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Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects

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Abstract

In a previous clinical study, a probiotic formulation (PF) consisting of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 (PF) decreased stress-induced gastrointestinal discomfort. Emerging evidence of a role for gut microbiota on central nervous system functions therefore suggests that oral intake of probiotics may have beneficial consequences on mood and psychological distress. The aim of the present study was to investigate the anxiolytic-like activity of PF in rats, and its possible effects on anxiety, depression, stress and coping strategies in healthy human volunteers. In the preclinical study, rats were daily administered PF for 2 weeks and subsequently tested in the conditioned defensive burying test, a screening model for anti-anxiety agents. In the clinical trial, volunteers participated in a double-blind, placebo-controlled, randomised parallel group study with PF administered for 30 d and assessed with the Hopkins Symptom Checklist (HSCL-90), the Hospital Anxiety and Depression Scale (HADS), the Perceived Stress Scale, the Coping Checklist (CCL) and 24 h urinary free cortisol (UFC). Daily subchronic administration of PF significantly reduced anxiety-like behaviour in rats ($P < 0.05$) and alleviated psychological distress in volunteers, as measured particularly by the HSCL-90 scale (global severity index, $P < 0.05$; somatisation, $P < 0.05$; depression, $P < 0.05$; and anger–hostility, $P < 0.05$), the HADS (HADS global score, $P < 0.05$; and HADS-anxiety, $P < 0.06$), and by the CCL (problem solving, $P < 0.05$) and the UFC level ($P < 0.05$). *L. helveticus* R0052 and *B. longum* R0175 taken in combination display anxiolytic-like activity in rats and beneficial psychological effects in healthy human volunteers.

Key words: Probiotics; Depression; Anxiety; Stress; Coping strategies

There is a well-established link between stress, mood disorders and gastrointestinal (GI) disease⁽¹⁾. While the organism is generally capable of adapting to stressors, chronic overload can result in GI and mood disorders^(2–4). Indeed, several studies have indicated that stressful events

are associated with the onset of chronic GI disturbances⁽⁵⁾, functional ones^(6–8), inflammatory bowel disease^(9–12) or peptic ulcers^(13–16), as well as anxiety and depression depending on the genetic background^(17–19). Since depression reduces the capacity of coping with stress⁽²⁰⁾,

Abbreviations: CCL, Coping Checklist; GI, gastrointestinal; HADS, Hospital Anxiety and Depression Scale; HADS-A, HADS-anxiety; HADS-D, HADS-depression; HPA, hypothalamic–pituitary–adrenal; HSCL-90, Hopkins Symptom Checklist; MWT, Mann–Whitney *U* test; PF, probiotic formulation; PL, placebo; PSS, Perceived Stress Scale; WT, Wilcoxon test.

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GI disorders may be accelerated or exacerbated. For example, inflammatory bowel disease was associated with mood disorders in more than 50% of patients, but with more pronounced psychological disturbances during periods of active intestinal distress^(21,22). Depression is sometimes the primary culprit, as demonstrated by the successful use of antidepressants in treating inflammatory bowel disease⁽²³⁾.

There is emerging evidence from preclinical studies of a role for gut microbiota on the central nervous system function^(1,23). GI bacterial infection induced anxiety-like behaviour in mice, probably due to the stimulation of brain areas implicated in integrating viscerosensory information and mood via the vagus nerve, such as paraventricular hypothalamus, amygdala and bed nucleus of the stria terminalis⁽²⁴⁾. Moreover, germ-free mice have an increased responsiveness of the hypothalamic–pituitary–adrenal (HPA) axis and modified serotonin and noradrenaline levels compared with specific pathogen-free mice⁽²⁵⁾. Mono-association with probiotics in these germ-free mice before 6 weeks of age reversed HPA hyper-reactivity. Neonatal maternal separation predisposed adult rats to intestinal mucosal dysfunction in response to stress⁽²⁶⁾ and the development of visceral hyperalgesia⁽²⁷⁾. Probiotics restored gut physiology in this stress model by regulating the interaction between mucosa and bacteria and reducing HPA hyperreactivity⁽²⁸⁾. Moreover, probiotics reversed apoptosis markers in the limbic system following myocardial infarction in rats⁽²⁹⁾. Monkeys exposed to stress during setting up of the intestinal microflora had an altered gut colonisation⁽³⁰⁾.

The adult human GI tract is the natural habitat of a large and dynamic population of micro-organisms thriving in the relationship between external and internal environments. It comprises at least 160 of different bacterial species per individual from the pool of 1000 and 1150 prevalent species of bacteria⁽³¹⁾, among which probiotics administered in adequate amounts are proposed to confer a health benefit⁽³²⁾ and as a novel therapeutic strategy⁽³³⁾, particularly for mood disorders^(1,23).

One probiotic formulation (PF), a combination of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175, showed beneficial effects on GI symptoms in patients subjected to chronic stress⁽³⁴⁾. When administered separately, these two strains have also showed beneficial effects^(35,36). For example, several strains of *Lactobacillus* displayed anti-inflammatory properties *in vitro* in human intestinal epithelial cells⁽³⁷⁾, while oral treatment with *B. longum* R0175 showed beneficial properties in human subjects with ulcerative colitis⁽³⁸⁾. Moreover, *L. helveticus* had favourable actions on sleep efficiency in elderly subjects⁽³⁹⁾. To evaluate the role of PF on anxiety, we first assessed its effects in the conditioned defensive burying test in the rat^(40,41), in which rats exposed to a probe associated with a single footshock show anxiety-related probe burying, head stretchings and approaches/escape

sequences towards the probe. The potential anxiolytic effects of PF were then assessed on human distress, anxiety and depression evaluated with the Hopkins Symptom Checklist (HSCL-90)^(42,43), the Hospital Anxiety and Depression Scale (HADS)⁽⁴⁴⁾, the Perceived Stress Scale (PSS)⁽⁴⁵⁾ and the coping checklist (CCL)^(46,47). Moreover, 24 h urinary free cortisol was assayed as a physiological index of stress level^(48,49).

Subjects and methods

Preclinical study

Animals. Thirty-six male Wistar rats (HsdBrlHan, Harlan, The Netherlands) weighing 200 g were housed three per cage inside polycarbonate cages measuring 48 × 27 × 20 cm (U.A.R., Epinay-Sur-Orge, France) in a regulated environment (temperature 22 ± 2°C; humidity 50 ± 10%; lights on from 21.00 to 09.00 hours). After a 7 d adaptation period and tail marking, the rats were weighed and randomly distributed into three groups (*n* 12): probiotic preparation (PF), placebo (0.5% methylcellulose solution) and diazepam (Valium® 1%; Roche, Neuilly-sur-Seine, France) as the reference substance. The rats had free access to food pellets (Teklad diet no. 2016; Harlan Teklad, Oxon, UK) and tap water until the day before anxiety testing, when deprived of food at 06.00 hours until the following day (day 14). The present experiment adhered to the guidelines provided by the ASAB Ethical Committee for the use of animals in behavioural research⁽⁵⁰⁾ and by the Canadian Council on Animal Care⁽⁵¹⁾. All procedures complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Study design. The behaviours were recorded by experimenters unaware of the administered products. The rats were placed under a dim red light inside a clear Plexiglas chamber (44 × 28 × 18 cm), whose floor was evenly covered with a 5 cm high bedding of wooden sawdust. On days 12 and 13, the rats were familiarised with the chamber for 20 min/d. At the centre of one wall, 2 cm above the bedding material, a shock probe (7 × 2 × 0.5 cm) overlaid with a copper wire-integrated circuit connected to a two-pole shock generator (Intellibio, Nancy, France) was inserted on day 14, facing away from where the rat was placed. When the animal touched the probe with its forepaws, a single 2 mA electric shock was delivered, and its behaviour was recorded for 5 min, evaluated from videotapes (Sony™ video camera and recorder) by a trained observer: duration of probe burying (piling sawdust with forelimbs in the direction of the probe); head stretchings towards the probe; approaches towards the probe and retreats away from the probe. The percentage of approaches followed by escapes was then calculated (escapes/approaches × 100), followed by a global stress/anxiety score by adding the ranks of duration of probe

burying, head stretchings and percentage of approaches/escapes⁽⁴¹⁾.

Products. The test product is a proprietary PF from Institut Rosell-Lallemand, Blagnac, France, containing a mixture of freeze-dried lactic acid bacteria and excipients. The lactic acid bacteria strains are *L. helveticus* R0052 (strain number I-1722 in the French National Collection of Cultures of Microorganisms (CNCM), Institut Pasteur, Paris, France) and *B. longum* R0175 (CNCM strain number I-3470). Excipients are xylitol, maltodextrin, flavour and malic acid. PF contains three billion colony-forming units/1.5 g sachet. The genetic identification of *L. helveticus* R0052 has been described previously⁽⁵²⁾. Strain R0175 was identified as a *B. longum* by 16S rRNA and *tuf* gene sequencing. To be brief, genomic DNA was extracted from an overnight broth culture of R0175 described previously for R0052⁽⁵³⁾. Extracted DNA was diluted one-twentieth for PCR to a final concentration of 100 ng/μl. DNA from R0175 was used as a template in PCR to amplify approximately 1370 nucleotides of the 16S rRNA gene using the primers P0 and P6 as described by Ventura *et al.*⁽⁵⁴⁾. The *tuf* genes, approximately 970 nucleotides, were amplified with BIF-1 and BIF-2⁽⁵⁵⁾. PCR products were sent to Genome Quebec (Montreal, QC, Canada) according to the guidelines of the DNA Sequencing Platform. Nucleotide sequences for the 16S rDNA and *tuf* genes of strains R0175 were compared with the BLASTN database available on GenBank⁽⁵⁶⁾ and were deposited under accession numbers (HM009032 and HM009033, respectively).

All products were freshly prepared every day and administered by gavage at a volume of 5 ml/kg. PF was dissolved in a 0.9% NaCl solution and stirred until homogenisation just before its administration at a dose of 250 mg/rat per d (10⁹ colony-forming units/d). Diazepam was suspended in a 0.5% methylcellulose solution and administered at 3 mg/kg 60 min before the test session on day 14. The placebo (PL) group received the 0.5% methylcellulose vehicle from days 1 to 14.

Statistical analyses. Comparisons between treated groups and controls were performed by the Kruskal–Wallis test and the Mann–Whitney *U* test (MWT). The results are expressed as medians with inferior and superior quartile values. Differences were considered to be

significant at the $P < 0.05$ level. All statistical analyses were carried out with the StatView[®]5 statistical package (SAS Institute, Inc., Cary, NC, USA).

Clinical study

Subjects. After written informed consent was obtained from all subjects, healthy Caucasian men and women (age and sex distribution of the sample on initial examination are summarised in Table 1) were recruited from the general population from a database of former research participants (Biofortis Clinical Investigation Center) and from a variety of sources including Internet, newspaper and radio advertisements. The formalities were performed in accordance with the rules of Good Clinical Practices (Guidelines GCP ICH), the Helsinki Declaration and French government guidelines ‘Code de la Santé Publique, titre II du livre premier’ relating to biomedical research. The protocol was favourably received by the following ethics committee: ‘Comité de Protection des Personnes (CPP) Ouest IV-Nantes’ on 14 November 2008.

Sixty-six subjects were included from the pool of ninety-nine subjects based on standard biological safety parameters and a score of ≤ 12 in the HADS-anxiety subscale (HADS-A) and in the HADS-depression subscale (HADS-D) and equal to or less than 20 in the HADS total score on initial examination (see Results section and Table 3). Fifty-five of them participated and finished the clinical trial. Subjects were excluded when suffering from neurological, psychiatric, renal, hepatic, cardiovascular and respiratory diseases, or food allergy, or when taking psychotropic drugs during the previous month, stimulating nutritional supplements (vitamin C), ginger, guarana, ginseng, dehydroepiandrosterone, melatonin, antioxidants, anxiolytics, antidepressants, selenium, narcotics, replacement hormones, more than 5 cups of coffee or tea/d, 0.2 litres of cola, 30–40 g of chocolate, three glasses of wine, or two fermented dairy products, or else when smoking more than twenty cigarettes. Pregnant women and subjects who had participated in another clinical study over the past 2 months were also excluded.

Calculation of the sample size, randomisation and blinding. Calculation of the sample size is based on the anxiety dimension of the HSCL-90 scale. A difference

Table 1. Age and sex of the subjects taking the probiotic formulation (PF) (*n* 26) or placebo (*n* 29) (Mean values and standard deviations with minimum–maximum values)

	<i>n</i>	Age (years)			Both sexes		
		Mean	SD	Minimum–maximum	Mean	SD	Minimum–maximum
PF							
Men	7	38.3	8.3	30–55	42.4	7.5	30–55
Women	19	43.8	6.7	34–55			
Placebo							
Men	7	46.1	9.1	33–60	43.2	8.5	31–60
Women	22	42.2	8.3	31–59			

from 1.5 to 2 points in the score in this dimension is considered as significant. The results of published studies indicate a mean of 3 (SD 2). To detect such a treatment effect with 80% power at a 5% level of statistical significance, it seems necessary to include twenty-eight subjects in each of both groups. Thus, the protocol plans the participation of fifty-six to sixty subjects distributed into two groups: probiotic formulation (PF) and placebo (PL).

After eligibility determination, subjects were then randomised based on age and sex according to a computer-generated randomisation list in sealed, opaque envelopes into two groups: PF and PL groups. The randomisation list was generated and kept by a project nurse not involved in the clinical trial. Subjects and clinical staff involved in the trial experiments were blinded to the treatment group assigned. The codes for the treatment groups were revealed only after the completion of the whole study and statistical analysis.

Study design. The clinical trial was designed as a double-blind, controlled, randomised, parallel study lasting 30 d. There were three visits to the Biofortis Clinical Investigation Center: preliminary examination; baseline (14 d later); follow-up (30 d after baseline). During preliminary medical examination, to be included, the volunteers were subjected to blood sampling in order to verify whether their safety biological parameters were within normal ranges, and the HADS. Two subjects did not participate in a satisfactory way and were discarded. The sample of fifty-five subjects was divided into two groups: test product (PF) or PL. At 2 weeks after the preliminary examination at baseline, the subjects completed the HSCL-90, the PSS and the CCL. Each participant then received thirty sticks of the probiotic preparation or placebo for 30 d. At follow-up, the subjects received a second medical examination and completed the rest of the tests. In addition, the day before baseline and follow-up, the subjects collected their urine samples over a period of 24 h to dose the urinary free cortisol.

Products (for the probiotic product characterisation, see above). During or just after breakfast, all volunteers took one stick of 1.5 g/d of PF (ProbioStick®: batch no. 6533308; Institut Rosell-Lallemand, Blagnac, France) containing *L. helveticus* R0052 and *B. longum* R0175 (3×10^9 colony-forming units/stick) or placebo (xylitol, maltodextrin, plum flavour and malic acid) of identical taste and appearance for 30 d. The first treatment was taken in the evening of baseline, and the last in the morning of the final test period. Study compliance was assessed by counting the number of sticks returned by participants to the study coordinator.

Testing methods

Hopkins Symptom Checklist-90. The HSCL-90 is a 90-item self-reported multidimensional questionnaire^(42,43) screening a broad range of psychopathological disorders. The

HSCL-90 measures nine primary symptom dimensions (somatisation, obsessive-compulsive, interpersonal sensitivity, depression, anxiety, anger-hostility, phobic anxiety, paranoid ideation and psychoticism). Each item is rated on a five-point scale, ranging from 'not at all' to 'extremely'. The subject's overall psychological distress was evaluated by the global severity index.

Hospital Anxiety and Depression Scale. The HADS, a four-point scale⁽⁴⁴⁾ that ranges from 0 (never) to 4 (very often), is a fourteen-item self-assessment instrument, often applied and convenient for measuring psychological distress in subjects with somatic or psychosomatic disorders⁽⁵⁷⁾. Three subscores were obtained: HADS global score, HADS-A and HADS-D.

Perceived Stress Scale. The PSS is a fourteen-item self-reported questionnaire⁽⁴⁵⁾ assessing the degree to which recent life situations are appraised as stressful. Respondents indicate how often they have felt or thought a certain way over the past month on a five-point scale that ranges from 0 (never) to 4 (very often). Responses are then summed to indicate the level of perceived stress.

Coping Checklist. The CCL, derived from the 'Ways of Coping Check-list' of Lazarus & Folkman⁽⁵⁸⁾, is a validated twenty-nine-item questionnaire^(47,48) measuring five types of coping strategies when confronting an adverse event: 'problem solving'; 'avoidance with wishful thinking'; 'seeks social support'; 'positive re-evaluation'; 'self-blamed'. Coping is currently defined as 'the various cognitive or behavioural efforts intended to master or tolerate the internal or external demands which threaten or go beyond the resources of a subject'⁽⁵⁸⁾.

Urinary free cortisol. Free cortisol in urine represents a direct filtration fraction of blood-free cortisol and tends to parallel the cortisol production rate. Cortisol is usually referred to as the 'stress hormone' as it is involved in response to stress and anxiety^(48,49). The 24 h collection time reflects the amount of cortisol that is released over a complete circadian cycle. This measure is insensitive to over- or underestimates obtained on moment-to-moment sampling that can occur due to transient fluctuations. R&D Systems' Cortisol Immunoassay (Ref KGE008) is a competitive enzyme immunoassay designed to measure cortisol in urine. This assay is based on competitive binding of cortisol with a fixed amount of horseradish peroxidase-labelled cortisol for sites on a mouse monoclonal antibody. During the incubation, the monoclonal antibody becomes bound to the goat anti-mouse antibody coated on the microplate. Following a wash to remove excess conjugate and the unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The colour development is stopped, and the absorbance is read at 450 nm.

Statistical analyses. Per protocol evaluations were carried out for all efficacy parameters. Raw data from subjects completing all tests on both sessions were delivered on case report form paper and entered with double data

entry. Data are expressed as the means and standard deviations (age) and as medians with interquartile ranges with inferior and superior quartile values. The parameters included the HSCL-90 global severity index and subscores of the HSCL-90, the HADS global score and HADS-A and HADS-D subscores, the PSS score, the CCL score and the cortisol level. As the assumptions required for parametric tests were not met, the comparisons between groups and repeated measures in each group were performed with the non-parametric MWT and Wilcoxon test (WT), respectively. Differences were considered significant at $P < 0.05$. The SPSS statistical software package version 11.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses.

Results

Preclinical study

Conditioned defensive burying. The Kruskal–Wallis test shows a group difference in the stress/anxiety score ($H(df = 2) = 13.76$; $P = 0.001$), which was lower in rats treated with PF (47.50 (36.25–68.75)) and diazepam (33.50 (33.50–41.13)) than with vehicle (62.25 (53.00–84.75)) (MWT: $U = 36$; $P = 0.04$ and $U = 9.5$; $P = 0.0004$, respectively).

Clinical study

Hopkins Symptom Checklist-90. As shown in Table 2, the percentage change in the global severity index after 30 d between baseline and follow-up was higher in the PF-treated subjects than in the PL-treated subjects (MWT: $z = 1.98$; $P < 0.05$), particularly due to improved somatisation, depression and anger–hostility subscales (MWT: $z = 2.16$; $P = 0.03$, $z = 1.96$; $P < 0.05$ and $z = 2.41$; $P = 0.02$, respectively).

Hospital Anxiety and Depression Scale. The percentage changes in HADS and HADS-A scores were higher in the PF-treated subjects (MWT: $z = 2.19$; $P = 0.03$ and $z = 1.92$; $P = 0.06$, respectively) with baseline scores being equivalent (Table 3). No significant differences were observed for HADS-D scores between the two groups at baseline (MWT: $z = 1.66$; $P < 0.10$) and over time (MWT: $z = 0.02$; $P = 1$). However, the HADS-D subscore of PF-treated subjects decreased between the two sessions (WT: $z = 2.65$; $P = 0.008$), whereas that of the control subjects remained stable (WT: $z = 0.60$; $P = 0.55$) (Table 3).

Perceived Stress Scale. As indicated in Table 3, no group differences were observed for PSS scores at baseline (MWT: $z = 0.36$; $P = 0.72$) and over time (MWT: $z = 0.36$; $P = 0.72$).

Coping Checklist. PL subjects increased their positive re-evaluation score between baseline and follow-up (WT: $z = 2.79$; $P = 0.005$), borderline for their problem solving score (WT: $z = 1.91$; $P = 0.06$), while PF subjects decreased their self-blame score and displayed a higher problem

Table 2. Effects of the probiotic formulation (PF) (n 26) and placebo (PL) (n 29) on Hopkins Symptom Checklist-90 (HSCL-90) scores at baseline (BL) and follow-up (FU) (Medians with inferior quartile (IQ) and superior quartile (SQ) values)

	PF						PL									
	BL			FU			BL			FU						
	Median	IQ–SQ	Change between BL and FU (%)	Median	IQ–SQ	Change between BL and FU (%)	Median	IQ–SQ	Change between BL and FU (%)	Median	IQ–SQ	Change between BL and FU (%)				
Global severity index	49	31–67	44.4	25	12–40	22.6–64.2	42	23.5–75.5	30.7*	17.5–55.5	5.5–51.8	32	5	32	30.7*	5.5–51.8
Somatisation	7.5	4–13	57.1	3.5	1–7	33.5–75	5.5	3–8.5	25*	1–8.5	0–57.5	5	5	5	25*	0–57.5
Obsessive–compulsive	8	3–11	34.8	4.5	1–8	0–66.7	8.5	4–13	28.6	3–8	–3.4–55	5.5	5.5	5.5	28.6	–3.4–55
Interpersonal sensitivity	4.5	2–6	22.5	2	1–6	0–79.2	4	3–9.5	20.8	1.5–7.5	–25–55.6	5	5	5	20.8	–25–55.6
Depression	8	5–11	50	4	1–7	22.7–71.1	7	4–11.5	25*	2–11.5	–10.7–50	6	6	6	25*	–10.7–50
Anxiety	5	3–9	50	2	1–4	27.7–82.2	4.5	2–6	50	1–4	0–76.4	2	2	2	50	0–76.4
Anger–hostility	2.5	1–5	60	1	0–3	38.2–100	3	1–5	33.3*	1–4	0–62.5	2	2	2	33.3*	0–62.5
Phobic anxiety	0	0–2	41.7	0	0–1	–25–100	1	0–2.5	66.7	0–1	0–100	0	0	0	66.7	0–100
Paranoid ideation	2.5	1–5	50	1	0–4	0–100	3.5	0.5–5.5	33.3	0–5	–20–60.6	2	2	2	33.3	–20–60.6
Psychoticism	2	0–4	37.5	0	0–3	0–100	2	1–5	50	0–4	2.8–84.3	1	1	1	50	2.8–84.3
Additional items	4	3–9	33.3	3	2–5	0–60.6	4.5	3–8.5	26.1	2–6	0–54.2	4	4	4	26.1	0–54.2

* Median values were significantly different when PF is compared with PL: $P < 0.05$ (Mann–Whitney U test).

Table 3. Effects of the probiotic formulation (PF) (*n* 26) and placebo (PL) (*n* 29) on Hospital Anxiety and Depression Scale (HADS), HADS-anxiety (HADS-A), HADS-depression (HADS-D) and Perceived Stress Scale (PSS) scores at baseline (BL) and follow-up (FU) (Medians with inferior quartile (IQ) and superior quartile (SQ) values)

	PF						PL					
	BL		FU		Change between BL and FU (%)		BL		FU		Change between BL and FU (%)	
	Median	IQ–SQ	Median	IQ–SQ	Median	IQ–SQ	Median	IQ–SQ	Median	IQ–SQ	Median	IQ–SQ
HADS	14	12–18	9	7–14	30.4	16.7–52.9	12	11–15.3	9	8–13.5	18.8**	–8.5–36.6
HADS-A	8	7–10	6	4–7	36.9	20–50	8	6–10.3	6	4–8	25*	–2.8–38
HADS-D	6	3–7	3.5***	2–7	31.7	0–57.1	5	3–6	4	2–6	16.7	–27.8–50
PSS	43	38–45	36.5	29–39	16.5	5.3–29.5	41	37–45.3	35	30.5–40	13	4.9–19.9

* Median values tended to be different when PF is compared with PL: $P < 0.10$ (Mann–Whitney *U* test).

** Median values were significantly different when PF is compared with PL: $P < 0.05$ (Mann–Whitney *U* test).

*** Median values were significantly different when BL is compared with FU in each group: $P < 0.01$ (Wilcoxon test).

solving score between the two test sessions (WT: $z = 2.50$; $P = 0.01$ and $z = 2.05$; $P = 0.04$, respectively) (Table 4).

Urinary free cortisol. Four PF subjects and two PL ones were discarded from analysis for not collecting their urines during 24 h. No significant differences were observed between the cortisol levels at baseline and over time (MWT: $z = 1.11$; $P = 0.27$ and $z = 0.01$; $P = 1$, respectively). However, the median urinary free cortisol level in ng/ml of PF-treated subjects decreased between baseline (50.5 (39.8–68)) and follow-up (43.7 (29.2–56.6)) (WT: $z = 2.03$; $P = 0.04$), whereas that of controls did not (47.4 (33.1–57.7) and 44.2 (31.7–52.7), respectively; WT: $z = 1.08$; $P = 0.28$).

Discussion

In the conditioned defensive burying test of anxiety, in which rats pile bedding on the source of perceived stress^(40,41), PF was better than PL, and similar to diazepam as the standard reference substance. These results favour the hypothesis of anxiolytic properties for this compound. It remains to be determined whether other anxiety tests will be equally sensitive, as one test does not necessarily generalise to others.

We next assessed whether a daily dose of *L. helveticus* R0052 and *B. longum* R0175 taken in combination over 30 d influenced the psychological impact of everyday life events in normal volunteers. PF-treated subjects had a lower global severity index of the HSCL-90 over time than PL-treated controls, due to lower values for somatisation, depression and anger–hostility. The potential usefulness of PF as an anti-stress/anti-anxiety agent is further supported by diminished HADS global scores over time, due to a lower HADS-A subscore. Taken together, PF appears to show a beneficial effect on general signs of anxiety and depression, which did not generalise to the PSS, although all three tests comprise self-reported measures. It remains to be determined whether the PSS is sensitive during a longer treatment period. The CCL provides an assessment of coping strategies used to counter the stress of daily life. The two groups differed in emotional

reactivity, with subjects administered PF reducing their self-blame score, while controls increasing their positive re-evaluation score. Moreover, PF-treated volunteers reported being more focused on the problem solving dimension than controls. In addition, cortisol values of PF-treated subjects decreased over time, while that of controls remained stable. Diop *et al.*⁽³⁴⁾ reported beneficial effects of the same mixture administered for 3 weeks on self-reported stress-related GI disturbances. But unlike the present results, they observed no effect of treatment on psychological symptoms. This discrepancy may be due to the duration of the period of administration of the preparation and/or to the use of a different questionnaire on stress-induced symptoms at the beginning and the end of the trial⁽³⁴⁾.

Other probiotics provide favourable results on behaviour. *L. helveticus* was demonstrated to favour sleep in elderly subjects⁽³⁹⁾. The *Lactobacillus casei* Shirota strain improved mood scores in normal subjects⁽⁵⁹⁾ and decreased anxiety in patients with chronic fatigue syndrome⁽⁶⁰⁾. It is interesting to note that the latter treatment increased the GI content of *Lactobacillus* and *bifidobacteria*.

The beneficial effects of probiotics on anxiety and depression may be explained by competitive exclusion of deleterious gut pathogens, decreases in pro-inflammatory cytokines and communication with the central nervous system via vagal sensory fibres, leading to changes in neurotransmitter levels or function^(1,61–63). As for the first explanation, marked alterations of the GI microflora occur in autism, including increases in various *Clostridium* spp., competitively displaced as other potentially pathogenic gut bacteria by *Lactobacillus*^(63,64). It has been shown that the addition of *B. longum* R0175, one of the strains used here, increased the number of bifidobacteria in the GI content of pigs⁽³⁵⁾.

Clostridium and *Bacteroides* spp. produce propionic acid, a SCFA increasing anxiety and aggression in animals⁽⁶⁵⁾, as well as increasing social isolation and stereotypes while decreasing play⁽⁶⁶⁾. While *L. helveticus* R0052 had never been tested in competition with *Clostridium*,

Table 4. Effects of the probiotic formulation (PF) (n 26) and placebo (PL) (n 29) on Coping Checklist scores (coping strategies) at baseline (BL) and follow-up (FU) (Medians with inferior quartile (IQ) and superior quartile (SQ) values)

	PF						PL					
	BL		FU		Change between BL and FU (%)		BL		FU		Change between BL and FU (%)	
	Median	IQ-SQ	Median	IQ-SQ	Median	IQ-SQ	Median	IQ-SQ	Median	IQ-SQ	Median	IQ-SQ
Problem solving	18	16-22	21**	17-23	6.5	-20-0	19	16-22	20*	17.8-23	4.3	-18.4-5.1
Avoidance with wishful thinking	14	11-16	13	11-15	13.3	-9.1-27.3	14	11-16.3	13	10.8-16	7.1	-9.1-18.3
Seeks social support	12.5	10-15	12	11-16	3.3	12.5-7.1	13	10.8-14.3	14	11-16	0	-18.3-8.3
Positive re-evaluation	12	10-13	12	10-13	4.2	-22.2-12.5	11	10-12	12***	10-14	8.3	-27.6-0
Self-blame	9	7-10	8***	7-9	10.6	0-22.2	9	7.8-10.3	8	7-10	0	-11.7-14.9

WT, Wilcoxon test.

* Median values tended to be different when PF is compared with PL: $P < 0.10$ (Mann-Whitney U test).

** Median values were significantly different when BL is compared with FU in each group: $P < 0.05$ (WT).

*** Median values were significantly different when BL is compared with FU in each group: $P < 0.01$ (WT).

this strain was recently demonstrated to protect GI microflora against the invasion of pathogenic bacteria⁽³⁶⁾.

The role of inflammatory processes on emotion is indicated by findings of a link between depression and elevated levels of IL-6, TNF and C-reactive protein⁽⁶⁷⁾. Systemically injected cytokines induce depressive symptoms^(68,69), prevented by antidepressants⁽⁷⁰⁾. It has been suggested that antidepressants act in part via generation of perhaps the most potent immunoregulatory cytokine, IL-10, thereby suppressing inflammation and depressive mood⁽⁷¹⁾. *Lactobacillus* and *Bifidobacterium* strains attenuated inflammatory responses or else induced IL-10 production in rodents⁽⁷²⁻⁷⁴⁾. In accordance with this finding, both *L. helveticus* R0052 and *B. longum* R0175 showed anti-inflammatory properties in human cell lines⁽³⁷⁾. Thus, bacteria may be used to influence mood in patients with elevated inflammatory chemicals⁽⁷⁵⁾.

The normal activity of the HPA axis is regulated by diurnal excitatory inputs, stress-induced stimulation and various negative feedback loops, mediated by corticotrophin-releasing hormone, adrenocorticotrophin hormone and to a large extent by cortisol⁽⁷⁶⁾. However, the ability of cortisol to regulate its own production may be impaired during chronic stress, resulting in sustained increase in its plasma level⁽⁷⁷⁾. In the present study, the daily administration of PF for 30d significantly decreased urinary free cortisol levels in subjects under daily life events as a source of stress. The administration of bacteria may support resilience and positively alter stress-related emotional behaviour in stressed animals⁽⁷⁸⁾. To our knowledge, no clinical study has yet reported on measurements of cortisol evolution following oral subchronic treatment with probiotics. However, in preclinical studies, corticosterone levels decreased in rat pups in response to lactobacilli strains⁽²⁸⁾. Likewise, germ-free mice had an increased responsiveness of the HPA axis compared with specific pathogen-free mice, reversed with a probiotic treatment before 6 weeks of age⁽²⁵⁾. Enterochromaffin cells, the source of serotonin in the bowel, may be involved, since these are affected by enteric flora and release neuroendocrine mediators activating afferents to the HPA axis as well as the paraventricular hypothalamus, amygdala and bed nucleus of the stria terminalis controlling stress responses and mood⁽²⁴⁾.

Conclusion

Consumption of the PF containing *L. helveticus* R0052 and *B. longum* R0175 in combination mitigated psychological distress in three tests without displaying any adverse event. These results provide further evidence that gut microflora play a role in stress, anxiety and depression, perhaps via the enteric nervous system as well as centrally. Subject to the confirmation of these results, probiotics might offer a useful novel therapeutic approach to neuropathological disorders and/or as adjunct therapies in psychiatric disorders⁽⁷⁵⁾. Though these data are preliminary,

preclinical and clinical investigations should be extended to examine specific gut microbes and physiological markers associated with psychological distress.

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