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Title	Lost in translation? The potential psychobiotic Lactobacillus rhamnosus (JB-1) fails to modulate stress or cognitive performance in healthy male subjects
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Publication date	2016-11-16
Original Citation	Kelly, J. R., Allen, A. P., Temko, A., Hutch, W., Kennedy, P. J., Farid, N., Murphy, E., Boylan, G., Bienenstock, J., Cryan, J. F., Clarke, G. and Dinan, T. G. (2017) 'Lost in translation? The potential psychobiotic Lactobacillus rhamnosus (JB-1) fails to modulate stress or cognitive performance in healthy male subjects', Brain, Behavior, and Immunity, 61, pp. 50-59. doi: 10.1016/j.bbi.2016.11.018
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1016/j.bbi.2016.11.018
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Download date	2026-11-15 06:28:33
Item downloaded from	https://hdl.handle.net/10468/3316

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PII: S0889-1591(16)30520-7
DOI: http://dx.doi.org/10.1016/j.bbi.2016.11.018
Reference: YBRBI 3020

To appear in: *Brain, Behavior, and Immunity*

Received Date: 8 September 2016
Revised Date: 28 October 2016
Accepted Date: 16 November 2016

Please cite this article as: Kelly, J.R., Allen, A.P., Temko, A., Hutch, W., Kennedy, P.J., Farid, N., Murphy, E., Boylan, G., Bienenstock, J., Cryan, J.F., Clarke, G., Dinan, T.G., Lost in Translation? The potential psychobiotic *Lactobacillus rhamnosus* (JB-1) fails to modulate stress or cognitive performance in healthy male subjects, *Brain, Behavior, and Immunity* (2016), doi: http://dx.doi.org/10.1016/j.bbi.2016.11.018

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Lost in Translation? The potential psychobiotic *Lactobacillus rhamnosus* (JB-1) fails to modulate stress or cognitive performance in healthy male subjects

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Abstract

Background: Preclinical studies have identified certain probiotics as psychobiotics - live microorganisms with a potential mental health benefit. *Lactobacillus rhamnosus* (JB-1) has been shown to reduce stress-related behaviour, corticosterone release and alter central expression of GABA receptors in an anxious mouse strain. However, it is unclear if this single putative psychobiotic strain has psychotropic activity in humans. Consequently, we aimed to examine if these promising preclinical findings could be translated to healthy human volunteers.

Objectives: To determine the impact of *L. rhamnosus* on stress-related behaviours, physiology, inflammatory response, cognitive performance and brain activity patterns in healthy male participants.

Methods: An 8 week, randomized, placebo-controlled, cross-over design was employed. Twenty-nine healthy male volunteers participated. Participants completed self-report stress measures, cognitive assessments and resting electroencephalography (EEG). Plasma IL10, IL1β, IL6, IL8 and TNFα levels and whole blood Toll-like 4 (TLR-4) agonist-induced cytokine release were determined by multiplex ELISA. Salivary cortisol was determined by ELISA and subjective stress measures were assessed before, during and after a socially evaluated cold pressor test (SECPT).

Results: There was no overall effect of probiotic treatment on measures of mood, anxiety, stress or sleep quality and no significant effect of probiotic over placebo on subjective stress measures, or the HPA response to the SECPT. Visuospatial memory performance, attention switching, rapid visual information processing, emotion

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recognition and associated EEG measures did not show improvement over placebo. No significant anti-inflammatory effects were seen as assessed by basal and stimulated cytokine levels.

Conclusions: *L. rhamnosus* was not superior to placebo in modifying stress-related measures, HPA response, inflammation or cognitive performance in healthy male participants. These findings highlight the challenges associated with moving promising preclinical studies, conducted in an anxious mouse strain, to healthy human participants. Future interventional studies investigating the effect of this psychobiotic in populations with stress-related disorders are required.

Keywords: Psychobiotic, brain-gut axis, stress, cognition, memory, EEG.

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Introduction

An abundance of preclinical studies have shown that probiotics acting via the brain-gut-axis can affect brain development, function and behaviour (Bercik et al., 2011a; Buffington et al., 2016; Cryan and Dinan, 2015; Desbonnet et al., 2014; Desbonnet et al., 2010; Hsiao et al., 2013). This has prompted a growing interest in the possibility of targeting the gut microbiota to beneficially impact human brain function and behaviour. Psychobiotics have been defined as bacteria that ingested in adequate amounts produce a positive mental health benefit (Dinan et al., 2013).

Considering the potential impact of putative psychobiotics upon central nervous system processes, especially stress, mood, anxiety and cognition (Cryan and Dinan, 2012; Dinan et al., 2015), the prospect of targeting the gut microbiota as a potential modifiable risk factor for stress-related disorders is appealing (Kelly et al., 2015). Preclinical research has indicated that chronic probiotic administration can reduce anxiety-like and depressive-like behaviour, and can normalise associated physiological outputs such as corticosterone, noradrenaline, brain-derived neurotrophic factor (BDNF) and immune function (Bercik et al., 2011b; Bravo et al., 2011; Desbonnet et al., 2010; Janik et al., 2016; Messaoudi et al., 2011). There is a growing appreciation of the need to translate this promising preclinical work to the clinic while at the same time recognising the challenges inherent in this process (Kelly et al., 2016b).

To date, there are indications from a number of sources that highlight the opportunities in this regard, for example, probiotic use in irritable bowel syndrome

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(IBS) (O'Mahony et al., 2005; Whorwell et al., 2006), a stress-related brain-gut axis disorder associated with high rates of psychopathology (Whitehead et al., 2002) as well as altered hypothalamic-pituitary-adrenal (HPA) axis activity (Kennedy et al., 2014b) and cognition (Kennedy et al., 2015; Kennedy et al., 2014a). A number of proof-of-principle studies in healthy human volunteers have demonstrated that multi-strain probiotics, fermented drinks containing probiotics, or prebiotics, can alter resting brain activity, cognitive performance, baseline physiological stress outputs and self-reported psychological variables (Benton et al., 2007; Chung et al., 2014; Messaoudi et al., 2011; Mohammadi et al., 2015b; Schmidt et al., 2015; Steenbergen et al., 2015; Tillisch et al., 2013). More recently, *Bifidobacterium longum* **1714**, selected based on pre-clinical evidence (Savignac et al., 2014; Savignac et al., 2015), was shown to reduce stress levels and to produce a neurocognitive profile associated with enhanced memory in healthy volunteers (Allen et al., 2016).

By utilizing a well-validated preclinical screening platform, developed to inform efficient selection of prospective psychobiotic strains, we identified *L. rhamnosus* (JB-1). In these studies, which were carried out in the stress-sensitive BALB/c mice, ingestion of the JB-1 strain reduced anxiety in the elevated plus maze and despair-like behaviour in the forced swim test. Moreover, there was enhanced learning in a fear conditioning paradigm and reduced stress-induced corticosterone levels. At a brain level there were marked alterations in central GABAA and GABAB receptor levels (Bravo et al., 2011). Furthermore, a magnetic resonance spectroscopy study, also conducted in BALB/c mice showed that treatment with the JB-1 strain significantly

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elevated central GABA levels by 25% after four weeks of treatment (Janik et al., 2016). In addition, *L. rhamnosus* treatment modulates the immune system (Forsythe et al., 2012; Karimi et al., 2009; Kozakova et al., 2016; Ma et al., 2004), intestinal motility (Wang et al., 2010), gut barrier function (Patel et al., 2012; Wang et al., 2012) and enteric nervous system (Kamiya et al., 2006; Ma et al., 2009). Taken together, these preclinical studies identify *L. rhamnosus* as a candidate psychobiotic with the one of the most comprehensive behavioural, physiological and neurobiological profiles.

We employed a randomized, placebo-controlled, cross-over, repeated measures design to examine the effects of the JB-1 strain compared to placebo on the psychobiological response to an acute, controlled stressor (Schwabe et al., 2008; Schwabe and Wolf, 2010) and assessed cognitive performance on tests assessing memory, sustained attention, social cognition and emotional processing. In addition, we measured the immune response to this candidate psychobiotic by measuring a panel of cytokines. Finally, to ascertain if the JB-1 strain effected brain activity patterns, we assessed brain activity in frontal, parietal and central regions using EEG following 4-week supplementation with the JB-1 strain in comparison to placebo, as these regions have been associated with memory and sustained attention (Coull et al., 1996; Hales et al., 2009) and are sensitive to anxiolytics (Fukami et al., 2010) and psychobiotics (Allen et al., 2016).

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Methods

Subjects

Approval of the study protocol was granted by the Cork University Hospital ethics committee (Protocol Number: APC057) and conducted in accordance with the ICH Guidelines on Good Clinical Practice, and the Declaration of Helsinki. Written informed consent was obtained from all subjects before any study procedures were conducted.

Participants were aged between 20 and 33 years of age. Inclusion criteria were as follows: aged between 18 - 40 years, able to speak English, in good health as determined by the investigator. Male participants were selected to avoid the influence of the menstrual cycle, which can impact upon cortisol output and other readouts and all preclinical studies with this bacteria to date have focused on male animals (Bravo et al., 2011). Exclusion criteria were as follows: having a significant acute or chronic illness, following a diet or taking a medication that would interfere with the objectives of the study, pose a safety risk or confound the interpretation of the study results; to include, probiotics, antibiotics, antipsychotics, anxiolytics, laxatives, enemas, anti-coagulants and over-the counter non-steroidal anti-inflammatorys (NSAIDS), antidepressants or any other psychotropic medication. Evidence of immunodeficiency, bleeding disorder or coagulopathy, colour blindness, dyslexia or dyscalculia, or receiving any treatment involving experimental drugs.

Design

A repeated measures cross-over design was employed. Participants were screened at an initial visit for psychiatric disorder using the MINI International Neuropsychiatric

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Interview (MINI) (Sheehan et al., 1998) and demographic and baseline psychological information was collected. Following screening, participants completed neurocognitive visits and acute stress visits utilizing the socially evaluated cold pressor test (SECPT) at baseline, at 4 weeks and at 8 weeks. See **Supplementary Figure S1** for the study design. Participants were administered placebo capsules for four weeks or *L. rhamnosus* capsules for four weeks and they then switched to the alternative treatment. See **Table 1** for detailed participant characteristics.

Materials

Both active and placebo capsules contained corn starch, magnesium stearate and silicon dioxide. The count for *L. rhamnosus* (JB-1) in the active capsules was $1x10^9$ colony-forming units (CFU). Participants were instructed to take one capsule each morning.

Tests from the CANTAB Battery

Tests from the Cambridge Neuropsychological Test Automated Battery (CANTAB) were presented on a touch-screen monitor, Sahara i440D Slate Tablet PC (Sand Dune Ventures, Tablet Kiosk) running CANTABeclipse™ software (Cambridge, UK) as previously described (Allen et al., 2016; Kennedy et al., 2014a). The researcher provided verbal instructions to participants from a standardised script, and had full control of a keyboard used to start, pause or abort each test. As a test battery of multiple cognitive tests was employed, test order was counterbalanced, using a Latin square design, to avoid effects of fatigue for tests completed later in the session. The test battery lasted approximately 45 minutes in total. Participants were assessed on

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the following tests: Motor Screening Test (MOT), Paired Associates Learning (PAL), Attention Switching Task (AST), Rapid visual information processing (RVP), Emotion Recognition Task (ERT) and Emotional Stroop. See **Supplementary Methods section** for details of CANTAB tests.

Acute Stress Procedure

We employed the socially evaluated cold pressor test (SECPT) (Schwabe et al., 2008) as a combined psychological and physiological stressor procedure, which has been shown not to induce HPA axis habituation across repeated exposures (Minkley et al., 2014). Participants were required to avoid alcohol for 24 hours prior to the visit, as well as caffeinated beverages on the day of the stress procedure and strenuous exercise from 2pm the day before, and to fast for 2 hours prior to testing. See **Supplementary Methods section** for details of SECPT procedure.

Neurocognitive assessment

Prior to EEG testing participants were asked to refrain from caffeine on the morning of their experimental session, to remove any piercings and avoid wearing hair gel. All EEG measurements were made using a Neuroscan®, SynAmps 2 Amplifier and Neuroscan 4.3.1 acquisition software. EEG was recorded at a sampling rate of 1,000Hz. Scalp electrodes were attached at midline positions Fz, Pz, Cz, and F1, F2, F3, F4, F5, F6, F7, F8, according to the international 10/20 system, as well as mastoid electrodes and a reference electrode on the nose. Vertical eye movements were detected using electrodes attached above and below the orbit of the left eye, simultaneously

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horizontal eye movements were monitored by electrodes at the right and left outer canthi. EEG recordings were made using Neuroscan® Quick-Cap (containing AgCl sintered electrodes and Neuroscan Quick-Cell technology) therefore ensuring reduced impedance levels for optimized recordings at each electrode. Following a resting EEG recording, the cognitive tasks were completed (see Cognitive tasks).

Resting EEG

EEG measures of absolute power in the delta (2-4Hz), theta1 (4-6Hz), theta2 (6-8 Hz) alpha1 (8-10Hz), alpha2 (10-12Hz), beta (15-30Hz) frequency bands were taken for five minutes with eyes closed. Participants were requested to relax and sit still with their eyes closed while resting EEG was recorded.

EEG analysis

The EEG signal was down sampled from 1000Hz to 256Hz with an antialiasing filter set at 128Hz. The filtered EEG signal was segmented into 1s windows without overlap. Curve length, root mean squared amplitude, Hjorth parameters (activity, mobility, complexity) (Hjorth, 1970), zero crossings (raw epoch, first and second derivative), autoregressive modelling error (model order 1-9), nonlinear energy, variance (first and second derivative), entropy (Shannon entropy, spectral entropy, singular value decomposition entropy), Fisher information, and wavelet energy (Daubechy 4) were calculated using MATLAB. EEG measures of absolute power were extracted in the delta (2-4Hz), theta1 (4-6Hz), theta2 (6-8 Hz) alpha1 (8-10Hz), alpha2 (10-12Hz), beta (15-30Hz) frequency bands.

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Sample analysis

Cortisol sampling & analysis

Salivettes were centrifuged at 1000 g for 5 min and aliquoted and stored at -80^{o}C until analysis. Cortisol concentrations were determined using the Cortisol Enzyme Immunoassay Kit as per manufacturers' instruction (Enzo®, Life Sciences). Assay detection limit was 0.16 nmol/L. Inter and intra-assay % C.Vs were 11.24% and 8.2% respectively.

Cytokine sampling & analysis

10ml of whole blood was collected in an EDTA tube. Samples were centrifuged at 1000 g for 15 minutes and then aliquoted and stored at -80^{o}C until analysis. Plasma levels of IL1β, IL6, IL8, IL10 and TNFα were assayed in duplicate using high sensitivity commercially available electrochemiluminescence MULTI-SPOT® Meso Scale Discovery kits (MSD, Rockville, MD, 75USA) as per manufacturer's instructions. The median lower limits of detection for each cytokine are; IL-1β; 0.04 pg/ml, IL-6; 0.06 pg/ml, IL-8; 0.04 pg/ml, IL-10; 0.03 pg/ml, TNF-α 0.04 pg/ml.

TLR-4 cytokine release

TLR cytokine release was determined as previously described (McKernan et al., 2011). Whole blood was collected in lithium heparin tubes and diluted 1:10 with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) and 5% penicillin streptomycin. Each blood sample was cultured with and without the TLR-4 receptor ligand - lipopolysaccharide (LPS), from the Human TLR agonist kit

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(InvivoGen, San Diego, CA, USA) for 24 hours. After the 24 hour culture period the supernatant from both untreated and stimulated cells was aspirated and stored at -80°C. Levels of IL1β, IL6, IL8, I-10 and TNFα were assayed in duplicate using high sensitivity commercially available electrochemiluminescence MULTI-SPOT® Meso Scale Discovery kits (MSD, Rockville, MD, 75USA) as per manufacturer's instructions.

Statistical analysis

With a power of 0.8 for a one-way ANOVA, a minimum sample size of 20 was required to demonstrate an effect sized f = 0.3 at alpha = 0.05 (Allen et al., 2016; Buchner et al., 1997). Data were analysed using SPSS 21. Repeated measures ANOVA and pairwise t-tests using post-hoc Fisher's least significant difference (LSD) were used to examine differences between conditions, and non-parametric equivalents (Friedman and Wilcoxon respectively) were used where parametric assumptions were violated. Areas under the curve with respect to ground (AUCg) were also calculated (Pruessner et al., 2003), and analysed in the same manner.

Results

Subjective stress measures

A repeated measures ANOVA with a Greenhouse-Geisser correction determined that there was no overall effect of treatment phase on the Beck Depression Inventory ($p = 0.75$), the Beck Anxiety Inventory ($p = 0.95$), the Perceived Stress Scale ($p = 0.053$), the State Anxiety Inventory ($p = 0.09$), the Trait Anxiety Inventory ($p = 0.72$), the Symptom Checklist-90 ($p = 0.87$) or the Pittsburgh sleep quality index ($p = 0.07$). In the coping

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checklist, there was a reduction in wishful thinking (p = 0.03) in both the placebo (p = 0.04) and probiotic phase (p = 0.02), see **(Figure 1).** For pairwise comparisons see **Supplementary Table 1.**

Acute Stress response to the SECPT

There was no overall effect of treatment phase on subjective stress measures pre or post the SECPT **(**see **Figure 2A-H)**, and no significant overall effect of probiotic over placebo in HPA response to the SECPT **(Figure 2I-J).** See **Supplementary Table 2** for pairwise comparisons.

Immune response

There was no overall treatment effect on the concentrations of IL10 (p = 0.32), IL1β (p = 0.23), IL6 (p = 0.13) or IL8 (p = 0.16) **(Figure 3A-D).** The concentration of TNFα increased from baseline during the placebo phase (p = 0.02), but there was no significant change in baseline versus probiotic (p = 0.08) or placebo versus probiotic (p = 0.18) **(Figure 3E).** There was no overall treatment effect on the IL1β:IL10 (p = 0.99), IL6:IL10 (p = 0.12), IL8:IL10 (p = 0.97), or TNFα:IL10 (p = 0.99) ratios **(Figure 3F-I).**

TLR-4 cytokine release

In the TLR-4 stimulated cytokines, there was an increase in the level of IL1β (p = 0.02) **(Figure 3J)** and TNFα (p = 0.01) **(Figure 3K)** during the placebo phase compared to baseline (p = 0.01) but no effect of probiotic (p = 0.03). There was no effect of either treatment phase on TLR-4 stimulated IL10 (p = 0.12), IL6 (p = 0.22) or IL8 (p = 0.25)

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cytokine release **(Figure 3L-M).** See **Supplementary Table 3** for pairwise comparisons of inflammatory Measures.

Cognitive Measures

Paired Associates Learning (PAL)

There was no overall treatment effect on the total errors made (p = 0.06) **(Figure 4A)**, however at the 8 shape stage **(Figure 4B)**, there was a significant reduction in errors from baseline in the placebo (p = 0.04) and probiotic phases (p = 0.04), but no significant difference between the placebo and probiotic. There was no significant difference in the mean trials to success (p = 0.13) **(Figure 4C)**.

Attention Switching Task (AST)

There was an increase in the correct response in the placebo (p = 0.03) **(Figure 4D)** and probiotic treatment phases (p = 0.01) compared to baseline, and a decrease in the reaction time to correct response **(Figure 4E)** in the probiotic phase compared to baseline (p = 0.006), however the differences between placebo and probiotic were not significant.

Rapid visual information processing (RVP)

The total correct hits increased following treatment with placebo and probiotic (p = < 0.001) **(Figure 4F)**, but there was no overall effect in the total false alarms (p = 0.53) **(Figure 4F)**, or the reaction time (p = 0.48) **(Figure 4H).**

Emotional Stroop

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There was an increase in the percentage of correctly identified neutral words in the probiotic phase of treatment (p = 0.03) **(Figure 4I)**, but this was not significantly greater than baseline (p = 0.54). There was no difference in reaction time to identify neutral words (p = 0.85) **(Figure J).** There were no significant differences in positive percent correct, positive reaction time, negative percent correct, negative reaction time (data not shown).

Emotion Recognition task

The total correctly identified emotions increased in the placebo and probiotic phase compared to baseline (p = < 0.001) **(Figure 4K)**, manifest in the disgust (p = 0.02) and fear (p = < 0.001) categories, but no differences between placebo and probiotic. In addition, there was a non-significant decrease in time taken to correctly identify emotions in the placebo and probiotic phases **(Figure 4L).** See **Supplementary Table 4** for pairwise comparisons.

EEG

There was a significant difference between placebo and probiotic for F3 zero crossings (second derivative) (p = 0.015), however, there was no significant difference in this index between baseline and placebo (p = 0.693) or between baseline and probiotic (p = 0.058). There were no significant differences between placebo and probiotic in any of the other measures. See **Supplementary Table 5** for pairwise comparisons.

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Discussion

Preclinical data strongly supports the view that *L. rhamnosus* (JB-1) treatment has the capacity to alter central GABA transmission by acting through the vagus nerve (Bravo et al., 2011) and in so doing impact significantly on stress responses and behaviour. In this translational study conducted in healthy volunteers we failed to replicate the preclinical findings, which were conducted in an anxious mouse strain. In contrast to the preclinical data, this cross-over study found that *L. rhamnosus* treatment was not superior to placebo in improving cognitive performance and did not attenuate reported stress in healthy male subjects. Furthermore, probiotic treatment did not have a clear anti-inflammatory effect and did not attenuate the subjective stress response or HPA axis response during an acute stress procedure. This study highlights the challenges in translating the findings from candidate psychobiotics in stress-susceptible animals, to healthy human populations.

The candidate psychobiotic used in this study displayed a strong behavioural signal across multiple aspect of behaviour in well-validated screening assays in an anxious mouse strain (Bravo et al., 2011). However, over the eight week period of this trial, self-reported mood, anxiety, stress and sleep were constant and not significantly altered from baseline during the placebo or probiotic phases **(Figure 1A-H).** The data from other studies is mixed. For example, our results are consistent with a study by (Benton et al., 2007), albeit in an older age group, that showed no overall effect of *Lactobacillus casei Shirota* on mood and only a small improvement when post-hoc analysis of the lowest tertile mood scores were considered. After a 6 week, randomized, double-blind, placebo-controlled trial in petrochemical workers, there

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was a significant improvement in the general health questionnaire score in the probiotic yogurt group (*L. acidophilus LA5 and B. lactis BB12*) and in the probiotic capsule group (*L. casei, L. acidophilus, L. rhamnosus, L. bulgaricus, B. breve, B. longum, S. thermophiles*), as well as a significant improvement in the depression anxiety and stress scale score in the probiotic yogurt and the multispecies probiotic capsule group. The improvement in scores in these scales were not seen in the conventional yogurt group (containing the starter cultures of *S.thermophilus and L. bulgaricus*). Probiotic treatment did not alter HPA axis function or the kynurenine/tryptophan ratio (Mohammadi et al., 2015b). The same group did not observe a significant effect between the groups in oxidative stress markers (Mohammadi et al., 2015a).

A more recent study, that used a multi-species probiotic (*B. bifidum W23, B. lactis W52, L. acidophilus W37, L. brevis W63, L. casei W56, L. salivarius W24, and Lactococcus lactis (W19 and W58*), did not find significant changes in mood or anxiety as measured by the Beck Depression Inventory or Beck Anxiety Inventory, but reported a reduction on subscales of the Leiden index of depression for rumination and aggressive thoughts (Steenbergen et al., 2015). Another study in healthy controls, using *L. helveticus R0052* and *B. Longum R0175* found no change in stress, as measured by the perceived stress scale, but did report a reduction in anxiety scores using the Hospital Anxiety and Depression Scale and a reduction in the global severity index, somatisation, depression and anger–hostility scores in the Hopkins Symptoms Checklist (HSCL-90) (Messaoudi et al., 2011). Interestingly, we have recently shown that treatment with *B. Longum* resulted in an improvement in stress related behaviour and cognition in BALB/c mice (Savignac et al., 2014; Savignac et al., 2015) and this

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probiotic treatment modulated behaviour and stress responses in healthy male volunteers (Allen et al., 2016).

From a physiological perspective, *L. rhamnosus* (JB-1) treatment also exhibited the capacity to reduce acute stress responses in mice (Bravo et al., 2011). Our participants exhibited an increased cortisol output in response to the acute stressor **(Figure 2I)**. However, probiotic treatment did not attenuate cortisol output and there were no differences in subjective stress reports **(Figure A-H).** Although not utilizing an acute stress procedure, Messaoudi and colleagues found a significant difference in urinary cortisol levels between the *L. helveticus R0052* and *B. longum R0175* group and placebo groups (Messaoudi et al., 2011). In a study administering a prebiotic (galactooligosaccharide) to healthy controls for three weeks a significant decrease in the salivary cortisol awakening response compared to placebo was found (Schmidt et al., 2015).

Our results suggest that *L. rhamnosus* (JB-1) treatment doesn't affect either basal or stimulated immune responses. In contrast to our findings, both preclinical and clinical studies have previously shown that *L. rhamnosus* treatment has anti-inflammatory effects (Forsythe et al., 2012; Mortaz et al., 2015; Pessi et al., 2000). Thus, two key pillars of brain-gut axis signalling were not modified following psychobiotic treatment. In terms of cognition, the parallel mode of the PAL test (which presents different shapes at each visit) was used in order to avoid practice effects, and to assess conditional learning of pattern-location associations. PAL test performance is dependent upon the hippocampus (de Rover et al., 2011; Eichenbaum and Bunsey,

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1995), which has a high proportion of glucocorticoid receptors (McEwen, 1999). A deficit in visuospatial memory performance, evident in PAL test performance, has been demonstrated in stress-related brain-gut axis disorders with a cognitive component such as IBS (Kennedy et al., 2015; Kennedy et al., 2014a). In this study, probiotic treatment was not superior to placebo across multiple cognitive domains including memory, attention, executive function and emotion recognition. Similarly, there were no significant differences of relevance in EEG measures between the probiotic treatment and the placebo.

We employed a rigorous cross-over trial with a repeated measures design to control for potential effects of individual differences. Given that our study consisted of young healthy males, with normal mood, stress and anxiety scores and no deficits in HPA, inflammatory or cognitive function, demonstrating a clear probiotic effect over placebo in this population may be challenging. This inability to demonstrate superiority of treatment over placebo is not unusual, either in the assessment of psychotropics in general or in microbiota-directed interventions. For example, a novel and initially promising spore based microbiome therapy (SER-109) in *Clostridium difficile* infection (Khanna et al., 2016), was shown not to be statistically superior to placebo in a larger phase II trial (Seres, 2016). At each study visit, participants were asked whether they experienced any side effects from consumption of the capsules. Side effects were negligible, however, formal assessment of gastrointestinal function, was not carried out and is thus a limitation of the study.

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There is an important difference in vulnerability between the anxious mouse strain used in the preclinical study and the healthy human volunteers that make up the clinical sample. It is worth noting in this regard that psychobiotics may be of limited benefit in healthy populations. Comparably, antidepressants also have a limited beneficial effect in healthy controls (Serretti et al., 2010). Moreover, antidepressants have a delayed onset of action (Taylor et al., 2006) and we acknowledge that more than four weeks of psychobiotic treatment may be required in future studies in populations with stress-related psychiatric disorders. A recent systematic review indicated that the impact of probiotic supplementation on gut microbiota structure, including an assessment across features such as α-diversity, richness and evenness, in healthy controls was minimal (Kristensen et al., 2016). However, it is important to consider that probiotic treatment may impact the function of colonizing microbes or promote homeostasis of the gut microbiota, rather than change its composition (Sanders, 2016).

A more defined role for probiotic intervention may be in populations with some degree of pathology, for example IBS (Didari et al., 2015). Recently, several studies have demonstrated altered gut microbiota composition in depression (Jiang et al., 2015; Kelly et al., 2016a; Naseribafrouei et al., 2014) and suggest that this altered gut microbiota composition may play a causal role in the development of certain features of depression (Kelly et al., 2016a; Zheng et al., 2016), though the precise mechanisms have yet to be elucidated. To date, only one small study has investigated a probiotic intervention in depressed patients (Akkasheh et al., 2016). In this eight week study, treatment with a multispecies probiotic containing *L. acidophilus, L. casei and B.*

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bifidum, reportedly reduced depressive symptoms in moderately depressed patients compared to placebo.

Despite the momentum provided by preclinical microbiome studies, there is a growing appreciation of the challenges in moving this work from bench to bedside (Arrieta et al., 2016; Dinan and Cryan, 2016). This includes the fact that the rodent gastrointestinal tract and microbiota composition differs from the human equivalent (Nguyen et al., 2015). It is worth noting that the effects of *L. rhamnosus* treatment were dependent on the vagus nerve (Bravo et al., 2011). The precise mediators between the gut microbiota and the vagus nerve have not been defined and could not therefore be assessed in this study. Moreover, it is important to note that the preclinical analysis of *L. rhamnosus* was carried out in BALB/c mice which are innately anxious and have different brain and gut responses to stress (Browne et al., 2011; Julio-Pieper et al., 2012; O'Mahony et al., 2010; Savignac et al., 2011).

Moreover, Bercik and colleagues have shown alterations in microbiota composition in this strain compared with strains with normal stress responses. Further, when these mice were transplanted with microbiota from a normo-anxious mouse their behaviour normalised suggesting a connection between host microbiota and behaviour (Collins et al., 2013). Recently, *B. longum* **1714,** an alternative candidate psychobiotic selected following a similar preclinical screening battery in BALB/c mice, has been reported to reduce stress and improve memory (Allen et al., 2016), although a detailed mechanistic understanding of its effects in this regard is currently lacking. These

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diverging outcomes highlight the issue of different putative psychobiotics likely exhibiting different mechanisms of action. Ultimately, this study, together with the SER-109 study illustrate the need to better understand the mechanisms, for effective translation. Whether the JB-1 strain has potential in the treatment of stress-related psychiatric disorders, either as a single agent, or in combination with other potential psychobiotics, remains an open question and further investigations are warranted.

Conclusions

This eight week randomized cross-over trial did not show that *L. rhamnosus* (JB-1) was superior to placebo in modifying stress-related measures, HPA responses, inflammation or cognitive performance in healthy male participants. These results suggest that some caution is required regarding expectations of targeting the gut microbiome in healthy populations and that there may be challenges in translating candidate psychobiotics with promising preclinical signals in anxious mouse strains into healthy human subjects. Future interventional studies investigating the effect of this probiotic in populations with stress-related disorders are required.

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Acknowledgements

The APC Microbiome Institute is funded by Science Foundation Ireland (SFI). This publication has emanated from research conducted with the financial support of Science Foundation Ireland (SFI) under Grant Number SFI/12/RC/2273. The authors and their work were also supported by the Health Research Board (HRB) through Health Research Awards (grants no HRA_POR/2011/23; TGD, JFC and GC, HRA_POR/2012/32; JFC, TGD and HRA-POR-2-14-647: GC, TGD, APA) and through EU GRANT 613979 (MYNEWGUT FP7-KBBE-2013-7). They have ongoing research collaborations with several companies including Suntory Wellness, Danone Nutritia, Mead Johnson Nutrition, Cremo and 4D-Pharma. GC is supported by a NARSAD Young Investigator Grant from the Brain and Behaviour Research Foundation (Grant Number 20771). The placebo and probiotic containing *L. rhamnosus* was provided by Alimentary Health Ltd. The authors would like to thank Aoife Collery, Clara Seira, Pauline Luczinski, Livia Morais, Dr. Karen Scott for acting as confederates for the socially evaluated cold pressor test.

Financial Disclosures

Dr Kelly reported that his research is supported by Science Foundation Ireland (grant number SFI/12/RC/2273). Dr Kennedy reported that his research supported by Science Foundation Ireland (grant number SFI/12/RC/2273) and EU GRANT 613979 (MYNEWGUT FP7-KBBE-2013-7). Dr Allen reported that his research is supported by Science Foundation Ireland (grant number SFI/12/RC/2273) and HRA-POR-2-14-647. Dr Clarke reported that his research is supported by SFI (grant number SFI/12/RC/2273) and by the Health Research Board (HRB) through Health Research Awards (grants no

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HRA_POR/2011/23, and HRA-POR-2-14-647: GC, TGD). GC is supported by a NARSAD Young Investigator Grant from the Brain and Behaviour Research Foundation (Grant Number 20771). Prof Cryan reported that his research is supported by SFI (grant number SFI/12/RC/2273) and by the Health Research Board (HRB) through Health Research Awards (grants no HRA_POR/2011/23, HRA_POR/2012/32) and through EU GRANT 613979 (MYNEWGUT FP7-KBBE-2013-7). Prof Cryan has been an invited speaker at meetings organized by Mead Johnson, Yakult, Alkermes, and Janssen and has ongoing research collaborations Suntory Wellness, Danone Nutritia, Mead Johnson Nutrition, Cremo and 4D-Pharma. Prof Dinan reported that his research is supported by SFI (grant number SFI/12/RC/2273) and by the Health Research Board (HRB) through Health Research Awards (grants no HRA_POR/2011/23, HRA_POR/2012/32 and HRA-POR-2-14-647) and through EU GRANT 613979 (MYNEWGUT FP7-KBBE-2013-7). Prof Dinan has been an invited speaker at meetings organized by Servier, Lundbeck, Janssen, and AstraZeneca and has ongoing research collaborations with Suntory Wellness, Danone Nutritia, Mead Johnson Nutrition, Cremo and 4D-Pharma.

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Figure Legends

Figure 1: Subjective Stress Measures

There was no overall effect of treatment phase in the **(A)** Beck Depression Inventory ($p = 0.75$), **(B)** the Beck Anxiety Inventory ($p = 0.95$), **(C)** the Perceived Stress Scale ($p = 0.053$) **(D),** the State Anxiety Inventory ($p = 0.09$), **(E)** the Trait Anxiety Inventory ($p = 0.72$), **(F)** the Symptom Checklist-90 ($p = 0.87$) or **(G)** the Pittsburgh sleep quality index ($p = 0.07$). **(H)** In the coping checklist, there was a reduction in wishful thinking ($p = 0.03$) in the placebo ($p = 0.04$) and probiotic phase ($p = 0.02$).

Figure 2: Acute Stress response to the Socially Evaluated Cold Pressor Test (SECPT)

In the primary appraisal/secondary appraisal scale, there was no significant effect of probiotic the **(A)** primary appraisal ($p = 0.73$), **(B)** secondary appraisal ($p = 0.14$) in **(C)** control expectancy ($p = 0.16$), **(D)** self-control ($p = 0.12$), **(E)** threat ($p = 0.27$), **(F)** challenge ($p = 0.51$), **(G)** or stress index ($p = 0.35$). **(H)** There was no significant effect of treatment in pre-stress ($p = 0.09$), post-stress, ($p = 0.51$), difficulty ($p = 0.29$), unpleasantness ($p = 0.12$), or pain reports ($p = 0.28$). **(I)** There were no significant differences in the HPA response to the SECPT ($p = 0.54$), the **(J)** Area under the curve with respect to ground (AUCg) ($p = 0.35$) or the **(K)** delta cortisol response ($p = 0.28$).

Figure 3: Immune response

There was no overall treatment effect on the concentrations of **(A)** IL10 ($p = 0.32$) **(B)** IL1β = ($p = 0.23$), **(C)** IL6 ($p = 0.13$) or **(D)** IL8 ($p = 0.16$). **(E)** The concentration of TNFα increased from baseline during the placebo phase ($p = 0.02$), but there was no significant change in baseline versus probiotic ($p = 0.08$) or placebo versus probiotic (p

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= 0.18). There was no overall treatment effect on the **(F)** IL1β:IL10 (p = 0.99), **(G)** IL6:IL10 (p = 0.12) **(H)** IL8:IL10 (p = 0.97), or **(I)** TNFα:IL10 (p = 0.99) ratios. Pairwise comparisons showed that the probiotic decreased the IL6:IL10 ratio (p = 0.03), though not significantly over placebo (p = 0.13). In the Toll-like 4 stimulated cytokines, there was an increase in the level of **(J)** IL1β (p = 0.02) and **(K)** TNFα (p = 0.01) during the placebo phase compared to baseline (p = 0.01). There was no effect of either treatment phase on **(L)** IL10 (p = 0.12) **(M)** IL6 (p = 0.22) or **(N)** IL8 (p = 0.25) cytokine release.

Figure 4: Cognitive Measures

In the paired associates learning task, there was no overall treatment effect on the **(A)** total errors made (p = 0.06), however at the **(B)** 8 shape stage, there was a significant reduction in errors from baseline in the placebo (p = 0.04) and probiotic group (p = 0.04), but no significant difference between the placebo and probiotic. There was no significant difference in the **(C)** mean trials to success (p = 0.13). **(D)** In the attention switching task, there was an increase in the correct response in the placebo (p = 0.03) and probiotic phases (p = 0.01) compared to baseline, and a decrease in the **(E)** reaction time to correct response in the probiotic phase compared to baseline (p = 0.006), however the differences between placebo and probiotic were not significant. In the rapid visual information processing task, the placebo and probiotic improved the **(F)** total correct hits (p = < 0.001), but there was no overall effect in the **(G)** total false alarms (p = 0.53) or the **(H)** reaction time (p = 0.48). In the emotional stroop task there was an increase in the percentage of correctly identified neutral words in the probiotic phase of treatment **(I)** (p = 0.03), but this was not significantly greater than

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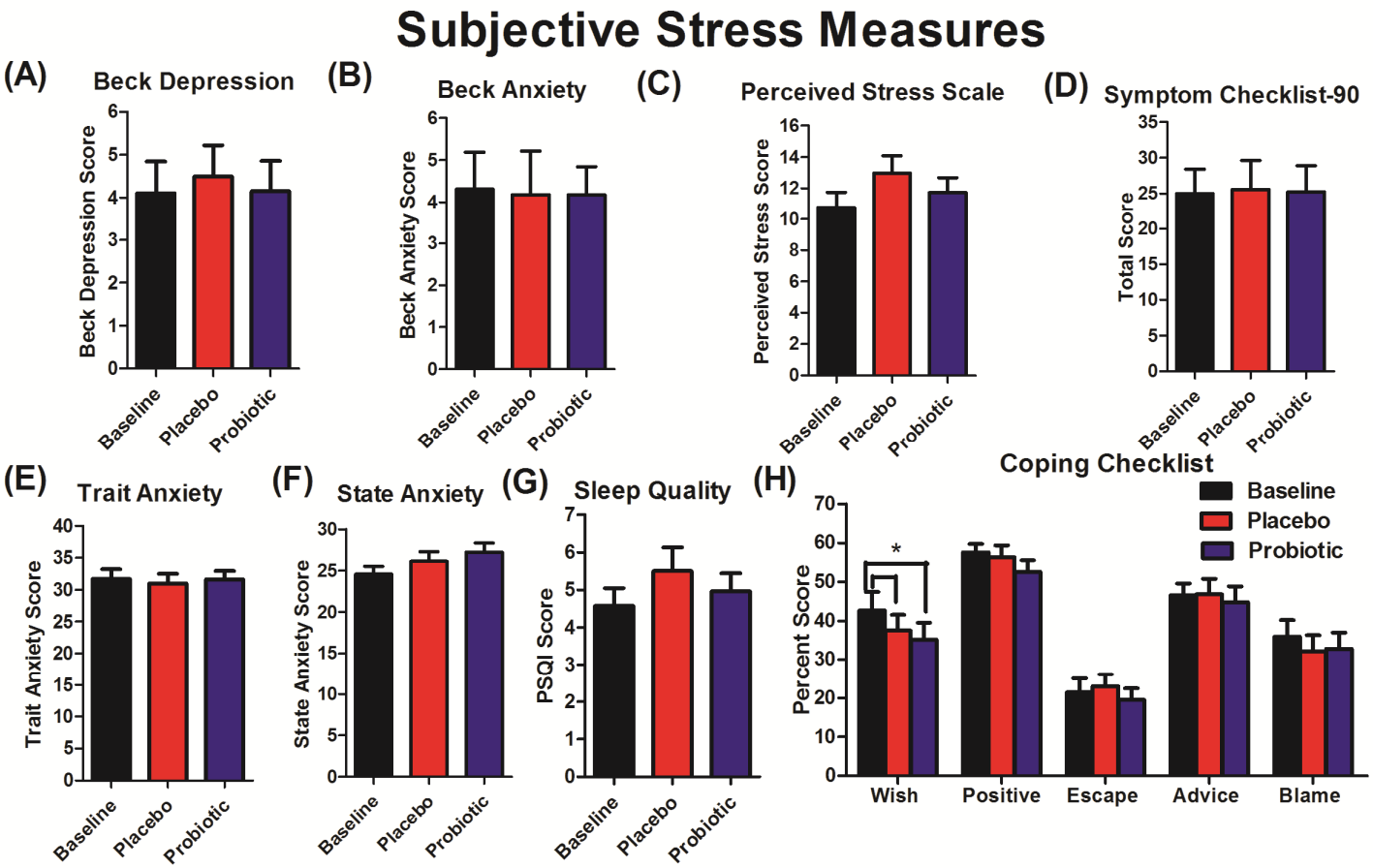
baseline (p = 0.54). There was no difference in **(J)** reaction time to identify neutral words (p = 0.85). In the emotion recognition task, the **(K)** total correctly identified emotions increased in the placebo and probiotic phase compared to baseline (p = < 0.001), manifest in the disgust (p = 0.02) and fear (p = < 0.001) emotion sub-categories. **(L)** In addition, there was a non-significant decrease in time taken to correctly identify emotions in the placebo and probiotic phases.

Table 1: Participant characteristics. Mean values (standard errors of the mean in parentheses). We assessed diet using a modified version of the food frequency questionnaire (Harrington et al., 2010) and presented the data in daily grams in **Supplementary Table 6.**

Participant characteristics	Total sample (n=29)	Placebo / Probiotic (n=15)	Probiotic / placebo (n=14)	p-value
Age (mean)	24.59 (0.75)	23.6 (0.97)	25.64 (1.14)	0.22
Ethnicity				
Caucasian	25	13	12	
Asian	4	2	2	1.00
BMI	24.55 (0.58)	24.8 (0.69)	24.29 (0.96)	0.66
Alcohol (units per week)	10.14 (1.85)	11.85 (2.68)	8.42 (2.56)	0.36
Years of Education	18.45 (0.49)	17.87 (0.71)	19.07 (0.65)	0.22

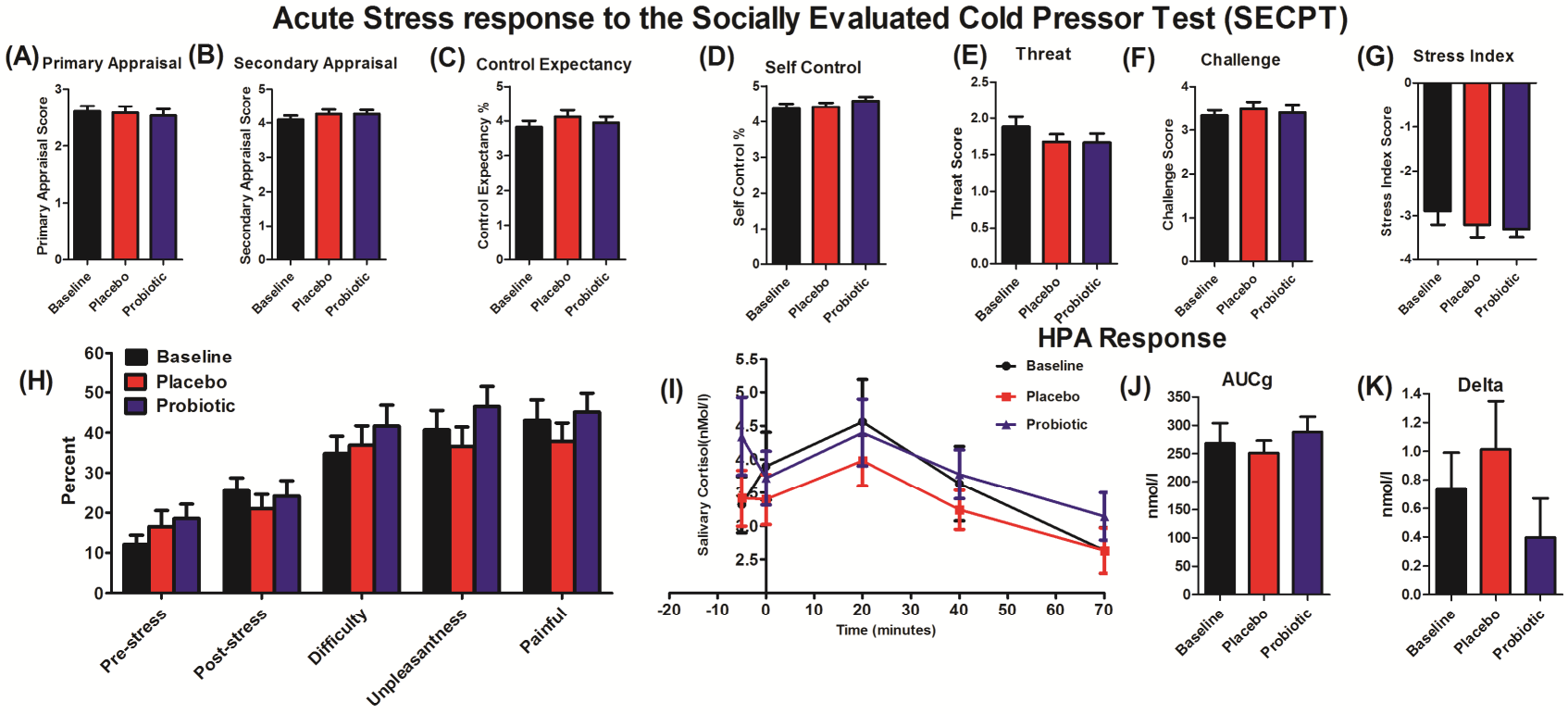
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Figure 1



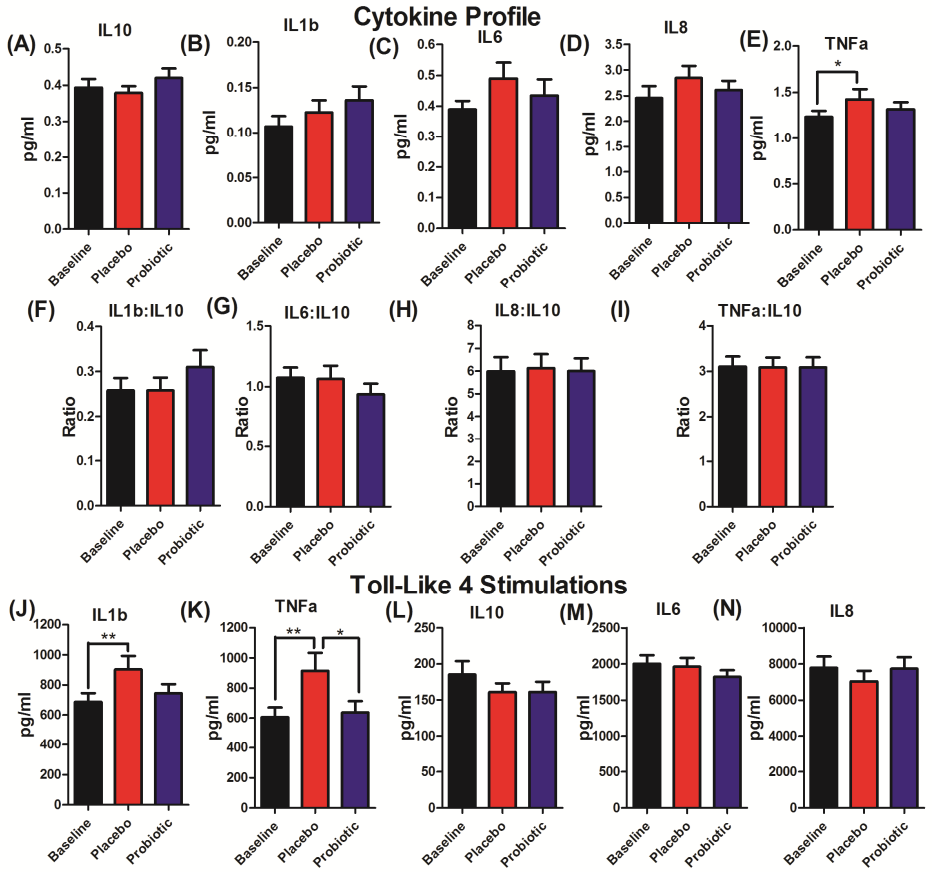
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Figure 2



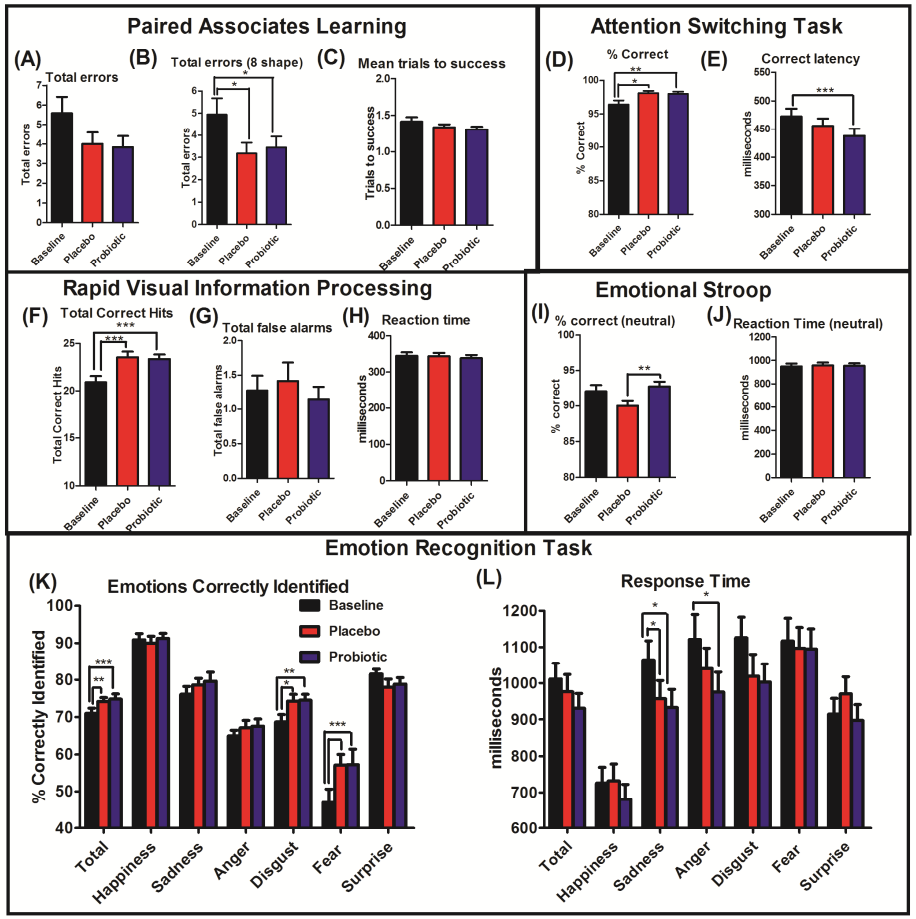
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Figure 3



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Figure 4: Cognitive Measures



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Research highlights

- *Lactobacillus rhamnosus* (JB-1) has been shown to reduce stress-related behaviour, corticosterone release and alter central expression of GABA receptors in mice.
- An 8 week, randomized, placebo-controlled, cross-over design trial was employed to assess the impact of *L. rhamnosus* on stress-related behaviours, physiology, inflammatory response, cognitive performance and brain activity patterns in 29 healthy male participants.
- *L. rhamnosus* was not superior to placebo in modifying stress-related measures, HPA response, inflammation or cognitive performance.
- These findings highlight the challenges associated with moving promising preclinical studies from bench to bedside.

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