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Paper

Oral toxicity evaluation of *Bacillus clausii* M31 isolated from the children's feces in the northern province of Vietnam

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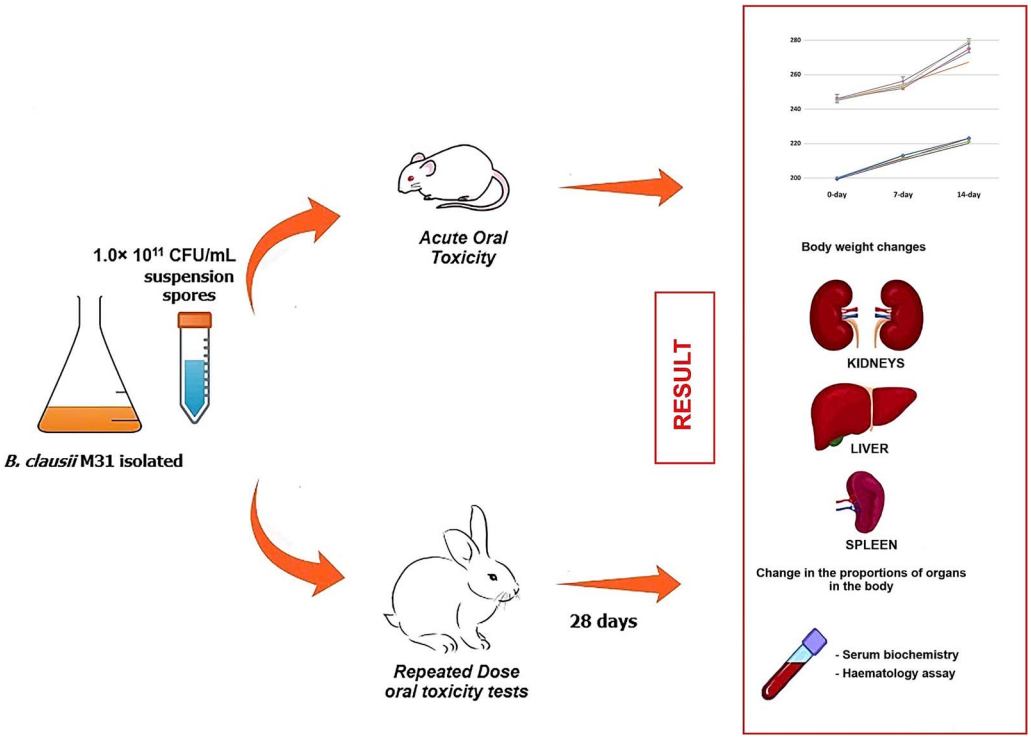
This study investigated the acute and repeated 28-day dose toxicity profiles of *Bacillus clausii* M31, isolated from children's feces, in Swiss rats and New Zealand rabbits. To investigate acute toxicity, rats were given varied doses of *B. clausii* M31 (1×10^{11} CFU/mL, 3×10^{11} CFU/mL, and 5×10^{11} CFU/mL) orally once daily for 14 days, in accordance with OECD recommendations No. 423. To evaluate toxicity, rabbits were given either a low dosage (1×10^{11} CFU/mL) or a high dose (5×10^{11} CFU/mL) during a 28-day period using the OECD Test Guideline 407 protocol. Neither death nor significant abnormalities were observed in the rats during the experiment. The microscopic examination of key organs revealed no substantial changes in organ morphology. Furthermore, analyses of serum biochemistry and hematological parameters did not reveal any treatment-associated variations. In sum, these findings suggest that the oral intake of *B. clausii* M31 at concentrations up to 5×10^{11} CFU/mL for 28 days poses no discernible risks.

Graphical Abstract



Key words: B. Clausii M31; Probiotic safety; Rat; Rabbit.

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Introduction

According to the World Health Organization (WHO) mentioned since 2000, probiotics refer to living microorganisms that offer benefits to the host when administered in adequate quantities.[1] The close association between gut microbiota and the overall health of the host, particularly in relation to metabolic disorders, is well-established.[2] Many studies and clinical trials increasingly support the notion that modifying the gut microbiota to restore balance can contribute to reinstating the body to a normal, healthy condition.[2,3] *Lactobacillus*, *Bifidobacterium* and *Saccaromyches* widely consumed globally, are recognized for their health-promoting effects. Notably, spore-forming bacteria, primarily *Bacillus*, are gaining attention due to their probiotic properties. Scientific literature has provided evidence supporting the efficacy, safety, and historical consumption of spore-forming bacteria strains belonging to widely known. *Bacillus* species like *B. subtilis*, *Bacillus clausii*, *B. coagulans*, and *B. Amiloliquefaciens*.[4,5] Furthermore, *Bacillus* strains have been utilized for the production of extracellular enzymes such as amylase, protease, cellulase, and pectinase.[6]

However, *Bacillus* species, unlike *Lactobacillus* and *Bifidobacterium*, *Bacillus* does not possess the "Generally Recognized as Safe" (GRAS) status, as some *Bacillus* strains have been identified as pathogenic.[7] Consequently, it is crucial to assess the safety of *Bacillus*, particularly concerning its impact on human health through ingestion. Various studies have investigated the toxicity of *Bacillus* species, particularly *B. subtilis*, *B. coagulans*, *Bacillus licheniformis*, *B. clausii*,[6] and more recently, *Bacillus amyloliquefaciens*.[8] Notably, these studies reported no adverse effects on the tested animals. However, it is essential to acknowledge that the benefits of probiotics and their potential for pathogenicity are specific to the individual strain.[9] Consequently, routine and individual evaluations of strain toxicity are imperative.

The human digestive tract is home to a diverse range of microbes, including *B. clausii*,[8] which thrives in stool after ten days. *B. clausii* M31 strain was isolated by the laboratory. However, in addition to examining probiotic properties such as spore-forming capability, heat resistance, acid and bile salt tolerance, non-hemolytic activity, and so on, it is critical to evaluate *B. clausii* M31's toxicity. Furthermore, there is little previous research on the toxicity of *B. clausii* strains.

The purpose of this study was to assess the toxicity of *B. clausii* M31, which was isolated from children's feces, using acute and repeated dose 28-day oral toxicity tests in rats and rabbits using OECD guidelines No. 423 and No. 407.[10,11] This research is part of our ongoing project focused on the development of probiotic products for future applications.

Materials and methods

The Appraisal Council, along with the Scientific Council, approved all experimental procedures involving laboratory animals, including rats and rabbits, as well as housing and feeding conditions, in accordance with Decision 563/QĐ-UBND on February 25th, 2022. Furthermore, the experiments were carried out in accordance with generally accepted ethical standards in animal treatment, as established by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986)[12] and the Convention required by Directive 2010/63/EU of the European Parliament and of the Council of 2010 September 22 on the protection of animals used for scientific purposes.[13] The experimental animals were divided into groups based on principles that considered their age, body weight, and gender. Body weight deviation vs The average value is allowed to be no more than 10% for animals of the same group. Factors such as physiological state, behavior and the work of ensuring animal safety during the quarantine period is monitored and implemented daily.

Animal

This study utilized adult male and female white rats of the Swiss strain in acute test. The Repeated Dose 28-day test in rabbits New Zealand strain. The experimental animals were supplied by the National Institute of Hygiene and Epidemiology.

Prior to the commencement of the study, the rats underwent acclimatization to standard laboratory conditions, including a temperature of 22 ± 2 °C, relative humidity maintained between 45%–60%, and a 12-h dark–light cycle. The animals were provided with standard food and had unrestricted access to water.

Preparation of culture for *B. clausii* M31

The lyophilized powder form of *B. clausii* M31, isolated in a laboratory in Vietnam, is stored in a − 80 °C deep refrigerator (*Panasonic, Japan*). The *B. clausii* M31 strain, originally isolated from children's feces in the laboratory following the methods detailed by Lee.[14] *B. clausii* M31 was cultivated in a Nutrient Broth (*NB, Himedia, India*) and underwent a 24 h incubation at 37 °C in a shaker incubator. Subsequently, the culture was centrifuged at 6,000 rpm for 10 min at 4 °C, leading to the removal of supernatants. The resulting cell pellets underwent two washes before being resuspended in phosphate-buffered saline (PBS) (*Merk, Germany*) at pH 7.2. For standardizing the bacterial concentration, optical density (OD) measurements were conducted at 600 nm using a UV–VIS spectrophotometer (*Evolution 60S, Thermo, USA*). The recorded readings were OD 7.3, 8.5, and 10.8, corresponding to viable counts of approximately 1.8×10^9 CFU/mL, 2.0×10^{10} CFU/mL, and 1.0×10^{11} CFU/mL, respectively. The culture pellets were preserved in 20% glycerol (*Merck, Germany*) (and stored at −20 °C until further use. Fresh cell suspensions were prepared from the glycerol stocks daily before being administered to the animals through syringe feeding. After centrifugation (*Hettich Eba, Germany*) at 6,000 rpm for 10 min at 4 °C, the resulting pellet was resuspended in water.

Acute toxicity study

Acute toxicity studies were conducted according to the OECD Guideline for the Testing of Chemicals No. 423, Acute Oral Toxicity - Acute Toxic class Methods, adopted on 17th 2001 December 10 Animals were randomly assigned to five different groups (n_group = 5) based on body weight, with males (205–249 g) and females (190–214 g). A total of 90 rats were used (5 groups) × (3 males +3 females)/groups x 3 times (the experiment was repeated 3 times to have mean). *B. clausii* M31 biomass suspension (Batch number: BCM31.02.2023) had a concentration of 1×10^{11} CFU/mL, and a volume of 5 mL/kg body weight was administered. The control group (Group 1) received 5 mL/kg of drinking water. The positive control group (Group 2) was orally administered *B. clausii* 088AE (in the commercial products in Vietnam, spore preparation of *B. clausii* 088AE (5 billion colony-forming unit per gram, CFU/g) at a dose of 5,000 mg/kg (equivalent to 25×10^{10} CFU/kg) body weight/rats once.

Group 1: Control group (C) - Normal rat, received a normal diet.

Group 2: Positive control group (P) used 5 mL *B. clausii* 088AE (25×10^{10} CFU/kg) in water.

Group 3: Low Dose (LD) - received a normal diet and 1.0 mL *B. clausii* M31 (1.0×10^{11} CFU/mL) in water.

Group 4: Medium Dose (MD) - received a normal diet and 3.0 mL *B. clausii* M31 (3.0×10^{11} CFU/mL) in water.

Group 5: High Dose (HD) - received a normal diet and 5.0 mL *B. clausii* M31 (5.0×10^{11} CFU/mL ~ 5,000 mg/kg body weight) in water.

Repeated dose 28-day oral toxicity study

This oral toxicity study was performed according to the OECD Guideline for the Testing of Chemicals No. 407, repeated dose 28-day oral toxicity study. This experiment was conducted over 28 days. Related the product Enterogermina containing *B. clausii* 2 billion CFU/5 mL (*Sanofi, France*) is 3 vials per day (15 mL of Enterogermina/ person/day or 6 x 10^9 CFU spores/person/day), which is equivalent to 0.01 mL *B. clausii* M31 spores/person/day.

A total of 18 rabbits were used for both sexes, 9 male rabbits (2025–2,234 g) and 9 female rabbits (1918–2096 g). Animals were randomly selected based on body weight and divided into 3 groups (n = 6 rabbits/group, 3 males, 3 females).

Group 1: Control group (drank sterilized water, 5 mL/kg body weight/day).

Group 2: Low dose-received a normal diet and 5 mL *B. clausii* M31 (1×10^{11} FU/mL) in water. [5.0 mL × (1×10^{11} CFU/mL) is equivalent to 5×10^{11} CFU/kg body weight/day].

Group 3: High dose—receive a normal diet and 5 mL *B. clausii* M31 concentrated 2 times after centrifugation [2x(5×10^{11} CFU/mL) ~ 10×10^{11} CFU/kg body weight/day ~5,000 mg/kg body weight].

Test doses for each group were prepared. Antiseptic water was used as the control medium. The *B. clausii* M31 biomass suspension was appropriately diluted with distilled water. Rabbits in the control group were administered distilled water for consumption. The rabbits underwent daily monitoring for food and water intake, physical condition, behavior, as well as the examination of feces and urine. We recorded the weights of the rabbits on days 0, 7, 14, and 28 of the observation period. Hematological indices related to hematopoietic function (number of red blood cells, white blood cells, platelets, hemoglobin, hematocrit), liver function (aspartate transaminase - AST, Alanine transaminase - ALT, total protein, cholesterol), kidney function (creatinine, urea), and glucose levels were measured on days 0, 14, and 28 days. On day 28, histopathological examination of the liver, kidney, spleen, heart, lung was conducted on three rabbits from each group for microscopic evaluation immediately after treatment. Upon the study's conclusion, the remaining animals underwent dissection to observe the general condition of organs such as the heart, liver, kidneys, lungs, and spleens and the difference of animals between groups tested.

General observations

The overall observations made during the oral toxicity study encompass alterations in skin and fur conditions, respiratory activity, movement patterns, behavior, occurrence of tremors, convulsions, salivation, diarrhea, fatigue, sleep patterns, and changes in posture.[15,16] Throughout the experimental period, the examination of physical parameters includes monitoring body weight, assessing local injuries, and recording any instances of mortality.

Histopathological examination

All preserved tissues were fixed in a buffered formalin solution (10% v/v) (*Merck, Germany*) and subsequently embedded in paraffin. Sections of 5 μm thickness were cut using a microtome (Leica RM2145, Germany), mounted on albumin-coated glass slides, and allowed to dry overnight. The sections were deparaffinized in xylene, rehydrated through a graded series of alcohols, stained with hematoxylin and eosin (Mayer's), mounted with DPX mounting medium, and examined under a microscope.(*HHM-2414 W Novel, Japan*).

Biochemical analyses of blood

At the conclusion of the experiment, rats were anesthetized with carbon dioxide, and blood samples were obtained via cardiac puncture for subsequent hematology and serum biochemistry analyses. Blood collected for biochemistry analysis was placed in non-heparin tubes and allowed to clot at room temperature before serum separation through centrifugation (4,000 rpm for 10 min) at 4 °C. The obtained serum was then stored at −4 °C until analysis using an automated clinical chemistry analyzer. The parameters assessed in serum biochemistry analysis included albumin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatinine, total bilirubin, urea, and total protein. Blood collected in K2EDTA tubes and underwent analysis using an automated hematology analyzer (*Nihon Kohden MEK-7300 K, Japan*). The parameters examined for whole blood cells encompassed red blood cell count, hemoglobin levels, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin concentration, thrombocytes, and white blood cell count.

Statistical analysis

The statistical analysis was carried out using SPSS software. All data were presented as Mean ± standard deviation (SD). Various datasets, including body weight, hematological parameters, clinical chemistry parameters, urine analysis, and organ-to-body weight percentages, were analyzed. The statistical significance was determined using one-way analysis of variance at the 5% level ($P < 0.05$).

Result

Results of oral acute study

Results of observations

During the 14-day administration period, no abnormal changes in hair appearance, watery eyes, or runny nose were noted in either male or female rats in both the *B. clausii* M31 and control groups. No signs of abnormalities or deceased rats were observed in any of the test groups. Additional observations of the animals were not displayed signs of toxicity. Observations included no changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatic motor activity and behaviour pattern. There were no statistically significant ($P > 0.05$) variations observed in absolute body weight gain between the rats in three *B. clausii* M31 groups and those in the control group and *B. clausii* 088AE group, irrespective of gender (*refer to* Fig. 1). Consequently, the growth pattern of rats subjected to *B. clausii* M31 treatments mirrored that of the control group. This result did not appear unexpectedly when using *B. clausii* M31 orally compared to the drinking water control group and the positive control group. This result is no different from previous studies on *Bacillus* and some other probiotics.[9,14,17,18]

Results of body rat's weight changes

The mean weight of rats throughout the 7-day trial did not exhibit a statistically significant difference between the test groups (L1, L2, L3 in both sexes) and the control group (C), with preceding pANOVA values all exceeding <0.05. Refer to Table 1 and Fig. 1,

Table 1. Comparison of the rat weight between the control and the test groups.

	Weight of rat (gam)		
Group	**Time test**		
Female	0-day	7-day	14-day
Control (Cf)	199.34 ± 1.88	210.45 ± 3.28	220.20 ± 3.12
Positive control (Pf)	200.06 ± 1.85	211.23 ± 2.21	223.10 ± 2.85
Low dose (L1f)	200.04 ± 2.27	211.70 ± 2.15	221.29 ± 3.24
Medium dose (L2f)	199.51 ± 2.90	213.08 ± 1.90	223.17 ± 2.16
High dose (L3f)	200.16 ± 2.07	213.26 ± 3.51	223.16 ± 2.61
Male	0-day	7-day	14-day
Control (Cm)	245.63 ± 1.34	254.45 ± 3.23	267.34 ± 1.88
Positive control (Pm)	245.06 ± 2.87	253.20 ± 2.18	273.15 ± 2.63
Low dose (L1m)	246.17 ± 1.21	252.06 ± 3.15	275.04 ± 2.27
Medium dose (L2m)	245.52 ± 2.06	253.41 ± 2.78	279.51 ± 2.60
High dose (L3m)	246.13 ± 2.85	256.26 ± 2.81	278.16 ± 2.07

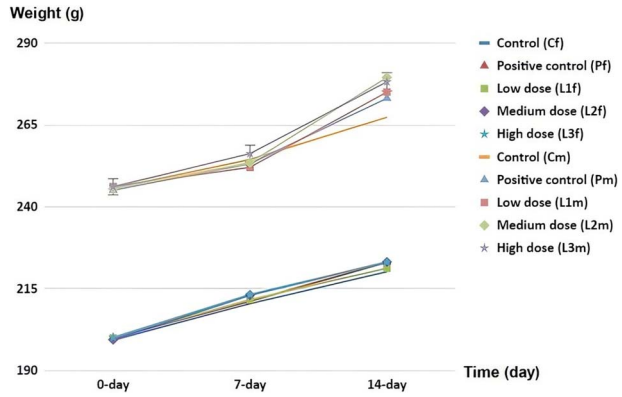

Fig. 1. Body weight changes among male and female rats during 14 days of oral administration of *Bacillus clausii* M31 note: Values are presented as mean ± standard deviation. Data groups are compared with each other and with the standard group, the comparison groups show $P < 0.05$.

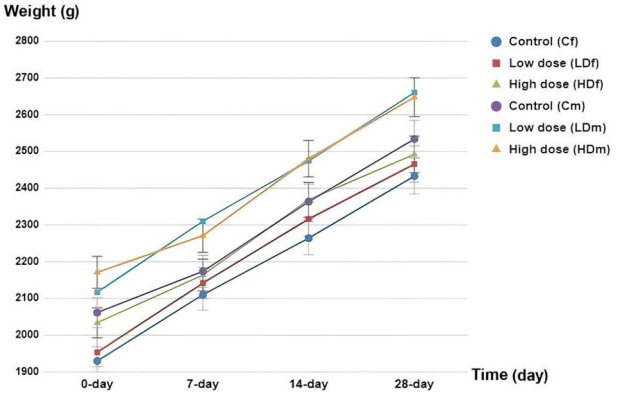

Fig. 2. Body weight changes among male and female rabbits during 28 days of oral administration of *B. clausii* M31 note: Values are presented as mean ± standard deviation. Data groups are compared with each other and with the standard group, the comparison groups show $P < 0.05$.

over the 7-day period, all control and test groups showed a consistent weight gain compared to day 0 within each respective group (p before-after <0.001). Notably, by day 28, a statistically significant disparity in average weight emerged among the test groups compared to the control group, supported by pANOVA values after <0.05, (*see* Table 1). The experiment established that the lethal dose 50 (LD_{50}) for the test animals exposed to the suspension of *B. clausii* M31 spores was greater than 5.0 mL sample/kg, equivalent to 25×10^{10} CFU/kg or approximately 5,000 mg/kg. In line with the toxicity classification outlined in the Globally Harmonized System (GHS) of 2019, the *B. clausii* M31 spores suspension exhibited acute toxicity below the GHS threshold.

During the study period, rats in all groups exhibited normal behavior, had smooth fur, and showed no changes in excretion of stool formation. All rats experienced an increase in weight throughout the study period ($P < 0.01$). Specifically, a significant change in weight was observed when comparing batches using *B. clausii* M31 with the control batch after 14 days ($P > 0.05$). The data indicate that rats drinking *B. clausii* M31 for 14 days exhibited a different weight change compared to the rats in the control group that consumed only water. Thus, *B. clausii* M31 did not affect metabolism during the experiment but did have an impact on the weight of rats after 14 days, differing from the control group that only drank water. This result is also consistent with previous studies.[8,9]

Results of organ of rat's weight changes

Addition, there were no significant ($P > 0.05$) differences in terminal organ weights of heart, liver, kidney, lungs, spleen and kidney between the *B. clausii* M31 and control groups in both sexes and their changes over time: 7 days, 14 days until the end of the experiment (*see* Table 2).

Results of repeated dose 28-day study

Results of body rabbit's weight changes

During a 28-day dosing period, both sexes of rabbits (male and female) showed no aberrant changes in behavior, body temperature, hair appearance, lacrimation, or nasal discharge. There was no significant difference ($P > 0.05$) in absolute body weight growth between *B. clausii* M31 and the control group for both sexes (Fig. 2). Rabbits treated with *B. clausii* M31 showed identical growth patterns to the control group.

Results of organ of rat's weight changes

In addition to weight changes in the rabbit, there were no differences in terminal organ weights of the heart, liver, kidney, lungs,

Table 2. Comparison of the organ of rat's weight between the control and the test groups.

Organ	Male/Treatment (CFU/mL) of Acute Toxicity				
	Control	Positive control (P) *Bacillus clausii* 088AE	*B. clausii* M31		
			1×10^{11} (LD)	3.0×10^{11} (MD)	5.0×10^{11} (HD)
Lung	0.54 ± 0.02	0.56 ± 0.13	0.56 ± 0.10	0.61 ± 0.07	0.56 ± 0.12
Heart	0.35 ± 0.02	0.52 ± 0.15	0.41 ± 0.45	0.40 ± 0.06	0.53 ± 0.05
Liver	4.15 ± 0.24	4.81 ± 0.24	4.34 ± 0.30	4.94 ± 0.42	4.70 ± 0.73
Spleen	0.23 ± 0.02	0.22 ± 0.06	0.25 ± 0.03	0.30 ± 0.10	0.24 ± 0.05
Right kidney	0.38 ± 0.06	0.46 ± 0.12	0.43 ± 0.04	0.45 ± 0.10	0.44 ± 0.11
Left Kidney	0.37 ± 0.03	0.43 ± 0.13	0.38 ± 0.03	0.38 ± 0.07	0.42 ± 0.15
	Female/Treatment (CFU/mL) Acute Toxicity				
Organ	**Control**	**Positive control (P) *B. clausii* 088AE**	***B. clausii* M31**		
			1×10^{11} (LD)	3.0×10^{11} (MD)	5.0×10^{11} (HD)
Lung	0.52 ± 0.11	0.54 ± 0.04	0.50 ± 0.16	0.59 ± 0.17	0.48 ± 0.21
Heart	0.39 ± 0.07	0.51 ± 0.10	0.36 ± 0.09	0.45 ± 0.08	0.52 ± 0.12
Liver	4.23 ± 0.25	4.81 ± 0.24	3.76 ± 0.51	3.63 ± 0.42	4.00 ± 0.62
Spleen	0.27 ± 0.04	0.21 ± 0.03	0.25 ± 0.06	0.26 ± 0.07	0.22 ± 0.03
Right kidney	0.43 ± 0.09	0.44 ± 0.10	0.38 ± 0.08	0.40 ± 0.12	0.44 ± 0.03
Left Kidney	0.40 ± 0.07	0.42 ± 0.13	0.38 ± 0.06	0.37 ± 0.07	0.41 ± 0.04

There were no statistical differences in each strain at the level of $p < 0.05$. Values are expressed as mean ± S.D. ($n = 5$). Statistical analysis was performed by one-way ANOVA. (C- drink water, P-positive control *B. clausii* 088AE; test: *B. clausii* M31 LD-low dose, MD-medium dose, HD-high dose).

Table 3. Comparison of the organ of rabbit's weight between the control and the test groups.

Treatment, CFU/mL			
Organ	**Male/ Repeated Dose 28-day oral toxicity tests**		
	Control	***Bacillus clausii* M31**	
		1.0×10^{11} (LD)	10.0×10^{11} (HD)
Lung	10.51 ± 0.52	9.52 ± 0.20	10.5 ± 0.52
Heart	5.32 ± 0.13	5.56 ± 0.25	5.69 ± 0.17
Liver	67.15 ± 1.12	66.21 ± 1.40	65.34 ± 1.62
Spleen	5.41 ± 0.41	5.62 ± 0.41	5.31 ± 0.40
Right kidney	9.30 ± 0.61	9.20 ± 0.21	9.50 ± 0.34
Left Kidney	8.30 ± 0.32	9.01 ± 0.20	8.83 ± 0.42
Organ	**Female/Repeated Dose 28-day oral toxicity tests**		
	Control	***B. clausii* M31**	
		1.0×10^{11} (LD)	10.0×10^{11} (HD)
Lung	7.32 ± 0.47	7.25 ± 0.36	7.50 ± 0.50
Heart	4.12 ± 0.15	4.16 ± 0.20	4.69 ± 0.19
Liver	50.10 ± 1.19	49.21 ± 1.51	50.34 ± 1.10
Spleen	3.65 ± 0.11	3.67 ± 0.92	3.71 ± 0.36
Right kidney	7.32 ± 0.23	7.50 ± 0.20	7.47 ± 0.25
Left Kidney	6.74 ± 0.22	6.31 ± 0.35	6.46 ± 0.41

Different superscript letters in the same row indicate statistical differences in each strain at the level of $P < 0.05$. Values are expressed as mean ± S.D. ($n = 8$). Statistical analysis was performed by one-way ANOVA followed by Dunnet's *t*-test (LD-low dose, HD-high dose).

spleen, and kidney between test group and control groups in both sexes, and their changes over time: 7 days, 14 days, and the end of the experiment (*see* Table 3).

Serum biochemistry profile

The serum biochemical parameters, including albumin, total protein, creatinine, cholesterol, triglyceride, ALP, ALT, AST, glucose did not exhibit significant differences between the treatment and control groups in both genders (*refer to* Table 4).

In comparing the blood biochemical parameters between the control group and the test group, it is crucial to acknowledge that the observed differences are not statistically significant $P < 0.05$. The meticulous analysis of the data reveals that the variations in biochemical markers such as albumin, total protein (TP), urea nitrogen (Urea N), creatinine, cholesterol, triglycerides, alanine

Table 4. Serum biochemistry profile of rabbits after repeated dose 28-day oral toxicity tests.

Sex/treatment		Serum biochemistry parameters									
		Albumin (g/dL)	TP (g/dL)	Urea N (mg/dL)	Creatinine (mg/dL)	Cholesterol (mg/dL)	Triglyceride (mg/dL)	ALP (IU/L)	ALT (IU/L)	AST (IU/L)	Glucose (mmol/L)
Female	Control a1	3.09 ± 0.18	6.23 ± 0.21	46.25 ± 0.97	0.96 ± 0.05	154.92 ± 3.90	169.50 ± 10.11	57.42 ± 2.41	50.82 ± 4.80	23.50 ± 1.65	6.50 ± 0.24
	Low dose b1	3.24 ± 0.16	6.36 ± 0.30	45.30 ± 2.00	1.05 ± 0.07	124.20 ± 5.76	116.10 ± 8.21	57.90 ± 1.30	49.89 ± 2.48	24.31 ± 2.35	6.24 ± 0.25
	Medium dose c1	3.16 ± 0.14	6.49 ± 0.29	36.92 ± 1.73	1.08 ± 0.03	125.25 ± 4.07	110.83 ± 10.35	53.42 ± 3.77	47.71 ± 2.76	25.01 ± 1.68	6.16 ± 0.41
	High dose d1	3.03 ± 0.09	6.43 ± 0.17	38.17 ± 1.27	0.91 ± 0.03	110.67 ± 8.41	120.83 ± 14.30	59.33 ± 2.47	47.67 ± 2.08	24.65 ± 2.15	6.22 ± 0.35
Male	Control a2	4.15 ± 0.21	6.87 ± 0.32	38.08 ± 2.07	0.89 ± 0.05	166.92 ± 5.73	176.58 ± 4.21	57.00 ± 1.79	75.56 ± 0.88	39.50 ± 3.60	7.62 ± 0.61
	Low dose b2	4.08 ± 0.35	6.95 ± 0.36	37.67 ± 1.56	0.93 ± 0.08	128.67 ± 8.66	128.83 ± 6.77	60.92 ± 2.64	69.00 ± 2.69	39.71 ± 3.81	7.50 ± 0.40
	Medium dose c2	3.92 ± 0.39	6.93 ± 0.45	38.83 ± 2.25	0.92 ± 0.06	118.50 ± 6.29	125.50 ± 5.90	60.83 ± 2.23	67.75 ± 1.83	40.35 ± 4.28	7.60 ± 0.69
	High dose d2	3.89 ± 0.21	6.84 ± 0.28	40.58 ± 2.75	1.10 ± 0.10	127.17 ± 4.76	130.92 ± 5.52	58.50 ± 1.92	62.00 ± 1.96	39.14 ± 2.53	7.46 ± 0.30

Values are presented as mean ± standard deviation. SD, Swiss strain; TP: total protein; ALP: alkaline phosphatase; urea-N: urea-nitrogen; ALT: alanine aminotransferase; AST: aspartate transaminase.

transaminase (ALT), aspartate transaminase (AST), and glucose activities do not surpass the established threshold for significance. In the absence of a statistically significant distinction, it is reasonable to infer that the experimental intervention did not exert a substantial impact on the measured blood parameters. These results underscore the importance of rigorous statistical analysis and emphasize the need for further research to explore potential underlying factors contributing to the observed non-significant differences.

Haematology assay

The haematology results indicated no statistically significant ($P > 0.05$) disparities between the *B. clausii* M31 and control groups for parameters including hemoglobin, WBC count, RBC counts, platelet counts, MCV, MCH and MCHC in both male and female rabbits between the control group and the test group (*refer to* Table 5).

Upon scrutinizing the blood hematological parameters between the control group and the test group, it became evident that the differences observed were not statistically significant. Rigorous analysis of hematological markers, including red blood cell count, white blood cell count, hemoglobin levels, and platelet count, fails to breach predetermined threshold for statistical significance. In the absence of a statistically significant distinction, it s reasonable to assert that the experimental intervention did not induce a substantial impact on the measured hematological parameters. These findings emphasize the importance of a meticulous approach to statistical analysis, as well as the need for further investigation to elucidate potential underlying factors contributing to the observed non-significant differences. While the numerical variances may exist, the lack of statistical significance underscores the stability and consistency in hematological profiles between the control and test groups.

In addition, the image of microscopic observation showed no significant changes in histological structure of the liver, kidney and spleen of rabbits between the control group and other groups (including low dose and high dose) following Figs 3–5.

The results of measuring creatinine in the blood of rats (Table 5) in the treatment groups showed no statistically significant difference compared to the control group as well as comparison between the before and after taking *B. clausii* M31 ($P > 0.05$). These results are consistent with the results of kidney histopathology. While observing the macroscopic kidney and microscopic structure of the kidneys of experimental rats (Fig. 3) showed that in two groups drinking *B. clausii* M31, the macroscopic image of the microscopic structure of the functional areas of the kidney was as normal as that of control rats. The structure of the kidney function areas is normal, the renal tubular epithelial cells are not degenerated.

The ALT, AST, total protein, albumin, ure, creatinine (Table 5) in rat blood from the two groups drinking *B. clausii* M31 showed no significant differences compared to the control group, both before and after the test (Figs 4 and 5). This suggests that *B. clausii* M31 did not cause damage to liver, kidney and spleen cells. Histopathological results were consistent with the blood biochemical test results. Rabbits drank *B. clausii* M31 all exhibited a normal liver cell structure, normal portal spaces, and blood vessels similar to the control group. Liver cells were arranged in strips and trabeculae, with vascular sinuses between them. In the treatment groups, liver cells did not degenerate. Additionally, *B. clausii* M31 did not affect the biochemical indices of rat blood. Thus, this evidence demonstrates that *B. clausii* M31 was not toxic to the liver and

Table 5. Heamatological parameter of male and female rabbits after repeated dose 28-day oral toxicity tests.

Sex/treatment		Hematological parameters						
		HB (g/dL)	WBC x 10^3/mm^3	RBC x 10^6/mm^3	PLT	MCV (mcm^3)	MCH (pg)	MCHC (g/dL)
Female	Control a1	15.02 ± 0.18	6.03 ± 0.21	7.25 ± 0.47	296 ± 15.05	64.92 ± 1.90	22.50 ± 0.11	36.42 ± 0.41
	Positive b1	14.94 ± 0.16	5.96 ± 0.30	7.05 ± 0.10	315 ± 14.07	64.20 ± 3.76	21.10 ± 0.21	36.90 ± 0.30
	Low dose c1	14.96 ± 0.14	5.79 ± 0.29	6.92 ± 0.73	338 ± 17.07	65.25 ± 3.07	22.83 ± 0.35	35.42 ± 0.77
	High dose d1	14.03 ± 0.09	6.01 ± 0.17	7.17 ± 0.27	331 ± 10.03	63.67 ± 4.41	22.24 ± 0.30	34.33 ± 0.47
Male	Control a2	12.35 ± 0.21	7.87 ± 0.32	6.08 ± 0.67	341 ± 10.15	64.92 ± 1.73	20.58 ± 0.21	28.00 ± 1.79
	Positive b2	12.48 ± 0.35	7.95 ± 0.16	6.67 ± 0.56	343 ± 12.08	64.67 ± 2.66	20.83 ± 0.77	30.92 ± 0.64
	Low dose c2	11.20 ± 0.39	7.93 ± 0.25	7.13 ± 0.25	342 ± 11.19	68.50 ± 1.29	19.50 ± 0.90	30.83 ± 2.23
	High dose d2	11.33 ± 0.21	8.04 ± 0.28	6.58 ± 0.75	340 ± 15.10	87.17 ± 2.76	20.92 ± 0.52	32.50 ± 2.92

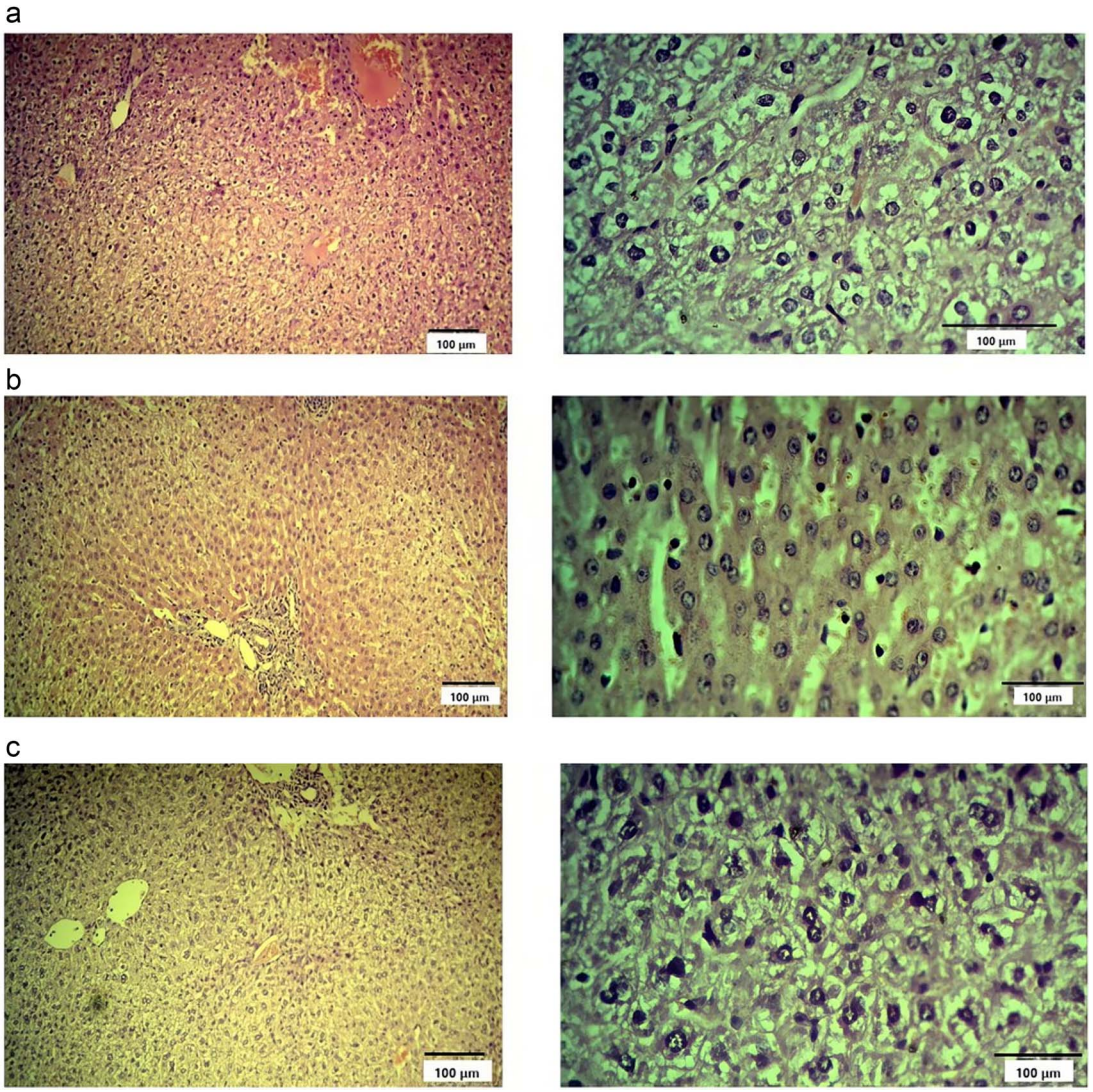

Fig. 3. Histopathological analysis of rabbit liver organs with *B. clausii* M31 culture from repeated dose 28-day oral toxicity tests (a1. Control; b1. Low dose; c1. High dose).

kidneys, and it did not impact the function of these organs. These results are also consistent with previous studies.[19,20]

Discussion

Results of oral acute toxicity tests in rats and Repeated Dose 28-day Oral Toxicity Study tests in rabbits demonstrated the complete safety of *B. clausii* M31 spores. The administration of *B. clausii* M31 did not adversely impact the overall health, growth, and development of the test animals. This was evident in both the Repeated Dose 28-day toxicity study in rabbits, where the dose administered was three times higher than the suggested dosage in humans, and in the acute toxicity study in rats. The study did not record any animals dying during the experiment. When

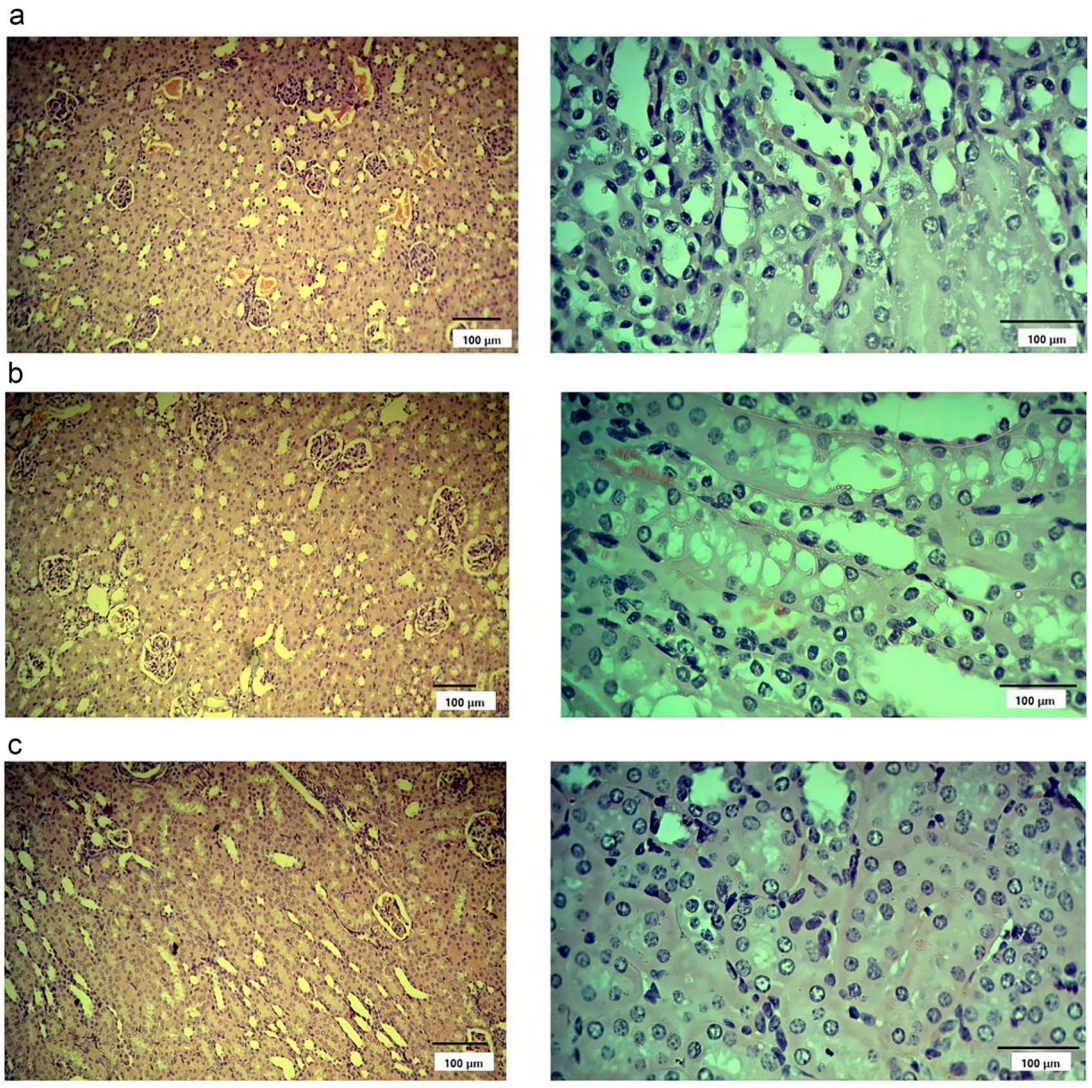

Fig. 4. Histopathological analysis of rabbit organs kidney with *B. clausii* M31 from repeated dose 28-day oral toxicity tests (a2. Control; b2. Low dose; c2. High dose).

comparing the rat weight between the control and the test groups, rats gained weight evenly, with no difference from the control group. No lethal LD_{50} was found for rats and rabbits. Our results also coincide with another toxicity study in Swiss rats using *B. clausii* UBBC 07 (>5,000 mg/kg body weight)[8,15,21] and other probiotic such as *Lactobacillus* and *Bifidobacterium*[22] Acute toxicity is dose-dependent and a 28-day subchronic toxicity study did not show treatment-related mortality. These results are also consistent like other probiotics such as *B. subtilis*.[22,23] No significant differences ($P > 0.05$) were observed in the terminal organ weights of the heart, liver, kidneys, lungs, and spleen between the *B. clausii* M31 and control groups in both sexes across the 7-day, 14-day, and final periods of the experiment.

About body rabbit's weight changes, results of Repeated Dose 28-day study showed the rabbits exhibited uniform weight gain, showing no discernible difference from the control group. Similarly, in rats also gained weight steadily, and no statistically significant data were tested using pANOVA. And result's test of serum biochemistry profile, in the present investigation, the oral administration of *B. clausii* M31 at low, medium, and high doses did not elicit adverse effects on the kidneys or liver. Furthermore, alterations in urea-nitrogen, as well as AST and ALT levels, remained within the normal physiological range for the subjects. The hematological analysis revealed no statistically significant differences ($P > 0.05$) between the *B. clausii* M31 and control groups for parameters such as hemoglobin, white blood cell (WBC) count, red blood cell (RBC) count, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) in both male and female rabbits. This had proved that the effects of *B. clausii* M31 on the two sexes of rabbits are no different. Histopathological examination further indicated that *B. clausii* M31 did not induce discernible changes in the kidneys and liver, affirming the normal functionality of these organs. Lifetime data analysis revealed no detrimental impact on body weight gain in both male and female subjects within the *B. clausii* M31 group compared to the control group after 7 days. However, a statistically significant difference in body weight emerged between the test and control groups after 28 days ($P < 0.05$). This observation suggests that the administered treatments were well-tolerated by the animals, implying a potential role for *B. clausii* M31 in supporting weight gain. Additionally, variations in certain serum biochemical and hematological parameters may be attributed to individual biological factors, variations in blood sampling

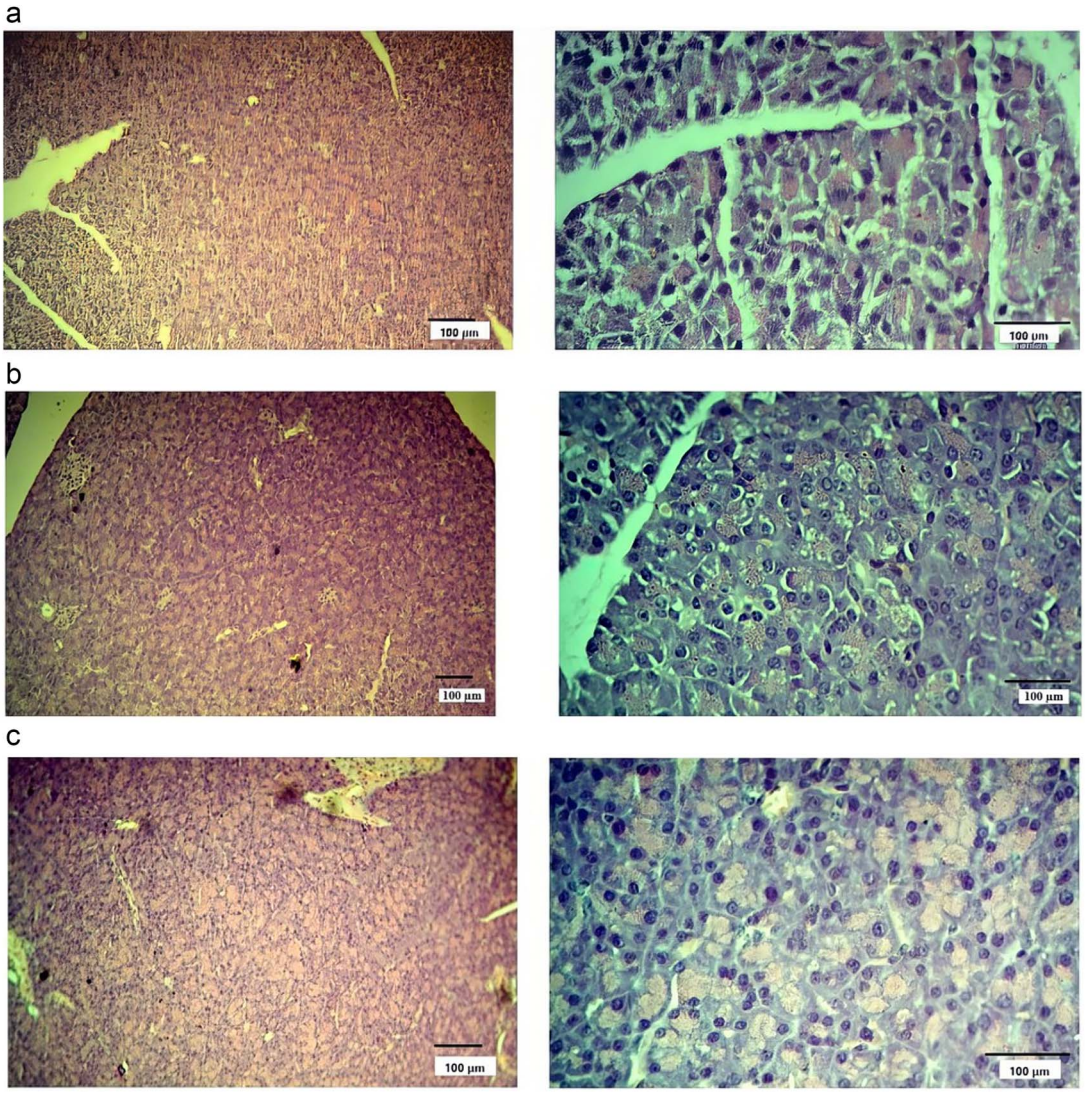

Fig. 5. Histopathological analysis of rabbit organ pancreas with *B. clausii* M31 culture from repeated dose 28-day oral toxicity tests (a3. Control; b3. Low dose; c3. High dose).

methods, or differences in processing techniques. The trials in the study also had designs and results consistent with previous studies.[24] The studies conducted according to OECD guidelines 423 and 407 offer a rigorous evaluation of toxicity.

Our data is consistent with the findings of several comparable studies on the toxicity of probiotics, revealing that daily doses within the range of 10^8–10^{10} colony-forming units (CFU) do not exhibit pathogenic or acute oral toxicity effects in experimental rats. Notable *Bacillus* examples before of such probiotics include *Lactobacillus*, *Bifidobacterium*. Our findings align with those reported in Lakshmi's oral toxicity study and the safety assessment of *B. clausii* UBBC07 in a mouse model, thus reinforcing the extensive history of safety associated with *B. coagulans* and *B. licheniformis*.[25,26]

In conclusion, in this investigation, a comprehensive examination of the safety profile of *B. clausii* M31 was conducted and establishes that *B. clausii* M31 did not induce adverse effects in male and female rabbits and rats. Thus, a dose of 25×10^{10} CFU/kg body weight of *B. clausii* M31 is identified as the no-observed-adverse-effect-level for both sexes in our investigation, representing the highest dose administered in the present study.

Conclusion

Overall, oral administration of *B. clausii* M31 with the highest dose 5×10^{11} CFU/kg body weight did not produce any mortality or severe abnormalities. No noticeable body weight reduction, toxicity symptoms, or death occurred. No obvious gross disruption or distortion of the organs was reported. No LD_{50} was found when administered orally in experimental animals. *B. clausii* M31 was safe in acute toxicity tests on Swiss rats and Repeated Dose 28-day study on New Zealand rabbits at different dose levels, continuously for 28 days.

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Author contributions

Anh Nguyen Quynh Ngo: Writing of the original draft, conceptualization, methodology, Writing—review & editing, Supervision;

Huong Thi Nguyen: Methodology, Data curation, Formal analysis, Writing—review & editing, and Project administration; Thanh Xuan Dam: Methodology, Formal analysis, and Project administration; Thao Thi Minh: Methodology, Writing—review & editing; Nhi Dinh Bui: Writing of the original draft, Writing—review & editing.

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Data availability

The data that support the findings of this study are available from the corresponding author, [Anh Nguyen Quynh Ngo], upon reasonable request.

Ethics approval

Ethical approval for this study was obtained from Ethics Committee in Biomedical Research of Hai Duong Central College of Pharmacy (563/QĐ-UBND).

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