Effect of a Multi-Strain Probiotic Supplementation to Manage Stress during the COVID-19 Pandemic: A Randomized, Double-Blind, Placebo-Controlled, Cross-Over Clinical Trial

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Abstract

Introduction: The COVID-19 pandemic strongly affected every aspect of the modern society, from health to socio-economics, leading people to experience high levels of stress. ***Methods:*** A double-blind, cross-over, placebo-controlled clinical study was performed to investigate the ability of a food supplement containing two probiotic strains, *Limosilactobacillus reuteri* PBS072 and *Bifidobacterium breve* BB077, in supporting 33 healthy adults, working at a university, in stress management. The efficacy of the tested strains in influencing the stress response, in terms of mood and sleep behavior, was assessed using the following validated questionnaires: Profile of Mood State (POMS) and Pittsburgh Sleep Quality Index (PSQI). ***Results:*** Outcomes of the POMS and the PSQI demonstrated a significant reduction of the questionnaire's scores both versus baseline and placebo after 30 days of probiotic intake. ***Conclusions:*** According to the results, the probiotic food supplement investigated showed a remarkable effect on stress management by improving the quality of sleep and the mood.

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Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused a global, unprecedented crisis that involved public health, economic system, and society in general, affecting populations all over the world [1]. As a consequence, people had to face not only the health emergency and the economic crisis but also the insecurity of the future and the fear for their health and employment [2, 3]. Furthermore, restrictions like social distancing and lockdowns led to a worsening of the quality of life, both from a physical and a psychological point of view [4, 5]. In Italy, the ongoing uncertainty due to the oscillation from strict to less strict restrictions (and vice versa) produced an increasingly unstable psychological condition, leading to a situation of high anxiety and stress level in the population [6, 7]. Moreover, the impact of introducing changes in work habits (i.e., smart working, layoffs, children at home, and distance learning) to manage the so-called "new normal" further exacerbated this mental state [8–10]. In this context, the need of new strategies for managing stressful conditions has become warranted.

Scientific evidence pointed out that stress induces several physiological alterations such as cortisol increases and sleep disturbances. Indeed, long-duration stress

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stimuli result in a response that involves the hypothalamic-pituitary-adrenal axis, leading to an impaired production of cortisol [11]. A compromised communication along this axis may cause structural changes in the brain with long-term effects on the nervous and immune system [12]. Furthermore, stress hormone levels correlate positively with reduced sleep duration, which in turn is associated with a higher incidence of metabolic disorders such as obesity and type 2 diabetes [13]. Stress can also affect the gastrointestinal functions, increasing mucosal permeability that causes an enhanced uptake of potentially harmful molecules such as lipopolysaccharide and pro-inflammatory agents (TNF-α, IL-1b, IL-6) [14]. These compounds can overstimulate the immune system, triggering autoimmune and inflammatory responses [15].

Microbiota plays a pivotal role in maintaining gut microbiota integrity, also known as *eubiosis* status, a condition of dynamic equilibrium of resident gut microorganisms [16]. Besides intestinal functionality, gut microbial communities are also actively involved in immune system regulation through the production of bioactive molecules that act as signals at immunological and neurological level [17–19]. Indeed, in the past decades, the link between the intestinal microbiota and the brain, along the so-called gut-brain axis, has been confirmed by several studies indicating an intimate connection between gut health and brain functions, including behavior and sleep [20–22]. A gut microbiota imbalance, as a consequence of stress, may lead to gut-brain miscommunication. Thus, maintaining a microbiota in *eubiosis* could be a valid approach to cope with such alteration.

In the last decades, clinical evidence indicates that probiotics can influence the gut-brain axis and modulate mood and stress responses [23, 24]. Indeed, probiotics are able to maintain mucosal barrier functions, mitigate stress-induced glucocorticoids and inflammatory cytokine responses, and increase neurochemicals such as tryptophan, dopamine, serotonin, gamma-aminobutyric acid known to modulate host neural activities and functions such as stress management, emotions, and cognition [25–27]. Moreover, there is increasing evidence that probiotics can restore the gut microbiota balance through several mechanisms [28–30]. For these reasons, probiotic intake to modulate microbiota could contribute to mental health in stressful conditions.

In this double-blind, cross-over, placebo-controlled clinical study, we investigated the ability of a food supplement containing two probiotic strains, *Limosilactobacillus reuteri* PBS072 and *Bifidobacterium breve* BB077, in supporting healthy adults, working alternately from home/

university, in stress management, monitoring their mood state and sleep quality. These two probiotic strains have been selected among different strains for their in vitro performance and further tested in a proof of concept clinical study reporting an improvement of cognitive functions and sleep quality in stressed students [31].

Materials and Methods

A randomized, double-blind, placebo-controlled, cross-over clinical study was carried out at the Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Italy. The study was performed in accordance with the principles of the Declaration of Helsinki, good clinical practice, and applicable national regulatory requirements. All procedures involving human subjects were approved by the Ethics Committee of the University of Calabria. The study was registered at ISRCTN registry with the following registration number: ISRCTN15033071.

Study Participants
Subjects participating in this study were healthy men and women, 25–60 years old, working at the Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, with an initial Pittsburgh Sleep Quality Index (PSQI) score in the range 4–6 [32] and Profile of Mood State (POMS) in the range 10–30. To the best of our knowledge, to discriminate between good and bad mood, no Total Mood Disturbance (TMD) score guidelines are available. Thus, considering that the TMD score can run from 0 to 200, the range 10–30 has been chosen to enroll only subjects with non-pathological mood disturbance.

The most important exclusion criteria were the use of oral antibiotics within 30 days before the screening visit and use of drugs, food, or herbal supplements for the mood or sleep. A complete list of the inclusion and exclusion criteria is reported in Table 1. A total of 33 subjects were considered eligible to participate in the study.

Study Design
The study was conducted with a cross-over design with the primary aim to reduce perceived stress during the pandemic period in terms of improvement of mood and sleep quality. At the screening visit, eligible subjects were enrolled in a two-treatment period (probiotic and placebo or vice versa) of 30 days each, alternated with a washout period of 30 days. The total duration of the study was 90 days. Outcome measures were evaluated at each of the four visits: visit 1 (beginning of the study), visit 2 (end of the first intervention), visit 3 (start of the second intervention, after a washout period of 30 days), and visit 4 (end of the second intervention). The flowchart of the study is reported in Figure 1.

Study Product
Subjects were randomly assigned to receive one capsule a day of the active or the placebo product, away from meals and stored at room temperature. The active capsules contained two probiotic strains, *Limosilactobacillus reuteri* PBS072 and *Bifidobacterium breve* BB077, for a final dose of $4×10^9$ CFU/day ($2 × 10^9$ CFU for each strain) and maltodextrin, while the placebo capsules were

Table 1. Inclusion and exclusion criteria

Inclusion criteria
    Good general health
    Female or male subjects
    Aged between 25 and 60 years
    Subjects who have not been recently involved in any other similar study
    Commitment to complete questionnaire
    Commitment to use during the entire study period only the products to be tested
    Commitment to not use products likely to interfere with the product to be tested
    Commitment to not vary the normal daily routine (i.e. lifestyle, physical activity, etc.)
    Subjects aware of the study procedures and having signed an informed consent form
    Profile of Mood State score between 10 and 30 at the baseline
    Pittsburgh Sleep Quality Index score between 4 and 6 at the baseline
Exclusion criteria
    Current antibiotic administration
    Treatment with probiotics in the 6 months preceding enrolment
    Subjects with known or suspected sensitization to one or more test formulation ingredients
    Subjects who do not meet the inclusion criteria
    Women who are pregnant/breast feeding or not using adequate contraceptive methods
    Any condition that the principal investigator deems inappropriate for participation
    Adult 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
    Suffering from other psychiatric disorders such as schizophrenia, other psychotic disorders, bipolar disorder or substance use disorder
    Herbal remedies or psychotropic drugs that are intended for depression taken within the last 2 weeks prior to baseline or during the study
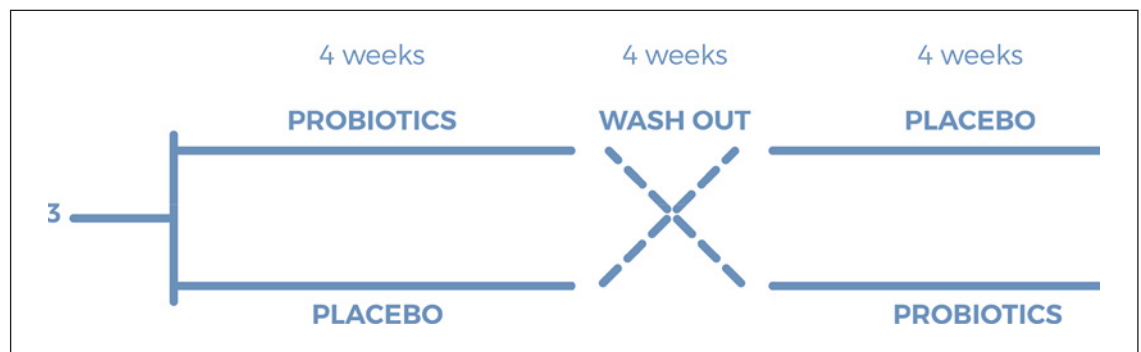

Fig. 1. Flowchart of the study.

composed only of maltodextrin. The active and placebo products presented a similar appearance and taste. The products were manufactured by ROELMI HPC, Origgio (VA), Italy. Compliance was evaluated at the end of each treatment period, when the subjects were asked to return all treatment capsules, and the number of unused capsules was counted.

Outcome Measures

The endpoint was to assess the ability of the probiotic formulation in enhancing mood and sleep behavior. All participants completed a battery of validated psychological questionnaires,

the Profile of Mood State (POMS) and the Pittsburgh Sleep Quality Index (PSQI), at the beginning and at the end of each treatment, for a total of four time points. Sessions were carried out half in person and half online. Each session lasted about 25 min.

Profile of Mood State

The Profile of Mood State (POMS) is a self-report questionnaire that assesses mood. The version used in the present study is the Italian adaptation of the original text [33]. It consists of 58 items (words or sentences) that describe feelings that people usually have. For each item, participants rate on a 5-point scale from
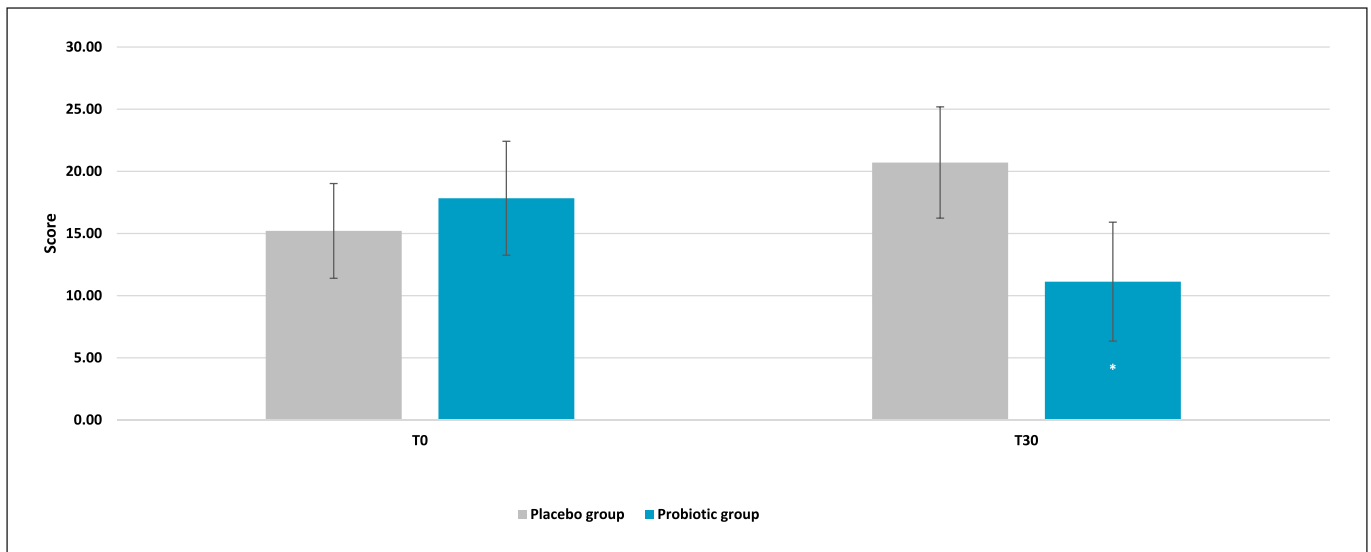
Fig. 2. Profile of Mood State: Total Mood Disturbance (TMD). Results are reported as mean values. *$p < 0.05$ versus baseline.

0 (not at all) to 4 (extremely) how they have been feeling in the past week including the current day. The items are grouped into 6 categories: tension (9 items), depression (15 items), anger (12 items), fatigue (7 items), confusion (7 items), and vigor (8 items). The Vigor scale is in inverted relationship with the other scales since it assesses positive feelings (e.g., to be lively, active, energetic). The total score, defined as the Total Mood Disturbance (TMD), can be calculated by adding the scores for tension, depression, anger, fatigue, and confusion and then subtracting the score for vigor as explained by the following equation: TMD = (Tension + Depression + Anger + Fatigue + Confusion) – Vigor (1)

The TMD global score ranges from 0 to 200: the reduction of TMD score over time is expected as a reflection of an improvement of general mood. A subgroup analysis of each category has been assessed to evaluate a possible modification toward a specific feeling category.

Pittsburgh Sleep Quality Index
The Pittsburgh Sleep Quality Index (PSQI) is a self-report questionnaire that assesses sleep quality for the majority of days and nights in the past month. The test used was the validated Italian version [34]. It consists of 19 questions. The first four investigate usual bedtime, the number of minutes needed to fall asleep, the usual getting up time, and the number of hours of sleep per night. The other questions are related to other aspects of sleep quality rated on scales appropriate for the specific question. The items are weighted on a 0–3 interval scale and grouped in seven component scores (i.e., subjective sleep quality, sleep latency, sleep duration, use of sleeping medication, daytime dysfunction, habitual sleep efficiency). A global PSQI score (range 0–21) was obtained by adding up the component scores, wherein the lower the PSQI global score, the better the sleep quality. A subgroup analysis of the different categories was assessed to determine if probiotic intake specifically modulates a certain sleep condition.

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 W normality test and data shape. Intragroup (vs. baseline) statistical analysis was carried out using the Wilcoxon signed-rank test, while intergroup statistical analysis was carried out using the Mann-Whitney U test. A $p < 0.05$ was considered statistically significant. Statistical analysis output was reported as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Results

Subjects of the Study
The cross-over study was conducted between March 2021 and June 2021. Recruitment was carried out via email invitation among subjects working at the Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Quattromiglia, Cosenza. A total of 33 male and female subjects, employees of university (researchers, professors, administrative employees) working alternately from home/university were successfully enrolled. At the end of the study, a total of 24 subjects completed the treatment. Nine subjects discontinued the study, five withdrew because of their inability to collect the last questionnaires of the second treatment period, and four had to take antibiotics during the washout period. The population was composed of 15 women and
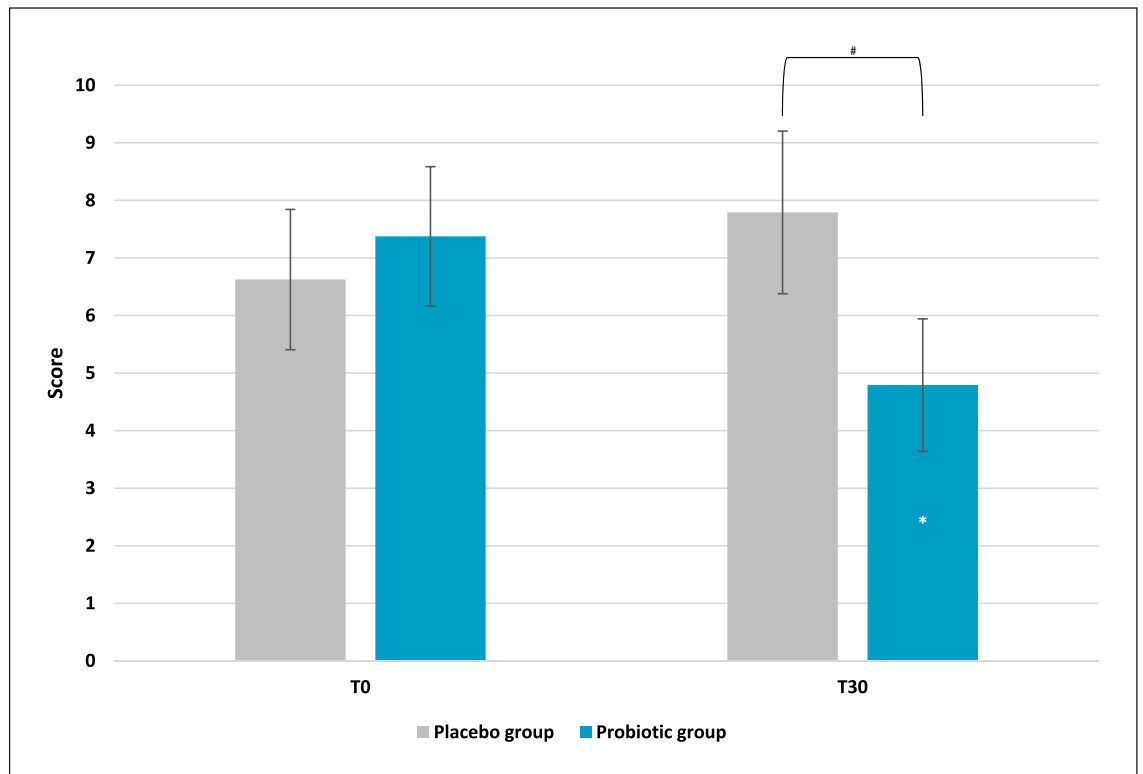
Fig. 3. Profile of Mood State: anger subscale score. Results are reported as mean values. *$p < 0.05$ versus baseline, #$p < 0.05$ versus placebo.

9 men with an average age of 36 ± 9 (±SD) years. In general, both products used in this clinical study were well tolerated, and the level of compliance to the treatment was high.

Mood-Related Aspects

Results obtained from the global score, the TMD, showed a strong reduction of the overall mood score. Indeed, after 30 days of treatment, the active group showed a significant reduction of 44% ($p = 0.025$ vs. T0), while the placebo reported a higher score (+27%), reflecting a worsening of the general mood state (Fig. 2). The comparison between active and placebo results reported a significance of $p = 0.0572$, meaning that there was a borderline statistical significance versus placebo. In addition, a subanalysis of each questionnaire category was assessed (see M&M section for categories' description). Anger evaluation showed a significant effect on the scores for the experimental group (Fig. 3). Results showed a lower score in the active group after probiotic intervention (mean ± SE, 4.79 ± 1.14; $p = 0.028$ vs. T0) compared to baseline (T0) and placebo ($p = 0.032$). Also, in this case, the placebo

group showed a higher value compared to the active treatment. No significant results on the anger category for the placebo group were recorded. A significant effect was also found on the tension subscale scores for the experimental group (Fig. 4). Post hoc comparisons showed that this effect was lower after 30 days of probiotic intake (T30) (mean ± SE, 7.04 ± 0.82; $p = 0.011$ vs. T0) compared to baseline (T0) and placebo ($p = 0.046$). The placebo group reported a light but no statistical reduction (mean ± SE, 8.42 ± 0.73; $p = 0.72$ vs. T0) of tension score with respect to the active group. For the other categories, results showed no significant variation (data not shown).

Sleep Quality

Sleep quality presented a significant improvement in the active group compared to the placebo. Results demonstrated a lower score after 30 days of probiotic intake (mean ± SD, 4.00 ± 1.64) compared to baseline (T0) (mean ± SE, 5.5 ± 0.56; $p = 0.002$ vs. T0) and placebo ($p = 0.009$) as reported in Figure 5. On the same trend, the placebo group reported a higher score if compared to the active
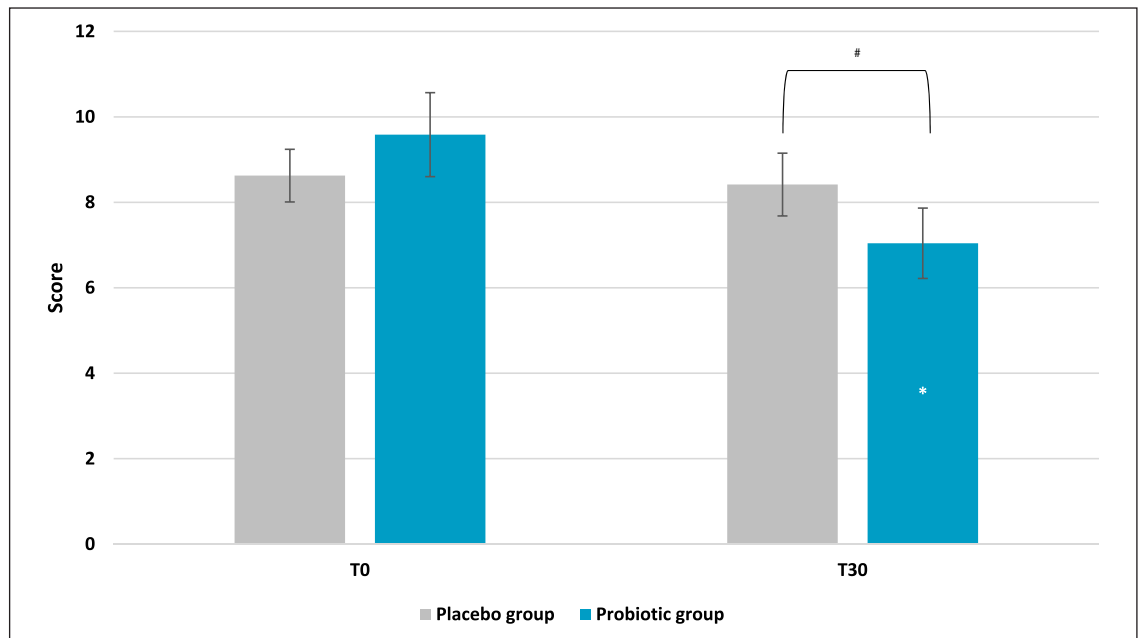
Fig. 4. Profile of Mood State: tension subscale score. Results are reported as mean values. **$p < 0.01$ versus baseline, #$p < 0.05$ versus placebo.
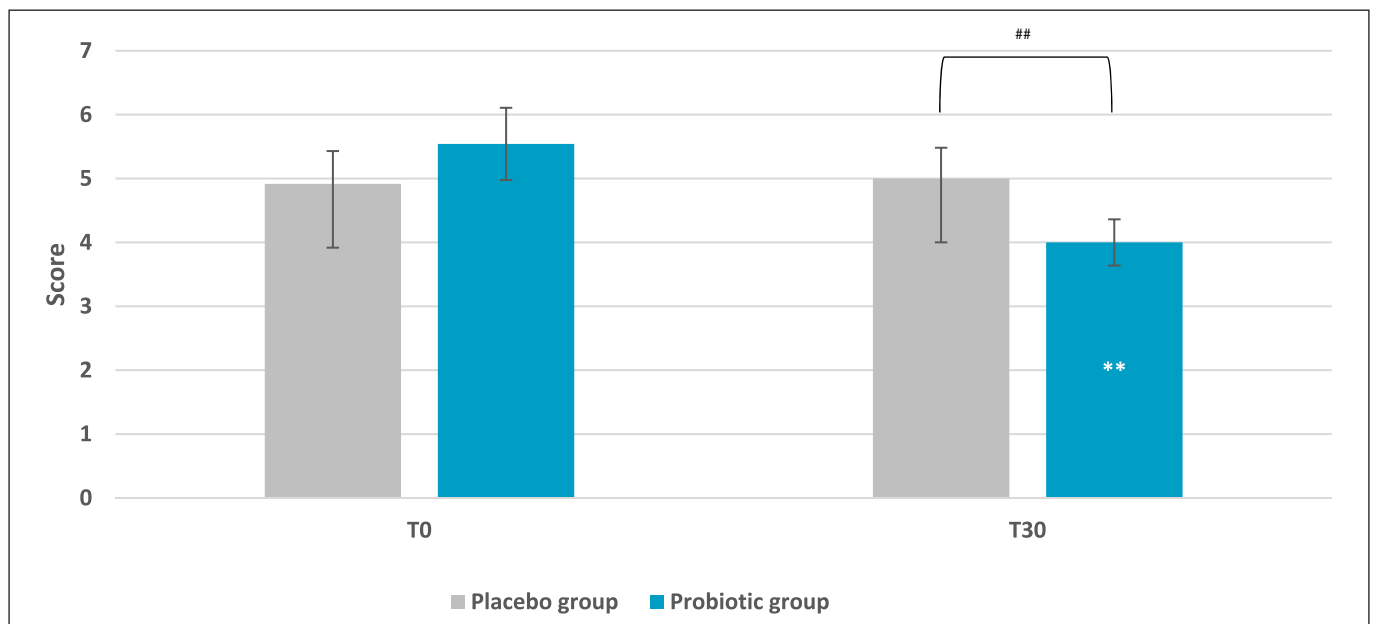

Fig. 5. Pittsburgh Sleep Quality Index score. Results are reported as mean values. **$p < 0.01$ versus baseline, ##$p < 0.01$ versus placebo.

treatment, which is linked to a worse sleep quality (+20% and −20%, respectively). No significant results were noted for the placebo group. Subscale analysis reported that among all the categories composing the questionnaire, only subjective sleep quality and sleep latency showed statistically significant results. Subjective sleep quality subscale evaluation indicated a slight but significant reduction in the active group (mean ± SE, 0.71 ± 0.13; $p = 0.047$ vs.
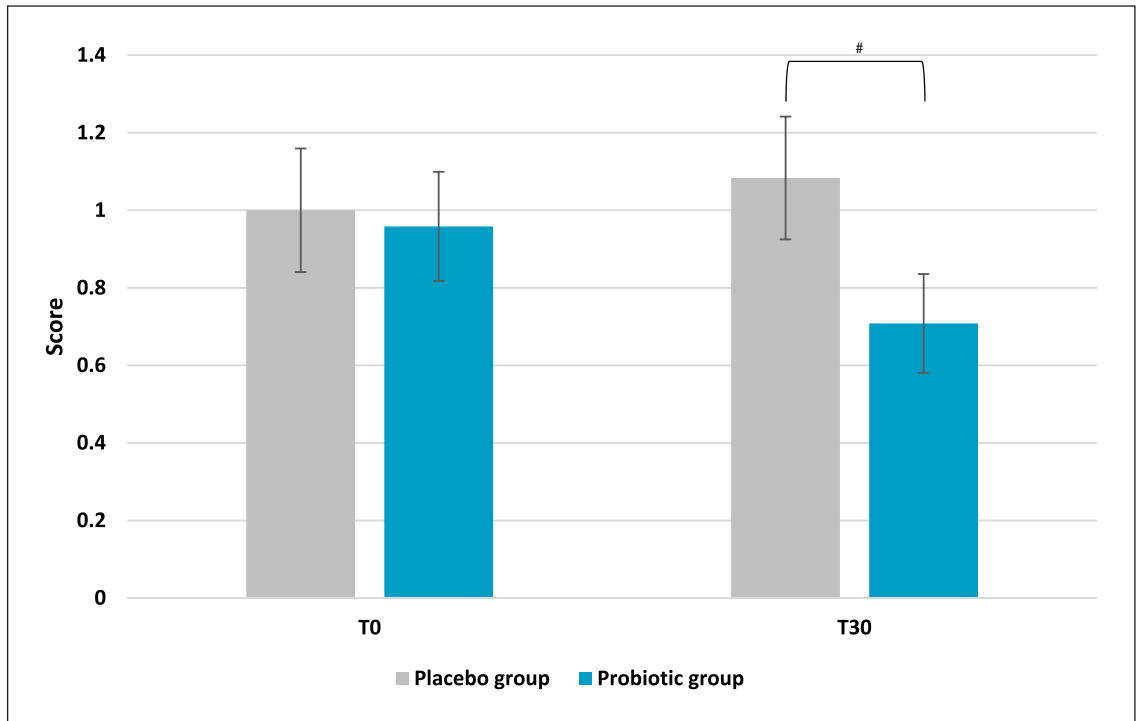
Fig. 6. Pittsburgh Sleep Quality Index: subjective sleep quality subscale score. Results are reported as mean values. $^\#p < 0.05$ versus placebo.
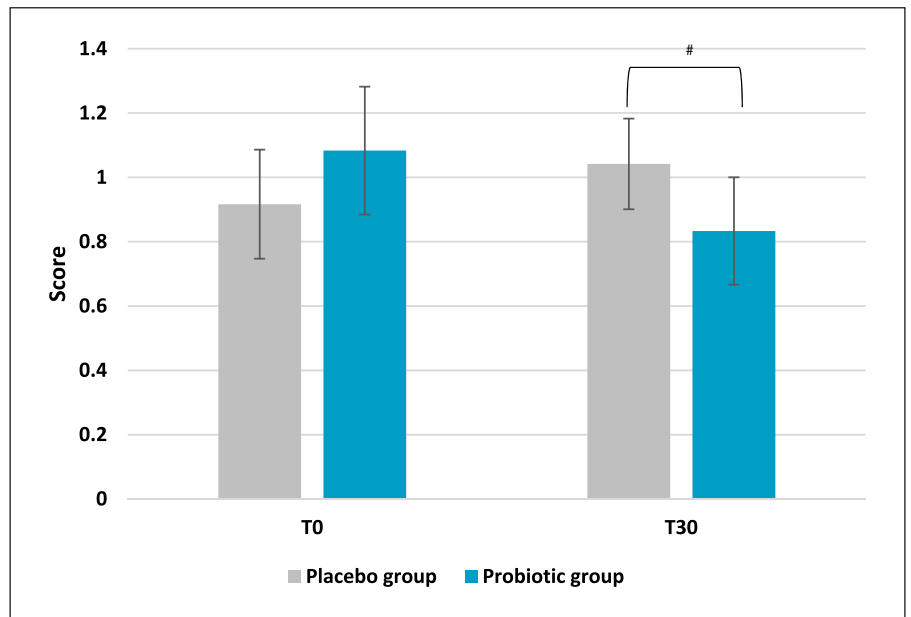

Fig. 7. Pittsburgh Sleep Quality Index: sleep latency subscale score. Results are reported as mean values. $^\#p < 0.05$ versus placebo.

placebo) compared to placebo (Fig. 6). Concerning the placebo group, no statistical results were observed at the end of the 30 days of treatment. The initial condition of subjects in the placebo group seemed to worsen, as indicated by an increase in the questionnaire score. The same trend was observed for sleep latency (Fig. 7). The statistical analysis showed a low but significant reduction of the score in the active group compared to the placebo.

Comparisons showed that the score was lower after 30 days of probiotic intake (T30) (mean ± SE, 0.83 ± 0.17; p = 0.035 vs. placebo) compared to placebo. The placebo group did not show any improvement and no statistical reduction of sleep latency score.

Discussion

The recent pandemic caused by the severe acute respiratory syndrome coronavirus 2 has had a dramatic impact on modern global society [35]. The panic caused by threats to health and the future uncertainty related to socioeconomic crisis had a devastating effect on many individuals and many aspects of everyday life [36]. Furthermore, the necessary restriction measures contributed to the worsening of the emotional state of many, leading to increased stress and anxiety [37, 38]. Several scientific papers highlighted how COVID-19 raised the level of perceived anxiety and tension all over the world [39–43]. In Italy, the first western country to experience lockdown, effects were overwhelming [44–46]. Even before this pandemic, stress represented an enormous burden for many people, being defined as the "Health Epidemic of the 21st Century" by the World Health Organization [47]. For these reasons, the need of a novel approach to influence mood and well-being is even more urgent. In recent years, it has become more and more evident that, through the gut-brain axis, gut microbiota can influence response to stress and that probiotics are useful to affect in a positive manner these commensal bacteria [48]. During the pandemic, probiotics were largely suggested as a possible tool to manage respiratory tract infection, gastrointestinal symptoms, and inflammation caused by severe acute respiratory syndrome coronavirus 2 [49–54]. However, to the best of our knowledge, no evidence of actual usage for stress management was proposed.

Results obtained from this cross-over, double-blind clinical study demonstrated how probiotic intake could contribute to the modulation of mood and sleep during a stressful period. Profile of Mood State questionnaire showed a decrease of the Total Mood Disturbance score, underlining a significant reduction compared to T0 of the active group. It is worth noting that the result was close to the significance (p = 0.0572) when compared to the placebo. Therefore, we can speculate that, with a larger cohort of subjects, these results may reach a p value <0.05. The TMD is used as a marker of negative feelings: a higher TMD score is indicative of a greater mood disturbance (i.e., greater negative mood) [55, 56]. To the best of our knowledge, no other clinical studies with probiotics have focused their attention on TMD but rather on the improvement of the single category [57, 58]. The anger category showed a significant reduction in score in the active group compared to the placebo. The same trend was observed for the tension category: in the active group, there was improvement of this negative feeling with respect to both T0 and placebo. In this context, it is difficult to make a comparison with other clinical studies since there are only few using POMS with probiotics, and they show conflicting results [59, 60]. The current circumstances caused by the pandemic mean that people returned to their workplaces after months of lockdown. They have done so with paradoxical emotions; thus, it is without question that working on negative feelings is now becoming a prerogative to support worker's mental health [60–62].

Sleep quality was assessed through the validated questionnaire Pittsburgh Sleep Quality Index. Also, in this case, a reduction of the total score indicated an improvement compared to the initial condition. Several clinical trials with probiotics have focused their attention on this topic using the same questionnaire; however, no significant effect has been found [63]. According to the meta-analysis performed by Irwin and colleagues, only subgroup analysis suggested a greater but no significant effect in healthy subjects with an average treatment period of 8 weeks. On the contrary, in this cross-over clinical study, the active group experienced a significant reduction of the global PSQI score, denoting a general improvement of the sleep condition in a shorter period of time (4 weeks of probiotic intake). In addition, subscale analysis of the single category evidenced a significant reduction of perceived sleep quality as well as sleep latency score, which corresponds to an improvement of the abovementioned category. Furthermore, it is worth noting that, although we enrolled healthy subjects, the current living situation has further affected the normal life routine of many people, which is fundamental for circadian rhythms. Indeed, the study was carried out during a period when the government decided to alternate stricter to softer restrictions due to the fluctuations of contagions. It is worth noting that, even though we performed a cross-over study, we did not observe significant variations between the two groups, according to the period they took the probiotic supplement. Furthermore, as reported in several probiotic cross-over clinical trials, 4 weeks of washout were sufficient to avoid any putative bias linked to the design of the study [64–66].

In the last decade, several clinical trials have been carried out to evaluate the role of probiotics in the gut-brain axis, pointing out their ability to improve cognitive functions, ameliorate stress management, as well as sleep quality, and underlining their positive effect toward negative feelings such as anxiety [67]. Other studies demonstrated that probiotics can improve psychological and physiological markers of stress and anxiety. However, it is interesting to note that, in those studies, probiotic concentrations were dramatically higher compared to the one presented in this clinical study [68–71]. Furthermore, the same concentration of the product was already tested in a proof of concept study to evaluate stress-related parameters in a group of stressed students, showing significant positive effect on mood and cognitive functions [31].

The results obtained in this study support the clinical evidence of a beneficial effect on mood and sleep quality of the tested strains, *L. reuteri* PBS072 and *B. breve* BB077, in stressed subjects. However, a core limitation of the study was related to the uncertainty of the moment that made it difficult to track fluctuations of life habits (diets, physical activity, etc.) due to frequent ministerial decree modifications according to the pandemic evolution. Furthermore, during the design of the protocol, no validated questionnaire linked to COVID-19 was available. In addition, even though everyday chronic stresses can be in part responsible for general mood and sleep alteration, our results are mainly related to COVID-19 and to the uncertainty of that period. Restrictions like social distancing and lockdowns were responsible for an increased state of anxiety and fear for the future, affecting the quality of life physically and psychologically. Any volunteer was infected during the study period.

Even though the trial was carried out on a small cohort of volunteers, data resulted sufficient to obtain significant results. For the reasons mentioned above, the choice of a cross-over clinical trial was the optimal solution to overcome the enrollment of a high number of people during pandemic and also to minimize the potential emotional bias intrinsic to each volunteer.

Another limitation observed was related to the correlation between physical and psychological parameters and the gut microbiota composition. Actually, the main outcome of the study was to investigate an improvement of mood and sleep through the oral administration of specific multi-strain probiotic formulation. The mutual connection between gut microbiota and psychophysiological aspects will be for sure investigated; further studies are needed to more deeply explore the effect and the mechanism of action of the tested probiotics in this field of research.

Author Contributions

Conceptualization: Francesco Puoci and Vincenzo Nobile; methodology, investigation, resources, writing – original draft preparation, supervision, project administration, and funding acquisition: Francesco Puoci; formal analysis, data curation, and writing – review and editing: Vincenzo Nobile. All authors have read and agreed to the published version of the manuscript.

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 backup. All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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