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Safety Assessment of Potential Lactic Acid Bacteria *Bifidobacterium longum* SPM1205 Isolated from Healthy Koreans

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The safety assessment of *Bifidobacterium longu*m SPM1205 isolated from healthy Koreans and this strain's inhibitory effects on fecal harmful enzymes of intestinal microflora were investigated. The overall safety of this strain was investigated during a feeding trial. Groups of SD rats were orally administered a test strain or commercial reference strain *B. longum* 1×10^9 CFU/kg body weight/day for four weeks. Throughout this time, their feed intake, water intake and live body weight were monitored. Fecal samples were periodically collected to test harmful enzyme activities of intestinal microflora. At the end of the four-week observation period, samples of blood, liver, spleen, kidney, and gut tissues were collected to determine for hematological parameters and histological differences. The results obtained in this experiment demonstrated that four weeks of consumption of this *Bifidobacterium* strain had no adverse effects on rat's general health status, blood biochemical parameters or histology. Therefore, it is likely to be safe for human use. Fecal harmful enzymes such as β-glucosidase, β-glucuronidase, tryptophanase and urease, were effectively inhibited during the administration of the *B. longum* SPM1205. These results suggested that this *B. longum* SPM 1205 could be used for humans as a probiotic strain.

Key words: fecal harmful enzymes *B. longum*, haematology, histology, probiotic, safety assessment

Lactic acid bacteria (LAB) have been considered as the most beneficial probiotic organisms contributing to the inhibition of harmful intestinal bacteria. They improve lactose malabsorption in humans, and led to the enforcement of immune functions (Park *et al*., 1999) and prevention of cancer (Salminen *et al*., 1974). *Lactobacillus* and *Bifidobacteria* have been used in fermented foods by humans for several centuries without adverse effects (Fuller, 1992; Yoon *et al*., 2004). They have been afforded Generally Recognized as Safe (GRAS) classification by many scientific groups because of their long history of safe use, particularly in dairy foods (Donohue and Salminen, 1996; Salminen and Donohue, 1996; Marteau and Salminen, 1997; Donohue *et al*., 1998). It has been demonstrated that most *Lactobaclillus* species and *Bifidobactrium* strains show no pathogenecity, including no acute oral toxicity to animals (Monose *et al*., 1979; Donohue *et al*., 1993) or humans (Saxelin *et al*., 1996; Kailasapathy and Rybka, 1997). With a growing consumer awareness concerning diet and health, and the increasing interest of food producers to develop functional foods, the health prompting properties of LAB have become the focus of active research (Luis E. N. Quadri, 2003). As a result, new and more specific LAB strains with probiotic attributes are being introduced into food products. These newly isolated organisms often have no previous history of food product use and do not necessarily share the GRAS status of traditional LAB strains. Furthermore, the safety of LAB has been recently questioned due to occasional infections, which have been associated with some indigenous LAB strains. Although the International Platform has confirmed the GRAS status of LAB for Lactic Acid Bacteria (LABIP) on the basis of an extensive review of the published data, confirmation of safety (especially the safety of the newly isolated probiotics), prior to their incorporation into food products, has been strongly recommended.

One of the LAB, *Bifidobacterium* spp. is a well-known probiotic organism in humans, due to its various biological activities. In order to develop a probiotic *Bifidobacterium* spp. having inhibitory effects on fecal harmful

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enzymes of intestinal microflora, we isolated *Bifidobacterium* spp. from fecal samples of healthy Koreans. One of the isolated strains is a well-known *Bifidobacteium longum*. This study aims to investigate the effects of feeding of this strain on animal health, growth and blood biochemical parameters for four weeks. We investigated the safety of this strain regarding human use and tested *in vivo* inhibitory effects on fecal harmful enzymes of intestinal microflora to show potential probiotic activity.

Materials and Methods

Bacterial strains and Media

B. longum SPM1205 isolated from healthy Koreans was used as a test strain and Duolac^{TM} (Cellbiotech, Korea), which consists of *L. casei*, *L. rhamnosus*, *L. lactis*, *L. plantarum* and *B. longum*) was used as a commercial reference strain. General anaerobic medium (GAM) and Blood-Liver (BL) medium were purchased from Nissui Pharm (Japan). The other reagents used were analytical/analytic grades.

Isolation and identification of Bifidobacterium spp.

Fecal samples for 20 healthy Koreans (20-30 years old) were collected by BBL's anaerobic sample collection and transport system in order to maintain anaerobic conditions and were used within 24 h. Fecal samples were serially diluted 10-fold from 10^{-1} to 10^{-8} and 100 μl of appropriate dilutions was spread onto the selective BL agar containing 5% sheep blood. After 48 h of incubation in anaerobic conditions (Bactron Anaerobic Chamber, Sheldon MFG Inc., USA), brown or reddish-brown colonies 2-3 mm in diameter were selected for further study (Scardovi, 1986). A fructose-6-phosphate phosphoketolase (F6PPK) test was performed (Lee *et al.*, 2001) to ensure that the colonies selected were *Bifidobacteria.* One of the isolated *Bifidobacteria* strains having good growth appearance in a large scale culture was selected for this experiment. To identify this *Bidifobacterium* spp., at the level of species, 16S rRNA sequencing was performed by Bioleaders (Korea).

Animals

Male SD rats aged four weeks were purchased from Orient Co (Korea). and individually housed in stainless steel cages. A 12 h light-dark cycle in a controlled atmosphere was maintained through the study. Rats were acclimatized for 1 week prior to commencing experiments. Diets and water were freely supplied.

Experimental design

Rats were adopted for 1 week in experimental conditions, following which 18 rats with optimal growth were selected and randomly assigned into three different groups. As a control group, one of the groups was orally inoculated with 0.3 ml of sterile phosphate buffer (pH 6.8). Two of the groups were orally inoculated with 0.3 ml of *B. longum* SPM 1205 and commercial LABs respectively. The treated groups received inoculums size of 1×10^{9} cfu per day for four weeks. During the treatment period, food and water were supplied freely and water intake, feed intake and body weight were monitored periodically.

Blood Biochemical assay

Blood samples were collected by cardiac puncture and collected into EDTA non-treated tubes for biochemical analyses. The biochemical analyses were performed by Samkwang Lab. (Korea).

Histology

The gross anatomy of visceral organs of each rat was inspected and recorded. The weight of each organ was compared between test and control groups.

The number of fecal Bifidoabcteium spp.

To compare the number of fecal *Bifidobacterium* spp. between the LAB fed groups and control group, fecal samples were periodically collected. 0.1 g of feces was suspended with 0.9 ml of 0.1 M phosphate buffer (pH 6.8 containing 0.5% cystein) and serially diluted 10-fold from 10^{-1} to 10^{-8}. 100 μl of appropriate dilutions was spread onto the selective BL agar containing 5% sheep blood. After 48 h of incubation under anaerobic conditions (Bactron Anaerobic Chamber, Sheldon MFG. Inc., USA), brown or reddish-brown colonies at least 2-3 mm in diameter were counted as *Bifidobacteium* spp.

In vivo inhibitory effect on harmful enzyme of rat intestinal microflora.

Enzyme activities in fecals samples, which are related to colon cancer, were tested in the following methods (Gutman and Bergmeyer, 1974; Kim *et al.*, 1992; Kim *et al.*, 1995).

Preparation of enzyme solution

0.1g of each fecal sample were suspended with 0.9 ml of 0.1 M phosphate buffer (pH 6.8 containing 0.5% cysteine) using micro-sonicator and this suspension was used as an enzyme source.

Assay of β-glucosidase activity

β-Glucosidase activity was assayed as follows; 2 ml of each reaction mixture consisting of 0.8 ml of 2 mM p-nitrophenyl-D-glucopyranoside and 0.2 ml of the enzyme solution (suspended fecal sample) was incubated for 30 min. at 37°C and then stopped by adding 1 ml of 0.5 N NaOH. The stopped reaction mixture was centrifuged at 3,000 rpm (Vision, VS-15000) for 10 min. The activity was measured by monitoring the absorbance at 405 nm.

Table 1. Blood biochemistry measurements of rat fed with different LABs

	Control	*B. lomgum* SPM1205	Duolac™
S-GOT (IU/l)	98 ± 13	103 ± 11	108 ± 15
S-GPT (IU/l)	45 ± 11	46 ± 13	40 ± 7
Total protein (g/dl)	6.4 ± 0.2	6.12 ± 0.3	6.05 ± 0.3
Albumin (g/dl)	2.76 ± 0.12	2.78 ± 0.09	2.77 ± 0.14
Total cholesterol (mg/dl)	74 ± 8	69 ± 7	78 ± 12

Assay of β-glucuronidase

β-Glucuronidase activity was assayed as follows; 2 ml each reaction mixture consisting of 0.8 ml of 2 mM p-nitrophenyl-β-D-glucuronide and 0.2 ml of enzyme solution (suspended fecal sample) was incubated for 30 min and then stopped by adding 1 ml of 0.5 N NaOH. The stopped reaction mixture was centrifuged at 3,000 rpm (Vision, VS-15000) for 10 min and measured the activity by monitoring the absorbance at 405 nm.

Assay of tryptophanase activity

Tryptophanase was assayed as follows; the reaction mixture containing 0.2 ml of complete reaction mixture (2.75 mg pyrophosphate, 19.6 mg disodium EDTA dihydrate and 10 mg of bovine serum albumin in 100 ml of 0.05 M potassium phosphate, pH 7.5), 0.2 ml of 20 mM tryptophan and 0.1 ml of enzyme solution was incubated for 1 h at 37°C. The reaction mixture was stopped by adding 2 ml of color reagent solution (14.7 g p-dimethylaminobenzaldehyde, 52 ml of H_2SO_4 and 948 ml of 95% ethanol) and centrifuged at 3,000 rpm for 10 min. The enzyme activity was measured by monitoring the absorbance at 550 nm.

Assay of urease activity

Urease activity was assayed by the indophenol method with minor modifications. Briefly, 100 µl of enzymes were added to 0.3 ml of urea substrate solution (4 mM urea in 20 mM phosphate buffer, pH 7.0) and incubated at 37°C for 30 min. The reaction mixture was terminated by 0.1 ml of 1 $(NH_4)_2SO_4$, added with phenolnitroprusside reagent and alkaline hypochlorite reagent, and incubated at 65°C for 20 min. The quantity of ammonia liberated from reaction mixture was determined from the standard curve, correlating the absorbance at 630 nm.

Statistics

All statistical analyses for comparison were performed by SAS software (version 8.12).

Results and Discussion

Identification of Isolated strain

One of the isolated *Bifidobactreium* strains having good growth appearance in the large-scale culture was identified at the level of species using the 16S rRNA sequencing. According to the sequence analysis (Fig. 1) and BLAST search, the 5' and 3' fragments of 16S rRNA sequence in this strain indicated the 100% homology with those of *B. longum* species in the database. Therefore, this strain was identified as *B. longum* and labeled *B. longum* SPM1205.

A

AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGA
ACGGGATCCATCAGGCTTTGCTTGGGGTGAGAGTGGCGAACGGGTGAGTAATGCGTG
ACCGACCTGCCCCATACACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGC
TCCAGTTGATCGCATGGTCTCCTGGGAAAGCTTTCGCGGTATGGGACCGGCCACATTG
GGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT
GGGCGCAAGCCTGATGC (383 bp)

B

AGAAAGGAGGTGGTCCAGCCGCACCTTCCGGTACGGCTACCTTGTTACGACTTAGTCC
CAATCACGAGCCTCACCTTAGACGGCTCCATCCCACAAGGGGTTAGGCCACCGGCTTC
GGGTGCTGCCCACTTTCATGACTTGACGGGCGGTGTGTACAAGGCCCGGGAACGCATT
CACCGCGACGTTGCTGATTCGCGATTACTAGCGACTCCGCCTTCAGCGAGTCGAGTTG
CAGACTGCGATCCGAACTGAGACCGGTTTTCAGGGATCCGCTCCGCGTCGCCGCGTCG
CATCCCGTTGTACCGGCCATTGTAGCATGCGTGAAGCCCTGGACGTAAGGGGCAGATG
ATCTGACGTCATCCCCACCTTCCTCCGAGT (379 bp)

Fig. 1. Sequence analyses of the 5'-fragment (A) and 3'-fragment (B) of *B. lomgum* SPM1205-16S rRNA gene.

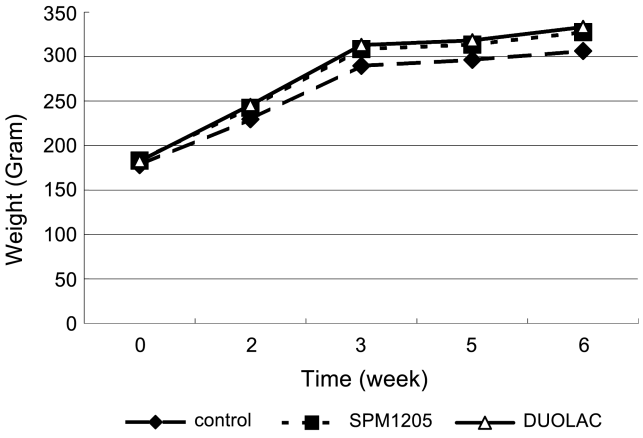

Fig. 2. Time dependent changes of rat body weight.

Feed intake and water intake

All animals were healthy and no noticeable abnormal behavior, changes in activity, or decline in hair luster was observed after four weeks of feeding the LABs. There was no statistically significant difference in daily feed and water intake among animals treated with the probiotic strain, reference strain and control diet (data not shown). These results indicated that the test strain have no adverse effects on the general health status of the rats when orally administered for four weeks.

Growth

Data on weekly live body weight gain is shown in Fig. 2. Growth rates were not affected by the administration of *B. longum* SPM1205. There was no statistically significant difference in the specific growth rate between the treatment groups and control groups ($p > 0.05$). The rat given probiotics exhibit an identical growth pattern to those of the control group.

Blood biochemical assay

To detect adverse sub-clinical effects of test strain on the experimental animals, the general blood biochemical parameters were monitored. Biochemical assays can be used for detecting moderate to mild deficiencies in nutrients or an imbalance in nutrient metabolism (Swendseid, 1987). The metabolism of protein, carbohydrate and lipid was not affected by feeding with the test strains. Tortuero *et al.*, (1995) also found that the administration of LAB had no effect on plasma glucose, total protein and albumin. The levels of S-GOT, S-GPT, total protein, albumin and total cholesterol were checked and the results are shown in Table I. There were no significant differences in blood biochemistry profiles between the LAB fed groups and control group (non fed group). This suggests that feeding with test LAB strains for a substained four-week period has no adverse effects on rat blood biochemical parameters.

Table 2. Comparison of organ weight between control group and LAB-fed groups

Organs	Control	*B. longum* SPM 1205	Duolac^{TM}
Kidney	2.25 ± 0.21 g	2.35 ± 0.24 g	2.35 ± 0.33 g
Spleen	0.55 ± 0.09 g	0.60 ± 0.11 g	0.60 ± 0.10 g
Liver	11.60 ± 0.62 g	13.00 ± 1.14 g	13.8 ± 1.21 g

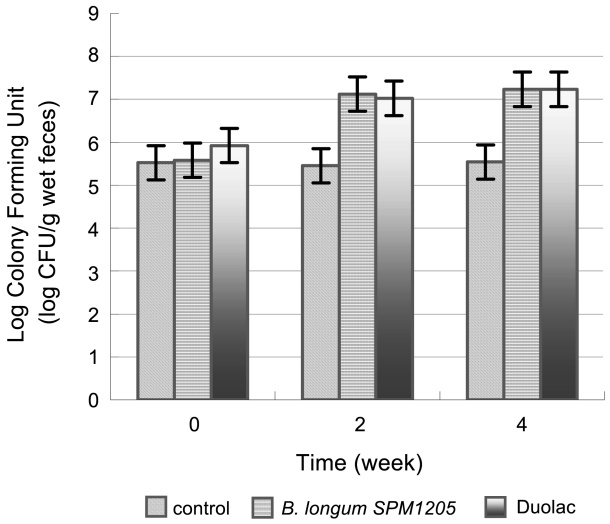

Fig. 3. The comparison of bifidobacterial population in the feces of rat fed with different LABs.

Histology

After four weeks of feeding of *B. longum* SPM1205, the animals were sacrificed and their organs were collected for macroscopic observation. Macroscopic observation did not reveal any obvious differences in the size and appearance of the visceral organs between each group, and there was no observable hepatomegaly and splenomegaly enlargement (Table 2).

The number of fecal Bifidobacterium spp.

During the experiments, we collected the fecal samples of the rats to compare the fecal *Bifodobacterial* numbers between control and LAB fed groups. BL media supplemented with 5% sheep blood was used as a *Bifidobacteria* selection media. As shown in Fig. 3, the LAB fed groups showed 100 times more *Bifidobacteriim* spp. in numbers than the control group. This result was attributed to the LAB feeding (Fig. 3). It showed that *B. longum* SPM 1205 might be used as a probiotic strain.

In vivo inhibitory effects on harmful fecal enzymes of intestinal microflora

The *in vivo* inhibitory effects of *B. longum* SPM1205 on the harmful enzymes of rat intestinal microflora were invested. During the experiments, the fecal samples of each animal group were collected each week. These fecal samples were sonicated and used as enzyme sources. According to the results, the activities of β-glucosidase, β-

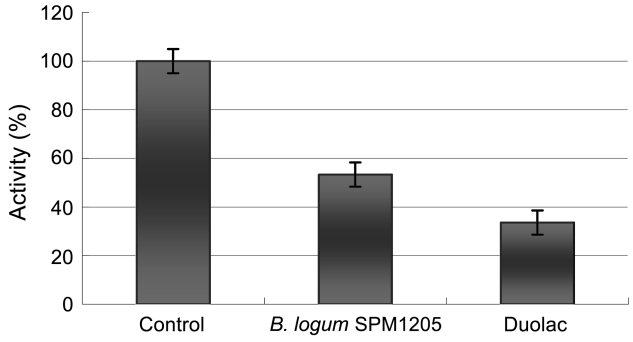

Fig. 4. β-glucosidase activities in the feces of rat fed with different LBAs.

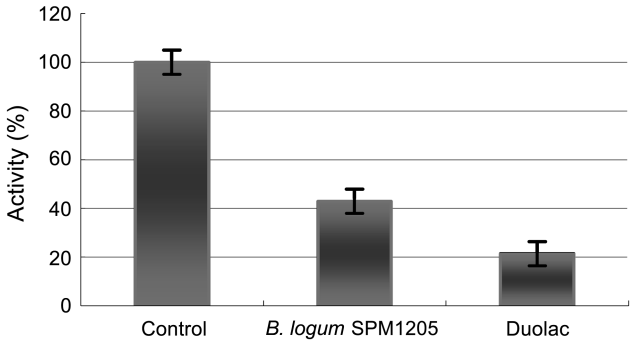

Fig. 5. β-glucuronidase activities in the feces of rat fed with different LBAs.

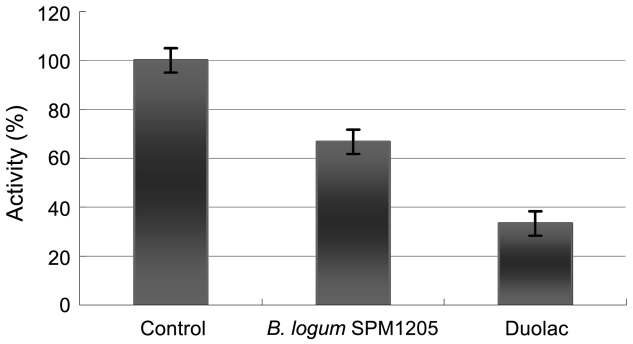

Fig. 6. Tryptophanase activities in the feces of rat fed with different LBAs.

glucuronidase, tryptophanse and urease were potently inhibited by the administration of *B. longum* SPM1205 and commercial reference strains. In the case of commercial reference strains, these enzymes were more potently inhibited than *B. longum* SPM1205 fed group. This may be due to the composition of the commercial references strain. The commercial reference strain is composed of five different LAB strains (*L. casei*, *L. rhamnosus*, *L. lactis*, *L. plantarum* and *B. longum*)(Figs. 4 - 7).

In this experiment, safety assessment of *B. longum* SPM1205 isolated from healthy Koreans was investigated. Feeding rats with potential probiotic *B. longum* SPM1205 for four weeks had no adverse effects on their

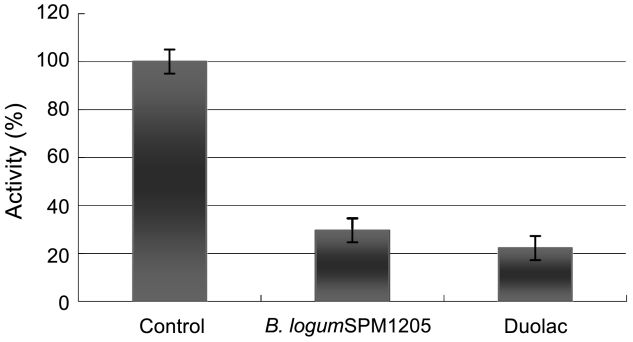

Fig. 7. Urease activities in the feces of rat fed with different LBAs.

general health status, growth, blood biochemistry and histology parameters. This *B. longum* SPM1205 showed a strong inhibitory effect on harmful enzymes of rat intestinal microflora. These results suggest that the *B. longum* SPM1205 are non-toxic for rats and therefore, may be suitable for human use as a probiotic strain.

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