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Safety of *Bifidobacterium breve*, Bif195, employing a human exercise-induced intestinal permeability model: a randomised, double-blinded, placebo-controlled, parallel group trial

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Abstract

We have previously shown that the probiotic *Bifidobacterium breve* strain Bif195 alleviates mucosal injury including ulcer formation in the upper intestine induced by non-steroid anti-inflammatory drugs (NSAIDs). Here, we report additional safety use of Bif195 in 126 healthy humans undergoing an exercise-induced intestinal permeability challenge in a double-blinded, placebo-controlled randomised 6-week intervention trial. Intestinal permeability was assessed by urinary lactulose/rhamnose (L/R) ratio. L/R ratio, plasma intestinal fatty acid binding protein (I-FABP) and gastrointestinal symptom rating scale (GSRS) questionnaire were measured resting and after a 1 h treadmill challenge, prior to and at the end of the intervention. To be able to compare the equivalence of resting state at baseline, of this cohort of well-trained subjects, to non-trained subjects, a cohort of 63 healthy and non-trained subjects (<2 h/week of endurance sports) was included. Study subjects (well-trained) were 35.7% women with a mean age and body mass index (in kg/m^2) of 35.0 years and 24.8, respectively. There were no differences between the Bif195 and placebo groups in effects on L/R ratio, I-FABP and GSRS questionnaire score. In addition, there were no differences between Bif195 and placebo in number of adverse events and change in cytokines, liver or kidney biomarkers. The exercise model successfully induced intestinal permeability by statistically significantly increasing L/R ratio by ~100% (P<0.0001) and cytokines after the exercise challenge. No significant difference was found between well-trained and non-trained subjects in baseline resting L/R ratio. In conclusion, the reported cytoprotective effects of Bif195 are unlikely to be primarily related to small bowel permeability, and the safety of Bif195 in individuals with increased permeability is supported by the present data.

ClinicalTrials.gov: NCT03027583

Keywords: *Bifidobacterium breve*, probiotics, gastrointestinal permeability

1. Introduction

In a recent randomised controlled trial (RCT), we found significant clinical efficacy of the probiotic strain, *Bifidobacterium breve* Bif195, in alleviating mucosal injury induced by a non-steroidal anti-inflammatory drug (NSAID) including erosions and ulcer formation in the upper small intestine (Mortensen *et al.*, 2019). Alleviating side effects of drugs, improving general health and reducing infections are among the potential features of probiotic strains, that are generally regarded as safe for the general population (Boyle *et al.*, 2006; Koutsoumanis

et al., 2020). However, the safety profile of healthcare products containing bacteria including new probiotics for humans needs robust assessment (Pariza *et al.*, 2015). While Bif195 was found to be safe in humans with NSAID-induced mucosal injury and presumed increased intestinal permeability, we performed an additional safety evaluation of Bif195 in humans with non-pharmacologically increased permeability using exercise. Subjects able to complete a 1 h treadmill challenge at a velocity of 80% of each individual participants VO_2max were selected. In addition, we included a cohort of non-trained subjects (Cohort B) to perform only the baseline small intestinal permeability test in order to compare whether resting state was equivalent to well-trained subjects (Cohort A).

Strenuous physical exercise is a recognised stressor, the effects of which include increased gut permeability (Chantler *et al.*, 2021; Pals *et al.*, 1997; Van Nieuwenhoven *et al.*, 2004). A meta-analysis of clinical studies indicated that an acute bout of exercise induces a significant increase in the urinary tracer disaccharide/monosaccharides ratio and circulating intestinal fatty acid binding proteins (I-FABP) (Chantler *et al.*, 2021). Intensity of running is a significant determinant of small intestinal permeability (Pals *et al.*, 1997). Pals *et al.* (1997) showed that running at a velocity of 80% of VO_2max (in contrast to 60 and/or 40%) significantly increased the small intestinal permeability compared to the resting state, measured by the lactulose/rhamnose (L/R) sugar test. Strenuous exercise at a velocity of 70-80% of VO_2max decreases the splanchnic blood flow (Costa *et al.*, 2017; Van Wijck *et al.*, 2012). This reduction in gastrointestinal blood flow can increase susceptibility to ischemic injury and increase mucosa permeability (De Oliveira and Burini, 2009). With this study we aim to prove the safe use of Bif195 in a cohort of healthy men and women representing resting state as well as exercise challenged state of small intestinal permeability.

2. Materials and methods

Study design and recruitment

This clinical trial was a single site, randomised, double-blinded, two-armed, placebo-controlled trial. In total, subjects attended 5 visits including a 2-week run-in period followed by a 6-week intervention period (Figure 1). L/R sugar test were measured resting and after a 1 h tread mill challenge, prior to (visit 2 and 3) and at the end of (visit 4 and 5) the intervention. Resting and exercise challenge visits were separated by one week. In addition to the well-trained subjects included in the trial described above (Cohort A), non-trained subjects receiving no intervention and only performing screening visit (visit 1) and visit 2 (after one-week run in) (Cohort B) were also included in the study. The trial was conducted at the contract research organisation Atlantia Food Clinical Trials and Mardyke Arena (Cork, Ireland). The trial was conducted in accordance with the ethical principles set forth in the current version of the Helsinki Declaration and the International Conference on Harmonisation E6 Good clinical Practice and was approved by the Clinical Research & Ethics Committee of the Cork Teaching Hospitals (Cork, Ireland). The trial period was from March 2017 to July 2017 and the trial was registered at ClnicalTrials.gov (NCT03027583).

Participants

All participants gave their written informed consent after receiving careful oral and written information about the trial. Inclusion criteria were adults between the ages of

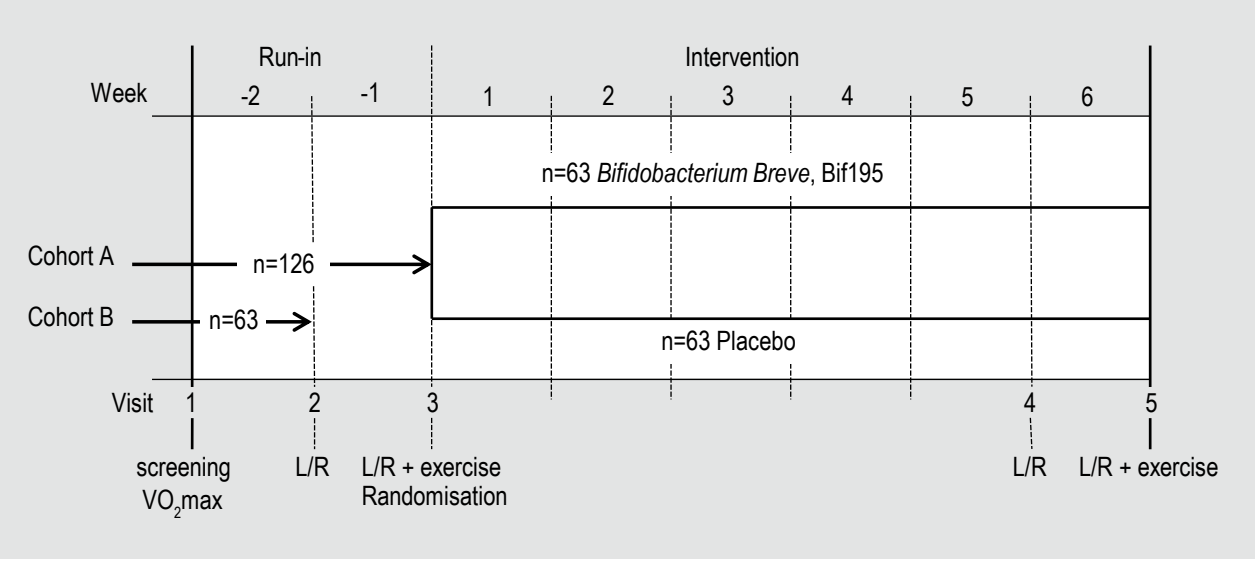

Figure 1. Trial design.

18 and 50 years, healthy although gastrointestinal (GI) symptoms were allowed. Subjects had to be willing to refrain from any probiotic products or medication known to alter GI function throughout trial participation. Participants were recruited to Cohort A or Cohort B with different inclusion criteria in terms of physical activity. In Cohort A, participants' weekly training load had to be ≥4 h of endurance sports, within which a minimum of 1.5 h had to be running activity. Further, the subjects should (to their own perception) be able to complete a 10 km run on a treadmill within 60 min at a velocity of 80% of VO_2max (well-trained subjects). In Cohort B (non-trained subjects), weekly training load had to be <2 h of endurance sports. Thus, we consider both Cohort A and B as part of the healthy population with Cohort A as a subgroup being well-trained. The main exclusion criteria were abdominal surgery which might affect gastrointestinal function (except appendectomy and cholecystectomy), diagnosis of inflammatory gastrointestinal disease or any other disease that could interfere with the intestinal barrier function of the subject, current diagnosis of psychiatric disease, pregnancy, lactation, diastolic blood pressure ≥90 mmHg, systolic blood pressure ≥140 mmHg, smoking or use of other nicotine products, lactose intolerance and regular use of probiotics, laxatives, anti-diarrhoeal medication, anti-cholinergics, non-steroidal anti-inflammatory drugs (NSAIDs), systemic antibiotics, steroids (except contraceptives) or antimicrobial medication in the last two months, use of immunosuppressant drugs in the last month, use of any medications except contraceptives in the last two weeks prior to screening. Subjects were instructed to maintain their habitual lifestyle with regards to diet, physical activity, and sleep throughout the entire trial.

Experimental design

All participants were instructed to ingest two capsules once daily with breakfast with or without *B. breve* Bif195 starting the day after visit 3 for a duration of six weeks. The active and placebo capsules were visually identical. The daily dose of Bif195 was minimal 50×10^9 cfu. A strain safety assessment of Bif195 was completed as described in Supplementary Material S1.

At screening, a test to determine the individual VO_2max was performed. Screened subjects completed a two-week run-in period to wash-out possible pre-trial probiotics. After baseline assessment at visit 2 and 3, Cohort A subjects were randomly assigned to six weeks intervention of Bif195 or placebo product. At visit 2-5, blood samples were collected, and subjects performed a standardised L/R sugar permeability test to assess intestinal permeability. At visit 3 and visit 5 the L/R sugar permeability tests were performed in combination with an exercise challenge where the subject ran 60 min on a treadmill at a velocity corresponding to the velocity of 80% of the subjects individual VO_2max in a room with a stable room temperature. During the exercise challenge the L/R drink was administered quickly during a very short break after completion of 30 min of the 60 min exercise challenge (Pals *et al.*, 1997). All urine was collected during the 5 h following intake of 1.0 g lactulose (CAS Number 4618-18-2; Sigma-Aldrich, St. Louis, MO, USA) and 0.5 g L-rhamnose (CAS Number 10030-85-0; Sigma-Aldrich) dissolved in 150 ml of tap water. During the 5 h of urine collection the subjects fasted and only consumed 1.5 l tap water. Subjects stayed on site for 5 h following oral administration of the L/R drink to ensure urine collection and compliance with respect to food intake. Subject stored the urine in electrical coolers during the 5 h collection period and delivered the samples in the coolers to the staff. The total volume of urine produced was recorded and after centrifugation (1,500×*g* for 10 min at 4 °C), aliquots of urine (>20 ml) were pipetted directly into 5 ml polypropylene tubes without disturbing the precipitate. Samples were stored at -80 °C until shipping for L/R analysis.

Determination of the L/R ratio in human urine samples was measured with LC-MS/MS by Synlab Pharma Institute AG, Birsfelden, Switzerland. Storage temperature of samples at Synlab Pharma Institute AG was -25 °C until analysis. To comply with Good Clinical Practice (GLP) and criteria for precision and accuracy, Synlab analysed the two sugars (rhamnose and lactulose) in two separate methods/columns instead of one with a specific separate assay developed for quantification of rhamnose in urine with dericatization with 1-phenyl-3-methyl-5-pyrazolone. The analytical methods to quantify the concentrations of lactulose and rhamnose in human urine by LC-MS/MS was successfully validated by Synlab Pharma Institute AG.

The GI symptom rating scale (GSRS) questionnaire was used to assess GI symptoms (Svedlund *et al.*, 1988). The 18 questions from the GSRS questionnaire were converted to five domains: abdominal pain syndrome, dyspeptic syndrome, indigestion syndrome, bowel dysfunction syndrome and GI symptoms total score. The questionnaire was based on the subject's assessment at visit 2 and visit 4 when filling out the questionnaire and at visit 3 and visit 5 for the duration of the 1 h run.

Dietary standardisation

To standardise the diet before permeability tests subjects were instructed to complete a food and fluid diary the last two days before the first permeability test (visit 2). The subjects were instructed to consume the same amount and type of food and liquids prior to visit 3, 4 and 5 based on the recorded diet. In addition, all subjects received a standardised dinner meal and evening snack prior to visit 2 (Cohort B also), visit 3-5 and instructed to only drink water as well as a standardised breakfast 2 h prior to the planned intake of the L/R sugar solution.

Blood collection and analysis

All laboratory analyses were performed in triplicate and to GLP standards. Blood samples were drawn before L/R administration during visit 2 and visit 4, and immediately after exercise (≤5 min after ended exercise) during visit 3 and visit 5 in the following order: serum, heparin plasma and K_3EDTA plasma. Serum and plasma samples were stored at -80 °C. Intestinal fatty acid-binding protein (I-FABP) was measured by Nordic BioSite (Tampere, Finland) in triplicate heparin plasma samples by using the HK406 Human I-FABP ELISA kit (Hycult Biotech Inc., Wayne, PA, USA) according to the manufacturer's instructions. Cytokine concentrations were quantified from heparin plasma. Secreted levels of interferon (IFN)-γ, interleukin (IL)-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8 and tumour necrosis factor (TNF)-α were quantified using the V-PLEX human proinflammatory panel 1 from Meso Scale Discovery (Cat. # K15049D; Meso Scale Diagnostics, Rockville, MD, USA) according to the manufacturer's protocol, using a 1:1 dilution. C-Reactive Protein (CRP) levels were also quantified using a V-PLEX single assay (Cat. # K151STD) according to the manufacturer's instructions, using a 1:1000 dilution. Inter-plate variation was assessed using a bridge control sample. All assays were read using a MESO QuickPlex SQ 120 and analysis was performed using Discovery Workbench 4.0 (Meso Scale Diagnostics). Liver and kidney safety biomarkers including albumin, bilirubin, creatinine, potassium, sodium, alpha-amylase, alanine aminotransferase (ALT) and urea-N were analysed by Synlab Pharma Institute AG according to standard protocol.

Randomisation and masking

Prior to trial initiation, the allocation of subject numbers to Bif195 or placebo in a 1:1 ratio was performed according to randomisation lists. Randomisation was stratified by gender and the list was drawn up to 90 subjects for each stratum using the SAS PROC PLAN procedure (SAS Institute Cary, NC, USA). The randomisation list and unblinding list were produced by an external statistician, who was not otherwise involved in the trial. At screening, each subject was assigned a 4-digit screening number according to the chronological entry into the trial. If the subject was deemed suitable for trial participation, he/she received a randomisation number by a blinded trial staff after all baseline assessments were finalised at visit 3. Randomisation numbers included the stratification number and were allocated sequentially in the order in which the subjects finalised visit 3.

The test and reference products were produced by the sponsor to be similar in smell, taste, and appearance. All trial products were packaged in identical packs with identical labelling except for the randomisation number. Trial subjects, the clinical team, statisticians, and the sponsor were blinded during the entire trial until after database lock. Only one assigned person at Atlantia Food Clinical Trials, who was not otherwise involved in the conduct of the trial was un-blinded as necessary to perform the labelling of the product. An emergency un-blinding procedure using emergency code break envelopes was established to allow the investigator the option of disclosing the product assignment for an individual subject if clinical circumstances required such an un-blinding. No subjects were un-blinded throughout this trial. Cohort B subjects were not part of the randomisation.

Statistical analysis

A sample size of 57 completing participants in each arm was estimated to be sufficient based on a power calculation assuming 80% power, significance level of 5% (two-sided) performed on intervention effects on L/R ratio and corresponding standard deviations, -0.022 and 0.04 respectively observed in a previous, smaller trial (unpublished data).

Statistical analyses were predefined in the statistical analysis plan which were finalised and signed before unblinding. No imputation of data was carried out in case of missing data, but all available data was used. Participant characteristics and all efficacy data presented are based on the intention-to-treat population.

In general, statistical differences for outcome endpoints (L/R ratio, I-FABP and GSRS pain domain score and GSRS total score) were analysed as the dependent variable (difference between visit 5 and visit 3 or between visit 4 and visit 2) with linear mixed models that included baseline value (visit 3 or visit 2) and age as the covariate and gender as factors. In addition, analysis on the difference between (visit 5 – visit 4) and (visit 3 – visit 2) as dependent variable was done in a linear mixed model that included visit 2 as baseline value and age as covariate and gender as factor. Post-hoc analyses were done for cytokines and safety biomarkers by unadjusted Mann-Whitney U test for the difference between visit 5 and visit 3, visit 4 and visit 2 and delta-delta (visit 5-visit 3 minus visit 4-visit 2) as dependent variables for each parameter. A cross-sectional analysis of difference in small intestinal permeability measured at visit 2 by lactulose-rhamnose sugar test in urine between well-trained subjects (Cohort A) and non-trained subjects (Cohort B) as well as analysis of difference between visit 2 and 3 in Cohort A were analysed with a linear mixed model including age as covariate and gender as factor. Analysis of difference in serum cytokine biomarkers between visit 2 and visit 3 for Cohort A was done by Wilcoxon signed-rank test. For all data sets model check were assessed using Q-Q residual plots together with Kolmogorov-Smirnov test for normality. If datasets did not meet normal distributions and after logarithmic transformation of the variables

normal distribution was still not obtained appropriate non-parametric statistical methods were used.

3. Results

Subjects

Between March 16, 2017 and July 29, 2017, 188 subjects were screened for eligibility. Among the 153 enrolled subjects, 27 dropped out during run-in and thus 126 were randomly assigned to the intervention groups. For Cohort B 63 subjects were screened for eligibility and all were enrolled. See Figure 2 for Consort flow diagram.

In general, the Bif195 group and the placebo group were similar in terms of baseline parameters as shown in Table 1 including sex distribution, age, ethnicity, body mass index, blood pressure, alcohol consumption as well as weekly running hours, VO_2max and degree of exercise intensity. Baseline characteristics for Cohort B is also described in Table 1. Subjects from Cohort B were in general a few years younger than subjects from Cohort A and had a higher bodyweight (P<0.05). As expected, there were statistically significant differences between Cohort A and B in terms of weekly running hours, VO_2max and weekly hours of exercise (total) at medium and high intensity (P<0.0001).

A complete list of major protocol deviations is provided in Table 2. In total, 13 major protocol deviations were reported. Besides accountability, protocol deviations were exercise test not completed, lack of breakfast, the health of the participants and visits out of window. Accountability of trial product was high in both groups with no more than six subjects consuming <80% of expected capsules (four were from the Bif195 group and two were from the placebo group).

The Bif195 intervention was well-tolerated with few adverse events reported of which none were considered related to intake of trial product. A complete list of adverse events is provided in Table 3, with three in the Bif195 groups and two in the placebo group. Among the five adverse events reported, two were described as flu-like symptoms while the other three were described as exercise-related injuries.

The exercise challenge model

The effect of the exercise challenge to induce intestinal permeability as measured by L/R ratio before intervention for Cohort A is shown in Figure 3. The L/R ratio was ~100% higher post exercise (visit 3) as compared to resting L/R ratio (visit 2) (P<0.0001). There was no statistically significant difference (P=0.078) in intestinal permeability between well-trained subjects (Cohort A) and non-trained subjects (Cohort B) in resting L/R ratio at visit 2. The exercise challenge also resulted in a statistically significant increase in cytokine biomarkers (IFN-γ, IL-10, IL-6, IL-8 and TNF-α) from visit 2 to visit 3, see Supplementary Table S2.

Lactulose:rhamnose ratio, plasma I-FABP and GSRS questionnaire score

Results of urinary lactulose and rhamnose concentrations are shown in Supplementary Table S3. For the primary endpoint of this clinical trial, there was no statistical difference between Bif195 and placebo in small intestinal permeability after six-weeks oral supplementation as measured by change in urinary L/R ratio following a 1 h

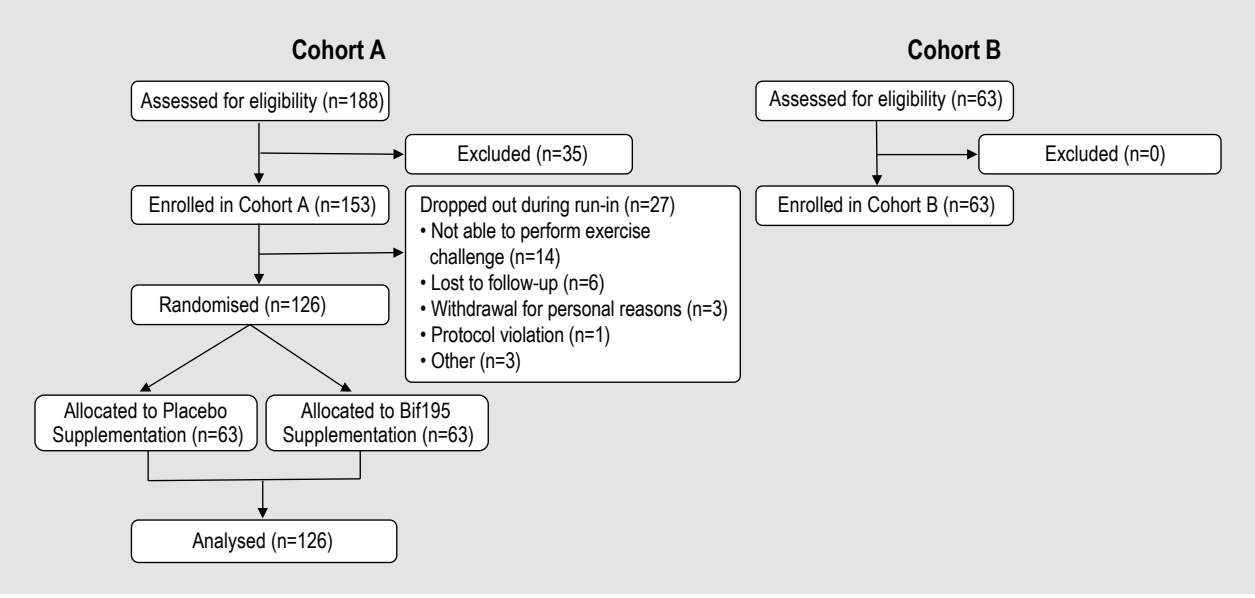

Figure 2. CONSORT flow diagram of the number of participants who were enrolled and randomised.

Table 1. Subject baseline characteristics of the analysis population.[1]

	Cohort A		Cohort A	Cohort B	*P*-value	
	Bif195	placebo	total	total	Bif195 vs Placebo	Cohort A vs Cohort B
n	63	63	126	63		
Age (years)	35.7±9.1	34.3±9.2	35.0±9.2	32.1±9.0	0.383	0.041
Gender (m/f)	41/22	40/23	81/45	39/24	0.853	0.749
Ethnicity (non-caucasians)	0	1	1	2	0.315	0.217
Height (cm)	173.7±13.5	174.3±9.4	174.0±11.6	173.8±9.1	0.764	0.908
Weight (kg) Visit 1	74.9±12.7	75.1±14.1	75.0±13.4	79.6±16.9	0.935	0.043
Body mass index (kg/m^2)	25.1±6.3	24.5±3.2	24.8±5.0	26.3±5.2	0.526	0.064
Blood pressure, systolic (mmHg)	123.8±10.9	125.0±10.6	124.4±10.7	123.1±13.1	0.547	0.487
Blood pressure, diastolic (mmHg)	75.2±7.1	76.3±9.2	75.7±8.2	75.9±9.6	0.428	0.892
Alcohol consumption (d/w)	5.1±4.4	5.3±4.3	5.2±4.3	5.6±4.6	0.811	0.681
Running (h weekly)	4.2±2.4	5.0±2.4	4.6±2.5	0.3±0.5	0.070	<0.0001
VO_2max (treadmill running ml O_2/kg/min)	46.9±6.8	47.1±6.9	47.0±6.9	37.8±1.9	0.888	<0.0001
Lactate ear prick test (mmol)	9.1±2.1	9.2±2.3	9.1±2.2	8.8±1.9	0.881	0.276
Exercise, light intensity (h/w)	1.0±1.5	0.8±1.4	0.9±1.4	0.7±0.7	0.321	0.243
Exercise, medium intensity (h/w)	3.8±3.4	4.1±3.1	3.9±3.2	0.7±1.0	0.603	<0.0001
Exercise, high intensity (h/w)	2.9±2.5	3.1±2.7	3.0±2.6	0.1±0.4	0.706	<0.0001

[1] Values are mean ± standard deviation unless noted otherwise. h/w = hours/week; d/w = drinks/week. Statistical differences were analysed with Chi-squared for categorical data and a Student's t-test for continues data.

Table 2. Major protocol deviations.[1]

	Bif195 n(%)/E	Placebo n(%)/E	Total n(%)/E
Number of participants	63 (100.0)	63 (100.0)	126 (100.0)
All deviations	6 (9.5)/7	6 (9.5)/6	12 (9.5)/13
Accountability	4 (6.3)/4	2 (3.2)/2	6 (4.8)/6
Exercise test not completed	1 (1.6)/1	2 (3.2)/2	3 (2.4)/3
No breakfast	1 (1.6)/1	0 (0.0)/0	1 (<1)/1
Not healthy	0 (0.0)/0	1 (1.6)/1	1 (<1)/1
Visit window	1 (1.6)/1	1 (1.6)/1	2 (1.6)/2

[1] n = number of subjects in the group having the deviation. Subjects can be counted in more than one group. E = number of deviations.

Table 3. Overview of trial adverse events after randomisation.[1]

Treatment	Bif195 (n)	Placebo (n)	Total (n)
Number of participants	3 (60%)	2 (40%)	5 (100%)
Cold and flu symptoms	1	0	1
Flu	1	0	1
Injured Achilles tendon when jogging	1	0	1
Ligament injury	0	1	1
Strained back	0	1	1

[1] n = number of subjects in the group having the event. All AE's were categorised as mild or moderate.

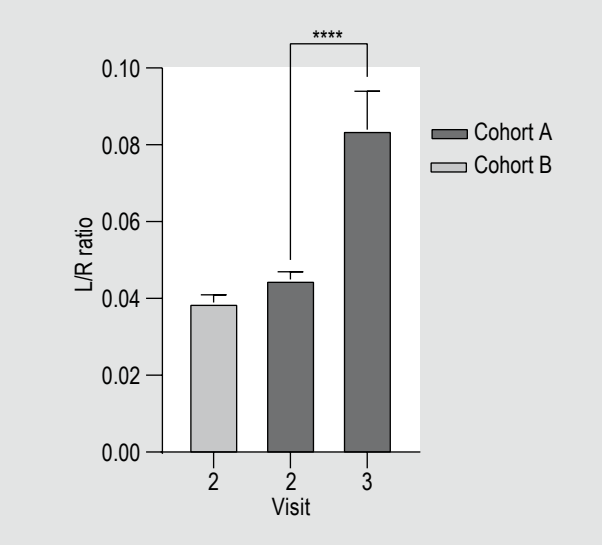

Figure 3. Resting L/R ratio for well-trained subjects (Cohort A) and non-trained subjects (Cohort B) and after exercise challenge on L/R ratio in Cohort A before Bif195 intervention.

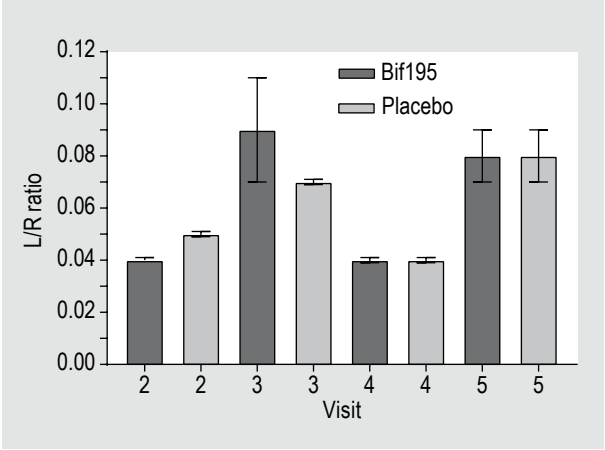

Figure 4. Urine L/R ratio at visit 2-5 in the Bif195 and the placebo groups.

exercise challenge from visit 3 to visit 5, see Figure 4. The room temperature was stable during the exercise challenge and statistical analyses of room temperature showed no differences between the groups at visit 3 and 5 (P=0.762 and P=0.774, respectively). Body weight was maintained during the trial and statistical analysis showed no difference between the groups in change in weight when comparing weight at visit 1, 3 and 5 (P=0.935, P=0.863 and P=0.893, respectively).

In addition, the trial did not show an effect of Bif195 on the secondary endpoint of a change in urinary L/R ratio from visit 2 to visit 4, after 5 weeks oral supplementation. The other secondary endpoints, plasma I-FABP and GSRS questionnaire score (Abdominal pain domain) as well as the exploratory end point of GSRS questionnaire score (Total) are listed in Table 5. There were no statistically significant differences between Bif195 and placebo for I-FABP or GSRS score measured as change from visit 2 to visit 4 and as change from visit 3 to visit 5. In addition, analysis of a difference in L/R ratio, I-FABP and GSRS scores (Abdominal Pain and Total) between the change from visit 4 to visit 5 and the change from visit 2 to visit 3 also showed no effect of Bif195.

Safety and cytokine biomarkers

Results of the safety biomarkers for liver and kidney, and cytokine biomarkers are shown in Supplementary Table S3 and S4, respectively. There were no statistically significant differences between Bif195 and placebo of the measured cytokines and liver and kidney biomarkers after adjustment for baseline or resting values (δV4-V2 – δV5-V3). For TNF-α there was a tendency for a statistically significant difference between the groups (P=0.051) where the concentration decreased with placebo and was unchanged in the Bif195 group. From visit 3 to visit 5 (after challenge) the concentration of CRP decreased statistically significantly more in the placebo group compared to the Bif195 group (P=0.013). From visit 3 to visit 5 the concentration of ALT was statistically significantly different between the two groups (P=0.006), with a decrease in concentration in the placebo group and an increase in the Bif195 group. For IL-12p70, IL-13, IL-1β, IL-2 and IL-4 between 49.6-90.6% of samples were below half-lower limit of detection (LLOD) and therefore no statistical analysis were done.

4. Discussion

In a model of exercise-induced intestinal permeability, we observed that six-weeks oral supplementation of *B. breve* Bif195 is safe, as we found no difference compared to placebo-controlled subjects in changes in intestinal permeability, intestinal integrity or GI symptoms in recreational runners as measured by L/R ratio, plasma I-FABP, the GSRS questionnaire score as well as safety and cytokine biomarkers. In addition, we found that no adverse events reported were related to intake of Bif195 which were in agreement with another Bif195 clinical trial (Mortensen *et al.*, 2019). The adverse events were equally divided between placebo and Bif195, with two and three adverse events, respectively. The low incidence of GI related AEs, such as reflux and diarrhoea, agrees with the fact that we found no difference in change in GSRS score endpoints between the Bif195 group and placebo group after six-weeks intervention. Accordingly, the results indicate that consumption of Bif195 at 50×10^9 cfu/day by healthy people for six weeks is safe and well-tolerated.

The fact that acute exercise induce a significant increase in the urinary disaccharide/monosaccharide ratio as well as I-FABP is well documented (Chantler *et al.*, 2021).

Treadmill running, at a velocity of 80% of VO_2max (Ribeiro *et al.*, 2021) can be regarded as a human challenge model capable of inducing reversible intestinal tissue damage, where the actual structure of the cellular tight junction is altered by hypoxia and/or temperature increase around the intestines. Intestinal ischemia and/or reperfusion can cause increase in oxidative stress through the production of e.g. hydrogen peroxide, which results in increased intestinal permeability through influence on the tight junction proteins, which are necessary for forming the para-cellular barrier in the intestinal epithelium (Zuhl *et al.*, 2014) and to prevent infiltration of pathogenic organisms into the systemic circulation (Costa *et al.*, 2017). The purpose of the exercise challenge model in this trial was to perform a safety evaluation of Bif195 by inducing a non-pharmacologically increased permeability in humans. The population in Cohort A was able to complete a 1 h running challenge on a treadmill at a velocity of 80% of their individual VO_2max. The exercise model succeeded as a model for a specific alteration of the intestinal tissue that led to a statistically significant increase in small intestinal permeability measured by L/R-ratio and cytokine biomarkers before and after a 1 h exercise challenge. In order to validate baseline values of such a 'recreational exercise' population, and to address the possibility that their lifestyle may not be comparable with the normal population, we included Cohort B. With Cohort B we established baseline of a less active, healthy population in terms of small intestinal permeability. Besides the ability to complete the running challenge in Cohort A, the characteristics of the two cohorts were similar. There was a statistically non-significant tendency for a higher resting L/R ratio in Cohort A compared to Cohort B. Baseline characteristics describes Cohort A as more physically active. Although acute exercise is known to increase intestinal permeability (Chantler *et al.*, 2021), weight loss studies and studies including pre-diabetics examining the effect of chronic exercise on gut barrier integrity at rest indicate that chronic exercise may improve the gut barrier integrity in physically active people at rest (Pasini *et al.*, 2019). However, studies done in weight stable and healthy subjects are lacking (Keirns *et al.*, 2020). Still, the comparison of Cohort A and Cohort B from this trial might indicate a difference in resting permeability. Due to the free-living design of the study the level of rest prior to the resting L/R ratio test is not fully controlled, which might partly explain the difference in resting L/R ratio.

Cytokines, liver, and kidney biomarkers remained unaffected by the six-week intervention with Bif195, as there were no statistically significant differences in concentration on any markers compared to placebo after adjustment for baseline or resting values. As cytokines and liver and kidney biomarkers were considered safety markers and post-hoc secondary endpoints no corrections for multiplicity were done. Nevertheless, statistical analysis indicated a tendency for the TNF-α concentration to be different between the groups; however, visual inspection of means indicated this was a result of a decrease in concentration in the placebo groups, whereas the concentrations in the Bif195 group remained stable during the intervention. Concentration of CRP decreased significantly more in the placebo group compared to the Bif195 group from visit 3 to visit 5 (after exercise challenge); however, pre-intervention concentrations (visit 3) in the two groups indicated that the CRP concentration in the placebo group were higher compared to the Bif195 group and this might be the reason for the difference between the groups. Visual inspection of IFN-γ concentration means indicated that the Bif195 group responded to the exercise challenge with a higher increase in concentration from visit 4 to visit 5 compared with placebo. However, the means does not indicate an effect of the intervention as the same response was observed before the Bif195 intervention (visit 2 to visit 3). All liver and kidney biomarkers were within the normal range throughout the trial. There was a statistically significant difference between the groups in effect of the intervention on ALT concentration, which decreased in the placebo group and increased in the Bif195 group from visit 3 to visit 5. Still, there was no difference when resting baseline (visit 2 and visit 4) values were included in the analysis. Therefore, we consider the difference in placebo as an effect of baseline and not an effect of the intervention. In general, there were no indications of that these differences in cytokines, liver and kidney biomarkers were related to intake of Bif195.

Thus far, Bif195 has been tested in two different clinical settings, the first being an aspirin-induced challenge model (Mortensen *et al.*, 2019) and the second being an exercise-induced intestinal permeability challenge model described herein. This strenuous physical exercise model represents a transient challenge inducing intestinal permeability, likely through pericellular passage of luminal molecules, which do not include ulcer formation and is thereby differentiated from the NSAID challenge model. NSAID induced intestinal ulcers, are at least in part caused by COX1/2 inhibition, followed by reduction of downstream products including prostaglandin and other eicosanoids (Bjarnason *et al.*, 2018). We have shown that NSAID induced ulceration was counteracted by Bif195, but that this do not include modulation of eicosanoid production. Nor have we observed any major change in the microbiome composition, save presences of the species itself (Mortensen *et al.*, 2019). Whereas it is possible that immunological components are changed in the microenvironment including the mucus layer and epithelial tissue, we did not observe any changes in the present study in the serum biomarker panel. It could be speculated, that reversal of NSAID associated ulceration by Bif195, may include stimulation of epithelial growth and regeneration of gastrointestinal tissue. However, clinical studies designed for such evaluation, remain to be conducted.

Comparison of the results from the two studies indicate a regenerative and protective effect of supplementation with Bif195 compared to placebo in the NSAID-induced model of GI enteropathy known to induce small intestinal damage leading to inflammation, erosions and ulcers (Bjarnason *et al.*, 2018), whereas the results show that supplementation of Bif195 in the setting of exercise-induced increased permeability is safe with no evidence of difference from placebo-controlled subjects.

In conclusion, the results of the present study show no effect of six-weeks daily, oral supplementation with Bif195 on resting and/or exercise-induced intestinal permeability, intestinal integrity, or GI symptoms in a recreational exercise population. In addition, the results of the RCT show that Bif195 compared to placebo did not change safety biomarkers and cytokines and that no adverse events with intake at 50×10^9 cfu/day for six weeks were reported. Accordingly, we conclude that the Bif195 strain is well tolerated and safe.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2021.0173.

Supplementary Material S1. Strain safety assessment of Bif195.

Table S2. The effect of the exercise challenge on serum cytokine biomarkers before (visit 2) and after (visit 3) the exercise challenge in Cohort A.

Table S3. Urine L/R ratio, plasma I-FABP and GI score at visit 2-5 and the difference between the Bif195 and the placebo groups after six weeks intervention as measured resting (five weeks), after a running challenge and after a resting adjusted running challenge.

Table S4. Liver and kidney serum safety biomarkers at visit 2-5 and the difference between Bif195 and placebo groups after six weeks intervention as measured resting (five weeks) and after a running challenge and after a resting adjusted challenge.

Table S5. Serum cytokine biomarkers at visit 2-5 and the difference between Bif195 and placebo groups after six weeks intervention as measured resting (five weeks), after a running challenge and after a resting adjusted running challenge.

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Conflict of interest

SE, BM, AW, NV, CS and AD are employed by the sponsor Chr. Hansen. The other authors declare no conflict of interest.

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