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Safety Evaluations of Long-Term and Excessive Intakes of *Bifidobacterium longum* CLA8013: A Placebo-Controlled, Randomized, Double-Blind Study

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Abstract

Heat-killed *Bifidobacterium longum* CLA8013 has been demonstrated to improve the frequency of defecation, straining, and pain during defecation in human placebo-controlled, double-blind, parallel-group studies. We conducted a randomized, double-blind, placebo-controlled, parallel-group study to evaluate the safety of both long-term and excessive intakes of heat-killed *B. longum* CLA8013, when used as a food with functional claims. In both tests, 30 healthy volunteers were divided into two groups: an active group that ingested heat-killed *B. longum* CLA8013 and a placebo group. In the long-term intake safety study, participants in the active group ingested 25 billion cells/day for 12 weeks. In the excessive intake safety study, participants in the active group ingested 125 billion cells/day for 4 weeks. Physical, hematological, biochemical, and urine examinations were conducted, and adverse events were evaluated in both studies. The studies revealed no abnormalities in any of the safety tests. In conclusion, no safety-related issues were identified with long-term or excessive intake of heat-killed *B. longum* CLA8013.

Keywords

Constipation, Safety, *Bifidobacterium longum*, Long-Term, Excessive

1. Introduction

Constipation is not simply defined as a small number of bowel movements. Treatment is required because patients with constipation have a significantly

lower survival rate [1] and more impediments to their daily activities [2] than in those with other gastrointestinal diseases. Constipation can be either organic (associated with morphological changes in the large bowel; e.g., inflammatory bowel disease, intestinal obstruction) or functional (not associated with large bowel morphological changes; e.g., irritable bowel syndrome, drug-induced constipation) [1] [3]. Functional constipation is a health problem that negatively impacts the quality of life (QOL) [4], according to systematic review and meta-analysis results, its global prevalence among adults is between 14%, and its prevalence increases modestly with increasing age [5] [6]. Therefore, constipation is a health problem that must be addressed. Typically, various agents, such as laxatives and fibers, are used to treat constipation, and probiotics have also been reported to be effective [7]. The mechanism of action by which probiotics such as *Bifidobacterium* improve constipation includes normalizing the intestinal environment [8] [9] [10], decreasing methanogenic bacteria [11], and increasing serotonin production [12] [13]. However, the effectiveness of probiotics varies significantly among individuals because the human intestinal environment is diverse.

Our previous double-blind study of healthy volunteers prone to constipation revealed that heat-killed *B. longum* CLA8013 effectively increased the frequency of defecation when ingested continuously over a 2-week period at a dose of 25 billion cells/day [14]. The present trial comprised two studies aimed at evaluating the safety of *B. longum* CLA8013 in healthy volunteers to verify the potential of *B. longum* CLA8013 as a food or supplement: a long-term intake safety study and an excessive intake safety study.

2. Participants and Methods

The long-term intake and excessive intake safety studies were approved by the Ethics Review Committee of the Kobuna Orthopedic Clinic (approval date: November 10, 2021; approval numbers: MK-2211-03 and MK-2211-02, respectively) and were registered and published in the UMIN clinical trial registration system (clinical trial registration numbers: UMIN000049596 and UMIN000049771, respectively). The trial was conducted in accordance with the principles of the Declaration of Helsinki and the Ethical Guidelines for Medical Research Involving Human Participants. The principal investigator adequately explained what the study entailed to the participants who voluntarily provided written consent to participate.

The study was conducted at a clinic in Tokyo, Japan. Medical interviews and examinations were conducted by physicians and nurses at the Shinagawa Season Terrace Health Care Clinic. The studies were conducted in collaboration with the KSO Corporation, a contract research organization that was responsible to recruit participants.

2.1. Participants

Healthy adult male and female volunteers (age: 20 - 65 years old) were recruited

by the KSO Corporation's participants panel. The recruitment criteria is subjective symptoms that prone to constipation with 3 - 5 spontaneous bowel movements per week or normal defecation exceeding 5 times per week. All participants were provided with adequate explanation of the study, including its purpose, contents, methods, and potential adverse events. Written informed consent was obtained from all participants before study initiation.

The inclusion and exclusion criteria are presented in Table 1. These criteria were set up to recruit for healthyorprone to constipation participants, three–five spontaneous bowel movements per week was set because one of the criteria for functional constipation is fewer than three bowel movements per week [3]. Discontinuation criteria are listed in Table S1.

2.2. Study Design

The two safety studies were placebo-controlled, randomized, double-blind, and parallel. Ingestion of the study substance began after selecting the participants

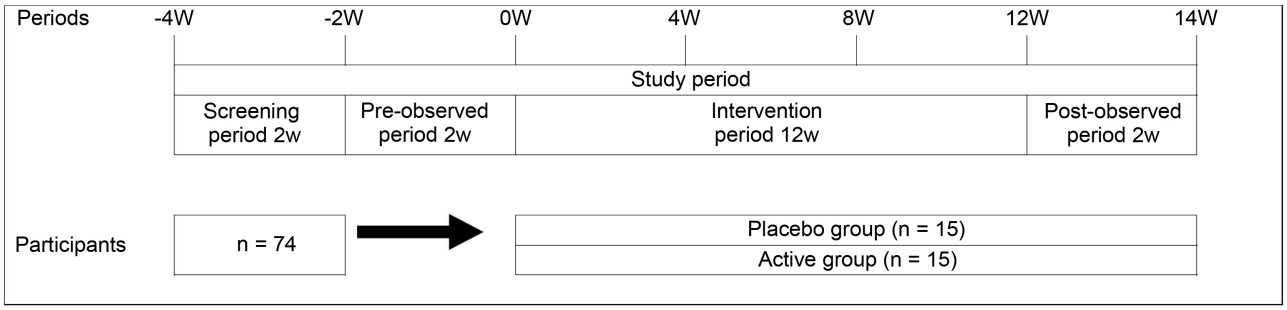
Table 1. Inclusion and exclusion criteria.

Inclusion Criteria	1). Healthy men and women aged 20 - 65 years at the time of providing consent to participate in the study.
	2). Participants prone to constipation with 3 - 5 spontaneous bowel movements per week or normal defecation exceeding 5 times per week.
	3). Participants who were fully informed of the purpose and content of the study, had the ability to provide consent, and provided written consent to participate in the study based on a thorough understanding of its purpose and content.
Exclusion Criteria	1). Participants who regularly take oral medicine (especially laxatives, antiflatulents, laxatives, etc. that affect bowel movements).
	2). Participants with a history of chronic diseases such as diabetes, kidney/liver disease or heart disease, and/or thyroid diseases, adrenal diseases, and other metabolic diseases or who were under medical treatment.
	3). Participants being treated for chronic diseases.
	4). Participants with digestive organ disease or surgical history that may influence digestive absorption and defecation (excluding appendicitis).
	5). Participants with other serious diseases.
	6). Participants unable to avoid ingesting foods containing live bacteria such as lactic acid bacteria, bifidobacteria, and *Bacillus subtilis* natto, foods fortified with oligosaccharides and dietary fiber, health foods reputed to improve constipation, and foods containing large amounts of sugar alcohol during the study period.
	7). Participants consuming large amounts of alcohol (≥40 g of pure alcohol) on a daily basis.
	8). Participants with a history of drug or alcohol dependence or with a current medical history.
	9). Participants with food and medicine allergy.
	10). Participants who are pregnant or breast-feeding, or may become pregnant during the study period.
	11). Participants participating in other clinical trials or who have participated in other tests within one month of obtaining consent, or who are willing to participate in other tests.
	12). Participants judged unsuitable for the study at the discretion of the investigator for other reasons.

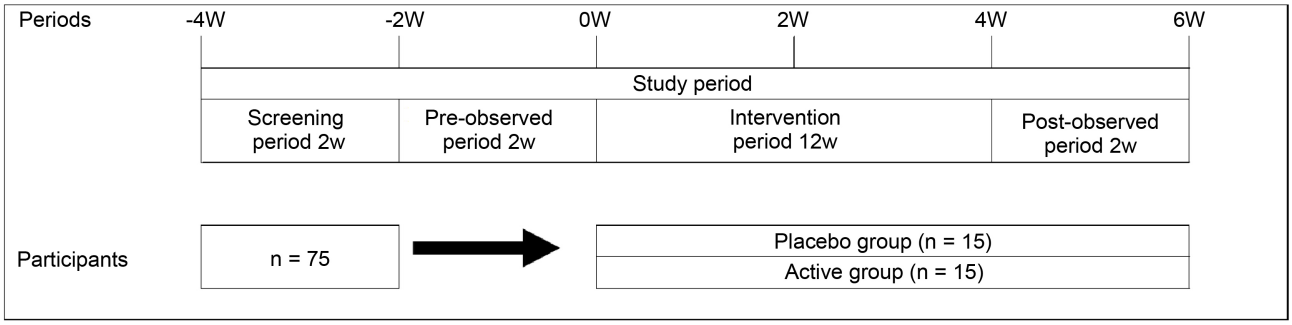
via screening and after a 2-week pre-observation period. The intervention periods were 12 and 4 weeks for the long-term and excessive intake safety studies, respectively. Post-study observation of the participants was conducted for 2 weeks after the conclusion of both studies (Figure 1). Participants were randomly assigned to one of the two groups at the time of screening to preclude biases based on age, sex, body mass index, or defecation frequency. In the long-term intake safety study, participants ingested one capsule once per day, whereas in the excessive intake safety study, participants ingested five capsules once per day. The participants were instructed to drink large amounts of water while ingesting the capsules. If the participants forgot to take the capsules at the designated time, they were instructed to take the capsules as soon as they became aware of the missed dose, rather than doubling the dose the following day.

The study substance comprised capsules containing heat-killed *B. longum* CLA8013 at a dose of 25 billion cells, in a formulation containing crystalline cellulose, calcium stearate, and silicon dioxide. The placebo group received capsules that did not contain heat-killed *B. longum* CLA8013. Both sets of capsules were manufactured by API Co., Ltd. (Gifu, Japan; Table 2). In the long-term intake safety study, participants in the active group ingested 25 billion cells/day for 12 weeks, while in the excessive intake safety study, participants in the active group ingested 125 billion cells/day for 4 weeks.

In the long-term intake safety study, we measured the parameters indicated below at the baseline; at ingestion weeks 4, 8, and 12; and subsequently at 2 weeks following the end of ingestion. In the excessive intake safety study, we



(a)



(b)

Figure 1. Intervention schedule. (a) The long-term intake safety study was conducted from November 2022 to March 2023; (b) The excessive intake safety study was conducted from January 2023 to March 2023.

Table 2. Composition, nutrients, duration and dosage of capsules.

		Active	Placebo
Composition	Capsule contents	Heat-killed *B. longum* CLA8013 (25 billion cells), Microcrystalline cellulose, Calcium stearate, Silicon dioxide	Microcrystalline cellulose, Calcium stearate, Silicon dioxide
	Capsule shell	Pork gelatin, Titanium dioxide, Lecithin from soybean	Pork gelatin, Titanium dioxide, Lecithin from soybean
Nutrients	Calories (kcal)	0.3766	0.3121
	Carbohydrates (g)	0.0115	0.0009
	Proteins (g)	0.0698	0.0649
	Lipids (g)	0.006	0.0057
	Salt equivalents (g)	0.0003	0.0002
Ingestion	Long-term intake safety study	1 capsule/day (25 billion cells/day)	1 capsule/day (0 cells/day)
	Excessive intake safety study	5 capsules/day (125 billion cells/day)	5 capsules/day (0 cells/day)

measured the same parameters at baseline; at ingestion weeks 2 and 4; and at 2 weeks after the end of ingestion.

In addition, we instructed the participants to maintain diaries containing data on their physical condition, meal content, and defecation status. We confirmed the safety of participants using this data. The parameters examined were as follows: 1) a medical interview conducted by a physician, 2) physical examination (body weight, body mass index [BMI], systolic blood pressure, diastolic blood pressure, and pulse rate), 3) hematological examination (white blood cell count, red blood cell count, hemoglobin (Hb), hematocrit (Ht), platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), differential leucocyte count [neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EOSINO), and basophils (BASO)], 4) blood biochemical examination (total protein, albumin, aspartate aminotransferase (AST (GOT)), alanine aminotransferase (ALT (GPT)), lactic dehydrogenase (LD (IFCC)), total bilirubin, alkaline phosphatase (ALP (IFCC)), gamma-glutamyl transferase (gamma-GT), urea nitrogen (UN), creatinine, uric acid (UA), sodium (Na), chloride (Cl), potassium (K), calcium (Ca), total cholesterol, low density lipoprotein (LDL)-cholesterol, high density lipoprotein (HDL)-cholesterol, triglyceride (TG), glucose, and HbA1c (NGSP value)), 5) urinalysis (urine protein, urine glucose, urobilinogen, urine bilirubin, urine pH, urine specific gravity, urine ketone body, and occult blood reaction) 6) defection frequency (defecation frequency, number of defecation days, stool volume, and stool consistency (Bristol Stool Form Scale)).

2.3. Statistical Analysis

The means and standard deviations were calculated for all the baseline parameters measured during the screening period before intake. Statistical analysis was performed using one-way analysis of variance (ANOVA), and the measured values were tabulated.

The full analysis set (FAS) at the start of the ingestion of the study substance was used to analyze all the safety evaluation parameters. If a participant had ingested the study substance, tests were performed at the time of dropout as necessary, and the results of the parameters related to safety were included in our evaluation. All data were presented as mean values and standard deviations. The study data that were believed to be outliers were also included in our evaluation. Statistical analysis was performed using the unpaired *t*-test to compare the active treatment groups with the placebo groups, and the Wilcoxon rank-sum test was performed on the qualitative evaluation parameters. Statistical significance was set at 5%. Statistical analyses were performed using SPSS ver. 27 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Long-Term Intake Safety Study

1) Participant characteristics

A Consolidated Standards of Reporting Trials (CONSORT) flow chart for this study is shown in Figure 2, and participant characteristics are listed in Table 3. Of the 30 participants, there was one discontinuation/dropout from the active group. Therefore, 29 participants (placebo group: 15; active group: 14) completed the study. One participant withdrew consent to participate in the study for personal reasons.

2) Physical, Hematological, Urinalysis, and Biochemical Examinations

No significant changes were observed in the physical, (Table S2), hematological (Table S3), or urine examination parameters (Table S5). The biochemical examinations revealed that the active group exhibited significantly higher glucose levels and HbA1C than the placebo group. No significant differences were observed in other parameters (Table S4). Although significant changes were observed in several parameters during the study, these were within the range of physiological fluctuations and were deemed clinically irrelevant. Based on a comprehensive analysis of the examination results, the principal investigator determined that there were no safety concerns regarding the long-term or excessive intake of heat-killed *B. longum* CLA8013.

3) Defecation frequency

Compared with the placebo group, the active group exhibited significant changes in the following parameters: higher defecation frequency at week 14 and higher stool volume at weeks 1 and 14. No significant changes were observed in other parameters (Table S6). Based on the examination results, the principal investigator concluded that there were no safety concerns.

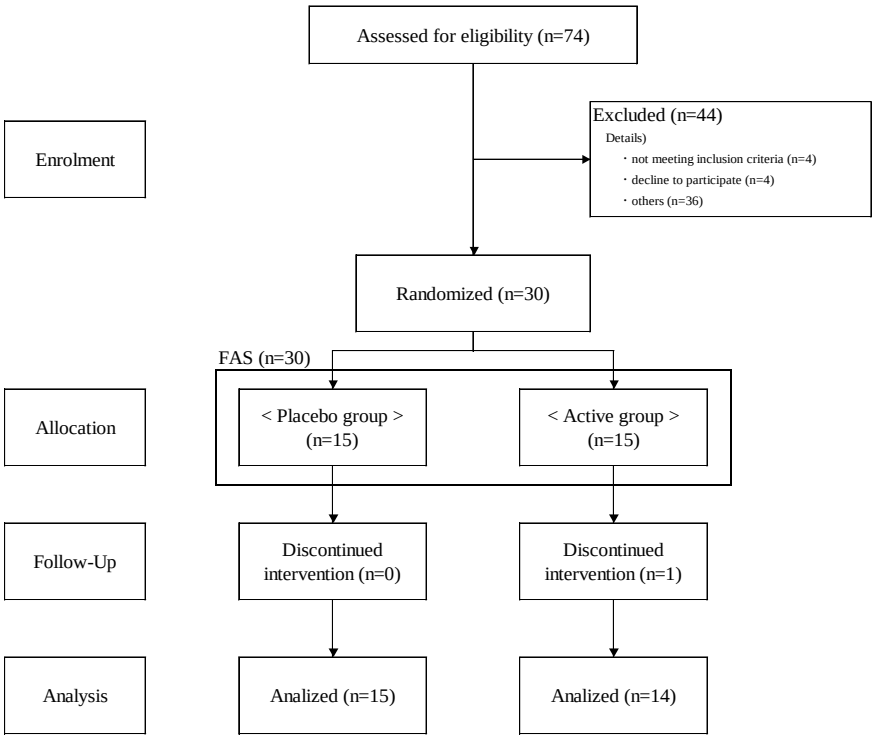


Figure 2. Flow diagram of the long-term intake safety study.

4) Adverse events

Twelve adverse events (active group: seven events in five participants, placebo group: five events in four participants) were observed during the study period (Table 4). The principal investigator concluded that none of the adverse events were related to the study capsules because the events were accidental or occurred outside the intervention period.

3.2. Excessive Intake Safety Study

1) Participant characteristics

A CONSORT flow diagram for this trial is shown in Figure 3, and the participant characteristics are listed in Table 5. None of the participants met the predefined discontinuance criteria during the study period. Therefore, data obtained from all 30 participants were included in the analysis.

2) Physical, Hematological, Urinalysis, and Biochemical Examinations

No significant changes were observed in the physical (Table S7) or hematological examination parameters (Table S8). However, biochemical examinations revealed significant changes in the active group compared with the placebo group in the following parameters: higher uric acid level at week 2, lower sodium level at week 6, lower chloride level at week 6, and higher potassium level at week 6. No significant changes were observed in any other parameters (Table S9). Urinalysis revealed that the active group had significantly lower urine pH at weeks 2 and 6 than that in the placebo group. No significant changes were observed in other parameters (Table S10). Although significant changes were

Table 3. (a) Baseline parameters of physical, hematological, and biochemical examinations (Long-term intake safety study). (b) Baseline parameters of urine examinations (Long-term intake safety study).

(a)

Parameter		(Unit)	Placebo			Active			*p* value
			Mean	±	SD	Mean	±	SD	
Gender	Male	(n)		7			7		
	Famale			8			8		
Age			45.9	±	7.1	45.0	±	9.7	0.782
Height		(cm)	163.7	±	9.9	165.7	±	8.5	0.555
Body weight		(kg)	60.2	±	12.3	61.6	±	8.5	0.735
BMI			22.3	±	2.9	22.5	±	2.9	0.880
Systolic blood presure		(mmHg)	119.0	±	13.1	119.1	±	15.4	0.980
Diastolic blood pressure		(mmHg)	73.8	±	9.1	73.3	±	10.8	0.899
Pulse rate		(bpm)	73.5	±	8.8	71.9	±	10.3	0.651
White blood cell count		(/μL)	5227	±	758	5200	±	1315	0.946
Red blood cell count		($x10^4$/μL)	468.4	±	64.1	459.9	±	25.9	0.636
Heamoglobin		(g/dL)	14.2	±	2.0	13.8	±	1.2	0.477
Hematocrit		(%)	44.0	±	5.3	42.7	±	2.6	0.418
Platelet count		($x10^4$/μL)	26.0	±	4.7	22.3	±	5.5	0.057
MCV		(fL)	94.1	±	4.0	93.1	±	5.9	0.568
MCH		(pg)	30.4	±	1.7	30.0	±	2.6	0.651
MCHC		(%)	32.3	±	0.9	32.2	±	1.4	0.903
NEUT/leukocyte fractionation		(%)	57.0	±	7.6	58.5	±	5.9	0.566
LYMPH/leukocyte fractionation		(%)	32.9	±	6.7	32.1	±	5.8	0.756
MONO/leukocyte fractionation		(%)	5.2	±	1.3	5.7	±	1.2	0.266
EOSINO/leukocyte fractionation		(%)	4.1	±	3.0	3.0	±	1.5	0.214
BASO/leukocyte fractionation		(%)	0.9	±	0.3	0.8	±	0.3	0.296
Total protein		(g/dL)	7.1	±	0.5	7.0	±	0.3	0.398
albumin		(g/dL)	4.3	±	0.2	4.3	±	0.3	0.797
AST		(U/L)	18.9	±	3.5	21.2	±	7.5	0.300
ALT		(U/L)	17.8	±	10.3	20.6	±	13.8	0.533
LD (IFCC)		(U/L)	167.5	±	25.0	176.0	±	30.0	0.404
Total bilirubin		(mg/dL)	0.7	±	0.3	0.7	±	0.3	0.734
ALP (IFCC)		(U/L)	60.7	±	15.3	61.3	±	11.1	0.903
g-GT		(U/L)	29.3	±	41.8	18.7	±	9.0	0.345
Urea nitrogen (UN)		(mg/dL)	13.2	±	3.5	12.9	±	2.4	0.756
Creatinine		(mg/dL)	0.78	±	0.13	0.74	±	0.16	0.497
Ureic acid (UA)		(mg/dL)	4.9	±	1.0	5.4	±	1.7	0.359
Sodium (Na)		(mEq/L)	140.5	±	2.0	141.0	±	1.7	0.495

Continued

Chloride (Cl)	(mEq/L)	103.7	±	2.5	104.5	±	1.7	0.271
Pottasium (K)	(mEq/L)	4.3	±	0.4	4.3	±	0.3	0.771
Calcium (Ca)	(mg/dL)	9.3	±	0.4	9.3	±	0.3	0.869
Total cholesterol	(mg/dL)	214.6	±	41.5	201.3	±	35.0	0.352
LDL-cholesterol	(mg/dL)	128.4	±	33.2	116.9	±	27.4	0.309
HDL-cholesterol	(mg/dL)	71.9	±	19.0	70.5	±	11.2	0.807
Triglyceride (TG)	(mg/dL)	68.8	±	35.0	69.1	±	32.2	0.979
Glucose	(mg/dL)	84.8	±	7.1	88.4	±	8.9	0.233
HbA1c (NGSP)	(%)	5.2	±	0.2	5.3	±	0.2	0.123
Ureic pH		6.4	±	0.6	6.0	±	0.4	0.052
Urine specific gravity		1.018	±	0.007	1.018	±	0.006	0.977

p value: ANOVA.

(b)

Urine protein	0.07	±	0.26	0.00	±	0.00	0.317
Urine glucose	0.00	±	0.00	0.00	±	0.00	1.000
Urobillinogen	0.00	±	0.00	0.00	±	0.00	1.000
Urine bilirubin	0.00	±	0.00	0.00	±	0.00	1.000
Urine ketone body	0.00	±	0.00	0.13	±	0.52	0.317
Occult blood reaction	0.27	±	0.80	0.07	±	0.26	0.524

p value: Wilcoxon's rank sum test (asymptotic significance probability).

observed in uric acid, sodium, chloride, potassium, and urine pH levels during the study, these changes were within the range of physiological fluctuations and were deemed clinically irrelevant. Based on the examination results, the principal investigator concluded that there were no safety concerns.

3) Defecation frequency

The active group showed a significantly lower difference in stool volume per week at week 2 than that of the placebo group. No significant changes were observed in other parameters (Table S11).

4) Adverse events

Three adverse events (active group: 1, placebo group: 2) were observed during the study period (Table 6). The principal investigator concluded that none of the adverse events were related to the study capsules because the events were accidental or occurred outside the intervention period.

4. Discussion

To develop a food with a functional claim that would be effective for constipation, we focused on hard stools as a cause of difficulty with bowel movements and conducted screening to evaluate the effectiveness of the intervention in

Table 4. Adverse events (Long-term intake safety study).

Group	Participant number	Adverse event (symptom)	Severity	Treatment	Causal relation with the study substance	Reason for causal conclusion	Continuation or discontinuation of the trial
Active	1	Fever, Pharyngeal pain	Moderate	Yes	Not related	Occasional event due to an infectious disease and was probably not associated with the study substance.	continued
Active	1	Gastroenteritis	Mild	No	Not related	The participant's symptoms improved even while continuing to ingest the study substance. The study substance was deemed to be unrelated to the symptoms.	continued
	2	Cough	Moderate	Yes	Not related	Occasional event due to an infectious disease and was probably not associated with the study substance.	continued
	3	Acute otitis media	Moderate	Yes	Not related	Occasional event and was probably not associated with the study substance.	continued
Active	1	Common cold	Moderate	Yes	Not related	Occasional event due to an infectious disease and was probably not associated with the study substance.	continued
Active	1	Treatment of pus from gums	Moderate	Yes	Not related	Occasional event and was probably not associated with the study substance.	continued
Active	1	Increase of TG, Increase of body weight	Mild	No	Not related	The participant's triglyceride level was believed to be temporarily elevated because of diet. This was determined to be unrelated to the study substance.	continued
Placebo	1	Pollinosis	Moderate	Yes	Not related	Occasional event and was probably not associated with the study substance.	continued
Placebo	1	Pollinosis	Mild	No	Not related	Occasional event and was probably not associated with the study substance.	continued
Placebo	1	Loose bowel movement	Mild	No	Not related	The symptoms occurred after conclusion of ingesting the study substance. It was concluded that the study substance was unrelated to the symptoms.	continued

Continued

2	Loose bowel movement	Mild	No	Not related	The symptoms occurred even after the conclusion of ingesting the study substance It was concluded that the study substance was unrelated to the symptoms.	continued
Placebo 1	Bleeding piles	Moderate	Yes	Not related	Occasional event and was probably not associated with the study substance.	continued

Table 5. (a) Baseline parameters of physical, hematological, and biochemical examinations (Excessive intake safety study). (b) Baseline parameters of urine examinations (Excessive intake safety study).

(a)

Parameter		(Unit)	Placebo			Active			*p* value
			Mean	±	SD	Mean	±	SD	
Gender	Male	(n)		9			9		
	Famale			6			6		
Age			44.8	±	10.6	45.7	±	14.0	0.838
Height		(cm)	167.2	±	8.0	165.6	±	5.6	0.524
Body weight		(kg)	65.4	±	10.7	64.2	±	9.4	0.759
BMI			23.3	±	2.7	23.4	±	2.7	0.916
Systolic blood presure		(mmHg)	118.0	±	15.9	112.7	±	12.9	0.322
Diastolic blood pressure		(mmHg)	74.0	±	11.1	72.1	±	9.5	0.611
Pulse rate		(bpm)	71.9	±	9.6	68.0	±	7.2	0.214
White blood cell count		(/μL)	5400	±	975	5840	±	1442	0.336
Red blood cell count		($\times 10^4$/μL)	461.7	±	44.2	461.0	±	33.7	0.963
Heamoglobin		(g/dL)	13.8	±	1.5	14.1	±	1.4	0.547
Hematocrit		(%)	43.0	±	4.2	43.5	±	3.6	0.691
Platelet count		($\times 10^4$/μL)	26.1	±	6.7	26.2	±	5.0	0.953
MCV		(fL)	93.1	±	4.9	94.5	±	3.4	0.375
MCH		(pg)	29.8	±	2.0	30.5	±	1.3	0.270
MCHC		(%)	32.0	±	0.7	32.3	±	0.7	0.277
NEUT/leukocyte fractionation		(%)	55.7	±	6.0	56.6	±	9.9	0.756
LYMPH/leukocyte fractionation		(%)	33.7	±	5.3	32.8	±	9.5	0.759
MONO/leukocyte fractionation		(%)	5.4	±	1.1	5.7	±	1.1	0.421
EOSINO/leukocyte fractionation		(%)	4.2	±	2.8	4.0	±	4.0	0.878
BASO/leukocyte fractionation		(%)	1.0	±	0.5	0.8	±	0.4	0.208
Total protein		(g/dL)	7.1	±	0.4	7.1	±	0.3	0.880
albumin		(g/dL)	4.3	±	0.3	4.3	±	0.2	0.942
AST		(U/L)	19.5	±	5.7	20.6	±	5.5	0.583

Continued

ALT	(U/L)	17.4	±	8.0	23.2	±	13.3	0.159
LD (IFCC)	(U/L)	164.7	±	19.3	180.9	±	31.5	0.101
Total bilirubin	(mg/dL)	0.8	±	0.3	0.7	±	0.3	0.807
ALP (IFCC)	(U/L)	65.8	±	16.7	71.4	±	13.2	0.318
g-GT	(U/L)	22.2	±	15.4	27.6	±	24.9	0.481
Urea nitrogen (UN)	(mg/dL)	13.3	±	2.4	12.4	±	3.8	0.430
Creatinine	(mg/dL)	0.75	±	0.16	0.77	±	0.17	0.755
Ureic acid (UA)	(mg/dL)	4.6	±	1.4	5.3	±	1.1	0.165
Sodium (Na)	(mEq/L)	141.7	±	1.4	141.3	±	1.5	0.533
Chloride (Cl)	(mEq/L)	105.1	±	0.9	104.5	±	1.3	0.116
Pottasium (K)	(mEq/L)	4.4	±	0.3	4.4	±	0.4	0.548
Calcium (Ca)	(mg/dL)	9.3	±	0.3	9.4	±	0.3	0.310
Total cholesterol	(mg/dL)	204.7	±	34.0	199.1	±	27.2	0.626
LDL-cholesterol	(mg/dL)	121.4	±	29.2	118.9	±	23.8	0.796
HDL-cholesterol	(mg/dL)	63.7	±	18.2	62.6	±	17.2	0.870
Triglyceride (TG)	(mg/dL)	101.7	±	54.0	92.9	±	51.0	0.647
Glucose	(mg/dL)	86.2	±	5.6	84.6	±	6.8	0.486
HbA1c (NGSP)	(%)	5.2	±	0.2	5.2	±	0.3	0.732
Ureic pH		6.4	±	0.5	6.1	±	0.5	0.116
Urine specific gravity		1.021	±	0.008	1.017	±	0.008	0.150

p value: ANOVA.

(b)

Urine protein	0.13	±	0.35	0.00	±	0.00	0.150
Urine glucose	0.00	±	0.00	0.00	±	0.00	1.000
Urobillinogen	0.00	±	0.00	0.00	±	0.00	1.000
Urine bilirubin	0.00	±	0.00	0.00	±	0.00	1.000
Urine ketone body	0.00	±	0.00	0.00	±	0.00	1.000
Occult blood reaction	0.00	±	0.00	0.00	±	0.00	1.000

p value: Wilcoxon's rank sum test (asymptotic significance probability).

promoting fluid secretion within the intestinal tract. This allowed us to identify the human-derived *Bifidobacterium* known as *Bifidobacterium longum*. *B. longum* is a species listed on the European Food Safety Authority (EFSA) "list of qualified presumption of safety (QPS)-recommended biological agents intentionally added to food or feed as notified to EFSA" [15]. *In vivo* efficacy evaluation showed that heat-killed *B. longum* CLA8013 improved the moisture content of feces and promoted bowel movements (unpublished data). An in-house safety evaluation was performed using approximately 2.1 billion units of heat-killed *B. longum* CLA8013 as the study substance. Single-dose toxicity testing using

Table 6. Adverse events (Excessive intake safety study).

Group	Participant number	Adverse event (symptom)	Severity	Treatment	Causal relation with the study substance	Reason for causal conclusion	Continuation or discontinuation of the trial
Active	1	Dental caries	Moderate	Yes	Not related	Occasional event due to an infectious disease and was probably not associated with the study substance.	continued
Placebo	1	Premenstrual syndrome	Mild	No	Not related	Occasional event due to an infectious disease and was probably not associated with the study substance.	continued
Placebo	1	Dental caries	Moderate	Yes	Not related	Occasional event due to an infectious disease and was probably not associated with the study substance.	continued

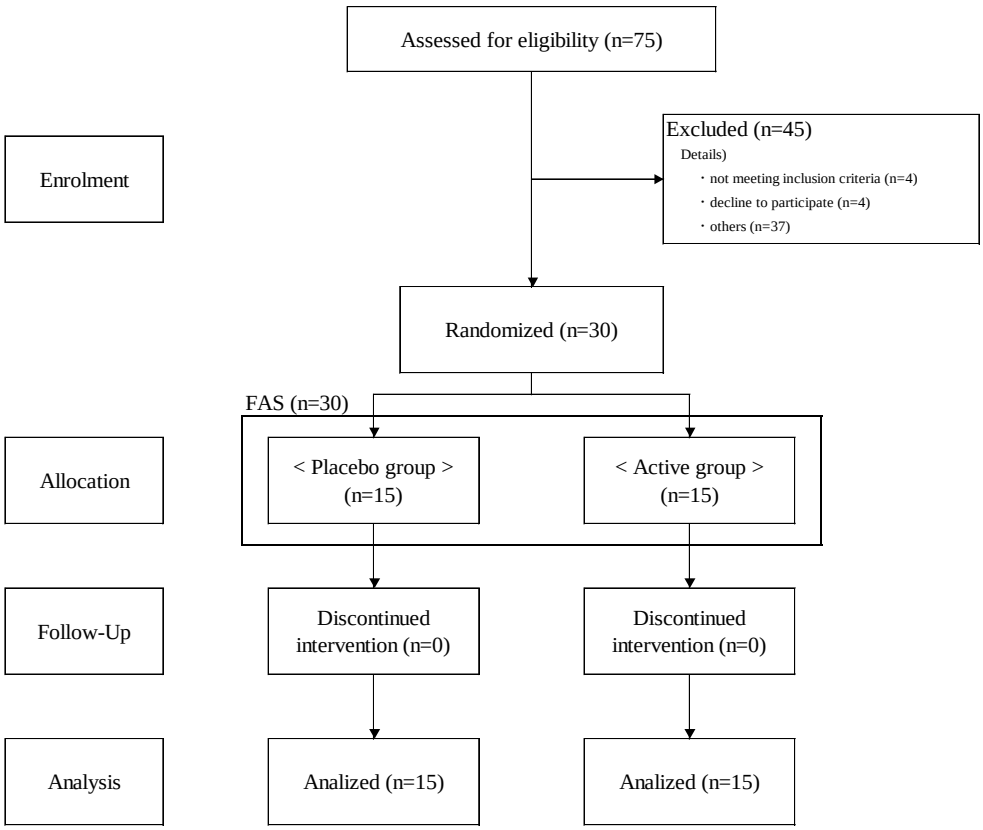


Figure 3. Flow diagram of the excessive intake safety study. A total of 75 individuals were registered and assessed for eligibility; thirty participants participated in the study.

Sprague–Dawley rats revealed that, at a maximum dose of 2000 mg/kg, the general condition, body weight, and autopsy results were normal. Repeated oral dosage toxicity testing at doses of 0, 500, 1000, and 2000 mg/kg showed no toxic changes in any of the following: general condition, body weight, amount of intake, ophthalmological tests, urinalysis, hematological tests, blood chemical tests, organ weight, autopsy results, or histopathologic tests. Based on these results, the no observed adverse effect level (NOAEL) for both men and women was determined to be 2000 mg/kg. The results of chromosome aberration and Ames tests of *Salmonella typhimurium* and *Escherichia coli* using the Chinese hamster lung cell line (CHL/IU) were negative.

The effectiveness of heat-killed *B. longum* CLA8013 was previously evaluated in a double-blind, placebo-controlled study of healthy volunteers prone to constipation, which was conducted after confirming its safety in animals. The study showed that ingesting heat-killed *B. longum* CLA8013 at a dose of 25 billion cells/day for 2 weeks effectively improved bowel movements [14]. After confirming the usefulness of heat-killed *B. longum* CLA8013, we conducted the two safety studies: long-term intake safety study and excessive intake safety study. Based on our study findings, we conclude that heat-killed *B. longum* CLA8013 is not associated with safety-related issues.

5. Conclusion

Our randomized, double-blind, placebo-controlled, parallel-group study confirmed the safety of long-term (25 billion cells/day for 12 weeks) and excessive (125 billion cells/day for 4 weeks) intake of heat-killed *B. longum* CLA8013.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Appendixs (Tables S1-S11)

https://zenodo.org/records/10016356/files/Supplemental%20Tables.xlsx?download=1