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Safety evaluation for multispecies probiotics in a 28-day feeding study in Sprague-Dawley rats

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This experiment evaluated the 28-day feeding study in rats after they were continuously fed with multi-species probiotics dosages in the low-dosage, medium-dosage, and high-dosage groups: 2,000 mg/kg body weight (B.W.), 4,000 mg/kg B.W., and 6,000 mg/kg B.W., respectively, for 28 days as a reference for the safety of its repeated usage. Each group consisted of 10 male and 10 female Sprague-Dawley (SD) rats and the test substance was administered for 28 continuous days. After the experiment, the rats were sacrificed, and their blood and viscera were collected for haematological analysis, serum bio-chemical analysis, and pathological examination. The test results showed that, during the experiment, none of the tested rats exhibited abnormal clinical symptoms; the rats in all dosage groups gained weight normally. At the end of the test, the results of urological testing, haematological testing, and serum biochemical testing revealed no significant differences between the dosage groups and the control group. The results of the pathological dissection, the macroscopic inspection of pathological changes, and the histopathological inspection revealed no significant pathological changes related to the tested substance in the dosage groups and the control group.

Key words: Probiotics, Sprague-Dawley rat, haematological analysis, serum biochemical analysis, pathological examination.

INTRODUCTION

Traditional dairy strains of lactic acid bacteria (LAB) are commonly regarded as safe because of their long history of use and have been given a “generally recognised as safe” (GRAS) status (Donohue, 2006). Probiotics have been generally defined as viable microorganisms which could confer a beneficial health effect on the host when administered in adequate amounts (Fuller, 1989). In order to demonstrate the efficacy of probiotics in improving human health, safety characteristics must be taken into consideration. However, new isolate-specific species or strains of probiotics, and novel probiotics cannot be assumed to share the historical safety of traditional strains (Salminen et al., 1998). Prior to incorporating new strains into products, their efficacy should be carefully assessed, and a case-by-case evaluation should be conducted to determine whether they share the safety status of traditional food-grade organisms (Salminen et al., 1998).

Different aspects of the safety of probiotics can be studied

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using *in vitro* and *in vivo* methods, including the Ames test, animal models, and human subjects (Salminen et al., 1998). Many countries, including those in the European Community, are currently developing more de-tailed guidelines with respect to regulations for novel and functional foods and related probiotic preparations. Conventional safety evaluation approaches, such as those for toxicological testing proposed by the Organisation for Economic Cooperation and Development (OECD), are appropriate as a first step in the evaluation of new probiotics (OECD, 2001). Therefore, a useful starting point could be a 28-day conventional rat-feeding study.

The available safety studies on LAB strains demonstrated that some *Lactobacillus* species, such as *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, and *Enterococcus faecium*, have no oral toxicity to animals (Tsai et al., 2004a; Tsai et al., 2004b; Hirose et al., 2009; Szabo et al., 2011; Jones et al., 2012; Sulemankhil et al., 2012; Jia et al., 2011; Jia et al., 2013), but no research has evaluated the safety of multiple species or strains in the same product. In this study, the freeze dried powder of a novel multispecies probiotic mixture (PROBIO S-23) including *Lactobacillus rhamnosus* LCR177*, Bifidobacterium adolescentis* BA286*,* and *Pediococcus acidilactici* PA318, were isolated from pickled vegetables and human feces, respectively.

In a previous study, we suggested that exposure to multispecies probiotics did not cause mutagenicity, such as did not provoke reverse mutation, chromosomal aberrations, and micronucleated reticulocytes in bacteria, mammalian cells, and mouse peripheral blood, respectively (Chiu et al., 2013). The aim of this study was to evaluate the safety of multispecies probiotics based on the methods suggested for the safety evaluation of novel probiotics. We confirmed the safety of the multispecies mixed powder product by a 28-day feeding study using Sprague-Dawley (SD) rats.

MATERIALS AND METHODS

Test substance

Stock culture collections were maintained at -80°C in Lactobacilli MRS broth (DIFCO, Detroit, Michigan, USA) containing 25% glycerol. Cells were propagated twice in Lactobacilli MRS broth containing 0.05% L-cysteine by incubation at 37°C for 20 h. Bacterial counts were determined by plating serial dilutions of the culture on MRS agar. Plates were incubated at 37°C for 48 h anaerobically. The test substance “PROBIO S-23” is a mixture including *L. rhamnosus* LCR177*, B. adolescentis* BA286, and *P. acidilactici* PA318, Isomalto-oligosaccharide, xylo- oligosaccharide, Fibersol 2, and the final total lactic acid bacteria cells was $5.0x10^{10}$ CFU/g.

Animals and experimental design

Eighty Sprague-Dawley (SD) rats (40 males and 40 females) with ages of 7 to 8 weeks were used. The animals were obtained from the Experimental Animal Centre of the National Yangming University (Taipei, Taiwan). The housing temperature was 22 ± 4°C, with a relative humidity of 40-70% and alternate light and darkness of 12 h each. The female and male rats were kept separately, with two animals per cage. The animals were fed Laboratory Autoclavable Rodent Diet® 5010 (PMI Nutrition International, USA). Sterile plastic water bottles were placed in the breeding cages, and the animals were not restricted (ad libitum) from drinking reverse osmosis (RO) water.

The animals were processed in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures were in accordance with requirements of the Institutional Animal Care and Use Committees (IACUC) of the Super Laboratory Co., Ltd (IACUC NO.: 100-9C, New Taipei City, Taiwan). This study tested control (RO water), low-dosage, medium-dosage, and high-dosage groups. The tested substance dosages in the low, medium, and high-dosage groups were 2,000 mg/kg body weight (B.W.), 4,000 mg/kg B.W., and 6,000 mg/kg B.W., respectively. With a sterile orogastric tube, three of the four groups were orally inoculated with the tested substance in RO water at three different doses.

During the testing period, clinical observation was conducted daily, and abnormal clinical symptoms or deaths after administration of the tested substance were recorded. All rats exhibiting abnormal clinical symptoms or death were recorded on log sheets for individual-animal clinical observation. The eye examinations first employed macroscopic observation to find any external abnormalities and then utilised an ophthalmoscope to examine the eyes’ internal structures. The eyes of all animals were examined before the administration of the probiotics and 1 day before being sacrificed at the end of testing. The body weight (B.W.) of each test animal was measured before the administration and once a week during the study.

Urinalysis

On the day before sacrifice, the animals were placed in a metabolism cage for 16 h, and their urine was collected. Specific gravity (SG), color, protein, urobilinogen, pH, ketone, bilirubin, glucose, nitrite, and occult blood were determined with a semi-quantitative biochemical urinalysis system (Urisys 2400, Roche, Basel, Switzerland). White blood cells (WBCs), red blood cells (RBCs), epithelial cells (EPs), crystals, and microorganisms were observed with microscopy.

Haematology

Before being sacrificed, the test animals were fasted overnight and anesthetised with carbon dioxide (CO_2). Blood samples were collected from the heart. The blood was placed in an anticoagulation tube containing ethylenediaminetetraacetic acid (EDTA), mixed evenly at room temperature, and analysed with an automatic blood analyser (Gen.S™, Beckman, USA). Hematocrit, hemoglobin, RBCs, WBCs, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), lymphocyte, neutrophil, and monocyte were determined. Another anticoagulation tube containing sodium citrate was used to collect the blood, and a blood prothrombin analyser (CA-1500, Sysmex) was employed to test the prothrombin time.

Blood biochemistry

The blood was kept at room temperature for solidification and then centrifuged to isolate the serum. The serum was analysed using a serum biochemistry analyser (LX®-20, Beckman). Plasma concentrations of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltranspeptidase (γ-GT), albumin, total protein, total bilirubin,

Table 1. The body weight of SD rats orally administrated PROBIO S-23 at three different dose levels for 28 days.

Time	Body weight (g)			
	Control (0 mg/kg) (n=10)	Low dose (2,000 mg/kg) (n=10)	Medium dose (4,000 mg/kg) (n=10)	High dose (6,000 mg/kg) (n=10)
Male				
Day 0	264.5±9.4[a]	264.8±9.5	264.5±10.0	264.1±9.1
Week 1	306.0±11.5	306.2±16.0	309.2±14.2	299.5±17.7
Week 2	347.8±18.3	344.3±19.2	345.1±15.1	340.9±14.2
Week 3	365.2±19.1	365.2±19.9	366.8±18.9	362.6±16.7
Week 4	389.6±21.4	392.9±25.8	394.5±19.4	381.6±19.2
After fasting	360.8±21.6	365.4±24.7	365.1±20.8	353.4±19.0
Female				
Day 0	162.0±7.7	162.2±7.7	162.2±7.6	162.1±7.8
Week 1	191.1±12.3	189.7±10.4	188.3±10.7	189.2±9.9
Week 2	211.4±13.8	208.9±9.7	207.4±10.6	209.1±13.9
Week 3	222.7±19.8	225.1±13.9	223.6±15.4	228.3±17.4
Week 4	241.8±18.3	244.1±16.3	240.0±15.7	244.1±21.4
After fasting	220.8±18.3	222.1±15.1	220.5±14.0	223.3±19.3

[a]Values are mean±S.D.

creatinine, blood urea nitrogen (BUN), glucose, cholesterol, triglyceride, phosphorus, calcium, chloride, potassium, and sodium were determined.

Histopathology

The following main organs (brain, heart, kidneys, liver, spleen, adrenal glands, and testes or ovaries) were removed from all surviving rats. After removal of the peripheral adipose tissue, the absolute weights of the above-mentioned organs were weighed and recorded. Next, the ratio of organ weight to B.W. (on day 29 of the experiment) was calculated for all organs. The ratio of organ weight to B.W. (%) = [organ weight (g) / B.W. (g)] x 100.

The brain, heart, kidneys, liver, spleen, adrenal glands, testes, and ovaries from all experimental groups were removed and fixed in 10% neutral formalin for preservation (the testes were fixed in modified Davidson`s solution for 24 h and then fixed in 10% neutral formalin for preservation). After fixation in 10% neutral formalin solution, sections were cut from the adrenal glands, brain, heart, kidneys, liver, spleen, and testes or ovaries of the control group and the high-dosage (6,000 mg/kg/day) group of 40 rats and examined for histopathology. The formalin-solution-fixed adrenal glands, brain, heart, kidneys, liver, spleen, and testes or ovaries were coarsely repaired and were subsequently processed via dehydration, clarification, paraffin infusion, and embedding steps to prepare paraffin tissue blocks. A microtome (Leica RM 2145, Nussloch, Germany) was then used to slice the blocks into 5-μm-thick tissue slices. The slices were stained with haematoxylin & eosin (H&E) and were observed under an optical microscope (Opticphot-2, Nikon, Tokyo, Japan) for histopathological changes in all organs. If any organ and/or tissue in the high-dosage group displayed pathology related to the tested substance, then that organ and/or tissue in the medium-dosage group (4,000 mg/kg/day) and low-dosage group (2,000 mg/kg/day) of animals had to undergo histopathological inspections.

Statistics

The experimental data were expressed as the mean and the standard deviation (S.D.). All data of the animals' B.W., food intake, organ weight, haematological analysis, and serum biochemical analysis were analysed by the Duncan test method of one-way analysis of variance (ANOVA) using the SPSS statistical software package. This method analysed the variance in the data among the different groups, and a probability (p) value less than 0.05 was taken as the standard for significance.

RESULTS AND DISCUSSION

Mortality rate, observation of clinical symptoms, B.W., food intake, and eye examination

During the experiment, no rats in any experimental group or in the control group died. During the experiment, two daily inspections of all experimental rat groups by the veterinarians revealed that no rats in any dosage group or the control group exhibited any abnormal clinical symptoms. The mean B.W. values for the male rats and female rats in all dosage groups and in the control group are listed in Table 1. The experimental results indicated that, compared with the control group, the B.W. values for the rats in all dosage groups exhibited no significant difference (p>0.05), suggesting that the rats in all dosage groups were able to gain weight normally.

The mean food-intake values for the male rats during the experimental period are listed in Table 2. The experimental results indicated that the food-intake values of male rats in the high-dosage group were significantly lower than those in the control group (p<0.05) during the 1st week of the experiment; there was no significant difference in the remaining periods for any of the dosage groups in comparison with the control group. The mean food-intake values for female rats in the experimental period are also presented in Table 2. There was no

Table 2. The feed intake of SD rats of three different PROBIO S-23 doses groups and the control set among 28 days.

Time	Feed intake（g/rat/day）			
	Control (0 mg/kg) (n=10)	Low dose (2,000 mg/kg) (n=10)	Medium dose (4,000 mg/kg) (n=10)	High dose (6,000 mg/kg) (n=10)
Male				
Week 1	28.9±1.6[a]	27.8±1.6	27.9±1.3	25.2±2.5*
Week 2	32.2±1.6	31.7±1.6	31.1±2.5	29.7±0.8
Week 3	30.3±2.3	29.9±1.8	30.4±2.1	30.5±1.7
Week 4	29.5±1.8	28.9±3.1	26.8±0.8	26.6±0.6
Female				
Week 1	24.0±2.3	24.7±1.6	23.7±2.8	23.0±2.1
Week 2	23.8±1.5	23.7±0.7	24.1±1.1	22.7±2.2
Week 3	20.2±1.0	19.0±1.0	18.8±1.4	18.8±1.2
Week 4	17.5±1.0	17.8±0.4	16.8±1.3	16.3±1.6

[a]Values are means ± S.D (n=5, two rats in each cage). *Significantly different from control group at $p<0.05$.

significant difference ($p>0.05$) in the mean food-intake values for any of the dosage groups compared with those of the control group. Eye examinations by naked eye and an ophthalmoscope were performed before the initial administration of the tested substance and at the end of the test; no abnormalities were found.

Urinalysis

Results of the microscopic examination of the urine sediment were the number of EPs, RBCs, WBCs, and urinary crystals exhibited no significant differences between any of the dosage groups and the control group. Conventional urine tests revealed no significant differences between any of the dosage groups and the control group regarding the colour, glucose, bilirubin, ketobodies, specific gravity, pH value, protein, urobilinogen, and nitrite.

Haematological analysis

The haematological analysis results for the male rats demonstrated that, except for a significant rise ($p<0.05$) in the number of reticulocytes in the low-dosage and medium-dosage groups compared with the control group, there was no significant difference ($p>0.05$) between any of the dosage groups and the control group for the remaining test items (Table 3). Regarding the female rats, the experimental results showed no significant difference ($p>0.05$) between the dosage groups and the control group (Table 3).

Serum biochemistry analysis

The serum biochemistry analysis results for the male rats revealed no significant difference between any of the dosage groups and the control group (Table 4). Concerning the female rats, except for the significantly higher ($p<0.05$) serum alkaline phosphatase (ALP) concentration in the high-dosage group compared with the control group, there was no significant difference ($p>0.05$) between any of the dosage groups and the control group for the remaining items (Table 4).

Organ weight

At the end of the experiment, the surviving rats were euthanized, and their brains, livers, kidneys, spleens, hearts, adrenal glands, and testes or ovaries were extracted and weighed. The results revealed no significant difference in the absolute organ weight of the male rats or female rats in any of the dosage groups compared with those in the control group (Table 5). There was no significant difference ($p>0.05$) in the relative weight of the male rat organs between the dosage groups and the control group, except for a significant decrease ($p<0.05$) in the relative weight of the livers in the high-dosage group (Table 6). There was no significant difference ($p>0.05$) in the relative weight of the main organs in the female rats between any of the dosage groups and the control group (Table 6).

Histopathological interpretation

After the experiment, the results of the pathological dissection and macroscopic pathological examination of all groups demonstrated that no macroscopic pathology related to the tested substance was detected in the heart, lungs, liver, spleen, kidneys, digestive tract, brain, and geni-tourinary system (Figure 1).

Results of the nonspecific pathological interpretation, the heart of one male rat from the control group exhibited focal, minute, focal mononuclear inflammatory-cell infiltration, with an incidence of 1/10 among the male rats in the control group (Figure 2A). There was no positive correlation

Table 3. Haematological findings in rats treated orally with PROBIO S-23 for 28 days.

Parameter	Control (0 mg/kg) (n=10)	Low dose (2,000 mg/kg) (n=10)	Medium dose (4,000 mg/kg) (n=10)	High dose (6,000 mg/kg) (n=10)
Male				
WBC (10^3/μL)	11.8±5.0[a]	11.7±4.4	11.3±4.8	11.5±3.6
RBC (10^6/μL)	8.6±0.3	8.3±0.3	8.5±0.3	8.4±0.3
Hemoglobin (g/dL)	16.6±0.4	16.1±0.8	16.6±0.6	16.4±0.7
Hematocrit (%)	52.3±1.1	51.2±2.0	52.0±1.8	51.7±1.7
MCV (fL)	61.1±1.9	61.4±2.0	61.0±1.4	61.3±1.1
MCH (pg)	19.4±0.6	19.3±0.6	19.4±0.4	19.4±0.4
MCHC (g/dL)	31.7±0.5	31.4±0.6	31.9±0.5	31.7±0.5
Platelet (10^3/μL)	872.2±130.0	957.2±150.2	923.1±126.3	1016.6±196.6
Neutrophil (%)	13.9±5.7	13.1±3.5	14.5±3.7	12.5±4.1
Lymphocyte (%)	81.0±6.5	82.3±4.0	80.3±4.4	82.4±5.0
Monocyte (%)	3.8±0.9	3.5±1.4	3.9±1.3	3.8±1.1
Eosinophil (%)	1.2±0.6	0.9±0.4	1.2±0.3	1.2±0.4
Basophil (%)	0.2±0.1	0.2±0.2	0.2±0.1	0.2±0.1
Reticulocyte (%)	3.8±0.9	5.2±1.0*	4.9±0.8*	4.0±0.9
PT (s)	12.5±1.3	12.2±1.2	12.7±1.1	12.9±0.8
Female				
WBC (10^3/μL)	9.0±3.5	9.5±2.2	8.5±4.4	8.1±2.7
RBC (10^6/μL)	8.4±0.4	8.5±0.2	8.3±0.2	8.4±0.3
Hemoglobin (g/dL)	16.5±0.5	16.4±0.6	16.3±0.6	16.5±0.6
Hematocrit (%)	53.3±1.3	53.6±2.0	53.2±2.3	53.7±1.6
MCV (fL)	63.4±3.0	63.4±2.0	64.4±1.9	64.2±1.5
MCH (pg)	19.6±0.5	19.4±0.5	19.8±0.4	19.7±0.4
MCHC (g/dL)	30.9±0.8	30.6±0.5	30.7±0.5	30.6±0.6
Platelet (10^3/μL)	877.6±110.8	955.3±102.4	870.7±123.0	905.7±94.4
Neutrophil (%)	11.2±3.8	11.5±3.9	11.7±3.6	10.4±2.7
Lymphocyte (%)	83.6±3.7	83.1±4.8	82.6±4.1	83.7±3.5
Monocyte (%)	3.9±1.6	4.0±1.9	4.5±1.7	4.6±1.5
Eosinophil (%)	1.1±0.4	1.2±0.3	1.1±0.3	1.1±0.4
Basophil (%)	0.3±0.4	0.2±0.2	0.2±0.1	0.3±0.2
Reticulocyte (10^3/μL)	3.8±0.8	4.2±0.9	4.3±0.4	4.3±0.5
PT (s)	10.2±0.2	10.2±0.3	10.5±0.6	10.2±0.2

MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; PT, Prothrombin time. a Values are means ± S.D. *Significantly different from control group at $p<0.05$.

of the tested substance to the occurrence of pathological changes in the female and male rats in the control group and the high-dosage group. In female rats from the control and high-dosage groups, the renal cortex and medulla exhibited a focal, minute-to-small, tubular mineral deposit, with an incidence of 7/10 for each group (Figure 2B). There was no positive correlation between the tested substance and the occurrence of pathological changes in the control and high-dosage groups of female rats. In female rats of the control and high-dosage groups, the liver exhibited multiple, minute-to-small fat-droplet infiltration, with an incidence of 4/10 and 7/10, respectively (Figure 2C). There was no positive correlation of the tested substance to the occurrence of pathological changes in the control and high-dosage groups.

In the previous study, the multispecies probiotic mixture showed no mutagenic potential that leads to bacteria reverse mutation, *in vitro* chromosome aberration, and micronucleated reticulocytes in mouse peripheral blood (Chiu et al., 2013). In this study, the 28-day administration of low, medium and high-dose mixed LAB strains did not produce any deaths or clinical signs of toxicity. Hepatomegaly and splenomegaly are usually indirect indicators of infection and invasion. Clinical chemical assays can be used to detect moderate to mild deficiency of nutrients or imbalances in nutrient metabolism, and these deficiencies

Table 4. Blood biochemistry in rats treated orally with PROBIO S-23 for 28 days.

Parameters	Control (0 mg/kg) (n=10)	Low dose (2,000 mg/kg) (n=10)	Medium dose (4,000 mg/kg) (n=10)	High dose (6,000 mg/kg) (n=10)
Male				
AST (U/L)	131.6±45.5[a]	124.8±18.9	119.1±14.9	120.6±13.8
ALT (U/L)	53.9±26.9	46.3±8.4	43.6±7.8	44.7±8.1
ALP (U/L)	125.6±25.0	148.1±32.2	153.9±43.3	149.0±15.5
T. bilirubin (µg/dL)	49.1±14.8	50.1±17.1	40.7±8.3	51.9±15.0
T. protein (g/dL)	6.7±0.3	6.7±0.4	6.6±0.3	6.6±0.3
Albumin (g/dL)	4.3±0.2	4.3±0.3	4.3±0.2	4.2±0.2
Globulin (g/dL)	2.4±0.1	2.4±0.2	2.4±0.2	2.4±0.1
BUN (mg/dL)	16.1±2.0	15.7±1.9	16.7±2.4	16.2±1.8
Creatinine (mg/dL)	0.40±0.04	0.36±0.07	0.37±0.07	0.36±0.06
Glucose (mg/dL)	181.0±37.0	167.9±26.3	175.0±44.8	168.2±31.7
Triglyceride (mg/dL)	28.0±3.3	28.0±4.2	30.8±8.1	28.3±8.2
Cholesterol (mg/dL)	58.8±16.5	66.1±13.6	61.5±16.8	53.9±13.9
Sodium (meq/L)	149.0±1.9	149.9±3.2	149.2±1.7	149.0±2.9
Potassium (meq/L)	7.6±0.7	7.5±1.1	7.4±0.8	7.7±0.6
Calcium (meq/L)	11.3±0.3	11.4±0.6	11.6±0.6	11.6±0.5
Chloride (meq/L)	99.3±1.5	100.6±3.0	100.0±2.2	100.4±1.6
Phosphorus (mg/dL)	12.3±0.9	12.6±1.2	12.3±0.7	12.3 ±1.0
Female				
AST (U/L)	174.4±149.4	142.7±59.0	153.9±28.8	141.9±32.9
ALT (U/L)	59.2±48.6	50.5±19.2	50.1±17.3	47.3±12.4
ALP (U/L)	108.0±20.6	117.4±23.6	122.2±33.8	146.2±36.2*
T. bilirubin (µg/dL)	59.5±17.6	64.1±18.6	61.3±14.5	55.2±21.5
T. protein (g/dL)	7.0±0.2	7.1±0.4	6.7±0.3	6.9±0.3
Albumin (g/dL)	4.5±0.2	4.6±0.2	4.4±0.2	4.5±0.2
Globulin (g/dL)	2.4±0.2	2.5±0.2	2.3±0.2	2.4±0.1
BUN (mg/dL)	17.4±2.4	15.4±2.1	15.5±3.9	15.7±3.0
Creatinine (mg/dL)	0.44±0.04	0.43±0.05	0.40±0.05	0.41±0.07
Glucose (mg/dL)	124.6±18.4	135.3±39.3	132.8±9.5	120.1±41.2
Triglyceride (mg/dL)	38.3±5.0	42.1±9.7	43.1±4.6	40.9±7.8
Cholesterol (mg/dL)	86.7±15.2	87.7±13.0	88.7±10.2	91.9±14.4
Sodium (meq/L)	146.8±1.2	148.0±1.1	147.6±1.1	147.6±1.3
Potassium (meq/L)	7.8±0.8	7.6±0.5	8.0±1.5	7.4±0.9
Calcium (meq/L)	11.8±0.3	11.9±0.4	11.9±0.3	11.9±0.3
Chloride (meq/L)	100.5±1.7	101.5±1.4	101.1±0.7	100.6±1.0
Phosphorus (mg/dL)	13.0±1.3	12.9±1.6	13.0±1.2	13.0±1.6

[a] Values are means Mean ± S.D. * Significantly different from control group at $p<0.05$.

are usually apparent before any clinical symptoms or changes in body weight (Swendseid et al., 1987). In this study, we did not find macroscopic changes in liver and spleen morphology of animals treated with test strains *in vivo*. Animals in these groups showed similar daily feed when compared with the control group. There was no significant difference in SGR between the treatment groups and the control group for rats ($p>0.05$). These results also suggest that the rats experienced no infections resulting from the 28-day treatment with multiple LAB strains.

Szabo et al. (2011) demonstrated the safety of *Lactobacillus pentosus* strain b240. Although statistical significance was shown for several parameters in this study, such as haematology, clinical chemistry or organ weights, among others, the authors hypothesised that none of these changes were attributable to treatment because they remained within the range of historical

Table 5. Absolute organ weight in SD rats treated orally with PROBIO S-23 at three different dose levels for 28 days.

Organ	Absolute organ weight (g)			
	Control (0 mg/kg) (n=10)	Low dose (2,000 mg/kg) (n=10)	Medium dose (4,000 mg/kg) (n=10)	High dose (6,000 mg/kg) (n=10)
Male				
Testes	2.99±0.12[a]	3.14±0.26	3.12±0.21	2.95±0.31
Spleen	0.68±0.10	0.71±0.14	0.63±0.07	0.65±0.09
Kidney	3.21±0.20	3.23±0.36	3.21±0.19	3.06±0.22
Adrenals	0.056±0.008	0.055±0.012	0.055±0.005	0.060±0.010
Liver	11.8±0.9	11.8±1.3	11.7±1.0	10.7±1.1
Heart	1.44±0.12	1.40±0.11	1.42±0.12	1.35±0.12
Brain	1.92±0.07	1.95±0.03	1.94±0.08	1.92±0.08
Female				
Ovaries	0.092±0.015	0.093±0.017	0.089±0.011	0.090±0.009
Spleen	0.50±0.05	0.49±0.08	0.54±0.06	0.54±0.09
Kidney	2.12±0.22	2.04±0.20	2.10±0.16	2.07±0.18
Adrenals	0.069±0.009	0.072±0.011	0.075±0.009	0.073±0.008
Liver	8.36±0.86	8.16±0.78	8.20±0.63	8.04±0.65
Heart	0.90±0.11	0.89±0.04	0.89±0.07	0.89±0.11
Brain	1.83±0.10	1.80±0.13	1.79±0.10	1.82±0.08

[a] Values are means mean ± S.D.

Table 6. Organ relative weight in SD rats treated orally with PROBIO S-23 at three different dose levels for 28 days.

Organ	Organ relative weight (g/ 100 g b.w.)			
	Control (0 mg/kg) (n=10)	Low dose (2,000 mg/kg) (n=10)	Medium dose (4,000 mg/kg) (n=10)	High dose (6,000 mg/kg) (n=10)
Male				
Testes	0.83±0.07[a]	0.86±0.10	0.86±0.07	0.83±0.07
Spleen	0.19±0.02	0.20±0.04	0.17±0.02	0.18±0.02
Kidney	0.89±0.05	0.88±0.08	0.88±0.07	0.87±0.05
Adrenals	0.016±0.003	0.015±0.003	0.015±0.001	0.017±0.002
Liver	3.28±0.13	3.23±0.20	3.21±0.18	3.02±0.23[*]
Heart	0.40±0.05	0.39±0.04	0.39±0.04	0.38±0.04
Brain	0.53±0.03	0.54±0.03	0.53±0.04	0.55±0.03
Female				
Ovaries	0.042±0.008	0.042±0.006	0.040±0.005	0.041±0.005
Spleen	0.23±0.02	0.22±0.03	0.24±0.02	0.24±0.03
Kidney	0.96±0.08	0.92±0.04	0.96±0.06	0.93±0.05
Adrenals	0.031±0.004	0.033±0.005	0.034±0.004	0.033±0.003
Liver	3.78±0.18	3.68±0.22	3.72±0.16	3.61±0.16
Heart	0.41±0.02	0.40±0.02	0.41±0.02	0.40±0.03
Brain	0.84±0.08	0.81±0.05	0.81±0.07	0.82±0.08

Relative weight = (organ weight/body weight) x 100; [a] Values are means Mean ± S.D. * Significantly different from control group at $p<0.05$.

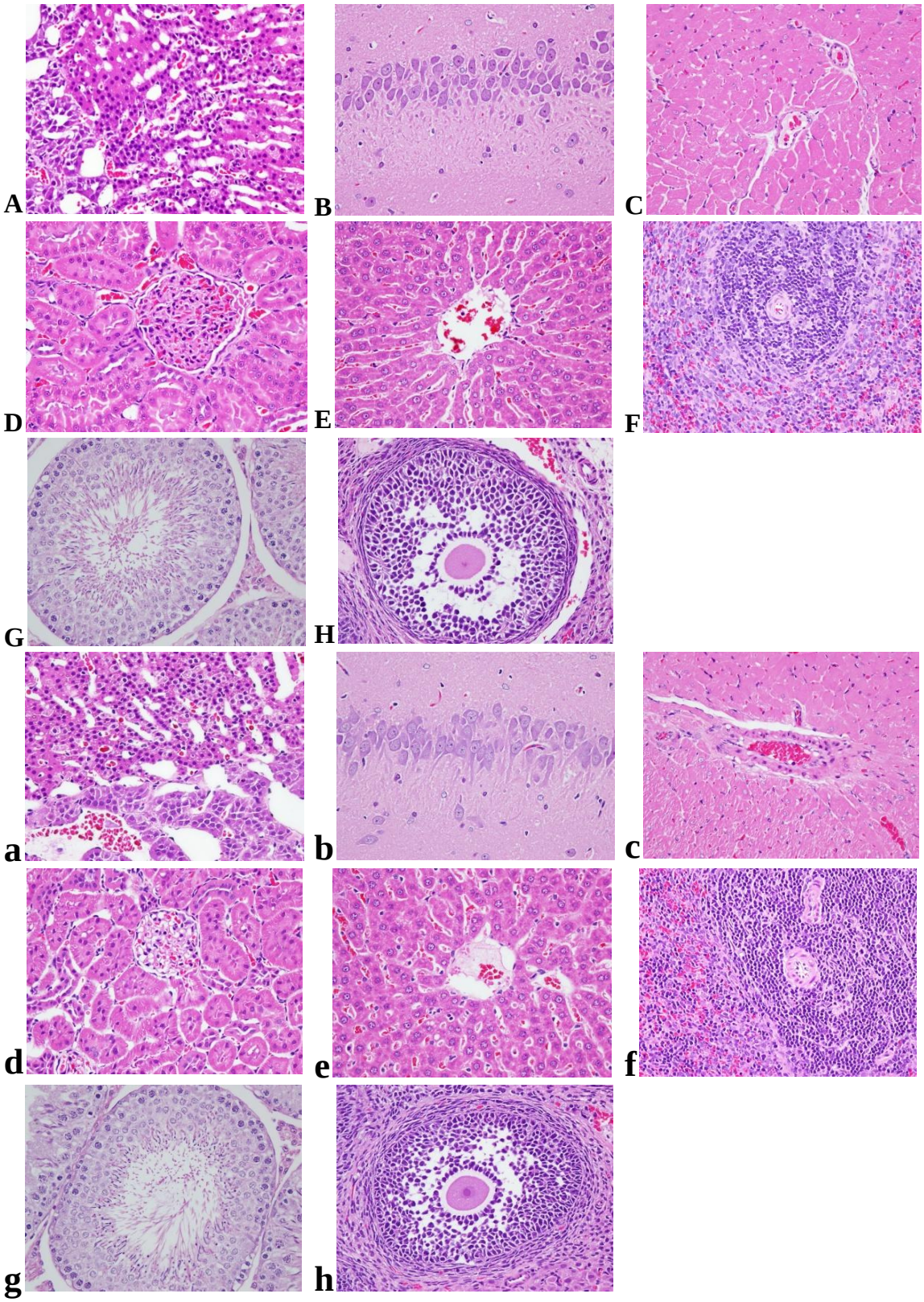

Figure 1. Photomicrographs of (A) adrenal glands, (B) brain, (C) heart, (D) kidneys, (E) liver, (F) spleen, (G) testes, and (H) ovaries histopathology from representative SD rats treated with RO water (control group), (a) adrenal glands, (b) brain, (c) heart, (d) kidneys, (e) liver, (f) spleen, (g) testes, and (h) ovaries histopathology from representative SD rats treated with PROBIO S-23 (6,000 mg/kg B.W, the high-dosage group) (Hematoxylin and eosin stain, 400x).

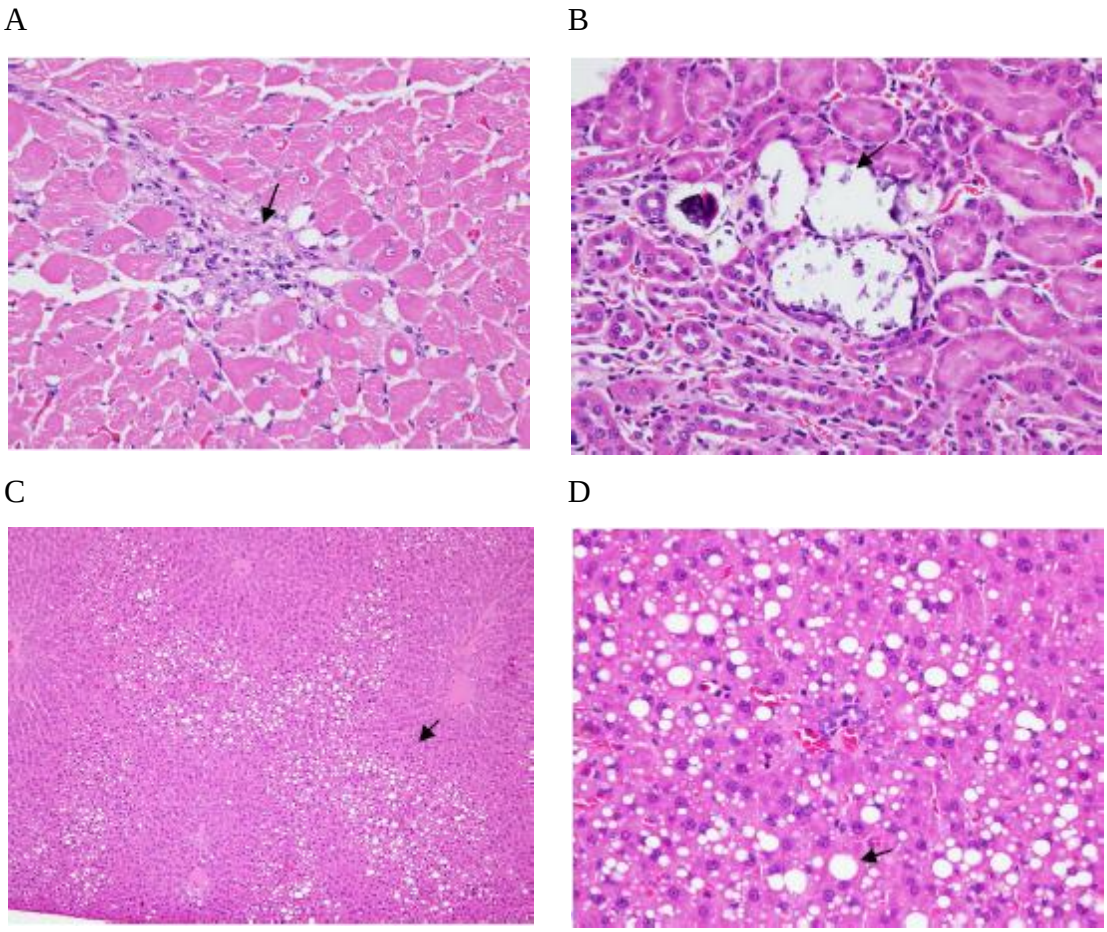

Figure 2. The nonspecific pathological interpretation of the organs in rats treated orally with purified water or PROBIO S-23 for 28 days. The heart of one male rat from the control group exhibited focal, minute, mononuclear inflammatory-cell infiltration (A, animal No. 05M, 400x). In female rats, the renal cortex and medulla exhibited a focal, minute-to-small, tubular mineral deposit (B, animal No. 04F, 400x). In female rats, the liver exhibited multiple, minute-to-small fat-droplet infiltration (C, 100x; D, 400x, animal No. 04F) (Hematoxylin and eosin stain).

controls, presented in only one sex, or noted only sporadically. In our study, some statistically significant results, such as the number of reticulocytes (male rats, the low and medium dose groups), serum alkaline phosphatase (female rats, the high dose group) and the relative weight of the livers (male rats, the high dose group), were observed as mentioned above.

Some rats exhibited focal, minute-to-small renal-tubular mineral deposits. The incidence of calcite deposition is high in SD, Wistar, and F344 female rats, which may be related to oestrogen (Reeves et al., 1993). Moreover, when the ratio of calcium (Ca^{++}) to phosphorus (P^{-}) in the feed is less than 1, magnesium (Mg^{++}) deficiency, chlorine (Cl^{-}) deficiency, or a high-pH urine can also induce a high incidence of calcite deposition (Schoenmakers et al., 1989). The calcite deposition in the renal pelvis can block the kidney tubules, which slows the speed of urination and thus leads to chronic progressive nephropathy, such as renal tubule enlargement and the accumulation of protein-like homogeneous exudates in the renal glomerulus, Bowman's capsule, and renal tubular cavity. However, long-term experiments on chronic toxicity have revealed no direct correlation between calcite deposition pathology and cancer (Rao, 2002; Owen and Heywood, 1986). In addition, the focal, minute mononuclear-cell infiltration in the heart of one male rat from the control group and the multiple, minute-to-small fat-droplet infiltrations in the hearts of female rats from the control and high-dosage groups were physiological phenomena.

Conclusions

Based on above-mentioned test results, the no-observed-adverse- effect level (NOAEL) for 28-day repeat dosages of PROBIO S-23 for rats was greater than 6,000 mg/kg/day. The margin of safety was greater than 80-fold safety factor. In Taiwan, the Food and Drug Administration

allows a 60-fold safety factor in the 28-day toxicity testing for natural extracts and Probiotic products. We conclude that consumption of the probiotic mixture is safe.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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