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Oral toxicity evaluation of kefir-isolated *Lactobacillus kefiranofaciens* M1 in Sprague–Dawley rats

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ABSTRACT

Lactobacilli kefiranofaciens M1 has shown novel immunomodulation and anti-allergy probiotic attributes in cell and animal models. An acute oral toxicity assessment of *L. kefiranofaciens* M1 was evaluated in Sprague–Dawley rats. The rats were randomly assigned to four groups (12 rats/sex/group): the low dose group was orally gavaged with *L. kefiranofaciens* M1 at 3.0×10^8 cfu/kg bw while the medium dose and high dose groups received 9.0×10^9 cfu/kg bw and 1.8×10^{10} cfu/kg bw, respectively, for 28 days. The control group received phosphate buffer saline. The body weights were measured weekly while blood samples were collected for haematology and serum biochemistry tests. Histopathology of the organs (heart, liver, kidney, adrenal glands, spleen, ovary, testis), and urinalysis were conducted on study termination. The body weight gain of the *L. kefiranofaciens* M1 and control groups were comparable during the administration period. Overall, *L. kefiranofaciens* M1 did not induce adverse effects on haematology, serum biochemistry, and urinalysis parameters. Gross and microscopic histopathology of the organs revealed no toxicity effect of *L. kefiranofaciens* M1. In conclusion, 1.8×10^{10} cfu/kg bw of *L. kefiranofaciens* M1 was considered as the no-observed-adverse-effect-level (NOAEL), which was the highest dose tested in the present study.

1. Introduction

In recent years, there has been increased reports on the beneficial health effects of probiotics in alleviating or preventing several disorders such as acute gastroenteritis (Francavilla et al., 2012), antibiotic-associated diarrhea (Hickson, 2011), lactose intolerance (Ojetti et al., 2010), inflammatory bowel syndrome (Stephani et al., 2011; Clarke et al., 2012), colorectal tumorigenesis (Zhu et al., 2011), constipation (Tabbers et al., 2011), allergy and atopic diseases (Kalliomaki et al., 2010; Wu et al., 2012). Consequently, new lactic acid bacteria strains (*Lactobacillus* and *Bifidobacterium* genus) with potential probiotic attributes are continuously being isolated and supplemented into food products. *Lactobacillus kefiranofaciens* M1 is one of the predominant lactic acid bacteria in *kefir*, a fermented milk beverage (Chen et al., 2008) and several studies have indicated the novel utility of this bacterium as a probiotic due to its immunomodulation and anti-allergic potential (Hong et al., 2010, 2011; Chen et al., 2012). Recent studies of *L. kefiranofaciens* M1 using sensitized mouse models showed diminished total and ovalbumin-specific IgE levels (Hong et al., 2010, 2011), and reduced airway inflammation (Hong et al., 2011). Moreover, *L. kefiranofaciens* M1 decreased IL-5 (T helper, Th1 cytokines) and increased IL-12, IL-2, IFN-γ, TNF-α, IL-1β levels (Th2 cytokines) in murine splenocyte and macrophage cells (Hong et al., 2009, Hong et al., 2010). It is widely suggested that the probiotic antiallergic effects are mediated via improvement of the T helper cells (Th)

Abbreviations: NOAEL, no-observed-adverse-effect-level; GRAS, generally recognized as safe; cfu, colony forming units; PBS, phosphate buffer saline; SD, Sprague–Dawley rats; ALP, alkaline phosphatase; GOT, glutamate oxaloacetate transaminase; GPT, glutamic-pyruvic transaminase; γGT, gamma glutamyl transferase; RBC, red blood cells; MCV, Mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; APTT, activated partial thromboplastin time; INR, international normalized ratio; Urea-N, urea-nitrogen; TP, total protein; WBC, white blood cells; SPSS, Statistics Package for Social Science.

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1/Th2 immunobalance by inducing the Th1 cytokines and suppressing Th2-skewed immuno-response. Furthermore, Hong et al. compared the immunostimulatory effects between live and heat-inactivated *L. kefiranofaciens* M1 and found insignificant differences, thereby suggesting that the cell wall components may be essential in the immunomodulation effects of the heat-inactivated analog (Hong et al., 2010).

Traditional lactic acid bacteria strains have been used for ages in food production without adverse health effects and hence are commonly designated GRAS status of food ingredients (Mattia and Merker, 2008). However, isolated case reports have shown some probiotics can cause infections, particularly in patients with organ failure, immunocompromised status and dysfunctional gut barrier (Liong, 2008; Whelan and Myers, 2010). Therefore, due to limited information on the newly isolated lactic acid bacteria strains, major safety concerns regarding their probiotic potential still remain (Borriello et al., 2003). To our knowledge, no comprehensive toxicological assessment data for *L. kefiranofaciens* M1 is available in published studies. It is necessary that an in depth investigation on safety be conducted as a prerequisite for determination of its suitability for human consumption. Therefore, the purpose of this study was to evaluate acute oral toxicity of *L. kefiranofaciens* M1 in male and female Sprague–Dawley rats.

2. Materials and methods

2.1. L. kefiranofaciens M1 culture

Heat-killed cultures of *L. kefiranofaciens* M1 were kindly provided by Prof. Ming-Ju Chen from Department of Animal Science and Technology, National Taiwan University (Taipei, Taiwan). The cultures were previously isolated from *kefir* and identified as described elsewhere (Chen et al., 2008). Briefly, the lactobacilli were grown and harvested, then inactivated by heating at 85 °C for 40 min as illustrated by Chen et al. (2010). The samples were supplied in powder form with original concentration of heat-inactivated *L. kefiranofaciens* M1 at 8.0×10^{12} cfu. Using PBS buffer, appropriate dilutions were prepared for low dose treatment (8.0×10^{7} cfu/ml), medium dose treatment (2.4×10^{9} cfu/ml), and high dose treatment (4.8×10^{9} cfu/ml) for further use in the experimental design. These dosages are equivalent to 3×10^{8} cfu/kg bodyweight (bw) (low dose), 9×10^{9} cfu/kg bw (medium dose) and 1.8×10^{10} cfu/kg bw (high dose). Based on the previous anti-allergy functional tests for *L. kefiranofaciens* M1 using sensitized allergy mouse models, the beneficial effects were evident at dosage ranges equivalent to 5.63×10^{7}–2.25×10^{8} cfu/kg bw (Hong et al., 2010). Thus 3×10^{8} cfu/kg bw was set as the baseline dosage representing the low dosage in the present toxicity study. The medium dosage was derived using 30-fold interval ascending dosage setting. However, following EPA (2000) guideline on interval ascending dose levels setting, and with reference to other published highest dosage ranges (10^{9}–10^{10} cfu/kg) used in other equivalent toxicity studies on probiotics (Zhou et al., 2000; Lara-Villoslada et al., 2007a,b; Yakabe et al., 2009), we derived the high dosage from the medium dosage value using a twofold interval ascending dose levels setting.

2.2. Animals

A total of 48 male and 48 female Sprague Dawley (SD) rats aged 6–7 weeks were supplied by Lasco Suppliers Ltd. (Taipei, Taiwan). The mean body weights (mean ± standard deviation) for males and females at receipt were 260.57 ± 10.99 g and 199.39 ± 7.03 g, respectively. All animals were examined for clinical signs on receipt and none had abnormalities suggestive of ill health. Sets of three rats were randomly assigned into polypropylene cages and the animals were acclimatized for one week before the start of the experiment. The animal chamber temperature and relative humidity were regulated within limits of 23 ± 2 °C and 30–70%, respectively. The rats were maintained in a 12-h light/dark cycle. Standard LabDiet rodent chow (Brentwood, USA) and water were offered *ad libitum*. All the animal treatments were carried out in accordance with the principles of laboratory animal care as outlined by the Institutional Animal Care and Use Committee.

2.3. Experimental design

The rats were randomly assigned to four groups (12 rats/sex/group): The low dose group was orally gavaged with *L. kefiranofaciens* M1 at 3.0×10^{8} cfu/kg bw while the medium dose and high dose group received 9.0×10^{9} cfu/kg bw and 1.8×10^{10} cfu/kg bw, respectively, for 28 days. The control group received PBS buffer. The rats in the treatment groups were each orally gavaged with 1 ml of *L. kefiranofaciens* M1 preparation once per day for 28 days.

Each rat was visually inspected daily for development of any physical appearance abnormalities during the study period. The body weights were recorded at pre-test and thereafter weekly. On day 28, the animals were fasted overnight and urine samples were collected for urinalysis. The animals were individually placed in metabolic cages, deprived of diet and water. On day 29, the animals were humanely sacrificed under diethyl ether (J. T. Baker, USA) anaesthesia. Blood was collected from the abdominal aorta and transferred into sampling tubes for blood biochemistry and haematology tests. The selected organs (liver, heart, spleen, kidney, testis, ovaries) were weighed and thereafter histopathological examinations were conducted.

2.4. Determination of serum biochemistry

The whole blood was centrifuged to obtain serum, which was analyzed at the Medical Laboratory Department, Taipei Medical University (TMU) Hospital using an auto-analyzer (Roche Modular P800, Hitachi, Japan). The following parameters were tested: albumin, total protein (TP), urea nitrogen (Urea-N), creatinine, cholesterol, triglyceride, alkaline phosphatase (ALP), glutamate oxaloacetate transaminase (GOT), glutamic-pyruvic transaminase (GPT), gamma glutamyl transferase (γGT), total bilirubin, chloride, sodium, potassium, calcium, glucose, and phosphorus.

2.5. Determination of haematology profile

The haematology profile was analyzed at Medical Laboratory Department, TMU Hospital using an automatic haematology analyzer (Roche Modular P800, Hitachi, Japan). The following parameters were measured: white blood cells counts (WBC), red blood cells counts (RBC), haemoglobin, heamocrit, mean corpuscular volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), platelet counts, neutrophils, lymphocytes, monocytes, eosinophils, basophil segments, prothrombin time, activated partial thromboplastin time (APTT), and international normalized ratio (INR).

2.6. Histopathological examination

Selected organs (liver, heart, spleen, kidney, testis, ovaries) were weighed and fixed in 4% formaldehyde solution (Mallinckrodt Chemicals, USA) for histopathological examination at the Department of Pathology, Taipei Medical University, Taiwan. The respective organs were embedded in paraffin, sectioned, stained with hematoxylin and eosin before microscopic examination for inflammation in the heart, liver, kidney, adrenal glands, spleen; and defective testis spermatogenesis and ovum production.

2.7. Determination of urinalysis profile

The analysis of blood, bilirubin, urobilinogen, ketone bodies, proteins, nitrite, glucose, pH, specific gravity, and leukocytes was conducted using urinary test strip (Uriscan SGL, Korea).

2.8. Statistical analysis

The PAWS Statistical System (SPSS for Windows 18.0, Chicago, USA) was used for data analysis. Data were expressed as the mean ± standard deviation for male and female rats. Two-way Analysis of variance was used for statistical evaluation while Tukey's multiple comparison was used to analyze the significance of differences between the control and *L. kefiranofaciens* M1 treatment groups. All statistical tests were performed at the $p < 0.05$ level of significance.

3. Results

3.1. General health status and body weight changes

No abnormal changes in hair appearance, watery eyes and runny nose were observed in both sexes of rats in the *L. kefiranofaciens* M1 and control groups during the 28-days administration period. Two male rats in the low-dose treatment group died during routine oral feeding in week 3 due to suffocation by the feeding tubes. There were no significant ($p > 0.05$) differences in absolute body weight gain between the *L. kefiranofaciens* M1 and control groups in both sexes (Fig. 1). Therefore, rats that received *L. kefiranofaciens* M1 treatments exhibited an identical growth pattern to those of the control group.

3.2. Serum biochemistry profile

Serum biochemistry parameters such as albumin, total protein, creatinine, cholesterol, triglyceride, ALP, GPT, γGT, total bilirubin, chloride, sodium, calcium, glucose, and phosphorus were not