STABILITY AND SAFETY STUDY OF LIQUID-SUSPENSION BACILLUS CLAUSII SPORE PROBIOTICS (LIVESPO CLAUSY)

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Bacillus clausii has been used as probiotic bacteria in dietary supplements because of its main probiotic activities, including its multi-antibiotic resistance, digestive enzyme production, vitamin synthesis, and immunomodulatory effects. In this study, heat, and acid-stability, acute and sub-acute toxicity were conducted to evaluate the stability and safety of liquid-suspension probiotics (LiveSpo® Clausy, LiveSpo Pharma) containing spores of Bacillus clausii ANA39 strain at ≥ 2 billion CFU/5 mL. The result showed that the B. clausii ANA39 spores were heat-resistant, acid pH-stable, and long-time survivors with the remaining survival rate of 80%, 60%, and 80% at treatment conditions of 80^{0}C, pH 2.0 for 20 min, and at 30^{0}C for 24 months respectively. Acute toxicity data in mice indicated LD^{50} was not detectable, even at an extremely high dosage of 60 mL/kg (equivalent to 2.4 x 10^{10} CFU/kg), indicating LiveSpo® Clausy was an unclassified category. The sub-acute toxicity results showed that the rabbits administrated with B. clausii ANA39 at the dosages of 0.93 mL/kg rabbit/day (equivalent to 0.37 x 10^9 CFU ANA39 spores/kg rabbit/day) and 2.80 mL/kg rabbit/day (equivalent to 1.12 x 10^9 CFU of B. clausii ANA39 spores/kg rabbit/day) were healthy and steadily gained weight without any abnormal physiology and anatomy. In conclusion, LiveSpo® Clausy probiotics are resistant to high temperatures and low pH acids, stable for at least 24 months at room temperature, and safe for use as a food supplement.

Keywords: Acid stability, acute toxicity, *Bacillus clausii*, heat stability, spore, sub-acute toxicity.

I. INTRODUCTION

According to WHO, probiotics are live microorganisms that benefit the host when added in sufficient quantities. It is well known that the gut microbiota is strongly correlated with the host's health status, especially concerning metabolic disorders in the body.[1] An increasing number of studies and clinical trials support the hypothesis that altering the gut microbiota to re-establish equilibrium can help return the body to a normal, healthy state.[2,3] *Lactobacillus* and *Bifidobacterium* are the two most consumed bacteria globally because of their health benefits. *Lactococcus, Enterococcus*, *Streptococcus*, *Pediococcus, Saccharomyces*, *Pitchia* are other examples of bacteria and yeast that can also be utilized as probiotics.[4] In addition, spore-forming bacteria, mostly *Bacillus*, are receiving more attention with the properties of a probiotic species. Scientific reports have also provided evidence of efficacy, safety, and a long history of consumption of spore-forming bacteria strains belonging to safe *Bacillus* species, such as *B. subtilis, B. clausii, B. coagulans, B. licheniformis, B. megaterium, B. amiloliquefaciens*.[5,6] *Bacillus* strains have been used to produce extracellular enzymes such as amylase, protease, cellulase, pectinase;[7]

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and some vitamins added to functional foods for human use.[8,9] Therefore, *Bacillus* bacteria are increasingly attracting attention as good probiotics due to their potent antimicrobial, antidiarrheal, and immunostimulatory effects, vital survival in unfavorable conditions like high temperature and low acidic pH of the stomach, growth stimulation of natural flora and protection of intestinal inflammation. Thus, *Bacillus* spp. has the potential to emerge as the "perfect multifunctional probiotic bacteria" for various clinical conditions in humans.

B. clausii, like most other *Bacillus*, is rod-shaped, Gram-positive, spore-forming, and commonly found in soil, air, water, human and animal intestines, and also in vegetables and fermented or non-fermented foods.[10,11] With a long history of research on its safety and effectiveness, *B. claussi* species have been widely used as probiotics to treat and prevent diseases associated with impaired intestinal barriers. Liquid-suspension spores of *B. clausii* (Sanofi–Aventis, Italy) have been used as over-the-counter (OTC) drugs since 1999.[12] The used strains have been investigated intensively for efficacy and safety in humans.[13] *B. clausii* is one of the immigrant strains of beneficial bacteria, initially absent from the intestinal microbiota, but has significant advantages. *B. clausii* spores have excellent tolerance to acidic pH, so they can live and overcome the stomach's acidic environment, reach the intestine, and germinate into beneficial bacteria.[12] *B. clausii* can be resistant to antibiotics,[14] therefore, during antibiotic therapy, it is always preferred to use probiotics with components containing *B. clausii*. Moreover, it also helps strengthen the host immune system,[15] supply vitamins and enzymes that stimulate digestion and enhance nutrient absorbance. Moreover, it can inhibit the growth of harmful bacteria through competition for the live environment or nutrient sources and secretion of antimicrobials, therefore, creates a double protective layer against the invasion of harmful bacteria into cells or intestinal mucosa.[16] Although studies on the stability and safety of *B. clausii* liquid-suspension spores are common worldwide, none have been undertaken in Vietnam. The stability and safety of LiveSpo® Clausy, a liquid-suspension *B. clausii* spore probiotic manufactured by LiveSpo Pharma (Vietnam), were presented in this research.

II. METHODS

1. Materials

Liquid-suspension *B. clausii* ANA39 spores at concentrations of 1-2 billion CFU/mL (3X stock suspension) and 2 billion CFU/5 mL (LiveSpo® Clausy, LiveSpo Pharma) in distilled water were used for tests in this study. LiveSpo® Clausy is available food probiotics produced under 4071/2021/ĐKSP registration approval by the Department of Food Hygiene and Safety, Vietnam Ministry of Health. 16S rRNA sequence (5′→3′) of the strain *B. clausii* ANA39 had been analyzed and deposited at the GenBank with accession number MT275656 in NCBI. BLAST analysis and phylogenetic relationship tree showed that ANA39 was closely related to *B. clausii* with 99.8% similarity to the strain *B. clausii* UBBC07 that have been proven to be safe.[13]

Swiss white mice 18 - 22 g (n = 40) supplied by the National Institute of Hygiene and Epidemiology were used in acute toxicity tests. Mature and healthy New Zealand rabbits, both male and female, 1.8 - 2.2 kg (n = 21) supplied by the Livestock unit, Pharmacology laboratory, National Institute of Drug Quality Control (NIDQC), were used in sub-acute toxicity test. The animals were fed in cages at a controlled breeding room temperature, and proper humidity, food, and drinking water were supplied as needed. A formula feed (for rabbits and

mice) consisting of approximately 16% protein, 65% carbohydrate, and 5% fat was provided in accordance with each animal species. All manipulations on test animals have followed the procedures for care and use of test animals by the Pharmacology laboratory, NIDQC.

Determination of heat and pH stability of liquid-suspension *B. clausii* ANA 39 spores

The pH stability of liquid-suspension containing high concentrations of 1-2 billion spores/mL of *B. clausii* ANA39 (3X stock suspension of LiveSpo® Clausy) was evaluated by adding the bacterial spore suspension into the appropriate buffers at 30°C ± 2°C and relative humidity (RH) of 65% ± 5% at pH 2.0 and 4.0 (citrate buffers), 6.0 and 8.0 (phosphate buffers). The samples' time-dependent viable count of bacterial spores was analyzed at 0, 3, 6, 9, 12, 18, and 24 months. For the heat stability evaluation, *B. clausii* ANA39 spore suspension in phosphate buffer saline (PBS) pH 7.4 was incubated in a water bath at 30, 60, 70, 80, and 90°C for 20 min. The treated suspension samples at different pH, time, and heat conditions were then serially diluted in sterilized NaCl 0.9%, and the viable count was enumerated by plating on LB agar. The plates were incubated at 37°C for 48 h to allow spores to germinate and form into colonies on each dish. Each analysis was performed twice in triplicate. The average mean of viable spore counts is expressed in log10 CFU mL^{-1}.

Determination of acute toxicity of LiveSpo Clausy

Acute toxicity of liquid-suspension *B. clausii* ANA39 spores at a concentration of 2 billion CFU/5 mL (LiveSpo® Clausy) was conducted in 40 Swizz mice supplied by the National Institute of Hygiene and Epidemiology (NIHE). The mice (n = 40) were divided into four groups, including one control group and three test groups (n = 10 each). For adults (50 kg weight on average), the recommended dosage of LiveSpo® Clausy and related product Enterogermina containing *B. clausii* 2 billion CFU/5 mL (Sanofi, France) is 3 vials per day (15 mL of LiveSpo® Clausy/ person/day or 6 x 10^9 CFU spores/person/day), which is equivalent to 0.3 mL per kg human weight. Thus, the dosages of test samples were determined to be 20.0 mL (level 1), 40.0 mL (level 2), and 60.0 mL (level 3) of LiveSpo® Clausy sample suspension per kg of mice weight. After administration, the toxic signs were monitored, including physiological signs, behavior, movement, eating, drinking, stool, urine every 15 min within the first hour and gradually decreased in frequency within the first 24 h. Mice were observed once a day for seven days. The number of dead mice in the test and control groups was recorded. The weight of mice was measured just before administration (day 0), day four, and day seven after administration to compare with the control group.

Determination of sub-acute toxicity of LiveSpo® Clausy

Twenty-one young adults and healthy New Zealand rabbits (n = 21) were divided into one control and 2 test groups with seven rabbits/ group. Each rabbit was kept in an individual cage and placed in the controlled temperature and humidity room. Food and water were supplied as needed. Based on the mentioned above dosages of LiveSpo® Clausy for human, the two-dose levels of 0.93 mL of LiveSpo® Clausy sample suspension/kg rabbit/day (equivalent to 0.37 x 10^9 CFU spores/kg rabbit/day, test 1) and 2.80 mL of sample suspension/kg rabbit/day, (3 folds higher and equivalent to 1.12 x 10^9 CFU spores/kg rabbit/day, test 2) were administered to rabbits for 28 days. Test solutions were prepared by diluting LiveSpo® Clausy sample with distilled water in appropriate proportions. The control group rabbits were given distilled water. The rabbits were checked daily for food

and water consumption, physical condition, behavior, stool, and urine. Rabbits' weight on days 0, 7, 14, 21, and 28 and 14 follow-up days were recorded. The hematological indices related to hematopoietic function (number of red blood cells, white blood cells, platelets, hemoglobin, hematocrit); to liver function (aspartate transaminase - AST, Alanine transaminase - ALT, total protein, total bilirubin, cholesterol); to kidney function (creatinine, urea); and glucose index at day 0, 14, 28, and 14 follow-up days were measured. On day 28, three rabbits in each group were randomly taken to make histopathological specimens of the liver, kidney, small and large intestine for microscopic evaluation of the organs immediately after treatment. At the end of the study, the remaining animals were dissected to observe the general organs of the heart, liver, kidney, lung, stomach, and intestine.

Statistical analysis

The data were presented as mean ± standard deviation (mean ± SD) and statistically analyzed using the Student test to compare the difference of the same indicator between the control and the test groups. p-value < 0.05 was considered to be statistically significant.

III. RESULTS

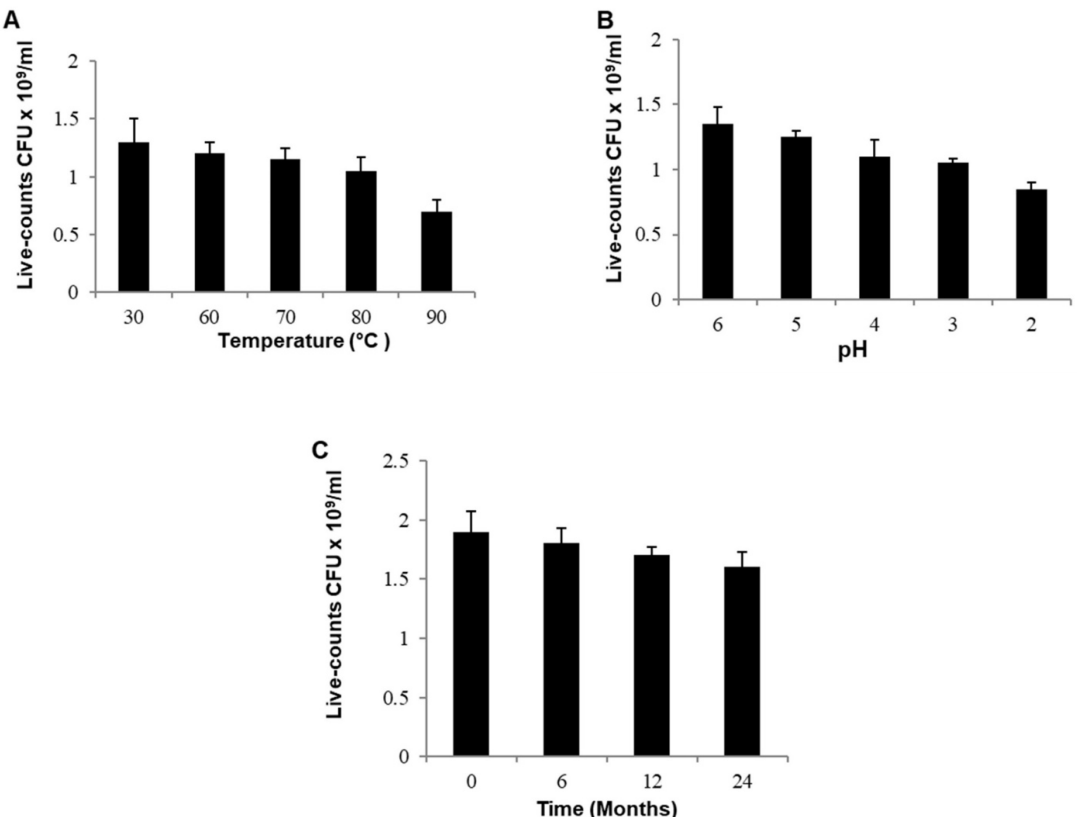

Figure 1. Stability of liquid-suspension *B. clausii ANA39 spores. A. Heat-stability of B. clausii ANA39 spores; B. pH-stability of B. clausii ANA39 spores; C. Time-dependent stability of B. clausii ANA39 spores at room temperature.*

High temperature and low acidic pH resistance of liquid-suspension *B. clausii* ANA 39 spores

The ideal probiotics should be stable at high temperatures and acidic pH to maintain their activity throughout storage and oral administration through the stomach if intended to be used as food supplements. Thus, we tested whether the spores of ANA 39 strain are stable at such challenging conditions. As a result, the spores retained about 60% of the viable count at even low pH 2.0 for two hours treatment, and the survival rate were about 80% at 80°C for 20 min. Interestingly, after 24-month storage at 30°C ± 2°C and in an RH of 65% ± 5%, about 80% of the viable spore count was still determined in the LiveSpo® Clausy liquid-suspension probiotics (Figure 1).

Non-detectable acute toxicity of LiveSpo® Clausy in mice

Liquid-suspension *B. clausii* ANA39 spores at a concentration of 2 billion CFU/5 mL (named LiveSpo® Clausy) was administrated into mice at the low dose level of 20.0 mL of sample (equivalent to 8 x 10^9 CFU/kg) to a high dose level of 60.0 mL of sample (equivalent to 2.4 x 10^{10} CFU/kg). No abnormal signs or dead mice were found in all the test groups. The average weight of mice during the 7-day test did not show a statistically significant difference between the test groups (T) and the control group (C) (previous $p_{ANOVA\ before} > 0.05$; p (TC); $p_{(T-C)\ before} > 0.05$; $p_{(T-T)\ before} > 0.05$). After seven days, the mice in both control and test groups steadily gained weight compared to day 0 in each group ($p_{\ before-after} < 0.001$). The difference in average weight among the test groups compared to the control group at day seven was not statistically significant ($p_{ANOVA\ after} > 0.05$; $p_{(T-C)\ after} > 0.05$; $p_{(T-T)\ after} > 0.05$) (Table 1). In this experiment, the lethal dose 50 of test animals (LD_{50}) was > 60.0 mL sample/kg (equivalent to 2.4 x 10^{10} CFU/kg). According to the toxicity classification of Globally Harmonized System (GHS, 2019), LiveSpo® Clausy containing *B. clausii* ANA39 spores was considered acute toxicity below the GHS threshold.

Table 1. Comparison of the mice weight between the control and the test groups fed with LiveSpo® Clausy

Group	Before test		After test		Gained weight (%)	$p_{before-after}$
	Weight of mouse (g)	p_{before}	Weight of mouse (g)	p_{after}		
Control (C)	19.70 ± 0.88	$p_{ANOVA} > 0.05$	30.21 ± 1.37	$p_{ANOVA} > 0.05$	153.4	$p < 0.001$
Level 1 (L1)	19.50 ± 1.04	$p_{T1-C} > 0.05$	30.33 ± 1.14	$p_{T1-C} > 0.05$	155.8	$p < 0.001$
Level 2 (L2)	19.23 ± 0.69	$p_{T2-C} > 0.05$	29.91 ± 1.39	$p_{T2-C} > 0.05$	155.6	$p < 0.001$
Level 3 (L3)	19.74 ± 0.93	$p_{T3-C} > 0.05$	30.26 ± 1.37	$p_{T3-C} > 0.05$	153.4	$p < 0.001$

Non-detectable sub-acute toxicity of LiveSpo® Clausy in rabbits

In the sub-acute toxicity study, no abnormality in the eating or movement of rabbits was found in all test groups. Before the experiment, the average weights of rabbits in the test groups (T) were not different from the control group (C) ($p_{\ before(T1-C)} > 0.05$; $p_{\ before(T2-C)} > 0.05$). After 14 and 28 days, rabbits gained weight steadily in both control and test groups

($p_{before-after} < 0.05$) compared to day 0, while no significant difference in the average weights was determined between the test group and the control group ($p_{(T1-C)\ after} > 0.05$; $p_{(T2-C)\ after} > 0.05$). After 14 follow-up days, the rabbits were still healthy and gained weight well. No statistically difference in average weight between the test groups and the control group was found ($p_{(T1-C)\ after} > 0.05$; $_{(T2-C)\ after} > 0.05$) (Table 2).

Table 2. Comparison of the rabbit weight between the control and the test groups fed with LiveSpo® Clausy

Group (n = 7)	Weight (kg)					p	Weight (kg)	$p_{(T-C)middle}$
	Before test (m_0)	After 7 days (m_1)	After 14 days (m_2)	After 21 days (m_3)	After 28 days (m_4)		14 follow-up days	
Control (C)	2.03 ± 0.12	2.09 ± 0.10	2.20 ± 0.10	2.28 ± 0.10	2.43 ± 0.08	$p_{before-after} < 0.001$	2.63 ± 0.10	
Test1 (T1)	2.02 ± 0.11	2.06 ± 0.12	2.18 ± 0.10	2.26 ± 0.08	2.36 ± 0.08	$p_{before-after} < 0.001$ $p_{(T1-C)\ before} > 0.05$ $p_{(T1-C)after} > 0.05$	2.58 ± 0.12	> 0.05
Test 2 (T2)	1.94 ± 0.08	1.98 ± 0.09	2.14 ± 0.11	2.22 ± 0.10	2.34 ± 0.13	$p_{before-after} < 0.001$ $p_{(T2-C)\ before} > 0.05$ $p_{(T2-C)after} > 0.05$	2.54 ± 0.09	> 0.05

At day 14, 28 and 14 follow-up days, the hematological indices were not statically significant difference between the control and the test groups ($p_{(T-C)\ before} > 0.05$); ($p_{(T-C)\ middle} > 0.05$; $p_{(T-C)after\ 28\ days} > 0.05$); $p_{(T-C)\ 14\ follow-up\ days} > 0.05$) (Figure 2A). The same results were also found for the liver function (Figure 2B) kidney function and blood glucose level (Figure 2C).

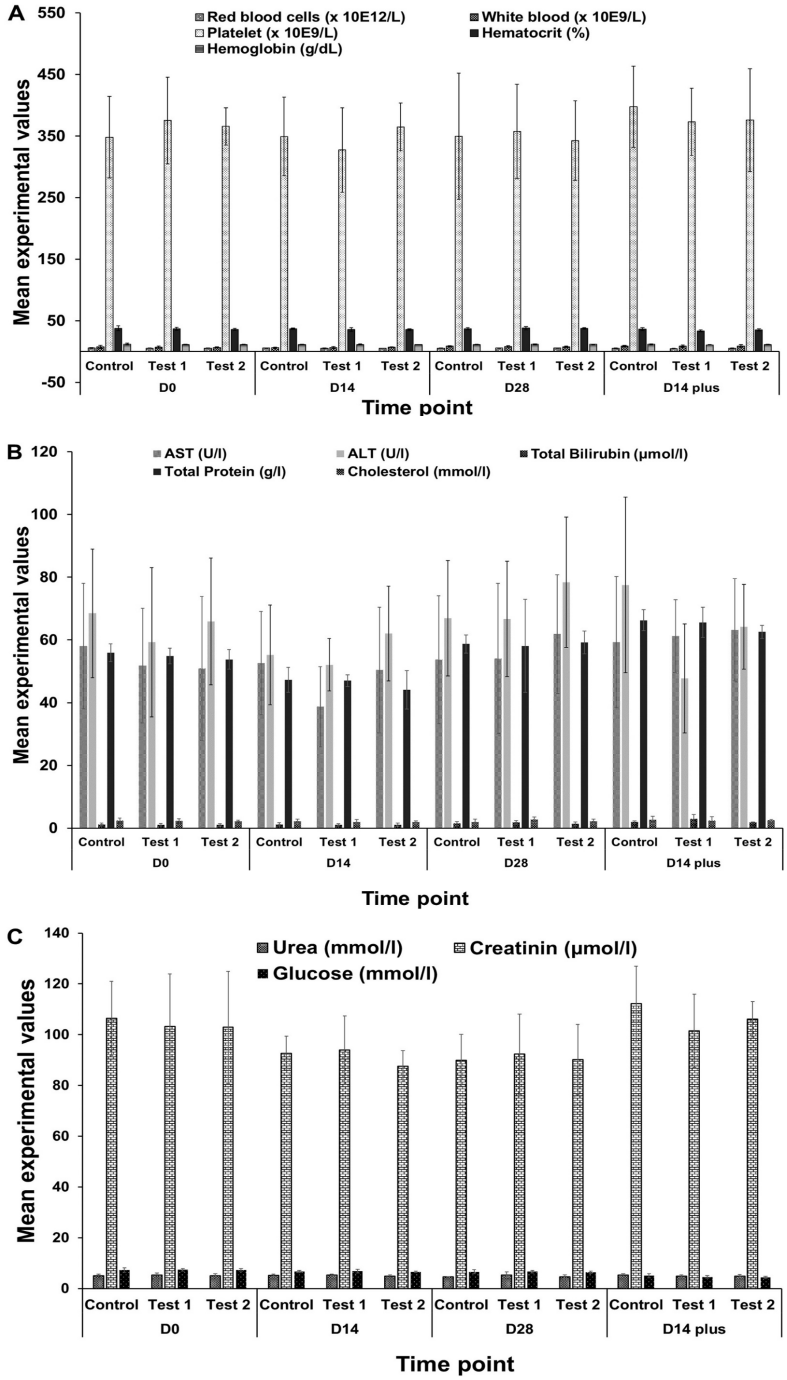

Figure 2. Sub-clinical indices in rabbits at day 14, 28, and 14 follow-up days of feeding LiveSpo® Clausy. *A. Hematological indices related to the hematopoietic function of rabbits; B. Biochemical indices related to the liver function of rabbits; C. Biochemical indices related to kidney function and blood glucose level of rabbits*

Hematoxylin and Eosin (H&E) stained liver, kidney, small intestine, and colon specimens were observed under an optical microscope. Macroscopic observation revealed normal appearance and color of the heart, liver, kidney, lung, stomach, intestines of rabbits in the test groups compared to the control group after the experiment (data not shown). The histopathology observation indicated the typical appearance of the liver, kidney, intestines, and colon of rabbits in the test groups. The morphological anatomy of the two test groups was within normal limits (Figure 3).

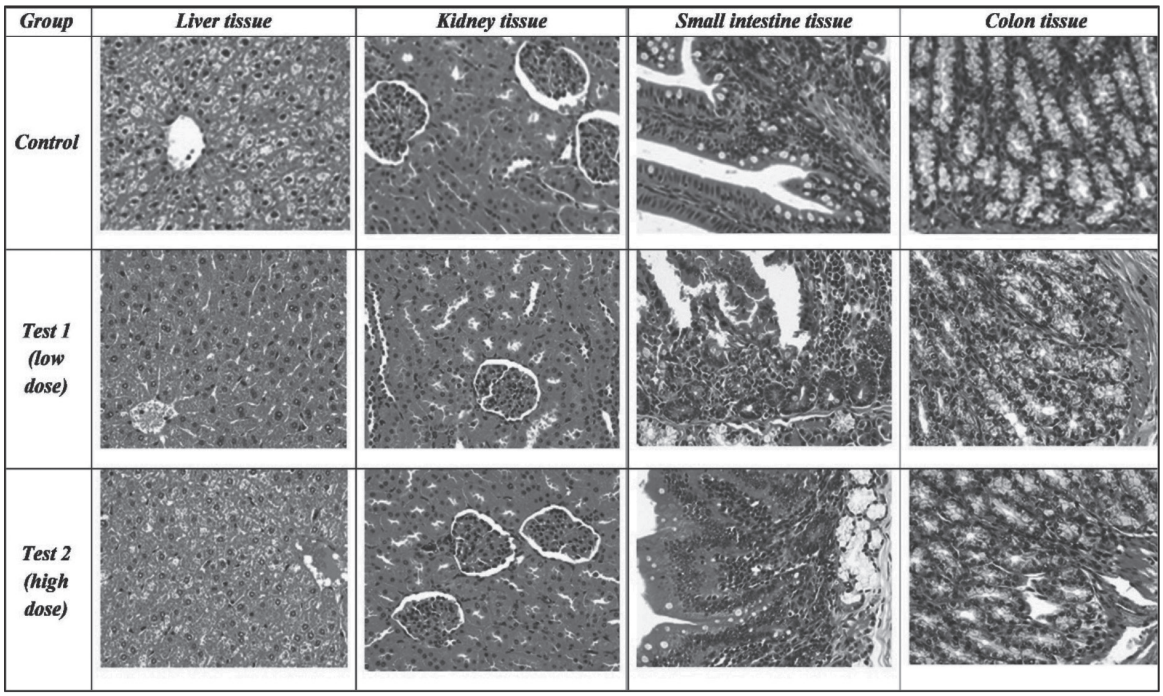

Figure 3. The histopathology observation of the liver, kidney, small intestine, and colon *(H & E stain x 400) from rabbits without probiotic feeding (Control), and supplemented with LiveSpo® Clausy at a low dose (Test 1) and at a high dose (Test 2)*

IV. DISCUSSION

Survival and germination are essential for spore-forming probiotics to exert beneficial effects on the gastrointestinal tract. The low acidic pH 2.0 resistance results suggest that *B. clausii* ANA39 spores can survive when transiting through the stomach (low acidic pH environment) to the small intestine. It senses a favorable environment for germinating into vegetable cells and proliferates to convey the host's benefits. This finding is similar to a previous study reporting that the spores of *Bacillus clausii* strains O/C, SIN, N/R, and T (Enterogermina) can survive at low pH 2 in the stomach environment,[17,18] something that not all *Lactobacillus* and other non-spore forming bacterial species can achieve.[19] Besides, the spores were stable to high temperature up to 80°C and for 24-month storage at 30°C without any loss of viability, confirming the stable quality of the commercial product LiveSpo® Clausy. The exceptional thermal stability of *B. clausii* ANA39 spores could be attributed to the strain's unique properties as well as the LiveSpo® Clausy product's spore-producing high

technology. In reality, some *B. clausii* probiotics are not as heat-resistant as LiveSpo® Clausy, retaining less than half of their live count at only 65°C treatment for 20 min[1,12,17,18]. Thus, LiveSpo® Clausy has an advantage over other conventional bacteria and yeast probiotics in that it may be utilized with warm or hot water, drinks, and food without losing their bacterial live-counts. In this study, we did an in-depth study on the safety of LiveSpo® Clausy. Acute toxicity tests in rats and sub-acute toxicity tests in rabbits have shown that *B. clausii* ANA39 spores of LiveSpo® Clausy (LiveSpo Pharma) is completely safe. *B. clausii* ANA39 did not affect the overall health, growth, and development of the test animals, both in sub-acute toxicity study in rabbits at the dose of 3-fold higher than suggested dosage in human, and in acute toxicity study in mice. Our results are similar to those of Lakshmi's oral toxicity study and the safety assessment of *B. clausii* UBBC07 in a mouse model, strengthening the long history of safety of *B. clausii* probiotics.[13]

V. CONCLUSIONS

Liquid-suspension *B. clausii* ANA39 spores of LiveSpo® Clausy were resistant to high temperature up to 80°C, low acidic pH of 2.0, stable for 24-month storage at 30°C. In terms of safety, LiveSpo® Clausy had LD_{50} under the GHS classification threshold. Moreover, no toxicity signs were found in the sub-acute toxicity test in the rabbits after 28 days of spore administration at the high dose of 1.12 x 10^9 CFU spores/kg rabbit/day, which is 3-fold higher than the suggested dosage in human. Macroscopic and microscopic observations did not reveal any damage in the internal organs of the test groups. Thus, liquid-suspension *B. clausii* ANA39 spores LiveSpo® Clausy is stable and safe for use as a dietary supplement.

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