Composition optimization and safety assessment of lactic-acid-bacteria-loaded composite film

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ABSTRACT
In this experiment, response surface methodology–central composite rotatable design was used to optimize the ratio of hydroxypropyl cellulose, konjac flour, and glycerol to obtain optimal composite film. Taking into account the importance of tensile strength, elongation at break and lactobacillus survivability, to the overall properties of composite film, a comprehensive index was proposed to evaluate comprehensive property by fuzzy comprehensive evaluation method. Optimization experiments showed that the optimal composition of the biocomposite membrane that maximized the combined response value was 0.586% HPC, 0.858% KF, and 1.620% GL. In vitro toxicity experiments were used for the safety evaluation of the membranes. In the 14-day feeding test, the mice in each experimental group grew well, and there were no obvious abnormalities in gross anatomical organs and related blood biochemical indicators. A 14-day mouse feeding experiment showed that the biocomposite film had no short-term toxicity, so the biocomposite film could be considered food-safe.

Optimización de la composición y evaluación de la seguridad de las películas compuestas cargadas con bacterias del ácido láctico

RESUMEN
Con el propósito de obtener una película compuesta óptima y a fin de optimizar la ratio de hidroxypropilcelulosa, la harina de konjac y el glicerol, en este experimento se utilizó la metodología de superficie respuesta-diseño rotativo centralizado y compuesto. Teniendo en cuenta la importancia de la fuerza tensil, el alargamiento a la rotura y la capacidad de supervivencia de los lactobacilos para las propiedades generales de la película compuesta, se propuso un índice integral que permitiera evaluar la propiedad integral empleando el método de evaluación integral difusa. Los experimentos de optimización permitieron comprobar que la composición óptima de la membrana de biocompuestos que maximizaba el valor de respuesta combinado era de 0.586% de HPC, 0.858% de KF y 1.620% de GL. Para la evaluación de la seguridad de las membranas, se utilizaron experimentos de toxicología in vitro. En la prueba de alimentación de 14 días, los ratones de cada grupo experimental crecieron bien, sin que se detectasen anomalías evidentes en los órganos anatómicos grandes ni en los indicadores bioquímicos sanguíneos relacionados. El experimento de alimentación de ratones durante 14 días demostró que la lámina de biocompuestos no tiene toxicidad a corto plazo, por lo que puede considerarse segura para la alimentación.

1. Introduction
In the past decades, composite films have been widely developed for food packaging, including degradable packaging, antimicrobial-coated films, antioxidant bioactive packaging, controlled-release packaging, pH-responsive packaging, probiotic edible films, etc. (Wang et al., 2022). Due to renewability and processability, cellulose-based films, such as hydroxypropyl cellulose (HPC) films, are expected to reduce consumption of synthetic packages and environmental pollution, and thus have received extensive attention (Milioto et al., 2021; Saini, Sharma, & Xia, 2021). Konjac glucomannan-enriched konjac flour (KF) has great potential to develop edible films, coatings, or packaging due to its biocompatibility and biodegradability (Man et al., 2022; Yangyang et al., 2022). The mechanical properties are very important because they determine whether the film maintains its integrity and functionality during food packaging, transportation, and storage. Tensile strength (TS) indicates resistance to direct pull, while elongation at break (EAB) indicates elasticity towards stretching, both of which are key indicators used to describe mechanical properties of biopolymer films (Aloui, 2015; Rouhi, 2017). It has been reported that the films formed by a pure biopolymer were hard to possess both good TS and EAB simultaneously, as pure HPC films with high elasticity but low mechanical strength, while pure KF films with high strength but low stretchability (Leuangsuksrik, 2014; El-Wakil, 2016). As reported by Wang et al., El-Wakil et al., and Leuangsuksrik et al., increasing the content of HPC increased the flexibility of the films, while increasing the content of KF significantly enhanced the
mechanical strength of the films (Leuangsuikerk, 2014; Wang, 2016). Therefore, it can be considered to compound different biopolymers and optimize the ratio to obtain a composite film with best comprehensive performance (Ghasempour et al., 2022; Herrera et al., 2022). In the presence of plasticizers, the intermolecular interactions of different components can change the structure of the composite film, thereby changing the mechanical properties of the composite film (Kalateh et al., 2021; Rouhi, 2017).

Recently, several researches have added natural active substances, like antioxidants or antibacterials, into composite films to develop novel food packaging. However, natural active ingredients, such as polyphenols, essential oils, and antimicrobial peptides, tend to be gradually deactivated during storage (Maroufi et al., 2022; Ramón et al., 2022; Shan et al., 2022). Therefore, some studies have reported directly encapsulating live microorganisms into composite films to ensure the antibacterial function of active packaging through the continuous production of metabolites by microorganisms. In these reports, the most commonly used microorganisms are lactic acid bacteria (LAB) (Li et al., 2020; Settler et al., 2020) and yeast (Guimaraes et al., 2020; Laura et al., 2022). In order to obtain a lasting bacteriostatic effect, it is necessary to provide a relatively suitable living environment for living microorganisms, that is to say, the components and ratios of composite membrane materials are particularly important.

Response surface methodology-central composite rotatable design (RSM-CCRD) is a commonly used experimental optimization design tool. As reported by Ajesh, K. V. et al., the RSM-CCRD was used to develop soybean aqueous-extract-based composite film and predict the effect of independent variables on the response (Ajesh et al., 2022). Liew, W. C. et al., reported that the optimum formulations for ZnO/GO nanocomposite-loaded polyactic acid active films were determined at 1.06 wt% ZnO, 1.11 wt% GO at 60°C, and 0.99 wt% ZnO, 1.28 wt% GO at 40°C (Liew et al., 2022). In the RSM-CCRD experiment, when there are multiple evaluation indexes with different importance, a comprehensive index can be obtained according to the fuzzy comprehensive evaluation method (Jiang, 2011). Therefore, the composite films can be optimized by RSM-CCRD combined with the fuzzy comprehensive evaluation method.

The objective of the present work was to obtain the optimal ratio of LAB-loaded HPC/KF/GL film by RSM-CCRD to obtain the composite film with optimal mechanical properties and LAB relative survival rate, and to evaluate the effect of different components on the overall performance of the film. In addition, the food safety of the prepared composite film was evaluated by in vitro toxicology experiments, laying a foundation for subsequent practical applications of the film.

2. Materials and methods

2.1. Materials

HPC (CSA 9004-64-2) was supplied by Shanghai Yuanye Biological Technology Co., Ltd. Commercial grade KF (76.3% KGM) was procured from Shaanxi Jintai Konjac Industry Development Co., Ltd. GL (CSA 56-81-5) obtained from Sichuan Xilong Chemical Co., Ltd. Lactobacillus paracasei (CICC 20,241) was acquired from China Center of Industrial Culture Collection. Polystyrene petri dishes (10 × 10 cm) were purchased from Haimen Chunbo Biological Experimental Equipment Inc.

2.2. Film preparation

The film was prepared by a casting method. A certain amount of HPC, GL, and KF were added to 100 mL sterile water in successive order, and all components were dissolved and mixed uniformly through sufficient magnetic stirring. Subsequently, the L. paracasei suspensions were incorporated into the preceding solution, and after homogenization and vacuum evacuation, the film-forming solution was obtained. Finally, the film-forming solution was poured into polystyrene petri dishes and then dried at 65% relative humidity (RH) and 25°C for 48 h. Prior to testing, the dried films were preconditioned at 65% RH and 5°C for 24 h to simulate refrigerated storage conditions.

2.3. Property measurement

Mechanical properties of the composite films were assessed by using a TA.XTPlus texture analyser (Stable Micro Systems Ltd, Surrey, UK). The TS and EAB of films (20 mm × 100 mm) were determined according to the ASTM Standard D882. Initial distance between the grips and cross-head speed was set at 50 mm and 1 mm/s, respectively. All tests were carried out on quintuplicate. The viability of L. paracasei added to the composite films was studied after a storage period of 7 days at 5°C and 65% RH. The films were transferred to quantitative tryptone phosphate broth with constant stirring. After sufficient dissolving, serial dilutions of the solution were made and then poured onto MRS agar. The MRS plates were cultured for 48 h at 37°C before colonies were counted. All tests were taken in triplicate. The RSR (%) of L. paracasei was calculated by regarding the maximum survival value as 100% (Lu et al., 2018).

2.4. Experimental design

2.4.1. Response surface methodology

RSM-CCRD was used as experimental optimization design. Three response surface factors were selected, and the range of values for HPC (0.25–0.75%), KF (0.46–1.14%), and GL (1.00–2.00%) was determined based on preliminary experiments (Table 1). The coded values for the design are “−1” for center point, “+1682” for maximum value, “−1682” for minimum value, and “1” and “−1” for interior points, covering 8 factorial points, 6 axial points, and 6 replicates at the central point, giving a total of 20 tests (Liew et al., 2022; Rouhi, 2017).

| Level | HPC(%)(X₁) | KGM(%)(X₂) | GL(%)(X₃) |
|-------|-----------|-----------|-----------|
| −1.68 | 0.25      | 0.46      | 1.00      |
| −1    | 0.35      | 0.60      | 1.20      |
| 0     | 0.50      | 0.80      | 1.50      |
| 1     | 0.65      | 1.00      | 1.80      |
| +1.68 | 0.75      | 1.14      | 2.00      |

Table 1. Factors and levels of response surface method (RSM) analysis.
2.4.2. Fuzzy comprehensive evaluation
Considering the different importance of each index to the overall property of composite film, fuzzy comprehensive evaluation method was used to combine multiple indexes into one comprehensive index, which could evaluate the overall property of the composite film. The membership functions of the indexes were characterized as:

\[ R_i = \frac{Y_i - Y_{i\min}}{Y_{i\max} - Y_{i\min}} \]

R were defined as the membership functions for TS, EAB, and RSR (%).
Each index was given a reasonable weighted value, which indicates the magnitude of the effect of each index on the comprehensive index. Combining with rank correlation analysis and practical purposes, the weighted values for TS, EAB, and RSR were set as \( k_i = (k_1, k_2, k_3) = (0.40, 0.33, 0.27) \) (\( k_1 + k_2 + k_3 = 1 \)).
Then, the fuzzy comprehensive evaluation values can be calculated as:

\[ D = \sum_{i=1}^{3} k_i R_i \]

2.4.3. Optimal regression model
The polynomial regression equation was obtained following the least-squares method and nonlinear regression technique.

\[ Y_i = k_0 + \sum_{i=1}^{k} k_i X_i + \sum_{i=1}^{k} k_2 X_i^2 + \sum_{i=1}^{k} k_3 X_i X_j \]

\( Y_i \) was the predicted response, and \( X_i \) and \( X_j \) were the coded independent variables of HPC, KF, and GL. In this model, \( k \) were the regression coefficients, \( k_0 \) was the offset term, \( k_i \) were the regression coefficients for linear effect terms, \( k_{ii} \) were quadratic effects, and \( k_{ij} \) are interaction effects.
The software provided coefficient of determination (\( R^2 \)) and lack of fit data to validate the model through analysis of variance (ANOVA). The suitability of the polynomial model equation’s fit was tested by the \( R^2 \), adjusted \( R^2 \), predicted \( R^2 \), coefficient of variation (CV) and adequate precision (AP). The non-significant terms were removed to obtain a reduced model (\( p < 0.05 \)). The fitted polynomial equation was further expressed as response surface plots to visualize the relation between the response and independent variables. Six additional experiments were conducted to verify the validity of the statistical experimental strategies.

2.5. Safety assessment
SPF-class Kunming mice were randomly divided into control group and four experimental groups, with eight males and eight females in each group. The four dose groups were equivalent to 10,000 times, 1000 times, 100 times and 10 times the adult daily intake, respectively. Each group was gavaged with 1.0 ml/100 g BW (body weight) of film-forming solution per day, and the control group was gavaged with distilled water. During the experiment, the general performance, behavior, toxic symptoms, and death of the experimental mice were observed. After a 14-day feeding, the serum biochemical indexes of the experimental mice were measured, and organ pathological examination was performed.

2.6. Data analysis
Statistical analysis and regression models were performed by using Design-Expert program (Version 8.0, Stat-Ease, Inc., USA). Analysis of variance (ANOVA) was used to evaluate the significance of experimental variables.

3. Results and discussion
3.1. Model development
A second-order polynomial model of dependent comprehensive responses on coded independent variables (HPC, KF, and GL) was established following nonlinear regression technique. Least-squares technique was employed to determine the multiple regression coefficients. The quadratic nonlinear equation proposed for optimizing the fuzzy comprehensive evaluation index was expressed as follows:

\[
Y = 0.804 + 0.0669X_1 + 0.160X_2 + 0.0647X_3 - 0.0338X_1X_2 + 0.0238X_1^2 + 0.0113X_2X_3 + 0.0616X_1^2 + 0.127X_2^2 - 0.0492X_3^2
\]

Both the actual values and the membership values of dependent variables are included in Table 2. Regression and variance analysis were applied to fit the model and to assess the statistical significance of the terms. The “Model F-value” of 368.88 indicated that the quadratic nonlinear model was significant, and the “Lack of Fit F-value” of 1.47 indicated that the lack of fit was not significant relative to the pure error.
The analysis of variance of the polynomial models for the response variables, along with the corresponding R-Squared, Adj R-Squared, Pred R-Squared, C.V., and Adeq Precision, is shown in Table 3. The satisfactory \( R^2 \) value of 0.997 implied that a high percentage of response variations were explained by the response surface equation. A high value of \( R^2 \) did not always

| X1 | X2 | X3 | Y1 | Y2 | Y3 | R1 | R2 | R3 | D |
|---|---|---|---|---|---|---|---|---|---|
| -1.00 | -1.00 | 1.00 | 0.00 | 0.00 | 1.00 | 0.56 | 0.18 | 0.42 | 0.40 |
| 0.00 | 0.00 | 0.00 | 0.94 | 44.15 | 0.44 | 0.88 | 0.62 | 0.91 | 0.80 |
| 3.00 | -1.68 | 0.00 | 0.52 | 27.01 | 0.35 | 0.00 | 0.72 | 0.70 | 0.70 |
| 1.00 | 1.00 | 1.00 | 0.91 | 53.55 | 0.38 | 0.81 | 0.96 | 0.78 | 0.78 |
| 1.00 | 1.00 | 0.75 | 34.31 | 0.48 | 0.48 | 0.26 | 0.55 | 0.55 |
| -1.00 | -1.00 | 1.00 | 0.13 | 0.01 | 0.94 | 0.24 | 0.77 | 0.77 |
| 1.00 | 1.00 | -1.00 | 0.81 | 54.11 | 0.29 | 0.60 | 0.98 | 0.59 | 0.72 |
| 0.00 | 0.00 | -1.68 | 0.79 | 45.81 | 0.22 | 0.57 | 0.68 | 0.43 | 0.57 |
| 0.00 | 0.00 | 0.00 | 0.95 | 44.71 | 0.45 | 0.90 | 0.64 | 0.93 | 0.82 |
| -1.68 | 0.00 | 0.00 | 0.96 | 39.73 | 0.02 | 0.92 | 0.46 | 0.52 |
| 1.00 | 0.00 | 0.00 | 0.94 | 44.63 | 0.44 | 0.88 | 0.63 | 0.91 | 0.81 |
| 0.00 | 0.00 | -1.68 | 0.86 | 54.66 | 0.21 | 0.71 | 1.00 | 0.40 | 0.72 |
| 0.00 | 1.00 | 1.00 | 1.68 | 40.56 | 0.45 | 0.92 | 0.49 | 0.93 | 0.78 |
| 0.00 | 0.00 | 0.00 | 0.93 | 43.58 | 0.43 | 0.85 | 0.60 | 0.89 | 0.78 |
| 1.68 | 0.00 | 0.00 | 0.85 | 45.72 | 0.46 | 0.69 | 0.68 | 0.96 | 0.76 |
| -1.00 | -1.00 | 1.00 | 0.64 | 32.46 | 0.07 | 0.25 | 0.20 | 0.11 | 0.20 |
| 1.00 | 0.00 | 0.00 | 0.94 | 44.14 | 0.45 | 0.88 | 0.62 | 0.93 | 0.81 |
| -1.00 | 1.00 | -1.00 | 0.86 | 52.17 | 0.07 | 0.70 | 0.91 | 0.10 | 0.61 |
| 20.00 | 1.00 | -1.00 | 0.64 | 39.46 | 0.36 | 0.24 | 0.45 | 0.73 | 0.44 |

The values were coded values of hydroxypropyl cellulose, konjac flour, and glycercol. \( Y_1, Y_2, \) and \( Y_3 \) were indicator values of tensile strength, elongation at break, and relative survival rate. \( R_1, R_2, \) and \( R_3 \) were membership functions of \( Y_1, Y_2, \) and \( Y_3 \). D was fuzzy comprehensive evaluation values.

\( X_1, X_2, \) and \( X_3 \) were coded values of hydroxypropyl cellulose, konjac flour, and glycercol. \( Y_1, Y_2, \) and \( Y_3 \) were indicator values of tensile strength, elongation at break, and relative survival rate. \( R_1, R_2, \) and \( R_3 \) were membership functions of \( Y_1, Y_2, \) and \( Y_3 \). D represents the values of evaluation integral difusa.
adequately elucidate the true model. Therefore, it was more suitable to apply Adj $R^2$ value to estimate the model adequacy, which was found to be 0.994. According to Table 3, the Pred $R^2$ value of 0.985, a measure of how well the model predicts a response value, was in reasonable agreement with the Adj $R^2$ value. The CV value for the responses was 2.365. The AP value of 58.536, which is greater than 4, was desirable, indicated an adequate signal for model discrimination. Conclusively, the statistical analysis ensured that the polynomial models fitted satisfactorily for the response.

Comparison of the respective significant coefficient of a model in terms of coded value helps to evaluate the relative impact of the independent variables on the response. The following section describes the effect of each independent variable on response.

### 3.2. Effect of independent variable on comprehensive indicator

Comprehensive index corresponding to all the experimental ran ranged from 0.19 to 0.82 (Table 2). Values of “Prob>F” less than 0.0500 indicate model terms are significant. In Table 4, it was apparent that all the linear terms ($X_1$, $X_2$, $X_3$), square terms ($X_1^2$, $X_2^2$, $X_3^2$) along with the interaction terms $X_1X_2$ and $X_1X_3$ were found to be highly significant in controlling overall properties, and the linear effect of KF concentration ($X_4$) demonstrated the most significant effect with the largest coefficient and value. Based on the sum of squares, the importance of the independent variables on the combination property of films could be ranked in the following order: $KFCGL$.

The positive linear terms and negative quadratic terms of all factors indicated that with the increase of $X_1$, $X_2$, $X_3$, comprehensive index response increased up to a critical value after which a further increase in the factors resulted in a decrease in the response. The comprehensive effect of HPC, KF, and GL could be further illuminated with the response surface plots presented in Figure 1(a–c). In general, the independent variable not visible in a response surface plot was kept at “0” coded level. Thus, GL for Figure 1(a), KF for Figure 1(b), and HPC for Figure 1(c) were kept fixed at 2.00%, 0.50%, and 0.80%, respectively.

As curvature in a response surface plot suggested the involvement of a significant quadratic term, the concave curvature of the surface in Figure 1(a–b) indicated the negative quadratic effect of HPC on comprehensive response in addition to positive linear effect. The negative square contribution of KF could be revealed from Figure 1(a–c) and it could also be observed that the change trend of the surfaces altered greatly with the variation of KF, further confirming the significant effect of KF mentioned above. As shown in Figure 1(b–c), the concave curvature of the surface displayed the involvement of negative GL² except linear term as well. Moreover, these figures showed that interactions of $X_1$-$X_2$, $X_1$-$X_3$, and $X_2$-$X_3$ in lower concentration resulted in lower comprehensive responses, whereas the interactions at higher concentration region led to the reduction of responses.

### 3.3. Optimization and verification of the model

Numerical optimization was performed to predict the optimum level of independent variables to obtain the maximum response value. According to the analysis of response optimizer and response surface plots, the optimum ratio corresponding to maximum comprehensive value would be obtained at 0.586% HPC, 0.858% KF, and 1.620% GL. The corresponding predicted comprehensive response value under the recommended optimum ratio was 0.876.

After verifying by six experimental tests with the predicted values, no significant difference between the experimental and predicted values was observed (data not shown). Thus, the experimental values were found to be in agreement with the predicted ones. This indicated the suitability of the developed model and ensured the suitability of optimized level of HPC, KF, and GL in the blends for achieving maximum comprehensive value of films.

According to the related studies (Lu et al., 2018), the L. paracasei-loaded composite film obtained a good balance between TS and EAB, showing sufficient mechanical strength and elasticity. As previously reported by Lu, D. et al. (Lu et al., 2018), the composite film was suitable for the survival of L. paracasei and exhibited antibacterial activity against some foodborne pathogens.

### 3.4. Toxicology experiments in vivo

During the 14-day feeding test, the experimental mice in each group grew well, and their body weight increased gradually. No abnormalities of the mice in diet, behavioral activities, and appearance were observed, and neither toxic symptoms nor death was observed.

By performing serum biochemical analysis, organ damage and pathological changes could be detected. As this experiment mainly focused on the liver and kidney of mice, the alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (γ-GT), and alkaline phosphatase (ALP) were selected to indicate the effects of different dose groups on liver tissue, as well as the urea nitrogen (BUN), uric acid (URIC), and creatinine (CRE) indicated the effects of different dose groups on kidney tissue. It can be seen from Table 5 that there were no abnormalities in serum biochemical indexes of

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### Table 3. Analysis of variance (ANOVA) and determination coefficients.

| Parameter | Std. Dev. | R-Squared | Adj R-Squared |
|-----------|-----------|-----------|---------------|
| Mean      | 0.0152    | 0.997     | 0.994         |
| CV %      | 0.642     | 2.365     | 0.985         |
| PRESS     | 0.0117    | 58.536    |               |

### Table 4. Regression model and analysis of variance (ANOVA).

| Source | Squares | df | Square | Value | Prob > F |
|--------|---------|----|--------|-------|----------|
| Model  | 0.76    | 9  | 0.085  | 368.88 | <0.0001  |
| $X_1$  | 0.061   | 1  | 0.061  | 265.61 | <0.0001  |
| $X_2$  | 0.35    | 1  | 0.35   | 1514.12| <0.0001  |
| $X_3$  | 0.057   | 1  | 0.057  | 248.2  | <0.0001  |
| $X_4$  | 0.11E-03| 1  | 0.11E-03| 39.6  | <0.0001  |
| $X_5$  | 0.45E-03| 1  | 0.45E-03| 19.6  | 0.0013   |
| $X_6$  | 0.10E-03| 1  | 0.10E-03| 4.4   | 0.0623   |
| $X_7$  | 0.005   | 1  | 0.005  | 237.73 | <0.0001  |
| $X_8$  | 0.23    | 1  | 0.23   | 1010.4 | <0.0001  |
| $X_9$  | 0.035   | 1  | 0.035  | 151.82 | <0.0001  |
| Residual| 0.23OE-03| 10 | 2.30E-04|       |
| Lack of Fit | 1.37E-03 | 5 | 2.74E-04 | 1.47  | 0.3426   |
| Pure Error | 9.33E-04 | 1 | 1.87E-04|       |
| Cor Total | 0.077  | 19 |         |       | not significant |
Figure 1. Response surface plots and contour plots showing the interaction effects of HPC and KF (a), HPC and GL, KF and GL (c) on comprehensive property of the biocomposite films.

Figura 1. Gráficos de superficie de respuesta y gráficos de contorno que muestran los efectos de interacción de HPC y KF (a); HPC y GL (b); y KF y GL (c) sobre la propiedad integral de las películas de biocompuestos.

Table 5. The values of blood biochemical analysis on experimental mice.

| Sex       | Dose | ALT (U/L)     | AST (U/L)     | γ-GT (U/L) | ALP (U/L) | BUN (mmol/L) | URIC (mmol/L) | CRE (umol/L) |
|-----------|------|---------------|---------------|------------|-----------|--------------|---------------|--------------|
| Male      | CK   | 49.24 ± 8.02  | 130.93 ± 16.47| 0.31 ± 0.06| 142.55 ± 16.69| 6.52 ± 0.28 | 0.14 ± 0.03  | 12.68 ± 2.20 |
|           | 10   | 53.81 ± 7.32  | 125.43 ± 22.43| 0.27 ± 0.04| 148.37 ± 20.45| 6.10 ± 0.22 | 0.12 ± 0.01  | 11.37 ± 2.15 |
|           | 100  | 43.48 ± 10.31 | 118.05 ± 13.37| 0.29 ± 0.03| 131.06 ± 11.32| 7.59 ± 0.34 | 0.11 ± 0.01  | 14.59 ± 1.88 |
|           | 1000 | 56.33 ± 6.54  | 114.45 ± 37.42| 0.33 ± 0.04| 129.70 ± 18.15| 5.74 ± 0.15 | 0.09 ± 0.02  | 15.22 ± 3.05 |
|           | 10,000| 48.65 ± 8.44  | 117.38 ± 8.54 | 0.40 ± 0.02| 137.06 ± 7.26  | 6.33 ± 0.16 | 0.13 ± 0.03  | 12.83 ± 2.66 |
| Female    | CK   | 48.67 ± 9.56  | 123.08 ± 30.81| 0.28 ± 0.05| 130.28 ± 9.88  | 5.38 ± 0.12 | 0.15 ± 0.03  | 13.23 ± 1.57 |
|           | 10   | 59.71 ± 4.30  | 125.43 ± 22.43| 0.35 ± 0.01| 121.30 ± 15.14| 4.61 ± 0.10 | 0.12 ± 0.02  | 14.41 ± 2.18 |
|           | 100  | 45.40 ± 7.04  | 140.85 ± 9.55 | 0.36 ± 0.03| 135.80 ± 22.45| 5.75 ± 0.35 | 0.13 ± 0.05  | 13.53 ± 0.99 |
|           | 1000 | 56.36 ± 11.19 | 118.83 ± 21.74| 0.29 ± 0.04| 149.63 ± 13.76| 4.39 ± 0.26 | 0.14 ± 0.02  | 15.43 ± 1.64 |
|           | 10,000| 60.93 ± 10.74 | 114.35 ± 15.13| 0.42 ± 0.03| 153.60 ± 18.47| 3.88 ± 0.20 | 0.15 ± 0.04  | 12.6 ± 2.45  |

ALT, AST, γ-GT, ALP, BUN, URIC, and CRE represent for alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, alkaline phosphatase, urea nitrogen, uric acid, and creatinine, respectively.

ALT, AST, γ-GT, ALP, BUN, URIC y CRE representan alanina aminotransferasa, aspartato aminotransferasa, γ-glutamil transpeptidasa, fosfatasa alcalina, nitrógeno ureico, ácido único y creatinina, respectivamente.
each dose group and control group, and there was no significant difference between the dose groups and the control group ($P > .05$).

The gross anatomical results of the experimental mice showed that there were no significant abnormalities in morphology and weight of the organs in each of the dose groups. The liver and kidney of the maximum-dose group presented uniform colour, smooth surface, and no tumefaction, and there were no significant differences compared with the control group. As shown in Figure 2, the hepatic lobules were polygonal, and the hepatocytes were arranged radially around the central vein, as well as the glomerular and tubular structures were clear and closely arranged. Microscopic observations of the liver and kidney tissue sections of the maximum-dose group showed no abnormalities. The toxicology experiments in vivo indicated that the composite film had no short-term toxicity. As the previous in vitro cytotoxicity experiments (CCK8 method) also showed that composite film was not cytotoxic (Lu et al., 2018); therefore, it could be concluded that the *L. paracasei*-loaded composite film was food safe.

4. Conclusions

In this study, response surface methodology and fuzzy comprehensive evaluation method were successfully used to optimize the comprehensive properties of composite films. The results showed that the response values were more influenced by the KF content. When the optimum values of the independent variables were 0.586% HPC, 0.858% KF, and 1.620% GL, the optimal comprehensive response value was 0.876. The toxicology experiments in vivo indicated that the composite film had no short-term toxicity and could be considered as food-safe. The safety evaluation can be further improved in subsequent experiments. The optimized composite films currently developed had good mechanical strength, flexibility, and lactic acid bacteria viability; therefore, they had the potential to develop antibacterial food packaging, and could also be used to develop edible films containing probiotics.

Disclosure statement

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