Article:
Pinto-Sanchez, MI, Hall, GB, Ghajar, K et al. (2017) Probiotic Bifidobacterium longum NCC3001 Reduces Depression Scores and Alters Brain Activity: a Pilot Study in Patients With Irritable Bowel Syndrome. Gastroenterology, 153 (2). 448-459.e8. ISSN: 0016-5085

https://doi.org/10.1053/j.gastro.2017.05.003

Accepted Manuscript

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Maria Ines Pinto-Sanchez, MD, Geoffrey B. Hall, PhD, Kathy Ghajar, BSc, Andrea Nardelli, MD, Carolina Bolino, MD, Jennifer T. Lau, BSc, Francois-Pierre Martin, PhD, Ornella Cominetti, PhD, Christopher Welsh, BSc, Amber Rieder, BA, Jenna Traynor, BSc, Caitlin Gregory, MD, Giada De Palma, PhD, Marc Pigrau, MD, Alexander C. Ford, MD, Joseph Macri, PhD, Bernard Berner, PhD, Gabriela Bergonzelli, PhD, Michael G. Surette, PhD, Stephen M. Collins, MD, Paul Moayyedi, MD, Premysl Bercik, MD.

PII: S0016-5085(17)35557-9
DOI: 10.1053/j.gastro.2017.05.003
Reference: YGAST 61167

To appear in: *Gastroenterology*
Accepted Date: 2 May 2017

Please cite this article as: Pinto-Sanchez MI, Hall GB, Ghajar K, Nardelli A, Bolino C, Lau JT, Martin F-P, Cominetti O, Welsh C, Rieder A, Traynor J, Gregory C, De Palma G, Pigrau M, Ford AC, Macri J, Berner B, Bergonzelli G, Surette MG, Collins SM, Moayyedi P, Bercik P, Probiotic *Bifidobacterium longum* NCC3001 Reduces Depression Scores and Alters Brain Activity: a Pilot Study in Patients With Irritable Bowel Syndrome, *Gastroenterology* (2017), doi: 10.1053/j.gastro.2017.05.003.

Probiotic *Bifidobacterium longum* NCC3001 Reduces Depression Scores and Alters Brain Activity: a Pilot Study in Patients With Irritable Bowel Syndrome.

Maria Ines Pinto-Sanchez[1] MD, Geoffrey B Hall[2] PhD, Kathy Ghajar[2] BSc, Andrea Nardelli[1] MD, Carolina Bolino[1] MD, Jennifer T Lau[1] BSc, Francois-Pierre Martin[3] PhD, Ornella Cominetti[3] PhD, Christopher Welsh[1] BSc, Amber Rieder[2] BA, Jenna Traynor[2] BSc, Caitlin Gregory[2] MD, Giada De Palma[1] PhD, Marc Pigrau[1] MD, Alexander C Ford[4] MD, Joseph Macri[5] PhD, Bernard Berner[6] PhD, Gabriela Bergonzelli[6] PhD, Michael G. Surette[1] PhD, Stephen M Collins[1] MD, Paul Moayyedi[1] MD, Premysl Bercik[1] MD.

[1]Department of Medicine, Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, ON, Canada

[2]Department of Psychology, Neuroscience & Behavior, McMaster University, Hamilton, ON, Canada.

[3]Nestlé Institute of Health Sciences SA, Lausanne, Switzerland

[4]Leeds Gastroenterology Institute, St. James's University Hospital, & Leeds Institute of Biomedical and Clinical Sciences, University of Leeds, Leeds, UK.

[5]Department of Pathology, McMaster University, Hamilton, ON, Canada.

[6]Nestlé Research Center, Nutrition Institute, Lausanne, Switzerland.

Short Title: *B. longum* decreases depression in IBS patients

Funding source: Nestlé SA, Switzerland,

Corresponding author: Premysl Bercik, MD

Department of Medicine, Farncombe Family Digestive Health Research Institute,

McMaster University, Hamilton, ON, Canada

e-mail: bercikp@mcmaster.ca

CONFLICT OF INTEREST: MIPS, AN, GH, ACF, KG, AR, JT, CW, GDP, MP, JM, CG, CB, JL, MS and PM have no conflicts of interest concerning this paper. GB, and BB are employees of Nestec SA, Switzerland. MFP and OC are employees of Nestlé Institute of Health Sciences SA. PB and SMC received research support from Nestlé.

DISCLOSURE: MIPS received a CIHR-CAG Health Professional Fellowship Award. PB is recipient of HHS Early Career Research Award and holder of Richard Hunt-AstraZeneca Chair in Gastroenterology.

MANUSCRIPT CONTRIBUTION

MIPS: acquisition, analysis and interpretation of data; statistical analysis, writing of the manuscript; *GH*: study concept and design; acquisition, analysis and interpretation of fMRI data; *KG AR, JT, CG:* fMRI data acquisition, analysis and interpretation; *AN:* clinical data acquisition, *CB*: study concept and design; *CW:* database development and data acquisition; *JL, MS:* microbiota analysis, critical review of manuscript; *FPM, OC*: NMR metabolomic analysis and data interpretation, *BB:* microbiota analysis, critical revision of the manuscript for important intellectual content, *GB:* study design, critical revision of the manuscript for important intellectual content: *AF, SMC:* critical review of the manuscript and important intellectual content; *PM*: Study design, statistical analysis, critical revision of the manuscript for important intellectual content; *PB:* Study design, critical revision of the manuscript and supervision of the study. All authors reviewed and approved the last version of the manuscript.

Acknowledgments:

The authors thank Peter McLean and Mirna Del Valle for their invaluable support and study monitoring and oversight. We also thank Tiago Nunes for thoughtful comments on the manuscript, Mireille Goillard for neurotransmitter analysis, Francis Foata for the detection of *BL* and Margaret Fahnestock for advice on BDNF analysis and Laeticia Da Silva and Dr. Sebastiano Collino for their contribution in metabonomics data generation.

ABSTRACT

Background & Aims: Probiotics can reduce symptoms of irritable bowel syndrome (IBS), but little is known about their effects on psychiatric comorbidities. We performed a prospective study to evaluate the effects of *Bifidobacterium longum* NCC3001 (*BL*) on anxiety and depression in patients with IBS.

Methods: We performed a randomized, double-blind, placebo-controlled study of 44 adults with IBS and diarrhea or a mixed-stool pattern (based on Rome III criteria) and mild to moderate anxiety and/or depression (based on the Hospital Anxiety and Depression scale) at McMaster University in Canada, from March 2011 to May 2014. At the screening visit, clinical history and symptoms were assessed and blood samples were collected. Patients were then randomly assigned to groups and given daily *BL* (n=22) or placebo (n=22) for 6 weeks. At week 0, 6 and 10, we determined patients' levels of anxiety and depression, IBS symptoms, quality of life, and somatization using validated questionnaires. At week 0 and 6, stool, urine and blood samples were collected, and functional magnetic resonance imaging (fMRI) test was performed. We assessed brain activation patterns, fecal microbiota, urine metabolome profiles, serum markers of inflammation, neurotransmitters and neurotrophin levels.

Results: At week 6, 14/22 patients in the *BL* group had reduction in depression scores of 2 points or more on the Hospital Anxiety and Depression scale, vs 7/22 patients in the placebo group (P=.04). *BL* had no significant effect on anxiety or IBS symptoms. Patients in the *BL* group had a mean increase in quality of life score compared with the placebo group. The fMRI analysis showed that *BL* reduced responses to negative emotional stimuli in multiple brain areas, including amygdala and fronto–limbic regions, compared with placebo. The groups had similar fecal microbiota profiles, serum markers of inflammation, and levels of neurotrophins and neurotransmitters, but the *BL*

group had reduced urine levels of methylamines and aromatic amino acids metabolites. At week 10, depression scores were reduced in patients given *BL* vs placebo.

Conclusion: In a placebo-controlled trial, we found that the probiotic *BL* reduces depression but not anxiety scores and increases quality of life in patients with IBS. These improvements were associated with changes in brain activation patterns that indicate that this probiotic reduces limbic reactivity. ClinicalTrials.gov no. NCT01276626.

Key words: IBS, anxiety, depression, fMRI

BACKGROUND

Irritable bowel syndrome (IBS), characterized by abdominal pain and altered bowel habits, affects 11% of the world-wide population[1], has a significant socioeconomic impact[2] and its current treatments have limited efficacy[1]. Its pathophysiology is incompletely understood but is considered to be a disorder of gut-brain interaction[3] and is frequently accompanied by psychiatric disorders[1,4].

Accumulating evidence suggests that commensal bacteria play a role in IBS, as multiple studies have demonstrated an abnormal composition or metabolic activity of gut microbiota in patients with IBS[5]. Dysbiosis, triggered by acute bacterial gastroenteritis, antibiotics or dietary factors, which are known to affect the composition of microbiota, may drive not only the gastrointestinal component of IBS but also contribute to its psychiatric comorbidity[6]. Furthermore, specific probiotic bacteria have been shown to improve gastrointestinal symptoms in IBS[7].

We have previously demonstrated that administration of *B. longum* NCC3001 *subspecies longum* strain (*BL*) normalized anxiety-like behavior and hippocampal Brain Derived Neurotrophic Factor (BDNF) levels in mice with low-grade gut inflammation, through vagal dependent pathways[8,9]. Based on these results, we hypothesized that *BL* will improve psychiatric comorbidity in patients with chronic bowel disorders and thus we performed a pilot study in IBS patients. As anxiety and depression are rather difficult to distinguish in animal models, they frequently co-exist in patients and altered central BDNF levels were reported in both conditions, we chose as our primary objective to evaluate the impact of *BL* on co-morbid anxiety and depression. The secondary objectives were then to assess the effect of *BL* on IBS symptoms and quality of life, and to explore changes in brain activation patterns, circulating inflammatory markers, neurotransmitters, neurotrophins, gut microbiota profile and urine metabolites as a measure of host-microbial

metabolic interactions. Considering the large heterogeneity of IBS we decided to restrict our study to IBS patients with diarrhea or mixed stool pattern, as they apper to share similar sensory neuro-imune interaction and are more likely to present with low-grade gut inflammation and similar microbiota compared with IBS patients with constipation[10,5].

Although several clinical studies investigated effects of probiotic bacteria on behavior and brain function[11-12], mostly in healthy individuals, our study is the first one to show that probiotics can improve depression scores as well as alter brain activity patterns in IBS patients with comorbid depression and anxiety.

METHODS

Study oversight

We conducted a randomized, double-blind, placebo-controlled, single center pilot study from March 2011 to May 2014. The study was approved by the Hamilton Health Sciences and St. Joseph's Health Care Research Ethics Boards, all participants signed the informed consent. The study was registered in clinicaltrials.gov under NCT01276626. All authors had access to the study data and reviewed and approved the final manuscript.

Participants

We recruited adult patients with a diagnosis of IBS with diarrhea or mixed-stool pattern (Rome III criteria)[13], and mild to moderate anxiety and/or depression scores based on the Hospital Anxiety and Depression (HAD) scale[14] (HAD-A or HAD-D score 8-14). Patients with a history of organic diseases, immune deficiency, major abdominal surgery, a psychiatric condition other than anxiety or depression, use of immunosuppressants, glucocorticosteroids, opioids, antidepressants or anxiolytics in regular doses, alcohol or illicit drug consumption, were excluded. Loperamide and laxatives were allowed as rescue medications. Other probiotics in any form were forbidden during the 1-month run-in period and the trial. Antibiotics were forbidden during the 3 months prior to the run-in period and the trial.

Design of the study

The study involved four hospital visits (Supplementary figure 1). At the screening visit (-4 weeks), clinical history and symptoms were assessed and physical exam and complete bloodwork performed. At the baseline visit (week 0), the inclusion and exclusion criteria and symptoms were re-assessed, stool, urine and blood samples were collected, and an fMRI study performed.

The patients were then randomised to receive 42 sachets of either spray dried *BL* (1.0E+10 CFU /1gram powder with maltodextrin) or placebo containing 1 gram of maltodextrin. Treatment products were indistinguishable in terms of package, color, taste and consistency. Patients were instructed to dissolve the content of the sachet in 100-200 ml of lactose-free milk, soy milk or rice milk, preheated to 20° Celsius. Patients were asked not to change their eating habits or fibre intake. Participants recorded the treatment intake, the empty sachets were used to assess the compliance at the next visit (week 6), where their symptoms were assessed, blood, urine and stool samples collected and fMRI test performed. Finally, patients' symptoms were re-assessed at a follow-up visit (week 10).

In addition to the regular hospital visits, HAD scores were also assessed at 3 weeks of treatment following request of Health Canada. HAD questionnaires were provided to patients at Visit 1 and then mailed or e-mailed to the investigators.

Study endpoints

The primary endpoint was a reduction in anxiety and/or depression scores of ≥2 points on HAD scale[13] at 6 weeks. This was based on the previously established mean clinically important difference for the anxiety and depression score on the HAD scale of 1.3 and 1.4, respectively[14]. Secondary endpoints included improvement in anxiety and depression scores (HAD, continuous data), anxiety (State-Trait Anxiety Inventory, STAI), IBS global adequate relief, IBS symptoms, somatization, quality of life, changes in brain activation patterns (functional Magnetic Resonance Imaging, fMRI), serum inflammatory markers, neurotransmitters and BDNF, and urine metabonomic and stool microbiota profiles.

Randomization

The randomization sequence was performed using a computer program (Proc Plan, SAS, V. 9.1). A block randomization was stratified by gender and IBS status (diarrhea or mixed stool pattern). The codes were kept in sealed opaque envelopes allocated to patients according to strata. Each pack was assigned a number according to the randomization sequence. On recruitment, the patients were assigned into one of four strata and given the next consecutive randomization number available for that stratum. Treatment allocation was concealed from participants and study staff.

Treatment products indistinguishable in terms of package, color, taste and consistency, were identified with two non-speaking codes per arm. Their identity was blind to subjects, investigators and support staff, known only by the manufacturer, Nestlé Product Technology Centre Konolfingen Switzerland.

Study Measurements

Anxiety and depression were assessed by the HAD score. As an additional measure of anxiety we used the STAI[15,] which assesses both state and trait anxiety. IBS symptoms and signs were assessed by the Birmingham IBS score[16] and Bristol stool scale[17]. To evaluate an overall improvement of IBS symptoms, patients were asked a validated question: "Over the past week have you had adequate relief of your IBS symptoms?" with a dichotomous option for responses[18]. Health-related quality of life (QoL) was measured by the SF-36[19] and somatization by the PHQ-15 questionnaires[20].

Brain activity was assessed by functional magnetic resonance imaging (fMRI) using General Electric 3-Tesla Discovery MR 750, whole body short bore scanner with 32 parallel receiver channels (General Electric, Milwaukee, WI). The 1-hour protocol included a seven minute T1 weighted structural scan, followed by four repetitions of a fearful face backward masking

paradigm[21] (Supplementary figure 2) during four fMRI Blood Oxygen Level Dependent scans[22] (BOLD EPI; TR/TE=2800/35 ms, flip angle=90º, 3 mm thick slices, no gap, field of view=24 cm, matrix=64x64). Pre-processing of MRI data was completed using Brain Voyager QX Version 2.8.2, 32-bit (Brain Innovation, Maastricht, Netherlands). Anatomic and functional data were inspected and scans with artefacts or fMRI scans with movement greater than 5 mm in any of 6 planes were excluded from analysis. Anatomical scans were transformed into standard sagittal orientation, and underwent spatial normalization into standard Talaraich space. Slice scan time correction and 3D motion correction were carried out on the fMRI data and spatial smoothing applied using a Gaussian filter (FWHM=6 mm). The amygdala was selected as region of interest (ROI), initially derived from the WFUPick Atlas and refined according to anatomic landmarks on the full group average transformed T1 image.

Blood and urine samples were collected after an overnight fast. After processing, the samples were stored at -80 C until assessment. Samples for BDNF were collected using the PAXgene Blood RNA (PreAnalytiX, Qiagen BD, Toronto, Canada). Serum cytokines and CRP levels were assessed by Human ProInflammatory 7-Plex Ultra-Sensitive Kit (MSD, Gaithersburg, MD) and CRP Abbott Architect kit (Abbott Laboratories, IL), respectively. BDNF protein level was assessed by Human BDNF DuoSet ELISA kit (R&D Systems, Minneapolis, MN). Plasma neurotransmitters were quantified using following kits: 5-HT: IBL, Hamburg, Germany; Substance P: Abcam, Cambridge, UK; CGRP: Cloud-Clone Corp, Houston, TX.

Urine metabolites were assessed by ^{1}H NMR profiling using a Bruker Avance II 600 MHz spectrometer equipped with a 1.7 mm probe at 300 K (Bruker Biospin, Rheinstetten, Germany), using a standard pulse sequence with water suppression, and processed using TOPSPIN (version 2.1, Bruker, Germany) software package. The metabolite identification was achieved using in house database and 2D ^{1}H NMR spectroscopy experiments. Chemometric analysis was performed using

the software package SIMCA-P+ (version 14.0, Umetrics AB, Umeå, Sweden) and in-house developed MATLAB routines. Orthogonal Projection to Latent Structures (OPLS) and OPLS discriminant analysis (OPLS-DA) were employed for exploring the variance in the metabonomics data that may explain statistical differences between groups of samples. The classification accuracy of the OPLS-DA was established from the predicted samples in the 7-fold cross-validation cycle. To highlight the weight of individual variables in the model, Variable Importance in Projection (VIP) was used, with a value above 1 used as a threshold by convention. For additional details, see Supplementary Methods.

Microbiota analysis was performed using Illumina sequencing of the V3 region of 16S rRNA gene as described previously[23], for details see Supplementary Methods. Bacterial strain-specific PCR[24] was used on fecal DNA extracts to detect the presence of *BL* at the end of the treatment period.

Statistical analysis

Statistical analyses were performed using IBM-SPSS (IBM-SPSS Statistics v20, Chicago, IL). We performed *post-hoc* power calculations based on our previous animal data, which showed strong therapeutic potential of this probiotic[8-9]. We estimated that a sample size of 19 in each group would have 80% power using a two-group $\chi 2$ test with 0.05 two-sided significance level assuming 30% have an improvement in depression and/or anxiety in the placebo group and 75% in the *B. longum* group.

Data from all randomized subjects were analyzed according to intention to treat (ITT) principles for the primary outcome. To deal with missing data, we used the extreme case analysis assuming that all missing subjects had no improvement in symptoms. Per protocol evaluation (PP) excluded data from subjects who did not complete the trial due to consumption of proscribed medication or non-

compliance with the study protocol, and was used for the primary and secondary outcomes. For testing the effects on the two primary endpoints, Pearson Chi-Squared and Mann-Whitney U test were used as appropriate. In addition, the HAD scores were analyzed at baseline, week 3, 6 and 10 post-treatment using ANOVA repeated measures. ANCOVA was used to adjust for baselines differences in HAD depression scores. A two-sided test was used and $p<0.05$ was considered statistically significant.

fMRI data were analyzed by the general linear model (GLM) with experimental events convolved with the hemodynamic response function. Activation maps were constructed identifying clusters of activity associated with peak differences in activation for experimental conditions (fear, happy, fixation)[21]. BOLD signal contrasts were submitted to random-effect analyses and corrected for multiple comparisons using the False Discovery Rate approach[25]. Second-level random effects-GLM analyses assessed differences in-group responses to the fearful faces before and after treatment. *A priori* predictions that *BL* will modulate activation in the amygdala were tested with region of interest (ROI) analysis. A standard Brodmann map (WFU Pic Atlas)[26] was co-registered to the average composite anatomic data set and used to prescribe Regions of Interest (ROI) in the right and left amygdala. An event related deconvolution model for each participant was used to examine % BOLD signal change at each and every voxel within the ROI. Contrasts were corrected for multiple-comparisons at the cluster level using the false discovery rate methodology (FDR(q)<.05)[25] and the average statistical value for ROI reported.

For metabonomic analaysis, representative signals of the identified metabolites were integrated and tested using non-parametric Mann Whitney test.

Role of the Funding source

Nestec SA was not involved in collection, analysis or interpretation of the clinical data. The corresponding author was in charge of collection and analysis of data and had final responsibility for the decision to submit for publication.

RESULTS

Study patients

Sixty patients were enrolled in the study (Figure 1). Sixteen patients failed the screening due to: 1) very mild IBS symptoms (n=2) or low HAD score (n=1); 2) use of antibiotics (n=2); 3) unwillingness to follow the protocol (n=7), or 5) loss to follow-up (n=4). Thus, 44 patients were randomized (22 in each study arm), from whom 38 completed the study (*BL*=18, placebo=20). During the treatment, six patients dropped out due to use of antibiotics (n=4), or antidepressants (n=2). No differences were observed between the groups in baseline characteristics (Table 1), except for higher HAD-D scores in the *BL* group (Table 2).

Primary outcome: Improvement in depression and/or anxiety scores

At 6 weeks, 14 out of 22 (64%) patients in the *BL* group had decreased depression scores (HAD-D ≥2 points) compared with 7 out of 22 (32%) patients given placebo (relative risk (RR) 1.98; 95% confidence interval (CI) 1.16-3.38; p=0.04) in the ITT population (Table 3A). This response was more prominent in the PP analysis with 78% in the *BL* group compared with 35% of the placebo group having lower depression scores (RR 2.4; 95% CI 1.26-4.58; p=0.016). The improvement in HAD-D scores was sustained in both the ITT (RR 2.05; 95% CI 1.07-3.93; p=0.04) and the PP analysis (RR 2.14; 95% CI 1.11-4.12; p=0.04) at 10-week follow-up. The results at 6 weeks were similar when the analysis was performed in only the subgroup of patients with baseline scores indicative of depression (HAD-D ≥8), (RR 3.75; 95% CI 0.6-22.1; p=0.047). No significant differences in number of patients with decreased anxiety (HAD-A ≥2 points) were found between the groups at 6 or 10-week follow-up.

A sensitivity analysis performed to explore the relationship between depression scores and gastrointestinal symptoms showed that the beneficial effect of *BL* on depression scores at 6 and 10

weeks was more likely to occur in those patients who reported adequate relief of IBS symptoms (Table 3B).

Secondary outcomes

HAD scores

After treatment, there were no significant differences on HAD-D scores when assessed as a continuous outcome between the two groups (Table 2), but when adjusting for baseline differences a greater improvement was found in the *BL* group (ANCOVA p=0.049). Anxiety scores assessed as a continuous outcome were similar between the two groups (Table 2).

IBS symptoms

There was no difference in adequate relief of IBS symptoms in the ITT analysis at 6 weeks (RR 1.6, 95% CI 0.86-2.91; p=0.22) but the PP analysis showed a statistically significant benefit of *BL* over placebo (RR 2.03 95% CI 1.13-3.65; p=0.02). This beneficial effect was not, however, maintained at 10-week follow-up (RR 0.70, 95% CI 0.24-2.09; p=0.52). No significant differences were observed in the overall Birmingham score or sub scores for constipation, diarrhea or pain at 6 or 10 weeks (Table 2).

State and trait anxiety and somatization

No differences in STAI scores were observed between groups at 6-week or 10-week follow-up (Table 2). Somatization scores were also similar between groups at 6-week or 10-week follow up (Table 2).

Quality of life

QoL improved in the physical subdomain in the *BL* group compared with placebo (p=0.03; 95% CI 0.01-0.90) (Table 2), with amelioration in general physical health (physical functioning; p=0.04;

95% CI 0.43-0.51) and problems with work or other daily activities (role physical; p=0.01; 95% CI 0.009-0.013).

Brain activation patterns by fMRI

Brain activation patterns differed in response to fearful versus happy faces in all subjects studied (Supplementary Figure 3, Supplementary Table 1). Before treatment, there was no difference in response to fear stimuli versus fixation between placebo and the *BL* group, except for greater engagement of the visual association and parietal cortices in the latter group (Figure 2A-B). However, after treatment, compared with placebo, the *BL* group showed reduced engagement of the amygdala, frontal and temporal cortices, as well as heightened engagement of occipital regions in response to the fear stimuli (compared with fixation) (Figure 2B-C, Table 4). Overall, the change in engagement of the amygdala correlated with the change in depression scores (r=0.52, p=0.004; Figure 2E). Within the *BL* group, reduced engagement of the amygdala correlated with decreased depression scores (r=0.58, p=0.03), but this was not observed in the placebo group (r=0.20, p=0.46). In the *BL* group, reduced engagement of the amygdala was more likely to occur in patients with adequate relief of IBS symptoms than in those without it (RR 3.07 95% CI 0.89-10.59; p=0.03), but this was not observed in the control group (RR 1.5 95% CI 1.00-2.23; p=0.51; Figure 2E).

There was no difference when comparing response to fearful versus neutral faces.

Inflammatory markers, neurotransmitters and BDNF levels

No differences in serum inflammatory markers (CRP, TNF-α, IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL12 and IL-10/12 ratio) or neurotransmitters (5-HT, substance P and CGRP) were found between the groups at 6 weeks (Supplementary table 2). Similarly, no changes in blood BDNF mRNA or BDNF protein levels were identified.

Intestinal microbiota composition

There were no major differences in taxa, compositional distance or alpha diversity (Shannon, Chao1 and Observed Species indices) before or after the treatment between the two groups. Microbiota profiles were also similar when assessed by Bray-Curtis Principle Coordinate analysis or Bray-Curtis Distance comparisons (Supplementary Figure 4). *BL* was detected at the end of treatment in 15 out of 18 (80%) patients of the probiotic group.

Urine metabonomics

OPLS discriminant analysis was applied using one predictive and two orthogonal components to model urine metabolic differences between the two groups (Supplementary Figure 5). The model was statistically robust only for post-treatment analysis (R2X=0.17, R2Y=0.84, Q2Y=0.20, where R2X: explained variance in the metabonomics data (urine metabolites), R2Y: explained group variance (placebo and probiotic) and Q2Y: robustness of the model). Before treatment, there was no difference between the two groups (Q2Y<0). After treatment, however, the *BL* group showed a lower urinary excretion of phenylacetyglutamine (PAG), creatine, 4-cresol sulfate and trimethylamine-N-oxide (TMAO) (Supplementary Figure 5, Table 6). Levels of 4-cresol sulfate after treatment correlated with depression scores in the *BL* group (r=-0.53, p=0.03) but not in the control group. No other correlation was found between the metabolites and depression scores or the amygdala activity.

Adverse events

No serious adverse events, with a probable or certain relationship to the study product, were noted (Supplementary table 3).

DISCUSSION

In this randomized, placebo controlled study we found that 6-week administration of *Bifidobacterium longum* NCC3001 *(BL)* decreased depression but not anxiety scores, which was our primary outcome, and decreased responses to fearful stimuli in multiple brain areas involved in the processing of emotions, including the amygdala and fronto–limbic regions. Patients given *BL* also reported improvement in overall symptoms of IBS and physical domain of QoL. Despite being a pilot study with limited subject numbers, this is the first trial to show that a specific probiotic improves depression scores in IBS patients and induces pronounced changes in brain activity in regions that have been previously implicated in depression, and that are influenced by anti-depressant therapy[27,28]. Indeed, both depression and anxiety disorders have been associated with amygdala hyperactivity and converging evidence suggests that one mechanism by which Selective Serotonin Re-uptake Inhibitors (SSRIs) exert their beneficial action is by downregulating its activity[27,28]. Our study also validates the use of some murine models[8,9] to screen probiotics for their potential therapeutic benefit in humans, as *BL* was shown previously to improve anxiety-like behavior and brain chemistry in mice.

There is growing interest in the role of the intestinal microbiota in health and disease. Gut bacteria not only instruct and shape the host immune system, and impact its metabolism, but also affect function in the gut and central nervous system[29,30]. Animal studies have demonstrated that changes in microbiota composition[31] or administration of specific probiotics, including bifidobacteria, can alter behavior and brain chemistry of the host[8,9,32]. Multiple mechanisms are likely involved in this microbiota-brain communication, including immune, neural and metabolic pathways[30]. We have previously extensively investigated the beneficial effect of *BL* on behavior in murine models and shown that this probiotic normalizes inflammation-induced anxiety-like behavior and hippocampal

BDNF levels[8,9]. The beneficial effect on behavior was mediated through vagal signaling, possibly through release of neuroactive metabolites acting on enteric neurons[9].

Despite accumulating evidence of the influence of microbiota on behavior in animal models, data from humans are rather limited[30]. Two studies in healthy volunteers reported no major effects of probiotic on anxiety and depression scores[33,34]. A very recent pilot study in patients with major depression showed that depression improved in both the placebo and probiotic groups, although the improvement appeared to be greater in the latter[35]. The only study, which directly linked the gut microbiota to brain function, assessed effects of mixture of probiotic bacteria in healthy females. Although no change in behavior in that trial was observed, fMRI showed decreased BOLD activity in the limbic and sensory brain regions in response to facial expressions, which stimulate emotional responses[12]. We used a similar emotive challenge that is known to induce activation in several brain regions, including the amygdala, where the fear response is particularly salient[36], and which was thus chosen as a predetermined ROI. We found that *BL* administration decreased activation of the amygdala and the fronto–limbic complex compared with placebo, which was paralleled by decreased depression scores. The amygdala is not only involved in regulation of fear and anxiety, but also in activation of the hypothalamic-pituitary-adrenal axis (HPA)[37] and modulation of the visceral sensitivity[38]. In our study, pain scores appeared to improve more in the *BL* group compared to the placebo group, although the difference was not statistically significant. However, the *BL* group was more likely to report the adequate relief of IBS symptoms and this was associated with decreased amygdala activation. Thus changes in pain perception could explain the overall improvement of IBS symptom in the *BL* group but this should be further investigated in future, better powered trials.

Multiple studies have demonstrated beneficial effects of different probiotics in patients with IBS, and a recent meta-analysis[7] has shown that bifidobacteria are more likely to improve gastrointestinal symptoms than lactobacilli. Underlying mechanisms may involve improvement of low-grade gut inflammation present in a subset of patients with IBS[39]. Inflammation has also been linked to psychiatric disorders as pro-inflammatory cytokines can affect mood by a number of mechanisms, including activation of the HPA axis and alteration of the metabolism of neurotransmitters[40]. We found no differences in serum CRP or cytokines levels between placebo and patients given *BL*, indicating that the beneficial effect of this probiotic is not mediated by improvement in inflammation, in agreement with the previous study in mice[8].

Our murine study demonstrated that hippocampal BDNF was upregulated by *BL*[8]. Neurotrophin BDNF influences survival and differentiation of neurons, formation of functional synapses and neuroplasticity[41] and is decreased in major depressive disorder[42]. We found no differences in serum BDNF or neurotransmitter levels, including serotonin, in patients given *BL* compared with patients in the placebo group but this does not rule out the possibility that BDNF or neutransmitters levels in specific brain regions were modulated by the probiotic.

As changes in gut microbiota composition have been linked to altered behavior and brain chemistry[31], we assessed fecal bacterial profiles before and after the treatment. Analysis of fecal 16S rRNA gene sequencing suggests that the beneficial effect of *BL* is independent of major alterations in microbioal composition. However, *BL* altered the urinary metabolic profile suggestive of downregulated bacterial degradation of methylamines and aromatic amino acids[43], including a decrease in host-bacterial co-metabolite 4-cresol sulfate, a by-product of tyrosine fermentation[44]. Interestingly, 4-cresol sulfate is known to inhibit dopamine β-hydroxylase[45], which is a constituent of the catecholamine biosynthetic pathway involved in mood regulation[46]. This enzyme converting

dopamine into noradrenaline is expressed in the central and peripheral nervous system as well as in chromaffin cells of the adrenal medulla[47], and its decreased activity has been associated with depression[46]. In our study, levels of 4-cresol sulfate correlated with improved depression scores in *BL* group suggesting that dopamine/noradrenaline pathway may play an important role in the effect of this probiotic. In future studies, a targeted metabolomic approach should provide further insight into the impact of *BL* on the bacterial metabolism of aromatic amino acids and the subsequent modulation of the host's catecholamine production.

Although this is the first randomized trial to show that probiotics decrease depression scores in IBS patients there are limitations that are important to emphasize. We used the HAD scale to identify patients with psychiatric comorbidity, which is well validated and widely used in studies investigating IBS as a simple tool to screen for comorbid anxiety or depression[3]. Its psychometric properties may be inferior to clinician-administered rating scales for depression and anxiety, but its main advantage is that it does not measure somatic symptoms[13,48], a strong confounding factor in any study of IBS patients. Indeed, there is no superior validated questionnaire that can be used in patients with a concurrent medical disorder. However, a confirmatory trial using additional specific psychometric tools in a larger cohort of patient is needed to substantiate our current results. A weakness of our study was the difference in the baseline depression scores between the two groups with lower values in the placebo group. The improvement in depression in the probiotic group could relate to regression to the mean or reflect a floor effect. We believe this is unlikely, however, as a statistically significant result in favor of *BL* remained when adjusting for baseline differences using, and when performing analysis only in the subgroup of patients with baseline scores indicative of depression (HAD-D $\geq$8). Also, there was a linear decrease in the depression subscale of the HAD score in the *BL* group that did not asymptote with the placebo HAD score

(Supplementary Figure 6). Furthermore, the observed changes in brain activation patterns in the areas involved in mood regulation in the *BL* group support the notion that this probiotic has anti-depressive properties. We have not shown any effect of *BL* on individual gut symptoms but our study was not adequately powered to detect these changes. Thus, a larger, appropriately powered trial with patients with IBS and comorbid depression is needed to verify our data on psychiatric and gut symptoms before *BL* can be recommended in clinical practice.

In conclusion, *B. longum NC3001* has longstanding beneficial effects on mild to moderate comorbid depression and it temporarily improves overall symptoms of IBS and QoL. This is associated with changes in activity of multiple brain areas involved in emotional processing, but no improvement in individual gut symptoms, suggesting that central effects of this probiotic underlies its therapeutic effect, possibly through modulation of host catecholamine production.

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LEGENDS

Figure 1: ***Flowchart study population***

Figure 2: Brain activation patterns assessed by fMRI

Functional Magnetic Resonance Imaging (fMRI) was used to assess the BOLD response to fearful stimuli compared with fixation. Group differences in activation pattern are displayed. At baseline, when examining all brain regions (A), there was slightly greater engagement of the visual association (A1) and parietal cortices (A2) *in B longum* (*BL)* group compared with the placebo group, with no difference in predetermined region of interest (ROI), amygdala (B). After treatment (at 6 weeks), the *BL* group displayed lesser engagement of the amygdala, as well as frontal and temporal cortices that are involved in anxiety and mood regulation (C, D; in yellow-orange) and increased engagement of occipital regions (in blue) compared with the placebo group. At 6 week, the amygdala activation correlated with the depression scores in the whole cohort and the *BL* group (E, middle and right panel). Patients with adequate relief of IBS symptoms (blue dots) were more likely to have lesser engagement of the amygdala than the patients without improvement in their IBS symptoms (red dots).

Table 1: Demographics characteristics of study population

	B. longum **n=22**	**Placebo** **n=22**
Study status, n (%)		
Completed	18 (82)	20 (91)
Dropped out	4 (18)	2 (9)
Age, median (IQR)	46.5 (30-58)	40.0 (26-57)
Female, n (%)	12 (54)	12 (54)
Ethnicity n (%)		
Caucasian	19 (86)	21 (95)
Other	3 (14)	1 (5)
Smoking status *Smokers, n (%)*	3 (14)	3 (14)
Alcohol consumption *Consumers, n (%)*	8 (36)	11 (50)
Fibre consumption, g/day, median (IQR)	18.0 (12.0-23.2)	13.5 (10.0-18.2)
BMI, median (IQR)	25.1 (21.5-28.4)	24.6 (22.3-29.5)
IBS subtype n (%)		
Female Diarrhoea	6 (27)	6 (27)
Female Mixed	6 (27)	6 (27)
Male Diarrhea	8 (37)	7 (32)
Male Mixed	2 (9)	3 (14)
Anxiety and depression, n (%)		
Anxiety (HAD-A ≥8)	21 (95)	18 (82)
Depression (HAD-D ≥8)	13 (59)	8 (36)
Anxiety and depression	12 (54)	6 (27)

No significant differences between groups for demographics characteristics (P value for all comparisons between groups >0.05)

Table 2. Depression, anxiety, IBS symptoms, quality of life and somatization scores

Test/ Mean (SD)	***Before treatment***		***Post-treatment, 6 weeks***		***Mean difference 95% CI***	***Follow-up, 10 weeks***		***Mean differenc 95% CI***
	Placebo	***B. longum***	***Placebo***	***B. longum***		***Placebo***	***B. longum***	
Depression HAD-D	5.2 (3.0)	7.6 (3.7)	4.5 (3.1)	3.9 (3.1)	0.6 (-1.6 to 2.6)	4.7 (3.5)	4.7 (3.8)	0.15 (-2.5 to 2.5)
Anxiety HAD-A	9.3 (2.6)	10.2 (3.2)	7.1 (3.9)	6.5 (3.8)	0.6 (-1.9 to 3.2)	8.0 (4.3)	7.6 (4.8)	0.39 (-2.7 to 3.5)
Anxiety STAI	40.4 (12.3)	41.3 (13.9)	38.8 (12.9)	33.1 (9.5)	5.7 (-2.0 to 13.5)	37.6 (11.9)	38.4 (15.3)	-0.83 (-10.1 to 8.4)
Anxiety TAI	44.0 (11.9)	47.7 (10.5)	42.5 (11.3)	39.4 (11.6)	3.1 (-4.5 to 10.6)	42.2 (11.3)	32.3 (12.7)	2.84 (-5.2 to 10.9)
IBS-Birmingham: total	17.8 (7.9)	17.7 (7.1)	12.6 (9.2)	8.8 (9.2)	3.8 (-2.4 to 9.9)	13.0 (6.9)	12.4 (9.5)	0.55 (-4.9 to 6.1)
Birmingham: constipation	3.8 (3.6)	3.0 (3.1)	3.1 (3.5)	1.4 (1.6)	1.7 (-0.1 to 3.6)	3.2 (3.5)	2.5 (3.8)	0.71 (-1.7 to 3.2)
Birmingham: diarrhea	8.2 (4.3)	8.5 (4.8)	4.7 (4.3)	4.1 (5.8)	0.5 (-2.8 to 3.9)	5.1 (3.2)	5.2 (4.9)	-0.17 (-2.9 to 2.6)
Birmingham: Pain	6.3 (3.9)	6.2 (3.2)	4.9 (4.1)	3.4 (3.5)	1.5 (-1.1 to 4.1)	4.7 (3.4)	4.7 (3.8)	0.15 (-2.4 to 2.4)
QoL-SF-36: Physical	43.9 (10.8)	45.0 (10.1)	43.1 (9.9)	49.9 (8.8)	-6.8 * (-13.2 to -0.4)	46.9 (10.0)	46.4 (9.6)	0.52 (-6.3 to 7.4)
QoL-SF-36 Mental	41.9 (11.1)	39.4 (11.8)	43.3 (9.7)	47.1 (9.9)	-3.80 (-10.4 to 2.8)	41.3 (12.0)	46.4 (12.8)	-5.06 (-13.7 to 3.6)
QoL-SF-36: Physical functioning	78.5 (22.5)	78.3 (24.2)	76.8 (22.7)	94.5 (9.5)	-14.7* (-26.7 to -2.7)	79.2 (23.1)	83.3 (19.8)	-4.17 (-18.7 to 10.4)
QoL-SF-36: Role physical	51.3 (38.4)	61.1 (43.1)	47.5 (38.8)	80.8 (35.9)	-33.4* (-58.5 to -8.2)	59.7 (38.5)	62.5 (42.2)	-2.77 (-30.1 to 24.6)
QoL-SF-36: Bodily pain	61.2 (24.2)	51.0 (20.1)	58.2 (23.3)	65.1 (20.7)	-6.9 (-21.7 to 7.9)	62.2 (25.5)	60.1 (20.4)	2.11 (-13.5 to 17.7)
QoL-SF-36: General health	55.2 (19.2)	59.6 (20.9)	60.9 (22.1)	68.1 (17.0)	-7.3 (-20.6 to 6.1)	59.9 (20.7)	65.6 (21.1)	-5.61 (-19.8 to 8.5)
QoL-SF-36: Vitality	45.5 (19.2)	40.3 (18.7)	43.7 (18.9)	55.3 (17.4)	-11.5 (-23.8 to 0.7)	46.4 (20.4)	49.7 (25.0)	-3.33 (-18.8 to 12.1)
QoL-SF-36: Social functioning	61.9 (21.6)	56.9 (26.8)	68.1 (24.4)	77.2 (19.4)	-9.08 (-24.0 to 5.8)	63.9 (26.7)	72.2 (22.5)	-8.33 (-25.1 to 8.4)
QoL-SF-36: Role emotional	60.0 (39.9)	55.6 (39.6)	56.7 (40.6)	78.4 (38.9)	-21.8 (-48.5 to 4.9)	59.3 (43.6)	68.5 (43.5)	-9.25 (-38.7 to 20.2)
QoL-SF-36: Mental health	62.0 (20.2)	59.6 (18.7)	65.6 (17.6)	71.5 (16.4)	-5.9 (-17.4 to 5.5)	64.0 (20.6)	69.3 (20.6)	-5.33 (-19.3 to 8.6)
PHQ15: Somatization	11.1	11.9	10.0	8.4	1.61	9.4	10.0	-0.63

	(2.9)	(3.5)	(3.9)	(3.2)	(-0.8 to 4.0)	(3.5)	(4.8)	(-3.4 to 2.2)

*p<0.05 vs placebo; HAD-D: Hospital Anxiety and Depression score for depression, HAD-A: Hospital Anxiety and Depression score for anxiety, STAI/TAI: State and Trait Anxiety Inventory, QoL SF-36: Quality of Life Short Form 36; PHQ-15: Patient Health Questionnaire;

Table 3: Decrease ≥2 points in depression and anxiety HAD scores at 6 and 10 weeks in the whole group (A) or when stratified by adequate relief of IBS symptoms (B)

A

Outcome	*B. longum* n	Placebo n	ITT Analysis RR	95% CI	p value	PP Analysis RR	95% CI	p value
Decrease HAD-D ≥2 at 6 weeks	14	7	1.98	1.16-3.38	0.04	2.40	1.26-4.58	0.01
Decrease HAD-A ≥2 at 6 weeks	14	11	1.31	0.72-2.42	0.54	1.69	0.76-3.77	0.19
Decrease HAD-D ≥2 at 10 weeks	13	6	2.05	1.07-3.93	0.04	2.14	1.11-4.12	0.04
Decrease HAD-A ≥2 at 10 weeks	12	10	1.4	0.65-2.82	0.50	1.6	0.77-3.17	0.34

B

Outcome	*B. longum* n	Placebo n	ITT Analysis RR	95% CI	p value	PP Analysis RR	95% CI	p value
Adequate relief of IBS symptoms								
Decrease HAD-D ≥2 at 6 weeks	11	2	3.07	0.89-10.6	0.03	3.07	0.89-10.6	0.03
Decrease HAD-A ≥2 at 6 weeks	11	4	1.53	0.72-3.27	0.66	1.53	0.72-3.27	0.34
Decrease HAD-D ≥2 at 10 weeks	4	1	6.00	1.03-35.9	0.04	6.00	1.06-35.9	0.04
Decrease HAD-A ≥2 at 10 weeks	4	4	1.5	0.85-2.64	0.46	1.50	0.85-2.64	0.46
No Adequate relief of IBS symptoms								
Decrease HAD-D ≥2 at 6 weeks	3	5	0.93	0.29-2.98	1.0	1.6	0.56-4.54	0.56
Decrease HAD-A ≥2 at 6 weeks	3	7	0.66	0.23-1.92	0.34	1.14	0.45-2.90	0.98
Decrease HAD-D ≥2 at 10 weeks	9	5	1.60	0.67-3.78	0.31	1.80	0.80-4.02	0.25

Decrease HAD-A ≥2 at 10 weeks	8	6	1.18	0.52-2.68	0.73	1.33	0.62-2.84	0.70

Chi^2 test, HAD-D: Hospital Anxiety and Depression score for depression, HAD-A: Hospital Anxiety and Depression score for anxiety, ITT: Intention-to-treat analysis, PP: Per-protocol analysis

Table 4: Brain activation patterns in all brain regions (top) and the amygdala (bottom).

Left/right	Brain region	Brodmann area	T-value	p-value	No. voxels
Right	Middle Temporal Gyrus	BA 21	4.62796	0.000013	818
Right	Middle Temporal Gyrus	BA 21	3.860474	0.000222	278
Right	Cerebellum	*	3.964027	0.000154	452
Right	Inferior Frontal Gyrus	BA 47	4.564617	0.000017	4657
Right	Cuneus	BA 19	-3.889998	0.0002	1268
Right	Middle Occipital Gyrus	BA 19	-4.455264	0.000026	898
Right	Middle Frontal Gyrus	BA 10	4.099587	0.000095	526
Right	Amygdala/ Parahippocampal Gyrus	BA 28	3.983939	0.000144	1474
Right	Cuneus	BA 19	-3.709217	0.000372	1527
Right	Medial Frontal Gyrus	BA 11	4.73075	0.000009	365
Left	Middle Occipital Gyrus	BA 19	-4.04685	0.000115	1196
Left	Amygdala/ Parahippocampal Gyrus	BA 35	4.451565	0.000026	1727
Left	Middle Frontal Gyrus	BA 10	3.687655	0.0004	1080
Left	Middle Occipital Gyrus	BA 19	-4.466287	0.000025	776
Left	Middle Temporal Gyrus	BA 21	4.097827	0.000096	689
Left	Middle Occipital Gyrus	BA 19	-3.282744	0.001499	472

Left/right	Brain region	T-value	p-value	No. voxels
Right	Amygdala	3.98394	0.000144	690
Left	Amygdala	4.031566	0.000121	212

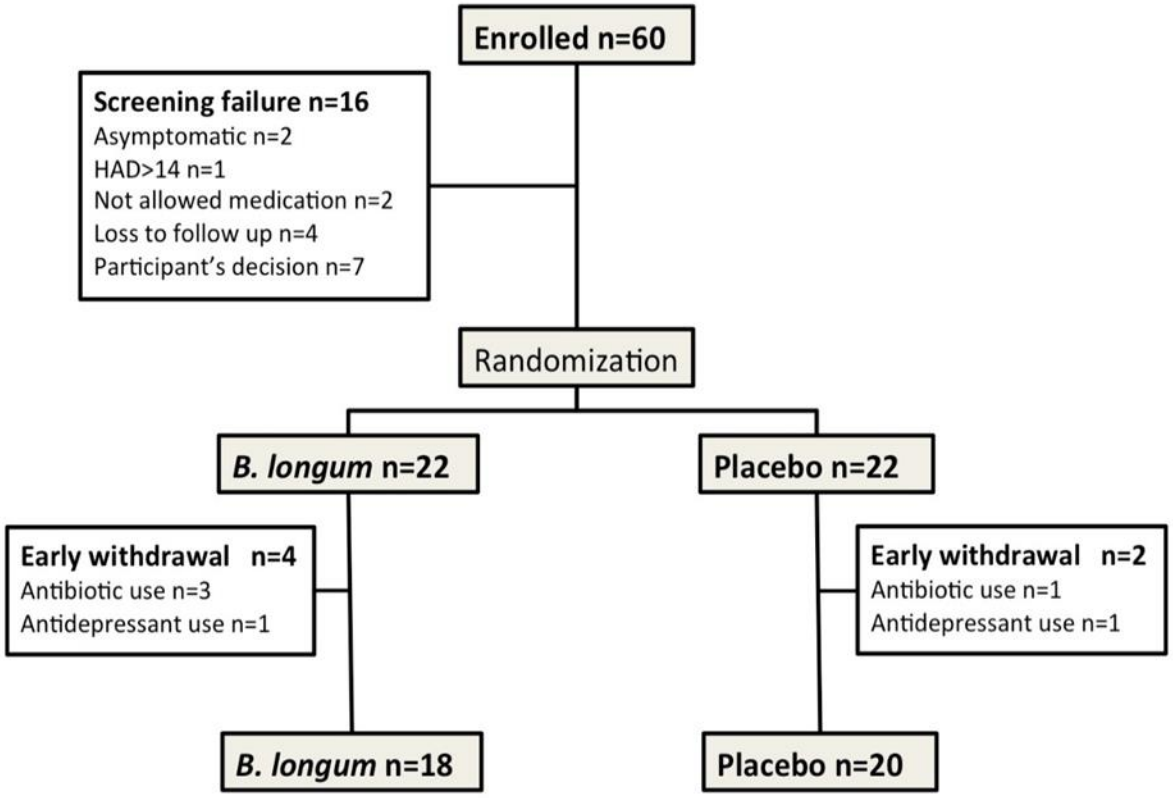
Brain activation patterns assessed by fMRI for post-treatment group differences (placebo vs treatment; fear vs. fixation) in all brain regions (top) and for ROI- amygdala analyses (bottom). All data are corrected for multiple comparisons; FDR(q)<.05. Positive T-values identify regions of greater activation in response to fear stimuli for the placebo group compared to *BL* group.

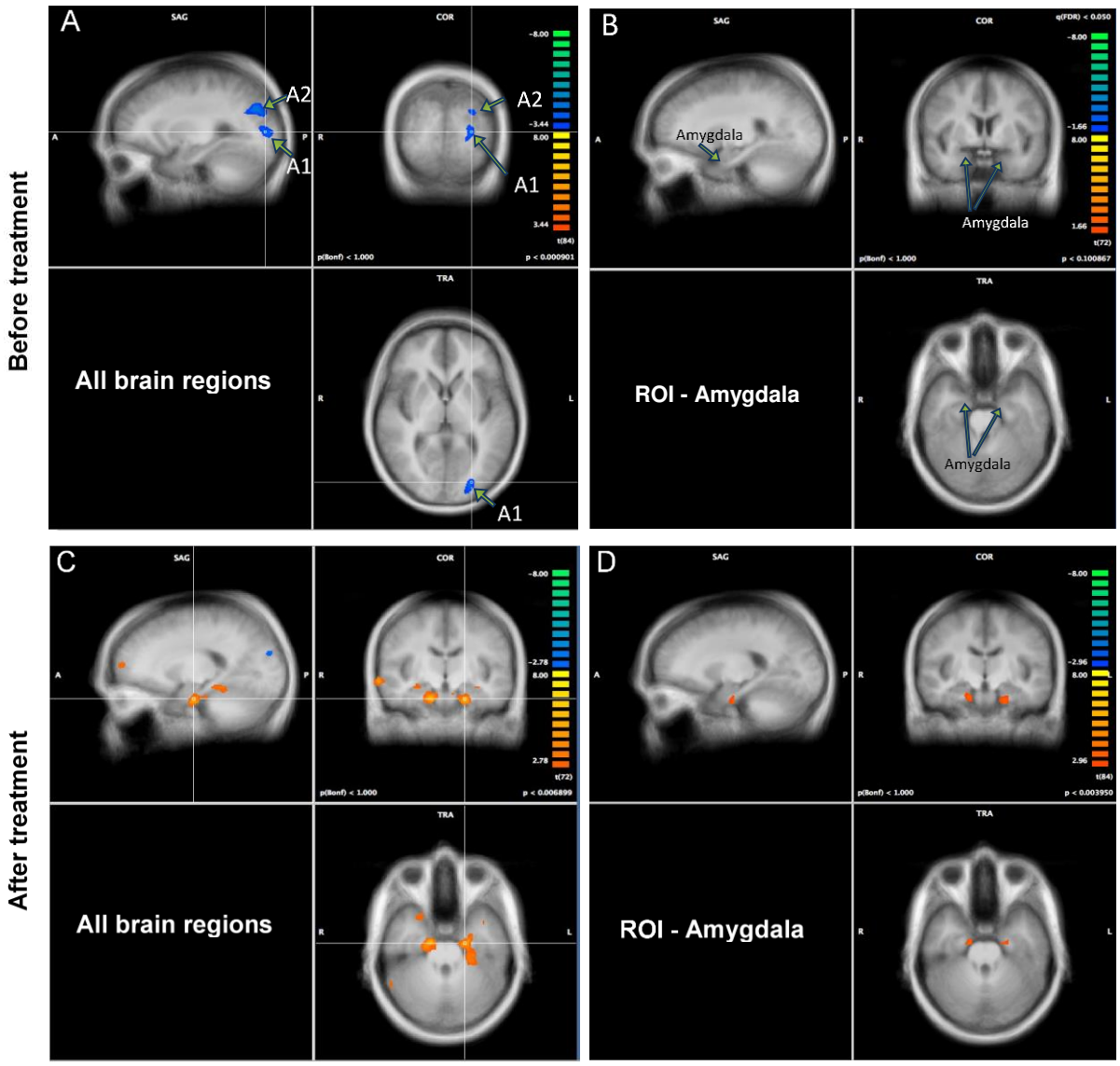
Table 5: Urinary metabolites after 6 weeks of treatment assessed by ^{1}H NMR

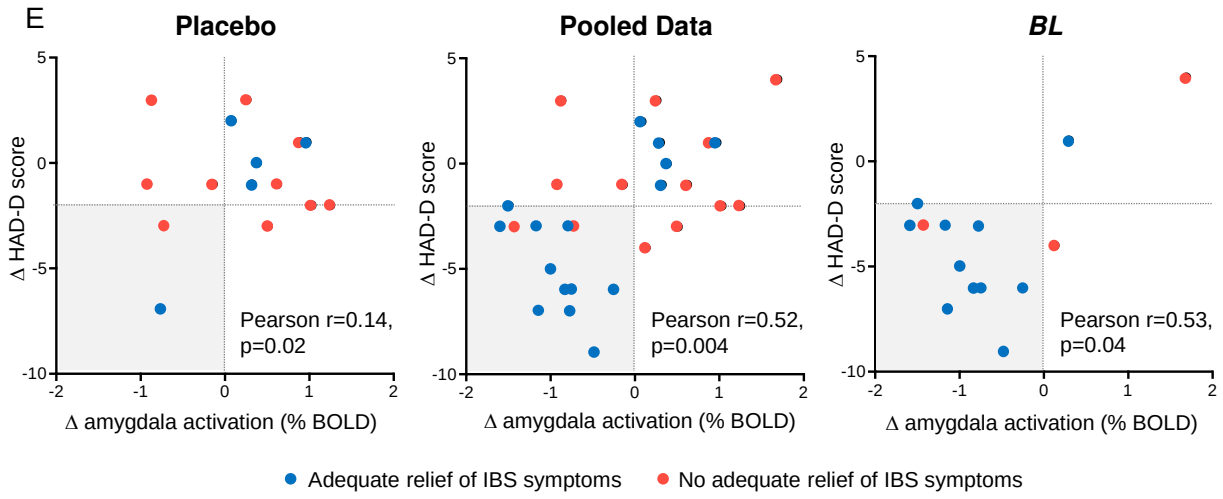
Urinary metabolites	**^{1}H NMR signal (multiplicity)**	**OPLS Correlation coefficient (VIP)**	**Relative concentration (au)**		**p-value**
			Placebo	***B. longum***	
Creatine	3.04 (s)	-0.44 (1.36)	14.49 (10.3)	7.44 (3.0)	0.007
Phenylacetylglutamine	7.43 (m)	-0.50 (1.76)	4.5 (1.8)	3.01 (1.3)	0.013
4-cresol sulfate	2.35 (s)	-0.36 (1.42)	5.38 (2.3)	3.9 (1.7)	0.022
Trimethylamine-N-Oxide	3.27 (s)	-0.32 (1.18)	44.0 (61.2)	15.48 (4.5)	0.002

Metabolite data are reported as mean (SD) with an arbitrary unit (au) derived from ^{1}H NMR spectral peak area. VIP: Variable Importance in Projection; m: multiplet; s: singlet.

Figure 1.




A
B
C
D
Before treatment
After treatment
All brain regions
ROI - Amygdala
A1
A2
Amygdala
SAG
COR
TRA

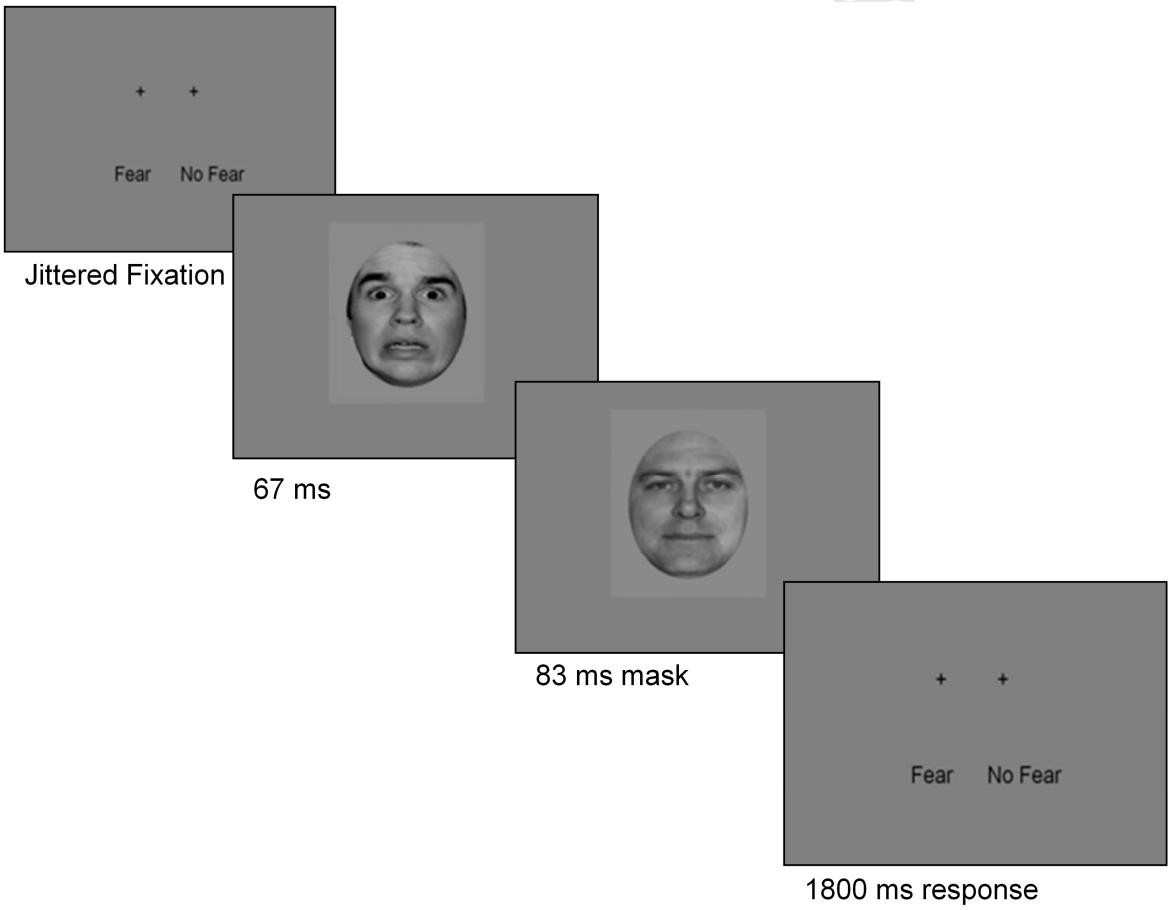

E
Placebo
Pooled Data
BL
Δ HAD-D score
Δ amygdala activation (% BOLD)
Pearson r=0.14, p=0.02
Pearson r=0.52, p=0.004
Pearson r=0.53, p=0.04
Adequate relief of IBS symptoms
No adequate relief of IBS symptoms

Supplementary Figure 1: ***Design of the study***

		Treatment x 6 weeks			
visit	**V0: Screening**	**Visit 1**		**Visit 2**	**Visit 3 End of study**
weeks	**-4**	**0**		**6**	**10**
Procedures	1. Informed consent 2. Questionnaires (HAD, Birmingham, fiber score) 3. General bloodwork	1. Questionnaires (HAD, Birmingham, STAI, PHQ-15, SF-36) 2. Stool, urine and blood samples 3. fMRI	Questionnaires (Birmingham weekly, HAD week 3)	1. Questionnaires (HAD, Birmingham, STAI, PHQ-15, SF-36) 2. Stool, urine and blood samples 3. fMRI	1. Questionnaires (HAD, Birmingham, STAI, PHQ-15, SF-36) 2. End of study

Supplementary Figure 2: The backward masking paradigm.

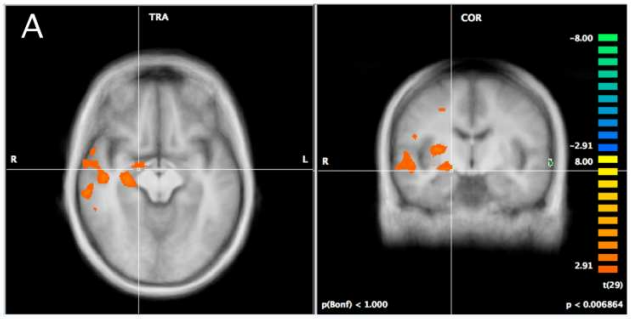
The backward masking paradigm: The fixation was jittered, followed by the presentation of a fearful or happy face for 67 ms, followed by the masking of the stimuli with a neutral face for 83 ms. Participants had 1800 ms to respond. Four presentations of the paradigm were collected, with 84 trials per presentation (scan).



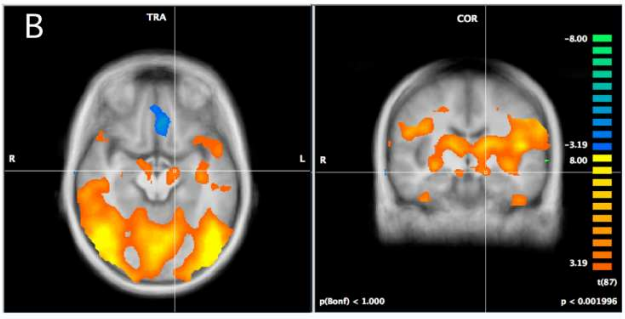
Supplementary Figure 3. Group responses to fear stimuli vs. happy or fixation stimuli.

A. Full group response to fear face stimuli at both time points contrasted with the full group response to happy face stimuli. The analysis identified a number of brain regions that showed greater engagement in response to the fear stimuli including the amygdala, insula and regions in the frontal cortices.

B. Full group response to fear stimuli contrasted with full group response to fixation stimuli, at both time points revealed broad activation including the fusiform gyri, thalamus, striatal regions, parietal cortices and hippocampal/amygdala complex.

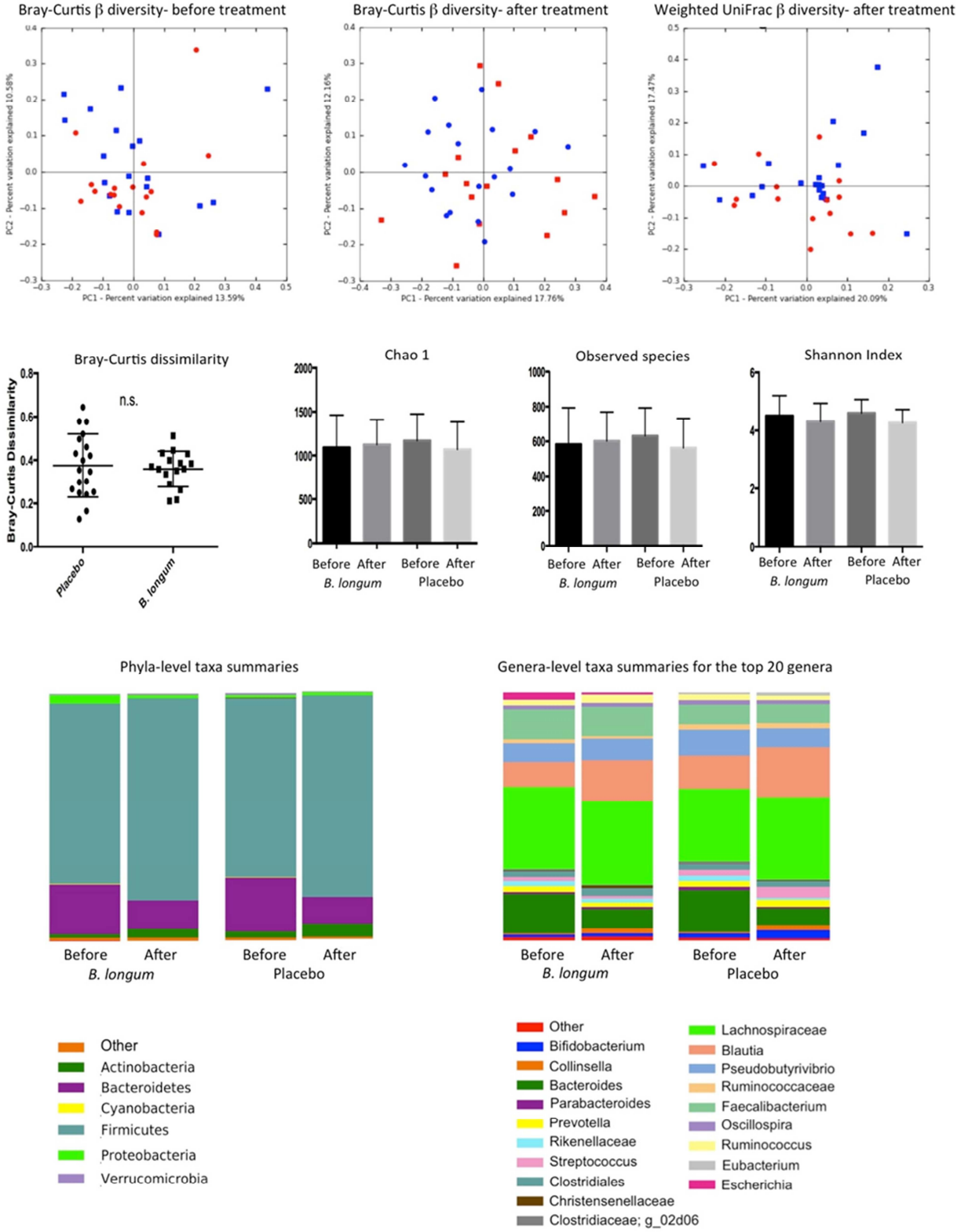


fear stimuli vs. happy stimuli



fear stimuli vs. fixation stimuli

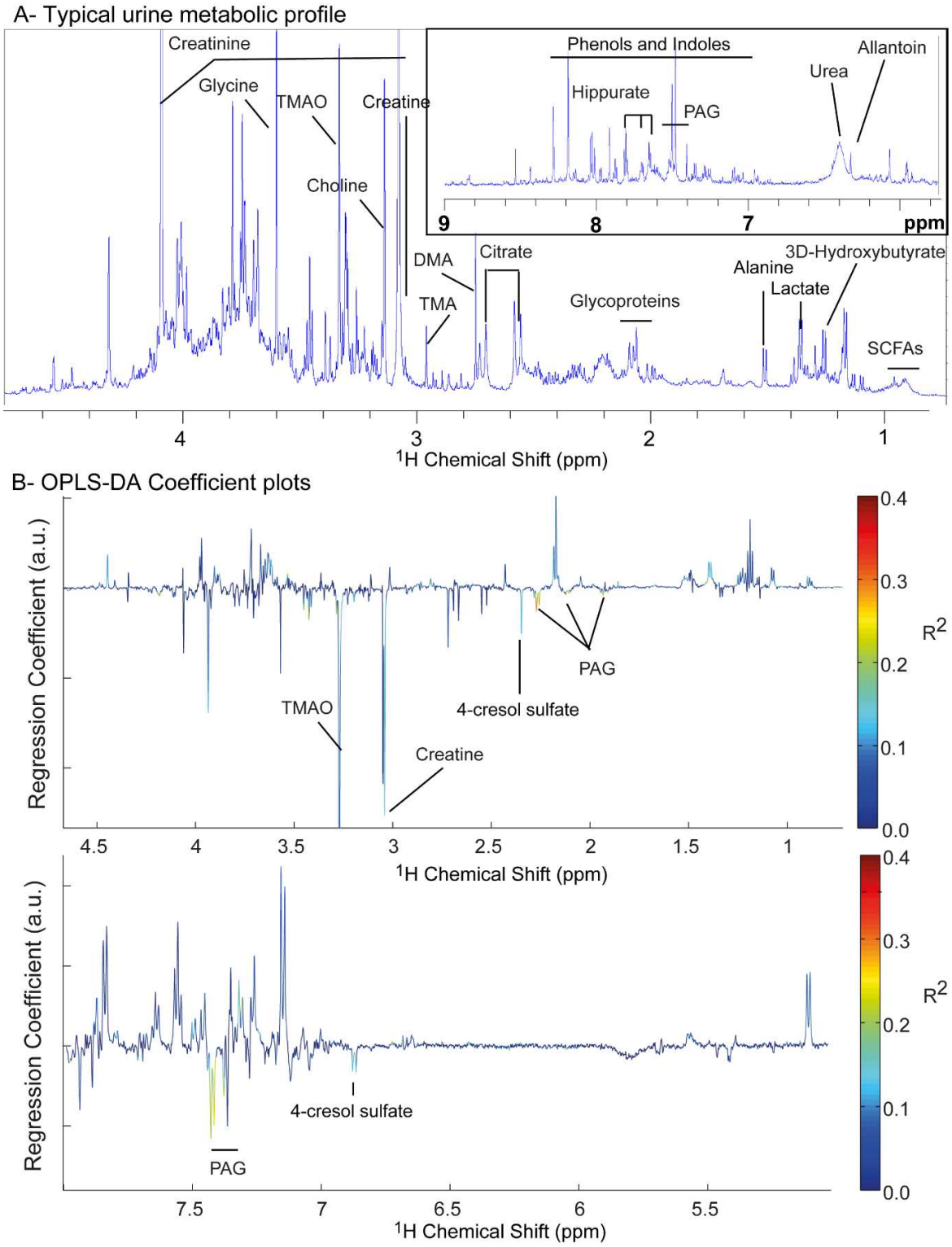
Supplementary Figure 4: Gut microbiota analysis



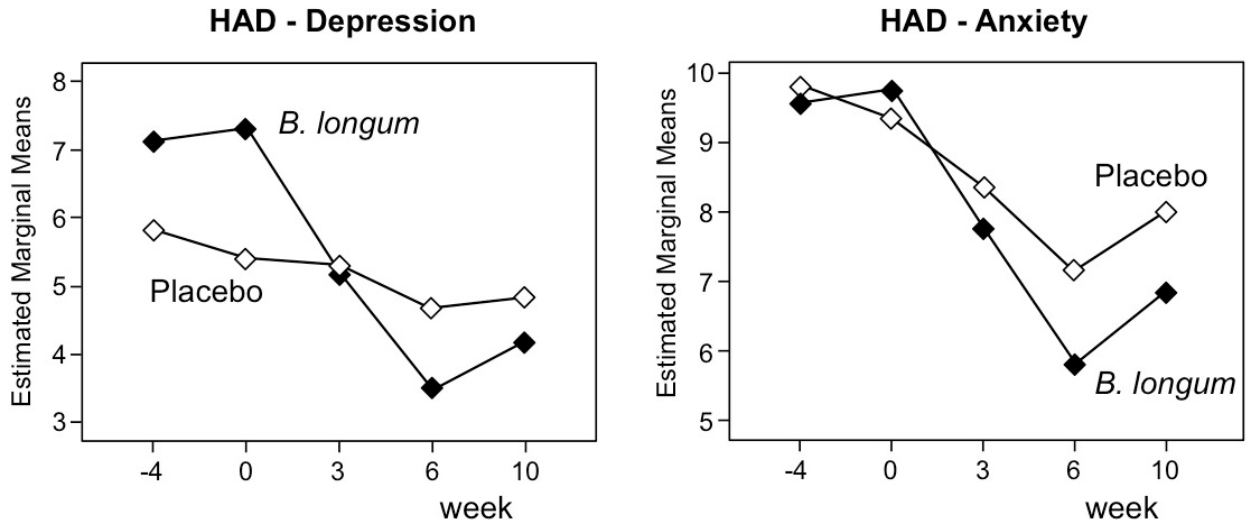
Supplementary Figure 5: Overview of 1H NMR urine metabolic profile and multivariate data analysis

A: Typical 1H NMR urine metabolic profile with selected metabolite signature highlighted in aliphatic (0.5 – 4.5 ppm) and aromatic spectral areas (6.5 – 9.0 ppm).

B: Overview of OPLS-DA coefficient plots resulting for urine NMR data and group discriminant analysis (placebo top, probiotics bottom). The OPLS coefficients plots are presented using a back-scaling transformation and projection to aid biomarker visualization. The direction of the signals in the plots relative to zero indicates positive or negative covariance with the group of interest. Each variable is plotted with a color code which indicates its discriminating power as calculated from the correlation matrix thus highlighting biomarker rich spectral regions.


A- Typical urine metabolic profile
Creatinine
Glycine
TMAO
Creatine
Choline
DMA
TMA
Citrate
Glycoproteins
Alanine
Lactate
3D-Hydroxybutyrate
SCFAs
Phenols and Indoles
Hippurate
PAG
Urea
Allantoin
9
8
7
ppm
4
3
2
1
1H Chemical Shift (ppm)
B- OPLS-DA Coefficient plots
Regression Coefficient (a.u.)
TMAO
Creatine
4-cresol sulfate
PAG
R2
0.4
0.3
0.2
0.1
0.0
4.5
4
3.5
3
2.5
2
1.5
1
1H Chemical Shift (ppm)
Regression Coefficient (a.u.)
4-cresol sulfate
PAG
7.5
7
6.5
6
5.5
1H Chemical Shift (ppm)

***Supplementary Figure 6: Differences in estimated marginal means for HAD-D and HAD-A (ANOVA* multiple measurements)**



HAD-D: Hospital Anxiety and Depression score for depression, HAD-A: Hospital Anxiety and Depression score for anxiety

Supplementary table 1: **Full group responses at both time points in response to fear vs. happy stimuli**

Left/ right	Brain region	Brodmann area	T	P value	No. voxels
Right	Insula	BA 13	5.4106	0.000008	3136
Right	Superior Temporal Gyrus	BA 22	4.8403	0.00004	7845
Right	Inferior Temporal Gyrus	BA 20	4.1807	0.000244	469
Right	Inferior Parietal Lobule	BA 40	3.9513	0.000456	1144
Right	Superior Temporal Gyrus	BA 21	3.3300	0.002375	159
Right	Middle Temporal Gyrus	BA 22	3.7361	0.000815	1410
Right	Superior Temporal Gyrus	BA 39	3.4202	0.001879	115
Right	Superior Temporal Gyrus	BA 38	-4.6615	0.000065	181
Right	Cingulate Gyrus	BA 24	4.4890	0.000105	1416
Right	Insula	BA 13	3.3460	0.002279	186
Right	Lentiform Nucleus	Lat. Globus Pallidus	4.7033	0.000058	4401
Right	Thalamus	Pulvinar	4.6475	0.000067	1116
Right	Paracentral Lobule	BA 5	4.2963	0.000178	855
Left	Parahippocampal Gyrus	BA 27	4.3273	0.000163	306
Left	Precuneus	BA 19	3.6435	0.001043	589
Left	Amygdala	Amygdala	3.9502	0.000458	144
Left	Caudate	Caudate Tail	3.3883	0.002041	156
Left	Insula	BA 13	3.6715	0.000968	567
Left	Lentiform Nucleus	Putamen	4.2270	0.000215	978
Left	Middle Occipital Gyrus	BA 19	3.5165	0.00146	231
Left	Inferior Frontal Gyrus	BA 46	3.4428	0.001771	142
Left	Middle Temporal Gyrus	BA 21	3.78414	0.000717	505
Left	Superior Temporal Gyrus	BA 22	4.2784	0.000187	683
Left	Inferior Temporal Gyrus	BA 20	3.5100	0.001485	209

Supplementary table 2: Inflammatory cytokines, BDNF and neurotransmitter levels at six weeks

Test / Median (IQR)	***Baseline***		***After treatment (6 weeks)***		**U**	**p**
	B. longum	**Placebo**	***B. longum***	**Placebo**		
CRP	1.12 (0.4-2.3)	0.85 (0.4-0.9)	1.32 (0.45-1.7)	1.70 (1.0-1.9)	120.5	0.18
TNF-α	0.98 (0.82-1.17)	0.96 (0.89-1.25)	0.97 (0.82-1.05)	1.06 (0.95-1.29)	116.0	0.33
IFN-γ	0.13 (0.07-0.22)	0.11 (0.08-0.15)	0.14 (0.10-0.18)	0.13 (0.09-0.18)	136.0	0.79
IL-6	0.22 (0.14-0.27)	0.20 (0.15-0.24)	0.18 (0.13-0.26)	0.23 (0.18-0.40)	90.0	0.06
IL-8	0.79 (0.64-1.42)	0.90 (0.65-1.17)	0.97 (0.70-1.14)	0.85 (0.66-1.11)	119.0	0.39
IL-1β	0.97 (0.70-1.14)	0.05 (0.02-0.07)	0.04 (0.02-0.05)	0.04 (0.01-0.06)	116.0	0.33
IL-10	1.32 (1.10-1.61)	1.39 (1.17-1.48)	1.45 (1.05-1.66)	1.69 (1.32-2.02)	101.0	0.14
IL-12 p70	0.09 (0.04-0.15)	0.05 (0.04-0.07)	0.06 (0.05-0.08)	0.06 (0.04-0.09)	136.0	0.78
IL10/12 ratio	26.5 (18.7-36.4)	24.1 (20.9-31.1)	19.7 (11.6-27.7)	24.4 (16.8-36.0)	114.0	0.30
BDNF / β actin	20 (20-28)	29 (17-52)	25 (17-41)	37 (21-55)	124.0	0.10
BDNF ELISA	6.61 (2.43-8.41)	4.87 (3.21-11.11)	7.00 (3.60-10.56)	5.83 (3.15-10.37)	148.0	0.65
CGRP	23.6 (17.1-28.8)	25.2 (18.8-30.8)	23.8 (17.4-30.3)	26.5 (20.9-32.7)	147.0	0.34
Substance P	1.01 (0.61-1.42)	1.26 (0.75-1.60)	1.03 (0.75-1.49)	1.19 (0.59-1.51)	176.0	0.91
Serotonin	12.3 (5.8-14.5)	10.2 (6.6-19.3)	8.3 (4.2-14.9)	8.9 (5.9-12.1)	165.0	0.67

Concentrations of individual biomarkers: CRP (mg/L), TNF-α, IFN-γ, IL-6, IL-8, IL-1β, IL-10 (all pg/mL), BDNF/β actin $Log10^{-5}$ (copies/ng RNA), BDNF ELISA (ng/mL) CGRP (pg/mL), Substance P (ng/mL), Serotonin (ng/mL),

Supplementary table 3: Most common adverse events

Adverse Event	***B. longum* n**	**Placebo n**	**P value**	**Causality**	**Outcome**
Constipation	0	2	0.48	NR NR	Recovered Recovered
Rectal bleeding	0	1	1.0	NR	Recovered
Rhinitis	2	1	1.0	NR NR NR	Recovered Recovered Recovered
Headaches	4	1	0.34	Possible Possible Possible Possible NR	Recovered Recovered Recovered Recovered Recovered
Oral vesicles	1	0	1.0	NR	Recovered
Anal fissure	1	0	1.0	NR	Recovered
Neck pain	0	1	1.0	NR	Recovered
Urine infection	1	0	1.0	NR	Dropped*
Nausea	0	1	1.0	NR	Recovered
GERD symptoms	2	1	1.0	NR NR NR	Recovered Recovered Recovered
Abdominal pain	0	1	1.0	NR	Recovered
Diarrhoea	0	1	1.0	NR	Recovered
Cold	2	2	1.0	NR NR NR NR	Recovered Recovered Recovered Recovered
Otitis	1	0	1.0	NR	Dropped*
Food allergy	1	0	1.0	NR	Recovered
Streptococcus pharyngitis	1	0	1.0	NR	Dropped *
Back pain	1	0	1.0	NR	Recovered
Iritis	1	0	1.0	NR	Recovered
Anxiety attack	0	1	1.0	NR	Recovered
Total	**18**	**14**	**0.31**		

NR: Not related, *Dropped from the study due to use of antibiotics

Supplementary methods:

Microbiota analysis:

Microbiota analysis was performed using Illumina sequencing of the V3 region of 16S rRNA gene as described previously[1]. The data were processed by an in-house bioinformatics pipeline that incorporates quality filtering, Cutadapt[2], PandaSeq[3], AbundantOTU[4], mothur[5] and QIIME[6]. Abundant OTU provide output of clustered sequences in operational taxonomic units (OTUs). Taxonomic assignments use the RDP classifier[7] with the Greengenes training set[8]. Analysis includes alpha-diversity measures for each sample and estimates of total diversity using QIIME, as well as β-diversity measures (weighted and unweighted Unifrac, Bray-Curtis) and other statistical analysis using QIIME and the PhyloSeq[9] package implemented in R.

Metabonomics analysis:

1H NMR metabolite profiling approach was applied to urine samples, since this biofluid contains useful time-averaged representations of the recent homeostatic metabolic history of the individual and also carry indirect information on the gut microbial metabolic activities via the excretion patterns of many polar microbial-mammalian co-metabolites[10, 11] (Supplementary Figure 5A).

Forty µL of urine were mixed with 20 µL of deuterated phosphate buffer solution 0.6 M KH2PO4, containing 1 mM of sodium 3-(trimethylsilyl)-[2,2,3,3-2H4]-1-propionate (TSP, chemical shift reference δH = 0.0 ppm). 60µL of the mixture were transferred into 1.7mm NMR tubes. 1H NMR spectra were acquired with a Bruker Avance II 600 MHz spectrometer equipped with a 1.7 mm probe at 300 K (Bruker Biospin, Rheinstetten, Germany), using a standard pulse sequence with

water suppression, and processed using TOPSPIN (version 2.1, Bruker, Germany) software package. The metabolite identification was achieved using in house database and 2D 1H NMR spectroscopy experiments.

Chemometric analysis was performed using the software package SIMCA-P+ (version 14.0, Umetrics AB, Umeå, Sweden) and in-house developed MATLAB routines. Orthogonal Projection to Latent Structures (OPLS)[12] and OPLS discriminant analysis (OPLS-DA) were employed for exploring the variance in the metabonomics data that may explain statistical differences between groups of samples. The classification accuracy of the OPLS-DA was established from the predicted samples in the 7-fold cross-validation cycle. To highlight the weight of individual variables in the model, Variable Importance in Projection (VIP) was used, with a value above 1 used as a threshold by convention. In addition, influential NMR variables that are correlated to the group separation are identified using the variable coefficients according to a previously published methods[13]. Representative signals of the identified metabolites were integrated and tested using non-parametric Mann Whitney test.

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