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Acute Oral Toxicity and Pathogenicity of a Potential Probiotic *Bacillus* sp. A9184 Isolated from Soybean Paste

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ABSTRACT. This study provides more information about the potential toxicological risk of *Bacillus* sp. A9184 isolated from soybean paste. The evaluation was based on the guidelines of acute oral toxicity/pathogenicity for microbial pesticide and was to get more comprehensive understanding on the acute toxicity of the potential probiotic in Sprgue-Dawley rats. No dead animal was observed in rats after single oral administration with bacteria (10^8 CFU per animal). There were neither no treatment-related changes in clinical signs, nor changes in body weight and body temperature as compared with the untreated group. All tested animal showed the increase in body weight with time. The results obtained in this study suggest that the potential probiotic, *Bacillus* sp. A9184, is non-toxic for rat.

Keywords: *Bacillus* sp., Acute oral toxicity, Pathogenicity, Rats.

INTRODUCTION

The use of antibiotics in farm animals has caused tissue residue of the antibiotics, an imbalance of normal intestinal flora, reduction of beneficial intestinal microbial populations, and generation of antibiotic resistant bacteria (Elmer, 2001; Reid and Friendship, 2002). In order to overcome the above mentioned problems, the utility and development of probiotics has been increased in veterinary sectors (Reid and Friendship, 2002; Verstegen and Williams, 2002). There is, however, a potential risk associated with the introduction of a novel probiotic strain into foods for human and animal consumption. Before any novel probiotic strain can be incorporated into products to be consumed, the strain should be carefully assessed and tested for its safety. No general guidelines for the safety assessment of a novel probiotic strain exist at this stage, and the type of tests that should be included has warranted much debate (Saarela *et al.*, 2002).

Many studies have promoted probiotic specific safety evaluation criteria, especially the infectivity, metabolic activity and immune function of a probiotic strain (Salminen *et al.*, 1998; Elmer, 2001). Bacterial translocation is the first step in the pathogenesis process for many bacterial strains, and the results of bacterial translocation could provide information for direct assessment of infectivity (Steffen and Berg, 1983). Bacterial enzymes, including azoreductase, nitroreductase, nitrate reductase, β-glucuronidase, and β-glycosidase, which have been found to be involved in generating mutagens, carcinogens and tumor promoters from dietary and endogenously produced precursors in animal gut, may indicate toxicity of bacterial strains, and have been used to test probiotic safety (Rowland *et al.*, 1983; Perez-Chaia *et al.*, 1999).

Bacillus sp. is a gram-positive, spore-forming organism normally found in the soil, and the robustness of spores is thought to enable passage across the gastric barrier, where a proportion of spores germinate in the small intestine and populate, albeit briefly, the intestinal tract (Farrar, 1963; Ihde and Armstrong,1973). The clinical effects of *Bacillus subtilis* as an immunostimulatory agent in a variety of diseases, as an *in vitro* and *in vivo* stimulant of secretory immunoglobulin A, and as an *in vitro* mitogenic agent have been documented (Due *et al.*, 2004). In addition, *Bacillus pumilus* and of a laboratory strain of *Bacillus subtilis* were found to induce the

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pro-inflammatory cytokine interleukin-6 in a cultured macrophage cell line, and *in vivo*, *Bacillus pumilus* and *Bacillus subtilis* induced the pro-inflammatory cytokine tumor necrosis factor alpha and the gamma interferon (Mazza *et al.*, 1992; Mazza, 1994, Due *et al.*, 2004). The results that provided evidence of colonization, immunostimulation, and antimicrobial activity support the hypothesis that the organisms have a potential probiotic effects.

As no guidelines are available for safety testing of probiotics, the safety assessment in this study was based on the guidelines of acute oral toxicity/pathogenicity for microbial pesticide (EPA, 1996). The aim of this study was to evaluate the *in vivo* safety of orally administered *Bacillus* sp. A9184 isolated from soybean paste using a Sprague-Dawley (SD) rat model.

MATERIALS AND METHODS

Bacterial strains

The newly isolated potential probiotic, *Bacillus* sp. A9184 from soybean paste, was used in this study. *Bacillus* sp. A9184 was serially transferred twice in tryptic soy broth and incubated at 30°C for 24 h. The bacterial cells were collected by centrifugation (3500 g, 5 min), and washed twice in phosphate buffered saline (PBS), and then resuspended in PBS to a final concentration of 10^{10} CFU/ml and stored at 4°C.

Animals

Male and female SD rats, weighing between 160 and 220 g at the age of 6~7 weeks, were used for this study. They were obtained from Samtaco Biokorea Co. (Anyang, Korea) and acclimated for one week before experiments in the Animal Environmental Control Unit (temperature, 23 ± 3°C; relative humidity, 50 ± 10%). Lighting was controlled to give a 12 h light-dark cycle. The animals were housed in a polycarbonate cage (28 cm × 42 cm × 18 cm). The animals were supplied with laboratory animal feed pellet (Samtaco, Korea) and filtered water *ad libitum*.

Healthy SD rats were randomly allocated into 2 groups of each 18 male and female rats for toxicity and pathogenicity test. To examine the pathogenicity and toxicity of *Bacillus* sp. A9184, a single dose of the strains (10^8 CFU of the *Bacillus* sp. A9184 per test animal) or PBS was administered to rats intragastrically using a polyethylene cannula attached to a disposable syringe.

Observation of animals

After single oral administration of *Bacillus* sp. A9184, the animals were monitored daily for two weeks. Any abnormalities of activities, behaviors, and general health status including ruffled coat, hunched posture, unstable movement, tremors/shaking, coughing/breathing difficulty, color of extremities, and presence of porphyrin staining were recorded if present. The presence of diarrhea or unformed feces was also monitored. Throughout the experiment, the food and water intakes were recorded everyday. As water and food was provided *ad libitum*, total food and water intake per cage was measured and an average intake per rat was determined. The individual weight of the rats was monitored at day 1, 3, 5, 7 and 14 post-infection.

Translocation of bacterial strain

The study for translocation of *Bacillus* sp. A9184 to the blood and tissues of rats was designed based on the methods of Zhou *et al.* (2000). For evaluating the infectivity, three treated rats per gender in each group sacrificed at 3, 7 and 14 days after dosing. The rats were euthanized by carbon dioxide, blood samples were taken by cardiac puncture and then autopsied. Before excising tissue samples, the surface of viscera was swabbed with a sterile bacteriological swab. The swabs were then streaked onto tryptic soy agar (TSA) plates to test the microbial sterility of the viscera surface. Following swabbing, the size and appearance of visceral organs were examined macroscopically. The mesenteric lymph nodes, spleen and a sample of liver tissues were then excised aseptically. The tissue suspensions and samples of blood were plated separately on TSA plates. The TSA plates were incubated at 37°C for overnight.

Blood analysis

Blood samples were either collected into EDTA-added bottles for hematological test at day 0 (pre), 3, 7 and 14 post-infection and analyzed within 20 min using HEMAVET (CDC, USA) for the determination of hematological parameters. Red blood cell (RBC), hemoglobin concentration (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and white blood cell (WBC) were determined.

Statistics

Continuous variables such as food and water consumption, body weight, body temperature and blood parameters were processed by a variance analysis (ANOVA). Statistical analyses were performed using a computer software program (Microsoft Excel, Microsoft, USA). The differences between the treatments were

performed using Student's *t*-test. Differences were considered statistically significant at $p<0.05$.

RESULTS

General health status

Throughout the experiment, SD rats appeared healthy, inquisitive and active. No illness or death occurred. No observable difference in the animals hair luster was noticed between the groups, and there were no signs of gastrointestinal upsets including diarrhea or vomiting (Table 1). There were no increasing or decreasing body temperatures by *Bacillus* sp. A9184 among all animals.

Translocation of *Bacillus* sp. A9184

There was no bacterial growth from swabs taken from the visceral surfaces of the rats of the experimental groups, which indicates that the visceral surfaces of the rats were not contaminated with bacteria. No bacteremia was detected in any animals. There was no growth of *Bacillus* sp. A9184 in any cultures of blood, spleen, mesenteric lymph nodes and liver samples of rats of any groups (Table 2).

Table 1. Clinical signs in SD rats after oral administration of *Bacillus* sp. A9184 (10^8 CFU per animal)

Groups[a]	Gender	Clinical signs at the indicated times (post-dose)				
		Day 1	Day 3	Day 5	Day 7	Day 14
Control	Male	-	-	-	-	-
	Female	-	-	-	-	-
Treatment	Male	-	-	-	-	-
	Female	-	-	-	-	-

-, normal.
[a]Control, a single oral dose of PBS was administered to rats (n=6); treatment, a single oral dose of the 10^8 CFU of the *Bacillus* sp. A9184 per test animal was administered to rats (n=6).

Table 2. Translocation of *Bacillus* sp. A9184 in SD rats after oral administration of *Bacillus* sp. A9184 (10^8 CFU per animal)

Groups[a]	Organ	Bacteria isolated at the indicated times (post-dose)			
		Pre-dose	Day 3	Day 7	Day 14
Control	Blood	-	-	-	-
	Liver	-	-	-	-
	Lymph node	-	-	-	-
	Spleen	-	-	-	-
Treatment	Blood	-	-	-	-
	Liver	-	-	-	-
	Lymph node	-	-	-	-
	Spleen	-	-	-	-

-, not isolated.
[a]Control, a single oral dose of PBS was administered to rats (n=12); treatment, a single oral dose of the 10^8 CFU of the *Bacillus* sp. A9184 per test animal was administered to rats (n=12). Three treated rats per gender in each group sacrificed at pre- (0), 3, 7 and 14 days after dosing.

Table 3. The changes of body weight in SD rats for 2 weeks after oral administration of *Bacillus* sp. A9184 (10^8 CFU per animal)

Post-dose	Control[a]		Treatment[a]	
	Male	Female	Male	Female
Pre-dose	241.2 ± 2.1	205.6 ± 1.7	241.3 ± 1.6	206.3 ± 1.0
Day 1	249.8 ± 5.8	207.6 ± 2.8	251.9 ± 5.6	207.3 ± 2.1
Day 3	256.0 ± 6.3	214.2 ± 3.6	256.3 ± 6.1	214.2 ± 3.3
Days 7	264.8 ± 5.8	220.8 ± 4.0	264.2 ± 9.4	221.5 ± 3.9
Day 14	279.0 ± 5.8	234.3 ± 5.4	281.5 ± 8.7	234.0 ± 5.5

Values are mean ± SD.
Values of Day 1, Day 3, Day 7, and Day 14 after dosing were compared with that of pre-dose within each group ($p>0.05$).
[a]Control, a single oral dose of PBS was administered to rats (n=6); treatment, a single oral dose of the 10^8 CFU of the *Bacillus* sp. A9184 per test animal was administered to rats (n=6).

Body weight changes

During the 14 days treatment, no significant difference ($p>0.05$) in the body weight gains between the experimental group and control group (Table 3). All tested animal showed the increase in body weight with time. There were no increasing or decreasing body weights by *Bacillus* sp. A9184 among all animals. In addition, total food and water intakes had no relevance to oral administration of *Bacillus* sp. A9184 in both of control and treatment group ($p>0.05$).

Gross pathological findings

At the end of the observation period, all animals were sacrificed and autopsied. Macroscopic examination did not reveal any obvious differences in the size and appearance of visceral organs between experimental group and control group. No hepatomegaly or splenomegaly occurred.

Hematological parameters

Hematological parameters did not change from baseline after oral administration of *Bacillus* A9184 (10^8 CFU per animal). The hematological levels of each gender were shown in Table 4. Hematological levels of experimental group were not different as compared to the pre-dose levels during the experimental periods ($p>0.05$).

Table 4. Hematological values in SD rats for 2 weeks after oral administration of *Bacillus* sp. A9184 (10^8 CFU per animal)

Gender	Parameters[a]	Post-dose			
		Pre-dose	Day 3	Day 7	Day 14
Male	RBC (× 10^6/mm^3)	7.6 ± 0.6	7.7 ± 0.3	8.0 ± 0.7	7.9 ± 0.9
	Hb (g/dl)	13.7 ± 0.6	14.0 ± 1.4	15.4 ± 1.9	13.6 ± 1.9
	HCT (%)	45.7 ± 3.2	42.6 ± 2.4	45.5 ± 3.8	44.3 ± 6.0
	MCV (fl)	60.0 ± 4.1	55.3 ± 2.3	57.4 ± 1.6	56.3 ± 1.9
	MCH (pg)	17.9 ± 0.4	15.9 ± 5.4	16.7 ± 3.4	17.3 ± 1.5
	MCHC (g/dl)	30.2 ± 3.5	28.5 ± 9.0	29.1 ± 5.3	30.7 ± 2.1
	WBC (× 10^3/mm^3)	15.0 ± 1.4	14.5 ± 2.7	13.5 ± 2.0	13.6 ± 1.3
Female	RBC (× 10^6/mm^3)	7.4 ± 0.9	7.4 ± 0.7	7.2 ± 0.1	7.7 ± 0.6
	Hb (g/dl)	15.3 ± 1.7	14.1 ± 0.6	14.6 ± 0.8	14.1 ± 2.3
	HCT (%)	46.3 ± 2.7	44.2 ± 2.6	44.4 ± 1.2	42.2 ± 3.7
	MCV (fl)	62.6 ± 4.3	59.7 ± 2.0	61.7 ± 1.0	55.2 ± 8.0
	MCH (pg)	20.6 ± 2.1	19.0 ± 1.4	20.3 ± 0.7	18.3 ± 1.9
	MCHC (g/dl)	33.0 ± 2.8	31.9 ± 1.2	32.9 ± 1.6	33.6 ± 6.2
	WBC (× 10^3/mm^3)	13.0 ± 2.1	14.6 ± 2.7	13.5 ± 1.0	13.5 ± 1.1

[a]RBC, red blood cell; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cell.

DISCUSSION

The safety of probiotic strains has been discussed in the last few years (Donohue and Salminen, 1996a, b; Salminen *et al.*, 1998; Elmer, 2001; Saarela *et al.*, 2002) but there are still no general guidelines or specific policy requirements on this issue. Acute oral toxicity has been advocated as a fundamental test for assessing safety (Donohue and Salminen, 1996a). With no safety guidelines in place, some probiotic researchers have recommended that new probiotic strains should be isolated from the host animal of its intended use (Conway, 1996; Donohue and Salminen, 1996a; Salminen *et al.*, 1998; Elmer, 2001; Saarela *et al.*, 2002). When a strain belongs to a species for which no pathogenic strains are known but which does not have a history of safe use, it may be safe as a probiotic strain but should be confirmed the safety for novel probiotic strain (Salminen *et al.*, 1998). Some *Bacillus* species are pathogenic (e.g., *Bacillus anthracis* and some *Bacillus cereus*), *Bacillus subtilis* has, at most, been associated with opportunistic infections of immunocompromised patients (Farrar, 1963; Ihde and Armstrong, 1973). For these reasons, it has received relatively little clinical interest. Therefore, we undertook the oral toxicity and pathogenecity of *Bacillus* sp. A9184 using commonly recommended criteria. In the present study, we investigated the acute toxicity of single oral administration (10^8 CFU per test animal) with *Bacillus* sp. A9184 to SD rats as a part of the development of a new probiotic for industrial animlas. As the results, no mortality was observed in rats treated with *Bacillus* sp. A9184 and no abnormal clinical signs were shown in animals.

Bacterial translocation is a further highly recommended indicator for probiotic safety assessment (Marteau *et al.*, 1997; Zhou *et al.*, 2000). Bacterial translocation refers to the phenomenon in which the intestinal bacteria pass through the mucosal epithelium to be transported to the lamina propria, mesentery lymph nodes and other organs, and may further cause bacteremia, septicemia and even multiple organ failure (Berg, 1992). Translocation is also an indicator of potential pathogenicity for most obligate and facultative pathogens (Zhou *et al.*, 2000). In this study, no translocation of *Bacillus* sp. A9184 from the gut to tissues including spleen, liver, mesenteric lymph nodes and blood occurred.

In conclusion, as the novel strains of probiotics are developed, the requirement for the regulated guidelines for safety testing needs to be addressed. The results on the acute toxicity of potential probiotics, *Bacillus* sp. A9184, indicate that it is not possible to reach oral dose levels related to death or dose levels with any harmful side-effects. It is unlikely that *Bacillus* sp. A9184 will cause adverse affects in any other animal species, including humans, further clinical trials need to be undertaken.

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