

The Effect of a Probiotic Complex on the Gut-Brain Axis: A Translational Study

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Keywords

Gut-brain axis · Probiotics · Cognitive functions · Sleep · Students

Abstract

Background: The gut-brain axis refers to the network of connections that involve multiple biologic systems, allowing bidirectional communication between the gut and the brain. This communication is mainly mediated by gut microbiota, thanks to its ability to modulate several processes like the production of neurotransmitters. As such, keeping a balanced gut microbiota through probiotic intake could be a valid solution in supporting the right gut-brain communications. **Methods:** A two-step in vitro screening of five different probiotic strains was carried out to select the best performers in the modulation of stress markers. A first selection on SK-N-DZ neuronal cell lines was performed to evaluate the inhibition of the epigenetic enzyme LSD1, promotion of GABA, and expression of serotonin. Three out of five strains were tested for their ability to promote serotonin synthesis in the Caco2 cell line. As a result, *Limosilactobacillus reuteri* PBS072 and *Bifidobacterium breve* BB077 were selected as the best performing strains. To confirm their effects in hu-

mans, a proof-of-concept trial was carried out to evaluate stress-related parameters for 28 days of product intake in a group of 30 stressed students. **Results:** A significant improvement of cognitive functions, in terms of short-term memory, attention, and executive performance, as well as of psychophysiological markers, such as salivary cortisol level, skin conductance, sleep quality, and anxiety, were observed. **Conclusions:** According to the results, *L. reuteri* PBS072 and *B. breve* BB077 are potential probiotic candidates for improving stress resilience, cognitive functions, and sleep quality.

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Introduction

The existence of bidirectional communication between the gut and the brain, known as the gut-brain axis, has long been recognized [1]. On the one hand, the brain modulates the gastrointestinal tract by regulating motility, secretion, absorption, and blood flow [2]. On the other hand, the gut can affect the brain functions through neurological, immunological, and endocrine pathways [3]. Microorganisms present in the gut are responsible for the production of different immunological and neurolog-

ical signaling molecules, such as short-chain fatty acids (SCFAs) and neurotransmitters [4, 5]. In recent years, the primary role of the gut microbiota in this interaction has become increasingly evident. Microbial metabolism by-products are able to reach the brain through the central, enteric, and autonomic nervous system as well as the hypothalamic-pituitary-adrenal axis, affecting not only brain physiology but also its adaptation to psychological conditions like anxiety and stress [6, 7]. For this reason, the gut microbiome has recently been recognized as a new hallmark linked to mental health, with the latest studies focusing their attention on the cause-effect relationship between gut microbiota dysbiosis and acute and/or chronic stress, as well as on cognitive dysfunctions [8–11]. Finally, recent advances in metagenomics sequencing have revealed that dysregulation in the composition of gut microbiota (dysbiosis) is present in a variety of neurological diseases [12].

In this context, the use of probiotics is becoming a validated approach as support in stress management [13, 14]. Probiotics are linked to the biosynthesis and metabolism of serotonin (5-hydroxytryptamine) and γ -aminobutyric acid (GABA), both associated with a positive impact on the central nervous system, as well as to the production of post-biotics like short-chain fatty acids, microbial cell fractions, functional proteins, and other compounds that can reach and exert their effect on different body districts [15–17]. In particular, GABA and serotonin are the main neurotransmitters involved in mood and sleep cycle regulation. GABA, the main inhibitory neurotransmitter of the brain, plays a crucial role in modulating the response to stressful stimuli by the regulation of brain circuits in the amygdala [18]. On the other hand, serotonin is also important in the modulation of several behaviors: impaired serotonin neurotransmission appears to be involved in depression and anxiety symptoms, as well as in sleep regulation [19]. Another aspect that has been recently taken into consideration is that epigenetic phenomena might contribute to the regulation of mental and physical well-being [20, 21]. Recent findings report that lysine-specific demethylase 1 (LSD1) has a major role in the transduction pathway that translates social stress into altered transcriptional physiology of plasticity genes in the hippocampus, indicating LSD1 as a putative biomarker for stress management including mood-related conditions and sleep disorders [22].

Taking this into account, the connection between the gut and the brain reflects the ideal target for a new era of microbiome-driven approaches in preventing and/or re-

storing both gastrointestinal symptoms and stress-related conditions [23, 24]. As a result, various animal and human clinical trials have been carried out using several species of probiotics like *L. casei*, *L. helveticus*, *B. longum*, *L. rhamnosus*, and *L. plantarum*, providing interesting results [25–29]. However, such outcomes are often contradictory and carried out on undefined study populations, ranging from healthy volunteers to persons exposed to various levels of stress [30–35]. For this reason, further evidence on specific cohorts of subjects and homogenous outcome measures are needed to clarify the potential effects of probiotics on stress symptoms. The present work has evaluated the efficacy of a probiotic complex (*L. reuteri* PBS072 and *B. breve* BB077) in exerting a positive modulation of stress-related markers in vitro and corresponding effects and symptoms in a human trial.

Materials and Methods

In vitro Study

The in vitro screening was performed on the following strains: *Limosilactobacillus reuteri* PBS072, *Limosilactobacillus fermentum* PBS073, *Bifidobacterium animalis* subsp. *lactis* BL050, *Bifidobacterium breve* BB077, and *Bifidobacterium longum* subsp. *longum* BLG240. The strains were first tested on nervous cells (SK-N-DZ, ATCC® CRL-2149™ batch 61952456), and the resulting best-performing strains (*L. reuteri* PBS072, *B. lactis* BL050, and *B. breve* BB077) were selected for the second stage carried out on intestinal cells (Caco2, colon adenocarcinoma human ATCC HTB-37, batch 62381028). Both experimental steps were carried out under the same protocol as follows. Suspensions of probiotic strain cells with a concentration of 10^9 CFU/mL were used to inoculate the culture medium specific for the experimental model. Nervous cells and Caco2 were singularly cultured on EMEM (ATCC, Catalog No. 30-2003 supplemented with 20% fetal bovine serum, glutamine 5 mM) and maintained at standard culture conditions (37°C, 95% RH, and 5% CO₂). For the study execution, cells were seeded in 12-well plates until full confluence. Fetal bovine serum and glutamine were from Sigma-Aldrich (catalog No. F7524 batch BCBW9576 and catalog No. 59202C batch SLBZ7361, respectively). The study involved the concomitant treatment of the experimental models for 48 h with 1 mL of the medium in which probiotic strains were cultured and with cortisol as a stressor agent (Alfa Aesar, catalog No. A16292 batch 10206891, 0.2 mg/L). Cells treated only with culture medium were used as a negative control. Cells treated only with stressor agent (cortisol) were used as positive control. ELISA assays using commercial kits were used to determine the amount of serotonin (Abnova, catalog No. KA1894), LSD1 (Abcam, catalog No. ab113459), and GABA (Abnova, catalog No. KA4912), produced and released by the cells. The quantitative determination used a calibration curve made up of known concentrations of each standard. Results of LSD1 and GABA dosages were expressed as pg/mL, while the results of serotonin concentrations as ng/mL.

Table 1. Inclusion and noninclusion criteria

Inclusion criteria

Good general health

Female or male subjects

Age between 18 and 30 (± 2) years

Limited alcohol consumption, comparable to not more than two glasses of wine per day throughout duration of the test and abstention from alcohol in the 24 h preceding the tests

Absence of neurologic and psychiatric deficits

Absence of deficits in taste perception or pathologies that compromise it temporarily (e.g., colds and respiratory diseases)

Subjects who have not been recently involved in any other similar study

Willingness to follow the proposed alimentary supplement for all the study time

Willingness to use during all the study period only the product to be tested

Willingness to not use products likely to interfere with the product to be tested

Willingness to not vary the normal daily routine (i.e., lifestyle and physical activity)

Subject is under effective contraception (oral/not oral); not expected to be changed during the trial

Subject is aware of the study procedures and has signed an informed consent form

Noninclusion criteria

Current antibiotic administration

Known history of chronic medical condition such as congenital heart disease, liver or kidney disease, or immune deficiency

Treatment with probiotics in the 6 months preceding enrollment

Acute or chronic infectious diseases

Preexisting hypersensitivity to components contained in the probiotic

Subject does not meet the inclusion criteria

Pregnant women or women intending to become pregnant during the study

Breastfeeding women

Any condition that the principal investigator deems inappropriate for participation

Adults protected by the law (under guardianship or hospitalized in a public or private institution, for a reason other than the research, or incarcerated)

Volunteer unable to communicate or cooperate with the investigator due to language problems, poor mental development, or impaired cerebral function

Clinical Study

Subjects of the Study

The proof-of-concept clinical study was carried out at the University of Calabria (Quattromiglia, CS, Italy) in compliance with the Helsinki Declaration (1964) and its amendment. All subjects provided written informed consent before participating in the study. Thirty eligible subjects, adult males and females aged between 18 and 30 (± 2) were selected based on self-reported stress condition during an exam session. Inclusion and noninclusion criteria are reported in Table 1.

Study Design and Protocol

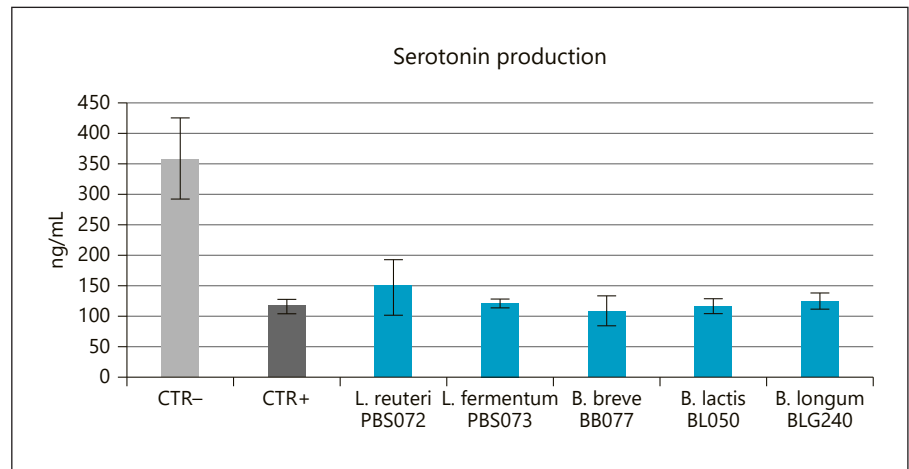
The objective of this study was to evaluate the clinical efficacy of the intake of a multi-strain probiotics combination as support for stressed students during exams. Enrolled subjects were randomly assigned to receive one stick of a multi-strain probiotic supplement to be taken once a day for 28 days. The tested product was composed of 2×10^9 CFU *L. reuteri* PBS072 and 2×10^9 CFU *B. breve* BB077, FOS tit. 93% (3.6%), inulin tit. 90% (3.703%), folic acid (0.003%), vitamin B₁₂ (0.035%), vitamin B₆ (0.022%), sorbitol (9%), sucralose (0.08%), aroma (1.3%), silicon dioxide (1%), and maltodextrin (73,479%). Clinical visits were performed by a technician and scheduled as follows: initial visit (T0) and final check visit (T28). During the initial visit, a screening test was carried out to evaluate the cognitive abilities of the eligible students, followed

by a series of cognitive tests, whereby physiological parameters were collected. At the end of the study, cognitive tests were repeated and physiological parameters collected again. Tests used are described below. Administered tests concerned attention, memory, and executive functions. Quality of sleep and anxiety levels were measured by validated questionnaires as follows: Athens Insomnia Scale and State-Trait Anxiety Inventory, respectively. Furthermore, instrumental parameters such as skin conductance and salivary swabs, for cortisol measurement, were collected during both the clinical visits. Each experimental session lasted 50 min. Only healthy subjects, experiencing a high level of stress due to exams period, were selected for this human study.

Test Measuring Cognitive Functions

Short-Term Memory Test. To evaluate short-term memory, two tests were selected [36]. The first required participants to memorize a random sequence of 15 words composed of 6 letters for 2 min. Next, the list was withdrawn and subjects underwent an interference task in order to avoid memory strategies or reiteration processes of the different items. Three minutes after the end of the interference task, the participants were asked to write as many random words as possible. During the second test, the participants had to memorize a random list of names associated with the corresponding surname. Similar to the previous test, the list was withdrawn and students were subjected to the interference task. After

Fig. 1. Serotonin levels in the culture medium of the neuroblast cell line SK-N-DZ after stress response induced by cortisol and treatment with probiotics. CTR–, not treated line, basal level; CTR+, cortisol-induced stress condition.



3 min, the participants were provided with a list of names and were asked to associate the right surname.

Problem-Solving Flexibility: WCST. The Wisconsin Card Sorting Test (WCST) is a neuropsychological test of “set-shifting.” The test measures the ability to show cognitive flexibility in decision-making processes. This cognitive function is mainly borne by the supervisory systems of the anatomical brain structures located at the level of the frontal lobes [37]. To evaluate problem-solving flexibility, a number of stimulus cards were presented to the subjects. The participant was instructed to match the cards, but not how to match; however, they were told whether a particular match was right or wrong. The test takes approximately 12–20 min and generates a number of psychometric scores, including numbers, percentages, and percentiles of categories achieved, trials, errors, and perseverative errors.

The Divided Attentional Performance Test. The divided attentional performance test measures the capacity to focus attention on two different irregular stimuli. Subjects responded to visual and acoustic stimuli (irregularities in an alternating series of high and low tones) that occurred at the same time. This test was extracted from Zimmermann et al. [38].

Questionnaires Measuring Psychophysiological-Related Aspects

Anxiety: STAI. The State-Trait Anxiety Inventory (STAI) questionnaire measures two types of anxiety: state anxiety (anxiety about an event) and trait anxiety (anxiety as a personal characteristic). High scores are positively correlated to higher levels of anxiety (concerning perceived stress levels). Participants answered 40 questions using a 4-point Likert scale [39].

Sleep Quality: Athens Insomnia Scale. Sleep quality was assessed using the Athens Insomnia Scale, assessing nocturnal sleep performance and daytime dysfunction. It is based on 8 parameters and rated on a 4-point Likert scale. Positive results are evaluated from the cumulative score of all factors and reported as an individual's sleep outcome [40].

Instrumental Parameters

In order to evaluate an improvement of physiological stress levels, two different parameters have been investigated. Specifically,

salivary specimens have been processed for salivary cortisol title, identifying alterations in hormone production that are physiologically related to stressful conditions [41]. Likewise, skin conductance was recorded aiming to detect changes of psychophysiological activation of sympathetic/parasympathetic systems as a marker of stress-related anxiety [42].

Salivary Cortisol. Salivary samples were collected at the beginning and at the end of the trial. Samples were collected directly by the participants that were provided with collection tubes (SALI SET 100, LDN – Labor Diagnostika Nord, Germany). Before being analyzed, each sample was centrifuged for 15 min at 3,000 rpm, placed at -20°C for 1 h, and centrifuged again for 15 min at 3,000 rpm. For quantification of cortisol an immunoradiometric Assay (Salivary Cortisol Kit, Pantec S.r.l. Italy) was performed following manufacturer instructions. The analysis was duplicated for each sample and the spectrophotometric data (absorbance) obtained were then processed for quantification in ng/mL.

Skin Conductance. Conductance was measured with the UFI Model 2701 BioDerm™ Skin Conductance Meter (UFI, USA) as follows: Ag-AgCl electrodes were connected to the phalanges of the nondominant hand from which the signal was obtained and the electric impulse was generated using the constant-voltage method (0.5 V), originally proposed by Lykken and Venables [43]. An external PC controlled the system and provided the trial start/end markers. The raw data were filtered through MATLAB® software (MathWorks Inc., USA), and a convolution analysis was performed on the signal using the Simple Scope™ software of the device (UFI, USA). Average values of the skin conductance level, expressed in micro-ohms, were then converted on a logarithmic scale and subjected to statistical analysis through multivariate ANOVA test.

Statistical Analysis

Statistical analysis was performed using NCSS 8 (version 8.0.4 for Windows; NCSS, Kaysville, UT, USA) running on Windows Server 2008 R2 Standard SP1 64-bit edition (Microsoft, USA). Data normality was checked using the Shapiro-Wilk normality test and data shape. Intragroup (vs. baseline) statistical analysis was carried out using Wilcoxon signed rank test. A $p < 0.05$ was considered statistically significant. Statistical analysis output was reported as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Fig. 2. Quantification of the neurotransmitter GABA levels in the culture medium of the neuroblast cell line SK-N-DZ after stress response induced by cortisol and treatment with probiotics. CTR–, not treated line, basal level; CTR+, cortisol-induced stress condition; GABA, gamma-aminobutyric acid.

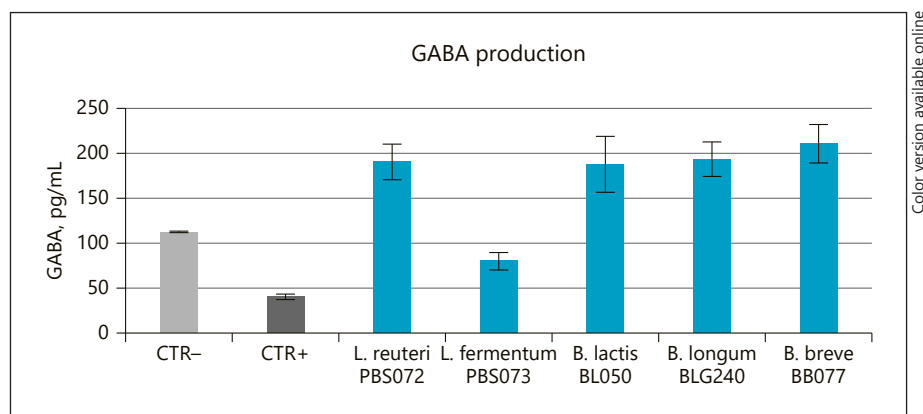
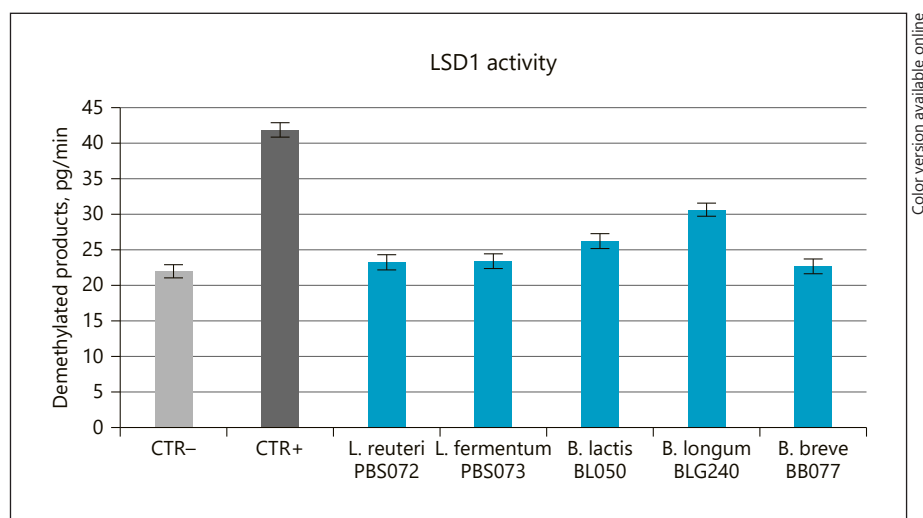


Fig. 3. LSD1 activity determination by quantification of the demethylated product in the culture medium of the neuroblast cell line SK-N-DZ after stress response induced by cortisol and treatment with probiotics. CTR–, not treated line, basal level; CTR+, cortisol-induced stress condition; LSD 1, lysine-specific demethylase 1.



Results

In vitro Evaluation

In order to select the best performing strains, different *in vitro* screening models have been evaluated. The first screening assay has been carried out on a neuroblast cell line SK-N-DZ in order to measure the following: (i) an improvement of serotonin synthesis, (ii) an improvement of GABA expression, and (iii) a downregulation of LSD1 activity.

Serotonin levels detected in the experimental model showed a significant decrease after inducing the neuronal response by adding the stressor agent (CTR+ vs. CTR–). Results demonstrated that no significant improvement in serotonin levels is mediated by the addition of probiotics to the neuronal cells line (Fig. 1).

The quantification of GABA was significantly influenced by the action of probiotics which underlined an opposite trend with respect to serotonin. After adding the

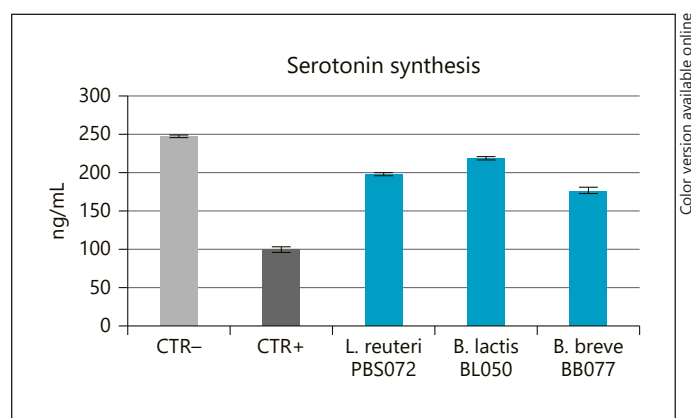
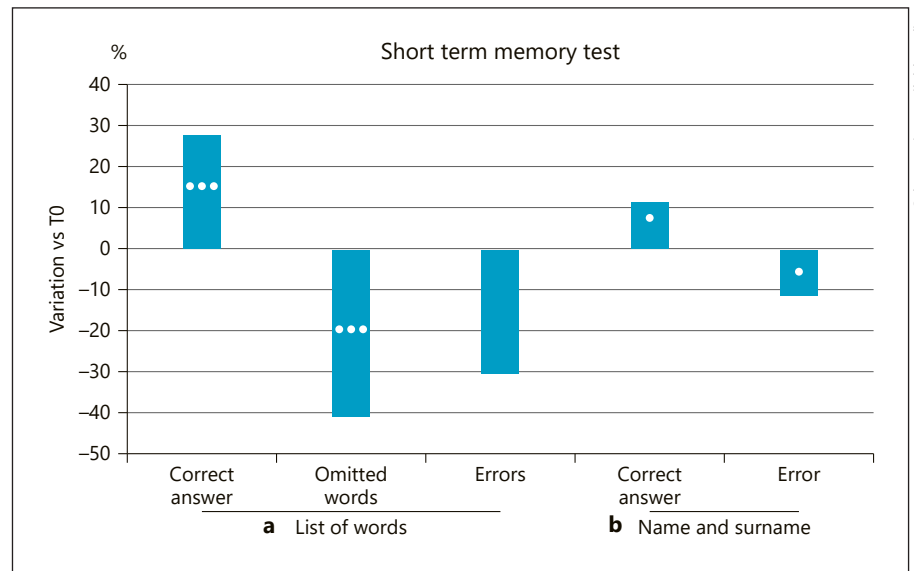


Fig. 4. Serotonin levels in the culture medium of the intestinal Caco2 cell line after stress response induced by cortisol and treatment with probiotics. CTR–, not treated line, basal level; CTR+, cortisol-induced stress condition.

Fig. 5. Short-term memory test. **a** Improvement in the memorization of a list of words. Results are reported as percentage of correct answers (number of reached words), reduction of omitted words, and reduction of errors. **b** Improvement in the association of name/surname pairs. Results are reported as percentage of correct answers (number of reached pairs) and reduction of errors. * $p < 0.05$ versus baseline (T0), *** $p < 0.001$ versus baseline (T0).



stressor agent (cortisol), GABA levels strongly decreased in the positive control. *L. fermentum* PBS073 was able to reverse the effect induced by cortisol, while *L. reuteri* PBS072, *B. breve* BB077, *B. lactis* BL050, and *B. longum* BLG240 were able to significantly increase it over the basal level (Fig. 2).

The LSD1 activity assay showed that probiotics have a positive effect in the epigenetic modulation of the enzyme in the neuroblast cell line. *L. fermentum* PBS073, *L. reuteri* PBS072, *B. breve* BB077, and *B. lactis* BL050 were able to strongly reduce the demethylase activity of LSD1, bringing it back to baseline values after stress induction by a stressor agent (cortisol). *B. longum* BLG240, however, only partially reduced the activity of LSD1 (Fig. 3).

As the strains of *B. longum* BLG240 and *L. fermentum* PBS073 were not significantly able to enhance GABA production nor to modulate LSD1 expression, they were excluded from the second screening step. Furthermore, measurement of serotonin in neuroblast cell line SK-N-DZ was not in line with the other markers. Taking into consideration that up to 90% of serotonin is produced in the gut, the next experimental model was carried out on an enteric cell line. The selected experimental model evaluated whether the probiotic strains were able to restore serotonin level in Caco2 cell line after treatment with cortisol. Indeed, cortisol is used as a stressor agent, and it decreases the serotonin level (CTR+). *L. reuteri* PBS072 and *B. breve* BB077 were able to restore the serotonin level after treatment with cortisol more effectively than *B. lactis* BL050. Results are reported in Figure 4. Taking into account the results achieved in the in vitro experimental

models, *B. breve* BB077 and *L. reuteri* PBS072 were selected as, on the tested cell lines, they were the best performing strains in modulating the stress induced by the presence of cortisol.

Human Study

The study was carried out from September 2019 to December 2019, at which time participants were in the winter exams session. Out of the 30 enrolled subjects, 3 volunteers did not complete the study. The discontinuation of the study was due to pharmacological treatment that the subjects needed for medical reasons. Thus, 27 patients completed the study. Among them, 8 were males and 18 females, with an average age of 23.4 ± 4.6 years. Treatments with food supplements were well tolerated by all the subjects, and no adverse events were reported during the study period.

Results obtained from the two short-term memory tests showed a significant improvement in observational time in terms of correct answers together with a significant reduction of the omitted words and/or errors. The short-term memory test based on common words (Fig. 5a) demonstrated an improvement of correct answers in terms of the number of reached words, of almost 28% and a reduction of omitted words of 41%, during the study period. Results were statistically significant ($p < 0.001$). Moreover, a reduction of errors of 30% was observed when compared to the baseline, although the result was not statistically significant. The second short-term memory test (Fig. 5b), based on names paired with surnames, demonstrated a similar significant trend in

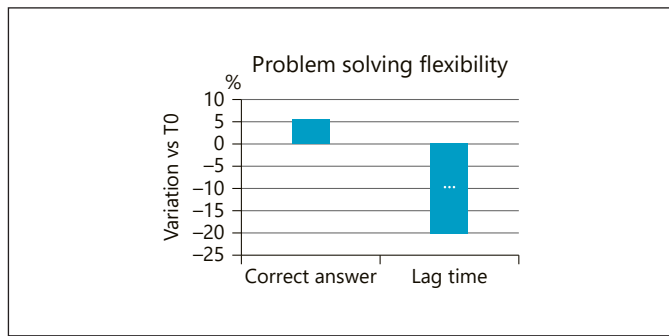


Fig. 6. Problem-solving flexibility through WCST. Improvement in the flexibility of choice in the decision-making processes. Results are reported as percentage of correct answers and reduction of latency between one answer and the following (lag time). *** $p < 0.001$ versus baseline (T0). WCST, Wisconsin Card Sorting Test.

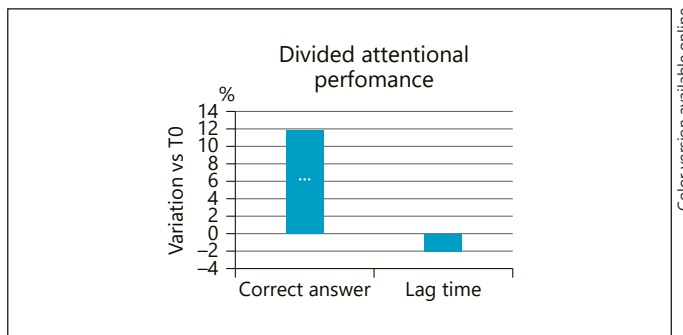


Fig. 7. Divided attentional performance. Improvement in the capacity to focus attention on two different irregular stimuli. Results are reported as percentage of correct answers and reduction of latency between one answer and the following (lag time). *** $p < 0.001$ versus baseline (T0).

terms of correct answers (+11.17%; $p < 0.05$) and number of errors (−11.56%; $p < 0.05$).

Through the use of the WCST, problem-solving flexibility was observed (Fig. 6). Although results did not show a significant improvement of the correct answers, the latency between one answer and the following was significant with $p < 0.001$ and was reduced by 21% from the beginning of the trial.

Results obtained from the divided attentional performance test (Fig. 7) showed that correct answers significantly improved up to almost 12% with respect to the baseline ($p < 0.001$) and that the lag time between answers was reduced, though the result was not statistically significant. With regards to the psychological parameters (Fig. 8), during the treatment, participants reported an improvement of sleep quality as evaluated by the Athens Insomnia Scale, where a lower score is equivalent to better

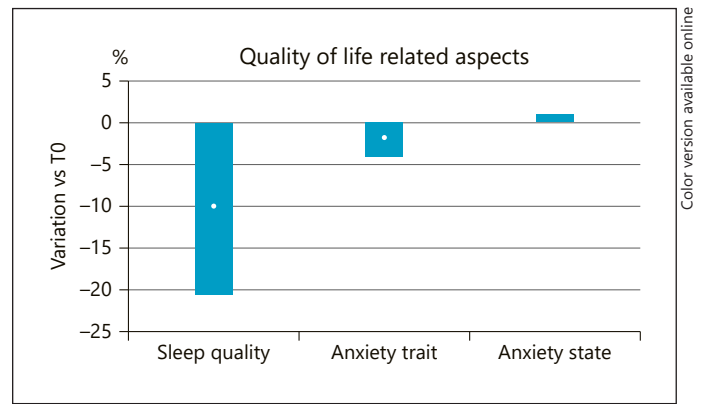


Fig. 8. Psychophysiological-related aspects. Sleep quality: percentage of reduction assessed with the Athens Insomnia Scale questionnaire. Results are calculated as cumulative scores of 8 parameters associated to nocturnal sleep performance and daytime dysfunction. Positive variations are related to poor sleep quality. Anxiety: percentage of the variation assessed with STAI questionnaire. The questionnaire measures both anxiety about an event (state anxiety) and anxiety as a personal characteristic (trait anxiety): higher scores are positively correlated with higher levels of anxiety. * $p < 0.05$ versus baseline (T0). STAI, State-Trait Anxiety Inventory.

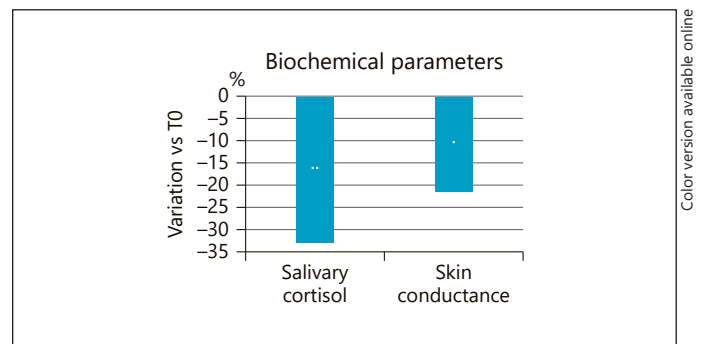


Fig. 9. Biochemical parameters. Salivary cortisol: percentage of reduction of hormone production related to stressful conditions and sleep disturbances. Skin conductance: percentage of reduction in the activation of sympathetic/parasympathetic systems as a marker of stress-related anxiety. * $p < 0.05$ versus baseline (T0), ** $p < 0.01$ versus baseline (T0).

sleep quality (−21% at T0–T28, $p < 0.05$). Anxiety, as a trait score, indicated a slight but significant reduction ($p < 0.05$), while the state score remained the same.

Instrumental parameter outcomes demonstrated a strong decrease. Cortisol values were significantly reduced by up to 33% ($p < 0.01$) together with skin conductance, exhibiting 21% less ($p < 0.05$) in the overall observational period (Fig. 9).

Discussion

The interconnection between the nervous and the gastrointestinal systems has a direct impact on health, psychological states (such as stress and anxiety), and overall well-being [1, 44]. Exposure to social stressors induces microbiota alteration and depletion of its composition [45, 46].

Working on the gut microbiota through probiotic administration is now becoming an innovative solution to improve the quality of life [47]. An increasing body of evidence highlights the supporting role of probiotics in the relationship between the gut and the brain due to their ability to produce and stimulate the expression of useful molecules such as SCFAs, serotonin, and GABA [48–50]. As an example, butyric acid has neuroactive properties and can improve brain health [51]. On the other hand, it is worth mentioning that most of the evidence has been acquired using animal subjects [52, 53]. Hence, understanding the bidirectional role of psychological processes of probiotics in humans is still under investigation. From this perspective, to gain evidence on probiotics and the gut-brain axis, five probiotic strains (*L. reuteri* PBS072, *B. breve* BB077, *B. longum* BLG240, *B. lactis* BL050, and *L. fermentum* PBS073) have been screened in two steps in an in vitro model to select the best performing strains in the modulation of stress markers serotonin, GABA, and LSD1.

A very important effect of probiotics in increasing GABA levels was observed. This is a positive achievement both for the modulation of sleep disorders and for the influence on mood. GABA acts as an inhibitory neurotransmitter and switches off excitatory signals involved in the awakening mechanisms and those generated in response to stress stimuli [18]. Many scientific studies have described the in vitro ability of different probiotic strains to produce GABA [54–56]. Others have also reported a possible antidepressant effect of probiotic supplements in mice following induced stress [57, 58]. The results reported in the present study are consistent with the abovementioned outcomes, suggesting the potential ability of the selected probiotic strains to overstimulate their own GABA production or to promote the endogenous one; however, more pieces of evidence would be needed to clarify the mechanism of action.

Concerning the epigenetic modulation patterns, probiotics were able to restore the basal enzymatic activity of LSD1 after induced stress. LSD1 is involved in the histone demethylation that, among others, regulates the genetic transcription of neuronal plasticity genes [59]. On the

contrary, decreased LSD1 activity is associated with maintenance of the correct transcriptional activity of the genes involved in social stress response. In the current study, when stress was induced by cortisol, the demethylase activity of LSD1 raised over the basal levels. This result is particularly interesting since, to the best of our knowledge, this is the first study reporting probiotic activity in the modulation of epigenetic response.

Surprisingly, serotonin results in the neuronal cell line did not show the same positive trend obtained for the other two selected markers. This outcome could be explained by the fact that the neuronal model was not the best system to determine the improvement of serotonin levels. Indeed, serotonin is mostly contained in the intestinal epithelium, where it is produced by enterochromaffin cells [60, 61]. Thus, the second screening was carried out, focusing on the enhancement of serotonin levels by probiotic strains in an enteric Caco2 cell lines. As expected, when compared to the positive control, probiotic strains were able to strongly increase serotonin levels, almost reaching the basal values. The previous scientific studies have described that bacteria are able to synthesize and/or induce serotonin production by the host. Some authors have investigated the ability of gut microbes in producing serotonin and its impact on health in germ-free mice, suggesting that the increased plasma serotonin levels observed in conventional mice compared with germ-free mice could indirectly result from a possible host-microbe interaction [62–64].

Given the results of the in vitro screening process and the proven colonization and persistence of these strains in the gut [57], *L. reuteri* PBS072 and *B. breve* BB077 were selected to be used in a proof-of-concept study involving stressed students for 28 days. Cognitive functions were our first outcome since stress can negatively lead to a cognitive failure affecting concentration and the ability to make decisions. Results demonstrated that, in only 28 days, subjects showed an improvement in short-memory, attention, and executive performance. The short-term memory test reported a significant improvement of correct answers, in terms of memorized words, and accuracy. The best result was obtained with the list of the randomized words rather than name and surname: this outcome could be explained since it is easier to remember only one word with respect to a coupled name and surname without any mismatch in gender (which was considered as an error).

The same trend was observed for the divided attention task, with a significant improvement of 12% in correct answers when compared to the baseline. However, the lag

time between one answer and the following was not statistically significant. In contrast, problem-solving flexibility showed no significant improvement of correct responses but a strong significant reduction (−21% vs. baseline) of the lag time. This is an interesting result which demonstrates students' reactivity in answering questions.

Finally, in order to gain a better understanding of the subjects' psychophysiological response, different tests were carried out to evaluate both physical (by skin conductance and salivary cortisol) and psychological (sleep quality and anxiety questionnaires) factors. Cortisol levels increase in a stressful situation, in turn, affecting sleep. The significant reduction achieved during the treatment (−33%) is an important outcome reflecting a decrease of the physiological stress level [65]. Likewise, a reduction (−21%) in skin conductance was observed, which is linked to the change of psychophysiological activation of sympathetic/parasympathetic systems, closely related to concentration and stress [66]. Lower levels are typically related to higher relaxation status: subjects were largely relaxed and consequently more focused during the last experimental section. In terms of sleep quality and anxiety, after 28 days of probiotic intake, subjects perceived a better sleep quality. These results are in line with the latest scientific research [67, 68]. Anxiety, on the other hand, showed a slight reduction when compared to the other parameters. This was not surprising since our participants did not present any kind of neurologic pathology, suggesting that stressed students felt much more pressure in terms of lack of concentration with respect to anxiety.

In the present study, we investigated connections between probiotics and stress. As a first step, we selected two probiotic strains among five as the best performer in modulating stressor markers *in vitro*. Subsequently, we tested *L. reuteri* PBS072 and *B. breve* BB077 in a human proof-of-concept clinical study with preliminary promising results, supporting the use of these probiotics as potential allies for stressful situations occurring in everyday life. To the best of our knowledge, this is the first study that links the *in vitro* positive results with efficacy in a human trial, also highlighting a putative mechanism of action for the positive effect exerted by probiotics. Furthermore, the results obtained from this exploratory study highlighted the potential and valuable impact of probiotics intake on different aspects of mood and cognitive functions in a specific cohort of subjects facing a stressful period.

A core limitation of the study is related to the low number of subjects as well as the absence of a placebo ref-

erence group which limited the opportunity to discuss and compare our results. For these reasons, more scientific evidence to confirm the outcomes of this first trial and placebo-controlled studies is needed.

Acknowledgment

The authors would like to acknowledge Roelmi HPC for providing the probiotic complex.

Statement of Ethics

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Ethical Committee approval was not considered necessary since this study was a proof-of-concept performed on healthy subjects with the only purpose of confirming the possible efficacy of the tested product.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Francesco Puoci was the principal investigator responsible for designing and conducting the clinical study. Silvana Giardina performed all the *in vitro* testing. Vincenzo Nobile was responsible for the calculations and the statistical analyses.

Data Availability Statement

Data supporting reported results are stored at the Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, and subjected to regular back-up.

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