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Fermented milk containing *Lactobacillus casei* strain Shirota prevents the onset of physical symptoms in medical students under academic examination stress

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RESEARCH ARTICLE

Abstract

This pilot study investigated the effects of the probiotic *Lactobacillus casei* strain Shirota (LcS) on psychological, physiological, and physical stress responses in medical students undertaking an authorised nationwide examination for promotion. In a double-blind, placebo-controlled trial, 24 and 23 healthy medical students consumed a fermented milk containing LcS and a placebo milk, respectively, once a day for 8 weeks until the day before the examination. Psychophysical state, salivary cortisol, faecal serotonin, and plasma L-tryptophan were analysed on 5 different sampling days (8 weeks before, 2 weeks before, 1 day before, immediately after, and 2 weeks after the examination). Physical symptoms were also recorded in a diary by subjects during the intervention period for 8 weeks. In association with a significant elevation of anxiety at 1 day before the examination, salivary cortisol and plasma L-tryptophan levels were significantly increased in only the placebo group ($P<0.05$). Two weeks after the examination, the LcS group had significantly higher faecal serotonin levels ($P<0.05$) than the placebo group. Moreover, the rate of subjects experiencing common abdominal and cold symptoms and total number of days experiencing these physical symptoms per subject were significantly lower in the LcS group than in the placebo group during the pre-examination period at 5-6 weeks (each $P<0.05$) and 7-8 weeks (each $P<0.01$) during the intervention period. Our results suggest that the daily consumption of fermented milk containing LcS may exert beneficial effects preventing the onset of physical symptoms in healthy subjects exposed to stressful situations.

Keywords: probiotics; physical symptoms; serotonin; tryptophan; salivary cortisol

1. Introduction

A bidirectional communication network between the gut and the brain has been recognised as the gut-brain axis. Signals from the brain modify the motor, sensory, and secretory modalities of the gastrointestinal tract; in turn, signals from the gut and/or gut microbiota can affect emotional behaviour, stress-, and pain-modulation systems. Neural, endocrine, and immune pathways are involved in the gut-brain axis (Forsythe *et al.*, 2014; Mayer *et al.*, 2015). It is particularly interesting to consider the possibility that probiotics, which are live microorganisms, could affect emotion by modulating bidirectional interactions between the gut and brain, and confer a health benefit on the host when administered in adequate amounts (Hill *et al.*, 2014).

Several animal studies have demonstrated that the administration of probiotics maintains mucosal barrier function under stressful situations (Agostini *et al.*, 2012; Ait-Belgnaoui *et al.*, 2012) and mitigates stress-induced glucocorticoid and inflammatory cytokine responses in association with a reduction of depression- and anxiety-related behaviour (Ait-Belgnaoui *et al.*, 2012, 2014; Bercik *et al.*, 2011; Bravo *et al.*, 2011; Gareau *et al.*, 2007; Messaoudi *et al.*, 2011). It has also been shown that probiotics reduce the mRNA expression of gamma-aminobutyric acid receptors

and c-Fos in the brain (Ait-Belgnaoui *et al.*, 2014; Bravo *et al.*, 2011), possibly by modulating the gut-brain axis (Bercik *et al.*, 2011; Bravo *et al.*, 2011). Several clinical trials have also demonstrated that probiotics have beneficial effects by alleviating psychological distress in healthy subjects (Messaoudi *et al.*, 2011) as well as normalising the stress-induced reduction in the number of natural killer (NK) cells (Marcos *et al.*, 2004) and gastrointestinal symptoms (Diop *et al.*, 2008). A brief naturalistic stress, that is, an academic examination, has been employed frequently to examine psychological stress responses together with alterations of gut microbiota (Hughes *et al.*, 2011; Kamezaki *et al.*, 2012; Katsuura *et al.*, 2012; Knowles *et al.*, 2008; Kurokawa *et al.*, 2010; Langkamp-Henken *et al.*, 2015; Marcos *et al.*, 2004; Segerstrom and Miller, 2004) and to assess the effects of prebiotics and probiotics on gastrointestinal dysfunction and/or upper respiratory tract infections (URTIs) (Hughes *et al.*, 2011; Langkamp-Henken *et al.*, 2015).

Lactobacillus casei strain Shirota (LcS) is a well-known probiotic strain which has been approved as 'generally recognised as safe' by the Food and Drug Administration of the USA. LcS provides many health benefits through balancing the gut microbiota, improving gastrointestinal dysfunction, preventing infection and cancer, and modulating inflammatory and immune responses (Hori, 2010; Miyazaki and Matsuzaki, 2008). Previous studies have demonstrated that LcS reduces the incidence of URTIs in athletes (Gleeson *et al.*, 2011) and the duration of URTIs in the elderly (Fujita *et al.*, 2013). Moreover, some clinical trials have found that LcS improves mood disturbance in the elderly (Benton *et al.*, 2007) and decreases anxiety symptoms in patients with chronic fatigue syndrome (Rao *et al.*, 2009). However, it has not been examined fully whether LcS relieves psychological stress-induced symptoms in healthy subjects.

This pilot study was conducted to examine the effect of fermented milk containing LcS on the psychological, physiological, and physical stress responses of healthy medical students undergoing an authorised nationwide examination for promotion in a double-blind, placebo-controlled, parallel-group trial.

2. Materials and methods

Test beverages

Milk fermented with LcS and a placebo milk were compared in this trial as test beverages. LcS YIT 9029 was obtained from the Culture Collection Research Laboratory of Yakult Central Institute. The placebo milk, not containing LcS, had the same nutritional content, colour, flavour, taste, and pH due to its preparation using the same ingredients as the LcS fermented milk by the addition of lactic acid. Between placebo milk and LcS fermented milk, the contents of protein (1.4 g/100 ml), fat (0.1 g/100 ml), carbohydrate (13.9 g/100 ml), water (83.8 g/100 ml), others (0.8 g/100 ml) and calorie (62.0 kcal/100 ml) were nearly the same. The major components of 'others' were minerals such as potassium, calcium, phosphorus, and sodium that were derived from milk. Mineral content was not different between both beverages. The beverages were distributed and stored at 0-10 °C. The LcS fermented milk contained more than 1.0×10^9 cfu/ml LcS during the intervention.

Subjects

The subjects comprised 57 fourth-year medical students at Tokushima University, Japan, undertaking an authorised nationwide (computer-based) examination for promotion, covering all subjects in both basic and clinical medicine. As described previously (Kamezaki *et al.*, 2012; Katsuura *et al.*, 2012; Kurokawa *et al.*, 2010) in Japan, passing the examination is required for promotion to the clinical bedside training level (fifth year). In all Japanese medical schools, this one-day examination is one of the most stressful events for students. From the 57 students, a total of 30 male and 21 female healthy students were included who did not correspond to the exclusion criteria of over 30 years of age, habitual smoking, taking medication, mental and other diseases, and milk allergy or other allergies for 3 months prior to enrolment. During the trial, the subjects complied with dietary restrictions to avoid the intake of other fermented milks, yoghurt, lactic acid bacteria beverages, and probiotic and 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 laid down 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 subjects.

Design

A double-blind, placebo-controlled, parallel-group trial was conducted from October 2012 to January 2013. According to its randomised design, the subjects were allocated to the LcS or placebo group based on their background data, as shown in Table 1. Either a fermented milk containing LcS or placebo milk in a bottle (100 ml) was consumed once a day for 8 weeks until the day before the examination. Daily intake was self-recorded in a diary to check the compliance rate of intake. The trial was composed of a pre-intervention period for 2 weeks, an intervention period for 8 weeks (pre-examination period), and a post-intervention period (post-examination period) for 2 weeks, as shown in Figure 1.

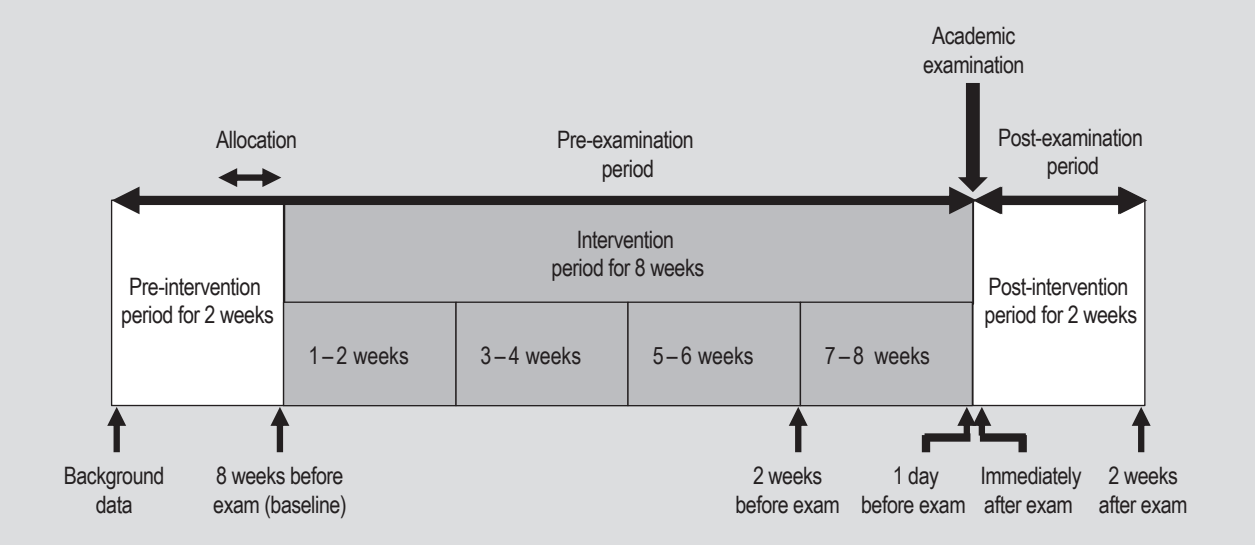

Figure 1. Experimental design.

Psychological parameters

The subjects answered questionnaires from the Japanese version of the State-trait Anxiety Inventory (STAI) (Nakazato and Mizuguchi, 1982; Spielberger *et al.*, 1970) to evaluate state and trait anxiety levels, the Japanese versions of the Hospital Anxiety and Depression Scale (HADS) (Hatta *et al.*, 1998; Zigmond and Snaith, 1983) and Zung Self-rating Depression Scale (SDS) (Fukuda and Kobayashi, 1973; Zung, 1965) to evaluate anxiety and depression levels for the previous 2 weeks, and the Japanese version of the Pittsburgh Sleep Quality Index (PSQI) (Buysse *et al.*, 1989; Doi *et al.*, 2000), which was modified to survey sleep levels for the previous 2 weeks.

Salivary cortisol and secretory immunoglobulin A levels

Saliva was collected for 2 min between 16:00 and 17:00 to avoid diurnal fluctuations using a Salivette® sampling device (Sarstedt, Inc., Rommelsdorf, Germany) prior to the collection of blood (Kurokawa *et al.*, 2011). After storage at -80 °C, salivary cortisol and secretory immunoglobulin A (SIgA) levels were assayed using a commercial enzyme-linked immunosorbent assay kit (Salimetrics, LLC, Carlsbad, CA, USA).

Plasma L-tryptophan and L-kynurenine levels

Venous blood was collected after the sampling of saliva and poured immediately into plasma separator tubes (Becton-Dickinson, Franklin Lakes, NJ, USA). The plasma levels of L-tryptophan (Trp) and L-kynurenine (Kyn) were analysed by a high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA), which was equipped with an octadecylsilyl column (Unison UK-C18 column, 3.0×150 mm; Imtakt Co., Kyoto, Japan), eluted with 50 mM acetic acid, 100 mM zinc acetate, and 3% acetonitrile, and monitored for UV absorbance at 280 nm wavelength for Trp and 330 nm wavelength for Kyn.

Faecal-living *Lactobacillus casei* strain Shirota

The subjects were instructed to collect faecal samples by themselves at home during 3 days before the indicated time points. Around 0.5 to 1 g of faeces was taken and suspended in 3 ml of anaerobic dilution solution (Matsumoto *et al.*, 2006). The faecal samples were kept at around 4 °C and transported to the Yakult Central Institute under cold condition. The faecal samples were weighed and serially diluted with sterilised phosphate buffered saline, and then spread on LLV agar medium (Yuki *et al.*, 1999). The agar plates were incubated at 37 °C for 72 h to determine the number of faecal-living LcS. Cells forming colonies on the agar plate were identified by colony PCR using an LcS-specific PCR primer set (Fujimoto *et al.*, 2008), and the cfu of LcS per g of faeces were determined.

Faecal serotonin levels

To assay faecal serotonin levels, faecal samples, stored at -80 °C, were weighed and diluted with one-tenth volume of 0.2 M perchloric acid containing 10 μM pargyline hydrochloride (Cayman Chemical Company, Ann Arbor, MI, USA), 100 μM disodium EDTA, and 500 ng/ml N-methylserotonin (internal standard; Sigma-Aldrich, St Louis, MO, USA). After incubation in an ice bath for more than 30 min, the mixture was centrifuged at 15,000×*g* for 15 min at 4 °C to obtain faecal supernatant. The supernatant was mixed with one-fourth volume of 1 M sodium acetate and filtered through a membrane filter (Centricut Ultramini, 0.45 μm; Kurabo, Osaka, Japan). The filtrate was stored at -80 °C until the following analysis. Faecal serotonin was

quantified using an HPLC system (Waters, Milford, MA, USA) equipped with an octadecylsilyl column (EICOMPAK SC-5ODS, 3.0×150 mm; Eicom, Kyoto, Japan), eluted with 16% methanol and 84% 0.1 M acetate/citrate buffer (pH 3.2) containing 190 mg/l sodium 1-octanesulphonate and 5 mg/l disodium EDTA at a flow rate of 0.5 ml/min at 25 °C, and monitored with an electrochemical detector (ECD-300, applied potential +650 mV vs Ag/AgCl; Eicom).

Physical symptoms

Physical symptoms, such as common abdominal and cold symptoms, if the subjects had any, were recorded daily in a diary based on self-evaluation from 8 weeks to 1 day before the examination, corresponding to the intervention period. Among the reported symptoms, fever, headache, sore throat, runny or stuffy nose, abdominal pain and discomfort (excluding menstrual cramps), diarrhoea, and constipation were counted as physical symptoms. Both the total number of days experiencing physical symptoms per subject (days with physical symptoms) and the rate of subjects experiencing physical symptoms in each group were tallied and analysed every 2 weeks during the intervention period.

Questionnaires of lifestyle and dietary habits

During screening, lifestyle and dietary habits were assessed by questionnaires using the Japanese versions of the Health Practice Index (HPI) (Morimoto, 2000) and Eating Attitudes Test-26 (EAT-26) (Garner *et al.*, 1982; Mukai *et al.*, 1994), respectively.

Other questionnaires

At the end of the trial, questionnaires about the rate of vaccination against influenza, habitual intake of probiotics or yoghurt before starting the trial, and the feeling of the severity of examination grind were administered. The question 'Which do you think you took, active or placebo?' was also asked.

Statistics

Data are shown as mean ± standard error. All data were analysed using SAS preclinical package ver. 5.0. (SAS Institute Japan, Tokyo, Japan). Questionnaire data and the total number of days experiencing physical symptoms per subject were analysed by Wilcoxon's rank-sum test between the groups and non-parametric Dunnett's multiple comparisons test within a group. The salivary, plasma, and faecal data were analysed by Student's *t*-test between the groups and Dunnett's multiple comparisons test within a group. Fisher's exact test was used to analyse the rate of subjects experiencing physical symptoms and the rate of subjects in other questionnaires between the groups. Two-sided *P*-values below 0.05 were considered to indicate statistical significance.

3. Results

Inclusion of subjects

Figure 2 shows the inclusion and exclusion of the subjects in this study. From the 57 subjects who consented, 2 and 4 students were excluded due to habitual smoking and

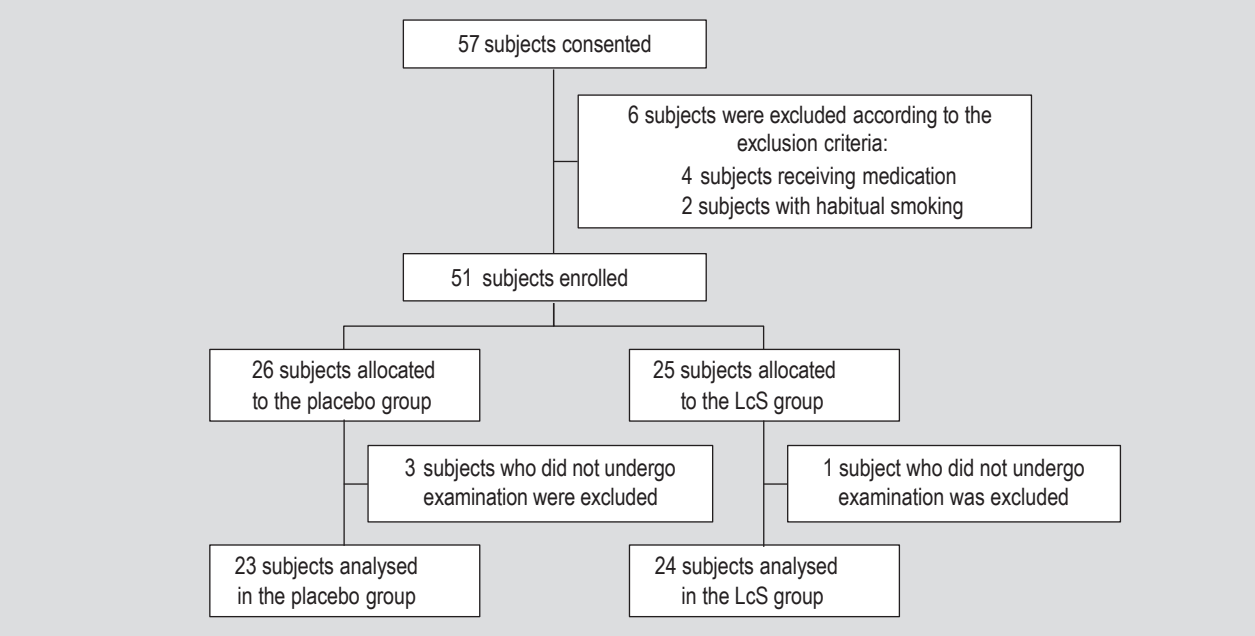

Figure 2. Participants in the present trial.

medication, respectively. As a consequence, 51 subjects were enrolled and allocated to the placebo (26 subjects) or LcS group (25 subjects). Between both groups, there was no bias in the following background data: age, sex, body mass index, STAI-state, STAI-trait, HADS-anxiety, HADS-depression, SDS, PSQI, HPI, and EAT-26 scores at the point of screening. In addition, we excluded 4 students who failed to gain enough credits to take the examination for promotion. Finally, 23 (12 male and 11 female) and 24 (14 male and 10 female) students were included in the placebo and LcS groups, respectively. As shown in Table 1, there was no significant difference in the background data between both groups, and screening detected no unhealthy subjects with an abnormal background.

Compliance and faecal *Lactobacillus casei* strain Shirota

The compliance rates for the intake of placebo and LcS milk were 97 and 96%, respectively, during the intervention period. As shown in Table 2, living LcS was detected in all faecal samples collected at 2 weeks and 1 day before the examination in the LcS group. The averaged logarithmic cfu values per g faeces were from 7.4 to 7.6, which were compatible with those in a previous report (Matsumoto *et al.*, 2006). In the LcS group, the average cfu value of LcS and the number of LcS-detectable subjects were reduced from 7.4 to 3.5 and from 23 to 2, respectively, at 2 weeks after the examination (2 weeks after stopping the consumption of LcS fermented milk). In contrast, living LcS was not detected in any faecal sample during the intervention period in the placebo group. However, it was detected in 1 subject with averaged logarithmic cfu values per g faeces of 3.3 and 3.5 before and at 2 weeks after the intervention period, respectively. The averaged cfu of LcS and the numbers of LcS-detectable subjects were similar between the two groups before and at 2 weeks after the intervention period. As several subjects routinely consumed a fermented drink containing LcS, the pre-intervention period of 2 weeks might not be sufficient to completely wash out LcS in the 2 subjects who had a lower level of LcS at baseline. Therefore, it was indicated that the present trial was performed in compliance with the intake of test beverages in subjects.

Table 1. Backgrounds of the subjects at screening.[1,2]

	Placebo group (n=23)	LcS group (n=24)	*P*-value
Age	22.7±0.4	23.0±0.4	>0.1
Sex (male/female)	12/11	14/10	>0.1
Body mass index	20.6±0.5	21.0±0.3	>0.1
STAI-state score	39.9±2.3	40.2±1.9	>0.1
STAI-trait score	42.0±2.2	43.1±2.3	>0.1
HADS-anxiety score	4.4±0.7	4.6±0.6	>0.1
HADS-depression score	4.8±0.6	4.8±0.6	>0.1
SDS score	36.0±1.6	37.0±1.4	>0.1
PSQI score	4.2±0.6	4.7±0.6	>0.1
HPI score	5.3±0.3	5.3±0.2	>0.1
EAT-26 score	40.0±2.5	39.3±2.1	>0.1

[1] LcS, *Lactobacillus casei* Shirota; STAI, state-trait anxiety inventory; HADS, hospital anxiety and depression scale; SDS, self-rating depression scale; PSQI, Pittsburgh sleep quality index; HPI, health practice index; EAT-26, eating attitudes test-26.

[2] Values indicate mean ± standard error. Data were analysed by Wilcoxon's rank-sum test for score, Fisher's exact test for the ratio of males/females, and Student's *t*-test for the others.

Psychological parameters

Figure 3 shows the time-dependent changes of STAI-state scores in the placebo and LcS groups. In both groups, STAI-state scores were normal at 8 weeks before the examination (baseline), significantly elevated at 1 day before the examination compared with the baseline level ($P<0.01$, non-parametric Dunnett's multiple comparisons test), and returned to the baseline level at 2 weeks after the examination. Since the STAI-state scores measured as the baseline level at 8 weeks before the examination were significantly lower in the placebo group than in the LcS group ($P<0.05$, Wilcoxon's rank-sum test), the time-dependent changes in its levels were compared between both groups. However, there was no significant difference in the changes at the analysed points between both groups (data not shown). The HADS-anxiety, HADS-depression, SDS, and PSQI scores were normal at baseline in both groups (Supplementary Table S1). We could not detect any time-dependent changes in the HADS-anxiety, HADS-depression, SDS, and PSQI scores in either group or any difference in these scores between both groups.

Table 2. Living cell counts of *Lactobacillus casei strain* Shirota (LcS) in faeces.[1]

	8 weeks before exam	2 weeks before exam	1 day before exam	2 weeks after exam
Placebo group	3.5 (1/20)	<3.3 (0/21)	<3.3 (0/21)	3.3 (1/22)
LcS group	3.6 (1/22)	7.6±0.2 (22/22)	7.4±0.2 (23/23)	3.5 (2/22)

[1] Faecal samples were collected at 8 weeks before (baseline), 2 weeks before, 1 day before, and 2 weeks after the examination, in spite of no collection from some subjects. Values show log cfu/g faeces and indicate mean ± standard error. The detection limit was 3.3 log cfu/g faeces. Values in parentheses show the detected number/analysed number of subjects.

Physiological and biochemical parameters

Figure 4 shows the changes in salivary cortisol and SIgA levels in both groups during the experimental period. Compared with the baseline level measured at 8 weeks before the examination, salivary cortisol levels were significantly increased at 1 day before the examination ($P<0.01$, Dunnett's multiple comparisons test) in the placebo group. In the LcS group, there was no significant elevation of salivary cortisol during the pre-examination period. However, there was no significant difference in salivary cortisol levels between both groups, because the levels were already different at baseline in this trial (Figure 4A). There was also no significant time-dependent change in salivary SIgA levels within each group and no difference was observed between both groups during the experimental period (Figure 4B).

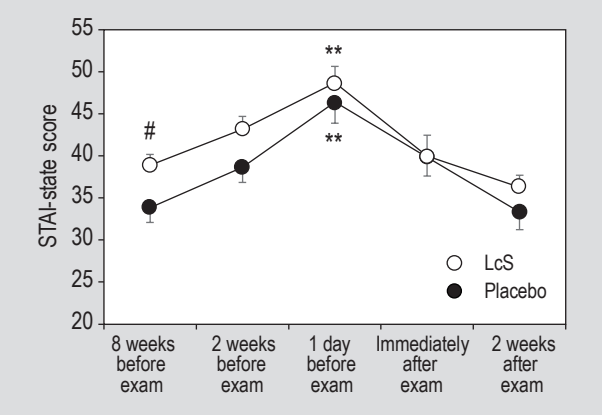

Figure 3. Changes in the state-trait anxiety inventory (STAI) score. Values indicate mean ± standard error. Data were analysed by Wilcoxon's rank-sum test between the groups and non-parametric Dunnett's multiple comparisons test within the group. # $P<0.05$ vs placebo, ** $P<0.01$ vs baseline (8 weeks before).

We also examined the plasma concentrations of Trp and its metabolite Kyn, and the amount of serotonin secreted into faeces. Figure 5 shows the changes in plasma Trp and Kyn levels, and faecal serotonin levels in both groups during the experimental period. In the placebo group, plasma Trp levels were significantly increased at 1 day before the examination ($P<0.05$, Dunnett's multiple comparisons test) without a change in plasma Kyn levels. In contrast, plasma Trp levels were kept at a constant level in the LcS group (Figures 5A and 5B). Both the placebo and LcS groups had reduced logarithmic levels of serotonin in faeces during the pre-examination period (2 weeks and/or 1 day before the examination), although these changes were not significant. At 2 weeks after the examination, faecal serotonin seemed to increase in the LcS group; however, it was maintained at a lower level in the placebo group. Consequently, the logarithmic level of faecal serotonin was significantly higher in the LcS group than in the placebo group at 2 weeks after the examination (Figure 5C, $P<0.05$, Student's t-test).

Physical symptoms

Figure 6 shows the effects of LcS on physical symptoms during the intervention period. Most of the physical symptoms reported were common abdominal and cold symptoms such as abdominal pain, diarrhoea, constipation, catching a cold, a runny nose, body temperature over 37 °C, and headache. As shown in Figure 6A, the number of days experiencing physical symptoms per subject over a 2 week period was approximately 1 day from 1-2 weeks to 5-6 weeks in the placebo group, and it increased to approximately 2 days at 7-8 weeks. In contrast, the corresponding number in the LcS group was approximately 0.6 days at 1-2 weeks, and it was maintained at approximately 0.2 days from 3-4 weeks to 7-8 weeks. There was a significant reduction in the total number of days experiencing physical symptoms at 5-6 weeks and 7-8 weeks in the LcS group compared

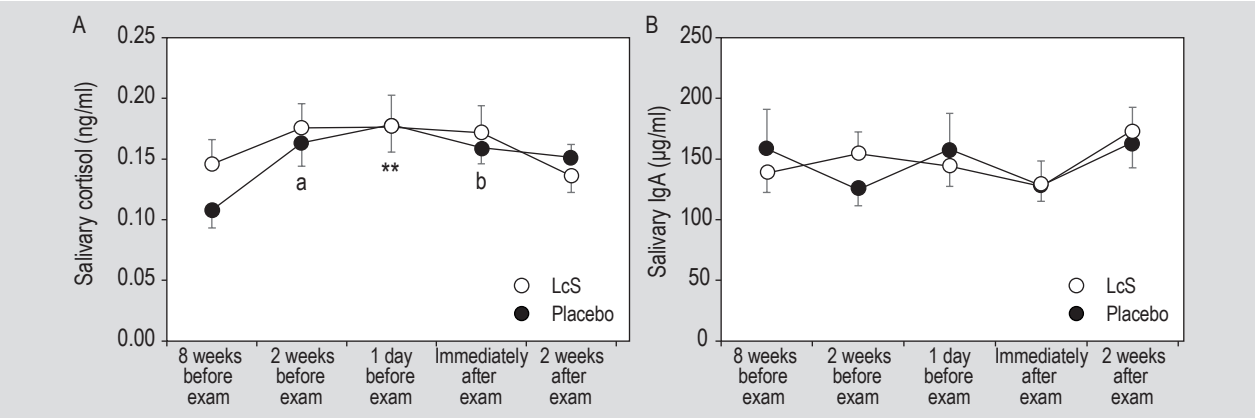

Figure 4. Changes in the levels of (A) salivary cortisol and (B) secretory immunoglobulin A (SIgA). Values indicate mean ± standard error. Two subjects in the placebo group were excluded from the analysis of salivary cortisol and SIgA, one because of blood contamination and the other because the amount of saliva was too small to assay. Data were analysed by Dunnett's multiple comparisons test within a group. ** $P<0.01$ vs baseline (8 weeks before), [a] $P=0.06$ and [b] $P=0.09$ vs baseline (8 weeks before).

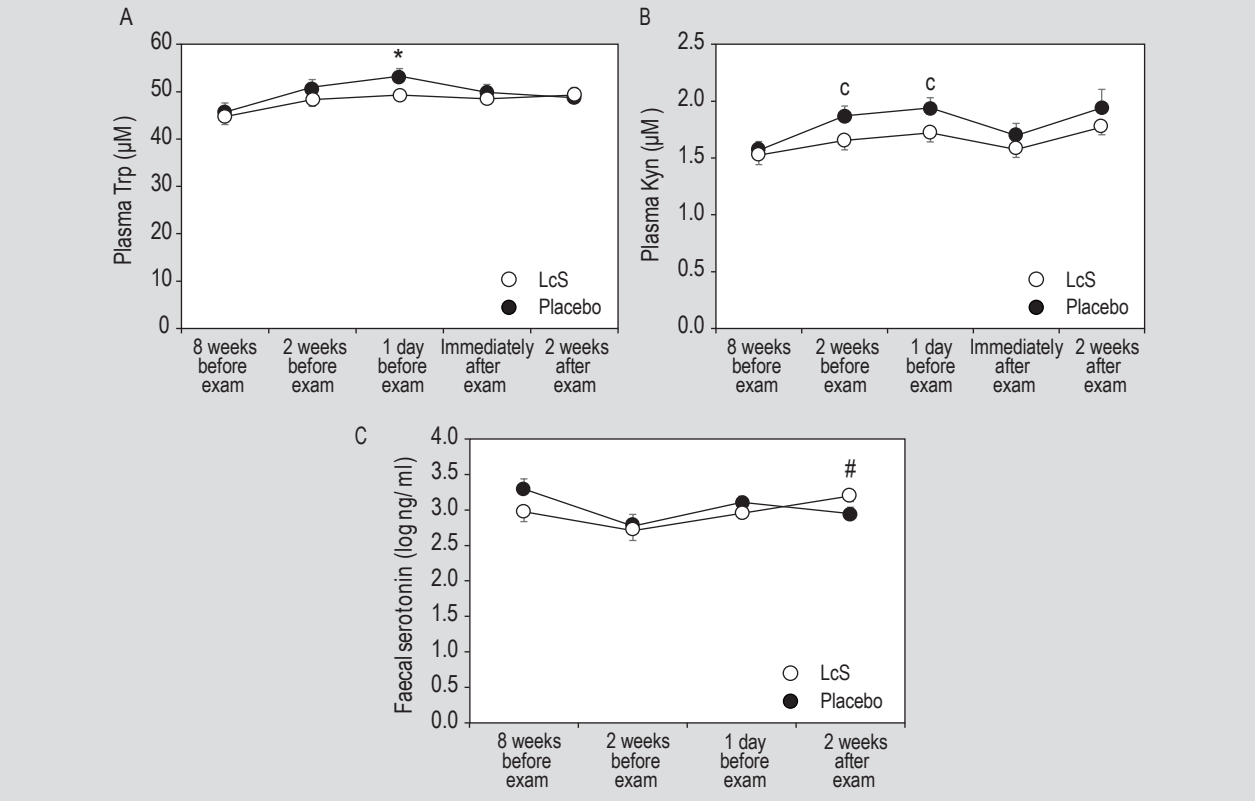

Figure 5. Changes in the levels of plasma (A) L-tryptophan (Trp) and (B) L-kynurenine (Kyn), and (C) faecal serotonin. Values indicate mean ± standard error. Data were analysed by Student's *t*-test between the groups and Dunnett's multiple comparisons test within a group. # *P*=0.05 vs placebo, * *P*<0.05 vs baseline (8 weeks before), c *P*=0.07 vs baseline (8 weeks before).

with the placebo group ($P<0.05$ and $P<0.01$, respectively, Wilcoxon's rank-sum test). As shown in Figure 6B, the rate of subjects experiencing physical symptoms for 2 weeks ranged from 35 to 52% from 1-2 weeks to 7-8 weeks in the placebo group. In contrast, the rate in the LcS group was approximately 33% at 1-2 weeks and was maintained below 17% from 3-4 weeks to 7-8 weeks. There were also significant lower rates of subjects experiencing physical symptoms at 5-6 weeks and 7-8 weeks in the LcS group than in the placebo group ($P<0.05$ and $P<0.01$, respectively, 2×2 Fisher's exact test). During the intervention period, the rate of subjects experiencing physical symptoms tended to be lower in the LcS group (50%) than in the placebo group (about 80%) (Figure 6C, $P=0.07$, 2×2 Fisher's exact test).

Other questionnaires

Between both groups, there was no significant difference in the rate of vaccination against influenza, frequency of habitual intake of probiotics and yoghurt before the trial, and feeling of the severity of examination grind ($P>0.1$, Fisher's exact test, Supplementary Table S2). For the question 'Which did you take, active or placebo?' at the end of the trial, the numbers of subjects who answered active, placebo and unknown were 12, 7 and 4, respectively, in the placebo group (n=23) and 10, 6 and 8, respectively, in the LcS group (n=24), suggesting that there was no significant difference in the accuracy rate of the answer between both groups (Supplementary Table S2, $P>0.1$, Fisher's exact test). Moreover, there was no significant difference in the examination pass rate between both groups (data not shown). Thus, we confirmed there was no bias in this double-blind trial.

4. Discussion

A double-blind, placebo-controlled, parallel-group pilot trial was conducted to examine the effect of a fermented milk containing probiotic LcS on the psychological, physiological, and physical stress responses in healthy medical students undergoing an authorised nationwide examination for promotion, which has been used as a brief naturalistic stress model (Kamezaki *et al.*, 2012; Katsuura *et al.*, 2012; Kurokawa *et al.*, 2010). This pilot study demonstrated that the daily intake of LcS fermented milk significantly reduced both the total number of days experiencing physical symptoms, such as common abdominal and cold symptoms, per subject and the rate of subjects experiencing these symptoms under stressful situations.

Consistent with previous reports (Kamezaki *et al.*, 2012; Katsuura *et al.*, 2012; Kurokawa *et al.*, 2010). the students

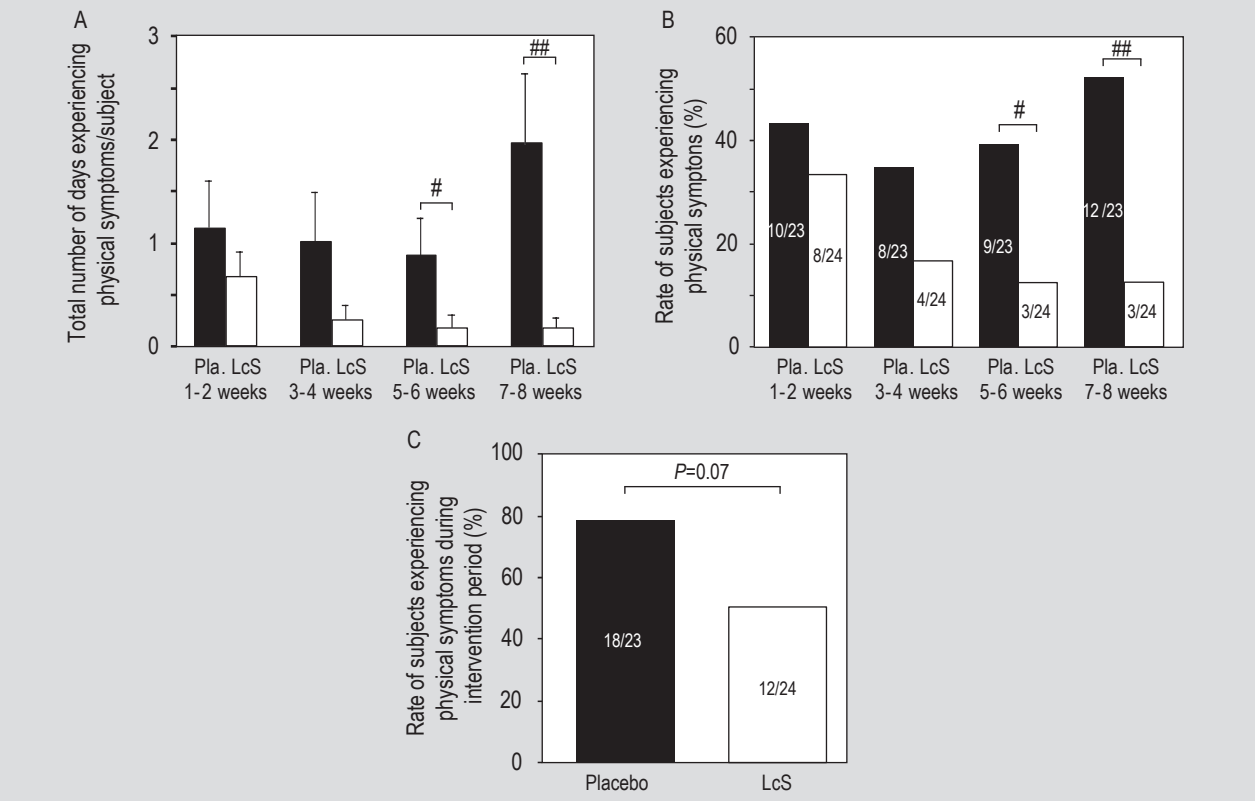

Figure 6. Effects of *Lactobacillus casei* strain Shirota (LcS) on physical symptoms during the intervention period. Values are (A) total numbers of days experiencing physical symptoms per subject every 2 weeks and (B) the rates of subjects experiencing physical symptoms in each group every 2 weeks or (C) during the intervention period. Values indicate the mean ± standard error. Data were analysed by Wilcoxon's rank-sum test for the total number of days and Fisher's exact test for the rate of subjects experiencing physical symptoms. # *P*<0.05 and ## *P*<0.01 between both groups. Pla = placebo.

consuming the placebo milk had increased STAI-state scores at 1 day before the examination, and the scores returned to the baseline level at 2 weeks after the examination. Although there was no difference in anxiety (STAI-state score) between both groups during the pre-examination period, a significant increase in salivary cortisol levels was observed at 1 day before the examination only in the placebo group. Animal studies have revealed that the oral administration of LcS suppressed the stress-induced increase of glucocorticoid levels in rats (unpublished data). These findings support the reported results that several probiotic strains mitigate the stress-induced increase of glucocorticoid levels, indicating hyper-activation of the hypothalamic-pituitary-adrenal (HPA) axis in animal models (Ait-Belgnaoui *et al.*, 2012, 2014; Bravo *et al.*, 2011; Gareau *et al.*, 2007), and the probiotic *L. casei* tended to reduce the salivary cortisol response in healthy subjects under academic examination stress (Marcos *et al.*, 2004). Further studies are necessary to clarify the effect of LcS on the hyper-activation of the HPA axis caused by brief naturalistic stressors.

The present study also found that the rate of subjects experiencing physical symptoms was approximately 80% in the placebo group during the intervention period, but it was reduced to 50% in the LcS group. Especially, at 7-8 weeks after starting the intervention (corresponding to from 2 weeks to 1 day before the examination), approximately 50% of subjects in the placebo group suffered from physical symptoms, and LcS completely blocked the onset of these physical symptoms. Because the subjects felt more stress, as the day of academic examination approached, the rate of subjects experiencing physical symptoms was considered to be increased in the placebo group. Also, the elongation of intervention period might be associated with the high preventive effects at 7-8 weeks after starting the intervention. Therefore, it is suggested that the preventive effect of LcS on the onset of physical symptoms may become much more pronounced during times of stress. This is consistent with the finding that acute psychological stress due to academic examinations is directly related to the onset of symptoms of gastrointestinal dysfunction as well as URTIs (Hughes *et al.*, 2011; Langkamp-Henken *et al.*, 2015), and another probiotic strain maintained the number of healthy days without URTIs under stress situations (Langkamp-Henken *et al.*, 2015). These findings suggest that a brief naturalistic stress model is suitable to evaluate the preventive effects of probiotics on physical

symptoms, gastrointestinal dysfunction, and URTIs in healthy subjects.

SIgA and NK cells play critical roles in mucosal and cellular immunity, respectively, against bacterial and/or viral infections. Previous reports have demonstrated that LcS reduces the incidence of URTIs via maintenance of salivary SIgA levels in athletes (Gleeson *et al.*, 2011) and augments NK cell activity in healthy subjects, especially those who have lower NK cell activity at baseline (Reale *et al.*, 2012; Takeda and Okumura, 2007). A meta-analysis also suggested the effectiveness of probiotics on the duration of illness in healthy children and adults who develop common acute respiratory infectious conditions (King *et al.*, 2014). Therefore, it is assumed that LcS augments immune activity to prevent the onset of physical symptoms in the present study. However, SIgA levels did not change significantly in the present study. It is possible that acute stress preferentially suppresses cellular immunity, but not humoral immunity, while chronic stress suppresses both systems in healthy students (Segerstrom and Miller, 2004). The athletes examined may have been in a chronically stressful condition. In contrast, acute psychological stress changes NK cell activity (Schedlowski *et al.*, 1993) and increases serum cortisol levels (Mavoungou *et al.*, 2005). One possible mechanism underlying the prevention of the onset of physical symptoms is the immune-augmenting activity of LcS on NK cells. Further studies are necessary to test this hypothesis.

Stress profoundly affects the gut-brain axis, in which serotonin, a key neurotransmitter, functions as an important regulator of the enteric and central nervous systems. In fact, intestinal serotonin activates vagal afferents and modulates brain functions such as food intake, motivation, stress (Cui *et al.*, 2012), and sleep (Carley *et al.*, 2004). Recently, it has been reported that the gut microbiota influences Trp metabolism and serotonin biosynthesis (O'Mahony *et al.*, 2015; Yano *et al.*, 2015), and the reduction of serum and faecal serotonin levels is accompanied with an increase of serum and faecal Trp levels (Wikoff *et al.*, 2009; Yano *et al.*, 2015); the serotonergic system may be one of the potential nodes in the regulation of the gut-brain axis (O'Mahony *et al.*, 2015). On the basis of these findings, we examined the effects of LcS on plasma Trp and Kyn levels and faecal serotonin levels. The placebo group had significantly increased plasma Trp levels with a peak at 1 day before the examination without changing Kyn levels; however, the LcS group did not show a Trp response. There was also no significant difference in free Trp and total Trp levels between both test beverages (data not shown). However, the LcS group had significantly higher faecal serotonin levels than the placebo group at 2 weeks after the examination. These findings suggest that the daily intake of LcS may suppress the stress-induced modulation of Trp metabolism and can enhance serotonin biosynthesis together with normalising Trp metabolism during the post-examination period. Previous clinical trials have demonstrated that LcS improves mood disturbance in the elderly (Benton *et al.*, 2007) and decreases anxiety symptoms in patients with chronic fatigue syndrome (Rao *et al.*, 2009). A recent study (Tanida *et al.*, 2014) revealed that an intragastric injection of LcS suppresses sympathetic nerve activity in anesthetised rats via the vagal nerve pathway. LcS-mediated neuronal signalling may influence gut to brain communications, which may be involved in the prevention of the onset of physical symptoms in association with the enhancement of serotonin production.

In conclusion, the daily intake of fermented milk containing probiotic LcS provides beneficial effects to prevent common abdominal and cold symptoms and to maintain the quality of life in subjects exposed to brief naturalistic stressors. The nerve- and/or immune-modulating activities of LcS may be involved in the underlying mechanism.

Supplementary materials

Supplementary material can be found online at http://dx.doi.org/10.3920/BM2015.0100.

Table S1. Changes of HADS-anxiety, HADS-depression, SDS, and PSQI scores.

Table S2. Other questionnaires at the end of the trial.

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Conflict of interest

This study was performed based on a collaboration between Yakult Central Institute in Yakult Honsha Co., Ltd and Tokushima University with the sponsorship and supply of test beverages from Yakult Honsha Co., Ltd. There are no known conflicts of interest associated with this publication.

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