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Daily intake of *Lactobacillus gasseri* CP2305 improves mental, physical, and sleep quality among Japanese medical students enrolled in a cadaver dissection course

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ABSTRACT

Increasing recognition of the interaction between the brain and gut microbiota has spurred interest in the efficacy of probiotics on stress-related behaviours. This study aimed to elucidate the effects of *Lactobacillus gasseri* CP2305 (CP2305) on mental and physical states in healthy male students enrolled in a cadaver dissection course in a double-blinded, placebo-controlled, crossover-designed trial. Daily administration of CP2305 for 4 weeks significantly improved anxiety, depressive mood and global sleep quality compared with placebo. CP2305 also suppressed salivary cortisol release. In the faecal microbiota, CP2305 suppressed the growth of Enterobacteriaceae. Differential changes in gene expression in peripheral blood leukocytes during the administration period were observed between the placebo and CP2305 groups. Eukaryotic initiation factor 2-related genes were down-regulated only in the placebo group. Thus, the ingestion of CP2305 may have beneficial effects on stress-associated behaviours.

1. Introduction

Several lines of evidence have suggested that the gut microbiota can profoundly modulate communication between the gut and the brain (De Palma, Collins, Bercik, & Verdu, 2014) via neural, endocrine, and immune signalling pathways (Cryan & Dinan, 2012; Montiel-Castro, González-Cervantes, Bravo-Ruiseco, & Pacheco-Lopez, 2013). This concept is now interpreted broadly as the microbiota–gut–brain axis (Bercik, 2011). The gut microbiota affect host stress sensitivity via brain-gut interactions. The relationship between stress and gut microbiota has begun to garner attention (De Palma et al., 2014). With respect to stress management, it is important to uncover whether and how probiotics can influence stress-related behaviours. Animal studies have demonstrated that the administration of probiotics mitigates stress-induced glucocorticoid and inflammatory cytokine responses in association with reductions in depression- and anxiety-related behaviours (Ait-Belgnaoui et al., 2012, 2014; Bercik et al., 2011; Bravo et al., 2011; Gareau, Jury, MacQueen, Sherman, & Perdue, 2007; Messaoudi et al., 2011). Clinical trials have demonstrated that probiotics exert beneficial effects by alleviating psychological distress in healthy subjects (Messaoudi et al., 2011) and normalizing stress-induced reductions in natural killer (NK) cell numbers (Marcos et al., 2004) and gastrointestinal symptoms (Diop, Guillou, & Durand, 2008).

We employed medical students enrolled in a cadaver dissection course to test the stress-relieving effect of a probiotic, *Lactobacillus gasseri* CP2305 (CP2305). Gross anatomy is one of the most important subjects in the preclinical part of medical education. A number of studies around the world have investigated student responses to the dissection of a human corpse and how to mitigate mental distress in the dissection room (Bob, Popescu, Armean, Suciu, & Buzoianu, 2014; Russa & Mligiliche, 2014). Bernhardt, Rothkötter, and Kasten (2012) reported that approximately 50% of German medical students began a dissection course with emotional stress, and approximately one-tenth of them were very concerned about being confronted with corpses. Although the attitudes of students in Japanese medical schools towards the dissecting room have been found to be consistently positive during the course, the cadaver dissection course is unquestionably the most stressful curriculum for pre-clinical students.

We originally isolated a unique strain, CP2305, from stool samples of a healthy volunteer in 1994. CP2305 has an antiflatulent

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effect, particularly in constipated volunteers, with significant changes to the composition of their microbiota. It can colonize the digestive tracts of volunteers at a rate of approximately 40% after oral administration 3 times at the dose of 1.0×10^{11} CFU (Sawada et al., 2016; Sugawara et al., 2016). Recently, we showed that a 4-week administration of CP2305 significantly improved the clinical symptoms of patients with irritable bowel syndrome (IBS) and significantly improved their QOL through the reduction of "health-related worry" (Nobutani et al., 2017). IBS symptoms are closely associated with psychosocial stress. Based on these findings, we conducted a double-blinded, placebo-controlled, parallel- and crossover-group trial to examine whether CP2305 alleviated stress-associated behaviours in medical students enrolled in a cadaver dissection course.

2. Materials and methods

2.1. Probiotics

CP2305 was originally isolated in 1994 from stool samples of a healthy volunteer at the R&D Center of Asahi Group Holdings, Ltd. (Kanagawa, Japan). Whole 16S ribosome DNA (16S-rDNA) sequence analysis and DNA-DNA hybridization of its genomic DNA with that of the type strain *L. gasseri* (ATCC 33323^T) identified this strain as *L. gasseri*. The safety of *L. gasseri* has been authorized by the European Food Safety Authority, and it is listed under the Qualified Presumption of Safety. CP2305 was cultured at 37 °C for 24 h in medium containing 10% skim milk and 0.25% yeast extract. The cultured medium was lyophilized and disintegrated by a food processor mill (TESCOM, Tokyo, Japan). The powder (2.5 g) containing CP2305 (1.0×10^{10} CFU) was packed in a disposable aluminium bag for single ingestion. The same amount of lyophilized powder prepared from the dispersion liquid containing skim milk (20%) and yeast extract (0.50%) was packed into a similar bag to serve as the placebo supplement. Subjects consumed the powder after dissolving it in water. The appearance and taste of both the test and placebo drinks could not be distinguished by sensory assessment. The CP2305 and placebo formulations were prepared in accordance with Japan's Food Sanitation Law and passed safety inspection.

2.2. Subjects and protocol

A double-blinded, placebo-controlled, crossover trial was conducted. We recruited 24 s-year medical students at Tokushima University, Tokushima, Japan. All participants were male students. Participants were not habitual smokers and had not taken medication for 3 months prior to enrolment. None of the students had mental or other diseases or allergies to milk or other foods. The students were taking the cadaver dissection course from September 1st to December 9th. As part of the crossover trial design, the students were randomly divided into 2 groups. As shown in Fig. 1, participants allocated to group 1 consumed the placebo once a day throughout the first experimental period of 4 weeks. After a 3-week wash out period, they consumed the CP2305 supplement once a day for 4 weeks during the second administration period. Participants allocated to group 2 followed the opposite protocol. Daily consumption was self-recorded in a diary to determine the compliance rate of ingestion. 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 probiotic or prebiotic products. Medications and hospital visits were allowed and recorded in a diary if these events occurred. This study was conducted according to the guidelines given in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Institutional Review Board of Tokushima University Hospital, Tokushima, Japan. Written informed consent was obtained from all participants prior to enrolment.

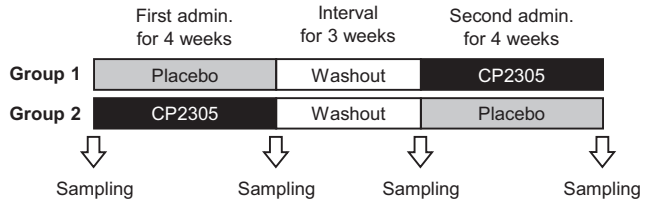

Fig. 1. Examination schedule. Participants were divided into 2 groups. Participants in group 1 were first administered the placebo, entered a wash-out period, and subsequently received CP2305. Those in group 2 were administered CP2305 and the placebo in the opposite order. Biological samples and questionnaire responses were obtained at the start and end of each experimental period.

2.3. Self-reported measurements of mental and physical states

The physical and mental health of the participants was assessed using the following questionnaires: GHQ28, the 28-item General Health Questionnaire (Goldberg & Hillier, 1979); Zung-SDS, the Zung Self-rating Depression Scale (Zung, 1965); HADS, the Hospital Anxiety and Depression Scale (Zigmond & Snaith, 1983); and STAI, the Spielberger State-Trait Anxiety Inventory (Kvaal, Ulstein, Nordhus, & Engedal, 2005). Sleep was evaluated using the Pittsburgh Sleep Quality Index (PSQI) (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989), which assesses sleep quality and disturbances over a 1-month time interval. The PSQI is a 19-item questionnaire; individual items were combined to form the following seven components: sleep disturbance, overall sleep quality, sleep latency, duration of sleep, daytime dysfunction due to sleepiness, sleep efficiency, and need for sleep medication A total score, ranging from 0 to 21, was obtained by adding these seven component scores. Scores >5 suggest poor sleep quality, whereas scores $\leqslant 5$ suggest good sleep quality (Buysse et al., 1989, 2008). These questionnaires were given to the subjects at the time of blood sampling.

Gastrointestinal symptoms of the participants were assessed using a 100-mm visual analogue scale (VAS) concerning abdominalgia, feelings of indigestibility, anorexia, borborygmus, abdomen distension, abdominal discomfort, diarrhoea, constipation, frequency of defecation, and colour of faeces. Eating behaviour was assessed using the Eating Attitudes Test with 26 items (EAT-26), which is a self-report measure of eating disorder symptoms that is widely used for the screening and measurement of symptoms and characteristics of eating disorders (Garner, Olmsted, Bohr, & Garfinkel, 1982).

2.4. Measurements of salivary cortisol, alpha-amylase and chromogranin A

Saliva was collected for 2 min between 16:00 and 17:00 to avoid diurnal fluctuations using a Salivette® sampling device (Sarstadt Inc., Rommelsdorf, Germany) prior to the collection of blood (Kurokawa et al., 2010), and samples were stored at −80 °C until analysis. Salivary chromogranin A (CgA), cortisol, and alpha-amylase were assayed using kits (YK070 Human CgA EIA Kit, Yanaihara Institute, Shizuoka, Japan; Salivary Cortisol EIA Kit and Alpha-Amylase Assay Kit, Salimetrics Inc., LLC, Carlsbad, CA, USA).

2.5. Analysis of faecal microbiota

Faecal samples were collected once during the 3 days prior to the previously appointed date before and after the administration

periods. The faecal microbiota composition was analysed by a cultivation method developed by Mitsuoka (Mitsuoka, Ohno, Benno, Suzuki, & Nanba, 1976; Mitsuoka, Sega, & Yamamoto, 1965).

2.6. Gene expression profiling of peripheral blood cells

Venous blood (2.5 ml) was collected from each subject between 16:00 and 17:00 and immediately poured into PAXgene blood RNA tubes (Becton Dickinson, Franklin Lakes, NJ, USA). After sufficient mixing, the tubes remained at room temperature for 2 h and were then stored at −80 °C until analysis. RNA was isolated using a PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Contaminated DNA was removed using a DNase kit (Qiagen). Purified RNA quality was assessed with an Agilent 2100 Bioanalyzer using an RNA 6000 Nano Labchip Kit (Agilent Technologies, Santa Clara, CA, USA), and RNA samples with >8.5 RNA integrity number (RIN) were used for further measurements.

The RNA samples were subjected to gene expression analysis using a whole human genome microarray (4 × 44 k ver. 2.0, Agilent Technologies) as previously described (Kuwano et al., 2011). Microarray data were analysed with GeneSpring 11.5.1 (Agilent Technologies). The functional pathways related to the set of differentially expressed genes were assessed using Ingenuity Pathway Analysis (IPA) 9.0 (http://www.ingenuity.com) (Kuwano et al., 2011). The probability of a relationship between each biological function and the identified genes was calculated by Fisher's exact test. The level of significance was set at a p value of 0.05.

2.7. Statistical analyses

All statistical analyses were performed using SAS ver. 9.2 and JMP11 Pro (SAS Institute Japan Ltd., Tokyo, Japan). We analysed the results of all the questionnaires and assessed differences in salivary hormones and faecal microbiota before and after intervention using a mixed-model analysis of variance (ANOVA) for crossover design. We paid close attention to the statistical significance of the factor "Treatment". Values less than 0.05 indicated a significant difference in both treatments. If the statistical significance of the factor "Period" was equal to or greater than 0.05, Welch's t-test was applied to compare the final and initial data for the ingestion periods with each treatment over both experimental periods.

3. Results

3.1. Effects of CP2305 ingestion on mental state

We first assessed whether CP2305 administration influenced the mental state of the participants. Using box-whisker plots, we plotted the time-dependent changes in STAI-state scores for groups 1 and 2 (Fig. 2A). There were marked individual variations in the scores during the course. Students taking placebo tended to

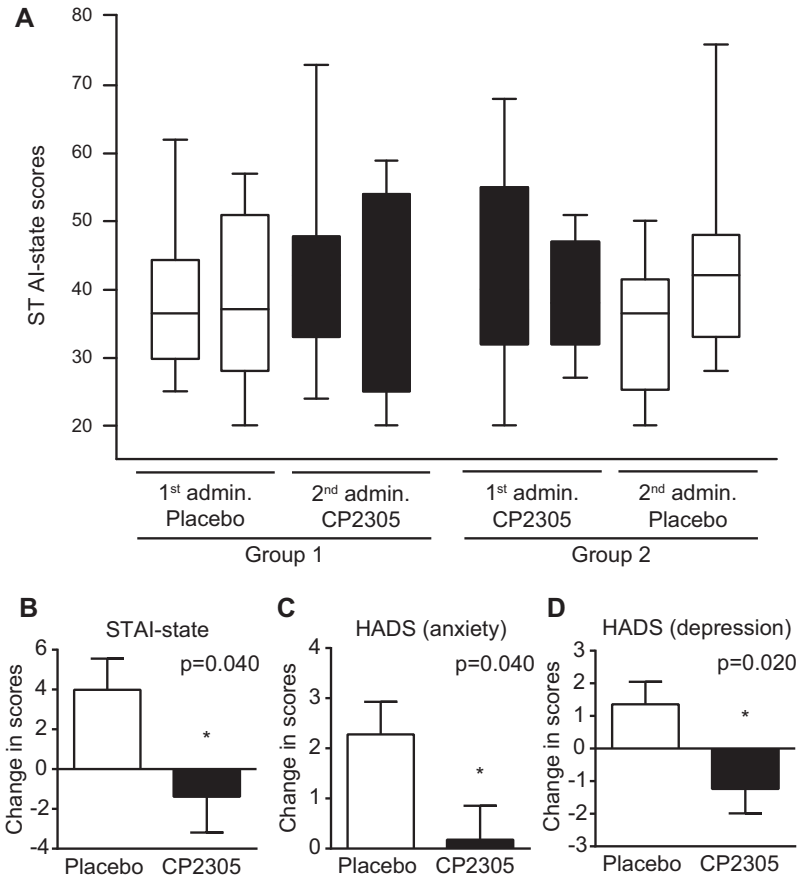

Fig. 2. Changes in stress-related behaviours as inferred from questionnaires. (A) Time-dependent changes in STAI-state anxiety scores. Scores before and after each experimental period are presented as box-and-whisker plots. Open and filled boxes represent placebo and CP2305 treatments, respectively. (B) Changes in STAI-state anxiety scores are shown. Changes in HADS-anxiety scores (C) and HADS-depression scores (D) are shown. Data are presented as the mean ± SEM of the changes in the scores before and after treatment in panels (B) to (D). Open and filled bars indicate placebo and CP2305 treatments, respectively. Statistical analysis was performed by mixed-model ANOVA for crossover design, and the p values indicate the significant probabilities of the term "Treatment". Data from both groups receiving the same treatments were combined because the probability of the factor "Period" was not significant, panels (B) to (D).

show increases in STAI-state scores, and some students reported moderate to severe anxiety. In contrast, CP2305 ingestion appeared to reduce STAI-state scores in both group 1 (1st administration) and group 2 (2nd administration). The CP2305- and placebo-treated groups during the first and second administration periods were mixed together because the probabilities of all items falling under the term of "Period" were not significant. The initial scores for all of the questionnaires, including STAI scores, and all

Table 1
ANOVA results for changes in the scores of mental health questionnaires before and after the intervention periods in the crossover design.

Questionnaire	Probability for each factor[a]				Treatment	Initial scores		Changes in scores		
	Group	Individual	Period	Treatment		Mean	SEM	Mean	SEM	Probability[b]
GHQ28										
Total score	0.290	0.104	0.912	0.978	Placebo	18.65	2.10	1.08	1.45	0.465
					CP2305	20.22	2.61	1.14	1.58	0.480
Physical symptom	0.087	0.556	1.000	0.311	Placebo	4.91	0.62	−0.08	0.59	0.891
					CP2305	4.96	0.79	0.82	0.62	0.205
Depression	0.020	0.038	0.589	0.589	Placebo	0.87	0.30	0.40	0.31	0.212
					CP2305	1.26	0.38	0.15	0.34	0.667
Anxiety and insomnia	0.409	0.013	0.839	0.207	Placebo	3.70	0.54	1.08	1.45	0.465
					CP2305	4.13	0.65	1.14	1.58	0.480
HADS										
Depression	0.798	0.437	0.626	0.020*	Placebo	4.48	0.81	1.36	0.69	0.063
					CP2305	6.96	1.04	−1.24	0.75	0.116
Anxiety	0.781	0.350	0.917	0.040*	Placebo	4.20	0.92	2.28	0.65	0.150
					CP2305	5.75	1.16	0.18	0.68	0.631
STAI										
State anxiety	0.157	0.041	0.335	0.040*	Placebo	36.70	1.85	3.99	1.56	0.075
					CP2305	40.26	3.28	−1.39	1.80	0.231
Trait anxiety	0.993	0.019	0.566	0.147	Placebo	38.20	2.69	2.88	0.77	0.462
					CP2305	41.79	3.57	1.08	0.89	0.517
Zung-SDS										
Depression	0.003	0.448	0.734	0.975	Placebo	36.87	1.75	−0.27	1.10	0.808
					CP2305	37.57	1.83	−0.32	1.15	0.783
EAT26										
Eating behaviour	0.929	0.455	0.097	0.278	Placebo	42.96	2.43	−0.94	1.07	0.392
					CP2305	40.48	2.30	−2.71	1.16	0.031*
PSQI										
Global PSQI	0.034	0.761	0.059	0.046*	Placebo	4.09	0.72	1.79	0.56	0.005*
					CP2305	4.00	0.44	0.09	0.56	0.880
Disturbance	0.233	0.604	0.345	0.041*	Placebo	0.64	0.22	0.29	0.11	0.069
					CP2305	1.44	0.28	−0.14	0.11	0.782

[a] ANOVA with a mixed model for crossover design was applied.
[b] Welch's *t*-test was applied to compare the final and initial data for each ingestion period and each treatment over both experimental periods where the probability of the factor "Period" was not significant.
* The probability was less than 0.05.

Table 2
ANOVA results for changes in the scales of abdominal symptom questionnaires before and after the intervention periods in the crossover design.

Questionnaire	Probability for each factor[a]				Treatment	Initial values (cm)		Changes (cm)		
	Group	Individual	Period	Treatment		Mean	SEM	Mean	SEM	Probability[b]
Abdominal pain	0.031	0.520	0.615	0.050	Placebo	13.76	5.17	5.30	3.39	0.146
					CP2305	13.64	5.63	−5.36	3.54	0.156
Heavy stomach	0.096	0.569	0.728	0.055	Placebo	12.76	5.25	5.68	3.61	0.144
					CP2305	12.72	5.66	−5.62	3.71	0.158
Anorexia	0.041	0.749	0.671	0.071	Placebo	5.33	2.31	8.74	4.23	0.065
					CP2305	7.85	3.48	−4.38	4.74	0.377
Borborygmus	0.351	0.983	0.987	0.129	Placebo	12.78	4.53	4.84	6.33	0.460
					CP2305	23.38	5.80	−10.08	6.62	0.153
Colour tone	0.050	0.061	0.289	0.204	Placebo	8.79	3.22	1.98	2.20	0.385
					CP2305	13.12	3.98	−2.29	2.30	0.339
Defecation frequency	0.724	0.625	0.301	0.365	Placebo	14.18	4.09	6.10	6.50	0.366
					CP2305	15.09	5.01	−2.75	6.79	0.693
Abdominal fullness	0.513	0.834	0.806	0.396	Placebo	15.56	5.43	4.55	5.35	0.412
					CP2305	19.78	5.48	−2.25	5.58	0.694
Diarrhoea	0.945	0.973	0.628	0.633	Placebo	21.14	5.89	−0.45	7.28	0.952
					CP2305	23.53	7.00	−5.61	7.60	0.475
Abdominal discomfort	0.823	0.735	0.451	0.819	Placebo	18.43	4.53	−3.20	4.13	0.453
					CP2305	17.90	5.40	−1.81	4.31	0.682
Constipation	0.019	0.545	0.783	0.451	Placebo	16.02	5.56	−0.41	4.81	0.933
					CP2305	11.17	3.92	−5.90	5.23	0.280

[a] ANOVA with a mixed model for crossover design was applied.
[b] Welch's *t*-test was applied to compare the final and initial data for each ingestion period and each treatment over both experimental periods where the probability of the factor "Period" was not significant.

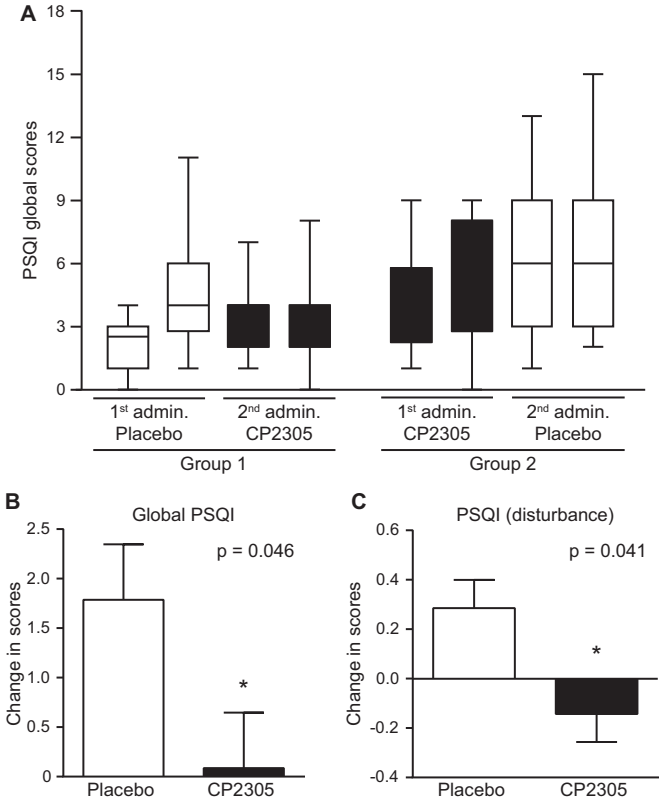

Fig. 3. Changes in sleep quality. (A) Time-dependent changes in PSQI-global scores. Scores before and after each experimental period are presented as box-and-whisker plots. Open and filled boxes represent placebo and CP2305 periods, respectively. Changes in PSQI-global scores (B) and PSQI-disturbance scores (C) are presented. Data are expressed as the mean ± SEM for the changes in the scores before and after treatments, panels (B) to (C). Open and filled bars represent data from the placebo and CP2305 periods, respectively. Statistical analysis was performed by mixed-model ANOVA for crossover design, and the *p* values indicate significant probabilities of the term "Treatment". In panels (B) and (C), the data from both groups are combined by treatment because the probability of the factor "Period" was not significant.

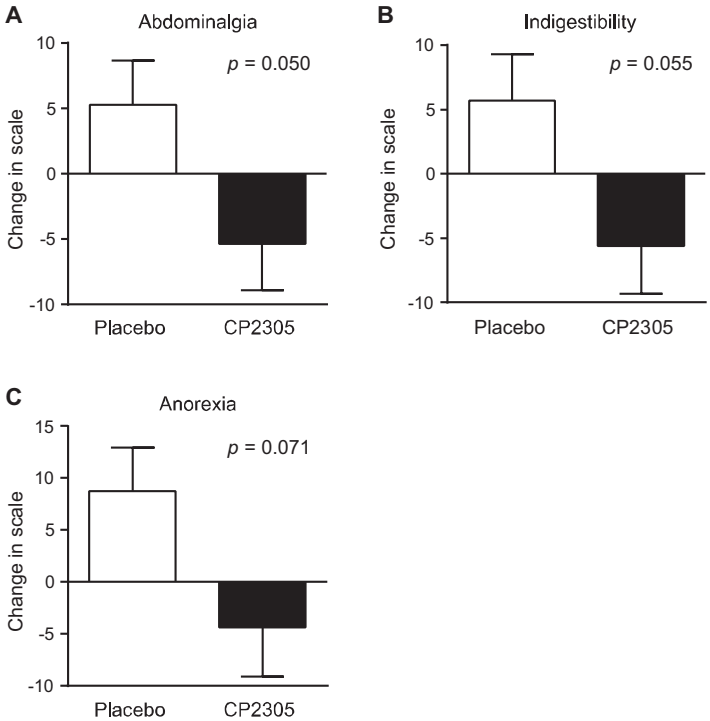

Fig. 4. Changes in abdominal symptom scores. Changes in the VAS scores for abdominalgia (A), indigestibility (B) and anorexia (C) are shown. Data are presented as the mean ± SEM for the changes in the scores before and after treatments. Open and filled bars represent placebo and CP2305 treatments, respectively. Statistical analysis was performed by mixed-model ANOVA for crossover design, and *p* values indicate significant probabilities of the term of "Treatment". Data from both groups were combined by treatment because the probability of the factor "Period" was not significant.

Table 3
ANOVA results for the changes in stress biomarker measurements before and after each intervention period in the crossover design.

Item measured	Probability for each factor[a]				Treatment	Initial values		Changes		
	Group	Individual	Period	Treatment		Mean	SEM	Mean	SEM	Probability[b]
Cortisol	0.529	0.350	0.620	0.023*	Placebo	0.64	0.10	0.12	0.09	0.203
					CP2305	0.68	0.07	−0.20	0.09	0.041*
Chromogranin A	0.014	0.043	0.613	0.081	Placebo	3.96	1.17	1.28	1.05	0.257
					CP2305	3.50	0.95	−1.51	1.05	0.167
Amylase	0.050	0.521	0.288	0.159	Placebo	158.40	26.55	114.86	60.82	0.073
					CP2305	185.30	54.05	−8.13	58.23	0.890

[a] ANOVA with a mixed model for crossover design was applied.
[b] Welch's *t*-test was applied to compare the final and initial data for each ingestion period and each treatment over both experimental periods when the probability of the factor "Period" was not significant.
* The probability was less than 0.05.

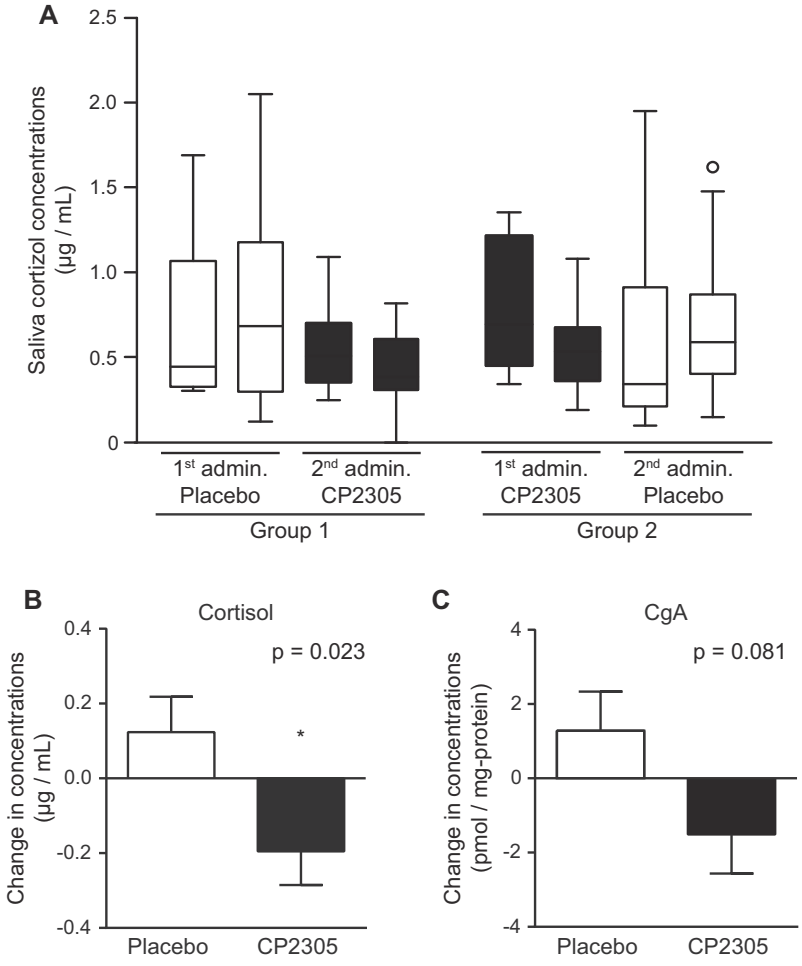

Fig. 5. Changes in concentrations of stress-related markers in saliva. (A) Time-dependent changes in salivary cortisol. Values before and after each experimental period are presented as box-and-whisker plots. Open and filled boxes represent the placebo and CP2305 treatments, respectively. Changes in the values of salivary cortisol (B) and chromogranin A (C) are represented. Data are presented as the mean ± SEM for the changes in the values before and after treatment in panels (B) and (C). Open and filled bars represent placebo and CP2305 treatments, respectively. Statistical analysis was performed by mixed-model ANOVA for crossover design, and *p* values indicate significant probabilities of the term of "Treatment". In panels (B) and (C), the data from both groups are integrated by treatment because the probability of the factor "Period" was not significant.

of the changes observed after the 4-week administration periods are summarized in Table 1. CP2305 tended to improve anxiety during the course, which was assessed using the two questionnaires STAI-state ($p = 0.040$; Fig. 2B) and HADS-anxiety ($p = 0.040$; Fig. 2C). Although CP2305 administration did not affect depressive mood as assessed by SDS, it significantly improved depressive mood, estimated by the HADS-depression score ($p = 0.020$; Fig. 2D).

3.2. Effects of CP2305 ingestion on stress-induced sleep disturbance

The box-whisker plots of global PSQI scores shown in Fig. 3A indicate that after starting the course, students taking placebo developed sleep disturbances that gradually progressed during the cadaver dissection course. CP2305 ingestion appeared to prevent the development of sleep disturbance (Fig. 3A). The daily intake of CP2305 significantly prevented increases in PSQI global

Table 4
ANOVA results for changes in the microbiota composition before and after each intervention period in the crossover design.

Bacterial group	Probability for each factor[a]				Treatment	Initial values (log cfu/g)		Changes (log cfu/g)		
	Group	Individual	Period	Treatment		Mean	SEM	Mean	SEM	Probability[b]
Enterobacteriaceae	0.157	0.744	0.925	0.018*	Placebo	6.73	0.26	0.87	0.30	0.007*
					CP2305	7.40	0.19	−0.22	0.30	0.465
Lactobacillus	0.363	0.708	0.260	0.073	Placebo	4.57	0.45	−0.42	0.30	0.297
					CP2305	4.58	0.34	0.65	0.41	0.127
Veillonella	0.0001	0.851	0.842	0.094	Placebo	2.71	0.15	1.16	0.49	0.029*
					CP2305	3.31	0.19	−0.07	0.49	0.891
Eubacterium	0.240	0.548	0.888	0.257	Placebo	9.56	0.13	0.16	0.18	0.407
					CP2305	9.41	0.13	−0.13	0.17	0.430
Megasphaera	0.155	0.501	0.329	0.329	Placebo	2.32	0.32	0.02	0.01	0.994
					CP2305	2.33	0.33	0.00	0.01	0.368
B. fragilis group	0.898	0.995	0.185	0.364	Placebo	9.05	0.28	0.47	0.35	0.197
					CP2305	9.01	0.18	0.01	0.35	0.985
Staphylococcus	0.687	0.385	0.685	0.445	Placebo	2.71	0.15	0.07	0.24	0.769
					CP2305	3.33	0.19	−0.20	0.24	0.430
M. hypermegas	0.261	0.501	0.431	0.519	Placebo	2.56	0.39	0.004	0.52	0.994
					CP2305	3.00	0.47	−0.48	0.52	0.368
Bacteroidaceae	0.228	0.0001	0.062	0.548	Placebo	9.93	0.14	0.19	0.08	0.024*
					CP2305	9.79	0.14	0.27	0.08	0.005*
Clostridium (Lecithinase-)	0.0003	0.535	0.521	0.666	Placebo	8.01	0.44	0.03	0.28	0.914
					CP2305	8.73	0.20	−0.13	0.24	0.595
Enterococcus	0.024	0.028	0.308	0.788	Placebo	6.49	0.31	0.04	0.27	0.871
					CP2305	6.63	0.32	0.15	0.27	0.588
Bacillus	0.981	0.808	0.099	0.651	Placebo	2.44	0.22	0.43	0.52	0.416
					CP2305	2.75	0.37	0.09	0.52	0.858
Yeast	0.860	0.398	0.335	0.713	Placebo	2.59	0.17	0.41	0.18	0.040*
					CP2305	2.65	0.18	0.31	0.20	0.148
Bifidobacterium	0.641	0.637	0.210	0.751	Placebo	9.70	0.13	−0.03	0.18	0.856
					CP2305	9.43	0.11	0.05	0.18	0.789
Clostridium (Lecithinase +)	0.300	0.005	0.742	0.870	Placebo	2.95	0.35	−0.13	0.45	0.780
					CP2305	3.45	0.36	−0.02	0.45	0.963
P. aeruginosa	0.491	0.601	0.212	0.912	Placebo	2.08	0.07	0.05	0.05	0.272
					CP2305	2.06	0.04	0.06	0.05	0.213

[a] ANOVA with a mixed model for crossover design was applied.
[b] Welch's *t*-test was applied to compare the final and initial data for each ingestion period and each treatment over both experimental periods where the probability of the factor "Period" was not significant.
* The probability was less than 0.05.

scores ($p = 0.046$; Fig. 3B). The global PSQI score is the sum of seven component scores (sleep disturbance, overall sleep quality, sleep latency, duration of sleep, daytime dysfunction due to sleepiness, sleep efficiency, and need for medicines to sleep). Among them, CP2305 significantly improved the PSQI-disturbance score ($p = 0.041$; Fig. 3C).

3.3. Effects of CP2305 ingestion on abdominal symptoms and salivary stress markers

All initial scores for abdominal symptoms and their changes before and after intervention are summarized in Table 2. Compared with the placebo group, the CP2305-treated group exhibited improvements in abdominalgia ($p = 0.050$), feelings of indigestibility ($p = 0.055$) and anorexia ($p = 0.071$; Fig. 4).

In addition to the above self-reported measurements, we assessed the effects of CP2305 by measuring salivary stress markers (cortisol, CgA, and α-amylase). All initial values and their changes before and after intervention are listed in Table 3. As shown in Fig. 5A, CP2305 intake appeared to reduce salivary cortisol levels in group 1 and group 2 during the second and first administration periods, respectively. In fact, CP2305 administration significantly reduced salivary cortisol levels compared with the levels before the intervention period ($p = 0.041$), and the changes in those values between the CP2305 and placebo groups were significantly different ($p = 0.023$; Fig. 5B). At the same time, CP2305 tended to suppress the increase in CgA levels observed in the placebo group ($p = 0.081$; Fig. 5C). Ingestion tended to increase salivary α-amylase release in the placebo group ($p = 0.073$), whereas this trend was not present in the CP2305-treated group, although the difference between the treatments was not significant ($p = 0.159$; Table 3).

3.4. Effects of CP2305 ingestion on faecal microbiota

Sixteen groups of microorganisms were identified by their growth on selective medium, colony shape, and Gram staining as follows: *Bacillus*, *Bacteroides fragilis* group, Bacteroidaceae, *Bifidobacterium*, *Clostridium* (Lecithinase+), *Clostridium* (Lecithinase-), Enterobacteriaceae, *Enterococcus*, *Eubacterium*, *Lactobacillus*, *Megamonas hypermegas*, *Megasphaera*, *Pseudomonas aeruginosa*, *Staphylococcus*, *Veillonella*, and yeasts. All of the changes are summarized in Table 4. Among these taxa, Enterobacteriaceae and *Veillonella* were significantly increased after intervention only in samples from the placebo subjects ($p = 0.007$, $p = 0.029$, respectively; Table 4). However, the increase in Enterobacteriaceae was significantly inhibited in the CP2305 group compared with the placebo group ($p = 0.018$; Fig. 6A). Tendencies towards increased *Lactobacillus* and suppressed increases in *Veillonella* were also observed ($p = 0.073$, $p = 0.094$, respectively; Fig. 6B and C).

3.5. Changes in gene expression in peripheral blood leukocytes

Finally, we tested whether stress-related gene expression signatures could be detected in peripheral blood leukocytes in the placebo group, and we examined how CP2305 administration

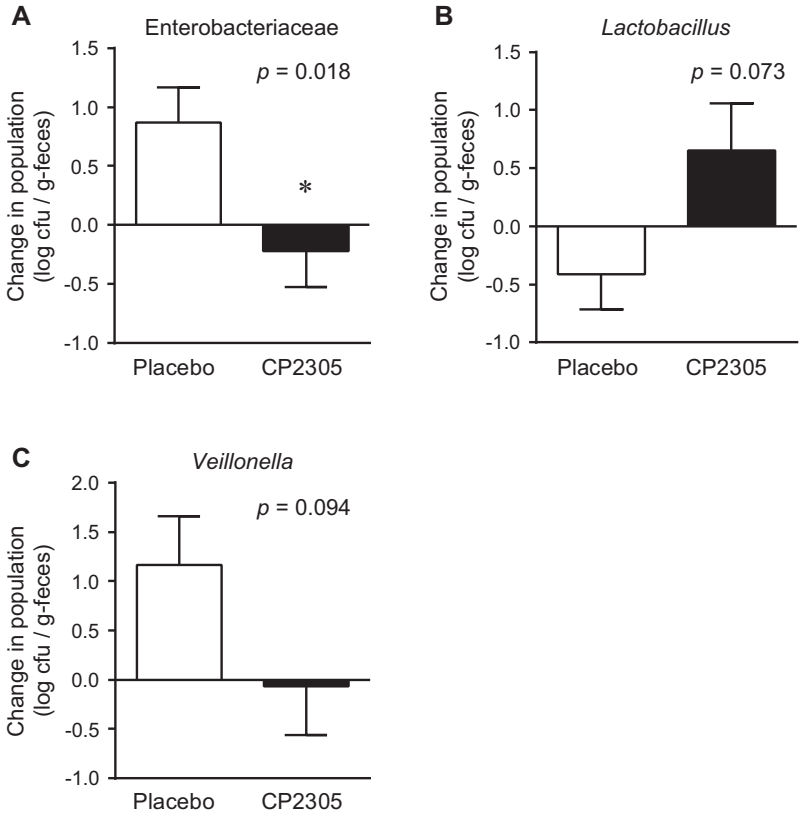

Fig. 6. Changes in the composition of the faecal microbiota. Changes in the populations of faecal Enterobacteriaceae (A), *Lactobacillus* (B) and *Veillonella* (C) are presented. Data are expressed as the mean ± SEM of the change in each population before and after treatment. Open and filled bars represent the placebo and CP2305 periods, respectively. Statistical analysis was performed by mixed-model ANOVA for crossover design, and *p* values indicate significant probabilities of the term of "Treatment". Data from both groups in the same treatment were integrated for evaluation because the probability of the factor "Period" was not significant.

modified these signatures. Individual variations in gene expression prior to administration significantly affected the assessment of gene expression changes in a limited number of subjects. We therefore compared the gene expression profile of each student before and after the administration of CP2305 or placebo.

The students in the placebo and CP2305 groups exhibited significant changes in the expression levels of 829 and 1083 genes, respectively. Among them, the expression of 327 genes changed in the same directions in both the placebo- and CP2305-treated groups. The 829 and 1083 affected genes were subjected to pathway analysis using IPA. As shown in Fig. 7A (left panel), IPA listed the top 5 canonical pathways related to the 829 affected genes in the placebo group as follows: (1) EIF2 Signalling ($p = 2.86E{-}14$), (2) mTOR Signalling (1.38E−06), 3) Regulation of eIF4 and p70S6K Signalling (1.57E−06), 4) TCA Cycle II (1.37E−04), and 5) Mitochondrial Dysfunction (1.79E−04). The 4-week administration of CP2305 significantly changed the expression of 1083 genes in total, and the top 5 canonical pathways related to the 1083 genes were the following: (1) TCA Cycle II (6.20E−04), (2) ERK5 Signalling (8.17E−4), (3) Hypoxia Signalling in the Cardiovascular System (1.05E−3), (4) Superpathway of Inositol Phosphate Compounds (5.48E−3), and (5) Lipid Antigen Presentation by CD1 (7.32E−03) (Fig. 7A, right panel). Considering the *p values* of the listed canonical pathways, the most prominent changes in gene expression were related to the EIF2 pathway in the placebo group. As shown in Fig. 7B (left panel), most of the EIF2-related genes were down-regulated in the placebo group. Notably, CP2305 administration prevented the down-regulation of EIF2-related genes (Fig. 7B, right panel).

4. Discussion

We report here that a unique enteric-colonizing strain, CP2305, exerted beneficial effects on physical and mental states of preclinical medical students enrolled in a cadaver dissection course. Similar to medical students in other countries, the students in the present study were expected to begin the cadaver dissection course under emotional stress. Unexpectedly, they reported only mild anxiety, as assessed by the questionnaires. Thus, our students may be able to rapidly cope with the stressful situation. However, compared with placebo administration, CP2305 administration resulted in a small but significant reduction of both STAI-state and HADS-anxiety scores. Because the course is mentaly demanding, our students progressively complained of abdominal pain and sleep disturbance, particularly in the placebo group. Notably, CP2305 administration significantly improved sleep quality. Sleep quality is an outcome that comprehensively reflects both physical and psychological conditions (Mollayeva et al., 2016). The beneficial effect of CP2305 on sleep quality is indicative of the potential benefits of this strain for health promotion.

The beneficial effects of CP2305 ingestion were confirmed not only subjectively by self-reported questionnaires but also objectively by the assessment of salivary cortisol release and gene expression profiling in peripheral blood leukocytes. Measurements of salivary stress markers reflect stress responses via both the HPA axis and the autonomic nerve system. Salivary cortisol, which is a well-known stress marker, is secreted via the activation of the HPA axis in response to psychological stress (Kirschbaum & Hellhammer, 1994). CgA and α-amylase are released in response to activation of

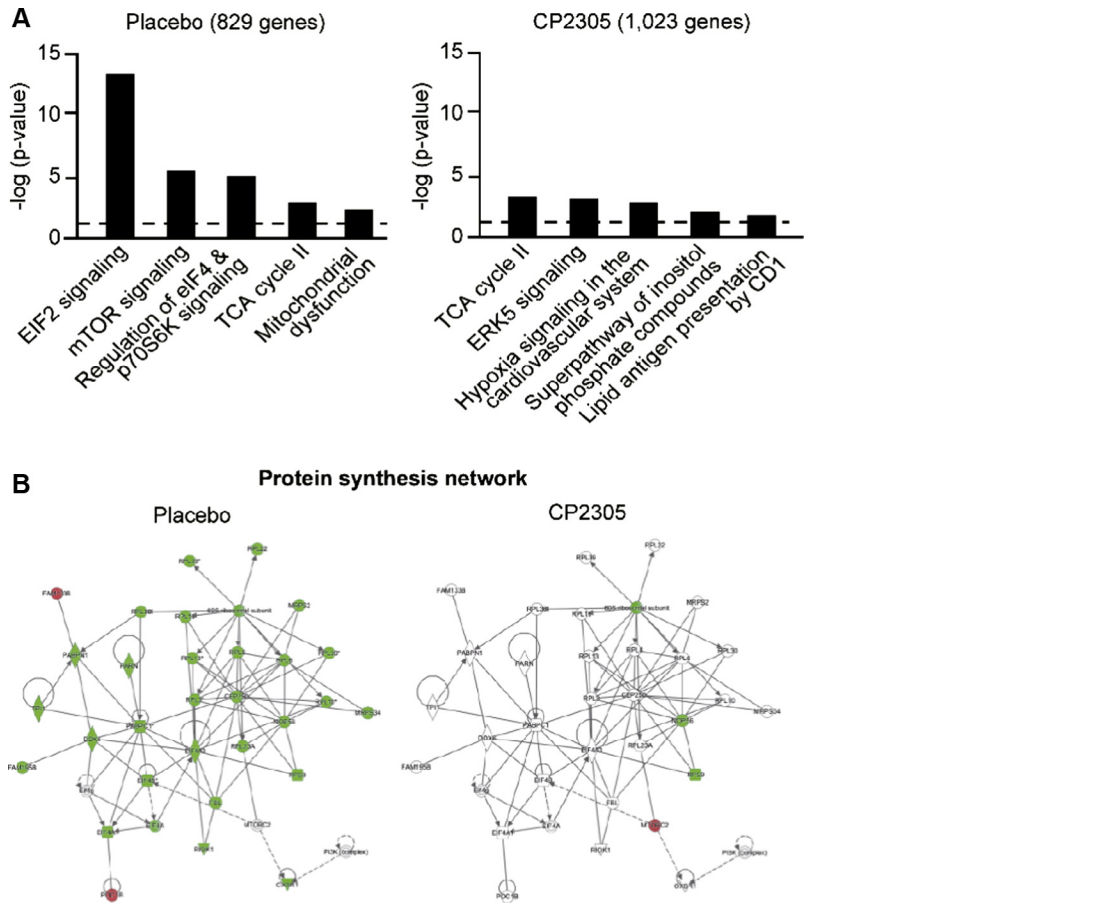

Fig. 7. Changes in gene expression profiles of peripheral blood leukocytes. Changes in gene expression in peripheral blood leukocytes were measured using a microarray before and after the 4-week administration of placebo or CP2305. The cadaver dissection course significantly changed the expression levels of 829 and 1083 genes in the placebo and CP2305 groups, respectively. (A) IPA analysis listed the top 5 canonical pathways related to the 829 and 1083 affected genes. Dotted lines show the level of significance ($p = 0.05$ by Fisher's exact test). (B) IPA listed "protein synthesis network" as the top network related to the 829 affected genes in the placebo group (left panel). Changes in these network-related genes in the CP2305 group are shown in the right panel. Up-regulated and down-regulated genes are shown in red and green, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the sympathetic nervous system (Nater & Rohleder, 2009). The reduction of salivary cortisol concentrations by the administration of CP2305 suggests that CP2305 suppressed the activation of the HPA axis. CP2305 ingestion also tended to suppress CgA and α-amylase levels and may affect autonomic nerve activity.

We employed transcriptome analysis of peripheral blood leukocytes to assess gene expression signatures associated with the beneficial effects of CP2305. Placebo-treated students showed a prominently reduced expression of EIF2-related genes, and this reduction was prevented by CP2305 administration. The observed modification of the EIF2-signal pathway by CP2305 administration is informative. In response to diverse stress stimuli, eukaryotic cells activate a common adaptive pathway, termed the integrated stress response, to restore cellular homeostasis. The core event in this pathway is the phosphorylation of eIF2α, which leads to a decrease in global protein synthesis and the induction of selected genes that together promote cellular recovery (Pakos-Zebrucka et al., 2016). Recently, we showed that patients with IBS preferentially down-regulated a group of genes related to the EIF2 signal and that daily intake of CP2305 was able to recover the expression of these genes, improving IBS severity and health-related worry (Nobutani et al., 2017). Given that IBS symptoms are also closely associated with psychosocial stress, the results of this previous study and the present study suggest that CP2305 may exert stress-relieving effects at least partially by maintaining EIF2 signal-related gene expression.

In the faecal microbiota, cadaver dissection significantly increased populations of Enterobacteriaceae and *Veillonella* in the placebo group, whereas CP2305 ingestion completely prevented the increases in these well-known inflammatory microbes (Lupp et al., 2007; Said et al., 2014). It has been suggested that exaggerated psychological stress induces inflammatory responses in the host, which in turn disrupt the intestinal microbiota and promote the overgrowth of Enterobacteriaceae and *Veillonella*. Thus, it is possible that the daily intake of CP2305 may correct the psychological stress-related imbalance in faecal microbiota.

Elucidating the mechanism for the stress-relieving action of CP2305 is an important next step. *Lactobacillus gasseri* strains, including CP2305, can colonize the human digestive tract (Fujiwara, Seto, Kimura, & Hashiba, 2001; Sawada et al., 2016). CP2305 efficiently regulates intestinal functions, even when this strain is administered as sterilized, washed bacterial cells (Sugawara et al., 2016). Moreover, elevated parasympathetic nerve activity has been observed after the continuous ingestion of sterilized CP2305 cells (Sugawara et al., 2016). These unique abilities of CP2305 imply that one or more bacterial cell components may provoke gut signalling by directly interacting with the intestinal mucosal epithelium or with other cells. These interactions may effectively regulate higher-order functions of the digestive tract and induce various biological responses. As a result, CP2305 may efficiently stimulate the brain-gut axis and influence the stress response. The improvements in physical and mental states in our

students in the cadaver dissection course suggest that this strain may sufficiently induce gut signals to produce effects that reach the central nervous system via the gut-brain axis. Further studies to test our hypothesis are currently underway.

In conclusion, CP2305 was demonstrated to exert beneficial effects on medical students under stressful conditions. Our results suggest that *L. gasseri* CP2305 has potential benefits for health promotion for mentally and physically distressed subjects.

Conflicts of interest

The authors declare that they have no conflicts of interest pertaining to this study.

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