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Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice

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Key Messages

- Take-home message: Bifidobacteria reduced stress-related behaviours in mice in a bacterial-strain dependent manner and more efficiently than a widely used antidepressant drug. This supports the concept of psychobiotic therapies for stress-related disorders.
- Research aims: To assess the psychobiotic potential of two Bifidiobacteria strains.
- Basic methodology: Innately anxious male BALB/c mice were fed with either of two Bifidobacteria, vehicle or an antidepressant (escitalopram) for 3 initial weeks before undergoing a battery of tests related to stress, anxiety and depression. Key stress-related physiological parameters were also measured..
- Results summary: *B. longum 1714* reduced stress, anxiety and depression-related behaviours whereas *B. breve 1205* reduced general anxiety behaviours and induced weight loss. Escitalopram had fewer or no effects on these parameters and induced weight gain.

Abstract

Background *Accumulating evidence suggests that commensal bacteria consumption has the potential to have a positive impact on stress-related psychiatric disorders. However, the specific bacteria influencing behaviors related to anxiety and depression remain unclear. To this end, we compared the effects of two different Bifidobacteria on anxiety and depression-like behavior; an antidepressant was also used as a comparator.* ***Methods*** *Innately anxious BALB/c mice received daily Bifidobacterium longum (B.) 1714, B. breve 1205, the antidepressant escitalopram or vehicle treatment for 6 weeks. Behavior was assessed in stress-induced hyperthermia test, marble burying, elevated plus maze, open field, tail suspension test, and forced swim test. Physiological responses to acute stress were also assessed.* ***Key Results*** *Both Bifidobacteria and escitalopram reduced anxiety in the marble burying test; however, only B. longum 1714 decreased stress-induced hyperthermia. B. breve 1205 induced lower anxiety in the elevated plus maze whereas B. longum 1714 induced antidepressant-like behavior in the tail suspension test. However, there was no difference in corticosterone levels between groups.* ***Conclusions & Inferences*** *These data show that these two Bifidobacteria strains reduced anxiety in an anxious mouse strain. These results also suggest that each bacterial strain has intrinsic effects and may be beneficially specific for a given disorder. These findings strengthen the role of gut microbiota supplementation as psychobiotic-based strategies for stress-related brain-gut axis disorders, opening new avenues in the field of neurogastroenterology.*

Keywords *anxiety, BALB/c mice, behavior, bifidobacteria, corticosterone, depression-related behavior, stress.*

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INTRODUCTION

Increasing evidence suggests that the microbiome-brain-gut axis, a major regulator of homeostasis and gastrointestinal (GI) functions, also plays a key role in the generation of stress and psychiatric disorders.[1,2] Indeed, alterations in microbiota have been linked to irritable bowel syndrome (IBS), a functional GI disorder highly comorbid with stress and anxiety.[2–5] Interestingly, exposure to early-life or social stress has recently been shown to be able to alter the enteric microbiota.[6,7] Moreover, it has been shown that germ-free mice or mice models of infections displayed enhanced stress axis responses and altered anxiety-related behavior.[8–11] Interestingly, mouse germ-free studies showed that the divergent innate anxiety-related phenotypes of two different mouse strains was transferred to the other strain once the corresponding microbiota was transplanted.[12]

Commensal bacteria, or probiotics, actively interact with the endogenous enteric microbiota and gut cells, therein conferring health benefit to the host.[13] Although data are somewhat limited, it has been shown that *Lactobacilli* (L.) and *Bifidobacteria* (B.) species have in particular been shown to display potential therapeutic properties in psychiatric disorders.[14,15] *Lactobacilli* strains have also been shown to normalize corticosterone release, reverse stress-induced colonic alterations[16] and improve anxiety associated with chronic fatigue syndrome.[17] Moreover, we have recently shown that *L. rhamnosus* was able to reduce the innate anxiety and stress response of healthy and non-manipulated BALB/c mice[18] and a *B. longum* was shown to decrease anxiety in both healthy and DSS-induced colitis AKR mice or mice infected with *T. muris*.[19,20]

Bifidobacteria are notably used as beneficial food supplements in dairy products and play a protective role against pathogenic bacteria and allergies.[21–23] Notably, *Bifidobacteria* of the genera *longum* and *breve* were shown, or hypothesized, to have positive effects on the immune system,[24] IBS,[25,26] depression,[27,28] anxiety[19] and neurodegenerative diseases.[29,30] Questions remain as to whether all *Bifidobacteria* strains are equal in their ability to have positive effects on behavior.

Therefore, in this study, we investigated the potential of two different *Bifidobacteria* strains, *B. longum 1714* and *B. breve 1205*, to alter the behavior of healthy BALB/c mice, as we have previously shown with *L. rhamnosus*.[18] This mouse strain was specifically selected as they have been proposed to be a model of pathological anxiety,[31] a good strain to model IBS in animals[32] and they have been used to characterize the *in vivo* effects of probiotics.[33]

MATERIALS AND METHODS

Animals

Male BALB/cOlaHsd (BALB/c) mice, aged 7 week-old were purchased from Harlan Laboratories, UK. Mice remained group-housed (3–4) in plexiglas cages (33 × 15 × 13 cm, L × W × H) under standard laboratory conditions (22 ± 1 °C, humidity 55 ± 5%) on a 12-h light/dark cycle (lights on 7.30 a.m.), and were provided with standard laboratory diet and water *ad libitum*. The sex and strain of mice was chosen to compare with our previous studies investigating the behavioral effects of potential probiotics.[18] Animals were housed in a specific room and treatments groups were separated from each other to avoid cross-contamination. All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork.

Commensal bacteria and antidepressant treatment

B. longum 1714 and *B. breve 1205* were kindly donated by Alimentary Health Ltd., Cork, Ireland as freeze-dried stocks (−80 °C). Bacteria were daily reconstituted in sterile phosphate buffered saline (PBS) to a final concentration of 1×10^9 CFU/mL ingested by mice.[25] The selective serotonin reuptake inhibitor (SSRI) escitalopram (S-enantiomer of Citalopram) Discovery Fine Chemicals (Dorset, UK) was used as a control antidepressant. Escitalopram was made up fresh daily in PBS and the dose administered was 20 mg/kg.[34] Vehicle-treated animals received PBS only. All treatments were given orally.

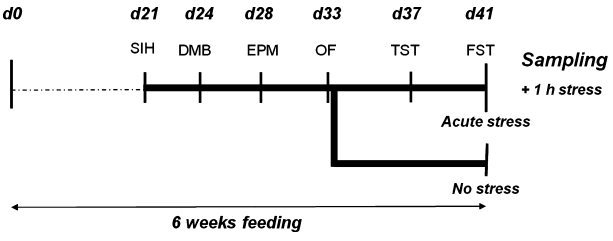

Figure 1 Representation of the study design. BALB/c mice were fed for a total period of 6 weeks with *Bifidobacterium longum 1714, B. breve 1205*, an antidepressant (escitalopram) or vehicle (PBS). All groups were weighed daily. After 3-week feeding, animals underwent a battery of testing relevant to anxiety. Half of the animals were then assessed for antidepressant-related behavior and acute stress, whereas the other half remained untested. All groups were sacrificed on the same day, either + 1 h post stress (forced swim test, FST) or 8 days post test (open field, OF). Blood and tissue were harvested for further physiological analysis. D, day; SIH, stress-induced hyperthermia; DMB, defensive marble burying; EPM, elevated plus maze; OF, open field; TST, tail suspension test; FST, forced swim test.

Study design

The experimental design is presented in Fig. 1. After at least 5-day habituation to the animal facility, mice were fed daily (between 6.00 and 7.00 p.m.) for 6 weeks with *B. longum 1714*, *B. breve 1205*, escitalopram or vehicle, using sterile gavage needles. Bodyweight was monitored throughout; behavioral testing was conducted starting with the least to the most stressful test,[35] from week 3 of feeding. Mice were tested in a random fashion regarding litters and treatments groups (four different litters per group), with an experimenter blind to conditions. Except for stress-induced hyperthermia, animals were brought to the experimental room 30 min prior testing, which occurred between 8.00 a.m. and 4.00 p.m. (8.00 a.m–12.00 p.m. for the forced swim test). Unless specified otherwise, experiments occurred under normal room lighting (100 lux at 1 m above the floor). All material were cleaned with 70% ethanol between animals. Briefly, at 10 weeks old, mice (n = 22 per group) were tested in a battery of anxiety tasks (see Fig. 1) and further split into two groups: half of the mice (n = 11) remained undisturbed in their home cage until the end of the experiments, whereas the other half (n = 11) was subjected to the tail suspension test and forced swim stress. All animals were sacrificed on the same day, thus1 h post stress for stressed mice.

Behavioral assessment

Stress-induced hyperthermia This test measures anxiety state as a function of body temperature increase in response to stress and was conducted as previously described.[36] Briefly, 24 h prior to testing, all mice were individually housed in standard plexiglas cages (33 × 15 × 13 cm, L × W × H). The following day, body temperature was measured between $T1$ (T = 0) and $T2$, 15 min later. A sterile mouse thermometer was gently inserted 20 mm in the rectum of mice hung by the tail until stable thermometer measurement was reached (~15 s). Body temperature was measured to the nearest 0.1 °C; difference between $T1$ and $T2$ (ΔT) reflected the stress-induced hyperthermia.

Defensive marble burying This test assesses compulsive and anxious behavior, and is based on mice defensive burying behavior and was conducted as previously described.[37] A higher number of marbles buried represents higher levels of anxiety. Mice were individually placed in a novel plexiglas cage (35 × 28 × 18.5 cm, L × W × H), filled up with sawdust (4 cms) and 20 marbles on top of it (five rows or marbles regularly spaced 2 cms away from the walls and 2 cms apart). Thirty minutes later, the number of marbles buried for more than 2/3 of their surface was counted.

Elevated plus maze This test assesses general anxiety, and is based on the conflict mice experience between the attraction and the fear for a novel environment; less anxious mice spend more time in fearful areas.[38] The set up was made of a gray plastic cross-shaped maze 1 m elevated from the floor, comprising two open (fearful) and two closed (safe) arms (50 × 5 × 15 cms walls or 1 cm no wall). Experiments occurred under red light (~5 lux). Mice were individually placed into the center of the maze facing an open arm (to avoid direct entrance into a closed one) and were allowed 5-min free exploration. Experiments were videotaped using a ceiling camera for further parameters analysis using Ethovision software (3.1 version, Noldus, TrackSys, Nottingham, UK). The percentage of time spent, distance moved and the number of entries in each arm were measured, for anxiety behavior and locomotor activity, respectively (entrance in an arm was defined as all four paws inside the arm).

Open field To assess the response to a novel stressful environment and locomotor activity, mice were placed into a brightly lit (400 lux) white open arena (43 × 35 × 25 cms, $L \times w \times h$) and allowed 10-min free exploration. Experiments were videotaped using a ceiling camera for further parameters analysis using Ethovision software. The distance travelled was scored for locomotor activity and the number of fecal outputs counted for stress-induced defecation.[39] The latency to enter a virtual central zone (defined at 2.5 cms away from the edges) was also scored to assess anxiety.

Tail suspension test This test assesses antidepressant activity and depression-like behavior and was conducted as previously described.[40] Mice were individually hung by the tail with adhesive tape (2 cms from tail tip) to a grid bar 30-cm elevated from the floor and the test lasted 6 min. Experiments were videotaped using a numeric tripod-fixed camera and data were further scored twice using the videos (Video Media Player software) and averaged by an experimenter blind to conditions. The time spent immobile (s) was scored; lower percentage of immobility reflecting lower depression-like behavior; immobility is defined as the absence of voluntary or escape-orientated movement.

Forced swim test This test assesses antidepressant-like behavior and was used as an acute stressor.[41] Mice were individually placed in a transparent plexiglas cylinder (24 × 21 cms diameter), containing 15-cm-depth water (25 ± 0.5 °C). Water was changed between each animal to remove odors. The test lasted 6 mins and experiments were videotaped using a numeric tripod-fixed camera; data were further scored twice using the videos (Video Media Player software) and averaged by an experimenter blind to conditions. The latency to immobility was scored. The time of immobility (s) was measured for the 4 last min of the test, with immobility being defined as a total absence of movement except slight motions to maintain the head above the water.

Tissue collection

Animals were sacrificed in a random fashion regarding treatment and testing condition; sampling occurred between 9.00 a.m. and 1:00 p.m. Trunk blood was collected in potassium EDTA (Ethylene Diamine Tetra Acetic Acid) tubes and spun for 15 min at 7000 g. Plasma was isolated and stored at −80 °C for further corticosterone analysis. As stress and anxiety are associated with physiological changes in peripheral organs, and as we hypothesized that *Bifidobacteria* may improve the stressed and anxious phenotype of BALB/c mice, additional routine stress-sensitive physiological parameters were scored.[32] The colon was removed, mechanically cleaned and its length measured to 0.1 cm precision, as colon length reduction is observed in case of colonic inflammation following stress[42]; thymus, heart, spleen, and adrenals were also weighed as thymus and adrenals hypotrophy, heart hypertrophy and splenomegaly are observed following chronic stress, due to the impact stress has on the immune system, immune cells survival, and interactions with the autonomic nervous system and metabolic pathways.[43–46]

Corticosterone assay

Corticosterone levels were measured in the plasma from all animals using an Enzyme Immunoassay Kit (Enzo Life Sciences,

Inc., Farmingdale, NY, USA) according to the manufacturer's instructions. Samples were analyzed in duplicate in a single assay using 20 μL plasma per sample; the threshold detection was less than 32 pg/mL; coefficient of variation limit=20%; the concentrations are expressed in ng/mL.

Data analysis

Data were analyzed using SPSS software, version 15 (IBM SPSS Statistics, IBM Corporation, Armonk, NY, USA). For bodyweight gain evolution, data were analyzed using a one-way ANOVA repeated measures. For individual day's comparison of bodyweight and all other data, a one-way ANOVA was conducted, followed by Fisher's *post hoc* test. Non-parametric data (Shapiro–Wilk test) were analyzed with a Kruskal–Wallis test followed by Dunn's *post hoc* test. Unpaired *t*-test, or Mann–Whitney test, were used for two-group comparison where appropriate. Statistical significance was set at $p < 0.05$. Data are expressed as mean ± SEM. Due to technical issues in the animal facility (fire alarms), a number of mice had to be excluded as they had been stressed at the time of testing. Thus, only data from 10 to 12 mice per group for the stress-induced hyperthermia test and from 15 to 17 mice per group for the marble burying were included in the analysis.

RESULTS

Bodyweight gain

Total bodyweight gain (Fig. 2A, between week 6 and 1) differed between groups ($F(3,80) = 7.968$, $p < 0.0001$), with *post hoc* test revealing that escitalopram increased it ($p < 0.05$) whereas *B. breve 1205* decreased it ($p < 0.01$). Further analysis of bodyweight gain over time (data not shown) revealed an effect of treatment ($F(3,83) = 9.43$, $p < 0.0001$), time ($F(5,415) = 149.18$, $p < 0.0001$) and a treatment x time interaction ($F(15,415) = 1.84$, $p < 0.05$). *Post hoc* test confirmed that escitalopram increased bodyweight gain at week 1, 3 and 4 ($p < 0.05$; week 6 $p = 0.054$) compared with vehicle, whereas *B. breve 1205* decreased it at week 2, 4 ($p < 0.05$), week 5 ($p = 0.01$), and 6 ($p < 0.05$). Although *B. longum 1714* had no overall effect in one-way ANOVA analysis, *post hoc* test on individual days showed that this bacterium induced lower bodyweight gain than vehicle treatment on week 1 and 5 ($p < 0.05$).

Anxiety-related behavior

Stress-induced hyperthermia (Fig. 2B) significantly differed between groups ($H(df = 3) = 9.970$, $p < 0.05$), as *B. longum 1714* induced a lower body temperature increase in mice than vehicle treatment ($p < 0.05$). Basal temperature (*T*1) did not differ between groups ($F(3,39) = 1.846$, $p = 0.1548$, data not shown).

In the marble burying test, the number of marbles buried (Fig. 2C) significantly differed between groups ($H(df = 3) = 13.18$, $p < 0.01$) as escitalopram, *B. longum 1714* and *B. breve 1205* all induced a reduction in the number of marbles buried compared with vehicle treatment ($p < 0.05$, $p < 0.001$, $p < 0.01$, respectively).

In the elevated plus maze, groups differed in their percentage of time spent (Fig. 3A), and distance moved (data not shown), in the open arms ($H(df = 3) = 8.821$, $p < 0.05$, and $H(df = 3) = 10.971$, $p < 0.05$, respectively), as *B. breve 1205*-treated animals spent more time, and travelled a greater distance, in the open arms than vehicle group ($p < 0.05$ for both parameters). However, locomotor activity (Fig. 3B) did not differ between animals as assessed by the total number of entries ($H(df = 3) = 3.164$, $p = 0.367$).

In the open field, the latency to enter the central zone differed between groups (Fig. 3C, $H(df = 3) = 8.457$, $p < 0.05$), as *B. longum 1714* induced a lower latency to enter the middle part of the arena than vehicle ($p < 0.05$). There was also no difference between treat-

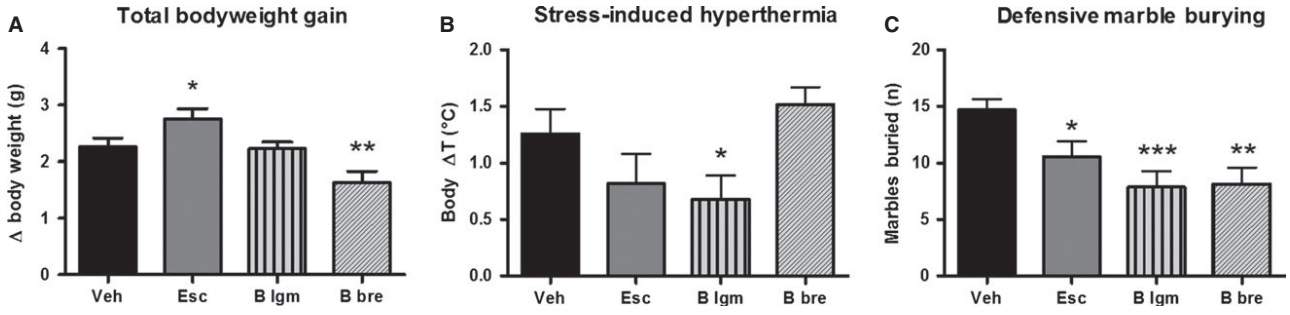

Figure 2 Effect of the two *Bifidobacteria* and escitalopram on bodyweight gain, stress-induced hyperthermia and defensive marble burying tests. *B. breve 1205* induced a decrease in bodyweight gain (A), whereas escitalopram increased it. *B. longum 1714* had no effect. $N = 20$–22 per group. *B. longum 1714* induced a lower body temperature increase in the stress-induced hyperthermia test compared with vehicle group, whereas *B. breve 1205* and escitalopram had no effect (B). $N = 10$–12 per group. All treatments induced a decrease in the number of marbles buried in the defensive marble burying test (C), but more significantly so in the two *Bifidobacteria*-fed animals. $N = 15$–17 per group. Veh, vehicle; Esc, escitalopram; B lgm, *B. longum 1714*; B Bre, *B. breve 1205*. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, treatment *vs* vehicle groups. Data are expressed as means ± SEM.

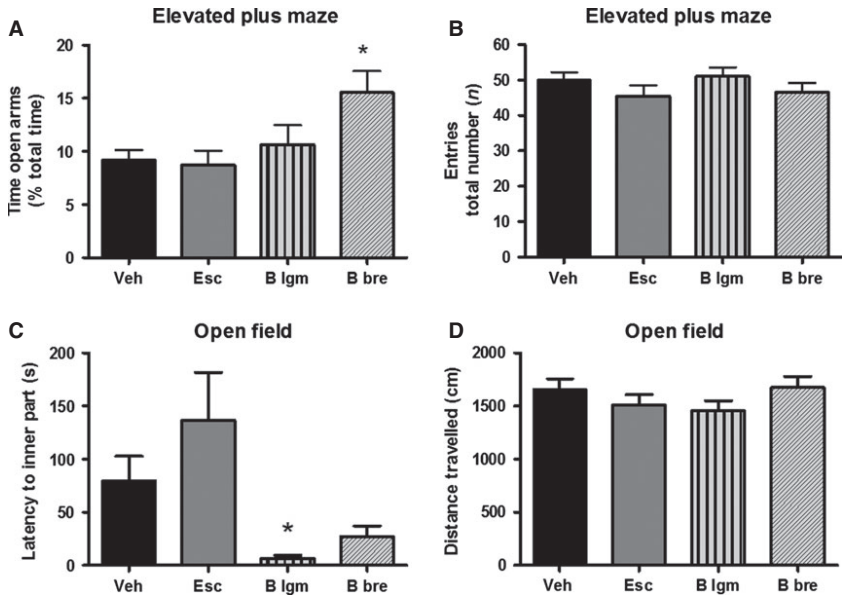

Figure 3 Effect of the two *Bifidobacteria* and escitalopram in the elevated-plus-maze and the open-field. *B. breve 1205* induced an increased % time spent (A) in the open arms, whereas *B. longum 1714* and escitalopram had no effect compared with vehicle-treated animals. The total number of entries did not differ between groups (B). $N = 19–21$ per group. *B. Longum 1714* induced a shorter latency to enter the inner part of the open-field than vehicle-treated animals (C), whereas *B. breve 1205* and escitalopram had no effect. There was no difference in the distance travelled (D) between treatments. $N = 19–22$ per group. Veh, vehicle; Esc, escitalopram; B lgm, *B. longum 1714*, B Bre, *B. breve 1205*. $^{*}p < 0.05$, treatment *vs* vehicle groups. Data are expressed as means ± SEM.

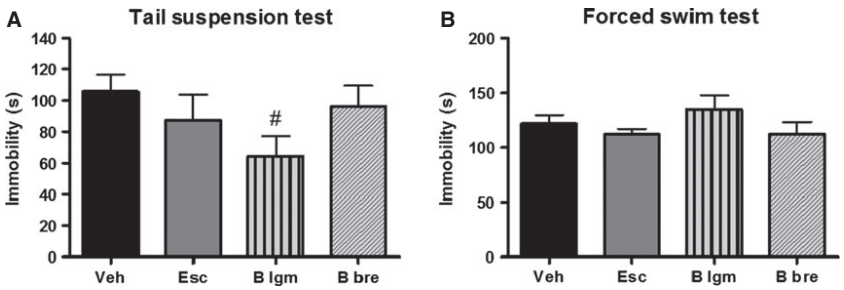

Figure 4 Effect of the two *Bifidobacteria* and escitalopram in the tail suspension test and the forced swim test. *B. Longum 1714* induced a reduced time of immobility in the tail suspension test compared with vehicle-treated animals (A), whereas *B. breve 1205* and escitalopram had no effect. However, there was no difference in the forced swim test between treatments (B). $N = 9–11$ per group. Veh, vehicle; Esc, escitalopram; B lgm, *B. longum 1714*; B Bre, *B. breve 1205*. $^{\#}p < 0.05$, *t*-test *B. Longum 1714 vs* vehicle groups. Data are expressed as means ± SEM.

ments in the distance travelled in the arena (Fig. 3D, $F(3,83) = 1.363$, $p = 0.26$) and in stress-induced defecation (data not shown, $H(df = 4) = 5.030$, $p = 0.1696$).

Antidepressant-related tests

Treatment groups did not statistically differ in the tail suspension test in the time spent immobile (Fig. 4A, $F(3,38) = 1.582$, $p = 0.210$), however *B. longum 1714* induced a significant 40% reduction in immobility time compared with vehicle treatment when assessed in an *a priori t*-test 2-group comparison ($p < 0.05$). In the forced swim test (Fig. 4B), none of the treatments induced any difference between animals in the time spent immobile ($H(df = 3) = 2.781$, $p = 0.4266$) or the latency to immobility ($H(df = 3) = 0.903$, $p = 0.825$, data not shown).

Physiological parameters

Results are presented in Table 1. There was a significant difference in spleen weight between groups ($F(3,73) = 7.66$, $p < 0.001$), as escitalopram decreased it ($p < 0.05$), whereas *B. breve 1205* increased it ($p < 0.05$). However, there was no difference between groups in thymus weight ($F(3,75) = 0.607$, $p = 0.613$), heart weight ($F(3,74) = 2.395$, $p = 0.075$), adrenal glands weight (right adrenal, $H(df = 3) = 4.52$, $p = 0.211$, left adrenal, $H(df = 3) = 1.304$, $p = 0.7282$), and colon length ($F(3,72) = 1.44$, $p = 0.238$).

Regarding corticosterone levels, there was a significant effect of stress ($F(1,69) = 21.30$, $p < 0.0001$), but no effect of treatment ($F(3,69) = 1.84$, $p = 1480$) or stress x treatment interaction ($F(3,69) = 2.36$, $p = 0.0788$). Specifically, stress induced a marked

increase in corticosterone and none of the treatment groups affected either basal or stress-induced levels.

DISCUSSION

Increasing evidences suggest that the microbiome-gut-brain axis can regulate behavior and emotions.[1,47–49] Here, we demonstrate that two *Bifidobacterium* strains, a *B. longum (1714)* and a *B. breve (1205)*, can improve behaviors relevant to stress in an innately anxious mouse strain.[31] A summary of the results is presented in Table 2. To our knowledge, this is the first study showing that *Bifidobacteria* can reduce anxiety in a normal unperturbed mouse, i.e. without previous stressed or physiological manipulation, as we showed with *L. rhamnosus*.[18]

Data from the defensive marble burying test suggest a positive effect of both bacteria on anxiety and compulsive disorders. Furthermore, *B. longum 1714* positive effects in the stress-induced hyperthermia and the tail suspension test suggests a positive role in sensitivity to acute stress and depression. *B. breve 1205* induced an anxiolytic effect in the elevated plus maze and reduced bodyweight gain, suggesting a role in general anxiety and metabolism. Of note, neither strain induced a change in locomotor activity or in antidepressant-related behavior in the forced swim test. Thus, these results confirm that each bacterial

Table 1 Effect of the two *Bifidobacteria* and escitalopram on tissue weight, colon length and corticosterone levels

% bodyweight	Vehicle	Escitalopram	*B. longum*	*B. breve*
Spleen	0.3608 ± 0.0049	0.3414 ± 0.0069*	0.3728 ± 0.0063	0.3803 ± 0.0062*
Thymus	0.1114 ± 0.004	0.1076 ± 0.0042	0.1038 ± 0.0042	0.1049 ± 0.005
Heart	0.4348 ± 0.0072	0.4156 ± 0.0053	0.4183 ± 0.005	0.4161 ± 0.006
Right adrenal	0.00647 ± 0.00118	0.00337 ± 0.00021	0.00462 ± 0.005	0.00556 ± 0.0011
Left adrenal	0.00563 ± 0.00096	0.00484 ± 0.00073	0.0048 ± 0.00073	0.00564 ± 0.0005
Colon length (cm)	9.8 ± 0.2	10.4 ± 0.3	9.7 ± 0.2	10.07 ± 0.3
Corticosterone basal levels (ng/ml)	6.29 ± 1.58	10.68 ± 2.81	14.34 ± 4.53	6.64 ± 0.57
Corticosterone stress levels (ng/ml)	66.76 ± 16 + 47^{+++}	40.74 ± 7.97^{++}	50 ± 12.36^{++}	127.2 ± 46.6^{+++}

Tissue weight data are expressed as % bodyweight. Colon length is expressed in cms. Corticosterone levels are expressed in ng/ml. Escitalopram reduced spleen weight whereas *B. breve 1205* increased it, compared with vehicle-treated animals. Stress induced a significant increase in corticosterone levels at + 1 hour posttest (forced swim test, FST), however, there was no difference between treatment groups for any other physiological parameter measured. N = 9–22 per group for tissue parameters and N = 8–11 per group for corticosterone levels. Veh, vehicle; Esc, escitalopram; B *longum*, *B. longum 1714*, B *Breve*, *B. breve 1205*. $^{*}p < 0.05$, treatment *vs* vehicle groups; $^{++}p < 0.01$, $^{+++}p < 0.001$, stress *vs* basal levels corticosterone. Data are expressed as means ± SEM.

Table 2 Summary of the effects of the two *Bifidobacteria* on anxiety, antidepressant-like behavior, acute stress

Feeding time	Test	Escitalopram	*B. longum*	*B. breve*
3 weeks	Stress-induced hyperthermia	Trend reduced ΔT	Reduced ΔT	= ΔT
3 weeks ½	Marble burying	Reduced marble buried	Reduced marbles buried	Reduced marbles buried
4 weeks	Elevated plus maze	= time open arms = activity	=time open arms = activity	Increased time open arms = activity
4 weeks ½	Open field	= activity = latency = fecal output	= activity Reduced latency = fecal output	= activity = latency = fecal output
5 weeks	Tail suspension test	= immobility	Reduced immobility	= immobility
6 weeks	Forced swim test	= immobility	= immobility	= immobility
6 weeks	Bodyweight gain (bwg)	Increased bwg	= bwg	Reduced bwg
6 weeks	Corticosterone	=basal and stress-induced levels	=basal and stress-induced levels	= basal and stress-induced levels
6 weeks	Tissue weight	Reduced spleen weight	= weight	Increased spleen weight

Study = 6 weeks feeding, behavior started at + 3 weeks feeding; anxiety + depression-like behavior + acute stress; *B. longum* = 1714; *B. breve* = 1205; n = 10–12 for stress sensitivity (stress-induced hyperthermia), n = 15–17 for anxiety/obsessive compulsive disorders (marble burying), n = 19–22 for general anxiety and locomotor activity (elevated plus maze, open field), n = 9–11 for acute stress and antidepressant-related behavior (tail suspension test, forced swim test), n = 8–11 for HPA-axis activity (corticosterone levels in the plasma), n = 9–22 for secondary stress-sensitive physiologic parameters (bodyweight gain, 20–22, and tissue weight, 9–22); ΔT = temperature increase.

strain displays specific characteristics and therapeutic potential, which emerge in only a few of the behavioral tasks assessed. This reinforces the crucial role of using a battery of tests, as has been widely done so for screening of pharmacological agents.[35]

We chose to assess the effects of two types of *Bifidobacteria* on behavior, due to the well-described beneficial role on health and their potential action on stress-related disorders.[15,22] Stress and anxiety are strongly linked to a dysfunction of the microbiome–brain–gut axis communication, therefore modulating the enteric microbiota is increasingly viewed as a new therapeutic approach for psychiatric and stress-related disorders.[2,48] Conversely, probiotics have been suggested to correct for stress-induced alterations,[16] post-infectious stress[19,50] and we have shown that *L. rhamnosus* have the potential to improve anxiety and stress response in BALB/c mice, along with changes in central GABA (gamma-aminobutyric acid) receptors expression.[18] Others also showed that both *Bifidobacteria* and *Lactobacilli* can have beneficial effects in anxiety in rodents and humans[17,19,51–54] and may have a potential in neurodegenerative disorders.[29,55]

Regarding the stress-sensitive physiological parameters measured in the present study, spleen, and body weight were altered by escitalopram and *B. breve 1205*, whereas the other parameters remained unchanged. These parameters are primary indicators of further stress-induced potential changes and give a first insight in the brain-gut axis and the potential systems involved behind the behavioral changes observed; as such, these parameters are routinely measured in stress studies, in our laboratory, and others.[32,42–44] Based on these studies, the fact that spleen, but not thymus weight, was changed, may reflect potential direct effects of escitalopram and *B. breve 1205* on a preferential subpopulation of immune cells (possibly B as opposed to T lymphocytes) recruited in the spleen. Given the fact that probiotics are widely reported to affect the immune system in a particular way for each bacterial strain,[56] our results provide valuable first insights and future directions in studying the potential mechanisms of action of the *Bifidobacteria* we used. As colon length and heart weight remained unchanged between groups, none of the treatments altered, at a macroscopic level, colon or heart tissue integrity. Although these parameters are key-features in understanding stress response, they have not been measured yet in other studies using probiotics; thus we highlight here the importance of such measurements. The meaning of our findings might be understood via deeper analysis and thus warrants further immunological and molecular studies. Regarding bodyweight, the fact that escitalopram increased weight is not surprising as this antidepressant, and in general, psychotropic drugs, have been reported to induce this effect,[57,58] although there is controversy in the literature.[59,60] It is possible that this effect is related to gut microbiota changes as bodyweight gain induced by antipsychotic treatment has been associated with microbiota modifications and microbiota has been shown to alter the efficacy of various drugs.[61–63] Moreover, obesity has recently been linked to changes in gut microbiota and to a specific gut population profile, high *Bifidobacteria* content being rather associated with lean individuals.[64–66] Moreover, probiotics are also hypothesized to have effects on bodyweight changes.[67,68] Therefore, it is possible that both escitalopram and *B. breve 1205* induced metabolic changes via gut microbiota modifications. These effects could be mediated via various mechanisms and notably gut hormones as gut hormones signaling to the brain has been linked to obesity.[69] However, the exact mechanisms behind all this remain unknown.

Furthermore, none of the probiotics altered baseline or stress-induced corticosterone levels compared with vehicle-treated animals. Other studies using *Bifidobacteria* also reported an absence of effect on corticosterone levels, either in healthy or stressed rats.[25,27,28] However, germ-free mice have been shown to display altered HPA-axis[11] and we, and others, observed that *Lactobacilli* blunted stress-induced corticosterone increase in rodents or humans.[16,18,50,53] Altogether, these data suggest that commensal bacteria effects may be strain-dependent and highlights and confirms that differences between *Lactobacilli* and *Bifidobacteria* strains exist at a neurobehavioral level. Very importantly, since all probiotics may have differential specific effects, and as evidenced in the literature, it will be very important in the future to investigate the effects of multiple bacterial strains in order to multiply the beneficial effects and cover a wider spectrum of psychiatric disorders.[16,53,70] Such cocktail-based strategies are also being adopted for human studies of probiotics in CNS indications[52,71].

We specifically chose to use the antidepressant escitalopram, which is a SSRI, as a comparator in this study. We administered escitalopram for a time and at a dose that are clinically relevant[33,58] and after 3 weeks of daily feeding, which is the time generally required for antidepressant onset of action.[72,73] We also used a battery of tests which constitute key-animal models of good predictability to screen potential anxiolytic and antidepressant treatments in human.[35,37,74–76] However, escitalopram only had a positive effect in the

defensive marble burying test and increased body-weight gain. Although these results could question the relevance of using this antidepressant, escitalopram was used due to its widely reported positive effects on anxiety and depression-related behavior[77–80] and its capability in reducing basal corticosterone levels in rats.[81] And also, escitalopram is one of the most widely used new-generation SSRIs in the clinic, which is described to give better outcome than other antidepressants with fewer negative side-effects and may have a neurotrophic role.[72,82] Moreover, at least 30% of depressed patients are reported to be resistant to SSRIs and up to 60% to overall therapeutics, such resistance being linked to deficits in the serotonin system.[83,84] Selective serotonin reuptake inhibitors such as escitalopram affect the serotonin system[79] and the anxious BALB/c mice naturally display lower serotonin rates than other mice, due to a mutation in the gene encoding for the serotonin precursor tryptophan.[85,86] As a result, it was particularly relevant for us to investigate the effects of escitalopram and the two *Bifidobacteria* in BALB/c mice. The lower serotonin levels in BALB/c mice are hypothesized to underlie their particular phenotype, for which either resistance or high sensitivity have been reported.[87–90] Therefore, there is a discrepancy in the literature regarding escitalopram and SSRIs effects, both in depressed patients and in rodents.[41,91]

It is also worth noting that there is a clear discrepancy in animal models of anxiety in their ability to detect the anxiolytic effects of chronic SSRIs preclinically.[76,92] Indeed, of the anxiety tests investigated, the defensive marble burying test is one of the few that has been shown to be sensitive to the positive effects of SSRIs on anxiety.[92] As a result, our data with escitalopram fit with the literature and we show here the important fact that the two *Bifidobacteria* we used have the potential to induce better results in anxiety and depressive-like behavior in BALB/c mice than SSRIs. Such findings may have crucial implications for all of these patients who are resistant to antidepressant therapy.

The mechanisms underlying commensal bacteria action on the microbiome–brain–gut axis remain so far poorly understood. A large amount of studies have shown that exogenous bacteria actively interact with the gut epithelial cells, the endogenous enteric microbiota and the host immune system, which all interact with the brain-gut axis.[47,48] Notably, studies showed that *L. reuteri* directly modulated colonic neurons potentially implicated in pain perception.[93–95] The fermentation products from a *B. longum* (*NCC3001*) were also able to decrease enteric neurons firing, suggesting that this bacteria communicates to the CNS via enteric nerves.[19] More generally, studies suggest that both *Lactobacilli* and *Bifidobacteria* genera could modulate enteric neurons.[19,94,95] And also, overall, studies converge to implicate the vagus nerve as one of the main routes of communication between the enteric microbiota, gut nerves and the CNS, with the vagus nerve being shown to be involved in both the anxiogenic or anxiolytic effects of bacterial infections or commensal bacteria, respectively.[18,19,96] Thus, it is possible that the *Bifidobacteria* we used in the present study directly interacted with colonic neurons, or to other gut (epithelial) cells signaling to the brain, therein ultimately inducing changes in behavior.

Furthermore, peripheral activation of the immune system induces cytokines release in the blood stream that can induce the activation of CNS neurons.[97] Thus, in the present study, the overall behavioral differences observed with the two *Bifidobacteria* may lie in differential effects on the immune system and possibly cell-interaction properties locally in the gut, which appear to be bacterial strain-dependent.[13,33] Such differences may then induce differential signaling to the brain, either via a humoral or neuronal route.[15] Indeed, although the mechanisms underlying the effects of these two bacteria are not known, other studies showed that microbes of the same genera may act via multiple mechanisms involving epithelial cells, dendritic cells, and T cells.[98,99] And also, *B. infantis* (*35624*), a *B. longum subspecies*, could promote specific anti-inflammatory T cells (Treg cell) conversion and protect against aberrant immune system reaction.[99] Moreover, in germ-free mice, a *B. longum (AH1206)* increased Treg cell numbers *in vivo*, whereas *B. breve 1205* and a *L. salivarius* (*AH102*) had no effect.[100] Altogether, these data suggest that *B. longum* bacterial strain may impact on behavior via their anti-inflammatory properties. However, a recent study[19] showed that the beneficial effects of a *B. longum* (*NCC3001*) on anxiety did not appear to be related to its anti-inflammatory properties, but rather to its impact on enteric neurons and vagus nerve signaling. Altogether, these findings suggest that the mechanisms underlying probiotics action are complex and may be intrinsic to each strain, warranting further investigations.

At a molecular level, *Bifidobacteria* may possibly act, among other candidates, on the serotonergic system. Indeed, 5-HT plays a key role in modulation of emotional and GI function[101] and we have previously shown that the serotonergic precursor tryptophan was elevated by *B. infantis 35624*.[27] The reason why *B. breve 1205* reduced anxiety in fewer tests than

B. longum 1714, and decreased bodyweight gain, is unclear but highlights that even within a species differential effects are evident on behavior and physiology. Interestingly, recent studies have shown that another *B. breve* strain was able to significantly impact brain fatty acids content and obesity.[30,102] However, all these hypotheses are based on a few investigations only as the literature lacks comprehensive mechanistic studies on the effects of probiotics on the CNS. Moreover, there are many other potential candidates involved in the effects of probiotics, which could be any of the neurotransmitters involved in stress, depression and anxiety such as GABA.[18] Hence, further detailed and deeper investigations in the levels of neurotransmitters in the brain following probiotics consumption are greatly needed, and would participate in the understanding of the effects of probiotics, along with the measurement of stress-sensitive physiological parameters. Moreover, future studies must focus on separating the differential neurobiological mechanisms underlying the differential changes observed here between the two *Bifidobacteria.*

CONCLUSIONS

The two *Bifidobacterium* strains used in this study were able to improve the anxious phenotype of innately anxious BALB/c mice in a strain-specific manner and to a larger extent than that induced by the antidepressant escitalopram. These findings give further credence to the concept of 'psychobiotics' recently proposed as a novel strategy for treating psychiatric disorders.[15] These data suggest that the two *Bifidobacteria* could be used to treat a wide range of psychiatric disorders in a strain-dependent manner for a given disease, just like conventional psychotropic treatments do. These data add to the recent and emerging literature showing that bacteria exert a crucial role on the microbiome-brain-gut axis, stress, and behavior.[1,103–105] The mechanisms underlying such action remain largely unknown; however, these findings open up new avenue for treating psychiatric diseases, in a way that may be better than current pharmacological treatments. Further studies characterizing the molecular and underlying mechanisms of bacteria action on behavior are now highly warranted.

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CONFLICTS OF INTEREST

The Center has conducted studies in collaboration with several companies including GSK, Pfizer, Alimentary Health, Cremo, Suntory Wellness, Danone-Nutricia, Wyeth and Mead Johnson. The authors have spoken at meetings sponsored by food and pharmaceutical companies.

AUTHOR CONTRIBUTION

HMS, BK, TGD and JFC designed the research study; HMS performed the research, analyzed the data and wrote the paper; TGD and JFC corrected and revised the paper; BK provided the probiotic strains via Alimentary Health Ltd.

REFERENCES

1 Collins SM, Surette M, Bercik P. The interplay between the intestinal microbiota and the brain. *Nat Rev Microbiol* 2012; **10**: 735–42.
2 Dinan TG, Cryan JF. Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology. *Psychoneuroendocrinology* 2012; **37**: 1369–78.
3 Carroll IM, Ringel-Kulka T, Keku TO, Chang YH, Packey CD, Sartor RB, Ringel Y. Molecular analysis of the luminal- and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2011; **301**: G799–807.
4 Jeffery IB, O'Toole PW, Ohman L, Claesson MJ, Deane J, Quigley EM, Simren M. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut* 2012; **61**: 997–1006.
5 Lutgendorff F, Akkermans LM, Soderholm JD. The role of microbiota and probiotics in stress-induced gastro-intestinal damage. *Curr Mol Med* 2008; **8**: 282–98.
6 Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M. Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain Behav Immun* 2011; **25**: 397–407.
7 O'Mahony SM, Savignac HM, O'Brien T, Scully P, Quigley EM, Marchesi J, O'Toole PW, Dinan TG *et al.* Early-life dysbiosis-induced visceral hypersensitivity in adulthood. *Gas-*

troenterology 2010; **138**: S-1-S-906-Supplement 901.
8 Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, Dinan TG, Cryan JF. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol Psychiatry* 2013; **18**: 666–73.
9 Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* 2012; **13**: 701–12.
10 Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterol Motil* 2011; **23**: 255–64 e119.
11 Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, Kubo C, Koga Y. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol* 2004; **558**: 263–75.
12 Collins SM, Kassam Z, Bercik P. The adoptive transfer of behavioral phenotype via the intestinal microbiota: experimental evidence and clinical implications. *Curr Opin Microbiol* 2013; **16**: 240–5.
13 Shanahan F, Dinan TG, Ross P, Hill C. Probiotics in transition. *Clin Gastroenterol Hepatol* 2012; **10**: 1220–4.
14 Bercik P, Collins SM, Verdu EF. Microbes and the gut-brain axis. *Neurogastroenterol Motil* 2012; **24**: 405–13.
15 Dinan TG, Stanton C, Cryan JF. Psychobiotics: a novel class of psychotropic. *Biol Psychiatry* 2013; **74**: 720–6.
16 Gareau MG, Jury J, MacQueen G, Sherman PM, Perdue MH. Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. *Gut* 2007; **56**: 1522–8.
17 Rao AV, Bested AC, Beaulne TM, Katzman MA, Iorio C, Berardi JM, Logan AC. A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. *Gut Pathog* 2009; **1**: 6.
18 Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci U S A* 2011; **108**: 16050–5.
19 Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, Deng Y, Blennerhassett PA *et al.* The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut-brain communication. *Neurogastroenterol Motil* 2011a; **23**: 1132–9.
20 Bercik P, Verdu EF, Foster JA, Macri J, Potter M, Huang X, Malinowski P, Jackson W *et al.* Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. *Gastroenterology* 2010; **139**: 2102–12 e2101.
21 Ishibashi N, Yaeshima T, Hayasawa H. Bifidobacteria: their significance in human intestinal health. *Mal J Nutr* 1997; **3**: 149–59.
22 Leahy SC, Higgins DG, Fitzgerald GF, van Sinderen D. Getting better with bifidobacteria. *J Appl Microbiol* 2005; **98**: 1303–15.
23 Wopereis H, Oozeer R, Knipping K, Belzer C, Knol J. The first thousand days - intestinal microbiology of early life: establishing a symbiosis. *Pediatr Allergy Immunol* 2014; **25**: 428–38.
24 O'Mahony L, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B *et al.* Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology* 2005; **128**: 541–51.
25 McKernan DP, Fitzgerald P, Dinan TG, Cryan JF. The probiotic *Bifidobacterium* infantis 35624 displays visceral antinociceptive effects in the rat. *Neurogastroenterol Motil* 2010; **22**(1029–1035): e1268.
26 Whorwell PJ, Altringer L, Morel J, Bond Y, Charbonneau D, O'Mahony L, Kiely B, Shanahan F *et al.* Efficacy of an encapsulated probiotic *Bifidobacterium* infantis 35624 in women with irritable bowel syndrome. *Am J Gastroenterol* 2006; **101**: 1581–90.
27 Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG. The probiotic Bifidobacteria infantis: an assessment of potential antidepressant properties in the rat. *J Psychiatr Res* 2008; **43**: 164–74.
28 Desbonnet L, Garrett L, Clarke G, Kiely B, Cryan JF, Dinan TG. Effects of the probiotic *Bifidobacterium* infantis in the maternal separation model of depression. *Neuroscience* 2010; **170**: 1179–88.
29 O'Sullivan E, Barrett E, Grenham S, Fitzgerald P, Stanton C, Ross RP, Quigley EM, Cryan JF *et al.* BDNF expression in the hippocampus of maternally separated rats: does *Bifidobacterium breve* 6330 alter BDNF levels? *Benef Microbes* 2011; **2**: 199–207.
30 Wall R, Ross RP, Shanahan F, O'Mahony L, Kiely B, Quigley E, Dinan TG, Fitzgerald G *et al.* Impact of administered bifidobacterium on murine host fatty acid composition. *Lipids* 2010; **45**: 429–36.
31 Belzung C, Griebel G. Measuring normal and pathological anxiety-like behaviour in mice: a review. *Behav Brain Res* 2001; **125**: 141–9.
32 Savignac HM, Hyland NP, Dinan TG, Cryan JF. The effects of repeated social interaction stress on behavioural and physiological parameters in a stress-sensitive mouse strain. *Behav Brain Res* 2011; **216**: 576–84.
33 Dunne C, Murphy L, Flynn S, O'Mahony L, O'Halloran S, Feeney M, Morrissey D, Thornton G *et al.* Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antonie Van Leeuwenhoek* 1999; **76**: 279–92.
34 Ji Y, Hebbring S, Zhu H, Jenkins GD, Biernacka J, Snyder K, Drews M, Fiehn O *et al.* Glycine and a glycine dehydrogenase (GLDC) SNP as citalopram/escitalopram response biomarkers in depression: pharmacometabolomics-informed pharmacogenomics. *Clin Pharmacol Ther* 2011; **89**: 97–104.
35 Crawley JN. Behavioral phenotyping strategies for mutant mice. *Neuron* 2008; **57**: 809–18.
36 Cryan JF, Kelly PH, Neijt HC, Sansig G, Flor PJ, van Der Putten H. Antidepressant and anxiolytic-like effects in mice lacking the group III metabotropic glutamate receptor mGluR7. *Eur J Neurosci* 2003; **17**: 2409–17.
37 Jacobson LH, Bettler B, Kaupmann K, Cryan JF. Behavioral evaluation of mice deficient in GABA(B(1)) receptor isoforms in tests of unconditioned anxiety. *Psychopharmacology* 2007; **190**: 541–53.

38 Rodgers RJ, Johnson NJ. Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. *Pharmacol Biochem Behav* 1995; **52**: 297–303.

39 Barone FC, Barton ME, White RF, Legos JJ, Kikkawa H, Shimamura M, Kuratani K, Kinoshita M. Inhibition of phosphodiesterase type 4 decreases stress-induced defecation in rats and mice. *Pharmacology* 2008; **81**: 11–7.

40 Cryan JF, Mombereau C, Vassout A. The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. *Neurosci Biobehav Rev* 2005; **29**: 571–625.

41 Jacobson LH, Cryan JF. Feeling strained? Influence of genetic background on depression-related behavior in mice: a review. *Behav Genet* 2007; **37**: 171–213.

42 Reber SO, Obermeier F, Straub RH, Falk W, Neumann ID. Chronic intermittent psychosocial stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced colitis and impairs regeneration. *Endocrinology* 2006; **147**: 4968–76.

43 Bartolomucci A, Palanza P, Sacerdote P, Panerai AE, Sgoifo A, Dantzer R, Parmigiani S. Social factors and individual vulnerability to chronic stress exposure. *Neurosci Biobehav Rev* 2005; **29**: 67–81.

44 Engler H, Engler A, Bailey MT, Sheridan JF. Tissue-specific alterations in the glucocorticoid sensitivity of immune cells following repeated social defeat in mice. *J Neuroimmunol* 2005; **163**: 110–9.

45 Krishnan V, Han MH, Graham DL, Berton O, Renthal W, Russo SJ, Laplant Q, Graham A *et al.* Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* 2007; **131**: 391–404.

46 Reber SO, Birkeneder L, Veenema AH, Obermeier F, Falk W, Straub RH, Neumann ID. Adrenal insufficiency and colonic inflammation after a novel chronic psycho-social stress paradigm in mice: implications and mechanisms. *Endocrinology* 2007; **148**: 670–82.

47 Dinan TG, Cryan JF. Melancholic microbes: a link between gut microbiota and depression? *Neurogastroenterol Motil* 2013; **25**: 713–9.

48 Forsythe P, Kunze WA. Voices from within: gut microbes and the CNS. *Cell Mol Life Sci* 2013; **70**: 55–69.

49 Rhee SH, Pothoulakis C, Mayer EA. Principles and clinical implications of the brain-gut-enteric microbiota axis. *Nat Rev Gastroenterol Hepatol* 2009; **6**: 306–14.

50 Gareau MG, Wine E, Rodrigues DM, Cho JH, Whary MT, Philpott DJ, Macqueen G, Sherman PM. Bacterial infection causes stress-induced memory dysfunction in mice. *Gut* 2011; **60**: 307–17.

51 Ait-Belgnaoui A, Durand H, Cartier C, Chaumaz G, Eutamene H, Ferrier L, Houdeau E, Fioramonti J *et al.* Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology* 2012; **37**: 1885–95.

52 Arseneault-Breard J, Rondeau I, Gilbert K, Girard SA, Tompkins TA, Godbout R, Rousseau G. Combination of Lactobacillus helveticus R0052 and *Bifidobacterium longum* R0175 reduces post-myocardial infarction depression symptoms and restores intestinal permeability in a rat model. *Br J Nutr* 2012; **107**: 1793–9.

53 Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejdi A, Bisson JF, Rougeot C *et al.* Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. *Br J Nutr* 2011; **105**: 755–64.

54 Ohland CL, Kish L, Bell H, Thiesen A, Hotte N, Pankiv E, Madsen KL. Effects of Lactobacillus helveticus on murine behavior are dependent on diet and genotype and correlate with alterations in the gut microbiome. *Psychoneuroendocrinology* 2013; **38**: 1738–47.

55 Matthews DM, Jenks SM. Ingestion of Mycobacterium vaccae decreases anxiety-related behavior and improves learning in mice. *Behav Processes* 2013; **96**: 27–35.

56 Ashraf R, Shah NP. Immune system stimulation by probiotic microorganisms. *Crit Rev Food Sci Nutr* 2014; **54**: 938–56.

57 Hasnain M, Vieweg WV. Weight considerations in psychotropic drug prescribing and switching. *Postgrad Med* 2013; **125**: 117–29.

58 Wade AG, Crawford GM, Yellowlees A. Efficacy, safety and tolerability of escitalopram in doses up to 50 mg in Major Depressive Disorder (MDD): an open-label, pilot study. *BMC psychiatry* 2011; **11**: 42.

59 Uher R, Mors O, Hauser J, Rietschel M, Maier W, Kozel D, Henigsberg N, Souery D *et al.* Changes in body weight during pharmacological treatment of depression. *Int J Neuropsychopharmacol* 2011; **14**: 367–75.

60 Unterecker S, Deckert J, Pfuhlmann B. No influence of body weight on serum levels of antidepressants. *Ther Drug Monit* 2011; **33**: 730–4.

61 Davey KJ, Cotter PD, O'Sullivan O, Crispie F, Dinan TG, Cryan JF, O'Mahony SM. Antipsychotics and the gut microbiome: olanzapine-induced metabolic dysfunction is attenuated by antibiotic administration in the rat. *Transl Psychiatry* 2013; **3**: e309.

62 Kang MJ, Kim HG, Kim JS, Oh do G, Um YJ, Seo CS, Han JW, Cho HJ *et al.* The effect of gut microbiota on drug metabolism. *Expert Opin Drug Metab Toxicol* 2013; **9**: 1295–308.

63 Viaud S, Saccheri F, Mignot G, Yamazaki T, Daillere R, Hannani D, Enot DP, Pfirschke C *et al.* The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. *Science* 2013; **342**: 971–6.

64 Delzenne NM, Cani PD. Interaction between obesity and the gut microbiota: relevance in nutrition. *Annu Rev Nutr* 2011; **31**: 15–31.

65 Million M, Angelakis E, Maraninchi M, Henry M, Giorgi R, Valero R, Vialettes B, Raoult D. Correlation between body mass index and gut concentrations of Lactobacillus reuteri, *Bifidobacterium* animalis, Methanobrevibacter smithii and Escherichia coli. *Int J Obes (Lond)* 2013; **37**: 1460–6.

66 Scarpellini E, Campanale M, Leone D, Purchiaroni F, Vitale G, Lauritano EC, Gasbarrini A. Gut microbiota and obesity. *Intern Emerg Med* 2010; **5**(Suppl. 1): S53–6.

67 Angelakis E, Bastelica D, Ben Amara A, El Filali A, Dutour A, Mege JL, Alessi MC, Raoult D. An evaluation of the effects of Lactobacillus ingluviei on body weight, the intestinal microbiome and metabolism in mice. *Microb Pathog* 2012; **52**: 61–8.

68 Delzenne NM, Neyrinck AM, Backhed F, Cani PD. Targeting gut microbiota in obesity: effects of prebiotics and probiotics. *Nat Rev Endocrinol* 2011; **7**: 639–46.

69 Schellekens H, Dinan TG, Cryan JF. Ghrelin at the interface of obesity and reward. *Vitam Horm* 2013; **91**: 285–323.

70 Diop L, Guillou S, Durand H. Probiotic food supplement reduces stress-induced gastrointestinal symptoms in volunteers: a double-blind, placebo-controlled, randomized trial. *Nutr Res* 2008; **28**: 1–5.

71 Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, Ebrat B, Guyonnet D, Legrain-Raspaud S *et al.* Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology* 2013 **144**: 1394–401, 1401 e1391–1394.

72 Cipriani A, Santilli C, Furukawa TA, Signoretti A, Nakagawa A, McGuire H, Churchill R, Barbui C. Escitalopram versus other antidepressive agents for depression. *Cochrane Database Syst Rev* 2009; (2). doi: 10.1002/14651858. CD006532.pub2.

73 Sekar S, Verhoye M, Van Audekerke J, Vanhoutte G, Lowe AS, Blamire AM, Steckler T, Van der Linden A *et al.* Neuroadaptive responses to citalopram in rats using pharmacological magnetic resonance imaging. *Psychopharmacology* 2011; **213**: 521–31.

74 Cryan JF, Markou A, Lucki I. Assessing antidepressant activity in rodents: recent developments and future needs. *Trends Pharmacol Sci* 2002; **23**: 238–45.

75 Cryan JF, Slattery DA. Animal models of mood disorders: recent developments. *Curr Opin Psychiatry* 2007; **20**: 1–7.

76 Cryan JF, Sweeney FF. The age of anxiety: role of animal models of anxiolytic action in drug discovery. *Br J Pharmacol* 2011; **164**: 1129–61.

77 Bhagya V, Srikumar BN, Raju TR, Shankaranarayana Rao BS. Chronic escitalopram treatment restores spatial learning, monoamine levels, and hippocampal long-term potentiation in an animal model of depression. *Psychopharmacology* 2011; **214**: 477–94.

78 Fish EW, Faccidomo S, Gupta S, Miczek KA. Anxiolytic-like effects of escitalopram, citalopram, and R-citalopram in maternally separated mouse pups. *J Pharmacol Exp Ther* 2004; **308**: 474–80.

79 Sanchez C, Bergqvist PB, Brennum LT, Gupta S, Hogg S, Larsen A, Wiborg O. Escitalopram, the S-(+)-enantiomer of citalopram, is a selective serotonin reuptake inhibitor with potent effects in animal models predictive of antidepressant and anxiolytic activities. *Psychopharmacology* 2003; **167**: 353–62.

80 Zomkowski AD, Engel D, Gabilan NH, Rodrigues AL. Involvement of NMDA receptors and l-arginine-nitric oxide-cyclic guanosine monophosphate pathway in the antidepressant-like effects of escitalopram in the forced swimming test. *Eur Neuropsychopharmacol* 2010; **20**: 793–801.

81 Uys JD, Muller CJ, Marais L, Harvey BH, Stein DJ, Daniels WM. Early life trauma decreases glucocorticoid receptors in rat dentate gyrus upon adult re-stress: reversal by escitalopram. *Neuroscience* 2006; **137**: 619–25.

82 Bhagya V, Srikumar BN, Raju TR, Shankaranarayana Rao BS. Chronic escitalopram treatment restores spatial learning, monoamine levels, and hippocampal long-term potentiation in an animal model of depression. *Psychopharmacology* 2010; **214**(2), 477–94.

83 Fava M. Diagnosis and definition of treatment-resistant depression. *Biol Psychiatry* 2003; **53**: 649–59.

84 Zhang X, Gainetdinov RR, Beaulieu JM, Sotnikova TD, Burch LH, Williams RB, Schwartz DA, Krishnan KR *et al.* Loss-of-function mutation in tryptophan hydroxylase-2 identified in unipolar major depression. *Neuron* 2005; **45**: 11–6.

85 Browne CA, Clarke G, Dinan TG, Cryan JF. Differential stress-induced alterations in tryptophan hydroxylase activity and serotonin turnover in two inbred mouse strains. *Neuropharmacology* 2011; **60**: 683–91.

86 Cervo L, Canetta A, Calcagno E, Burbassi S, Sacchetti G, Caccia S, Fracasso C, Albani D *et al.* Genotype-dependent activity of tryptophan hydroxylase-2 determines the response to citalopram in a mouse model of depression. *J Neurosci* 2005; **25**: 8165–72.

87 Crowley JJ, Blendy JA, Lucki I. Strain-dependent antidepressant-like effects of citalopram in the mouse tail suspension test. *Psychopharmacology* 2005; **183**: 257–64.

88 Jacobsen JP, Nielsen EO, Hummel R, Redrobe JP, Mirza N, Weikop P. Insensitivity of NMRI mice to selective serotonin reuptake inhibitors in the tail suspension test can be reversed by co-treatment with 5-hydroxytryptophan. *Psychopharmacology* 2008; **199**: 137–50.

89 Kobayashi T, Hayashi E, Shimamura M, Kinoshita M, Murphy NP. Neurochemical responses to antidepressants in the prefrontal cortex of mice and their efficacy in preclinical models of anxiety-like and depression-like behavior: a comparative and correlational study. *Psychopharmacology* 2008; **197**: 567–80.

90 Siesser WB, Zhang X, Jacobsen JP, Sotnikova TD, Gainetdinov RR, Caron MG. Tryptophan hydroxylase 2 genotype determines brain serotonin synthesis but not tissue content in C57Bl/6 and BALB/c congenic mice. *Neurosci Lett* 2010; **481**: 6–11.

91 Trkulja V. Is escitalopram really relevantly superior to citalopram in treatment of major depressive disorder? A meta-analysis of head-to-head randomized trials. *Croat Med J* 2010; **51**: 61–73.

92 Borsini F, Podhorna J, Marazziti D. Do animal models of anxiety predict anxiolytic-like effects of antidepressants? *Psychopharmacology* 2002; **163**: 121–41.

93 Kunze WA, Mao YK, Wang B, Huizinga JD, Ma X, Forsythe P, Bienenstock J. Lactobacillus reuteri enhances excitability of colonic AH neurons by inhibiting calcium-dependent potassium channel opening. *J Cell Mol Med* 2009; **13**: 2261–70.

94 Ma X, Mao YK, Wang B, Huizinga JD, Bienenstock J, Kunze W. Lactobacillus reuteri ingestion prevents hyperexcitability of colonic DRG neurons induced by noxious stimuli. *Am J Physiol Gastrointest Liver Physiol* 2009; **296**: G868–75.

95 Wang B, Mao YK, Diorio C, Wang L, Huizinga JD, Bienenstock J, Kunze W. Lactobacillus reuteri ingestion and IK(Ca) channel blockade have similar effects on rat colon motility and myenteric neurones. *Neurogastroenterol Motil* 2010; **22**: 98–107 e133.

96 Tanida M, Yamano T, Maeda K, Okumura N, Fukushima Y, Nagai K. Effects of intraduodenal injection

of Lactobacillus johnsonii La1 on renal sympathetic nerve activity and blood pressure in urethane-anesthetized rats. *Neurosci Lett* 2005; **389**: 109–14.
97 Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci* 2008; **9**: 46–56.
98 Ng S, Hart AL, Kamm MA, Stagg AJ, Knight SC. Mechanisms of action of probiotics: recent advances. *Inflamm Bowel Dis* 2009; **15**: 300–10.
99 O'Mahony C, Scully P, O'Mahony D, Murphy S, O'Brien F, Lyons A, Sherlock G, MacSharry J *et al.* Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. *PLoS Pathog* 2008; **4**: e1000112.
100 Lyons A, O'Mahony D, O'Brien F, MacSharry J, Sheil B, Ceddia M, Russell WM, Forsythe P *et al.* Bacterial strain-specific induction of Foxp3 + T regulatory cells is protective in murine allergy models. *Clin Exp Allergy* 2010; **40**: 811–9.
101 Spiller R. Recent advances in understanding the role of serotonin in gastrointestinal motility in functional bowel disorders: alterations in 5-HT signalling and metabolism in human disease. *Neurogastroenterol Motil* 2007; **19**(Suppl. 2): 25–31.
102 Kondo S, Xiao JZ, Satoh T, Odamaki T, Takahashi S, Sugahara H, Yaeshima T, Iwatsuki K *et al.* Antiobesity effects of *Bifidobacterium breve* strain B-3 supplementation in a mouse model with high-fat diet-induced obesity. *Biosci Biotechnol Biochem* 2010; **74**: 1656–61.
103 Foster JA, McVey Neufeld KA. Gut-brain axis: how the microbiome influences anxiety and depression. *Trends Neurosci* 2013; **36**: 305–12.
104 Moloney RD, Desbonnet L, Clarke G, Dinan TG, Cryan JF. The microbiome: stress, health and disease. *Mamm Genome* 2014; **25**: 49–74.
105 Clarke G, O'Mahony S, Dinan TG, Cryan JF. Priming for health: gut microbiota acquired in early life regulates physiology, brain and behaviour. *Acta Paediatr* 2014; **103**: 812–9. doi:10.1111/apa.12674.