Safety and efficacy of using heat-killed \textit{Lactobacillus plantarum} L-137: High-dose and long-term use effects on immune-related safety and intestinal bacterial flora

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ABSTRACT
Heat-killed \textit{Lactobacillus plantarum} L-137 (HK L-137) promotes immune function in animals. In healthy people, T-cell proliferation was shown to be enhanced by taking 10 mg HK L-137 daily for 12 weeks. However, the safety and efficacy of higher doses or longer treatments have not yet been investigated in humans. To investigate the high-dose and long-term use effects of HK L-137 on immune-related safety and on host intestinal bacterial flora, 15 healthy volunteers took a daily HK L-137 (50 mg) preparation for 4 weeks. An additional 29 participants who regularly visited a clinic for health care took HK L-137 (10 mg) daily for 12 months. Measures for anthropometrics, hematology, biochemistry, and urinalysis were taken at scheduled timepoints for all participants. Stool and blood samples were also collected and evaluated for microbes and short-chain fatty acids (SCFA); isolated T-cells were assessed for levels of proliferation induced by phytohemagglutinin in the long-term study. Adverse events or shifts in clinical measures from normal ranges due to the dietary intervention were not observed in the high-dose or long-term studies. Long-term intake also did not result in immune exhaustion due to any chronic immunostimulation; ex vivo T-cell proliferation was significantly greater at 12 months than at baseline ($p<0.01$). In addition, the Firmicutes/Bacteroidetes ratio in stool samples was significantly lower at 12 months than at baseline ($p<0.05$) due to the long-term intake of the HK L-137. Lastly, fecal SCFA concentrations were significantly greater ($p<0.05$) at 6 months than at baseline. From these data, it can be concluded that the efficacy of HK L-137 is maintained with no overt adverse effects as a result of high-dose and/or long-term consumption.

Introduction

\textit{Lactobacillus plantarum} L-137 was first isolated from fermented fish and rice in Southeast Asia (Olympia et al. 1992). Heat-killed \textit{L. plantarum} L-137 (HK L-137) potently induces T-helper-Type 1 cell-related cytokines, including interleukin (IL)-12 and interferon (IFN)-\gamma, \textit{in vitro} as well as \textit{in vivo} in mice (Murosaki et al. 1998; Nakai et al. 2019). Studies in mice have revealed that administration of HK L-137 suppresses the IgE response in a food allergy model (Murosaki et al. 1998), inhibits tumor growth (Murosaki et al. 2000), and enhances protection against influenza virus infection (Maeda et al. 2009). In human studies, daily intake of HK L-137 improved health-related quality of life in healthy participants assessed using a self-rating questionnaire with 26 items (Hirote et al. 2006). It also decreased the incidence of upper respiratory tract infection in participants with high levels of psychological stress who were otherwise healthy but potentially susceptible to catching a cold (Hirote et al. 2013). In addition, HK L-137 helped improve the status of host systemic inflammation (decreased leukocyte count) and hepatic inflammation (decreased aspartate aminotransferase [AST] and alanine transaminase [ALT]) as well as lipid metabolism (decreases in cholesterol) in overweight adults, possibly through an improvement in the condition of their intestinal barrier (Tanaka et al. 2020). Thus, HK L-137 might contribute to these effects, in part, by enhancing immune function in the host.

In studies examining the effectiveness of HK L-137, when human subjects took daily oral doses of 10 mg for a relatively short period (12 weeks), this resulted in significantly-augmented proliferative capacities of T-cells isolated from their peripheral blood; these changes in T-cell activity occurred without any notable adverse events in these hosts (Hirote et al. 2006, 2013; Tanaka et al. 2020). In fact, during its 15 years on the market, there have been “virtually” no apparent HK L-137-related health problems in long-term users of the product. However, the immune-related safety of HK L-137 as a result of longer treatments has not yet been investigated in humans. Animal models studies reflected this same lack of adverse outcome; one study in mice given high-dose HK L-137 (at 10 mg/kg, suggested acceptable daily intake for humans) by a single gavage did not evidence any safety concerns (Hirote et al. 2009). In fact, some effects appeared to be beneficial. For example, in Red Sea bream juveniles, providing these hosts diets containing HK L-137 at 10–2000 ppm (extrapolates to daily intake of \approx 5–1000 mg in humans using body surface area normalization method) led to augmented natural immunity irrespective of the dose of HK.
L-137 used (Dawood et al. 2015). Similarly, Nile tilapia juveniles given 10–50 ppm HK L-137-containing diets, (corresponding to 5–25 mg/day in humans) also showed augmented natural immunity irrespective of dose (Nguyen et al. 2019). Nevertheless, even in light of the above-noted studies, the safety and efficacy of higher doses or longer treatment periods have not been systematically investigated in humans. Of concern is whether long-term consumption of HK L-137 might induce immune unresponsiveness, since chronic immune stimulation can trigger T-cell exhaustion (Saedi et al. 2018).

Apart from the effects on host immune status, HK L-137 consumption has also been shown to lead to improved hepatic inflammation status and in hepatic lipid metabolism in mice and humans; these changes are believed due, in part, to enhancements in intestinal barrier function (Tanaka et al. 2020; Yoshitake et al. 2021). The relationship between intestinal barrier and microbiota is complex, but recent studies showed that oral intake of heat-killed probiotics, such as Enterococcus faecalis EC-12, L. gasseri CP2305, and L. kunkeei YB38, influenced the composition of the intestinal microbiota and the intestinal environment in healthy adults (Terada et al. 2004; Asama et al. 2016; Sugawara et al. 2016). The intestinal microbial ecosystem is a key regulator of intestinal conditions and host immune function. Moreover, recent studies have reported that abnormal alterations in the intestinal microbial flora are implicated in several human diseases such as obesity, cardiovascular disease, inflammatory bowel disease, and autism (Kinross et al. 2011). Therefore, it is meaningful to investigate the effects of HK L-137 on intestinal microbiota in humans.

Accordingly, in the study reported here, the safety of high-dose and long-term intake of HK L-137 was evaluated. In these experiments, the effects of long-term intake on immune function and on the intestinal microbiota of the dosed hosts were monitored.

Materials and methods

Preparation of heat-killed L. plantarum L-137

LP20 (House Wellness Foods Corp., Hyogo, Japan), containing 20% HK L-137 and 80% dextrin, was used in this study. HK L-137 was prepared by the method described previously in Fujiki et al. (2012). In brief, L. plantarum L-137 was incubated in maltose, yeast extract and peptone-based modified medium (modified GYP medium) at 32°C for 18 hr, harvested and then washed by microfiltration. The preparation was then heated to 80°C for 20 min and spray-dried.

High-dose consumption of HK L-137

Participants

Sixteen healthy volunteers (8 men, 8 women; mean age 44.3 [± 10.3]) participated in this study. Their eligibility was assessed by interviews and consulting with their health care providers. The inclusion criteria for the study were general good health, ages between 20 and 64 years, and a willingness to follow the trial guidelines. Exclusion criteria were: prior daily consumption of lactic acid bacteria used in this study, milk allergy, pregnancy, breastfeeding, current medical treatment, a history of serious illness, abnormal laboratory findings, or cardiopulmonary dysfunction. Eating habits were not considered as part of the criteria, and participants were instructed to continue their usual diets throughout the study.

This study was approved by the Ethics Committee of Aiseikai Hospital Ueno Clinic in accordance with the Helsinki Declaration of 1975 as revised in 2013 and the ethical guidelines for epidemiological research proposed by the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) and the Ministry of Health, Labor and Welfare (MHLW). The procedures were fully explained to the participants, and written informed consent was obtained from each participant before the study commenced.

Experimental design

This study was designed as a single-arm, non-randomized, open-label study to evaluate the safety of the short-term use of high-dose HK L-137 (i.e. 50 mg/day for 4 weeks) in healthy participants. Here, participants consumed five-times the effective dose of HK L-137 (see Hirose et al. 2006, 2013; Tanaka et al. 2020) each day (5 capsules each containing 10 mg of HK L-137) for 4 weeks. The study was conducted in accordance with the International Conference on Harmonization of Good Clinical Practice.

Anthropometric measures (e.g. body height, body weight, body mass index [BMI], systolic blood pressure [SBP], diastolic blood pressure [DBP], and pulse rate) were measured at scheduled timepoints. Safety assessments (hematologic examination, biochemical analysis, and urinalysis) were performed before the start of treatment (baseline), 2 and 4 weeks after the start of treatment, and 2 weeks after the end of treatment. Safety assessments were conducted by LSI Medicine (Tokyo, Japan). Physicians examined results of the medical reviews/self-record diaries at scheduled timepoints. The study was conducted at Medical Station Clinic (Tokyo, Japan) from February to April 2015.

The hematological parameters measured here were: white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb), hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets. The biochemical parameters measured were blood: total protein, albumin, total bilirubin, AST, ALT, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GTP), total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-Chol), low-density lipoprotein cholesterol (LDL-Chol), glucose, creatinine, blood urea nitrogen (BUN), uric acid, sodium, potassium, and chloride. The urinalysis parameters included measures of glucose, protein, and occult blood.

Long-term consumption of HK L-137

Participants

Thirty participants (15 men, 15 women; mean age 65.5 [± 10.3] years), who had regularly visited the study clinic for health care, participated in this study. Eligibility was assessed by interviews and consulting with their health care providers. Inclusion criteria for the study were age > 20 years and a low bifidobacteria composition in past stool samples (as judged by the physician). Exclusion criteria were: prior daily consumption of the lactic acid bacteria used in this study, milk allergy, pregnancy, breastfeeding, or childbearing desire, current pharmaceutical treatment or radiotherapy for cancer, thyroid disorder, or arthritic rheumatism, or being judged “unsuitable” for this study by the investigators. Eating habits were not considered for inclusion or
This study was approved by the Ethics Committee of Wakamatsukawada Clinic in accordance with the Helsinki Declaration of 1975 as revised in 2013 and the ethical guidelines for epidemiological research proposed by MEXT and MHLW. The procedures were fully explained to the participants, and written informed consent was obtained from each participant before the study commenced. This study was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN000034695).

**Experimental design**

This was a 12-mo single-arm, non-randomized, open-label study to evaluate the safety, primarily immune-related safety, of long-term consumption of a previously-determined effective dose of HK L-137 (i.e., 10 mg/day; see Hirose et al. 2006, 2013; Tanaka et al. 2020) in adults, as well as its potential effects on host intestinal bacterial flora. Here, participants consumed one tablet containing 10 mg HK L-137 daily for 12 months. The study was conducted in accordance with the International Conference on Harmonization of Good Clinical Practice.

Anthropometric measurements, immune function analysis, and safety assessments (i.e. hematologic examination, biochemical analysis, and urinalysis) were performed before and 3, 6, and 12 months after start of treatment. Safety assessments and immune function analysis were conducted by LSI Medicine. Subjective physical symptoms were evaluated using self-described questionnaires at scheduled timepoints. stool samples were collected at scheduled timepoints and analyzed by Techno Suruga Laboratory (Shizuoka, Japan). Participant general health was assessed via direct interviews with health care providers at scheduled timepoints. The study was conducted at Wakamatsukawada Clinic (Tokyo, Japan) from November 2018 to October 2019.

**Immune-related safety**

An ex vivo proliferation assay for participant whole blood lymphocytes stimulated by phytohemagglutinin (PHA) was done by LSI Medicine. In brief, blood samples were diluted to a fixed dilution rate and cultured with or without an optimal dose of PHA, and then pulse-labeled with [3H]-thymidine. At the end of this period, DNA synthesis reflecting cellular division/proliferation was assessed by measuring thymidine uptake in cells. White blood cell sub-populations (percentages of lymphocytes, neutrophils, eosinophils, basophils, monocytes), and relative numbers of CD2⁺ T-cells and CD20⁺ B-cells were determined via flow cytometry performed at LSI Medicine using standard labeling protocols with fluorochrome-labelled antibody against each specific marker.

**Anthropometric, hematologic, biochemical, and urinary measurements**

Body height, body weight, BMI, waist circumference, SBP, and DBP were measured according to the protocol. The hematological parameters measured in isolated blood samples were WBC, RBC, hemoglobin, hematocrit, MCV, MCH, MCHC, and platelets. The biochemical parameters measured in blood were levels of total protein, albumin, total bilirubin, AST, ALT, LDH, ALP, γGTP, total cholesterol, triglycerides, HDL-Chol, LDL-Chol, glucose, creatinine, BUN, uric acid, creatine kinase (CK), hemoglobin A1c (HbA1c), C-reactive protein (CRP), sodium, potassium chloride, and calcium. Urinalysis parameters evaluated in isolated samples were total glucose, protein, occult blood, urobilinogen, bilirubin, and ketones.

**Subjective physical symptoms**

Subjective physical symptom status of study participants were evaluated based on responses provided in self-described questionnaires. A total of 20 items were scored as present or absent, e.g. cough, phlegm, slight fever, headache, dizziness, tinnitus, lisp, hand-foot numbness, chest pain, palpitations, dry mouth, stiff shoulders, swelling of limbs, cold limbs, backache, fatigue, night urination, depression, and hot flashes. Three items in the questionnaire were scored on a 3-point scale, i.e. appetite (large, normal, poor), defecation (soft feces, normal, constipation), and sleep (deep, normal, night waking).

**Evaluation of stool samples**

Fecal samples for intestinal microbiota analysis were kept in collection tubes containing guanidine thiocyanate solution and analyzed at Techno Suruga. DNA extraction and fecal microbiota analysis via a terminal restriction fragment length polymorphism (T-RFLP) method were done as previously described (Nagashima et al. 2003, 2006; Takahashi et al. 2014). In brief, the 16S rRNA gene in DNA isolated from each sample was amplified with fluorescent-labeled primers. The amplified products were then digested with Bst 1 restriction enzyme and classified by distribution of operational taxonomic units (OTU). Each OTU was quantified as a percent of total OTU area, and expressed as percentage of the area under the curve. T-RFLP was used to classify microbes into the following groups: Lactobacillales, Clostridium sub-cluster XIVa, Clostridium clusters IV, IX, XI, and XVIII, Bacteroides, Prevotella, Bifidobacterium, and others. Firmicutes/Bacteroidetes (F/B) ratios were also calculated (Emoto et al. 2016; Saji et al. 2019). The phyllum Firmicutes included Lactobacillales and Clostridium, while Bacteroidetes included Bacteroides and Prevotella.

Fecal samples for short-chain fatty acid (SCFA) content measurement were stored at −20°C in collection tubes (without storage solution) until analyzed (Techno Suruga). Fecal levels of acetate, propionic, iso-butyric, n-butyric, iso-valeric, n-valeric, and n-capric acids were measured using a 7890B gas chromatography-flame ionization system (Agilent Technologies, Santa Clara, CA).

**Statistical analysis**

In consideration of statistical power, a Student’s paired t-test for assessing safety and efficacy at each timepoint rather than over the entire study period was utilized. Values at baseline and at each timepoint was used to analyze all anthropometric, biochemical, and hematologic measures, as well as the composition of intestinal microbiota, fecal SCFA, and biomarkers of immune functions. A Wilcoxon signed-rank test was used to compare subjective physical symptom scores (ranked 0, 1 or 0, 1, 2) at baseline and at each timepoint. Statistical analyses were performed using SPSS Statistics 25 (IBM Japan, Tokyo, Japan). A p-value < 0.05 was considered significant.
Results

High-dose consumption of HK L-137

Among the 28 potential recruits, 16 were eligible to participate in the study. One dropped out due to personal reasons (scheduling difficulties) before completing the study. The other 15 participants completed the study and were included in the safety analysis (Figure 1). Adverse events were assessed in all 16 participants who had taken HK L-137 at least once. A total of 11 adverse events were recorded in 7 out of 16 study participants. Four were symptoms of a common cold, and the remaining were one each of abdominal pain, hangover, hay fever, sputum, diarrhea, chills, and rhinitis. All adverse events were either not caused by HK L-137 or were mild and transient. As a result, all adverse events were judged by the responsible physicians to be unrelated to HK L-137 use.

Anthropometric, hematological, and biochemical measures are shown in Tables 1–3, respectively. Compared to baseline, body weight and BMI were significantly higher at 4 weeks, pulse rate was significantly lower at 2 weeks, hemoglobin was significantly higher at 2 weeks, and both MCV and MCH at 2 and 4 weeks were significantly higher. Also compared to baseline, platelet levels were lower at 2 weeks, total bilirubin, HDL-cholesterol, and sodium were significantly lower at 4 weeks, and both potassium at 2 weeks and chloride at 4 weeks were each significantly higher. These changes were all considered “inconsequential” by

| Assessed for eligibility (n = 28) |
|------------------|
| Ineligible |
| Did not meet inclusion criteria (n = 12) |
| Allocated (n = 16) |
| Dropped out (scheduling difficulties) (n = 1) |
| Completed follow-up (n = 15) |
| Analyzed (n = 15) |

Figure 1. Flowchart of participants who took high-dose HK L-137.

Table 1. Anthropometric measures in participants who took high-dose HK L-137 for 4 weeks.

| Weight (kg) | 57.8 ± 9.2 | 58.9 ± 9.3 | 58.3 ± 9.4 |
| BMI (kg/m²) | 21.1 ± 1.8 | 21.2 ± 1.8 | 21.3 ± 1.8 |
| SBP (mmHg) | 14.2 ± 1.1 | 14.5 ± 1.0 | 14.7 ± 1.2 |
| Hematocrit (%) | 44.2 ± 3.3 | 45.0 ± 2.7 | 44.3 ± 3.1 |
| Hemoglobin (g/dl) | 94.1 ± 3.1 | 95.4 ± 4.2 | 94.9 ± 4.0 |
| Platelets (x10⁹/μl) | 24.6 ± 3.8 | 23.6 ± 3.6 | 24.1 ± 4.5 |

Values are means ± SD; N = 15. *p < 0.05, **p < 0.01 vs. baseline (paired t-test).

Table 2. Hematological measures in participants who took high-dose HK L-137 for 4 weeks.

| WBC (>10³/μl) | 56.1 ± 22.5 | 57.3 ± 15.8 | 53.4 ± 18.6 |
| RBC (>10⁹/μl) | 471.0 ± 43.0 | 473.0 ± 40.0 | 469.0 ± 39.0 |
| Hemoglobin (g/dl) | 14.2 ± 1.1 | 14.5 ± 1.0 | 14.7 ± 1.2 |
| Hematocrit (%) | 44.2 ± 3.3 | 45.0 ± 2.7 | 44.3 ± 3.1 |
| MCV (fl) | 32.2 ± 0.6 | 32.2 ± 0.5 | 32.1 ± 0.4 |
| MCH (pg) | 95.4 ± 4.2 | 95.4 ± 4.0 | 95.0 ± 3.8 |
| Platelets (x10⁹/μl) | 24.6 ± 3.8 | 23.6 ± 3.6 | 24.1 ± 4.5 |

Values are means ± SD; N = 15. *p < 0.05, **p < 0.01 vs. baseline (paired t-test).

WBC: white blood cells; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

Table 3. Biochemical measurements in participants who took high-dose HK L-137 for 4 weeks.

| Total protein (g/dl) | 7.07 ± 0.42 | 7.05 ± 0.37 | 7.05 ± 0.44 |
| Albumin (g/dl) | 4.43 ± 0.33 | 4.45 ± 0.26 | 4.38 ± 0.32 |
| Total bilirubin (mg/dl) | 0.74 ± 0.18 | 0.72 ± 0.21 | 0.65 ± 0.12 |
| AST (U/L) | 19.2 ± 4.2 | 19.3 ± 6.3 | 19.2 ± 4.2 |
| ALT (U/L) | 16.8 ± 7.0 | 16.8 ± 6.9 | 16.7 ± 10.0 |
| LDH (U/L) | 160.0 ± 25.0 | 160.0 ± 26.0 | 160.0 ± 26.0 |
| ALP (U/L) | 183.0 ± 47.0 | 190.0 ± 48.0 | 181.0 ± 47.0 |
| γ-GTP (U/L) | 21.8 ± 16.2 | 20.7 ± 14.0 | 21.1 ± 15.3 |
| Cholesterol (mg/dl) | 21.1 ± 39.0 | 21.0 ± 41.0 | 210.0 ± 37.0 |
| Triglycerides (mg/dl) | 70.10 ± 28.40 | 80.20 ± 38.10 | 73.50 ± 21.20 |
| HDL-Chol (mg/dl) | 75.40 ± 13.70 | 74.0 ± 15.50 | 71.30 ± 12.60 |
| LDL-Chol (mg/dl) | 120.0 ± 34.00 | 121.0 ± 35.00 | 120.0 ± 34.00 |
| Glucose (mg/dl) | 84.80 ± 7.30 | 83.90 ± 7.50 | 83.50 ± 5.30 |
| Creatinine (mg/dl) | 0.74 ± 0.15 | 0.75 ± 0.15 | 0.74 ± 0.14 |
| Uric acid (mg/dl) | 4.89 ± 1.39 | 4.92 ± 1.12 | 4.63 ± 1.28 |
| Sodium (mEq/L) | 141.00 ± 2.00 | 141.50 ± 1.80 | 139.50 ± 1.60 |
| Potassium (mEq/L) | 4.29 ± 0.20 | 4.45 ± 0.27 | 4.26 ± 0.25 |
| Chloride (mEq/L) | 103.90 ± 1.70 | 103.50 ± 2.10 | 104.90 ± 2.10 |

Values are means ± SD; N = 15. *p < 0.05, **p < 0.01 vs. baseline (paired t-test).

AST: aspartate aminotransferase; ALT: alanine transaminase; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; γ-GTP: γ-glutamyl transpeptidase; HDL-Chol: high-density lipoprotein cholesterol; LDL-Chol: low-density lipoprotein cholesterol; BUN: blood urea nitrogen.
the study physicians because each remained within reference ranges. Among urinalysis parameters, there was no positive test result during the test period. The study physicians concluded that daily intake of 50 mg HK L-137 for 4 weeks was not associated with overt safety problems.

Long-term consumption of HK L-137

Sixty people were assessed for eligibility; 30 were deemed eligible and enrolled in the study. Before completing the study, one participant dropped out due to preexisting chronic gastrointestinal upset. The other 29 participants completed the study and were included for the analysis of safety and efficacy (Figure 2). Adverse events were assessed in all 30 participants who had taken HK L-137 at least once. One adverse event was recorded during the study. It was mild gastrointestinal upset and was judged to be unrelated to the dietary intervention due to the participant having preexisting chronic gastrointestinal upset.

Immune-related safety

WBC populations, numbers of T- and B-cells in the blood, and ex vivo PHA-induced T-cell proliferation values for with the participants who took the HK L-137 for 12 months are shown in Table 4. The percentages of WBC that were lymphocytes, neutrophils, eosinophils, basophils, and monocytes did not change significantly during the 12-months dietary intervention. There was also no change in numbers of T-cells. However, there was significant transient decrease in B-cell numbers at 3 months, but this returned to baseline levels thereafter. There was no decline in ex vivo T-cell responsiveness due to long-term intake of HK L-137; instead, PHA-induced proliferation was significantly increased vs. baseline at 12 months.

Overall safety

Anthropometric, hematological, and biochemical measures are shown in Tables 5–7, respectively. Compared to baseline, body weight and BMI were significantly increased at 3 months, SBP was significantly decreased at 6 months, and MCHC was significantly increased at 12 months, though all parameters remained within reference ranges. Compared to baseline, HbA1c was significantly higher at 12 months, and sodium at 3 and 6 months and chloride at 3 months were significantly higher; potassium at 3 months was significantly lower. These changes were considered as age-related or seasonal variations. Among urinalysis parameters, positive urine ketones were observed in 1 participant at 6 months and in 2 participants at 12 months; these outcomes were deemed unrelated to the dietary intervention. The responsible physician concluded that a daily intake of 10 mg HK L-137 for 12 months was not associated with safety problems.

Subjective physical symptoms

There was no significant deterioration in 23 subjective physical symptoms during the dietary intervention; this supported a claim for the safety of long-term HK L-137 consumption. Night urination was significantly better at 6 months than at baseline; this correlated with a trend toward improved sleep at 6 and 12 months compared to baseline (p = 0.058 and 0.059, respectively).

Effects on intestinal microbiota

Intestinal microbiota composition analysis results are shown in Table 8. Abundance of Clostridium sub-cluster XIVa was significantly increased vs. baseline at 12 months, though all parameters remained within reference ranges. Compared to baseline, BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure.
significantly lower at 12 months than at baseline, while that of *Bacteroides* was significantly greater at 6 and 12 months than at baseline. As a result, vs. baseline, *Firmicutes* abundance was significantly lower at 12 months whereas *Bacteroidetes* was significantly higher at 3 and 12 months. Accordingly, the F/B ratio (relevant to metabolic syndrome) was significantly lower at 12 months. Fecal concentrations of SCFA involved in gut homeostasis are shown in Table 9. The concentrations of n-butyric acid, iso-butyric acid, n-valeric acid, and iso-valeric acid were significantly higher at 6 months than at baseline, whereas those at 12 months were higher than at baseline. However, the difference was no longer significant at that point.
Table 9. Effects of long-term intake of HK L-137 on fecal short-chain fatty acids.

| Acid Type   | Baseline | 3 months | 6 months | 12 months |
|-------------|----------|----------|----------|-----------|
| Acetonic acid | 45.90 ± 22.2 | 46.80 ± 18.0 | 54.50 ± 31.20 | 48.40 ± 20.90 |
| Propionic acid | 17.60 ± 9.90 | 15.70 ± 7.60 | 21.60 ± 13.10 | 19.10 ± 10.80 |
| n-Butyric acid | 14.20 ± 9.30 | 12.90 ± 9.30 | **19.80 ± 12.30** | 16.20 ± 13.20 |
| iso-Butyric acid | 2.03 ± 1.34 | 1.69 ± 1.06 | **2.92 ± 1.65** | 2.36 ± 1.33 |
| n-Valeric acid | 1.88 ± 1.08 | 1.54 ± 0.96 | **2.92 ± 1.74** | 2.36 ± 1.33 |
| iso-Valeric acid | 2.99 ± 2.14 | 2.37 ± 1.63 | **4.20 ± 2.47** | 3.27 ± 2.24 |

Values are means (µmol/g) ± SD; N = 29. *p < 0.05, **p < 0.01 vs. baseline (paired t-test).

Discussion

In the present study, no adverse events or unfavorable changes in clinical measurements were observed in participants who took 50 mg HK L-137 orally daily for 4 weeks or 10 mg daily for 12 months. In the 12-months study, *ex vivo* T-cell proliferation did not decrease but instead was significantly higher at 12 months than at baseline. In addition, fecal samples showed significantly lower proportions of phylum Firmicutes and significantly higher proportions of Bacteroides after participants had taken HK L-137. This was associated with a significant decrease in F/B ratio and a temporal increase in fecal SCFA levels. From these outcomes, it can be assumed that any immunostimulatory effect from HK L-137 might be maintained for prolonged periods without adverse reaction.

The present study also showed that HK L-137 taken at a dose of 50 mg (5-times the effective dose) was not associated with safety issues in humans. One study of safety evaluation in mice given a single oral dose of HK L-137 at 1000 mg/kg body weight suggested that the acceptable daily intake of HK L-137 corresponded to 600 mg/person. Animal studies, in which both rodent sea bream juveniles given diets containing 2000 ppm HK L-137 for 56 days (Dawood et al. 2015) and tilapia fingerlings given 1000 ppm HK L-137-containing diets for 12 weeks (Dawood et al. 2019) grew soundly and showed improved survival rates, have suggested to investigators that the human daily intake of HK L-137 at extrapolated doses of 500–1000 mg/person could be safe and potentially augment host immune defenses. This would be in keeping with the findings of human studies in which 11–32 healthy participants consumed L. plantarum 299v daily at a 2 × 10^11 CFU (≈200 mg/day) for 2–4 weeks (Goossens et al. 2003, 2005, 2006). Together, these results seem to suggest that a daily intake of HK L-137 at a dose of up to 200 mg/person would be safe and effective.

The present study showed long-term consumption of immunostimulatory HK L-137 for 12 months had no overt adverse effect. Recently, Maldonado et al. (2019) demonstrated the safety of two strains of probiotics given to infants for 12 months, i.e. *L. fermentum* CECT5716 LC40 and *Bifidobacterium breve* CECT7263. As live and heat-killed probiotics are able to induce immunomodulation (Pique et al. 2019), intake of immunostimulatory bacteria like HK L-137 for long periods should also be safe and well-tolerated. Another concern with long-term consumption of immunostimulatory bacteria is if continued immune stimulation reduces the responsiveness of the host immune system. This concern is based on reports that chronic viral infections and/or cancers can trigger T-cell exhaustion due to persistent antigenic exposure and inflammation (Wherry and Kurachi 2015; Saeidi et al. 2018). Considering the results of the present study that showed significant increases in *ex vivo* T-cell proliferative capacity of cells from participants who consumed HK L-137 for 12 months, and the previous study that showed beneficial health effects in infants given probiotics for 12 months, it would seem the efficacy from intake of immunostimulatory bacteria (including HK L-137) may be maintained during their long-term use. Although augmentation of T-cell proliferation by HK L-137 might have a negative impact on allergies or autoimmune diseases, HK L-137 has been shown to mitigate both pathologies in animal models of food allergy and inflammatory bowel disease (Muroski et al. 1998; Fujiki et al. 2012). Nevertheless, further studies are needed to clarify the influences of HK L-137 on humans susceptible to allergies and autoimmune diseases.

The adult human gut microbiome is dominated by the *Firmicutes* and *Bacteroidetes* phyla which comprise > 80% of the total intestinal microbiota. It has been reported the F/B ratio is higher in people who are obese than those who are a healthy weight or underweight, mainly due to a reduced *Bacteroidetes* proportion (Kasai et al. 2015). It was also reported the F/B ratio was increased and *Bacteroidetes* levels decreased in patients with coronary artery disease (Emoto et al. 2016). In addition, patients with dementia reportedly have a heavily reduced enterotype I (*Bacteroides* > 30%) and significantly higher F/B ratio than people without the disease (Saji et al. 2019). Taking into account previous findings that oral administration of HK L-137 alleviated obesity and associated metabolic abnormalities in high-fat diet-induced obese mice (Yoshitake et al. 2021), the present findings that F/B ratios decreased significantly due to HK L-137 dosing for 12 months suggest this material helps lead to an attenuation of obesity and associated metabolic disorders through improvements induced in the intestinal microbiota balance.

SCFA are produced by the intestinal bacteria mainly in colon as fermentation products from undigested dietary polysaccharides. Among them, acetate, propionate, and butyrate are the most abundant in the large intestine. SCFAs regulate tissue-specific homeostasis, including gastrointestinal motility, gut barrier function, colitis, energy and lipid metabolism, carcinogenesis, and immune function (Correa-Oliveira et al. 2016; Rios-Covian et al. 2016). In the present study, repeated dosing with HK L-137 increased levels of fecal SCFA such as n-butyric acid and propionic acid. Daily HK L-137 intake was already shown to improve inflammation status and lipid metabolism in mice and humans, possibly by causing enhanced maintenance of intestinal barrier integrity. Thus, it is possible that the increases in SCFA in the intestines of the subjects here could in turn contribute to improved gut barrier function.

Since HK L-137 is heat-inactivated dead bacteria, HK L-137 could not influence the intestinal environment by producing metabolites. The effects of administration of heat-killed lactic acid bacteria (without fermented foods) on intestinal microbiota composition have been studied (Terada et al. 2004; Asama et al. 2016; Sugawara et al. 2016). Nevertheless, the mechanisms of action through which dead bacteria act on local microbiota and their metabolites is still not clear. Kobayashi et al. (2019) recently reported that oral administration of heat-killed *L. bulgaricus* 2038 and *Streptococcus thermophilus* 1131 increased the gene expression of bactericidal lectin Reg3γ in intestinal epithelial cells of mice through the induction of interleukin (IL)-22 production in the lamina propria. In addition, Usui et al. (2018) reported that mice fed yogurt containing these strains for 20 months developed decreased F/B ratios and an elevated abundance of *Bacteroides* in their intestines. These changes occurred in association with an up-regulation of intestinal Reg3γ gene expression. Production of Reg3 family peptides is induced by IL-22, which is in turn induced by IL-23, a product of dendritic cells and
macrophages. Since IL-23 production is stimulated by bacterial cell components via Toll-like receptor (TLR) on antigen-presenting cells (Kastelein et al. 2007; Zindl et al. 2013), HK L-137 – which highly expresses a TLR2 ligand lipoteichoic acid on its cell surface (Hirose et al. 2010) – could potentially induce IL-23 production and so influence the microbiota balance via induction of IL-22 and Reg3 peptides. Clearly, further studies are needed to clarify the mechanisms of action through which HK L-137 impacts on intestinal microbiota.

In conclusion, the present study demonstrated that daily intake for 4 weeks of HK L-137 at 5-times the effective dose was safe in adult participants. The study also showed that taking the effective dose of HK L-137 for 12 months significantly increased the ex vivo proliferate capacity of host T-cells and decreased fecal F/B ratios in association with temporal increases in fecal SCFA levels. From this, it is possible to conclude that daily intake of HK L-137 for a prolonged period likely imparts beneficial effects on the intestinal tract not associated with immune-related safety issues.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content of this manuscript.

Funding

No funding was received.

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