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Biosafety measures for *Alicyclobacillus* spp. strains across various levels of biohazard

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ABSTRACT

Alicyclobacillus bacteria are important contaminants in the beverage industry because their spores remain in the product after usual pasteurization. At the same time, their impact on human health has yet to be characterized, as it is generally assumed to be low or non-existent. However, these bacteria are causing quality concerns mainly due to odor and taste changes of the product. Since potential health effects are not precisely known, an experimental assessment was performed, including a biosafety assessment of six viable and non-viable vegetative and spore forms of *Alicyclobacillus* spp. strains using cell cultures and rodent study. The monolayer of Caco-2 (Cancer coli-2) cells was investigated for its adsorption effect on the epithelium of the small intestine of mice. Lactate dehydrogenase leakage (LDH) and transepithelial electrical resistance (TEER) tests were used to ensure the integrity of the cell membrane and tight junctions. The methylthiazole tetrazolium bromide (MTT) assay examined *in vitro* cytotoxicity in Caco-2 and HepG2 cell lines. The hemolysis of erythrocytes was spectrophotometrically measured. The results showed negligible cytotoxicity or non-toxic response in mice. In conclusion, *Alicyclobacillus* spp. exhibited biocompatibility with negligible cytotoxicity and minimal safety concerns.

1. Introduction

Gram-positive *Alicyclobacillus* spp. are isolated from soil and hot springs and appear as rod-shaped, aerobic, nonpathogenic, thermoacidophilic, and endospore-forming microorganisms (Sourri et al., 2022; Zhao et al., 2021; Molva and Baysa, 2016; Gobbi et al., 2010). They are responsible for incidents of spoilage that produce taint compounds in pasteurized and heat-treated apple juice (Shang et al., 2023; Smit et al., 2011; Vieira et al., 2002a,b). Furthermore, worldwide health problems associated with *Alicyclobacillus* spp. are the main cause of significant economic losses to beverage industries (Neggazi et al., 2023; Torlak,

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2014; Smit et al., 2011; Steyn et al., 2011). A profitable biological control strategy to reduce mycotoxin patulin from contaminated apple fruit juice by the potential use of different strains of *Alicyclobacillus* spp. could provide a suitable control strategy for the treatment of contaminated apple juice. Therefore, the rapid and low-cost sustainable management of apple juice waste can be achieved by direct collection of *Alicyclobacillus* from contaminated apple juice (Hu et al., 2020; Sajid et al., 2018).

In addition, microbial toxicity is a significant food safety threat.

Abbreviations

A_s	Absorptive value of hemolysis test for the sample
ALT	Alanine amino transferase
AAM	*Alicyclobacillus acidocaldarius* medium
ALP	Alkaline phosphatase
AST	Aspartate amino transferase
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
Caco-2	Cancer coli-2, Human epithelial colorectal adenocarcinoma Caco-2 cell lines
CK	Creatine kinase
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's modified eagle medium
E_m	Emission wavelength
EDTA	Ethylenediamine tetraacetic acid
E_x	Excitation wavelength
FBS	Fetal bovine serum
FI	Fluorescence intensity
HBSS	Hank's buffered salt solution
HI	Heat-inactivated
H&E	Hematoxylin and eosin
HepG2	Hepatoblastoma cell line, Human hepatocellular carcinoma cell lines
LDH	Lactate dehydrogenase
LD_{50}	Lethal dose 50 or median lethal dose
MTT	Methylthiazole tetrazolium bromide
MEM	Minimum essential medium eagle
A_n	Negative control absorptive value of hemolysis test
1-way ANOVA	One-way analysis of variance
OD	Optical density
PBS	Phosphate buffered saline
A_p	Positive control absorptive value of hemolysis test
RBC	Red blood cells
RPMI 1640	Roswell Park Memorial Institute 1640
SD	Standard deviation
$TEER_{post}$	TEER value across Caco-2 cell monolayers after treatment Ωcm^2
$TEER_{pre}$	TEER value across Caco-2 cell monolayers before treatment Ωcm^{-2}
TEER	Transepithelial electrical resistance
TP	Total Protein
λ	Wavelength

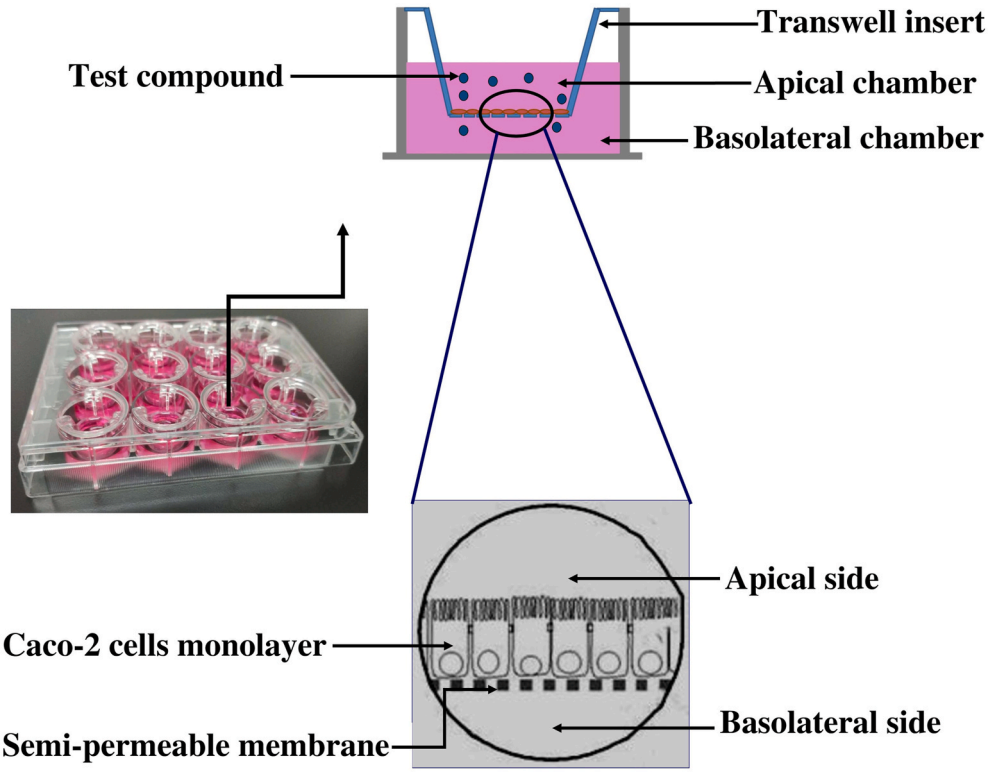

Fig. 1. Caco-2 cell monolayer model. The human small intestine cancer-derived Caco-2 cell lines were generated on a transwell insert. The model mimics processes for the directional transport of the apical-basolateral region, the test compound solution is added to the apical chamber and the transport media, as the working solution of the receiver, is added to the basolateral section.

Although *Alicyclobacillus* bacteria are significant contaminants in the

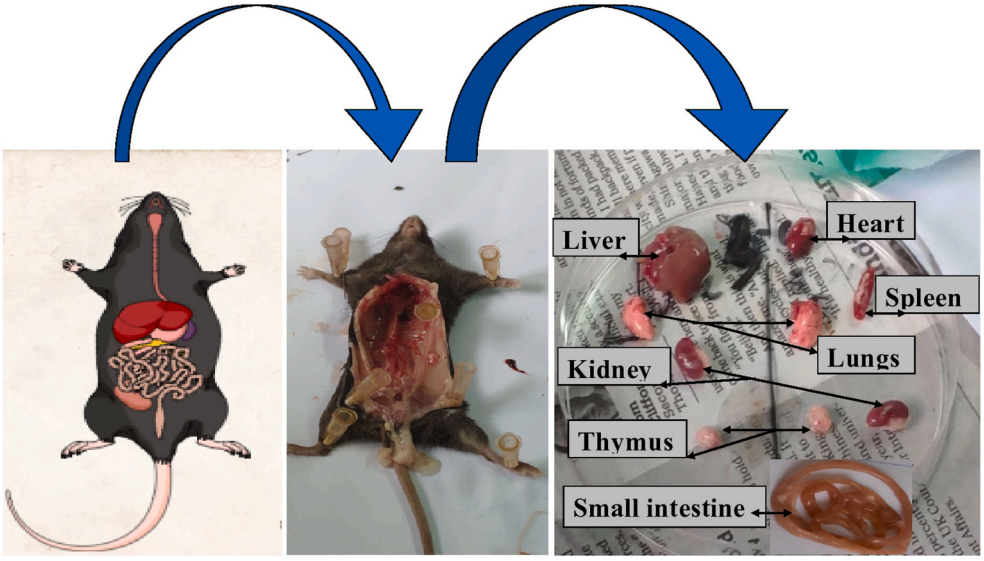

Fig. 2. Mice dissection: C57BL/6J mice were killed and body organs (heart, liver, spleen, kidneys, lungs, thymus and small intestine) were weighed and hematoxylin and eosin (H&E) stained for histological analysis.

beverage industry due to their spores surviving standard pasteurization processes, their impact on human health remains unclear. It is generally assumed, however, that their effect is minimal. *In vivo,* numerous microorganisms, including *Streptomyces californicus*, *Mycobacterium terrae* and *Aspergillus versicolor*, have shown inflammatory and toxic responses (Sourri et al., 2022; Jussila et al., 2001, 2002a, 2002b). However, to date, no reports address the *in vitro* and *in vivo* safety of *Alicyclobacillus* spp. cells and spores. Therefore, the possible effect of *Alicyclobacillus* spp. strains on human health, designed for the adsorption of mycotoxins in fruit juices, needs to be considered.

In the human body digestive system, two vital organs are the liver and the small intestine, which plays an important role. During digestion and absorption of food, inactivated cells and spores from *Alicyclobacillus* strains could openly interact through the small intestine epithelium. It is possible that after absorption with food particles in the small intestine, the *Alicyclobacillus* spp. inactivated cells and spores could be carried to the liver through the portal vein. As a result, the potential toxicity of inactivated cells and spores of the *Alicyclobacillus* strains must be studied in these two organs.

In vitro toxicity or cytotoxicity can be examined using human cancer-derived Caco-2 cell lines. About 3 weeks of Caco-2 cell culture, distinguish to separate by producing microvilli on the apical side of the cell membrane, and form tight junctions between adjacent cells (Hidalgo et al., 1989). Due to these physical characteristics, Caco-2 cell monolayers are generally used to mimic the epithelium of the small intestine. *In vivo,* absorption of any foreign material is based on the permeability of Caco-2 cell monolayers (Cheng et al., 2023; Lopez-Escalera and Wellejus 2022; Hubatsch et al., 2007). Meanwhile, *in vitro,* the toxicity was evaluated by HepG2 (hepatoblastoma cell line) human liver cancer cell lines (Lingfa et al., 2023; Lu and Cederbaum, 2006; Bort et al., 1999). This research was designed to examine the biosafety assessment of viable and non-viable cells and spores of *Alicyclobacillus* spp. strains with different biohazard levels and their ultimate effects on the internal organs of rodents.

2. Materials and methods

2.1. Materials and reagents

Alicyclobacillus acidocaldarius medium (AAM) broth, AAM agar, phosphate buffered saline (PBS), methylthiazole tetrazolium bromide (MTT), Roswell Park Memorial Institute 1640 (RPMI 1640) media and dimethylsulfoxide (DMSO) were obtained from Solarbio Science & Technology Co. Ltd. Beijing, China. Human epithelial colorectal adenocarcinoma Caco-2 cell lines and human hepatocellular carcinoma HepG2 cell lines were obtained from the Department of Preventive Veterinary, College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi, China. Dulbecco's modified Eagle's medium (DMEM), minimum essential medium eagle (MEM), Hank's buffered salt solution (HBSS), fetal bovine serum (FBS) were obtained from Basalmedia Technologies Co., Ltd. Shanghai, China. Other reagents and chemical including 100 × non-essential amino acids, 100 × penicillin and streptomycin and bovine serum albumin (BSA) were purchased from Solarbio (Beijing, China). Transwell permeable polycarbonate inserts (0.4 μm) were obtained from Coring (Arizona, USA).

2.2. Bacterial strains, cultivation conditions and estimation of viable and non-viable bacterial cell and spore concentrations

Six *Alicyclobacillus* spp. were studied, *viz. Alicyclobacillus acidocaldarius* (DSM 449), *Alicyclobacillus contaminans* (DSM 17975), *Alicyclobacillus fastidiosus* (DSM 17978), *Alicyclobacillus hesperidum* (DSM 12489), *Alicyclobacillus herbarius* (DSM 13609) and *Alicyclobacillus sendaiensis* (DSM 17614), coded as AA DSM 449, AC DSM 17975, AF DSM 17978, AH DSM 12489, AH DSM 13609 and AS DSM 17614, respectively. All these *Alicyclobacillus* spp. were purchased from the German Resource Centre for Biological Materials (DSMZ, Germany). Standard glycerol was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China) and used to preserved stock cultures at −40 °C in 30% (v/v) glycerol or on potato dextrose agar (PDA) slants in a 4 °C refrigerator for routine use. Viable cells and spores of *Alicyclobacillus* spp. were cultivated according to the method previously described (Sajid et al., 2018). All strains were cultured in AAM broth for 24–48 h according to their specific cultivation conditions (Supplementary material: Table S1). All compounds of each culture media were purchased from Solarbio Science & Technology Co. Ltd. Beijing, China. The concentration of active cells and spores of *Alicyclobacillus* spp. was determined by plating 100 μL of appropriate dilutions onto AAM agar, adjusted to 1×10^6 cells and spores/mL using distilled water. To obtain cells and spores of non-viable bacterial species, the pellets (bacterial pastes) of each species were autoclaved at 121 °C for 20 min. Subsequently, viable and non-viable *Alicyclobacillus* spp. cells and spores were lyophilized at

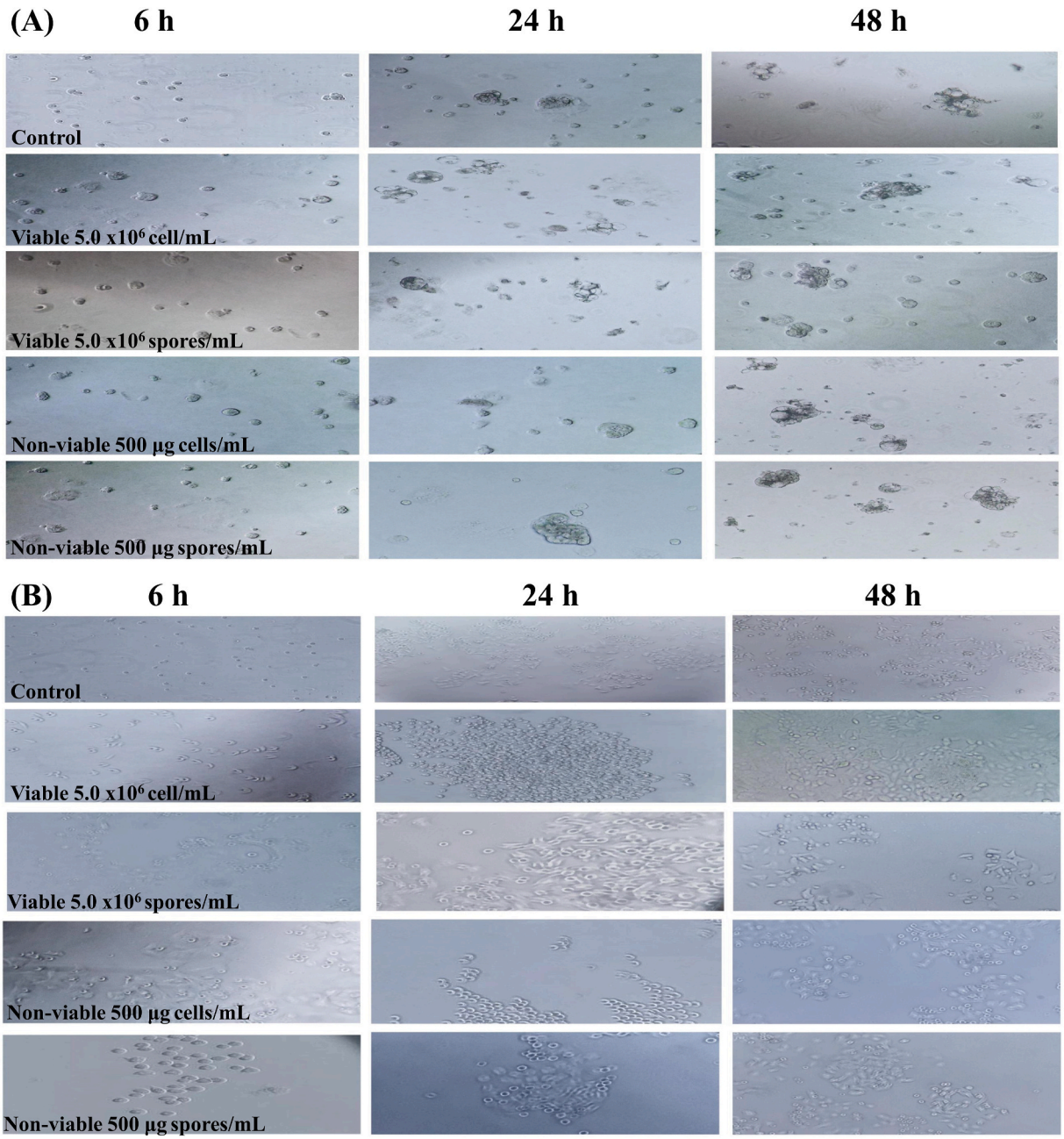

Fig. 3. Morphology of human cancer-derived cell lines: Control cells of (**A**) Caco-2 and (**B**) HepG2 cells compared before and after exposure to different concentrations of viable and non-viable cells and spores at different times 6, 24, and 48 h. Magnification: 100 × , bar scale = 10 µm

−54 °C for 26 h (vacuum degree, 5 mtorr [79.3 Pa]) by a vacuum freeze-dryer (MCFD5505, SIM International Group Co. Ltd, Beijing, China) and sieved (BK-TS200, BIOBASE, Jinan, Shandong, China) for further experiments.

2.3. Maintenance of Caco-2 and HepG2 cell cultures

The cell cultivation of Caco-2 was carried out according to the method described by Yu and Huang (2013). Caco-2 cells (passage 35–45) were cultured in 75 cm^2 tissue culture flask T-75 (Coring, Arizona, USA) with Dulbecco's modified Eagle's medium (DMEM, Basalmedia Technologies Co., Ltd. Shanghai, China) including 10% fetal bovine serum (FBS, NOVA Medical Science and Technology Co., Ltd. Shanghai, China), 1% non-essential amino acids (Solarbio, Beijing, China), 1.5% L-glutamine solution (200 mM) (Solarbio, Beijing, China) and 100 µg/mL antibiotics (penicillin and streptomycin) (Solarbio, Beijing, China). Meanwhile, HepG2 cells (passages 7–10) were cultured in minimum essential medium eagle (MEM, Basalmedia Technologies Co., Ltd. Shanghai, China) with 10% FBS, 100 µg/mL penicillin and streptomycin under a carbon dioxide incubator (Zhejiang FUXIA Medical Technolgy Co., Ltd. Zhejiang China) of humidified atmosphere at 5% CO_2 with 95% air and incubated at 37 °C. Trypsinization was done with trypsin (0.25%, w/v) Basalmedia Technologies Co., Ltd. Shanghai, China) and ethylenediamine tetraacetic acid (EDTA) (0.2%%, w/v) (Basalmedia Technologies Co., Ltd. Shanghai, China)) at 80% of a confluence of cells.

2.4. Generation and treatment of Caco-2 cell monolayers

The Caco-2 cell monolayer model was built according to the protocol

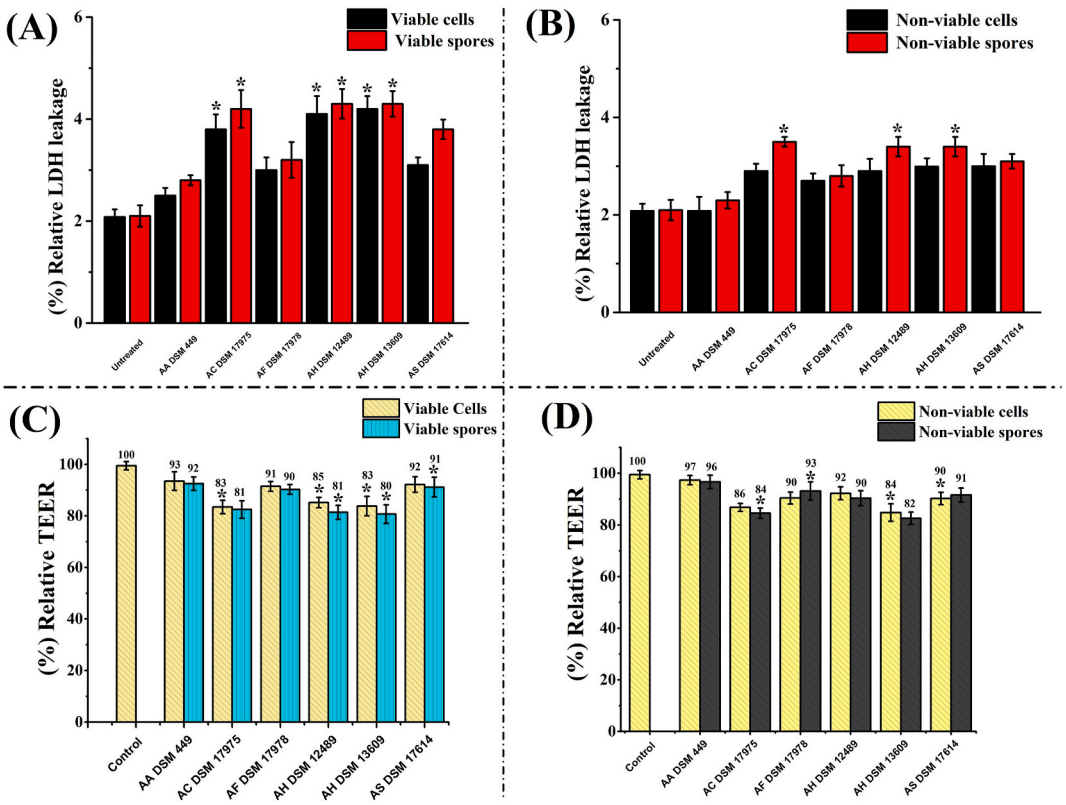

Fig. 4. Cytotoxicity of viable/non-viable cells and spores of *Alicyclobacillus* spp. strains in Caco-2 cells monolayer: Different *Alicyclobacillus* spp. strains treated and untreated with different LHD leakage levels of Caco-2 cell monolayers (**A**) viable cells and spores, and (**B**) non-viable cells and spores. Relative TEER (%) of Caco-2 cell monolayer controls and treated with different *Alicyclobacillus* spp. (**C**) viable cells and spores, and (**D**) non-viable cells and spores. The calculations of the sample data are shown as means ± SD (n = 3). $*P < 0.05$ compared with control samples.

described by Yu and Huang (2013) with slight modifications. Initially, 0.5 ml of Caco-2 cells were plated onto inserts (apical compartment) of 12-well plates (Coring, Arizona, USA) at the density of 6×10^5 cell/mL. Subsequently, 1.5 ml of cell culture medium was added to the lower chamber of each well (Fig. 1). The cell culture medium was changed every two days. *In vitro* cytotoxicity experiments were performed after 21–29 days of plating. Before treatment, Hank's buffered salt solution (HBSS, Basalmedia Technologies Co., Ltd. Shanghai, China) was used to wash Caco-2 cell monolayers three times with HBSS. The Caco-2 cell monolayers were then kept in HBSS (0.5 ml in an apical compartment and 1.5 ml in the basolateral chamber) for 30 min at 37 °C.

After washing, 500 μL (500 μL) of viable and non-viable *Alicyclobacillus* spp. cells and suspensions of spores with different concentrations were added separately to the apical compartment. The Caco-2 cell monolayers were then kept in an incubator set at 37 °C for 2 h.

2.5. Lactate dehydrogenase (LDH) leakage assay

Lactate dehydrogenase (LDH) leakage assay was carried out by measuring the activity of LDH in cells and media as described by Yu and Huang (2013) with minor modifications. After treatment of Caco-2 cell monolayers with different concentrations of viable and non-viable *Alicyclobacillus* spp. cells and spores, a CytoTox-ONETM homogeneous membrane integrity assay kit (Promega, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine lactate dehydrogenase leakage in culture media. Viable and non-viable *Alicyclobacillus* spp. cells and spores in HBSS were used as a negative control (−). Cell lysates of Caco-2 cell monolayers were used as a positive (+) control. The percentage (%) of relative LDH leakage was calculated using the following **Equation (1)**:

$$\%\ \text{relative LDH leakage} = \frac{\text{FI (treated sample)} - \text{FI (negative control)}}{\text{FI (positive control)} - \text{FI (negative control)}} \quad (1)$$

Where, FI = the fluorescence intensity with excitation (E_x) wavelength (λ) at 560 nm and emission (E_m) wavelength at 590 nm, as mentioned in the kit protocol.

2.6. Measurement of transepithelial electrical resistance (TEER)

The integrity of each Caco-2 cell monolayer was verified by measuring its transepithelial electrical resistance (TEER) immediately before treatment of viable and non-viable *Alicyclobacillus* spp. cells and spores, using the Evom^2 epithelial voltmeter (World Precision Instrument, WPI, Shanghai Trading Co., Ltd. Shanghai, China). After treatment, the Caco-2 cell monolayers were washed and incubated in HBSS for 25–35 min, before post-treatment TEER measurement. Changes in TEER were expressed as percentage (%) relative TEER and calculated using the following **Equation (2)** by Yu and Huang (2013):

$$\%\ \text{relative TEER} = \frac{\text{TEER}_{\text{post}}}{\text{TEER}_{\text{pre}}} \times 100 \quad (2)$$

Where, $\text{TEER}_{\text{post}}$ = The TEER value across Caco-2 cell monolayers after treatment Ωcm^2; TEER_{pre} = The TEER value across Caco-2 cell monolayers before treatment Ωcm^{-2}.

2.7. Methylthiazole tetrazolium bromide (MTT) assay in Caco-2 and HepG2 cells

The MTT assay (Mosmann, 1983) on Caco-2 and HepG2 cells was

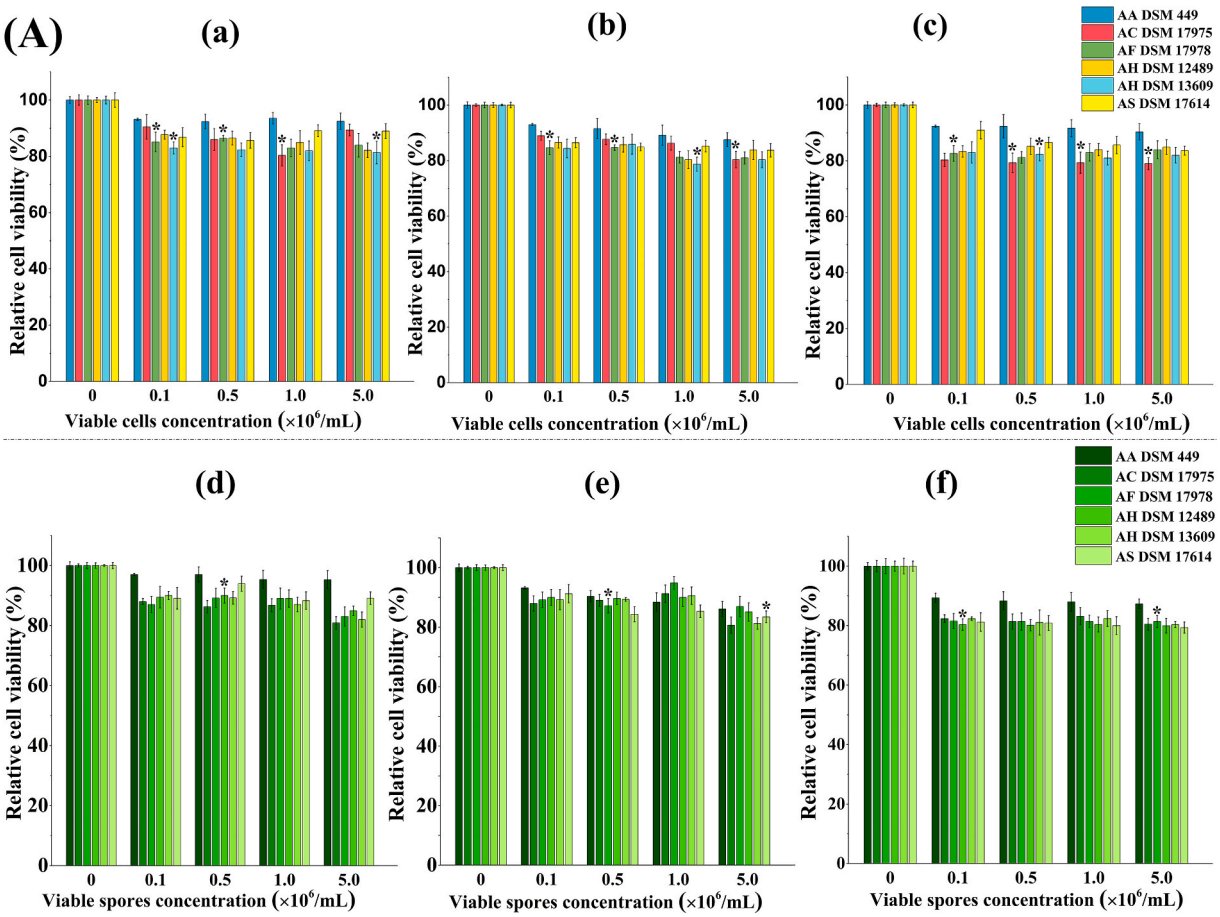

Fig. 5. *In vitro* cytotoxicity of *Alicyclobacillus* spp. in Caco-2 and HepG2 cells: Cell viability of Caco-2 cells tested with different *Alicyclobacillus* spp. **A:** viable cells 6 h **(a)**, 24 h **(b)**, and 48 h **(c)**; viable spores 6 h **(d)**, 24 h **(e)**, and 48 h **(f)**. **B:** non-viable cells 6 h **(g)**, 24 h **(h)** and 48 h **(i)**; non-viable spores 6 h **(j)**, 24 h **(k)**, and 48 h **(l)**, and cell viability of HepG2 cells treated with different *Alicyclobacillus* spp. **C:** viable cells 6 h **(m)**, 24 h **(n)**, and 48 h **(o)**; viable spores 6 h **(p)**, 24 h **(q)**, and 48 h **(r)**. **D:** non-viable cells 6 h **(s)**, 24 h **(t)**, and 48 h **(u)**; non-viable spores 6 h **(v)**, 24 h **(w)**, and 48 h **(x)** at different concentrations and reaction times. Data are shown as means ± SD (n = 3). $*P > 0.05$ compared with control samples

carried out using previously published methods by Yu and Huang (2013). Initially, 100 μL of Caco-2 and HepG2 cells (1×10^4) were seeded separately, in each well of a 96-well plate (Coring, Arizona, USA). The cells were then treated with different concentrations of *Alicyclobacillus* spp. from 0.1 to 5 × 10^6/mL (viable cells and spores) and 5–500 μg/mL (non-viable cells and spores) separately. Caco-2 and HepG2 treated cells were kept at 37 °C in an incubator (5% CO_2 and 95% relative humidity) for a period of 6, 24 and 48 h. The cells were then incubated for 2 h with 100 μL/mL MTT solution [10% (v/v) of 5 mg/ml of MTT agent with 90% (v/v) of RPMI-1640 medium]. After incubation, the cells were dissolved in 100 μL of DMSO per well for 10 min and absorbance (Bio-Rad, Hercules, CA, USA) was recorded. All samples were taken in triplicate. Supplementary Table S2 shows the *in vitro* cell viability and cytotoxicity level of *Alicyclobacillus* cells and spores. Caco-2 and HepG2 cells grown in a medium without any treatment were taken as control. An iMark Microplate Reader (Bio-Rad, Hercules, CA, USA) was used to measure the optical density (OD) at a wavelength of 570 nm. The relative cell viability was calculated using the following **Equation (3)**:

$$\%\ \text{relative cell viability} = \frac{\text{Sample mean OD}}{\text{Control OD}} \times 100 \quad (3)$$

2.8. Microscopic observations

After incubation with viable and non-viable *Alicyclobacillus* spp. cells and spores, changes in morphology and detachment of Caco-2 and HepG2 cells from the plate were observed using a Nikon inverse phase contrast microscope (Nikon TMS, Nikon, Japan) equipped with an objective (Plan 10/0.30DL/Ph1, Nikon, Japan) of 100 × magnification.

2.9. Hemolysis test

The hemolytic evaluation test was carried out using the operation steps with slight modifications as described by Lin and Haynes (2009). The blood of heparinized C57BL/6J mice was diluted to 1/10 of the original blood concentration using an aseptic PBS and then the mice erythrocyte (red blood cells, RBC) was isolated by centrifugation (PM180R, SIM International Group Co. Ltd, Newark, DE, USA) at 3000 rpm for 10 min, at room temperature. Purified RBCs were washed and dissolved in PBS solution 5 times to ensure that no heme derivatives were found in the isolated RBCs to avoid interfering with hemolytic evaluation results. All *Alicyclobacillus* spp. viable cells and spores (0.1–5 × 10^6) and non-viable cells and spores (10–1000 μg/mL) pairing with different concentrations separately, were added to the RBC and incubated for 3 h at 37 °C in a shaking water bath (DFS; KW-1000DC, Wincom Company Ltd. Changsha, Hunan, China). Finally, the mixture was separated by centrifugation at 13000 rpm for 3 min, at room temperature, and, then, the sample supernatant was transferred to a 96-well plate. An iMark microplate reader (Bio-Rad, Hercules, CA, United States) was used to measure the *in vitro* hemolysis rate at an absorbance

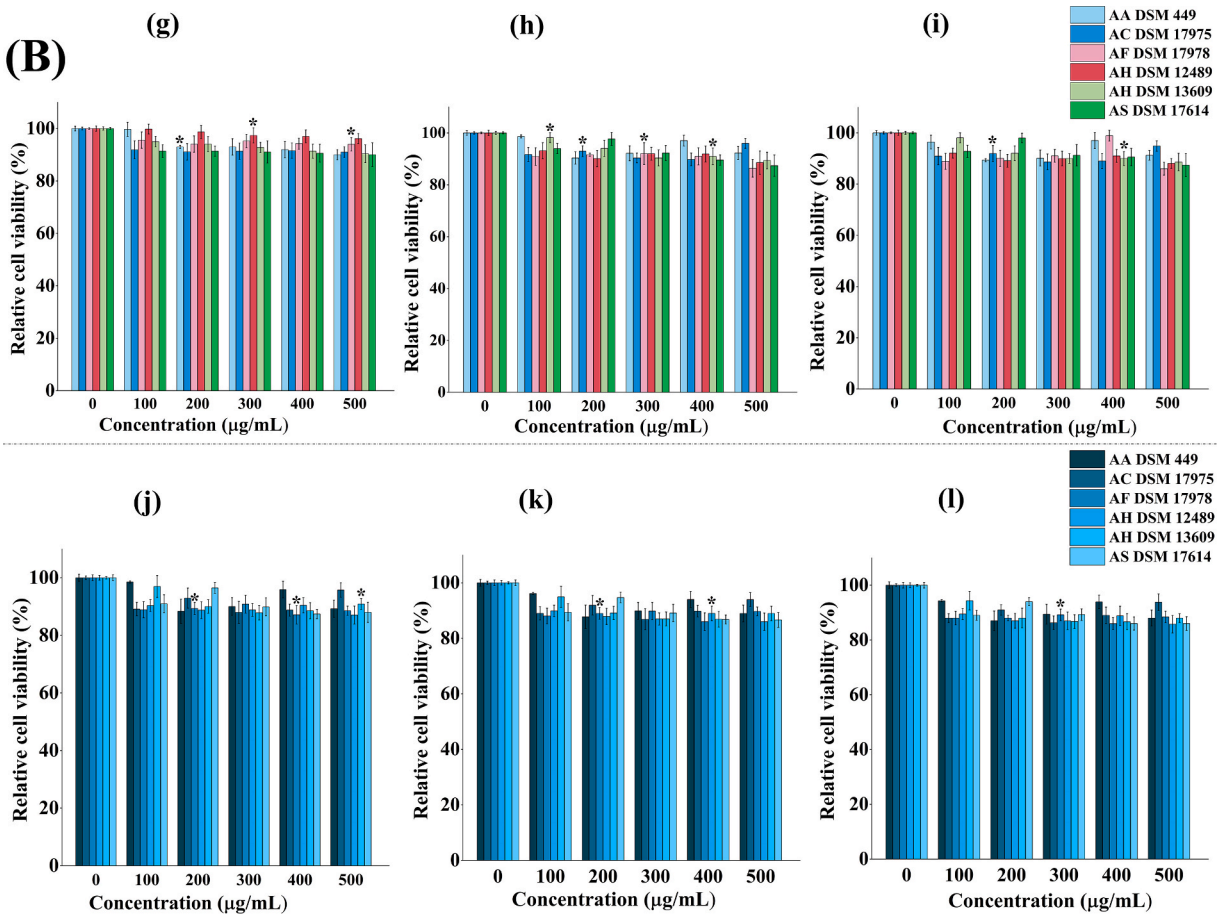

Fig. 5. (*continued*).

wavelength of 540 nm. The RBCs were mixed with 1% Triton X-100 (Sigma-Aldrich, Thermo Fisher Scientific Co., Ltd., Shanghai, China) and PBS solution to be used as a positive (+) and negative (−) control in hemolytic evaluation test, respectively. The RBCs in the positive control were assumed to be completely dissolved in 1% Triton X-100. All of the experiments were performed in triplicate. The percentage of hemolysis rates of viable and non-viable *Alicyclobacillus* spp. cells and spores were calculated using the following **Equation (4)**:

$$(\%)\ \text{Hemolysis rate} = \frac{A_s - A_n}{A_p - A_n} \times 100 \quad (4)$$

Where, A_p = positive control absorptive value of hemolysis test; A_n = negative control absorptive value of hemolysis test; A_s = absorptive value of hemolysis test for the sample. For the hemolytic evaluation test, if the hemolytic rate of the sample is less than 5%, it can be considered that there is no hemolytic reaction in the sample to be tested, which is in accordance with the requirements of the hemolytic test (Zhou et al., 2014; Ronny et al., 2008; Wu et al., 2010).

2.10. *In vivo* acute toxicity assay

The mouse experiment protocol was approved (AEWC-2021-0205) by the ethics committee of Northwest Agriculture & Forestry University, Yangling, China on care and use of laboratory animals in accordance with the Animal Welfare Legislation of China. Six-week-old C57BL/6J mice (16–18 g) were purchased from the Fourth Military Medical University (Xi'an, Shaanxi, China). All these mice used to evaluate the acute toxicity of cells and spores from *Alicyclobacillus* spp. *in vivo* via oral diet.

In the experimental animal study mice were randomly divided into 12 groups (n = 5). For an initial period of one week, the basal diet was administered to the mice to acclimate them to the laboratory conditions. In our previous publication of Sajid et al. (2018), the results of patulin adsorption from apple juice using heat-inactivated (HI) *Alicyclobacillus acidocaldarius* DSM 449 cells and spores were valuable and supportive in this study. One selected *Alicyclobacillus acidocaldarius* cells and spores (AA DSM 449) to evaluate an acute toxicity assay *in vivo* and, then, administered at a dose volume of 0.5mL/10 g of body weight. The tested groups were administered at doses of 100, 200, 300, 400, and 500 μg/mL. Consequently, the control group was administered equal volumes of tap water without *Alicyclobacillus acidocaldarius* cells and spores (AA DSM 449). Thereafter, the mice were monitored for clinical signs (toxicity and mortality) for eight consecutive hours, followed by a once-daily examination, which was continued for 14 days. Feed and water intake were recorded daily while body weight was recorded on study days 0, 8 and 14 (Luo et al., 2017; Shih et al., 2013).

At the end of the animal study, fasting mice were sacrificed overnight (Fig. 2) and blood was collected in EDTA coated tubes for hematological research, by removing the retroorbital plexus, and blood serum was collected in non-coated tubes by centrifugation (Hitachi Koki Co., Ltd., Japan) at 13,000 × *g* for 5 min at 4 °C. Plasma enzyme activities, such as alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), Blood urea nitrogen (BUN), Total Protein (TP), and Creatine kinase (CK) were determined by an automatic biochemical analyzer (7180, Hitachi, Japan). After the mice were sacrificed, the weights of the body organs including the heart, liver, kidneys, spleen, thymus and lungs were recorded to calculate the organ to body weight ratio. The liver, spleen, lung and small intestine samples of mice were taken for histological analysis. The samples were first fixed in phosphate buffered formalin 10% solution (Sigma-Aldrich, Thermo Fisher Scientific Co., Ltd., Shanghai, China) and, then, dehydrated,

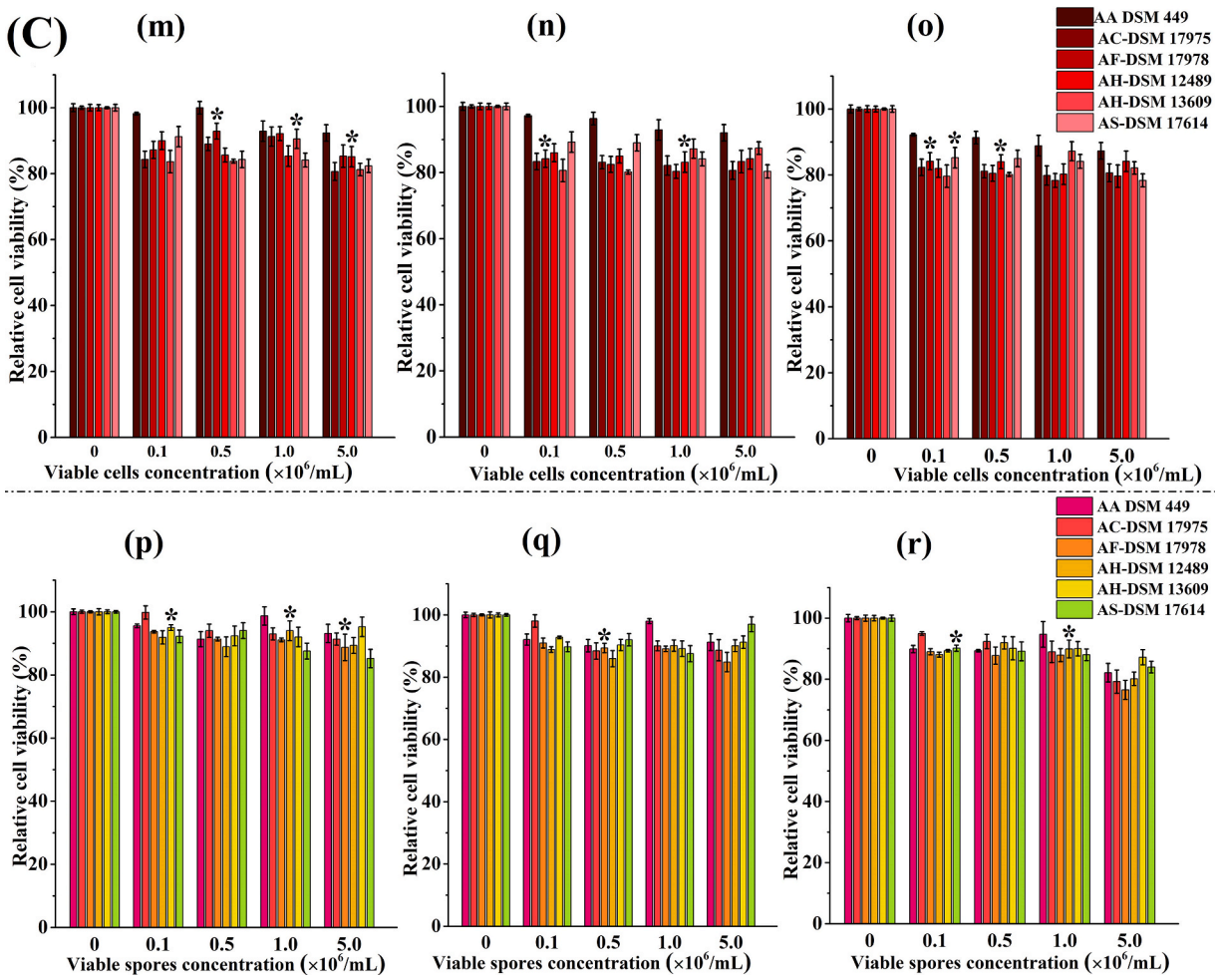

Fig. 5. (*continued*).

embedded in paraffin. Afterwards, hematoxylin and eosin (H&E) stained tissue sections of the liver were observed under an optical microscope (CX31RTSF, Olympus Optical Co., Cebu, Philippines) and to monitor possible histological changes (Shih et al., 2013).

2.11. Statistical analysis

All experiments were performed in triplicate. The results were presented as means ± standard deviation (SD). Data were subjected to one-way analysis of variance (1-way ANOVA) followed by Dunnett's test with IBM SPSS Statistics software 23.0 (IBM Corp., Armonk, NY, USA). Statistical significance was considered to exist at $P < 0.05$.

3. Results and discussion

3.1. General

In this work, *in vitro* and *in vivo* safety analysis of *Alicyclobacillus* spp. was studied. *In vitro* cytotoxicity of all viable and non-viable *Alicyclobacillus* spp. cells and spores with different concentrations were evaluated in various assays and cell types. For *in vivo* examination of acute toxicity, *Alicyclobacillus acidocaldarius* (AA DSM 449) was used from the six strains tested based on its significant amount of biomass and 50% absorbed patulin rate according to the former study made by Sajid et al. (2018). The overall safety evaluation results showed that all viable and non-viable *Alicyclobacillus* spp. cells and spores tested are noncytotoxic and nontoxic.

3.2. Evaluation of morphological changes in Caco-2 and HepG2 cells

Usually, in suspension, Caco-2 and HepG2 cells are small, spherical, adherent and growing as a confluent monolayer (Jokhadar et al., 2009; Wakatsuki et al., 2003). Cell morphology was detected using a microscope after an incubation period of 6, 24 and 48 h. The results exposed that viable *Alicyclobacillus* spp. cells and spores at 0.1 to 5.0 × 10^6/mL and non-viable at 100–500 μg/mL for Caco-2 and HepG2 cells after 6, 24 and 48 h of exposure separately showed that more than 80% cell viability in morphology photos compared to control cells (Fig. 3). Moreover, the cell fragments and changes in morphology were not observed at given incubation times and concentrations.

3.2.1. *In vitro* investigation of the membrane integrity of Caco-2 cell monolayers

Caco-2 cell monolayers were treated with strains of *Alicyclobacillus* spp. cells and spores to mimic the exposure of the small intestine epithelium to the strains. Cell membrane integrity was examined by an LDH leakage assay. During the experimental procedures, rupture of the cell membrane was found by releasing the enzyme from the Caco-2 cell monolayers without treatment, with a range of 1–3%. The treated Caco-2 cell monolayers with viable and non-viable *Alicyclobacillus* spp. cells and spores showed 3–6% of cells with LDH leakage (Fig. 4A and **B**). The results showed no significant differences in LDH leakage between untreated cells and most of the *Alicyclobacillus* spp. treated Caco-2 cell monolayers, except for the viable cells and spores of strains AH DSM 13609, AH DSM 12489 and AC DSM 17975. Furthermore, non-significant changes were observed between all six pairs of

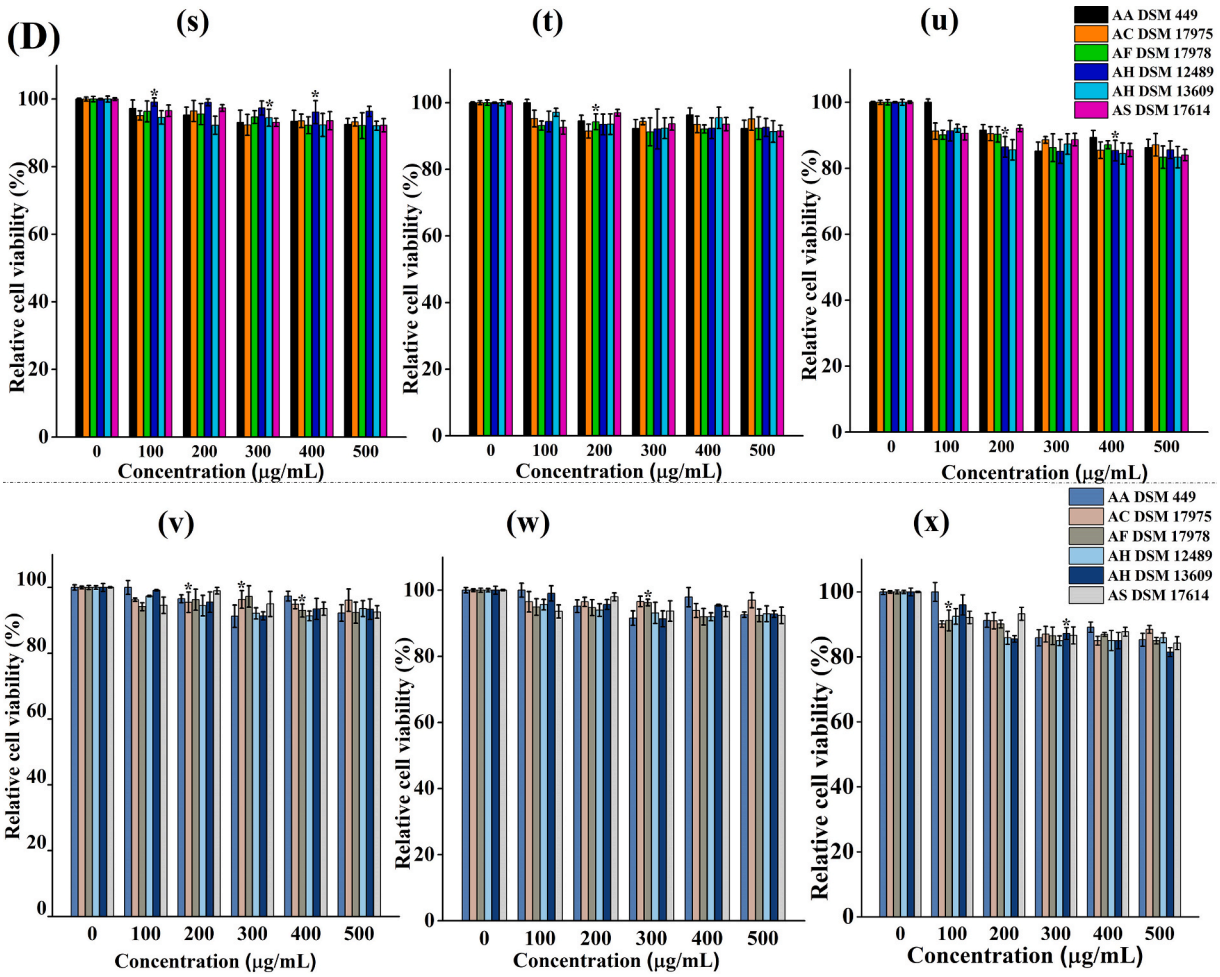

Fig. 5. (*continued*).

Alicyclobacillus spp., revealing that the viable cells and spores of strains AH DSM 13609, AH DSM 12489 and AC DSM 17975 did not possess more cytotoxicity for Caco-2 cells than other *Alicyclobacillus* spp. strains.

In one hand, *Alicyclobacillus* spp. are generally labeled as spoilage bacteria for acidic drinks. On the other hand, the use of non-viable *Alicyclobacillus* strains as adsorbents to decrease the patulin level in apple juice (Sajid et al., 2018; Yuan et al., 2014). It is expected that after the adsorption of patulin from fruit juices, *Alicyclobacillus* spp. residues may present and undergo the digestion process, which can cause health risks in humans. Our research results, illustrated that all tested viable and non-viable *Alicyclobacillus* strain cells and spores were nontoxic to small intestine Caco-2 cells. The possible explanation is that during digestion with apple juice, viable and non-viable *Alicyclobacillus* strain cells and spores are digested (once ingested) more rapidly on larger surface area of small intestine than other consistent food particles. In the *in vivo* environment, only small portion of viable and non-viable *Alicyclobacillus* strain cells and spores may have the chance of interaction with the small intestine epithelium directly. This interaction in the *in vitro* did not reveal the apparent toxicity. The explanations of the results are in agreement with previous experimental studies conducted by Steyn et al. (2011), Li and McClements (2010) and Yu and Huang (2012) using different particles for the membrane integrity of Caco-2 cell monolayers.

3.3. Examination of the tight junction integrity in the Caco-2 cell monolayers

After good differentiation, similar tight junctions in the epithelium of the small intestine were observed between adjacent Caco-2 cells. Under normal physiological conditions, disruption of the tight junction should be avoided for water-soluble (toxic) compounds. The TEER values of Caco-2 cells were examined with viable and non-viable *Alicyclobacillus* spp. cells and spores (Fig. 4C and **D)**. The one-way ANOVA results showed that six viable and non-viable *Alicyclobacillus* spp. strain cells and spores (AA DSM 449, AC DSM 17975, AF DSM 17978, AH DSM 12489, AH DSM 13609 and AS DSM 17614) did not have a deceptive effect on the tight junction integrity and caused no decrease in TEER. More importantly, comparing all viable and non-viable six *Alicyclobacillus* spp. cells with the corresponding spores, there was not any significant difference in the TEER change except AH DSM 13609 strain viable cells and spores. The hypothesis is that all tested *Alicyclobacillus* spp. are considered nontoxic and display a little distraction from the tight junctions.

In the previous section, the results of LDH leakage showed that no elevated toxicity was detected for non-viable *Alicyclobacillus* spp. strain cells and spores on Caco-2 cell monolayers, proposing that *Alicyclobacillus* spp. strain cells and spores are nontoxic to the small intestine epithelium. These results are reliable with the other research group's findings that some emulsifiers and surfactants might interrupt the tight junctions (Ohno et al., 2006; Yamashita et al., 2000).

3.4. Evaluation of cytotoxicity in Caco-2 and HepG2 cells

Regarding to the possible *in vitro* toxicity of *Alicyclobacillus* spp. on the epithelium of the small intestine or liver, Caco-2 monolayers and HepG2 cells was taken as a model, respectively. Furthermore, cells were tested with different concentrations of viable and non-viable

Table 1
Percentage of Hemolysis of RBCs (mean ± SD) incubated at 37 °C ($n = 3$) with different concentrations of viable and non-viable cells and spores of *Alicyclobacillus* spp. strains.

Viability	Viable								Non-viable							
Concentration	0.1×10^6/mL		0.5×10^6/mL		1.0×10^6/mL		5.0×10^6/mL		100 µg/mL		200 µg/mL		300 µg/mL		400 µg/mL		500 µg/mL	
Strains	Cells	Spores	Cells	Spores	Cells	Spores	Cells	Spores	Cells	Spores	Cells	Spores	Cells	Spores	Cells	Spores	Cells	Spores
AA DSM 449	2.14 ± 0.58	3.31 ± 0.23	3.43 ± 0.32	3.76 ± 0.38	3.92 ± 0.40	3.74 ± 0.38	3.41 ± 0.48	3.10 ± 0.48	2.07 ± 0.25	2.13 ± 0.25	2.01 ± 0.50	2.57 ± 0.53	2.33 ± 0.55	2.85 ± 0.54	3.08 ± 0.65	3.51 ± 0.62	2.20 ± 0.24	2.76 ± 1.29
AC DSM 17975	2.63 ± 0.15	2.83 ± 0.18	2.68 ± 0.25	2.91 ± 0.26	1.36 ± 0.37	3.57 ± 0.37	3.41 ± 0.50	3.51 ± 0.53	2.03 ± 0.12	2.48 ± 0.12	2.61 ± 0.25	2.81 ± 0.25	1.80 ± 0.37	2.90 ± 0.37	2.62 ± 0.54	3.12 ± 0.56	3.10 ± 0.76	3.20 ± 0.84
AC DSM 17978	2.74 ± 0.20	3.05 ± 0.23	2.90 ± 0.30	2.5 ± 0.32	2.71 ± 0.35	3.02 ± 0.38	3.87 ± 0.42	3.82 ± 0.47	1.83 ± 0.24	2.03 ± 0.25	2.16 ± 0.30	1.90 ± 0.32	2.03 ± 0.50	1.84 ± 0.53	2.50 ± 0.62	2.36 ± 0.64	2.90 ± 0.87	3.61 ± 1.93
AH DSM 12489	2.19 ± 0.21	2.54 ± 0.25	2.31 ± 0.34	3.26 ± 0.33	2.52 ± 0.35	3.42 ± 0.35	2.66 ± 0.48	3.73 ± 0.51	2.30 ± 0.23	2.41 ± 0.23	2.11 ± 0.32	3.06 ± 0.62	1.47 ± 0.58	2.61 ± 0.57	2.05 ± 0.61	2.51 ± 0.32	2.43 ± 0.64	3.12 ± 0.92
AH DSM 13609	2.21 ± 0.12	2.71 ± 0.13	2.64 ± 0.25	2.61 ± 0.27	2.52 ± 0.30	3.12 ± 0.34	3.70 ± 0.47	3.79 ± 0.52	2.14 ± 0.23	2.16 ± 0.25	1.70 ± 0.28	2.27 ± 0.28	2.47 ± 0.42	2.83 ± 0.46	2.41 ± 0.51	2.07 ± 0.53	3.31 ± 0.24	3.61 ± 1.29
AS DSM 17614	2.14 ± 0.17	2.44 ± 0.25	2.42 ± 0.23	3.46 ± 0.36	2.68 ± 0.44	3.70 ± 0.38	2.83 ± 0.62	4.08 ± 0.51	2.08 ± 0.245	2.45 ± 0.42	2.19 ± 0.38	1.74 ± 0.35	2.53 ± 0.38	1.7 ± 0.394	2.52 ± 0.44	1.53 ± 0.42	2.24 ± 0.51	3.26 ± 0.53

Mice blood hemolysis assay using Triton X-100 as the positive control and phosphate-buffered saline (PBS) as the negative control.
The values presented are the means ± standard deviation (SD) from three independent assays.

Alicyclobacillus spp. strain cells and spores (Fig. 5). On the other hand, the safety of *Alicyclobacillus* spp. cells and spores was the primary focus in applying their commercial-level usage as biosorbent for mycotoxins from fruit juices.

Furthermore, residues of viable and non-viable *Alicyclobacillus* cells and spores could be absorbed by the stomach and pass through the intestinal mucosa, which can cause adverse health effects (Nutt et al., 2015). The exposure assessment of viable and non-viable *Alicyclobacillus* spp. cells and spores were, thus, restricted to the digestive tract, particularly the small intestine, considering its large surface area. The liver, where the food is digested and absorbed, is carried for possible metabolism and storage. Based on these observations, Caco-2 cell monolayers and HepG2 cells were selected for the small intestine epithelium and liver hepatocytes, respectively.

The results of the one-way ANOVA illustrated a gradual decrease in cell viability after 6, 24 and 48 h of contact with *Alicyclobacillus* spp. viable cell and spore concentrations at 0.1 to 5.0 × 10^6/mL, and non-viable cell and spore concentrations at 100–500 µg/mL for Caco-2 and HepG2 cells, separately. The results revealed that comparing all viable and non-viable six *Alicyclobacillus* spp. cells with the corresponding spores, there was not any significant difference in the Caco-2 and HepG2 cells viability. The observations of the morphology of Caco-2 and HepG2 cells (Fig. 3) showed no cytotoxicity and the cell viability was still greater than 80%. According to the cytotoxicity classification (Supplementary material: Table S2), *Alicyclobacillus* spp. cells and spores were qualified as non-cytotoxicity.

3.5. Determination of the hemolytic activity

Hemolysis is the liberation of red blood cells, resulting in the release of hemoglobin due to decomposition. In biological contexts, hemolysis can precipitate conditions such as anemia, jaundice, and other severe illnesses. Consequently, it is imperative to thoroughly analyze and assess the impact of drug substances on blood upon entry into the body (Oberdorster et al., 2005). The bacterial biomass material utilized ultimately enters the human bloodstream or the lymphatic circulatory system. Viable and non-viable *Alicyclobacillus* spp. cells and spores enter the body's circulatory system and access organs such as the spleen, liver, kidney, small intestine and muscle. Therefore, a red blood cell hemolysis test was necessary to check their toxicity (Dobrovolskaia et al., 2008).

The release of hemoglobin was used to measure the biocompatibility or membrane-damaging properties of any drug intake. The erythrocytes were incubated with different concentrations using six different viable (0.1–5.0 × 10^6/mL) and non-viable (100–500 µg/mL) *Alicyclobacillus* spp. for 3 h at 37 °C. The Triton X-100 solution as 100% and PBS 0% values were used for treated erythrocytes, respectively. The results revealed that under these conditions, all tested pairs of viable and non-viable *Alicyclobacillus* spp. cells and spores did not have hemolytic effects up to 500 µg/mL, as shown in Table 1, which does not represent any detectable disturbance of the red blood cell membranes. It can be clearly seen in Fig. 6A, B, C and **D** that the hemolysis of RBCs is caused by viable and non-viable cells and spores of the *Alicyclobacillus acidocaldarius* strain coded as AA DSM 449. Also a dose-response curve was presented in Supplementary material: Fig. S1. Moreover, the hemolytic rate, below the safety standard of 5%, shows that tested viable and non-viable *Alicyclobacillus* spp. cells and spores do not have harmful effects on RBCs.

No statistical difference was found for each different concentration of viable and non-viable cells and spores of *Alicyclobacillus* spp.

3.6. Examination of an acute toxicity study

We used C57BL/6J mice in the *in vivo* study, as they play a critical role in the testing of new drugs because their biological, genetic and behavioral characteristics are closely related to those of humans and many similar symptoms in humans can be replicated in mice. After

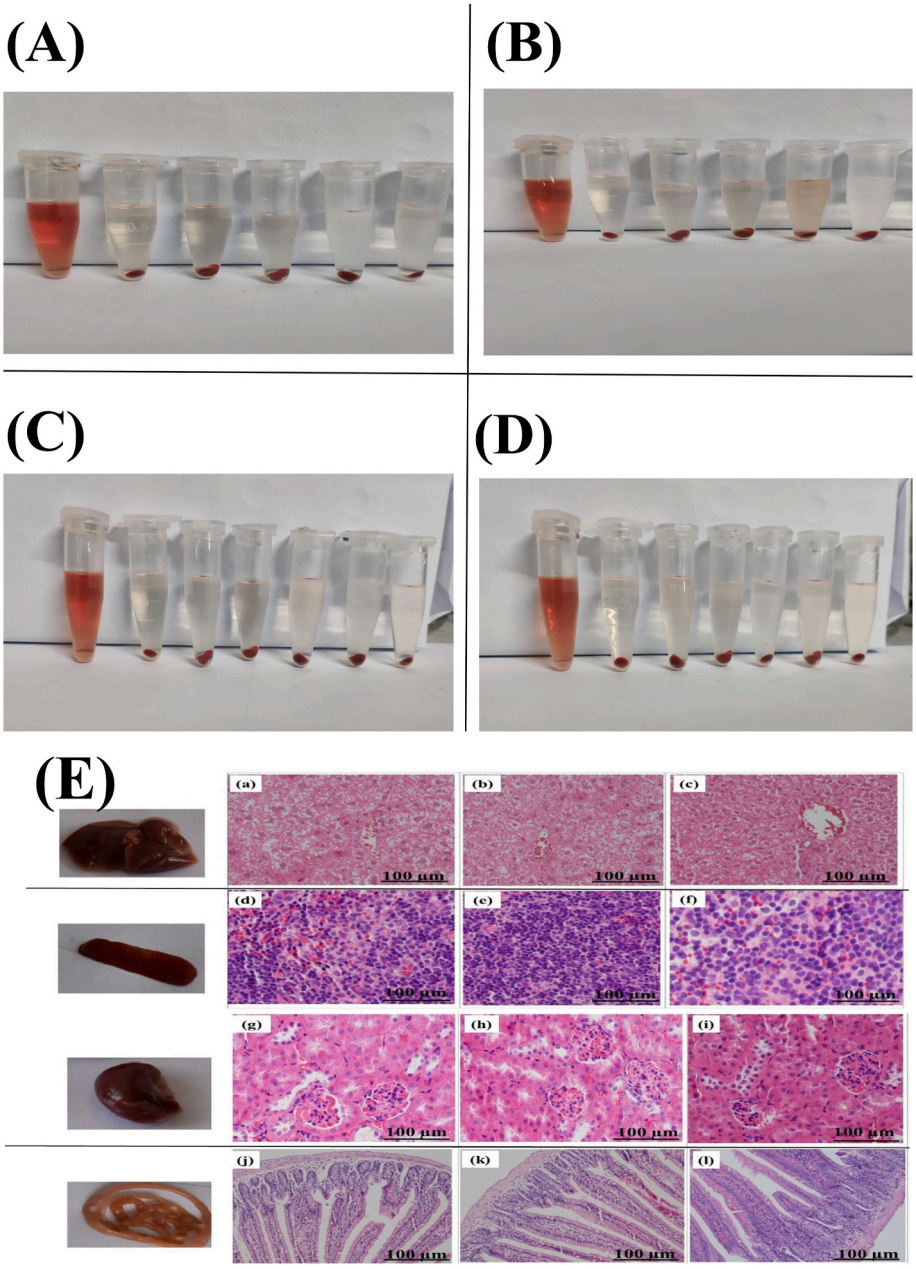

Fig. 6. Hemolysis of erythrocytes and H&E stained photomicrographs of mice body organs: Photography of hemolysis of C57BL/6J mice red blood cells (RBC) in the presence of *Alicyclobacillus acidocaldarius* (AA DSM 449) (viable cells **A** and viable spores **B**) and (non-viable cells **C** and non-viable spores **D**) with different concentrations. **(E)** Photomicrographs of C57BL/6J mice body organs H&E stained at Magnification 10 × and scale bar = 100 µm for all images: liver (**a**), spleen (**d**), kidney (**g**) and small intestine (**j**) showing control groups; and treated liver (**b**, **c**), spleen (**e**, **f**), kidney (**h**, **i**) and small intestine (**k**, **l**) with maximum dose concentration (500 µg/mL) of *Alicyclobacillus* cells and spores, respectively.. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

administering viable and non-viable cells and spores of AA DSM 449 to mice, no statistically significant clinical effects were observed in any group of mice. Meanwhile, the treated mice did not show abnormal behavior. During the acute oral toxicity study (14 days), body weights were also measured (Table 2A), and no statistically significant differences ($P > 0.05$) were found between all treated and control groups. On the other hand, the effect of viable and non-viable *Alicyclobacillus* spp. cells and AA DSM 449 spores was observed in organ tissue morphology of mice, as shown in Fig. 6E (a, b, c, d, e, f, g, h, I, j, k, and l). Additionally, the organ body weight ratios for each organ are shown in Table 2B for each dose group. The results showed that the proportions in different dose groups were not significant compared to the control group. In addition, routine blood hemodynamic and biochemical parameters showed that the values of the treated groups were relatively average compared to the control group (Table 2C).

The results of an acute oral toxicity study showed that viable and non-viable *Alicyclobacillus* spp. cells and spores of AA DSM 449 had no safety concerns, which specified that *Alicyclobacillus* spp. are novel and promising biosorbents and could be applied commercially for mycotoxin (for example, patulin) adsorption from fruit juice. The *in vitro* and *in vivo* results of viable and non-viable cells and spores of AA DSM 449 could be used to explain the possible reason for the nontoxicity of *Alicyclobacillus*spp. Furthermore, the LD_{50} (lethal dose 50) of *Alicyclobacillus* metabolites was greater than the concentration of residual *Alicyclobacillus* spp. cells and spores, and these results agree with the previous study by Zhao et al. (2012).

Table 2a
The body weight of experimental animals (C57BL/6J mice, n = 5) after exposure of *Alycyclobacillus* spp. cells and spores of AA DSM 449 strain at different time intervals.

Strains	Dose group (μg/mL)	Day 1 body weight (g)		Day 8 body weight (g)		Day 14 body weight (g)	
		Cells	Spores	Cells	Spores	Cells	Spores
AA DSM 449	**Control**	20.80 ± 1.26	20.15 ± 1.91	21.56 ± 1.63	22.39 ± 1.75	23.94 ± 1.57	23.59 ± 1.83
	100	20.75 ± 3.02	20.78 ± 2.05	21.70 ± 2.59	21.80 ± 1.62	24.72 ± 2.46	23.90 ± 3.04
	200	19.84 ± 1.21	20.91 ± 1.86	21.03 ± 1.95	21.00 ± 2.35	23.55 ± 1.90	23.78 ± 1.74
	300	20.65 ± 1.38	19.50 ± 3.15	21.65 ± 3.01	20.74 ± 1.55	23.92 ± 2.08	23.00 ± 2.50
	400	19.83 ± 2.70	20.86 ± 1.40	21.35 ± 1.32	20.96 ± 2.20	23.60 ± 1.15	24.63 ± 1.55
	500	20.36 ± 1.42	20.07 ± 2.63	21.61 ± 1.75	21.90 ± 1.14	23.84 ± 1.50	23.50 ± 2.51

Data were analyzed by one-way analysis of variance (1-way ANOVA) and no differences were found within each organ *versus* controls at 100–500 μg/mL doses of AA DSM 449 cells and spores, separately. All values represent the means ± standard deviation (SD).

4. Conclusions

The present study provides the first approach to examining the *in vitro* and *in vivo* safety of viable and non-viable *Alicyclobacillus* spp. cells and spores under different assays, cell types, and rodent studies. *In vitro,* Caco-2 and HepG2 cells were used for possible toxicity. Next, all cells and spores tested for *Alicyclobacillus* spp. compared to control samples, the integrity of the Caco-2 cell membrane was not affected. In addition, the percentage of relative proliferation/viability of Caco-2 monolayers and HepG2 cells were not affected by viable and non-viable *Alicyclobacillus* spp. cells and spores, and cell viabilities were still higher than 80%. In an acute toxicity study, *Alicyclobacillus acidocaldarius* AA DSM 449 did not induce harmful effects and showed a non-toxic response in treated mice. Moreover, *in vitro* studies showed that viable and non-viable *Alicyclobacillus* spp. strain cell and spores did not show harmful effects on red blood cells having a negligible hemolysis rate. The findings of this research study suggested that potential biosorbents such as *Alicyclobacillus* spp. could be used commercially to remove the level of mycotoxin patulin from fruit juices.

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CRediT authorship contribution statement

Marina Sajid: Writing – original draft, Validation, Methodology, Data curation. **Sajid Mehmood:** Methodology, Formal analysis, Data curation. **Yahong Yuan:** Data curation, Conceptualization. **Tianli Yue:** Validation, Supervision, Conceptualization. **Muhammad Zubair Khalid:** Writing – review & editing, Methodology, Formal analysis. **Ahmad Mujtaba:** Software. **Sulaiman Ali Alharbi:** Writing – review & editing, Software. **Mohammad Javed Ansari:** Writing – review & editing, Software. **Abdellah Zinedine:** Writing – review & editing, Formal analysis. **João Miguel Rocha:** Writing – review & editing, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Table 2b
The weight of experimental animal (mice) organs for example heart, liver, spleen lung, kidneys and thymus after oral administration of different doses (100–500 μg/mL) for 14 days.

Strain	Body organs		Dose groups (μg/mL)					
		Units	Control	100	200	300	400	500
AA DSM 449 cells	**Heart**	(g)	0.152 ± 0.06	0.150 ± 0.21	0.155 ± 0.05	0.158 ± 0.25	0.160 ± 0.35	0.154 ± 0.26
		(%) of body weight	0.634 ± 0.11	0.719 ± 0.15	0.658 ± 0.08	0.689 ± 0.18	0.684 ± 0.10	0.645 ± 0.12
	Liver	(g)	1.267 ± 0.25	1.204 ± 0.06	1.290 ± 0.04	1.305 ± 0.22	1.269 ± 0.03	1.311 ± 0.14
		(%) of body weight	6.392 ± 0.38	5.770 ± 0.35	5.477 ± 0.60	5.693 ± 0.37	5.432 ± 0.83	5.499 ± 0.35
	Spleen	(g)	0.084 ± 0.05	0.082 ± 0.08	0.082 ± 0.11	0.080 ± 0.07	0.081 ± 0.05	0.083 ± 0.06
		(%) of body weight	0.424 ± 0.03	0.393 ± 0.11	0.348 ± 0.10	0.349 ± 0.06	0.346 ± 0.06	0.348 ± 0.05
	Lungs	(g)	0.192 ± 1.85	0.173 ± 0.25	0.201 ± 0.06	0.195 ± 0.22	0.231 ± 0.21	0.198 ± 0.22
		(%) of body weight	0.962 ± 0.16	0.830 ± 0.18	0.853 ± 0.12	0.850 ± 0.09	0.988 ± 0.04	0.830 ± 0.11
	Kidneys	(g)	0.292 ± 0.11	0.270 ± 0.07	0.290 ± 0.06	0.287 ± 0.15	0.301 ± 0.11	0.287 ± 0.04
		(%) of body weight	1.239 ± 0.18	1.295 ± 0.10	1.231 ± 0.17	1.253 ± 0.11	1.288 ± 0.13	1.203 ± 0.25
	Thymus	(g)	0.015 ± 0.06	0.011 ± 0.02	0.012 ± 0.04	0.013 ± 0.02	0.015 ± 0.05	0.013 ± 0.08
		(%) of body weight	0.075 ± 0.03	0.052 ± 0.02	0.050 ± 0.01	0.056 ± 0.06	0.065 ± 0.03	0.054 ± 0.02
AA DSM 449 spores	**Heart**	(g)	0.158 ± 0.10	0.153 ± 0.24	0.160 ± 0.08	0.157 ± 0.06	0.155 ± 0.26	0.159 ± 0.35
		(%) of body weight	0.669 ± 0.12	0.669 ± 0.10	0.672 ± 0.16	0.682 ± 0.12	0.652 ± 0.08	0.676 ± 0.17
	Liver	(g)	1.260 ± 0.04	1.253 ± 0.20	1.271 ± 0.03	1.268 ± 0.16	1.270 ± 0.03	1.255 ± 0.05
		(%) of body weight	5.341 ± 0.83	5.317 ± 0.83	5.344 ± 0.34	5.513 ± 0.33	5.347 ± 0.18	5.340 ± 0.15
	Spleen	(g)	0.080 ± 0.01	0.084 ± 0.06	0.081 ± 0.15	0.082 ± 0.03	0.080 ± 0.06	0.082 ± 0.04
		(%) of body weight	0.339 ± 0.28	0.338 ± 0.06	0.340 ± 0.03	0.347 ± 0.11	0.336 ± 0.17	0.348 ± 0.08
	Lungs	(g)	0.190 ± 0.12	0.182 ± 0.11	0.185 ± 0.04	0.170 ± 0.05	0.195 ± 0.18	0.191 ± 0.06
		(%) of body weight	0.805 ± 0.11	0.774 ± 0.09	0.777 ± 0.04	0.739 ± 0.12	0.821 ± 0.15	0.812 ± 0.05
	Kidneys	(g)	0.291 ± 0.03	0.295 ± 0.05	0.289 ± 0.03	0.278 ± 0.04	0.285 ± 0.04	0.293 ± 0.35
		(%) of body weight	1.233 ± 0.10	1.209 ± 0.11	1.215 ± 0.16	1.208 ± 0.18	1.205 ± 0.10	1.246 ± 0.13
	Thymus	(g)	0.013 ± 0.04	0.013 ± 0.18	0.011 ± 0.06	0.014 ± 0.11	0.012 ± 0.08	0.013 ± 0.15
		(%) of body weight	0.055 ± 0.02	0.046 ± 0.01	0.046 ± 0.05	0.060 ± 0.03	0.050 ± 0.02	0.055 ± 0.05

Table 2c
The values of blood routine test ① and biochemical analysis ② for *Alicyclobacillus* spp. cells and spores.

Strains	Dose groups (μg /mL)	Hematological and biochemical parameters								
		RBC (1×10^{12} /L)	WBC (1×10^{9} /L)	PLT (1×10^{9} /L)	ALT (U /L)	AST (U /L)	ALP (U /L)	BUN (mmol /L)	TP (g/L)	CK (U /L)
AA DSM 449 cells	**Control**	10.57 ± 1.25[a]	3.62 ± 1.80[a]	1153.20 ± 146.02[a]	28.73 ± 8.46[a]	95.63 ± 45.69[a]	138.17 ± 20.51[a]	5.78 ± 1.35[a]	58.26 ± 4.30[a]	873.50 ± 61.43[a]
	100	9.68 ± 1.85[a]	2.28 ± 1.92[a]	1240.33 ± 116.75[a]	23.20 ± 2.36[a]	130.46 ± 36.65[a]	162.43 ± 11.03[a]	6.58 ± 0.95[a]	50.41 ± 3.15[a]	750.23 ± 65.50[a]
	200	10.42 ± 1.25[a]	4.58 ± 1.53[a]	1087.45 ± 169.40[a]	30.51 ± 4.50[a]	92.48 ± 7.13[a]	173.81 ± 18.50[a]	5.45 ± 1.32[a]	55.66 ± 4.30[a]	810.17 ± 54.91[a]
	300	9.98 ± 1.90[a]	4.17 ± 1.74[a]	1138.20 ± 152.12[a]	26.03 ± 3.56[a]	99.38 ± 32.90[a]	140.93 ± 25.68[a]	6.53 ± 0.90[a]	51.10 ± 3.34[a]	1058.84 ± 70.62[a]
	400	8.85 ± 1.26[a]	2.94 ± 1.23[a]	1190.15 ± 110.86[a]	26.85 ± 7.90[a]	76.69 ± 36.76[a]	138.25 ± 43.69[a]	6.90 ± 0.89[a]	49.88 ± 5.50[a]	792.45 ± 55.17[a]
	500	10.71 ± 1.47[a]	3.83 ± 1.57[a]	1275.47 ± 150.64[a]	28.90 ± 11.5[a]	103.40 ± 42.87[a]	160.33 ± 15.20[a]	5.61 ± 1.35[a]	49.61 ± 2.46[a]	850.60 ± 62.33[a]
AA DSM 449 spores	**100**	10.81 ± 1.62[a]	3.14 ± 1.98[a]	1175.35 ± 152.02[a]	30.56 ± 6.50[a]	117.64 ± 42.78[a]	150.25 ± 18.02[a]	6.86 ± 0.97[a]	58.10 ± 2.80[a]	824.10 ± 70.37[a]
	200	10.59 ± 1.94[a]	3.03 ± 1.25[a]	1140.43 ± 131.15[a]	24.36 ± 11.31[a]	99.16 ± 5.20[a]	155.64 ± 22.37[a]	6.55 ± 0.83[a]	50.24 ± 4.33[a]	711.50 ± 62.15[a]
	300	9.84 ± 1.31[a]	2.76 ± 1.30[a]	1221.63 ± 165.04[a]	28.42 ± 8.53[a]	92.28 ± 39.15[a]	177.35 ± 20.41[a]	5.90 ± 0.93[a]	55.53 ± 6.82[a]	862.47 ± 58.40[a]
	400	10.15 ± 1.85[a]	3.55 ± 1.86[a]	1095.50 ± 150.16[a]	30.66 ± 11.24[a]	117.27 ± 45.36[a]	139.49 ± 11.58[a]	5.61 ± 0.90[a]	49.30 ± 5.24[a]	1015.62 ± 50.94[a]
	500	9.77 ± 1.51[a]	2.90 ± 1.55[a]	1157.45 ± 115.78[a]	37.58 ± 9.60[a]	95.18 ± 6.40[a]	151.20 ± 12.33[a]	6.38 ± 1.29[a]	50.55 ± 5.50[a]	841.75 ± 68.55[a]

① RBC count/L = N × 25/50 × 200× 10^6; WBC count/L = N/4 × 10× 20× 10^6; PLT count/L = N × 25/5× 10× 20× 10^6.
② Blood biochemical indexes such as ALT, AST, ALP, BUN, TP, and CK were analyzed by an automatic biochemical analyzer. Lowercase letters indicate significant differences according to Dunnett's test ($P < 0.05$).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2024.114840.

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