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Safety and Toxicology Evaluation of Novel Multiple Strains of Lactic Acid Bacterial in Laboratory Rats

Jing Tian, Wang Xin, Ma Kai and Fang Shu-guang
Department of Research and Development, Jiangsu Wecare Biotechnology Co., Ltd, Songling Town, Wujiang Bridge Road, 1033, Suzhou, Jiangsu, China

Corresponding Author: Wang Xin, Department of Research and Development, Jiangsu Wecare Biotechnology Company Co., Ltd, Songling Town, Wujiang Bridge Road, 1033, Suzhou, Jiangsu, China Tel: +86051286856990

ABSTRACT

Multiple Strains of lactic acid bacterial, *Lactobacillus acidophilus* LA85, *Lactobacillus plantarum* Lp90, *Bifidobacterium longum* BL21 and *Bifidobacterium lactis* BLa80 exhibit probiotic potential as antiallergy agents, both *in vitro* and *in vivo*. Although, some lactic acid bacteria have exhibit probiotic effect, as new strains, their safety still requires evaluation when isolated from infant faeces or pickled cabbage. The safety and toxicology evaluation of this Lactic Acid Bacterial (LAB) powder was subject to a bacterial reverse mutation assay and a short-term oral toxicity study. The LAB powder product exhibited mutagenic potential in *Salmonella Typhimurium* strains TA97, TA98, TA100 and TA102 (with or without metabolic activation). In the short-term oral toxicity study, rats received a normal dosage of 70 mg kg^{-1} day^{-1} (approximately CFU kg^{-1} day^{-1}) or a high dosage of 700 mg kg^{-1} day^{-1} (approximately CFU kg^{-1} day^{-1}) for 30 days. The result of 30 days feed test indicated that rat growth in good condition, including behaviour, growth, feed and water consumption, haematology, clinical chemistry indices, organ weights or histopathologic analysis of the rats. These studies have demonstrated that the consumption of multiple bacterial strains (LA85, Lp90, BL21, BLa80) is not associated with any signs of mutagenicity of *S. typhimurium* or toxicity in rats, even after consuming large quantities of bacteria.

Key words: Lactic acid bacterial, LAB powder, probiotic potential, Ames test, feeding test on 30 days

INTRODUCTION

Many strains of Lactic Acid Bacteria (LAB) are typically regarded as safe because of their long history of use and their status is generally recognized as safe (Donohue, 2006). In addition to demonstrating the efficacy of probiotics in improving human health, safety characteristics must be considered. For new isolate-specific species or strains of probiotics, novel probiotics cannot be assumed to share the historical safety of traditional strains (Donohue, 2006; De Flora *et al*., 1992; Salminen *et al*., 1998). New or specific strains of probiotics are continually being identified. The efficacy of new strains should be carefully assessed prior to incorporating them into products and a case-by-case evaluation should be conducted to determine whether they share the safety status of traditional food-grade organisms (De Flora *et al*., 1992; Jones *et al*., 2012). Various aspects associated with the safety of probiotic bacteria can be studied using *in vitro* and *in vivo* methods such as Ames test and 30 days fed test. Safety and toxicology evaluation are appropriate as an initial step in the evaluation of new probiotics (Hudault *et al*., 1997).

Lactobacillus plantarum Lp90 was isolated from Chinese Traditional pickled vegetables, *Lactobacillus acidophilus* LA85, *Bifidobacterium longum* BL21 and *Bifidobacterium lactis* BLa80

were isolated from health infant faeces. *Lactobacillus plantarum* is a crucial industrial starter culture in many fermented food such as fermenting vegetables (Meng *et al.*, 1993). *Bifidobacterium longum* is non-pathogenic and is often added to food products for its beneficial probiotic health effects, it is considered part of the gut flora and its production of lactic acid is believed to prevent growth of pathogenic organisms. *Bifidobacterium lactis* is present in many food products and dietary supplements, the probiotic is mostly found in dairy products. The intestinal microbiota of host have some differences between human, the average effect of single strain is very limited, a variety of complex bacteria may have synergistic effect (Coconnier *et al.*, 1998).

This study was conducted to evaluate the safety of multiple strains based on the methods recommended for the safety evaluation of novel probiotics. We confirmed the safety of the multiple-strain mixed powder product by using a bacterial reverse mutation assay and a 30 day feeding study on rats. We investigated the effects of the consumption of viable mixtures of multiple LAB strains on the health, growth, haematology and blood chemistry in rats daily.

MATERIALS AND METHODS

Material: Test LAB powder, composed by *Lactobacillus acidophilus* LA85, *Lactobacillus plantarum* Lp90, *Bifidobacterium longum* BL21 and *Bifidobacterium lactis* BLa80. The LAB powder (1×10^{10}-1×10^{11} CFU g^{-1}) was produced by fermentation, freeze-dried (Jiangsu Wecare Biotechnology Co., Ltd., Suzhou, China) and refrigerated at –20°C until it was needed for testing. The bacterial counts were determined by plating serial dilutions of the culture in Phosphate Buffered Saline (PBS) or MRS agar. The plates were incubated anaerobically at 37°C for 48 h. Experiments using distilled water as solvent to prepare the sample solution with different concentrations of the tested. Laboratory rats were approved by the Department of Animal Laboratory, Shanghai Medical University.

Ames test: *Salmonella Typhimurium* strains TA97, TA98, TA100 and TA102, were provided by Department of Biochemistry, University of California. Mutagenicity tests were conducted using *S. Typhimurium* strains TA97, TA98, TA100 and TA102 , the S9 fraction as the metabolic activation system, as described previously (Follmann and Lucas, 2003). The suspension mixture (total volume, 500 μL) comprised of 4 mM NADP, S9 fraction (total protein, 170 μg), 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate and phosphate buffer (pH 7.4), promutagen (NC, positive control, 0.4-4.0 μg of BP, 2-AA, SA, or NQNO), test compound (5 mg of probiotic- combination mix) and 100 μL of *S. Typhimurium* in overnight culture. The components were sequentially added to 2 mL of warm soft agar. The mixture was poured into a petri dish containing Vogel-Bonner minimal medium (1.5% agar in Vogel-Bonner E medium with 20 mg mL^{-1} glucose). After incubating for 2-3 days at 37°C, the revertant colonies (His+) were counted. The toxicity of the tested agents was assessed by observing the background bacterial growth on minimal agar plates caused by traces of histidine in the medium (Hirose *et al.*, 2009). All of the experiments were performed in triplicate.

Feeding test on 30 days: The rats are divided into 4 groups randomly, 20 rats for each group and the weight is 70-80 g. The rats were provided diet and water ad libitum. A sterile gastric feeding tube was used for orally inoculating 2 of the 3 groups with mixed LAB strains in PBS at 2 doses; the control group received PBS only. For subsequent feeding, each rat received 1 mL of suitably diluted LAB suspensions for obtaining 5×10^{9} and 5×10^{10} CFU kg^{-1} b.wt. The animal room was ventilated and maintained at 25±2°C with a relative humidity of 60±5%. Artificial lighting was sequenced to provide 12 h light/dark cycles. The treatments lasted for 30 days; during this period, the activity, behaviour and hair luster of each rat were observed and recorded daily. Water Intake

(WI) and Feed Intake (FI) were measured. The Specific Growth Rate (SGR) was expressed as the average weekly weight gain (g). On day 30, all the animals were sacrificed humanely, blood and tissue samples were collected for further laboratory analysis. Test hematology, biochemistry and weigh liver, kidney and spleen and count the ratio of organ to body, histological examination for mainly organ.

Statistical analysis of data: All data using SPSS 11.0 for Windows to take statistical software chi-square test. t-Test (two-tail) was used for statistical analysis of all the data. The $p \leq 0.05$ was considered statistically significant. The statistical analysis was performed using Microsoft Excel 2010.

RESULTS

Ames test: The number of revertant colonies of each strain is shown in Table 1, the results indicated that the number of revertant colonies for different doses of samples in the S9 and non S9 condition were not more than the blank control group, solvent control group (distilled water) two times and each dose group has no dose-response relationship obviously, with the positive control results, the results of Ames test is negative for the LAB powder.

Thirty days feeding

Growth and food use ratio: Growth curve shown in Fig. 1 for male rats were fed for 30 days, the growth curve of female rat in 30 days feeding similar to male rats. Food utilization rate of rats as shown in Table 2. Therefore, the growth situation of each tested rats is fine.

Table 1: No. of revertant colonies of each strain with and without treatment of LAB powder

	Revertant (number/vessel)							
	TA97		TA98		TA100		TA102	
Dose (mg/vessel)	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0.1	156±11[b]	165±9[b]	34±3[b]	39±5[b]	143±11[b]	164±11[b]	284±8[b]	275±16[b]
0.5	169±8[b]	171±7[b]	32±2[b]	32±13[b]	146±11[b]	175±19[b]	277±15[b]	279±8[b]
1.0	156±20[b]	171±3[b]	32±2[b]	39±7[b]	151±23[b]	169±12[b]	273±10[b]	269±6[b]
2.5	169±11[b]	164±12[b]	29±2[b]	35±4[b]	149±13[b]	160±10[b]	265±15[b]	271±14[b]
5.0	169±10[b]	168±11[b]	35±4[b]	40±5[b]	147±25[b]	166±18[b]	261±8[b]	263±14[b]
Blank	154±15[b]	160±10[b]	34±4[b]	44±7[b]	136±14[b]	162±18[b]	272±11[b]	279±10[b]
SC	145±13[b]	151±8[b]	31±5[b]	39±8[b]	131±10[b]	155±16[b]	268±11[b]	275±10[b]
PC	1180±85[a]	1450±42[a]	600±28[a]	2010±99[a]	1320±85[a]	1310±71[a]	3200±141[a]	600±28[a]

[a,b]$p<0.05$ compared with the control group, SC: Blank solvent, PC: Positive control, values are given Means±SD

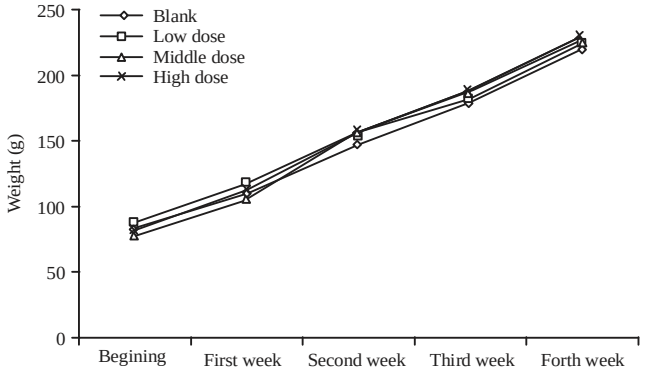

Fig. 1: Thirty days feeding growth curve for male rats

Table 2: Food use ratio for each test

Genders	WOB (g)	WOF (g)	DY (g)	Intake (g)	UR (%)
Male					
Blank	82±8[a]	229±17[a]	147[a]	617[a]	24[a]
Low dose	78±7[a]	230±16[a]	152[a]	589[a]	26[a]
Middle dose	80±8[a]	228±37[a]	148[a]	617[a]	24[a]
High dose	76±8[a]	227±40[a]	151[a]	571[a]	27[a]
Female					
Blank	75±4[a]	193±19[a]	118[a]	601[a]	20[a]
Low dose	80±8[a]	200±10[a]	122[a]	632[a]	19[a]
Middle dose	78±9[a]	203±12[a]	125[a]	650[a]	19[a]
High dose	76±4[a]	202±15[a]	126[a]	668[a]	19[a]

[a]:$p<0.05$ compared with the control group, WOB: Weight of beginning, WOF: Weight of final, DY: Dynamiting, values are given Means±SD

Table 3: Blood routine examination

Genders and groups	WC (109/L)	RC (1012/L)	HH (g L^{-1})	LY (%)	NG (%)
Male					
Blank	10.2±1.6[a]	5.5±0.1[a]	156±5.3[a]	65±1.9[a]	35±1.9[a]
Low dose	10.6±1.6[a]	5.5±0.3[a]	156±3.1[a]	66±3.6[a]	34±3.6[a]
Middle dose	10.8±1.1[a]	5.2±0.2[a]	157±3.1[a]	64±2.3[a]	36±2.3[a]
High dose	10.6±1.2[a]	5.4±0.1[a]	156±4.7[a]	67±2.5[a]	33±2.5[a]
Female					
Blank	9.7±1.1[a]	5.3±0.2[a]	148±3.8[a]	66±1.9[a]	34±1.9[a]
Low dose	9.8±0.8[a]	5.3±0.2[a]	148±4.8[a]	66±2.7[a]	34±2.7[a]
Middle dose	9.6±0.7[a]	5.1±0.2[a]	148±2.9[a]	65±3.6[a]	35±3.6[a]
High dose	10.1±1.4[a]	5.1±0.2[a]	148±2.9[a]	65±1.8[a]	35±1.8[a]

[a]:$p<0.05$ compared with the control group, WC: White cell, RC: Red cell, HH: Hemochrome Hb, LY: Lymphocyte, NG: Neutrophil granulocyte, values are given Means±SD

Hematological examination: Rats fed for 30 days, the effects of feeding various doses of mixed LAB strains on the hematological parameters shown in Table 3. Haematological analysis revealed no treatment-related changes in the white or red blood cell counts, Hemochrome Hb, Lymphocyte, or Neutrophil granulocyte among the rats in the various dosage groups and sex groups. However, significant increases were observed in the hemoglobin levels of the high-dose males in comparison with those of the other groups. Significant decreases were observed in haematocrit in the normal-dose males in comparison with those in the high-dose males, but no significant difference for the control group. The differences in hemoglobin or haematocrit were nonsignificant for the female groups. All values were within normal physiological ranges.

Biochemistry: Rats fed for 30 days, test blood and shown in Table 4. As it is shown, Clinical chemistry values at the terminal sampling time for the rats treated with normal or high-dose mixed LAB strains or those for the control groups indicated that no statistically significant differences existed in the plasma concentrations of the serum glucose, albumin, cholesterol, urea nitrogen, glutamic-pyruvic aminase, total protein, triglyceride and other biochemical examination.

Weight of visceral organ: The rats were killed after 30 days of continuous feeding. Weigh liver, kidney and spleen and calculate the ratio of organ to body, as shown in Table 5. There were no significant differences in the relative heart, kidney, testicle or ovary weights among the rats in the various dosage groups or sex groups.

Histological examination: The rats were killed after 30 days of continuous feeding, weigh liver, kidney and spleen and the main organ for histological examination, the results in Table 6-7. No abnormality for each animal examined, there is no experiment related lesions for liver, kidney and other major organs.

Table 4: Biochemistry test for rats by 30 days feeding

Genders and groups	GL (mmol L^{-1})	AL (g /DI)	CR (mmol L^{-1})	UN (mmol L^{-1})	GT (IU L^{-1})	TP (g dL^{-1})	TY (mmol L^{-1})
Male							
Blank	5.5±0.8[a]	3.8±0.5[a]	2.1±0.1[a]	5.1±0.5[a]	30±7.7[a]	7.4±0.7[a]	1.2±0.1[a]
Low dose	5.3±0.7[a]	4.0±0.7[a]	2.2±0.2[a]	4.7±0.4[a]	32±8.1[a]	7.2±0.7[a]	1.2±0.1[a]
Middle dose	5.2±0.4[a]	4.2±0.8[a]	2.2±0.2[a]	4.7±0.3[a]	32±5.4[a]	7.2±0.7[a]	1.2±0.1[a]
High dose	5.4±0.8[a]	4.4±0.4[a]	2.3±0.2[a]	4.7±0.2[a]	35±5.8[a]	7.5±0.8[a]	1.2±0.1[a]
Female							
Blank	5.6±0.8[a]	4.2±0.4[a]	2.2±0.2[a]	5.2±0.2[a]	37±7.6[a]	7.6±1.0[a]	1.2±0.1[a]
Low dose	5.7±0.5[a]	4.3±0.3[a]	2.3±0.2[a]	4.6±0.1[a]	27±8.3[a]	6.8±0.8[a]	1.2±0.1[a]
Middle dose	5.8±0.7[a]	4.0±0.5[a]	2.2±0.1[a]	4.7±0.3[a]	32±6.4[a]	6.9±0.9[a]	1.3±0.1[a]
High dose	5.5±0.7[a]	3.9±0.5[a]	2.2±0.2[a]	4.7±0.4[a]	32±8.5[a]	6.9±0.7[a]	1.2±0.1[a]

[a]p<0.05 compared with the control group, GL: Glucose, AL: Albumin, CR: Cholesterol, UN: Urea nitrogen, GT: Glutamic pyruvic transaminase, TP: Total protein, TY: Triglyceride , values are given Means±SD

Table 5: Ratio of organ to body for rats by 30 days feeding

Genders and groups	LB (%)	KB (%)	SB (%)
Male			
Blank	5.33±0.26[a]	0.95±0.05[a]	0.50±0.04[a]
Low dose	5.31±0.39[a]	0.97±0.15[a]	0.46±0.07[a]
Middle dose	5.20±0.24[a]	0.91±0.07[a]	0.48±0.11[a]
High dose	5.35±0.45[a]	0.96±0.11[a]	0.48±0.07[a]
Female			
Blank	5.30±0.28[a]	0.99±0.04[a]	0.50±0.04[a]
Low dose	5.34±0.52[a]	1.03±0.14[a]	0.54±0.16[a]
Middle dose	5.32±0.51[a]	0.97±0.08[a]	0.52±0.14[a]
High dose	5.28±0.38[a]	0.94±0.15[a]	0.47±0.12[a]

[a]p<0.05 compared with the control group, LB: Liver/body, KB: Kidney/body, SB: Spleen/body

Table 6: Result of experiment related lesions for liver

Groups	HG	Blank
Animal number	20	20
Tunica change	0	0
Hepatic lobule		
Necrosis	0	0
Bleed	0	0
Vacuolar degeneration	3	4
Granular degeneration	2	2
Liver cell line breaks	0	0
Liver cell swelling	0	2
Inflammatory cell infiltration	0	0
Other	0	0
Portal area		
Necrosis	0	0
Bleed	0	0
Ductular proliferation	0	0
Inflammatory cell infiltration	3	3
Other	0	0

HG: No significant lesions by gross examination, so only choose high dose group for histological examination

DISCUSSION

Regarding the Ames test, the results from the promutagen treatment were similar to those reported by Zhang *et al.* (2012). In this study, treatment with a probiotic-combination mix did not induce mutagenicity of the *S. typhimurium* strains TA97, TA98, TA100 and TA102 with or without metabolic activation, indicating that the probiotic-combination mix was free of mutagenic activity. Previous studies on LAB strains have demonstrated that certain *Lactobacillus* species, such as *L. acidophilus*, *L. pentosus*, *L. plantarum*, *L. reuteri* and *Enterococcus faecium*, produce no oral toxicity in animals (Hirose *et al.*, 2009; Tsai *et al.*, 2004a, b; Szabo *et al.*, 2011; Jones *et al.*, 2012), but few researchers have evaluated the safety of multiple strains in a single product.

Table 7: Result of experiment related lesions for kidney

Groups	HG	Blank
Animal number	20	20
Cortex tunica change	0	0
Epithelial cells necrosis	0	0
Degeneration swell	0	0
Kidney vacuolar degeneration	0	0
Tubules cast	4	4
Inflammatory cell infiltration	0	0
Glomerulus degeneration	0	0
Within the interstitial lesions	2	2
Other	0	0
Medulla epithelial necrosis	0	0
Cells swell	0	0
Degeneration vacuolar degeneration	0	0
Curved tube cast	0	0
Inflammatory cell infiltration	0	0
Mesenchyme inflammatory cell infiltration	0	0
Renal pelvis of nipple change	0	0
Transitional epithelial change	0	0

HG: No significant lesions by gross examination, so only choose high dose group for histological examination

The 30-day administration of normal- and high-dose mixed LAB strains did not cause death or produce any clinical signs of toxicity. Hepatomegaly and splenomegaly are usually indirect indicators of invasion and infection (Swendseid, 1987). In this study, we did not observe macroscopic change in the liver or spleen morphology of the animals treated with the test strains *in vivo*. These results indicate that the rats experienced no infections resulting from the 30 day treatment with multiple LAB strains. Clinical chemical assays can be used to detect moderate to mild deficiency of nutrients or imbalances in nutrient metabolism and these deficiencies are usually apparent before any clinical symptoms or changes in host body weight (Swendseid, 1987).

CONCLUSION

In this study, the relative weights and morphology of the major organs did not exhibit any macroscopic changes or significant differences. The 30 day feeding test show that, good growth of rats and no significant differences between the LAB powder and blank for hematological examination, biochemical examination, main organ and histological examination. In summary, the LAB powder with multiple LAB strains (*Lactobacillus acidophilus* LA85, *Lactobacillus plantarum* Lp90, *Bifidobacterium longum* BL21 and *Bifidobacterium lactis* BLa80) is nonpathogenic and safe for animal or human consumption.

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