Brain, Behavior, and Immunity 122 (2024) 256–265

Contents lists available at ScienceDirect

Brain Behavior and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Full-length Article

Gaba-producing lactobacilli boost cognitive reactivity to negative mood without improving cognitive performance: A human Double-Blind Placebo-Controlled Cross-Over study

Melania Casertano [a,b], Matthijs Dekker [a], Vincenzo Valentino [b], Francesca De Filippis [b], Vincenzo Fogliano [a,*], Danilo Ercolini [b]

[a] Food Quality & Design Group, Wageningen University & Research, NL-6708 WG, the Netherlands
[b] Department of Agricultural Sciences of the University of Naples "Federico II", Università 100, 80055 Portici (NA), Italy

ARTICLE INFO

Keywords:
Psychobiotics
Stress
Depression
Cognition
Gut −microbiota-brain-axis

ABSTRACT

Background: Psychobiotic bacteria are probiotics able to influence stress-related behavior, sleep, and cognitive outcomes. Several *in vitro* and human studies were performed to assess their physiological potential, to find strains having psychotropic activity in humans, and to elucidate the metabolic pathways involved. In our previous *in vitro* study, we identified two strains *Levilactobacillus brevis* P30021 and *Lactiplantibacillus plantarum* P30025, able to produce GABA and acetylcholine, being promising candidates to provide an effect on mood and cognitive performance.

Aim: To investigate the effects of probiotics in the alleviation on the cognitive performance of moderately stressed healthy adults. Secondary outcomes were related to mood improvement, production of GABA, glutamate, acetylcholine, and choline and modification of the microbiota composition.

Methods: A 12-week randomized, double-blind, placebo-controlled, cross-over study investigated the effects of a probiotic formulation (*Levilactobacillus brevis* P30021 and *Lactiplantibacillus plantarum* P30025) on psychological, memory, and cognition parameters in 44 (Probiotic = 44, Placebo = 43) adults with a mean age of 29 ± 5.7 years old by CogState Battery test. Subjects-inclusion criteria was a mild-moderate (18.7 ± 4.06) stress upon diagnosis using the DASS-42 questionnaire.

Results: Probiotic treatment had no effect on subjective stress measures. The probiotic formulation showed a significant beneficial effect on depressive symptoms by reducing cognitive reactivity to sad mood ($p = 0.034$). Rumination significantly improved after intake of the probiotic ($p = 0.006$), suggesting a potential benefit in reducing the negative cognitive effects associated with depression and improving overall mental health. When stratifying the treated subjects according to the response, we found an increase in the abundance of the probiotic genera in the gut microbiota of positive responders ($p = 0.009$ for *Lactiplantibacillus* and $p = 0.004$ for *L.brevis*). No relevant correlations were observed between the neurotransmitter concentration in the faecal sample, scores of LEIDS, DASS-42, and cognitive tests.

Conclusion: We highlight the potential of this probiotic preparation to act as psycobiotics for the relief of negative mood feelings. The assessment of the psychotropic effects of dietary interventions in human participants has many challenges. Further interventional studies investigating the effect of these psychobiotic bacteria in populations with stressed-related disorders are required including longer period of intervention and larger sample size in order to verify the effects of the treatment on further stress-related indicators.

Abbreviations: ACC, Acceptance/Coping; AGG, Aggression; CON, Control/Perfectionism; CBB, CogState Brief Battery; DASS-42, Depression Anxiety Stress Scale; DET, Detection performance; GABA, Gamma-aminobutyric acid; HOP, Hopelessness; IDN, Identification Test; LEIDS-r, Leiden Index of Depression Sensitivity-Revised Test; MSG, MonoCsodium glutamate; OCL, One card Learning Test; ONB, One Back Test; RAV, Risk aversion; RCT, randomized controlled trial; RUM, Rumination; SCFA, Short-Chain Fatty Acid; SEC, Social-Emotional Cognition Test.

* Corresponding author.
E-mail address: vincenzo.fogliano@wur.nl (V. Fogliano).

https://doi.org/10.1016/j.bbi.2024.08.029
Received 3 May 2024; Received in revised form 1 August 2024; Accepted 11 August 2024
Available online 18 August 2024

1. Introduction

Stress is an emotional and physiological reaction to major life events and demanding circumstances and it is becoming very common in modern society (Kriakous et al. 2021). A successful stress response can be useful to stay alert and focused, while repeated exposure to stressful conditions can easily tip over the stress from being an important motivational mechanism into a maladaptive and inconvenient response that can have a detrimental effect on both physical and mental health (Morgado and Cerqueira 2018). Social stress is also known to induce depressive-like behaviors and to have well-documented effects on cognitive processes, such as working memory (Sample et al., 2019). Cognitive dysfunctions, including impaired attention, learning, memory, planning, and problem-solving, are now recognized as core symptoms of depression and other psychiatric disorders, and recent findings suggest a role for alteration in GABAergic inhibition in cognitive symptoms. Stress adaptation may be dysfunctional in vulnerable individuals, potentially contributing to the risk for psychopathology like the Major Depressive Disorders (MDD). The current pharmacological treatment approaches rely on monoaminergic-based drugs inhibitors (SSRI); however there are many reasons to limit their use (van Leeuwen et al., 2018) and promote dietary approaches that can help to tackle these conditions before they become psychiatric disturbances.

Mounting evidence shows the connections among the brain, and gut, including the intestinal microbiota. Evidence for a link between microbiota and stress has been gathered mostly from experiments carried out in the microbiota-depleted model, either through treatment with a combination of antibiotics or use germ-free (GF) lines (Sarkar et al., 2016). Developing a novel therapeutic method targeting the gut microbiota for the treatment of mood disorders, especially derived from outside stressors, is emerging as an appealing strategy to moderate their impact on the quality of life and prevent cognitive impairment.

A novel class of probiotic microorganisms, called psychobiotics, is able to convey benefit upon the host's mental health via the bidirectional dynamic microbiota-gut-brain crosstalk (Casertano et al., 2022). To date no probiotic bacteria with positive effects on mental health were able to obtain the specific health claim from the European Food Safety Authority (EFSA) (Lockyer,2020), calling for new studies providing more compelling evidence.

According to recent clinical research, some probiotic strains—specifically, those from the *Lactobacillus* and *Bifidobacterium* genera—may be able to reduce symptoms of stress, anxiety, and depression (Allen, 2016; Moloney, 2021; Wu, 2021b). Although some research provided mechanistic insight in humans, most of the literature focuses on rodent models. Evidence from animal models suggests that the probiotic-brain connection is related by microbiota-mediated mechanisms (such as vagus nerve, the immune system, the hypothalamus–pituitary–adrenal axis, and tryptophan metabolism) (McVey Neufeld et al., 2018; Hashikawa-Hobara et al. 2022; Sun et al. 2019; Ding et al. 2021, Huang et al. 2023). Some encouraging studies in humans examined the effectiveness of a microbiota-targeted probiotic strategy on anxiety, depression, or cognition and only few of them investigated changes in microbiota composition. Results demonstrated that specific probiotic strains can influence symptoms of the stress-related gut-brain disorder and alter resting brain activity, cognitive performance, and memory (Lew et al., 2019; Bloemendaal et al. 2021; Wang et al. 2019; Allen et al. 2016). A strain of *Bifidobacterium longum* 1714 was able to reduce stress levels and to enhance memory in healthy volunteers (Allen et al., 2016). In another independent interventional study, Wang et al., showed that the same strain reduced mental fatigue and might be involved in the activation of brain coping centers to counter-regulate negative emotions (Wang et al. 2019).

Data are still preliminary and some of them portray conflicting results, or difficulty in moving promising preclinical animal studies to humans. Kelly and colleagues could not confirm the effects obtained on anxious mouse regarding the ability of *Lacticaseibacillus rhamnosus* in modifying stress-related measures, and cognitive performance in healthy male participants (Kelly et al. 2017). Most of the research used questionnaires, behavior tests, and cognitive evaluations to evaluate symptom reduction (Allen et al., 2016). Some studies (Bloemendaal et al., 2021; Ma et al., 2023) trying to characterize probiotic-induced changes in the taxonomic (16S rRNA-based) profiles of intestinal microbiota were unable to clarify the role of the gut microbiota and its neuroactive potential due to the lack of corresponding reference genomes . According to (Kyrpides et al., 2014), microbial reference genomes are essential resources for microbial profiling, metagenome annotation, and understanding the physiological role of metagenomes.

Previously, we identify two *Lactobacillus* strains (*Levilactobacillus brevis* P30021 and *Lactiplantibacillus plantarum* P30025) able to produce GABA, and acetylcholine when cultured with their precursor monosodium glutamate (MSG) and choline, respectively (Casertano et al., 2024). These strains were assessed *in vitro* batch fermentation, and in the Simulator of Human Intestinal Microbial Ecosystem (SHIME). Results showed positive effect on the gut microbiota structure and GABA production (Casertano et al., 2024) prompted us to perform a human trial aiming to elucidate the effect of these probiotic strains on cognitive performance and depression related parameters. In this study a double-blind placebo controlled cross-over study was performed administering a psychobiotic formulation containing the two lactobacilli for 12 weeks to adults showing a mild-moderate stress. Considering that GABA is a neurotransmitter able to modulate the anxiety and stress level we aimed to select a precisely targeted population to investigate the effects of the specific strains in alleviation of stress in stressed adults. Stress is supposed to affect the cognitive performance chosen as our primary outcome as main reliable reference found in previous study for the sample size calculation. Beside the cognitive and mood tests, we analyzed the production of neurotransmitters as well as the modulation of the intestinal microbiota.

2. Materials and Methods

2.1. Screening

Healthy study participants were recruited via advertisement, direct contact, and mailing list, from and around the Wageningen (The Netherlands) area. One hundred and seven volunteers responded to the advertisement and direct contact; Participants were screened based on the inclusion and exclusion criteria, after receiving the informed consent file. Inclusion criteria were: age of 18–60 years, a BMI between 18.5–25 kg/m2, men and women, a mild/moderate stress score in the DASS-42 questionnaire. Participants received the DASS-42 questionnaire and a medical information questionnaire as screening tests to assess their eligibility. Decision of the subjects-inclusion based on the mild-moderate stress score, in the scoring range 1of 5–25, and on the answers of the medical questionnaire was made in collaboration with a medical specialist based on eligibility. Participants with stress scoring level on DASS-42 considered as normal (<15) and severe (>25) we excluded from the study. The flowchart of the study design is reported in Fig. 1.

2.2. Randomization and masking

Randomization was done by a researcher who had no contact with the participants, in this way the study was double-blinded. A random sequence was randomly assigned to a confidential treatment number linked to the two treatment arms (first probiotic product and second placebo or vice versa). The allocation sequence was concealed from the researchers and details of the allocated group were given on code containing the sequential number which was placed on the product. The products of identical appearance were labelled with the treatment number by another independent researcher. The Independent study coordinator dispensed either placebo or probiotic sticks according to a

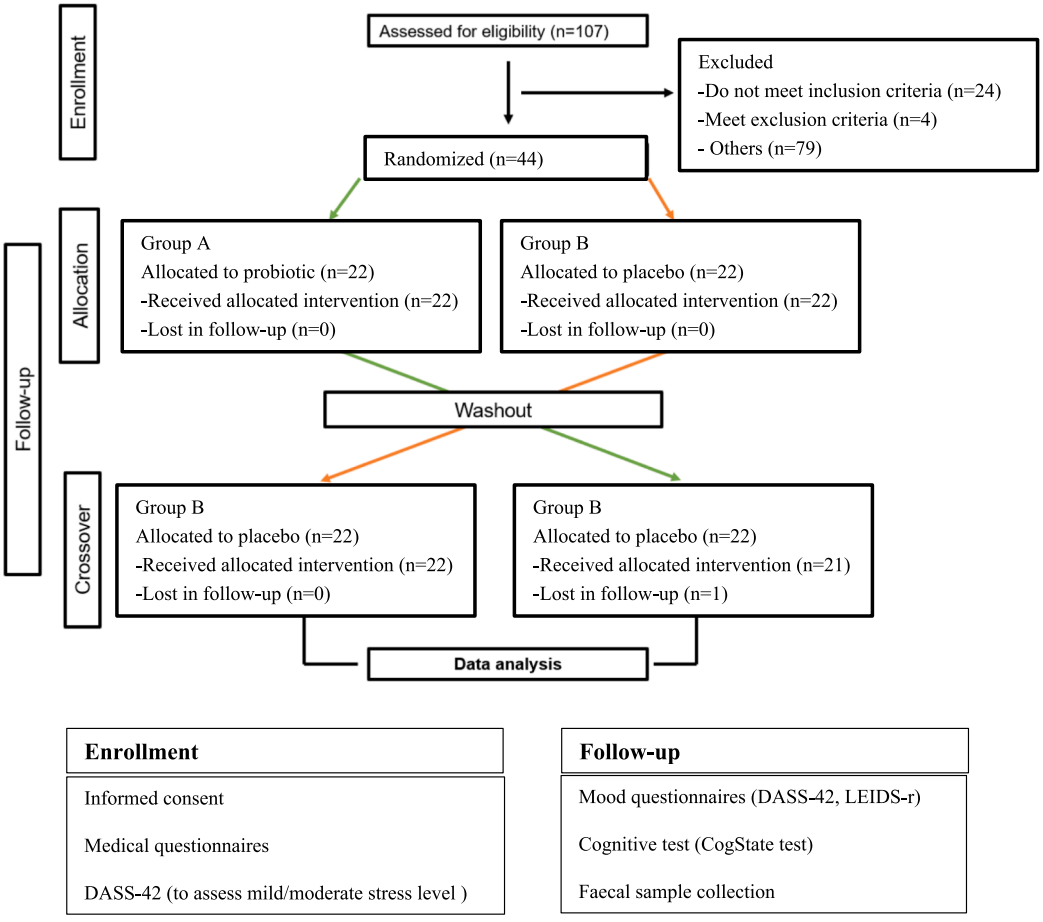

Fig. 1. Study design.

computer-generated randomized sequence.

2.3. Study protocol

All participants, study coordinators, and researchers were blinded throughout the entire study. The study was unblinded along with the entire study and available in the case of an emergency or at the end of the study when all statistical analyses were completed.

The study was conducted as a randomized double-blind placebo-controlled cross-over intervention trial, to test the effect of a supplement consisting of probiotics, vitamins, and zinc on cognitive functioning in adult women and men. The crossover design allow to study the differences in treatments yielding to a more efficient comparison of treatments than a parallel design as the subjects are on their own controls and the within-patient variation is less than between-patient variation (Solito et al. 2021). A washout of 4 weeks was considered enough not to have carry-out effects. Furthermore, no habituation effects which could interfere with the outcomes were expected since the testing software is designed to limit learning effects.

Recruited individuals were randomized into two groups (Fig. 1).

A. Probiotic supplementation – Placebo supplementation (PRO-PLA; $n = 44$).

B. Placebo supplementation – Probiotic supplementation (PLA-PRO; $n = 43$).

One group received supplementation with probiotic sachets containing $> 2x10^9$ CFU/day *Levilactobacillus brevis* P30021 and *Lactiplantibacillus plantarum* P30025 and the other group a placebo (PLC) formulation, containing the same excipients without the probiotic strains, for 4 weeks. Probiotic (PRB) formulation was a 1:1 mixture of the 2 strains.

All the analyses were performed at the recruitment (T0), after the first 4 weeks of treatment (T1), at the beginning of the second phase (T2) and end of washout, and after the last 4 weeks of treatment (T3). Case report forms (CRF) were completed. Patients were asked to report any adverse reaction, antibiotic therapy or other drug administration that occurred. The adherence was monitored counting the returned sachets.

Ethical statement

The study protocol was approved by the Medical Ethics Review Committee of Brabant (Tilburg, The Netherlands; Protocol number: NL76751.028.21). The study was conducted in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki. Written informed consent was obtained from all participants at the screening visit before any study procedures were conducted. The consent form was included in the PIF (Participant Information Form). Participants were free to withdraw from the study at any time.

2.4. Dietary intervention: Probiotics and placebo products

The supplement under investigation was GABAflor, produced by the company Progefarm (Novara, Italy) GABAflor is a stick of powder that consists of: *Lactiplantibacillus plantarum* P30025 and *Levilactibacillus brevis* P30021 (Both 10^9 CFU/stick) which both have been shown to be beneficial for gut health, vitamin B6 included as pyridoxine hydrochloride (2,8 mg/stick), vitamin D3 as cholecalciferol (5 ug/stick), and zinc (7,5 mg). In order to give the supplement a pleasant taste both fructose and cacao powder were added. Participants consumed one stick per day for 4 weeks. The powder sticks could be dissolved in milk or any other liquid to make consumption easier.

The placebo was not able to be distinguished by package, color, taste, or smell in order to maintain treatment allocation concealed from the participants. The placebo had the same taste since it also consisted of cacao powder and fructose.

To ensure compliance during the study, we counted the sticks left in the box from the subjects after each treatment.

2.5. Sample size

The appropriate sample size was estimated by a priori analysis for a clinical research study using G*Power software (Kang 2021). G*Power is a tool to compute statistical power analyses for many different tests and can also be used to compute effect sizes and to graphically display the results of power analyses. The primary objective of the study is to evaluate verbal learning and memory, processing speed, visual attention, learning, and working memory. The cognitive test assessment was chosen as primary outcome as the results obtained from Lew et al.(2019) and Chong et al. (2019) could be used as reference for the calculation of the sample size. With this tool was not possible to calculate the sample size based on a quality assessment like the mood questionnaire. Furthermore the DASS-42 was used as screening test as several studies (Kelly et al. 2017; Allen et al.,2016; Papalini et al., 2018) observed that without stress induction, probiotics did not affect brain, behavioral or related self-report measures, thus we tested whether probiotics can buffer against the detrimental effects of stress on cognitive outcomes. With a power of 0.95 for a one-way ANOVA, a minimum sample size of 36 was required to demonstrate an effect size of 0.25 at alpha 0.05 and a power of 0.95. Allowing for a 20 % drop-out, as seen in similar studies, at least 44 participants should be recruited, divided between 22 males and 22 females. This ensured enough power in case some of the assumptions were not met or in case of some incomplete results (i.e., missing data). A p-value of < 0.05 was considered significant.

2.6. Primary outcome: Cognitive function assessment

All subjects were assessed for memory and cognitive functions using the computerized CogState Brief Battery (CBB) (Mielke et al. 2015). Administration of the CogState battery test was conducted in a personal laptop, installed with the CogState ClinicalTrials software. All subjects first underwent one initial practice prior to the actual test battery. The study coordinator was available to help the subjects understand the tasks during sessions. From the CogState test software, several tests were chosen. These tests were chosen because they have been shown to indicate a significant improvement in the studies from Lew et al. (2019) and Chong et al. (2019), Outcome parameters of the tests include correct and incorrect responses, response speed, and accuracy of performance.

The tests were administered in this order:

- *Detection (DET) task* – The subject is asked to press a key as soon as a playing card, that is displayed on the screen, turns over. The outcome of this task is speed and accuracy of each response. The outcome variable is measured in milliseconds for correct responses which is normalised using a logarithmic base 10 (Log 10 transformation).
- *Identification (IDN) task* – The subjects are asked to press the "yes" key when the card that has flipped over is red. If the card is not red, they have to press the "no" key. The task ends as soon as the subject has given 30 correct answers. The correct outcome is recorded in milliseconds and normalised using a logarithmic base 10 (log 10 transformation).
- *One Card Learning (OCL) task* – The subjects have to press "yes" when the card has appeared before and "no" if it has not. The task ends when 42 trials have been recorded. The outcome variable is the proportion of correct responses (accuracy) normalised with the help of an arcsine root transformation.
- *One Back (ONB) task* – a task that assesses working memory by asking the subject whether the presented card is the same as the previous presented card. The subject can either press "no" or "yes". The primary outcome variable of this task is accuracy. The task ends upon recording 30 trials.
- *Social Emotional Cognition (SEC) task* – 4 pictures of faces are presented on the screen. By clicking the odd-one-out, the task measures emotional recognition. The task end upon recording 30 trials. The outcome measures are accuracy and reaction speed.

The composition of each battery and their respective outcomes are listed in Table 1.

2.7. Secondary outcome: Questionnaires measuring mood-related aspects

2.7.1. Leiden index of depression Sensitivity-Revised Test (LEIDS-r)

The secondary outcome of our study was the difference in the score at the Leiden Index of Depression Sensitivity-Revised test (LEIDS-r) between and within the subjects belonging to the experimental group and the control group. The LEIDS-R is a self-report questionnaire that tests cognitive reactivity to sad mood, which is an index of cognitive vulnerability to depression. LEIDS-r scores have been found in multiple longitudinal studies to predict depression incidence and to correlate with depression risk factors, such as depression history, genetic markers of depression. It consists of 34 items describing different situations. Before answering the items, participants are asked to take a few minutes to imagine their feelings and thoughts when they experience a sad mood. They then rate how much each item applies to themselves on a 5-point scale ranging from 0 (*not at all*) to 4 (*very strongly*). Of note, the experimenter emphasizes that each item describes a situation happening on *a day that is not good, but you don't feel depressed*. The LEIDS-R consists of 6 subscales: Hopelessness/Suicidality (5 items), Acceptance/Coping (6 items), Aggression (5 items), Control/Perfectionism (6 items), Risk aversion (6 items), and Rumination (6 items). The total score for each subscale is obtained by adding the scores from the corresponding item. The range of the total score for the Aggression and Hopelessness/Suicidality subscales is from 0 to 20. The range of the total score for the other three scales is from 0 to 24. The higher the total subscale score, the higher the vulnerability to the assessed dimension.

Table 1
Test information and cognitive domain assessed.

CogState Test (TCode)	Cognitive Domain	Test Description	Primary outcome and Interpretation
Detection Test (DET)	**Psychomotor function** Has the card been turned over?	Detecting and responding to a cue of a card turning over as quickly as possible	Speed of performance; mean of the log10 transformed reaction times for correct responses (**lmn**) **Lower score = better performance**
One Back Test (ONB)	**Working memory** Is this card the same as the previous card?	Remembering the previous card and identifying whether the presented card is the same	
Identification Test (IDN)	**Attention** Is the card red?	Identifying the color of a card and clicking the right button in accordance with it	
One Card Learning Test (OCL)	**Visual learning** Have you seen this card before?	Identifying whether the presented card was seen before	Accuracy of performance; arcsine square root proportion correct (**acc**) **Higher score = better performance**
Social-Emotional Cognition Test (SECT)	**Emotional Cognition** Which picture is different?	Identifying which of the four presented faces is the odd-one-out	

2.7.2. Depression anxiety stress scale (DASS-42)

DASS-42 is a 42 item self-report validated inventory comprising of three scales designed to measure the negative emotional states of depression, anxiety, and stress, where each of the three scales contained 14 items. DASS-42 has been widely used in both clinical and non-clinical settings. The depression scale assessed dysphoria, hopelessness, devaluation of life, self-deprecation, lack of interest/involvement, anhedonia, and inertia. The anxiety scale assessed autonomic arousal, skeletal muscle effects, situational anxiety, and subjective experience of anxious affect, while the stress scale assessed difficulty in relaxing, nervous arousal, and being easily upset/agitated, irritable/overreactive, and impatient. Subjects were assessed based on a 4- point Likert scale (0 ¼ did not apply to me at all, 1 ¼ applied to me to some degree or some of the time, 2 ¼ applied to me to a considerable degree or a good part of the time, 3 ¼ applied to me very much or most of the time). Scores for each subscale were categorized into five severity ranges, namely normal, mild, moderate, severe, and extremely severe. DASS-42 was used as a screening test and for assessment at intervals of 4-weeks (week 4, 8, 12).

2.8. Fecal sample analysis

Faecal samples of the volunteers were collected at baseline and every four weeks (during every test session). For analysis, fecal samples were diluted 10 times with deionized water (w/v) and homogenized. After centrifugation at 2,000 RPM for 10 min, the supernatant was filtered through a 0.45 μm syringe filter Phenex (Phenomenex, Aschaffenburg, Germany). Samples were further diluted in 50 % ACN and 5 μL injected on a SeQuant® ZIC HILIC 3.5 μm, 4.6 x 150 mm (Merck KGaS, 64271, Darmstadt, Germany) attached to a SeQuant® ZIC HILIC PEEK coated guard column 20 x 2.1 mm (Merck KGaS, 64271, Darmstadt, Germany). The analysis has been carried out with a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) coupled with a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The flow rate set at 0.7 mL/min and the column temperature at 40 °C. The mobile phases consist of 0.1 % formic acid (solvent A), acetonitrile with 0.1 % formic acid (solvent B) with the following elution profile (t in [min]/[% B]): (0.0/90), (4.0/70), (10.0/20), (13.0/20), (15.0/90) and (18.0/90). MS data is collected for 18 mins. Data is processed with LabSolutions (Shimadzu Corporation, Kyoto, Japan).

2.9. Fecal microbiota analysis

DNA extraction from fecal samples was carried out following the SOP 07 developed by the International Human Microbiome Standard Consortium (https://www.microbiome-standards.org). The V3-V4 region of the 16S rRNA gene was amplified by using the primers S-D-Bact-0341F5-CCTACGGGNGGCWGCAG and S-D-Bact-0785R5-GACTACHVGGGTATCTAATCC and protocol previously described (Quast et al. 2013). PCR mixtures were initially heated to 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and completed at 72 °C for 5 min. Barcoded amplicons were pooled at equimolar concentration and library preparation and sequencing was carried out according to the Illumina metagenomic sequencing library preparation protocol on a MiSeq platform (leading to 2 × 250 bp reads). Reads were imported into QIIME 2 (q2cli version 2020.11.1; Bolyen et al., 2019), and Amplicon Sequence Variant (ASV) table was obtained following the pipeline recently reported (Sequino et al., 2022). The ASV table was collapsed at genus level, and relative abundances of each taxon were computed. Alpha-diversity indices were calculated in a R environment (https://www.r-project.org) using the function 'diversity' from the 'vegan' package.

2.10. Statistical analysis

To characterize the relationship between treatments and cognitive function performance, as well as mood survey, linear mixed-effects analyses were performed using the lme4 (Version 1.1–17) package in R (Version 3.5.0) and SPSS. Mixed-effects analyses allowed for the modeling of variation in how individual participants reacted to each treatment. Treatment was included as a categorical fixed effect with three levels (baseline, probiotic, placebo) in models of all outcomes. Models of the DASS-42 and LEIDS-r measures included the fixed effect of group and interaction between treatments and group to identify differences in the outcomes between groups under a different sequence of treatment. Models of cognitive tasks included the fixed effect of the language. To account for potential moodiness or depression due to the female period, changes in DASS-42 and LEIDS-r were modeled over the course of the experiment period by including a fixed effect of gender and considering the female period calendar. Following current best practices to evaluate the significance of fixed effects of models fit with lme4, p-values were derived using Satterthwaite approximations for degrees of freedom with the lmerTest package (Version 3.0–1). The 'wilcox.test()' function from the 'base' R package compared the relative abundance of *Lactiplantibacillus* sp., *Lactobacillus crispatus* and *Levilactobacillus brevis* between the groups in positive and negative responders. P-values were adjusted using the Benjamini-Hochberg procedure. The corr.test function in the 'psych' package was used to perform Spearman correlation analysis among the variables. Intercepts for the random effect of participants for the effect of treatment and group (when applicable) were included in each model. We though assessed the effects of probiotics versus placebo (pre vs. post intervention).

3. Results

43 out of 44 subjects completed the first and the second part of the study. No adverse events were reported in any part of the study. Baseline characteristics were similar between groups. The compliance was high; only one subject returned 6 placebo sachets. The self-reported levels of product consumption did not differ significantly between groups (27 ± 1.6 for the probiotic mix and 27 ± 1.4 for placebo).

3.1. Cognitive assessment

No significant differences were observed between treatments and with the baseline in all the cognitive test performed, measured as speed in performance (ONB, OCL, IDN) and accuracy of performance (SEC) (Fig. 2).

3.2. Subjective stress measures

Statistical analysis performed on the DASS-42 total score revealed no main effect of treatment by group interaction ($p = 0.16$). Similarly, for Acceptance/Coping (ACC), Aggression (AGG), Control/Perfectionism (CON), Risk aversion (RAV), and Rumination (RUM) scores, no effect was observed for treatment on the group. Hopelessness (HOP) showed significant differences in the baseline between Group A and Group B ($p = 0.043$). No significant differences were observed between baselines of treatments in the DASS-42, showing no carry-over effects. Mixed-effects model analyses were performed for DASS-42, LEIDS-R, and cognitive outcomes, taking into consideration interaction between the crossover arm and the treatment type, and also by both of them with the period. Thus, the two groups of participants (A and B) were comparable in terms of depression, anxiety, and stress scores at baseline and follow-up for the DASS-42. Further exploitation of the probiotic supplementation on psychometric status came from self-reported measures using the DASS-42 questionnaire. Overall, the participants self-reported no changes after intervention.

To investigate the influence of probiotics supplementation on cognitive reactivity to sad mood, the Leiden index of depression sensitivity was used (LEIDS-r). Significant differences were observed between treatments ($p = 0.034$) (Fig. 3). Rumination is the subscale in LEIDS-r that showed significant improvement compared to the placebo after

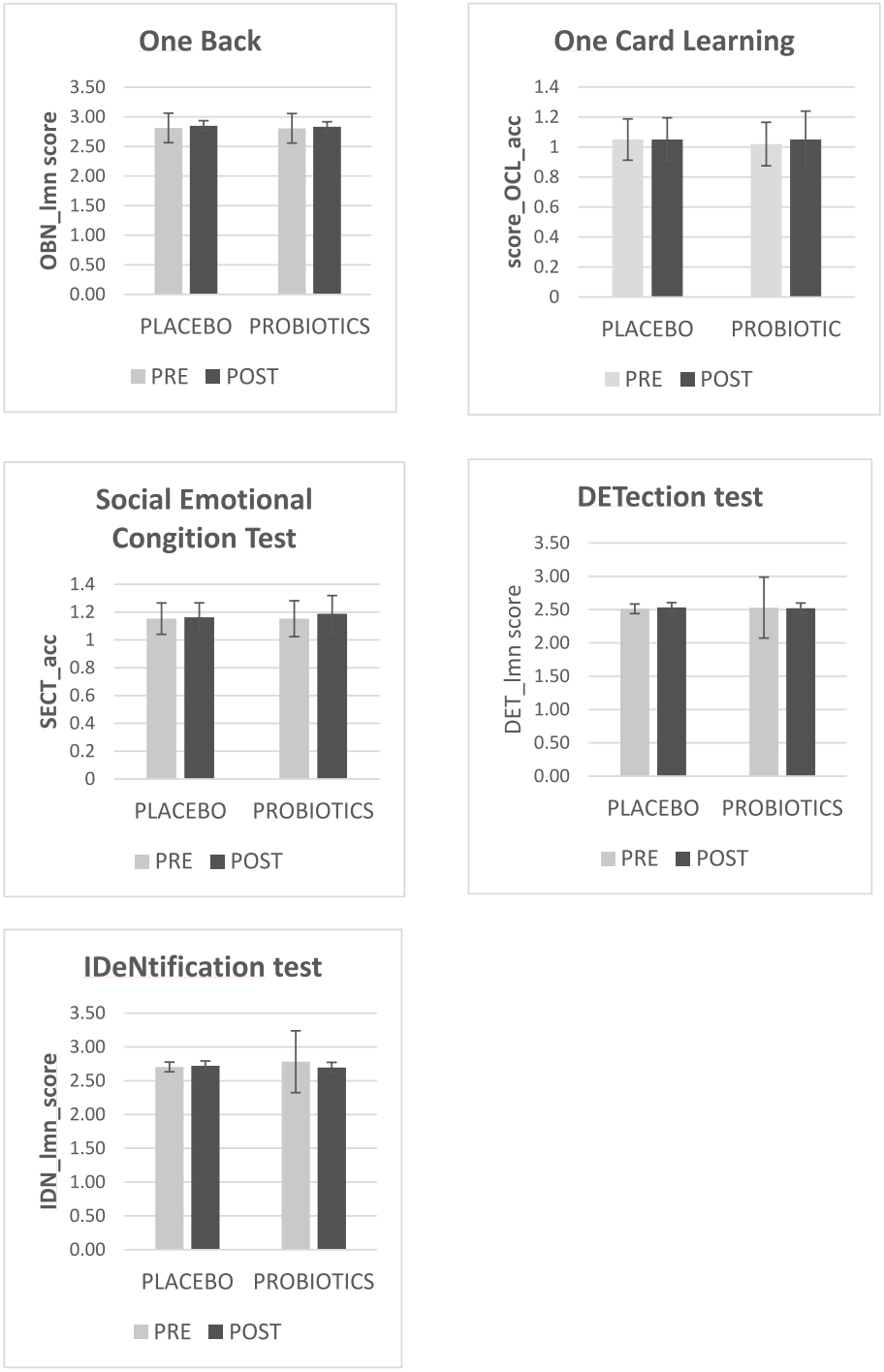

Fig. 2. Cognitive outcomes: Detection performance; Identification Test; One card Learning Test; One Back Test; One card Learning Test; Social-Emotional Cognition Test; Detection performance; Identification Test.

intake of the probiotic formula ($p = 0.006$) (Fig. 4) .

3.3. Neurotransmitters analysis

To investigate the microbiome activity for GABA and acetylcholine production, fecal GABA, acetylcholine, choline and glutamate levels were evaluated in 44 participants. No significant differences were observed between the treatments on acetylcholine, while GABA concentration was reduced with respect to the baseline both with placebo and with probiotics (data no shown). Correlation between fecal neurotransmitters' concentration and mood/cognitive outcomes was not observed.

3.4. Intervention effect on gut microbiota

No significant differences in the overall microbiota composition between the participants treated with probiotics and placebo was detected. Therefore, to investigate the effect of the probiotic intervention on gut microbiota composition, participants were divided in positive responders and negative responders to the treatment. Positive responders were the participants which outscored in more than half of the cognitive tasks and mood self-questionnaires. When the value

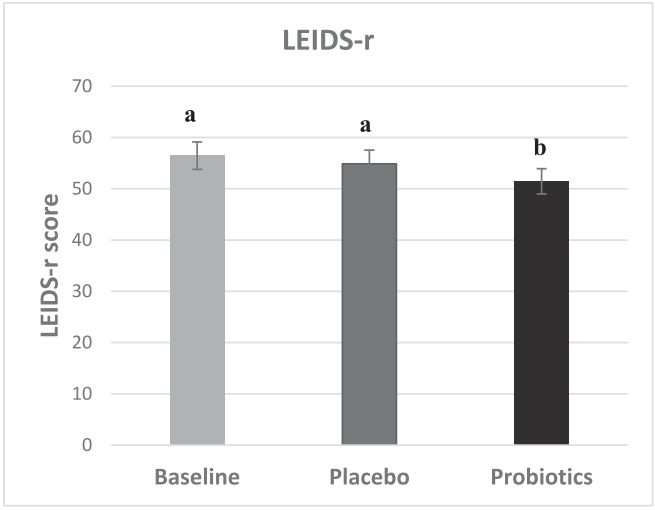

Fig. 3. Mood outcomes measured by LEIDS-r.

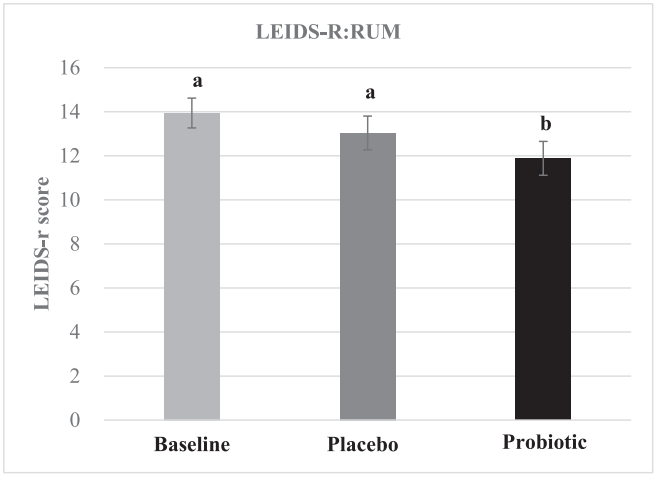

Fig. 4. Mood results measured by DASS-42 and LEIDS-r. Bar charts represent changes in self-reported scores of Rumination (RUM) measured by the mood questionnaire LEIDS-r.

obtained was negative they were considered as negative responders, thus the proportion of the positive reactions after probiotic intake between positive responders and negative is 10.8 vs 6.3. Positive responders showed a significant increase in genera and species of lactobacilli upon probiotic treatment (Fig. 5). In particular, *Lactiplantibacillus* sp. and *Levilactobacillus brevis*, both contained in the probiotic supplement administered, significantly increased in responders, but did not in negative responders.

4. Discussion

Probiotics showed an array of gut health benefits and recent evidence on mental well-being, along the gut-brain axis was also been reported. In this frame, the first aim of our study was to assess the neurocognitive effects of a mixture of GABA-producing *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* on general cognitive control, and whether these effects on cognition (i.e. working memory) were visible by improving stress score. We used DASS-42 and LEIDS-r questionnaires which are validated psychological instruments that correlated with psychological, clinical emotional, and behavioral measures. All subjects were recruited based on mild/moderate stress levels as assessed by DASS-42.

The cognitive outcomes were our primary outcome to investigate. Cognition encompasses a variety of cognitive domains including attention, executive function, memory, visuospatial function, psychomotor speed and social cognition, which include both short and long-term information acquisition process. The effects of probiotics and stress on cognition share common pathways of action (Sarkar et al. 2016), it is still to clarify whether probiotics might affect cognitive performance independent or dependent of the detrimental effects of stress. We observed that over the 12-week period of treatment, several cognitive tasks were constant and not significantly altered by placebo or probiotic administration, which could have resulted in ceiling effects, i.e. not enough room for improvement; these results are overall in line with what reported by other studies (Kelly et al.2017; Lew et al. 2019). Kelly et al. (2017) performed a cross-over clinical study with a small sample size of healthy volunteers (n = 29) and observed no effect of *L.rhamnosus* JB-1 on cognitive performance, as well as inflammatory response, stress-related behaviors and brain activity. In the context of cognitive impairment, the role of psychobiotic can be more evident. Several studies performed on humans with cognitive impairment demonstrate the efficacy of that probiotic supplementation for protecting cognitive health in aging (Kao et al. 2016, Sanborn et al. 2020, Xiao et al. 2020; Aljumaah et al., 2022 Nov). Sanborn et al. (2020) showed in a double-blind, placebo-controlled randomized study that three- month supplementation of *Lactobacillus rhamnosus* GG (LGG) was associated with improved cognitive function measured in middle-aged and older adults meeting the criteria for mild cognitive impairment, measured by NIH Toolbox Total Cognition Score. Similar results were observed by Xiao et al. (2020) which study showed improved scored of the cognitive functions measured by Neuropsychological Status (RBANS) in adults with mild cognitive impairments after 16 weeks of consumption of *B. breve* A1 (MCC1274). In this work significant improvements were observed in domain scores of immediate memory, visuospatial/constructional, and delayed memory (Xiao et al. 2020). Based on several studies performed on humans with cognitive impairment, probiotic

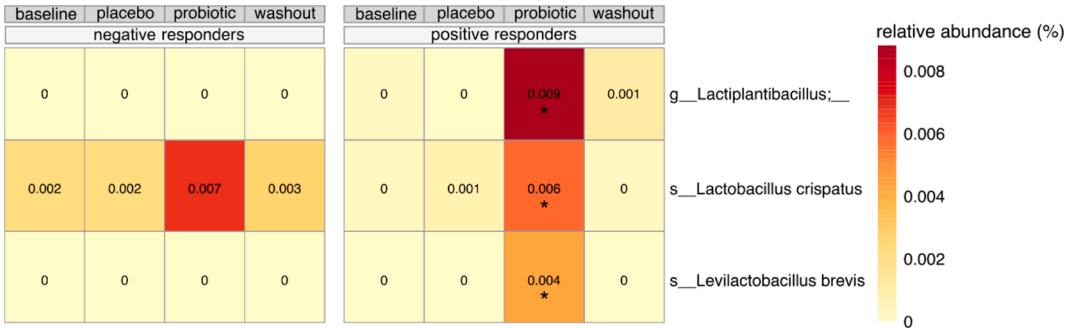

Fig. 5. Heatplot showing the average relative abundance (%) of selected taxa in positive and negative responders. Significance was tested using the Wilcoxon rank-sum test. The asterisk (*) indicates a significantly higher abundance of the taxon in the group compared to all the others ($p < 0.05$).

supplementation may be the novel method for protecting cognitive health in aging. However, complexities increase with disorders like schizophrenia and bipolar disorder. Therefore, these conditions require a more intricate exploration of how psychobiotics influence them, suggesting that some mental health conditions may be more receptive to psychobiotic treatment than others, and the need for personalized treatment approaches (Skowron et al. 2022; Del Toro-Barbosa et al 2020).

In the cross-over study by Lew et al. (2019), the administration of the probiotic formula did not yield significant changes in stress, anxiety levels as compared to placebo or baseline after 4 weeks. Similarly, Steenbergen et al. (2015) in a randomized placebo-controlled study with 20 healthy participants did not find significant changes in mood or anxiety as measured by the Beck Depression Inventory or Beck Anxiety Inventory. Our results are consistent with the work of Chong et al. (2019) who did not observe significant differences in the DASS-42 scores after 4 weeks. However, they were able to see a difference after 12 weeks of treatment suggesting that the length of psychobiotics intake can be a key factor to exert an effect on mental well-being in stress-susceptible healthy humans.

Moving to the mood parameters, the main modification caused by the psychobiotic administration was the reduction of the score on the subscale of LEIDS for rumination thoughts, index of repetitive negative thinking, suggesting an influence of probiotic mix intake on the mechanisms associated with vulnerability to mood disorders. Our results were aligned with previous studies (Wu et al., 2021a; Steenbergen et al. 2015) indicating that improvements in the overall score of cognitive reactivity after probiotic supplementation.

A recent cross-over intervention study examined the effect of *B.logum* on measures of stress, cognitive performance, and mood in twenty male students during the university exam period used as a naturalistic chronic stress (Moloney et al. 2021). They observed stress and depression scores increased in both placebo and probiotic treated groups during the exam period, while overall sleep quality improved significantly in probiotic treatment compared to placebo. In line to our results they showed no efficacy in improving measures of visual memory, sustained attention and neither alleviate symptoms of chronic stress and depression (Moloney et al. 2021). The same bacterial strain was also assessed in healthy adults undergoing an acute stressor, showing attenuation in psychological reaction along with enhanced frontline midline electroencephalographic mobility (Allen et al., 2016). These results suggested f strains could also be specific for the type of stress.

Given the evidences of reduction in levels of GABA synthetic enzymes during chronic stress, and that the concentration GABA/Glutamate is associated with microbiota composition, we decided to investigate potential correlations between human fecal neurotransmitters and stress/depression (Altaib et al. 2021). Altaib et al. (2021) analyzed the relationship between fecal GABA concentration and microbial composition in more than 70 human participants, demonstrating that *Bifidobacterium* abundance was associated with high fecal levels of GABA in healthy human subjects. We showed, in a recent study, that *Levilactobacillus brevis* P30021 and *Lactiplantibacillus plantarum* P30025 were able to produce GABA and acetylcholine *in vitro* batch fermentation, and in the Simulator of Human Intestinal Microbial Ecosystem (SHIME) when added with their precursor glutamate and choline (Casertano et al. 2024). In this study we also looked at the effect of the psychobiotic administration *in vivo* study on the production of acetylcholine and GABA. Unfortunately, we did not observe any trend of correlation of the neurotransmitters concentration in the feaces, with the microbiota composition and mood and cognitive performance within positive and negative responders. The lack of significant results suggests that within the scope of our study, no detectable relationships exist between these variables. This might imply that any potential interactions might be subtle in healthy participants. A previous study in MDD (Zhao et al.2022) exploring the connection between the microbiome metagenomics, immunology, and metabolomics, could observe a multifactorial effect of the gut microbiota.

To elucidate the mechanisms of action of specific probiotics strains on the mental health it would be very important to underpin the results on mood scores as well as the outcomes of cognitive measured by DASS-42 and LEIDS-r as by detecting a significant variation of the concentration of specific neurotransmitters like GABA/Glutamate, acetylcholine and choline in biological fluids.

In our previous study, we observed that the amount of GABA produced into the SHIME from the mix of *L.plantarum* and *L.brevis* was between 400 and 800 ug/L (Casertano et al. 2024). The effect of orally administered GABA on human able to reduce stress and enhance sleep have been previously investigated, even though no studies has been done to understand the amount of GABA able to reach the intestine. Abdou et al. (2006) investigated the effect of 100 mg GABA intake on relaxation and immunity during stress by evaluating the electroencephalogram (EEG) and immunoglobulin A (IgA) levels. They concluded that GABA could work effectively as a natural relaxant, and its effects could be seen within 1 h of its administration to induce relaxation, diminish anxiety, and enhance immunity under stress conditions. With respect to the mental task, Kanehira et al. (2011) observed that 25 or 50 mg GABA taken orally had an anti-fatigue effect observed by measuring the salivary secretion levels of chromogranin A (CgA) and cortisol in nine participants who were diagnosed with chronic fatigue.

Beside the direct measure of neurotransmitters, we also looked at the microbiota modification induced by the probiotic treatment. We observed an increase of *Saccharomonospora* genus in line with the study by Yu et al. (2023) who observed an increased abundance of *Saccharomonospora* bacteria in mice fed with lactic acid bacteria. We also observed a significant increase of *L.plantarum* in the treatment arm, a specie exhibiting ecological and metabolic flexibility in the mammalian gastrointestinal tract niche, and it has gained popularity for alleviating the intensity of anxiety and depression (Ma et al. 2023). Notably, we also detected higher abundance of *Lactobacillus crispatus* after treatment with the probiotic formula, which can be beneficial for the gut owing to the strong lactate production, that in presence of complex microbiota, it is further converted by cross-feeding into propionate and butyrate (Ríos-Covián et al., 2016). There are no studies that has observed a relation of *L.crispatus* with mental health or production of GABA, however, in our study we could observe an increase of *Lactiplantibacillus* and *Levilactobacillus brevis* due to the probiotic intake.

Intestinal microflora balance is very important for healthy and physiological function of the host intestine. It is reported that there is great differences in the composition of the intestinal microbial community between healthy people and patients with mental illness. The healthy gut microbiota is predominantly comprised of the phyla Firmicutes and Bacteroidetes, collectively constituting more than 90 % of the total population (Jandhyala et al., 2015). However, this composition undergoes alterations along the gastrointestinal tract (Adak and Khan, 2019). When considering psychotic disorders overall compared to healthy controls, Vindegaard et al. 2021 observed a higher relative abundance of Actinobacteria (4 of 7 studies reporting on Actinobacteria) and lower relative abundance of Firmicutes (lower relative abundance of *Lachnospiraceae* and of *Faecalibacterium* at genus level) (Vindegaard et al. 2021).

In conclusion, 4 weeks of our intervention was safe, well-tolerated, and effective in improving cognitive reactivity to mood. However, the potential benefits might be also due to the synergic activity of the probiotics with the vitamins. Vitamin D, which has known influence on the brain functioning, (Akpınar and Karadağ, 2022; Singh, 2020; Steinert et al., 2020) has been explored in several studies for its efficacy on prevention and treatment of mood disorders when administrated in combination with probiotics (Mohammadi et al. 2024; Amirani et al. 2020; Jamilian et al. 2019). The analysis performed on the microbiota could confirm the impact of the treatment with *Levilactobacillus brevis* and *Lactiplantibacillus plantarum* on the gut microbiota composition, changing significantly the relative abundance of the specific taxa.

Further studies should consider the effect of this probiotic combination in larger test populations and broader period of intervention taking in account differences between men and women, age, microbiota composition and levels of neurotransmitters. Several *meta*-analysis suggested that utilizing probiotic may be more useful and effective as adjunctive treatment in patients with certain co-morbidities, such as irritable bowel syndrome (Noonan et al. 2020), or in Major Depressive Disorder (MDD) (Misera et al. 2021), in which there is more room for improvement. Hence, efforts should aim to incorporate a more thorough screening to investigate the influence of comorbidity and include extensive background details (e.g., history of psychological treatment) will provide an important extension of our findings. Our study is not without limitations: we have more female than male participants; we did not investigate the sleep parameters and neither we examined brain imaging or EEG which has revealed to be a functional readout of efficacy in probiotic treatments (Allen et al. 2016).

The potential of the psychobiotic as treatment stress-related disorders by targeting the microbiota is much promising. Our findings suggest that caution is required regarding expectations of improving stress-related conditions by targeting the microbiota in healthy participants.

CRediT authorship contribution statement

Melania Casertano: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Matthijs Dekker:** Supervision. **Vincenzo Valentino:** Writing – review & editing, Supervision, Conceptualization. **Francesca De Filippis:** Formal analysis. **Vincenzo Fogliano:** Writing – review & editing, Supervision, Conceptualization. **Danilo Ercolini:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The PhD fellowship of Melania Casertano was supported by the Ministry of University and Research (MUR) in the frame of the National Operational Program ESF-ERDF Research and Innovation 2014–2020, action Innovative PhD with industrial characterization.

References

Abdou, A.M., Higashiguchi, S., Horie, K., Kim, M., Hatta, H., Yokogoshi, H., 2006. Relaxation and immunity enhancement effects of γ-aminobutyric acid (GABA) administration in humans. Biofactors 26 (3), 201–208.

Adak, A., Khan, M.R., 2019. An insight into gut microbiota and its functionalities. Cellular and Molecular Life Sciences 76, 473–493.

Akpınar, Ş., Karadağ, M.G., 2022. Is Vitamin D Important in Anxiety or Depression? What Is the Truth? Epub 2022 Sep 13. PMID: 36097104; PMCID: PMC9468237 Curr Nutr Rep. 11 (4), 675–681. https://doi.org/10.1007/s13668-022-00441-0.

Aljumaah, M.R., Bhatia, U., Roach, J., Gunstad, J., Azcarate Peril, M.A., 2022 Nov. The gut microbiome, mild cognitive impairment, and probiotics: a randomized clinical trial in middle-aged and older adults. Clin Nutr. 41 (11), 2565–2576. https://doi.org/10.1016/j.clnu.2022.09.012. Epub 2022 Sep 28 PMID: 36228569.

Allen, A.P., et al., 2016. Bifidobacterium longum 1714 as a translational psychobiotic: modulation of stress, electrophysiology and neurocognition in healthy volunteers. Translational Psychiatry 6 (11). https://doi.org/10.1038/tp.2016.191.

Altaib, H., et al., 2021. Differences in the concentration of the fecal neurotransmitters GABA and glutamate are associated with microbial composition among healthy human subjects. Microorganisms 9 (2), 1–15.

Amirani, E., Milajerdi, A., Mirzaei, H., Jamilian, H., Mansournia, M.A., Hallajzadeh, J., Ghaderi, A., 2020. The effects of probiotic supplementation on mental health, biomarkers of inflammation and oxidative stress in patients with psychiatric disorders: A systematic review and meta-analysis of randomized controlled trials. Complementary Therapies in Medicine 49, 102361.

Bloemendaal, M., Szopinska-Tokov, J., Belzer, C., Boverhoff, D., Papalini, S., Michels, F., van Hemert, S., Arias Vasquez, A., Aarts, E., 2021. Probiotics-induced changes in gut microbial composition and its effects on cognitive performance after stress: exploratory analyses. Transl Psychiatry. 11 (1), 300. https://doi.org/10.1038/s41398-021-01404-9. PMID: 34016947; PMCID: PMC8137885.

Bolyen, E., Rideut, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., et al., 2019. Reproducible, interactive, scalable, and extensible microbiome data science using QIIME 2. Nature Biotechnology 37, 852–857.

Casertano, M., Fogliano, V., Ercolini, D., 2022. Psychobiotics, gut microbiota and fermented foods can help preserving mental health. Food Res Int. 152, 110892 https://doi.org/10.1016/j.foodres.2021.110892. Epub 2021 Dec 22 PMID: 35181072.

Casertano, M., Fryganas, C., Valentino, V., Troise, A.D., Vitaglione, P., Fogliano, V., Ercolini, D., 2024. Gut production of GABA by a probiotic formula: an in vitro study. Beneficial Microbes 15 (1), 67–81. https://doi.org/10.1163/18762891-20230025.

Chong, H.X., et al., 2019. Lactobacillus plantarum DR7 alleviates stress and anxiety in adults: a randomised, double-blind, placebo-controlled study. Beneficial Microbes 10 (4), 355–373.

Del Toro-Barbosa, M., Hurtado-Romero, A., Garcia-Amezquita, L.E., García-Cayuela, T., 2020. Psychobiotics: mechanisms of action, evaluation methods and effectiveness in applications with food products. Nutrients 12, 3896.

Ding, Y., Bu, F., Chen, T., Shi, G., Yuan, X., Feng, Z., Duan, Z., Wang, R., Zhang, S., Wang, Q., Zhou, J., Chen, Y., 2021. A next-generation probiotic: Akkermansia muciniphila ameliorates chronic stress-induced depressive-like behavior in mice by regulating gut microbiota and metabolites. Appl Microbiol Biotechnol 105, 8411–8426.

Hashikawa-Hobara, N., Otsuka, A., Okujima, C., Hashikawa, N., 2022. Lactobacillus paragasseri OLL2809 improves depression-like behavior and increases beneficial gut microbes in mice. Frontiers in Neuroscience 16, 918953.

Huang, C.L., Chu, H.F., Wu, C.C., Deng, F.S., Wen, P.J., Chien, S.P., Chao, C.H., Chen, Y. T., Lu, M.K., Tsai, Y.C., 2023. Exopolysaccharide is the potential effector of Lactobacillus fermentum PS150, a hypnotic psychobiotic strain. PMID: 37469436; PMCID: PMC10352126 Front Microbiol. 14, 1209067. https://doi.org/10.3389/fmicb.2023.1209067.

Jamilian, H., Amirani, E., Milajerdi, A., Kolahdooz, F., Mirzaei, H., Zaroudi, M., Asemi, Z., 2019. The effects of vitamin D supplementation on mental health, and biomarkers of inflammation and oxidative stress in patients with psychiatric disorders: A systematic review and meta-analysis of randomized controlled trials. Progress in Neuro-Psychopharmacology and Biological Psychiatry 94, 109651.

Jandhyala, S.M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., Reddy, D.N., 2015. Role of the normal gut microbiota. World Journal of Gastroenterology: WJG 21 (29), 8787.

Kanehira, T., Nakamura, Y., Nakamura, K., Horie, K., Horie, N., Furugori, K., Sauchi, Y., Yokogoshi, H., 2011. Relieving occupational fatigue by consumption of a beverage containing γ-amino butyric acid. J Nutr Sci Vitaminol 57, 9–15.

Kang, Hyun. 2021. Sample Size Determination and Power Analysis Using the G * Power Software. : 1–12.

Kao, A.C.C., Harty, S., Burnet, P.W.J., 2016. Chapter two - the influence of prebiotics on neurobiology and behavior. International Review of Neurobiology 131, 21–48. https://doi.org/10.1016/bs.irn.2016.08.007.

Kelly, J.R., et al., 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, 50–59. https://doi.org/10.1016/j.bbi.2016.11.018.

Kriakous, S.A., Elliott, K.A., Lamers, C., Owen, R., 2021. The Effectiveness of Mindfulness-Based Stress Reduction on the Psychological Functioning of Healthcare Professionals: a Systematic Review. Mindfulness (N Y) 12 (1), 1–28. https://doi.org/10.1007/s12671-020-01500-9. Epub 2020 Sep 24. PMID: 32989406; PMCID: PMC7511255.

Kyrpides, N.C., Woyke, T., Eisen, J.A., et al., 2014. Genomic Encyclopedia of Type Strains, Phase I: The one thousand microbial genomes (KMG-I) project. Stand in Genomic Sci 9, 1278–1284. https://doi.org/10.4056/sigs.5068949.

Lew, L.C., et al., 2019. Probiotic Lactobacillus Plantarum P8 Alleviated Stress and Anxiety While Enhancing Memory and Cognition in Stressed Adults: A Randomised, Double-Blind, Placebo-Controlled Study. Clinical Nutrition 38 (5), 2053–2064. https://doi.org/10.1016/j.clnu.2018.09.010.

Lockyer, S., 2020. Effects of diets, foods and nutrients on immunity: Implications for COVID-19? Nutrition Bulletin 45 (4), 456–473.

Ma, J., Chen, Y., Wang, Z., Wang, R., Dong, Y., 2023. Lactiplantibacillus plantarum CR12 attenuates chronic unforeseeable mild stress induced anxiety and depression-like behaviors by modulating the gut microbiota-brain axis. ISSN 17564646 Journal of Functional Foods 107, 105710. https://doi.org/10.1016/j.jff.2023.105710.

McVey Neufeld, K.-A., Kay, S., Bienenstock, J., 2018. Mouse strain affects behavioral and neuroendocrine stress responses following administration of probiotic Lactobacillus rhamnosus JB-1 or traditional antidepressant fluoxetine. Front. Neurosci. 12, 294. https://doi.org/10.3389/fnins.2018.00294.

Mielke, Michelle M et al. 2015. "Performance of the CogState Computerized Battery in the Mayo Clinic Study on Aging." 11: 1367–76.

Misera, Agata, Paweł Li, Igor Łoniewski, and Karolina Skonieczna- Zydecka. 2021. "Effect of Psychobiotics on Psychometric Tests and Inflammatory Markers in Major Depressive Disorder : Meta-Analysis of Randomized Controlled Trials with Meta-Regression.".

Mohammadi, A., Sadighi, G., Nazeri Astaneh, A., Tajabadi-Ebrahimi, M., Dejam, T., 2024. Co-administration of probiotic and vitamin D significantly improves cognitive function in schizophrenic patients: A double-blinded randomized controlled trial. Neuropsychopharmacology Reports.

Moloney, G.M., et al., 2021. "Brain, Behavior, & Immunity - Health Improvements in Sleep Indices during Exam Stress Due to Consumption of a Bifidobacterium Longum". Brain, Behavior, & Immunity -. Health 10 (November 2020), 100174. https://doi.org/10.1016/j.bbih.2020.100174.

Morgado, P., Cerqueira, J.J., 2018. Editorial. The Impact of Stress on Cognition and Motivation. 12 (December), 1–2.

Noonan, Sanjay, Meena Zaveri, Elaine Macaninch, and Kathy Martyn. 2020. Food & Mood : A Review of Supplementary Prebiotic and Probiotic Interventions in the Treatment of Anxiety and Depression in Adults. : 1–12.

Papalini, S., Michels, F., Kohn, N., Wegman, J., van Hemert, S., Roelofs, K., Arias-Vasquez, A., Aarts, E., 2018. Stress matters: Randomized controlled trial on the effect of probiotics on neurocognition. Neurobiol Stress. 10 (10), 100141 https://doi.org/10.1016/j.ynstr.2018.100141. PMID: 30937347; PMCID: PMC6430409.

Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., De Los Reyes-gavilán, C.G., Salazar, N., 2016. Intestinal short chain fatty acids and their link with diet and human health. Frontiers in Microbiology 7, 185.

Sample, N.-D., et al., 2019. The Relationship of Anxiety and Stress with Working Memory Performance in a Large. 10 (January), 1–9.

Sanborn, V., Azcarate-Peril, M.A., Updegraff, J., Manderino, L., Gunstad, J., 2020. Randomized Clinical Trial Examining the Impact of *Lactobacillus rhamnosus* GG Probiotic Supplementation on Cognitive Functioning in Middle-aged and Older Adults. Neuropsychiatric Disease and Treatment 16, 2765–2777. https://doi.org/10.2147/NDT.S270035.

Sarkar, A., et al., 2016. Psychobiotics and the Manipulation of Bacteria–Gut–Brain Signals. Trends in Neurosciences 39 (11), 763–781. https://doi.org/10.1016/j.tins.2016.09.002.

Sequino, G., Valentino, V., Torrieri, E., De Filippis, F., 2022. Specific microbial communities are selected in minimally-processed fruit and vegetables according to the type of product. Foods 11, 2164.

Singh, P., et al., 2020. The Potential Role of Vitamin D Supplementation as a Gut Microbiota Modifier in Healthy Individuals. Scientific Reports 0123456789, 1–14. https://doi.org/10.1038/s41598-020-77806-4.

Skowron, K., Budzyńska, A., Wiktorczyk-Kapischke, N., Chomacka, K., Grudlewska-Buda, K., Wilk, M., Wałecka-Zacharska, E., Andrzejewska, M., Gospodarek-Komkowska, E., 2022. The Role of Psychobiotics in Supporting the Treatment of Disturbances in the Functioning of the Nervous System—A Systematic Review. Int. J. Mol. Sci. 23, 7820.

Solito, A., et al., 2021. Supplementation with Bifidobacterium Breve BR03 and B632 Strains Improved Insulin Sensitivity in Children and Adolescents with Obesity in a Cross-over. Randomized Double-Blind Placebo-Controlled Trial. *Clinical Nutrition* 40 (7), 4585–4594. https://doi.org/10.1016/j.clnu.2021.06.002.

Steenbergen, L., et al., 2015. Brain, Behavior, and Immunity A Randomized Controlled Trial to Test the Effect of Multispecies Probiotics on Cognitive Reactivity to Sad Mood Q. Brain Behavior and Immunity 48, 258–264. https://doi.org/10.1016/j.bbi.2015.04.003.

Steinert, R.E., Lee, Y.-K., Sybesma, W., 2020. Vitamins for the Gut Microbiome. Trends in Molecular Medicine 26 (2), 137–140. https://doi.org/10.1016/j.molmed.2019.11.005.

Sun, Y., Geng, W., Pan, Y., Wang, J., Xiao, P., Wang, Y., 2019. Supplementation with Lactobacillus kefiranofaciens ZW3 from Tibetan Kefir improves depression-like behavior in stressed mice by modulating the gut microbiota". Food & Function 10 (2), 925–937.

van Leeuwen, J.M.C., Vink, M., Fernández, G., Hermans, E.J., Joëls, M., Kahn, R.S., Vinkers, C.H., 2018. At-risk individuals display altered brain activity following stress. Neuropsychopharmacology. 43 (9), 1954–1960. https://doi.org/10.1038/s41386-018-0026-8. Epub 2018 Feb 26. PMID: 29483659; PMCID: PMC6046038.

Vindegaard, N., Speyer, H., Nordentoft, M., Rasmussen, S., Eriksen, B.M., 2021. Gut microbial changes of patients with psychotic and affective disorders: A systematic review. Schizophrenia Research 234, 41–50. https://doi.org/10.1016/j.schres.2019.12.014. ISSN 0920–9964.

Wang, H., Braun, C., Murphy, E.F., Enck, P., 2019. Bifidobacterium longum 1714™ Strain Modulates Brain Activity of Healthy Volunteers During Social Stress. Am J Gastroenterol. 114 (7), 1152–1162. https://doi.org/10.14309/ajg.0000000000000203. PMID: 30998517; PMCID: PMC6615936.

Wu, S.u.I., et al., 2021b. Psychobiotic Supplementation of PS128TM Improves Stress, Anxiety, and Insomnia in Highly Stressed Information Technology Specialists: A Pilot Study. Frontiers in Nutrition 8 (March), 1–10.

Wu, L., Shen, F., Wang, W., Qi, C., Wang, C., Shang, A., Xuan, S., 2021a. The effect of multispecies probiotics on cognitive reactivity to sad mood in patients with Crohn's disease. Journal of Functional Foods 82, 104431.

Xiao, J., Katsumata, N., Bernier, F., Ohno, K., Yamauchi, Y., Odamaki, T., Yoshikawa, K., Ito, K., Kaneko, T., 2020. Probiotic Bifidobacterium breve in Improving Cognitive Functions of Older Adults with Suspected Mild Cognitive Impairment: A Randomized, Double-Blind. Placebo-Controlled Trial. J Alzheimers Dis. 77 (1), 139–147. https://doi.org/10.3233/JAD-200488. PMID: 32623402; PMCID: PMC7592675.

Yu, J., Guo, H., Sun, M., Jiang, C., Jiang, S., Mu, G., Tuo, Y., Gao, P., 2023. Milk fermented by combined starter cultures comprising three Lactobacillus strains exerts an alleviating effect on loperamide-induced constipation in BALB/c mice. Food Funct. 14 (11), 5264–5276. https://doi.org/10.1039/d3fo00222e. PMID: 37194317.

Zhao, H., Jin, K., Jiang, C., et al., 2022. A pilot exploration of multi-omics research of gut microbiome in major depressive disorders. Transl Psychiatry 12, 8. https://doi.org/10.1038/s41398-021-01769-x.