Gut Pathogens

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The effects of probiotic treatment with *Bifidobacterium breve*, Bif195 for small intestinal Crohn's disease and the gut microbiome: results from a randomised, double-blind, placebo-controlled trial

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Abstract

Background The aetiology of Crohn's disease, a chronic inflammatory bowel disease, is multifactorial and not completely understood. However, the association with gut dysbiosis is well-established, and manipulation of the gut microbiota has gained interest as a treatment strategy. This study aimed to investigate the effects of the probiotic strain *Bifidobacterium breve*, Bif195™ (Bif195) on intestinal inflammation, symptoms, and the gut microbiome composition in patients with small intestinal Crohn's disease.

Methods This was a randomised, double-blind, placebo-controlled trial. Thirty-three patients with small intestinal Crohn's disease were assigned to eight weeks of treatment with Bif195 or placebo (1:1). The primary outcome was changes in bowel wall thickness measured by intestinal ultrasonography. Other outcomes were changes in symptom severity, quality of life, faecal calprotectin, fatigue, and specific inflammatory parameters on ultrasonography. Changes in the microbiome composition were also examined.

Results Bif195 did not affect the bowel wall thickness in the small intestine compared to placebo. Nor did we observe effects on secondary or clinical explorative outcomes. Analysis of the gut microbiome showed that the relative abundance of *B. breve* rose during the intervention in the Bif195 group, but the result was statistically non-significant. Surprisingly, we observed a clustering of baseline microbiome data into two groups that differed in several aspects including a statistically significant difference in the incidence of previous bowel resections among the participants. Furthermore, changes in symptom scores after eight weeks of intervention were significantly different across the two microbiome groups, with an interaction effect of $p = 0.04$.

Conclusions Eight weeks of treatment with Bif195 did not affect clinical outcomes for Crohn's disease. However, variations in baseline microbiome data influenced the results. This underscores the importance of assessing baseline microbiome data in intervention studies in Crohn's disease.

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Keywords Crohn's disease, Gut microbiota, Probiotic treatment, *Bifidobacterium breve*, Bif195™, Shotgun metagenomics

Background

Crohn's disease (CD) is an inflammatory bowel disease (IBD) characterised by segmental, transmural inflammation often located in the ileocaecal region, although it can affect the entire gastrointestinal (GI) tract [1]. Typical clinical and biochemical indicators of inflammatory flareup are stomach pain, diarrhoea, weight loss, fatigue, and increased faecal calprotectin (f-calprotectin), but more severe intestinal complications and extraintestinal manifestations, may also occur [2]. The primary goal for treating CD is to induce remission, avoid complications and surgery, and enhance quality of life through symptom relief [3].

The aetiology of CD is due to both genetic predisposition and environmental factors. However, the details still need to be clarified [4]. Intestinal mucosal immune regulation and barrier function disturbances may be involved in the pathogenesis of IBD, as well as dysbiosis in the gut microbiota [4, 5]. Multiple studies have found an association between IBD and reduced bacterial diversity and increased bacterial translocation [4], leading to research into new treatment strategies involving manipulation of the gut microbiota with faecal microbiota transplantation (FMT) and probiotics [5].

In recent years, intestinal ultrasonography (IUS) has gained recognition as a valuable tool for diagnosing and monitoring CD. It is a non-invasive procedure requiring little preparation for both patients and clinicians [6]. With the introduction of the International Bowel Ultrasound Segmental Activity Score (IBUS-SAS) [7] standardised and reliable measurements to identify transmural segmental inflammatory activity are available by involving four key parameters: 1) bowel wall thickness (BWT) which is defined as abnormal when it is > 3 mm, 2) grade of intestinal hyperaemia using colour Doppler imaging (CDI), 3) presence of inflammatory mesenteric fat (I-fat), and 4) disturbances in the bowel wall stratification (BWS) [7]. All four parameters are assessed with individual scores corresponding with the deviance from the healthy intestine, eventually giving a total score of up to 100. This score has been validated and is currently the most responsive IUS score [8].

Thus, IUS is an objective modality to assess transmural inflammation. Objective measures are important elements in clinical trials investigating the clinical outcomes of probiotic treatments.

This study investigates the effects of the probiotic strain *Bifidobacterium breve*, Bif195™ (DSM 33360), GALENEX™, in the following referred to as Bif195. *Bifidobacterium* is a bacterial genus naturally occurring in the healthy human intestine which appears to have protective properties against pathogens and inflammation and is found to have a reduced abundance in patients with IBD [9]. Specifically, Bif195 has been shown to protect healthy individuals' stomachs and small intestines against mucosal damage caused by acetylsalicylic acid (ASA) in randomised controlled studies from 2019 [10] and 2024 [11].

We aimed to investigate the clinical effects of supplementing Bif195 for eight weeks on disease activity and the gut microbiome in patients with small intestinal Crohn's disease.

Methods

Study design

The study was a randomised, double-blind, placebo-controlled trial with a design illustrated in Fig. 1. Eligible participants were assigned to eight weeks of treatment with either Bif195 or placebo (1:1) followed by eight weeks without treatment (follow-up). Participants were screened for eligibility before randomisation at visit 1. They were found ineligible if they did not meet the inclusion criteria on IUS or other parameters mentioned beneath. At randomisation, all study products were handed out, and participants were instructed to consume one capsule daily until the end of the intervention eight weeks later when the remaining study product was returned to the investigator. To assess compliance, the capsules were counted after four and eight weeks of intervention. During the study period, from screening to end-of-study visit, participants completed two validated questionnaires regarding disease severity and quality of life and collected stool samples for gut microbiome analyses.

The trial was conducted in accordance with the International Conference on Harmonisation E6 Good Clinical Practice, approved by the National Committee on Health Research Ethics, Capital Region of Denmark, and registered at Clinicaltrials.gov as NCT04842149.

Study participants

Between May 2021 and September 2023, participants were recruited from three outpatient clinics at Copenhagen

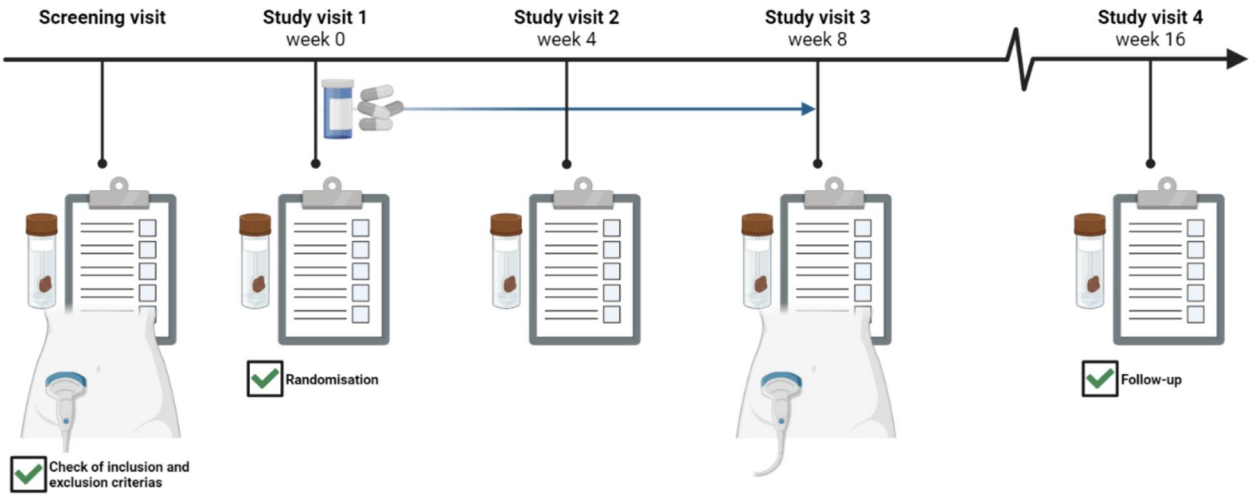

Fig. 1 The study timeline with an illustration of the activities for all five visits. Faecal samples were collected, and questionnaires completed at every visit. Intestinal ultrasonography was performed at the screening visit and after eight weeks of intervention

University Hospitals at Hvidovre, Bispebjerg, and Herlev, Denmark, and as self-referrals via advertisements on social media and in a patient-oriented magazine (Colitis-Crohn Foreningen Magazine). At the outpatient clinics, the recruitments were conducted in connection with medical appointments, but at Copenhagen University Hospital Hvidovre, patients in self-monitored treatment programmes were also reached by letter.

The aim was to include 60 participants based on the assumption that 40% and 80% of the participants in the placebo group and Bif195 group, respectively, would achieve the primary endpoint criterium, with a power (1-β) of 0.8 and an alpha level of 0.05 (two-tailed test) for intension-to-treat analysis.

Participants were adult patients with CD aged 18–75 with an IUS-verified small bowel wall thickness ≥ 4 mm. Other inclusion criteria were: 1) ability to read and speak Danish, 2) provided voluntary written informed consent, and 3) diagnosis of CD for at least six months.

The exclusion criteria were 1) positive findings of enteropathogenic microorganisms including *Clostridioides difficile*, 2) consumption of antibiotics, probiotics, non-steroidal anti-inflammatory drugs (NSAIDs) for more than three days, or systemic glucocorticosteroids within four weeks prior to inclusion, 3) non-stable treatment with immunosuppressants or anti-cancer drugs, e.g., anti-TNF-alpha agents, anti-integrin agents, azathioprine or 6-MP, 6-thioguanine, methotrexate, tacrolimus, cyclophosphamide, or cyclosporine within three months prior to inclusion, 4) participation in other clinical trials within 30 days prior to inclusion, 5) severe psychiatric disorder, 6) alcohol or drug abuse, and 7) pregnancy, planned pregnancy, or breastfeeding.

A f-calprotectin level < 250 mg/kg was initially defined as an exclusion criterion but was discarded because five participants with IUS-verified inflammation with BWT ≥ 4mm had been excluded only because of a f-calprotectin level < 250 mg/kg. Thus, we revised the study protocol with subsequent approval by the Scientific Ethics Committee for Copenhagen Regional Hospitals, Denmark.

All participants were instructed to maintain their usual lifestyle throughout the study.

Intestinal ultrasound

IUS was performed at Copenhagen University Hospitals Hvidovre and Bispebjerg using a Siemens Sequoia with a 10L4 probe. The procedure was performed by experienced doctors trained in IUS and certified by the International Bowel Ultrasound Group. The terminal ileum was identified, and the IBUS-SAS was assessed using a mean of two BWT measures in cross-section and two in longitudinal. CDI, I-fat and BWS were graded as previously described [7].

Study product description

The vegetable capsules used in both the Bif195 group and placebo group were in identical packaging and appeared similar in terms of taste, size, shape, colour, etc.

In the Bif195 group, capsules contained the probiotic *Bifidobacterium breve,* Bif195™ (DSM 33360) and excipients. The colony-forming unit (CFU) stability of the production batch of Bif195 was monitored in parallel with the study, and the daily dose of CFU was at least 15×10^9 at the end of the study intervention by intake of 1 capsule/day. The placebo capsules contained excipients only.

All capsules were stored in a temperature-monitored refrigerator at the research site. During the study intervention, the study participants were instructed to store the capsules in their refrigerator at home.

Outcomes and measures

The primary outcome of the study was reduction in BWT after eight weeks of treatment with Bif195 compared to placebo. BWT at the most affected bowel segment was assessed by IUS with a response defined as a reduction of BWT ≥ 25% OR 2 mm OR a 2-point CDI reduction as a binary outcome (reduction yes/no) [12].

Secondary outcomes were differences between the Bif195 and placebo groups regarding changes in f-calprotectin, total IBUS-SAS score, transmural remission, symptom severity, quality of life, and BWT after eight weeks of intervention. Transmural remission was defined as BWT ≤ 3 mm as a binary outcome (yes/no). The severity of symptoms was measured by the Harvey-Bradshaw Index (HBI) [13], which is a validated numerical index used for measuring the activity of CD containing five items regarding general well-being, abdominal pain, daily number of liquid stools, abdominal mass, and complications associated with CD. The higher the HBI score, the greater the disease activity. Quality of life was measured by the validated Inflammatory Bowel Disease Questionnaire (IBDQ) [14], which consists of 32 questions each scored 1–7, giving a total score range of 32–224 (lowest score = worst, highest score = best).

Explorative outcomes were differences between Bif195 and placebo regarding effects on fatigue, CDI, and I-fat. Information about fatigue was obtained via two items from the IBDQ: question 2: "How often has the feeling of fatigue or being tired and worn out been a problem for you during the last two weeks?" [14] and question 6: "How much energy have you had during the last two weeks?" [14]. The scores were evaluated separately and for the two questions combined. In the IBUS-SAS, CDI is ranked on a 4-point scale depending on the presence of hyperaemia in the bowel wall: none (0 points), discrete (1 point), moderate (2 points), and severe (3 points). The presence of I-fat is ranked on a 3-point scale: none (0 points), unsure (1 point), and present (2 points).

Lastly, the effect of Bif195 on the microbiome composition after eight weeks of treatment was also defined as an explorative outcome.

Because the IUS was used as a screening tool before inclusion, IUS was not repeated at visit 1, and IUS results from all screening visits were used as baseline data. For every other outcome, data obtained from visit 1 (i.e., at randomisation) was used as baseline data.

Statistical analysis of clinical data

Except for the microbiome analyses, statistical analyses were performed by Signifikans, Denmark, using the software SAS® Release 9.3 or later versions. The study investigators remained blinded until after the statistical analyses were performed.

A binomial logistics regression model was used for the primary outcome and transmural remission. The primary outcome included terms of treatment (Bif195 or placebo) and previous bowel resection, while transmural remission only included terms of treatment. A Chi-square test was used for the secondary outcome regarding the reduction in BWT. For all other secondary and explorative clinical outcomes, the differences between the Bif195 and placebo groups were found by ANCOVA analysis of the delta values from baseline and after eight weeks of treatment, with baseline values as covariate.

Participants were grouped into a full analysis set (FAS) and a per-protocol analysis set (PPS). PPS excluded all participants with records of major protocol deviations. Unless otherwise described, FAS will be reported for clinical outcomes. Safety data will also be reported on the FAS population, as all participants met the criteria for FAS.

DNA purification and sequencing

DNA was purified using the DNeasy PowerSoil Pro Kit (250) (cat. nr. 47016, Qiagen, Hilden, Germany) as recommended by the manufacturer. DNA concentration was measured on a 2.0 Qubit Fluorometer with the Qubit 1X dsDNA HS Assay Kit (Invitrogen, cat. nr. Q33231, Eugene, Oregon, USA). Negative and positive controls (water and ZymoBIOMICS Gut Microbiome Standard (cat. no. D6331, Zymo Research, Irvine, California, USA) respectively) were included in DNA purification. The library preparation was performed automatically on a Biomek i7 robot (Bechman Coulter, Indianapolis, USA) using the Illumina DNA prep—LP (M) Tagmentation reagents (cat. nr. 20060059, Illumina, San Diego, California, USA) and IDT® for Illumina® DNA/RNA UD Indexes Tagmentation (cat. nr. 20027213/20027214, Illumina, San Diego, California, USA). Libraries were sequenced on an Illumina NextSeq 500 with Illumina NextSeq 500/550 Mid Output Kit v2.5 (300 Cycles) (cat. nr. 20024905, Illumina, San Diego, California, USA) generating 2 × 150 base pair paired-end reads.

Microbiome data analysis

Microbiome analyses were performed on the PPS. Raw FASTQ read files were trimmed using fastp v. 0.20.1 [15] with –qualified_quality_phred of 20 and minimum read length of 50. FastQC v. 0.11.8 [16] was used to evaluate the quality of the reads before and after trimming.

Depletion of human sequences was performed by aligning the trimmed reads to the human genome (hg38, University of California, Santa Cruz) using bowtie2 v. 2.3.4.1 [17] with end-to-end alignment and maximum fragment length for valid paired-end alignments (-X) of 2000. Clade-based microbial profiling of the human-depleted reads was performed with MetaPhlAn v. 4.1.1 [18] (database version mpa_vJun23_CHOCOPhlAnSGB_202403) with the addition of the parameters –ignore_eukaryotes, –ignore_usgbs, and -t rel_ab_w_read_stats.

The taxonomic data was processed with R v. 4.3.2 [19] in RStudio v. 2023.12.0 [20], and ggplot2 v. 3.5.1 [21] was used for visualisations. Additionally, the microshades package v. 1.13 [22] was used to generate the stacked bar chart visualising the microbiome composition. Shannon diversity was calculated using the microbiome package [23]. Differences in richness and Shannon diversity between week 0 and week 8 within the Bif195 and the placebo groups were tested with the Wilcoxon signed rank test. Aitchison distances for beta diversity analyses were calculated in QIIME2 v. 2023.09 [24] with the diversity plugin adding a pseudocount of 1, using the species-level estimated read counts generated by MetaPhlAn as input. Aitchison distance was chosen as a beta diversity metric to account for the compositionality of the data [25]. Principal-coordinate analysis was calculated using the ecodist R-package v. 2.1.3 [26]. Differences in beta diversity between week 0 and week 8 within the Bif195 and the placebo groups were tested with PERMANOVA using the adonis2 function of the R package vegan v. 2.6.6.1 [27] and the permute package v. 0.9.7 to define restricted permutations (setting blocks to patient ID as the samples are paired). Differences in beta diversity between metadata variables at baseline (week 0) were also assessed. The included variables were: 1) age, 2) sex, 3) body mass index, 4) frequency of liquid stool, 5) travel abroad within the recent six months, 6) smoking habit, 7) alcohol consumption, 8) previous bowel resection, 9) treatment with biologics, immunosuppressives, and vitamin B12, 10) other chronic diseases including asthma and allergy, 11) f-calprotectin level, 12) crp level, 13) BWT, 14) IBUS-SAS score, and 15) inflammation in the colon. Differences in beta diversity for these variables were likewise tested using adonis2 and p-values were corrected for multiple testing using the Benjamini–Hochberg procedure. The same method was used to investigate the beta diversity in the study population when divided into two groups of participants with a reduction in BWT versus no reduction in BWT after eight weeks of treatment (regardless of intervention groups).

Differential abundance analysis was performed with the R package ALDEx2 v. 1.34.0[28] for week 0 vs. week 8 samples within the Bif195 and the placebo groups. The data was transformed using the aldex.clr function, which generates random instances of the centered log-ratio transformed values, with a model matrix representing the groups, and mc.samples = 1000 (number of Monte-Carlo instances) and denom = 'all' (using all features as denominator for the geometric mean calculation). The function aldex. ttest was used to generate test statistics for the output of aldex.clr (adding the parameter paired. test = TRUE). p values were adjusted using the Benjamini–Hochberg procedure. Effect sizes and differences between the groups were calculated using aldex.effect. Differential abundance analysis at week 0 was also performed as described above for the two clusters seen in the beta diversity analysis, and for patients with resections of the bowel vs. patients not having resection.

Results

Patient characteristics

Sixty-seven patients gave consent to participate in the study, but 34 did not meet the inclusion criteria at the screening visit and were excluded from the trial due to reasons listed in Fig. 2. Thirty-three participants were randomised; 16 were allocated to treatment with Bif195 and 17 to treatment with placebo. Five participants were excluded from PPS due to a study product consumption < 80% (n = 3) and use of antibiotics (n = 1) or probiotics (n = 1) during the intervention period (Fig. 2). No participants were withdrawn or lost to follow-up.

Table 1 presents baseline characteristics. The two groups were similar in all baseline characteristics except for the use of oral vitamins and dietary supplements, as participants in the Bif195 group consumed these significantly more than participants in the placebo group (mean (SD) Bif195 vs placebo; n = 12 (75%) vs n = 7 (41%), $p = 0.049$).

The IUS showed that 32 participants had inflammation of the terminal ileum and one had inflammation in the proximal small bowel. Additionally, three participants exhibited concurrent inflammatory segments in the colon.

Sixteen of the participants had previously undergone one or more bowel resections. Of these, 15 had ileocecal resection, and one had an ileostomy after a total colectomy.

Clinical outcomes

Primary and secondary outcomes are presented in Table 2. Our primary outcome showed that eight weeks of treatment with Bif195 did not reduce BWT compared

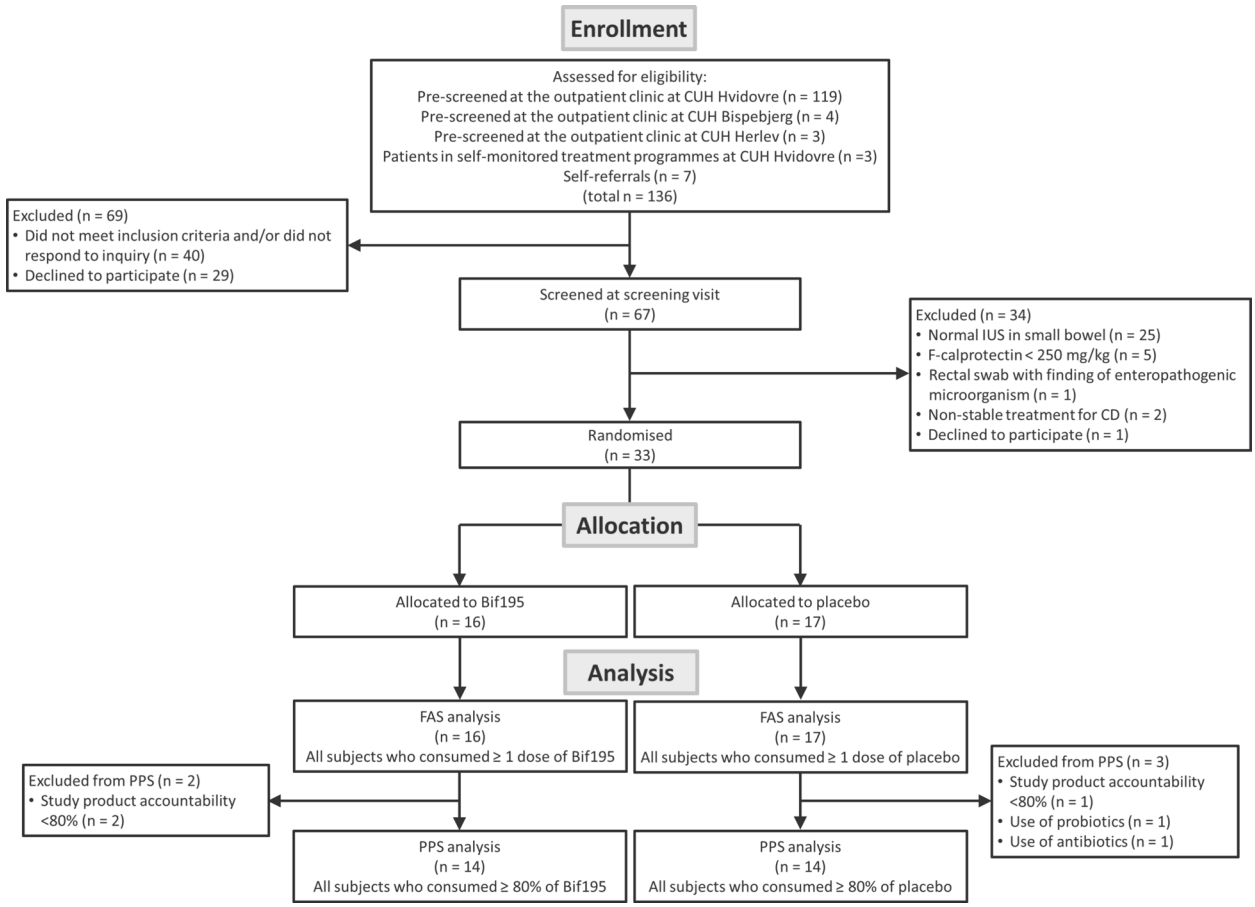

Fig. 2 Flowchart of patients who were included and excluded from analysis. *Bifidobacterium breve*, Bif195 (Bif195), Copenhagen University Hospital (CUH), Crohn's disease (CD), full analysis data set (FAS), intestinal ultrasonography (IUS), per-protocol data set (PPS)

to placebo. In the Bif195 group, the mean difference in the f-calprotectin level before and after the intervention was − 35 mg/kg, whereas the f-calprotectin level rose with a mean value of 116 mg/kg in the placebo group. However, the difference in change between the two groups was not statistically significant. There were no statistically significant differences between the Bif195 and the placebo group in any other secondary or clinical explorative outcomes.

The gut microbiome

We found no statistically significant differences in richness or Shannon diversity between baseline at week 0 and after eight weeks of treatment in any of the groups (richness Bif195 group and placebo group week 0 vs week 8, $p=0.98$ and $p=0.94$, respectively; Shannon diversity Bif195 group and placebo group week 0 vs week 8, $p=0.18$ and $p=0.89$, respectively). Nor did eight weeks of treatment affect beta diversity (Bif195 group and placebo group week 0 vs week 8, $p=0.36$ and $p=0.92$, respectively) (data not shown).

Additional investigations of the beta diversity were performed involving the primary outcome, as we divided the study population into two groups regardless of treatment: reduction in BWT versus no reduction in BWT and analysed changes in the microbiome within the groups. However, in both groups, there were no changes in the gut microbiome from baseline and after eight weeks (reduction in BWT week 0 vs week 8, $p=0.57$, no reduction week 0 vs week 8, $p=0.32$). The gut microbiome at week 8 for both groups is visualised in Fig. 3. There was no statistically significant difference in beta diversity between the groups at week 8 (reduction in BWT week 8 vs no reduction week 8, $p=0.32$) and no species were differentially abundant in one group compared to the other.

The differential abundance analysis in the Bif195 group showed a difference in *Bifidobacterium breve* (*B. breve*) between week 0 and week 8 with unadjusted p-values at 0.03 and 0.04 (Welch's t-test and Wilcoxon test, respectively). However, after adjustments, the p-values were statistically insignificant ($p=0.98$ (Benjamini–Hochberg corrected p-value of Welch's t-test), $p=0.90$

Table 1 Baseline characteristics

	Overall (n = 33)	Bif195 (n = 16)	Placebo (n = 17)
Age (mean (SD))	47.6 (16.5)	47.7 (16.3)	47.6 (17.1)
Sex, n (%)			
Male	14 (42)	7 (44)	7 (41)
Female	19 (58)	9 (56)	10 (59)
BMI (mean (SD))	27.6 (5)	27.0 (5)	28.1 (5)
Medication, n (%)	32 (97)	16 (100)	16 (94)
Immunosuppressives	12 (36)	5 (31)	7 (41)
Biological treatment	13 (39)	7 (44)	6 (35)
Allopurinol	6 (18)	3 (19)	3 (18)
Contraceptives	6 (18)	2 (13)	4 (24)
Hormone supplements	2 (6)	1 (6)	1 (6)
Asthma/allergy agents	6 (18)	2 (13)	4 (24)
Cardiovascular agents	5 (15)	2 (13)	3 (18)
Cholesterol-lowering agents	1 (3)	0	1 (6)
Antidepressants and anxiety medication	1 (3)	0	1 (6)
Colestyramin	6 (18)	3 (19)	3 (18)
Proton pump inhibitors	6 (18)	1 (6)	5 (29)
Peristalsis regulating agents	1 (3)	1 (6)	0
Vitamin B12 injection/oral	14 (42)	9 (56)	5 (29)
Other vitamin and nutritional supplements	19 (58)	12 (75)	7 (41)
Other	10 (30)	4 (25)	6 (35)
Alcohol intake [units/week], n (%)			
0	15 (46)	8 (50)	7 (41)
1–7	12 (36)	8 (50)	4 (24)
8–14	4 (12)	0	4 (24)
> 14	2 (6)	0	2 (12)
Smoking habits, n (%)			
Never smoker	15 (46)	9 (56)	6 (35)
Active smoker	7 (21)	3 (19)	4 (24)
Former smoker	11 (33)	4 (25)	7 (41)
HBI score at screening visit (mean (SD))	2.0 (2.4)	1.3 (1.3)	2.6 (2.9)
HBI score at baseline (visit 1) (mean (SD))	1.7 (2.5)	0.9 (1.0)	2.5 (3.2)
IBDQ score at screening visit (mean (SD))	190 (19)	190 (20)	190 (19)
IBDQ score at baseline (visit 1) (mean (SD))	192 (19)	193 (18)	192 (21)

n = number of subjects within the population

% = percent of subjects in the study

Harvey-Bradshaw Index (HBI), Inflammatory Bowel Disease Questionnaire (IBDQ), standard deviation (SD)

(Benjamini–Hochberg corrected p-value of Wilcoxon test), effect size 0.592) (Fig. 4). The relative abundance of *B. breve* returned to baseline levels at follow-up eight weeks after termination of the treatment.

Another species, *Anaerostipes hadrus* (*A. hadrus*), was also found in higher abundance at week 8 compared to baseline in the Bif195 group with an unadjusted p-value of 0.02 with the Wilcoxon test. Likewise, this finding was statistically insignificant after adjustments were performed.

No species were differentially abundant between baseline and after eight weeks of treatment in the placebo group.

We made an interesting observation of the baseline microbiome data visualised in Fig. 5A. The gut microbiome clustered into two groups from the beginning of the trial, regardless of randomisation groups (Fig. 5B). The two groups were named A and B. By performing relative abundance analyses, we found that 27 species were more abundant in Group A, and nine were more abundant in Group B (Table 3).

Table 2 Primary and secondary outcomes

	Overall (n = 33)	Bif195 (n = 16)	Placebo (n = 17)	*p-value*
Primary outcome				
Reduction in BWT				
1. A reduction of BWT by ≥ 25% OR 2 mm				
Yes (%)		4 (25)	4 (24)	
No (%)		12 (75)	13 (77)	
2. A 2-point CDI reduction				
Yes (%)		1 (6)	2 (12)	
No (%)		15 (94)	15 (88)	
Reduction in BWT (1 and 2)				
Yes (%)		5 (31)	5 (29)	0.73[a]
No (%)		11 (69)	12 (71)	
Secondary outcomes				
F-calprotectin (µg/g), median (IQR)				
Baseline	482 (639)	484 (566)	482 (947)	0.35[b]
After 8 weeks	446 (977)	425 (632)	446 (1183)	
Change from baseline	34 (391)	46 (451)	22 (299)	
IBUS-SAS score, mean (SD)				
Baseline	49 (22)	53 (23)	46 (22)	
After 8 weeks	41 (23)	42 (26)	40 (21)	
Change from baseline	− 8 (21)	− 11 (20)	− 6 (22)	0.75[b]
Transmural remission				
BWT ≤ 3 mm				
Yes (%)		4 (25)	3 (18)	0.61[c]
No (%)		12 (75)	14 (82)	
HBI score, mean (SD)				
Baseline	1.7 (2.5)	0.9 (1.0)	2.5 (3.2)	
After 8 weeks	1.6 (2.1)	1.1 (1.3)	2.1 (2.5)	
Change from baseline	− 0.2 (2.0)	0.2 (1.5)	− 0.5 (2.4)	0.78[b]
IBDQ score, mean (SD)				
Baseline	192 (19)	193 (18)	192 (21)	
After 8 weeks	194 (18)	195 (17)	194 (19)	
Change from baseline	2 (14)	2 (12)	2 (16)	0.88[b]

n = number of subjects within the populatio

% = percent of subjects in the study.

[a] Analysed using a binomial logistics regression model including terms of treatment and previous bowel resection

[b] ANCOVA analysis of the delta values (V3-V1, V3-V0 for IBUS-SAS), with V1/V0 values as covariate

[c] Analysed using a binomial logistics regression model including terms of treatment

Bowel wall thickness (BWT), International Bowel Ultrasound Segmental Activity Score (IBUS-SAS), Harvey-Bradshaw Index (HBI), Inflammatory Bowel Disease Questionnaire (IBDQ), standard deviation (SD)

After discovering this, we performed post hoc analyses investigating possible reasons for the cluster. Of all variables, we found that smoking habits, intake of vitamin B12, and previous bowel resection significantly differed between Group A and B. Still, after correction for multiple testing, only previous bowel resection was left as a statistically significant difference [$p = 0.01$ (Benjamini–Hochberg corrected)], as more participants in Group B had undergone bowel resection compared to group A (Fig. 6). We performed a relative abundance analysis and found that four species were more abundant in the group of participants who had never had bowel resection (Table 4).

The hypothesis that the gut microbiome profiles may be differently affected by the Bif195/placebo treatment led to further analyses of the factor effects and interaction effects between the cluster groups on all primary and

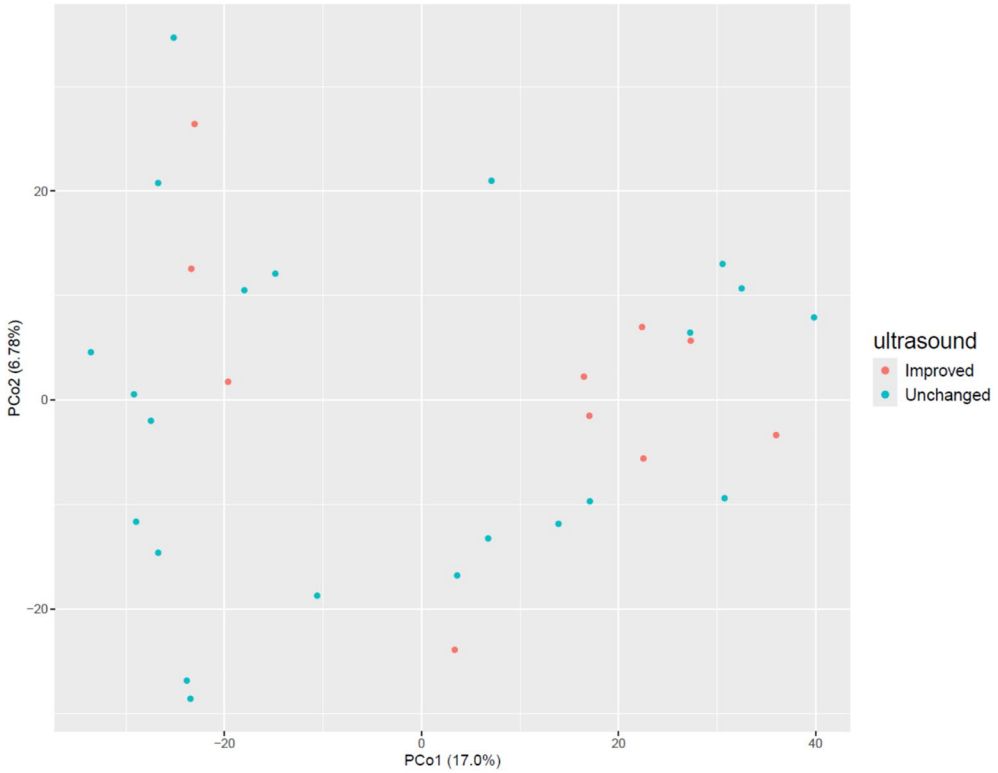

Fig. 3 Visualisation of the gut microbiome at week 8 divided into two groups: participants with a reduction in BWT (red) and participants with no reduction (blue/green). There were no differences in beta diversity between the groups after eight weeks of treatment (Bif195/placebo), and no species were differentially abundant

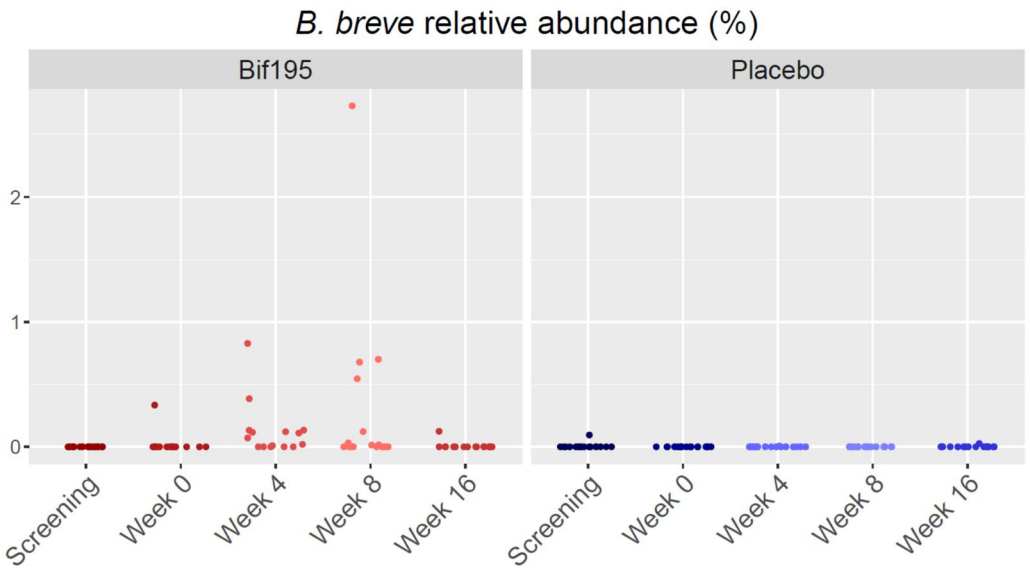

Fig. 4 Relative abundance (%) of *Bifidobacterium breve* divided into study visits in the Bif195 and placebo groups. In the Bif195 group, *Bifidobacterium breve* was differentially abundant at week 8 compared to week 0 with statistical significance before adjustments for multiple testing but not after

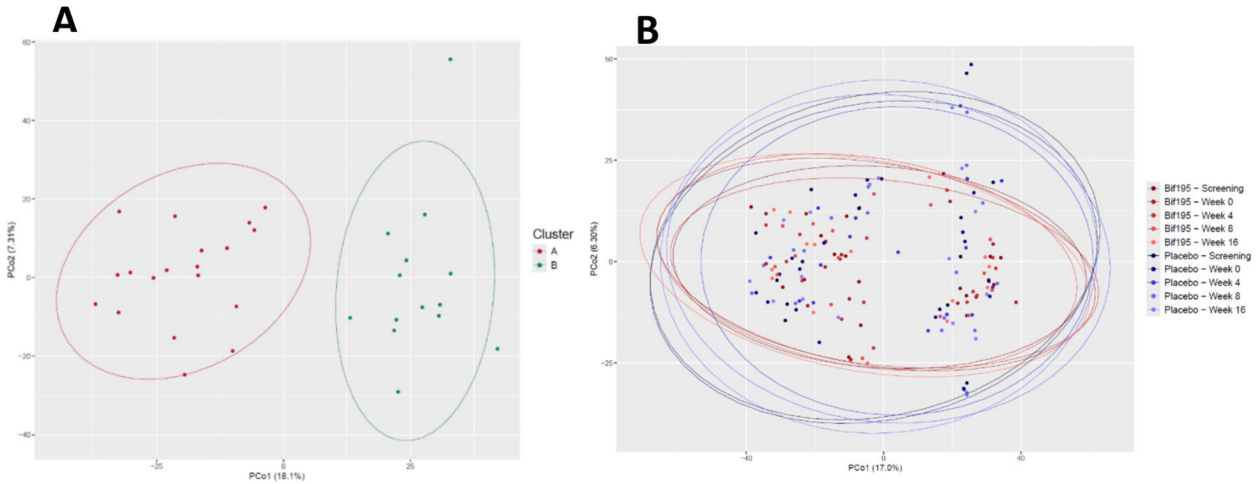

Fig. 5 Visualisation of the cluster of gut microbiome baseline data (**A**) and distribution over the study period in both randomisation groups (**B**)

secondary outcomes. The same analyses were performed for participants with and without previous bowel resection. The analyses showed two statistically significant outcomes: (1) changes in the severity of symptoms measured by HBI were significantly different between Cluster Group A and B with an interaction effect of $p = 0.04$, and (2) changes in f-calprotectin were significantly affected by previous bowel resection with a factor effect of $p = 0.004$.

Safety and adverse events

Eleven participants reported a total of 16 adverse events that were divided into eight categories (Table 5). The intensity of each event was ranked mild, moderate, or severe with a total distribution of six events, seven events, and three events, respectively. Of all reported events, eight were assessed as related to the study product intake. However, 7/8 of these events were reported from the placebo group and only one from the Bif195 group (data not shown). The event from the Bif195 group was described as "loose and more frequent stool", categorised as diarrhoea.

No adverse events were more prominent in one group than the other, and no serious adverse events were reported.

Discussion

This study aimed to investigate the effects of *Bifidobacterium breve*, Bif195 on small intestinal CD and the gut microbiome. Inflammation of the small intestine was explicitly chosen as an inclusion criterion because there is promising data on Bif195 as a probiotic strain for preventing mucosal damage in the upper GI tract after treatment with ASA in healthy individuals [10, 11].

The clinical effects of Bif195 were assessed by validated and recognised non-invasive monitoring tools for CD: IBUS-SAS, HBI, IBDQ, and f-calprotectin. Bif195 did not affect any of the primary or secondary outcomes. Although there was a drop in f-calprotectin in the Bif195 group and a rise in the placebo group after eight weeks of treatment, the analysis of the difference in change between the groups was non-significant. There was no placebo effect on any primary or secondary outcomes.

The evidence supporting the effectiveness of probiotic treatment for CD is limited and not as strong as for other conditions such as irritable bowel syndrome and ulcerative colitis (UC) [29, 30]. Despite both UC and CD being characterised by intestinal inflammation, the variations in the benefits of probiotic treatment between the two diseases may be due to differences in the pathophysiology and the complex immune-mediated mechanisms underlying each condition [30]. A growing amount of research suggests that no universal probiotic treatment can be recommended for IBDs. Instead, a personalised approach with tailored probiotic strains based on the individual's inflammatory changes and gut microbiota composition seems to have better therapeutic outcomes [31]. However, this approach requires a clearer understanding of the pathogenesis and microbial disturbances characterised by the disease.

To summarise, the beneficial effect of Bif195 on mucosal damage in healthy individuals was not reproduced in participants with CD in our study. The damage caused by ASA in the otherwise healthy small intestine differs from the inflammatory mucosal damage in IBD. While the mucosal injuries caused by ASA involve inhibition of the protective properties of the cyclooxygenase 1 enzyme [32], the chronic immune-mediated

Table 3 Species of differential abundance between Cluster Group A and Group B

More abundant in Group A	More abundant in Group B
Fusicatenibacter saccharivorans[c]	*Flavonifractor plautii*[b]
Dorea formicigenerans[c]	*Ruminococcus gnavus*[b]
Faecalibacterium prausnitzii[c]	*Clostridium innocuum*[b]
Candidatus Cibionibacter quicibialis[c]	*Blautia hansenii*[c]
Faecalibacillus intestinalis[c]	*Faecalimonas umbilicata*[c]
Blautia obeum[c]	*Enterocloster bolteae*[c]
Clostridium fessum[a]	*Erysipelatoclostridium ramosum*[c]
Gemmiger formicilis[a]	*Enterocloster aldenensis*[c]
Coprococcus catus[a]	*Enterocloster clostridioformis*[c]
Lachnospiraceae bacterium CLA AA H244[a]	
Mediterraneibacter faecis[a]	
Oscillospiraceae bacterium CLA AA H250[a]	
Eubacterium rectale[a]	
Blautia faecis[a]	
Coprococcus comes[a]	
Dorea sp AF36 15AT[a]	
Faecalicatena fissicatena[a]	
Clostridiaceae bacterium[a]	
Clostridium sp AM22 11AC[a]	
Anaerobutyricum hallii[a]	
Eubacterium ramulus[a]	
Clostridiales bacterium KLE1615[a]	
Blautia massiliensis[a]	
Oscillibacter sp ER4[a]	
Dorea longicatena[a]	
Ruminococcus bromii[a]	
Roseburia inulinivorans[a]	

[a] p-value < 0.05 with Benjamini–Hochberg corrected p-value of Welch's t-test

[b] p-value < 0.05 with Benjamini–Hochberg corrected p-value of Wilcoxon test

[c] p-value < 0.05 with Benjamini–Hochberg corrected p-value of both Welch's t-test and Wilcoxon test

inflammatory damages in IBD are more complex [33]. This difference cannot be ruled out as the reason for the lack of mucosal healing in participants with CD.

We also investigated the effects of Bif195 on the gut microbiome. We found no significant changes in alpha and beta diversity or relative abundance of species after adjustments of p-values, perhaps caused by the small sample size.

Interestingly, we found differences in the baseline microbiome data, as the microbiome clustered into two groups, which differed significantly in relative abundance in no less than 36 species. More of these species are mentioned in the literature as being associated with CD and IBD in general, e.g., *Ruminococcus gnavus*, frequently found in increased abundance in patients with IBD, particularly CD [34, 35]. In this study, *Ruminococcus gnavus* was more abundant in Cluster Group B, as well as *Flavonifractor plautii* which belongs to a genus also found in higher abundance in CD [35].

Genera that are previously found less abundant in IBD and CD are *Faecalibacterium*, *Eubacterium*, and *Roseburia* [35, 36]. In our study, the species *Faecalibacterium prausnitzii*, *Eubacterium rectale*, and *Roseburia inulinivorans,* as well as *Ruminococcus bromii* were all more abundant in Group A than in Group B. *Eubacterium rectale* and *Ruminococcus bromii* are believed to be symbiotic species that stimulate the growth of a third bacteria, *A. hadrus*, which, interestingly, is the same species found to be more abundant after the Bif195-treatment (before statistical adjustments). *A. hadrus* may have protective properties against inflammation of the colon [37, 38]. It is recognised, that the species produces butyrate, a short-chain fatty acid, essential for intestinal mucosal immune regulation and normal barrier function [37], but the evidence of the role of *A. hadrus* on overall human health is ambiguous [37]. Nevertheless, *A. hadrus* is known to be less abundant in the IBD microbiota [37, 38].

The distribution of species more abundant in Cluster Group A compared to Cluster Group B, leaves the impression that the microbiome of Group B shares more characteristics associated with severe inflammatory disease than Group A. From the data available for post hoc analyses, we found that more participants in Group B had undergone bowel resection, and there was a trend of fewer "never-smokers" and a higher intake of vitamin B12 supplements within this group. These trends correlate with the current knowledge of risk factors for CD and complications after bowel surgery, as smoking is a substantial predisposing factor for complications leading to resection of the bowel [39]. Also, vitamin B12 deficiency is not only a common manifestation of active intestinal inflammation, especially involving the terminal ileum; one of the main risk factors of B12 deficiency is ileal resection [40, 41]. In an extension of this, the species *Candidatus Cibionibacter quicibialis*, found more abundant in Cluster Group A, may be involved in the vitamin B12 synthesis [42]. We can only speculate whether this is linked to the lower need for B12 supplementation among the participants within Cluster Group A. In addition, based on our findings, it is impossible to conclude whether the bowel resections are causing the microbiome cluster or if the microbial composition is responsible for an increased risk of complications leading to bowel resection.

The hypothesis that variations of the microbiome composition may be differently affected by the probiotic treatment led to post hoc analyses of the factor- and interaction effects. Due to the outcomes of these

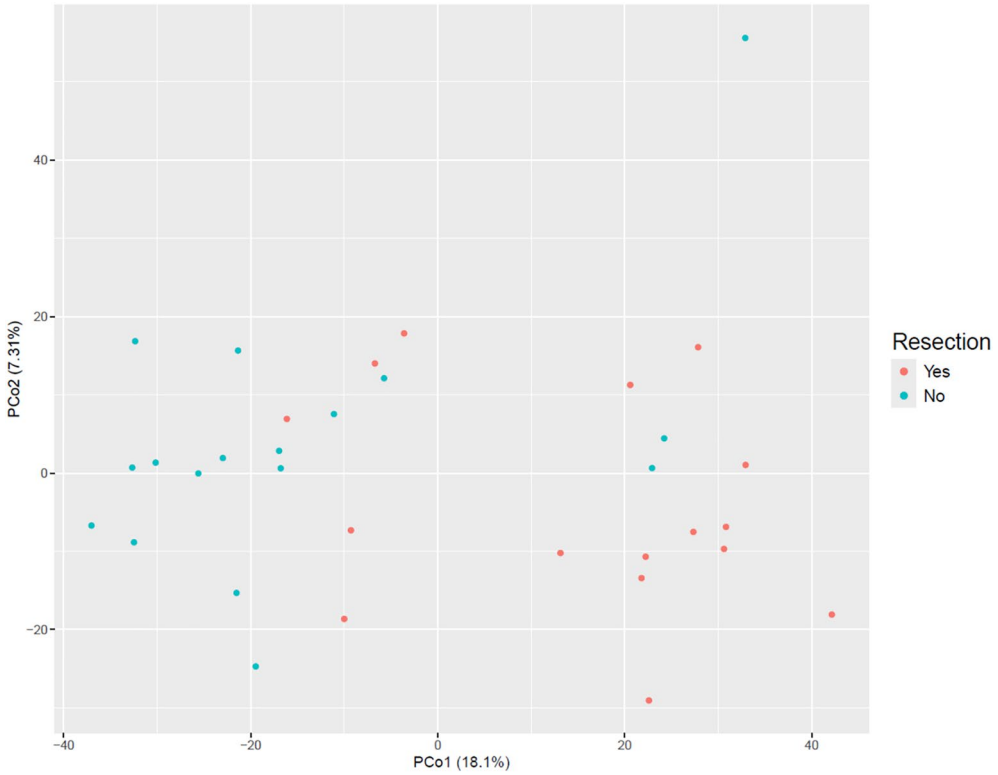

Fig. 6 Visualisation of the cluster of gut microbiome baseline data correlated to previous bowel resection

analyses indicating subgroups with different characteristics, we stress the importance of considering the baseline microbiome composition for subgrouping the study population when conducting clinical trials like the present.

A strength of this study is the collection of stool samples at every visit, which made it possible to evaluate the gut microbiome throughout the study period. Because of the relative abundance analysis, we were able to observe a high study product consumption in the Bif195 group. The fact that this was not statistically significantly different from baseline may be due to the small sample size. In general, we acknowledge the small cohort as a significant study limitation.

Still, by counting the study products two times during the intervention period, we observed that 90% of the participants in the FAS consumed at least 80% of the study product, and over 80% had consumed at least 90%.

While we consider the many stool samples a strength of the study, the lack of biopsies may be a limitation. IUS is a non-invasive procedure that offers insight into the terminal ileum without bowel cleansing, known to influence the microbiome [43, 44], but without the ability to collect biopsies. Thus, an accurate assessment of the microbiota within the terminal ileum was compromised.

Table 4 Species of differential abundance between participants with/without previous bowel resection

More abundant without resection	More abundant with previous bowel resection
Oscillospiraceae bacterium CLA AA H250†	
Alistipes shahii[a]	
Blautia obeum[b]	
Blautia faecis[a]	

[a] p-value < 0.05 with Benjamini–Hochberg corrected p-value of Welch's t-test

[b] p-value < 0.05 with Benjamini–Hochberg corrected p-value of both Welch's t-test and Wilcoxon test

Conclusions

In conclusion, eight weeks of treatment with Bif195 did not affect BWT in the small intestine compared to placebo. Nor did the treatment affect secondary or explorative outcomes or the gut microbiota in terms of richness, Shannon diversity, or beta diversity. In the Bif195 group, the relative abundance of *B. breve* increased after eight weeks of treatment. However, the result was statistically

Table 5 Adverse events in full analysis data set (FAS)

	Overall		Bif195	Placebo	*p-value
	E	(n = 33)	(n = 16)	(n = 17)	
All adverse events	16	11 (33)	5 (31)	6 (35)	
Abdominal pain	2	2 (6)	1 (6)	1 (6)	1.00
Diarrhoea	3	3 (9)	1 (6)	2 (12)	1.00
Constipation	2	2 (6)	1 (6)	1 (6)	1.00
Bloating and/or flatulence	3	3 (9)	0	3 (18)	0.23
Nausea	2	2 (6)	2 (13)	0	0.23
Borborygmi	1	1 (3)	0	1 (6)	1.00
Skin rash	1	1 (3)	0	1 (6)	1.00
Other	2	2 (6)	1 (6)	1 (6)	1.00

n = number of subjects within population

(%) = percent of subjects in the study

E = number of events

*Chi-squared test is used for categorical variables with expected counts greater than 5 and Fisher exact test is used for categorical variables with expected counts less than 5

Other: Bilateral chest pain, fever

non-significant. Post hoc analyses of the baseline microbiome composition showed correlations between variations in the microbiome profiles and the incidence of bowel resections. Furthermore, analyses showed that the treatment may have different effects depending on the microbiome composition. This suggests that the baseline microbiome composition should be considered in future studies with objective measures as endpoints.

Abbreviations

ASA	Acetylsalicylic acid
B. breve	*Bifidobacterium breve*
Bif195	*Bifidobacterium breve*, Bif195 (DSM 33360)
BMI	Body Mass Index
BWS	Bowel wall stratification
BWT	Bowel wall thickness
CDI	Colour Doppler imaging
CD	Crohn's disease
f-calprotectin	Faecal calprotectin
FMT	Faecal microbiota transplantation
GI	Gastrointestinal
HBI	Harvey-Bradshaw Index
IBD	Inflammatory bowel disease
IBDQ	Inflammatory Bowel Disease Questionnaire
I-fat	Inflammatory fat
IBUS-SAS	International bowel ultrasound segmental activity score
IUS	Intestinal ultrasonography
UC	Ulcerative colitis

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Availability of data and materials

Raw sequencing reads depleted of human reads are deposited in NCBI Sequence Read Archive under BioProject no. PRJNA1216426.

Declarations

Ethics approval and consent to participate

The study is approved by the Danish Data Protection Agency (P-2020-1136) and the Scientific Ethics Committee for Copenhagen Regional Hospitals, Denmark (Permission no.: H-20068527). The study was performed in accordance with the Revised Declaration of Helsinki. All participants were given verbal and written information and provided written informed consent to participate in the study. The study is registered at www.clinicaltrials.gov as NCT04842149.

Consent for publication

Not applicable.

Competing interest

Bifidobacterium breve, Bif195™ is marketed under the name GALENEX™ and is a registered trademark of Chr. Hansen A/S. Author SE is an employee of Chr. Hansen A/S, part of Novonesis.

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