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Effect of a probiotic fermented milk supplementation on behavior and sleep

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ABSTRACT

This study attempted to analyze the effect of supplementing Wistar-Kyoto rats with fermented milk containing the probiotic *Bifidobacterium animalis* BB-12 and pomegranate juice on the microbiota-gut-brain axis of rats, with special focus on their behavior, sleep patterns, and response to stress. This study was divided into two experiments: (1) For the behavioral analysis the animals were divided into two groups: Fermented probiotic milk (BB + 1) and control (BB-). (2) For the sleep analysis the animals were divided into two groups: Fermented probiotic milk (BB + 2) and control (H2O). For the behavioral analysis, the open field method was used, which evaluates the behavior after ten, twenty, and thirty days of supplementation. For sleep analysis, the animals were submitted to implantation of electrodes and 24 h polysomnography, followed by 48 h sleep deprivation (REM) and 48 h polysomnography, then euthanized 100 days after the beginning of the experiment. In addition, animal feces were collected before and after sleep deprivation to assess its effects on the microbiota. A decrease in anxiety-related behaviors was observed in the supplemented animals and an increase in sleep efficiency and a reduction in the number of awakenings of the animals before deprivation. It has also been observed that sleep deprivation decreased the amount of total bacterial DNA. The number of copies of genomes of the genus *Bifidobacterium* did not differ in both groups.

KEYWORDS

Animal model; *B. animalis*; sleep deprivation; supplementation; polysomnography; open field

Introduction

The intestinal microbiota exerts numerous functions in human central nervous systems, immune responses, and metabolism. It also synthesizes vitamins and enzymes to maintain the intestinal barrier [1–4]. Currently, we find growing interest in research that evaluates how microbial taxa influence the microbiota-gut-brain axis since their composition and structure exert significant effects on behavior, memory, mood, and sleep [5–11].

The microbiota-gut-brain axis is characterized by a bidirectional communication system between the hosts' intestinal microbiota and the central nervous system by specific pathways involving the endocrine, immune, and neural systems. Neural communication occurs via vagal and spinal afferent pathways [9,12]. This axis can modulate the hosts' sleep, mood, and cognitive responses, from neural and hormonal signals to the production of metabolites [10,13–15]. Among the metabolites involved, we can mention neurotransmitters and their precursors, such as GABA, serotonin, and tryptophan; short-chain fatty acids; and proteins, such as brain-derived neurotrophic factor, which can release neuropeptides and gut hormones [3,9,15,16].

Sleep is essential for the proper functioning of individuals' cognitive and physiological functions, immune and metabolic responses, as well as muscle recovery [14,17,18]. The stress caused by poor quality and insufficient sleep negatively affects individuals' circadian cycle and general health, and it can decrease cognitive responses and alter their behavior and mood [12,14,18,19].

Ingesting strains of the genus *Bifidobacterium* spp. is associated with improved sleep quality and mood in humans [10,12]. In a study by Moloney et al. [20], the authors observed that administering *B. longum* improved sleep quality and duration in healthy subjects which were subjected to stress periods [20]. The beneficial effects of strains belonging to the genus *Bifidobacterium* spp. during sleep may be associated with their modulation of hosts' intestinal microbial ecology and the bacterial metabolites produced [10]. The probiotic *Bifidobacterium animalis* subsp. *lactis* BB-12 is one of the most documented strains in the scientific literature, so it was chosen for this research due to its proven health-promoting effects [2,21–23], including alleviating tenseness and sleepiness in healthy young military individuals [11].

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Gallic acid, a phenolic compound present in pomegranate, can present neuroprotective properties [24], attenuating neuroinflammation induced by LPS endotoxin [25]. Our research group developed a functional beverage containing a probiotic strain and pomegranate juice. The product was evaluated for high-intensity acute exercise in Wistar rats, for which performance did not improve, but relative abundance of *Lactobacillus* species was maintained compared to the control group [2].

This study aimed to evaluate the effects of supplementation of fermented probiotic milk with pomegranate juice on the microbiota-gut-brain axis of Wistar-Kyoto rats, with emphasis on their sleep patterns and behavior.

Methodology

In this study, 60-day-old Wistar-Kyoto rats from the Animal Research Center of UNICAMP – Multidisciplinary Center for Biological Research (CEMIB) – were used. During the entire study, the animals were kept in air-conditioned rooms (22–24°C) with a constant light–dark cycle (12/12 h), with food and free access to water, where they shared cages in groups of three. All procedures were carried out following the regulations of the National Council of Animal Experimentation (CONCEA) and after approval by the Ethics Committee for Animal Experimentation at the University of Campinas – UNICAMP, Brazil (4946-1/2018-06/18/18; 5261-1/2019-07/31/19).

Experimental design

This study was divided into two experiments:

1° experiment (behavioral analysis):
The first aimed to analyze, by the open field method, the behavior of the animals via the effects caused by supplementation of fermented milk containing or not *Bifidobacterium animalis* subsp. *lactis* BB-12 on behavior parameters. Fermented milk without the probiotic was used as control to isolate the effect of the strain. Pomegranate juice and other ingredients (except the probiotic) were included in the placebo. To do this, 20 male Wistar-Kyoto rats were used. They were distributed into two groups of 10 animals: (1) BB- (control group), which was given a placebo fermented milk supplementation without probiotic and (2) BB + 1 (supplemented group), which was administered fermented milk with *Bifidobacterium animalis* subsp. *lactis* BB-12. The rats were allocated to the cages in groups of three.

At 60 days of life, the animals began their process of adaptation to gavage with water, and to the open field arena so they could acclimate to the supplementation and place of analysis. Gavage consists of an orogastric feeding method, through a rigid cannula with a ball at the end, connected to a syringe, which is placed carefully into the oral cavity, passing through the esophagus, and reaching the animal's stomach, ensuring the correct dosage of the supplement, and the adaptation to this method consisted of administering water by gavage once a day, for five days, to minimize stress from the novelty of the procedure. The supplemented daily volume was 2 mL per rat, per day, five days a week. After seven days, our first behavior analysis was performed, which we called baseline because it was performed before the start of supplementation. From the 70th day of life of these animals, the supplementation of both groups was started, for 30 days, performed by gavage, from Monday to Friday, between 12:00 and 14:00pm, at 2 mL per day. During supplementation, three behavioral analyses were performed: the first (1st M) after 10 days of supplementation; the second (2nd M), after 20 days; and the third (3rd M), after 30 days. After the end of the experiment, the rats were individually euthanized by deep sedation and decapitation.

2° Experiment (sleep analysis):
The second experiment of this study aimed to evaluate the effect of supplementation of the probiotic fermented milk containing pomegranate juice on the sleep patterns and recovery after subjection to stress. In this case, the control was water so the effect of the functional beverage with all ingredients could be assessed. For this, 16 male Wistar-Kyoto rats were used. The animals were divided into two groups with eight animals each: (1) H2O (control group), to which gavage with water was given, and (2) BB + 2 (supplemented group), which was given fermented milk with a probiotic containing *B. animalis*. Before surgery for electrode implantation (described below in Experimental Procedures), the rats were allocated to cages in groups of three, regardless of the experimental groups and, after surgery, they were placed in individual cages.

At 60 days of age, the animals of both groups began to adapt to gavage with water and our supplementation method. At 70 days, supplementation began and the supplemented group was given 2 mL of fermented milk containing the probiotic culture *B. animalis,* whereas the control group was given 2 mL of water. At 90 days of life, electrodes were surgically implanted. No other experimental interventions were performed for seven days so they could recover, except for gavage. After recovery, at 97 days of life, baseline polysomnography of the animals was performed for 24 h, followed by the collection of the first stool sample for the bacterial

quantification. At 98 days of age, the animals were deprived of REM sleep for 48 h, after which the second stool sample was collected. On their 100th day of life, a final 48-hour polysomnography was performed, followed by euthanasia and tissue collection.

Figure 1 illustrates our experimental timeline.

Experimental procedures

Preparation of the fermented milk

In the behavioral analysis (1° experiment), two formulations of fermented milk were prepared: the first was supplemented with the probiotic *Bifidobacterium animalis* subsp. *lactis* BB-12 and used to the rats in the supplemented group (BB + 1), and the second was elaborated without the addition of the probiotic and was used for the rats in the control group (BB-).

In the sleep analysis (2° experiment), the probiotic formulation was the same used in experiment 1, but the placebo fermented milk formulation was not used and the rats of the control group (H2O) only received water.

The ingredients used to prepare the fermented probiotic milk and control for each experiment are described in Table 1.

Behavioral assessment (1° experiment)

Open field. The open field test is an exploratory behavior assessment test designed to emotionally evaluate rodents. It easily assesses well-defined behaviors. It consists of an area surrounded by opaque walls which stand tall enough to prevent the animals from escaping. The open field is usually circular or square and proportional in size to the tested subjects to provoke a feeling of openness in the center of the arena [26].

In this study, the open field test was used to evaluate the possible effect of supplementation with fermented milk containing the probiotic *B. animalis* on animal behavior. For this, each animal was placed individually in the center of the open field test (81 cm circular arena, 41 cm high walls, with open top and bottom divided into 12 quadrants, of which 4 are in the center of the arena and 8 are on the periphery, close to the walls), where they remained for 10 min. Only animals in their final five minutes of exposure were considered.

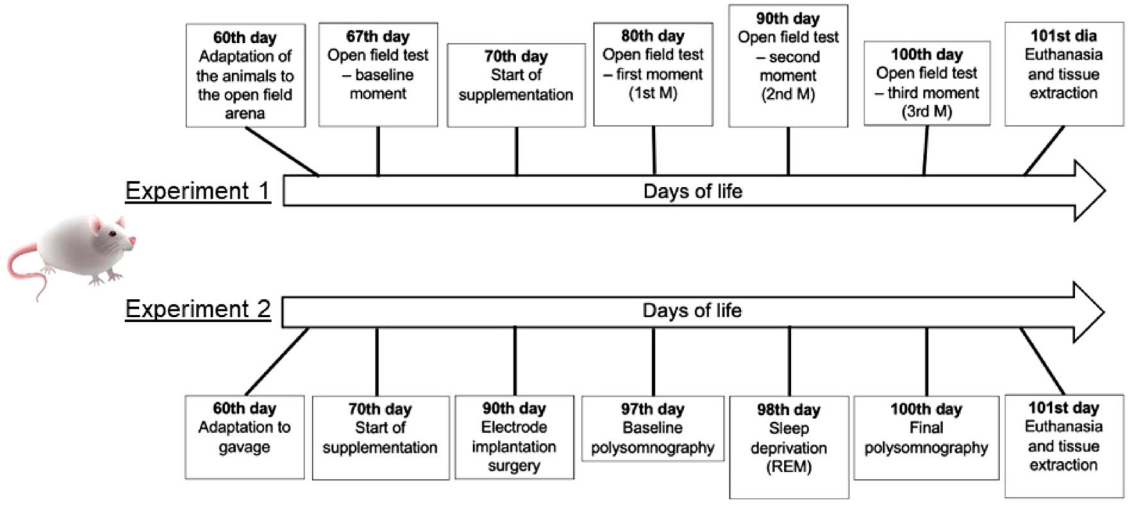

Figure 1. Experimental design.

Table 1. Ingredients used to prepare the fermented probiotic milk and control for each experiment.

Ingredients	Fermented probiotic milk (treatment)	Control
1° experiment	(BB + 1) Maltodextrin, fructose, powdered milk, whey protein (80% protein), potassium sorbate, pasteurized pomegranate juice, food coloring, *Streptococcus thermophilus* (starter culture) and *Bifidobacterium animalis* subsp. *lactis* BB-12 (probiotic).	(BB-) Maltodextrin, fructose, powdered milk, whey protein (80% protein), potassium sorbate, pasteurized pomegranate juice, food coloring, *Streptococcus thermophilus* (starter culture).
2° experiment	(BB + 2) Maltodextrin, fructose, powdered milk, whey protein (80% protein), potassium sorbate, pasteurized pomegranate juice, food coloring, *Streptococcus thermophilus* (starter culture) and *Bifidobacterium animalis* subsp. *lactis* BB-12 (probiotic)	(H2O) Water

At the end of each test, the arena was cleaned with 70% alcohol and dried with a paper towel.

The evaluated parameters were: total ambulation (total number of entries in each quadrant that the animal covered in the arena), central ambulation (number of entries in the quadrants covered in the center of the arena), peripheral ambulation (number of entries in quadrants covered in the periphery of the arena, that is, close to the walls), rearing (behavior in which the animals stand on their hind legs), total duration of grooming (self-grooming behavior) and total freezing time (behavior in which the animal is completely immobile, except for movements corresponding to breathing). All tests were performed four times from 16:00–18:00pm (light cycle) before supplementation (baseline) was started and at 10-day intervals after the start of supplementation (10 days – 1st M, 20 days – 2nd M, and 30 days – 3rd M).

Sleep assessment (2° experiment)

Surgery for electrode implantation. Electrode implantation surgery to evaluate the brain activity of the study animals was performed on their 90th day of life, according to the protocol described by Franco et al. [27]. Initially, all animals were anesthetized (2% isoflurane), their upper head was shaved, and placed in a stereotaxic device (David KopfTM). Stereotactic coordinates were plotted: In all, four stainless steel screws 152 (∅ 1.0 mm) were carefully fixed, just touching the dura mater (1.0 mm deep). A copper wire was attached to each of the 4 screws to record cortical electrical and formed two pairs of ipsilateral cortical long bipolar electrodes, to record cortical electrical activity (CEA). One of the pairs of screws was placed laterally to the sagittal plane of the upper head of the animals to record a minimum theta activity (1 mm posterior to the bregma, 3 mm lateral to the central suture, 1 mm anterior to the lambda and 4 mm lateral to the central suture) and the other was placed medial to the sagittal plane of the skull to record maximal theta activity (3 mm anterior to bregma, 1 mm lateral to central suture, 4 mm anterior to lambda, and 1 mm lateral to central suture). These were used for a better characterization of sleep phases, especially REM sleep. A pair of electrodes (copper wires) was also implanted in the dorsal neck muscle (trapezius) of the animals in our sample for electromyographic analysis [27,28].

After electrode implantation, a connector was fixed with a self-curing dental acrylic adhesive to the skull of the rats in our sample [27].

The rats were given 10 mg/kg diclofenac sodium for postoperative analgesia and to reduce surgical wound inflammation. They were taken to their cages and free access to water and food was provided for another seven days before any other experimental intervention was conducted [27].

Polysomnography. Polysomnography was evaluated at two moments: baseline (before deprivation), which occurred 7 days after recovery from the electrode implantation surgery, and the final moment (after deprivation), which occurred shortly after the 48 h sleep deprivation. The baseline analysis lasted 24 h and the final analysis lasted 48 h.

Electrophysiological signals were recorded on a digital polygraph (Neurofax QP 223A ©Nihon Kohden – EEG-1200 model) at a sampling rate of 200 Hz, using the following three channels: two for CEA and one for the electromyographic analysis of the cervical musculature in the sample. Raw signals were amplified of 125*x* and filtered with a 0.53 Hz high pass filter and 70 Hz low pass filter (for EEG) and a 5.3 Hz high pass and 35 Hz low pass filter (for EMG). Analysis was based on the predominant amplitude and frequency of the tracking, and periods of 10 s were considered, these classified according to their dominant state (i.e. awakening, slow-wave sleep, or paradoxical sleep) [29].

The analysis of sleep records was divided into two periods of 12 h (light/dark). The following sleep parameters were evaluated: total sleep time (TST), sleep efficiency (SE – percentage of total sleep time during data collection), wakefulness (W – percentage of all awake periods over data collection, characterized by fast desynchronized low-voltage cortical signals associated with high-voltage muscle signals), slow-wave sleep (SWS – percentage of all periods with little high voltage activity in the delta range (0.75–4.0 Hz) with very low muscle signals), and paradoxical sleep (REM – percentage of all paradoxical sleep periods during data collection, analyzed by desynchronized low voltage cortical signals in the theta range (4.0–8.0 Hz)). Interference was considered when the recorded waves did not fit the patterns determined for any sleep parameter. All records were analyzed by a single researcher, which were blindly and manually analyzed using the Rem Logic software programs. The EEG/EMG data in periods including artificial/electrical noise were excluded from analysis after checking the recording.

REM sleep deprivation

Stress induction was performed at 98 days of life after the animals had recovered from surgery and baseline polysomnography analysis. Stress was caused by paradoxical sleep deprivation (REM) for 48 h using the single-platform method. The rats were housed in a

container/box (22.0 cm long × 22.0 cm wide × 35.0 cm high) with water (a safe amount for the animals not to drown if they fell in). Platforms with a diameter of 6.5–7.0 cm were placed inside the container, remaining immersed in water up to 1 cm from the edge. The animals remained on the platforms, and when they would fall asleep, muscle atony would cause them to fall into the water and consequently wake them up. All animals were habituated and acclimated to this sleep deprivation room and its experimental environments [30].

Bacterial quantification

qPCR. Total bacteria and *Bifidobacterium* concentration in the feces of the animals in the studied sample were analyzed by the quantitative PCR method (qPCR). Feces were collected only in the second stage of the experiment at two time points: before and after REM sleep deprivation.

To quantify total bacteria and *Bifidobacterium* count in the feces of the animals, the DNA present in the sample was initially extracted using the QIAamp™ PowerFecal™ DNA Kit (QIAGEN Group) and quantified by Nanodrop, adding a mixture of Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific), primers (Forward and Reverse), and endonuclease-free ultrapure water (DEPC-treated). The primers used in the analysis are described in Table 2 [31,32]. Readings were taken by the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). *Bifidobacterium* spp. primers and total bacteria were used to amplify DNA. *Lactobacillus* spp. [33] was employed as a negative control of the reaction.

Analysis of results

General linear models were used to test differences and interaction between groups and time. Interactions were analyzed to observe the effect of supplementation and dependent variables were treated as linear scales. First, all data were analyzed for normality using the QQ plot and Shapiro–Wilk test. Equal variances (homoscedasticity) were determined using Levene's test. The open field test was analyzed considering group (BB- vs BB + 1) and time (baseline, 1st M, 2nd M, and 3rd M) as fixed effects. Sleep variables were assessed considering group (H2O vs BB + 2) and time (before and after sleep deprivation (on day 1 and day 2)). The cycle (before sleep deprivation: light and dark, day 1: light and dark, and day 2: light and dark) was considered as a fixed effect. The expression of *Bifidobacterium* spp. (genome copy number) and total bacteria (amount) by real-time PCR was examined for the groups (H2O vs BB + 2) before and after sleep deprivation. Sidak post hoc tests were used for multiple comparisons since the data had a normal distribution and homoscedasticity. A paired *t*-test was used to observe in-group differences (before and after sleep deprivation). The significance level was assumed to be $p < 0.05$ and data were expressed as means and standard deviations or minimum and maximum values. The results were analyzed using Statistica (version 7.0, StatSoft Inc, Tulsa, OK, USA) and SPSS Statistics (version 25 IBM corporation).

Results

The results obtained in the study were divided into three sections: behavior, sleep, and quantification of bacteria by qPCR.

Behavior (1° experiment)

Among the health benefits promoted by probiotics, the effect on the gut-brain axis must be proven individually for each strain so, in this study, we chose to isolate the effect of supplementation of *B. animalis* BB-12 on the behavior of the animals, with effects proven over all behavior parameters analyzed, as described below.

In the open field test, we used the linear mixed model to analyze central ambulation, peripheral ambulation, total ambulation, rearing, grooming, and freezing. Our analysis compared the two groups (with and without supplementation), time (baseline, 1st M, 2nd M, and 3rd M) and group × time. We log-transformed the data to statistically analyze the freezing parameter.

We found that probiotic supplementation influenced all behavior analyses, increasing central ambulation (Figure 2(A), $F(1.72) = 13.33$, $p = 0.001$), peripheral ambulation (Figure 2(B), $F(1.72) = 5.65$, $p = 0.028$), total ambulation (Figure 2(C), $F(1.72) = 8.53$, $p = 0.009$), rearing (Figure 2(D), $F(1.72) = 4.63$, $p = 0.045$),

Table 2. Sequences of primers used in the qPCR analysis.

Target	Oligonucleotide sequences 5′ – 3′	Annealing temperature (°C)	Base pairs (bp)	References
Total bacteria count	F- 5′-ACTCCTACGGGAGGCAGCAG-3′ R- 5′-ATTACCGCGGCTGCTGG-3′	60	200	[32]
Bifidobacterium spp.	F- 5′-TCGCGTC(C/T)GGTGTGAAAG-3′ R- 5′-CCACATCCAGC(A/G)TCCAC-3′	60	243	[31]

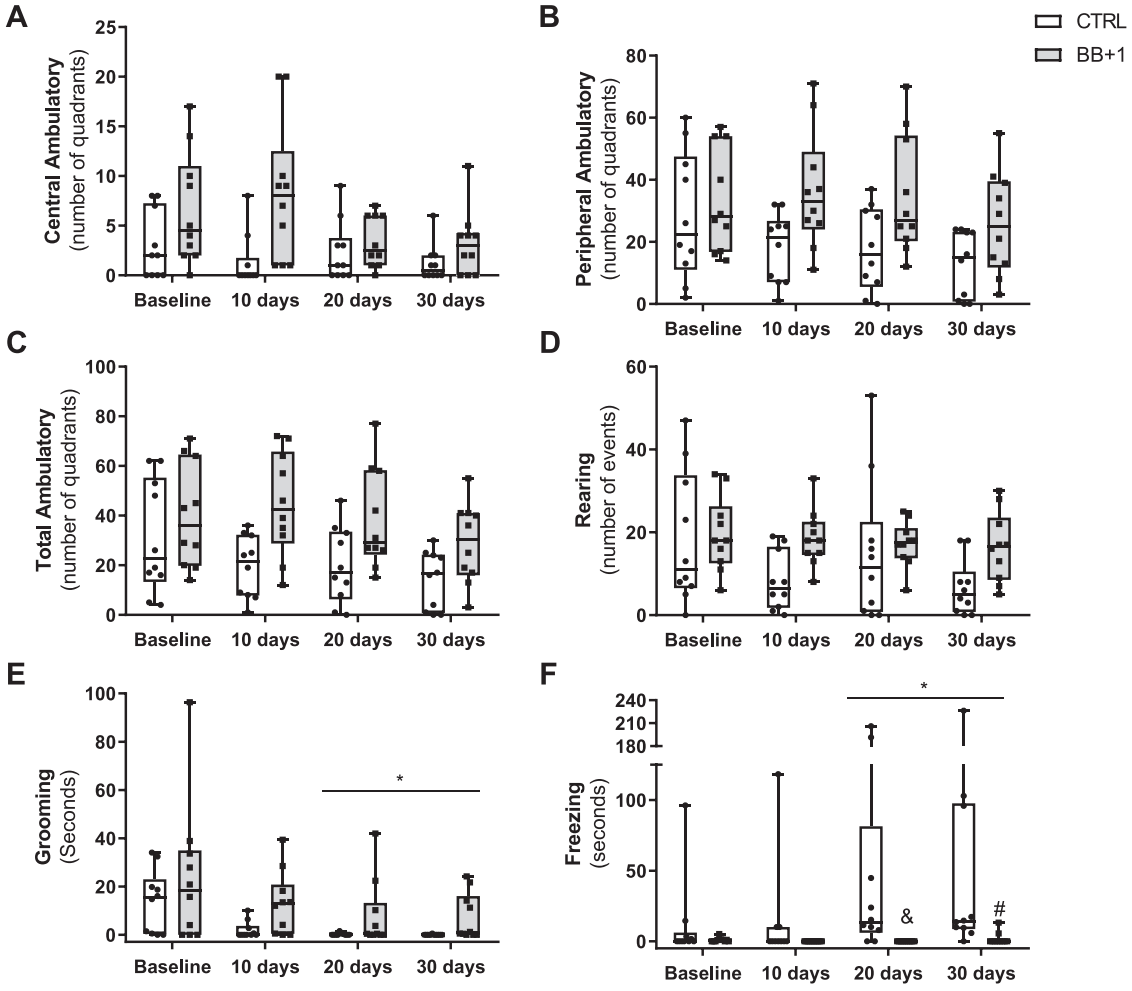

Figure 2. Open field test performed at 9–12am (light cycle) at the following moments: baseline, 1st M (10 days), 2nd M (20 days), and 3rd M (30 days). (A) Central ambulatory (number of quadrants); (B) Peripheral ambulatory (number of quadrants); (C) Total ambulatory (number of quadrants); (D) Rearing (events); (E) Grooming (seconds); (F) Freezing (seconds). All tests (A, B, C, D, E, and F) showed a significant difference between groups. We show each parameter as individual values, means, and minimum and maximum results (n = 10). We analyzed the data by mixed models with the Sidak post-hoc test ($p < 0.05$). *Difference for time from baseline; and & and # difference for group × time (in 2nd M and 3rd M from CTRL).

grooming (Figure 2(E), $F(1.72) = 6.24$, $p = 0.018$), and reduced freezing (Fig XE, $F(1.72) = 17.39$, $p = 0.001$) compared to the group which received no probiotic supplementation (BB + 1).

By analyzing grooming, we found a difference for time (Figure 2(E), $F(3.72) = 4.64$, $p = 0.010$), with a lower 2nd M and 3rd M than the baseline (2nd M $p = 0.027$; 3rd M $p = 0.018$; Sidak post hoc).

The freezing variable also showed differences for time (Figure 2(F), $F(3.72) = 7.87$, $p < 0.001$), with an increase in 2nd M and 3rd M compared to baseline (2nd M $p = 0.047$; 3rd M $p = 0.006$; Sidak post hoc) and to 1st M (2nd M $p = 0.046$; 3rd M $p = 0.001$; Sidak post hoc). Moreover, we found that freezing differed in the group x time interaction ($F(3.72) = 6.05$, $p = 0.002$), with longer freezing times in the BB- group at 2nd M than in the BB + 1 2nd M ($p < 0.001$, Sidak post hoc) and in BB- 3rd M than in the BB + 1 3rd M ($p < 0.001$, Sidak post hoc).

Sleep (2° experiment)

In addition to the probiotic culture, other components of the functional beverage developed by our research group, such as the pomegranate juice, may have effects on the central nervous system (added to increased antioxidant activity), which is compounded by the possible presence of bioactive peptides released during fermentation (some with opioid properties). Therefore, the entire fermented milk solution (and not just the probiotic) was evaluated in the sleep deprivation test.

We evaluated the sleep of the animals before deprivation (at baseline) and for 48 h after sleep deprivation, divided into 24 h (day 1 and day 2). In the first moment,

we analyzed sleep records in light and dark cycles separately, and the variables assessed were total sleep time (TST), sleep efficiency, number of arousals, slow-wave sleep (SWS), REM sleep, and wakefulness. We performed this analysis by comparing groups (with and without supplementation), time (baseline, 24, and 48 h), and the group x time interaction.

When analyzing the light cycle for a group, we found a significant difference in slow-wave sleep (Figure 3(D), $F(1.36) = 5.81$, $p = 0.030$), with an increase in SWS time for the group with fermented milk / BB + 2 ($p = 0.030$; Sidak post hoc). REM sleep differed for time (Figure 3(E), $F(2.36) = 5.72$, $p = 0.014$), increasing after deprivation (day 1 and day 2), compared to the baseline (day 1 $p = 0.021$; day 2 $p = 0.024$; Sidak post hoc). We found no difference for the interaction between group and time.

In the dark cycle of the sleep records, we found a significant difference in the parameters of sleep efficiency (Figure 4(B), $F(2.37) = 5.32$, $p = 0.013$), number of arousals (Figure 4(C), $F(2.37) = 9.08$, $p = 0.005$), REM sleep (Figure 4(E), $F(2.37) = 16.00$, $p < 0.001$), and wakefulness (Figure 4(F), $F(2.37) = 5.21$, $p = 0.014$) when we analyzed our mixed model for time. The sleep efficiency of the animals increased in day 1, in relation to the baseline ($p = 0.013$; Sidak post hoc), whereas, on day 1, wakefulness decreased in relation to the baseline ($p = 0.014$; Sidak post hoc). REM sleep increased on day 1 and day 2 compared to the baseline (day 1, $p < 0.001$; day 2, $p = 0.016$; Sidak post hoc), whereas the number of arousals decreased on day 1 and day 2, compared to baseline (day 1), ($p = 0.007$; day 2, $p = 0.005$; Sidak post hoc). We found no

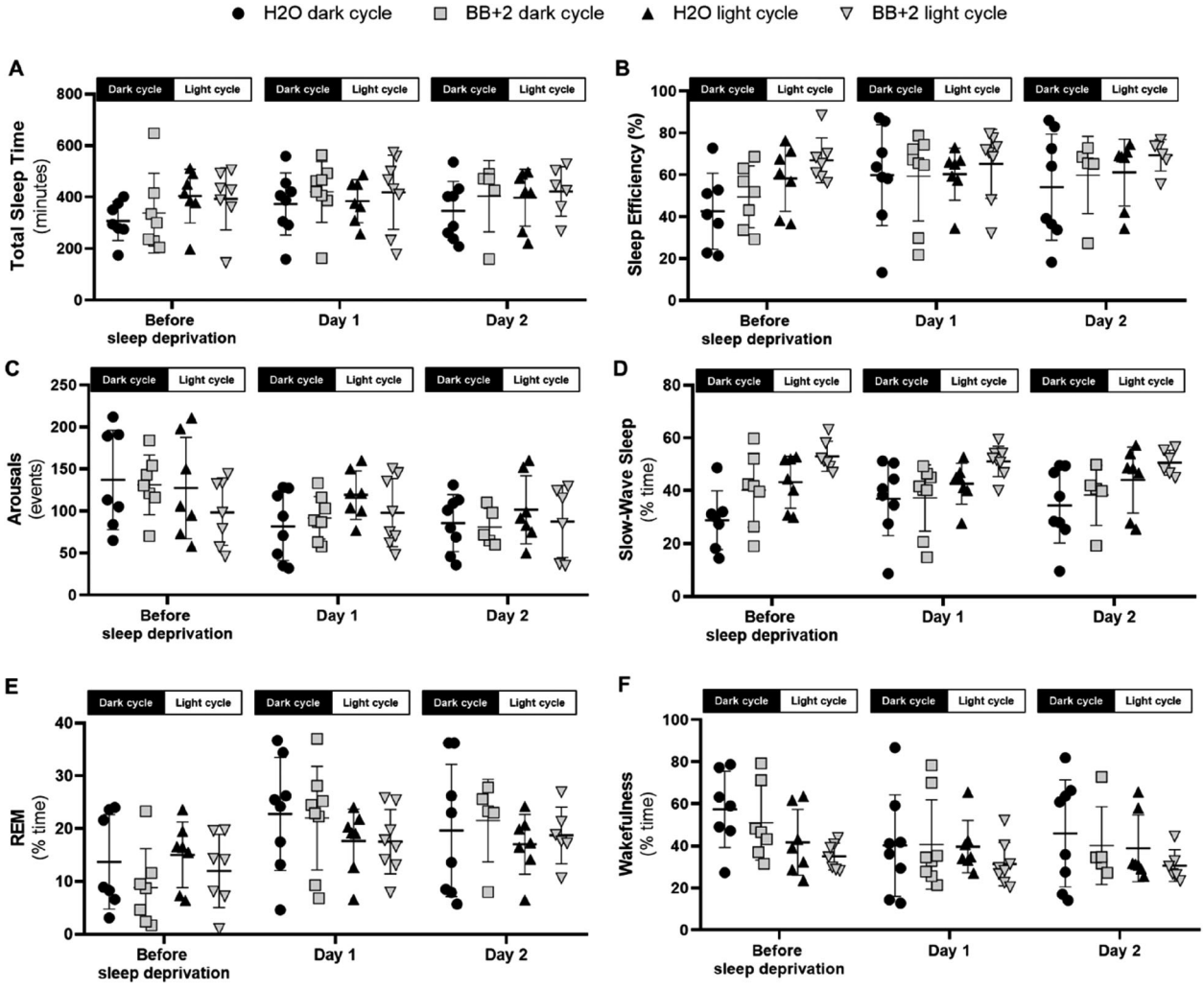

Figure 3. Sleep record (12-h dark cycle and 12-h light cycle): before sleep deprivation and after sleep deprivation (Day 1 and Day 2). **(A)** Total sleep time (min); **(B)** sleep efficiency (%); **(C)** arousals (events); **(D)** slow-wave sleep (% time), with a significant increase in the supplementation group; **(E)** REM sleep (% time); and **(F)** wakefulness (% time). We show each parameter as individual values, means, and standard deviations ($n = 6$–8). We analyzed data by mixed models and the Sidak post hoc test ($p < 0.05$); a differs from b; c differs from d.

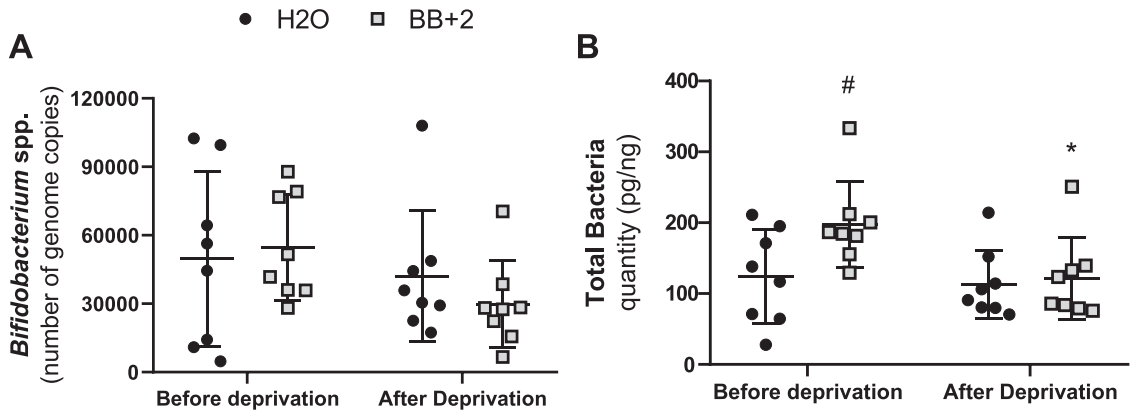

Figure 4. Quantification of bacteria in the feces of our sample. (A) *Bifidobacterium* spp. (number of genome copies); and (B) total bacteria count (pg/ng), shown as individual values, means, and standard deviations ($n = 8$). We analyzed data via the Student's paired *t*-test and two-way ANOVA ($p < 0.05$). *Difference in the intervention group between time; and # difference between groups at the same time.

difference between groups or for the interaction between group and time.

In a second moment, we analyzed the cycles together to understand sleep replacement. The group variable had no effect in this analysis.

Before deprivation, the light cycle differed from the dark one in sleep efficiency ($F(5.73) = 5.76$, $p < 0.001$; increased light cycle $p = 0.003$; Sidak post hoc), slow-wave sleep ($F(5.73) = 7.85$, $p < 0.001$; increased light cycle $p = 0.001$; Sidak post hoc), and wakefulness ($F(5.73) = 6.49$, $p < 0.001$; reduced light cycle $p = 0.004$; Sidak post hoc). However, after deprivation, analysis of day 1 (light x dark) and day 2 (light x dark) showed a difference only for slow-wave sleep on day 2, with longer ones in the light cycle than in the dark cycle ($p = 0.036$; Sidak post hoc).

The supplementary material shows detailed readings of the differences between sleep times.

Bacteria quantification by qPCR

We evaluated the expression of *Bifidobacterium* spp. (i.e. number of genome copies) and total bacteria (quantity) using real-time PCR (Figure 4(A and B), respectively). We failed to find any differences between groups (with and without supplementation) and, for the number of genome copies *Bifidobacterium* spp. over time ($p = 0.35$; $p = 0.65$, respectively). We found an increase ($p = 0.03$) in total bacteria count between groups (with and without supplementation) before sleep deprivation and a decrease ($p = 0.04$) in the BB + 2 group after sleep deprivation.

Regarding the genus *Bifidobacterium*, comparing the H2O group with the BB + 2 group, soon after the supplementation period and before sleep deprivation, we observed a slight increase in the number of genome copies for this genus but without a significant difference between the groups ($p = 0.75$). Before and after SD, we observed no differences between control animals and those supplemented with the probiotic beverage. In relation to total bacteria count, after supplementing the animals with the probiotic beverage, we observed a significant increase ($p = 0.03$) in the number of total bacteria (from 1245.54 to 1980.45 ng). However, sleep restrictions reduced their numbers in the supplemented group to a value like that in the control one / H2O (1214.5 and 1134.7 ng, respectively), showing that supplementation was unable to mitigate this effect.

Discussion

Considering the promising results regarding the interaction of the microbiota-gut-brain axis, and the importance of probiotics in this interaction, this study aims to evaluate the effect of supplementation with fermented milk containing probiotics and pomegranate juice on the central nervous system, focusing on behavioral and sleep patterns.

Behavior

Studies show that some behavioral changes are typically related to anxious animals. Among these variables, the reduction of central ambulation and increased defecation and freezing time stand out [26,34,35].

Rodents have a common behavior called thigmotaxis, which close to walls, which in turn is linked to the propensity of rodents to avoid open spaces [36,37]. Though common in these animals, this behavior tends to increase when anxiety levels rise, so its increase can be

directly associated to anxiety [26]. This statement is corroborated by a study that compared the behavior of rats that received anxiolytic and anxiogenic drugs, with an increase in the central ambulation of the animals that received the anxiolytic drugs [38], as well as in another study that evaluated the treatment with the anxiolytic Diazepam in the behavior of animals subjected to early deprivation (isolation in the cages), also finding an increase in central ambulation in the group that received treatment with anxiolytic [39]. The results of central ambulation in this study indicate that, despite the individual differences that led to the initial dissimilarity in central ambulation between groups, supplementation prevented a more robust decline of this behavior in animals from the SUPP group.

Regarding peripheral ambulation and total ambulation, the animals in the BB- group showed a decrease in both parameters in relation to the Baseline analysis, but without statistical significance. The decrease in these parameters may be an indicator of anxiety since it is described that anxious animals have low locomotor activity [35,40,41].

Rearing is generally described as a common exploratory behavior in rats that is not necessarily related to anxiety. As shown in Figure 2(D), in this study, we saw the occurrence of a large variation in the number of rearing events in the BB- group, while the BB + 1 group showed less variation, keeping the number of events similar over the 30 days of the experiment. There is still some divergence in the interpretations of rearing, and some authors consider that an increase in this behavior may also be related to anxiety resulting from an unfamiliar environment. However, as the literature has not been able to isolate interpretations, an evaluation is necessary according to the context and in conjunction with other open field variables [26, 42].

Finally, the animals were evaluated in relation to freezing time (Figure 2(F)). It is described in the literature that emotional and more anxious animals tend to have an increase in this behavior [34,35].

In this study, we saw that supplementation with fermented milk containing the probiotic *B. animalis* showed a probable protective effect, preventing increased anxious behavior since it helped maintain the baseline freezing time, whereas the BB- group tended to increase this behavior throughout the intervention. Several preclinical studies support this effect, showing a similar effect in animals supplemented with probiotic strains. A meta-analysis using preclinical studies showed that supplementing animals with probiotics significantly reduced anxiety-related behaviors and this reduction was independent of sample size, supplementation time, and probiotic dosage [43].

We emphasize that, in the present study, the open field test was performed several times, which may cause effects on test repetitions [44]. Therefore, the results referring to time may be due to different exposures to the test. And yet, we cannot rule out that different groups may have responded differently to these repetitions.

Sleep

The literature currently knows that sleep quality is an important component for body homeostasis, and some preclinical studies with rats have shown that sleep deprivation has several consequences for the body, such as decreased serum leptin, increased serum levels of TNF-α, corticosterone, and lipopolysaccharide, as well as increased intestinal permeability, oxidative stress, reduced levels of short-chain fatty acids, changes in the intestinal microbiota, and changes in sleep architecture, such as REM rebound [45,46].

We also observed that sleep deprivation caused an increase in REM sleep, and we also observed a significant increase in the sleep efficiency of both groups in the first dark cycle after deprivation, as well as a significant reduction in the number of awakenings and a reduction in wakefulness, which corroborates with the results from a study about total sleep deprivation in mice [46].

Dispersyn et al. [46] also mention that REM rebound occurs in two stages, respecting the circadian cycle, as observed in the data found in our evaluated mice. However, in this study, we noticed that REM rebound of the rats in our experiment did not depend on their circadian cycle; i.e. we clearly observed that there was a significant increase in REM sleep in the dark cycle of their sleep on both Day 1 and Day 2. This increase was even more expressive than that in the clear cycle of both groups after deprivation, which may be explained by the difference in species or deprivation time, considering that this study used a 48-hour paradoxical sleep deprivation, whereas Dispersyn et al. [46], a 24 h one, or, perhaps, because our dark cycle of Day 1 was the first recovery cycle after 48 h of deprivation [46].

Though we observed no influence of the probiotic fermented milk on improving sleep patterns after deprivation, when we observed the baseline, we found that supplemented animals had a greater sleep efficiency and a lower number of arousals than the animals in the control group, as in Graphs B and C of Figure 3. Such data point to a possible effect of fermented milk containing *B. animalis* and pomegranate juice on normal sleep, though it failed to influence typical sleep changes after deprivation.

In addition to its influence on sleep efficiency and number of arousals, we also found that, at baseline, the supplemented animals had an increase in their percentage of slow-wave sleep than the control group, although this was a statistically insignificant difference, with this increase perpetuating the Day 1 light cycle after sleep deprivation. These data bring an interesting discussion, as stated by Kim and Dimsdale [47] in their systematic review evaluating the effect of stressors on polysomnographic measures of sleep. They found, among other changes, a reduction in slow-wave sleep after subjection to experimental psychosocial stress [47]. Though this study worked with another type of stressor (in this case, sleep deprivation), such data show that the microbiota modulation of the rats may have attenuated the stress caused by deprivation, reflected in this higher (though statistically insignificant) percentage of slow-wave sleep in the supplemented rats.

Moreover, Herman [48] suggests that probiotic supplementation can contribute to maintaining intestinal permeability, favoring the expression of GABA receptors in the central nervous system and the synthesis of tryptophan and serotonin (melatonin precursors) in the gastrointestinal tract [48]. Hadizadeh et al. [49] suggested that probiotic supplementation may contribute to brain functions, which are linked to the microbiota-gut-brain axis [49]. Thus, there may be a relation between probiotic supplementation and the improvement of SWS in the SUPP group.

Bacteria quantification by qPCR

Recent studies have shown that sleep disorders can alter the composition of gut microbiota [50–52]. The literature described the association of these events with dysbiosis and, consequently, with a series of metabolic diseases, such as obesity; diabetes; and cardiovascular, neurological, and cognitive disorders [53]. Thus, administering probiotic microorganisms and prebiotic ingredients seems to be a beneficial alternative for promoting sleep quality and general health [54].

In our study, we observed a small reduction in the population of *Bifidobacterium* spp. in both groups after sleep restrictions. Contrary to expectations, after supplementing the animals with the probiotic beverage, the increase in the population of the *Bifidobacterium* genus failed to achieve statistical significance. Poroyko et al. [55] observed significant changes in the composition of the intestinal microbiota of rats after a long period of sleep deprivation (four weeks). The authors found an increase in highly fermentative families, such as Lachnospiraceae and Ruminococcaceae, and a decrease in the Lactobacilaceae and Bifidobacteriaceae families. for the researchers, however, it is unclear whether the observed changes in the composition of microbiota were due to sleep deprivation or increased food intake (and consequently increased adiposity).

Zhang et al. [56] observed a decrease in the richness of a single operational taxonomic unit (TM7a-3) in the intestinal microbiota of rats after seven days of sleep deprivation, whereas they maintained the proportion of other bacterial populations. El Aidy et al. [57] evaluated the impact of five hours of sleep deprivation on the composition of the intestinal microbiota of rats. The authors found that this period was insufficient to promote major changes in the composition of intestinal microbiota, but it caused subtle changes in the relative abundance of the Clostridiaceae and Lachnospiraceae families in the animals tested. Notably, the studies mentioned above did not include any type of supplementation with probiotics.

The findings regarding this subject are still scarce and divergent. According to the studies cited above, it is evident that short-term sleep deprivation induces subtle effects on the intestinal microbiota. Zhang et al. [56] proposed that the composition of the gut microbiota seems to be more affected by long-term sleep quality than by acute periods of sleep deprivation. This study used a 48-hour sleep deprivation protocol, which may have been insufficient to detect possible changes in the population of the analyzed sexes. Several factors may also explain the different outcomes found in the mentioned studies, such as duration of sleep deprivation, sample size, stool collection, and intervention methods. The literature therefore needs further studies to clarify how probiotic supplementation can contribute to altering the composition of the gut microbiota during periods of sleep deprivation.

In this study, we did not evaluate the genomic sequencing of intestinal microbiota to enable a more detailed interpretation of the effect of supplementation with the probiotic beverage, as well as the effect of sleep restriction on the relative abundance of different bacterial taxa, but this analysis may be useful for subsequent research steps.

Thus, this present study was not without limitations. The first limitation to be mentioned is that not only the probiotic strain B. animalis BB-12 but other ingredients present in the fermented probiotic milk could be responsible for the observed outcomes, such as milk proteins, maltodextrin, fructose, potassium sorbate and the coloring additive. The study of each of the ingredients individually would require many experimental groups. However, since our group wanted to develop a product that would meet consumer

expectations (improving sensory properties) and appropriate preservation (adding a food preservative), evaluating the beverage with all ingredients seemed more favorable at this stage of the research.

Conclusions

Based on the findings of this study, we can conclude that supplementation with fermented milk containing *Bifidobacterium animalis* subsp. *lactis* BB-12 showed beneficial effects on the general behavior of rats, especially in preventing anxiety-related behavioral changes, such as increased freezing and reduced total and central ambulation. Such results are seen in other studies, which underscores that probiotics can provide benefits that go beyond improving intestinal functioning. We also observed that supplementation with probiotic fermented milk containing BB-12 and pomegranate juice had a subtle effect on normal sleep, i.e. before the stressor effect of REM sleep deprivation, thus promoting better sleep efficiency, fewer awakenings and an increase in the percentage of slow wave sleep in supplemented animals. However, we did not observe significant effects of supplementation on stress caused by sleep deprivation. On the other hand, sleep deprivation seemed to decrease the total number of bacteria in the intestinal microbiota, an effect that occurred in both groups, with supplementation not being effective in preventing these changes in the microbiota, but it did reinforce the impact of psychopathologies and other changes in psychological effects on intestinal health.

This reduction in anxious behavior, observed in this and other studies, reinforces the existing bidirectional communication between microbiota/gut and brain and proves that probiotics have beneficial effects that go beyond intestinal health, with improvement in mental health and protection or attenuation of symptoms of psychopathologies, such as anxiety. However, further studies to evaluate the effect of probiotics on behavior, sleep, and stress response are needed to reinforce these findings.

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No potential conflict of interest was reported by the author(s).

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Data availability

All data presented in the manuscript can be shared upon request to the corresponding author.

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