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Safety evaluation of *Lactobacillus pentosus* strain b240

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ABSTRACT

Lactobacillus pentosus has a long history of use in cooked and uncooked fermented foods. Viable and heat-killed nonviable preparations of *L. pentosus* strain b240 were evaluated for short term and subchronic toxicity and genotoxic potential. Dose levels were determined through acute oral toxicity tests with viable (LD_{50} > 2500 mg/kg) and nonviable (LD_{50} > 2000 mg/kg) b240. In the short term study, rats received 2500 mg/kg/day (~1.7×10^{11} cfu/kg/day) viable b240 for 28 days. In the subchronic study, rats received 500, 1000 or 2000 mg/kg/day (up to ~3.0×10^{12} cfu equivalents/kg/day) nonviable b240 for 91 days followed by a 28-day recovery. No mortalities occurred. No treatment-related effects were identified for general condition, body weight, food–water consumption, ophthalmology, urinalysis, hematology, blood chemistry, organ weights, histopathology and gross pathology. Although statistically significant effects were noted for several endpoints in the short term and subchronic studies, none were related to the test materials. The NOAEL for nonviable b240 was 2000 mg/kg/day, the highest dose tested. Additionally, nonviable b240 (⩽5000 μg/plate) was not mutagenic in *Salmonella typhimurium* or *Escherichia coli* tester strains nor did nonviable b240 orally administered to rats at levels ⩽ 2000 mg/kg/day for two days, induce a clastogenic response.

1. Introduction

Lactobacilli have a long history of use in human foods and are naturally present among human gut flora (Guarner, 2006; Salminen et al., 1998). Utilized for centuries in the fermentation of food and dairy products, Lactobacilli are valued as a means of enhancing aromas and flavors, modifying the textures, increasing the nutritional value, and preserving foods (Edwards and Parrett, 2002; Kostinek et al., 2007; Rahayu, 2003; Salminen et al., 1998; Tamminen et al., 2004). *Lactobacilli* are generally considered non-pathogenic to humans (Guarner, 2006; Salminen et al., 1998; Wassernaar and Klein, 2008). Although a few species have been associated with opportunistic infection (Aguirre and Collins, 1993; Cannon et al., 2005; Gasser, 1994), many within the genus are commensal organisms found naturally within the human oral and vaginal cavities and throughout the intestinal tract (Falsen et al., 1999; Guarner, 2006; Okkers et al., 1999).

Isolated from sauerkraut in 1894 (Fred et al., 1921), *Lactobacillus pentosus* (*L. pentosus*) has since been identified as playing a traditional role in the preparation of many common foods, including raw and soured milks (Kim et al., 2006; Manolopoulou et al., 2003), cheeses (De Angelis et al., 2001; Manolopoulou et al., 2003; Sánchez et al., 2000), sourdough starters and breads (Catzeddu et al., 2006; Randazzo et al., 2005; Robert et al., 2009), fermented cereals and vegetables including sauerkraut, pickles, eggplant, cassava and olives (Bergqvist et al., 2006; Brenes et al., 2004; Panagou et al., 2008; Rahayu, 2003; Sánchez et al., 2000; Tamminen et al., 2004; Tanasupawat et al., 2007; Todorov and Dicks, 2007), fermented meats and fish (Klingberg et al., 2005; Kostinek et al., 2007; Mogensen et al., 2002; Pulido et al., 2007; Rahayu, 2003; Tanasupawat et al., 1998), and fermented beverages such as sake (Iino et al., 2003), tea (Tanasupawat et al., 2007)

Abbreviations: 2-AA, 2-aminoanthracene; 9-AA, 9-aminoacridine hydrochloride; AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; A/G, albumin–globulin; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; b240, *Lactobacillus pentosus* strain b240; bw, body weight; cfu, colony forming units; CP, cyclophosphamide monohydrate; GLP, Good Laboratory Practice; IACUC, Institutional Animal Care and Use Committee; *L. pentosus*, *Lactobacillus pentosus*; LD_{50}, the dose that produces 50% lethality in the test population; MCV, mean corpuscular volume; MHW, Ministry of Health and Welfare (Japan); MPCE, micronucleated polychromatic erythrocytes; NaN_3, sodium azide; NOAEL, no-observed-adverse-effect-level; PCE, polychromatic erythrocytes; PT, prothrombin time; SD, Sprague–Dawley; WFI, water for injection.

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and Scotch malt whiskey (Klingberg and Budde, 2006). *L. pentosus* is consumed as live viable colonies in uncooked foods (e.g. soured milks and cheeses) and as dead nonviable cells in fermented foods having a later cooked stage (e.g. sourdough breads); the intentional use and natural presence of *L. pentosus* in foods has been international in scope, encompassing countries throughout Europe, Africa and Asia. Similar to other *L. pentosus* strains, strain b240 was originally isolated from *miang*, a non-salted fermented tea traditional to northern Thailand (Okada et al., 1986). Initially identified as a strain of *L. plantarum* (a phenotypically similar species), the bacterium was later reclassified as *L. pentosus* by genetic analysis using recA (Personal communication; Otsuka Pharmaceutical Co. Ltd.).

To evaluate the general toxicity of *L. pentosus* strain b240 (b240) in the rat model, a short-term 28-day repeat dose study and a subchronic 91-day repeat dose study (with subsequent 4-week recovery period) were performed. Clinical observations, blood and urine assays, and gross and microscopic pathology were conducted to evaluate whether repeated ingestion of b240 induced any sign of toxicity in the rat model. Reversibility, progression and/or the appearance of delayed changes were also evaluated during the recovery period in a subset of treatment and control animals. To evaluate the mutagenic and clastogenic potentials of heat-killed nonviable b240, bacterial reverse mutation (Ames et al., 1975; Ohta et al., 1998) and rat micronucleus (Miller, 1973; Schmid, 1975) assays were performed. To determine dose levels for these tests, acute toxicity was determined in the rat using viable and nonviable bacteria. All studies were conducted following GLP or GLP-like protocols (described in Section 2.4.).

2. Materials and methods

2.1. Test articles

All studies were conducted by Otsuka Pharmaceutical Co. Ltd., Tokyo 101-8535, Japan. Both the viable test article, a white to slightly brownish yellow lyophilized powder, and the heat-killed nonviable test article, a white lyophilized powder, were supplied by Otsuka. The viable test material was prepared by adding 10% skim milk powder and 1% sodium glutamate as a vehicle to b240 after the harvested bacteria had been washed two times in saline but prior to lyophilization (viable count, 6.9×10^7 cfu/mg). During storage, the viable test material was shielded from light under refrigerated conditions (1–8 °C). The nonviable test material was prepared by suspending harvested b240 in distilled water after the bacteria had been washed two times in saline but before autoclave (viable count prior to sterilization, 1.5×10^9 cfu/mg) and lyophilization. The nonviable test material was stored with silica gel in light-resistant containers under refrigerated conditions (1–8 °C). No materials other than b240 bacteria were present in the nonviable test article. The placebo, a white to slightly brownish yellow lyophilized powder, consisted of powdered 10% skim milk with 1% sodium glutamate. Vehicle solvents, normal saline and water for injection (WFI), were supplied by Otsuka Pharmaceutical Factory Inc.

2.2. Chemicals and materials

2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2), 2-aminoanthracene (2-AA), cyclophosphamide monohydrate (CP), and sodium azide (NaN_3) were supplied by Wako Pure Chemical Industries Ltd. 9-Aminoacridine hydrochloride (9-AA) was supplied by ICN Biomedicals Inc. Components for the S9 metabolic activation mix used in the bacterial reverse mutation assay were obtained from Oriental Yeast Co. Ltd. and included the supernatant fraction of rat liver homogenate (derived from male Sprague–Dawley (SD) rats pretreated with the inducers phenobarbital and 5,6-benzoflavone) and Cofactor-1. Nutrient Broth No. 2 (nutrient medium for the test strains of the genotoxicity assay) was supplied by Oxoid Ltd. Unless otherwise specified, water was in-house and distilled.

2.3. Animals and organisms

Male and female Sprague–Dawley (SD) rats were supplied by the Atsugi and Hino Breeding Centers of Charles River Laboratories Japan Inc. for the acute, 28-day repeat dose and subchronic studies and for the micronucleus assay. In the acute and 28-day rat studies, the acclimatization period was seven to eight days before initial dosing. In the 91-day subchronic study, the acclimatization period was 15 days. The rats were 6 weeks old at first dosing in the acute, 28-day and subchronic studies and 8 weeks old at the beginning of the micronucleus assay. Initial body weights of the test animals for the viable and nonviable acute studies ranged from 153–165 g for the male rats and 119–137 g for the females. Weights for male and female rats in the 28-day repeat dose study ranged between 202–221 g and 141–160 g, respectively, and in the subchronic study between 213–260 g and 146–173 g, respectively. Male rats utilized in the micronucleus assay weighed between 295 and 325 g.

All animals were visually inspected at the time of delivery and during the quarantine-acclimation period by a veterinarian associated with the attendant Institutional Animal Care and Use Committee (details provided in Section 2.4.). Those animals found unsuitable due to obvious ill-health or to abnormality in general condition, behavior and/or body weight, were excluded. The animals were housed individually. Housing consisted of stainless steel wire mesh cages in rooms with 12-h light cycles (7:00 AM–7:00 PM). Room temperature was maintained at 21.0–24.9 °C with a relative humidity of 45.8–71.7% and 10–20 air changes/h. Potable water and a pellet diet (CRF-1) prepared by Oriental Yeast Co. Ltd were provided *ad libitum* prior to dosing. Rats in the acute studies were fasted 16–18 h before dosing. Rats in the 28-day repeat dose and subchronic studies were fasted during a 4-h urine collection and 16–24 h before necropsy. The *Salmonella typhimurium* and *Escherichia coli* test strains for the bacterial reverse mutation assays were obtained from the Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo 158-8501, Japan.

2.4. Guidelines

All studies were conducted in compliance with the "Partial Amendments to the Law for the Humane Treatment and Management of Animals (Law No. 68, Jun. 22, 2005, Japan)" and the "Guidance for Animal Care and Use (revised on Nov. 7, 2007)" of Ina Research Inc., Nagano 399-4501, and in accordance with the protocol reviewed by the Institutional Animal Care and Use Committee (IACUC) of Ina Research Inc., which is fully accredited by AAALAC International (Accredited Unit No. 001107).

The acute and 28-day repeat dose studies were conducted under the Revisions of Guidelines for Single and Repeated Dose Toxicity Studies (Notification No. 88) issued by the Ministry of Health and Welfare (MHW), Japan on August 10, 1993. The subchronic study and clastogenic assay were conducted under the Good Laboratory Practice (GLP) Standards for Non-Clinical Safety Studies on Drugs (Ordinance No. 21, March 26, 1997, partially revised by Ordinance No. 114, June 13, 2008) issued by MHW, Japan. The mutagenic assays were conducted in accordance with the MHW Guidelines for Genotoxicity Studies of Drugs (Notification No. 1604, November 1, 1999).

2.5. Experimental design

2.5.1. Acute oral toxicity studies

Viable b240 in normal saline was administered *via* gavage to five male and five female Crl:CD(SD) rats in a single oral dose of 2500 mg/kg ($\sim 1.7 \times 10^{11}$ cfu/kg). An equal number of animals served as placebo (2500 mg/kg single oral dose) and vehicle controls. Rats were observed for clinical signs before dosing, immediately after dosing, at 0.5, 1, 2, 3, 4, 5 and 6 h post-dosing, and daily thereafter for 14 days. Body weights were measured before dosing and on Days 1, 3, 7, 10 and 14, with food consumption measured post-dose for Days 1–3, 3–7, 7–10 and 10–14. On Day 14, all rats were euthanized by exsanguination under ether anesthesia and necropsied.

Heat-killed b240 in WFI was administered *via* gavage to five female Crj:CD(SD)IGS rats in a single oral dose of 2000 mg/kg ($\sim 3.0 \times 10^{12}$ cfu equivalents/kg). Rats were observed for clinical signs before dosing, immediately after dosing, at 0.25, 0.5, 1, 2, 4 and 6 h post-dosing, and daily thereafter for 14 days. Body weights were measured before dosing and on Days 1, 3, 7 and 14. On Day 14, all rats were euthanized by exsanguination under ether anesthesia and necropsied.

2.5.2. 28-Day repeat dose study

Ten male and 10 female Crl:CD(SD) rats each were administered 2500 mg/kg ($\sim 1.7 \times 10^{11}$ cfu/kg) of viable b240 in normal saline *via* gavage each day for 28 days. An equal number of animals served as placebo and vehicle controls. Clinical observations were made twice daily (before and 4 h after dosing) during the treatment period. Body weights were measured once per week during the treatment period and on the day of necropsy. Food consumption was measured once a week during the dosing period and on the last day of administration. On Day 29, all rats (having fasted for 18–24 h) were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital, euthanized by exsanguination and then necropsied.

2.5.2.1. Ophthalmologic observations. All rats underwent ophthalmological examination before the start of administration. At Week 4, five males and five females from each group were selected to repeat the eye exam. Each exam included gross examinations of optic media and the anterior and ocular fundus.

2.5.2.2. Urinalysis. Pre- and post-dose urine samples were collected from five males and five females of each group at Week 4. During urine collection periods, individual animals were housed in metabolism cages. Pre-dose urine was collected from fasted rats during the 4 h immediately prior to dosing. After dosing, urine was again collected from the same rats, having access to food and water, for 24 h thereafter. Urinalysis parameters examined in the 4-h collections were bilirubin, glucose, ke-