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Para-psychobiotic *Lactobacillus gasseri* CP2305 ameliorates stress-related symptoms and sleep quality

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Keywords
faecal microbiota, *Lactobacillus gasseri* CP2305, para-psychobiotics, sleep quality, stress-relieving effects.

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Abstract

Aims: To confirm the stress-relieving effects of heat-inactivated, enteric-colonizing *Lactobacillus gasseri* CP2305 (paraprobiotic CP2305) in medical students taking a cadaver dissection course.
Methods and Results: Healthy students (21 males and 11 females) took paraprobiotic CP2305 daily for 5 weeks during a cadaver dissection course. The General Health Questionnaire and the Pittsburgh Sleep Quality Index were employed to assess stress-related somatic symptoms and sleep quality respectively. The aggravation of stress-associated somatic symptoms was observed in female students ($P = 0{\cdot}029$). Sleep quality was improved in the paraprobiotic CP2305 group ($P = 0{\cdot}038$), particularly in men ($P = 0{\cdot}004$). Among men, paraprobiotic CP2305 shortened sleep latency ($P = 0{\cdot}035$) and increased sleep duration ($P = 0{\cdot}048$). Diarrhoea-like symptoms were also effectively controlled with CP2305 ($P = 0{\cdot}005$) in men. Thus, we observed sex-related differences in the effects of paraprobiotic CP2305. In addition, CP2305 affected the growth of faecal *Bacteroides vulgatus* and *Dorea longicatena*, which are involved in intestinal inflammation.
Conclusions: CP2305 is a potential paraprobiotic that regulates stress responses, and its beneficial effects may depend on specific cell component(s).
Significance and Impact of the Study: This study characterizes the effects of a stress-relieving para-psychobiotic in humans.

Introduction

Several lines of evidence suggest that certain lactic acid bacteria significantly modulate communication in the gut–brain axis through neural, endocrine and immune signalling pathways (Cryan and Dinan 2012), allowing them to alter stress sensitivity (De Palma *et al.* 2014). Studies employing experimental animals have demonstrated that the administration of specific probiotics eases stress-induced glucocorticoid and inflammatory cytokine responses in conjunction with the reduction of depression- and anxiety-related behaviours (Gareau *et al.* 2007; Bravo *et al.*, 2011; Bercik *et al.* 2011). In clinical trials, certain probiotics have alleviated psychological distress in healthy subjects by normalizing stress-induced reductions in natural killer cell numbers (Marcos *et al.* 2004) and gastrointestinal symptoms (Diop *et al.* 2008). However, the scientific evidence supporting this concept remains insufficient, particularly in humans.

There is considerable variety in the genetic diversity and microbial properties of probiotic microbes, particularly in terms of their physiological functions, which vary from genus to genus, from species to species and even between strains (Million *et al.* 2012). Probiotics were originally defined by Fuller (1989) and then redefined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Joint Food and Agriculture Organization of the United Nations/World Health Organization, 2016) based on research investigating the underlying mechanism. Furthermore, there is increasing evidence that nonviable microbial cells, cell lysates, semipurified microbial fractions or purified substances exert beneficial effects on human and animal health as "biogenics" (Mitsuoka 2014)

or "paraprobiotics" (Taverniti and Guglielmetti 2011). The underlying mechanisms of these paraprobiotic effects suggest a direct interaction between host cells and bacterial components or products (Taverniti and Guglielmetti 2011). Usage of these bacterial cell components or products derived from them has notable advantages for the development of safer and more stable products for industrial use (De Almada *et al.* 2016).

To elucidate the exact working mechanism(s) of beneficial microbes during stress relief, it is important to clarify which microbe-derived bioactive compound(s) exert stress-relieving effects. We are particularly interested in the probiotic *Lact. gasseri* strain CP2305 (CP2305). The unique CP2305 strain colonized the digestive tracts of *ca.* 40% of volunteers after three-time oral administration at a dose of $1{\cdot}0 \times 10^{11}$ CFU (Sawada *et al.* 2016). This strain had an antiflatulent effect, especially in constipated volunteers, and significantly changed the microbiota composition. Similar to live CP2305, heat-inactivated CP2305 improved bowel habits in healthy volunteers by correcting the balance between sympathetic and parasympathetic nerve activities (Sugawara *et al.* 2016). Thus, CP2305 may effectively modulate brain–gut axis activity and exert stress-relieving effects. Indeed, we reported that CP2305 relieves stress in healthy young adults under stressful conditions (Sawada *et al.* 2017) and improves the clinical symptoms of irritable bowel syndrome (IBS) (Nobutani *et al.* 2017). Moreover, we considered the possibility that bacteria-derived bioactive compound(s) exert the stress-relieving effects induced by CP2305 without requiring bacterial metabolic activity or product release. To investigate this hypothesis, we performed the current study to examine the effects of heat-inactivated CP2305 (paraprobiotic CP2305) in the form of a fermented milk-based beverage, using a beverage prepared from lactic acid-acidified, nonfermented milk as the placebo.

A brief, naturalistic stress, such as an academic examination, is a useful model for the investigation of physical and psychological stress responses in healthy subjects. However, examination stress is an acute stress model that activates the hypothalamus–pituitary–adrenal (HPA) axis for only a short period of time (Kurokawa *et al.* 2010; Kamezaki *et al.* 2012). To clarify the beneficial effects of probiotic CP2305 on stress-related behaviours under more stressful situations that last for longer periods of time, we employed medical students taking a cadaver dissection course as described in a previous study (Sawada *et al.* 2017), although there were two major differences. First, the present experimental design employs a parallel competitive design rather than a crossover design (Sawada *et al.* 2017) to eliminate carry-over effects. Second, the previous study included only male students, while both male and female students were employed in the present study to examine sexual differences. Thus, we performed this study to confirm the stress-relieving effects of the heat-inactivated paraprobiotic CP2305 in a double-blinded, placebo-controlled and parallel-group comparison trial as a first step.

Materials and methods

Supplementary beverages

Two types of acid milk beverages were prepared: a beverage containing sterilized CP2305-fermented milk and a placebo beverage. The constituents of the beverages are shown in Table 1. The placebo beverage was prepared from a bacteria-free, artificial sour milk base by adding flavouring and granulated sugar and performing acidification with lactic acid. The test and placebo beverages were indistinguishable based on an organoleptic examination performed by test panels. Both beverages were canned as 190-g (daily intake dosage) doses in 200-ml containers. The CP2503-containing test beverage contained 1×10^{10} bacterial cells per container. The primary CP2305-fermented milk was prepared with base constituents of 10% (w/w) skimmed milk plus 0·25% (w/w) yeast extract at 37°C for 18 h. After preparation of the final mixtures of the test beverages, including the intermediate materials and fermented milk, final sterilization was performed using a continuous sterilizer at 95°C for 30 s. No living bacteria were detected in either the test beverage or the placebo beverage.

Experimental design

We recruited 32 subjects who were second-year undergraduate medical students at Tokushima University, Tokushima, Japan. The students included both sexes (21 males and 11 females) and were 18–34 years of age. Participants were not habitual smokers and did not take any medication for 3 months prior to enrolment. None of the students had

Table 1 Constituents of the beverages

	Beverages	
Constituent (w/w %)	CP2305	Placebo
CP2305-fermented milk	52·63	0
Skim milk powder	0	5·263
Yeast extract	0	0·0132
Granulated sugar powder	8	8
Stabilizer	0·35	0·35
Lactate (50%)	1·012	1·137
Sodium citrate	0·1842	0·1842
Yoghurt flavour	0·1	0·1
Water	37·7238	84·9526
Total	100	100

psychological or physical disorders or milk or other food allergies (Table 2). All students took the cadaver dissection course from September 4 to December 12. The students were randomly divided into two groups: participants allocated into one group consumed the placebo beverage, and participants in the other group consumed the test beverage containing heat-inactivated CP2305. They consumed the provided beverage daily for 5 weeks from September 4 to October 9, 2007 (Fig. 1). Daily consumption was self-recorded in a diary to verify the compliance rate of ingestion. During the trial, the subjects complied with dietary restrictions by avoiding 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 performed in accordance with the ethical standards described in the 1964 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 subjects prior to enrolment.

Table 2 Characteristics of the participants

		Initial scores	
Questionnaire	Treatment	Mean	SEM
GHQ28			
Total score	Placebo	17·88	1·39
	CP2305	22·56	1·88
HADS			
Depression	Placebo	4·75	0·62
	CP2305	6·25	0·81
Anxiety	Placebo	5·00	0·58
	CP2305	6·06	0·89
STAI			
State anxiety	Placebo	37·88	2·61
	CP2305	43·81	2·69
Trait anxiety	Placebo	41·50	2·82
	CP2305	46·50	2·81
PSQI			
Sleep habit (global)	Placebo	4·38	0·52
	CP2305	5·43	0·68
Age		Mean	SEM
	Placebo	21·31	0·90
	CP2305	20·75	0·40
Sex		(M/F)	
	Placebo	(10/6)	
	CP2305	(11/5)	

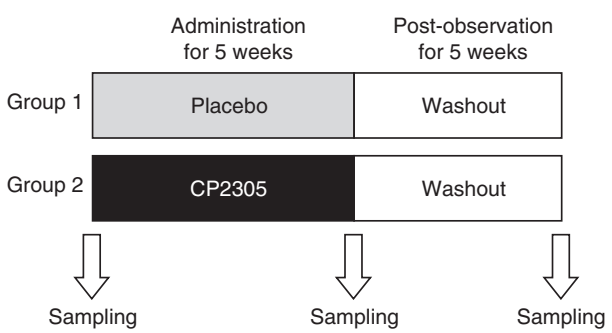

Figure 1 Examination schedule. Participants were divided into two groups. Participants in group 1 received the placebo, and those in group 2 received CP2305. Biological samples and questionnaire responses were obtained at the start and end of each experimental period and after a postobservation period of 5 weeks.

Questionnaires to assess mental and physical status

The physical and mental health of the participants were assessed using the following questionnaires: GHQ-28, the 28-item General Health Questionnaire (Goldberg and Hillier 1979); Zung-SDS, the Zung Self-rating Depression Scale (Zung 1965); HADS, the Hospitality Anxiety and Depression Scale (Zigmond and Snaith 1983); and STAI, the Spielberger State-Trait-Anxiety-Inventory (Kvaal *et al.* 2005). Sleep was evaluated using the Pittsburgh Sleep Quality Index (PSQI) (Buysse *et al.* 1989), which assesses sleep quality and disturbances over a 5-week time interval. These questionnaires were given to the subjects at the time of saliva sampling. We analysed the differences before and after the intervention using the independent *t*-test. The level of significance was set at a P value of 0·05. When determining the significance of sex differences, two-way analysis of variance was stratified to describe the background.

Questionnaires to assess gastrointestinal symptoms and eating disturbances

The gastrointestinal symptoms of the participants were assessed using a 100-mm visual analogue scale (VAS) concerning abdominalgia, feeling of indigestibility, anorexia, borborygmus, abdominal distension, abdominal discomfort, diarrhoea, constipation, frequency of defecation and colour features of faeces. Eating behaviour was also assessed using the Eating Attitudes Test with 26 items (EAT-26), a self-reported measure of eating disorder symptoms that is widely used to screen and measure the symptoms and characteristics of eating disorders (Garner *et al.* 1982). We analysed the differences before and after the intervention using the independent *t*-test. The comparison method chosen for analysis depended on the distribution of the data. The level of significance was set at a P value of 0·05. When determining the significance of sex differences, two-way analysis of variance was also applied to describe the background in detail.

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 was stored at −80°C until analyses were performed. Salivary chromogranin A (CgA), cortisol, and alpha-amylase were assayed using kits (YK070 Human CgA EIA kit, Yanaihara Institute, Shizuoka, Japan; cortisol EIA kit and alpha-amylase assay kit, Salimetrics Inc., LLC, Carlsbad, CA, USA). We analysed the differences before and after the intervention using the independent *t*-test. The level of significance was set at a P value of 0·05.

Analysis of the faecal microbiota

The faecal microbiota was analysed using faecal bacterial 16S rRNA V6–V8 region-targeted pyrosequencing. Faecal samples (1–2 g) were collected in sterilized plastic tubes by the patients. A pyrosequencing-based analysis based on the method described by Nakayama (Nakayama 2010) was performed to assess the compositional changes in the faecal microbiota. Briefly, whole bacterial DNA was extracted from 100 mg of faeces. Bacterial cells were lysed by beating with zirconium beads in a 100 mmol l^{-1} Tris-HCl buffer (pH 9·0) containing 40 mmol l^{-1} EDTA and 1% sodium dodecyl sulphate. Bacterial DNA was first extracted using a phenol/chloroform/isoamyl alcohol mixture and then precipitated with a polyethylene glycol solution. Purified DNA was used as a template for the following two-step polymerase chain reaction (PCR) procedure. In the first round of PCR, the V6–V8 fragment of 16S rDNA was amplified with the 968F and 1390R primers without a barcode tag. The second round of PCR was performed to attach a sample ID tag using primers containing four-base barcode sequences. The amplicon was purified, and its concentration was measured using an IMPLEN NanoPhotometer (Implen GmbH, Munich, Germany). Equal amounts of amplicons derived from different samples were subjected to oil emulsion PCR to produce millions of copies of clonal DNA fragments captured on fine beads. The beads were spread onto a pico-titre plate to include one bead per well. Pyrosequencing reactions were performed in each well to generate sequential luminescence that was monitored using a CCD camera. A one million-well titre plate containing 2 × 1/8 regions was used to analyse 128 samples. Pyrosequencing was performed using a Roche GS FLX (Roche Diagnostics Japan, Tokyo, Japan). Individual changes in the per cent composition of identified bacteria before and after treatment were compared using Welch's *t*-test. Next, a discrimination analysis was performed to assess the significance using JMP11 Pro (SAS Institute Japan Inc., Tokyo, Japan).

Results

Mental status and sex differences

Paraprobiotic CP2305 significantly suppressed the aggregation of physical and mental health assessed by changes in total GHQ-28 scores (Fig. 2a). The scores remained unchanged changed in the paraprobiotic CP2305 group, while the average scores changed from 17·9 to 20·3 in the placebo group (P = 0·380; Fig. 2a). Although there was no significant change in the GHQ-28 total score when all subjects were included, significant improvement with paraprobiotic CP2305 ingestion was observed in females (P = 0·046) (Fig. 2c). Two-way ANOVA indicated a significant probability of the "interaction of the treatment and sex" (P = 0·022), suggesting significant differences in the responses to paraprobiotic CP2305 between males and females. Paraprobiotic CP2305 significantly suppressed the aggregation of somatic symptom scores of the GHQ-28. The average score changed from 4·31 to 4·20 in the paraprobiotic CP2305 group, while the placebo group exhibited a change from 3·88 to 4·06 (P = 0·029; Fig. 2b). By sex, the improvement of somatic symptoms assessed with the GHQ-28 was significant in females (P = 0·042; Fig. 2d) but not in males (P = 0·340; Fig. 2d). Two-way analysis of variance (two-way ANOVA)-stratified analysis showed that the significances of the factors of 'treatment', 'sex' and 'interaction of the two factors' were P = 0·007, P = 0·049 and P = 0·062 respectively. Significant changes in the other components of the GHQ-28, namely, 'anxiety and insomnia', 'social disorders' and 'severe depression', were not observed.

Paraprobiotic CP2305 administration significantly improved sleep quality as evaluated by changes in global PSQI scores (P = 0·038; Fig. 3a and d). The scores changed from 5·44 to 4·04, which were close to the threshold value, in the paraprobiotic CP2305 group. The improvement in the PSQI scores was observed in male students (P = 0·004; Fig. 3d) but not in females (P = 0·760; Fig. 3d). In stratified analysis of the males, of the seven components of the PSQI, paraprobiotic CP2305 significantly improved the 'latency' (P = 0·035, Fig. 3e) and 'duration' (P = 0·048, Fig. 3f) items. No significant differences were observed in the other questionnaires.

Abdominal symptoms and salivary stress markers

We assessed stress-related abdominal symptoms using questionnaires. While analyses that included all subjects or only females did not show particular trends for the tendency of diarrhoea (P = 0·390, P = 0·240 respectively;

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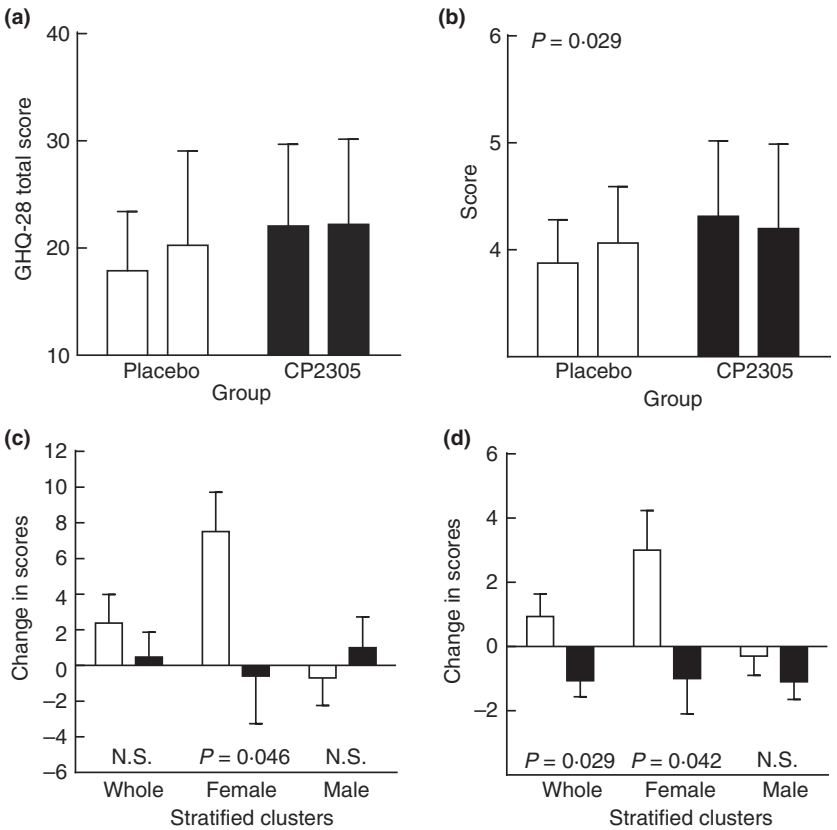

Figure 2 Changes in stress-related behaviours as determined by questionnaires. (a) Changes in the GHQ-28 total scores are shown. Scores before (left bar) and after each experimental period (right bar) in each group are presented as the mean ± SE. (□) Open and (■) filled bars represent the placebo and CP2305 treatments respectively. (b) Changes in the GHQ-28 somatic symptom scores induced by stress are shown. Differences in the GHQ-28 total scores (c) and the GHQ-28 somatic symptom scores (d) before and at the end of each treatment are denoted. Data are presented as the mean ± SE of the changes in the scores before and after treatment in panels (c) and (d). (□) Open and (■) filled bars represent the placebo and CP2305 treatments respectively. Statistical analysis was performed using ANOVA for the time-dependent changes in panels (a) and (b) and Welch's *t*-test for the difference data in panels (c) and (d). The *P* values indicate significant probabilities.

Fig. 4a and b), paraprobiotic CP2305 administration effectively suppressed the tendency of diarrhoea among male subjects during the cadaver dissection course. The scores changed from 4·61 to 2·07 in the paraprobiotic CP2305 group ($P = 0·005$; Fig. 4b).

No significant differences were observed between the CP2305 and placebo groups for salivary stress markers (cortisol, CgA, and alpha-amylase).

Effects of paraprobiotic CP2305 ingestion on faecal microbiota

Changes in the proportions of faecal bacteria were significantly different between the placebo and CP2305 groups for one phylum (*Bacteroidetes*), two specific genera (*Corynebacterium* and *Raoultella*) and 15 specific species (*Bact. vulgatus, Barnesiella intestinihominis, Bifidobacterium bifidum, Cl. hathewayi, Cl. irregular, Cl. ruminantium, Cl. straminisolvens, Eggerthella sinensis, Eubacerium plautii, Hesperia porcina, Lact. animalis, Marvinbryantia formatexigens, Robinsoniella peoriensis, Streptococcus agalactiae* and *Weissella confusa*) as determined by phylum-level, genus-level and species-level analyses respectively. Sex differences were not observed in these analyses.

Among the changes in bacterial group proportions at the phylum level, only that of Bacteroidetes was significantly decreased in the paraprobiotic CP2305 group ($P = 0·040$; Fig. 5a). Actinobacteria and Firmicutes appeared to change but not significantly because of their wide dispersion. Next, discrimination analyses using genus-level data, in which differences in genera with probabilities of less than 0·229 were incorporated, provided a clear discriminant criterion ($P = 0·024$; Fig. 5b). Figure 5b shows the changes in the occupancy rates for two species of bacteria, *Bact. vulgatus* and *Dorea longicatena*, that greatly contributed to this discrimination along with the canonical species. *Bact. vulgatus* occupancy was significantly decreased ($P = 0·022$), while that of *Dorea longicatena* was increased ($P = 0·054$) in the paraprobiotic CP2305 group (Fig. 5c).

Discussion

Recently, we reported that live, enteric-colonizing CP2305 relieved stress-associated behaviours in healthy medical students taking a cadaver dissection course (Sawada *et al.* 2017) and clinical symptoms in patients with IBS (Nobutani *et al.*, 2017). Here, we show for the first time that heat-inactivated paraprobiotic CP2305 also exhibits favourable effects on physical symptoms and sleep quality in medical students during the same course. In this study, paraprobiotic CP2305 did not significantly improve STAI

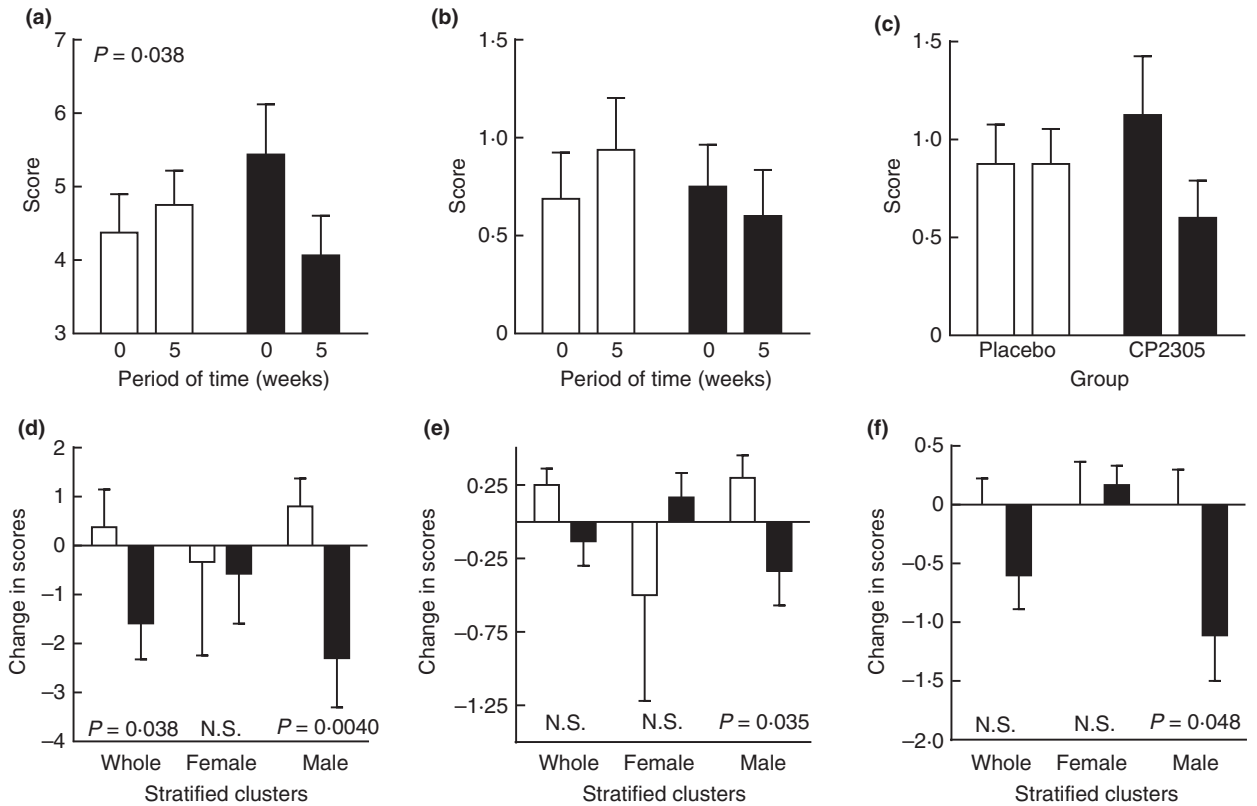

Figure 3 Changes in sleep quality. (a) Time-dependent changes in PSQI global scores. Scores before and after each experimental period are presented as the mean ± SE. (□) Open and (■) filled bars represent the placebo and CP2305 treatments respectively. In each group, the left bar shows the mean score before treatment, and the right bar represents the mean score after treatment. Changes in the PSQI sleep latency scores (b) and the PSQI sleep duration scores (c) are presented. Data are expressed as the mean ± SE for the scores before and after treatment for panels (a), (b) and (c). (□) Open and (■) filled bars represent data from the placebo and CP2305 treatments respectively. Statistical analysis was performed using ANOVA, and the p values indicate significant probabilities. Differences in the PSQI global scores (c), the PSQI sleep latency (d) and the PSQI sleep duration (e) before and end of each treatment are denoted. Data are presented as the mean ± SE of the changes in the scores before and after treatment. (□) Open and (■) filled bars represent the placebo and CP2305 treatments respectively. Statistical analysis was performed using Welch's *t*-test for the difference data in panels (d), (e) and (f), and the p values indicate the significant probabilities.

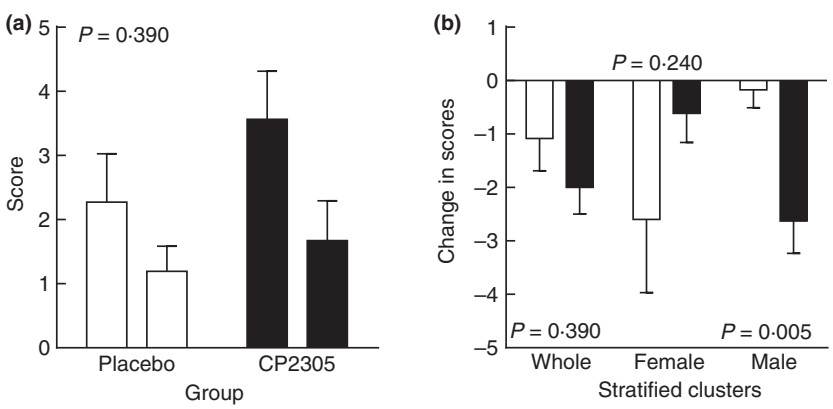

Figure 4 Changes in abdominal symptom scores. Time-dependent changes in the VAS scores for diarrhoea (a) are shown. The left and right bars in each group represent the scores before and after each treatment respectively. Differences in the scores before and after the treatments (b) are shown. Data are presented as the mean ± SE. (□) Open and (■) filled bars represent the placebo and CP2305 treatments respectively. The left and right bars in the same group (a) represent the scores before and after each treatment respectively. Statistical analysis was performed using ANOVA (A) and Welch's *t*-test (b).

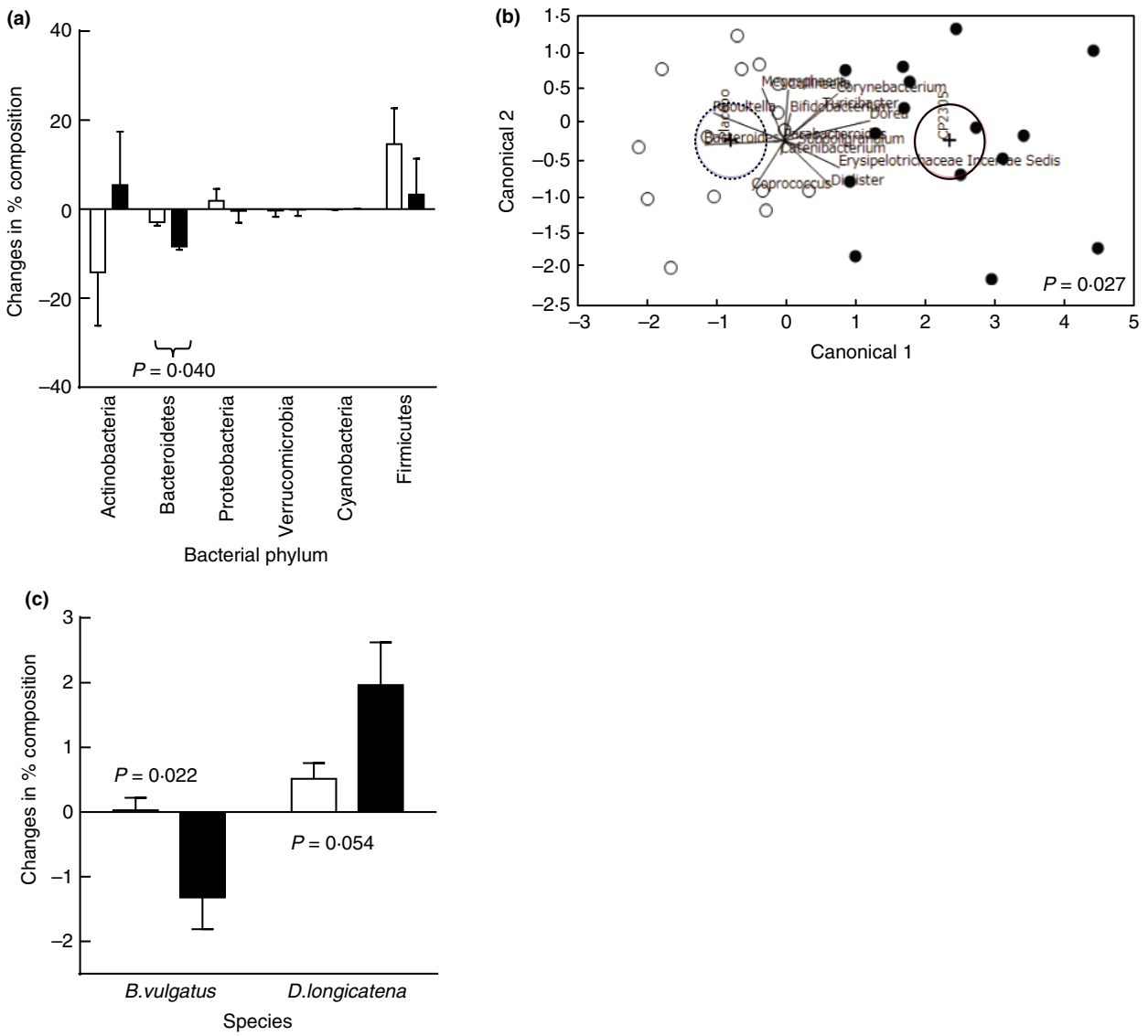

Figure 5 The effects of paraprobiotic CP2305 on the composition of the intestinal microbiota. The compositional changes in the microbiota at the phylum level (a) and the increase in *Dorea longicatena* and decrease in *Bact. vulgatus* (c) are presented. Data are presented as the mean ± SE of the differences in the % composition data before and after each treatment. (□) Open and (■) filled bars represent the placebo and CP2305 treatments respectively. Statistical analysis was performed using Welch's *t*-test in panels (a) and (c). The results of the discrimination analysis using genus-level data (b) are presented. (○) Open and (●) closed small circles show individuals in the placebo and CP2305 groups respectively. Dotted and solid large circles show the 0·95 density ellipses of the multivariate means for the placebo and CP2305 groups respectively.

state and HADS scores, both of which were significantly reduced with live CP2305. However, the differing results observed for live and heat-inactivated CP2305 must be interpreted cautiously as there may be other causes for these inconsistencies. One possible cause is the lack of statistical power in this study due to the small number of participants in the competitive parallel-group trial. In the former trial, a small number of participants were also employed, but the experimental design was a crossover trial. Another important cause may be sex-related differences. Subjects of both sexes were employed in the present trial, while only male students were examined in the previous study (Sawada *et al.* 2017). There are likely sex-related differences in the response to paraprobiotic CP2305 (Spanakis *et al.* 2016). The paraprobiotic CP2305 improved sleep quality and bowel habits in male students more effectively than in female students. There are sex differences in several aspects of sleep behaviour, such as quality, duration and chronotype, and in the risk of progressing from sleep disorder to insomnia (Adan and Natale 2002; Bailey and Silver 2014). We also observed the sleep disturbance-ameliorating effects of paraprobiotic

CP2305 as well as sex-related differences associated with these effects; daily paraprobiotic CP2305 intake resulted in significant improvement only in male participants (Fig. 2c). A previous study with IBS patients showed that a probiotic composed of a complex of eight bacterial strains increased melatonin synthesis and sleep quality only in male patients (Wong *et al.* 2015). Differences in stress responses resulting from the sex hormone balance are well documented (Stephens *et al.* 2016). Although the mechanism of action of paraprobiotic CP2305 is unknown, it may be similar to the mode of action of the eight-strain probiotic. In addition, we observed that the paraprobiotic CP2305 ameliorated diarrhoea-like symptoms only in men. These sex dimorphisms in the paraprobiotic response may reveal its mode of action. However, sex-related differences may also lead to a possible reduction in statistical detection power.

The microbiota profile is a dynamic entity that may be influenced by many factors, including host genetics, age, geography, diet, antibiotic treatment and exposure to stressful conditions (Lozpone *et al.* 2012). Changes in the gut microbiota may cause low-grade inflammation and intestinal immune activation through their effects on cytokine levels and toll-like receptor activity (Bercik 2011; Hughes *et al.* 2013), thereby generating gut-related symptoms. Furthermore, the oral intake of beneficial bacteria such as probiotics may help maintain intestinal permeability (Camilleri *et al.*, 2012), normalize circulating cytokine levels (O'Mahony *et al.* 2005) and improve peristalsis (Cani *et al.* 2013). The paraprobiotic CP2305 significantly affected the faecal microbiota as observed in our previous studies (Sawada *et al.* 2016; Sugawara *et al.* 2016). At the phylum level, the occupation ratio of Bacteroidetes was significantly reduced after the ingestion of paraprobiotic CP2305 ($P = 0{\cdot}040$; Fig. 5a). At the genus level, microbial community structures apparently differed between the placebo and paraprobiotic CP2305 groups ($P = 0{\cdot}027$; Fig. 5b). The constituent genera in the microbiota that effectively contributed to this distinction were *Bacteroides* and *Dorea*. In addition, at the species level, *Bact. vulgatus* and *Dorea longicatena* showed characteristic changes in the microbiota along the canonical 1 axis ($P = 0{\cdot}022$ and $P = 0{\cdot}054$ respectively; Fig. 5c). Interestingly, according to a recent study, unrestricted *Bact. vulgatus* expansion evokes a subclinical inflammatory response, including IFN-γ production (Ramanan *et al.* 2014). Although further investigation is required, the decrease in *Bact. vulgatus* occupancy in the paraprobiotic CP2305 group may reduce the risk of intestinal inflammation (Kishi *et al.* 2000). In addition, the population of *Dorea longicatena* is increased during the remission phase in patients with Crohn's disease (Mondot *et al.* 2015), suggesting that this species curbs inflammation in the digestive tract. The community structure and bacterial networks in both groups indicated that daily administration of the paraprobiotic CP2305 significantly modified the gut microbiome despite its low administered dose (1×10^{10} counts per day). Moreover, targeting specific species, including *Bact. vulgatus* and *Dorea longicatena*, may facilitate precise modulations in the overall microbial ecosystem resulting in the downregulation of inflammation in the gut mucosa, which may be related to stress response regulation. The genus *Dorea* also showed quite similar changes in our clinical trial with IBS patients (Nobutani *et al.*, 2016); thus, this phenomenon might be closely related to the stress-relieving effect of CP2305.

Elucidation of the working mechanism underlying the stress-relieving effects of paraprobiotic CP2305 is an important next step. It has long been believed that lactic acid bacteria are not retained but instead pass through the human digestive tract when ingested. However, strains of *Lact. gasseri,* including CP2305, have been reported to colonize the intestine based on an analysis of the established microbiota after oral administration (Fujiwara *et al.* 2001; Sawada *et al.* 2016). The discovery that CP2305 is a typical enteric colonizer may provide new insights given the closeness of its interaction with the intestinal tract.

In conclusion, CP2305 exerts stress-relieving effects, even after sterilization. Based on these results, bacterial cell component(s) may contribute to this activity. This study is the first step to determine the possible microbial bioactive compounds involved in the stress-relieving effects. Although sex-related differences in the action of CP2305 were observed, heat-inactivated CP2305 treatment effectively and favourably alleviated stress-associated symptoms. Here, we propose the use of heat-inactivated CP2305 as the first stress-relieving paraprobiotic. CP2305 is advantageous given its potentially wide range of applications. To further confirm the stress-relieving effects of paraprobiotic CP2305, another trial of heat-inactivated, washed CP2305 cells encompassing a higher number of participants of both sexes as well as a longer period of administration (12 weeks) is currently underway.

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Conflict of interest

The authors declare no conflicts of interest.

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