

Letter to the Editor

A Subchronic Toxicity Study on *Lactobacillus Fermentum* GM 090 in Rat*

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Lactobacillus fermentum (*L. fermentum*) is one of seven species in the genus *Lactobacillus*^[1]. With a long history of safe use in fermented food production, *Lactobacillus* is considered as one of the most beneficial probiotics^[2-3]. The most well-known potential health benefit is improving digestion and immune function^[4-5]. Other beneficial functions of *Lactobacillus* strains include managing lactose intolerance^[5], lowering cholesterol and blood pressure^[5], reducing inflammation^[6], and prevention of cancer^[5,7]. *L. fermentum* is usually found during malt whisky fermentation^[8]. *L. fermentum* CP34 was found to have the significant effect of decreasing the serum antigen-specific IgE levels compared to a control group^[9].

In recent years, many new *Lactobacillus* strains with probiotic attributes are being introduced into food products due to the growing consumer awareness concerning diet and health. For traditional *Lactobacillus* strains, they have an excellent history of safe use in the formation of dairy products and other foods and some have "generally recognized as safe" (GRAS) status^[10], however, newly isolated organisms often have no previous history of food product use, thus, they do not necessarily share the GRAS status of traditional *Lactobacillus* strains. Therefore, it is necessary and essential to conduct the safety assessment on any new strain with the intent to be added into foods or used as a dietary supplement. We have conducted a 90-day feeding study on a new strain of *L. paracasei* in our lab and the results confirmed that no subchronic toxicity was observed^[11]. *L. fermentum* GM 090 has the similar morphological characteristics like rod-like shape with round edge with *L. paracasei* GM 080, and both of them belong to the the genus *Lactobacillus*. However, they have obvious difference: *L. paracasei* GM 080 is a strain of *L. paracasei*, and *L. fermentum* 090 is one of strains of *L. fermentum*. Yeung et al.^[12] analyzed the 16s rDNA sequence and randomly amplified polymorphic DNA (RAPD analysis) of this strain. The result found that GM 090 belongs to *Lactobacillus*

fermentum, but has a specific RAPD patterns. Given this, GM 090 is a novel *Lactobacillus fermentum* strain. Therefore, the present study aims to evaluate the subchronic toxicity of *L. fermentum* GM 090 when administered daily by gavage to Sprague Dawley rats for 90 days.

Groups of 10 male and 10 female weaning Sprague-Dawley rats were given 0, 1.25, 2.5, and 5.0 g/kg body weight *Lactobacillus fermentum* GM 090 by gavage, respectively. Clinical observations were recorded daily. Body weights and food consumption were measured weekly. Blood samples were obtained in the middle of the study (day 46) and at the end of the study for measurement of hematology and clinical chemistry. At the end of the study, all animals were euthanized for necropsy. Selected organs were weighted and recorded. Histological examination was performed on all tissues from animals in the control and high dose groups. The study was conducted at the National Institute for Nutrition and Food Safety (Beijing, China) in compliance with the Food and Drug Administration (FDA) principles of GLP and in accordance with the FDA Guidance for Industry and Other Stakeholders, "Toxicological Principles for the Safety Assessment of Food Ingredients Redbook 2000-Subchronic Toxicity Studies with Rodents"^[13]. This protocol has been approved by the Office of Laboratory Animal Welfare, National Institute for Nutrition and Food Safety.

L. fermentum GM 090 (Lot No. 20070618001), a brown powder, provided by GenMont Biotech Incorporation (Taiwan, China) and stored at 2-8 °C in a refrigerator, was used in this study. The concentration of the tested bacteria is $\geq 2 \times 10^9$ cfu/g. The viability was tested throughout the study, and the *L. fermentum* GM 090 counts on Day 0, Day 30, Day 60, and Day 90 were constant ($\geq 2 \times 10^9$ cfu/g), which confirmed that the bacteria were alive during the study.

Sprague-Dawley rats were received from Vital River Laboratory Animal Technology Co, Ltd (Beijing, China). All animals were examined for clinical signs of

doi: 10.3967/0895-3988.2013.08.008

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ill health on receipt and observed within 5 days of arrival. After 5 days of acclimation, healthy rats were randomly divided into four groups. The test material was suspended into water to make different concentrations as shown in the study design and given to rats by gavage, once per day for 90 days. The gavage volume was 2 mL/100 g body weight. The body weights were measured weekly and the gavage volume was adjusted based on the weekly body weight of rats. Rats were individually housed in suspended stainless steel, open-mesh cages in environmentally controlled rooms. The room temperature was maintained at 23 ± 2 °C and the relative humidity was controlled within the range of 30%-70%. Air was changed 10-15 times per hour. Light was set for a 12 h light/dark cycle.

Each animal was observed twice daily for abnormalities, physical appearance and mortality.

The body weight of each rat was measured pre-test, weekly thereafter and at sacrifice after fasting. Food consumption for each animal was determined weekly.

On day 46 and day 91, following fasting for 16-18 h, rats were anesthetized with 3% sodium pentobarbital solution and blood was collected from tail vein. Blood for hematology studies was collected into tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. A COULTER Ac.T diff2 Hematology Analyzer (Beckman Coulter Corporation) was employed to measure the following parameters: red blood cell count, hemoglobin, platelet count, white blood cell count, and differential blood cell count.

Blood for clinical chemistry studies was collected into tubes containing no anticoagulant and centrifuged to obtain serum. Serum chemistry parameters included: alanine aminotransferase, aspartate aminotransferase, total protein, albumin, glucose, blood urea nitrogen, creatinine, cholesterol, triglyceride, and alkaline phosphatase. Parameters were analyzed using an automatic clinical analyzer (Hitachi 7080, Hitachi High-Technologies Corporation).

All rats were humanely sacrificed at the end of the test, and a complete necropsy was performed. Organ weights were obtained for the heart, kidneys, liver, spleen, testes and thymus. Paired organs were weighed together. Organ-to-body weight ratios (relative weight) were also calculated. In addition to the above-mentioned organs, the following tissues (when present) were sampled and fixed in 10% neutral-buffered formalin: cecum, colon, duodenum,

esophagus, femur with bone marrow, ileum, jejunum, lacrimal gland, lung, lymph node, mammary gland, nasal turbinates, pancreas, pituitary gland, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle (thigh), skin, spinal cord, sternum with bone marrow, trachea, urinary bladder, and vagina.

All stored organs and tissues from each animal in the control group and high-dose group were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and subjected to microscopic examination. Macroscopic lesions observed at necropsy were also examined from each animal in other dose groups.

The SPSS Statistical System (SPSS for Windows 11.0, Chicago, USA) was used to analyze body weights, food consumption, clinical pathology, and organ weights, followed by testing for variance homogeneity. Data were presented as the mean \pm SD. Standard analysis of variance (ANOVA) was used for statistical evaluation of the data and Dunnett's multiple comparisons were used to analyze the significance of differences between control and treated groups. All statistical tests were performed at the $P < 0.05$ level of significance.

No mortality or treatment related adverse clinical reactions were found during the study.

Body Weights and Food Consumption

There were no statistically significant differences in body weights between the treatment groups and the control group in each week (Figure 1). No significant differences were observed between the treatment groups and the control group in weekly food consumption of females and males (Figure 2).

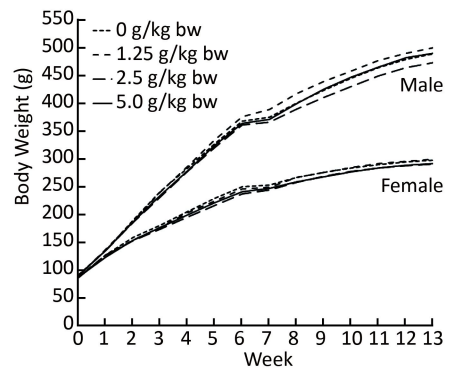


Figure 1. Mean body weights of female and male rats received different levels of *L. fermentum* GM 090 for 90 days.

Clinical Pathology

There were some sporadic, statistically significant changes in some hematology and clinical chemistry parameters (Table 1).

On Day 46, for males at 2.5 g/kg body weight group, red blood cell counts were significantly higher than the control group. At the end of the study, urea nitrogen and creatinine at 2.5 g/kg body weight group of females were significantly decreased as compared with the control group.

There were no statistically significant differences between the treatment groups and the control group for all other hematology and clinical chemistry parameters measured.

There were no statistically significant differences in all absolute organ weights and relative organ weights (organ-body weight ratios) between treatment groups and control group for male and females. No macroscopic pathology findings were observed in all males and females. Slightly sporadic focal necrosis in liver was found in three animals in the control group and four rats in the 5.0 g/kg body

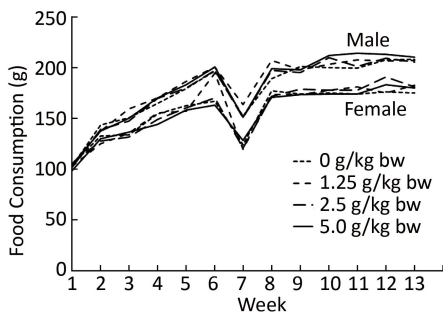


Figure 2. Mean food consumption of female and male rats received different levels of *L. fermentum* GM 090 for 90 days.

Table 1. Summary of Statistically Significant Hematology and Clinical Chemistry Parameters

Dose (g/kgbw)	n	Male (Day 46)	Female (Day 91)	
		Red blood cell count ($\times 10^{12}/L$)	Urea nitrogen (mmol/L)	Creatinine ($\mu\text{mol}/L$)
0.00	20	7.23 \pm 0.47	5.99 \pm 0.99	77.2 \pm 6.6
1.25	20	7.34 \pm 0.17	5.69 \pm 0.69	71.2 \pm 9.0
2.50	20	7.63 \pm 0.22*	5.13 \pm 0.70*	68.5 \pm 5.4*
5.00	20	7.42 \pm 0.25	6.23 \pm 0.44	79.6 \pm 7.3

Note. * $P < 0.05$, as compared with the control.

weight treatment group. Except for the findings mentioned above, no microscopic lesions were observed from the other tissues.

Probiotics including *Lactobacillus* strains are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. Many *Lactobacillus* strains have been afforded GRAS classification because of their long history of safe use in food production^[10]. No pathogenicity or acute oral toxicity to animals^[14-15] or humans^[16-17] was confirmed for most *Lactobacillus* species. Still, strong recommendation has been suggested to confirm the safety of any newly isolated strains before their incorporation into food products. So, the potential toxicity of *L. fermentum* GM 090 was assessed in our study.

In our present study, *L. fermentum* GM 090 did not lead to any general organ or systemic toxicity when fed to rats at dietary concentrations as high as 5.0 g/kg body weight.

Isolated statistically significant changes of some hematology and clinical chemistry parameters in *L. fermentum* GM 090 treatment groups were observed. Red blood cell counts for males at 2.5 g/kg body weight group were significantly higher than the control group, and urea nitrogen and creatinine at 2.5 g/kg body weight group of females were significantly decreased as compared with the control group; however, the changes were not dose-responsive and within the laboratory's historical normal range of controls (the normal ranges for red blood cell counts, nitrogen and creatinine are 7.07-8.05, 4.96-7.14, and 65.7-82.1, respectively), therefore, the effects were not considered to be toxicological significance.

Similarly, microscopic changes in the liver and heart, within the range of normal background lesions and randomly distributed among different groups, were considered incidental and reflected the usual individual variability without any relationship to treatment.

In conclusion, the results of the present study demonstrate that *L. fermentum* GM 090 are non-toxic up to a level of 5.0 g/kg body weight, when given orally. The No-Observed-Adverse-Effect-Level (NOAEL) for *L. fermentum* GM 090 was 5.0 g/kg body weight (approximately equivalent to 1×10^{10} cfu/kg-bw) in male and female rats, the highest dose tested.

*This work was supported by the National Science and technology support program (2012BAK01B04).

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Received: September 21, 2012;

Accepted: February 12, 2013

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