THE ACUTE TOXICITY AND SEMI-CHRONIC TOXICITY OF BACIMIX PRODUCT CONTAINING TWO STRAINS OF *BACILLUS SUBTILIS* BS 304.04 AND *BACILLUS COAGULANS* BC 304.06 ON EXPERIMENTAL ANIMAL MODEL

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Summary

Objectives: To examine the safety of a BaciMix product, containing two strains of *Bacillus subtilis* BS 304.04 and *Bacillus coagulans* BC 304.06 on mice and rats. ***Materials and methods:*** The acute toxicity using the Litchfield-Wilcoxon method on Swiss mice and semi-chronic toxicity of the BaciMix product on Wistar rats was performed. ***Results***: LD50 of the mice which were administered orally with the highest dose of 4 x 10^{11} CFU/kg of the product was not determined. The BaciMix product was well-tolerated and did not show any effects on the growth or food intake in animals. The differences in the number of red blood cells, white blood cells, hemoglobins, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, cholesterol, protein, bilirubin and creatinine were not statistically significant in rats serum dosed continuously for 28 days with of 1.68 x 10^9 CFU/kg and 8.4 x 10^9 CFU/kg of the product. In addition, no histopathological abnormalities or changes were observed in all the groups of animals. ***Conclusion:*** These results suggested that the BaciMix product could be safe for human use.

** Keywords: Acute toxicity; Bacillus subtilis; Bacillus coagulans; Probiotics; Semi-chronic toxicity.*

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INTRODUCTION

Probiotics are live and beneficial microorganisms that are added to the human gastrointestinal tract in sufficient amounts to improve and balance the intestinal flora and inhibit harmful microorganisms, thereby improving human health [3]. Some bacteria genera are commonly used as probiotics such as *Bacillus, Lactobacillus, Bifidobacterium*, *Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Saccharomyces e.g.* [2]. The strains used as probiotics must be safe, survive and grow in the host's digestive system, inhibit harmful microorganisms, increase host metabolic efficiency and immunity [3]. *Bacillus* is widespread bacteria in nature such as in soil, air, fermented foods, and in the human intestinal tract [9]. Especially, *Bacillus* spores can survive in extremely harsh environmental conditions. *Bacillus* can compete with pathogens through the mechanism of immunosuppression, competition for adhesion sites, and production of bacteriocins or other substances [1]. In addition, *Bacillus* is popular for use due to its low cost, easy to make, and heat resistance. *Bacillus subtilis*, *B. clausii, B. coagulans* and *B. licheniformis* are normally used as probiotics. Although *Bacillus* strains are considered safe and belong to the GRAS group but several species of this group have been reported in certain infections showing a risk when used as antimicrobial agents or biological products. Besides probiotic characteristics, the safety of probiotic products is very important. Therefore, safety assessments have to perform in both *in-vitro* and *in-vivo* conditions. According to FAO/WHO guidelines, it is necessary to examine the safety of probiotic products on animal models before using for human [3]. Therefore, this study was conducted: *To examine a safety assessment of BaciMix, containing two strains Bacillus subtilis BS 304.04 and Bacillus coagulans BC 304.06, on mice and rats using acute and semichronic oral toxicity tests.*

MATERIALS AND METHODS

1. BaciMix product

BaciMix was a mixture of two probiotic strains of *Bacillus subtilis* BS 304.04, and *Bacillus coagulans* BC 304.06 in a ratio of 1:1 with the density of 3 x 10^9 CFU/g for each strain. BaciMix was a product of the National Science and Technology Project, code ĐTĐL.CN-61/19 of the Ministry of Science and Technology of Vietnam. The product was manufactured according to GMP (Standards of Good Manufacturing Practice) add Nam Viet biotechnology Joint Stock company.

The BaciMix product was strictly controlled in both quality and quantity. The product was used for animal by mixing with distilled water with a ratio of 1:1.5 (w:v).

2. Animals

Adult Swiss mice and Wistar rats 8 to 10 weeks old (healthy with a weight of 20g ± 2g for mice and a weight of 200 ± 20g for rats) were provided by the Center for Experimental Animal Research, Military Medical University. Before the experiment, they were housed in laboratory conditions for 5 - 10 days and fed according to animal feeding standards for the research subjects.

** Acute oral toxicity study:*

The study was conducted according to the guidelines of the World Health Organization (WHO), Organization for Economic Co-operation and Development (OECD), and Vietnam Ministry of Health for drug toxicity research [5, 6]. After housing days, the mice were starved for 12 hours and then dosed with the BaciMix product with a specially curved needle. Mice were randomly divided into 4 groups of 10 individuals for each group and dosed with BaciMix product or water as follows: i) Control group: dosed with 0.1 ml of distilled water/10 g body weight (WB); ii) Group 1; dosed with BaciMix 1 x 10^{11} CFU/kg BW; iii) Group 2; dosed with BaciMix 2 x 10^{11} CFU/kg BW; Group 3: dosed with BaciMix 4x10^{11} CFU/kg BW. Each dose was administered at the interval of 2 hours and the maximal dosing number per day was 4. Among the doses tested, the interval between the highest doses that did not kill an individual mouse and the lowest dose that killed 100% of the mice in the group was used for calculation. After administration of the product, the mice were fed by foods provided by the Laboratory Animal Research Center, and drank water freely. They were observed for signs continuously for 72 hours and 14 days thereafter. The ratio of dead mice was used to calculate LD50.

General observations: Number of mice dead, daily mouse weights, percentage of mice with abnormalities in movement (staying in a corner of the cage, movement disorders), and with signs of convulsions, tremors, sweating, cyanosis, as well as proportion of mice with abnormal changes in digestion (diarrhea) were observed. The above criteria were monitored before dosing, after dosing of 3 hours, 1 day, 2 days, 3 days, 6 days, 9 days, and 13 days. At the end of the study, mice were anesthetized with diethylether and operated on to get their livers, spleens, and kidneys for microscope observations.

** Semi-chronic oral toxicity study:*

Experimental design: The study was performed following the guidelines of the Vietnamese Ministry of Health on Research into the Safety and Efficacy of Traditional Medicines and Substances of Natural Origin (2015) and by the guidelines of the OECD (2018) [5, 7]. After housing days, rats were randomly divided into 3 groups of 10 individuals for each group, and then dosed with BaciMix product or water as follows: Control group: Dosed with distilled water in a volume of 5 mL/kg/24h; Group 1: dosed with 1.68 x 10^9 CFU/kg of BaciMix (equivalent to the human dose of 2g/50 kg/24 hours); Group 2: Dosed with 8.4 x 10^9 CFU/kg of BaciMix (5 times higher than the human dose). The rats were orally dosed once daily in the morning, and this continued for 28 days.

General observations: Changes in the skin, fur, respiration, excretion of mice, general signs, food intake, weight, injury, and mortality of rats during the experimental period were observed.

Hematological and serum biochemistry analyses: For hematological studies, blood samples were collected in tubes containing K2EDTA and analyzed by an automatic machine (Erba Elite - 3, Germany) for counting red blood cells, hemoglobins, white blood cells, and platelets. For biochemical studies, blood was collected into an appropriate anticoagulant tube and then centrifuged at 3.000 x g for 10 minutes to get sera. The sera were analyzed by an automatic machine (AU480 - Beckman Coulter, Japan) for the following parameters: ALT, AST, total bilirubin, total protein, cholesterol, creatinine, and blood glucose. Blood samples were collected at 3 time-points: i) before the experiment (T1), ii) after 14 days (T2), and iii) after 28 days (T3) of the product intake.

Histopathology: At the end of the experiment, the rats were anesthetized with diethyl ether. In each group, 30% of the rats were dissected to get their livers, kidneys, and spleens for weighing and histological analysis to assess the gross and microscopic damages. Visceral tissues of the livers, kidneys, and spleens were fixed in 10% formalin and then cut into thick slices of 4 µm, stained with hematoxylin and eosin and after that examined under the microscope.

** Statistical analysis:*

The collected data was processed by statistical algorithms and Microsoft Excel 2013, SPSS 20.0 software. The mean of two variable values was compared using two tests, including the paired-samples t-test and the one-way ANOVA. The difference was considered statistically significant when $p < 0.05$.

RESULTS

1. Acute oral toxicity study

The results of the 14-day acute oral toxicity study in mice with the doses of 1 x 10^{11}, 2 x 10^{11}, and 4 x 10^{11} CFU/kg BW of BaciMix product showed that the product did not cause any deaths or signs of toxicity in all of the groups. No movement disorders, seizures, cyanosis, disheveled hair, or digestive disorders occurred during the experimental period. All groups of mice increased their body weights compared to that before dosing, but the differences in their weights between the groups were not statistically significant ($p > 0.05$) (*Table 1*). Histopathological images showed normal liver, kidney, and spleen viscera and no damages were observed in the organs at the time of surgery (the results not shown here). In general, there was no evidence of acute oral toxicity when the BaciMix product was administered to the mice.

Table 1: Body weight of mice (*).

Group	Day 0	Day 3	Day 6	Day 9	Day 13
Control	17.50 ± 0.84	18.00 ± 0.94	19.30 ± 1.49	20.50 ± 1.58	23.30 ± 2.31
Group 1	18.00 ± 1.05	18.20 ± 1.03	20.00 ± 1.33	21.30 ± 1.25	24.40 ± 2.17
Group 2	18.00 ± 1.43	18.40 ± 1.43	21.10 ± 1.73	21.40 ± 1.84	23.80 ± 2.39
Group 3	18.10 ± 0.99	18.30 ± 0.95	20.10 ± 1.37	21.56 ± 1.27	24.10 ± 1.97

(: mean ± SD, n =10)*

Control group: Dosed with distilled water; Group 1: Dosed with 1 x 10^{11} CFU/kg BW; Group 2: Dosed with 2 x 10^{11} CFU/kg BW, Group 3: Dosed with 4 x 10^{11} CFU/kg BW

2. Semi-chronic oral toxicity study

There were no deaths and no signs of disturbances in motility, digestion, and excretion during the experiment. The mean weights of the rats did not show statistically significant difference between the control group and the tested groups ($p > 0.05$) (*Table 2*). Additionally, there was no statistically significant difference in the weights of the livers, kidneys, and spleens between the control group and the tested groups ($p > 0.05$) (*Table 3*).

Table 2: Body weight of rat (*).

		Group		
		Control (a)	**Group 1 (b)**	**Group 2 (c)**
Body weight (g)	Before the experiment	157.90 ± 26.53	155.70 ± 13.97	153.00 ± 17.25
	After 02 weeks	198.60 ± 15.41	199.90 ± 13.24	195.00 ± 16.97
	After 04 weeks	214.00 ± 13.26	212.70 ± 16.34	203.90 ± 17.83

(: mean ± SD, n =10)* $P_{a\text{-}b,a\text{-}c,b\text{-}c} > 0.05$

Control group: Dosed with distilled water, Group 1: Dosed with 1.68 x 10^9 CFU/kg BW, Group 2: Dosed with 8.4 x 10^9 CFU/kg BW

Table 3: Weight of liver, kidney and spleen of rat (*).

	Liver (g)	**Kidney (g)**	**Spleen (g)**
Control	6.43 ± 1.84	1.23 ± 0.15	0.55 ± 0.12
Group 1	6.52 ± 1.05	1.15 ± 0.12	0.78 ± 0.29
Group 2	6.76 ± 1.16	1.40 ± 0.13	0.56 ± 0.08

(: mean ± SD, n = 10)*

Control group: Dosed with distilled water, Group 1: Dosed with 1.68 x 10^9 CFU/kg BW, Group 2: Dosed with 8.4 x 10^9 CFU/kg BW

Hematological analysis: The results of counting hematological parameters such as red blood cells (RBC), hemoglobins (HGB), white blood cells (WBC), and platelets (PLT) showed that there was no statistically significant difference between the control group and the tested groups at all the time points of T_0, T_1 and T_2 (*Table 4*).

Table 4: Hematological parameters[1] of rats.

Group	Before experiment (T_0)				After 14 days (T_1)				After 28 days (T_2)			
	RBC (10^{12}/L)	HGB (g/L)	WBC (10^9/L)	PLT (10^9/L)	RBC (10^{12}/L)	HGB (g/L)	WBC (10^9/L)	PLT (10^9/L)	RBC (10^{12}/L)	HGB (g/L)	WBC (10^9/L)	PLT (10^9/L)
Control (a)	8.32 ± 0.38	15.68 ± 0.97	10.39 ± 2.45	403.60 ± 79.19	7.24 ± 0.37	13.60 ± 0.87	8.59 ± 2.04	538.50 ± 125.76	8.32 ± 0.47	15.01 ± 0.83	8.05 ± 1.81	528.30 ± 114.10
Group 1 (b)	8.50 ± 0.40	15.74 ± 0.93	11.66 ± 2.47	438.20 ± 158.79	7.28 ± 0.49	13.72 ± 0.82	10.18 ± 3.20	538.90 ± 93.99	8.11 ± 0.48	14.56 ± 0.77	6.87 ± 1.27	490.70 ± 124.27
Group 2 (c)	8.32 ± 0.60	15.65 ± 1.14	10.30 ± 2.57	456.90 ± 161.16	7.51 ± 0.30	14.05 ± 0.55	8.95 ± 1.79	574.20 ± 95.78	8.17 ± 0.57	15.59 ± 0.88	6.72 ± 0.96	524.00 ± 75.49

(mean ± SD, n = 10), $p_{a-b,\ a-c,\ b-c} > 0.05$)*

Control group: Dosed with distilled water, Group 1: Dosed with 1.68 x 10^9 CFU/kg BW, Group 2: Dosed with 8.4 x 10^9 CFU/kg BW

Biochemical analysis: Results of serum indices such as ALT, AST, total bilirubin (BIL), total protein (PRO) and cholesterol (CHO), creatinine (CRE), and blood glucose (GLU) showed that there was also no statistically significant difference between the control group and the tested groups at T0 T1 and T2 (*Table 5*).

Table 5: Serum biochemical parameters[1] of rats.

Group	Before experiment (T_0)						
	AST (U/L)	**ALT (U/L)**	**BIL (umol/L)**	**CHO (mmol/L)**	**CRE (µmol/L)**	**GLU (mmol/L)**	**PRO (g/L)**
Control (a)	115.00 ± 48.85	31.94 ± 9.67	2.52 ± 1.30	1.44 ± 0.13	42.31 ± 2.93	6.46 ± 0.63	71.79 ± 2.66
Group 1 (b)	108.07 ± 20.26	33.41 ± 14.43	3.32 ± 1.46	1.54 ± 0.33	40.81 ± 3.55	6.59 ± 0.66	70.07 ± 3.63
Group 2 (c)	102.14 ± 18.33	37.19 ± 7.84	3.79 ± 1.61	1.49 ± 0.33	41.78 ± 10.62	6.42 ± 0.66	69.71 ± 5.14
	After 14 days (T_1)						
Control (a)	122.61 ± 31.72	70.47 ± 13.12	3.82 ± 0.57	1.31 ± 0.18	44.62 ± 4.50	5.63 ± 0.59	68.28 ± 5.36
Group 1 (b)	123.56 ± 22.16	66.49 ± 17.94	3.38 ± 0.72	1.32 ± 0.26	46.74 ± 5.37	5.64 ± 0.70	70.94 ± 2.80
Group 2 (c)	113.09 ± 18.93	67.37 ± 12.20	3.52 ± 0.55	1.28 ± 0.24	44.25 ± 4.80	5.00 ± 0.75	71.12 ± 3.40
	After 28 days (T_2)						
Control (a)	108.31 ± 25.45	52.06 ± 25.90	2.75 ± 0.67	1.61 ± 0.35	69.86 ± 6.20	3.27 ± 1.08	85.36 ± 4.74
Group 1 (b)	120.21 ± 20.49	67.93 ± 16.04	2.81 ± 0.37	1.44 ± 0.26	68.65 ± 5.48	3.28 ± 0.59	82.92 ± 3.93
Group 2 (c)	108.09 ± 23.90	53.86 ± 10.63	2.95 ± 0.36	1.53 ± 0.16	64.82 ± 4.53	3.28 ± 0.32	82.56 ± 4.29

(mean ± SD, n = 10, $p_{a-b,\ a-c,\ b-c} > 0.05$)*

Control group: Dosed with distilled water, Group 1: Dosed with 1.68 x 10^9 CFU/kg BW, Group 2: Dosed with 8.4 x 10^9 CFU/kg BW

Histopathological examination: There were no abnormalities or histological changes in the organs of all groups of rats. There was no necrosis, fibrosis, or

loss of normal structure in internal organs such as liver, kidney, spleen, or intestine in the control and tested groups (*Figure 1*).

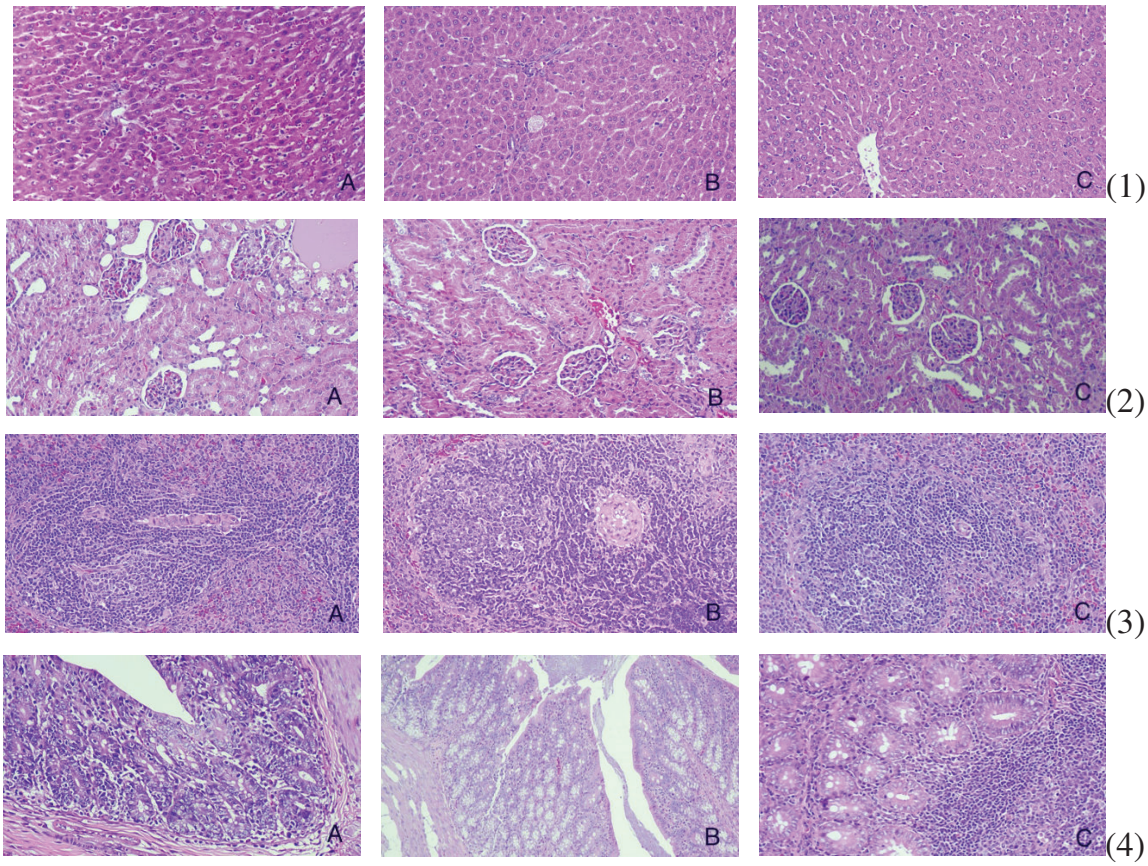

Figure 1: Light micrographs of liver (1), kidney (2), spleen (3) and intestine (4) of the groups of rats

A: Dosed with distilled water, B: Group 1: Dosed with 1.68 x 109 CFU/kg BW, C: Group 2: Dosed with 8.4 x 109 CFU/kg BW.

In particular, hepatocytes were arranged in bands, the liver rafts had vascular sinuses, and liver cells were not broken down. The renal cortex had glomeruli, tubules, and blood vessels between the tubules. Renal tubulars and epithelial cells were not degenerated**.** Splenic parenchyma was with white and red pulp. The white medulla had fairly uniform lymphoid follicles with a central quill artery. The red pulp region showed the Billroth cord and the vascular sinuses. The intestinal mucosa was thick with superficial papillary and ductal glands. Epithelial cells in intestine had small and regular nuclei and the cells of the lymphatic system were observed.

DISCUSSION

The safety assessment in animal model is an important step before applying a probiotic product for human use. In this study, we evaluated the safety of the BaciMix product through acute and subchronic oral toxicity tests. The animals used are mice since the mice have the same physiological conditions as humans, and are easy to control. The experimental conditions were strictly followed according to the guidelines of the OECD/OCDE 2008 and the guidelines of the Ministry of Health of Vietnam 2015 as well as the national guidelines for the care and use of laboratory animals. The 14-day acute oral toxicity results help us to calculate the LD50 (the dose that kills 50% of the test animals) to estimate the dose used in the semichronic and human toxicity tests. The results of the acute oral toxicity test showed that no mice died and there were no signs of abnormalities in movement (100% of the mice walked normally). No disorders such as cramps, tremors, increased sweating, or cyanosis, and digestive disorders such as decreased appetite, diarrhea, etc. were observed in all the tested groups. Therefore, the LD50 of the BaciMix product in white mice was not determined even at the dose of 4 x 10^{11} CFU/kg BW, which was equivalent to the dose of 68g/kg BW of the product.

In addition to acute toxicity data, we also performed a semi-chronic toxicity test on rats that were administered orally with the doses of 1.68 x 10^{9} CFU/kg BW for 28 days (equivalent to the human dose) and 8.4 x 10^{9} CFU/kg BW (5-fold higher than the human dose). The results of the semi-chronic toxicity study also showed no abnormalities in the tested groups, even at the high dose of 8.4 x 10^{9} CFU/kg BW. Our results showed that in the 28-day test, there were no statistically significant differences between the tested groups and the control group in terms of body weight, general conditions such as movement, excretion, gait, skin, hair; hematological, biochemical, and pathological indicators.

The results of our study are consistent with some other studies. Sorokulova et al., (2008) reported that acute and semi-chronic toxicity of two *Bacillus* strains in animals showed the LD50 of two strains more than 2 x 10^{11} CFU/kg BW, and the semi-chronic toxicity studies in rats and rabbits showed no signs of toxicity, no histological changes in experimental animals [4]. Lucas Wauters et al., 2021 studied the effect and

safety of a probiotic product consisting of two strains of *B. coagulans* MY01 and *B. subtilis* MY02 in human and showed that the product was effective and safe for human use [10]. A study by Soman et al., 2019 on the efficacy and safety of the product for human use of 3 strains of *B. coagulans*, *B. clausii*, and *B. subtilis* showed that the product was safe and the symptoms of gastrointestinal tract discomfort improved [8]. For our BaciMix product, LD50 was not determined and abnormalities of the tested safety indicators were not observed between the control and experimental groups.

CONCLUSION

The results of the study showed that BaciMix was safe in the acute and semi-chronic oral toxicity tests in laboratory animals. BaciMix was safe up to the level of 4 x 10^{11} CFU/kg BW mice/day in a 14 - day treatment period. The LD50 of the BaciMix product in laboratory animals was not determined. In addition, regarding overall assessments, body weights, and biochemical and hematological parameters, no abnormalities were observed in the probiotic tested groups with 1.68 x 10^9 CFU/kg and 8.4 x 10^9 CFU/kg of the product. This suggests that BaciMix could be safe for human use. However, it is necessary to further evaluate the product's effects on animal models to use for human.

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