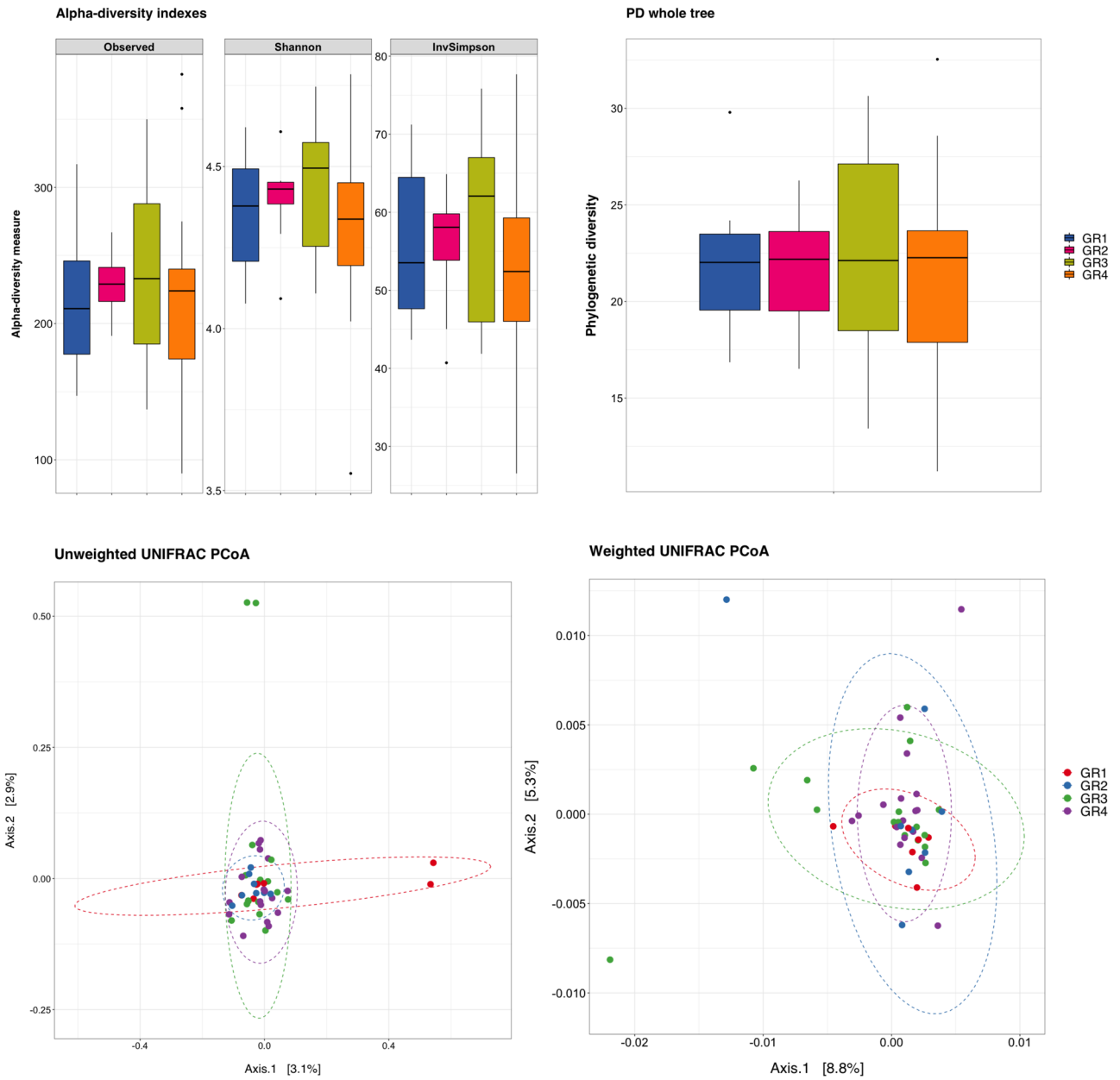

Fig. 3 Alpha-diversity indexes of microbiome samples from SB patients. Top left and right panels: boxplots with whiskers show the comparison of alpha-diversity measures between SB patients GR1 ($n=7$), GR2 ($n=8$), GR3 ($n=16$), and GR4 ($n=17$). Median, first and third quartile, and p values with FDR correction and outliers are shown. Bottom left and right panels: principal coordinate analysis (PCoA) on unweighted and weighted UniFrac distance metric at the ASV level calculated on SB patients from GR1 (red dots), GR2 (blue dots), GR3 (green dots), and GR4 (purple dots). Each sample is represented by a dot. Axis 1 explained 3.1% and 8.8% of the variation observed, in the left and right graph, respectively, while Axis 2 explained 2.9% and 5.3% of the variation, in the left and right graph, respectively

[26]. Nevertheless, recent studies investigating broad cohorts of patients [27, 28] and characterizing specific confounder effects [29–31] highlighted that multiple factors are associated with the variation of microbiome-derived biomarkers, including richness, in both health and disease status. Among healthy subjects, microbiome was found to be mostly influenced by transit time and stool consistency [30, 31], age [32], body mass index [33], dietary habits [34], and medical treatment [27]. These variables seem to cause important inter- and intra-individual variation in gut microbiota composition and richness, regardless of host health [26].

In the present study, fecal microbial richness was unexpectedly found to be greater in SB patients with neurogenic bowel than in healthy subjects. Previous studies reported

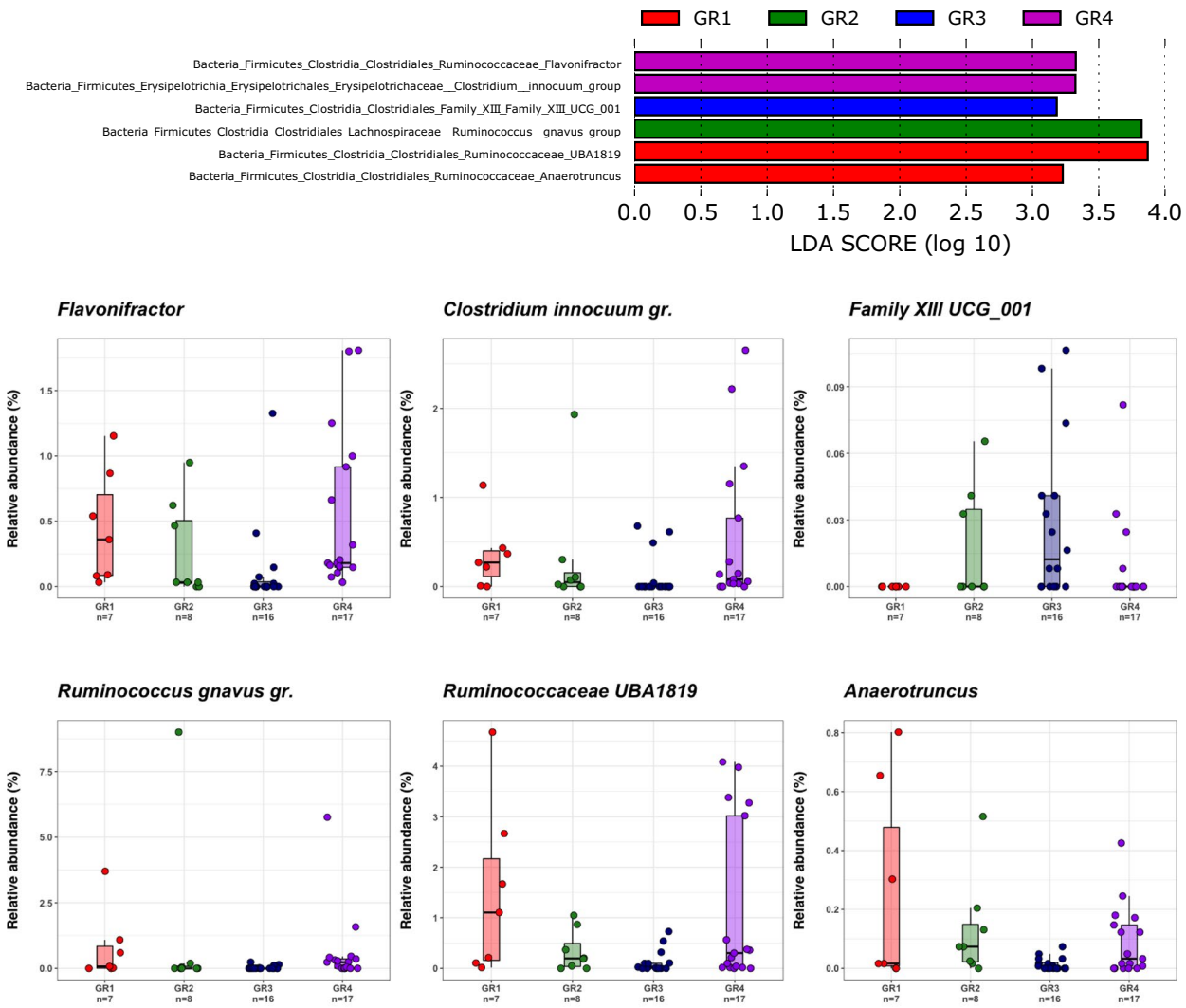

Fig. 4 Top panel: plot from LDA LEfSE analysis on SB patients divided in four groups by treatment. The plot was generated using the online Galaxy web platform tools at https://huttenhower.sph.harvard.edu/galaxy/. The length of the bar column represents the LDA score. The figure shows the microbial taxa with significant differences between the SB patients' subgroups GR1 (red), GR2 (green), GR3 (blue), and GR4 (purple) (LDA score > 3.0). Bottom panel: dot plots with box and whiskers of bacterial biomarkers relative abundances (RA%)

increased gut microbe diversity and richness in patients suffering from depression [35], autism [36], and HIV-1 infection [37], but the consequences of these results remain unexplained. Considering factors which influence gut microbiota richness, the results may be explained by age and dietary differences between the two study groups. Unfortunately, data on the dietary habits of SB and healthy subjects are mainly lacking, this representing a limitation of the study. Indeed, statistically significant differences between age and BMI of the two study groups could support this finding.

In line with previous evidence [13], the analysis of gut microbiota composition revealed significant differences between SB patients and healthy controls, confirming that dysbiosis is related to bowel dysfunctions which characterized spinal cord defects like SB. This study showed that numerous taxa were positively associated with the absence of SB condition, and among them, we found some anaerobic butyrate-producing bacterial taxa. For example, a positive association was found between healthy subjects and *Coprococcus 2*, comprising the species *C. eutactus*, whose presence is a biomarker for good language development in children [38], and *Roseburia*, in accordance with the fact that these bacteria have been indicated as scarcely present in the fecal microbiome of SB patients [13]. Another bacterial genus negatively linked with the SB condition is *Dialister* [13], whose presence in the healthy human gut, together

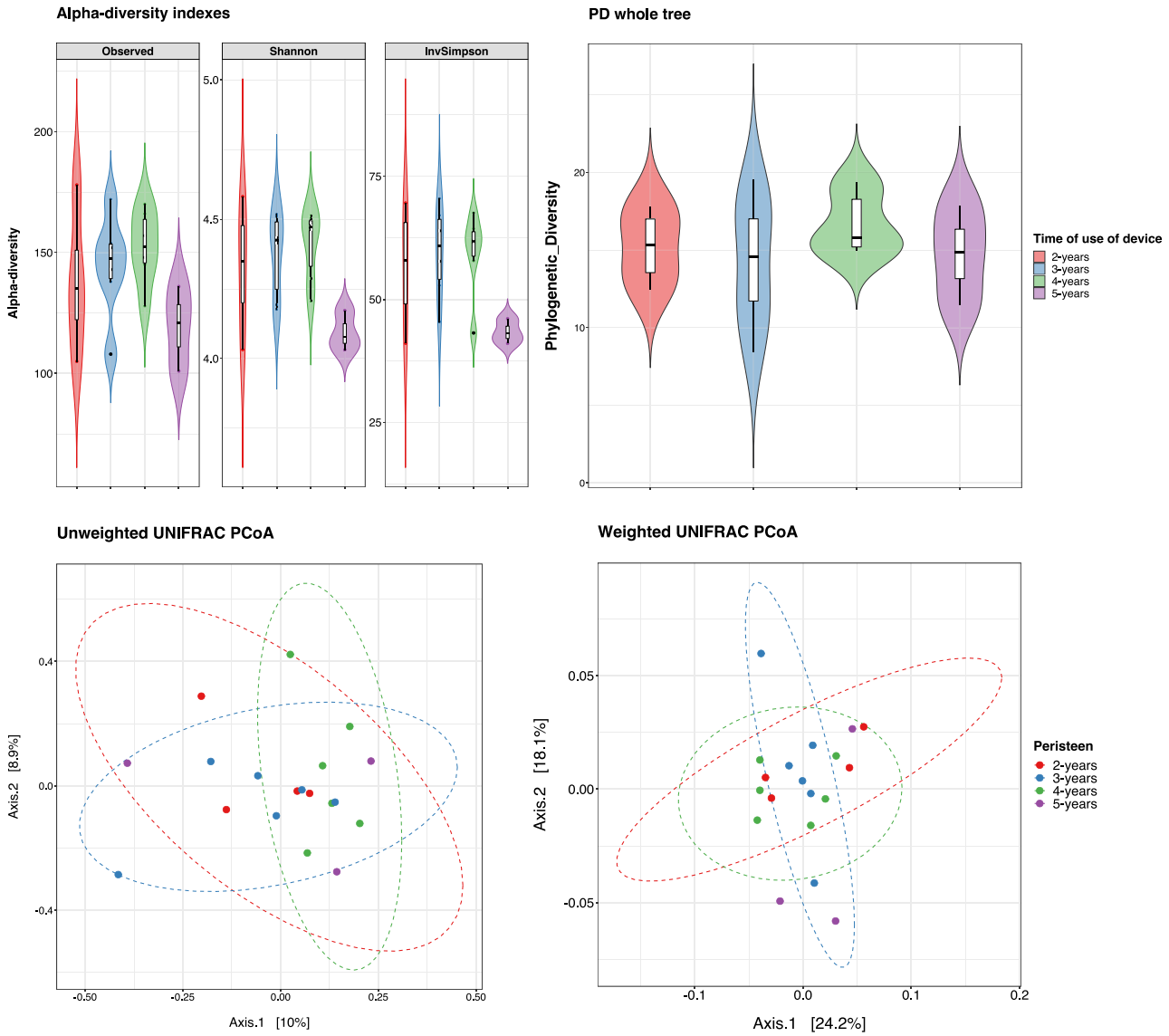

Fig. 5 Alpha-diversity indexes of microbiome samples from SB patients who have used TAI for several years. Top left and right panels: violin plots with box and whiskers show the comparison of alpha-diversity measures between SB patients who used TAI for 2 years ($n=4$), 3 years ($n=6$), 4 years ($n=6$), and 5 years ($n=3$). Median, first and third quartile, and outliers are shown. p values with FDR correction are not shown since we found no significant differences among the values. Bottom left and right panels: principal coordinate analysis (PCoA) on unweighted and weighted UniFrac distance metric at the ASV level calculated on SB patients who used TAI for 2 years (red dots), 3 years (blue dots), 4 years (green dots), and 5 years (purple dots). Each sample is represented by a dot. Axis 1 explained 10% and 24.2% of the variation observed, in the left and right graph, respectively, while Axis 2 explained 8.9% and 18.1% of the variation, in the left and right graph, respectively

with *Coprococcus*, has been formerly linked with mental health and a higher quality of life [39]. Of note, a positive association was also detected between healthy control fecal microbiota and [*Eubacterium*] *coprostanoligenes* group, which has been found capable of metabolizing cholesterol to coprostanol, thus possibly helping the modulation of host cholesterol levels [40].

On the other hand, the SB patients were found to be associated with *Blautia*, an acetic acid- and bacteriocin-producing genus. The latter bacteria have been negatively associated with visceral fat accumulation [41] but also positively associated with ulcerative colitis (UC) and irritable bowel syndrome (IBS) [42]. At the same time, microbiota analysis of SB patients showed an expansion of the relative abundance of subdominant taxa such as *Collinsella*, a genus whose increased presence has been associated with atherosclerosis [43], type 2 diabetes [44], and gut permeability alteration [45]. Other increasing taxa were *Intestinibacter* and *Romboutsia* genera,

whose presence has been associated with neurodevelopmental disorders (NDD) in children [46]; [*Clostridium*] *innocuum* group, a vancomycin-resistant microorganism associated with antibiotic-induced diarrhea [47]; and *Clostridium* sensu *strictu 1*, a butyrate-producing bacterium, whose increase has been linked to IBS [48]. *Bifidobacterium*, a widely known health-associated probiotic bacterial genus, was present at higher levels in these patients. In addition, [*Eubacterium*] *hallii* group, a butyrate- and propionate-producing [49] genus of intestinal bacteria, was found positively associated with SB gut microbiome in this study.

Regarding the management of neurogenic constipation in SB patients and effect on their intestinal microbiota, TAI and laxatives used together or alone were found not to affect bacterial taxa biodiversity in comparison with the absence of treatments. Nevertheless, differences were detected among microbial communities associated to the four treatment groups. Specifically, the use of Peristeen® device for transanal irrigation combined with laxatives was positively associated with the *Ruminococcaceae* UBA1819 group, a taxon which has been linked to rheumatoid arthritis [50], and with *Anaerotruncus*, a common intestinal bacterial commensal genus. The use of Peristeen® device alone was associated with *Family XIII UCG001* unknown genus, which is a common intestinal bacterial commensal group and has been found to be negatively associated with hepatic glycogen storage diseases (GSD) [51]. Finally, the complete absence of treatment was positively associated with *Flavonifractor* genus, a flavonoid-degrading gut bacterial commensal genus, and [*Clostridium*] *innocuum* group, whose increase has been linked to antibiotic-associated diarrhea [47].

Remarkably, the microbiota analysis of SB patients using Peristeen® device for long periods of time (2–5 years) highlighted that TAI do not alter their intestinal microbial composition. This evidence could be even more important when considering that the use of laxatives was instead demonstrated to create profound long-term changes in the gut microbiome, according to experiments performed in mice [52]. These side effect consequences of laxative treatment are particularly important considering the increased use of laxatives like polyethylene glycol (PEG) in the treatment of neurogenic constipation in children with spina bifida [53], with the long-term impact of laxative-related immune response being currently unknown. Based on that, the recurrence to transanal irrigation devices like Peristeen appears to be a safer therapy for neurogenic constipation in SB patients, providing clinical benefits and avoiding the risk of addiction on laxatives.

Finally, the main limitations of this study regard the comparison of SB patients with healthy subjects from database, with different average age, as well as the small sample size of SB patient subgroups considered for the analysis of TAI and laxatives effects on gut microbiota.

Conclusions

Overall, this work represents a significant step forward a better knowledge of SB pathological implications and related bowel dysfunctions, describing gut microbiota richness and composition in a sample of pediatric SB patients compared to healthy subject. In these patients, the management of neurogenic constipation by the use of laxatives and Peristeen was found not to alter gut microbiota, with TAI device revealing to be a safe treatment option even in the long term. Nevertheless, further studies are needed to characterize gut microbiota in SB patients and understand how it is affected by the medical treatment of neurogenic constipation associated with this pathology.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00381-022-05688-0.

Acknowledgements The Authors thank Margherita Capriati for the coordination of the study and Clariscience S.r.l. for the support in manuscript preparation.

Author contribution C. R. devised the project and the main conceptual ideas for the study. V. F. P., V. B., G. C., C. D. A., G. L., L. L., A. M., G. M., L. M., S. G.N., A. R., D. S. B, E. V., and A. C. acquired and analyzed the data. E. A. conducted the work. All authors approved the final draft submitted.

Availability of data and material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval and consent to participate This study was a multicenter clinical trial involving 11 different national hospitals and medical centers treating SB pediatric patients, namely, (1) Fondazione Policlinico Universitario A. Gemelli-IRCCS, Roma; (2) Ospedale Regina Margherita, Torino; (3) Ospedale Casa del Sollievo della Sofferenza, San Giovanni Rotondo (Foggia); (4) Azienda Provinciale Sanitaria, Caltanissetta; (5) Azienda Ospedaliera – Universitaria, Centro Spina Bifida, Parma; (6) Azienda Ospedaliera – Università degli Studi della Campania Luigi Vanvitelli, Napoli; (7) Ospedale San Bortolo, Vicenza; (8) ASST Papa Giovanni XXIII, Bergamo; (9) Fondazione IRCCS Policlinico San Matteo, Pavia; (10) Presidio Ospedaliero Santo Spirito, Pescara; and (11) Azienda Ospedaliera Brotzu, Cagliari. The study protocol was approved by the ethical committees of all the participating hospitals.

Consent for publication All authors approved the final draft submitted. All of the material is owned by the authors, and/or no permissions are required.

Conflict of interest The authors declare that they have no conflict of interest.

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