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## Safety assessment of *Lactobacillus brevis* KB290 as a probiotic strain

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### ABSTRACT

*Lactobacillus brevis* KB290 (KB290), a plant-derived probiotic lactic acid bacterium, reportedly improves gut health and stimulates immune function. Here we extensively investigated the geno-, acute, subacute, and subchronic toxicity of KB290 and its bacterial translocation potential. KB290 was non-mutagenic in the bacterial reverse mutation assay by the preincubation method. In the single oral dose toxicity test, KB290 at  $\geq 10^9$  cfu/ml was nontoxic at maximum capacity (20 ml/kg). When  $10^8$ ,  $10^9$ , or  $10^{10}$  cfu/kg was administered daily to rats by gavage for 2 weeks (subacute assay), we observed no clear treatment-related effect and no evidence of bacterial translocation from the gastrointestinal tract. When it was administered for 13 weeks (subchronic assay), we again observed no clear treatment-related effect and no significant toxicological effect. Based on those results, we consider  $10^{10}$  cfu/kg per day, the highest dose tested, to be the no observed adverse effect level (NOAEL). These results suggest that KB290 is safe for human consumption.

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### 1. Introduction

Probiotics are live microorganisms that confer a health benefit on the host when administered in adequate amounts (FAO/WHO, 2001). Probiotics have received increasing attention in recent years and have been shown to be useful to humans. They are beneficial for viral diarrhea (Allen et al., 2003), antibiotic-associated diarrhea (D'Souza et al., 2002), allergic diseases (Ouweland, 2007), inflammatory bowel disease (Mach, 2006; Ewaschuk and Dieleman, 2006; Bai and Ouyang, 2006), irritable bowel syndrome (Drouault-Holowacz et al., 2008; McFarland and Dublin, 2008), and constipation (Hamilton-Miller, 2004). Probiotics exhibit strain-specific differences in acid and bile resistance, ability to colonize the gastrointestinal tract, clinical efficacy, and health benefit to the host (Pham et al., 2008).

Lactic acid bacteria (LAB) have proven useful for food fermentation and have been ingested safely with fermented foods throughout history. Safety concerns have been raised, however, because probiotic LAB have been isolated from patients with endocarditis (Presterl et al., 2001; Wallet et al., 2002; Zé-Zé et al., 2004), sepsis

(Salminen et al., 2004; Ha et al., 1999; Aguirre and Collins, 1993), liver abscesses (Rautio et al., 1999; Cukovic-Cavka et al., 2006) and urinary tract infections (Aznar et al., 2004). Recently, probiotics were associated with an increased risk of mortality in patients with severe acute pancreatitis (Besselink et al., 2008).

In our laboratory, we have isolated a plant-derived LAB, *Lactobacillus brevis* KB290 (KB290), from *suguki*, a traditional Japanese fermented vegetable. Since KB290 tolerates digestive juices, stimulates immune function (Kishi et al., 1996), and improves gut health (Nobuta et al., 2009), it meets the criteria for a probiotic strain. In this study, we performed a safety assessment of KB290 to determine its suitability for human consumption. We evaluated its geno-, acute, subacute, and subchronic toxicity. We did not perform a chromosome aberration because KB290 is a live microorganism and would inhibit the growth of the test cell. We did not conduct a mouse micronucleus assay or a toxicokinetic study because ingested live microorganisms, other than invasive pathogenic species, are not absorbed from the intestinal tract (Edmiston and Condon, 1991), and negative results of the bacterial translocation test indicate that a toxicokinetic study was not needed.

### 2. Materials and methods

#### 2.1. Cell culture

KB290 (International Patent Organism Depository Accession Number, FERM BP-4693), a gift of Dr. T. Kishida (Louis Pasteur Center for Medical Research, Japan), was inoculated into 500 ml carrot juice medium (Wm. Bolthouse Farms, US) and

Abbreviations: LAB, lactic acid bacteria; KB290, *Lactobacillus brevis* KB290; BT, bacterial translocation; NOAEL, no observed adverse effect level; cfu, colony forming units; GLP, the good laboratory practice; MHW, the Ministry of Health and Welfare of Japan; *L.*, *Lactobacillus*.

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cultured 32 °C for 18 h to the maximum achievable concentration of 10<sup>9</sup> cfu/ml. Dilutions of 10<sup>8</sup> and 10<sup>7</sup> cfu/ml were prepared in carrot juice medium and stored at 4 °C until use.

## 2.2. Bacterial reverse mutation assay

We performed the bacterial reverse mutation assay at Hatano Research Institute, Food and Drug Safety Center, by the preincubation method with and without S9 mix (Kikkoman, Japan) using *Salmonella typhimurium* TA100, TA1535, TA98, and TA1537 and *Escherichia coli* WP2 *uvrA* as the tester strains (Ames et al., 1975; Ohta et al., 1998). We used water for injection JP (Otsuka Pharmaceutical Factory Co. Ltd., Japan) as the negative control and 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (Wako Pure Chemical Industries, Japan), sodium azide (Wako), 9-aminoacridine (Sigma Chemical, US), and 2-aminoanthracene (Wako) as positive controls.

## 2.3. Animals

We obtained 4-week-old (acute toxicity assay) or 29–35-day-old (subacute and subchronic toxicity assay) male and female Crl:CD (SD) rats from Charles River (Japan or UK). The animals were housed in cages with appropriate space and allowed free access to solid diet (CE-2, CLEA Japan, Inc. [acute toxicity assay]; Rat and Mouse No. 1 Maintenance Diet, Special Diet Services, UK [subacute and subchronic toxicity assay] and tap water and were acclimatized for 8 days (acute toxicity assay), 26 days (subacute toxicity assay), or 11 days (subchronic toxicity assay). They were administered the test substances through a stomach tube.

## 2.4. Acute toxicity

For the acute toxicity assay, 10 rats of each sex were fed KB290 at dose levels based on the maximum achievable concentration and the maximum dose volume (20 ml/kg) according to the guideline for single and repeat dose toxicity studies (Notification #88, the Ministry of Health and Welfare of Japan [MHW], 1993).

The animals were fasted for 18 h, weighed, given the test substance, and permitted solid diet 3 h later. We recorded the animals' general appearances and bodyweight. On day 15, we anesthetized them with pentobarbital, killed them by bleeding, and performed a macroscopic examination. This study conformed to the following Japanese guidelines: Good Laboratory Practice standards for safety studies on drugs (Ordinance #21, MHW, 1997), and the guideline for application and evaluation of foods for specified health uses (Notification #21, MHW, 1998). It was conducted at Hatano Research Institute, Food and Drug Safety Center, with approval of the institutional animal care and use committee.

## 2.5. Subacute toxicity and bacterial translocation

The subacute toxicity study consisted of a control group (six rats of each sex), which was fed carrot juice medium, and three treatment groups that were fed KB290 daily for 2 weeks at the following doses (cfu/kg): 10<sup>8</sup> (three rats), 10<sup>9</sup> (three rats), and 10<sup>10</sup> (six rats). The dose levels were based on the maximum achievable concentration and the maximum dose volume for repeat dosing (10 ml/kg). We inspected the animals visually at least twice daily and recorded their weight and food consumption.

Feces from three rats of each sex in the control and high dose groups were collected on days 4, 8, and 15. On Day 15, 1 ml blood was drawn from each animal into heparinized containers and the animals were killed by carbon dioxide asphyxiation. We removed and weighed the brain, kidney, spleen, liver, heart, lungs, and mesenteric lymph nodes, and we removed the contents of the stomach, small intestine (7th loop), and cecum. We cultured 1-g samples of the organs, blood, intestinal contents, and feces anaerobically at 32 °C for 72 h on MRS agar, counted colonies typical of KB290, and calculated the cfu/g in the sample.

This study was conducted at Huntingdon Research Centre, Huntingdon Life Sciences Ltd., in compliance with the UK GLP regulations, OECD Principles of GLP (1997 revision) and EC Commission Directive 2004/10/EC of 11th February 2004. The institutional animal care and use committee approved the study.

## 2.6. Subchronic toxicity and neurotoxicity

We based dose levels for the subchronic and neurotoxicity assay on the results of the subacute toxicity study. This study consisted of the same experimental groups described in 2.4.2 except each group consisted of 10 rats of each sex. The test substance was administered for 13 weeks.

We inspected the animals visually at least twice daily and recorded their weight and food consumption weekly. We examined their eyes before treatment commenced and during week 12 of the study. During week 13, we drew blood samples into EDTA-treated or heparinized tubes and collected overnight urine samples for hematology, blood chemistry, and urinalysis investigations.

We tested the sensory reactivity (approach response, touch response, auditory startle reflex, tail pinch response), grip strength, and motor activity of all animals before commencement of the study and before week 6 and 12. On day 92, we killed the animals by carbon dioxide asphyxiation, dissected the or-

gans, weighed them, and conducted detailed macroscopic and microscopic examinations. This study was conducted at Huntingdon Research Centre, Huntingdon Life Sciences Ltd. and followed the guidelines and requirements described in Section 2.5.

## 2.7. Statistical analysis

We analyzed the subacute, subchronic, and neurotoxicity results separately for males and females. We used the following sequence of statistical tests: if 75% of the data (across all groups) were the same, the pairwise Fisher Exact test (Fisher, 1973). If the Bartlett test (Bartlett, 1937) was not significant at the 1% level, the Williams (Williams, 1971, 1972) or Dunnett test (Dunnett, 1955, 1964). If the Bartlett test was still significant after logarithmic and square-root transformations, the Shirley (Shirley, 1977) or Steel test (Steel, 1959). For organ weight data, we initially performed analysis of covariance using terminal bodyweight as the covariate. We analyzed the data using Startox version 3.2 and SAS version 8.2. Data were expressed as mean ± SD.

## 3. Results

### 3.1. Bacterial reverse mutation assay

We did not observe a doubling of the number of revertant colonies in the bacterial reverse mutation assay, regardless of the presence of S9 mix (data not shown). All positive controls showed mutagenic activity, and the mean number of revertant colonies in the positive and the negative controls was within the range of historical background data.

### 3.2. Acute toxicity

Weight gains (male; 151 ± 16.5 g, female; 76 ± 11.9 g) were consistent with the background range, and we observed no adverse effects and no deaths. Necropsy showed no anomalous findings.

### 3.3. Subacute toxicity

One female in the intermediate dose group was found dead on day 11. No clinical signs were noted in the animal prior to death. It gained weight during the first week of the study but lost weight (3.3 g) between day 8 and the day of death. Macroscopic examination revealed congested and partially collapsed lungs and gaseous distension in the stomach. We saw no indication of the death being related to treatment with KB290. No clinical signs were noted for any other study animal.

For males, mean bodyweight gain in the high dose group (52 ± 15.6 g) was slightly less than in the control group (61 ± 15.6 g), but weight gain in the low (57 ± 13.3 g) and intermediate (66 ± 4.1 g) dose groups was similar to that in the control group. For females, mean bodyweight gain in all treatment groups (low dose; 24 ± 5.4 g, intermediate dose; 24 ± 12.0 g, high dose; 28 ± 9.4 g) significantly exceeded that of the control group (20 ± 5.2 g) ( $P < 0.05$ ). Mean food consumption did not differ significantly between the treatment and control groups.

Mean organ weight did not differ significantly between the treatment and control groups except that males in the intermediate and high dose groups had significantly elevated mean adjusted spleen weights ( $P < 0.05$ ). The weight differences were not related to dose, however, and all the individual values for animals in the high dose group were within the concurrent control range. Males in the intermediate and high dose groups had statistically significantly lower mean absolute thyroid and parathyroid weights, but there was no dose relationship and no similar effect in females.

Macroscopic examination revealed no lesions attributable to KB290 treatment. The incidence and distribution of all findings were consistent with the background range of macroscopic changes.

3.4. Bacterial translocation

We did not detect viable KB290 in samples of blood, brain, kidney, spleen, liver, heart, or lymph nodes from animals in the high dose group. We detected low to moderate numbers of commensal bacteria in the lungs of the high dose and control groups and high numbers in the intestinal tracts and feces of the control group. We found slightly increased numbers of bacteria in the cecum and feces of the high dose group (data not shown).

3.5. Subchronic toxicity and neurotoxicity

We detected no deaths and no clinical signs. The overall mean bodyweight gains were similar in the treatment and control groups (control; 302 ± 37.9 g [male], 109 ± 15.1 g [female], low dose; 320 ± 39.3 g [male], 117 ± 21.7 g [female], intermediate dose; 298 ± 32.4 g [male], 108 ± 19.7 g [female], high dose; 298 ± 34.5 g [male], 115 ± 15.6 g [female]). The overall mean food consumptions were also similar in the treatment and control groups. The ophthalmoscopic findings were within normal limits for animals of this age, breed, and strain.

Mean platelet counts were significantly lower in females in the intermediate and high dose group than in control group females, but the decreases were not dose related and all individual values were within the background data ranges.

Blood chemistry results revealed no significant differences between treatment and control groups in alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, total bilirubin, creatinine, total cholesterol, triglycerides, potassium, chloride, calcium, total protein, albumin, α1 globulin, globulin, β globulin, and γ globulin levels or the albumin/globulin ratio (data not shown). Mean glucose values were significantly elevated in both sexes in the high dose group and males in the intermediate dose group (Table 1). We found no corroborative histopathological

differences between the groups, however. Furthermore, the majority of individual values were within background data ranges. We found statistically significant differences in the mean levels of sodium, phosphorus, and α2 globulin in three treatment groups, but the levels were not related to dose, and all individual values were within background data ranges (Table 1).

Urinalysis revealed no statistically significant difference between treatment and control groups in volume, specific gravity, or protein (data not shown). Mean urinary sodium values were significantly elevated for females in the intermediate and high dose groups as were mean urinary chloride levels for females in the high dose group (Table 2). Mean urinary sodium and chloride values were slightly elevated in males, although not to the level of statistical significance (Table 2).

Absolute mean brain weights of males were significantly lower in the intermediate and high dose groups than in the control group ( $P < 0.05$ ), but there was no dose–response relationship and no similar finding in females. Female bodyweight adjusted mean thymus weight was significantly higher in the high dose group than in the control group ( $P < 0.05$ ), but there was no dose–response relationship and no similar finding in males (data not shown).

Macroscopic examination revealed no changes attributable to KB290 treatment. The nature and incidence of all the findings, both macroscopic and microscopic, were consistent with background historical data.

Sensory reactivity was unaffected by treatment. During treatment week 6, forelimb grip strength for both sexes was significantly lower in the high dose group than in the control group ( $P < 0.05$ ); hindlimb strength was unaffected. Mean values in the low and intermediate dose groups were also slightly decreased, but the differences did not reach statistical significance. For females during treatment week 12, forelimb grip strength in the high dose group was still lower than in the control group, although less markedly so (data not shown).

**Table 1**  
Remarkable blood chemistry results for rats treated with *L. brevis* KB290 for 13 weeks.

Sex	Dose (cfu/kg/day)	Gluc (mmol/l)	Na (mmol/l)	Phos (mmol/l)	a2 (g/l)
Male	0 (control)	6.81 ± 0.713	142 ± 1.4	2.11 ± 0.134	4 ± 0.8
	10 <sup>8</sup>	7.13 ± 0.565	140 ± 1.6*	1.99 ± 0.091*	4 ± 0.6
	10 <sup>9</sup>	7.55 ± 0.794*	140 ± 1.7*	1.92 ± 0.155*	5 ± 0.7
	10 <sup>10</sup>	7.93 ± 0.840**	140 ± 1.0*	2.05 ± 0.089*	4 ± 0.5
Female	0 (control)	6.38 ± 0.840	141 ± 0.9	1.65 ± 0.170	4 ± 0.5
	10 <sup>8</sup>	6.62 ± 0.750	141 ± 1.2	1.68 ± 0.229	4 ± 0.4
	10 <sup>9</sup>	6.27 ± 1.504	141 ± 1.6	1.53 ± 0.145	4 ± 0.6
	10 <sup>10</sup>	8.03 ± 0.942**	140 ± 1.1	1.56 ± 0.073	4 ± 0.7*

Values are mean ± SD ( $n = 10$ /dosage group).  
Gluc, glucose; Na, sodium; Phos, inorganic phosphorus; a2, α2 globulin.  
\*  $P < 0.05$ .  
\*\*  $P < 0.01$  (compared to control, Williams test).

**Table 2**  
Remarkable urinalysis results for rats treated with *L. brevis* KB290 for 13 weeks.

Sex	Dose (cfu/kg/day)	n	U–Na (mmol/l)	U–K (mmol/l)	U–Cl (mmol/l)
Male	0 (control)	10	7.4 ± 0.19	176.7 ± 73.21	48.5 ± 33.92
	10 <sup>8</sup>	10	7.2 ± 0.35	151.1 ± 25.46	56.8 ± 17.26
	10 <sup>9</sup>	10	7.5 ± 0.44	154.2 ± 32.21	55.7 ± 15.28
	10 <sup>10</sup>	10	7.3 ± 0.34	169.9 ± 30.31	61.7 ± 13.42
Female	0 (control)	10	6.8 ± 0.49	162.9 ± 38.28	45.7 ± 23.22
	10 <sup>8</sup>	10	6.4 ± 0.47	180.5 ± 44.95	54.2 ± 27.63
	10 <sup>9</sup>	10	6.3 ± 0.32	202.1 ± 69.06	64.9 ± 43.09
	10 <sup>10</sup>	8	6.5 ± 0.31	184.8 ± 60.35	80.4 ± 25.39*

Values are mean ± SD.  
U–Na, sodium; U–K, potassium; U–Cl, chloride.  
\*  $P < 0.05$ .  
\*\*  $P < 0.01$  (compared to control, Williams test).

Motor activity scores varied from group to group during treatment weeks 6 and 12, with a few of the 6-min interval scores achieving statistical significance, but no differences were associated with treatment (data not shown).

#### 4. Discussion

*L. brevis* is seen widely in nature and has been found in fermented foods of both plant and animal origin (Mante et al., 2003; De Vuyst et al., 2002; Sánchez et al., 2000) as well as in human intestinal flora (Gu et al., 2008; Delgado et al., 2005). Several laboratories have reported the utility of *L. brevis* as a probiotic (Soo et al., 2008; Rönkä et al., 2003; Ouwehand et al., 2001). The KB290 strain was isolated from a Japanese traditional fermented vegetable (*suguki*) that has been consumed for over 1000 years. Because of this history, *L. brevis* KB290 can be considered generally safe. The present study further demonstrated that safety. We showed that KB290 was not genotoxic, and at doses as high as  $10^{10}$  cfu/kg/day for up to 13 weeks, we did not detect bacterial translocation, neurotoxicity, or immunotoxicity in the rat. We therefore conclude that KB290 is safe for probiotic consumption in humans.

#### 5. Uncited reference

Nam et al. (2007).

#### Conflict of interest statement

All authors have a financial relationship with the sponsor of the studies, Kagome Co., Ltd.

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