Research report

Strain specific stress-modulating effects of candidate probiotics: A systematic screening in a mouse model of chronic restraint stress

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ABSTRACT

Background: Changes in the gut microbiota have been implicated in mood and cognition. In rodents, supplementation with certain bacteria have been shown to alleviate adverse effects of stress on gut microbiota composition and behaviour, but little is known of how the performance of different strains compare to each other. We took a systematic approach to test the efficacy of twelve candidate probiotic strains from ten species/subspecies of Bifidobacterium and Lactobacillus on behaviours and neuroendocrine responses of chronically stressed mice.

Methods: The strains were tested in four screening experiments with non-stressed and chronically stressed vehicle groups. The three most efficacious strains were re-tested to validate the results. Mice were administered a daily oral gavage containing either 1 × 10⁹ colony forming units (CFU) of selected candidate probiotic or saline solution for one week prior to and for three weeks during daily chronic restraint stress. Behavioural tests including the elevated plus maze, open field, novel object recognition, and forced swim test were applied during week five. Corticosterone and adrenocorticotropic hormone (ACTH) were analysed to measure the neuroendocrine response to stress. Plasma and tissue samples were collected for biomarker analyses.

Results: Of the twelve candidate probiotics, Lactobacillus paracasei Lpc-37, Lactobacillus plantarum LP12407, Lactobacillus plantarum LP12418 and Lactobacillus plantarum LP12151 prevented stress-associated anxiety and depression-related behaviours from developing compared with chronically stressed vehicle mice. In addition, Lpc-37 improved cognition.

Conclusion: This systematic screening indicates species- and strain-dependent effects on behavioural outcomes related to stress and further suggests that strains differ from each other in their effects on potential mechanistic outcomes.

1. Introduction

In 1936, Hans Selye, a Hungarian endocrinologist who dedicated most of his life to stress research defined stress as "the non-specific response of the body to any demand for change" [1]. The psychological threat to the body proceeding this demand disrupts normal homeostasis through activation of the hypothalamic-pituitary adrenal (HPA) axis and ultimately culminates in the production of cortisol, our body’s fight-or-flight response to an acute stressor [2]. In time, daily chronic stress can entirely dysregulate the neuroendocrine response to stress and becomes a major risk factor in the development of neuropsychiatric disorders, including anxiety and depression, with persistent disruption to brain function and physiology [3,4], behaviour [5,6] and the gut microbiota [4,5]. Many of these stress-associated disruptions in the gut microbiota are not exclusively identified in animal studies, but are also relevant in humans, for example [7,8].

The gut is inhabited by a vast community of microbes that develop important commensal and symbiotic relationships with their host [9]. The microbiota-gut-brain axis refers to the bidirectional dialogue between the gut microbiota and the central nervous system (CNS), the extent of which has only recently begun to be unravelled [10]. It is now known that this communication system can impact on stress [11]. Germ-free mice also display an altered behavioural profile, compared with that of conventional mice, with altered anxiety-like behaviour.
coming to the forefront [12–15]. In line with this, germ-free mice also display an exaggerated HPA axis response to stress [12,16]. These findings highlight an intriguing role for the gut microbiome in altering neurophysiology and behaviour.

Considerable efforts have been leveraged to identify specific microbes that interact with the CNS and to develop such microbes into candidate probiotics suitable for human consumption [17]. Psychobiotics have been defined as live organisms that, when ingested in adequate amounts, produce a health benefit in patients suffering from psychiatric illness [18]. Although numerous bacterial strains have demonstrated psychobiotic efficacy on the CNS [19] and effects are generally considered strain specific, there is a lack of studies that investigate comparisons between strains of the same species and only a few have compared different species to each other. With the current limited understanding of mechanisms and therefore lack of in vitro screening methods for mood outcomes, behavioural tests are considered the gold standard for testing candidate probiotics for future clinical trials.

We took a systematic and comparative approach to test the effect of twelve candidate probiotic strains on stress-related behaviour and biomarkers of stress in a mouse model of chronic restraint stress. We used a behavioural test battery designed to include tests necessary to investigate anxiety- and depression-related behaviours and cognitive function. The three most effective strains were then re-tested in a follow-up validation experiment, designed in the same manner. Potential mechanisms were explored by measuring biomarkers of the immune system, neuroendocrine response, as well as the expression of GABA receptors in jejenum and prefrontal cortex and concentration of brain-derived neurotransfactor (BDNF) in hippocampus.

2. Materials and methods

2.1. Candidate probiotic strains

Twelve candidate probiotic strains representing ten different species/sub-species were selected for experiments: Bifidobacterium longum BG0014 (DuPont Global Culture Collection (DGCC) 2956), Bifidobacterium longum ssp. infantis BI11471 (DGCC11471), Bifidobacterium animalis BL0005 (DGCC2962), Bifidobacterium animalis ssp. lactis 420 (DGCC420), Lactobacillus paracasei Lpc-37 (DGCC4981), Lactobacillus salivarius LS-33 (DGCC9868; ATCC: SD5208), Lactobacillus plantarum LP12418 (DGCC12418), Lactobacillus plantarum LP12151 (DGCC12151), Lactobacillus plantarum LP12407 (DGCC12407), Lactobacillus acidophilus LA11873 (DGCC11873), Lactobacillus rhamnosus LX11881 (DGCC11881) and Lactobacillus helveticus LH0138 (DGCC451; ATCC: SD5587). All strains were owned and were produced for the experiments by DuPont Nutrition & Biosciences.

2.2. Animals

Five-week-old male Swiss mice weighing 30–35 g were purchased from JANVIER (Saint Berthevin, France) and group housed in a temperature and humidity-controlled animal facility with a 12 h/12 h light/dark cycle. Mice were housed six per cage with food (SAFE A04C, SAFE, Route de Saint Bris, 89290 AUGY, France) and water ad libitum and all mice in a cage receiving the same treatment. This study was conducted in Amylgen’s animal facility (Approval #A-34-169-002) in accordance with the recommendations of Directive 2010/63/EU, European Union commission.

The protocol #14423 was approved by the Languedoc Roussillon Ethic Committee CE2A-36. Routinely, the overall health and condition of the mice was visually monitored daily. Across all five experiments, n = 324 mice in total were used. Of these, n = 21 were euthanised prematurely due to poor general health or injuries sustained / heightened anxiety from aggressive behaviour, caused by group housing of adult male mice. A list of these mice is provided in Table S1. Unless otherwise shown in Table S1, n = 12 per group in each experiment.

2.3. Scheduling of procedures

The scheduling of procedures is captured in Fig. 1. Mice were treated with candidate probiotics for a total of five weeks by daily oral gavage. The chronic stress procedure was performed daily during weeks 2–4, and behavioural assessments were performed on the fifth week of candidate probiotic intervention over five consecutive days: Day 1) Elevated plus maze (EPM), Day 2) Open field (OF), Day 3) Novel object recognition (NOR) (same object session), Day 4) NOR (novel object session), and Day 5) Forced swim test (FST). Mice were then sacrificed by decapitation and plasma was collected for further analyses in all experiments. In experiment 5 (validation experiment), tissue samples including hippocampus, prefrontal cortex, colon, colon content, caecum, caecum content and jejunum were collected (validation experiment).

2.4. Treatments

2.4.1. Groups and administration

There were five groups of mice in each experiment: Non-stressed vehicle (no stress/vehicle), chronically stressed vehicle (stress/vehicle), and three stressed groups supplemented with candidate probiotics. Mice were randomly assigned to their groups (n = 12 per group) and perorally gavaged with one of the candidate probiotics (1 × 10^9 CFU per day). Freeze-dried bacterial cultures were produced by Danisco Cultures, a division of Danisco USA Inc. (DuPont Nutrition & Biosciences; Madison, WI) and freshly diluted into 100 μl of saline each day to give a concentration of 1 × 10^9 CFU per day. Vehicle mice were perorally gavaged with 100 μl per day of saline solution only. The peroral gavage was performed using a disposable 1 ml syringe (SS + 01T1, Terumo Europe NV, Belgium) fitted with a stainless-steel cannula (075486, Dominique Dutscher, France). Either the bacterial cultures or saline solution were administered in a volume calculated according to the individual body weight of each mouse (5 ml/kg). The mouse was held by the neck and the back skin and the cannula was placed in the mouth of the mouse on one side of the tongue and moved down slightly to pass the tongue and then the content was injected slowly directly
2.4.3. Candidate probiotics in the validation experiment

Each including three candidate probiotics and compared with stress/vehicle and no stress/vehicle control groups in each experiment:

Experiment 1) BG0014, BI11471 and BL0005;
Experiment 2) Lpc-37, Ls-33 and B420;
Experiment 3) LP12418, LP12151 and LP12407;
Experiment 4) LA11873, LX11881 and LH0138.

2.4.3. Candidate probiotics in the validation experiment

Based on the results of the screening experiment, three candidate probiotics which demonstrated efficacy to improve stress-induced behavioural deficits were selected for a validation experiment; Lpc-37, LP12407 and LP12418. The design of the validation experiment followed that of the screening experiment, and its purpose was simply to validate the reproducibility of the results from the screening experiment.

Due to the premature euthanasia of n = 12 mice from one candidate probiotic intervention group (outlined in Table S1), an additional n = 6 mice in each control group were taken together with 12 new mice in the probiotic intervention group. These groups were run in parallel to experiment 5, with a two-week delay. No significant differences were observed across any parameters when comparing between the original n = 12 mice per control group and the additional n = 6 mice per control group (Figure S1). Therefore, the data from original (n = 12 per group) and additional (n = 6 per group) control mice in the no stress/vehicle and stress/vehicle groups were pooled together and presented as a single experiment (n = 18 in total for each control group).

2.5. Chronic stress procedure

The chronic restraint stress procedure was initiated one week after intervention and continued for three weeks. Chronic stress was induced as previously described [20]. Briefly, the mice were inserted into plexiglass transparent cylinders (12 cm long, 3 cm in diameter) under a bright light from 11:00 am to 2:00 pm, five days per week from Monday to Friday during weeks 2-4. The no stress/vehicle group remained undisturbed. Body weight evolution of the stressed and non-stressed mice for all experiments is shown in Figure S2.

2.6. Behavioural test battery

Following four weeks of candidate probiotic intervention and three weeks of the chronic restraint stress procedure in stressed groups, mice in all experiments underwent a behavioural test battery, selectively designed to screen for stress-associated behaviours (anxiety- and depression-related behaviour and deficits in cognitive function). For all behaviour tests, mice were habituated to the testing room by placing home-cages there for at least 30 min prior to testing. The same mice were assessed across all behavioural tests. The behaviour tests were completed over five days. All apparatus were cleaned with 70 % ethanol between mice in each test. A researcher who was blinded to the group designations remained in the testing room during each behavioural test. All outputs were measured by an experimenter blinded to the experimental groups. The EPM and OF tests were used to measure anxiety-related behaviour. The NOR test was used to measure depression-related behaviour. A detailed description of the methods for each behaviour test can be found in Supplementary Methods.

2.7. Collection of samples

In the screening experiments, plasma was isolated following pretreatment with ethylenediaminetetraacetic acid (EDTA) during blood collection for measurement of corticosterone and stored at −20 °C until analysis.

The validation experiment included biomarker analyses: plasma corticosterone, plasma adrenocorticotrophic hormone (ACTH), hippocampal BDNF, gamma-aminobutyric acid (GABA) receptor expression from prefrontal cortex and whole-thickness jejunum, and cytokine determination from plasma, colon and caecum tissue samples. Plasma and tissue samples were stored at −80 °C until subsequent analyses.

2.8. Biochemical measurements

2.8.1. Plasma corticosterone and ACTH determination

Plasma corticosterone was assayed from 25 μl of plasma using a commercial colorimetric kit (Corticosterone (CSCI) ELISA kit, ab108821, Abcam, France). Samples were diluted 1:200 in 1X diluent M and assayed according to the manufacturer’s instructions. Typical intra- and inter-assay coefficients of variation were 5 % and 7 %, respectively. Samples were assayed in duplicate.

Plasma ACTH was also assayed with a commercial colorimetric kit (ACTH ELISA kit, ENZ-KIT138, Enzo Life Science, France). A 25 μl sample was diluted 1:1 in 1X diluent M and assayed according to the manufacturer’s instructions in singlet. Due to lack of duplicate analyses, the ROUT method [21] was used to identify outliers that were left out of the data analyses using GraphPad Prism software v.6 (GraphPad Software Inc., La Jolla, CA, USA). The ROUT method was developed as a method to identify outliers from nonlinear regression. Briefly, it first fits a model to the data using a robust method where outliers have little impact. Then it uses a new outlier detection method, based on the false discovery rate, to decide which points are far enough from the prediction of the model to be called outliers.

2.8.2. GABA receptor expression in prefrontal cortex and jejunum

GABA_{α2} and GABA_{β3} receptor expression were measured from the prefrontal cortex from mice in the validation experiment. In addition, GABA_{α1} receptor expression was measured from the jejunum of mice, also in the validation experiment. Tissues were immersed in 1 ml of lysis reagent (QiAzo1 and homogenised using high-speed shaking with a single 1 mm stainless steel bead (tissue Lyzer Qiagen, 2 × 2 min at 20 °C). Chloroform was added and the sample was centrifuged to collect the RNA-containing aqueous phase. RNA was then purified using a Qiachorpe robotic workstation and the miRNeasy Mini Kit (Qiagen). Residual DNA removal was performed using the Turbo DNase kit (Ambion), and RNA was then transcribed to cDNA using ReadyScript cDNA Synthesis Mix (Sigma-Aldrich) following the manufacturer’s protocol. Individual qPCR was performed in triplicate using SYBR Green, using β-actin (ACTB) as the housekeeping gene. The primer sequences were as follows: GABA_{α2} forward: GGAAGCTACGCTTACACAACC, reverse: CCATCCTGGGAGCAACCTGAA, GABA_{α1} forward: CGCACCCTCCTCAAGAC, reverse: GTCTTCCAGGCCGCTCCTC, ACTB forward: ATGCTCCCCGGGCTGTAT, reverse: CATAGGATCCTTCTTGACATT.

2.8.3. Analysis of immune markers in plasma and intestinal tissue

Proteins were extracted from tissue samples prior to cytokine analyses. In brief, 1 ml of T-PER protein extraction reagent (Thermo Fisher Scientific, Vantaa, Finland) and 0.1 ml of Halt Protease Inhibitor Cocktail (Thermo Scientific) were added onto tissue samples in CKMix bead tubes (Bertin Technologies SAS, France). The samples were homogenised using Precells 24 (Bertin Technologies SAS) 6500 rpm for 23 s and centrifuged at +4 °C 10,000 g for 5 min. Samples were stored at −80 °C.

Cytokine levels were measured from EDTA plasma and tissue
3.2. The commercial ELISA kit (Promega, #G7610). The tissue was thawed and tumor necrosis factor (TNF)-α extracts by multiplex enzyme linked immunosorbent assay (ELISA) system (Aushon Biosystems, Billerica, MA) following manufacturer’s instructions. Mouse cytokines included in the assay were interferon-γ (IFN-γ), interleukin (IL)-1β (IL-1β), IL-2, IL-6, IL-10, IL12p70, IL-17, and tumor necrosis factor (TNF)-α.

2.8.4. BDNF measurement from hippocampus

BDNF concentration was measured from the hippocampus with a commercial ELISA kit (Promega, #G7610). The tissue was thawed and homogenised in 50 mM Tris-150 mM NaCl buffer, pH 7.5, and sonicated for 20 s. The samples were then centrifuged (16,100 g for 15 min at 4 °C) to collect the supernatant for analysis according to the manufacturer’s instructions. Samples were assayed in singlet. Due to lack of duplicate analyses, the ROUT method [21] was used to identify outliers that were left out of the data analyses.

2.9. Statistical analyses

All values are expressed as mean ± SEM. All data were first checked for normality using the Shapiro-Wilk test, and then analysed using one-way ANOVA and Dunnett’s test for multiple comparisons if the data were normally distributed. Non-normally distributed data were analysed using the non-parametric Kruskal-Wallis test, followed by Dunn’s test for multiple comparisons. To analyse GABA receptor gene expression data, the 2-DDCT algorithm was first used to quantify relative differential expression, and data were then analysed non-parametrically with the Kruskal-Wallis test and the Nemenyi post hoc test. All data except gene expression data were analysed using GraphPad Prism software v.6 (GraphPad Software Inc., La Jolla, CA, USA). A p-value lower than 0.05 was considered statistically significant. Unless otherwise stated in Table S1, n = 12 for all groups were analysed in each screening experiments (1–4) for behaviour and corticosterone. In the validation experiment 5, behaviour data, corticosterone and ACTH were analysed for the control groups by pooling the data from the original (n = 12 per group) and the additional (n = 6 per group) to give a total n = 18 for each control group. In the validation experiment 5, n = 12 mice per group were analysed for BDNF concentration in the hippocampus. All bacterial intervention groups in the validation experiment were analysed with n = 10–12, depending on the final numbers of mice in each group. GABA receptor expression was analysed in the prefrontal cortex and jejunum from n = 6 mice per group. Statistical analyses for cytokine concentrations were conducted with GraphPad Prism v7.03 (GraphPad Software Inc.). The difference between the treatment groups was tested with one-way ANOVA, followed by Tukey’s test for multiple comparisons.

3. Results

3.1. Daily chronic restraint stress induced a neuropsychiatric disorder-associated behavioural profile in the stress/vehicle group

The model of three weeks daily administration of chronic restraint stress was sufficient to induce significant persistence of anxiety-related behaviour in the EPM (Figs. 2 and S4) and OF (Figs. 3, S5, and S6) tests, disruption to cognitive function in the NOR test (Figs. 4 and S7), and depression-related behaviour in the FST (Figs. 5 and S8).

3.2. The effect of candidate probiotics are species- and strain-specific and significantly improved anxiety- and depression-related behavioural responses to stress and stress-associated disruption to cognitive function compared with the stress/vehicle group

3.2.1. Anxiety-related behaviour

In the screening experiment, Lpc-37 (p < 0.01; Fig. 2A), LP12407 (p < 0.05; Fig. 2B) and LP12418 (p < 0.05; Fig. 2B) significantly increased the time spent in the open arms of the EPM test, with similar results identified in the validation experiment (p < 0.001; Fig. 2C). Bacterial intervention had no effect on the number of open arm entries (p > 0.05; Figs. 2D, E, F, S4C and S4D) or locomotor activity in any of the experiments (p > 0.05; Figs. S3A, S3B, S3C, S4E and S4F).

Lpc-37, LP12151, LP12407 and LP12418 increased the amount of time spent and locomotion in the centre of the OF arena in the screening experiments (p < 0.001; Figs. 3A, B, S6A and S6B). These findings were reproduced for Lpc-37, LP12407 and LP12418 in the validation experiment (p < 0.001; Figs. 3C and S6C). LH0138 increased the locomotion in the centre of the OF arena in the screening experiment (p < 0.05; Fig. S5E). Bacterial intervention had no effect on overall locomotor activity during the OF test in any of the experiments (p > 0.05; Figs. S6D, S6E, S6F, S5C and S5F).

3.2.2. Cognitive function

Bacterial intervention had no significant impact on the amount of time mice spent interacting with either object during the first day the NOR test in the screening (p > 0.05; Figs. 4A, D, S7A and S7D) or validation (p > 0.05; Fig. 4G) experiments. In the screening experiment, Lpc-37 was the only strain to increase object interaction time with the novel object during the NOR test (p < 0.01; Fig. 4B) and this result was also displayed in the discrimination index (p < 0.001; Fig. 4C). In the validation experiment, all three candidate probiotics increased object recognition time with the novel object during the NOR test (p < 0.001; Fig. 4H) and the discrimination index (p < 0.001; Fig. 4I).

3.2.3. Depression-related behaviour

In the screening experiment, Lpc-37, LP12151, LP12407 and LP12418 significantly increased the swimming time (p < 0.001; Figs. 5A and B) and decreased immobility time (p < 0.001; Figs. S5D and E). These results were reproduced in the validation experiment for Lpc-37, LP12407 and LP12418 (p < 0.001; Figs. 5C and F).

3.3. In some cases, intervention with candidate probiotic strains normalised the behavioural profile associated with stress to levels that were non-significantly different to the no stress/vehicle group

3.3.1. Anxiety-related behaviour

Time spent in the centre of the OF arena was similar following intervention with Lpc-37 and LP12407 to the no stress/vehicle group in the screening experiment (p > 0.05; Figs. 3A and B). Lpc-37 was also not significantly different to the no stress/vehicle group in the screening experiment for locomotion in the centre of the OF arena (p > 0.05; S6A). In the validation experiment, Lpc-37, LP12407 and LP12418 were not significantly different to the no stress/vehicle group for either time spent or locomotion in the centre of the OF arena (p > 0.05; Figs. 3C and S6C).

3.3.2. Cognitive function

In the screening experiment, Lpc-37 (p > 0.05; Fig. 4B), BL0005 (p > 0.05; Fig. S7B) and BG0014 (p > 0.05; Fig. S7B) were not significantly different to the no stress/vehicle group in the amount of time spent interacting with the novel object during the NOR test. None of the three strains in the validation experiment were significantly different to the no stress/vehicle group in the amount of time spent interacting with the novel object (p > 0.05; Fig. 4H). In the discrimination index, Lpc-37 (p > 0.05; Fig. 4C), LP12151, LP12407, LP12418 (p > 0.05; Fig. 4F), BL0005 and BG0014 (p > 0.05; Fig. S7C) did not significantly differ from the no stress/vehicle group. This result was reproduced for Lpc-37, LP12407 and LP12418 in the validation experiment (p > 0.05; Fig. 4I).

3.3.3. Depression-related behaviour

In the screening experiment, Lpc-37 (p > 0.05; Fig. 5A), LP12151, LP12407, LP12418 (p > 0.05; Fig. 5B) and BI11471 (p > 0.05; Figure S8A) did not significantly reduce swimming time compared with the no.
stress/vehicle group. This result was reproduced for Lpc-37, LP12407 and LP12418 in the validation experiment (p > 0.05; Fig. 5C). In the screening experiment, Lpc-37 (p > 0.05; Fig. 5D), LP12151, LP12407, LP12418 (p > 0.05; Fig. 5E) and BL11471 (p > 0.05; Fig. S8B) did not significantly increase immobility time compared with the no stress/vehicle group. This result was reproduced for Lpc-37, LP12407 and LP12418 in the validation experiment (p > 0.05; Fig. 5F).

### 3.4. Plasma corticosterone and ACTH

Plasma corticosterone was analysed as a biomarker of the neuroendocrine response to stress in all five experiments and values from the screening experiment are displayed in Table S2. In the validation experiment, chronic stress significantly increased levels of corticosterone (p < 0.001; Fig. 6A) and decreased levels of ACTH (p < 0.05; Fig. 6B) in the stress/vehicle group compared with the no stress/vehicle group. In the Lpc-37 group, corticosterone levels did not differ significantly from the no stress/vehicle group (Fig. 6A; p > 0.05).

In the validation experiment, bacterial intervention with LP12418 completely normalised the stress-induced reduction in ACTH concentration, displaying a significant difference to the stress/vehicle group (p < 0.01; Fig. 6B). There was no effect of any other strain on ACTH or corticosterone in the validation experiment.

### 3.5. GABA receptor expression in the prefrontal cortex and jejunum

Chronic stress significantly reduced the expression of GABA_A_2 (p < 0.05; Fig. 7A) and GABA_A_1p (p < 0.05; Fig. 7B) receptors in the prefrontal cortex of mice in the stress/vehicle group compared with the no stress/vehicle group. Following intervention with LP12418, both GABA_A_2 (p < 0.05; Fig. 7A) and GABA_A_1p (p < 0.05; Fig. 7B) receptor expression were significantly increased compared with the stress/vehicle group and not did not statistically differ from the no stress/vehicle group. Neither stress nor bacterial intervention had any significant impact on GABA_A_1p receptor expression in jejunal tissue (Fig. 7C).
3.6. Hippocampal BDNF concentration

Neither stress nor bacterial intervention had any significant impact on hippocampal BDNF concentration in the validation experiment (p > 0.05; Fig. S9).

3.7. Plasma and intestinal tissue extract cytokines

Cytokines IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL2p70, IL-17, and TNF-α were measured from plasma, and from caecum and colon tissue extracts in the validation experiment. Only IL-10 and TNF-α were detected from plasma in adequate number of samples for statistical analyses. Neither chronic stress nor bacterial intervention had any significant effect on cytokine levels in plasma (p > 0.05; Fig. S10), caecum tissue (p > 0.05; Fig. S11) or colon tissue (p > 0.05; Fig. S12) extracts, except that TNF-α level in the colon tissue extract of the Lpc-37 group was significantly higher than in the stress/vehicle group (p < 0.05; Fig. S12).

4. Discussion

Exploring the complex role of the microbiota-gut-brain axis in the pathogenesis and treatment of mood disorders has been given major consideration in nutritional neuropsychopharmacology and probiotics are at the forefront of such research. The purpose of this study was to examine the individual effects of twelve candidate probiotic strains from ten different species/sub-species on the behavioural and physiological responses to chronic stress. The results suggest that the beneficial effects of the candidate probiotics on behavioural outcomes associated with chronic stress are species- and strain-specific. The most prominent strains for preventing stress-related behaviours from developing in these experiments were Lactobacillus paracasei Lpc-37, Lactobacillus plantarum LP12418 and Lactobacillus plantarum LP12407. Most of the data obtained from the screening experiment following intervention with these strains was reproduced in the validation experiment for each behavioural outcome. Lactobacillus plantarum LP12151 also demonstrated benefits in some, but not all the behavioural tests during the screening experiment. Anxiety-related behaviour was measured from data collected during the EPM and OF tests. Intervention with Lpc-37, LP12418 and LP12407 completely prevented anxiety-related behaviours from developing, as demonstrated in the EPM test. Daily chronic stress, regardless of intervention had no impact on the number of open arm entries in the EPM or on overall locomotor activity. This indicates that chronic stress did not disrupt the natural exploratory behaviour of these mice but that activities challenging anxious behaviour were exposed. A similar result was obtained for anxiety-related behaviour in the OF test. In the screening experiment, Lpc-37, LP12418, LP12407 and LP12151 prevented anxiety-related behaviours from developing. All these significant improvements in anxiety-related behaviour in the EPM and OF tests were reproduced for Lpc-37, LP12407 and LP12418 in the validation experiment.

Coupled with the chronic stress-associated development of anxious behaviour was a disruption of normal cognitive function. Deficits in cognitive function following stress have previously been reported in rodents [22] and in humans [28] and probiotics have proven efficacious in alleviating such cognitive dysfunction [22,29]. When cognition is functioning as expected in rodents, the animal can discriminate between the novel and familiar objects within the NOR test, however, chronic stress had the capacity to diminish the ability to discriminate between the objects. The results showed that Lpc-37 prevented the chronic stress-associated deficits in recognition memory in the screening experiment and this result was confirmed in the validation experiment. Meanwhile, LP12407 and LP12418 prevented chronic stress-associated deficits in recognition memory in the validation experiment only. These same results were not identified for either strain in the screening experiment, thus highlighting the objectivity of identifying stress-associated behaviours in rodents following various dietary interventions, including prebiotics and probiotics, for example [6,24,25]. Notably, chronic stress in humans is also associated with the development of neuropsychiatric disorders, most commonly, anxiety and depression (extensively reviewed in [26,27]).
behavioural datasets and the essentiality of validating results. It should be noted that the discordant results for LP12407 and LP12418 between the screening experiment and validation experiment is an indication that further experimental evidence is needed before hypothesising that these strains have any significant impact on cognitive function.

Furthermore, the impact of candidate probiotic intervention on stress-associated, depression-related behaviour was assessed. The FST is commonly used to measure depression-related behaviour and using this test, several microbial interventions have proven successful at improving despair behaviour in rodents [4,30]. Lpc-37, LP12151, LP12407 and LP12418 prevented despair behaviour induced by chronic stress from developing in the screening experiment and this result was reproduced for Lpc-37, LP12407 and LP12418 in the validation experiment.

While the positive impact that probiotic supplementation can have on behavioural profiles in pre-clinical models are now well established, to our knowledge no other published research has compared strains of the same species in such experiments while dozens of studies have been

![Fig. 4. Cognitive function in the novel object recognition (NOR) test.](A, D and G) Object interaction time (%) during the same object session in experiments 2, 3 and the validation experiment, respectively. (B, E and H) Object interaction time (%) during the novel object session in experiments 2, 3 and the validation experiment, respectively. (C, F and I) Discrimination index (time of interaction) as a graphical representation of cognitive function in experiments 2, 3 and the validation experiment, respectively. All data were analysed using either the one-way ANOVA (F value) and Dunnett's test for multiple comparisons or the Kruskal-Wallis test (H value), followed by Dunn's test for multiple comparisons, depending on normality, checked using the Shapiro-Wilk test. Unless otherwise stated, n = 12 for all groups. Experiment 2; Stress/Lpc-37 (n = 11). Experiment 3; Stress/vehicle (n = 11), stress/LP12418 (n = 11). Validation experiment; No stress/vehicle (n = 18), stress/vehicle (n = 18), stress/LP12407 (n = 10) and stress/LP12418 (n = 10). *, **, *** p < 0.05, p < 0.01, p < 0.001 vs no stress/vehicle. ##, ###, p < 0.01, p < 0.001 vs stress/vehicle.

![Fig. 5. Depression-related behaviour in the forced swim test (FST).](A, B and C) Time spent swimming (s) in the FST in experiments 2, 3 and the validation experiment, respectively. (D, E and F) Time spent immobile (s) in the FST in experiments 2, 3 and the validation experiment, respectively. All data were analysed using either the one-way ANOVA (F value) and Dunnett’s test for multiple comparisons or the Kruskal-Wallis test (H value), followed by Dunn’s test for multiple comparisons, depending on normality, checked using the Shapiro-Wilk test. Unless otherwise stated, n = 12 for all groups. Experiment 2; Stress/Lpc-37 (n = 11). Experiment 3; Stress/vehicle (n = 11), stress/LP12418 (n = 11). Validation experiment; No stress/vehicle (n = 18), stress/vehicle (n = 18), stress/LP12407 (n = 10) and stress/LP12418 (n = 10). ***, p < 0.001 vs no stress/vehicle. ###, p < 0.001 vs stress/vehicle. Data are presented as mean ± SEM.
conducted on single strains and strain combinations [5,25,31]. In addition, only a few studies have compared strains representing different species and showed species-specific differences [32,33]. Based on the dataset herein, candidate probiotic effects on behavioural responses are strain-specific. While *L. plantarum* LP12151 had a significant impact on some behavioural outcomes, the effects of *L. plantarum* strains LP12418 and LP12407 more often improved stress-related behaviours. Furthermore, while all the candidate probiotics tested in this study were derived from species / sub-species of *Bifidobacterium* and *Lactobacillus*, it should be noted that other groups are now exploring alternative next generation candidate probiotics to alter brain function and behaviour [34]. Further evidence to substantiate the role of the gut microbiota in stress-associated neuropsychiatric disorders have been described by the presence of an altered gut microbiota homeostasis in rodents [4,5] and in humans [7,8]. Thus, it has been proposed that the microbiome can determine the stress response. Understanding the mechanisms through which bacterial intervention or altering the gut microbiota composition can influence brain behaviour is crucial for the future development of candidate probiotics.

Chronic stress is associated with dysregulation of the HPA axis response to everyday stressors (e.g., caregiver stress [35]). This shift in cortisol reactivity together with a maladaptive suppression of both types of the immune response [36] are risk factors for the development of chronic stress-related psychological disorders. In this study, we further addressed strain-specificity by exploring the impact of our candidate probiotics on some physiological outcomes associated with stress. Plasma corticosterone was measured in all experiments and while chronic stress had no consistent effect on corticosterone levels across the experiments, there was a significant increase in corticosterone production in the validation experiment in the stress/vehicle group compared with no stress/vehicle group. There was no effect of candidate probiotics on corticosterone production. That said, the association between corticosterone release and behavioural outcomes are often conflicting following probiotic intervention. While some studies have

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**Fig. 6. Endocrine response to stress.** (A) Corticosterone (ng/mL) in the validation experiment. (B) Adrenocorticotropic hormone (ACTH; pg/mL) in the validation experiment. All data were analysed using either the one-way ANOVA (F value) and Dunnett’s test for multiple comparisons or the Kruskal-Wallis test (H value), followed by Dunn’s test for multiple comparisons, depending on normality, checked using the Shapiro-Wilk test. Unless otherwise stated, n = 12 for all groups. Validation experiment; No stress/vehicle (n = 18), stress/vehicle (n = 18), stress/LP12407 (n = 10) and stress/LP12418 (n = 10). *, **, *** p < 0.05, p < 0.01, p < 0.001 vs no stress/vehicle. ##, p < 0.01 vs stress/vehicle. Data are presented as mean ± SEM.

**Fig. 7. Prefrontal cortex and jejunum gene expression of gamma-aminobutyric acid (GABA) receptor expression.** (A) GABAα2 receptor expression in the prefrontal cortex (fold change) in the validation experiment. (B) GABAβ1 receptor expression in the prefrontal cortex (fold change) in the validation experiment. (C) GABAβ receptor expression in the jejunum (fold change) in the validation experiment. To analyse relative GABA receptor gene expression data, the 2-DDCT algorithm was first used to quantify relative differential expression, and data were then analysed non-parametrically with the Kruskal-Wallis test and the Nemenyi post hoc test. The 2-DDCT algorithm is the gold standard for differential expression analysis. This algorithm allows the user to quantify relative differential expression between two or more conditions. n = 6 mice per group. *, p < 0.05, vs no stress/vehicle. #, p < 0.05 vs stress/vehicle. Data are presented as mean ± SEM.
proven that stress-induced corticosterone release coincides with improvements in anxiety- and depression-related behaviours [30,37], others have found no association between these outcomes [32]. In this study, there were limitations in our plasma sampling schedule and perhaps it may have been more accurate to have taken a series of samples over time in response to an acute stress (for example before, during and after the FST [5]), rather than at a single timepoint during the experiments. In line with the increase in corticotropin production following chronic stress, ACTH was significantly reduced in the stress/group vehicle group compared with the no stress/vehicle group. ACTH was measured in the validation experiment only and the data showed that intervention with LP12418 increased the ACTH concentration compared with the stress/vehicle group. Termination of the stress response occurs via a negative feedback loop, as corticosterone levels become elevated they start to slow down the release of corticotrophin-releasing hormone from the hypothalamus and ACTH from the pituitary gland which usually results in decreased levels of ACTH. While this is the case observed in the stress/vehicle group, the effect of LP12418 intervention on the HPA axis response to stress is unusual and should be investigated further in future studies.

Further, the topic of microbial endocrinology, i.e. the production of neuroendocrine hormones or neurotransmitters by the gut microbiota has been extensively explored [38]. GABA is the main inhibitory neurotransmitter in the brain. The prefrontal cortex is a distinct brain region involved in stress adaptation and here, the balance of excitatory and inhibitory neurotransmission plays a crucial role in maintaining psychiatric health. Chronic stress is a contributing factor in the dysregulation of GABAergic inhibitory neurotransmission in the prefrontal cortex and therefore a vital brain region whereby probiotic intervention may mediate anxiolytic effects (reviewed in [39]). Many commensal lactic acid-producing bacteria have been reported to produce GABA [40,41]. While, it is currently undetermined whether intestinally-produced GABA is relevant to CNS signaling, especially since GABA has long been thought to lack the ability to cross the blood-brain barrier [42], intestinal-GABA could bind to afferent nerve endings in the gastrointestinal tract, where GABAB receptors are abundantly expressed to transmit signals to the CNS [43]. In the present study, LP12418 normalised the stress-induced reduction in GABAβ and GABAβ receptor expression in the prefrontal cortex. This implies that LP12418, which demonstrated anxiolytic effects on mouse behaviour, has the potential to modulate brain biochemistry. Since LP12418 did not alter GABAB receptor expression in the small intestine, whether this modulation of GABA receptors in the brain is caused by microbial-production of GABA in the small intestine remains to be explored further. Lpc-37 and LP12407 had no effect on GABA receptor expression, despite positively influencing behaviour and therefore, further mechanistic pathways should be explored.

The experiments herein expose the delicate nature of conducting behavioural tests in mice and supports the concept that caution must be taken when interpreting such datasets. The results for the NOR test were not entirely repeatable, since there was no effect with any of the L. plantarum strains in the screening experiment, but we achieved significant improvements in cognitive function in the validation experiment. This discrepancy underlines the importance of repeating behavioural tests to validate the robustness of the data. For example, it is known that behavioural tests can be influenced by external factors, whereby even the gender of the experimenter has been shown to influence the outcome [44]. One main limitation to our study was the loss of an entire group of mice from the validation experiment due to aggressive behaviour, caused by group housing of male mice. Group housing is widely recommended to give mice the opportunity to behave as “social animals” but the despotic social systems of mice leads to the establishment of dominance-subordinate relationships during group housing under experimental conditions [45]. Persistent competition for dominance coupled with repeated trespassing within each-others social space is a daily challenge to the dominant male and the inability of subordinates to escape is not a natural behaviour [45]. These social stresses often result in excess stress, fighting, injury and in severe cases, even death [45]. On the other hand, social isolation in male mice leads to changes in behavioural, neuro-endocrinological and neuro-physiological parameters (reviewed in [46]). Both housing systems have their advantages and disadvantages but in experiments measuring behavioural outcomes, group housing is by far the most favored option. When the data for additional mice in the control groups was compared with that of the original mice in control groups, there were no significant differences detected, thus making it possible to combine the datasets. Furthermore, the results of this study are restricted to male mice. Sex differences in the gut microbiota and behavioural response have previously been detected in various mouse models [47–49] and sex-specific changes in brain neurochemistry have been identified in germ-free mice [12,15]. Therefore, future studies should identify whether the effects mediated by the candidate probiotics in this study are male-specific or can also be further translated to female mice. The primary limitation of this study and indeed with many probiotic intervention pre-clinical trials exploring the microbiota-gut-brain connection is the currently unknown translatability to humans. Although experiments on single strains and strain combinations have shown benefits, for example in rodents [33,50] and humans [51–53], translating these results to humans presents a major challenge. For example, promising pre-clinical results on L. rhamnosus JB-1 in an anxious mouse model [30,54] and in mice exposed to chronic social stress [5] demonstrated that this strain could protect against stress-related behaviours and some mechanistic potential was described. However, there was no effect of the strain in either mice with normal phenotypes for anxiety-like behaviour [54] or healthy humans on outcomes related to stress and cognitive performance [55]. This example is key evidence demonstrating that careful consideration should be taken when selecting the appropriate model in rodents to best predict the outcome in humans. Since the specific mechanisms by which gut microbes exert their effects on mood are largely unknown, it is not yet possible to attempt to improve translatability to humans by searching for relevant mechanistic evidence.

5. Conclusions

The study results described herein demonstrate that intervention with Lactobacillus paracasei Lpc-37, Lactobacillus plantarum LP12418 and Lactobacillus plantarum LP12407 can prevent behavioural impairments in mice caused by chronic daily restraint stress from developing. Each of these strains had a unique profile in terms of mechanistic biomarkers related to the HPA axis and prefrontal cortex GABA receptor expression. The results of the systematic screening of twelve probiotic candidate strains indicate that certain species and specific strains maybe superior to other bacterial strains for improving behavioural outcomes associated with chronic stress. Future work will determine whether probiotic effects on stress and anxiety-related behavioural outcomes can be clinically translated.

Author contributions statement

LKS, MJL, FJR and JM designed the research; FJR and JM conducted the research. LKS, EP, MJL, FJR and JM analysed the data. EP and LKS drafted the manuscript. MJL, FJR and JM contributed to the writing of the manuscript. EP, LKS and MJL had primary responsibility for final content.

Declaration of Competing Interest

EP and MJL are employed by DuPont Nutrition & Biosciences, which is a manufacturer and provider of cultures and probiotics. At the time the research was undertaken, LKS was also employed by DuPont Nutrition & Biosciences. JM and FJR are employed by Amylgen SAS,
which provides research services in the field of cognitive health.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jbbr.2019.112376.

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