Article

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Safety evaluation of *Bifidobacterium breve* MCC1274 via oral toxicity tests in rats

Satoshi Arai[1], Jun-ichi Minami[1], Masamichi Muto[1], Noriyuki Iwabuchi[1], Koji Yamauchi[1], Jin-zhong Xiao[2], and Fumiaki Abe[1]

Abstract

In this study, the safety of *Bifidobacterium breve* MCC1274, a probiotic bifidobacterial strain, was assessed by single-dose and 90-day repeated-dose oral toxicity studies. In the single-dose oral toxicity assay using 6000 mg/kg of *B. breve* MCC1274 corresponding to 8.4×10^{11} colony-forming unit (CFU)/kg, mortality and adverse effects were not observed. Furthermore, the administration of 1000 mg/kg of *B. breve* MCC1274 by oral gavage in saline for 90 days did not induce any signs of toxicity, such as changes in clinical signs, body weight (BW), food consumption, ophthalmoscopy, urinalysis, hematology, blood chemistry, organ weight, gross pathology, and histopathology compared to the control group given cornstarch in saline (10/sex/group). The no-observed-adverse-effect-level of *B. breve* MCC1274 in the 90-day repeated-dose toxicity study was greater than 1000 mg/kg corresponding to 1.3×10^{11} CFU/kg. Based on the findings of this study, the acceptable daily intake of *B. breve* MCC1274 was calculated to be 1.3×10^{9} CFU/kg BW/day.

Keywords

Bifidobacterium breve, MCC1274, safety, toxicity study

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Introduction

Bifidobacteria are a major member of the human intestinal microflora and have been reported to promote various physiological functions, such as improvement of gastrointestinal conditions, protection against infections, promotion of immunomodulatory activity, and prevention of allergic diseases.[1,2] With the increasing interest in the many health-promoting effects of bifidobacteria, certain *Bifidobacterium* strains have been used for various nutritional and medical applications. However, a few safety issues associated with probiotics have been reported,[3–6] thus demonstrating the need to investigate both the beneficial effects and the safety of probiotics with regard to human health. Safety remains one of the most important criteria for the selection and industrial application of probiotics.

In the United States, the Food and Drug Administration (FDA) has responded favorably with "no questions" to generally recognized as safe (GRAS) notifications for a number of *Bifidobacterium* strains.[7–13] Similarly, certain *Bifidobacterium* species, such as *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum*, have been given a qualified presumption of safety (QPS) status in the European Union.[14] However, scientists have proposed that the safety of probiotics should be evaluated on a strain-by-

[1] Food Ingredients and Technology Institute, R&D Division, Morinaga Milk Industry Co., Ltd, Zama-City, Kanagawa, Japan
[2] Next Generation Science Institute, R&D Division, Morinaga Milk Industry Co., Ltd, Zama-City, Kanagawa, Japan

Corresponding author:
Satoshi Arai, Food Ingredients and Technology Institute, R&D Division, Morinaga Milk Industry Co., Ltd, 5-1-83, Higashihara, Zama-City, Kanagawa 252-8583, Japan.
Email: s-arai@morinagamilk.co.jp

strain basis because features of probiotic strains vary among strains.[15]

The probiotic *B. breve* MCC1274 (B-3) has been used in animal studies and clinical investigations involving adults with a tendency for obesity.[16,17] Minami et al. reported that no serious adverse events were observed in medical interviews or by measurements of blood parameters in clinical investigation.[17,18] Although the clinical investigation reported no abnormal signs during the study, basic safety investigations using animals are important to ensure that harmful effects do not occur. Generally, single-dose oral toxicity studies in animals are conducted as an acute toxicity test, whereas 90-day repeated-dose oral toxicity studies are used as subchronic toxicity tests to evaluate the safety of food ingredients or food additives.

In the present study, *B. breve* MCC1274 was evaluated for safety in single-dose and 90-day repeated-dose oral toxicity assays.

Materials and methods

Bifidobacterium powder

The test substance used in this study, *B. breve* MCC1274 powder, supplied by the Morinaga Milk Industry Co., Ltd (Kanagawa, Japan) was sold as dietary ingredient. The pure cell was freeze-dried with cornstarch to achieve the desired concentration. The lot numbers 2009.12.24 and 2010.02.10 were used for the single- and repeated-dose toxicity studies, and they contained 1.4×10^{11} and 1.3×10^{11} viable cells per gram, respectively. For enumeration of test products, products were serially diluted, Mitsuoka's buffer and Reinforced Clostridial Medium Agar was used as the plating media. Plates were incubated at 37°C for 72 h anaerobically in an anaerobic chamber. The colonies formed in plates were counted and viable counts were calculated. The test substance was suspended in physiological saline (Japanese Pharmacopoeia, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) immediately prior use. Physiological saline and cornstarch suspended in physiological saline (purified, dried, and sterilized cornstarch white; Matsutani Chemical Industry Co., Ltd, Hyogo, Japan) were used as the control substances in the single- and repeated-dose toxicity studies. A dose analysis was performed once for the single-dose toxicity study and twice for the repeated-dose toxicity study, and the results were confirmed to be acceptable.

Animals

Male and female Sprague-Dawley rats (Crl:CD(SD); Atsugi Breeding Center, Charles River Laboratories Japan, Inc., Kanagawa, Japan) were obtained at 5 weeks of age. The animals were quarantined and acclimated for 7 or 8 days at the test facility, and healthy animals were used in the studies at 6 weeks of age. The animals were randomly assigned to each group to ensure the homogeneity of the group mean values for body weight (BW), which was calculated using a computer. The animals were housed in an animal room maintained under the following conditions: 23 ± 3°C, 50 ± 20% relative humidity, 12–17 air changes per hour, and 12 h of light per day (07:00–19:00). The animals were housed individually in bracket-type stainless steel wire mesh cages (W 250 × D 350 × H 200 mm^3 or W 254 × D 350 × H 170 mm^3) and allowed free access to a pelleted diet of CRF-1 or CR-LPF (radiation-sterilized; Oriental Yeast Co., Ltd, Tokyo, Japan) and Gotemba City tap water via water bottles or an automatic water supply system.

The studies were conducted at Gotemba Laboratory, Bozo Research Center Inc. (Shizuoka, Japan), under Good Laboratory Practice conditions for Non-Clinical Safety Studies on Drugs (Ordinance no. 21, March 26, 1997; partially revised by Ordinance no. 114, June 13, 2008) issued by the Ministry of Health and Welfare (MHW), Japan. The animal experiments were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of the test facility and in accordance with the laws and guidelines related to animal welfare, including the "Act on Welfare and Management of Animals" (Law no. 105, October 1, 1973), the "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain" (Notification no. 88 of the Ministry of the Environment, Japan, April 28, 2006) and the "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan, June 1, 2006).

Single-dose oral toxicity study

This study was conducted in accordance with the toxicity guideline "Revision of Guidelines for Single-Dose and Repeated-Dose Toxicity Studies" (Notification no. 88 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, August 10, 1993).

Two test groups were used, a control group and a dose group, and each contained five males and five females. Rats deprived of food overnight were administered the test substance suspended in physiological saline once via oral gavage at a dose level of 6000 mg/kg corresponding to 8.4×10^{11} colony-forming unit (CFU)/kg, which is the maximum feasible dose level with respect to dose concentration and dose volume. The animals in the control group received physiological saline (20 mL/kg BW). The intervention dose was administered in the same volume as the control. All animals were allowed free access to food 4 h after dosing.

The animals were observed for any clinical signs, including changes in external appearance, emaciation, posture, behavior, and excretions, for 14 days after dosing (frequently on the day of administration and once a day thereafter), and their BWs were recorded on days 1, 2, 3, 7, 10, and 14. After the 14-day observation period, the animals were euthanized by exsanguination via the abdominal aorta under isoflurane

anesthesia and subsequently necropsied by carefully observing the external appearance of organs/tissues in the cranial, thoracic, and abdominal cavities.

Ninety-day repeated-dose oral toxicity study

This study was conducted in accordance with the toxicity guideline "Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives" (Notification no. 29 of the Environmental Health Bureau, Ministry of Health and Welfare, Japan, March 22, 1996).

Two test groups, a control group and a dose group, were used in this study, and each consisted of 10 males and 10 females. The test substance was administered once daily by oral gavage for 91 days at a dose level of 1000 mg/kg corresponding to 1.3×10^{11} CFU/kg. The animals in the control group received cornstarch (equivalent to 75% of the amount in the test suspension).

The animals were observed for any clinical signs, including changes in external appearance, emaciation, posture, behavior, and excretions, three times a day during the administration period prior to dosing and immediately after and between 1 h and 3 h after dosing (twice a day on Saturdays, Sundays, and holidays prior to and immediately after dosing). The BWs and food consumption were recorded every day.

Ophthalmoscopy was conducted on six animals of each sex per group during week 13 of administration. First, the mydriatic agent Mydrin P (Santen Pharmaceutical Co., Ltd, Osaka, Japan) was applied to dilate the pupil, and then the anterior portion, optic media, and fundus oculi were examined using an ophthalmoscope (Omega 200; HEINE Optotechnik GmbH & Co. KG, Germany).

Urinalysis was conducted on all animals during week 13 of administration. After dosing, the animals were individually placed in cages equipped with a urine collector, deprived of food but allowed free access to water, and then 4-h urine samples were collected. Subsequently, the animals were given free access to food and water, and then 20-h urine samples were collected. The following parameters were examined using the 4-h urine samples: pH, protein, ketones, glucose (GLU), occult blood, bilirubin, urobilinogen (AUTION Sticks-7EA test paper, AUTION MINI™ AM-4290; ARKRAY Inc., Kyoto, Japan), urine volume, color, and urinary sediments. The following parameters were assessed using the 20-h urine samples: urine volume, osmotic pressure (Automatic Osmometer AUTO & STAT OM-6030; ARKRAY Inc.), sodium (Na), potassium (K), and chloride (Cl) (Clinical Chemistry Autoanalyzer PVA-α II; A&T Corp., Kanagawa, Japan). The 24-h urine volume was calculated by totaling the 4-h and 20-h urine volumes. The daily excretion of electrolytes was calculated from the determined electrolyte concentration and the 24-h urine volume. In addition, the daily water consumption for each animal was measured at the time of urine sample collection using a water bottle.

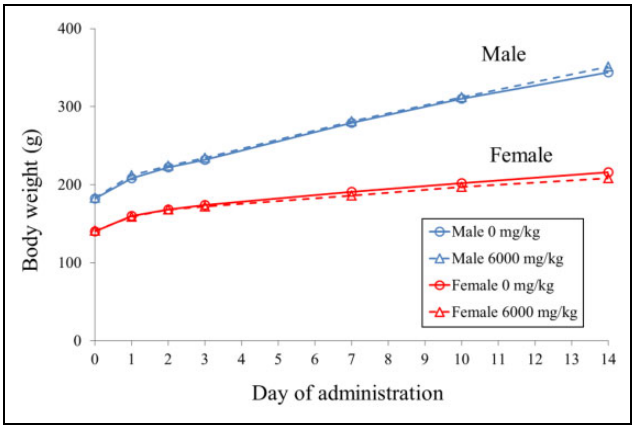

Figure 1. Changes in the average body weights of male and female rats treated orally with *Bifidobacterium breve* MCC1274 for 14 days. (○) Control rats and (Δ) rats administered 6000 mg/kg corresponding to 8.4×10^{11} CFU/kg. CFU: colony-forming unit.

At the time of the scheduled necropsy on the day following the end of the administration period, blood samples were collected from the abdominal aorta under isoflurane anesthesia for hematology and blood chemistry analyses. The animals were deprived of food overnight prior to blood sample collection. The following hematological parameters were determined using a Hematology Analyzer ADVIA 120 (Siemens Healthcare Diagnostics Inc., Hyogo, Japan) on the blood samples treated with EDTA-2K: red blood cell count (RBC), hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration, reticulocyte percentage (Retic), platelet count, white blood cell count (WBC), and differential leukocyte count and percentage (lymphocyte, neutrophil, eosinophil, basophil, monocyte, and large unstained cell). In addition, the blood coagulation parameters prothrombin time, activated partial thromboplastin time, and fibrinogen were determined using a coagulometer ACL Elite Pro (Instrumentation Laboratory, Massachusetts, USA) on the plasma obtained by centrifuging the blood samples treated with sodium citrate. The following parameters for blood chemistry were determined using a Clinical Chemistry Autoanalyzer TBA-120FR (Toshiba Medical Systems Corporation, Tochigi, Japan) on the plasma obtained by centrifuging at $1600 \times g$ for 10 min from the blood samples treated with heparin sodium: aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, γ-glutamyl transpeptidase, alkaline phosphatase, total cholesterol, triglyceride, phospholipid, total bilirubin, GLU, blood urea nitrogen, creatinine, Na, K, Cl, calcium, inorganic phosphorus, total protein, albumin (ALB), and the albumin/globulin ratio. In addition, protein fractionation was conducted by agarose gel electrophoresis using a QuickScan Densitometer (K.K. Helena Kenkyujyo, Saitama, Japan) to determine the percentages of albumin (ALB-E), α_1-globulin, α_2-globulin, β-globulin, and γ-globulin.

Table 1. Pathological findings in male and female rats treated orally with *Bifidobacterium breve* MCC1274.

	Sex	Male		Female	
	Dose (mg/kg)	0	6000	0	6000
	Dose (CFU/kg)	0	8.4×10^{11}	0	8.4×10^{11}
Organs/finding	Number of animals	5	5	5	5
External appearance					
No abnormality		5	5	5	5
Viscera of cranial cavity					
No abnormality		5	5	5	5
Viscera of thoracic cavity					
No abnormality		5	5	5	5
Viscera of abdominal cavity					
No abnormality		5	5	5	5

CFU: colony-forming unit.
Values are the number of animals with the indicated findings.

After collecting blood samples, all animals were euthanized by exsanguination via the abdominal aorta and thoroughly necropsied, after which the following organs were weighed (absolute weight), and the organ weight per 100 g BW (relative weight) was calculated: the brain, pituitary gland, thyroid gland (with parathyroid gland), adrenal gland, thymus, spleen, heart, lung (including bronchus), salivary gland (submandibular + sublingual gland), liver, kidney, testis, prostate, seminal vesicle, ovary, and uterus. Subsequently, the following organs/tissues were dissected and fixed with phosphate buffered 10% formalin (the eyeball and optic nerve were fixed with a mixture of 3% glutaraldehyde and 2.5% formalin, and the testis and epididymis were fixed with Bouin's solution and subsequently preserved in phosphate buffered 10% formalin), embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically: the cerebrum, cerebellum, spinal cord (thoracic), sciatic nerve, eyeball, optic nerve, Harderian gland, pituitary gland, thyroid gland, parathyroid gland, adrenal gland, thymus, spleen, cervical lymph node, mesenteric lymph node, heart, thoracic aorta, trachea, lung (with bronchus), tongue, esophagus, stomach, duodenum, jejunum, ileum (including Peyer's patch), cecum, colon, rectum, submandibular gland, sublingual gland, liver, pancreas, kidneys, urinary bladder, testis, epididymis, prostate, seminal vesicle, ovary, uterus, vagina, oviduct, mammary gland (inguinal), sternum (including bone marrow), femur (including bone marrow), femoral skeletal muscle, skin (inguinal), nasal cavity, and Zymbal gland.

Statistical analysis

For the numerical data (BW, food consumption, quantitative urinalysis parameters (including water consumption), hematology, blood chemistry, and organ weights), the homogeneity of variance was analyzed by the F test (significance level: 5% one-tailed). Homogeneous data were assessed by Student's t test, and heterogeneous data were

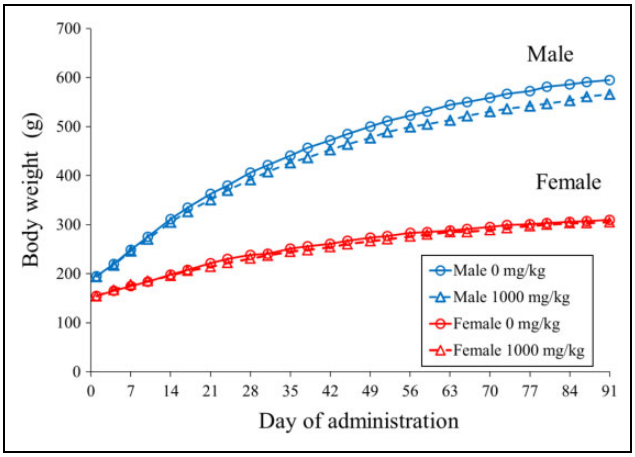

Figure 2. Changes in the average body weights of male and female rats treated orally with *Bifidobacterium breve* MCC1274 for 90 days. (○) Control rats and (Δ) rats administered 1000 mg/kg corresponding to 1.3×10^{11} CFU/kg. CFU: colony-forming unit.

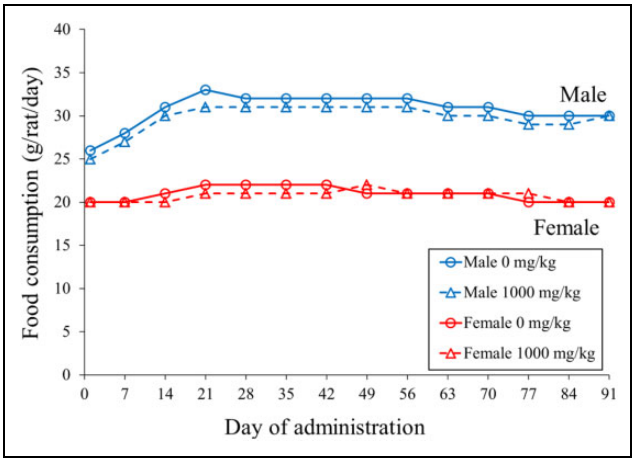

Figure 3. Changes in the average food consumption of male and female rats treated orally with *Bifidobacterium breve* MCC1274 for 90 days. (○) Control rats and (Δ) rats administered 1000 mg/kg corresponding to 1.3×10^{11} CFU/kg. CFU: colony-forming unit.

Table 2. Urinalysis in male and female rats treated orally with *Bifidobacterium breve* MCC1274 for 90 days.

Items	Sex	Male		Female	
	Dose (mg/kg)	0	1000	0	1000
	Dose (CFU/kg)	0	1.3×10^{11}	0	1.3×10^{11}
	Number of animals	10	10	10	10
pH[a]	5.5	0	0	0	1
	7.0	0	0	1	0
	7.5	0	0	0	2
	8.0	0	1	2	3
	8.5	6	4	6	3
	9.0	4	5	1	2
Protein[a]	−	1	0	9	7
	+/−	5	4	0	1
	1+	4	5	1	2
	2+	0	1	0	0
Ketone[a]	−	6	6	9	8
	+/−	4	2	0	2
	1+	0	2	1	0
Glucose[a]	−	10	10	10	10
Occult blood[a]	−	10	10	10	10
Urobilinogen[a]	+/−	10	9	9	10
	1+	0	1	1	0
Bilirubin[a]	−	10	10	10	10
Color[a]	Yellow	10	10	10	10
Sediments[a]					
RBC	−	10	10	10	10
WBC	−	10	10	10	10
Squamous epithelial cell	+/–	10	10	10	10
Small round epithelial cell	−	10	10	10	10
Cast	−	10	10	10	10
Phosphate salt	−	2	1	6	6
	+/−	8	9	4	4
Calcium oxalate	−	10	10	10	10
Urine volume[b]	mL/24 h	13.5 ± 2.6	12.1 ± 4.2	9.0 ± 5.0	11.2 ± 7.5
Water consumption[b]	mL/24 h	36 ± 3	33 ± 6	37 ± 12	31 ± 8
Osmotic pressure[b]	mOsm/kg	1807 ± 273	1888 ± 376	1792 ± 662	1581 ± 731
Na[b]	mmol/24 h	2.6 ± 0.4	2.2 ± 0.5	1.4 ± 0.3	1.3 ± 0.2
K[b]	mmol/24 h	4.0 ± 0.6	3.6 ± 1.0	2.2 ± 0.5	2.2 ± 0.5
Cl[b]	mmol/24 h	3.3 ± 0.4	2.9 ± 0.7	1.8 ± 0.4	1.7 ± 0.3

CFU: colony-forming unit.
Protein (mg/dL) −: <10, +/−: 10–25, 1+: 26–85, 2+: 86–250, 3+: 251–600, 4+: >600; ketone (mg/dL) −: <5, +/−: 5–7.5, 1+: 7.6–30, 2+: 31–70, 3+: 71–125, 4+: >125; glucose (mg/dL) −: <30, +/−: 30–60, 1+: 61–125, 2+: 126–250, 3+: 251–750, 4+: >750; occult blood (mg/dL) −: <0.03, +/−: 0.03–0.05, 1+: 0.06–0.15, 2+: 0.16–0.75, 3+: >0.75; urobilinogen (Ehrlich U/dL) +/−: <2.0, 1+: 2.0–3.5, 2+: 3.6–7.0, 3+: 7.1–12.0, 4+: >12.0; bilirubin (mg/dL) −: <0.5, 1+: 0.5–1.5, 2+: 1.6–5.0, 3+: 5.1–10.0, 4+: >10.0; sediments −: negative, +/−: slight, 1+: mild, 2+: moderate, 3+: severe.
[a]Values in the table indicate the number of animals with the indicated findings.
[b]Values in the table are the mean ± SD.

assessed by the Aspin-Welch's *t* test (levels of significance: 5% and 1%, two-tailed). Analyses were performed using the integrated statistical package SAS Release 9.1.3 (SAS Institute Inc., North Carolina, USA).

Results

Acute oral toxicity study

To investigate the acute oral toxicity of *B. breve* MCC1274 in mammals, a single dose of 6000 mg/kg *B. breve* MCC1274 was administered to male and female SD rats by oral gavage, which was followed by 14 days of monitoring. Mortality did not occur among the control rats or the rats administered 6000 mg/kg *B. breve* MCC1274 corresponding to 8.4×10^{11} CFU/kg, and no treatment-related changes in clinical signs were observed, including the external appearance, emaciation, posture, behavior, excretions, and BW, among the animals of either sex during the 14-day observation period (Figure 1). All animals were necropsied at the end of the observation period, and none of the organs showed abnormal findings attributable to the administration of *B. breve* MCC1274 (Table 1).

Table 3. Hematology in male and female rats treated orally with *Bifidobacterium breve* MCC1274 for 90 days.

	Sex	Male		Female	
	Dose (mg/kg)	0	1000	0	1000
	Dose (CFU/kg)	0	1.3×10^{11}	0	1.3×10^{11}
Items	Number of animals	10	10	10	10
RBC	10E4/μL	894 ± 44	893 ± 56	857 ± 34	824 ± 22[a]
HGB	g/dL	15.3 ± 0.4	15.1 ± 0.7	15.1 ± 0.4	14.9 ± 0.5
HCT	%	44.9 ± 1.4	44.5 ± 2.0	43.3 ± 1.5	42.6 ± 1.4
MCV	fL	50.2 ± 1.4	49.9 ± 1.5	50.5 ± 0.9	51.7 ± 1.8
MCH	pg	17.2 ± 0.6	17.0 ± 0.6	17.6 ± 0.3	18.1 ± 0.6[a]
MCHC	g/dL	34.1 ± 0.5	34.0 ± 0.4	34.9 ± 0.4	34.9 ± 0.4
RET	%	2.2 ± 0.4	2.5 ± 0.4	1.9 ± 0.3	2.1 ± 0.3
PLT	10E4/μL	114.1 ± 12.0	116.7 ± 10.8	113.0 ± 10.4	113.1 ± 18.1
WBC	10E2/μL	90.2 ± 29.6	87.0 ± 26.7	61.2 ± 15.7	54.6 ± 24.2
LYMP	%	71.7 ± 10.4	71.0 ± 9.4	70.6 ± 7.4	66.4 ± 13.0
	10E2/μL	64.5 ± 23.4	61.8 ± 21.6	42.9 ± 11.1	34.4 ± 11.9
NEUT	%	23.4 ± 10.8	23.3 ± 8.3	23.8 ± 7.9	28.1 ± 13.1
	10E2/μL	21.2 ± 13.3	20.5 ± 10.7	14.9 ± 7.7	17.4 ± 16.5
EOS	%	1.4 ± 0.5	1.7 ± 0.7	1.9 ± 0.7	1.9 ± 0.8
	10E2/μL	1.3 ± 0.5	1.3 ± 0.5	1.1 ± 0.3	1.0 ± 0.4
BASO	%	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
	10E2/μL	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
MONO	%	2.7 ± 0.7	3.0 ± 1.1	2.9 ± 0.9	2.9 ± 1.2
	10E2/μL	2.5 ± 1.1	2.5 ± 1.0	1.7 ± 0.5	1.5 ± 0.7
LUC	%	0.6 ± 0.3	0.8 ± 0.6	0.7 ± 0.5	0.6 ± 0.3
	10E2/μL	0.5 ± 0.5	0.7 ± 0.6	0.5 ± 0.4	0.3 ± 0.1
PT	s	13.2 ± 1.0	13.1 ± 0.6	12.9 ± 0.7	12.6 ± 0.8
APTT	s	18.9 ± 1.9	20.5 ± 2.8	18.6 ± 1.4	17.9 ± 2.9
FIB	mg/dL	299 ± 34	281 ± 22	221 ± 35	241 ± 103

RBC: red blood cell count; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RET: reticulocyte; PLT: platelet; WBC: white blood cells; LYMP: lymphocyte; NEUT: neutrophil; EOS: eosinophil; BASO: basophil; MONO: monocyte; LUC: large unstained cell; PT: prothrombin time; APTT: activated partial thromboplastin time; FIB: fibrinogen; CFU: colony-forming unit.
Values in the table are the means ± SD.
[a]$p \leq 0.05$ (significantly different from the control group).

Subchronic oral toxicity study

Oral administration of 1000 mg/kg *B. breve* MCC1274 corresponding to 1.3×10^{11} CFU/kg for 90 days did not cause any mortality or abnormal clinical signs in animals of either sex throughout the observation period. The average BW in the group-administered *B. breve* MCC1274 was comparable to that of the control group for the male and female rats, and significant differences were not observed (Figure 2). Food consumption in the group-administered *B. breve* MCC1274 was comparable to that in the control group for both male and female rats, and significant differences were not observed (Figure 3). Significant differences were not observed in the ophthalmoscopy or urinalysis results (Table 2). In addition, significant differences were not observed between the control and test rats (both male and females) with respect to urine color, dipstick test results for pH, protein, ketone, glucose, occult blood, urobilinogen, bilirubin, and microscopic sediment analysis for RBC, WBC, squamous epithelial cells, small round epithelial cells, cast, phosphate salts, and calcium oxalate. All the values remained within the normal range throughout the study. The hematology results are presented in Table 3. A significant decrease in the RBC (−4% of the control mean) was observed in females given 1000 mg/kg *B. breve* MCC1274, and it was accompanied by a significant increase in the MCH (+3% of the control mean). The blood chemistry showed that significant differences did not occur between the control and treated groups (Table 4). Table 5 presents the absolute and relative organ weights of the rats. Significant differences were not observed between the control and treated groups in the brain, pituitary, thyroid, salivary gland, thymus, heart, lung, liver, spleen, kidney, adrenal, testis, prostate, seminal vesicle, ovary, and uterus. The necropsy and histopathological examinations revealed none of the studied organs showed obvious changes related to the administration of *B. breve* MCC1274 (Tables 6 and 7).

Discussion

Probiotic bifidobacteria and lactobacilli have been widely used for their beneficial effects, and many products containing these organisms have gained popularity

Table 4. Blood chemistry in male and female rats treated orally with *Bifidobacterium breve* MCC1274 for 90 days.

	Sex	Male		Female	
	Dose (mg/kg)	0	1000	0	1000
	Dose (CFU/kg)	0	1.3×10^{11}	0	1.3×10^{11}
Items	Number of animals	10	10	10	10
AST	IU/L	63 ± 9	60 ± 12	65 ± 19	59 ± 15
ALT	IU/L	26 ± 5	26 ± 4	33 ± 18	24 ± 7
LDH	IU/L	55 ± 23	51 ± 18	47 ± 16	47 ± 23
ALP	IU/L	310 ± 100	319 ± 64	163 ± 36	148 ± 28
γ-GTP	IU/L	1 ± 1	1 ± 0	1 ± 0	1 ± 0
T-CHO	mg/dL	70 ± 10	73 ± 16	79 ± 13	83 ± 11
TG	mg/dL	76 ± 42	74 ± 36	27 ± 13	29 ± 9
PL	mg/dL	112 ± 14	117 ± 18	147 ± 20	159 ± 19
T-BIL	mg/dL	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
GLU	mg/dL	145 ± 17	141 ± 16	124 ± 17	132 ± 15
BUN	mg/dL	14 ± 2	15 ± 1	16 ± 2	15 ± 2
CRNN	mg/dL	0.26 ± 0.03	0.25 ± 0.03	0.30 ± 0.03	0.30 ± 0.03
Na	mmol/L	144 ± 1	145 ± 1	143 ± 1	143 ± 1
K	mmol/L	4.5 ± 0.2	4.5 ± 0.2	3.9 ± 0.2	4.0 ± 0.3
Cl	mmol/L	106 ± 2	107 ± 2	109 ±1	109 ± 1
Ca	mg/dL	9.9 ± 0.4	9.9 ± 0.3	10.0 ± 0.2	10.1 ± 0.5
P	mg/dL	5.7 ± 0.8	5.9 ± 0.8	4.6 ± 0.7	4.2 ± 0.8
TP	g/dL	6.3 ± 0.2	6.3 ± 0.3	6.8 ± 0.3	7.0 ± 0.5
ALB	g/dL	3.3 ± 0.1	3.2 ± 0.1	3.8 ± 0.2	4.0 ± 0.4
A/G ratio		1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
ALB-E	%	52.6 ± 3.1	51.8 ± 2.8	57.0 ± 1.8	59.2 ± 4.3
α_1-globulin	%	20.3 ± 3.0	21.4 ± 3.3	14.0 ± 2.5	12.9 ± 2.5
α_2-globulin	%	8.7 ± 0.8	8.9 ± 1.2	9.9 ± 1.0	9.8 ± 2.2
β-globulin	%	13.4 ± 1.4	13.2 ± 1.1	11.4 ± 1.5	11.1 ± 1.3
γ-globulin	%	5.1 ± 1.6	4.8 ± 1.3	7.7 ± 2.1	7.1 ± 2.3

AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactic dehydrogenase; ALP: alkaline phosphatase; γ-GTP: γ-glutamyl transpeptidase; T-CHO: total cholesterol; TG: triglycerides; PL: phospholipid; T-BIL: total bilirubin; GLU: glucose; BUN: blood urea nitrogen; CRNN: creatinine; TP: total protein; ALB: albumin; A/G ratio: albumin/globulin ratio; CFU: colony-forming unit.
Values in the table indicate mean ± SD.

worldwide.[19] However, recent studies have raised concerns about the safety of probiotics. Interestingly, only a few reports have focused on the safety issues surrounding probiotics relative to the large number of articles focused on their efficacy. Bacterial infections related to probiotic use, including bacteremia and sepsis, have been reported.[20–23] Furthermore, *Lactobacillus* strains isolated from blood samples could not be distinguished from a probiotic strain ingested by clinical patients.[24] Therefore, the safety of probiotic strains must be investigated in detail. One aspect of this safety assessment is a toxicology evaluation. Toxicological studies are performed in many parts of the world to assess the safety of foods. To assess the safety of probiotic strains, even for probiotic species with a long history of safe consumption, detailed animal studies are warranted to confirm the lack of potential toxicity for human consumption.

Bifidobacteria primarily inhabit the large intestine from the lower part of the small intestine.[1] Ahmed et al. reported that the number of bifidobacteria in the adult intestinal mucosa was larger in the large intestine than in the terminal ileum quantitative polymerase chain reaction (PCR) analysis.[25] Generally, several Bifidobacterial species inhabit the human infant intestine, such as *B. longum*, *B. breve*, *B. infantis*, *B. bifidum*, and they are dominant bacteria of the intestinal microbiota.[26] *B. breve* strain M-16V has acquired GRAS notification from the FDA in the United States,[11–13] and it has also been listed with a QPS status since 2007.[27] Therefore, *B. breve* is generally considered safe for food use. In the present report, both single-dose and 90-day repeated-dose oral toxicity studies were conducted, and detailed observations and analyses provide an additional evidence that *B. breve* may be considered safe; yet additional research for this particular strain is warranted.

In the single-dose oral toxicity study, mortality and treatment-related changes in clinical signs were not observed. Therefore, the minimal oral lethal dose (LD_{Lo}) of *B. breve* MCC1274 was determined to be greater than 6000 mg/kg BW (corresponding to 8.4×10^{11} CFU/kg BW). Because acute toxicity studies alone are insufficient for evaluating the safety of the subject of interest, a subacute toxicity or repeated-dose study should be carried out after obtaining initial toxicity information via an acute oral toxicity study.[28] In the 90-day oral repeated-dose toxicity study, female rats administered 1000 mg/kg MCC1274

Table 5. Organ weights in male and female rats treated orally with *Bifidobacterium breve* MCC1274 for 90 days.

		Sex	Male		Female	
		Dose (mg/kg)	0	1000	0	1000
		Dose (CFU/kg)	0	1.3×10^{11}	0	1.3×10^{11}
Organs		Number of animals	10	10	10	10
Body weight at necropsy		g	564 ± 63	539 ± 60	295 ± 27	287 ± 22
Brain	Absolute	g	2.18 ± 0.07	2.17 ± 0.08	1.97 ± 0.06	1.97 ± 0.07
	Relative	g/100 g	0.39 ± 0.05	0.41 ± 0.04	0.67 ± 0.06	0.69 ± 0.05
Pituitary	Absolute	mg	13.1 ± 10.7	14.0 ± 1.6	16.1 ± 1.6	16.0 ± 2.8
	Relative	mg/100 g	2.3 ± 0.2	2.6 ± 0.4	5.5 ± 0.7	5.5 ± 0.6
Thyroid	Absolute	mg	22.3 ± 2.1	22.7 ± 2.9	17.2 ± 2.9	15.7 ± 1.9
(R+L)	Relative	mg/100 g	4.0 ± 0.6	4.2 ± 0.6	5.9 ± 1.1	5.5 ± 0.8
Salivary gland	Absolute	mg	714 ± 72	685 ± 64	444 ± 62	447 ± 37
(R+L)	Relative	mg/100 g	127 ± 12	128 ± 13	151 ± 14	157 ± 21
Thymus	Absolute	mg	317 ± 68	293 ± 125	293 ± 58	249 ± 60
	Relative	mg/100 g	56 ± 11	53 ± 17	101 ± 24	87 ± 21
Heart	Absolute	g	1.56 ± 0.13	1.57 ± 0.20	0.93 ± 0.06	0.94 ± 0.09
	Relative	g/100 g	0.28 ± 0.02	0.29 ± 0.02	0.31 ± 0.02	0.33 ± 0.02
Lung	Absolute	g	1.52 ± 0.13	1.51 ± 0.12	1.13 ± 0.06	1.14 ± 0.15
	Relative	g/100 g	0.27 ± 0.02	0.28 ± 0.02	0.39 ± 0.03	0.40 ± 0.04
Liver	Absolute	g	14.18 ± 2.20	14.30 ± 2.36	7.19 ± 0.52	7.22 ± 1.04
	Relative	g/100 g	2.51 ± 0.18	2.64 ± 0.21	2.45 ± 0.08	2.50 ± 0.20
Spleen	Absolute	g	0.84 ± 0.12	0.85 ± 0.15	0.50 ± 0.07	0.52 ± 0.09
	Relative	g/100 g	0.15 ± 0.01	0.16 ± 0.02	0.17 ± 0.02	0.18 ± 0.03
Kidney	Absolute	g	3.13 ± 0.25	3.19 ± 0.37	1.89 ± 0.12	1.90 ± 0.16
(R+L)	Relative	g/100 g	0.56 ± 0.05	0.59 ± 0.04	0.65 ± 0.04	0.66 ± 0.02
Adrenal	Absolute	mg	63 ± 12	60 ± 12	64 ± 9	68 ± 15
(R+L)	Relative	mg/100 g	11 ± 2	11 ± 2	22 ± 3	24 ± 4
Testis	Absolute	g	3.43 ± 0.27	3.50 ± 0.23	—	—
(R+L)	Relative	g/100 g	0.62 ± 0.10	0.65 ± 0.07	—	—
Prostate	Absolute	g	1.28 ± 0.22	1.33 ± 0.22	—	—
	Relative	g/100 g	0.23 ± 0.06	0.25 ± 0.03	—	—
Seminal vesicle	Absolute	g	1.33 ± 0.26	1.35 ± 0.16	—	—
	Relative	g/100 g	0.24 ± 0.05	0.25 ± 0.03	—	—
Ovary	Absolute	mg	—	—	82.1 ± 12.8	75.1 ± 12.8
(R+L)	Relative	mg/100 g	—	—	28.1 ± 5.7	26.2 ± 4.7
Uterus	Absolute	mg	—	—	523 ± 100	580 ± 66
	Relative	mg/100 g	—	—	180 ± 47	203 ± 28

CFU: colony-forming unit.
Values are the means ± SD.

Table 6. Necropsy findings in male and female rats treated orally with *Bifidobacterium Breve* MCC1274 for 90 days.

	Sex	Male		Female	
	Dose (mg/kg)	0	1000	0	1000
	Dose (CFU/kg)	0	1.3×10^{11}	0	1.3×10^{11}
Organs/findings	Number of animals	10	10	10	10
Kidney					
Focus, white		0	1	0	0
Liver					
Focus, raised		0	0	1	0
Focus, dark red		0	0	0	1
Hepatodiaphragmatic nodule		0	0	1	0
Stomach					
Focus, white, glandular stomach		0	1	0	0
Focus, dark red, glandular stomach		0	1	0	0

CFU: colony-forming unit.
Values are the number of animals with the indicated findings.

Table 7. Histopathological findings in male and female rats treated orally with *Bifidobacterium breve* MCC1274 for 90 days.

	Sex	Male		Female	
	Dose (mg/kg)	0	1000	0	1000
	Dose (CFU/kg)	0	1.3×10^{11}	0	1.3×10^{11}
Organs/findings	Number of animals	10	10	10	10
Cerebrum					
Cell infiltration, perivascular	Minimal	0	1	0	0
Epididymis					
Cell infiltration, interstitial	Minimal	1	3	—	—
Harderian gland					
Porphyrin secretion, increased	Minimal	1	1	0	0
Cell infiltration, interstitial	Minimal	1	1	1	1
Heart					
Myocarditis, focal	Minimal	1	1	0	0
Intestine, cecum					
Cell infiltration, mucosal	Minimal	1	0	0	0
Kidney					
Dilatation, tubular, cystic	Minimal	0	1	0	1
Regeneration, tubular	Minimal	3	4	2	0
Urinary cast, hyaline	Minimal	2	2	0	0
Mineralization	Minimal	4	0	4	6
Cell infiltration, interstitial	Minimal	2	1	2	0
Liver					
Microgranuloma	Minimal	7	8	5	4
Altered cell focus, eosinophilic	Minimal	1	0	0	0
Hepatodiaphragmatic nodule	Minimal	0	0	1	0
Hemorrhage, focal	Mild	0	0	0	1
Lung (bronchus)					
Mineralization, arterial wall	Minimal	2	1	2	1
Accumulation, alveolar macrophage	Minimal	1	3	0	0
Pneumonia, focal	Mild	0	0	0	1
Nasal cavity					
Rhinitis	Minimal	0	0	0	1
Pancreas					
Atrophy, acinar, focal	Minimal	1	0	0	0
Cell infiltration, interstitial	Minimal	1	2	1	0
Pituitary					
Aberrant craniopharyngeal tissue	Mild	1	0	0	0
Cyst, anterior	Mild	0	1	0	0
Prostate					
Cell infiltration, interstitial	Minimal	6	6	—	—
Salivary gland, sublingual					
Atrophy, acinar	Minimal	0	1	0	0
Skeletal muscle, femoral					
Degeneration/necrosis, muscular, focal	Minimal	4	2	1	0
Stomach					
Erosion, glandular stomach	Minimal	0	1	0	0
Ectopic forestomach tissue, glandular	Minimal	0	1	0	0
Thyroid					
Remnant, ultimobranchial body	Minimal	0	1	1	2
Cell infiltration, interstitial	Minimal	0	0	1	2

CFU: colony-forming unit.
Values are the number of animals with the indicated findings.

corresponding to 1.3×10^{11} CFU/kg showed a minimal decrease in the RBC (−4% of the control mean). However, this dosage did not have toxicological significance since histopathological changes were not observed in the femur, sternal bone marrow, hematopoietic organ, spleen, or liver, where extramedullary hematopoiesis could occur in response to anemia. In addition, the differences in RBC and MCH were within the historical control ranges. The increased MCH was also judged to have no toxicological significance since it was considered to be associated with variations in the RBC. Thus, the no-observed-adverse-effect level (NOAEL) of *B. breve* MCC1274 was

1000 mg/kg BW/day (corresponding to 1.3×10^{11} CFU/kg BW/day). In order to translate results from rats to human, a 100-fold safety factor is commonly used. Hence, acceptable daily intake of *B. breve* MCC1274 would be 1.3×10^{9} CFU/kg BW/day.

Conclusion

In this study, the safety of *B. breve* MCC1274 was evaluated in single- and repeated-dose toxicity studies. An acute dose of 6000 mg/kg corresponding to 8.4×10^{11} CFU/kg showed no signs of toxicity in male or female rats (LD_{Lo} > 6000 mg/kg). Rats supplemented with 1000 mg/kg/day of *B. breve* MCC1274 corresponding to 1.3×10^{11} CFU/kg during a 90-day subchronic study did not show adverse effects compared with the control animals (NOAEL > 1000 mg/kg/day).

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ORCID iD

Satoshi Arai http://orcid.org/0000-0002-0700-6830

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