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CLINICAL CASE STUDY

VIDEO CAMERA ENDOSCOPY (VCE) CLINICAL TRIALS

How to asset the ability of the probiotic strain to attenuate NSAIDs effect in deterioration of small intestinal mucosa tissue

Executive Summary

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are commonly used worldwide, both as prescription-only medicines and as "over-the-counter" preparations. However, low dose use of NSAIDS, is associated with gastrointestinal (GI) injury. Strategies to prevent GI complications associated with NSAID use included are generally associated with undesired side effects, whereas live bacteria formulated as probiotics may offer a safe alternative to prevent or at least decrease negative side effects of NSAIDS. The present clinical trial is aiming to bring a product containing a probiotic strain able to attenuate and/or reverse NSAIDs-induced small intestinal damage and GI symptoms in NSAIDs users.

We established a clinical challenge model aiming at investigating the ability of the probiotic strain in attenuating and/or reversing deterioration in the healthy human gastrointestinal tract

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Keep reading about the challenges and objectives

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About the Sponsor

Probiotic bacteria have been demonstrated to have possible therapeutic effects against intestinal inflammation and the sponsor, a world-class innovative company, focused on bringing to market a treatment. They have previously performed several in vitro screening assays in order to characterize approximately 200 different strains. Five strains were then selected based on their characteristics in vitro, the selected 5 strains were then tested in a rat model of colitis. The present clinical trial was the first in a clinical development program aiming to bring a product containing the selected strain to marked able to attenuate and/or reverse NSAIDs-induced small intestinal damage and GI symptoms in NSAIDs users. The investigational product were vegetable capsules containing the probiotics per daily dose. All capsules were produced in the same batch by the sponsor, which is certified for food production.

Challenges and Objectives

The Sponsor needed a highly experienced research partner able to assess the deterioration of small intestinal mucosa tissue however they needed a method not too invasive in order to overcome the Ethical burdens. The main objectives were:

- To investigate the ability of the probiotic strain to attenuate and/or reverse low-dose, long-term NSAIDs-induced deterioration of small intestinal mucosa tissue as assessed by capsule endoscopy in healthy volunteers
- To investigate the ability of the probiotic strain to attenuate and reverse low-dose, long term NSAIDs-induced GI symptoms as assessed AUC ulcer number as well as assessed by AUC of pain syndrome score for GSRS.
- To investigate co-administration of the probiotic strain to low-dose, long term NSAIDs on changes in multiple biomarkers of general intestinal barrier function in blood and faecal samples.

How Atlantia's Solution Helped

Consequently, we established a clinical challenge model aiming at investigating the ability of the probiotic strain in attenuating and/or reversing deterioration in the healthy human gastrointestinal tract. The deterioration was induced by a chemical agent commonly used and with well-established deteriorating effects on the small intestine. For the primary endpoint, we used the method capsule endoscopy (CE) to assess the small intestinal damage. Capsule endoscopy has been reviewed in a technology status evaluation report by the American society for gastrointestinal endoscopy and it is now the gold standard for assessing occult gastrointestinal bleeding, and indications for its use are continuing to expand. Current uses include exploration and surveillance of bowel pathology such as in Crohn's disease, polyps, small bowel malignancy and drug-induced mucosal injury. Capsule endoscopy (CE) is generally a safe and well tolerated procedure. Atlantia has a highly expert team on managing these technologies when conducting trials and was a perfect fit for the sponsor.

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About the Study

This trial was a single-site, randomised, double-blind, placebo-controlled, two-armed, parallel-group trial in healthy, adult volunteers. It investigated the effect of daily intake of the probiotic strain or placebo when co-administered with daily intake of 300mg of Aspirin.
The trial was conducted in accordance with the ethical principles set forth in the current version of the Declaration of Helsinki (seventh revision; October 2013), the International Conference on Harmonization E6 Good Clinical Practice (ICH-GCP, 10 June 1996) and all applicable local regulatory requirements.
The trial included a run-in period of two weeks duration followed by a six-week intervention period where the probiotic/placebo and NSAIDs were co-administered. After the 6-weeks, the probiotic/placebo was given for two additional weeks to investigate the potential effects of the probiotic on intestinal healing after long-time NSAIDs use. Subjects participated in the trial for a total duration of 10-weeks including the run-in phase.
The primary efficacy variable was to investigate the effect of oral supplement of the probiotic strain versus placebo on small intestinal mucosa damage when co-administered with a NSAIDs challenge for measured as the area-under-the-curve (AUC) for Lewis Score obtained by capsule endoscopy. The sample size of 30 completing participants in each arm was estimated based on a power calculation performed on intervention on percent difference of AUC between two normalized curves (active vs. placebo) as an approximation. To account for a potential drop-out rate of approximately 15%, a total of 35 subjects were randomized in each group. A cclinically significant decrease was seen in the AUC data following treatment with the Probiotic versus placebo.

Recruitment	Number of subjects
Planned	75
Screened	140
Randomized	75
Dropouts	17
Completed	66

Throughout the entire trial, subjects were instructed to maintain their habitual life style with regard to diet, physical activity level and sleep habits. Intake of probiotic products as well as food and food supplements containing probiotics were not allowed from the screening visit and until the end of the intervention period. Subjects were not withdrawn from the trial due to single violations, but violations were recorded as protocol deviations.
Small intestine mucosa deterioration was evaluated using video capsule endoscopy as well as indirect biomarkers in feaces and blood samples At these visits, subjects also filled out the GSRS questionnaire which assessed GI symptoms and pain.
Each subject underwent a video capsule endoscopy over the course of the 8 week intervention. Capsule endoscopy was deemed to be a relevant and acceptable method to evaluate both mild and severe intestinal damage caused by low dosage of Aspirin measured as area-under-the-curve for damage.

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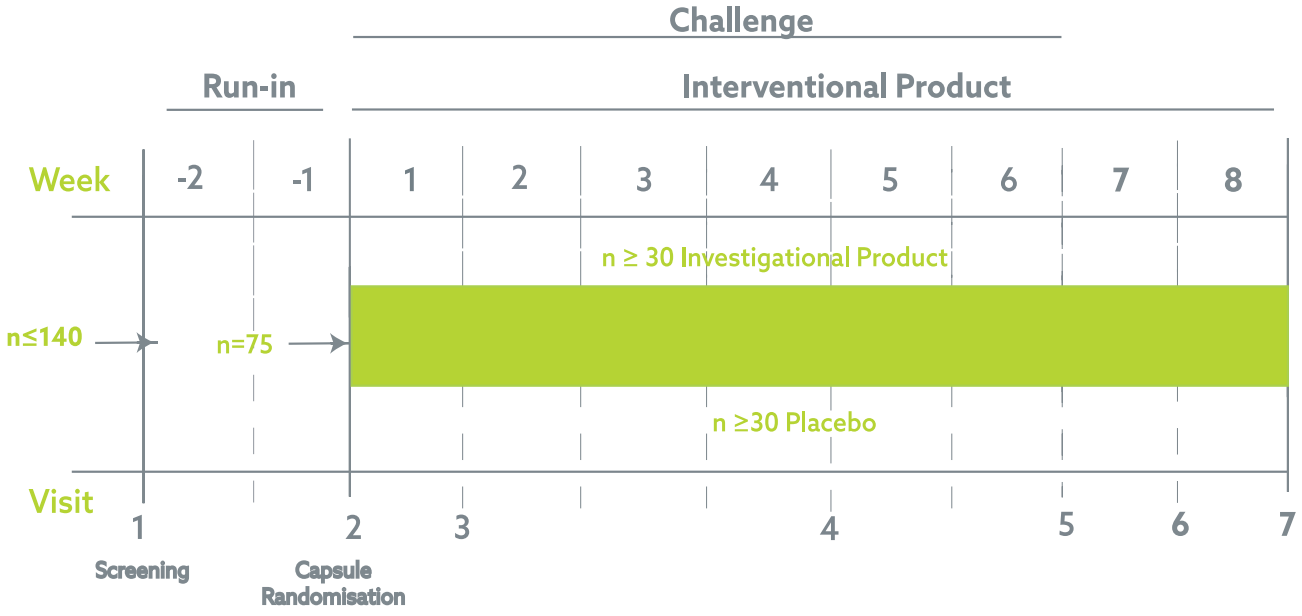

Future plans and Return on Investiment

Our findings support that capsule endoscopy is a robust and reliable method for measuring intestinal damage. The GSRS questionnaire showed a response in some of the categories, but the challenge signal was in general very small and no effect of the intervention was observed in the overall data. While fecal calprotectin and blood I-FABP responded to the NSAIDs challenge, VCE should remain the preferred method used for all clinical activities above any biomarkers or questionnaires. The AUC approach allowed the sensitivity required to observe intervention effects and should be used again in future clinical activities relating to this project. Further studies are necessary to determine if probiotics can aid in reversal of GI damage in the period after NSAIDs intake (recovery). A number of statistically significant findings in exploratory endpoints related to ulcers, further support further clinical development of the pobiotio selected.

This clinical trial demonstrates that subjects who were randomized to receive the probiotic responded significantly better to the Aspirin challenge model in relation to the primary outcome measure, as well as a number of the secondary and exploratory outcomes related to ulcers. The dataset was robust with few protocol violations and excellent product accountability. This study was published in a high impact journal after completion.

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Accepted Manuscript

Bifidobacterium breve Bif195 Protects Against Small-intestinal Damage Caused by Acetylsalicylic Acid in Healthy Volunteers

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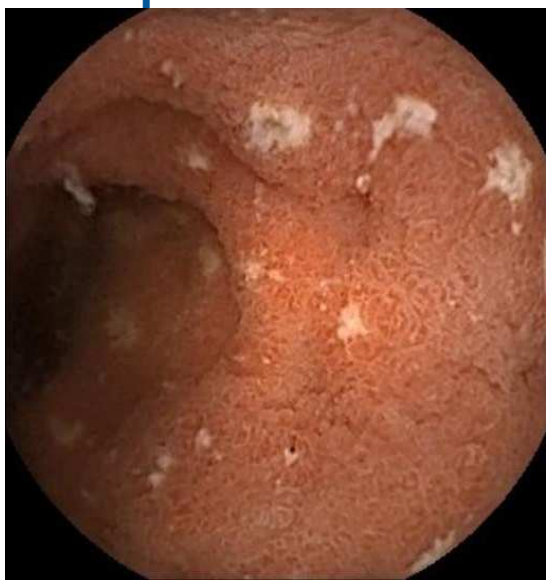
Bifidobacterium breve Bif195 Protects Against Small-intestinal Damage Caused by Acetylsalicylic Acid in Healthy Volunteers

A randomized, placebo-controlled, double-blind clinical trial

Aspirin + Placebo



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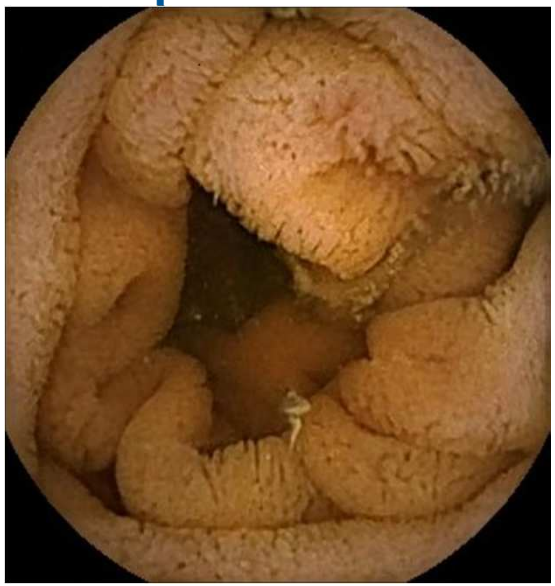
Aspirin + Bif195



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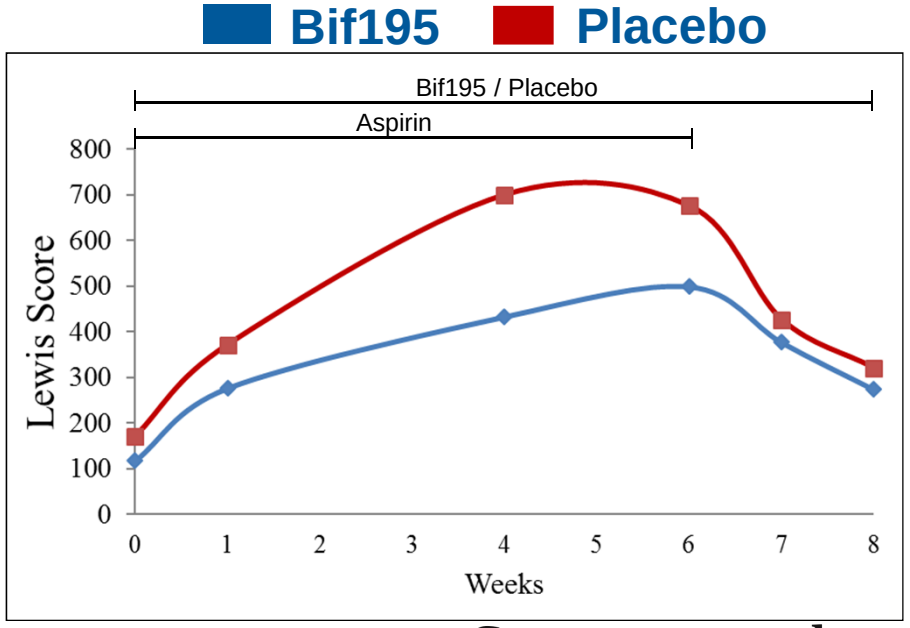

Gastroenterology

Bifidobacterium breve Bif195 Protects Against Small-intestinal Damage Caused by Acetylsalicylic Acid in Healthy Volunteers

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Contributors: BM contributed to the trial conception and trial design, wrote the trial protocol and prepared and managed the trial on behalf of the sponsor, drafted the article and contributed to the final version. CM reviewed the VCE data and contributed to the final version. JOG reviewed the VCE data and contributed to the final version. ML reviewed the VCE data and contributed to the final version. GE reviewed the VCE data and contributed to the final version. LB oversaw the VCE procedure, reviewed the VCE data and contributed to the final version. VW contributed to the trial conception and trial design and contributed to the final version**.** AW contributed to the trial conception and trial design and contributed to the final version. OL performed analysis and statistics on microbiome data obtained from fecal samples and contributed to the final version. ACE performed analysis and statistics on microbiome data obtained from fecal samples and contributed to the final version. HBN performed analysis and statistics on microbiome data obtained from fecal samples, drafted parts of the article and contributed to the final version. AB contributed to the trial conception and trial design and contributed to the final version. AD contributed to the trial conception and trial design and contributed to the final version. JETVHV contributed to the trial conception and trial design and contributed to the final version. FS contributed to the trial conception and trial design, was Co-PI during the trial, oversaw the VCE procedure, reviewed the VCE data, wrote in part the manuscript and contributed to the final version**. MB** contributed to the trial conception and trial design, was PI during the trial, oversaw the VCE procedure, reviewed the VCE data and contributed to the final version**.**

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Abbreviations:

AUC	Area-under-the-curve
ASA	Acetylsalicylic Acid
NSAID	Non-steroidal anti-inflammatory drugs
PPIs	Proton pump inhibitors
VCE	Video Capsule Endoscopy

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Abstract

Background & Aims: Enteropathy and small-intestinal ulcers are common side effects of non-steroidal anti-inflammatory drugs such as acetylsalicylic acid (ASA). Safe, cytoprotective strategies are needed to reduce this risk. Specific Bifidobacteria might have cytoprotective activities, but little is known about these effects in humans. We used serial video capsule endoscopy (VCE) to assess the efficacy of a specific Bifidobacterium strain in healthy volunteers exposed to ASA.

Methods: We performed a single-site, double-blind, parallel-group, proof of concept analysis of 75 heathy volunteers given ASA (300 mg) daily for 6 weeks, from July 31 through October 24, 2017. The participants were randomly assigned (1:1) to groups given oral capsules of *Bifidobacterium breve* (Bif195; $\geq 5*10^{10}$ colony forming units) or placebo, daily for 8 weeks. Small-intestinal damage was analyzed by serial VCE at 6 visits. The area under the curve (AUC) for intestinal damage (Lewis score) and the AUC value for ulcers were the primary and first-ranked secondary endpoint of the trial, respectively.

Results: Efficacy data were obtained from 35 participants given Bif195 and 31 given placebo. The AUC for Lewis score was significantly lower in the Bif195 group (3040 ± 1340 arbitrary units) than the placebo (4351 ± 3195 arbitrary units) (*P*=.0376). The AUC for ulcer number was significantly lower in the Bif195 group (50.4 ± 53.1 arbitrary units) than in the placebo group (75.2 ± 85.3 arbitrary units) (*P*=.0258). Twelve adverse events were reported from the Bif195 group and 20 from the placebo group. None of the events were determined to be related to Bif195 intake. ClinicalTrials.gov no: NCT03228589

Conclusions: In a randomized double-blind trial of healthy volunteers, we found oral Bif195 to safely reduce the risk of small-intestinal enteropathy caused by ASA.

KEY WORDS: aspirin, bacteria, microbiota, bleeding

Introduction

Non-Steroidal Anti-Inflammatory Drugs *(*NSAIDs) are used worldwide both as prescription and over-the-counter products for their analgesic, anti-inflammatory and cardiovascular disease (CVD) risk-reduction properties, and are among the most used pharmaceuticals in the world today[1]. Chronic, low-dose use (commonly defined as 75-325 mg daily) of the NSAID Acetylsalicylic Acid (ASA) is widely recommended for both primary and secondary prevention of CVD. More than 30% of the US population aged above 40 are estimated to be on chronic, daily, low-dose ASA for that reason alone[2]. However, chronic use of ASA is also associated with adverse side effects including small-intestinal mucosal lesions and ulcers, perforations, major hemorrhage and in rare instances death[3,4,5]. A recent review and meta-analysis addressing both the efficacy of ASA in prevention of CVD and also bleeding-related side-effects concluded that a balanced, cautious approach should be taken in the case of primary CVD prevention due to these side-effects[6], highlighting the unmet need to reduce the risk of side-effects of chronic ASA use.

For decades endoscopists have acknowledged the vulnerability of the gastroduodenal mucosa to NSAID-induced enteropathy. Complications include ulceration, blood loss, protein loss, perforation and occasional strictures. The pathogenesis of tissue injury at the gastric and small-intestinal sites appears to differ[7,8], and therefore distinct and separate preventative strategies are probably required to combat enteropathy and gastropathy. For example, the risk of gastropathy can be offset by acid suppression, usually with proton pump inhibitors (PPIs). However, the pathogenesis of NSAID-induced damage in the small bowel seems to be much more complex and has been shown to involve microbiota composition, bile and enterohepatic circulation of the certain NSAIDs[8]. Moreover, there is evidence to suggest that PPIs may actually increase the risk of NSAID-associated small bowel injury[9], possibly by disturbing the composition of the small bowel microbiota[10]. The importance of the microbiota is emphasised by the fact that administration of NSAIDs to germ-free animals is

associated with minimal damage to the small intestinal mucosa and co-administration of antibiotics reduces NSAID-induced injury[8,7]. Besides the well-established inhibitory effect of cyclooxygenase (COX), ASA specifically has been recognised to compromise the phospholipid layer in mucus[11] increasing access to luminal aggressors like lipopolysaccharide and bile as well as disrupt intestinal permeability and cause inflammation[12]. Given that deleterious compositional changes to the microbiota, in addition to direct effects on mucus and epithelial tissue, may increase the risk of NSAID-enteropathy, we hypothesised that an intervention targeting microbiome-host interactions may offer an attractive, preventative strategy. Our strain selection was based on the anti-inflammatory properties of certain bifidobacteria[13,14] and experimental pre-clinical evidence for a role of bifidobacteria in NSAID-associated ulceration[15,16,17] as well as unpublished pre-clinical screening data suggesting a particular potential of efficacy for the specific strain belonging to this genus. In addition, another *Bifidobacterium breve* has been shown to express a pilus-associated protein (Tad E) *in vivo,* but not *in vitro,* which promotes colonic epithelial proliferation[18].

Here, we describe the development of a clinical model to assess the quantitative and time-resolved induction of small intestinal injury upon ASA administration. Using this model, we addressed whether oral co-administration of a single bacterial strain of *Bifidobacterium breve* (Bif195) can reduce the risk of low-dose ASA-induced intestinal ulceration in humans in a randomized, placebo-controlled, parallel-group, double-blind trial using serial video capsule endoscopy (VCE) as a rigorous demonstration of efficacy.

Methods

Study design

This clinical trial was a single-site, randomized, double-blind, placebo-controlled, parallel-group, proof-of-concept trial. The trial was conducted at the CRO Atlantia Food Clinical trials (Cork, Ireland). The trial was conducted in accordance with the ethical principles set forth in the current version of the Declaration of Helsinki, the International Conference on Harmonisation E6 Good Clinical Practice (ICH-GCP). The trial was approved by The Clinical Research Ethics Committee of the Cork Teaching Hospitals (Cork, Ireland) prior to trial conduct. The trial conduct period was July – December 2017. The trial was registered at ClinicalTrials.gov under the ID number NCT03228589.

Participants

All subjects were carefully informed about the trial before they signed the informed consent form and were screened for participation criteria. Main inclusion criteria for participation were: Age between 18 and 40 years, healthy and without GI symptoms, sedentary lifestyle and willingness to refrain from other bacterial products and medication known to alter GI function throughout trial participation.

Main exclusion criteria were: History of abdominal surgery (except appendectomy and cholecystectomy), history of peptic ulcers, known bleeding disorders, known allergy to ASA, history of diseases related to *H. pylori* infection, diastolic blood pressure ≥ 90 mmHg, systolic blood pressure ≥ 140 mmHg, BMI > 27, smoking or use of other nicotine products, lactose intolerance, pregnancy, lactation and regular use of probiotics, systemic antibiotics, steroids (except contraceptives), NSAIDs, laxatives, anti-diarrheals, PPIs, and/or immunosuppressant drugs prior to

screening. After inclusion, subjects went through a two-week run-in period before baseline data were obtained at Visit 2 with randomization being performed at the very end of visit 2.

Randomization and masking

Prior to trial conduct, the allocation of subjects in a 1:1 ratio to Bif195 or placebo intervention was planned according to randomization lists. The randomization procedure was stratified by gender and the lists were drawn up to n=50 for each strata using the proc plan procedure in SAS.

Randomization blocks of n=6 was used throughout and trial site and sponsor were kept blinded to the use of randomization blocks. The randomization list and unblinding list were produced by a third party not otherwise involved in the trial.

At screening, subjects were assigned a 4-digit screening number according to their chronological entry into the trial. If a subject was found eligible and enrolled for trial participation, they received their randomization number by blinded trial staff after all baseline assessments performed at Visit 2. Randomization numbers included the stratification number and was allocated sequentially by trial staff in the order in which the subjects completed Visit 2.

Test and placebo product were produced by the sponsor to be similar in smell, taste and appearance. All trial product was packaged in identical packs with identical labelling, except for the randomization number. All trial subjects, the clinical team, statisticians and the sponsor were all blinded during the entire trial until database lock and signature of the request for unblinding document.

An emergency unblinding procedure using emergency code break opaque sealed envelopes was established to allow the investigator the option of disclosing the product assignment for any individual subject if clinical circumstances required such unblinding. This option was not used in the conduct of this trial. The randomization list and production of emergency code break envelopes

were performed by a third party not otherwise involved in the trial. The labeling of product vials, based on the randomization list, was also performed by a third-party not otherwise involved in the trial.

Procedures

Bif195 or placebo were administered in a 1:1 ratio daily to 75 randomized subjects for 8 weeks. To induce damage to the small intestine, all subjects were co-treated daily with 300 mg of ASA for the first 6 weeks of the 8 week Bif195/placebo intervention period.

In order to document small-intestinal damage, we performed VCE at 6 visits during the 8 week intervention period (Suppl. Figure 1 and Suppl. Figure 4). The time course kinetics of ASA-induced damage, as well as a potentially protective effect by Bif195, were expressed as area-under-the-curve (AUC) for the 8 week intervention period for all datasets obtained.

All subjects were given 2 hypromellose capsules daily with or without *Bifidobacterium breve* Bif195 starting the day after visit 2 with a duration of 8 weeks. The product stability was monitored in parallel to trial conduct and showed at least $5*10^{10}$ Colony forming units (CFU) of Bif195 per daily dose during the period of trial conduct. Detailed trial product and placebo description is provided in Supplementary Table 1.

All randomized subjects were also given 300 mg of ASA (Alliance Pharmaceuticals, Ireland) to induce small-intestinal damage. This dose was taken daily from the day after visit 2 with a duration of 6 weeks.

VCE is the widely accepted reference standard for assessment of occult gastrointestinal bleeding. Current use include exploration and surveillance of bowel pathology such as in Crohn's disease, polyps, small bowel malignancy and drug-induced mucosal injury[19]. To standardise the findings from VCE, we used a reproducible, clinical scoring system to categorise small intestinal mucosal damage, the Lewis score. The Lewis score is a validated tool that evaluates villous edema, ulcers

and stenosis in order to quantify small bowel inflammatory change in one score[20]. This scoring system uses specific definitions for each of the recorded parameters to reduce inter-reviewer variability. In addition, we also counted red spots as observed during VCE.

For all VCE analyses (visit 2-7), data were recorded using the SB3^{TM} Pillcam video recording capsule (Medtronic, Ireland). For all visits, subjects met fasting in the morning and the Pillcam capsule was swallowed with water. Video images were recorded for a total of 8 hours during each visit, after which the capsule was verified in the video to have passed the small intestine.

Four experienced gastroenterologists, blinded to intervention and not allowed to communicate internally regarding obtained VCE data, reviewed the video material retrieved from the capsules using the Pillcam^{TM} Reader Software Version 9.0 from Medtronic. The VCE video material from all 6 VCE visits for each of the subjects were evaluated by two randomized reviewers, and mean values for each subject visit were calculated. In cases where the data from a specific visit differed with 4 or more number of ulcers, a third reviewer would review the VCE dataset. A mean value of all 3 datasets was then calculated and used as the final data point for that specific visit. All VCE reviews were performed prior to database lock and unmasking of the randomization key.

Representative pictures of the VCE material obtained are shown in Supplementary Figure 4.

Fecal samples and blood samples were obtained during all visits from visit 2 to visit 7 for secondary and exploratory analyses.

At all visits, subjects completed the GI symptoms rating score (GSRS) questionnaire to assess GI symptoms[21].

Intestinal fatty acid binding protein (I-FABP) was measured by Nordic Biosite, Finland, in triplicate heparin plasma samples using the HK406 human I-FABP ELISA kit from Hycult Biotech under GLP conditions.

Serum calprotectin was measured in duplicate serum samples under GLP conditions by Nordic Biosite, Finland, using the HK379 Human Calprotectin ELISA kit from Hycult Biotech.
Fecal calprotectin was measured in duplicates under GLP conditions by Synlab, Switzerland, using an ELISA kit from Immundiagnostik AG, Germany.

Outcomes

The primary outcome of this trial was the effect of the Bif195 intervention on the AUC Lewis score obtained by VCE from visit 2 (randomization) to visit 7 (end of treatment). As the first secondary endpoint, the effect of the Bif195 intervention on the AUC number of ulcers obtained by VCE from visit 2 to visit 7 was tested. Other secondary endpoints were, in hierarchical order: AUC of the pain module from the GSRS questionnaire, AUC of the total score from the GSRS questionnaire, AUC of blood I-FABP, AUC of red spots from the VCE procedure, AUC of fecal calprotectin and AUC of blood calprotectin.
As exploratory endpoints, data stratified into tertiles (small intestine divided into thirds) on effects of the Bif195 intervention on ulcerations observed by VCE was analysed and further post-hoc analyses on intervention effects on prostaglandin E2 (PGE2) and thromboxane B2 (TBX2) in serum samples downstream of COX were studied.
Safety was assessed by means of adverse events. A complete list of adverse events is provided in Table 2.

Statistical analysis

For all data obtained, area-under-the-curve (AUC) was calculated in order to evaluate the intervention effects by comparing the AUC in the Bif195 arm versus the placebo arm. For this purpose, the kinetics of Lewis score for each subject over the 6 VCE visits, was fitted to a third-

degree polynomial and the total AUC was calculated by computing the integral. This approach was taken for all VCE-obtained data.

Statistical tests were pre-defined and agreed in the statistical analyses plan finalised and signed prior to unblinding of the randomization key. The randomization list was made, and the labelling of trial product was performed by third parties not otherwise involved in the trial. No imputation of data was carried out in cases of missing data, but all available data were used.

Subject characteristics and all efficacy data presented are based on the Full Analysis Set (FAS) population. Criteria for inclusion in FAS was defined as maximum one missing visit in between the randomization visit (visit 2) and end of trial (Visit 7). The safety reporting by listing of adverse events included all subjects that were randomized (n=75)."

A sample size calculation was performed prior to trial initiation based on the primary endpoint of the trial. The curve shapes were assumed to fit with a third-degree polynomial. We considered a 30% lower AUC following treatment of Bif195 compared to placebo to be clinically relevant and aimed at a trial design that would have 80% power in detecting an intervention effects of this size as statistical significant. No previously knowledge exists on AUC values and SD. Sample size calculation was therefore performed on percent difference of AUC between two normalised curves (Active vs. placebo) as an approximation. We assumed similar standard deviation in each arm and planned for two-sided testing with a significance level of 5%. Given the above assumptions the number of subjects needed in each arm was 30. To account for potential drop-out subjects, we aimed to randomize a total of 75 subjects. Subjects who withdrew within one week of randomization were replaced by standby-subjects.

In general, datasets were modelled as the dependent variable in a linear mixed model. The model included the baseline value as covariate and gender and Bif195/placebo intervention as factors. Model check was always assessed for all datasets using QQ residual plots together with

Kolmogorov-Smirnov test for normality. In cases where datasets did not meet a normal distribution, a log transformation was performed and check for normality performed again. In cases where a normal distribution was still not obtained, the dataset was tested for intervention effects using a non-parametric Mann-Whitney test. Curves in Figure 2,3 and 5 are shown as mean values or medians, depending on normality. Bars in Figure 2-5 are always shown as mean ± SEM.

All authors had access to the study data and reviewed and approved the final manuscript.

Results

Between July 31st, 2017 to October 24th 2017, 109 subjects were screened for eligibility, of whom 75 were enrolled and randomized. Among the 75 randomized subjects, 9 subjects discontinued during the intervention (n=3 active and n=6 placebo) and therefore efficacy data was obtained in a total of 66 subjects, the analysis population (n=35 active arm and n=31 placebo, Figure 1).

The arms were in general similar in their baseline parameters as shown in Table 1, including gender distribution, age, BMI and blood pressure. Accountability of both ASA and trial product were in general very high in both two arms (Table 1).

This clinical trial met its primary endpoint with a statistically significantly (p=0.0376) lower AUC Lewis Score, as captured by VCE, during the 8 weeks intervention in the Bif195 arm versus the placebo arm (3040 ± 1340 arbitrary units (au) in the Bif195 arm vs 4351 ± 3195 au in the placebo arm, Figure 2A and B). In addition, the trial met its secondary endpoint with a significantly (p=0.0258) lower AUC ulcer number as captured using VCE during the intervention in Bif195 subjects versus the placebo group (50.4 ± 53.1 au in the Bif195 arm vs 75.2 ± 85.3 au in the placebo arm, Figure 2C and D).

An exploratory tertile stratification of VCE data showed that the damage induced by ASA occurs primarily in the first tertile (Figure 3) where a significant Bif195 protective effect (p=0.03) was also observed (31.0 ± 16.8 au in the Bif195 arm vs 41.6 ± 25.2 au in the placebo arm, Figure 3A and B). The other secondary endpoints GSRS pain AUC, GSRA total score AUC, plasma I-FABP AUC, red spots from VCE AUC and serum calprotectin AUC did not meet statistical significance (Figure 4) while fecal calprotectin AUC was significantly lower (p=0.0347) in the Bif195 arm compared to the placebo arm (Figure 4E.)

ASA and trial product were both generally well-tolerated by the subjects. In total, 32 adverse events were registered from 22 different subjects included in the n=75 safety analysis set. Twelve of these adverse events were reported from the Bif195 arm and 20 from the placebo arm (Table 2). None of the adverse events were related to Bif195 intake, while in total 10 of them were assumed related to ASA intake, as assessed by the principal investigator. The number of adverse events related to ASA did not differ between the two intervention arms (4 and 6 in the Bif195 and placebo arm, respectively).

DNA sequencing of all fecal samples obtained showed an increase after randomization in abundance of *Bifidobacterium breve* in fecal samples obtained from subjects in the Bif195 arm compared to the placebo arm, confirming trial product compliance (Supplementary Figure 2). The Bif195 intervention was not associated with significant changes in abundance of specific microbial taxa nor in the changes of the overall microbiome composition (as revealed by Bray-Curtis dissimilarity index, Supplementary Figure 3).

Serum PGE2 and TXB2 concentrations showed a robust decline during ASA intake and a reversal to baseline levels during the final 2 weeks recovery period. The Bif195 intervention did not have significant effects on these datasets (Figure 5).

Discussion

The trial results indicate that *Bifidobacterium breve* Bif195 confers significant and objectively verifiable protection against small-intestinal damage caused by a 6 week ASA challenge in healthy volunteers. The primary and first secondary efficacy criteria for the trial were met, thereby highlighting the potential of Bif195 co-treatment in future prevention strategies for a growing population experiencing silent or overt small-intestinal enteropathy from chronic ASA use.

Although prior studies have described gastric damage from NSAIDs, this is, to the best of our knowledge, the first trial to record the detailed time-resolved kinetics of ASA-induced, and reversal of, small-intestinal damage. This dataset shows a gradual increase in damage observed by VCE during the 6 weeks of daily ASA intake and a partial reversal towards baseline levels over a 2-week recovery period. Furthermore, the small-intestinal tertile stratification clearly shows that ASA-induced enteropathy is mainly a duodenal phenomenon. This site coincides with localisation of the main effect of the Bif195 intervention on ulceration, further highlighting the potential of protective intervention with this strain. The strategy to perform serial capsule endoscopies in this trial, enabled us to obtain the sensitivity needed to observe a significant effect in a dynamic environment where damage formation and healing co-exists. Thus, it represents a superior and more sensitive form of assessment than the more usually adopted before/after intervention trial design.

The efficacy of Bif195 in NSAID-associated small intestinal injury may be partly explained by the difference in pathogenesis between NSAID-associated small intestinal injury and NSAID-associated gastropathy. Whereas acid and pepsin are the principal luminal aggressors in NSAID-gastropathy, bile and indeed bacteria are the luminal factors in NSAID-enteropathy[22]. Although pre-clinical studies in experimental animals have been encouraging, previous trials in humans of putative probiotics in NSAID-enteropathy have been inconsistent. Certain strains of Bifidobacteria, are known to strengthen the intestinal epithelium layer, to modulate the local immunoinflammatory

response as well as compete with potential bacterial aggressors. The molecular details of bifidobacterial-mediated protection against small-intestinal epithelial injury are currently under investigation, but one candidate includes the pilus-associated protein Tad E which exerts a proliferative effect on host colonic epithelium following oral consumption of B. breve[18]. This appears to be a characteristic of all B. breve and supports our choice of the stain used in this trial. Interestingly, fecal microbiome analysis revealed changes were limited to a marked increase in the total *B. breve* population in the Bif195 arm. These data provide further evidence that microbial intervention strategies targeting the microbiome can be clinically efficacious without inducing major alterations in the overall microbial population structure.

Our 6-week ASA challenge model yielded minor responses in the GSRS questionnaire and in the biomarkers of damage, I-FABP in blood and calprotectin in blood and feces. Although trends were observed for I-FABP, only the fecal calprotectin endpoint reached statistical significance indicating a modest Bif195 protective effect. Our data suggest that VCE is the method of choice when conducting human challenges with mild induction of small-intestinal damage by NSAIDs over a limited time period.

Although encouraging, the present clinical trial has limitations in terms of translation to a real-life clinical setting. The relatively short-term challenge in healthy volunteers, for proof-of-concept, used a higher dose of ASA than is most commonly prescribed for primary CVD prevention. However, it is a dose that is readily available for over-the-counter usage. It is also noteworthy, that a recent report suggested that the current cardioprotective dosage of ASA may be insufficient and recommended doses based on a mg/kg basis[23].

Due to our AUC approach based on a polynomic curve fitted to data-points obtained from 6 different visits, data imputation is not feasible for drop-out subjects where only baseline data are available. Therefore, we acknowledge that long-term intervention clinical trials will be needed to

confirm if Bif195 has long-term clinical efficacy in a larger intention-to-treat population of chronic users of ASA taking lower doses for CVD prevention.

In addition, we acknowledge that the division of the small intestine into tertiles by VCE is based on assumptions and that tertile-specific data are an approximation.

As expected, the ASA intake was associated with robust inhibition downstream of the COX enzyme on serum PGE2 and TXB2 concentrations. It is noteworthy that the Bif195 intervention did not alter these well-described ASA-induced changes in metabolites downstream of COX[24,25]. This suggests that the small-intestinal protective actions of Bif195 is unlikely to interfere with the specific cardiovascular-protective properties of ASA. Close monitoring of adverse events during this trial suggests that daily, oral intake of Bif195 is safe and without side-effects. Further clinical trials are required to test whether the strain has clinical efficacy also in other settings and populations, i.e. in chronic users of ASA.

Figure legends:

Figure 1. Enrollment and randomization of subjects according to the CONSORT Flow Diagram.

Figure 2. Primary and Secondary endpoint. Mean Lewis Score per visit (A) and the primary endpoint mean Lewis Score AUC ± SEM (B) per treatment arm. Median number of ulcers per visit (C) and the secondary endpoint ulcer number AUC ± SEM (D) per treatment arm. * indicates $p < 0.05$. Effects sizes were 30% lower AUC in Bif195 arm (B) and 33% lower AUC in Bif195 arm (D).

Figure 3. Tertile-stratification of ulceration. Median ulcer numbers, both per visit (A, C and E) and mean ulcer number AUC ± SEM (B, D and F) from Video Capsule Endoscopy stratified on small-intestinal tertiles (thirds of small intestine). * indicates $p < 0.05$.

Figure 4. Other secondary endpoints measured in the trial. AUC ± SEM of the Pain module (A) and total score (B) from in the GSRS questionnaire. AUC ± SEM of blood I-FABP (C), AUC ± SEM of red spots from VCE (D), AUC ± SEM of fecal (E) and blood (F) calprotectin. * indicates $p < 0.05$.

Figure 5. Mean serum concentrations of Prostaglandin E2 per visit (A) and AUC ± SEM (B). Mean serum concentrations of Thromboxane B2 per visit (C) and AUC ± SEM (D).

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Table 1. Subject baseline characteristics and trial compliance of the analysis population.

	Bif195	Placebo
N	35	31
Age (years)	30.5 ± 6.8	31.2 ± 6.4
Gender (m/f)	16/19	14/17
Ethnicity (non-caucasians)	2	0
Height (cm)	172.2 ± 12.1	173.4 ± 10.2
Weight (kg)	73.5 ± 12.5	72.0 ± 11.4
BMI (kg/m^2)	24.6 ± 2.1	23.8 ± 2.2
Blood pressure, Systolic (mm hg)	124.1 ± 7.8	121.6 ± 10.2
Blood pressure, Diastolic (mm hg)	78.7 ± 6.9	77.1 ± 7.6
alcohol consumption (“drinks” per week)	5.1 ± 3.2	5.5 ± 3.7
Compliance of ASA intake (%, 100% = product subj. should have taken during trial)	98.7 ± 2.4	99.1 ± 1.9
Compliance of trial product (%, 100% = product subj. should have taken during trial)	98.6 ± 2.4	99.0 ± 1.9

Body-mass index, BMI, is the weight in kilograms divided by the square of height in meters. Numbers are given as Mean ± SD.

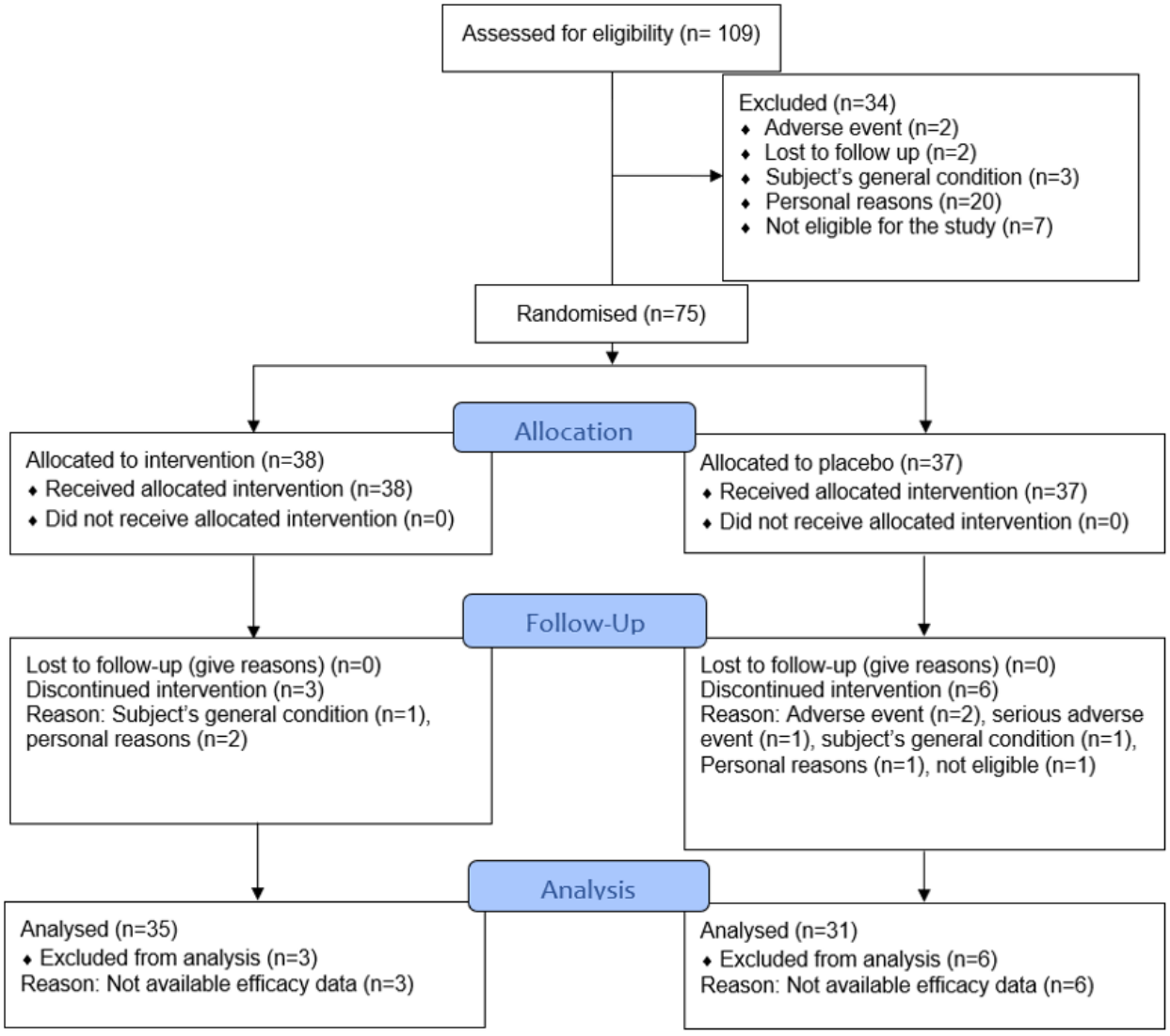
Table 2. Trial adverse events overview.

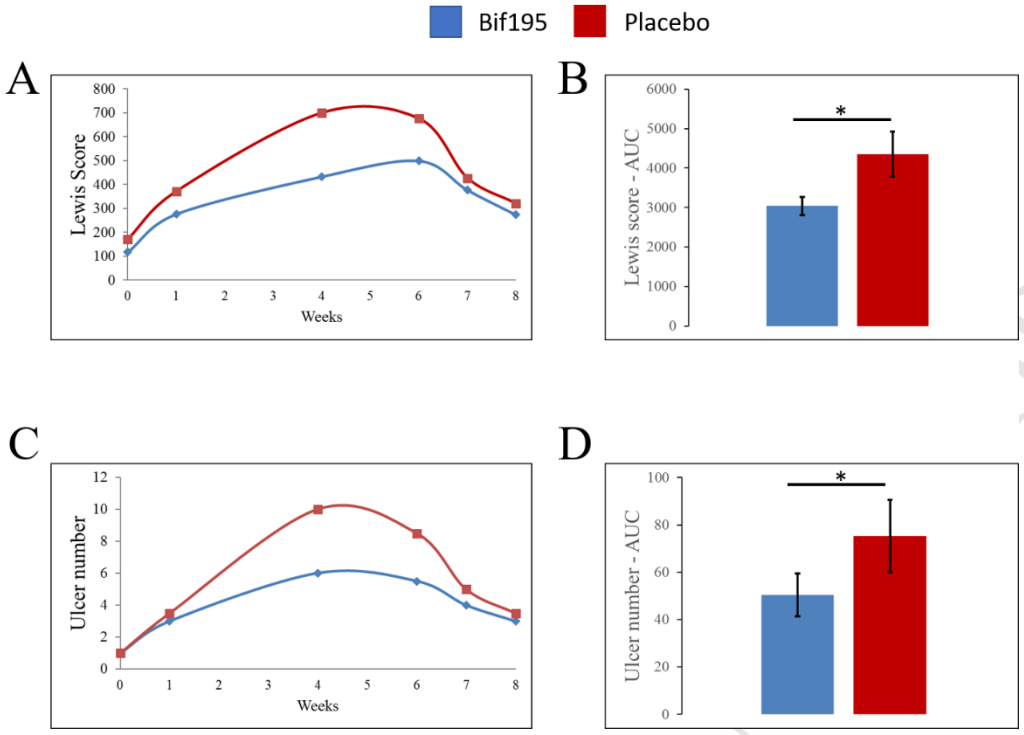
Treatment	Bif195	Placebo	Total
	N (%) E	N (%) E	N (%) E
Number of subjects	38	37	75
All adverse events	8 (21.1) 12	14 (37.8) 20	22 (29.3) 32
Back pain	1 (2.6) 1	0 (0) 0	1 (1.3) 1
Blocked sinuses	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Chest infection	1 (2.6) 1	0 (0) 0	1 (1.3) 1
Cold and flu	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Cold flu symptoms	1 (2.6) 1	0 (0) 0	1 (1.3) 1
Cold/flu symptoms including a nose bleed.	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Cough, nasal congestion	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Cramping in the stomach	1 (2.6) 1	0 (0) 0	1 (1.3) 1
Cystitis	1 (2.6) 1	0 (0) 0	1 (1.3) 1
Headache	1 (2.6) 1	2 (5.4) 2	3 (4.0) 3
Headache, sore throat, rhinorrhea.	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Heartburn	0 (0) 0	2 (5.4) 2	2 (2.7) 2
Inflammation in kidneys due to kidney stones	1 (2.6) 1	0 (0) 0	1 (1.3) 1
Lower abdominal pain	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Nasal congestion	1 (2.6) 1	0 (0) 0	1 (1.3) 1
Nausea	1 (2.6) 1	0 (0) 0	1 (1.3) 1
Nausea, vomiting, headache, fatigue	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Pain and discomfort in the stomach and gut region.	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Pain and discomfort in the stomach/gut region.	1 (2.6) 1	0 (0) 0	1 (1.3) 1
Pain/discomfort in the stomach and gut region.	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Painful headache which caused vomiting, thigh and back pain.	0 (0) 0	1 (2.7) 1	1 (1.3) 1

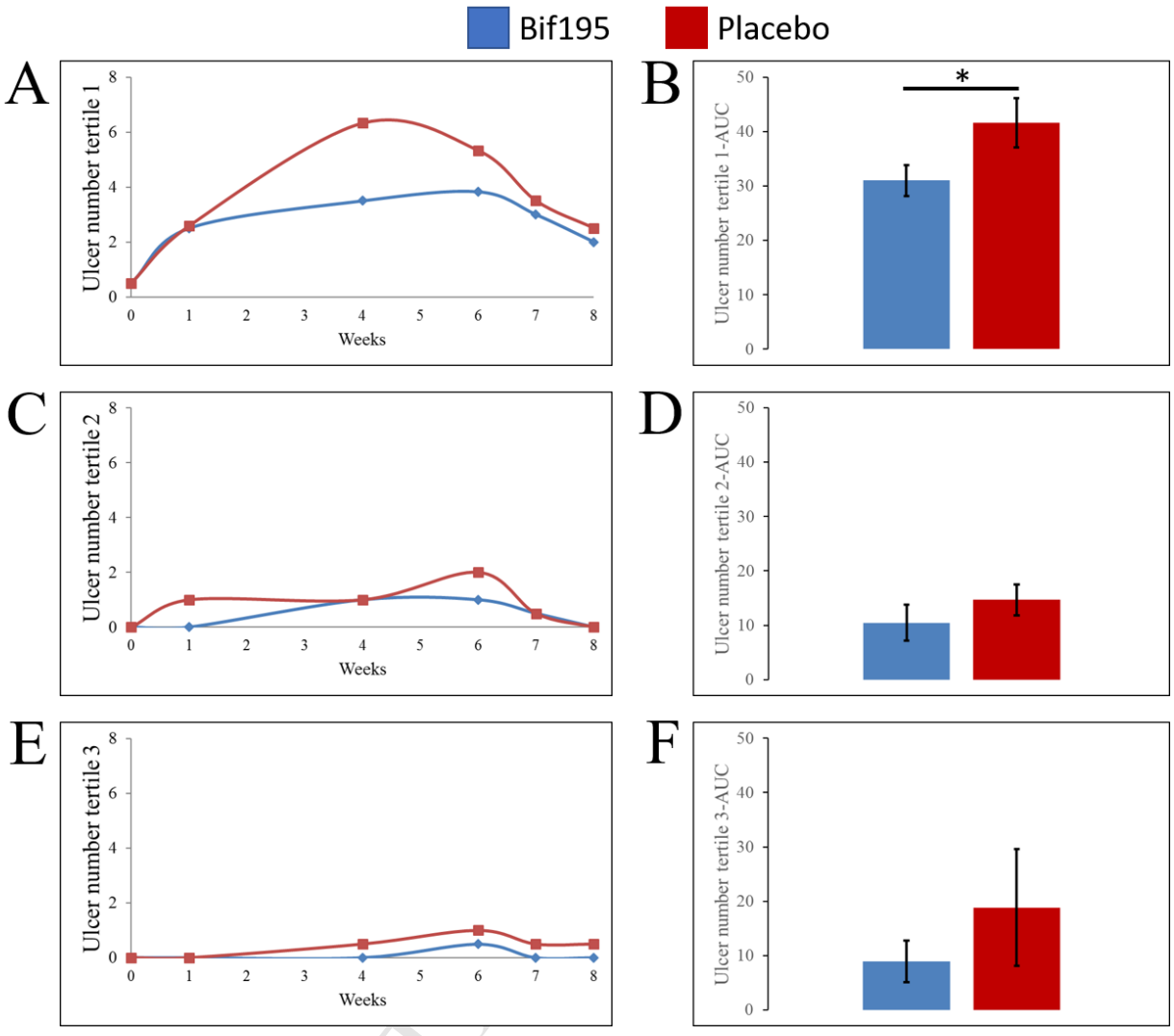
Sore throat and flu symptoms	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Stomach cramps	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Stomach cramps and loose stools	1 (2.6) 1	0 (0) 0	1 (1.3) 1
Stomach discomfort	1 (2.6) 1	0 (0) 0	1 (1.3) 1
Subject became pregnant	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Subject was physically assaulted and suffered facial injuries	0 (0) 0	1 (2.7) 1	1 (1.3) 1
Vomiting bug	0 (0) 0	2 (5.4) 2	2 (2.7) 2

N = Number of subjects in the group having the event. E = Number of events in total in the group.

() = Percentage of subjects in the group having the event.






Bif195
Placebo
A
Ulcer number tertile 1
Weeks
B
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*
C
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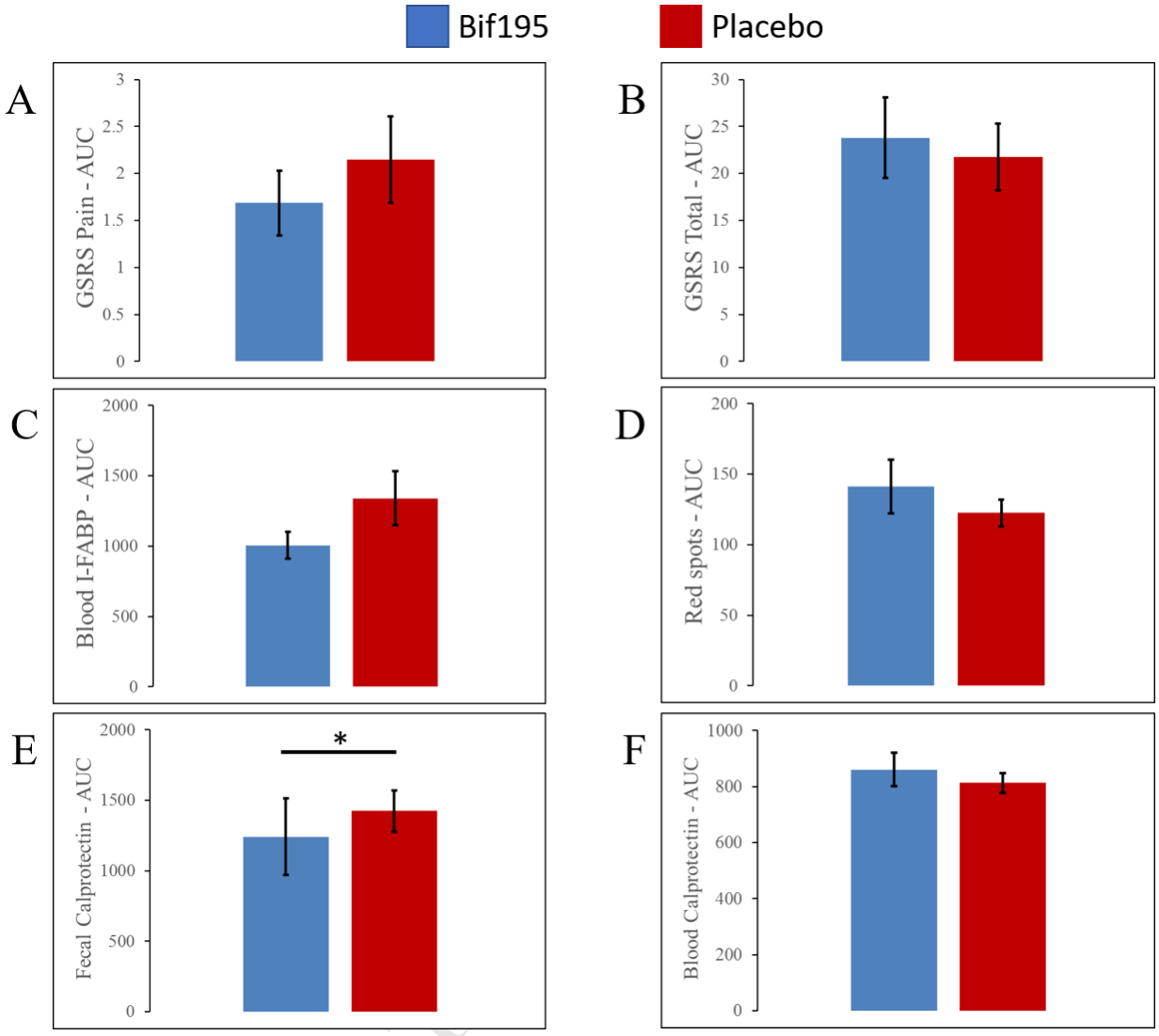
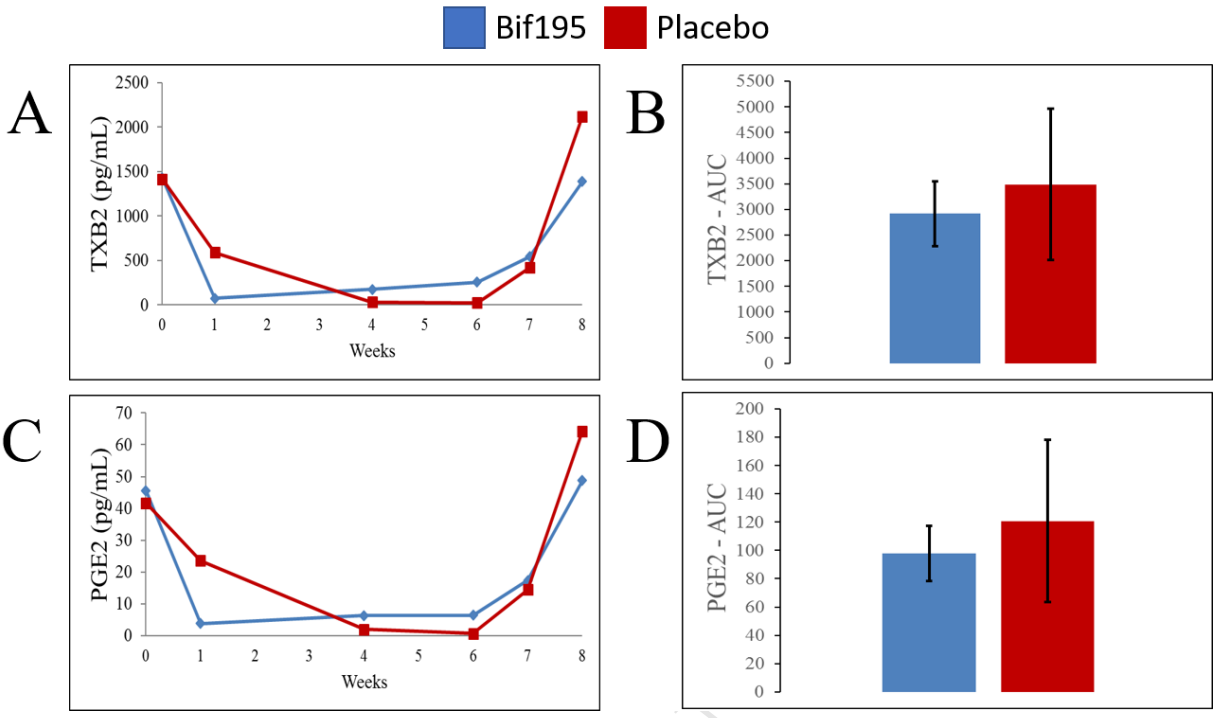
F
Ulcer number tertile 3-AUC





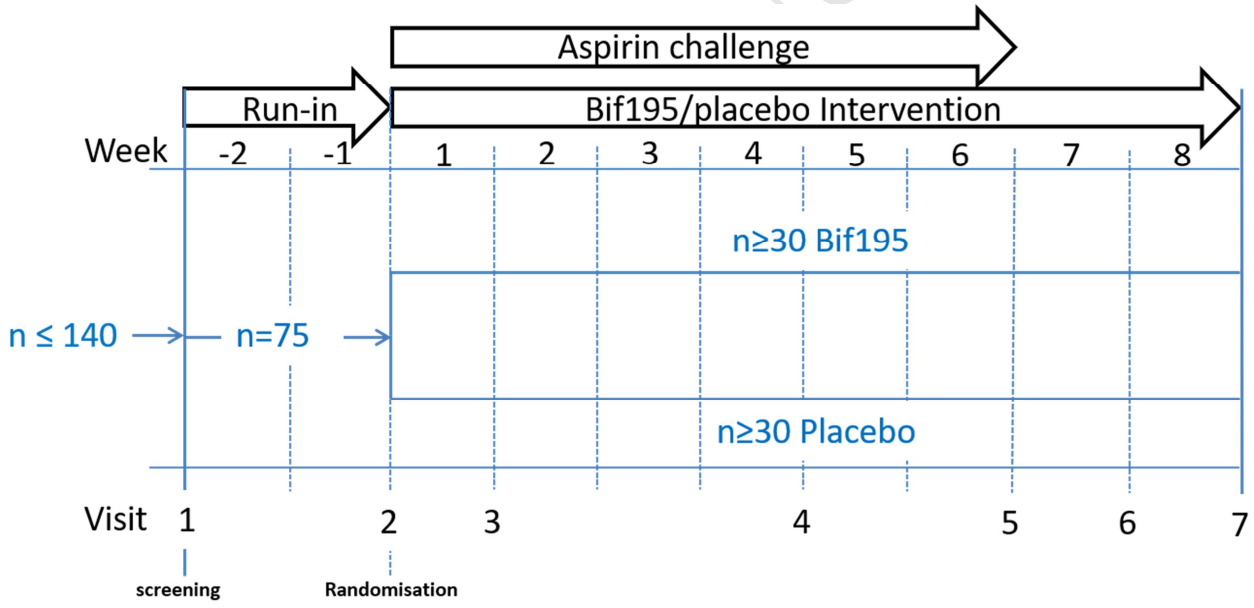
Supplementary table 1. Trial product composition

	Placebo capsules	**Probiotic capsules Bifidobacterium breve**
Manufacturing	Chr. Hansen A/S, Denmark	Chr. Hansen A/S, Denmark
Brief description	Capsules with excipients only	Capsules containing Bifidobacterium breve and excipients
Capsules	Size 1 HPMC capsules	Size 1 HPMC capsules
Capsules shell	73.6 mg Hypmellose 1.4 mg Titanium dioxide	73.6 mg Hypmellose 1.4 mg Titanium dioxide
Active Ingredients	None	Bifidobacterium breve Bif195
Excipients	Microcrystalline Cellulose 6 mg per capsule Magnesium Stearate 1.5 mg per capsule Maltodextrin 277.8 mg per capsule Sodium Ascorbate 14.7 mg per capsule	
Supplied as	CSP Activ-Vials containing 24 capsules in each vial	
Storage conditions	Store at +2-8 ºC	

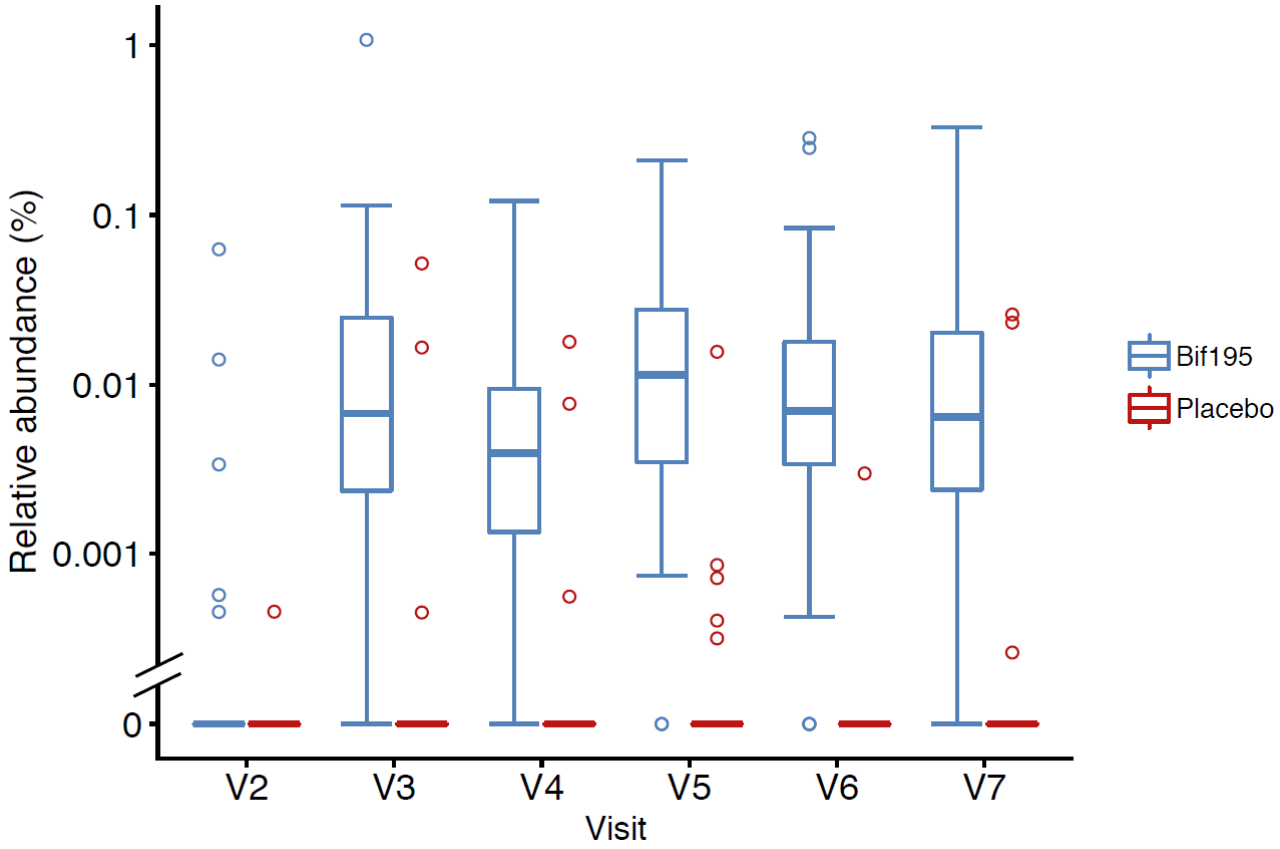
Supplementary table 2. Discontinued subjects overview.

Subject ID	Reason for trial discontinuation	Bif195/placebo	Trial product taken	Aspirin taken	Baseline Lewis score
1013	Subject became pregnant. Contraindicates continuation.	Placebo	yes	yes	0
1026	Withdrawal for personal reasons	Placebo	yes	yes	184
1029	Baseline VCE capsule did not reach caecum. Contraindicates continuation.	Bif195	No	No	-
1042	Baseline VCE capsule retained in stomach. Contraindicates continuation.	Placebo	No	No	-
1048	Withdrawal for personal reasons	Bif195	yes	yes	0
1077	Baseline VCE capsule retained in stomach. Contraindicates continuation.	Placebo	No	No	-
1083	Baseline VCE capsule did not reach caecum. Contraindicates continuation.	Placebo	No	No	-
1089	SAE due to prolonged hospitalisation (back pain). Event unrelated to intake of trial product.	Placebo	yes	yes	67.5
1108	Withdrawal for personal reasons	Bif195	yes	yes	67.5

Supplementary Figure 1: Trial design.



Supplementary Figure 2:



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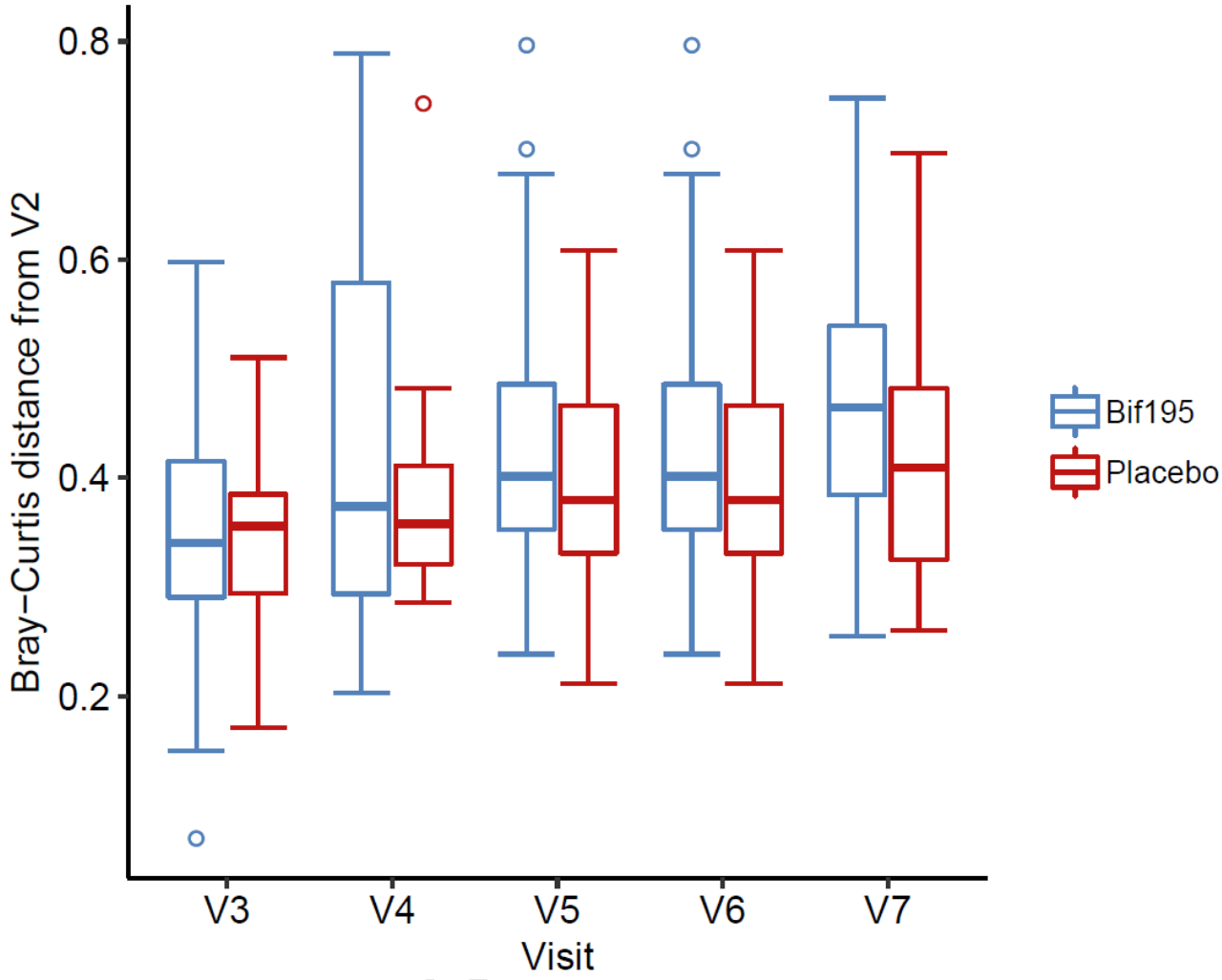

Supplementary figure 4. Representative Video Capsule Endoscopy data.

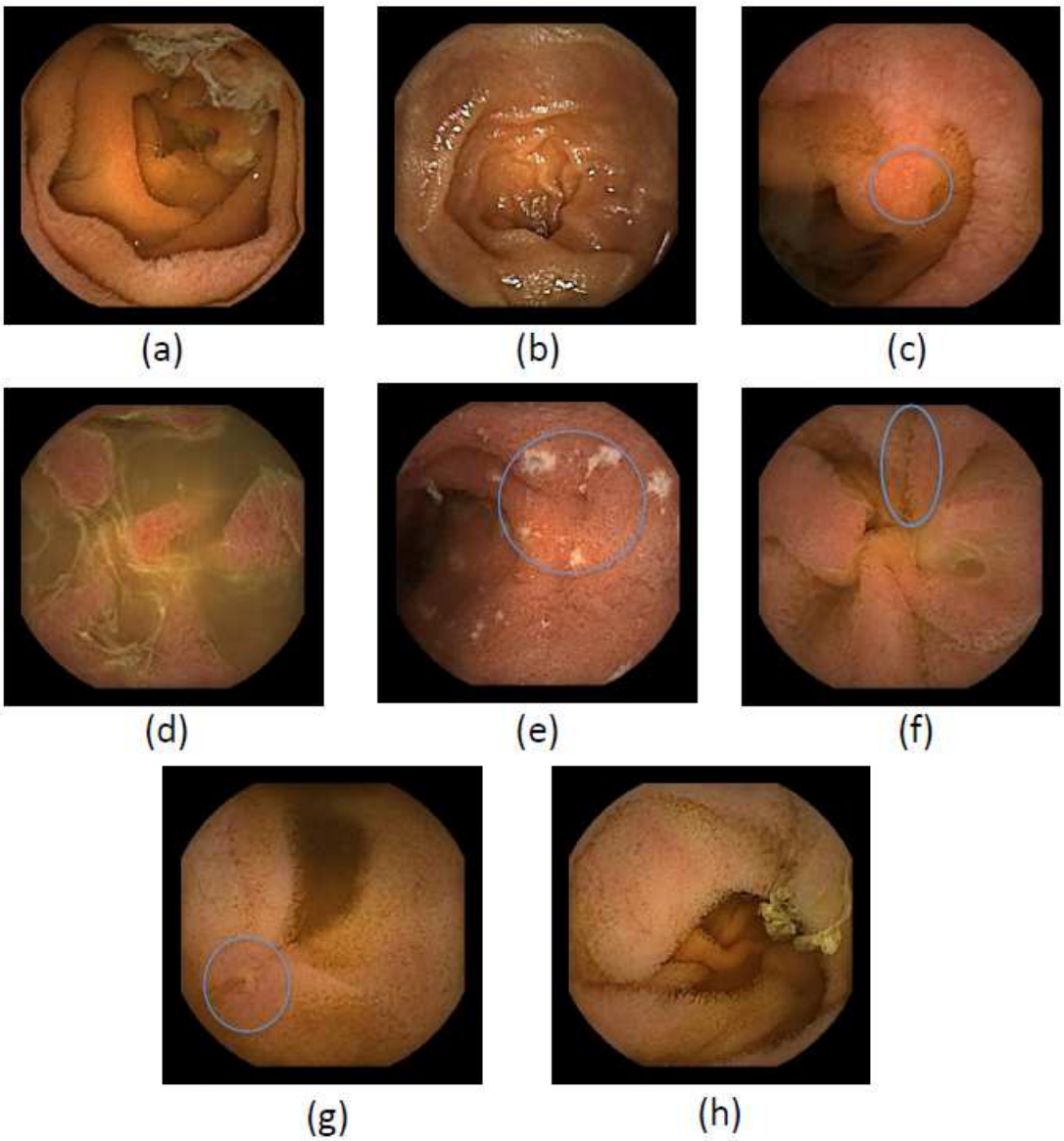

Figure legends:

Supplementary Figure 1. The trial consisted of a 2 week run-in phase after the screening visit. Subjects were then randomised to 8 weeks of Bif195 or placebo intervention for 8 weeks. The first 6 of these 8 weeks, all subjects took 300 mg ASA daily. In total 6 visits with video capsule endoscopies were performed during the 8 week intervention period.

Supplementary Figure 2. Box-plot showing the relative abundances of Bifidobacterium breve in stool at visits 2-7. The boxed extends from the first quartile (Q1) to the third quartile (Q3) and the line within the box shows the median value. The lower whisker extends to the smallest value within Q1 - 1.5 x inter-quartile range (IQR) and the upper whisker extends to the largest value within Q3 + 1.5 x IQR. Values outside the whiskers are shown as circles. After unblinding, a post-hoc lab and bioinformatic analysis was performed on DNA extracted from all obtained fecal samples using a NucleoSpin 96 Soil kit (Macherey-Nagel) and randomly sheared into 350 bp fragments. Libraries were constructed using NEBNext Ultra Library Prep Kit for Illumina (New England Biolabs) and sequenced to at least 30 million read pairs per sample (2 x 150 bp paired-end Illumina sequencing). Sequencing reads were filtered to remove human and low-quality reads, mapped to the Clinical Microbiomics Human Gut 22M gene catalog, and summarised as a taxonomic relative abundance table as described previously[21]. The involved parties were kept blinded for intervention during analyses. Changes in relative abundances of taxa between visit 2 and the integral of later time-points was tested using Wilcoxon rank sum test and corrected for multiple comparison using a Bonferroni correction. Similarly, the Bray-Curtis distance between visit 2 and later time-points were compared between the two arms (t-test).

Supplementary Figure 3. Box-plot showing the Bray-Curtis dissimilarity of stool microbial composition, comparing Visit 2 with later visits (V3-V7). The boxed extends from the first quartile (Q1) to the third quartile (Q3) and the line within the box shows the median value. The lower whisker extends to the smallest value within Q1 - 1.5 x inter-quartile range (IQR) and the upper whisker extends to the largest value within Q3 + 1.5 x IQR. Values outside the whiskers are shown as circles.

Supplementary Figure 4. Representative images obtained by Video Capsule Endoscopy from one subject throughout intervention period. All images are obtained from the first tertile of the small intestine. The pictures shows: (a) Visit 2 with normal intestinal mucosa, (b) Visit 3 with normal intestinal mucosa, (c) Visit 4 with Ulcer highlighted by blue circle, (d) Visit 4 with villous edema, (e) Visit 5 with Ulcer highlighted by blue circle, (f) Visit 5 villous edema highlighted by blue circle, (g) Visit 6 with ulcer highlighted by blue circle and (h) Visit 7 with normal intestinal mucosa.