Lactobacillus plantarum induces innate cytokine responses that potentially provide a protective benefit against COVID-19: A single-arm, double-blind, prospective trial combined with an *in vitro* cytokine response assay

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Received March 31, 2021; Accepted September 22, 2021

DOI: 10.3892/etm.2021.10942

Abstract. Intestinal microbiota can indirectly modulate airway physiology and immunity through the gut-lung axis. Recent microbiome studies indicate that patients with coronavirus disease 2019 (COVID-19) exhibit a specific intestinal dysbiosis that is closely associated with the disease pathophysiology. Therefore, rebalancing the intestinal microbiome using probiotics may be effective for controlling COVID-19. However, the rationale for using probiotics in COVID-19 remains unclear. In the present study, an *in vitro* cytokine response assay was conducted, followed by a single-arm, double-blind, prospective trial to evaluate the immunological efficacy of probiotic lactic acid bacteria against COVID-19. The present study focused on *Lactobacillus plantarum* (*L. plantarum*), *Bifidobacterium longum* and *Lactococcus lactis* ssp. *lactis*, which exhibit robust protective effects against infection with respiratory RNA viruses. Considering the feasibility of long-term daily intake for prophylactic purposes, healthy uninfected individuals were enrolled as subjects. Our previous pilot trial demonstrated that oral Qingfei Paidu decoction (QFPD), a Chinese herbal medicine formulated specifically against COVID-19, upregulates plasma TNF-α, IL-1β, IL-18 and IL-8. Therefore, the present study utilized the cytokine changes induced by QFPD to define the innate cytokine index QICI [=(TNF-α) x (IL-1β) x (IL-18) x (IL-8)/(IL-6)] as an indicator of the anti-COVID-19 immunomodulatory potential of the lactic acid bacteria. A total of 20 eligible volunteers were enrolled, 18 of whom completed the intervention. *L. plantarum* demonstrated a strikingly high innate cytokine index in all subjects in the *in vitro* cytokine response assay. In the subsequent trial, oral intake of *L. plantarum* significantly increased the innate cytokine index (mean fold change, 17-fold; P=0.0138) and decreased the plasma level of IL-6 (P=0.0128), a key driver of complex immune dysregulation in COVID-19, as compared with the baseline. The cytokine index increased in 16 of 18 subjects (88.9%) with considerable individual differences in the fold change (1- to 128-fold). In line with these innate cytokine changes, *L. plantarum* ingestion significantly enhanced the activity of natural killer cells. By contrast, oral *B. longum* failed to induce a significant increase in the innate cytokine index (mean fold change, 2-fold; P=0.474) as compared with the baseline. In conclusion, *L. plantarum* demonstrated superior QFPD-like immunomodulatory ability and mimicked the blood cytokine environment produced by early immune responses to viral infection. Daily consumption of *L. plantarum* as an anti-COVID-19 probiotic may be a possible option for preventing COVID-19 during the pandemic. The present study was prospectively registered in the University Hospital Medical Information Network-Clinical Trials Registry under the trial number UMIN000040479 on 22 May 2020 (https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000046202).

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Abbreviations: QFPD, Qingfei Paidu decoction; ACE2, angiotensin-converting enzyme 2; TMPRSS2, transmembrane serine protease 2; LAB, lactic acid bacteria; QICI, QFPD-induced innate cytokine index; IFN, interferon; FSC, forward scatter; SSC, side scatter; NK, natural killer; IQR, interquartile range; BALF, bronchoalveolar lavage fluid; TLR, toll like receptor; NLRP3, NLR family pyrin domain containing 3; ssRNA, single-stranded RNA

Key words: coronavirus disease 2019, cytokines, dysbiosis, gut-lung axis, lactic acid bacteria, *Lactobacillus plantarum*, microbiota, natural killer cells, probiotics, severe acute respiratory syndrome coronavirus 2

Introduction

The coronavirus disease 2012 (COVID-19) pandemic is becoming an increasingly serious threat to global public health. The causative coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), primarily infects a subpopulation of airway epithelial cells that co-express the viral entry molecules angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) (1-3). Notably, several lines of evidence suggest that SARS-CoV-2 can also infect human intestinal epithelial cells, as ACE2 and TMPRSS2 are co-expressed in the lower gastrointestinal tract, particularly enterocytes and progenitor cells of the ileum and colon (2-5). Targeted infection and active replication of SARS-CoV-2 in ACE2-expressing enterocytes have been demonstrated using human intestinal organoids (5-8). SARS-CoV-2 RNA has been detected in stool specimens and anal/rectal swabs of patients with COVID-19 (9-11), and infectious viruses have been isolated from feces of patients (8). A considerable percentage of patients with COVID-19 present with concurrent gastrointestinal symptoms, such as diarrhea and abdominal pain (12-14). These findings raise the possibility that there are adverse effects between the enteric infection of SARS-CoV-2 and the intestinal microbiome.

Gut microbiota serves key roles in the crosstalk between the intestinal and respiratory tracts, which is called the gut-lung axis, via which gut microbe-derived molecules (including structural components, metabolites and toxins, among others) modulate airway physiology and immunity (15-17). Intestinal dysbiosis leads to aberrant immune tone in the airway mucosa, which can trigger dysregulated immune responses to respiratory viral infection (15-17). Recent microbiome studies have revealed that patients with COVID-19 have compositional changes in the specific taxa of enteric bacteria (18-21). Notably, a subset of the changes correlate with the serum levels of proinflammatory cytokines, symptom severity and fecal SARS-CoV-2 virus load, suggesting that COVID-19-related intestinal dysbiosis is closely associated with the disease pathophysiology (19-21).

Numerous animal and clinical studies have demonstrated that the oral intake of probiotic strains of various lactic acid bacteria (LAB) species exhibited prophylactic and therapeutic efficacy against infection by respiratory RNA viruses (22-26). Life-threatening symptoms and complications of COVID-19 are caused by hyperinflammation owing to complex immune dysregulation involving neutrophilia, lymphocytopenia, reduced T-cell immunity and excessive production of inflammatory mediators (27-29). Specific probiotic LAB strains, such as *L. plantarum* strain DR7 and *L. paracasei* strain 8700:2, have superior immunomodulatory and anti-inflammatory abilities against respiratory viral infection and may therefore be suitable for therapeutic use (30,31). On the other hand, certain proinflammatory LAB strains are known to induce innate cytokine changes that can trigger early antiviral immune responses and may therefore be employed prophylactically (32,33). Notably, previous preclinical studies and randomized controlled trials have demonstrated that among probiotic LAB, *Lactobacillus plantarum* (*L. plantarum*) (33-39), *Bifidobacterium longum* (*B. longum*) (40,41) and *Lactococcus lactis* ssp. *Lactis* (*L. lactis* ssp. *lactis*) (42) exhibit robust protective effects against influenza virus infection through enhancing host innate immunity.

The genera *Lactobacillus*, *Bifidobacterium* and *Lactococcus* are the most representative LAB and have been recognized as having probiotic properties beneficial for the human health (43,44). *L. plantarum* is a Gram-positive, facultatively anaerobic, rod-shaped bacterium with a plant origin and is distributed in the human intestinal tract and oral cavity. It is a heterofermentative LAB closely associated with various fermented plant foods, such as pickles, sauerkraut and kimchi. *B. longum* is a Gram-positive, obligatory anaerobic, heterofermentative bacterium with V- or Y-shaped morphology and inhabits the human intestine predominantly from newborns to elderly people. *L. lactis* ssp. *lactis* is a Gram-positive, facultatively anaerobic, spherical bacterium commonly present in raw milk and fermented dairy products.

Qingfei Paidu decoction (QFPD), the Chinese word for 'lung cleansing and detoxifying decoction', is a Chinese herbal medicine newly formulated and specifically optimized against COVID-19, and its therapeutic use has been encouraged in the Chinese official management guidelines (45). Clinical trials in China have demonstrated that QFPD accelerated recovery and prevented disease progression in mild to critical cases (46-49). A retrospective clinical study has also indicated that QFPD decreased the blood levels of COVID-19 biomarkers, such as C-reactive protein, creatine kinase and lactate dehydrogenase (50). Our previous study has demonstrated that the pharmacological action of QFPD was associated with the upregulation of the plasma levels of TNF-α, IL-1β, IL-18 and IL-8, which are key cytokines that mediate early innate immune responses to viral infection (51).

Therefore, rebalancing the gut microbiome using probiotics may be effective for the control of COVID-19. However, to the best of our knowledge, no studies have focused on the efficacy of probiotics in patients with COVID-19, and the rationale for using probiotics against COVID-19 remains unclear. In addition, there is a requirement for investigating diverse prophylactic options owing to the frequent emergence and rapid spread of novel SARS-CoV-2 variants carrying immune escape mutations. To explore the immunological efficacy of probiotics for preventing COVID-19, a single-arm, double-blind, prospective trial combined with an *in vitro* cytokine response assay was conducted using *L. plantarum*, *B. longum* and *L. lactis* ssp. *lactis*. The innate cytokine changes induced by QFPD were used as an indicator of the anti-COVID-19 immunomodulatory potential of the LAB. Furthermore, the effects of LAB ingestion on the activity of innate immune cells were examined.

Materials and methods

Subjects. Participants were recruited through the University Hospital Medical Information Network-Clinical Trials Registry website, Takanawa Clinic (Tokyo, Japan) website, announcements in an e-mail newsletter and personal contacts. Individuals who met all the following inclusion criteria were enrolled: i) Healthy adults between the ages of 20 and 70; and ii) having negative PCR and IgM/IgG antibodies tests for SARS-CoV-2 at study entry (no previous and current SARS-CoV-2 infection). Chest imaging tests were not used in

the present study. Individuals were excluded from this trial if they met any of the following exclusion criteria: i) Pregnant; ii) breastfeeding; iii) duplicate enrollment in other clinical trials; iv) history of infectious disease within 6 months before the enrollment; v) current or past history of chronic inflammatory, immune-related or neoplastic diseases; vi) history of medicinal drug use within 6 months before the enrollment; and vii) underlying conditions associated with higher risk of COVID-19, including hypertension, cardiovascular disease, cerebrovascular disease, diabetes, obesity (body mass index ≥30) (52,53), chronic obstructive pulmonary disease and chronic kidney disease. Therefore, the enrolled subjects had no recorded and reported comorbidities.

Subject recruitment. In our previous study using QFPD on 18 healthy subjects (51), the effect sizes r obtained were 0.816 (TNF-α), 0.881 (IL-1β), 0.724 (IL-18) and 0.796 (IL-8). The average value of 0.804 was employed as an estimated effect size for the present trial. *A priori* two-tailed power analysis was conducted with a level of significance α of 0.05, a desired power 1-β of 0.8 and the estimated effect size of 0.804, which suggested a required total sample size of 15 individuals.

Participant recruitment took place between 27 May 2020 and 2 June 2020 at Takanawa Clinic (Tokyo, Japan). A total of 20 volunteers were screened for eligibility, indicated to be eligible and enrolled in the present trial (Fig. 1). *In vitro* cytokine response assay was performed between 7 and 9 June 2020. LAB were administered to all the enrolled participants between 25 June and 17 August 2020, 2 of whom were excluded from the main analysis due to no visit to Takanawa Clinic following the LAB prescription. Consequently, 18 subjects (1 male and 17 females; age, 28-66 years; mean age ± SD, 44.2±10.1 years) completed the intervention, and the data were subjected to statistical analysis.

Study design. The present study comprised two sequential experimental procedures: An *in vitro* cytokine response assay and a single-arm, double-blind, prospective trial.

The optimal LAB in each subject were determined using co-culture of the peripheral blood with each LAB. QFPD-induced innate cytokine changes were used as an indicator to evaluate the anti-COVID-19 immunomodulatory potential of LAB. The QFPD-induced innate cytokine index (QICI) was defined as follows: QICI=(TNF-α) x (IL-1β) x (IL-18) x (IL-8)/(IL-6), where brackets represent the plasma level of the cytokine in pg/ml. IL-6 is a critical driver of complex immune dysregulation in patients with COVID-19 and was thus adopted as the denominator (54-56).

The LAB with the highest and lowest QICI were used in a subsequent clinical trial to examine whether the ingested LAB could reproduce the *in vitro* cytokine responses (*in vitro* QICI). The trial consisted of three consecutive sessions: i) Validation (intervention using the LAB with the highest QICI); ii) washout; and iii) control (intervention using the LAB with the lowest QICI) sessions. The primary outcome measure was the changes in the plasma levels of TNF-α, IL-1β, IL-18, IL-8 and IL-6 and the QICI after each 7-day LAB session compared with those at baseline. The secondary outcome measure was the changes in hematological parameters after each 7-day LAB session compared with those at baseline.

In vitro cytokine response assay. *L. plantarum* SNK12 [$2x10^{12}$ colony-forming unit/g (cfu/g); Bio-Lab Co., Ltd.] is a probiotic strain with potent immunomodulatory, anti-inflammatory and antiviral abilities (57,58). A probiotic *B. longum* BB536 strain ($1.5x10^{11}$ cfu/g; Morinaga Milk Industry Co., Ltd.) has been reported to exhibit various clinical benefits, such as anti-allergic effects (59,60), protection against viral infection (41,61) and modulation of gut microbiota (62-64). *L. lactis* ssp. *lactis* demonstrates immunomodulatory and antiviral effects through activating plasmacytoid dendritic cells and increasing their ability to produce interferons (IFNs) (42). The commercially available probiotic strain LLL970 ($1x10^{11}$ cfu/g; Synbio Tech, Inc.) was used in the present study.

Live LAB cells were disrupted by vibrating them twice at 4,600 rpm (the number of the figure-8-shaped movement of sample tubes per minute) for 3 min at room temperature with 0.5 g of 3.0-mm zirconia beads in a commercial bead vibrator (PS-2000; Kurabo Industries Ltd.) followed by heating at 60˚C for 20 min. Dead LAB were suspended at a 100 μg/ml concentration in Dulbecco's PBS (without Ca^{2+} and Mg^{2+}).

The ability of the LAB to stimulate QFPD-like cytokine production by peripheral blood immune cells was evaluated as described in previous studies (65-67). Briefly, heparinized peripheral blood (0.4 ml) from each subject was co-cultured with each killed LAB suspension (4 μg) in 1 ml RPMI-1640 medium (DS Pharma Biomedical Co., Ltd.) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA) in a humidified incubator with 5% CO_2 at 37˚C for 48 h. Concentrations of TNF-α, IL-1β, IL-8 and IL-6 in culture supernatants were measured using the V-PLEX Proinflammatory Panel 1 Human kit (cat. no. K15049D-1; Meso Scale Diagnostics, LLC) and the concentration of IL-18 was measured using the Human IL-18 ELISA kit (cat. no. ab215539; Abcam) according to the manufacturers' protocols. The data were used to calculate the QICI value for the characterization of the species with the highest or lowest QICI.

Clinical trial. The present trial was a single-arm, double-blind, prospective trial. Each subject was instructed to orally ingest the live LAB with the highest QICI ($1x10^{11}$ cfu/day) in the *in vitro* cytokine response assay twice daily in the morning and evening between meals for 7 days (days 1-7). After a 7-day washout period (days 8-14), a negative control trial was conducted, in which the LAB with the lowest QICI in the *in vitro* cytokine response assay ($1x10^{11}$ cfu/day) was orally administered twice daily in the morning and evening between meals for 7 days (days 15-21). Peripheral blood samples were obtained from each subject on days 0, 8 and 22. Neither the subjects nor physicians in charge were aware of the results of the *in vitro* LAB assessment, prescribed LAB or their QICI properties until the final blood sampling was completed. Concentrations of plasma TNF-α, IL-1β, IL-18, IL-8 and IL-6 were quantified as aforementioned. Hematological and blood biochemical tests (parameters as listed in Table SII) were outsourced to SRL, Inc.

Innate immune cell activity assays. A total of ~9 months after the completion of the trial, 10 healthy subjects were randomly selected from the 18 trial participants and randomly assigned

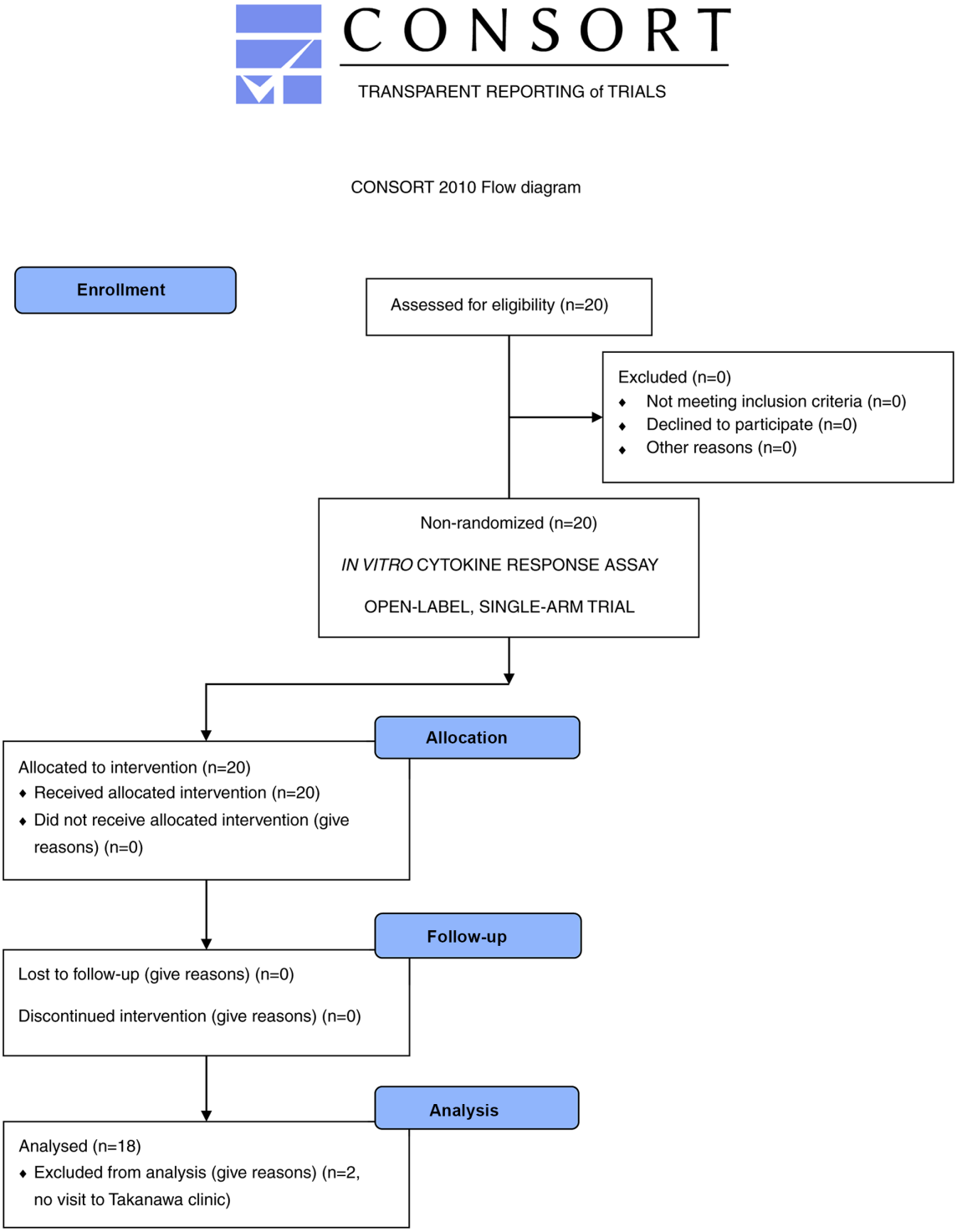

Figure 1. CONSORT flow diagram of participants in the present study. CONSORT; consolidated standards of reporting trials.

to either the *L. plantarum* group (n=5) or the *B. longum* group (n=5) through simple randomization. Ingestion of *L. plantarum* and *B. longum* and blood sampling were conducted with the same protocol as that of the trial ($1x10^{11}$ cfu/day; twice daily for 7 days). After a 7-day ingestion, innate immune cell activity was measured using standard methods as described below in detail.

Neutrophil activity. Measurement of the phagocytic activity of neutrophils was outsourced to BML, Inc. Briefly, heparinized peripheral blood (0.1 ml) from each subject was mixed with 40 μl fluorescent microbeads (Fluoresbrite® YG Carboxylate Microspheres 1.75 μm; Polysciences, Inc.) diluted 4-fold with Dulbecco's PBS [-(without Ca^{2+} and Mg^{2+})] and incubated with gentle agitation at 37˚C for 30 min. The samples were treated with 2 ml 10X FACS lysing solution (BD Biosciences) at 4˚C for 15 min to lyse erythrocytes under gentle hypotonic conditions, followed by flow cytometric analysis using FACSCalibur™ flow cytometer (BD Biosciences). Granulocytes were characterized as medium-sized cells with high granularity and

separated by setting a medium forward scatter (FSC)/high side scatter (SSC) gating. The percentage of fluorescence-positive granulocytes (granulocytes that phagocytosed the fluorescent microbeads) to the total count of granulocytes was calculated using the BD CellQuest™ Pro software version 6.0 (BD Biosciences).

Natural killer (NK) cell activity. Analysis of NK cell activity using chromium-51 (^{51}Cr) release assay was outsourced to SRL, Inc. Lymphocytes were isolated from 5 ml peripheral blood using density gradient centrifugation (Lymphosepar I; Immuno-Biological Laboratories Co., Ltd.) according to the manufacturer's instructions. The lymphocytes were washed twice with Dulbecco's PBS (-) and resuspended at 1x10^6 cells/ml in RPMI-1640 supplemented with 10% FBS. A total of 200-μl aliquots (effector cells; 2x10^5) were mixed with human chronic myelogenous leukemia K562 cells (target cells; 1x10^4 cells/10 μl; cat. no. CCL-243; American Type Culture Collection) radiolabeled with ^{51}Cr (PerkinElmer, Inc.) and incubated at 37˚C for 3.5 h in a 5% CO_2 incubator. The cells were collected by centrifugation, and the remaining ^{51}Cr radioactivity was measured using WIZARD® Automatic Gamma Counter (PerkinElmer, Inc.). ^{51}Cr-loaded K562 cells treated with effector-free culture medium (RPMI-1640; 10% FBS) were used for the quantification of spontaneously released ^{51}Cr.

Macrophage activity. The serum level of neopterin, an activation marker produced primarily by IFN-γ-stimulated monocytes and macrophages (68,69), was assessed for macrophage activity. Determination of serum neopterin was outsourced to SRL, Inc. Serum (0.3 ml) was analyzed by a reverse-phase high-performance liquid chromatography column-switching method (LC-2000Plus; JASCO Corporation) (70) using Wakosil GP-N6 4.6x150 mm as a pretreatment column and Wakosil-II 5C18 HG 4.6x250 mm as an analysis column (FUJIFILM Wako Pure Chemical Corporation). The neopterin level was determined by detecting its native fluorescence (excitation, 353 nm; emission, 438 nm) with a fluorescence detector (FP-2025; JASCO Corporation).

Statistical analysis. For the *in vitro* cytokine response assay, when IL-1β was undetectable in negative control samples (14 of 20 enrolled subjects; Table SI), 0.5x lower limit of detection (0.05 pg/ml) was used to calculate the QICI value.

In the analysis of the trial data, the interquartile range (IQR) method was used to identify outliers; any values that fell below Q1-1.5x IQR or above Q3 + 1.5x IQR (Q1, first quartile; Q3, third quartile) were considered outliers and removed from the statistical analysis (71,72). In order to perform statistical tests of matched pairs, the paired values of the outliers were removed, even if they fell into the non-outlier range. When calculating QICI scores, outliers and zero values were handled as follows: i) The outliers were replaced with mean values that were calculated from the non-outliers to avoid unreasonable reduction of QICI scores by simple removal of the outliers; and ii) zero values in the measurement of IL-1β and IL-18 were replaced with the values of 0.5x lower limit of detection (IL-1β, 0.05 pg/ml; IL-18, 8.30 pg/ml) (73,74).

The normality of the data was firstly examined using the normal quantile-quantile plots and the Shapiro-Wilk test. On the basis of the results from these normality tests, the Friedman test was used, followed by the Nemenyi post hoc test for the data from the clinical trial; a two-tailed paired Student's t-test was used for the data from the assays of innate immune cell activity.

All statistical analyses were performed with EZR v1.53 (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (R Foundation for Statistical Computing; https://www.R-project.org/) (75). *A priori* sample size calculation and post hoc power analysis were performed using G*Power v3.1.9.2 (Department of Experimental Psychology, Heinrich Heine University Düsseldorf; https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower) (76). Spearman's rank correlation coefficients for the post hoc power analysis were calculated with EZR v1.53 (75). P<0.05 was considered to indicate a statistically significant difference.

Results

Selection of LAB with the highest and lowest QICI. Firstly, the species with the highest and lowest QICI among the three probiotic LAB (*L. plantarum*, *B. longum* and *L. lactis* ssp. *lactis*) were determined in each of the 20 study subjects, using *in vitro* cytokine response assay. *L. plantarum* demonstrated the highest QICI in all subjects, whereas *B. longum* demonstrated the lowest QICI (Table SI). *L. plantarum* had a 52-7,210-fold (mean ± SD, 1,350±1,870; 95% CI, 458-2,250) higher QICI than *B. longum* and a 3-188-fold (mean ± SD, 33±44; 95% CI, 12-54) higher QICI than *L. lactis* ssp. *lactis*. The present results indicated that *L. plantarum* had a superior QFPD-like ability to stimulate innate cytokine production by blood immune cells. Therefore, *L. plantarum* and *B. longum* were selected in all subjects for the subsequent clinical trial.

L. plantarum ingestion induces QFPD-like innate cytokine responses in vivo. To investigate whether *L. plantarum* could reproduce *in vivo* the QFPD-like immunomodulatory activity observed *in vitro*, a single-arm, double-blind, prospective trial that included three consecutive sessions was conducted: i) A validation session using *L. plantarum* in the first 7 days; ii) a 7-day washout period; and iii) a control session using *B. longum* in the last 7 days. The peripheral blood samples that were obtained before (day 0) and after (day 8) *L. plantarum* ingestion and after *B. longum* ingestion (day 22) were evaluated for plasma TNF-α, IL-1β, IL-18, IL-8 and IL-6, as well as the QICI.

As indicated in Table I, oral intake of *L. plantarum* significantly increased plasma IL-1β [median (IQR), 0.000 (0.000-0.000) vs. 0.134 (0.092-0.292) pg/ml; P=0.0000310 after Friedman test; P=0.00284 after Nemenyi post hoc test] and decreased plasma IL-6 [median (IQR), 1.180 (0.812-2.130) vs. 0.495 (0.425-0.775) pg/ml; P=0.0131 after Friedman test; P=0.0128 after Nemenyi post hoc test]. There were no significant differences in the plasma levels of TNF-α, IL-18 and IL-8. The QICI value was significantly increased [mean fold change, 17-fold; median (IQR), 1,760 (680-3,550) vs. 12,300

Table I. Cytokine changes in *Lactobacillus plantarum*- and *Bifidobacterium longum*-administered subjects.

	Day 0		Day 8		Day 22		Friedman test	Nemenyi post hoc test	
								Lactobacillus plantarum (Day 0 vs. Day 8)	*Bifidobacterium longum* (Day 8 vs. Day 22)
Cytokines	Median	(IQR)	Median	(IQR)	Median	(IQR)	P-value	P-value	P-value
TNF-α, pg/ml	1.790	(1.300-3.160)	2.330	(1.780-3.150)	2.760	(2.530-3.020)	0.558		
IL-1β, pg/ml	0.000	(0.000-0.000)	0.134	(0.092-0.292)	0.000	(0.000-0.000)	0.0000310[a]	0.00284[a]	0.000706[a]
IL-18, pg/ml	154	(126-214)	155	(132-191)	324	(219-991)	0.146		
IL-8, pg/ml	232	(144-315)	155	(101-263)	134	(87-311)	0.584		
IL-6, pg/ml	1.180	(0.812-2.130)	0.495	(0.425-0.775)	1.420	(0.692-2.220)	0.0131[a]	0.0128[a]	0.105
QFPD-induced innate cytokine index, pg^3/ml^3	1,760	(680-3,550)	12,300	(5,440-42,200)	2,780	(940-9,820)	0.0173[a]	0.0138[a]	0.474

Data are presented as the median (IQR) and were analyzed using the Friedman test followed by the Nemenyi post hoc test. [a]P<0.05. TNF, tumor necrosis factor; IL, interleukin; IQR, interquartile range; QFPD, Qingfei Paidu decoction.

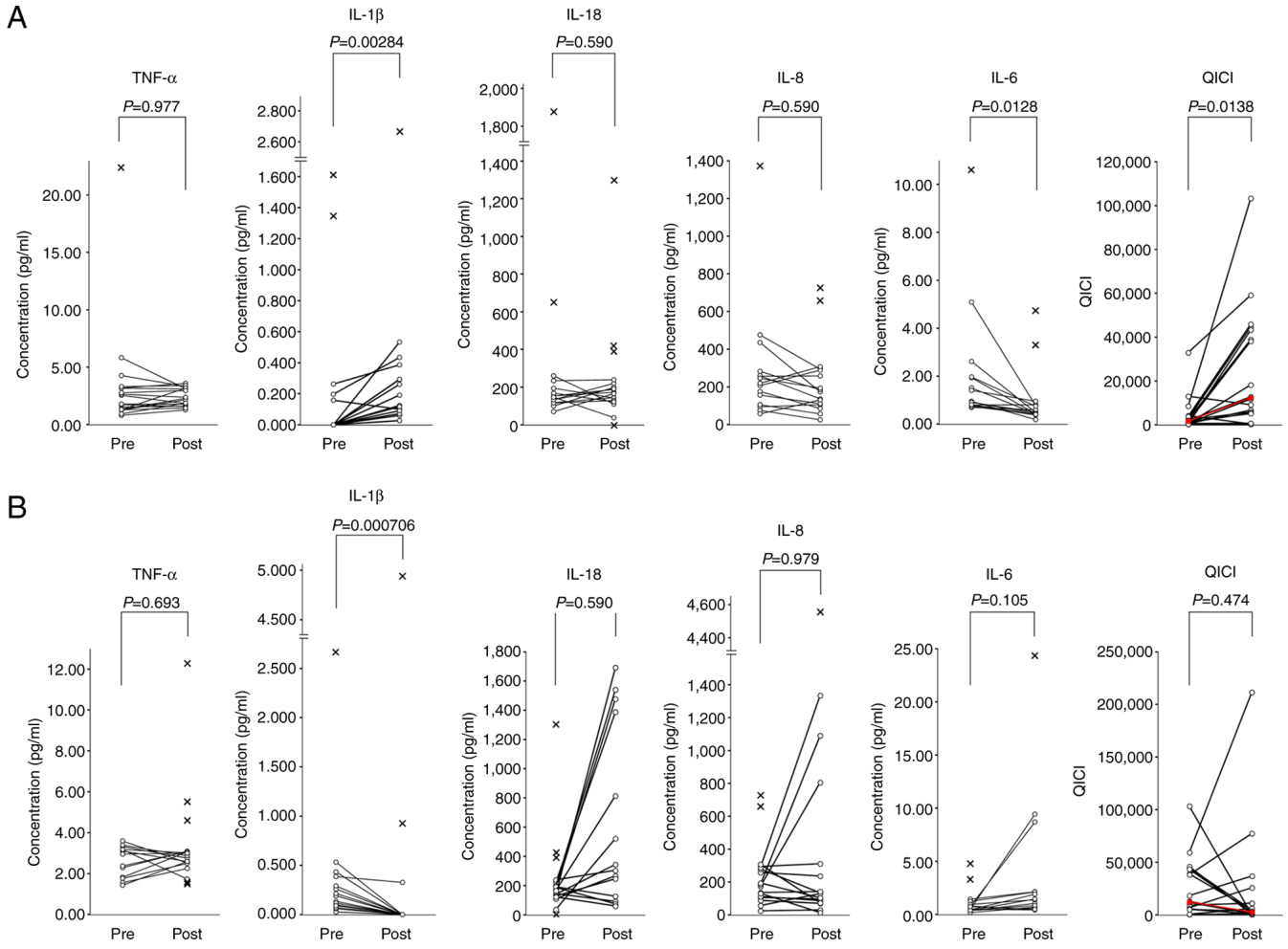

Figure 2. Changes in plasma cytokine levels and QICI values before (pre) and after (post) oral intake of (A) *Lactobacillus plantarum* SNK12 and (B) *Bifidobacterium longum* BB536. QICI was defined as follows: QICI=(TNF-α) x (IL-1β) x (IL-18) x (IL-8)/(IL-6), where brackets represent the plasma level of the cytokine in pg/ml. Cross marks denote outliers. Each red line represents the change of the medians. The data were statistically analyzed using the Friedman test followed by the Nemenyi post hoc test. QICI, Qingfei Paidu decoction-induced innate cytokine index.

(5,440-42,200); P=0.0173 after Friedman test; P=0.0138 after Nemenyi post hoc test]. The QICI values were increased in 16 of 18 subjects (88.9%) with considerable individual differences in the fold change (1-128-fold; mean fold change, 19-fold; Fig. 2A), suggesting that there are large variations in responsiveness to *L. plantarum* among individuals.

By contrast, oral intake of *B. longum* induced a significant decrease in plasma IL-1β [median (IQR), 0.134 (0.092-0.292) vs. 0.000 (0.000-0.000) pg/ml; P=0.0000310 after Friedman test; P=0.000706 after Nemenyi post hoc test]; however, the QICI value did not change significantly [mean fold change, 2-fold; median (IQR), 12,300 (5,440-42,200) vs. 2,780 (940-9,820); P=0.0173 after Friedman test; P=0.474 after Nemenyi post hoc test]. The QICI values increased in 8 of 18 subjects (44.4%), but the fold changes were markedly lower (1-9-fold; mean fold change, 3-fold; Fig. 2B) than those obtained during the *L. plantarum* session. The present results suggested that orally administered *L. plantarum* induced an *in vivo* cytokine change similar to that induced by oral QFPD in a previous experiment (51).

L. plantarum ingestion also caused a minor but significant change the mean corpuscular hemoglobin concentration [median (IQR), 33.3 (32.4-33.9) vs. 32.6 (32.1-33.1)%; P=0.00456 after Friedman test; P=0.00836 after Nemenyi post hoc test] (Table SII). No significant changes in the hematological parameters were observed during the *B. longum* session (Table SII).

The post hoc two-tailed power analysis revealed that satisfactory effect sizes were obtained [0.845 (QICI in the *L. plantarum* session)-1.20 (IL-1β in the *B. longum* session)], as well as statistical powers [0.908 (QICI in the *L. plantarum* session)-0.996 (IL-1β in the *B. longum* session)] after completion of the trial (Table SIII).

Effects of L. plantarum ingestion on the innate immune cell activity. Subsequently, the present study examined whether the *L. plantarum*-induced cytokine changes (increase in the QICI score) led to the increased activity of innate immune cells. *L. plantarum* ingestion significantly enhanced the activity of NK cells, which are key effectors of antiviral innate immunity that directly attack virus-infected host cells (Table II; Fig. 3A) (77,78). By contrast, *B. longum* ingestion significantly promoted the phagocytic activity of neutrophils (Table II; Fig. 3B). Neither *L. plantarum* nor *B. longum* ingestion activated macrophages, as assessed by the serum neopterin levels (Table II; Fig. 3C). The present results further supported the

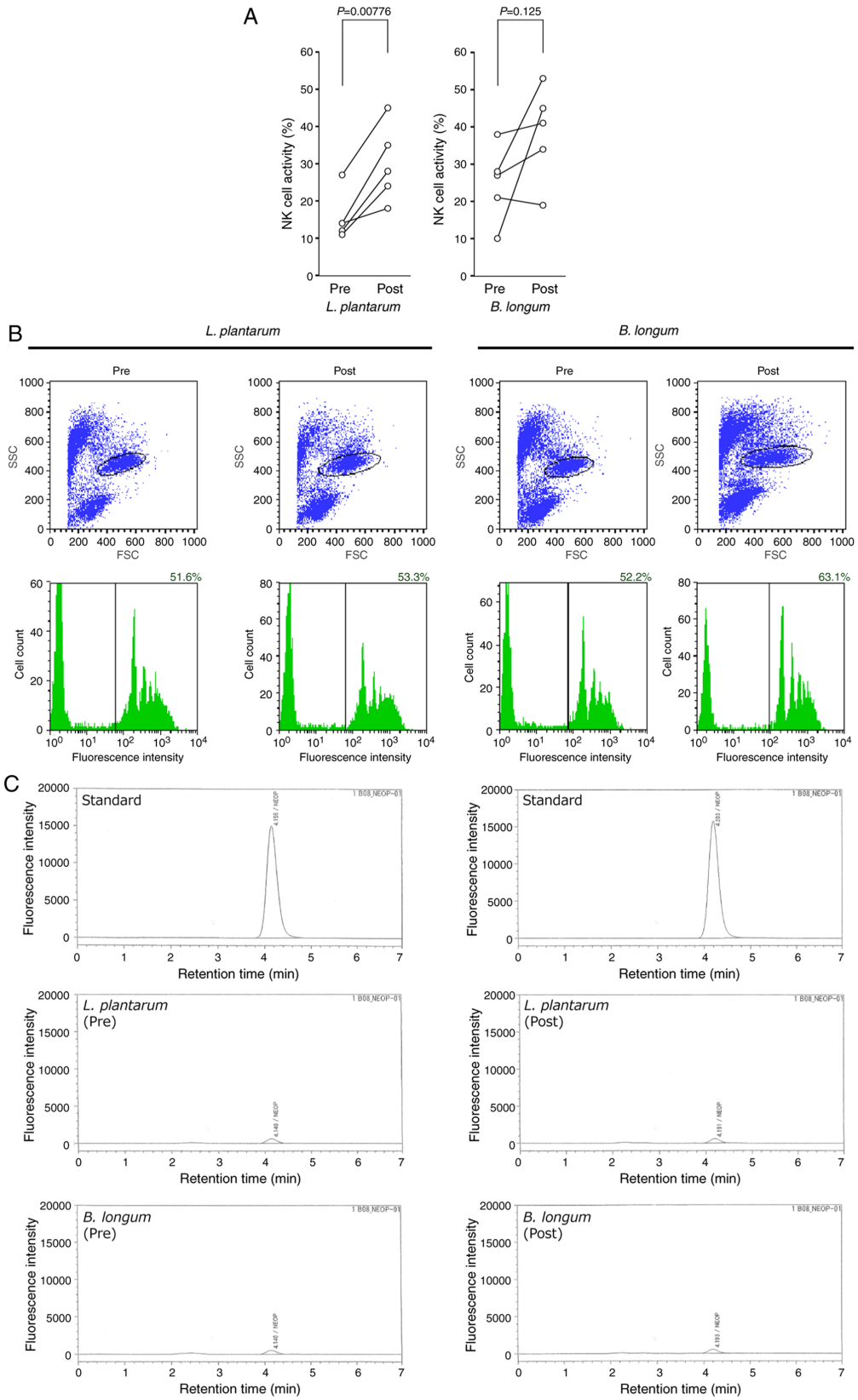

Figure 3. Effects of *L. plantarum* or *B. longum* ingestion on the innate immune cell activity. (A) NK cell activity before (pre) and after (post) the *L. plantarum* or *B. longum* ingestion. (B) Phagocytic activity of neutrophils. Representative flow cytometry plots are presented in the upper panels. The vertical and horizontal axes are SSC and FSC, respectively. Areas surrounded by black lines represent granulocyte populations characterized as medium FSC/high SSC (granulocyte gating). Histograms of fluorescence intensities of the granulocytes separated by the granulocyte gating are presented in the lower panels. The vertical and horizontal axes demonstrate cell count and fluorescence intensity, respectively. A black vertical line in each histogram indicates the threshold of fluorescence-positive granulocytes (granulocytes that phagocytosed the fluorescent microbeads). The phagocytic activity was calculated as the ratio of fluorescence-positive granulocytes to the total count of granulocytes and presented in the upper right corner of each histogram. (C) Macrophage activity. The serum levels of neopterin, an activation marker of monocytes and macrophages, were determined using reverse-phase high-performance liquid chromatography. Representative chromatograms are presented. The vertical and horizontal axes show the intensity of native fluorescence of neopterin and retention time, respectively. NK, natural killer; *L. plantarum*, *Lactobacillus plantarum*; *B. longum*, *Bifidobacterium longum*; FSC, forward scatter; SSC, side scatter.

Table II. Effects of *Lactobacillus plantarum* and *Bifidobacterium longum* ingestion on the activity of innate immune cells.

	Lactobacillus plantarum				*Bifidobacterium longum*			
Activities of innate immune cells	Baseline, mean ± SEM	Post-ingestion, mean ± SEM	P-value	95% CI	Baseline, mean ± SEM	Post-ingestion, mean ± SEM	P-value	95% CI
Phagocytic activity of neutrophils, %	54.50±4.39	57.80±4.47	0.512	-9.28-15.80	50.90±4.80	57.00±5.22	0.0154[a]	1.90-10.20
Natural killer cell activity, %	15.60±2.60	30.00±4.17	0.00776[b]	6.32-22.50	24.80±4.11	38.40±5.13	0.125	-5.91-33.10
Macrophage activity, pmol/ml	3.80±0.34	3.00±0.28	0.178	-2.16-0.56	4.20±0.34	4.00±0.28	0.374	-0.76-0.36

[a]P<0.05 and [b]P<0.01, analyzed using two-tailed paired Student's t-test. SEM, standard error of the mean; 95% CI, 95% confidence interval for the difference between two sample means.

immunological benefits of *L. plantarum* and *B. longum* against viral infection.

Discussion

A large body of evidence has suggested that *L. plantarum* strengthens several aspects of the host defense mechanism against the infection by respiratory viruses, particularly seasonal and highly pathogenic influenza viruses. For example, oral administration of *L. plantarum* in mice significantly suppressed viral replication in the lungs and reduced airway inflammation, thereby increasing survival rates (33-39). The underlying immunological effects are known to be diverse, including stimulation of type I IFN production (37,39), enhancement of NK cell activity (36,39), promotion of T helper type 1 cell-mediated immune responses (33,34,39) and activation of IgA-dependent mucosal immunity in the small intestine and lung (34,35). Similarly, randomized controlled trials have demonstrated that oral intake of *L. plantarum* reduced the risk of upper/lower respiratory tract infection and alleviated the respiratory symptoms of infected patients (30,79). The protective efficacy is associated with enhancement of the phagocytic activity of granulocytes, reduction of the plasma proinflammatory cytokines IFN-γ and TNF-α, elevation of the anti-inflammatory cytokines IL-4 and IL-10, activation of $CD8^+$ T cells and induction of the specific secretory IgA neutralizing antibodies in the bronchoalveolar lavage fluid (BALF) and sera (30,31).

In line with the wide variety of immunomodulatory abilities of *L. plantarum*, the SNK12 strain was indicated to exhibit protective effects against influenza A virus subtype H1N1 (57,58). The SNK12 strain has been isolated from traditional non-salted pickles of autochthonous red turnip that have been produced in the Kiso area of Nagano prefecture in Japan for >400 years (80). Recent animal studies demonstrated that oral administration of *L. plantarum* strain SNK12 to mice before influenza viral challenge suppressed the viral load in the BALF and lung, induced a higher titer of neutralizing antibodies in the BALF and sera and higher levels of specific IgA in BALF and feces compared with control mice and mice treated with the anti-influenza drug oseltamivir (57,58).

The present study revealed that *L. plantarum* SNK12 could also upregulate a subset of proinflammatory cytokines, as assessed both *in vitro* and in humans. This immunomodulatory effect is likely contradictory to its potential for clinical benefits against the influenza virus, since the patients present with a broad range of inflammatory symptoms. However, it is notable that acute, low-grade inflammation is a physiological basis for early stages of host defense mechanisms and has been demonstrated to serve a protective role against viral infection. Kechaou *et al* (32) demonstrated that a proinflammatory *L. plantarum* strain with superior ability to stimulate the production of IL-8 and IL-12 markedly inhibited viral proliferation in the lung and alleviated clinical symptoms in mice when orally administered before or after influenza virus challenge. Furthermore, Park *et al* (33) revealed that ingestion by mice of a probiotic *L. plantarum* strain conferred protection against influenza virus by elevating both IL-12 and IFN-γ levels in the BALF and inducing low-grade inflammation.

QFPD consists of 21 traditional Chinese herbs optimized specifically against the symptoms of COVID-19 (43). QFPD has demonstrated satisfactory therapeutic benefits in patients with mild-to-severe disease in clinical trials in China (46-50) and has been recommended officially for the treatment of COVID-19 (43). Our recent clinical study indicated that oral QFPD upregulated the blood levels of TNF-α, IL-1β, IL-18 and IL-8, which are key mediators of innate immune responses to viral infection (51). TNF-α, IL-1β and IL-18 are induced directly by toll like receptor (TLR)7/TLR8 and NLR family pyrin domain containing 3 (NLRP3), foreign single-stranded RNA (ssRNA) sensors in dendritic cells and macrophages (81-83). These 'immediate-early' cytokines initiate and coordinate a broad spectrum of downstream antiviral immune cascades (81-83). Notably, recent metagenomics studies demonstrated that the TLR7/8- and NLRP3/inflammasome-mediated inflammatory pathways are strongly suppressed in the upper airway of patients with COVID-19 and those non-responsive to SARS-CoV-2 infection early in the course of the disease (84,85). These findings highlight the importance of the active TLR7/8- and NLRP3-driven inflammatory pathways in the early stages of anti-SARS-CoV-2 immunity. We hypothesized that QFPD, which mimics the blood cytokine environment produced by TLR7/8- and NLRP3-driven early innate immune responses to ssRNA viruses, may be effective in preventing SARS-CoV-2 infection (51). The present study suggested that *L. plantarum*, which can stimulate innate cytokine changes similar to those induced by oral QFPD, may also potentially provide a protective benefit against COVID-19.

IL-6 serves key roles in complex immune dysregulation and systemic hyperinflammation, which are hallmarks of severe COVID-19, and IL-6 blood level has been associated with COVID-19 severity and mortality (54-56). The production of IL-6 and TNF-α is stimulated directly by common TLR7/8-driven intracellular signaling in response to ssRNA viruses (81-83). However, *L. plantarum* ingestion significantly increased the plasma level of TNF-α, whereas it downregulated IL-6 plasma level in the present trial. Although the mechanism of this opposite effect remains unknown, the ability of *L. plantarum* to reduce blood IL-6 may be indicative of its prophylactic administration to uninfected individuals.

Recent transcriptomic studies have demonstrated that exhausted NK cell responses determine severity and fatality of COVID-19. Liu *et al* (86) have identified IL-15-mediated attenuated inflammation in NK cells as being primarily associated with COVID-19 severity. A study by Sahoo *et al* (87) has also highlighted that IL15-mediated NK cell exhaustion, senescence and apoptosis are important determinants for severe/fatal COVID-19. *L. plantarum*-induced activation of NK cells may therefore be efficacious as an adjunctive therapy to improve NK cell exhaustion and dysfunction in severe or fatal COVID-19.

The main limitations of the current study are the small number of participants, the selection of uninfected individuals as subjects and a female-biased gender ratio in the subjects. Further clinical studies with larger cohorts are required to confirm the conclusions and determine generalizability. The uninfected subjects employed were healthy, in order to examine the feasibility of prophylactic, daily use of *L. plantarum*. As a result, no chest CT images of the subjects were obtained. Additional studies are essential to clarify whether *L. plantarum* can induce the similar cytokine changes and improve chest CT findings in patients with COVID-19. Further studies are also required to identify probiotic strains with the ability to strengthen innate immunity by inducing moderate physiological inflammation. In addition, in the control session using *B. longum* (day 15-21), blood samples were obtained at day 8 as baseline, not at day 14. Since the plasma IL-1β and IL-6 levels had been up- or down-regulated by *L. plantarum* by day 8, it is not possible to exclude the possibility that the IL-1β and IL-6 levels were spontaneously restored to the day 0 levels during days 8-22 without the effects of *B. longum*. Similarly, there was a significant increase in the QICI in the *L. plantarum* session (Table I, comparison between day 0 and day 8), whereas no significant change in the QICI was observed in the *B. longum* session (comparison between day 8 and day 22). *B. longum* sustained the QICI score that had been upregulated by *L. plantarum*. Therefore, it cannot be excluded either that *B. longum* also had positive effects on the QICI score *in vivo*.

Considering the recent emergence of novel SARS-CoV-2 variants with relevant mutations that potentially affect the efficacy of vaccines and therapeutic antibodies, there is an increasing need to prepare diverse prophylactic and therapeutic options against COVID-19. The present study indicated that *L. plantarum* exhibited a superior ability to mimic inflammatory innate cytokine responses essential for early stages of host defense mechanism against viral infection. Daily consumption of probiotic *L. plantarum* strains may be a reasonably safe, cost-effective and viable option to protect uninfected individuals against SARS-CoV-2 infection during the pandemic.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YK, YN, TE, TA and TN conceived the study. YK, YN, TE, TA and TN developed the methodology. TN performed formal analysis. YK, KA, KY and TE performed/interpreted the experiments. YK, KA, KY and TE provided resources. YK and TE curated data. TN wrote the original draft. YK, YN, KA, KY, TE and TA reviewed and edited the manuscript. TN was involved in visualization. YK, YN, TA and TN supervised the study. YK, KA and TE were involved in project administration. YK, TE and TN confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was carried out in accordance with The Code of Ethics of the World Medical Association (The Declaration of

Helsinki). All procedures were reviewed and approved by the Ethics Committees of Takanawa Clinic (approval nos. 2016-3, 20 December 2016 and 2020-2, 7 May 2020; Tokyo, Japan). A signed informed consent form was obtained from each participant prior to inclusion in the present study.

Patient consent for publication

Not applicable.

Competing interests

YK, KA, KY and TE are employees of Takanawa Clinic. TA and TN serve as research advisors to Takanawa Clinic and receive advisory fees.

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