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Physicochemical characterization and OECD-guided toxicological evaluation of a beer extract supplemented with *Monascus ruber*-fermented Rice

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ABSTRACT

This study investigated the physicochemical characteristics and performed an initial toxicological evaluation of a freeze-dried beer extract supplemented with *Monascus ruber*-fermented rice (BEMR). Two Pilsner-style beers were produced and characterized according to European Brewery Convention (EBC) analytical methods, including pH, density, bitterness, and color measurements (SRM and CIELAB). The freeze-dried extract was subsequently evaluated in Wistar rats using acute (OECD Test Guideline 423) and repeated-dose 28-day oral toxicity (OECD Test Guideline 407) protocols. No mortality, treatment-related clinical signs, or gross organ abnormalities were observed following acute administration up to 2000 mg/kg or repeated oral administration up to 1000 mg/kg/day. Hepatic, renal, and hematological parameters remained largely within physiological ranges throughout the study. Treated animals exhibited reduced body-weight gain without significant changes in feed or water intake. Modest increases in serum creatinine and reductions in creatine kinase activity were observed in the absence of corresponding clinical or pathological findings. Physicochemical analyses confirmed that incorporation of *Monascus*-fermented rice markedly modified beer color while maintaining the principal brewing parameters within acceptable ranges for the beer style. Under the experimental conditions evaluated, BEMR was well tolerated in both acute and repeated-dose toxicity screening assays. The biological significance of the observed physiological responses remains to be clarified through additional studies incorporating targeted chemical characterization (*e.g.*, citrinin and monacolin contents), histopathological evaluation, and longer-term repeated-dose investigations.

1. Introduction

Fermented beverages represent an important category of foods in which microbial activity contributes not only to preservation and sensory attributes, but also to the generation of bioactive compounds. Among these beverages, beer stands out as a complex fermented matrix composed of water, malted cereals, hops, yeast, and, in some formulations, adjunct raw materials and technological additives, according to national regulatory frameworks (Alves, 2014). Beyond its sensory relevance, beer has increasingly been investigated as a source of fermentation-derived compounds whose composition depends on raw materials, processing conditions, and fermentation control.

From a biochemical perspective, beer contains a variety of compounds originating from cereals, hops, and Maillard reactions, including

Abbreviations: a*, red/green color coordinate; ABV, alcohol by volume; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BEMR, freeze-dried beer extract supplemented with *Monascus ruber*-fermented rice; C*, chroma; CG, control group; CK, creatine kinase; CNS, central nervous system; EBC, European Brewery Convention; EFSA, European Food Safety Authority; GHS, Globally Harmonized System; γ-GT, gamma-glutamyl transpeptidase; H°, hue angle; IBU, International Bitterness Units; L*, lightness; LD50, median lethal dose; OECD, Organisation for Economic Co-operation and Development; RBC, red blood cells; SG, satellite group; SRM, Standard Reference Method; WBC, white blood cells..

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polyphenols, melanoidins, peptides, and vitamins, which have been associated with antioxidant activity and other biological effects (Koren et al., 2019; Martínez-Gomez et al., 2020; Šibalić et al., 2021). The concentration and chemical profile of these constituents are highly sensitive to formulation choices and technological steps during brewing, such as mashing conditions, fermentation parameters, and post-fermentation treatments (Koren et al., 2020). As a result, modifications in the brewing process or the incorporation of additional fermented ingredients can significantly alter the physicochemical characteristics of the final product.

In this context, filamentous fungi of the genus *Monascus* have attracted increasing attention due to their ability to produce a diverse range of secondary metabolites with technological and biological relevance, including pigments, γ-aminobutyric acid, sterols, enzymes, and monacolins (Shao et al., 2014). Monacolin K, a natural inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, is structurally identical to lovastatin and has been widely studied for its effects on lipid metabolism (Penson & Banach, 2021). In addition, *Monascus*-derived pigments have been applied as natural colorants and have been reported to exhibit antioxidant and antiproliferative activities in experimental models (Monu et al., 2022; Oliveira et al., 2022). These characteristics have contributed to the growing interest in *Monascus* fermentation as a strategy for developing functional food ingredients.

Red yeast rice, obtained through the fermentation of rice by *Monascus* species, is a traditional product with a long history of dietary use in several Asian countries (Lin et al., 2023; Sham et al., 2014). Beyond its traditional applications, *Monascus*-fermented rice and its derivatives have been incorporated into different food matrices, including alcoholic beverages, to modify color and technological properties (Sham et al., 2014). More recently, experimental studies have suggested possible effects on metabolic homeostasis and gut microbiota composition, although these responses appear to depend on the composition of the fermented product and the experimental model adopted (Yang et al., 2025). Nevertheless, the biological effects of these products are highly dependent on their chemical composition and the matrix in which they are delivered. Because the incorporation of Monascus-fermented ingredients into a beer matrix involves additional processing steps that may influence the stability, interactions, and bioavailability of fermentation-derived metabolites, toxicological evaluation of the final product represents an important component of its overall safety assessment.

Despite the technological and functional interest in *Monascus*-derived ingredients, their application in foods raises important toxicological and regulatory considerations. The main toxicological concern associated with *Monascus* fermentation is the possible co-production of citrinin, a nephrotoxic mycotoxin, together with pigments and other metabolites (Kamle et al., 2022; Shi et al., 2022). Although advances in strain selection, substrate optimization, and fermentation control have demonstrated that citrinin production can be substantially reduced or suppressed (Egea et al., 2023; Kang et al., 2014; Marič et al., 2019; Su et al., 2003; Vendruscolo et al., 2016), regulatory acceptance of *Monascus*-based ingredients remains heterogeneous worldwide. In Brazil, for instance, the use of *Monascus* species in food products is still restricted, highlighting the need for robust toxicological data to support future regulatory evaluation.

Importantly, while numerous studies have investigated the chemical composition and biological effects of red yeast rice and related products, there is a limited number of *in vivo* toxicological screening studies addressing the safety of *Monascus*-fermented ingredients when incorporated into complex food matrices such as beer. Likewise, studies integrating physicochemical characterization with standardized OECD-based toxicological screening remain limited. Addressing this gap may contribute to assessing the feasibility and biological tolerability of incorporating *Monascus*-derived ingredients into complex fermented beverage matrices.

Therefore, the present study aimed to characterize a freeze-dried beer extract supplemented with rice fermented by *Monascus ruber* and conduct an initial *in vivo* toxicological evaluation following internationally recognized OECD guidelines. The product was characterized by standard physicochemical and colorimetric analyses based on European Brewery Convention (EBC) methods, and its toxicological profile was evaluated through acute and subacute oral toxicity assays in Wistar rats. By integrating physicochemical characterization with biological endpoints, this work provides preliminary *in vivo* toxicological evidence to support future investigations on the incorporation of *Monascus*-derived ingredients into fermented beverage formulations.

2. Material and methods

2.1. Beer production

Two beer formulations were prepared: one supplemented with rice fermented with *Monascus ruber* CCT 3802 and a control beer without rice addition. Both beers were brewed according to conventional brewing specifications described for German Pilsner-style beers, including an original gravity of 1.044–1.050 g/mL (~11–12.5°Plato), final gravity of 1.008–1.013 g/mL, bitterness of 22–40 IBU, alcohol content of 4.4–5.2% (*v*/v), and color of 2–5 SRM, in accordance with standard brewing references (Briggs et al., 2004; European Brewery Convention, 2010; Kunze, 2014).

The beer supplemented with *Monascus*-fermented rice was produced using 4.95 kg of Agrária Pilsen malt (Ponta Grossa, PR, Brazil), 0.55 kg of *Monascus ruber*-fermented rice, and 15 g of Nugget hops (Barth Haas, 2019 harvest, USA; 15% α-acids), dissolved in 20 L of mineral water (Nativa, Goiânia, GO, Brazil). The control beer contained 5.5 kg of Agrária Pilsen malt and the same hop and water composition. The fermented rice was incorporated as a brewing adjunct during wort production prior to wort boiling. After boiling and cooling, alcoholic fermentation was initiated by inoculating the wort with *Saccharomyces cerevisiae* W 34/70.

The grist was milled using a Meurer Brew roller mill and mashed with water at 53 °C. The mashing schedule consisted of 20 min at 50 °C (proteolytic rest), 40 min at 62 °C (β-amylase activity), 20 min at 67 °C (α-amylase activity), and 15 min at 80 °C (mash out). At the end of mashing, the wort was separated by filtration, boiled for 60 min, whirlpooled, cooled, and transferred to a conical fermenter.

Alcoholic fermentation was performed using *Saccharomyces cerevisiae* W 34/70 (Fermentis, France) at an initial concentration of 2×10^6 cells/mL. Fermentation was conducted at 10 ± 2 °C for 5 days, followed by 12 ± 2 °C for 2 days, and maturation at 0 ± 1 °C for approximately 20 days. The beers were bottled in sanitized 600 mL glass bottles and carbonated by the addition of 5 g/L sucrose. Bottles were stored at 10 ± 2 °C until carbonation was achieved and subsequently maintained at 5 °C until physicochemical analyses and sample preparation.

2.2. Analytical determinations

All physicochemical analyses were performed according to the standardized methods of the European Brewery Convention (European Brewery Convention, 2010). Prior to analysis, beer samples (600 mL) were degassed and vacuum-filtered through Whatman No. 1 filter paper at 20 °C to remove suspended solids.

Alcohol content (% *v*/v) was determined by distillation (EBC method 9.2.1), and apparent density at 20 °C was measured using a calibrated density meter (EBC method 9.4). Beer color was determined spectrophotometrically according to EBC method 9.6 and converted to Standard Reference Method (SRM) units. Bitterness was quantified as International Bitterness Units (IBU) following EBC method 9.8, and pH was measured using a calibrated pH meter according to EBC method 9.35.

Beer color was further characterized using the CIELAB color space.

The parameters L* (lightness), a* (red–green coordinate), and b* (yellow–blue coordinate) were determined under total transmittance conditions using a Color Quest® XE colorimeter (HunterLab, USA). Chroma (C*) and hue angle (H°) were calculated from the measured a* and b* values using standard equations.

All analytical determinations were performed in sextuplicate ($n = 6$), and results were expressed as mean ± standard deviation.

2.3. Preparation of the freeze-dried beer extract

The two beer formulations were freeze-dried using an SP Scientific Genesis SQ Super XL-70 freeze-dryer to remove ethanol and concentrate non-volatile constituents. The beers were refrigerated at 6 ± 2 °C, transferred into 100-mL glass flasks (filled to approximately 75% of their capacity), and sealed with perforated PVC film (six 0.7-mm perforations). Freeze-drying was carried out at 0 °C and 760 Torr, with the condenser maintained at −80 °C. Freeze-drying of the production batch yielded approximately 4.0% dry extract. The resulting product, designated BEMR (freeze-dried beer extract supplemented with *Monascus ruber*-fermented rice), was used as the test material in all subsequent *in vivo* experiments.

2.4. Animals and ethical approval

The experimental protocol was approved by the Ethics Committee for the Use of Animals of the Federal University of Goiás (CEUA/UFG; Protocol No. 17/17). All animal procedures were conducted in accordance with Brazilian federal legislation (Law No. 11,794/2008), the ethical principles established by the Brazilian College of Animal Experimentation (Colégio Brasileiro de Experimentação Animal (COBEA), 2003), and internationally recognized guidelines for the care and use of laboratory animals, including the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Sixty-day-old female Wistar rats (*Rattus norvegicus*), weighing approximately 200 g, were obtained from the Central Animal Facility of the Federal University of Goiás. Animals were housed under controlled laboratory conditions (24 ± 2 °C, 12-h light/dark cycle, controlled humidity, and ventilated cages) with free access to standard commercial feed and water. A seven-day acclimation period was allowed before the start of the experimental procedures.

Group sizes were established according to the recommendations of OECD Test Guidelines 423 and 407 for acute and repeated-dose oral toxicity studies, respectively, while adhering to the principles of reduction and refinement in animal experimentation.

2.5. Acute oral toxicity

Acute oral toxicity was evaluated in accordance with OECD Test Guideline 423 (OECD, 2002a), which describes the Acute Toxic Class Method for the identification of potential adverse effects following single-dose oral exposure. Sequential doses of 5, 50, 300, and 2000 mg/kg body weight of the freeze-dried beer extract supplemented with *Monascus ruber*-fermented rice (BEMR) were administered orally to groups of three female Wistar rats, with progression to the subsequent dose level determined according to the mortality criteria established by the guideline.

The acute toxicity assay consisted of a control group receiving filtered water (10 mL/kg body weight) and a treatment group receiving BEMR at a dose of 2000 mg/kg body weight by oral gavage. Animals were observed individually during the first 30 min after administration, periodically during the first 24 h, and daily thereafter for a total observation period of 14 days, following the recommendations of OECD Test Guideline 423 (OECD, 2002a).

Clinical and behavioral observations were systematically performed using a standardized Hippocratic screening protocol, including assessment of general activity, posture, gait, reflex responses, motor coordination, tremors, convulsions, autonomic signs (lacrimation, salivation, urination, defecation, and piloerection), and thermoregulation. Clinical observations were recorded at 15 min, 30 min, 1 h, 2 h, 4 h, and 8 h after administration and subsequently once daily throughout the observation period. All observations were performed by the same investigator to ensure consistency in behavioral assessment.

Body weight was recorded periodically during the study. Feed and water consumption, as well as fecal and urinary output, were monitored daily throughout the experimental period. At the end of the study, animals were anesthetized with a ketamine/xylazine combination (0.2 mL/100 g body weight), euthanized by cervical dislocation, and subjected to a complete gross pathological examination. Major organs were examined during the gross pathological examination for treatment-related abnormalities in accordance with OECD Test Guideline 423 (OECD, 2002a).

2.6. Subacute oral toxicity

Subacute oral toxicity was evaluated in accordance with OECD Test Guideline 407 (OECD, 2025), which describes repeated-dose oral toxicity studies conducted over a 28-day exposure period. Female Wistar rats were randomly allocated into five experimental groups ($n = 6$ per group): a control group receiving filtered water (10 mL/kg body weight), three treatment groups receiving the freeze-dried beer extract supplemented with Monascus ruber-fermented rice (BEMR) at doses of 250, 500, or 1000 mg/kg body weight, and a satellite group receiving 1000 mg/kg/day for 28 consecutive days, followed by a 14-day treatment-free recovery period to evaluate the reversibility or persistence of any treatment-related effects.

Dose levels were selected in accordance with the OECD Test Guideline 407 (OECD, 2025), taking into account the exploratory nature of this repeated-dose toxicity study and the absence of treatment-related mortality or severe clinical signs in the preceding acute oral toxicity study (OECD Test Guideline 423). The selected dose range (250, 500, and 1000 mg/kg body weight/day) was intended to provide a graded assessment of potential toxicological effects over the 28-day exposure period. BEMR or vehicle was administered once daily by oral gavage for 28 consecutive days.

Animals were monitored daily for clinical signs and behavioral alterations using the same standardized Hippocratic screening protocol adopted for the acute toxicity study. Clinical observations were performed by the same investigator throughout the experimental period to ensure consistency in behavioral assessment. Body weight, feed consumption, water intake, and general clinical condition were monitored throughout the study. Clinical examinations were performed before the first administration to establish baseline health status.

At the end of the 28-day treatment period, animals were anesthetized with a ketamine/xylazine combination, and blood samples were collected by cardiac puncture for hematological and biochemical analyses. Animals were subsequently euthanized by cervical dislocation performed by a licensed veterinarian. A complete gross pathological examination, including evaluation of the liver, kidneys, heart, lungs, and stomach, was performed according to the recommendations of OECD Test Guideline 407 (OECD, 2025).

The satellite group remained untreated for an additional 14-day recovery period to evaluate the reversibility, persistence, or delayed onset of treatment-related effects.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) to compare treatment groups. When significant differences were detected, Tukey's multiple comparison test was applied to evaluate differences among experimental groups, and Dunnett's test was used for comparisons between each treated group and the control group, as

appropriate. Normality and homogeneity of variance were verified prior to ANOVA. Statistical significance was established at $p < 0.05$.

3. Results and discussion

3.1. Physicochemical characterization of beers

The physicochemical characteristics of the experimental beers are summarized in Table 1.

The beer supplemented with *Monascus*-fermented rice exhibited a significantly lower pH than the control beer (4.33 ± 0.03 *vs.* 4.44 ± 0.04, $p < 0.05$), although both values remained within the typical range reported for Pilsner-style lagers (3.8–4.7). This pH interval is commonly associated with appropriate microbial and colloidal stability in bottom-fermented beers (Briggs et al., 2004; European Brewery Convention, 2010; Koren et al., 2019). The slight reduction in pH may be associated with organic acids generated during the fermentation of rice by *Monascus spp.*, as previously reported (Shao et al., 2014; Su et al., 2003).

Titratable acidity was significantly higher in the *Monascus* beer (0.396 ± 0.002 g/100 mL) than in the control (0.320 ± 0.006 g/100 mL). Similar increases have been reported for fermented beverages enriched with microbial metabolites and are generally attributed to modifications in the organic acid profile of the final product rather than to deviations from expected brewing quality parameters (Martínez-Gomez et al., 2020; Merinas-Amo et al., 2016).

Final apparent density values (1.013–1.014 g/mL) were consistent with those expected for Pilsner-style beers (Briggs et al., 2004; Kunze, 2014), indicating satisfactory fermentation performance. The slightly higher density observed in the *Monascus* beer (1.014 ± 0.00003 g/mL) may be attributable to residual non-fermentable carbohydrates or polysaccharide fractions derived from the fermented rice adjunct, as reported for other *Monascus*-based fermented products (Lin et al., 2023).

Alcohol content reached 4.74% (*v*/v) in the *Monascus* beer and 4.14% (v/v) in the control beer, remaining within the range commonly reported for Pilsner-style lagers (4.4–5.2% v/v; Briggs et al., 2004; Kunze, 2014). The difference between formulations may be associated with differences in fermentable sugar availability resulting from the incorporation of pre-fermented rice during wort production (Lin et al., 2023). However, the present study was not designed to investigate the mechanisms underlying this difference.

Bitterness values were slightly lower in the *Monascus beer* (17.85 ± 0.20 IBU) than in the control (19.38 ± 0.23 IBU), although both formulations exhibited bitterness values slightly below the typical range reported for the style (22–40 IBU; European Brewery Convention, 2010; Kunze, 2014). This difference may reflect interactions between fermentation-derived metabolites and hop isoα-acids that influence bitterness perception or retention during brewing and fermentation (Vendruscolo et al., 2016). Nevertheless, both formulations remained compatible with the sensory profile expected for lightly bitter lager-style beers.

Beer color differed substantially between formulations (Table 1). The beer supplemented with *Monascus*-fermented rice presented a significantly higher color value (11.70 ± 0.02 SRM) than the control beer (5.01 ± 0.02 SRM), reflecting the incorporation of pigments produced during *Monascus* fermentation. These findings are compatible with the presence of azaphilone pigments, which have been widely described as characteristic secondary metabolites of *Monascus spp.* and are responsible for the yellow, orange, and red coloration observed in fermented food products (Shao et al., 2014; Vendruscolo et al., 2016).

To further characterize these chromatic differences, CIELAB color parameters were determined (Table 2). Compared with the control beer, the *Monascus* beer exhibited lower lightness (L*), higher redness (a*), higher yellowness (b*), and greater chroma (C*), indicating a darker and more saturated color profile. Hue angle values (H° = 78.56–89.98°) were consistent with yellow-to-orange color coordinates. The CIELAB system is widely employed in beer quality evaluation because it provides greater sensitivity to color variation than conventional SRM measurements (Koren et al., 2020; Martínez-Gomez et al., 2020). These observations are consistent with previous reports describing beers and other fermented products containing *Monascus*-derived pigments (Shi et al., 2022; Vendruscolo et al., 2016) and confirm that the incorporation of *Monascus*-fermented rice substantially modified the chromatic profile of the final beer.

3.2. In vivo safety evaluation — acute oral exposure

The administration of the freeze-dried beer extract supplemented with *Monascus ruber*-fermented rice (BEMR) at a dose of 2000 mg/kg body weight did not result in mortality or treatment-related clinical signs during the 14-day observation period, according to OECD Test Guideline 423 (OECD, 2002a). No alterations in body weight progression, general behavior, posture, locomotor activity, or clinical appearance were observed throughout the study, indicating no evidence of overt acute toxicity under the experimental conditions evaluated. These findings are consistent with previous studies reporting low acute oral toxicity of *Monascus*-fermented products and related extracts in rodent models (Lee et al., 2010; Mohan-Kumari et al., 2009).

The toxicological profile of *Monascus*-derived products has been associated with the occurrence of secondary metabolites whose production depends on fungal strain, substrate composition, and fermentation conditions. Among these metabolites, citrinin has received particular attention because of its recognized nephrotoxic potential (Marič et al., 2019; Shi et al., 2022; Vendruscolo et al., 2016). Although citrinin was not analytically quantified in the present study, the absence of mortality, treatment-related clinical signs, or gross pathological findings is consistent with the overall *in vivo* tolerability of the tested formulation. Accordingly, the present findings should be interpreted as an *in vivo* toxicological evaluation of the final freeze-dried beer formulation rather than as evidence regarding the presence or absence of specific fermentation-derived metabolites.

Final body weight and organ weight data are presented in Table 3. No statistically significant differences were observed between treated and control animals. Mean final body weights were 208.3 ± 3.5 g in the BEMR-treated group and 204.7 ± 4.0 g in the control group. Likewise, no significant differences were detected in the absolute or relative weights of the liver and kidneys, indicating no evidence of treatment-

Table 1
Physicochemical parameters of beers produced with and without addition of *Monascus ruber*-fermented rice.

Property	Control Beer	*Monascus Beer*
pH	4.44 ± 0.04^{a}	4.33 ± 0.07^{b}
Total titratable acidity (g/100 mL)	0.320 ± 0.006^{b}	0.396 ± 0.002^{a}
Final density (g/mL)	1.013 ± 0.00002^{b}	1.014 ± 0.00003^{a}
Alcohol (% v/v)	4.14 ± 0.04^{b}	4.74 ± 0.07^{a}
Bitterness (IBU)	19.38 ± 0.23^{a}	17.85 ± 0.20^{b}
Color (SRM)	5.01 ± 0.02^{b}	11.70 ± 0.02^{a}

Values are expressed as mean ± SD (n = 6). Means followed by the same letter in a row do not differ significantly (Tukey's test, $p < 0.05$).

Table 2
CIELAB color parameters of beers produced with and without addition of *Monascus ruber*-fermented rice.

Sample	L*	a*	b*	C*[1]	H°[2]	Color
Control beer	64.25 ± 0.09^{a}	0.01 ± 0.001^{b}	38.99 ± 0.02^{b}	38.99 ± 0.02^{b}	89.98 ± 0.01^{a}	Copper
Monascus beer	60.13 ± 0.44^{b}	19.74 ± 0.040^{a}	97.59 ± 0.50^{a}	99.57 ± 0.48^{a}	78.56 ± 0.07^{b}	Dark brown

Values are expressed as mean ± SD (n = 6). Means followed by the same letter in a row do not differ significantly (Tukey's test, $p < 0.05$). C* (chroma) = $\sqrt{(a^2 + b^2)}$; H° (hue angle) = $\tan^{-1}(b^*/a^*)$.

Table 3
Final body weight and absolute and relative liver and kidney weights of Wistar rats treated with water (control) or a single oral dose of 2000 mg/kg BEMR.

Group	Final Body Weight (g)	Liver		Right kidney		Left kidney	
		Absolute Weight (g)	Relative Weight (g/100 g)	Absolute Weight (g)	Relative Weight (g/100 g)	Absolute Weight (g)	Relative Weight (g/100 g)
Control	204.67 ± 4.04[a]	7.80 ± 0.52[a]	3.82 ± 0.32[a]	0.84 ± 0.07[a]	0.41 ± 0.04[a]	0.83 ± 0.11[a]	0.41 ± 0.06[a]
BEMR	208.33 ± 3.51[a]	7.84 ± 0.13[a]	3.76 ± 0.12[a]	0.87 ± 0.05[a]	0.42 ± 0.03[a]	0.78 ± 0.08[a]	0.37 ± 0.03[a]

Values are expressed as mean ± SD (n = 6). Means followed by the same letter in a row do not differ significantly (Tukey's test, $p < 0.05$). BEMR = freeze-dried beer extract supplemented with Monascus ruber-fermented rice (2000 mg/kg, p.o.).

related macroscopic effects on these major detoxification or excretory organs.

Neurobehavioral assessment using the Hippocratic screening protocol (Table 4) revealed no treatment-related abnormalities following BEMR administration. Animals from both groups exhibited only mild and transient agitation during the first 15 min after gavage, which resolved spontaneously. All remaining clinical parameters, including level of consciousness, reflex responses, motor coordination, muscle tone, central nervous system activity (*e.g.*, tremors and convulsions), and autonomic signs (lacrimation, salivation, respiration, urination, defecation, and piloerection), remained within physiological ranges throughout the observation period.

According to OECD Test Guideline 423 (OECD, 2002a), the absence of mortality following oral administration at 2000 mg/kg body weight allows BEMR to be classified within GHS Category 5 ($LD_{50} > 2000$ mg/kg), indicating low acute oral toxicity under the experimental conditions evaluated. This classification is consistent with previous reports describing low acute toxicity for *Monascus*-derived products produced under controlled fermentation conditions (Lee et al., 2010; Mohan-Kumari et al., 2009).

3.3. Subacute safety assessment

Body weight progression during the 28-day repeated-dose study is presented in Table 5 and Fig. 1. Animals receiving the freeze-dried beer extract supplemented with *Monascus ruber*-fermented rice (BEMR) at doses of 250, 500, and 1000 mg/kg/day exhibited a significantly lower percentage of body-weight gain than the control group ($p < 0.05$), which showed an average increase of approximately 21% over the experimental period. Although body-weight gain differed significantly among groups, differences in final body weight were comparatively small.

No significant differences were detected in daily feed intake, water consumption, or fecal and urinary output throughout the study (Table 6). These findings indicate that the lower body-weight gain observed in BEMR-treated animals was not accompanied by detectable differences in feed consumption, dehydration, or evident alterations in excretory function.

Changes in body-weight gain are considered a sensitive but nonspecific endpoint in repeated-dose toxicity studies and should therefore be interpreted together with the remaining clinical, biochemical, and pathological findings (OECD, 2002b; OECD, 2025). In the present study, the reduced body-weight gain occurred in the absence of treatment-related clinical signs, behavioral alterations, mortality, or gross pathological findings, indicating that this response should be interpreted cautiously within the overall toxicological profile of the tested product.

Table 4
Summary of behavioral and clinical findings during acute oral exposure to BEMR.

Parameter	Control group	BEMR
Mortality	Absent	Absent
Hippocratic Screening	Normal	Normal
Behavioral changes	None	None
Gross pathological findings	None	None
Organ-weight alterations	None	None

BEMR = freeze-dried beer extract supplemented with Monascus ruber-fermented rice (2000 mg/kg, p.o.).

Table 5
Body-weight evolution of Wistar rats treated with BEMR (p.o.) during the 28-day subacute safety assessment.

Parameter	CG	G1	G2	G3	SG
Initial weight (g)	178.60 ± 2.19[b]	195.60 ± 5.13[a]	197.40 ± 4.51[a]	199.80 ± 5.93[a]	197.40 ± 5.73[a]
Final weight (g)	217.40 ± 7.09[a]	207.40 ± 4.04[b]	212.60 ± 4.72[a]	216.60 ± 9.71[ab]	213.40 ± 6.35[a]
Weight gain (%)	21.75 ± 4.59[a]	6.06 ± 1.65[c]	7.71 ± 1.87[bc]	6.97 ± 4.20[bc]	8.11 ± 1.60[b]

Values are expressed as mean ± SD (n = 6). Means followed by the same letter in a row do not differ significantly (Tukey's test, $p < 0.05$). BEMR = freeze-dried beer extract supplemented with Monascus ruber-fermented rice. CG = control (water 10 mL/kg); G1 = BEMR 250 mg/kg; G2 = BEMR 500 mg/kg; G3 = BEMR 1000 mg/kg; SG = satellite group (BEMR 1000 mg/kg).

Previous studies have reported that certain *Monascus*-derived metabolites may influence lipid metabolism, adipogenesis, and energy homeostasis, leading to attenuated body-weight gain without affecting food intake in experimental models (Choe et al., 2020; Hong et al., 2017). Although these observations provide a plausible explanation for the pattern identified in the present study, no mechanistic endpoints were evaluated. Consequently, it is not possible to establish whether the reduction in body-weight gain reflects metabolic adaptation, altered energy utilization, or other physiological responses associated with the fermented product.

Consistent with the overall toxicological evaluation, no treatment-related mortality, clinical abnormalities, or gross pathological findings were observed during the 28-day exposure period. These observations are in agreement with previous repeated-dose studies reporting good tolerability of selected *Monascus*-derived products produced under controlled fermentation conditions (Lee et al., 2010; Mohan-Kumari et al., 2009). Nevertheless, comparisons among studies should be interpreted with caution, since the composition of *Monascus*-fermented products may vary substantially according to fungal strain, substrate, fermentation conditions, and the occurrence of secondary metabolites.

Taken together, repeated oral administration of BEMR at doses up to 1000 mg/kg/day resulted in reduced body-weight gain without concurrent alterations in food consumption, water intake, clinical condition, or gross pathological findings. Although this response may represent a physiological response associated with the fermented matrix, the absence of mechanistic investigations precludes definitive conclusions regarding its biological basis. Accordingly, additional studies integrating targeted chemical profiling, histopathological evaluation, and mechanistic approaches will be necessary to further elucidate the physiological significance of the observed responses.

3.4. Organ weights and behavioral observations

Absolute and relative organ weights following 28 days of repeated oral administration are presented in Tables 7 and 8, with relative heart weight illustrated in Fig. 2. No statistically significant differences were

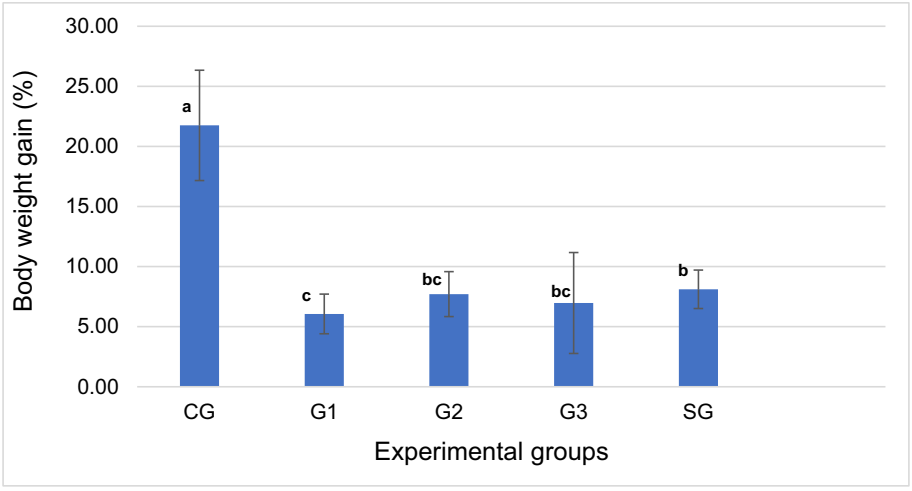

Fig. 1. Body-weight gain (%) of female Wistar rats following repeated oral administration of BEMR for 28 days. Values are expressed as mean ± SD ($n = 6$ per group). Bars with different letters differ significantly (Tukey's test, $p < 0.05$). CG = control (water, 10 mL/kg); G1 = BEMR 250 mg/kg; G2 = BEMR 500 mg/kg; G3 = BEMR 1000 mg/kg; SG = satellite group (BEMR 1000 mg/kg).

Table 6
Daily dietary and physiological parameters of Wistar rats treated with BEMR during the 28-day subacute safety assessment.

Parameter	CG	G1	G2	G3	SG
Feed Consumption (g)	231.67 ± 40.80[a]	213.00 ± 49.37[a]	222.42 ± 51.71[a]	215.08 ± 50.01[a]	224.25 ± 49.25[a]
Water Consumption (g)	681.58 ± 126.34[a]	563.75 ± 217.99[a]	554.42 ± 150.57[a]	629.08 ± 188.25[a]	567.33 ± 157.05[a]
Excreta output (g)	371.33 ± 117.01[a]	342.83 ± 275.17[a]	291.67 ± 113.21[a]	339.08 ± 279.75[a]	315.75 ± 106.57[a]

Values are expressed as mean ± SD (n = 6). Means followed by the same letter in a row do not differ significantly (Tukey's test, $p < 0.05$). BEMR = freeze-dried beer extract supplemented with Monascus ruber-fermented rice. CG = control (water 10 mL/kg); G1 = BEMR 250 mg/kg; G2 = BEMR 500 mg/kg; G3 = BEMR 1000 mg/kg; SG = satellite group (BEMR 1000 mg/kg).

Table 7
Absolute organ weights of Wistar rats treated with BEMR during the 28-day subacute safety assessment.

Organ (g)	CG	G1	G2	G3	SG
Liver	7.46 ± 1.15[a]	7.07 ± 0.38[a]	6.95 ± 0.56[a]	7.15 ± 0.87[a]	7.75 ± 0.77[a]
Right Kidney	0.86 ± 0.06[a]	0.92 ± 0.16[a]	0.89 ± 0.06[a]	0.86 ± 0.09[a]	0.94 ± 0.10[a]
Left Kidney	0.82 ± 0.06[a]	0.85 ± 0.06[a]	0.86 ± 0.07[a]	0.88 ± 0.08[a]	0.83 ± 0.07[a]
Stomach	1.60 ± 0.21[a]	1.73 ± 0.20[a]	1.55 ± 0.25[a]	1.65 ± 0.27[a]	1.73 ± 0.22[a]
Heart	0.99 ± 0.11[a]	0.85 ± 0.04[bc]	0.81 ± 0.08[c]	0.89 ± 0.07[abc]	0.97 ± 0.13[ab]
Lungs	1.62 ± 0.14[a]	1.71 ± 0.18[a]	1.65 ± 0.15[a]	1.85 ± 0.22[a]	1.64 ± 0.20[a]

Values are expressed as mean ± SD (n = 6). Means followed by the same letter in a row do not differ significantly (Tukey's test, $p < 0.05$). BEMR = freeze-dried beer extract supplemented with Monascus ruber-fermented rice. CG = control (water 10 mL/kg); G1 = BEMR 250 mg/kg; G2 = BEMR 500 mg/kg; G3 = BEMR 1000 mg/kg; SG = satellite group (BEMR 1000 mg/kg).

observed among experimental groups for the liver, kidneys, stomach, or lungs, indicating no evidence of treatment-related effects on these organs.

Table 8
Relative organ weights (g/100 g body weight) of Wistar rats treated with BEMR during the 28-day subacute safety assessment.

Organ (g/100 g)	CG	G1	G2	G3	SG
Liver	3.47 ± 0.49[a]	3.42 ± 0.22[a]	3.28 ± 0.26[a]	3.33 ± 0.28[a]	3.53 ± 0.35[a]
Right Kidney	0.40 ± 0.04[a]	0.45 ± 0.08[a]	0.42 ± 0.03[a]	0.40 ± 0.03[a]	0.43 ± 0.04[a]
Left Kidney	0.38 ± 0.02[a]	0.41 ± 0.03[a]	0.40 ± 0.03[a]	0.41 ± 0.02[a]	0.38 ± 0.02[a]
Stomach	0.74 ± 0.10[a]	0.84 ± 0.08[a]	0.73 ± 0.11[a]	0.77 ± 0.12[a]	0.79 ± 0.09[a]
Heart	0.46 ± 0.04[a]	0.41 ± 0.02[b]	0.38 ± 0.04[b]	0.42 ± 0.03[b]	0.44 ± 0.06[ab]
Lungs	0.76 ± 0.07[a]	0.83 ± 0.10[a]	0.78 ± 0.08[a]	0.87 ± 0.11[a]	0.74 ± 0.07[a]

Values are expressed as mean ± SD (n = 6). Means followed by the same letter in a row do not differ significantly (Tukey's test, $p < 0.05$). BEMR = freeze-dried beer extract supplemented with Monascus ruber-fermented rice. CG = control (water 10 mL/kg); G1 = BEMR 250 mg/kg; G2 = BEMR 500 mg/kg; G3 = BEMR 1000 mg/kg; SG = satellite group (BEMR 1000 mg/kg).

A small but statistically significant reduction in both absolute and relative heart weight was observed in animals receiving BEMR at 250 mg/kg (G1) and 500 mg/kg (G2) compared with the control group, whereas no significant difference was detected at the highest dose (1000 mg/kg). Although individual values partially overlapped among groups, this pattern was observed consistently at the two lower dose levels. Moreover, the absence of a dose-response relationship suggests that this isolated finding should be interpreted cautiously.

Organ-weight variations are considered sensitive indicators in repeated-dose toxicity studies but should not be interpreted in isolation. Their toxicological significance requires integration with clinical observations, pathological evaluation, and complementary laboratory findings, as recommended by OECD guidance for repeated-dose toxicity studies (OECD, 2002b; OECD, 2025). In the present study, the reduction in heart weight occurred in the absence of treatment-related clinical signs, behavioral abnormalities, mortality, or gross pathological findings.

Previous studies have reported biological activities of *Monascus*-derived metabolites associated with lipid metabolism, antioxidant responses, and cardiac physiology (Merinas-Amo et al., 2016; Penson & Banach, 2021). However, because histopathological examination and

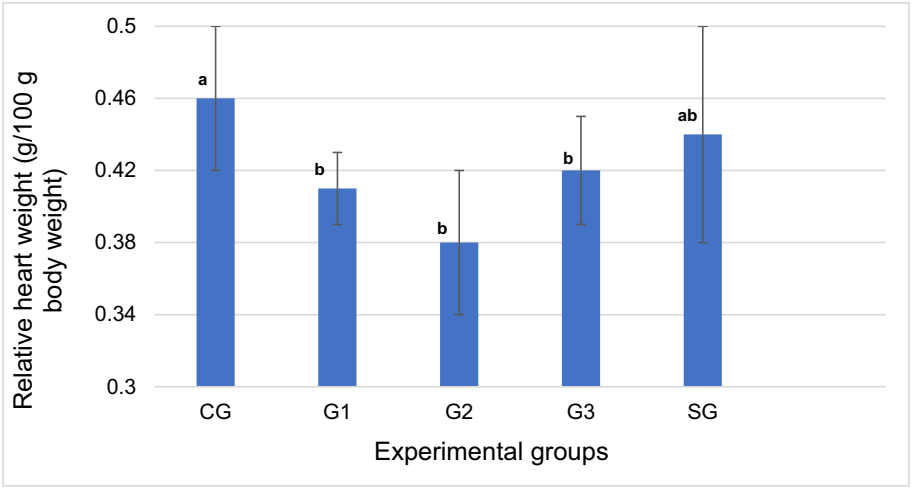

Fig. 2. Relative heart weight (g/100 g body weight) of female Wistar rats following repeated oral administration of BEMR for 28 days. Values represent mean ± SD (n = 6 per group). Bars with different letters differ significantly (Tukey's test, $p < 0.05$). CG = control (water, 10 mL/kg); G1 = 250 mg/kg; G2 = 500 mg/kg; G3 = 1000 mg/kg; SG = satellite group (1000 mg/kg).

mechanistic investigations were beyond the scope of the present study, the biological significance of the observed reduction in heart weight cannot be established from the available data.

Throughout the experimental period, animals remained clinically normal, with no evidence of altered behavior, autonomic dysfunction, or reduced spontaneous activity. Likewise, the Hippocratic screening protocol revealed no treatment-related abnormalities, and the gross pathological examination did not reveal visible pathological changes in either cardiac or extracardiac tissues.

Collectively, these findings indicate that repeated oral administration of BEMR produced no evidence of overt systemic toxicity based on the endpoints evaluated. Nevertheless, the isolated reduction in heart weight observed at the two lower dose levels should be interpreted cautiously, since its toxicological relevance cannot be determined in the absence of histopathological evaluation and complementary mechanistic investigations. Accordingly, additional studies incorporating cardiac histology, morphometric analyses, and functional assessments are warranted to clarify the biological significance of this finding.

3.5. Biochemical and hematological parameters

Biochemical and hematological parameters obtained after 28 days of repeated oral exposure to BEMR are summarized in Tables 9, 10 and 11, with creatine kinase (CK) activity and serum creatinine levels illustrated in Figs. 3 and 4.

Markers of hepatic function, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transferase (γ-GT), and bilirubin, remained within the expected physiological ranges for female Wistar rats across all experimental groups (Table 9). Likewise, no treatment-related alterations were observed in glucose concentration, lipid profile, uric acid, electrolyte balance, or urea levels, indicating preservation of the major biochemical endpoints associated with hepatic and renal function. These findings are consistent with previous repeated-dose studies reporting good hepatic tolerance of selected *Monascus*-derived products produced under controlled fermentation conditions (Lee et al., 2010; Mohan-Kumari

Table 9
Serum biochemical parameters of Wistar rats treated with BEMR during the 28-day subacute safety assessment.

Group	CG	G1	G2	G3	SG
Glucose (mg/dL)	194.33 ± 29.76^{a}	175.50 ± 22.29^{a}	231.00 ± 76.43^{a}	217.50 ± 36.57^{a}	240.67 ± 50.16^{a}
AST (U/L)	157.67 ± 28.07^{a}	114.83 ± 64.72^{a}	136.00 ± 40.16^{a}	117.83 ± 40.44^{a}	130.17 ± 31.34^{a}
γ-GT (U/L)	2.25 ± 1.50^{a}	1.67 ± 0.58^{a}	1.33 ± 2.31^{a}	2.25 ± 2.22^{a}	1.33 ± 0.58^{a}
Uric acid (mg/dL)	1.63 ± 0.66^{a}	1.55 ± 0.67^{a}	1.83 ± 0.77^{a}	1.74 ± 1.06^{a}	1.61 ± 0.91^{a}
ALP (U/L)	150.17 ± 54.40^{a}	143.50 ± 73.00^{a}	120.00 ± 41.87^{a}	114.33 ± 71.15^{a}	183.5 ± 68.10^{a}
ALT (U/L)	73.83 ± 30.64^{a}	60.17 ± 11.36^{a}	66.50 ± 23.72^{a}	57.00 ± 7.62^{a}	74.17 ± 11.30^{a}
Triglycerides (mg/dL)	85.67 ± 21.65^{a}	66.17 ± 27.74^{a}	74.83 ± 37.22^{a}	87.67 ± 20.16^{a}	88.83 ± 27.80^{a}

Values are expressed as mean ± SD (n = 6). Means followed by the same letter in a row do not differ significantly (Tukey's test, $p < 0.05$). BEMR = freeze-dried beer extract supplemented with Monascus ruber-fermented rice. CG = control (water 10 mL/kg); G1 = BEMR 250 mg/kg; G2 = BEMR 500 mg/kg; G3 = BEMR 1000 mg/kg; SG = satellite group (BEMR 1000 mg/kg).

Table 10
Biochemical parameters related to renal and muscular function in Wistar rats treated with BEMR.

Group	CG	G1	G2	G3	SG
Total Protein (g/dL)	6.52 ± 0.75^{a}	6.65 ± 0.81^{a}	6.63 ± 0.55^{a}	6.83 ± 0.83^{a}	7.08 ± 0.77^{a}
Albumin (g/dL)	3.10 ± 0.57^{a}	3.20 ± 0.28^{a}	3.20 ± 0.38^{a}	3.40 ± 0.43^{a}	3.58 ± 0.27^{a}
Total bilirubin (mg/dL)	0.92 ± 0.17^{a}	1.78 ± 2.48^{a}	0.88 ± 0.22^{a}	1.02 ± 0.43^{a}	1.18 ± 0.93^{a}
CK (U/L)	2038.33 ± 524.74^{a}	1048.67 ± 491.84^{bc}	1011.80 ± 458.31^{b}	483.00 ± 224.41^{d}	758.60 ± 399.06^{cd}
Urea (mg/dL)	45.17 ± 10.36^{a}	45.00 ± 3.46^{a}	45.83 ± 7.22^{a}	40.83 ± 3.19^{a}	51.67 ± 7.09^{a}
Creatinine (mg/dL)	0.39 ± 0.18^{a}	0.53 ± 0.03^{b}	0.53 ± 0.05^{b}	0.51 ± 0.10^{b}	0.59 ± 0.06^{b}
Cholesterol (mg/dL)	76.00 ± 8.39^{a}	79.33 ± 7.94^{a}	85.33 ± 15.78^{a}	83.33 ± 7.89^{a}	84.83 ± 5.34^{a}
Direct bilirubin (mg/dL)	0.43 ± 0.10^{a}	0.39 ± 0.13^{a}	0.38 ± 0.09^{a}	0.50 ± 0.27^{a}	0.55 ± 0.49^{a}

Values are expressed as mean ± SD (n = 6). Means followed by the same letter in a row do not differ significantly (Tukey's test, $p < 0.05$). BEMR = freeze-dried beer extract supplemented with Monascus ruber-fermented rice. CG = control (water 10 mL/kg); G1 = BEMR 250 mg/kg; G2 = BEMR 500 mg/kg; G3 = BEMR 1000 mg/kg; SG = satellite group (BEMR 1000 mg/kg).

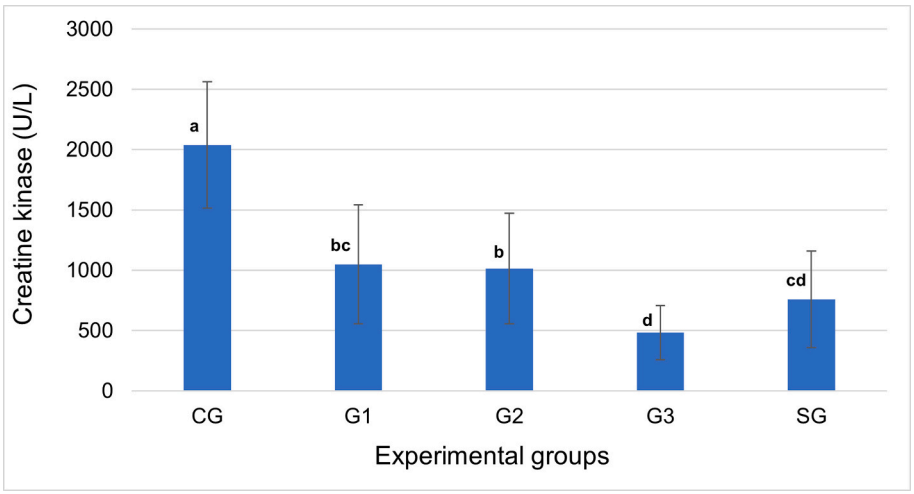

Fig. 3. Serum creatine kinase (CK) activity (U/L) of female Wistar rats following repeated oral administration of BEMR for 28 days. Values represent mean ± SD (n = 6 per group). Bars with different letters differ significantly (Tukey's test, $p < 0.05$).

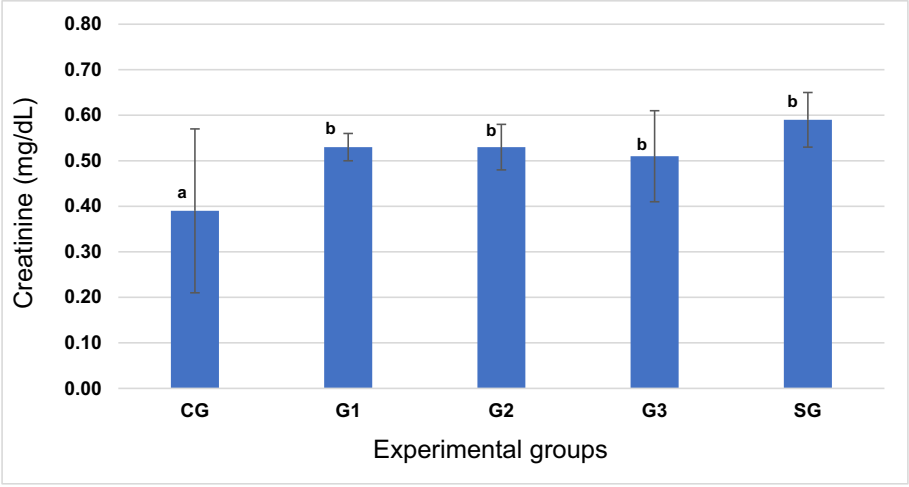

Fig. 4. Serum creatinine levels (mg/dL) of female Wistar rats following repeated oral administration of BEMR for 28 days. Values represent mean ± SD (n = 6 per group). Bars with different letters differ significantly (Tukey's test, p < 0.05).

et al., 2009).

Among the biochemical endpoints evaluated, statistically significant differences were observed only for creatine kinase (CK) activity and serum creatinine concentration (Table 10; Figs. 3 and 4). CK activity decreased by approximately 50–75% in all BEMR-treated groups compared with controls, whereas serum creatinine showed a modest increase of approximately 25–30%. These findings occurred without concurrent alterations in the remaining hepatic and renal biochemical parameters evaluated.

Creatine kinase plays a central role in cellular energy metabolism and is widely used as a biomarker of skeletal and cardiac muscle injury. However, total CK is a non-specific marker influenced by multiple physiological and metabolic factors. Consequently, isolated alterations in CK activity should be interpreted together with clinical observations and the remaining toxicological endpoints rather than considered independently, in accordance with the integrated approach recommended for repeated-dose toxicity assessment (OECD, 2002b; OECD, 2025). In the present study, the reduction in CK activity occurred in the absence of treatment-related clinical abnormalities, behavioral alterations, mortality, or gross pathological findings.

Previous experimental studies have suggested that certain *Monascus*-derived metabolites may influence mitochondrial function, lipid metabolism, and cellular energy homeostasis (Merinas-Amo et al., 2016; Penson & Banach, 2021). Although these observations provide a plausible context for the present findings, the present study did not include mechanistic investigations capable of determining whether the observed reduction in CK activity was related to physiological adaptation, altered energy metabolism, or other biological processes.

Serum creatinine was modestly but consistently higher in treated animals than in controls. However, this finding was not accompanied by changes in urea concentration, electrolyte balance, kidney weight, urine output, or gross pathological findings involving the kidneys. Therefore, the isolated increase in creatinine does not, by itself, demonstrate renal impairment and should likewise be interpreted within the context of the remaining toxicological endpoints evaluated (OECD, 2002b; OECD, 2025).

Hematological parameters (Table 11) revealed a limited number of statistically significant differences, including variations in hematocrit, platelet indices, and leukocyte subpopulations. Nevertheless, all values remained within the expected physiological ranges for the species and did not exhibit consistent dose-response relationships. According to OECD guidance for repeated-dose toxicity studies, isolated statistical differences without supporting clinical or pathological evidence should be interpreted cautiously, particularly when they lack biological

Table 11
Hematological parameters of Wistar rats treated with BEMR during the 28-day subacute safety assessment.

Parameter	CG	G1	G2	G3	SG
WBC ($\times10^3$/μL)	4.28 ± 0.71^{b}	4.68 ± 0.77^{b}	6.70 ± 1.39^{a}	6.70 ± 0.89^{a}	4.42 ± 1.36^{ab}
RBC ($\times10^6$/μL)	6.51 ± 0.38^{a}	6.16 ± 0.84^{a}	6.60 ± 0.40^{a}	6.59 ± 0.36^{a}	6.36 ± 0.52^{a}
Hb (g/dL)	13.50 ± 0.86^{a}	13.12 ± 1.27^{a}	13.82 ± 0.66^{a}	14.26 ± 0.55^{a}	13.05 ± 0.87^{a}
HCT (%)	36.60 ± 1.89^{a}	33.97 ± 4.39^{ab}	35.42 ± 1.62^{a}	37.02 ± 1.75^{a}	31.35 ± 2.13^{b}
PLT ($\times10^3$/μL)	470.6 ± 214.0^{b}	670.0 ± 194.8^{ab}	804.5 ± 144.7^{ab}	851.0 ± 89.3^{a}	791.8 ± 135.1^{ab}
PCT (%)	0.36 ± 0.13^{c}	0.49 ± 0.14^{bc}	0.65 ± 0.14^{a}	0.61 ± 0.08^{a}	0.61 ± 0.15^{ab}
LYM (%)	83.7 ± 3.5^{a}	85.1 ± 5.3^{a}	84.3 ± 4.0^{a}	84.5 ± 1.2^{a}	86.8 ± 2.6^{a}
MON (%)	11.36 ± 2.22^{a}	10.75 ± 3.13^{a}	11.00 ± 2.09^{a}	11.58 ± 1.13^{a}	10.45 ± 2.37^{a}
GRA (%)	4.94 ± 1.93^{a}	4.18 ± 2.27^{a}	4.68 ± 2.17^{a}	4.10 ± 0.84^{a}	2.80 ± 1.21^{a}
MCV (fL)	20.76 ± 0.79^{a}	26.88 ± 13.34^{a}	26.53 ± 13.49^{a}	28.44 ± 14.86^{a}	25.90 ± 13.28^{a}
MCH (pg)	20.76 ± 0.79^{a}	21.40 ± 1.17^{a}	21.07 ± 0.92^{a}	21.66 ± 0.66^{a}	20.58 ± 0.52^{a}
MCHC (g/dL)	36.94 ± 1.60^{c}	38.77 ± 1.70^{bc}	39.02 ± 0.99^{b}	36.78 ± 3.48^{bc}	41.70 ± 1.15^{a}
RDW (%)	13.96 ± 0.52^{a}	13.77 ± 0.29^{a}	13.73 ± 0.35^{a}	13.84 ± 0.18^{a}	13.73 ± 0.44^{a}
MPV (fL)	7.88 ± 0.89^{a}	7.45 ± 0.65^{a}	8.05 ± 0.71^{a}	7.16 ± 0.32^{a}	7.83 ± 1.71^{a}
PDW (%)	3.34 ± 3.16^{b}	5.57 ± 2.36^{b}	4.85 ± 1.28^{b}	8.34 ± 1.07^{a}	4.07 ± 1.32^{b}
LYM# ($\times10^3$/μL)	3.54 ± 0.70^{a}	9.93 ± 14.84^{a}	5.62 ± 1.27^{a}	5.62 ± 0.72^{a}	3.77 ± 1.14^{a}
MON# ($\times10^3$/μL)	0.42 ± 0.04^{b}	0.45 ± 0.21^{ab}	0.68 ± 0.19^{a}	0.70 ± 0.16^{a}	0.43 ± 0.24^{ab}
GRA# ($\times10^3$/μL)	0.32 ± 0.11^{a}	0.30 ± 0.09^{a}	0.40 ± 0.18^{a}	0.38 ± 0.04^{a}	0.22 ± 0.08^{a}

Values are expressed as mean ± SD (n = 6).
Means followed by the same letter in a row do not differ significantly (Tukey's test, $p < 0.05$).
BEMR = freeze-dried beer extract supplemented with Monascus ruber-fermented rice. CG = control group (water, 10 mL/kg); G1 = BEMR 250 mg/kg; G2 = BEMR 500 mg/kg; G3 = BEMR 1000 mg/kg; SG = satellite group (BEMR 1000 mg/kg).
Abbreviations: WBC = white blood cells; RBC = red blood cells; Hb = hemoglobin; HCT = hematocrit; PLT = platelet count; PCT = plateletcrit; LYM = lymphocytes (%); MON = monocytes (%); GRA = granulocytes (%); MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = red cell distribution width; MPV = mean platelet volume; PDW = platelet distribution width; LYM# = absolute lymphocytes; MON# = absolute monocytes; GRA# = absolute granulocytes.

consistency (OECD, 2002b; OECD, 2025). Similar hematological stability has been reported in previous repeated-dose studies involving *Monascus*-derived products (Lee et al., 2010; Mohan-Kumari et al., 2009).

Repeated oral administration of BEMR produced no evidence of treatment-related alterations in the majority of the biochemical and hematological parameters evaluated. The reductions in CK activity and the modest increase in serum creatinine represent isolated findings whose biological significance cannot be fully established based on the endpoints assessed in the present study. Additional investigations integrating targeted chemical analyses, histopathological evaluation, and mechanistic approaches will be necessary to determine the biological basis of these responses.

3.6. Toxicological and functional considerations

The integration of physicochemical characterization with acute and repeated-dose toxicological assessment provides an integrated overview of the biological behavior of the freeze-dried beer extract supplemented with *Monascus ruber*-fermented rice (BEMR). Overall, the evaluated endpoints indicate good tolerability within the experimental conditions evaluated, while also identifying physiological responses that warrant further investigation.

No mortality, treatment-related clinical signs, behavioral abnormalities, or gross pathological findings were observed following acute or repeated oral administration. Likewise, the majority of biochemical, hematological, and organ-weight parameters remained comparable to those of the control group, indicating that repeated exposure to the tested formulation was generally well tolerated during the experimental period, consistent with previous repeated-dose studies of selected *Monascus*-derived products (Lee et al., 2010; Mohan-Kumari et al., 2009).

One of the principal toxicological considerations regarding *Monascus*-fermented products is the possible occurrence of citrinin, a nephrotoxic mycotoxin, together with other secondary metabolites, including monacolins. The relative abundance of these compounds depends on multiple factors, including fungal strain, substrate composition, fermentation conditions, and processing. Consequently, current scientific evidence and regulatory assessments emphasize that the toxicological evaluation of *Monascus*-derived products should ideally be complemented by comprehensive chemical characterization, particularly regarding citrinin and monacolin content (EFSA Panel on Food Additives and Nutrient Sources Added to Food (ANS), 2018). Although such analyses were not included in the present study, this aspect should be considered when interpreting the findings and comparing them with other *Monascus*-based formulations.

Among the evaluated endpoints, the most consistent treatment-related findings were the reduction in body-weight gain, the decrease in creatine kinase activity, and the modest increase in serum creatinine. These responses occurred without concurrent alterations in feed or water intake, clinical condition, gross pathological findings, or the remaining biochemical indicators of hepatic and renal function. Previous experimental studies have shown that selected *Monascus*-derived metabolites may influence lipid metabolism, mitochondrial function, and energy homeostasis (Choe et al., 2020; Hong et al., 2017; Penson & Banach, 2021). Although these observations provide a biologically plausible context for the present findings, the current study did not include mechanistic investigations capable of determining whether the observed responses represent physiological adaptation, altered metabolic regulation, or other biological processes.

Similarly, the reduction in heart weight observed at the lower dose levels should be interpreted cautiously. Organ-weight variations represent sensitive indicators in repeated-dose toxicity studies but require interpretation in conjunction with complementary pathological, biochemical, and clinical findings, as recommended by OECD guidance for repeated-dose toxicity studies (OECD, 2002b; OECD, 2025). Because histopathological evaluation was not performed, the biological significance of this isolated finding cannot be established from the available data. Consequently, neither adaptive nor adverse interpretations can be conclusively supported based solely on the present results.

Taken together, the present findings should be interpreted within the scope of an initial OECD-guided toxicological evaluation. The absence of overt toxicity during both acute and repeated-dose exposure provides evidence of good tolerability under the experimental conditions evaluated. However, comprehensive chemical characterization, histopathological evaluation, and mechanistic investigations remain necessary to further elucidate the biological basis of the observed physiological responses and to support a broader toxicological characterization of this formulation.

Future studies integrating targeted chemical analyses—including

citrinin, monacolins, and pigment profiling—with histopathological examination and mechanistic approaches, such as metabolomic, lipidomic, transcriptomic, and microbiota analyses, will contribute to a more comprehensive understanding of the biological effects associated with this fermented beverage. Considering the known variability in metabolite production among *Monascus* strains and fermentation processes, the present findings should be interpreted within the context of the specific formulation and production conditions evaluated in this study.

4. Conclusions

This study integrated physicochemical characterization with OECD-guided *in vivo* toxicological screening to evaluate a freeze-dried beer extract supplemented with *Monascus ruber*-fermented rice (BEMR). The incorporation of fermented rice markedly modified the beer color profile while maintaining the principal physicochemical characteristics within values compatible with the intended beer style. Acute and repeated oral administration produced no evidence of overt toxicity under the experimental conditions evaluated.

No mortality, treatment-related clinical signs, behavioral abnormalities, or gross pathological findings were observed during either toxicity study. Likewise, most biochemical, hematological, and organ-weight parameters remained comparable to those of the control group. Although reductions in body-weight gain and creatine kinase activity, together with a modest increase in serum creatinine, were identified following repeated exposure, the biological significance of these isolated findings could not be fully established based on the endpoints evaluated in the present study.

The present findings provide an initial toxicological characterization of a freeze-dried beer formulation supplemented with *Monascus ruber*-fermented rice and contribute to current knowledge on the biological evaluation of *Monascus*-derived fermented products. Further studies integrating targeted chemical characterization of relevant fermentation-derived metabolites, histopathological evaluation, and mechanistic approaches will be important to clarify the physiological basis of the observed responses and to support a more comprehensive toxicological assessment of this formulation.

CRediT authorship contribution statement

Tainá Francisca Cordeiro de Souza: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Luiz Carlos da Cunha:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization. **Jerônimo Raimundo de Oliveira Neto:** Software, Investigation, Formal analysis, Data curation. **Michele Paula de Oliveira de Melo:** Investigation, Formal analysis, Data curation. **Anyelle Teixeira Vilas Boas:** Investigation, Formal analysis, Data curation. **Julio Cesar Colivet Briceno:** Writing – review & editing, Methodology. **Francielo Vendruscolo:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests and personal relationships which may be considered as potential competing interests: Tainá Francisca Cordeiro de Souza received financial support from the Federal University of Goiás (UFG) through a CAPES scholarship (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil). All other 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.

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