BRAZILIAN JOURNAL OF FOOD TECHNOLOGY

ISSN 1981-6723
Online version

ORIGINAL ARTICLE

Preclinical safety evaluation of a probiotic yogurt made with tumbo pulp (*Passiflora tripartita* Kunth)

*Avaliação da segurança pré-clínica de um iogurte probiótico feito com polpa de tumbo (*Passiflora tripartita *Kunth)*

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Cite as: Inocente Camones, M. A., Arias Arroyo, G. C., Flores López, O. B., Capcha Siccha, M. F., Bravo Araujo, G. T., & Zavaleta Ayala, J. J. (2024). Preclinical safety evaluation of a probiotic yogurt made with tumbo pulp (*Passiflora tripartita* Kunth). *Brazilian Journal of Food Technology*, *27*, e2022137. https://doi.org/10.1590/1981-6723.13722

Abstract

A probiotic yogurt made from the pulp of the tumbo fruit (*Passiflora tripartita* Kunth) possesses antioxidant capacity, physicochemical and microbiological quality and stability; however, it needs to be safe for later studies of clinical functionality. The present study would be considered as a precursor to the preclinical safety evaluation of a functional food by in vivo toxicological testing. The study aimed to evaluate the preclinical safety of yogurt made from the pulp of the tumbo fruit (*P. tripartita*). The toxicological studies proposed were based on the OECD 423:2001 standard on Acute Oral Toxicity, performing the peroral administration of the probiotic yogurt at a dose of 2000 mg/kg one-time in a group of randomized male rats, for its evaluation during 14 days compared to a control group; and on the OECD 407: 2008 on Repeated Dose Oral Toxicity, a daily dose of 2000 mg/kg of the yogurt was administered for 28 days in male rats, considering the evaluation of their clinical, hematological and biochemical parameters, to determine any possible toxic effect produced by the probiotic formulation. The results showed that in both toxicological studies, no adverse alterations were detected in their clinical signs, relative organ weights and hematological and biochemical profiles. In addition, a possible improvement in the immunological status, liver function and hematological profile was evidenced as a finding. In conclusion, it was possible to establish knowledge on the preclinical safety of the formulation of a probiotic yogurt made with tumbo fruit pulp, obtaining a NOAEL (No Observed Adverse Effect Level) value higher than 2000 mg/kg body weight in male rats.

Keywords: *Passiflora tripartita* Kunth; Probiotic yogurt; NOAEL; Preclinical toxicity; Hematological parameters; Biochemical parameters.

Resumo

Um iogurte probiótico feito com a polpa do fruto tumbo (*Passiflora tripartita* Kunth) apresentou capacidade antioxidante, qualidade físico-química e microbiológica, e estabilidade; entretanto, é necessário ser seguro para estudos posteriores de funcionalidade clínica. O presente estudo é considerado um precursor para a avaliação de segurança pré-clínica de um alimento funcional por meio de testes toxicológicos *in vivo*. O objetivo do estudo foi avaliar a segurança pré-clínica do iogurte feito com a polpa do fruto tumbo (*Passiflora tripartita* Kunth). Os estudos toxicológicos propostos foram baseados no padrão OECD 423:2001 sobre Toxicidade Oral Aguda, realizando uma única administração via oral do iogurte probiótico em uma dose de 2.000 mg/kg, em um grupo de ratos machos randomizados, para avaliação durante 14 dias, comparado a um grupo controle; e, no padrão OECD 407:2008 sobre Toxicidade Oral de Dose Repetida, administrando uma dose diária de 2.000 mg/kg do iogurte por 28 dias em ratos machos, considerando a avaliação de seus parâmetros clínicos, hematológicos e bioquímicos, para determinar qualquer possível efeito tóxico produzido pela formulação probiótica. Os resultados mostraram que, em ambos os estudos toxicológicos, não foram detectadas alterações adversas nos sinais clínicos, no peso relativo dos órgãos e nos perfis hematológicos e bioquímicos. Além disso, foi possível evidenciar uma possível melhora no status imunológico, na função hepática e no perfil hematológico. Em conclusão, foi possível estabelecer conhecimento sobre a segurança pré-clínica da formulação de um iogurte probiótico feito com a polpa do fruto tumbo, obtendo um valor de NOAEL (Nível Sem Efeito Adverso Observado) superior a 2.000 mg/kg de peso corporal em ratos machos.

Palavras-chave: *Passiflora tripartita* Kunth; Iogurte probiótico; NOAEL; Toxicidade pré-clínica; Parâmetros hematológicos; Parâmetros bioquímicos.

Highlights

- Preclinical safety of probiotic yogurt made with tumbo fruit pulp (*Passiflora tripartita* Kunth)

1 Introduction

According to the Food and Agriculture Organization of the United Nations (2006), probiotics in adequate concentrations have beneficial effects on health. Also, the use of probiotics is increasing daily and their consumption is implemented as adjuvant therapy in some pathologies such as the treatment of lactose malabsorption, infant colic, inflammatory bowel disease, necrotizing enterocolitis, against *Helycobacter pylori* (Castro et al., 2016), type 2 diabetes mellitus (Hu et al., 2017), metabolic syndrome (Rivero García & Monroy-Torres, 2022), dyslipidemias (González et al., 2020), periodontitis (Quintero-Rojas et al., 2022) and human mastitis (Aguilar et al., 2020).

Probiotics used as adjuvant treatment in the diseases described above, are achieving better prognosis of recovery in the patient's health (Hu et al., 2017; Rivero García & Monroy-Torres, 2022). Due to the consumption of nutrient-enriched probiotics, evaluating the minimal toxicity parameters is a priority to consolidate patient safety. Therefore, toxicity studies can be performed in animal models and subsequently extrapolated to humans considering qualitative and/or quantitative differences (Leenaars et al., 2019).

Also, food safety is important as part of public health standards, since it reduces the biological risks related to some possible toxic ingredients in food products. Concerning probiotics used in humans, the Food and Agriculture Organization (FAO) of the United Nations emphasizes that they must comply with safety profiles through safety protocols on their use as a food supplement (Food and Agriculture Organization of the United Nations, 2006).

Preclinical toxicological studies provide insight into the adverse effects of future administration of a drug or dietary supplement; similarly, functional foods should be evaluated before being consumed by the population (Vilas-Boas et al., 2021).

When this type of study is developed in animal models such as rodents, it facilitates the identification of severe, moderate or mild toxic damage with better feasibility and effectiveness in the results. Acute toxicological studies are related to the observation and evaluation of the alterations that may occur in less than 90 days, providing scientific evidence of the safety of the functional food and the changes it produces in the rodent's organism, being a fundamental part to achieve more reliable in vivo preclinical studies (Paladines-Santacruz et al., 2021). In addition, it is possible to test a wide range of doses in rodents, increasing the possibility of detecting adverse events.

For this reason, it was proposed to evaluate the toxicological risks at a preclinical level with a subsequent projection to a clinical study, of a probiotic developed based on the pulp of the fruits of tumbo (*P. tripartita*), whose previous results demonstrated its probiotic quality, It also meets the technical specifications of physicochemical and microbiological quality, and has antioxidant properties related to its chemical content such as flavonoids and carotenoids in the pulp of its fruits, whose plant grows in the inter-Andean valleys of Peru (Inocente-Camones et al., 2022).

2 Materials and methods

2.1 Materials for the production of probiotic yogurt

Raw cow's milk was obtained from a local dairy farm in the district of Lurin (Lima, Peru) during morning hours. The color, odor, flavor, and texture were analyzed to determine the quality of raw milk. In addition, the physicochemical characterization by evaluating pH, acidity, density and alcohol test was conducted (Inocente-Camones et al., 2022). Instant whole milk powder for milk standardization was purchased from a Peruvian dairy industry company. The commercial lactic cultures Nu-trish Lactobacillus casei 431 from Chr. Hansen (Denmark), Dri-Set yogurt 438 and Dri-Set Bioflora ABY 424 from Vivolac Cultures Corporation (USA) were used for milk inoculation. The fruits of tumbo (*P. tripartita*) were collected in Ancash (Peru), whose species was identified, and classified and a voucher specimen was deposited with the registration number USM 281623.

2.2 Elaboration and quality evaluation of probiotic yogurt with tumbo pulp

The preparation of the probiotic yogurt with tumbo pulp and the evaluation of its physicochemical and microbiological quality were carried out under the same procedures according to Inocente-Camones et al. (2022).

The tumbo fruits were washed and peeled, and the epicarp and mesocarp were removed. Then, it was distributed homogeneously in a polyethylene bag, vacuum sealed and stored at 3 °C before use.

Milk was pasteurized (90.0 °C, 10 minutes) cooled and filtered (48.0 ± 2.0 °C, 180 µm). Dehydrated sugarcane juice (panela) and instant whole milk powder were added, concentrated (85.0 ± 2.0 °C, 10 minutes), cooled and filtered (45.0 ± 2.0 °C, 180 µm). The lactic culture mixture was inoculated (1.0 µg/mL, constant agitation), and tumbo pulp (50.0 mg/mL) was added. It was incubated in a fermenter tank with a temperature regulator (42.0 ± 2.0 °C, 8 hours). It was churned, bottled and cooled to 3.0°C for physicochemical, microbiological and sensory analysis.

The yogurt made from the tumbo pulp was stored in glass containers with airtight lids, under refrigerated conditions (5.0 ± 1.0 °C), and samples were taken in a sterile environment to determine the physicochemical and microbiological quality (Inocente-Camones et al., 2022).

A sample of the prepared probiotic yogurt batch was used to evaluate the preclinical safety.

2.3 Conditioning of experimental animals

For the preclinical safety assessment of the probiotic yogurt, two *in vivo* toxicological tests were considered with albino rats: acute oral toxicity assessment (OECD, 2001; Hsu et al., 2021) and 28-day repeated dose toxicity assessment (OECD, 2008; Hsu et al., 2021).

Handling and experimentation with rats were performed according to the ethical standards for the care and use of experimental animals (Aller et al., 2000; Deutsche Forschungsgemeinschaft, 2019). The research protocol was previously approved by the Institutional Preclinical Research Ethics Committee of the Instituto de Investigación Traslacional y Biotransversal Ayru according to the provisions of Dictamen Nº CIEIP-014-2022.

A total of 24 male Holtzman rats of 9 to 10 weeks of age with a body weight of 210 to 300 grams were obtained from the Centro Nacional de Productos Biológicos del Instituto Nacional de Salud (INS - Bioterio, Chorrillos, Lima-Peru).

The rats were acclimatized in the Bioterio of the Instituto de Investigación Traslacional y Biotransversal Ayru (Comas, Lima, Peru), for 7 days in stainless steel cages of 55 x 30 x 30 cm with a metal base, with a maximum capacity of three rats per cage. They were kept at a temperature of 19 °C (± 5 °C) and humidity between 50 - 70% monitored with a digital LCD thermohygrometer (Boeco, Germany), sound levels below 70 decibels (dB) monitored with a sound level meter measuring 30 to 130 dB (UNI-T, UT35306) and 12-hour light/dark cycles. Rats received daily filtered distilled water and balanced feed ad libitum, and they were deprived of food 12 hours before the experiment and 3 hours after dosing.

2.4 Acute oral toxicity assessment

The study was conducted based on the OECD 423: 2001 Standard on Acute Oral Toxicity (Organisation for Economic Cooperation and Development, 2001; Hsu et al., 2021). Twelve male rats were randomized into two groups uniformly. Group 1 (control group) received filtered distilled water. Group 2 (experimental group) received the probiotic yogurt with the dose of 2000 mg/kg body weight because no toxic effect is expected due to the dietary nature of the probiotic yogurt.

Yogurt suspended in 1.5 mL of water was administered once perorally via an orogastric cannula and then the health status of the rats was observed twice daily for 14 days. Observation of each rat was frequent for the first 24 hours and continuous for the first 4 hours of each day. Changes in clinical appearance, behavior, body position, movements, reflexes, respiratory, and circulatory functions, and evaluation of fecal and urine color were documented daily. Body weight was evaluated daily and the weight before yogurt administration and the weights on days 7 and 14 of observation were considered for analysis.

At the end of the study, all rats were sacrificed by cervical dislocation. The liver and kidneys were removed and analyzed for their surface and consistency, considering any color change to detect possible damage, because it is considered the most affected depurative organ at the systemic level (Möller Bredo & Vásquez Odo, 2011).

2.5 Evaluation of oral toxicity by repeated dose for 28 days

The study was conducted based on the OECD 407: 2008 Standard on Repeated Dose Oral Toxicity for 28 days (Organisation for Economic Cooperation and Development, 2008; Hsu et al., 2021) and previous results obtained from acute oral toxicity assessment. Twelve rats were randomized into two groups uniformly. Group 1 (control group) received filtered distilled water. Group 2 (experimental group) received the probiotic yogurt with the dose of 2000 mg/kg body weight.

Yogurt suspended in 1.5 mL of water was administered daily perorally via an orogastric cannula for 28 days and then the health status of the rats was observed twice daily. The rats in the control and experimental groups were sacrificed 28 days after yogurt administration by cervical dislocation. The liver and kidneys were removed and analyzed for surface area and consistency, considering any color changes to detect possible organ damage.

2.5.1 Clinical signs and body weight

The animals were observed daily for signs of toxicity such as cyanosis, tremors, hypersalivation, piloerection, diarrhea, vomiting and death. Their body weight was monitored weekly from the initial dose until the end of the study. Body weight was measured using a digital balance accurate from 0.1 g to 5200 g (OHAUS, USA). The result was recorded in units of grams and the weight before administering the yogurt and the weights on days 7, 14 and 28 of observation were considered for the analyses.

2.5.2 Relative weight of organs

The heart, kidneys, liver and spleen were dried with filter paper before being weighed. The number that was recorded is the organ weight. The weight of the rats was then divided by the absolute weight to obtain the relative weight of the organs.

2.5.3 Necropsy and macroscopic examination of the liver and kidneys

The color, surface and consistency of the liver and kidneys of the rats in the experimental group were observed shortly after the rats were sacrificed for evidence of any abnormalities compared to the control group.

2.5.4 Hematological and biochemical analysis

After the sacrifice of the rats, blood was obtained for hematological and biochemical analysis. An automated hematology analyzer (Genrui KT6200) was used for hematological analyses: hematocrit, hemoglobin, red blood cells (RBC), white blood cells (WBC), platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), lymphocytes, neutrophils, monocytes, eosinophils, and basophils. An automated biochemical analyzer (Bioelab ES101C) was used for biochemical analyses: alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, globulin, total protein, bilirubin, creatinine, blood urea nitrogen (BUN), glucose, cholesterol and triglycerides.

2.6 Statistical analysis

IBM SPSS Statistic 26 (2021) software was used for the analysis. All experiments were performed in triplicate. Results were expressed as mean ± standard deviation. Data from body weight, organ weights, hematological analysis and biochemical analysis were analyzed using the t-test for independent samples. For those data that did not meet the assumptions of normality and homogeneity of variance, a nonparametric test (Mann-Whitney U test) was chosen to establish the significant difference ($p < 0.05$) between the experimental group and the control group.

3 Results and discussion

The use of probiotics as dietary supplements has increased due to their benefits such as having a high concentration of beneficial bacteria such as lactobacillus found as part of the intestinal flora, and they improve various pathologies in patients because they strengthen their immune system (Martínez et al., 2022; Xu et al., 2016). For example, probiotics improve the pathological condition of obesity, through the ability to reduce energy intake, and prevent weight gain and body fat (Rivero García & Monroy-Torres, 2022; Parra-Ruiz et al., 2019).

Alternative medicine considers the use of probiotics as an ally in the treatment of diabetes, obesity, digestive malabsorption, metabolic syndrome and dyslipidemias. Currently, the world population is opting for nutritional treatments to avoid the adverse effects of allopathic treatment (Magro Roque & Brandelli, 2021). However, studies with functional foods present only physicochemical and microbiological quality evaluations, being scarce the studies that evaluate preclinical toxicity in foods based on serum biochemical and hematological parameters of experimental animals, before clinical studies. Therefore, prior evaluations are necessary to detect any indication of toxicity and establish it as a background to the clinical study.

For this reason, to obtain a functional food product such as the probiotic formulation with tumbo pulp (*P. tripartita*) that has physicochemical and microbiological quality, as well as its evident antioxidant functional capacity due to the content of flavonoids and carotenoids (Inocente-Camones et al., 2022); the present study was conducted to establish its preclinical safety and then postulate new evaluations of its functional capacity at the preclinical and clinical level.

After administration of the yogurt by orogastric cannula in the acute oral toxicity study, each rat was observed daily for 14 days for clinical signs of toxicity and death. Clinical observation showed that consumption of the probiotic yogurt with tumbo pulp did not cause death, behavioral changes, or clinical signs of toxicity. Macroscopic examination of the liver and kidneys showed no abnormalities of color, surface and consistency, in the control and experimental groups. Figure 1 shows that the average body weights of male rats increased normally within the 14-day observation period. No significant difference was observed between the control and experimental groups.

For the acute oral toxicity study, the LD_{50} of probiotic yogurt is considered to be greater than 2000 mg/kg body weight.

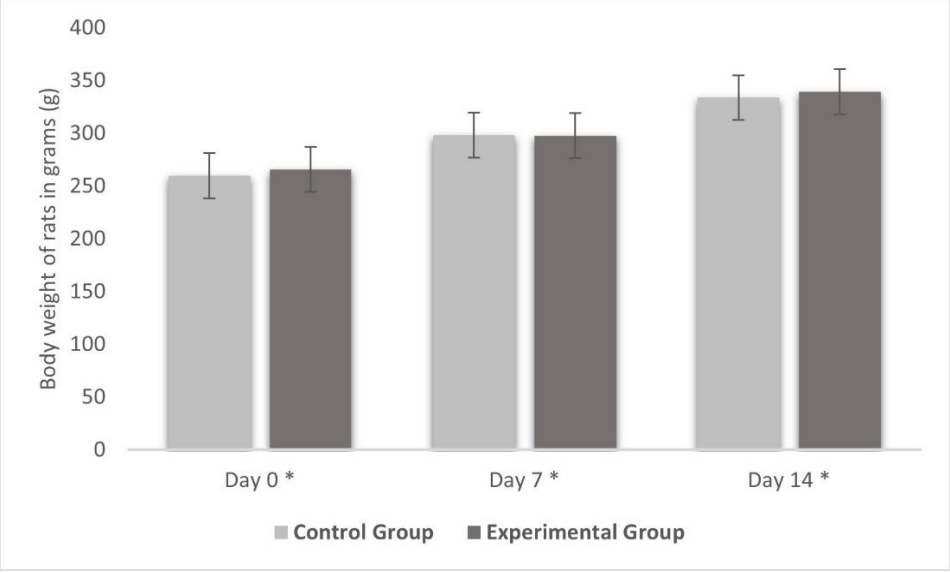

Figure 1. Effect of a single administration of tumbo probiotic yogurt on body weight of rats. (All values are expressed as mean value ± standard deviation. * The independent samples t-test was applied.)

In the 28-day repeated dose oral toxicity study, no signs of toxicity were observed due to the administration of the probiotic yogurt by orogastric cannula. There were no obvious signs of toxicity in the coat, eyes and mucous membranes, and no abnormal behaviors were observed in the control and experimental groups. Figure 2 shows that the average body weights of the male rats increased normally within the 28-day observation period. No significant difference was observed between the control group and the experimental group.

For the 28-day repeated dose oral toxicity study, the LD_{50} of the probiotic yogurt was considered to be greater than 2000 mg/kg body weight consumed daily for 28 days.

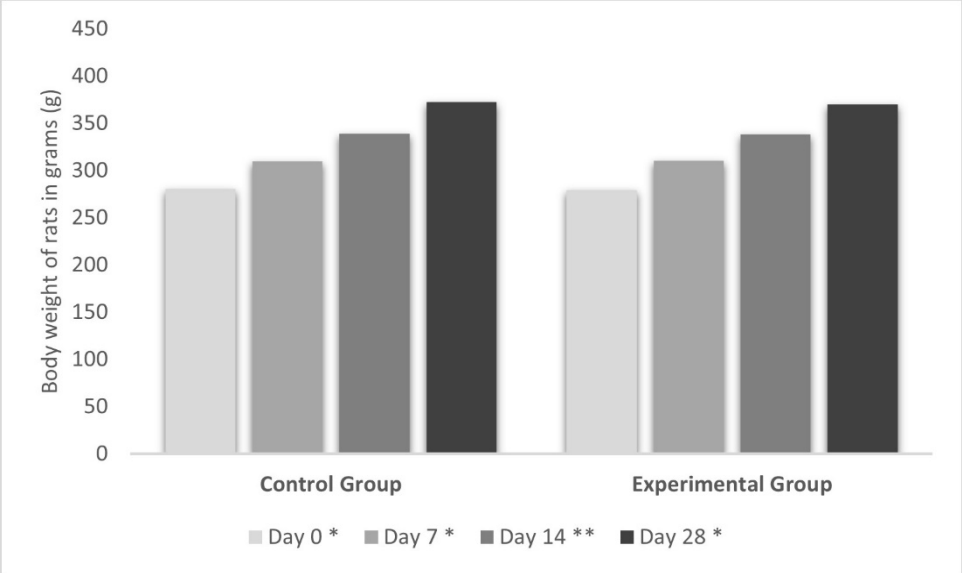

Figure 2. Effect of 28-day repeated dose administration of tumbo probiotic yogurt on body weight of rats. (All values are expressed as mean value ± standard deviation. * The independent samples t-test was applied; ** The Mann Whitney U-test was applied)

There were no statistically significant differences ($p > 0.05$) in the relative liver weight between the control and experimental groups presented in Table 1. There were no abnormalities in the macroscopic examination (color, consistency and surface) of the liver compared to the control group.

Table 1. Effect of repeated administration of probiotic tumbo yogurt on organ weights of rats.

Organ	Relative organ weight (g/100 g)	
	Control Group (n = 6; 0 mg/kg)	Experimental Group (n = 6; 2000 mg/kg)
Heart*	0.396 ± 0.014	0.401 ± 0.009
Kidney**	0.885 ± 0.026	0.909 ± 0.012
Liver*	3.611 ± 0.207	3.599 ± 0.118
Spleen*	0.204 ± 0.006	0.196 ± 0.005[a]

All values are expressed as mean value ± standard deviation. * Independent samples t-test was applied; ** Mann Whitney U-test was applied; [a] indicates significant difference ($p < 0.05$).

The effect of the administration of the probiotic yogurt on the weight of the animal models and their most important organs (heart, kidney, liver) of the experimental group with the control group, did not register significant differences. The spleen was the organ that evidenced a significant difference, considering slightly decreased values in size in the experimental group, which could generate new specific experimental studies to demonstrate decrease in spleen size (Silva-Maia et al., 2019).

The variations of hematological and biochemical serum parameters evidenced in animal models by continuous administration of probiotic yogurt provide valid information to determine its toxicity (Silva-Santana et al., 2020).

Table 2 shows that the hematological profiles between the control group and the experimental group present statistically significant differences ($p > 0.05$) in the values of hematocrit, hemoglobin, RBC, platelets, MCV, MCH, MCHC and neutrophils.

Table 2. Effect of repeated administration of probiotic yogurt on hematological parameters in rats.

Hematological parameter	Control Group (n = 6; 0 mg/kg)	Experimental Group (n = 6; 2000 mg/kg)
WBC (10^3/µL) **	13.88 ± 0.67	13.01 ± 0.52
RBC (10^6/ µL) *	8.96 ± 0.15	8.67 ± 0.12[a]
Hemoglobin (g/dL) *	16.89 ± 0.09	17.03 ± 0.07[a]
Hematocrit (%) *	51.32 ± 0.72	53.53 ± 0.42[a]
MCV (fL) *	56.72 ± 0.62	58.77 ± 0.54[a]
MCH (pg) *	19.35 ± 0.21	19.66 ± 0.24[a]
MCHC (g/dL) *	33.62 ± 0.22	32.94 ± 0.18[a]
Platelets (10^3/µL) *	353.83 ± 24.23	368.17 ± 25.62
Neutrophils (%) *	13.78 ± 0.41	14.51 ± 0.64[a]
Lymphocytes (%) *	80.78 ± 0.40	80.13 ± 0.69
Monocytes (%) *	4.31 ± 0.16	4.28 ± 0.07
Eosinophils (%) *	0.80 ± 0.05	0.78 ± 0.05
Basophils (%) *	0.34 ± 0.08	0.30 ± 0.05

WBC: white blood cells; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration. All values are expressed as mean value ± standard deviation. * Independent samples t-test was applied; ** Mann Whitney U test was applied; [a] indicates significant difference ($p < 0.05$)

The hematological serum parameters presented normal indices in both groups compared to the reference values according to Delwatta et al. (2018). With respect to lymphocyte, monocyte, eosinophil, basophil and white blood cell counts, a slight decrease was denoted in the experimental group, suggesting an improvement in the immune status, since their increase would be related to possible inflammatory or infectious events (Xu et al., 2013). A slight increase in platelets was denoted in the experimental group, which could be related to an improvement in the immune system. Likewise, hemoglobin, hematocrit, MCV and MCH showed an increase in their serum values, which could be related to better hematological profiles for an adequate nutritional status (Zakrzewska et al., 2022).

Table 3 shows that the biochemical parameters between the control group and the experimental group presented statistically significant differences ($p > 0.05$) in the values of ALP, AST, ALT, globulin, glucose, cholesterol and triglycerides.

Table 3. Effect of repeated administration of probiotic yogurt for 28 days on biochemical parameters in rats.

Biochemical parameter	Control Group (n = 6; 0 mg/kg)	Experimental Group (n = 6; 2000 mg/kg)
ALP (U/L) *	193.83 ± 20.72	153.21 ± 21.40[a]
AST (U/L) *	78.90 ± 12.97	58.55 ± 7.39[a]
ALT (U/L) *	40.73 ± 6.75	23.26 ± 4.95[a]
Albumin (g/dL) *	4.57 ± 0.71	4.28 ± 0.66
Globulin (g/dL) **	2.92 ± 0.14	2.55 ± 0.35[a]
Total proteins (g/dL) *	6.86 ± 0.34	6.95 ± 0.29
Total bilirubin (µg/dL) *	0.027 ± 0.016	0.033 ± 0.021
Creatinine (mg/dL) *	0.60 ± 0.07	0.63 ± 0.10
BUN (mg/dL) *	14.74 ± 0.57	14.79 ± 0.59
Glucose (mmol/L) **	5.94 ± 0.85	4.50 ± 0.41[a]
Cholesterol (mmol/L) *	1.81 ± 0.25	1.46 ± 0.26[a]
Triglycerides (mmol/L) *	1.12 ± 0.11	0.97 ± 0.08[a]

ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen. All values are expressed as mean value ± standard deviation. * Independent samples t-test was applied; ** Mann Whitney U-test was applied; [a] indicates significant difference ($p < 0.05$).

The biochemical serum parameters presented normal indexes in both groups compared to the reference values according to Goñi et al. (2011). The parameters that evaluate correct liver function, such as albumin, total protein, total bilirubin, creatinine, BUN and globulin, showed normal values. Likewise, the values of ALP, AST and ALT of the experimental group presented a decrease in serum levels, thus favoring the correct hepatic function; since the increase in their values would be related to possible damage at the liver level (Tang et al., 2019).

The values of glucose, cholesterol and triglycerides of the experimental group are lower than the values of the control group, which would indicate an improvement in nutritional status, whose values are similar to the study of Figueroa et al. (2013). The result could be due to the presence of *P. tripartita* fruits as reported by Coral Caycho et al. (2020), seeing that this fruit decreases glucose, cholesterol and triglyceride levels when exposed to *in vivo* and *in vitro* studies (Franco et al., 2014).

4 Conclusion

It was possible to establish knowledge on the preclinical safety of the formulation of a probiotic yogurt made with tumbo pulp (*P. tripartita*), obtaining a NOAEL (No Observed Adverse Effect Level) value higher than 2000 mg/kg body weight in male rats. The administration is safe in animal models, through the evaluation of biochemical and hematological profiles, according to tests of acute oral toxicity and toxicity at repeated doses for 28 days, making the probiotic yogurt safe and innocuous for further preclinical and clinical functionality studies.

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Funding: Carrera Profesional de Farmacia y Bioquímica de la Universidad Científica del Sur; Consejo Nacional de Ciencia, Tecnología (145-2018-FONDECYT-BM-IADT-MU); Inca Garcilaso de la Vega University; Innovación Tecnológica, Subsidized Project (145-2018-FONDECYT-BM-IADT-MU); Instituto de Investigación Traslacional y Biotransversal Ayru (017-FONAYRU)

Received: Nov. 16, 2022; Accepted: Nov. 16, 2023

Associate Editor: Airton Vialta.