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Daily administration of paraprobiotic *Lactobacillus gasseri* CP2305 ameliorates chronic stress-associated symptoms in Japanese medical students

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ABSTRACT

Administration of *Lactobacillus gasseri* CP2305 for 4 weeks improved stress-associated behaviours in healthy young adults and clinical symptoms in patients with irritable bowel syndrome. The present study was designed to confirm the stress-relieving effects of heat-inactivated, washed CP2305 (paraprobiotic CP2305) on 69 sixth-year medical students (40 males and 29 females) preparing to take the national examination for medical practitioners. Administration of the paraprobiotic CP2305 for 12 weeks significantly improved sleep quality assessed by both the Pittsburgh Sleep Quality Index and a one-channel sleep electroencephalogram during the pre-examination period compared with that of the placebo administration. The paraprobiotic CP2305 administration also prevented increases in basal salivary cortisol release and expression of stress-responsive microRNAs (miR-144 and miR-144*). In addition to the improvement in parasympathetic nerve activity, the paraprobiotic CP2305 normalized the bowel habits under the stressful conditions. Based on these results, we propose that paraprobiotic CP2305 may be used as a para-psychobiotic.

1. Introduction

There is growing evidence that the bidirectional communication between the brain and the gut, called "the gut-brain axis", plays a potential role in the maintenance of the brain function as well as the gut homeostasis (Aziz & Thompson, 1998). Recently, a number of studies with experimental animals have shown that gut microbes are crucial factors for normal neurodevelopment and behaviour (Cryan & Dinan, 2012; Sampson & Mazmanian, 2015). At the same time, distinct types of microbes, such as orally administered probiotics, have been shown to modify the gut-brain axis and control the stress response (Bercik, Collins, & Verdu, 2012). These recent findings suggest that probiotics may have a therapeutic role in the management of mood disorders, such as major depression or anxiety disorders, by regulating "the microbiota-gut-brain axis" (Dinan, Stanton, & Cryan, 2013).

Psychological stress causes a negative effect on the composition of the intestinal microbiota (Suzuki, Harasawa, Yoshitake, & Mitsuoka, 1983) and enteric environment and consequently increases the risk for the development of functional disorders, such as irritable bowel syndrome (IBS) (Wood, 2002). Several lines of evidence from animal and human studies have shown that intestinal inhabitants and orally introduced external microbes could change the activities of the autonomic nervous system and modulate gut functions (Obata & Pachnis, 2016; Tougas, 2000), serotonin biosynthesis (Yano et al., 2015), and mental health status (Sampson & Mazmanian, 2015). The living microbes used for these treatments are called "psychobiotics" (Dinan et al., 2013). The use of the paraprobiotic instead of probiotic CP2305 has potential benefits for the production of manufactured products. In addition, the heat-inactivated, washed bacterial cells is a starting material for

Abbreviations: ANOVA, analysis of variance; ANCOVA, analysis of covariance; BSS, Bristol Stool Scale; CNS, central nervous system; EEG, electroencephalograph; GHQ, General Health Questionnaire; HADS, Hospital Anxiety and Depression Scale; IBS, irritable bowel syndrome; LAB, lactic acid bacteria; miRNA, micro-ribonucleic acids; PSQI, Pittsburgh Sleep Quality Index; QOL, quality of life; STAI, State Trait Anxiety Inventory.

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identification of the active ingredient (s). However, the use of non-living, inactivated beneficial microbe(s) for this purpose has not yet been reported.

We originally isolated a unique *Lactobacillus gasseri* strain CP2305 (CP2305) from stool samples of a healthy volunteer (Sawada et al., 2016). CP2305 colonized the digestive tracts of approximately 40% of volunteers after 3-time oral administration at a dose of 1.0×10^{11} CFU (Sawada et al., 2016). Recently, we reported that CP2305 could relieve stress-associated behaviours in healthy young adults (Sawada et al., 2017) and improve clinical symptoms of IBS (Nobutani et al., 2017). IBS symptoms are closely associated with psychosocial stress. Subsequently, we showed that heat-inactivated CP2305 improved stool properties and bowel habits and balanced the autonomic nervous activity of healthy volunteers (Sugawara et al., 2016).

Based on these findings, this study was designed to assess paraprobiotic CP2305 as a stress-relieving para-psychobiotic. For this purpose, we enrolled 69 six-year undergraduate medical students preparing for the national examination for medical practitioners. We conducted a double-blinded, placebo-controlled, parallel-group trial. The participants daily took paraprobiotic CP2305 or placebo for 12 weeks during the pre-examination period. This period has a significant impact on psychological state of medical students and represents a suitable model for the analysis of chronic psychological stress (Honda et al., 2013; Kurokawa et al., 2011). In addition to psychological measures, we assessed biological stress responses by measuring basal salivary cortisol levels, sleep electroencephalogram (EEG), autonomic nervous activities, and stress-responsive microRNAs in circulating leukocytes.

2. Materials and methods

2.1. Study design

The present study was approved by the Institutional Review Board of Tokushima University Hospital, Tokushima, Japan. Informed consent was also certified by this committee. This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. We recruited 69 sixth-year undergraduate medical students (40 males and 29 females with a mean age of 25.0 years) at Tokushima University in Tokushima, Japan. After the experimental procedures were fully explained, written informed consent was obtained from all participants. All students were in good physical health, taking no medication for at least three months prior to study enrollment and during the experimental period, and had no history of psychiatric or somatic diseases, either past or present. Female students were not taking hormonal contraceptives. All subjects were nonsmokers. None of the students had allergies to milk or other foods.

The clinical trial was performed from November 2nd, 2015, to February, 5th, 2016. The students were in the preparation period for the national examination for medical practitioners (Fig. 1). The students were randomly divided into 2 groups (Table 1). Participants allocated to group 1 (n = 35; m/f = 20/15) received the placebo beverage once per day throughout the 12-week manipulation period. Participants allocated to group 2 (n = 34; m/f = 20/14, Table 1) consumed the beverage containing the paraprobiotic CP2305 in the same manner. Daily consumption was self-recorded in a diary to assess the compliance rate of the participants. During the trial, the subjects complied with dietary restrictions to avoid the consumption of other fermented milks, fermented foods, beverages containing living lactic acid bacteria, and other probiotic or prebiotic products. Medications and hospital visits were allowed and recorded in a diary if these events occurred.

2.2. Experimental beverages

A heat-inactivated CP2305 cell product was prepared as described below. CP2305 was cultured at 37 °C for 18 h in an original, food ingredients-based medium containing glucose, casein, fish protein peptone, yeast extract, a pH adjuster, and an emulsifier. The cultured CP2305 cells were concentrated using a ceramic filter, washed with excessive water, pasteurized at 90 °C, and freeze dried.

Test beverages containing CP2305 were prepared by blending high-fructose corn syrup, powdered skim milk, lactic acid, soybean polysaccharide, pectin, sodium citrate, flavours, sweeteners, and the heat-inactivated, washed CP2305 powder corresponding to 1×10^{10} bacterial cells in water, followed by pasteurization and packing into a 200-mL container. Placebo beverages were prepared using the same formula and procedure as for the CP2305 beverage, except this product did not contain the CP2305 powder. The nutritional content of the test and placebo beverages are described as follows. The CP2305 beverages provided a total of 214 kJ/d (51 kcal/d), 1.8 g protein, 11 g carbohydrates, and 73 mg sodium chloride in 200 mL. The placebo beverage provided a total of

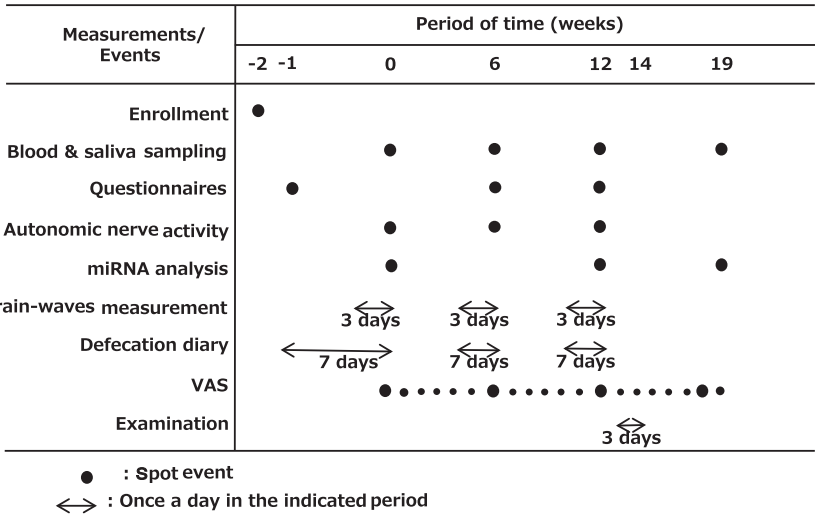

Fig. 1. Schedule for the clinical trial. Participants were divided into 2 groups. Biological samples and questionnaire responses were obtained prior to the start of treatment, 6 weeks after the start of ingestion, at the end of the ingestion period and at the end of the post-ingestion period.

Table 1
The clinical characteristics of the CP2305 and placebo groups.

Measurements	Placebo			CP2305		
	Whole group	Male	Female	Whole group	Male	Female
Numbers	35	21	14	34	19	15
Years of age	25.1 ± 0.4 (22–35)	25.7 ± 0.6 (23–35)	24.2 ± 0.4 (22–27)	24.9 ± 0.3 (23–32)	25.3 ± 0.5 (23–32)	24.3 ± 0.27 (23–27)
Exercise habits[a]	3.7 ± 0.1 (2–4)	3.6 ± 0.1 (3–4)	3.6 ± 0.1 (3–4)	3.6 ± 0.1 (2–4)	3.5 ± 0.1 (2–4)	3.8 ± 0.1 (3–4)
Height (cm)	167.8 ± 1.4 (154–189)	172.6 ± 1.2 (165–189)	160.5 ± 1.3 (154–168)	166.5 ± 1.3 (155–181)	171.0 ± 1.3 (158–181)	160.8 ± 1.2 (155–170)
Weight (kg)	58.9 ± 1.7 (43–81)	63.9 ± 1.8 (50–81)	51.4 ± 1.9 (43–68)	58.6 ± 1.5 (43–81)	64.4 ± 1.6 (54–78)	51.2 ± 1.3 (44–60)
BMI (kg/m^2)	20.8 ± 0.9 (16.5–25.3)	21.4 ± 0.4 (17.9–25.3)	19.9 ± 0.7 (16.5–26.6)	21.1 ± 0.4 (18.1–27.0)	22.1 ± 0.6 (18.1–27.0)	19.8 ± 0.4 (18.1–23.4)
GHQ total (Goldberg)	5.6 ± 0.9 (0–19)	4.2 ± 1.2 (0–19)	7.6 ± 1.3 (0–14)	5.8 ± 0.8 (0–17)	4.8 ± 1.3 (0–17)	7.1 ± 0.9 (1–13)
STAI state anxiety	41.7 ± 2.2 (21–64)	38.0 ± 2.8 (21–63)	47.3 ± 3.3 (26–64)	40.1 ± 1.9 (22–62)	35.7 ± 2.3 (22–57)	45.7 ± 2.5 (25–62)
STAI trait anxiety	44.4 ± 2.1 (20–67)	40.2 ± 2.6 (20–65)	50.7 ± 2.8 (34–67)	43.8 ± 2.3 (21–69)	39.7 ± 3.1 (21–68)	49.0 ± 2.9 (30–69)
PSQI	4.1 ± 0.5 (0–12)	3.8 ± 0.6 (0–12)	4.6 ± 0.7 (1–9)	4.9 ± 0.5 (0–12)	4.8 ± 0.7 (0–17)	4.9 ± 0.6 (0–12)
HADS anxiety	6.4 ± 0.8 (0–16)	5.1 ± 0.9 (0–14)	8.4 ± 1.1 (1–16)	6.3 ± 0.8 (0–16)	5.3 ± 1.0 (0–13)	7.6 ± 0.8 (0–16)
HADS depression	6.8 ± 0.7 (0–17)	6.3 ± 1.0 (0–17)	7.6 ± 0.9 (1–14)	6.3 ± 0.6 (0–12)	5.3 ± 0.8 (0–12)	7.5 ± 0.7 (1–11)

Data are expressed as the mean ± SEM.
Numbers in parentheses are the range of sample data.
[a] Exercise habits were evaluated by the records of the four-grade questionnaire. The higher the grade, the less the exercise habit is shown.

214 kJ/d (51 kcal/d), 1.8 g protein, 11 g carbohydrates, and 71 mg sodium chloride in 200 mL. These experimental beverages were produced at Asahi Soft Drinks Co., Ltd. (Tokyo, Japan).

2.3. Self-report measurements

The physical and mental health of the participants was subjectively assessed using the following questionnaires: GHQ28, the 28-item General Health Questionnaire (Goldberg & Hillier, 1979); HADS, the Hospital Anxiety and Depression Scale (Zigmond & Snaith, 1983); STAI, the Spielberger State-Trait Anxiety Inventory (Kvaal, Ulstein, Nordhus, & Engedal, 2005); and PSQI, the Pittsburgh Sleep Quality Index (PSQI) (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989). These questionnaires were given to each participants at three time points: one week prior to the start of administration of the beverages and 6 and 12 weeks after the administration. One week after finishing the administration, the students took the 3-day national examination and then went on vacation. We also assessed their mental and physical state 5 weeks after the examination (19 weeks after starting the manipulation) when they appeared to be relaxed while on vacation.

2.4. Measurement and assessment of sleep EEG

All subjects underwent overnight EEG monitoring using a single-channel EEG (SleepScope™; SleepWell Co., Osaka, Japan). This instrument is now approved as a medical equipment (certification No. 27ADBZX00087000) in Japan and has been widely used in recent sleep studies (Goto et al., 2015). The EEG data provided from the device show high agreement with those from polysomnography (86.89% with 0.753 k-value). The instrument is a palm-sized (6.3 cm width × 9.4 cm length × 3.4 cm depth), single-channel, portable electroencephalograph with two self-adhesive electrodes, one to be placed on the forehead and another to be placed behind the ear. After the participants were instructed and trained, they wore the portable EEG monitor at home on Tuesday, Wednesday, and Thursday nights in bed at 3 time points: prior to the ingestion, 6 and 12 weeks after starting the ingestion. We treated Tuesday night measurements as a practice. Data collected on Wednesdays were used for analysis, unless the EEG measurement was not successful on Wednesday in cases of failure in data-gathering, alcohol intake, and lack of sufficient sleep time recordings. In those cases, data from Thursday were used. The following indicators of sleep quality were obtained for each subject: sleep latency, total sleep time, sleep efficiency, percentage of wake after sleep onset (WASO), percentage of stage 3 non-REM (N3) sleep, and delta power during the first sleep cycle. Scoring of sleep stages was performed according to the AASM 2007 manual (Berry et al., 2012).

2.5. Assessments of stool properties and bowel habits

For seven-day periods before the start of administration (baseline), 6 weeks (mid-term) and 12 weeks (end of ingestion) after starting the administration, and 7 weeks after finishing the ingestion (19 weeks after starting the manipulation) as a relaxed, recovery time, the subjects recorded their physical conditions, stool properties (form, colour, output, and intensity of odour), frequency of defecation, feelings after evacuation, and abdominal symptoms. Faecal form and colour were evaluated in accordance with the Bristol Stool Scale (BSS) (Lewis & Heaton, 1997) and a faecal colour chart as previously described (Sugawara et al., 2016).

2.6. Assessment of autonomic nerve activities

Autonomic nervous system activity was examined after measuring the variation in the fingertip heart rate (Guideline: heart rate variability standards, 1996). A TAS9 VIEW (YKC Corporation, Tokyo, Japan) acceleration pulse wave measurement apparatus, which is an approved medical device (licence number: 13B3X00442I00001), was used to analyse the heart rate variability. The autonomic nerve balance analysis conducted at a frequency level from 0.04 to 0.15 Hz was classified as Low Frequency (LF), while the analysis conducted at 0.15 to 0.40 Hz was classified as High Frequency (HF). LF and HF were used as indicators for the activities of sympathetic and parasympathetic nerves, respectively. The ratio between LF and HF (LF/HF) or the HF norm parameter, [HF/(LF + HF)] revealed the overall balance between sympathetic and parasympathetic nerve activities. The total power (TP, ≤approximately < 0.4 Hz) was also measured.

2.7. Measurements of salivary cortisol and chromogranin A

Saliva was collected 3 days before and 6 and 12 weeks after starting test beverage ingestion, as well as 7 weeks after finishing the ingestion. Saliva samples were collected between 16:00 and 17:00 to avoid diurnal fluctuations using Salivette sampling devices (Sarstadt Inc., Rommelsdorf, Germany) prior to the

collection of blood as previously described (Kurokawa et al., 2011). Salivary chromogranin A (CgA), cortisol and total protein levels were measured using commercialized enzyme immunoassay kits (chromogranin A: YK070 Human CgA EIA kit, Yanaihara Institute, Shizuoka, Japan; cortisol: Expanded Range High Sensitivity Salivary Cortisol Enzyme Immunoassay kit, Salimetrics Inc., LLC, Carlsbad, CA, USA; total protein: Protein Quantification Kit-Wide Range, Dojindo Inc., Kumamoto, Japan Ciron, Tokyo, Japan).

2.8. Stress-responsive miRNA levels in peripheral blood leukocytes

We previously reported 7 chronic stress-responsive microRNA (miR-16, -20b, -26b, -29a, -126, -144, and -144*) in peripheral leukocytes from medical students preparing for the same national examination for medical practitioners (Honda et al., 2013). Among them, miR-16, miR-144 and miR-144* levels were also significantly increased in medical students taking the nationally authorized examination for promotion (Katsuura et al., 2012). Blood was collected after saliva sampling between 16:00 and 17:00 and immediately poured into PAXgene-blood RNA tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Total RNA was prepared, and levels of miR-16, miR-144 and miR-144* were measured using quantitative real-time reverse transcription PCR (qPCR) and TaqManR MicroRNA assays (Applied Biosystems, Foster City, CA, USA) as previously described (Honda et al., 2013; Katsuura et al., 2012). All miRNA data were normalized using RNU48 as the endogenous quantity control.

2.9. Statistical analyses

Analysis of variance (ANOVA) with split-plot design for repeated measures and analysis of covariance (ANCOVA) for repeated measures (panel analysis) was applied to the data obtained using JMP11 Pro (SAS Institute Japan Ltd., Tokyo, Japan) and IBM SPSS Statistics ver. 23 (IBM Japan Ltd., Tokyo, Japan). ANCOVA was used as needed at 12 weeks after the start of ingestion as the post hoc test with JMP11 Pro (SAS Institute Japan Ltd., Tokyo, Japan). The initial values of each measurement or the recorded items measured before the manipulation were employed as covariates.

3. Results

3.1. Effects of paraprobiotic CP2305 on physical and mental state

The results of the questionnaires are summarized in Table 2. The mean values of the STAI-state scores measured at 0 week (14 weeks before the examination) were already higher than the threshold value of 40 in the Japanese version of STAI (Nakazato & Shimonaka, 1989), gradually increased to 55–60 at 12 weeks (2 weeks before the examination), and returned to normal (baseline) levels at 19 weeks (5 week after the examination) (Fig. 2A). The mean values of GHQ-28 general score measured at 0, 6, and 12 weeks were above the threshold value of 5, and then, they returned to normal levels at 19 weeks (5 weeks after the examination) (Fig. 2B). Generally, a majority of medical students feel pressure and become anxious more than 6 months before the examination. The medical students felt anxiety and complained of physical symptoms for more than 14 weeks. Thus, we confirmed that the paraprobiotic CP2305 was administered during this long-term stressful situation.

The STAI-state scores were not significantly different between the paraprobiotic CP2305 and placebo groups through the trial period (Fig. 2A left). However, female students showed significantly higher initial STAI-state scores and more extensive elevation of the score compared with that of male students (Tables 1 and 2). Moreover, a significant difference in the scores between the two groups was observed in female students 12 weeks after the start of ingestion by ANCOVA with their initial scores as the covariance [$p = 0.0346$ (ANCOVA); Fig. 2A right]; the paraprobiotic CP2305 significantly prevented the elevation of the STAI-state scores 2 weeks before the examination. A significant difference in the total score ($p = 0.0468$; Fig. 2B left) of the GHQ-28 general score was also observed in female volunteers from the post hoc analyses

Table 2
Summary of the results of questionnaires.

Questionnaire	Detailed item	Factor							
		Primary			Secondary				
		Treatment	Sex	Treatment × Gender	Time	Time × Treatment	Time × Sex	Time × Group × Gender	Initial score
STAI	State anxiety	0.3433	0.0750	0.6815	<0.0001	0.8417	0.0197*	0.5607	<0.0001
	Trait anxiety	0.4541	0.1972	0.6049	0.2760	0.9309	0.6495	0.5301	<0.0001
HADS	Anxiety	0.3939	0.3700	0.7003	0.0430	0.6935	0.1782	0.7461	<0.0001
	Depression	0.4849	0.6381	0.4930	0.0101	0.8429	0.8601	0.2439	<0.0001
GHQ	Total score	0.1459	0.4402	0.8779	0.1479	0.8363	0.7936	0.9641	<0.0001
	Somatic symptoms	0.4686	0.3875	0.2916	0.8162	0.3968	0.8401	0.9656	<0.0001
	Anxiety/insomnia	0.0561	0.0013	0.0472	0.0147	0.7080	0.0748#	0.7361	<0.0001
	Social dysfunction	0.2715	0.4990	0.8793	0.5956	0.9855	0.9275	0.7301	<0.0001
	Severe depression	0.3434	0.8233	0.2692	0.6489	0.2857	0.2118	0.8320	<0.0001
PSQI	Total score	0.2794	0.5890	0.8706	0.4343	0.0281*	0.2807	0.7376	<0.0001
	Overall sleep quality	0.6950	0.8817	0.5838	0.5424	0.0333*	0.8700	0.7035	<0.0001
	Sleep latency	0.7999	0.1217	0.4998	0.7246	0.6773	0.1798	0.9835	<0.0001
	Duration of sleep	0.0475	0.4012	0.5097	0.1467	0.0286*	0.0379*	0.0920#	<0.0001
	Sleep efficiency	0.2975	0.8557	0.7667	0.2098	0.3242	0.9468	0.8385	<0.0001
	Sleep disturbance	0.8821	0.5809	0.9792	0.9223	0.4570	0.3151	0.1764	<0.0001
	Need medicine to sleep	0.0963	0.5335	0.5335	0.2408	0.2408	0.6646	0.6646	<0.0001
	Day dysfunction due to sleepiness	0.4745	0.8605	0.1778	0.7412	0.3541	0.5947	0.3551	<0.0001

Data are expressed as the significant probability of the factor calculated by mixed model ANOVA for repeated measures.
* $p < 0.05$.
# $p < 0.10$.

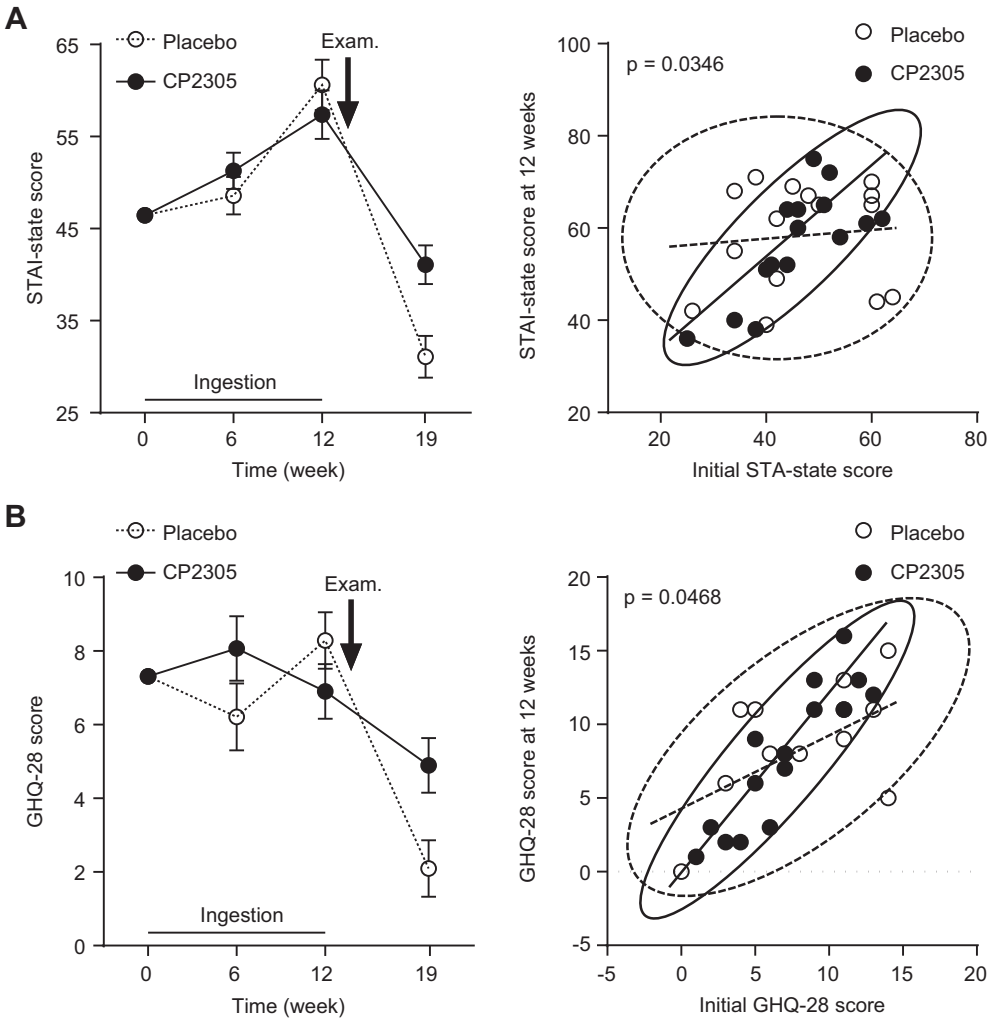

Fig. 2. Changes in stress-related behaviours as inferred from the questionnaires. (A) Time-dependent changes in the STAI-state anxiety score in female participants. ANCOVA showed a significant difference between the groups at the end of the ingestion period. (B) Changes in the GHQ28 total score in female volunteers. At the end of the ingestion period, ANCOVA indicated a significant difference between groups (right panel). Open circles (○) and short dashed lines represent the placebo group, and closed circles (●) and solid lines represent the paraprobiotic CP2305 group. Ovals express 0.95 density ellipses, and lines inside of ovals are regression lines.

with ANCOVA at 12 weeks after the start of ingestion (Fig. 2B right). Additionally, paraprobiotic CP2305 suppressed the increase in the general scores.

In the PSQI questionnaire, the paraprobiotic CP2305 group showed desirable changes compared with those of the placebo group in total score ($p = 0.0281$), overall sleep quality ($p = 0.0333$), and duration of sleep ($p = 0.0286$) (Fig. 3A-C). There was no significant difference between both groups in sleep efficiency, while a significant difference was observed at 12 weeks by ANCOVA when their initial values were used as covariates ($p = 0.0044$; Fig. 3D). The sleep latency was also significantly different in female volunteers at 12 weeks by ANCOVA ($p = 0.0234$; data not shown).

Both anxiety and depression scales of the HADS were significantly increased during the pre-examination, and its anxiety scale was more profoundly elevated in female than male students (Table 2). However, we could not detect any significant differences in the HADS scores between the CP2305 and the placebo groups (Table 2).

3.2. Assessment of sleep quality using EEG

We could not measure EEG after the examination because most students had long vacations. We therefore measured sleep EEG before (0) and 6 or 12 weeks after starting the ingestion and examined how the CP2305 administration affected their sleep. Compared with the placebo intake, the CP2305 intake significantly decreased sleep latency ($p < 0.001$; Fig. 4A), prevented the reduction of the total δ power (high-amplitude slow wave: 0.5–2 Hz, 75 μV) for one minute during sleeping time ($p < 0.001$; Fig. 4B), and increased the appearance of the fraction of stage N3 in the non-REM sleep period ($p < 0.001$; Fig. 4C) during the pre-examination period. In addition, the number of awakenings in the 2 h before wake-up was significantly decreased ($p < 0.001$; Fig. 4D). Notably, the incremental trend in the number of awakenings in volunteers who had a low initial number of awakenings with time was controlled by CP2305 administration.

3.3. Effects of paraprobiotic CP2305 on salivary stress markers

As shown in Fig. 5A, ANCOVA for repeated measures with the initial value of the concentration as the covariate revealed that the paraprobiotic CP2305 ingestion significantly suppressed the escalation of salivary cortisol levels as time progressed ($p < 0.001$) compared with that of the placebo intake. ANCOVA was applied to each time point as a post hoc test. Significant

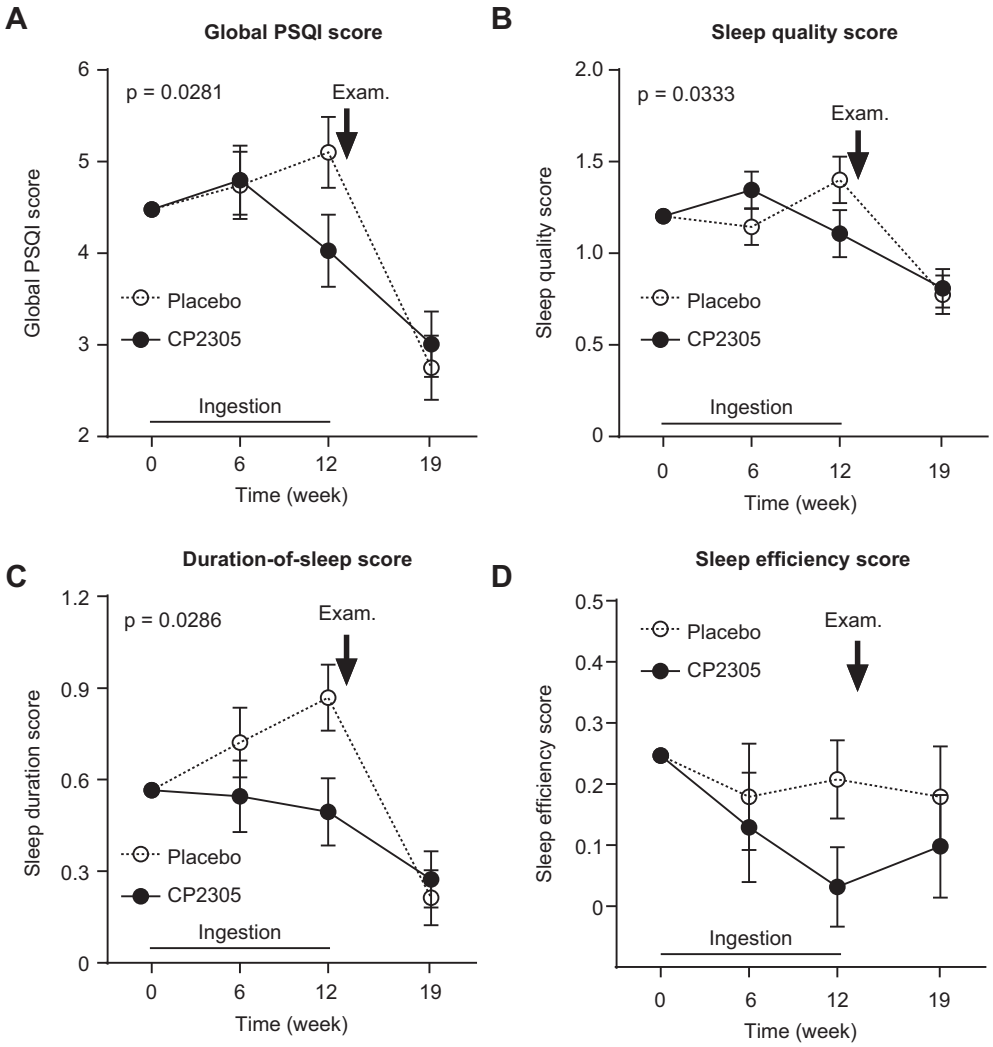

Fig. 3. Time-dependent changes in PSQI score. (A) Time-dependent changes in total PSQI scores are presented. (B) Time-dependent changes in the sleep quality scores are expressed. (C) Time-dependent changes in the duration of sleep scores are presented. (D) Time-dependent changes in the sleep efficiency scores are presented. Data are expressed as the mean ± SEM. Statistical analysis was performed with an ANOVA for repeated measures, and p values indicate significant probabilities for the term "Time × Group". In panels A, B and C, significant differences were observed. In contrast, there was no significant difference, but a tendency toward a decrease in the paraprobiotic CP2305 group was observed in panel D.

differences between the two groups were observed 6 ($p = 0.0161$) and 12 ($p = 0.0087$) weeks after starting the ingestions.

We could not detect any time-dependent change and group difference in salivary chromogranin A levels (data not shown).

3.4. Effects of paraprobiotic CP2305 on autonomic nervous activities

Repeated-measures ANCOVA showed a significant difference in the HF/(HF + LF) value (HF norm) between the paraprobiotic CP2305 and the placebo groups, as shown in Fig. 5B. The HF norm value, which represents the relative parasympathetic nervous activity, was increased by ingestion of the beverage containing the paraprobiotic CP2305 ($p < 0.001$; Fig. 5B).

3.5. Paraprobiotic CP2305 and bowel movement

Paraprobiotic CP2305 ingestion normalized the defecation frequency under stressful conditions; the frequency in the parabiotic CP2305 group was closer to the seven times per week of ideal bowel movements ($p < 0.001$; Fig. 6A). These results were similar to the results of past clinical trials (Sawada et al., 2016; Sugawara et al., 2016). Notably, there was clear decrement in stool frequency in male participants who had a high frequency of passage at the start of the trial (data not shown).

When stool properties were evaluated with the BSS, the shape of the faeces effectively moved closer to a value of 4 after taking the paraprobiotic CP2305, but not the placebo, even under stressful conditions ($p < 0.001$; Fig. 6B). Thus, stool shapes became normalized in the paraprobiotic CP2305 group. Consequently, daily faecal output was also favourably changed with the paraprobiotic CP2305 ($p < 0.001$; Fig. 6C); the average amount of defecation per day was altered to the equivalent of approximately 4 moquettes in the paraprobiotic CP2305 group.

3.6. Expression of stress-responsive microRNAs in peripheral blood leukocytes

MiR-144, miR-144*, and miR-16 in circulating leukocytes were identified as stress-responsive microRNAs in healthy medical students exposed to a chronic stress model (Honda et al., 2013) in this study and to an acute stress model (Katsuura et al., 2012). Daily intake of the paraprobiotic CP2305 significantly suppressed the

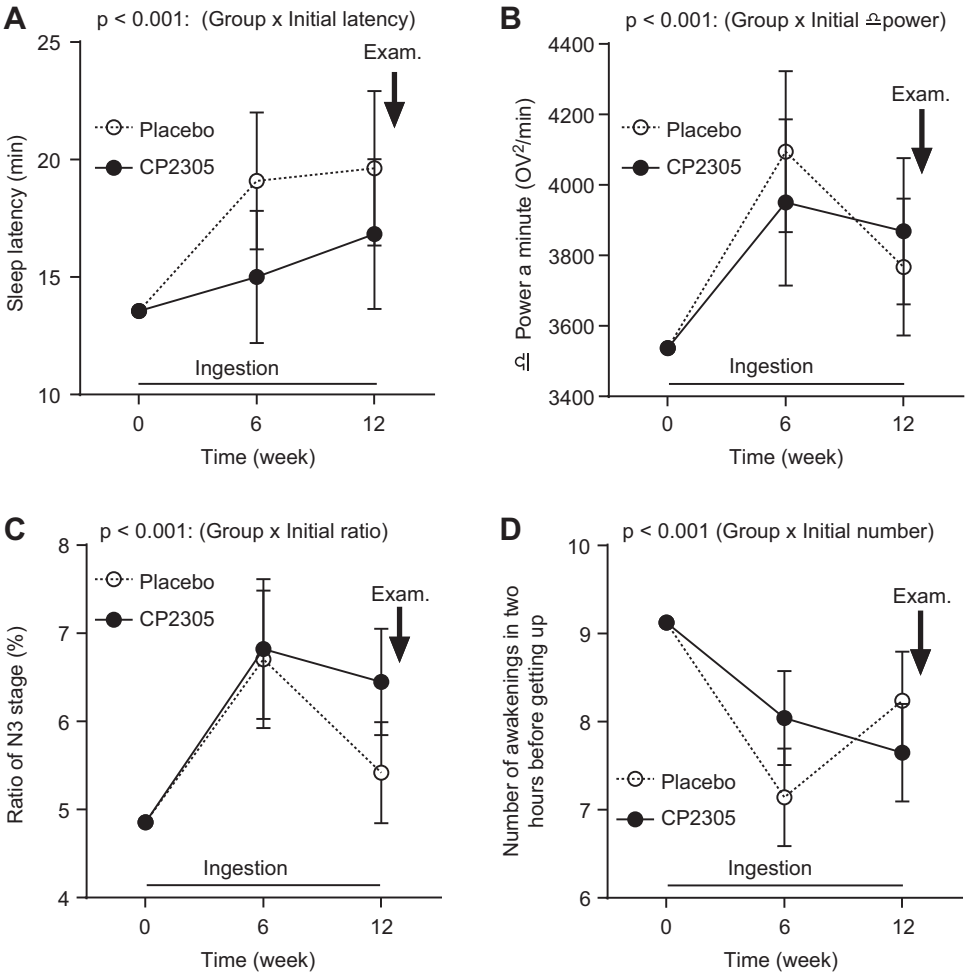

Fig. 4. Time-dependent changes in the EEG data. (A) Time-dependent changes in sleep latency are presented. (B) Time-dependent changes in total δ power in sleep time are presented. (C) Time-dependent changes in the ratio of the appearance of stage N3 in non-REM sleep. (D) Time-dependent changes in the number of awakenings in the 2 h before getting up. Open and closed circles represent individuals in the placebo (○) and paraprobiotic CP2305-fed (●) groups. Dotted and solid lines indicate the placebo and paraprobiotic CP2305-fed groups, respectively. Repeated-measures ANCOVA was applied to time-dependent changes of both items in the whole model. ANCOVA was applied to all time point data as a post hoc test.

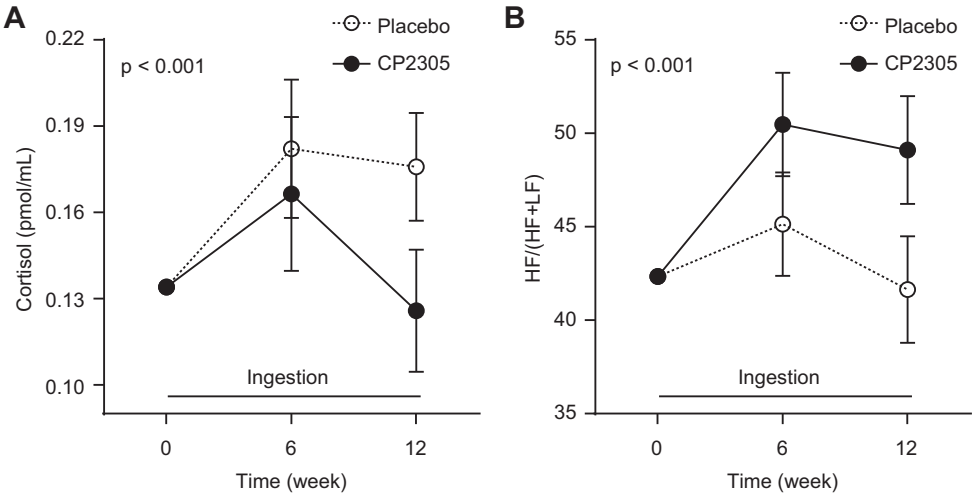

Fig. 5. Time-dependent changes in the salivary cortisol concentration (A) and the ratio of parasympathetic nerve activity (HF/HF + LF) (B). Open and closed circles represent individuals in the placebo (○) and paraprobiotic CP2305-fed (●) groups, respectively. Dotted and solid lines indicate the placebo and paraprobiotic CP2305-fed groups, respectively. Repeated-measures ANCOVA was applied to the time-dependent changes of both items in the whole model. ANCOVA was applied to each time point as a post hoc test.

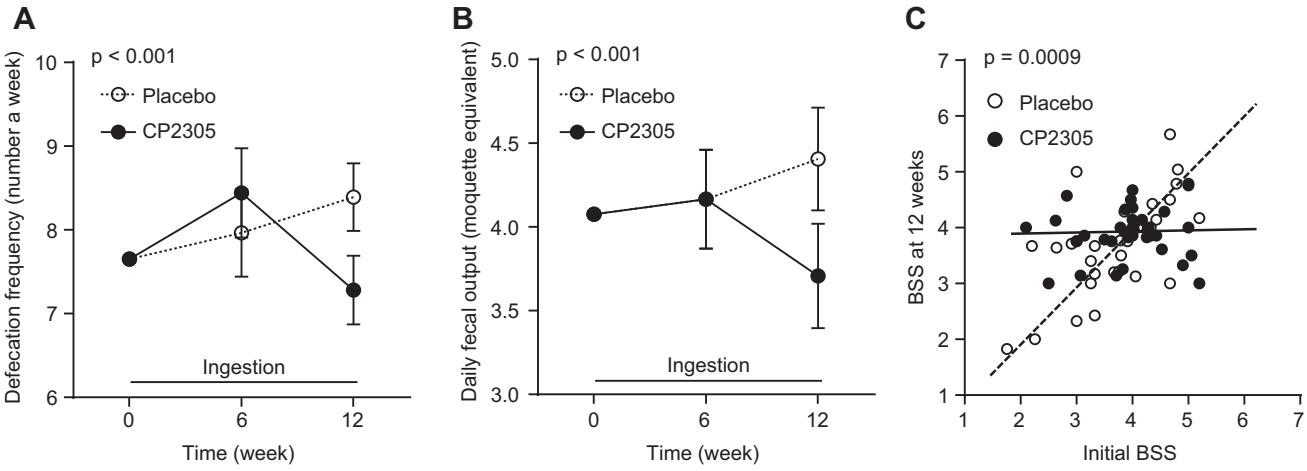

Fig. 6. Time-dependent changes in the frequency of weekly bowel movements (A), daily faecal output (B) and faecal shapes determined using BSS (C). Open and closed circles represent individuals in the placebo (○) and paraprobiotic CP2305-fed (●) groups, respectively. Dotted and solid lines in panels A and B are for the placebo and paraprobiotic CP2305-fed groups, respectively. Repeated-measures ANCOVA was applied to the time-dependent changes of both items in the whole model. ANCOVA was applied to each time point as a post hoc test. Lines inside of the data area in panel C are regression lines calculated by ANCOVA. ANCOVA was applied to the data in panel C.

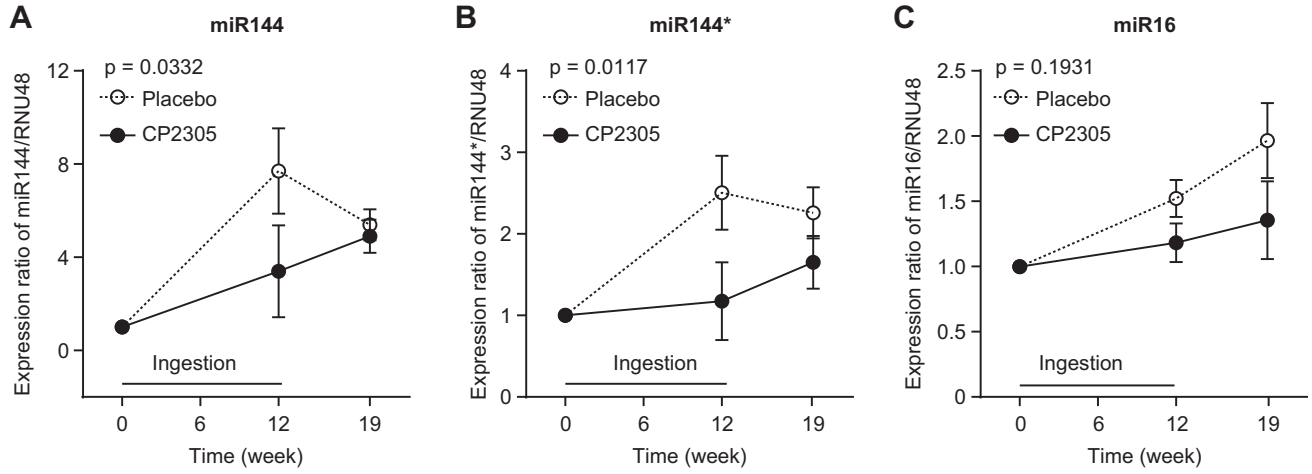

Fig. 7. Time-dependent changes in the expression of stress-responsive miRNAs with time. The changes in the expression of miR-144 (A) in volunteers of both genders are represented. The time-dependent expressions of miR-144* (B) and miR-16 (C) are also shown in the same manner. Data are expressed as the mean ± SEM. Statistical analysis was performed with ANOVA for repeated measures, and p values indicate significant probabilities of the term "Time × Group".

up-regulation of miR-144 ($p = 0.0332$; Fig. 7A) and miR-144* ($p = 0.0117$; Fig. 7B), compared with that of the placebo intake. This suppressive effect was not observed in miR-16 ($p = 0.1931$; Fig. 7C). A repeated-measures ANOVA showed that "sex" was an important factor for the changes in the expressions of these microRNAs. Although the paraprobiotic CP2305 similarly reduced the miR-144* expression in both male and female students (data not shown), the effect of paraprobiotic CP2305 on miR-144 was observed in female students ($p = 0.0118$) but was absent in male students ($p = 0.9357$).

4. Discussion

The term "psychobiotics" was recently defined as "live microbes that have a positive mental health benefit" (Dinan et al., 2013). Several recent studies in healthy subjects suggest that certain bacterial strains have a psychobiotic activity (Romijn & Rucklidge, 2015). In this manner, *B. longum* strain 1714 improved stress responses and enhanced cognition in healthy subjects while altering their EEG (Allen et al., 2016). In addition, a cocktail of probiotics was shown to significantly change brain function, as demonstrated with functional MRI (Tillisch et al., 2013). Until very recently, however, the probiotics literature for stress relief was dominated by animal studies with little effort to translate this work into humans. In addition, there is an example that a probiotic *Lactobacillus rhamnosus* JB-1 with promising preclinical evidence (Bravo et al., 2011) failed to translate to healthy human participants (Kelly et al., 2017). There is a strong need for translational studies in human subjects, and for that reason, the current study may be of interest.

In this study, we tried to expand the above concept of "psychobiotics" to "para-psychobiotics". For this purpose, we used heat-inactivated and washed CP2305 as a paraprobiotic to confirm its stress-relief effects on healthy young adults. The paraprobiotic CP2305 is also useful for understanding the mechanism(s) of psychobiotic actions. We previously reported the beneficial effects of live CP2305 on the clinical symptoms and the QOL of IBS patients (Nobutani et al., 2017). We also showed the beneficial effects of live CP2305 on anxiety, depression, and sleep disturbances in medical students taking a cadaver dissection course (Sawada et al., 2017). However, these trials were carried out with live CP2305 and only for a short ingestion period of 4 weeks. We therefore

tested whether paraprobiotic CP2305 could relieve chronic stress-associated behaviours in a long-term dosage period of 12 weeks.

Medical students preparing for the national examination for medical practitioners were accepted as a useful model for studying chronic psychological stress responses (Honda et al., 2013; Kurokawa et al., 2011). In this study, samples were taken at four time points: prior to ingestion, 6 and 12 weeks after ingestion had started, and 7 weeks after termination of the ingestion (post-ingestion period). All subjects in this study were newly recruited. Consistent with the previous reports (Honda et al., 2013; Kurokawa et al., 2011), the students in this study felt pressure even at the initial sampling time (0); the mean values of the STAI-state and the PSQI-general scores were above their threshold values. The STAI-state score gradually increased, and the general GHQ-28 score remained elevated during the pre-examination period. Both scores returned to normal levels 5 weeks after the examination (19 weeks). Thus, we confirmed that paraprobiotic CP2305 was administered under chronically stressed conditions.

We first examined whether the daily administration of the paraprobiotic CP2305 improved self-reported measures of physical and psychological state. As described above, the situation resulted in high scores in the questionnaires at the start of the trial; the initial scores in GHQ-28, STAI, and HADS were over their thresholds. The differences in these scores between both groups may not be distinguishable. Another important concern is to consider the sex-dependent symptoms and sensitivity to CP2305 intake. Female students showed greater increases in the STAI-state and the general GHQ-28 scores than male students did, and the CP2305 intake significantly suppressed these increases only in female students. However, female students did not show changes in their bowel habits and stool properties, while male students significantly increased the incidence of diarrhoea. The CP2305 intake effectively improve their bowel movements and stool properties. Thus, there may be sexual differences in the appearance of stress-associated symptoms. The paraprobiotic CP2305 intake appeared to effectively suppress the stress-associated symptoms in both sexes. To address these issues, we should analyse larger numbers of participants of both sexes at more appropriate sampling times.

All medical students have to pass the national medical license examination to become clinical physicians. This examination consists of a three-day test and is the most stressful and important event for medical students. Because of this difficult study period in a special environment, the students had progressively complained of sleep disturbance, particularly in the placebo group. Notably, paraprobiotic CP2305 administration significantly improved sleep disturbance under this long-term stressful situation. Sleep quality is an outcome that comprehensively reflects both physical and psychological conditions (Mollayeva et al., 2016). In a recent study (Sawada et al., 2017), we observed similar improvement of sleep disturbance with live CP2305 in medical students taking a cadaver dissection course, which was also assessed with the PSQI. The beneficial effects on sleep quality were again observed even with paraprobiotic CP2305, indicating the potential benefits of this strain for mental health promotion. This time, the effect was objectively confirmed by sleep EEG monitoring. The δ power is an indicator of depth of sleep. An epoch (30 s of sleep) that consists of 20% or more slow-wave δ sleep is considered to be stage three depth of sleep. Daily intake of paraprobiotic CP2305 increased the ratio of the slow-wave stage, even under chronically stressful conditions. Paraprobiotic CP2305 shortened sleep latency, increased δ power and N3 stage sleep, and maintained sleep, resulting in suitable sleep time.

In addition to this important finding on sleep EEG monitoring, we confirmed another interesting physiological effect of paraprobiotic CP2305 on the parasympathetic nervous system. Autonomic nervous activities were evaluated using a pulse analyser, TAS9-VIEW (YKC Group, Tokyo, Japan). Daily intake of paraprobiotic CP2305, but not placebo, preferentially increased the rate of parasympathetic nerve activity (HF norm) during the pre-examination period. This effect may also be associated with the stress-relief effects of the paraprobiotic CP2305. In fact, in association with the improvement of parasympathetic nerve activity, the paraprobiotic CP2305 intake improved bowel habits and stool properties, and it also effectively suppressed the escalation of basal salivary cortisol levels during the pre-examination period. Salivary cortisol level reflects the HPA axis activity and the sympathetic nervous system. Increased salivary cortisol levels measured in early evening (basal levels) are associated with mental distress. In the parameters of gut function, paraprobiotic CP2305 regulated the frequency of the bowel movement to the ideal condition. Paraprobiotic CP2305 acted as an effective antiflatulent, particularly in male students. Male students gradually developed loose bowel-like symptoms in the placebo group, which were ameliorated by the CP2305 administration, as observed in previous studies (Sawada et al., 2016; Sugawara et al., 2016). Consequently, after the 12-week administration, faecal samples of all participants had a normal stool shape (close to score 4 on the BSS). These data indicate that CP2305 could exert sufficient effects on the gut function even in the paraprobiotic form. The next important step is to clarify the active material(s) of the bacterium and the mechanism of its effect on the gut-brain axis.

Finally, we assessed the beneficial effects of paraprobiotic CP2305 by measuring a different type of biological stress marker. For this purpose, we measured psychological stress-responsive microRNAs. MicroRNAs play key roles in the regulation of cellular processes in response to changes in the environment. Using the same chronic stress model (students preparing for the national examination for medical practitioners), we identified miR-144, miR-144* and miR-16 as stress-responsive microRNAs (Honda et al., 2013). MiR-144 modulates oxidative stress, and miR-16 inhibits the expression of the serotonin transporter (Baudry, Mouillet-Richard, Schneider, Launay, & Kellermann, 2010; Katsuura et al., 2012; Sangokoya, Marilyn, & Chi, 2010). The daily intake of paraprobiotic CP2305 suppressed elevation of miR-144 and -144* expression compared with that of the placebo intake. The microRNA responses also showed sexual differences. When the effect of paraprobiotic CP2305 was examined independently in male and female students, CP2305 significantly suppressed the stress-induced elevation of miR-144 and miR144* in female students, while these effects were absent in male students.

The present study reports the potential stress-relieving effect of paraprobiotic CP2305 using several objective measurements. However, it is unclear how the daily intake of the heat-inactivated and washed bacterial cells could affect the gut-brain axis and alter the stress responses. One possible route of the communication is through the vagal nerve. We attempted to address this possibility and found that an intragastric ingestion of the preparation could activate the afferent vagal nerve from the stomach or the intestine in rats (unpublished observations). We also repeatedly confirmed that the CP2305 strain has an enteric-colonizing ability after its oral administration. These findings support the following novel hypothesis. This strain may bind a responsible receptor(s) on intestinal epithelial or other cells and promote the dispatch of a signal(s) driving the HPA axis. Studies on the purification of the bioactive substance(s) of CP2305 are now in progress.

5. Conclusions

In conclusion, paraprobiotic CP2305 was demonstrated to have beneficial effects on medical students under chronically stressful conditions. Administration of paraprobiotic CP2305 significantly

ameliorated their stress-related adverse behaviours and improved sleep disturbance. Our results suggest that the paraprobiotic has potential benefits for mental health promotion. To the best of our knowledge, the paraprobiotic strain described in this report is the first-reported ‘‘para-psychobiotic”.

Conflict of interest

No conflict of interest is declared.

Acknowledgements

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