Bioscience of Microbiota, Food and Health Vol. 43 (1), 81–91, 2024

Full Paper

Safety evaluation of a heat-treated *Bifidobacterium bifidum* OLB6378 concentrate

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Received June 9, 2023; Accepted October 11, 2023; Published online in J-STAGE November 3, 2023

Several bacterial strains, including probiotic strains, have undergone evaluations for their safety and potential beneficial health effects. Some of these strains have been introduced into various markets, including that for infant products. However, certain probiotic strains have been linked to serious infections in infants, such as septicemia and meningitis. Given this, it is crucial to assess the safety of each probiotic strain, including those of *Bifidobacterium*, which is a common genus of probiotics. One such strain, *Bifidobacterium bifidum* OLB6378 (NITE BP-31), referred to as OLB6378 hereafter, has been selected for use in infants. To determine its genotoxicity and general toxicity potential, a heat-treated OLB6378 concentrate was subjected to various tests, including the bacterial reverse mutation test, *in vitro* chromosome aberration test, *in vivo* micronucleus test, and single- and 90-day oral gavage toxicity studies in rats. No significant differences were observed compared with negative controls in any of genotoxicity tests. The single-dose toxicity study employed dose levels of 560, 1,693, and 5,092 mg/kg, representing the total solid contents of culture concentrates containing OLB6378 (equivalent to 8.1×10^{11}, 2.4×10^{12}, and 7.4×10^{12} cells/kg of *Bifidobacterium*, respectively). In the 90-day toxicity study, dose levels of 280, 853, and 2,546 mg/kg/day were used (equivalent to 4.0×10^{11}, 1.2×10^{12}, and 3.7×10^{12} cells/kg/day, respectively). Importantly, the heat-treated OLB6378 concentrate did not induce any signs of toxicity in any of the conducted toxicity studies. In conclusion, the heat-treated OLB6378 concentrate exhibited no genotoxicity potential, and the no-observed-adverse-effect level in the 90-day toxicity study was determined to be 2,546 mg/kg/day (equivalent to 3.7×10^{12} cells/kg/day). This suggests that heat-treated OLB6378 can be safely utilized as a food source.

Key words: *Bifidobacterium*, genotoxicity, repeated-dose oral toxicity, rats

INTRODUCTION

Several bacterial strains have been evaluated with regard to their safety and potential beneficial health effects and have been introduced in some markets [1]. The beneficial effects arise from secretions (e.g., enzymes) or metabolic products (e.g., lactic acid and acetic acid) of the live bacteria. Additionally, their bacterial cell components, such as the cell wall, have beneficial effects regardless of whether the bacteria are alive or dead. The cell wall components of gram-positive bacteria, including bifidobacteria, play important roles in maintaining health and mucosal barrier function in the intestinal epithelium and in repairing the intestinal epithelium [2–4]. Therefore, the incorporation of bacterial cell components into the intestines is considered beneficial for the integrity of the intestinal epithelium, even if they are derived from dead bacterial cells.

Bifidobacteria are the dominant bacteria in the gut flora of breastfed infants [5]. Similar to lactobacilli, bifidobacteria are extensively used as beneficial microbes and are considered safe. However, some bifidobacterial strains can cause serious infections, such as septicemia and meningitis in infants [6–8]. Therefore, even for bifidobacteria, it is essential to evaluate the safety of each probiotic strain. *Bifidobacterium bifidum* OLB6378 (NITE BP-31), hereafter referred to as OLB6378, is a bifidobacterial strain derived from a subculture of the original parent isolate *B. bifidum* OLB6139 [9], which was obtained from

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(Supplementary materials: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2480/)

doi: 10.12938/bmfh.2023-044

a human infant fecal sample. OLB6378 has been demonstrated to enhance secretory immunoglobulin A by increasing intestinal polymeric immunoglobulin receptor expression [10], and it was selected as a candidate industrial food ingredient for infants. In the present study, the non-observed adverse-effect level of a heat-treated OLB6378 concentrate was evaluated based on repeated-dose toxicity tests in rats, in addition to its genotoxic potential. The findings of the present study could facilitate the safe use of bifidobacteria as proteobacteria in various food applications.

MATERIALS AND METHODS

Test substance

The test substance was a heat-treated OLB6378 concentrate that had a milky white liquid appearance and the solid content is 12.3 g ± 0.615 g/100 g of total solid content of medium components (hereafter "g" is referred to as the solid content unless otherwise mentioned); it was obtained from Meiji Co., Ltd., Tokyo, Japan (lot number 160324). The heat-treated OLB6378 used in all experiments had $1.8 \times 10^{11} \pm 7.2 \times 10^{10}$ cells/g and a density of 1.035 g/mL at 24.1°C. The manufacturing process for the heat-treated OLB6378 concentrate consisted of culturing, heat treatment, and concentration. To prepare the mother starter, the frozen original OLB6378 stock was thawed and transferred to a starter medium for activation. The original OLB6378 stocks were maintained by the Food Microbiology and Function Research Laboratories of Meiji Co., Ltd. Activated OLB6378 cells were sub-cultured using three scaled-up procedures to establish the mother starter. During all the subculture steps, the same starter medium, which contained hydrolyzed whey protein, lactose, casein, yeast extract, sodium hydroxide, and potassium carbonate, was used as a manufacturing medium and cultured at 37°C under anaerobic conditions. Prior to use, the manufacturing medium was sterilized and cooled for inoculation with the mother starter. To begin the culture process, the mother starter was combined with the manufacturing medium. During the culturing process, the medium was gently agitated, and the temperature was held at 36–38°C. After culturing, the culture was cooled to 10°C and analyzed using microbiological tests for quality control. The cultured OLB6378 was then heat-treated (80°C, 10 min) and concentrated by centrifugation. The stabilities of the test substances during the experiments were verified by Meiji Co., Ltd.

Animals

Sprague Dawley Crl:CD(SD) rats were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and quarantined/acclimated for at least eight days. Healthy animals were used in *in vivo* studies. The animals were housed in an animal room where the temperature was maintained at 20 to 26°C, the relative humidity ranged from 30% to 70%, and air ventilation occurred 10 to 15 times per hour, with a 12-hr light period each day (07:00 to 19:00). They were housed in groups of two or three in solid-floored plastic cages with bedding and appropriate environmental enrichment. They were provided with free access to a γ-irradiated pelleted basal diet (CR-LPF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. To ensure the homogeneity of group-mean body weight values, the animals were assigned to each group using a computer. Animal studies were conducted from May 9, 2016, to February 16, 2017, with the approval of the Institutional Animal Care and Use Committee of BoZo Research Center Inc. This center has received full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International, and the studies were conducted in compliance with animal welfare regulations and guidelines in Japan, including the Act on Welfare and Management of Animals (Act No. 105 of October 1, 1973), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification No. 88 of the Ministry of the Environment), and Guidelines for Proper Conduct of Animal Experiments (June 1, 2006, Science Council of Japan).

Bacterial reverse mutation test

The testing was conducted at CMIC Pharma Science Co., Ltd., and was in compliance with the GLP regulations (Ordinance No. 21 of MHW, Japan), OECD Principles of Good Laboratory Practice (as revised in 1997), and OECD test guideline 471.

Assays were conducted using S. typhimurium tester strains (TA98, TA100, TA1535, and TA1537) and E. coli WP2 uvrA. The four S. typhimurium strains were obtained from the Japan Bioassay Research Center (Kanagawa, Japan), and the E. coli was obtained from the National Institute of Genetics (Shizuoka, Japan). Water was used as a negative control, and AF2, AZI, 9AA, and 2AA were used as positive controls. Positive controls were used for quality checks in the present study. AF2, AZI, and 2AA were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and 9AA was obtained from MP Biomedicals, LLC (Irvine, CA, USA). Rat liver homogenate 9,000 × g supernatant fraction (S9) mix, obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan), was used for metabolic activation.

A dose-finding test was performed in which six doses, 5,000, 1,250, 313, 78.1, 19.5, and 4.88 µg/plate, were tested and compared with the negative control. All doses, negative controls, and positive controls were plated in duplicate. The test substance did not inhibit the growth of any bacterial strain at any dose, with or without metabolic activation. Additionally, it did not induce a dose-dependent increase in the number of revertant colonies, with or without metabolic activation. It was precipitated with or without metabolic activation at 1,250 and 5,000 µg/plate, but this did not affect the colony count. Based on the results of the dose-finding test, five of the doses, 5,000, 2,500, 1,250, 625, and 313 µg/plate, were tested in the main and confirmatory tests and compared with the negative control. In the main and confirmatory tests, all doses, negative controls, and positive controls were plated in triplicate. The results were considered positive when biologically meaningful increases in the number of revertant colonies, i.e., reproducible, statistically significant, dose-dependent increases, were observed due to the mutagenicity of the test substance. The numbers of revertant colonies were compared with the corresponding negative controls, both in the presence and absence of metabolic activation, and statistically significant differences were analyzed using Dunnett's test ($p<0.05$). Dose dependence was analyzed using the linear regression method ($p<0.05$) when a statistically significant difference was detected using Dunnett's test. Means and standard deviations were calculated using MS Excel 2010 (Microsoft Corp., Redmond, WA, USA), and statistical analyses were performed in SAS for Windows v9.3 (SAS Institute Inc., Cary, NC, USA).

In vitro *mammalian chromosomal aberration test*

The testing was conducted at CMIC Pharma Science Co., Ltd., and was in compliance with the GLP regulations (Ordinance No. 21 of MHW, Japan), OECD Principles of Good Laboratory Practice (as revised in 1997), and OECD test guideline 473. Cell shape was classified according to the *Atlas of Chromosomal Aberrations Induced by Chemicals* [11]. If the results were positive, the representative data on structural or numerical abnormalities were recorded.

The assay was performed using a Chinese hamster lung fibroblast (CHL/IU) cell line obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Water was used as the negative control. Mitomycin C (MMC; Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) and cyclophosphamide (CP; Sigma-Aldrich Inc., St. Louis, MO, USA) were used as positive controls.

The doses were set based on the data obtained from cell growth inhibition tests. A culture medium was Eagle's MEM liquid medium (Wako Pure Chemical Industries, Ltd.) contained 1% of penicillin (5,000 U/mL) and streptomycin (5,000 µg/mL) solution (formerly Life Technologies, current Thermo Fisher Scientific, Waltham, MA, USA) and 10% of inactivated fetal bovine serum (formerly Life Technologies, current Thermo Fisher Scientific). Each test consisted of short-term treatment with or without metabolic activation and continuous treatment for 24 hr. A single plate was used for the cell growth inhibition test, and the corresponding negative control treatments were included. In the chromosome aberration test, three plates were used: two plates were used for the preparation of specimens, and the rest were used for the evaluation of cell growth inhibition in the satellite group. Negative and positive controls were included for all treatment conditions. The cell growth rate was calculated as the relative population doubling (RPD) rate from the numbers of cells at the beginning of the test substance treatment and at the end of the culture after the test substance treatment.

The highest dose used in the cell growth inhibition tests was 2,000 µg/mL, and 10 lower doses were prepared by diluting the sample from half to half the concentration. Negative control and test substance solutions were added to the culture medium as 10% of the volume. A 5-mL cell suspension of CHL/IL cells at a density of 4×10^3 cells/mL was seeded into a plate for the cell growth inhibition test and cultured for three days. The presence or absence of precipitation in the test substance was checked at the time of the addition of the test substance suspension and at the end of the treatment. In short-term treatments, the cells were treated with 0.3 mL of the test substance suspension, with or without 0.5 mL of S9 mix, after removing the appropriate volume of culture medium to adjust the total volume to 3 mL. The cells were cultured with the test substance suspension for 6 hr and washed with Dulbecco's phosphate-buffered saline (formerly Life Technologies, current Thermo Fisher Scientific), and then cultured in 5 mL fresh culture medium for an additional 18 hr.

In the 24-hr continuous treatment, the cells were treated for continuously 24 hr with 0.5 mL of the test substance suspension after removing the appropriate volume of culture medium to adjust the total volume to 5 mL. The cells were collected with a 0.1% ethylenediamine tetraacetic acid (EDTA)-supplemented 1.25% trypsin solution, and the RPD of each dose was calculated based on the number of cells. Based on the results of cell growth inhibition tests, a 50% cell growth inhibition dose was determined. The test substance was precipitated under all treatment conditions at the dose of 2,000 µg/mL; however, it did not decrease the cell growth rate by ≥50% at any dose under any treatment condition. Based on these results, three doses, 2,000, 1,000, and 500 µg/mL, were set under all treatment conditions. Negative control and test substance suspensions were added to the culture medium at 10% of the culture medium volume (0.5 mL), and the positive control solution was added at 1% of the culture medium volume (0.05 mL). The cells were treated using procedures similar to those used for the cell growth inhibition test.

The cells arrested in metaphase when 0.2 µg/mL colcemid was added two hours before the termination of culture. They were collected using a 0.1% EDTA-supplemented 1.25% trypsin solution and centrifuged. They were then resuspended in 0.075 mol/L potassium chloride and fixed in a 3:1 methanol-acetic acid solution. The cells were then dropped onto a slide, air-dried, and stained for 20 min with a 2% Giemsa solution. Duplicate slides were prepared for each plate and scored blindly by microscopic examination. For structural chromosomal aberration analysis, 300 well-spread metaphase cells (chromosome numbers ranging from 23 to 27) per dose, yielding 150 metaphase cells per plate, were scored at 1,000× magnification. For numerical aberrations, 400 well-spread metaphase cells per dose, yielding 200 metaphase cells per plate, were scored at 200× magnification. Structural aberrations were classified as chromatid breaks, chromatid exchange, chromosome breaks, chromosome exchange, fragmentation, and multiple aberrations (classified as polyploidy and endoreduplication). The frequencies of cells with structural aberrations, excluding the gap, were used to evaluate chromosomal aberrations. Differences between the negative control and test substance treatments and the positive control at each dose were analyzed using the chi-square test with Yates correction ($p<0.05$, one-tailed). When a statistically significant increase was observed in the test substance treatments, dose dependence was examined using the Cochran-Armitage trend test ($p<0.05$, one-tailed).

In vivo *bone marrow micronucleus test*

The testing was conducted at BoZo Research Center Inc. and was in compliance with GLP regulations (Ordinance No. 21 of MHW, Japan) and OECD test guideline No. 474.

Male rats were obtained at seven weeks old and used at eight weeks old. Three dose levels, 636.5, 1,273, and 2,546 mg/kg/day, were set, and the test substance was administered by oral gavage to groups of five males at doses of 5, 10, and 20 mL/kg/day for two consecutive days at intervals of approximately 24 hr. The high-dose level set in the present study was the maximum feasible dose calculated based on the dose volume (20 mL/kg/day for repeated doses) and concentration of the test substance. Additionally, the negative control group received 20 mL/kg of water-for-injection in a manner similar to that of the test substance group, and the positive control group received a single intraperitoneal dose of MMC at 2 mg/kg.

During the administration period, mortality and clinical signs were observed daily, and body weights were recorded once a day for all animals. The animals were euthanized by exsanguination via the abdominal aorta under isoflurane anesthesia approximately 24 hr after the second administration of the test substance. Bone marrow smears were prepared from the right femurs of all

doi: 10.12938/bmfh.2023-044

animals, stained with acridine orange, and examined under a fluorescence microscope at 600× magnification. The frequencies of immature erythrocytes (polychromatic erythrocytes [PCEs]) per 500 erythrocytes (PCEs plus normochromatic erythrocytes [NCEs]) and micronucleated immature erythrocytes (MNPCEs) per 4,000 PCEs were counted. The incidence rates (%) were also calculated.

The frequency of MNPCEs was compared between the concurrent negative control group and each test substance group and between the negative control group and the positive control group, using Fisher's exact test (significance level=0.05; one-tailed). The dose dependence of MNPCEs in treatments with the test substance was assessed using the Cochran-Armitage trend test (significance levels=0.01 and 0.05; one-tailed). Additionally, homogeneity of variance in the incidence of PCEs was analyzed using Bartlett's test (significance level=0.01). Homogenous data were analyzed using Dunnett's test in comparisons between the negative control group and each test substance group (significance levels=0.01 and 0.05; two-tailed), and heterogeneous data were analyzed using Steel's test (significance levels=0.01 and 0.05; two-tailed).

Single-dose oral toxicity study

The study was conducted at BoZo Research Center Inc. and was in compliance with the GLP regulations (Ordinance No. 21 of MHW, Japan) and toxicity study guidelines (Notification No. 88 of the PMSB, MHW, Japan).

Male and female rats were obtained at five weeks of age and used at six weeks of age.

Three dose levels, 560, 1,693, and 5,092 mg/kg, were tested, and the test substance was administered once by oral gavage to groups of five animals of each sex at doses of 4.4, 13.3, and 40.0 mL/kg, respectively. In addition, as a control group, five animals of each sex were treated with 40.0 mL/kg water. The animals were deprived of food for approximately 16 hr before dosing. The high-dose level used in the present study was the maximum feasible dose, which was calculated based on the dose volume (40.0 mL/kg) and the concentration of the test substance.

An approximate lethal dose was determined based on cumulative mortality during the 14-day observation period. Body weight and overall body weight gain during the observation period were also analyzed. First, the homogeneity of variance was analyzed using Bartlett's test (significance level=0.01). Homogeneous data were analyzed using Dunnett's test, and heterogeneous data were analyzed using Steel's test (levels of significance: 0.01 and 0.05; two-tailed). All analyses were performed using the statistical package SAS v9.1.3 (SAS Institute Inc., Cary, NC, USA).

90-day oral toxicity study

The study was conducted at BoZo Research Center Inc. and was in compliance with GLP regulations (Ordinance No. 21 of MHW, Japan) and OECD test guideline No.408.

Male and female rats were obtained at five weeks of age and used at six weeks of age.

Three dose levels, 280, 853, and 2,546 mg/kg/day, were applied. The test substance was administered once daily by oral gavage to groups of 10 animals of each sex at doses of 2.2, 6.7, and 20.0 mL/kg/day, respectively, for 91 days. The high-dose level set in the study was the maximum feasible dose calculated based on the dose volume (20 mL/kg/day for repeated doses) and concentration of the test substance. Water was administered to the control group at a dose of 20 mL/kg/day.

During the administration period, all animals were observed for mortality and clinical signs at least twice a day, and their body weights and food consumption were recorded at least once a week.

Detailed clinical observations were performed on all animals using a scoring system once before the beginning of administration and once a week during the administration period.

Functional tests (grip strength, locomotor activity, and sensory reactivity assessments) were performed on all animals 12 weeks after administration.

Ophthalmoscopy was performed before administration of the test substance in all animals, including the test animal candidates, to exclude animals with ocular lesions, which could have affected the study evaluation. It was also performed on all animals in the control and high-dose groups at week 13 of administration.

Urinalysis was performed 13 weeks after administration of the test substance in all animals. Each animal was placed in a separate cage, and urine was collected over a 4-hr period under fasting conditions with free access to water. Urine samples were collected over an additional 20 hr under non-fasting conditions. The following parameters were examined in the 4-hr urine samples: urinary sediment and urine color, pH, proteins, ketones, glucose (GLU), occult blood, bilirubin, and urobilinogen. The following parameters were determined in the 20-hr urine samples: urine volume, osmotic pressure, and sodium (Na), potassium (K), and chloride (Cl) concentrations. Urine volume was calculated by the total volume of 4-hr urine samples and 20-hr urine samples. Concentrations per 24-hr of Na, K and Cl were calculated by the concentration of 20-hr urine samples and the calculated urine volume.

Terminal blood samples were collected under anesthesia during necropsy after overnight fasting (approximately 16–21 hr) for hematological and blood chemistry analyses. The hematological parameters assessed in blood samples treated with EDTA-2 K included the red blood cell (RBC) count; hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) levels; and platelet (PLT), white blood cell (WBC), and differential leukocyte (lymphocyte, LYMP; neutrophil, NEUT; eosinophil, EOS; basophil, BASO; monocyte, MONO, and large unstained cell, LUC) counts. Blood coagulation parameters, including prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen (FIB), were assessed in plasma obtained by centrifuging blood samples treated with sodium citrate. Blood chemistry parameters, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), total cholesterol (T-CHO), triglyceride (TG), phospholipid (PL), total bilirubin (T-Bil), GLU, blood urea nitrogen (BUN), urea, creatinine (CRNN), Na, K, Cl, calcium (Ca), inorganic phosphorus (P), total protein (TP), albumin (ALB), and albumin/globulin ratio (A/G), were determined in the plasma obtained by centrifuging blood samples treated with heparin sodium.

Subsequently, all animals were euthanized by exsanguination via the abdominal aorta and necropsied. The following organs were weighed (absolute weight), and organ weight per 100 g

doi: 10.12938/bmfh.2023-044

body weight (relative weight) was calculated: brain, pituitary gland, thyroid gland, adrenal gland, thymus, spleen, heart, lung, salivary gland, liver, kidney, testis, epididymis, prostate, seminal vesicle, ovary, and uterus. For paired organs, evaluations were conducted based on the total value of the right and left organs. Thereafter, the following organs and tissues were fixed with phosphate-buffered 10% formalin (however, the eyeball with optic nerve was fixed in 3% glutaraldehyde and 2.5% formalin, and the testis and epididymis were fixed in Bouin's solution), embedded in paraffin, sectioned, and stained with hematoxylin/eosin: the cerebrum, cerebellum, medulla oblongata, pons, spinal cord (cervical, thoracic, and lumbar), sciatic nerve, eyeball, optic nerve, Harderian gland, pituitary gland, thyroid gland, parathyroid gland, adrenal gland, thymus, spleen, submandibular lymph node, mesenteric lymph node, heart, thoracic aorta, trachea, lung (including bronchus), tongue, esophagus, stomach, duodenum, jejunum, ileum (including Peyer's patch), cecum, colon, rectum, submandibular gland, sublingual gland, liver, pancreas, kidney, urinary bladder, testis, epididymis, prostate, seminal vesicle, ovary, uterus, vagina, oviduct, mammary gland (inguinal region), sternum and femur (including bone marrow), femoral skeletal muscle, and skin (inguinal region). All the organs and tissues in the control and high-dose groups were examined under a microscope.

For numerical data, such as body weight, food consumption, organ weight data and quantitative data from open-field observations, functional tests, urinalysis, hematology, and blood chemistry, pairwise comparisons were performed between the control group and each test substance group. The homogeneity of variance was analyzed using Bartlett's test (significance level=0.01). Homogeneous data were analyzed using Dunnett's test, and heterogeneous data were analyzed using Steel's test (significance levels=0.01 and 0.05; two-tailed). All analyses were performed using the statistical package SAS v9.1.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Bacterial reverse mutation test

The results of the main tests are presented in Table 1. The test substance did not inhibit the growth of any bacterial strain at any dose. Similarly, no dose-dependent increases in the number of revertant colonies were observed. Reproducibility was confirmed based on the results of the main and confirmatory tests. The results indicated that the mutagenicity of the test substance did not result in any biologically meaningful increases in the number of revertant colonies for any bacterial strain at any dose. The test substance was precipitated at concentrations ≥1,250 μg/plate with or without metabolic activation. The mean values of the revertant colonies in the positive controls were at least twice those of the negative controls in all bacterial strains with or without metabolic activation. The mean values for the colony counts of the negative and positive controls were within the range of the variation values calculated from historical control data, indicating that the test was properly performed.

In vitro mammalian chromosomal aberration test

The results are summarized in Table 2. The test substance was precipitated at a concentration of 2,000 μg/mL under all treatment conditions. It did not decrease the cell growth rate by 50% or more under any treatment conditions at any dose. The frequency of cells with structural aberrations and polyploidy in all the test substance treatments was within the variation range of historical control data in the negative control. Statistically significant increases in the frequency of cells with structural aberrations and polyploidy were not confirmed with any test substance treatment. Conversely, the frequency of cells with structural aberrations and polyploidy in each concurrent negative control was within the variation range of historical negative control data. The frequency of cells with structural aberrations in the positive controls was within the variation range of background data of the positive controls, and a statistically significant increase compared with the negative control was confirmed. The results demonstrate that the test was performed appropriately.

In vivo *bone marrow micronucleus test*

No deaths occurred in any test group, and no test substance-related changes were noted based on clinical signs or body weight. The incidences of MNPCEs per 4,000 PCEs at 636.5, 1,273 and 2,546 mg/kg were 0.16% ± 0.04% (mean ± SD), 0.14% ± 0.05%, and 0.15% ± 0.02%, respectively, and the values did not show a significant increase compared with the negative control group value, 0.19% ± 0.05%. In addition, no dose-dependent increases were observed after treatment with the test substances (Table 3). The incidence of PCEs per 500 erythrocytes was not significantly different between the test substance groups. Conversely, the incidence of MNPCEs in the positive control group was significantly higher (3.21% ± 0.56%) than in the corresponding negative control group, and the values in the negative control group and positive control groups were within the mean ± 1.96 SD of their respective historical control data, which confirmed that the test was performed appropriately.

Single-dose oral toxicity study

No deaths occurred in either sex at doses up to 5,092 mg/kg; therefore, the minimum lethal dose of the heat-treated OLB6378 concentrate was estimated to be greater than 5,092 mg/kg. No test substance-related changes were observed based on clinical signs, body weight, or necropsy.

90-day oral toxicity study

One male rat in the high-dose group (2,546 mg/kg/day; No. 4007) died 12 weeks after administration. The animal died from convulsions when restrained by a technician. Gross signs and histopathology revealed no lesions in any tissue or organ (Table 4).

One male in the middle-dose group (853 mg/kg/day; No. 3004) was euthanized as moribund in week 5 of administration. The animal exhibited body weight loss and decreased spontaneous movement from week 3 of test substance administration, in addition to bradypnea, emaciation, and unkempt fur from week 5 of test substance administration. Hematological analyses revealed marked decreases in PLT and WBC counts accompanied by a decrease in all leukocyte fractions. Blood chemistry analyses revealed marked increases in AST, ALT, LDH, ALP, γ-GTP, triglycerides, T-Bil, glucose, BUN, and urea, as well as marked decreases in ALB and the A/G ratio (Supplementary Table 1). Necropsy revealed unkempt fur; undernourishment; a small prostate, spleen, and thymus; small seminal vesicles and testes; dark red foci in the glandular stomach; and dilatation

doi: 10.12938/bmfh.2023-044

of the urinary bladder. The major histopathological findings (Supplementary Table 2) for this animal included atrophic changes in various organs and tissues, including the spleen, submandibular and mesenteric lymph nodes, ileal Peyer's patches, cecum, colon, rectum, pancreas, submandibular gland, sublingual gland, Harderian glands, femoral muscle, skin, mammary glands, prostate, and seminal vesicles. Erosions/ulcers were observed in the glandular stomach, corresponding to macroscopic dark red foci. Such changes in body weight, clinical signs, hematology, blood chemistry, and macroscopic and microscopic examinations revealed the poor physical condition of the animal.

Table 1. Bacterial reverse mutation test for the heat-treated *Bifidobacterium bifidum* OLB6378 concentrate in the main tests

With (+) or without (−) S9 mix	Dose (μg/plate)	Number of revertants per plate (number of revertants per dose, mean ± SD)									
		Base-pair substitution type						Frameshift mutation type			
		TA100		TA1535		WP2*uvr*A		TA98		TA1537	
S9 mix (−)	NC [a)]	136		16		29		16		10	
		148		11		14		12		10	
		134	(139 ± 7.6)	13	(13 ± 2.5)	36	(26 ± 11.2)	19	(16 ± 3.5)	10	(10 ± 0.0)
	313	159		13		24		18		13	
		116		10		21		15		14	
		140	(138 ± 21.5)	13	(12 ± 1.7)	20	(22 ± 2.1)	16	(16 ± 1.5)	9	(12 ± 2.6)
	625	111		11		21		18		12	
		130		8		20		26		9	
		129	(123 ± 10.7)	8	(9 ± 1.7)	21	(21 ± 0.6)	15	(16 ± 1.5)	12	(11 ± 1.7)
	1,250	148#		17#		24#		18#		11#	
		119#		8#		17#		16#		13#	
		100#	(122 ± 24.2)	10#	(12 ± 4.7)	20#	(20 ± 3.5)	15#	(16 ± 1.5)	11#	(12 ± 1.2)
	2,500	122#		6#		23#		14#		6#	
		130#		13#		30#		17#		5#	
		117#	(123 ± 6.6)	11#	(10 ± 3.6)	22#	(25 ± 4.4)	22#	(18 ± 4.0)	9#	(7 ± 2.1)
	5,000	127#		15#		19#		15#		8#	
		132#		12#		23#		16#		10#	
		111#	(123 ± 11.0)	14#	(14 ± 1.5)	18#	(20 ± 2.6)	16#	(16 ± 0.6)	13#	(10 ± 2.5)
S9 mix (+)	NC [a)]	123		9		26		32		11	
		129		13		20		28		14	
		118	(123 ± 5.5)	13	(12 ± 2.3)	27	(24 ± 3.8)	25	(28 ± 3.5)	15	(13 ± 2.1)
	313	151		15		32		21		17	
		117		11		31		19		11	
		127	(132 ± 17.5)	11	(12 ± 2.3)	27	(30 ± 2.6)	26	(22 ± 3.6)	12	(13 ± 3.2)
	625	116		12		34		27		9	
		133		17		25		16		16	
		136	(128 ± 10.8)	10	(13 ± 3.6)	21	(27 ± 6.7)	21	(21 ± 5.5)	11	(11 ± 3.6)
	1,250	128#		8#		21#		36#		14#	
		128#		4#		19#		27#		17#	
		122#	(126 ± 3.5)	8#	(7 ± 2.3)	29#	(23 ± 5.3)	34#	(32 ± 4.7)	14#	(15 ± 1.7)
	2,500	127#		11#		14#		24#		14#	
		106#		7#		24#		28#		14#	
		140#	(124 ± 17.2)	13#	(10 ± 3.1)	22#	(20 ± 5.3)	18#	(23 ± 5.0)	12#	(13 ± 1.2)
	5,000	128#		7#		23#		31#		11#	
		114#		11#		23#		24#		12#	
		132#	(125 ± 9.5)	13#	(10 ± 3.1)	22#	(23 ± 0.6)	22#	(26 ± 4.7)	11#	(11 ± 0.6)
Positive control S9 mix (−)	Name & Dose	AF-2 0.01 μg/plate		AZI 0.5 μg/plate		AF-2 0.01 μg/plate		AF-2 0.1 μg/plate		9AA 80.0 μg/plate	
	Number of colonies/ plate	432		519		129		229		214	
		569		524		105		260		237	
		555	(519 ± 75.4)	527	(523 ± 4.0)	110	(115 ± 12.7)	220	(236 ± 21.0)	177	(209 ± 30.3)
Positive control S9 mix (+)	Name & Dose	2AA 1.0 μg/plate		2AA 2.0 μg/plate		2AA 10.0 μg/plate		2AA 0.5 μg/plate		2AA 2.0 μg/plate	
	Number of colonies/ plate	1,417		458		884		403		205	
		1,282		417		858		424		127	
		1,156	(1,285 ± 130.5)	424	(433 ± 21.9)	834	(859 ± 25.0)	470	(432 ± 34.3)	166	(166 ± 39.0)

a): Negative control (water-for-injection).
#: Precipitation.
AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide; AZI: Sodium azide; 9AA: 9-Aminoacridine; 2AA: 2-Aminoanthracene; SD: standard deviation.

doi: 10.12938/bmfh.2023-044

In the survivors, convulsions were observed transiently in one male in the low-dose group (280 mg/kg/day) when the animal was restrained for dosing in week 11 of test substance administration.

Detailed clinical observations revealed no test substance-related changes in either sex. The following changes in the open field observations were considered incidental because the changes were temporal without any relationship with the dose level or the period of administration: an increase in the number of rearing events in females at 853 mg/kg/day in week 1 of administration and decrease in the number of rearing events in males at 2,546 mg/kg/day in week 2 of administration.

Table 2. Chromosome aberration test for the heat-treated *Bifidobacterium bifidum* OLB6378 concentrate in short-term treatment with/without metabolic activation and in continuous treatment for 24 hr

Treatment time	Dose (μg/mL)	RPD (%)		Number of cells with structural aberrations										Number of cells with numerical aberrations				
				Total (%)														
				observed	gap	ctb	csb	cte	cse	others	total	−gap	+gap	observed	pol	end	total	Total (%)
6 hr S9 mix (−)	NC[a)]	100		150	0	1	0	1	0	0	2			200	0	0	0	
				150	1	1	0	0	0	0	1			200	1	0	1	
			Total	300	1	2	0	1	0	0	3	(1.0)	(1.3)	400	1	0	1	(0.3)
	500	101.0		150	0	1	1	0	0	0	2			200	1	0	1	
				150	1	0	1	0	0	0	1			200	0	0	0	
			Total	300	1	1	2	0	0	0	3	(1.0)	(1.3)	400	1	0	1	(0.3)
	1,000	107.9		150	0	2	0	0	0	0	2			200	1	0	1	
				150	0	1	0	0	0	0	1			200	2	0	2	
			Total	300	0	3	0	0	0	0	3	(1.0)	(1.0)	400	3	0	3	(0.8)
	2,000#	86.5		150	0	2	0	0	0	0	2			200	0	0	0	
				150	1	0	1	0	0	0	1			200	0	0	0	
			Total	300	1	2	1	0	0	0	3	(1.0)	(1.3)	400	0	0	0	(0.0)
	MMC 0.05	−		150	0	5	1	13	0	0	18			200	0	0	0	
				150	0	6	1	9	0	0	16			200	0	0	0	
			Total	300	0	11	2	22	0	0	34	$(11.3)	(11.3)	400	0	0	0	(0.0)
6 hr S9 mix (+)	NC[a)]	100		150	1	2	0	0	0	0	2			200	0	0	0	
				150	1	2	0	0	0	0	2			200	2	0	2	
			Total	300	2	4	0	0	0	0	4	(1.3)	(2.0)	400	2	0	2	(0.5)
	500	84.2		150	0	0	0	0	0	0	0			200	1	0	1	
				150	0	1	0	0	0	0	1			200	0	0	0	
			Total	300	0	1	0	0	0	0	1	(0.3)	(0.3)	400	1	0	1	(0.3)
	1,000	87.9		150	1	2	0	0	0	0	2			200	0	0	0	
				150	0	0	0	0	0	0	0			200	0	0	0	
			Total	300	1	2	0	0	0	0	2	(0.7)	(1.0)	400	0	0	0	(0.0)
6 hr S9 mix (+)	2,000#	68.2		150	1	1	0	0	0	0	1			200	2	0	2	
				150	1	1	1	0	0	0	1			200	0	0	0	
			Total	300	2	2	1	0	0	0	2	(0.7)	(1.3)	400	2	0	2	(0.5)
	CP 5.0	−		150	1	10	1	29	0	0	36			200	0	0	0	
				150	1	9	1	38	0	0	43			200	0	0	0	
			Total	300	2	19	2	67	0	0	79	$(26.3)	(26.7)	400	0	0	0	(0.0)
24 hr	NC[a)]	100		150	1	0	0	0	0	0	0			200	0	0	0	
				150	0	1	0	0	0	0	1			200	0	0	0	
			Total	300	1	1	0	0	0	0	1	(0.3)	(0.7)	400	0	0	0	(0.0)
	500	93.1		150	0	2	0	0	0	0	2			200	1	0	1	
				150	0	1	0	1	0	0	2			200	1	0	1	
			Total	300	0	3	0	1	0	0	4	(1.3)	(1.3)	400	2	0	2	(0.5)
	1,000	93.9		150	0	0	0	0	0	0	0			200	3	0	3	
				150	0	0	1	0	0	0	1			200	1	0	1	
			Total	300	0	0	1	0	0	0	1	(0.3)	(0.3)	400	4	0	4	(1.0)
	2,000#	101.1		150	1	0	0	0	0	0	0			200	0	0	0	
				150	0	1	0	0	0	0	1			200	4	0	4	
			Total	300	1	1	0	0	0	0	1	(0.3)	(0.7)	400	4	0	4	(1.0)
	MMC 0.05			150	2	10	1	29	0	0	36			200	0	0	0	
				150	3	9	0	23	0	0	29			200	0	0	0	
			Total	300	5	19	1	52	0	0	65	$(21.7)	(23.0)	400	0	0	0	(0.0)

a): Negative control (water-for-injection).

#: Precipitation, $: $p \leq 0.05$.

RPD: Relative population doubling; MMC: Positive control (Mytomycin C); CP: Positive control (cyclophosphamide); ctb: chromatid break; csb: chromosome break; cte: chromatid exchange; cse: chromosome exchange; others: multiple aberrations; pol: polyploids; end: endoreduplication.

doi: 10.12938/bmfh.2023-044

Table 3. Micronucleus test for the heat-treated *Bifidobacterium bifidum* OLB6378 concentrate in male rat bone marrow cells

Dose (mg/kg)		No. of MNPCE in 4000 PCE	Incidence of MNPCE (%)	No. of PCE In 500 erythrocytes	Incidence of PCE (%)
	No. of animals examined	5	5	5	5
NC[a)]	Mean ± SD	8 ± 2	0.19 ± 0.05	272 ± 15	54.5 ± 2.9
	(Min/Max)		(0.13 / 0.28)		(51.4 / 58.4)
636.5	Mean ± SD	6 ± 2	0.16 ± 0.04	287 ± 18	57.5 ± 3.7
	(Min/Max)		(0.13 / 0.20)		(51.6 / 60.6)
1,273	Mean ± SD	6 ± 2	0.14 ± 0.05	283 ± 27	56.7 ± 5.3
	(Min/Max)		(0.10 / 0.23)		(50.4 / 64.0)
2,546	Mean ± SD	6 ± 1	0.15 ± 0.02	274 ± 47	54.8 ± 9.4
	(Min/Max)		(0.13 / 0.18)		(48.4 / 71.2)
MMC	Mean ± SD	128 ± 23	3.21 ± 0.56*	238 ± 20	47.7 ± 4.1
2	(Min/Max)		(2.63 / 3.90)		(42.6 / 52.4)

a): Negative control (water-for-injection).
*: $p \leq 0.05$.
MMC: Positive control (Mytomycin C); MNPCE: Micronucleated polychromatic erythrocytes; PCE: Polychromatic erythrocytes, including MNPCE; SD: standard deviation.

In the functional tests, no test substance-related changes were observed in either sex. The following changes were considered incidental because they were not dose related: a decrease in hind limb grip strength in males administered 853 mg/kg/day and a decrease in locomotor activity from 10 to 20 min after dosing in males administered 280 mg/kg/day.

There were no test substance-related changes in body weight (Supplementary Fig. 1), food consumption (Supplementary Fig. 2), ophthalmoscopy findings, or urinalysis results in either sex (Table 4). A decrease in 24-hr chloride output was observed in males at ≥280 mg/kg/day and an increase in 24-hr potassium output was observed in females at 2,546 mg/kg/day; however, they were deemed physiological variations because the values were within the historical control data. Increases in urine volume output and 24-hr potassium output in males at 280 mg/kg were also considered incidental because they were not dose related.

Hematology and blood chemistry analyses revealed no test substance-related changes in either sex (Table 5), and the following changes were considered physiological variations because the values were within the historical control data: decreases in HGB, HCT, PT and APTT in males at 2,546 mg/kg/day; an increase in EOS in females at ≥853 mg/kg; increases in BUN and urea and decreases in Cl, ALB, and A/G ratio in males at 2,546 mg/kg/day. Additionally, an increase in ALT in males treated with 853 mg/kg/day was deemed incidental because it was not dose related. No test substance-related changes were observed in either sex in terms of organ weights (Supplementary Table 3) or any of the organs or tissues during necropsy or histopathological examination (Supplementary Table 2).

DISCUSSION

The genotoxic potential of the heat-treated OLB6378 concentrate was assessed using the bacterial reverse mutation test, *in vitro* mammalian chromosomal aberration test, and *in vivo* mammalian micronucleus test. All the tests demonstrated that the test substance was negative for genotoxicity. Acute oral toxicity was assessed in male and female SD rats, and there were no signs of toxicity in either sex at doses up to 5,092 mg/kg, which is the maximum feasible dose.

Repeated-dose oral toxicity was assessed in male and female SD rats by oral gavage for 90 days. One male rat in the high-dose group died 12 weeks after administration. The animal died of convulsions when restrained by a technician during dosing. Gross signs and histopathological examination revealed no lesions in any tissue or organ. One male rat in the middle-dose group was euthanized as moribund at week five of administration. Although the animal was in poor physical condition, as mentioned in the Results section, the cause of moribundity, including the relationship with the test substance, was not determined. However, its moribundity was considered incidental because no such observations were made in other surviving rats of the middle-dose group or in any of the high-dose groups. Convulsion, which was triggered by contact with a technician attempting to restrain the animal during dosing, was also observed transiently in one surviving male in the low-dose group at week 11 of administration. Such convulsions have been reported to occasionally be induced by external stimuli, particularly contact stimuli, during animal restraint for dosing in the strain of rats used in the present study [12, 13]. Considering the similarity of the present study to studies in which convulsions and subsequent deaths were reported following contact, deaths associated with convulsions in the present study were considered incidental. Consequently, there were no indications of toxicity in the examinations, including clinical signs, detailed clinical observations, gross signs, and the results of functional tests, ophthalmoscopy, urinalysis, hematology, blood chemistry, and histopathology, and measurements of body weight, food consumption, and organ weight, when the heat-treated OLB6378 concentrate was repeatedly orally administered to the rats. Previous studies reported that the NOAEL for other live bifidobacterial strains was 10^{11} cfu/kg/kg/day [14, 15]. In this study, the NOAEL for heat-treated OLB6378 (10^{12} cells /kg/day) was higher than that of the other live bifidobacterial strains.

In conclusion, the heat-treated OLB6378 concentrate had no genotoxic potential, and the no-observed-adverse-effect-

level of the heat-treated OLB6378 concentrate in the 90-day toxicity study was 2,546 mg/kg/day (3.7×10^{12} cells /kg/day as *Bifidobacterium*). Heat-treated OLB6378 can be safely used as a food source.

FUNDING

This research received no external funding.

Table 4. Urinalysis in male and female rats treated with the heat-treated Bifidobacterium bifidum OLB6378 concentrate by oral gavage for 90 days

Sex		Male				Female			
Dose (mg/kg)		0	280	853	2,546	0	280	853	2,546
No. of animals		10	10	9	9	10	10	10	10
pH [a)]	6.5	0	0	0	0	0	0	0	0
	7	0	0	0	0	5	2	0	0
	7.5	1	0	0	0	1	1	0	0
	8	2	0	0	0	1	0	0	0
	8.5	7	5	0	1	2	4	2	1
	≥9.0	0	5	9	8	1	3	8	9
Protein [a)]	−	5	1	0	0	10	7	7	6
	+/−	5	3	2	6	0	2	2	3
	+	0	4	6	3	0	1	0	1
	+2	0	2	1	0	0	0	1	0
Ketone [a)]	−	6	1	0	7	10	8	9	10
	+/−	4	4	5	2	0	1	1	0
	+	0	5	4	0	0	1	0	0
Glucose [a)]	−	10	10	9	9	10	10	10	10
Occult blood [a)]	−	2	3	9	8	10	10	10	10
	+/−	8	7	0	1	0	0	0	0
Urobilinogen [a)]	±	10	10	9	9	10	10	10	10
Bilirubin [a)]	−	10	10	9	9	10	10	9	10
	+	0	0	0	0	0	0	1	0
Color [a)]	LY	0	0	0	0	0	0	0	0
	Y	10	10	9	9	10	10	10	10
Urinary sediments [a)]									
RBC	−	10	10	9	9	10	10	10	10
WBC	−	10	10	9	9	10	10	10	10
Ep.SEC	+/−	10	10	9	9	10	10	10	10
EP.SREC	−	10	10	9	9	10	10	10	10
Cast	−	10	10	9	9	10	10	10	10
PS	−	9	7	0	3	9	9	5	2
	+/−	1	3	9	6	1	1	5	8
CO	−	10	10	9	9	10	10	10	10
Volume [b)] (mL)		17.9 ± 4.1	11.9* ± 3.9	13.0 ± 4.5	18.9 ± 5.5	7.9 ± 3.8	5.9 ± 2.6	7.7 ± 3.2	11.0 ± 5.0
Water intake [b] (mL)		35 ± 6	32 ± 6	32 ± 6	38 ± 8	27 ± 7	27 ± 6	30 ± 7	32 ± 6
Osmotic pressure [b)] (mOsm/kg)		1,049 ± 269	2,096 ± 214	2,009 ± 386	1,958 ± 341	2,058 ± 560	2,299 ± 790	2,007 ± 448	1,892 ± 529
Na [b] (mmol/24h)		2.8 ± 0.5	2.2 ± 0.7	2.1 ± 0.7	2.1 ± 0.8	1.3 ± 0.5	0.9 ± 0.3	1.3 ± 0.3	1.4 ± 0.6
K [b] (mmol/24h)		6.1 ± 1.1	4.6* ± 1.2	4.8 ± 1.1	7.2 ± 1.8	2.6 ± 0.9	2.1 ± 0.6	2.8 ± 0.8	3.7* ± 1.2
Cl [b] (mmol/24h)		4.4 ± 0.7	3.2** ± 0.9	3.0** ± 0.8	3.0** ± 0.8	1.8 ± 0.6	1.4 ± 0.4	1.7 ± 0.5	1.8 ± 0.7

a): Numbers in the table are number of animals with respective findings.
b): Values in the table are group mean ± SD.
*: p≤0.05, **: p≤0.01 (significantly different from the control group).
Protein −: Negative, +/−: 15, +:30, 2+: 100, 3+: 300, 4+: 1,000 mg/dL.
Ketone −: Negative, +/−: 5, +: 15, 2+: 40, 3+: ≥500 mg/dL.
Glucose −: Negative, +: 100, 2+: 250, 3+: 500, 4;: ≥1,000 mg/dL.
Occult blood −: Negative, ±: 0.015, +:0.062, 2+: 0.135, 3+: 0.405 mg/dL.
Urobilinogen +/−: 0.1–1, +: 2.0, 2+: 4.0, 3+: ≥8.0 Ehrlich/dL.
Bilirubin −: Negative, +: 0.8, 2+: 1.6, 3+: 3.2 mg/dL.
Color LY: Light yellow; Y: Yellow.
RBC: −: Negative, +/−: slight, +: mild, 2+: moderate, 3+: severe.
WBC: −: Negative, +/−: slight, +: mild, 2+: moderate, 3+: severe.
Ep.SEC: Squamous epithelial cell; −: Negative, +/−: slight, +: mild, 2+: moderate, 3+: severe.
Ep.SREC: Small round epithelial cell; −:Negative, +/−: slight, +: mild, 2+: moderate, 3+: severe.
PS: Phosphate salts; −: Negative, +/−: slight, +: mild, 2+: moderate, 3+: severe.
CO: Calcium oxalate; −: Negative, +/−: slight, +: mild, 2+: moderate, 3+: severe.

doi: 10.12938/bmfh.2023-044

Table 5. Hematology and blood chemistry in male and female rats treated with the heat-treated *Bifidobacterium bifidum* OLB6378 concentrate by oral gavage for 90 days

Sex	Male				Female			
Dose (mg/kg)	0	280	853	2,546	0	280	853	2,546
No. of animals	10	10	9	9	10	10	10	10
RBC (10^4/μL)	881 ± 29	859 ± 30	866 ± 37	852 ± 30	775 ± 31	776 ± 35	773 ± 39	776 ± 37
HGB (g/dL)	15.7 ± 0.6	15.4 ± 0.4	15.4 ± 0.3	15.2* ± 0.4	14.6 ± 0.6	14.7 ± 0.7	14.6 ± 0.5	14.5 ± 0.6
HCT (%)	45.8 ± 1.7	44.2 ± 1.2	44.9 ± 1.3	44.0* ± 1.6	41.9 ± 1.5	42.1 ± 1.9	41.2 ± 1.3	41.4 ± 1.8
MCV (fL)	51.9 ± 0.4	51.4 ± 0.8	51.9 ± 1.5	51.6 ± 0.7	54.1 ± 1.5	54.3 ± 1.2	53.4 ± 1.9	53.4 ± 1.6
MCH (pg)	17.8 ± 0.4	17.9 ± 0.4	17.8 ± 0.6	17.9 ± 0.3	18.9 ± 0.5	18.9 ± 0.4	18.9 ± 0.9	18.7 ± 0.4
MCHC (g/dL)	34.4 ± 0.4	34.8 ± 0.5	34.3 ± 0.6	34.6 ± 0.5	34.9 ± 0.4	34.8 ± 0.5	35.4 ± 0.7	35.1 ± 0.5
Reticulocyte (10^9/L)	170.9 ± 24.2	179.2 ± 31.2	189.7 ± 40.2	180.7 ± 29.4	159.4 ± 30.2	148.4 ± 28.7	142.9 ± 41.9	176.6 ± 43.9
PLT (10^4/μL)	98.1 ± 7.1	100.0 ± 11.3	101.6 ± 8.3	115.5 ± 21.3	102.3 ± 12.2	103.8 ± 9.0	110.7 ± 13.0	110.4 ± 9.9
WBC (10^2/μL)	86.3 ± 20.8	84.7 ± 18.6	80.1 ± 19.6	90.3 ± 18.7	45.8 ± 10.1	60.6 ± 13.5	57.2 ± 16.9	59.5 ± 21.6
LYMP (10^2/μL)	60.6 ± 20.1	61.5 ± 14.2	56.1 ± 14.1	68.6 ± 15.3	34.5 ± 8.4	46.7 ± 11.6	43.8 ± 15.5	43.8 ± 16.4
NEUT(10^2/μL)	20.9 ± 9.3	18.2 ± 6.7	19.4 ± 7.7	16.2 ± 4.4	8.9 ± 2.4	10.3 ± 4.4	10.1 ± 3.0	12.1 ± 5.6
EOS (10^2/μL)	1.4 ± 0.3	1.2 ± 0.5	1.2 ± 0.6	1.2 ± 0.3	0.7 ± 0.2	0.9 ± 0.4	1.0** ± 0.3	1.2 ± 0.7
BASO (10^2/μL)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
MONO(10^2/μL)	2.6 ± 0.6	2.9 ± 1.2	2.5 ± 0.9	3.0 ± 1.4	1.4 ± 0.6	2.1 ± 1.0	1.6 ± 0.6	1.8 ± 0.7
LUC (10^2/μL)	0.7 ± 0.5	0.7 ± 0.4	0.7 ± 0.5	0.9 ± 0.5	0.4 ± 0.2	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.3
PT (s)	14.9 ± 2.6	13.8 ± 2.3	13.8 ± 2.0	11.9** ± 0.9	11.2 ± 1.0	10.9 ± 0.8	10.8 ± 0.5	11.0 ± 0.8
APTT (s)	17.3 ± 2.2	15.5 ± 2.1	17.3 ± 1.7	14.5* ± 1.9	14.4 ± 1.6	13.3 ± 1.3	13.5 ± 1.1	13.6 ± 1.9
FIB (mg/dL)	302 ± 38	300 ± 22	302 ± 43	330 ± 56	184 ± 19	182 ± 24	182 ± 24	191 ± 12
AST (IU/L)	60 ± 9	65 ± 8	65 ± 8	64 ± 7	58 ± 12	64 ± 17	67 ± 25	73 ± 30
ALT (IU/L)	27 ± 3	30 ± 3	31* ± 4	28 ± 3	23 ± 6	25 ± 10	29 ± 9	39 ± 28
LDH (IU/L)	45 ± 11	53 ± 13	52 ± 14	48 ± 13	30 ± 6	36 ± 17	41 ± 23	43 ± 28
ALP (IU/L)	265 ± 51	283 ± 27	294 ± 54	257 ± 41	124 ± 35	125 ± 22	136 ± 54	138 ± 28
γ-GTP (IU/L)	1 ± 0	1 ± 1	1 ± 0	1 ± 1	1 ± 0	1 ± 0	1 ± 0	1 ± 0
T-CHO (mg/dL)	73 ± 20	69 ± 13	64 ± 10	84 ± 18	78 ± 24	77 ± 15	79 ± 16	92 ± 22
TG (mg/dL)	52 ± 17	61 ± 21	54 ± 29	63 ± 23	19 ± 10	18 ± 9	19 ± 9	20 ± 8
PL (mg/dL)	107 ± 21	104 ± 13	99 ± 12	116 ± 16	143 ± 32	139 ± 27	142 ± 19	162 ± 34
T-BIL (mg/dL)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
GLU (mg/dL)	140 ± 14	140 ± 19	138 ± 11	154 ± 22	123 ± 11	118 ± 18	123 ± 12	121 ± 11
BUN (mg/dL)	13 ± 1	13 ± 1	13 ± 2	15* ± 1	17 ± 3	16 ± 2	16 ± 3	16 ± 2
Urea (mg/dL)	28 ± 3	27 ± 2	28 ± 4	31* ± 2	36 ± 7	34 ± 5	33 ± 6	35 ± 5
CRNN (mg/dL)	0.24 ± 0.04	0.24 ± 0.04	0.23 ± 0.04	0.25 ± 0.02	0.31 ± 0.03	0.31 ± 0.04	0.31 ± 0.05	0.29 ± 0.04
Na (mmol/L)	146 ± 1	146 ± 1	145 ± 1	145 ± 1	144 ± 1	144 ± 1	143 ± 1	143 ± 1
K (mmol /L)	3.8 ± 0.2	3.7 ± 0.2	3.8 ± 0.2	3.6 ± 0.2	3.5 ± 0.2	3.6 ± 0.2	3.5 ± 0.2	3.5 ± 0.3
Cl (mmol /L)	109 ± 1	108 ± 1	108 ± 2	107* ± 2	109 ± 1	109 ± 2	108 ± 1	108 ± 2
Ca (mg/dL)	10.5 ± 0.3	10.5 ± 0.3	10.4 ± 0.3	10.3 ± 0.4	10.4 ± 0.4	10.5 ± 0.2	10.5 ± 0.3	10.5 ± 0.4
P (mg/dL)	5.9 ± 0.7	6.1 ± 0.9	5.9 ± 0.6	5.5 ± 0.6	4.9 ± 0.7	5.2 ± 0.7	4.7 ± 0.9	5.0 ± 0.5
TP (g/dL)	6.2 ± 0.2	6.2 ± 0.3	6.0 ± 0.1	6.2 ± 0.2	6.6 ± 0.3	6.7 ± 0.3	6.9 ± 0.4	6.7 ± 0.4
ALB (g/dL)	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	3.0* ± 0,1	3.8 ± 0.2	3.7 ± 0.3	3.9 ± 0.4	3.7 ± 0.3
A/G	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.9* ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.2 ± 0.1

Values in the table are group mean ± SD.
*: p≤0.05, **: p≤0.01 (significantly different from the control group).
RBC: red blood cell; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet; WBC: white blood cell; LYMP: lymphocyte; NEUT: neutrophil; EOS: eosinophil; BASO: basophil; MONO: monocyte; LUC: large unstained cells; PT: prothrombin time; APTT: activated partial thromboplastin time; FIB: fibrinogen; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; γ-GTP: γ-glutamyl transpeptidase; T-CHO: total cholesterol; TG: triglyceride; PL: phospholipid; T-Bil: total bilirubin; GLU: glucose; BUN: blood urea nitrogen; CRNN: creatinine; Na: sodium; K: potassium; Cl: chloride; Ca: calcium; P: inorganic phosphorus; TP: total protein; ALB: albumin; A/G: albumin/globulin ratio.

CONFLICTS OF INTEREST

M. Tsuboi and Y. Nakamura are employed by Meiji Co., Ltd. M. Terahara and M. Nagata are employed by Meiji Holdings Co., Ltd. T. Katsumata and T. Ishii are employed by Bozo Research Center Inc. M. Kato was formerly employed by CMIC Pharma Science Co., Ltd.

REFERENCES

1. EFSA Panel on Dietetic Products, N. a. A. N. 2014. Scientific Opinion on the essential composition of infant and follow-on formulae. EFSA J 12: 3760. [CrossRef]
2. Latorre E, Layunta E, Grasa L, Castro M, Pardo J, Gomollón F, Alcalde AI, Mesonero JE. 2016. Intestinal serotonin transporter inhibition by toll-like receptor 2 activation. A feedback modulation. PLoS One 11: e0169303. [Medline] [CrossRef]
3. Cario E, Gerken G, Podolsky DK. 2004. Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. Gastroenterology 127: 224–238. [Medline] [CrossRef]

4. Hörmann N, Brandão I, Jäckel S, Ens N, Lillich M, Walter U, Reinhardt C. 2014. Gut microbial colonization orchestrates TLR2 expression, signaling and epithelial proliferation in the small intestinal mucosa. PLoS One 9: e113080. [Medline] [CrossRef]
5. Picard C, Fioramonti J, Francois A, Robinson T, Neant F, Matuchansky C. 2005. Review article: bifidobacteria as probiotic agents—physiological effects and clinical benefits. Aliment Pharmacol Ther 22: 495–512. [Medline] [CrossRef]
6. Bertelli C, Pillonel T, Torregrossa A, Prod'hom G, Fischer CJ, Greub G, Giannoni E. 2015. *Bifidobacterium longum* bacteremia in preterm infants receiving probiotics. Clin Infect Dis 60: 924–927. [Medline] [CrossRef]
7. Jenke A, Ruf EM, Hoppe T, Heldmann M, Wirth S. 2012. *Bifidobacterium* septicaemia in an extremely low-birthweight infant under probiotic therapy. Arch Dis Child Fetal Neonatal Ed 97: F217–F218. [Medline] [CrossRef]
8. Ohishi A, Takahashi S, Ito Y, Ohishi Y, Tsukamoto K, Nanba Y, Ito N, Kakiuchi S, Saitoh A, Morotomi M, Nakamura T. 2010. *Bifidobacterium* septicemia associated with postoperative probiotic therapy in a neonate with omphalocele. J Pediatr 156: 679–681. [Medline] [CrossRef]
9. Toshimitsu T, Nakamura M, Ikegami S, Terahara M, Itou H. 2013. Strain-specific identification of *Bifidobacterium bifidum* OLB6378 by PCR. Biosci Biotechnol Biochem 77: 572–576. [Medline] [CrossRef]
10. Nakamura Y, Terahara M, Iwamoto T, Yamada K, Asano M, Kakuta S, Iwakura Y, Totsuka M. 2012. Upregulation of polymeric immunoglobulin receptor expression by the heat-inactivated potential probiotic *Bifidobacterium bifidum* OLB6378 in a mouse intestinal explant model. Scand J Immunol 75: 176–183. [Medline] [CrossRef]
11. Group, J. E. M. S.-M. M. S. 1988. Atlas of chromosome aberration by chemicals. Asakura Publishing, Tokyo (in Japanese).
12. Satomoto K, Kuroiwa Y, Masubuchi Y, Uemura H, Oshima Y, Okazaki S. 2012. Spontaneous convulsions in Sprague-Dawley rats in carcinogenicity studies. J Toxicol Sci 37: 645–647. [Medline] [CrossRef]
13. Sugai T, Okamura T, Itayagoshi S, Enami T, Mochizuki M, Okazaki K, Okazaki S. 2004. Spontaneous convulsion in carcinogenicity study in IGS rats. J Toxicol Sci 29: 462.
14. Abe F, Yaeshima T, Iwatsuki K. 2009. Safety evaluation of two probiotic bifidobacterial strains, *Bifidobacterium breve* M-16V and *Bifidobacterium infantis* M-63, by oral toxicity tests using rats. Biosci Microflora 28: 7–15. [CrossRef]
15. Arai S, Minami Ji, Muto M, Iwabuchi N, Yamauchi K, Xiao Jz, Abe F. 2018. Safety evaluation of *Bifidobacterium breve* MCC1274 via oral toxicity tests in rats. Toxicol Res Appl 2: 2397847318807373.

