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〈Research Article〉

Lactococcus lactis subsp. *cremoris* H61 improved iron status in male distance runners

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Summary Iron status is related to aerobic capacity, and athletes are recommended to supplement iron more than its recommended dietary allowance. However, some adverse events such as abdominal pain, nausea, and vomiting may be observed with oral iron intake. Since iron has no specific excretion pathway, overdosing with iron supplements carries the risk of adverse events. Therefore, the effect of *Lactococcus lactis* subsp. *cremoris* H61 (H61 strain) on iron status was investigated as an alternative to iron supplements. The study included 22 male collegiate distance runners. For 30 days, the H61 group ingested a supplement containing a heat-killed H61 strain. Participants who took iron supplements (n = 3) and had low compliance (n = 3) were excluded from further analysis. Significant increases were observed in serum iron, red blood cell count (RBC), total iron-binding capacity (TIBC), and hemoglobin in the H61 group (n = 9), whereas a significant increase in TIBC was also observed in the control group (n = 7). As a result, the H61 strain was thought to improve serum iron, RBC, and hemoglobin. During the intervention, there was no difference in dietary iron intake. Therefore, the H61 strain was thought to improve iron bioavailability.

Key words: Serum iron, Hemoglobin, Red blood cell, Probiotics, Biogenics

1. Introduction

Iron deficiency is the most common malnutrition in the world. Iron deficiency is not the only cause of anemia, but it is the most common cause of anemia[1]. Because iron status is important for aerobic capacity, higher hemoglobin concentrations

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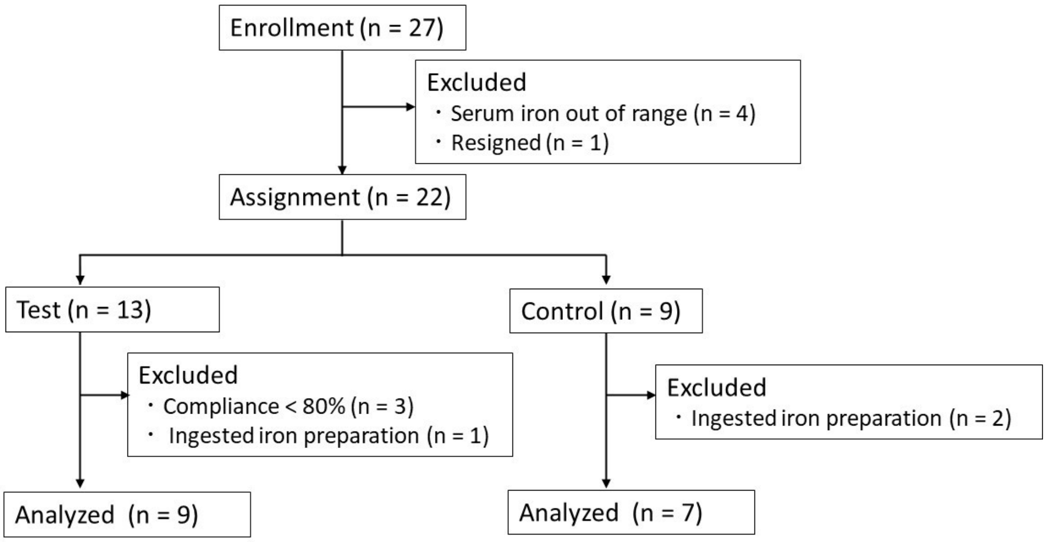

Fig. 1. Flow diagram of the participants

are associated with greater endurance capacity, even if they are not classified as anemic, for example, lower heart rate and blood lactate concentrations during exercise[2,3]. Thereby, athletes are advised to supplement iron at doses higher than their recommended dietary allowance (RDA) (i.e., >18 mg/day for women and >8 mg/day for men) to maintain adequate iron status[4]. However, oral ingestion of iron may cause clinical symptoms such as abdominal pain, nausea, and vomiting, as well as gastrointestinal disorders such as mucosal necrosis, ulceration, and ischemia observed endoscopically[5,6]. The IOC advised athletes not to take high-dose iron supplements unless they had an iron deficiency[4].In our previous study, we found a significant increase in serum iron after consuming yogurt fermented with *Lactococcus lactis* subsp. *cremoris* H61 (H61 strain) for 4 weeks[7]. However, because the changes in red blood cell count and hemoglobin concentration were not statistically significant, and the iron intake during the intervention period was unknown, the report did not discuss or appeal the findings[7].

Therefore, in this study, we aimed to examine the effect of H61 strain on iron status.

2. Materials and Methods

Participants

Male healthy collegiate distance runners were recruited. The inclusion criteria were as follows: (1)

Table 1 Characteristics of participants

		H61 (n = 9)		Control (n = 7)	
		Mean	SD	Mean	SD
Age	year	19.8	1.1	19.6	1.1
Height	cm	169.4	3.1	170.8	9.6
Bodyweight	kg	54.6	4.8	56.5	5.2
Body mass index	kg/m^2	19.0	1.4	19.4	1.0
Iron intake *	mg/day	9.60	2.74	9.09	1.29

* Daily iron intake was estimated from the iron intake per 1000 kcal and estimated energy requirement of Dietary Reference Intake for Japanese (2020) which was 3050 kcal/day for male aged 18-29 year with physical activity of 2.0.

aged 18–25 years old, (2) belonging to a same athletic club, and (3) residing in the club dormitory. The exclusion criteria were as follows: (1) suffering from a serious cardiovascular disorder, liver function disorder, renal dysfunction, respiratory disorder, an endocrine disorder, metabolic disorder, or those having a history of them, (2) having a history of chest pain or fainting, (3) being likely to cause allergies related to the test supplement, (4) having donated 200 mL within 1 month before the start of the study or 400 mL of blood (blood donation, etc.) within 3 months, (5) smokers, and (6) having serum iron levels out of normal range (54–200 µg/dL). They were given a thorough explanation of the study, including its purpose, methods, expected results, and method of outcome review, as well as personal information protection, potential benefits, and drawbacks of participating in the trial. They agreed to take part in the trial and provided written informed consent. This trial was designed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Juntendo University Graduate School of Sports and Health Sciences (Approval #28-76).

A total of 27 runners were enrolled in the trial. Four were excluded because their serum iron levels were out of the normal range. One resigned to participate before the intervention for his reasons. The remaining 22 participants (aged 18 – 21 year) were randomly assigned to test (H61) or control groups using Research Randomizer (https://www.randomizer.org/, accessed on July 26, 2017) and received the intervention. During the intervention, three participants (one in H61 and two in control groups) took iron supplements, and three participants in the H61 group took supplements less than 80% of the regimen (6 tablets/day for 30 days); they were excluded from the further analyses (Fig. 1). The characteristics of the remaining 16 participants were shown in Table 1. There was no statistically significant difference in age, height, bodyweight and body mass index between the H61 and control groups. There were no adverse events or complaints reported by any of the participants .

Experimental design

The study design was a two-arm, randomized parallel-group trial. The participants in the H61 group took six tablets per day of test supplement, while the control group did not take any supplement for 30 days. During the intervention period, participants were instructed to keep their dietary pattern and to refrain to take iron supplement.

The iron status was determined both before and after the intervention (pre and post). During the intervention period, participants were asked to record their supplement intake (H61 group only) and health status and were also told not to take iron supplements. Dietary iron intake during the intervention was measured using a brief-type self-administered diet history questionnaire (BDHQ). Daily iron intake was estimated from the observed iron intake (mg/1000 kcal) and estimated energy requirement of Dietary Reference Intake for Japanese (2020) which was 3050 kcal/day for male aged 18 – 29 year with high physical activity level (physical activity of 2.0).

Test supplement

A commercial dietary supplement "H61-Bio" (Kenko Plaza Pal Co., Ltd., Tokyo, Japan) was purchased from Yagi Pharmacy (Kobe, Japan). The supplement's six tablets each contained 250 mg of dried broth fermented with the H61 strain, which contained 1.6×10^8 cells before being heat-killed and dried. The ingredients were *Lactobacillales* H61 strain (heat-killed) with fermented broth (cow milk and soy), *Lactobacillus sporegenes*, oligo-saccharide, vitamin C, vitamin E, niacin, calcium panthotenate, vitamin B1, vitamin B6, vitamin B2, vitamin A, folic acid, vitamin D, vitamin B12, powdered rapeseed oil, and sucrose fatty acid ester. The nutrient contents of a daily dose (six tablets, 1.5 g) were as follows: energy, 6.78 kcal; protein, 0.045 g; fat, 0.193 g; carbohydrate, 1.215 g; sodium, 0.582 mg; and water, 0.021 mg.

Blood collection and measurements

Blood was collected from the cubital vein between 12:00 and 13:00. Blood and biochemical analyses were conducted by a certified clinical laboratory (SRL, Tokyo, Japan). Briefly, a Sysmex XE-2100 automated hematology analyzer (Sysmex Corporation, Hyogo, Japan) was used to measure red blood cell count (RBC), hemoglobin (Hgb), hematocrit (Hct), mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC). Serum ferritin, iron, and total iron-binding capacity (TIBC) were determined using latex agglutination turbidimetry, direct colorimetry, and 2-nitroso-5-(N-propyl-N-sulfopropylamino) phenol (nitroso-PSAP), respectively, with a JCA-BM8060 automatic analyzer (JEOL Ltd., Tokyo, Japan). The transferrin saturation and UIBC were calculated as serum iron/ TIBC ×100 and TIBC − serum iron, respectively.

Statistical analyses

Data were presented as mean ± standard

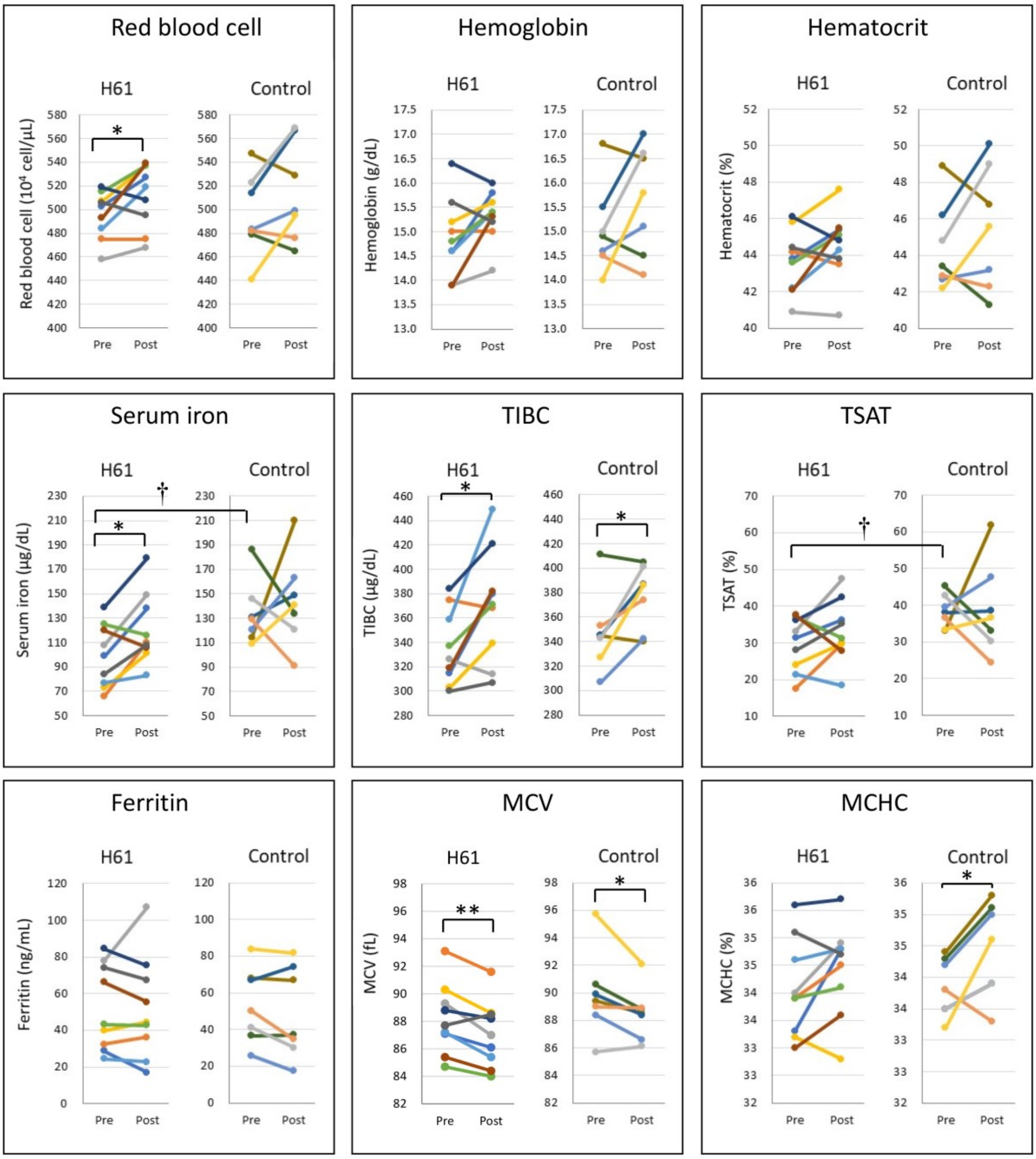

Fig. 2. Change in hematological parameters of the male distance runners
TIBC, total iron-binding capacity; TSAT, transferrin saturation; MCV, mean cell volume; MCHC, mean cell hemoglobin concentration*, $p < 0.05$; **, $p < 0.01$ pre vs. post, †, $p < 0.05$ H61 vs. control

deviation (SD). When equal variance was hypothesized, hematologic parameters were compared using Student's t-test; otherwise, Welch's t-test was used. Consequently, only hemoglobin was compared by Welch's t-test, because equal variance was not hypothesized. The paired t-test was used to determine the intragroup difference, pre vs. post.

The changes in hematological parameters were also analyzed by the generalized estimating equation of the generalized linear model. The model included subject ID as a subject variable, intervention (H61, control) and measure point (pre, post) as within-subject variables, and intervention × measure point as interaction.

Statistical significance was set at $p < 0.05$. Statistical analyses were conducted using SPSS Statistics Ver. 24 (IBM Japan, Tokyo, Japan).

3. Results

Iron intake during the intervention was 3.15 ± 0.89 (range: 1.96—4.58) and 2.98 ± 0.42 (range: 2.08—3.43) mg/1000 kcal in H61 and control groups, respectively; no significant difference was seen between the groups. The daily iron intake was estimated to be 9.6 ± 2.7 (range: 6.0—14.0) and 9.1 ± 1.3 (range: 6.3—10.5) mg/day in H61 and control groups, respectively. The daily iron intakes of two in H61 group (6.0 and 6.3 mg/day) and one in control group (6.3 mg/day) were less than the estimated average requirement of iron (6.5 mg/day for men aged 19–29 year) set in Dietary Reference Intake of Japanese (2020). The other participants met the recommended dietary allowance of iron (7.5 mg/day for men aged 19–29 year) set in Dietary Reference Intake for Japanese (2020). All participants' intakes were below the upper limit (50 mg/day) set in Dietary Reference Intake for Japanese (2020).

The iron status was summarized in Fig. 2. The participants were randomly allocated to H61 or control group before the blood collected for pre-intervention assessment. As a result, before the intervention (pre), control group showed accidentally higher serum iron ($p = 0.018$) and TSAT ($p = 0.015$) compared to H61 group. The 30 days of the intervention significantly elevated RBC (from 495.6 ± 20.0 × 10^4 cell/μL to 511.7 ± 27.1 × 10^4 cell/μL, $p = 0.045$), serum iron (from 99.0 ± 25.7 μg/dL to 121.1 ± 29.2 μg/dL, $p = 0.018$) and TIBC (from 335.3 ± 30.8 μg/dL to 370.1 ± 46.3 μg/dL, $p = 0.016$), while decreased MCV (from 88.2 ± 2.6 fL to 87.1 ± 2.4 fL, $p = 0.007$) in H61 group. Control group showed a significant increase in TIBC (from 347.3 ± 32.0 μg/dL to 376.4 ± 26.3 μg/dL, $p = 0.029$) and MCHC (from 33.8 ± 0.5% to 34.4 ± 0.8%, $p = 0.036$) and a significant decrease in MCV (from 89.8 ± 3.0% to 88.5 ± 1.9%, $p = 0.036$). UIBC did not show significant change in both H61 (from 236.3 ± 36.5 μg/dL to 249.0 ± 55.1μg/dL, $p = 0.149$) and control (from 213.6 ± 16.3 μg/dL to 232.3 ± 57.5μg/dL, $p = 0.564$) groups.

Because there were significant differences between groups in the initial serum iron and TSAT, the hematological parameters were also examined using the generalized linear model. The results demonstrated significant increases in RBC ($p = 0.012$), Hgb ($p = 0.031$), serum iron ($p = 0.002$), and TIBC ($p = 0.001$) and a significant decrease in MCV ($p < 0.001$) in H61 group after the intervention, while the control group showed significant increases in TIBC ($p = 0.002$) and MCHC ($p = 0.004$) and a significant decrease in MCV ($p = 0.004$) (Table 2).

During the intervention period, no adverse events were onserved in both groups including events related to iron supplementation; e.g. abdominal pain, nausea, and vomiting.

4. Discussion

In our previous study, healthy females who consumed yogurt fermented with the H61 strain (10^{10} cfu/day) for 4 weeks had a significant increase in serum iron[7]. However, mean RBC and Hgb increased from 441 × 10^4/μL to 460 × 10^4/μL and from 13.2 g/dL to 13.7 g/dL, respectively, but the changes were not significant[7]. In this study, male collegiate distance runners took a supplement containing heat-killed H61 strain for 30 days. Descriptive statistics showed increases in RBC, serum iron and TIBC and a decrease in MCV in the

Table 2 Changes in iron status of the male distance runners analyzed by generalized linear model

		Group	Pre EMM	Pre SE	Post EMM	Post SE	p	
Red blood cell	10^4 cell/mL	H61	510.4	3.2	526.5	3.2	0.012	*
		Control	476.5	5.6	495.3	5.6	0.096	
Hemoglobin	g/dL	H61	15.2	0.1	15.6	0.1	0.031	*
		Control	14.6	0.2	15.2	0.2	0.081	
Hematcrit	%	H61	44.1	0.2	45.0	0.2	0.091	
		Control	43.8	0.5	44.9	0.5	0.291	
Serum iron	μg/dL	H61	98.9	3.5	121.1	3.5	0.002	**
		Control	133.8	9.1	144.2	9.1	0.568	
TIBC	μg/dL	H61	316.1	5.4	350.9	5.4	0.001	**
		Control	372.0	4.7	401.1	4.7	0.002	**
UIBC	μg/dL	H61	236.3	11.5	249.0	17.3	0.418	
		Control	213.6	5.7	232.3	20.1	0.372	
TSAT	%	H61	31.3	1.3	34.9	1.3	0.164	
		Control	36.1	2.6	36.7	2.6	0.915	
Ferritin	ng/mL	H61	46.6	2.0	46.3	2.0	0.935	
		Control	60.6	1.4	56.3	1.4	0.115	
MCV	fL	H61	86.3	0.1	85.2	0.1	< 0.001	**
		Control	92.2	0.2	90.9	0.2	0.004	**
MCHC	%	H61	34.5	0.1	34.8	0.1	0.056	
		Control	33.3	0.1	33.9	0.1	0.004	**

EMM, estimated marginal mean; TIBC, total iron-binding capacity; TSAT, transferrin saturation
*, $p < 0.05$; **, $p < 0.01$ pre vs. post

H61 group following the intervention period. The generalized estimating equation of generalized linear model revealed that the H61 strain significantly increased in Hgb in addition to serum iron and TIBC. In the control group, the model also showed a significant change in TIBC, MCV, and MCHC. These findings showed that consuming a heat-killed H61 strain for 30 days increased RBC, Hgb and serum iron while having the influence on TIBC and MCV same as control group. As a result, a heat-killed H61 strain was shown to improve iron status in healthy young male distance runners.

In our previous study,11 healthy females (age = 19–21 years) ingested yogurt fermented with H61 strain (10^{10} cfu/day) for 4 weeks[7]. Increases in RBC, Hgb, and serum iron were observed but the change was significant only in serum iron[7]. In this study, 9 collegiate male distance runners (aged 18-21 years) ingested supplement containing heat-killed H61 strain (1.6 x 10^8 cells/day before heat killed) for 30 days, and RBC, Hgb, and serum iron were all significantly increased. Participants were of similar age, but the influence of H61 strain on RBC, Hgb and serum iron are inconsistent. This discrepancy could be related to the gender of the participant, and the preparation, the amount, and the duration of the H61 strain administered.

In general, yogurt is one of the probiotics that is defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" by the 2001 World Health Organization/Food and Agriculture Organization (WHO/FAO) expert consultation[8]. On the other hand,heat-killed probiotic bacteria are commonly referred to biogenics[9] which was defined as a "food ingredients that contain biologically active peptides and immunopotentiators (biological response modifiers) produced directly or indirectly by modulation of the intestinal microflora"[10]. Live *Lactobacillus reuteri*, a probiotic bacterium, inhibited the production and secretion of IL-8 induced by TNF-α in intestinal epithelial cells, but this effect was not seen in irradicated or heat-killed *L. reuteri*[11]. Thereby, the effect of a heat-killed probiotic bacterium cannot be simply predicted from the live probiotic bacterium. Heat-killed H61 strain improved iron status in this study, as observed by yogurt fermented with H61 strain. Therefore, both live and heat-killed H61 strains were discovered to improve iron status.

Orally ingested iron is absorbed in the duodenum and upper small intestine. Heme iron is absorbed as is. Non-heme iron (Fe^{3+}) is reduced to Fe^{2+} by ferric reductase on the apical membrane and absorbed by divalent metal transporter 1 (DMT1). Thereby, the absorption of non-heme iron competes with that of zinc and copper, which are also absorbed by DMT1. Iron absorbed in the small intestinal epithelium is converted to Fe^{3+} by a transmembrane copper-dependent ferroxidase hephaestin (HEPH), released into the blood by ferroportin (SLC40A1), an iron transporter of the basolateral membrane, and bound to transferrin in the serum[12]. In this study, the H16 group had a significant increase in serum iron. Therefore, the H61 strain is thought to have an effect somewhere in the preceding process.

The absorption rate of dietary iron is estimated to be 14% in the Swedish diet, 16% in the French diet, and 16.6% in the US diet[13]. Dietary non-heme iron (Fe^{3+}) must be reduced to divalent iron (Fe^{2+}) in the lumen before it can be absorbed. As a result of its iron-reducing properties, vitamin C promotes iron absorption[14,15]. Organic acids[16] including lactic acid[17,18] have also been reported to enhance iron absorption. Meanwhile, ingestion of heat-killed H61 strain has been reported to increase the abundance of *Lactobacillales* in the intestine[19]. *Lactobacillales*, commonly called lactic acid bacteria, produce lactic acid by fermenting carbohydrates. Considering the above, ingested heat-killed H61 strain may have increased iron absorption by increasing intestinal *Lactobacillales* abundance and lactate concentration. Therefore, enhanced iron absorption by the H61 strain may account for the increase in serum iron observed in this study.

In this study, a significant increase in TIBC and a significant decrease in MCV were observed after the intervention period in both the H61 and control groups; MCHC also increased in both groups, but the change was significant only in the control group. A decrease in MCV could be associated with an increase in MCHC. Both changes are within the reference range provided by SRL (MCV, 82.7–101.6 fL and MCHC, 31.6–36.6%). Thereby, the change may not affect the health status of the participants. The increase in TIBC may be due to the enhanced production of transferrin in liver. However, it should not be due to the ingestion of H61 strain, because the change was observed in both groups.

This research has some limitations. The test supplement in this study was a commercially available dietary supplement containing the H61 strain, which was also labeled as containing another *Lactobacilalles* and vitamins. Thereby, the possible contribution of these ingredients, alone or in combination with the H61 strain, cannot be excluded. Therefore, the results of this study should be confirmed with the heat-killed H61 strain alone. In this study, we discussed how the heat-killed H61 strain may improve iron absorption by increasing the abundance of intestinal *Lactobacillales*, but we did not measure the intestinal microbiota. The enhanced release from stored iron including upregulation of iron transport may be involved in the increase in serum iron, although not discussed in this paper because of the lack of supporting literature. In addition, in this study, significant differences were seen in serum iron and TSAT between the H61 and the control groups before intervention. Participants were randomly assigned prior to the intervention, but the assignment did not based on each participant's iron bioavailability or genetic predispositions involved in iron absorption. Thereby, we cannot rule out that an accidental allocation with different iron utilization capacities may have occurred. Furthermore, hepcidin[12] regulates the release of iron from intestinal epithelial cells into the blood, so this process must also be investigated. Therefore, further studies are needed to clarify the mechanism of H61 strain to improve iron status.

In conclusion, a 30-day ingestion of heat-killed H61 strain consumption improved the iron status in college male distance runners. The iron intake of H61 group during the intervention period did not differ from that of the control group. Therefore, H61 strain may improve iron status by increasing iron availability.

Conflicts of Interest

The authors have no conflicts of interest.

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