Effects of oral intake of plasmacytoid dendritic cells-stimulative lactic acid bacterial strain on pathogenesis of influenza-like illness and immunological response to influenza virus

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Abstract

Lactococcus lactis ssp. *lactis* JCM5805 has been shown to be a rare lactic acid bacterium that can activate plasmacytoid dendritic cells in both murine and human species. In this study, we carried out a randomised placebo-controlled double-blind experiment to evaluate its effect on the pathogenesis of influenza-like illness during the winter season. A total of 213 volunteers were divided into two groups, which received either yogurt made with *L. lactis* JCM5805 or a placebo beverage daily for 10 weeks. In the JCM5805 group, the cumulative incidence days of 'cough' and 'feverishness', which are defined as major symptoms of an influenza-like illness, were significantly decreased compared with the placebo group. In addition, peripheral blood mononuclear cells prepared from volunteers were cultured in the presence of inactivated human influenza virus A/H1N1 (A/PR/8/34). IFN-α elicited by A/H1N1 tended to be higher in the JCM5805 group compared with the placebo group, and an IFN-α-inducible antiviral factor, *interferon-stimulated gene 15* (*ISG15*), elicited by A/H1N1 was significantly higher in the JCM5805 group compared with the placebo group after the intake period. These results suggest that intake of JCM5805 is able to prevent the pathogenesis of an influenza-like illness via enhancement of an IFN-α-mediated response to the influenza virus.

Key words: Plasmacytoid dendritic cells: Type I interferon: *Lactococcus lactis* JCM5805: Influenza virus

Plasmacytoid dendritic cells (pDC) are a crucial subset of immune cells that act as the first line of defence against viral infection by producing large amounts of interferons (IFN)[1,2]. IFN induce the expression of numerous interferon-stimulated genes (ISG) responsible for the inhibition of viral replication and spread[3]. *Interferon-stimulated gene 15* (*ISG15*), a ubiquitin-like protein involved in protein modification, is one of the most highly induced ISG, and it has an indispensable role in antiviral immunity[3]. ISG15 can conjugate to viral proteins and inhibit replication and spread of viruses[4–7]. It can also modulate host-derived proteins and enhance other antiviral systems[3,8]. Furthermore, pDC and pDC-derived type I IFN act as inducers of antiviral factors and 'commanders' of antiviral immunity by controlling various immune factors such as T cells[9–17], natural killer (NK) cells[18] and B cells[19,20].

Probiotics are live micro-organisms present in intestinal flora or starter bacteria for dairy products that have beneficial effects on human health, and their diverse immunomodulatory effects of lactic acid bacteria (LAB) has been reported elsewhere[21]. The protective function of LAB against viral infection has been drawing much attention. Seasonal influenza is one of the major epidemic viral infectious threats around the world. Some LAB were reported to be effective in protecting mice against influenza virus infection: intranasal administration of *Lactobacillus pentosus* S-PT84 activated protective immune responses[22], oral administration of *Lactobacillus gasseri* TMC0356 enhanced gut and respiratory immune responses[23] and exopolysaccharides in yogurt fermented with *Lacobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 also activated immune responses leading to influenza virus protection[24]. Studies have reported that intake of LAB might be related to a reduction in pathogenesis of the common cold in humans: intake of yogurt fermented with *L. delbrueckii* ssp. *bulgaricus* OLL1073R-1 reduced the risk of pathogenesis of common cold in the elderly[25] and

Bifidobacterium longum BB536 reduced the incidence of influenza and fever in the elderly[26]. Gleeson et al.[27] suggested that *Lactobacillus casei* Shirota ingestion may reduce the frequency of upper respiratory tract infections in athletes. These reports are mainly based on up-regulation of the innate immune response, such as activity of NK cells and IgA secretion. However, previous reports do not discuss any mechanisms of how LAB affects antiviral immunity or antiviral function.

It is reported that normally LAB are not able to activate pDC directly[28]. However, *Lactococcus lactis* JCM5805 (*L. lactis* JCM5805) is originally selected as a unique LAB strain, which has a significant impact on murine pDC *in vitro* and *in vivo*[29]. This pDC stimulative activity of JCM5805 was confirmed using human peripheral blood mononuclear cells (PBMC)-derived pDC *in vitro*[30]. We also confirmed that intake of JCM5805 increased the ability to produce IFN-α in response to CpG oligodeoxynucleotide stimulation, but the response to human influenza virus and the effects of antiviral factor are still unknown[30]. Furthermore, we previously explored the preventive effects and mechanism of oral administration of JCM5805 using a parainfluenza-infected model and found that an enhanced lung immune response could be elicited by oral intake of JCM5805, and it exerted protective effects against parainfluenza virus infection[31].

In this study, a randomised placebo-controlled double-blind study was conducted to examine the effects of oral intake of JCM5805 on the pathogenesis of influenza-like illness and response against human influenza virus. Our results suggest that oral intake of JCM5805 was suggested to contribute to the inhibition of pathogenesis of influenza-like illness by stimulation of expression of the antiviral factor.

Methods

Volunteers

Volunteers in this study were recruited from Japanese who live in and around Tokyo (Tokyo, Kanagawa prefecture, Chiba prefecture and Saitama prefecture). All volunteers were healthy adults (30–59 years old) without serious illness (immune disease, hepatic disorder, renal disorder, cardiac disease, anaemia, including anamnesis), milk allergy and serious hay fever. Volunteers who had received the influenza vaccination within the previous 18 months, volunteers routinely taking supplements containing LAB or yogurt, pregnant women, lactating women and alcoholics were excluded. Furthermore, volunteers who were judged as unsuitable for the study by the medical doctor for other reasons were also excluded. This study was conducted at Shiba Palace Clinic in Tokyo. Informed consent was obtained before enrolling in this study. We explained sufficiently about the purpose of the test, parameters to be measured, test sample,

exclusion criteria, possible risk and so on. Volunteers visited the medical doctor three times during the study: pre-blood test, before and after the intake period.

To determine the sample size, we simulated using data derived from our previous clinical test on thirty-eight volunteers[30]. As the results, of simulation using data of individual symptoms, more than eighty volunteers in each group were expected to detect differences between groups at 5 % significance level. Therefore, in this trial, we set the number of volunteers to 100 in each group.

A total of 214 volunteers from 297 candidates were selected on the basis of a pre-blood test (WBC, RBC, Hb, Ht, MCV, MCH, MCHC, PLT, Total-cho, TG, LDL-cho, HDL-cho, BUN, UN, CRE, UA, AST, GOT, ALT, GPT, γ-GT, γ-GTP, LD, LDH, CPK, CK, GLU) and a background questionnaire.

Selected volunteers were divided into two groups by computerised randomisation based on age and sex (Table 1): JCM5805 group (107 adults) and placebo group (107 adults). As one volunteer in the JCM5805 group who could not visit the doctor dropped out, the final number of subjects in the JCM5805 group was 106. In this study, any adverse events were not reported.

This study was approved by the clinical research ethics committee of Kirin Holdings Co. Ltd. Informed consents were obtained from all participants after explanation of the study, according to the Declaration of Helsinki.

Test samples

The yogurt beverage fermented by only JCM5805 (100 ml) and the placebo beverage without LAB (100 ml) were prepared by Koiwai Dairy Products. A volume of 100 ml of JCM5805 yogurt contained approximately 1×10^{11} colony-forming units. The compositions of yogurt beverage and placebo beverage are the same, except for *L. lactis* JCM5805: milk, powdered skim milk, milk peptide, granulated sugar, pectin, lactic acid, flavouring agent and water. In addition, they were similar in terms of nutritional compositions: 67 kcal, protein 3·2 g, lipid 0·7 g and carbohydrate 12 g.

Study design

This study was conducted as a randomised placebo-controlled double-blind study. Volunteers received JCM5805 yogurt beverage or placebo beverage daily, for 10 weeks (January 2013 to March 2013). During the intake period, volunteers recorded body temperature, daily questionnaires with regard to symptoms based on influenza-like illness and incidence of influenza or common cold after diagnosis by a medical doctor. Blood samples were collected before and after the intake period. Volunteers were prohibited from receiving food containing LAB during the test period. We registered this study to UMIN-CTR (University

Table 1. Characteristics of volunteers in each group
(Number of subjects, and mean values with their standard errors)

Groups	Male number	Mean age (years)	SE	Female number	Mean age (years)	SE	Total number	Mean age (years)	SE
Placebo	47	45·8	7·7	60	44·5	7·6	107	45·1	7·6
JCM5805	45	46·0	8·3	61	45·2	7·1	106	45·5	7·6

Hospital Medical Information Network Clinical Trials Registry). The registered ID is UMIN000017274.

Outcomes

Primary outcome is derived from daily questionnaire symptoms based on influenza-like illness. Secondary outcome is derived from immunological parameters using PBMC collected before and after the intake period – that is, pDC activity and response against human influenza virus A/H1N1 (A/PR/8/34).

Daily questionnaire symptoms based on influenza-like illness

Volunteers recorded the severity of symptoms of influenza-like illness, such as cough and feverishness, and common cold symptoms, such as sore throat, runny nose, nasal congestion, sneezing and headache. Volunteers were asked to choose their severity from five grades: (1) normal, (2) slight, (3) mild, (4) moderate and (5) severe. Influenza-like illnesses were defined as 'an acute respiratory infection with measured fever of ≥38°C, and cough, with onset within the last 10 days' by the WHO Global Epidemiological Surveillance Standards for influenza (2014). The severity of feverishness was derived from the subjective symptom.

We evaluated the cumulative incidence days of each severity. The cumulative incidence days are effective to evaluate both the duration and frequency of severe symptom onset comprehensively. At first, we compared the cumulative incidence days of each of the five grades between two groups using Wilcoxon rank sum test. This analysis was done to test whether there were significant differences between two groups in terms of both severity and duration together. Next, we compared the cumulative incidence days by separating two severities, 'normal, slight, mild' and 'moderate, severe', using χ^2 test. This analysis was done to test whether intake of JCM5805 affected the duration of severe symptom onset.

Fluorescence-activated cell sorting analysis

PBMC were stained with fluorescent dye conjugated to Abs: CD123-FITC (AC145), BDCA4-APC (AD-17F6) (Miltenyi Biotec), CD86-PE (IT2.2) (eBioscience) and HLA-DR-PerCP (L243) (BD Pharmingen). After staining, cells were washed twice with fluorescence-activated cell sorting (FACS) buffer (0·5 % bovine serum albumin in PBS buffer) and suspended in 4 % paraformaldehyde for FACS analysis. Data were collected by FACS Cant II (BD Biosciences) and analysed by FCS Express software (De Novo Software). pDC were defined as CD123$^+$BDCA4$^+$, and the activation markers on pDC were measured. To eliminate the influence of analytical error, volunteers whose data were outliers (mean ± 2 SD) were excluded from the analysis.

Immunological response to human influenza virus A/H1N1 (A/PR/8/34)

Human influenza virus A/H1N1 (A/PR/8/34) was propagated and inactivated by thermal denaturation (56°C for 60 min) at the National Institute of Infectious Diseases.

Immunological response against virus sensitisation was evaluated as follows: PBMC were cultured at a density of 1×10^6 cells/ml in RPMI medium in 48-well plates, for 24 h at 37°C, with 20 ng/ml inactivated virus particle. Total RNA was extracted using an RNeasy Micro Kit (Qiagen), and complementary DNA (cDNA) was prepared using an iScript cDNA synthesis kit (BioRad), according to the manufacturer's protocol. Quantitative reverse transcription (qRT)-PCR was performed using SYBR Premix Ex Taq (TaKaRa) and LightCycler 480 (Roche). β-actin was used as the reference gene. The transcriptional level was normalised by placebo both before and after intake period in order to compare the transcriptional level between two groups without influence of storage term on the sample.

Primers for β-actin, IFN-α and ISG15 were originally designed as follows:

β-actin F (5'-gagcgggaaatcgtgcgtgacatt-3')
β-actin R (5'-tgcccaggaaggaaggctggaaga-3')
IFN-α F (5'-ctcccctgatgaatgcggactcca-3')
IFN-α R (5'-tgctctgacaacctcccaggcaca-3')
ISG15 F (5'-gcgggcaacgaattccaggtgt-3')
ISG15 R (5'-tcgcatttgtccaccaccagca-3')

Statistical analysis

The cumulative number of volunteers diagnosed with influenza or common cold infection during the trial was evaluated using a χ^2 test. The cumulative incidence days of each symptom were evaluated using the Wilcoxon rank sum test and a χ^2 test. The number of volunteers who marked on each scores were counted separately. The results from the biogenic markers were evaluated using a standard Student's t test.

Results

Number of influenza and common cold infections and clinical symptoms of influenza-like illness

First, we evaluated the cumulative number of volunteers diagnosed with influenza or common cold by a medical doctor: fourteen volunteers were diagnosed in the placebo group and seven in the JCM5805 group (Table 2). The number of volunteers diagnosed with influenza by the doctor is one in each group. The cumulative numbers diagnosed with influenza or common cold were lower in the JCM5805 group; however, there was no statistical difference between the two groups ($P = 0·127$, χ^2 test).

We also evaluated the effects of *L. lactis* JCM5805 on clinical symptoms using a daily questionnaire for symptoms of

Table 2. Cumulative numbers of volunteers diagnosed with influenza or common cold infection during the trial*

Groups	Infection (+)	Infection (−)	χ^2 test: P
Placebo	14	7476	0·127
JCM5805	7	7413	

* Comparison of the cumulative number of volunteers diagnosed with influenza or common cold by a medical doctor during the trial. *Lactococcus lactis* JCM5805 group: *n* 7420 (106 volunteers × 70 d); and placebo group: *n* 7490 (107 volunteers ×70 d). The cumulative numbers were evaluated using the χ^2 test.

influenza-like illness and common cold symptoms. As noted in the 'Daily questionnaire symptoms based on influenza-like illness' section, volunteers chose the severity of each symptom from five grades, and the cumulative incidence days of each grade were compared between the two groups using the Wilcoxon rank sum test. The cumulative incidence days of cough and feverishness, which are defined as influenza-like illness by the WHO, were significantly lower in the JCM5805 group compared with the placebo group (Table 3, Fig. 1(a) and (b)). Furthermore, the sum of 'severe' and 'moderate' symptoms of cough, feverishness and sore throat in the JCM5805 group were significantly lower compared with the placebo group (Table 4). There was no significant difference between the two groups on the score for headache. Other symptoms such as runny nose, nasal congestion and sneezing were also analysed; however, 61 % of the volunteers claimed that they developed pollen allergy symptoms during the intake period; therefore, the data were regarded as ineffective. These results suggest that intake of *L. lactis* JCM5805 would have an ameliorating effect on the subjective symptoms of influenza-like illness.

Effects of Lactococcus lactis JCM5805 on pDC activity in vivo

L. lactis JCM5805 was previously suggested to be a stimulator of pDC in mice and humans[28,29]. We analysed the pDC status in this study using CD86 as an activation marker. No changes were observed in the placebo group between before and after the intake period. However, CD86 in the JCM5805 group tended to be increased after the intake period ($P = 0.126$) (Fig. 2).

Effects of Lactococcus lactis JCM5805 intake on the immunological response against human influenza virus

To evaluate whether the antiviral immunity of volunteers could be affected by *L. lactis* JCM5805 consumption in accordance with the ameliorated symptoms of influenza-like illness, the transcriptional levels of *IFN-α*- and IFN-stimulated antiviral factor *ISG15* were analysed. PBMC were cultured with heat-inactivated human influenza virus A/H1N1 (A/PR/8/34), and *IFN-α* and *ISG15* transcriptional levels were determined by qRT-PCR.

No change was observed in the transcriptional level of *IFN-α* before the intake period between the two groups; however,

IFN-α transcriptional levels tended to be higher in the JCM5805 group compared with the placebo group after the intake period ($P = 0.140$) (Fig. 3(a)). The transcriptional level of *ISG15* tended to be lower in the JCM5805 group compared with the placebo group before the intake period ($P = 0.054$), but *ISG15* was significantly higher in the JCM5805 group compared with the placebo group after the intake period ($P = 0.019$) (Fig. 3(b)).

Discussion

We previously revealed that intake of JCM5805 can activate not only murine pDC but also human pDC both *in vitro* and *in vivo*[29,30]. A double-blind human clinical trial conducted during the summer season using yogurt made with JCM5805 revealed that cell surface markers on serum pDC with activation status were significantly higher in the JCM5805 group compared with the placebo group[30]. The results of this study indicated that intake of JCM5805 would be able to inhibit the development of subjective symptoms associated with influenza-like illness. It was suggested that the clinical improvement would be related to a rise in the transcriptional level of the *IFN-α* gene and IFN-stimulated antiviral factor *ISG15*.

We analysed the cumulative incidence days of symptoms (cough, sore throat, feverishness and headache) using five grades to evaluate the severity, as well as incidence. Symptoms relating to influenza-like illness, such as 'cough' and 'feverishness', were significantly inhibited in the JCM5805 group compared with the placebo group. The cumulative number of volunteers diagnosed by a medical doctor to have influenza, influenza-like illness or common cold tended to be decreased in the JCM5805 group compared with the placebo group; however, there were no significant statistical differences between the two groups. The reason why the difference was not significant might be the low infection rate of middle-aged volunteers. It is generally known that children, the young and the elderly are more susceptible to infection, and inclusion of them in the trial would show the difference more clearly. These results suggested that intake of JCM5805 would have preventive effects on respiratory viral infection, including influenza.

The immunological response test using PBMC cultured with influenza A virus suggested that intake of JCM5805 would increase the transcriptional level of *IFN-α* in response to influenza virus

Table 3. Cumulative incidence days of each symptom and its grade during the trial*

Symptoms	Group	Normal	Slight	Mild	Moderate	Severe	Wilcoxon rank sum test: *P*
Cough	Placebo	6440	866	141	24	19	< 0·001
	JCM5805	6708	624	65	19	4	
Sore throat	Placebo	6438	848	126	49	29	0·226
	JCM5805	6426	814	137	36	7	
Feverishness	Placebo	7124	286	51	21	8	< 0·001
	JCM5805	7194	191	23	10	2	
Headache	Placebo	6795	520	115	42	18	0·958
	JCM5805	6731	502	123	29	35	

* The grades marked for each symptom were counted separately. The cumulative incidence days of each symptom were evaluated using the Wilcoxon rank sum test. *Lactococcus lactis* JCM5805 group: *n* 7420 (106 volunteers × 70 d); and placebo group: *n* 7490 (107 volunteers × 70 d).
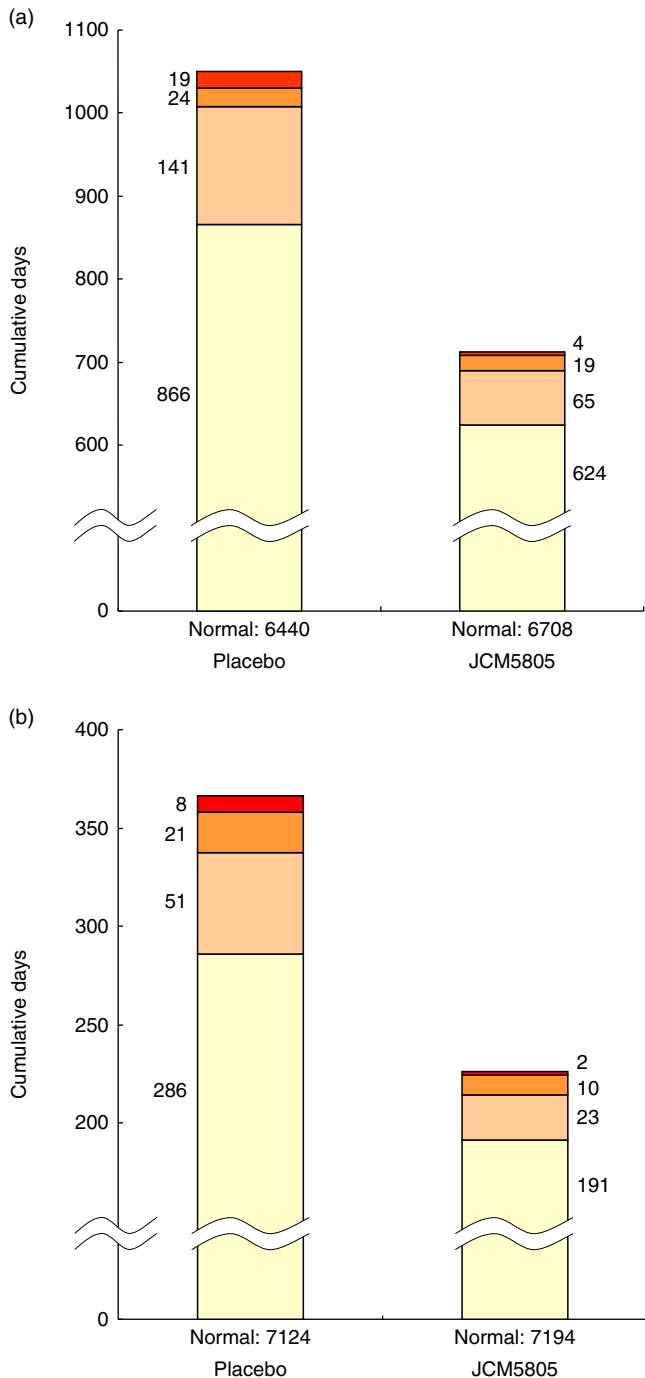
(a)

(b)

Fig. 1. Comparison of the cumulative incidence days of symptoms associated with influenza-like illness and common cold. (a) Cough and (b) feverishness. The severity was evaluated on a scale of 1 to 5, as follows: (1) normal, (2) slight, (3) mild, (4) moderate and (5) severe. *Lactococcus lactis* JCM5805 group: n 7420 (106 volunteers × 70 d); and placebo group: n 7490 (107 volunteers × 70 d).

Table 4. Cumulative incidence days of the grades as scores of severe–moderate and mild–normal in each group

Symptoms	Group	Normal, slight, mild	Moderate, severe	χ^2 test: P
Cough	Placebo	7447	43	0·015
	JCM5805	7397	23	
Sore throat	Placebo	7412	78	0·009
	JCM5805	7377	43	
Feverishness	Placebo	7461	29	0·009
	JCM5805	7408	12	
Headache	Placebo	7430	60	0·679
	JCM5805	7356	64	

The number that volunteers marked for each symptom was counted separately. The cumulative incidence days of each symptom were evaluated using the χ^2 test. *Lactococcus lactis* JCM5805 group: n 7420 (106 volunteers × 70 d); and placebo group: n 7490 (107 volunteers × 70 d).
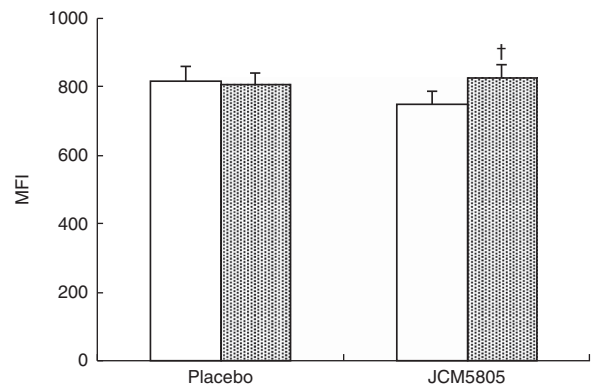

Fig. 2. Changes in CD86 expression level on plasmacytoid dendritic cells (pDC) before (□) and after (▨) the intake period. To evaluate pDC status, the expression level of CD86 was measured using fluorescence-activated cell sorting. To eliminate the influence of analytical error, volunteers whose data were outliers (mean ± 2 SD) were excluded from the analysis. As a consequence, the JCM5805 group and the placebo group consisted of ninety-eight and ninety-six volunteers, respectively. Numbers indicate median fluorescence intensity (MFI). Data are means, with their standard errors represented by vertical bars for each group, before and after the intake period. † Mean value was marginally significantly different from that before intake ($P = 0.13$).

stimulation. In our previous study, we demonstrated that JCM5805 could activate pDC to increase the transcriptional level of type I IFN (IFN-α, IFN-β), type III IFN (IFN-λ) and some interferon regulatory factor (IRF) genes (IRF3, IRF5, IRF7 and IRF8) *in vitro*. Furthermore, intake of JCM5805 could increase activation markers on pDC

in vivo in humans[30]. pDC are known to be proficient cells producing type I IFN[1,2], and IRFs are key factors in the production of type I and type III IFN. Therefore, increased IFN-α reaction against human influenza virus *ex vivo* suggests that the antiviral potential would be elevated *in vivo*. Indeed, it was also shown in this study that the transcriptional level of *ISG15* in response to influenza A virus infection was significantly higher in the JCM5805 group compared with the placebo group after the intake period. Type I IFN derived from pDC are known to induce numerous antiviral factors to restrict viral replication and spread[3]. Among them, ISG15 is one of the most important proteins in the inhibition of the influenza A virus. ISG15 protein can be conjugated to the NS1 protein of influenza virus A and inhibit its replication[4-7]; in addition, hundreds of host-derived proteins are modulated by ISG15, including interferon-related factors[32]. For example, the stability of IRF3, which has an important role in inducing type I IFN, is increased by Herc5 via ISG15 modification[33]. PKR (protein kinase R), which inhibits translation of viral protein by phosphorylating
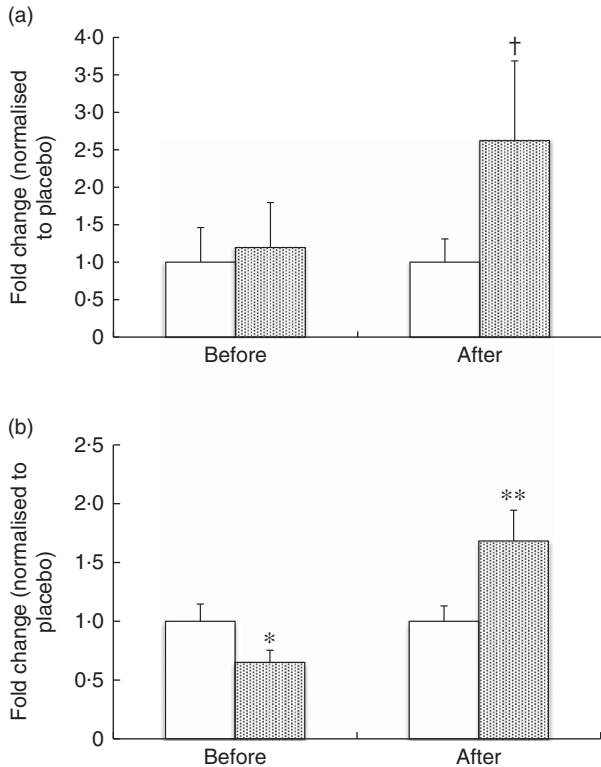
(a)

(b)

Fig. 3. Comparison of transcriptional levels of *IFN-α* and *interferon-stimulated gene 15* (*ISG15*) in peripheral blood mononuclear cells cultured with inactivated influenza virus A/H1N1 (A/PR/8/34), between the JCM5805 group (▨) and the placebo group (□). (a) Relative *IFN-α* gene transcriptional level normalised by placebo. (b) Relative *ISG15* gene transcriptional level normalised by placebo. Data were normalised using β-actin. Samples were excluded from the analysis if RNA extraction failed. JCM5805 group: *n* 102; and placebo group: *n* 105. Values are means, with standard errors represented by vertical bars. Mean value was significantly different from that of the placebo group: * $P=0\cdot05$, ** $P<0\cdot05$. † Mean value was marginally significantly different from that of the placebo group ($P=0\cdot14$).

eIF2α, is activated by ISG15 modification[34]. In addition, similar to cytokines, ISG15 secreted from immune cells activates IFN-γ production[35]. Therefore, daily intake of certain LAB strains would be beneficial for the inhibition of viral infection due to ISG15 up-regulation.

In this study, we indicated that oral administration of JCM5805 could significantly decrease the symptoms of influenza-like illness, such as 'cough' and 'feverishness'. Furthermore, we revealed that the transcription levels of *IFN-α* and *ISG15*, indicating responsiveness to influenza A virus, were up-regulated by administration of JCM5805. Further studies are required to understand the precise mechanisms and effect of JCM5805 on other immune systems.

Yogurt is widely accepted as healthy food these days, and it is well suited for daily intake. It is very valuable for us to promote immune function to prevent common cold and seasonal influenza by daily diet. However, yogurt form requires strict temperature control; therefore, it will be a significant step if the function will be obtained by various food forms. Indeed, the effects of JCM5805 are supposed to be not only dependent upon living LAB form, but also heat-killed LAB. We have demonstrated that oral administration of heat-killed JCM5805

could activate murine immune response and protect from viral infection[29,31]. Therefore, it is intriguing whether the heat-killed form also stimulates antiviral immunity to prevent common cold and seasonal influenza in humans.

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