

编号: ZKY-H-20260908-01

# 检索报告

根据委托人陈宣伯要求，通过网络检索，期刊《Food Frontiers》（ISSN: 2643-8429 / 2643-8429）是 2025 年《中国科学院期刊分区表（升级版）》数据库收录期刊，具体收录情况如下。

|                       |                       |
|-----------------------|-----------------------|
| 刊名                    | Food Frontiers        |
| 年份                    | 2025                  |
| ISSN / EISSN          | 2643-8429 / 2643-8429 |
| Review                | 否                     |
| OA Journal Index（OAJ） | 否                     |
| Open Access           | 是                     |
| Web of Science        | ESCI                  |

|    |                                |    |        |
|----|--------------------------------|----|--------|
|    | 学科                             | 分区 | Top 期刊 |
| 大类 | 农林科学                           | 1  | 是      |
| 小类 | FOOD SCIENCE & TECHNOLOGY 食品科技 | 2  | -      |

查证检索：

西北农林科技大学图书馆  
2026 年 9 月 8 日





## RESEARCH ARTICLE OPEN ACCESS

# Ligand Fishing for Xanthine Oxidase Inhibitors From *Brassica rapa* L. via Bioaffinity Ultrafiltration-HPLC-MS/MS: Mechanistic Insights and In Vitro Anti-Hyperuricemic Activity

Xuanbo Chen<sup>1</sup> | Haiyu Wu<sup>2</sup> | Ziwei Guo<sup>2</sup> | Zhuoyuan He<sup>1</sup> | Chuanying Pan<sup>1</sup> | Xianying Lan<sup>1</sup> | Lin Han<sup>2</sup>

<sup>1</sup>College of Animal Science and Technology, Northwest A&F University, Xianyang, People's Republic of China | <sup>2</sup>College of Food Science and Engineering, Northwest A&F University, Xianyang, People's Republic of China

Correspondence: Xianying Lan (lanxianying79@nwsuaf.edu.cn) | Lin Han (hanlin730@163.com)

Received: 22 June 2026 | Revised: 6 August 2026 | Accepted: 12 August 2026

Keywords: *Brassica rapa* L. | hypouricemic effects | UF-HPLC-MS/MS | xanthine oxidase inhibitors

## ABSTRACT

Hyperuricemia is a prevalent metabolic disorder arising from dysregulated purine metabolism, wherein xanthine oxidase (XOD) serves as the key rate-limiting enzyme catalyzing the final oxidation of hypoxanthine and xanthine to uric acid. Although *Brassica rapa* L. (chamagu) has been previously reported to exert antihyperuricemic effects, the specific bioactive constituents responsible for XOD inhibition and their underlying mechanisms remain elusive. In this study, UF-HPLC-MS/MS was employed to screen 183 potential XOD inhibitors from the water extract of chamagu. Through combined molecular docking and in vitro activity assays, psoralen was identified as a potent XOD inhibitor ( $IC_{50} = 0.237 \pm 0.003$  mg/mL) and selected for further characterization. Mechanistically, psoralen binds spontaneously to XOD at a 1:1 stoichiometric ratio, primarily driven by van der Waals forces and hydrogen bonding, which induce local conformational perturbations in the protein microenvironment and consequently suppress enzymatic activity. In cellular models, psoralen significantly reduced XOD activity and uric acid levels in xanthine-stimulated BRL-3A hepatocytes, while concurrently alleviating oxidative stress as evidenced by decreased superoxide anion ( $O_2^{\cdot-}$ ) levels and restored total antioxidant capacity. Moreover, in uric acid-treated HK-2 renal tubular cells, psoralen mitigated oxidative stress (decreased MDA levels and increased SOD activity) and inflammatory responses (reduced IL-1 $\beta$ , IL-6, and TNF- $\alpha$  levels, and increased IL-10 levels). Collectively, these findings elucidate the molecular basis of the uric acid-lowering action of chamagu via XOD targeting, providing experimental rationale for its further development and utilization as a functional food.

## 1 | Introduction

Hyperuricemia is a chronic metabolic disorder characterized by abnormally elevated serum uric acid (UA) level resulting from purine metabolism dysfunction (Du et al. 2024). In recent

years, with global economic development and changes in dietary patterns (e.g., the widespread consumption of high-purine and high-fructose diets), the prevalence of hyperuricemia has shown a sharp increase and a trend toward younger age groups (T. Zhang et al. 2020). Studies have indicated that hyperuricemia is closely

Xuanbo Chen and Haiyu Wu contributed equally to this work.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2026 The Author(s). *Food Frontiers* published by Nanchang University, Northwest University, Jiangsu University, Fujian Agriculture and Forestry University, International Association of Dietetic Nutrition and Safety and John Wiley & Sons Australia, Ltd.



associated with multiple diseases, including gout, kidney injury, cardiovascular disease, and metabolic syndrome (Kasahara et al. 2023; Wan et al. 2016; J. Wang et al. 2022; Zhong et al. 2024; Y. Zhu et al. 2014). In the UA production pathway, xanthine oxidase (XOD) is the key rate-limiting enzyme that catalyzes the hypoxanthine and xanthine to UA, thus representing an important therapeutic target for clinical UA-lowering agents (FitzGerald et al. 2020). Although currently used XOD inhibitors, such as febuxostat, topirostat, and allopurinol, exhibit favorable therapeutic efficacy, they still have certain clinical adverse effects and application limitations, including the increase of cardiovascular risk (White et al. 2018), hepatotoxicity (Dewi et al. 2025) and promotion of severe skin allergy (Ishikawa et al. 2020; Chia and Leung 2021). Therefore, screening XOD inhibitors with high safety and well-defined activity from natural products is of great significance for the adjunctive treatment of hyperuricemia and the development of functional foods. In the recent decades, cellular experiments are commonly used to investigate the XOD inhibitory activity of natural compounds. As XOD is mainly expressed in the liver, BRL-3A cells are widely employed as the ideal model to evaluate the anti-hyperuricemia activity (Ullah et al. 2024). Moreover, XOD catalyzes xanthine and hypoxanthine into UA, which is delivered to kidneys by blood to cause damages, including oxidative stress and inflammatory response (Ullah et al. 2024). Thus, HK-2 cells are always used to create UA-induced kidney damages model, and which is often employed to investigate the kidney protective activity of antihyperuricemic functional compounds (Hou et al. 2019).

Chamagu (*Brassica rapa* L.) is a representative medicinal and edible plant in the Xinjiang region of China with a long history of consumption and medicinal use (Cao et al. 2021). Previous studies have shown that chamagu contains various bioactive components, including flavonoids, polyphenols, polysaccharides, and glucosinolates, and exhibits multiple biological activities, such as antioxidant, anti-inflammatory, and immunomodulatory effects (Paul et al.; H. Zhu et al. 2022). The anti-hyperuricemic effect of chamagu has been perceived and recorded in several Chinese medical classics, such as Shiliao Bencao (Dietetic Meteria Medica) and Qianjin-Shizhi, which has gradually attracted attention in recent years. In the alpine regions of Europe and Central Asia, chamagu has been widely used to alleviate gout, arthritis, and rheumatic pain (Bussmann et al. 2020). Researchers have preliminarily explored the anti-hyperuricemic effect of chamagu using network pharmacology, metabolomics, and animal experiments. Sousa et al. (2009) found that the flavonoids and polyphenols-originated from chamagu in *Pieris brassicae* showed strong XOD inhibitory activity (Sousa et al. 2009). Zaabat et al. (2025) confirmed that polyphenolic components in chamagu root extract possess strong binding and inhibitory activities against metabolism-related enzyme targets, including gout-related targets through in vitro enzymatic assays and molecular docking analysis (Zaabat et al. 2025). In another study, researchers systematically identified the chemical constituents of chamagu by UPLC-Q/Orbitrap HRMS and predicted that chamagu exerts its anti-hyperuricemic effect primarily through the PI3K/AKT signaling pathway. They also identified eight core targets and nine core active components (Wei et al. 2025). These previous studies have mainly focused on the extract level or macro-mechanism analyses based on the database predictions, but the systematic

research on the key XOD-inhibiting compounds in chamagu, as well as their inhibitory mechanisms remain unclear.

Presently, ultrafiltration-liquid chromatography mass spectrometry (UF-HPLC-MS/MS) was employed to screen and identify the XOD-inhibitory compounds in chamagu. Key active molecules were further determined through molecular docking and in vitro XOD inhibition assays. Subsequently, the inhibitory kinetic characteristics and mechanisms of the screened key active compound were elucidated from structural perspectives, including enzyme inhibition kinetics, fluorescence spectroscopy, circular dichroism (CD), and atomic force microscopy. Finally, cellular experiments were conducted to validate the UA-lowering effect and the cellular protective activity of the identified XOD inhibitor with the aim of providing a natural alternative for the treatment of hyperuricemia.

## 2 | Materials and Methods

### 2.1 | Materials

Chamagu (*B. rapa* L.) was collected from Keping County, Aksu Prefecture, Xinjiang Uygur Autonomous Region (GPS location: E79.343, N40.520) of China in October 2024, and the product batch number is 10079358018775. XOD (10 U/mg, from *Arthrobacter* sp., product No. X80001-1KU) was purchased from Beijing PuXiTang Biotechnology Co. Ltd. (Beijing, China). Xanthine (Product No. B20561) was obtained from Shanghai Yuanye Bio-Technology Co. Ltd. Hydrochloric acid, sodium hydroxide, absolute ethanol, acetone, methanol, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, dimethyl sulfoxide (DMSO), and ammonium acetate were of analytical grade and purchased from Xilong Chemical Co. Ltd.

### 2.2 | Preparation of Chamagu Extracts

Fresh chamagu roots were washed, peeled, destemmed, cut into pieces, and dried at 50°C. The dried samples were pulverized using a high-speed grinder and passed through a 100-mesh sieve. Presently, three solvents (water, 70% ethanol [v/v], and 80% acetone [v/v]) with different polarities were employed to prepare the extracts from chamagu according to the previous reports (Han et al. 2023; H. Wu et al. 2026). The obtained powder (20 g each) was mixed separately with deionized water, 70% (v/v) ethanol, or 80% (v/v) acetone at a solid-to-liquid ratio of 1:20 (g/mL) and ultrasonically extracted (80 kHz) at 40°C for 20 min uniformly. After centrifugation (5000 r/min, 10 min), the supernatant was collected, respectively. The residue was re-extracted twice under the same conditions by the corresponding solvents, and all three supernatants were combined. The combined extract was filtered to remove solid impurities and concentrated to 50 mL by rotary evaporation at 45°C. The concentrate was transferred to a petri dish, sealed with plastic wrap, and pre-frozen at −80°C for 24 h. After that, the sample was freeze-dried in a freeze dryer with a stoppered organic-resistant system. The resulting lyophilized powder was stored at −20°C in sealed centrifuge tubes until use (Han et al. 2023).



### 2.3 | XOD Inhibitory Activity Assay

The assay was performed in a 96-well microplate. In each well, 100  $\mu\text{L}$  of PBS, 40  $\mu\text{L}$  of XOD solution (0.01 U/mL), and 20  $\mu\text{L}$  of sample working solution (various concentrations of water extract, ethanol extract, acetone extract, or allopurinol) were sequentially added. The mixture was incubated at 37°C for 15 min. Subsequently, 40  $\mu\text{L}$  of xanthine substrate solution (300  $\mu\text{mol/L}$ ) was mixed and the reaction mixture was further incubated at 37°C for 30 min. The reaction was terminated by adding 40  $\mu\text{L}$  of hydrochloric acid. XOD catalyzes the conversion of xanthine to UA, and the absorbance was measured at 290 nm. The UA production was used to reflect enzyme activity. The inhibition rate of XOD was calculated by the following formula:

$$\text{Inhibition rate (\%)} = \left(1 - \frac{A_1 - A_2}{A_3 - A_4}\right) \times 100$$

where  $A_1$  is absorbance of the sample test group (sample + XOD);  $A_2$  is absorbance of the sample control group (sample + PBS);  $A_3$  is absorbance of the control group (PBS + XOD);  $A_4$  is absorbance of the blank control group (PBS + PBS). The half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) for each extract was determined by nonlinear regression analysis using SPSS Statistics 26 software (Han et al. 2023).

### 2.4 | Ultrafiltration Coupled With HPLC-MS/MS

The ultrafiltration procedure was performed according to the method of Lu et al. (2021) and X.-D. Zhang et al. (2025) with slight modifications. Based on the previous reports and our preliminary experiments, the ratio of aqueous extract to XOD, as well as the working concentrations of aqueous extract and XOD were determined as 1:1, 50 mg/mL, and 0.5 U/mL, respectively. Briefly, the lyophilized powder was dissolved in 10 mmol/L ammonium acetate buffer (pH 6.86), filtered through a 0.45  $\mu\text{m}$  membrane, and adjusted to a concentration of 50 mg/mL. A 200  $\mu\text{L}$  aliquot of the sample solution was mixed with 200  $\mu\text{L}$  of XOD solution (0.5 U/mL) and incubated in an orbital shaker at 37°C and 500 r/min for 30 min. The mixture was transferred to an ultrafiltration tube and centrifuged at 10,000 r/min for 15 min. The filtrate was discarded, and the residue was washed three times with 100  $\mu\text{L}$  of ammonium acetate buffer by centrifugation. Subsequently, 100  $\mu\text{L}$  of 50% methanol-water (pH 3.3) was added, and after gentle vortexing, the tube was centrifuged at 10,000 r/min for 15 min to collect the filtrate. This elution step was repeated three times, and the combined filtrates were lyophilized. Prior to analysis, the lyophilized sample was reconstituted in 200  $\mu\text{L}$  of methanol-water (50:50, v/v) for HPLC-MS/MS analysis.

HPLC-MS/MS analysis was performed using an ultra-high performance liquid chromatography system (ExionLC AD) coupled with a tandem mass spectrometer. The liquid chromatographic conditions were as follows: an Agilent SB-C18 column (1.8  $\mu\text{m}$ , 2.1 mm  $\times$  100 mm) was used; Mobile Phase A was water containing 0.1% formic acid, and Mobile Phase B was acetonitrile containing 0.1% formic acid. The gradient elution program was: 0–9 min, 5%–95% B; 9–10 min, 95% B; 10–11.1 min, 95%–5% B; 11.1–14 min, 5% B. The flow rate was 0.35 mL/min, the column temperature was 40°C, and the injection volume was 2  $\mu\text{L}$ .

The mass spectrometric conditions were: an electrospray ionization source with a temperature of 500°C; ion spray voltage of 5500 V in positive ion mode and –4500 V in negative ion mode; nebulizer gas (GSI), auxiliary gas (GSII), and curtain gas (CUR) set at 50, 60, and 25 psi, respectively. Multiple reaction monitoring (MRM) mode was used with medium collision gas (nitrogen). Qualitative analysis was performed based on an in-house MWDB database (Metware Biotechnology Co. Ltd., Wuhan, China), excluding isotopic signals and interfering ions containing  $\text{K}^+$ ,  $\text{Na}^+$ , or  $\text{NH}_4^+$ . Quantitative analysis was carried out in MRM mode, and peak area integration and chromatographic peak alignment were performed using MultiQuant software (Fraga et al. 2010).

### 2.5 | Molecular Docking

The three-dimensional crystal structure of XOD was obtained from the RCSB PDB database (PDB ID: 3NRZ) and validated using the UniProt database. The three-dimensional structures of 12 core components were downloaded from the PubChem database in SDF format. Molecular docking was performed using CB-Dock2 (<https://cadd.labshare.cn/cb-dock2>). The lowest binding energy of each complex was extracted as the affinity indicator, with binding energies below –5 kcal/mol considered indicative of binding activity.

### 2.6 | Molecular Dynamics Simulation

Molecular dynamics simulation was performed using GROMACS 2022. The receptor was described with the AMBER14SB force field. Ligand topologies were generated by sobtop 1.0 (dev3.1) based on GAFF2, with atomic charges assigned via the RESP method. The system was placed in a cubic box with a minimum distance of 1.0 nm between the protein surface and the box boundary, solvated with TIP3P water, and neutralized by adding 0.15 M NaCl. Long-range electrostatic interactions were treated with the particle mesh Ewald (PME) method using a real-space cutoff of 1.0 nm. Bond constraints were handled by LINCS (with SETTLE for water molecules). Prior to production run, energy minimization was performed using steepest descent, followed by conjugate gradient, NVT equilibration (310 K, 100 ps) and NPT pre-equilibration (310 K, 1 bar, 100 ps). A 100 ns NPT production simulation was then conducted at 310 K and 1 bar, maintained by the Nosé–Hoover thermostat and Parrinello–Rahman barostat, respectively, with an integration time step of 2 fs. Post-simulation, root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), number of hydrogen bonds, radius of gyration (Rg), and solvent-accessible surface area (SASA) were calculated using GROMACS built-in tools to evaluate system stability, structural changes, and solvent effects.

### 2.7 | Kinetic Study of Enzymatic Inhibition

The inhibition kinetics assay was performed according to Han et al. (2023) with minor modifications. XOD inhibitor identified by ultrafiltration-HPLC-MS/MS was used at various concentrations. Different concentrations of each inhibitor were added to XOD solution (0.1 U/mL) and incubated at 37°C for 10 min.

The reaction was initiated by adding xanthine substrate, and the absorbance at 290 nm was recorded every 60 s. The kinetic parameters were quantified using the Lineweaver–Burk double-reciprocal plot to determine the type of enzyme inhibition. The following equation was used:

For competitive inhibition:

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \left( 1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \frac{1}{V_{\max}}$$

The secondary plot:

$$K_m^{\text{app}} = \frac{K_m[I]}{K_i} + K_m$$

For non-competitive and mixed-type inhibitions:

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \left( 1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \frac{1}{V_{\max}} \left( 1 + \frac{[I]}{\alpha K_i} \right)$$

The secondary plot:

$$\text{Slope} = \frac{K_m[I]}{K_i} + \frac{K_m[I]}{V_{\max} K_i}$$

For mixed-type inhibition:

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \left( 1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \frac{1}{V_{\max}} \left( 1 + \frac{[I]}{\alpha K_i} \right)$$

$$\text{Slope} = \frac{K_m[I]}{K_i} + \frac{K_m[I]}{V_{\max} K_i}$$

$$Y \text{ intercept} = \frac{1}{V_{\max}} + \frac{1}{\alpha K_i V_{\max}} [I]$$

where  $K_m$  is Michaelis–Menten constant;  $V_{\max}$  and  $V$  represent enzyme reaction velocity and maximum velocity, respectively;  $K_i$  is the inhibition constant;  $[I]$  and  $[S]$  are the concentration of inhibitors and substrate, respectively;  $\alpha$  is the apparent coefficient.

## 2.8 | Fluorescence Titration Analysis

The interaction between small molecule and XOD was investigated by steady-state fluorescence titration. In a quartz cuvette, 1.5 mL of XOD solution (10 U/mL) was added, followed by successive additions of the inhibitor. After each addition, the mixture was thoroughly mixed and allowed to equilibrate for 5 min. Fluorescence emission spectra were recorded from 300 to 460 nm with an excitation wavelength of 280 nm, and both excitation and emission slit widths were set to 5.0 nm. To examine the temperature effect, measurements were performed at 298, 304, and 310 K. All samples were measured under identical instrumental parameters to ensure comparability.

To further elucidate the mechanism of inhibitor-induced fluorescence quenching of XOD, the experimental data were fitted using

the Stern–Volmer equation:

$$F_0/F = 1 + K_{SV}[Q] = 1 + K_q\tau_0[Q]$$

where  $F_0$  and  $F$  are the fluorescence intensities of XOD in the absence and presence of the inhibitor, respectively;  $K_{SV}$  is the Stern–Volmer quenching constant;  $[Q]$  is the inhibitor concentration;  $K_q$  is the bimolecular quenching rate constant; and  $\tau_0$  is the average lifetime of the fluorophore in the absence of quencher (taken as  $10^{-8}$  s).

The binding constant ( $K_a$ ) and the number of binding sites ( $n$ ) were calculated using the double-logarithmic equation:

$$\log \frac{F_0 - F}{F} = n \log K_a + n \log [Q]$$

Thermodynamic parameters were determined using the van't Hoff equation and the Gibbs free energy equation:

$$\ln K_a = -\Delta H/RT + \Delta S/R$$

$$\Delta G = \Delta H - T\Delta S$$

where  $R$  is the molar gas constant (8.314 J/(mol·K));  $T$  is the absolute temperature (K). Based on the signs of the calculated enthalpy change ( $\Delta H$ ) and entropy change ( $\Delta S$ ), the predominant non-covalent driving forces between the inhibitor and XOD were determined according to the classical thermodynamic principles of Ross and Subramanian.

## 2.9 | CD Determination

CD determination was operated on the chirascan CD spectrometer (Chirascan V100, Applied Photophysics Ltd., Leatherhead, UK). The spectral band width was set to 2 nm, the response time of 1 s, the path length of 1.0 mm, and the experiments were conducted at a scanning rate of 30 nm/min. XOD (10 U/mg) was mixed to psoralen with different ratios (1:1, 5:1), and pure water was used as a blank for background correction. The obtained spectra were smoothed using CDNN software, and the secondary structure changes of the different CD spectral curves were calculated.

## 2.10 | Atomic Force Microscope (AFM) Test

AFM can assess morphological changes on the protein surface as well as distance, particle size, and force between binding molecules. Based on previous studies (Mehmood et al. 2022; Mehmood et al. 2020), 100  $\mu$ L of XOD solution (0.1 U/mL) was mixed with the inhibitor (500  $\mu$ g/mL) and incubated at 37°C for 30 min. The mixture was then uniformly spread onto a mica sheet and dried at room temperature for 12 h. The XOD solution without the ligand served as the control group. Surface structure images were obtained using a Multimode-8 atomic force microscope and SCANASYST-AIR probe (Bruker Co., Santa Barbara, USA). Nanoscope Analysis V1.18 software was then used to process and analyze the height image.



## 2.11 | The Anti-Hyperuricemia and Cellular Protective the Active Compound in Chamagu

### 2.11.1 | Cell Culture

BRL-3A rat hepatocytes were cultured in MEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. HK-2 human renal tubular epithelial cells were cultured in DMEM/F12 supplemented with 10% FBS and 1% antibiotics. Both cell lines were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. BRL-3A and HK-2 cells were subcultured every 1–2 days and 2–3 days, respectively. All experiments were performed using cells in the logarithmic growth phase without contamination.

### 2.11.2 | Cell Viability Assay

Cell viability was assessed using the CCK-8 assay. Logarithmic-phase cells were trypsinized, resuspended, seeded into 96-well plates (100 µL per well), and cultured for 12 h to allow attachment. The medium was then replaced with basal medium containing various concentrations of psoralen. After 48 h of treatment, 100 µL of medium containing 10% CCK-8 reagent was added to each well, followed by incubation for 1–2 h in the dark. Absorbance was measured at 450 nm using a microplate reader. Cell viability was calculated as:

$$\text{Cell viability (\%)} = \frac{A_{\text{exp}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100\%$$

where  $A_{\text{exp}}$  is the absorbance of treated wells;  $A_{\text{blank}}$  of wells containing only medium and CCK-8;  $A_{\text{control}}$  of untreated control wells.

### 2.11.3 | Cellular Treatments

To determine the XOD inhibitory activity of psoralen in BRL-3A cells, five groups, including control group (NC), model group (MD, treated by 100 µM PSO), positive control group (AP, treated by 10 µM allopurinol), psoralen low-dose group (PSO-100 µM + 100 µM psoralen), and psoralen high-dose group (PSO-200 µM + 200 µM psoralen), were divided and treated for 48 h. After that, the parameters (UA content, XOD activity, superoxide anion levels, and total antioxidant capacity [T-AOC]) were determined by the corresponding test kits. To investigate the protective activity of psoralen on UA-induced kidney damages, HK-2 cells were cultured and divided to five groups: control group (NC), model group (MD, treated by 400 µM UA), positive control group (AP, treated by 10 µM allopurinol), psoralen low-dose group (UA-400 µM + 100 µM psoralen), and psoralen high-dose group (UA-400 µM + 200 µM psoralen). After treatment for 48 h, the inflammatory factors and oxidative stress were determined.

### 2.11.4 | UA Content in BRL-3A Cells

BRL-3A cells were seeded in 6-well plates and cultured for 16 h. After washing with PBS, the medium was replaced with MEM basal medium containing xanthine and psoralen as described. Following 48 h of treatment, the culture supernatant was col-

lected and centrifuged. UA levels were determined using a commercial kit (Solarbio, BC1365).

### 2.11.5 | XOD Activity in BRL-3A Cells

BRL-3A cells were washed twice with PBS and lysed in XOD extraction buffer by sonication on ice. The lysate was centrifuged, and the supernatant was collected. XOD activity was measured using an activity assay kit (Solarbio, BC1095).

### 2.11.6 | Superoxide Anion Level and T-AOC in BRL-3A Cells

Treated BRL-3A cells were lysed by sonication on ice in extraction buffer. After centrifugation, the supernatant was collected. Superoxide anion content and T-AOC were measured using commercial kits (Solarbio, BC1295 and BC1315, respectively).

### 2.11.7 | Inflammatory Cytokine Levels in HK-2 Cells

After 48 h of treatment, the culture supernatant of HK-2 cells was collected and centrifuged (3000 r/min, 4°C, 10 min). The levels of IL-1β, IL-6, TNF-α, and IL-10 were determined using ELISA kits (Shanghai Jianglai, JL13662, JL14113, JL10208, JL19246) according to the manufacturer's instructions.

### 2.11.8 | MDA Content and SOD Activity in HK-2 Cells

After 48 h of treatment, HK-2 cells were washed twice with cold PBS and lysed in extraction buffer by sonication on ice (200 W, 3 s on, 10 s off, 30 cycles). The lysate was centrifuged, and the supernatant was collected. Protein concentration was determined by the BCA method. Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were measured using commercial kits (Solarbio, BC0025 and BC5165, respectively).

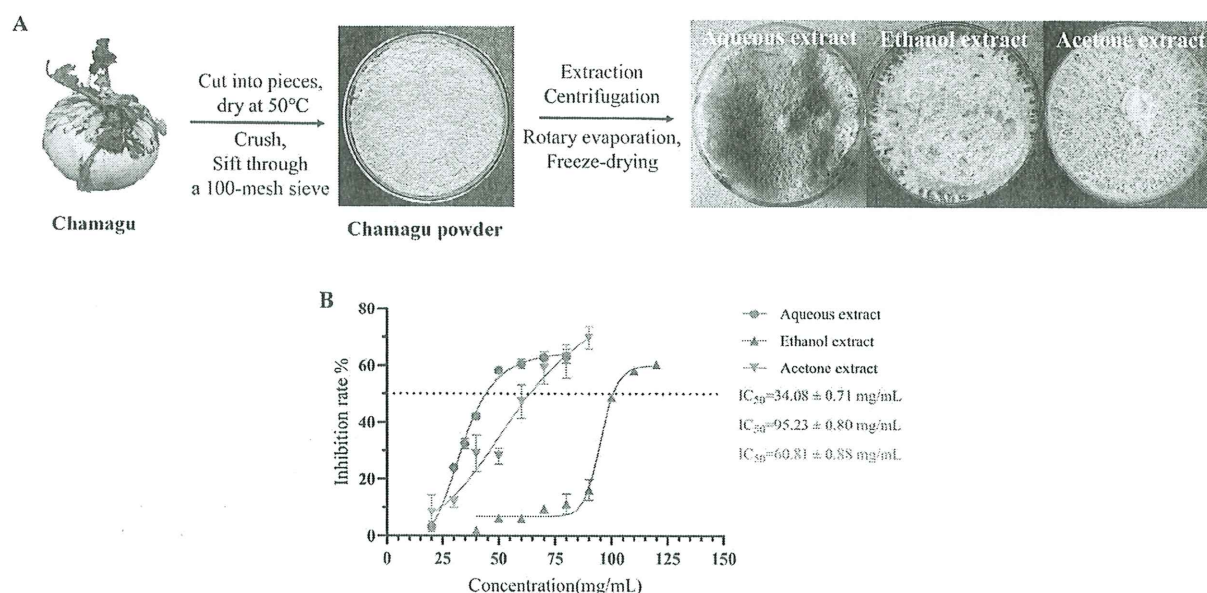
## 2.12 | Statistical Analysis

All experiments were carried out with three independent biological replicates ( $n = 3$ ). Each measurement was performed in triplicate. Data were presented as mean ± standard deviation. Significant differences between means were estimated by analysis of variance (ANOVA) followed by Duncan's multiple range test at a significance level of  $p < 0.05$  using SPSS 21.0 software. Graphs were prepared using GraphPad Prism 10.1.2.

## 3 | Results and Discussion

### 3.1 | Inhibitory Activity of Chamagu Extracts on XOD

The fresh chamagu plants were dried and ground, and the resulting powder was sieved through a 100-mesh sieve to obtain a pale yellow powder (Figure 1A). Subsequently, ultrasonic-assisted extraction was performed on the powder using water,



**FIGURE 1** | The (A) preparation process and (B) XOD inhibitory effect of chamagu extracts.

70% (v/v) ethanol, and 80% (v/v) acetone as extraction solvents. The obtained extracts were concentrated by rotary evaporation under reduced pressure and then freeze-dried to yield three types of freeze-dried powders: water, ethanol, and acetone extracts of chamagu.

To evaluate the inhibitory effects of the three chamagu extracts on XOD, each extract was incubated with XOD, followed by the addition of the substrate xanthine. The amount of UA generated was determined by measuring the absorbance at 290 nm, and the inhibition rates of each extract against XOD were calculated accordingly. As shown in Figure 1B, all three extracts exhibited *in vitro* XOD inhibitory activity, but their inhibitory potency exhibited markedly differences. The half-inhibitory concentration ( $IC_{50}$ ) values of the water, ethanol, and acetone extracts against XOD were  $34.08 \pm 0.71$ ,  $95.23 \pm 0.80$ , and  $60.81 \pm 0.88$  mg/mL, respectively. Aqueous extract, with lower value of  $IC_{50}$ , showed significant stronger inhibitory effect on XOD than that of ethanol and acetone extracts. Although the aqueous extract of chamagu did not revealed the very strong anti-XOD activity than some other plant-food due to the difference of specific experimental process, this fraction might contain potential active compounds that were responsible for its inhibitory capacity. Thus, the aqueous extract was selected as the primary candidate for further screen and identification of the potential XOD inhibitors.

### 3.2 | Ultrafiltration and Identification of the XOD Inhibitors From Chamagu

The above study showed that the water extract of chamagu exhibited the best inhibitory effect on XOD. However, the specific compounds responsible for this inhibition remained unclear. Therefore, ultrafiltration combined with non-targeted metabolomics, one of the effective method to screen and identify the active compounds, was used to screen XOD inhibitors from

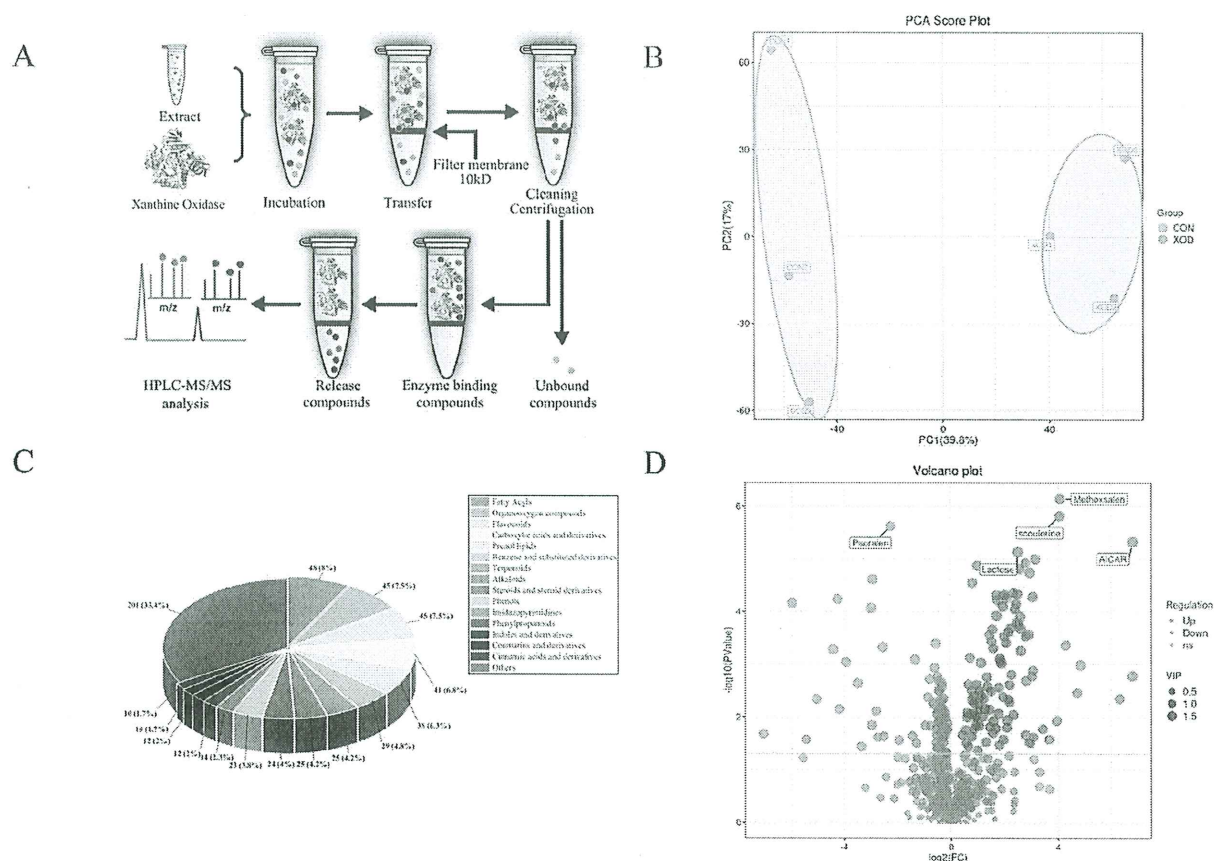
chamagu (Figure 2A) (Dong et al. 2026; Ren et al. 2024). Principal component analysis (PCA) results showed that samples from the CON group (control group) and the XOD group (experimental group) exhibited a distinct separation trend in the principal component space, with differentiation primarily occurring along the PC1 axis (Figure 2B). This indicated that the overall metabolic profile of the chamagu water extract underwent significant changes following XOD incubation and ultrafiltration, laying the foundation for subsequent screening of differential compounds. As shown in Figure 2C, a total of 602 compounds were identified by HPLC-MS/MS, spanning multiple chemical classes and exhibiting significant structural diversity.

Using screening criteria of  $p < 0.05$  and  $VIP > 1.0$ , a differential compound analysis was conducted between the CON and XOD groups, leading to the identification of 183 differential compounds. Volcano plot analysis further revealed that the distributions of these compounds were distinctly separated between the two groups, confirming significant differences in metabolic profiles. Among these, 78 compounds were significantly less abundant in the XOD group ( $\log_2 FC < 0$ ) (Table S1). Furthermore, the volcano plot clearly displayed the distribution of candidate compounds with potential XOD-binding capability (Figure 2D). Among them, psoralen ( $\log_2 FC = -2.28$ ) and several others exhibited evident affinity-based enrichment characteristics.

### 3.3 | Screening and Verification of Key XOD Inhibitors From Chamagu

Based on the differential compound screening results, further screening was conducted by combining molecular docking with *in vitro* XOD inhibition assays. First, molecular docking was performed on the 183 differential metabolites using CB-Dock2 with the XOD crystal structure (PDB ID: 3NRZ) as the receptor. Using allopurinol and febuxostat as positive control, the candidate compounds were ranked by binding energy, and the





**FIGURE 2** | (A) UF-HPLC-MS/MS flow chart, (B) PCA score plot, (C) pie chart of compound categories, and (D) differential compound volcano plot.

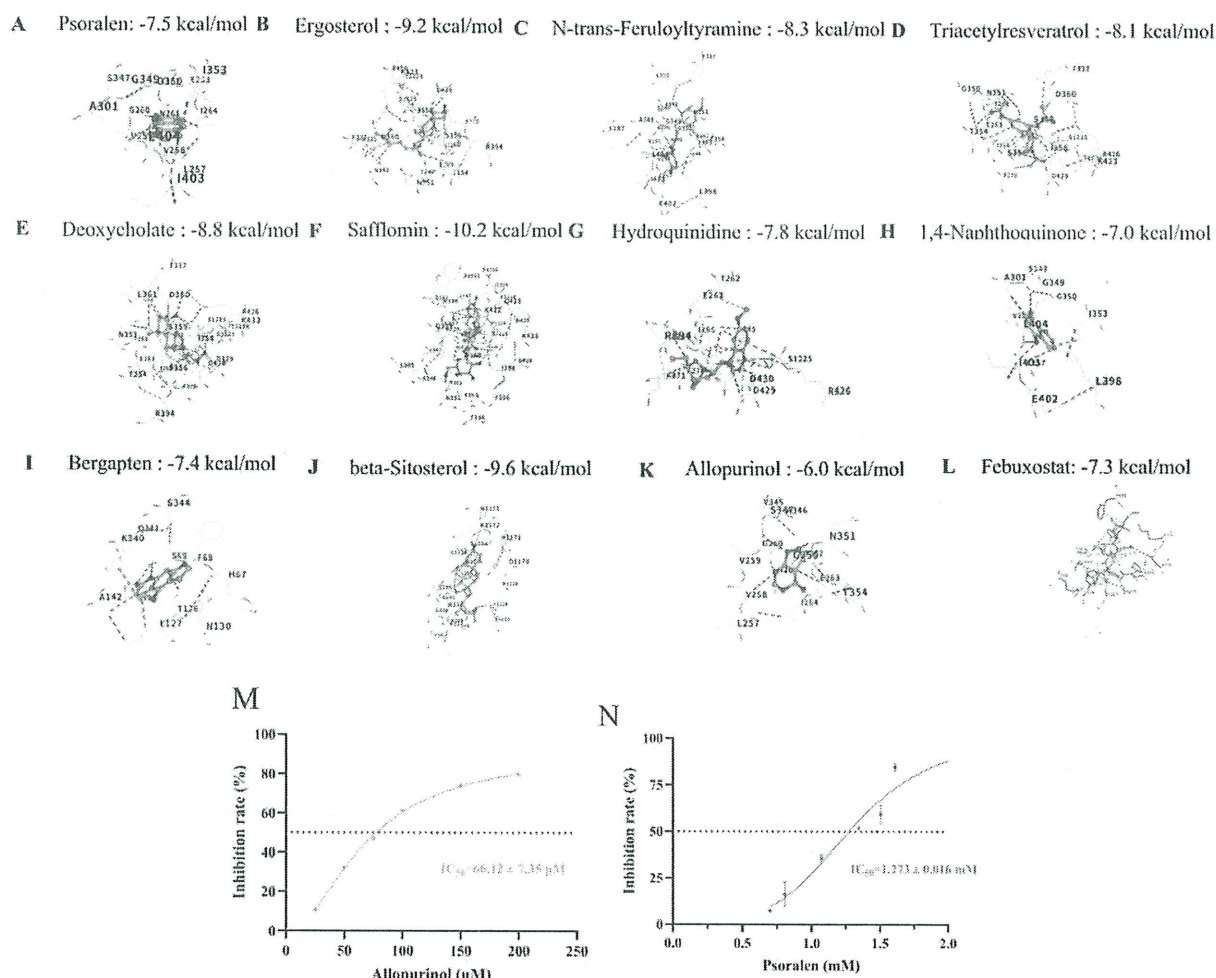
top 10 compounds were selected and the detailed HPLC-MS/MS information were shown in Table S2. As shown in Figure 3, these 12 compounds could effective enter to the active site of XOD and primarily bound to the surrounding amino residues through hydrogen bonding, hydrophobic interactions, van der Waals forces, and  $\pi$ - $\pi$  interactions. Previous studies showed that compounds with lower binding energies typically formed multi-site, multi-type non-covalent interactions with the receptor, thereby contributing to the stability of the ligand-receptor complex (Nguyen et al. 2026). Among these inhibitors, psoralen exhibited the lowest binding energy of  $-9.3$  kcal/mol. Psoralen not only formed hydrogen bonds and hydrophobic interactions with the receptor but also formed  $\pi$ - $\pi$  stacking interactions with aromatic residues. This multi-mode binding might account for its lowest binding energy. To further validate the molecular docking results, the XOD inhibitory activity of psoralen was evaluated in vitro, yielding an  $IC_{50}$  value of  $0.237 \pm 0.003$  mg/mL.

The results of molecular docking and in vitro assays demonstrated that psoralen possessed potential enzyme inhibitory activity. It was speculated that its planar aromatic conjugated skeleton and moderate polar functional groups might facilitate binding to XOD and exert inhibitory effects (Fais et al. 2023). However, psoralen exhibited moderate anti-XOD capacity compared to other polyphenols and flavonoids, such as quercetin, kaempferol, phloretin, pinosylvin, and daidzin, as well as the positive drug,

allopurinol (Ullah et al. 2024). This situation might be closely related to the specific experimental conditions, including the enzyme activity of XOD and the ratio for the inhibitors to XOD (V:V) that used in the experiment (Ullah et al. 2024). In the present study, psoralen was screened and identified as the key compound in chamagu with stronger XOD inhibitory activity, and which was selected to further explore the underlying mechanisms against XOD in vitro. Most importantly, more in vivo and clinical experiments are necessary to be performed to further confirm the anti-hyperuricemia activity of this compound.

### 3.4 | Molecular Dynamics Simulation Analysis

Molecular dynamics simulations showed that the XOD-psoralen complex reached a stable state after approximately 5 ns, with the RMSD remaining around 1.3 Å and exhibiting minimal fluctuations (Figure 4A). The radius of gyration (Rg) and SASA fluctuated minimally throughout the simulation, indicating that psoralen binding did not cause the overall structure of the protein to destabilize or induce significant changes in surface conformation (Figure 4B,C). These results suggested that the complex possessed good overall stability (Sahoo et al. 2025). Hydrogen bond analysis revealed that the number of hydrogen bonds formed between psoralen and XOD in the complex ranged primarily from 0 to 4, with 1 being the most frequent (Figure 4D).



**FIGURE 3** | Molecular docking analysis of the top ten compounds and two positive drugs based on binding energy (A–L), and the inhibitory effects of the (M) positive control and (N) psoralen on XOD.

The fluctuation in the number of hydrogen bonds during the simulation further indicated that the interaction between psoralen and XOD was not entirely rigid but maintained a certain degree of flexibility during dynamic processes. This dynamic variation suggested that the binding site might exhibit conformational adaptability, which was conducive to maintaining stable ligand binding within the pocket (M. Liu et al. 2024). Thus, the hydrogen bond analysis further demonstrated that psoralen formed a relatively stable interaction with XOD. RMSF analysis showed that the RMSF values of the vast majority of amino acid residues were below 4 Å (Figure 4E), indicating that local structural fluctuations of the protein were small and residue motions were relatively restricted, thereby helping to maintain the stability of the complex conformation (Nguyen et al. 2026). This finding indicated that psoralen binding did not cause extensive local structural perturbations in XOD. These results were consistent with the RMSD, Rg, and SASA analyses, further demonstrating that psoralen maintained a relatively stable binding state in the XOD binding pocket.

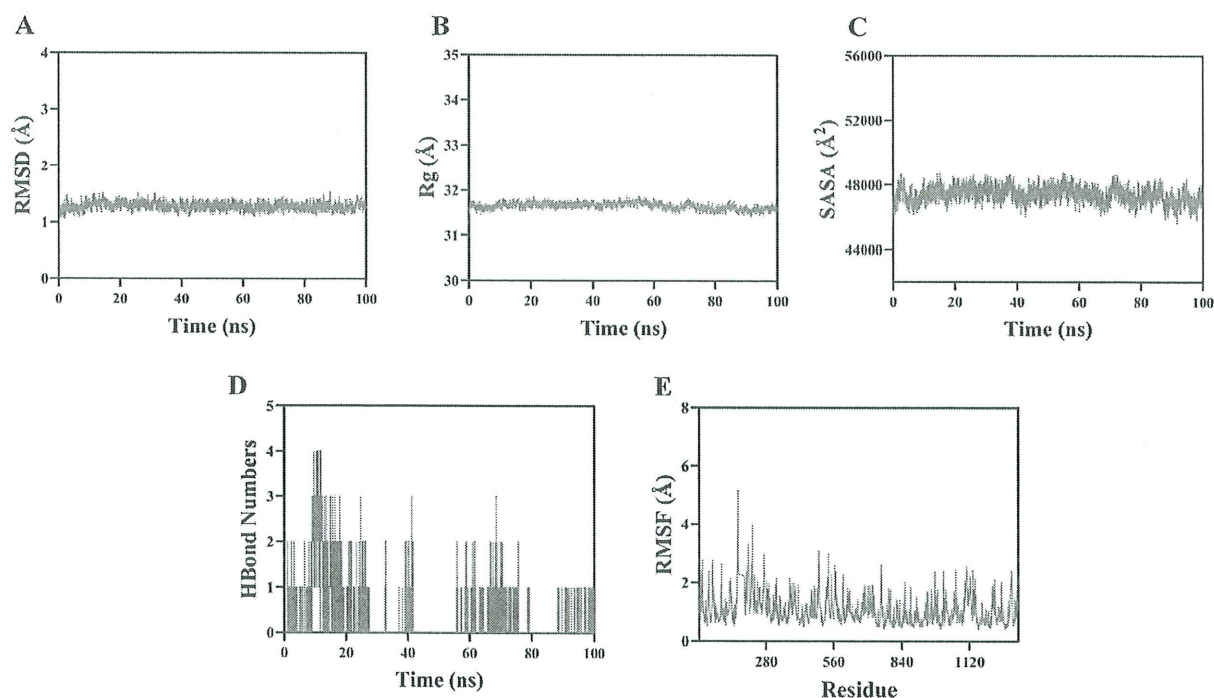
In summary, the XOD-Psoralen complex exhibited good conformational and binding stability throughout the molecular

dynamics simulations. The complex showed minimal overall structural fluctuations, a relatively compact conformation, sustained hydrogen bonding interactions, and low flexibility of most residues. Taken together, these results indicated that psoralen could form a stable complex with XOD to inhibit the enzyme's activity.

### 3.5 | Inhibition Kinetics

To elucidate the mechanism by which psoralen inhibits XOD, its inhibitory kinetic characteristics were analyzed. As shown in Figure 5A, in the Lineweaver–Burk plot obtained in the presence of psoralen, all straight lines intersected the y-axis. Moreover, as the psoralen concentration increased, the slopes of the lines gradually increased, while the y-intercepts remained essentially unchanged. Meanwhile, the value of  $K_i$  is  $2.813 \times 10^{-5}$  mol/L, which is lower than that of some other inhibitors, such as ferulic acid and Genistein (J. Zhang et al. 2024; Lou et al. 2023). This pattern indicated that psoralen primarily affected the apparent affinity between the enzyme and the substrate, with minimal impact on the maximum reaction rate ( $V_{max}$ ). These results





**FIGURE 4** | Molecular dynamics simulation of XOD-psoralen complex. (A) RMSD value, (B) Rg value, (C) SASA value, (D) HBonds value, and (E) RMSF value.

demonstrated that psoralen acted as a competitive inhibitor of XOD, likely binding to the active site of XOD and inhibiting the enzymatic reaction by competing with the substrate xanthine for the binding site (Di Stefano et al. 2018). Plotting the slopes and y-intercepts of the individual lines from the Lineweaver-Burk double-reciprocal plots against psoralen concentration revealed good linear relationships, implying that the inhibitory behavior of psoralen on XOD conformed to the classical enzyme inhibition kinetic model.

### 3.6 | Fluorescence Titration Analysis

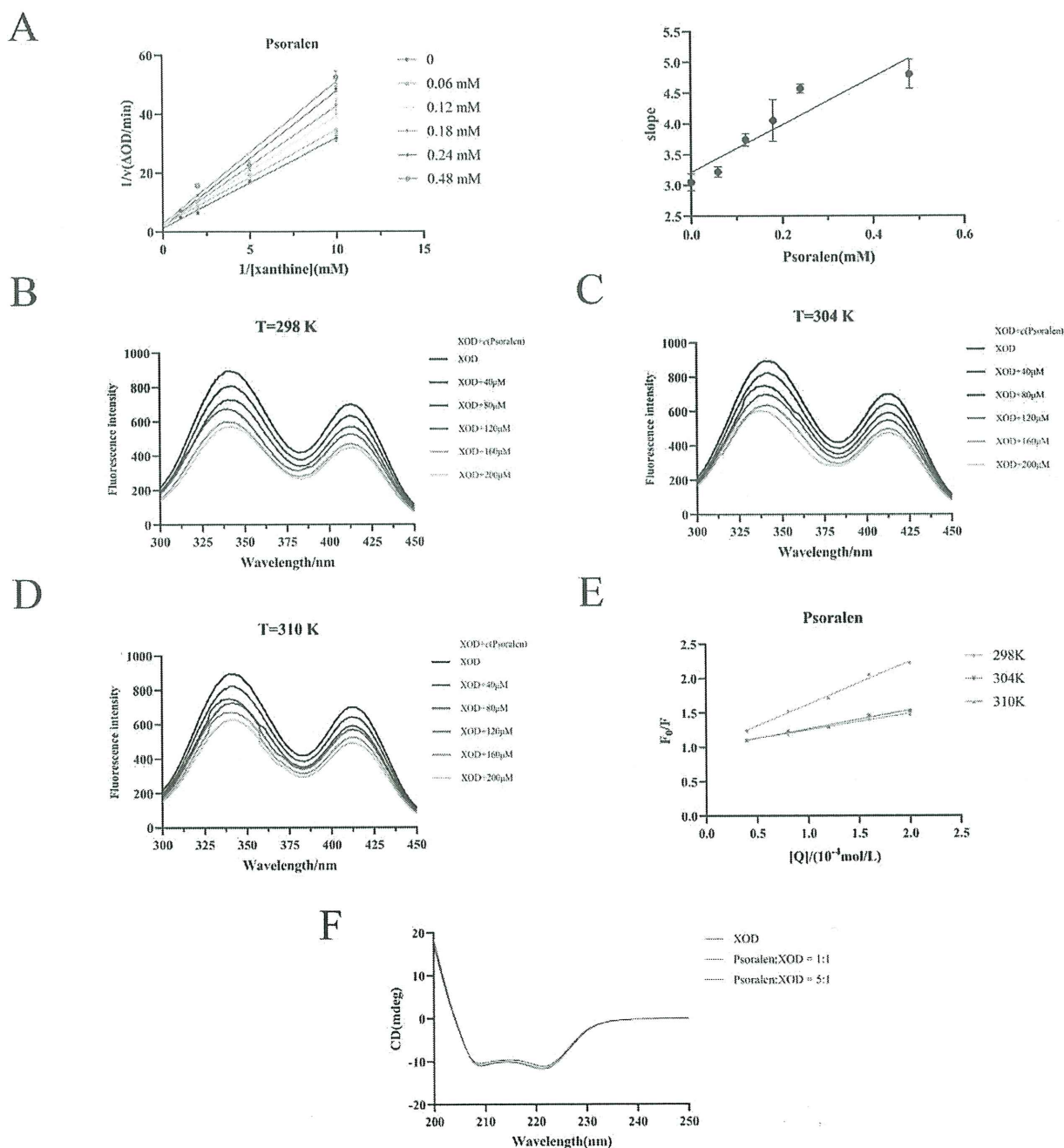
To further elucidate the interaction mechanism between psoralen and XOD at the molecular level, steady-state fluorescence titration was employed to investigate the quenching behavior of psoralen on the intrinsic fluorescence of XOD. As shown in Figure 5B–D, psoralen significantly quenched the intrinsic fluorescence of XOD in the experimental concentration, indicating a direct intermolecular interaction between the two. Furthermore, the quenching constant ( $K_{sv}$ ) of psoralen decreased with increasing system temperature (Table 1). Meanwhile, the bimolecular quenching rate constant ( $k_q$ ) at each temperature was on the order of  $10^{11}$  L·mol<sup>-1</sup>·s<sup>-1</sup>, which exceeded the maximum diffusion collision quenching rate constant for various biological macromolecules ( $2.0 \times 10^{10}$  L·mol<sup>-1</sup>·s<sup>-1</sup>) (M. Liu et al. 2024). This indicated that the interaction between psoralen and XOD was a typical static quenching process. Using a double-logarithmic fit ( $R^2 > 0.96$ ), the binding constants  $K_a$  of psoralen with XOD at 298, 304, and 310 K were determined to be  $9.82 \times 10^3$ ,  $3.98 \times 10^3$ , and  $1.66 \times 10^3$  L·mol<sup>-1</sup> (Table 1), respectively. All values were on the order of  $10^3$ – $10^4$ , indicating a moderately high binding

affinity and the ability to form relatively stable complexes. Moreover, as the temperature increased, the  $K_a$  values decreased, further supporting static quenching and suggesting that high temperatures were detrimental to complex stability. In addition, the number of binding sites ( $n$ ) at different temperatures was close to 1, indicating that psoralen bound to a single inhibitory site on XOD. These results provided more support for the observation of molecular docking, and further demonstrated that psoralen could effectively “occupy” the active site of XOD and interact with the surrounding amino residues by different molecular forces.

The thermodynamic parameters (Table 1) showed that at the three measured temperatures,  $\Delta G < 0$  (–23.0 to –19.3 kJ/mol),  $\Delta H = -113.2$  kJ/mol, and  $\Delta S = -302.9$  J/(mol·K), indicating that the binding process proceeded spontaneously, with van der Waals forces and hydrogen bonds serving as the primary driving forces (Yao et al. 2025). The exothermic nature of the binding process ( $\Delta H < 0$ ) also explained why  $K_a$  decreased with increasing temperature. Based on the comprehensive results of the fluorescence titration analysis, psoralen spontaneously bound to XOD in a 1:1 ratio via van der Waals forces and hydrogen bonds by forming a stable ground-state complex that statically quenched the fluorescence of the target enzyme. These results were consistent with the observation of inhibition kinetics.

### 3.7 | CD Analysis

To investigate the effect of psoralen binding on the conformation of XOD, CD spectroscopy was employed to measure the spectral changes of XOD in the presence of psoralen, and a fitting analysis of its secondary structure was performed. As shown



**FIGURE 5** | (A) Inhibition kinetics, (B–D) fluorescence spectrum, (E) Stern–Volmer plot, and (F) circular dichroism spectrum.

in Figure 5F, XOD exhibited distinct negative peaks at 208 and 222 nm, indicating that it contained a high proportion of  $\alpha$ -helices and possessed a relatively stable native conformation. Upon the addition of psoralen, the intensity of the negative peaks at these two characteristic wavelengths decreased only slightly, and the extent of this change remained relatively small as the psoralen concentration increased. This suggested that although psoralen could interact with XOD, its impact on the overall stability of the secondary structure was limited. The results in Table S3 showed

that psoralen caused some changes in the secondary structure composition of XOD, generally characterized by a decrease in the proportion of  $\alpha$ -helices, while the proportions of  $\beta$ -sheets,  $\beta$ -turns, and random coils increased to varying degrees. However, these changes were minor, suggesting that psoralen binding primarily caused local conformational adjustments without significantly disrupting the overall structural stability of the protein (Micsonai et al. 2015).



TABLE 1 | Fluorescence quenching binding parameters and thermodynamic parameters of the interaction between psoralen and XOD.

| Name     | T (K) | $K_{sv} \times 10^3$ (L·mol <sup>-1</sup> ) | $k_q \times 10^{11}$ (L·mol <sup>-1</sup> ·s <sup>-1</sup> ) | $R_{sv}^2$ | $K_a \times 10^3$ (L·mol <sup>-1</sup> ) | n           | $R_n^2$ | $\Delta G$ (kJ/mol) | $\Delta H$ (kJ/mol) | $\Delta S$ (J/mol·K) |
|----------|-------|---------------------------------------------|--------------------------------------------------------------|------------|------------------------------------------|-------------|---------|---------------------|---------------------|----------------------|
| Psoralen | 298   | 6.04 ± 0.18                                 | 6.04                                                         | 0.985      | 9.82 ± 0.22                              | 1.06 ± 0.05 | 0.963   | -23.0               | -113.2              | -302.9               |
|          | 304   | 2.72 ± 0.08                                 | 2.72                                                         | 0.986      | 3.98 ± 0.15                              | 1.04 ± 0.03 | 0.984   | -21.1               |                     |                      |
|          | 310   | 2.44 ± 0.05                                 | 2.44                                                         | 0.989      | 1.66 ± 0.09                              | 0.96 ± 0.04 | 0.985   | -19.3               |                     |                      |

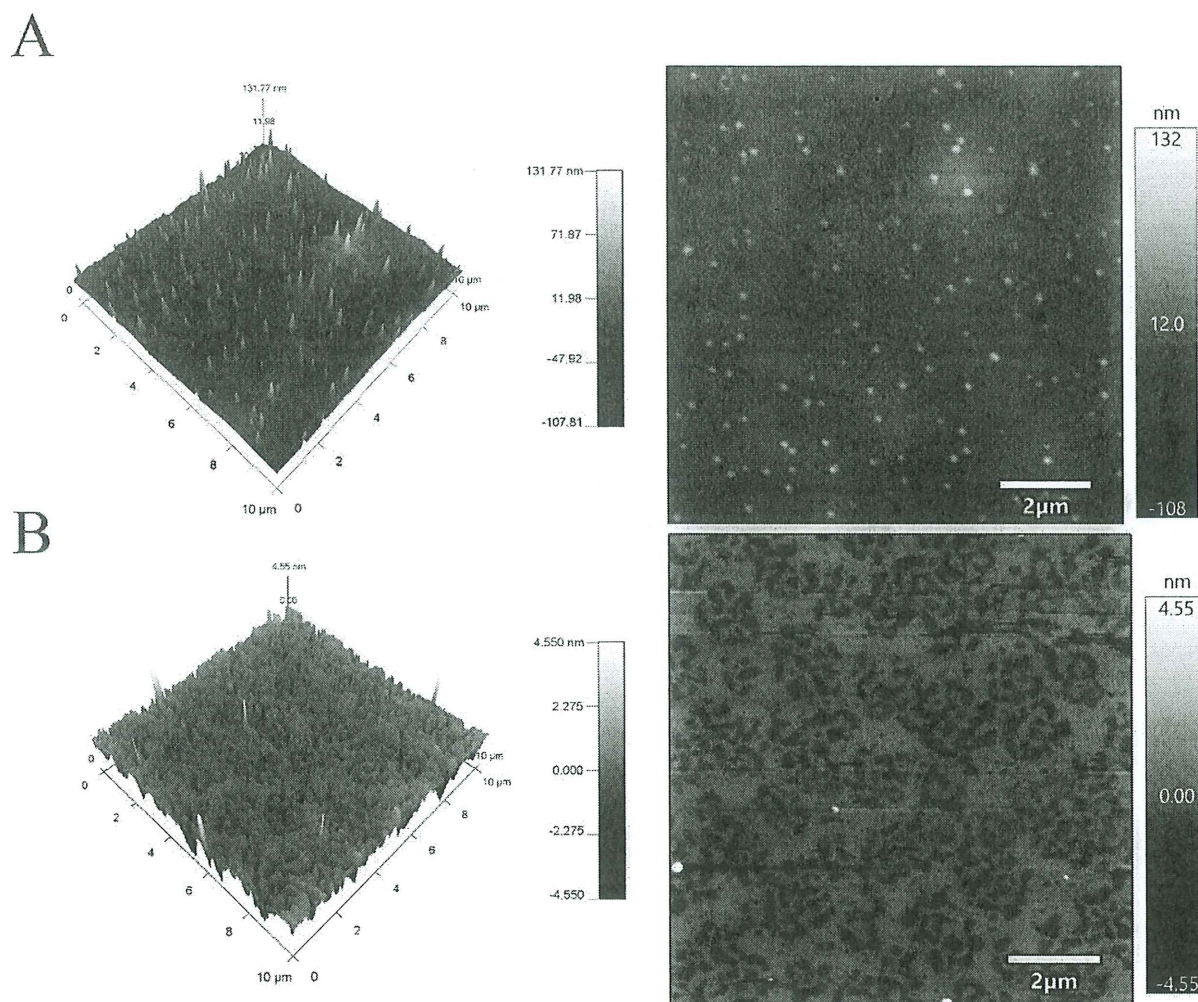
### 3.8 | Observation of Atomic Force Microscope (AFM)

Atomic force microscopy (AFM) was employed to investigate the effect of psoralen binding on the morphology of XOD at the microscopic level. As shown in Figure 6A, XOD was distributed relatively uniformly on the mica surface, with clear particle boundaries and an overall dispersed state. No obvious large-scale aggregation was observed, indicating that XOD maintained a relatively stable spatial conformation in its native state. In addition, both two-dimensional and three-dimensional topographic images showed that XOD primarily existed as relatively independent particles with high surface elevations, reflecting the relatively intact structural morphology of the native protein. After psoralen treatment, the surface topography of XOD underwent certain changes. The distribution of the complex on the substrate surface became denser, with particles arranged more closely together and a significant reduction in surface elevation, indicating that the binding of psoralen to XOD altered the protein's adsorption and arrangement on the substrate surface (Figure 6B). Overall, the samples retained good uniformity, with no obvious disordered aggregation observed. This result demonstrated that psoralen could interact with XOD directly to promote, but not disrupt the structural stability of the protein, which was consistent with the CD results.

### 3.9 | The Effect of Psoralen on Xanthine-Induced BRL-3A Cell Viability, UA Production, and XOD Activity

Liver is one of the main organs expressing XOD, so BRL-3A cells are widely used to investigate the anti-hyperuricemia activity of natural compounds. Presently, the CCK-8 assay was used to assess the effects of different concentrations of psoralen on BRL-3A cell viability, and the results were shown in Figure 7A. Compared with the blank control group, psoralen had no significant effect on the viability of BRL-3A cells within the concentration range of 0–200 μM, indicating good cellular compatibility within this range. Therefore, 100 and 200 μM were selected as the low and high intervention concentrations of psoralen for subsequent BRL-3A cell-related experiments.

Since the liver is an important site for purine metabolism and UA production and is also one of the major organs expressing XOD, the normal rat liver cell line BRL-3A was selected as an in vitro model to further validate the inhibitory effect of psoralen on XOD at the cellular level (Ullah et al. 2025). The results showed that, compared with the blank group (NC), the UA content in the supernatant of BRL-3A cells in the model group (MD) was significantly elevated, indicating that xanthine substrate induction significantly promoted cellular UA production. Concurrently, intracellular XOD activity in the model group was also significantly increased, confirming the successful construction of a hyperuricemic cell model. Compared with the model group, UA levels in the cell supernatants of the low- and high-concentration psoralen groups (PSO: 100 μM, PSO: 200 μM) were both significantly reduced, with the high-concentration group exhibiting a more pronounced effect (Figure 7B). At the same time, intracellular XOD activity was reduced to varying degrees compared with the model group, with the high-concentration group showing



**FIGURE 6 |** AFM surface morphology images of (A) XOD and (B) XOD-psoralen complex.

a stronger inhibitory effect than that of the low-concentration group (Figure 7C). These results indicated that psoralen could significantly inhibit xanthine-induced UA production in BRL-3A cells, and that this effect might be related to the inhibition of intracellular XOD activity. However, psoralen showed less inhibitory activity against XOD than that of the positive control drug-allopurinol, indicating that this active compound could not replace drug to treat hyperuricemia, but reveal the possibility to assist in improving this disease.

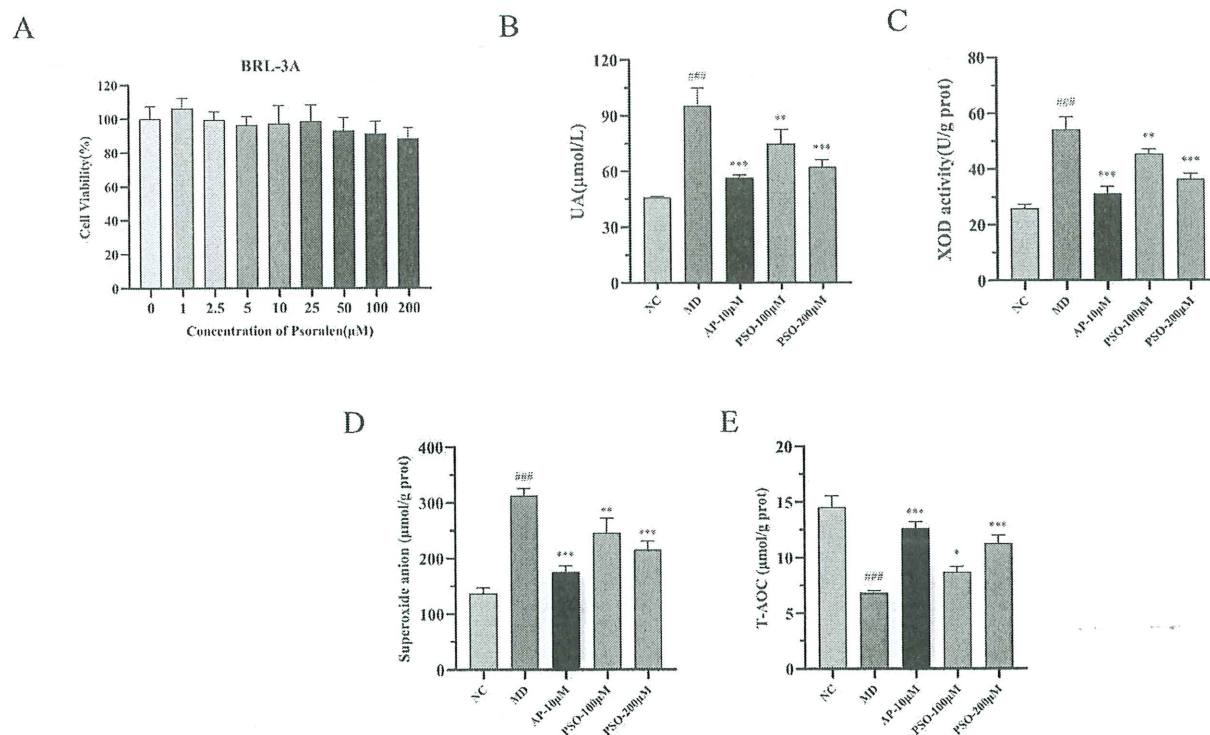
### 3.10 | The Effect of Psoralen on Xanthine-Induced BRL-3A Cell Superoxide Anion Level and T-AOC

To further investigate whether psoralen could alleviate oxidative stress damage in BRL-3A cells under hyperuricemic condition (Bortolotti et al. 2021), this study measured the levels of superoxide anion ( $O_2^{\cdot-}$ ) and T-AOC in the cells, and the results were shown in Figure 7D,F. Compared with the normal control

group, the  $O_2^{\cdot-}$  levels in BRL-3A cells of the model group were significantly increased, while the T-AOC levels were significantly reduced, indicating that the large amount of superoxide anions generated during xanthine catabolism disrupted the cellular redox balance, thereby inducing oxidative stress. Following psoralen intervention, the oxidative stress status in the treatment groups was significantly improved. Compared with the model group, both low- and high-concentration psoralen (PSO: 100  $\mu$ M, PSO: 200  $\mu$ M) significantly alleviated the abnormal accumulation of  $O_2^{\cdot-}$  within the cells, and T-AOC levels showed a marked recovery.

Taken together with the aforementioned results on UA and XOD activity, these findings indicated that psoralen not only reduced UA production by inhibiting XOD activity, but also effectively cleared the byproduct  $O_2^{\cdot-}$  by increasing the T-AOC. Consequently, it restored the total antioxidant defense capacity of hepatocytes and alleviated hyperuricemia-induced oxidative stress.





**FIGURE 7** | Effects of psoralen on (A) viability, (B) uric acid content, (C) XOD activity, (D) superoxide anion levels, and (E) total antioxidant capacity of BRL-3A cells (compared with the blank group,  $###p < 0.001$ ; compared with the model group,  $**p < 0.01$ ,  $***p < 0.001$ ).

### 3.11 | The Effect of Psoralen on UA-Induced HK-2 Cell Viability and Inflammatory Factor Secretion

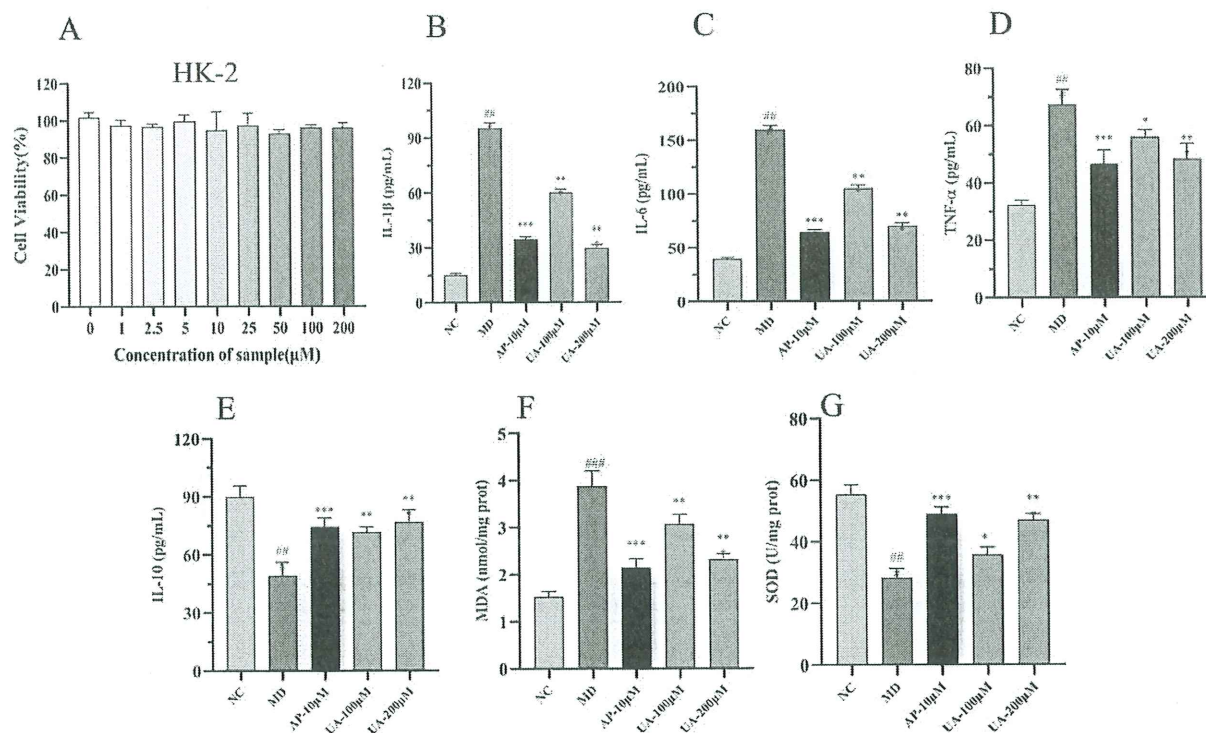
UA is mainly generated in liver, but which will be delivered to kidney to cause renal oxidative stress and inflammatory response (Ullah et al. 2024). The effect of different concentrations of psoralen on the viability of HK-2 cells was measured by the CCK-8 assay, as shown in Figure 8A. Compared with the blank control group, psoralen had no significant effect on cell viability within the concentration range of 0–200  $\mu\text{M}$ . Therefore, 100 and 200  $\mu\text{M}$  were selected as the low and high intervention concentrations for subsequent HK-2 cell experiments.

The kidney was the main organ for UA excretion, and damage to renal tubular epithelial cells was an important pathological basis of hyperuricemia-related kidney injury. Accordingly, an *in vitro* renal injury model was established by stimulating HK-2 cells with UA to evaluate the protective effect of psoralen on renal tubular epithelial cells. Inflammatory response was a key mechanism underlying hyperuricemia-induced renal tubular damage (M. Wu et al. 2021). IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were classic pro-inflammatory cytokines that reflect the degree of cellular inflammatory activation. As shown in Figure 8B–E, compared with the blank group, the levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the model group were significantly increased, while the content of IL-10 was significantly decreased, indicating that UA induced a marked inflammatory response in HK-2 cells. Compared with the model group, both the positive drug and the psoralen treatments

significantly reduced the levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , and obviously increased the level of IL-10. These results indicated that psoralen effectively ameliorated the abnormal secretion of inflammatory factors induced by UA in HK-2 cells and alleviated the cellular inflammatory response in kidney.

### 3.12 | The Effect of Psoralen on UA-Induced Oxidative Stress in HK-2 Cells

Given that oxidative stress and inflammatory response are closely intertwined in UA-induced renal tubular injury, the MDA content and SOD activity in HK-2 cells were further measured. The results were shown in Figure 8F,G. Compared with the blank group, MDA content in the model group was significantly increased, while SOD activity was significantly decreased, suggesting that UA stimulation induced significant oxidative stress injury in HK-2 cells. Compared with the model group, both the positive control group and the psoralen treatment groups exhibited significantly decreased MDA content and increased SOD activity. Following intervention, the oxidative stress level of the cells was ameliorated, as evidenced by reduced MDA content and elevated SOD activity. These results indicated that psoralen alleviated UA-induced oxidative stress in HK-2 cells and enhanced cellular antioxidant defense capacity to protect against hyperuricemia-stimulated kidney damages.



**FIGURE 8** | Effects of psoralen on (A) viability, (B) IL-1β levels, (C) IL-6 levels, (D) TNF-α levels, (E) IL-10 levels, (F) MDA levels, and (G) SOD levels of HK-2 cells (compared with the blank group, ###*p* < 0.001; compared with the model group, \*\**p* < 0.01, \*\*\**p* < 0.001).

## 4 | Conclusion

Among the chamagu extracts obtained using three different solvents, the water extract exhibited the strongest inhibitory activity against XOD, with an IC<sub>50</sub> value of 34.08 mg/mL. Through XOD affinity ultrafiltration-HPLC-MS/MS analysis, a total of 183 differential compounds were screened. By combining molecular docking and in vitro XOD inhibition assays, psoralen was ultimately identified as the key compound in chamagu responsible for its potent XOD inhibitory activity. Further studies revealed the mechanism and structure-activity relationship of psoralen with XOD. Molecular dynamics simulation results indicated that psoralen could form a stable complex with XOD. Enzyme inhibition kinetic analysis confirmed that the inhibitory effect of psoralen on XOD was competitive. Fluorescence titration analysis showed that psoralen spontaneously binds to XOD in a 1:1 ratio via van der Waals forces and hydrogen bonds, thereby statically quenching the target enzyme's fluorescence. CD spectroscopy and atomic force microscopy results showed that the interaction of psoralen with XOD primarily induces changes in the local protein microenvironment, with minimal overall impact on the secondary structure. Cell experiments demonstrated that psoralen could reduce intracellular XOD activity and UA levels in the supernatant of a xanthine-induced hyperuricemic BRL-3A cell model, decrease O<sub>2</sub><sup>•-</sup> levels, and restore T-AOC. In HK-2 cells, psoralen effectively reduced the levels of pro-inflammatory factors IL-1β, IL-6, and TNF-α, decreased the accumulation of the lipid peroxidation product MDA, and enhanced SOD activity. In summary, this study has revealed the chemical basis of the XOD-inhibitory effects of chamagu, providing a theoretical foundation for the development of functional products derived from chamagu.

However, the anti-hyperuricemia activity of chamagu and the identified compound-psoralen still need more in vivo and clinical experiments to further confirm their XOD inhibitory and kidney protective activities in the future.

## Author Contribution

**Xuanbo Chen:** writing – review and editing, writing – original draft, methodology, investigation, conceptualization. **Haiyu Wu:** writing – review and editing, investigation. **Ziwei Guo:** writing – review and editing, methodology. **Zhuoyuan He:** methodology, conceptualization. **Chuangyong Pan:** writing – review and editing, conceptualization. **Xianying Lan:** writing – review and editing, writing – original draft, supervision, conceptualization. **Lin Han:** writing – review and editing, writing – original draft, supervision.

## Funding

This work was financially supported by the Key Research and Development Program of Henan Province (No. 25111110200), Key Project on Frontier Exploration of the Natural Science Basic Research Program of Shaanxi Province (No. 2025JC-QYCX-027), and Shaanxi Provincial Innovation Leadership Program in Sciences and Technologies for Young and Middle-Aged Scientists (No. 2023SR205).

## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

Data will be made available on request.



## References

- Bortolotti, M., L. Polito, M. G. Battelli, and A. Bolognesi. 2021. "Xanthine Oxidoreductase: One Enzyme for Multiple Physiological Tasks." *Redox Biology* 41: 101882. <https://doi.org/10.1016/j.redox.2021.101882>.
- Batsatsashvili, K., Z. Kikvidze, and R. W. Bussmann, eds. 2020. *Ethnobotany of the Mountain Regions of Far Eastern Europe: Ural, Northern Caucasus, Turkey, and Iran*. Springer International Publishing, 219–224.
- Cao, Q., G. Wang, and Y. Peng. 2021. "A Critical Review on Phytochemical Profile and Biological Effects of Turnip (*Brassica rapa* L.)." *Frontiers in Nutrition* 8: 721733. <https://doi.org/10.3389/fnut.2021.721733>.
- Chia, S. L., and P. H. Leung. 2021. "Severe Cutaneous Adverse Drug Reactions Induced by Febuxostat: A Systematic Review of Case Reports." *Journal of Clinical Pharmacy and Therapeutics* 46, no. 4: 903–910. <https://doi.org/10.1111/jcpt.13407>.
- Dewi, C., F. Puspita, W. Insani, R. Sinuraya, I. Puspitasari, and N. Zakiyah. 2025. "Hepatic Safety of Febuxostat and Allopurinol in Gout Patients: An Updated Systematic Review and Meta-Analysis of Randomized Controlled Trials." *Therapeutics and Clinical Risk Management* 21: 1747–1759. <https://doi.org/10.2147/TCRM.S552281>.
- Di Stefano, E., T. Oliviero, and C. C. Udenigwe. 2018. "Functional Significance and Structure–Activity Relationship of Food-Derived  $\alpha$ -Glucosidase Inhibitors." *Current Opinion in Food Science* 20: 7–12. <https://doi.org/10.1016/j.cofs.2018.02.008>.
- Dong, J., B. D. Zhang, S. Li, et al. 2026. "Identification of Sesquiterpenes From *Curcuma longa* and Its Potential for Alcoholic Liver Disease Based on FBMN Strategy and Network Pharmacology." *Food & Medicine Homology* 3: 9420114. <https://doi.org/10.26599/FMH.2026.9420114>.
- Du, L., Y. Zong, H. Li, et al. 2024. "Hyperuricemia and Its Related Diseases: Mechanisms and Advances in Therapy." *Signal Transduction and Targeted Therapy* 9, no. 1: 212. <https://doi.org/10.1038/s41392-024-01916-y>.
- Fais, A., F. Pintus, B. Era, et al. 2023. "Design of 3-Phenylcoumarins and 3-Thienylcoumarins as Potent Xanthine Oxidase Inhibitors: Synthesis, Biological Evaluation, and Docking Studies." *ChemMedChem* 18, no. 21: e202300400. <https://doi.org/10.1002/cmde.202300400>.
- FitzGerald, J. D., N. Dalbeth, T. Mikuls, et al. 2020. "2020 American College of Rheumatology Guideline for the Management of Gout." *Arthritis Care & Research* 72, no. 6: 744–760. <https://doi.org/10.1002/acr.24180>.
- Fraga, C. G., B. H. Clowers, R. J. Moore, and E. M. Zink. 2010. "Signature-Discovery Approach for Sample Matching of a Nerve-Agent Precursor Using Liquid Chromatography–Mass Spectrometry, XCMS, and Chemometrics." *Analytical Chemistry* 82, no. 10: 4165–4173. <https://doi.org/10.1021/ac1003568>.
- Han, L., H. Wang, J. Cao, et al. 2023. "Inhibition Mechanism of  $\alpha$ -Glucosidase Inhibitors Screened From Tartary Buckwheat and Synergistic Effect With Acarbose." *Food Chemistry* 420: 136102. <https://doi.org/10.1016/j.foodchem.2023.136102>.
- Hou, C., D. Liu, M. Wang, et al. 2019. "Novel Xanthine Oxidase-Based Cell Model Using HK-2 Cell for Screening Antihyperuricemic Functional Compounds." *Free Radical Biology and Medicine* 136: 135–145. <https://doi.org/10.1016/j.freeradbiomed.2019.03.011>.
- Ishikawa, T., T. Maeda, T. Hashimoto, et al. 2020. "Long-Term Safety and Effectiveness of the Xanthine Oxidoreductase Inhibitor, Topiroxostat in Japanese Hyperuricemic Patients With or Without Gout: A 54-Week Open-Label, Multicenter, Post-Marketing Observational Study." *Clinical Drug Investigation* 40: 847–859. <https://doi.org/10.1007/s40261-020-00941-3>.
- Kasahara, K., R. L. Kerby, Q. Zhang, et al. 2023. "Gut Bacterial Metabolism Contributes to Host Global Purine Homeostasis." *Cell Host & Microbe* 31, no. 6: 1038–1053.e1010. <https://doi.org/10.1016/j.chom.2023.05.011>.
- Liu, M., X. Wu, H. Chen, et al. 2024. "Exploring the Mechanism and Inhibitory Effect of Robinin on Xanthine Oxidase Using Multi-Spectroscopy and Molecular Dynamics Simulation Methods." *Journal of Molecular Liquids* 408: 125373. <https://doi.org/10.1016/j.molliq.2024.125373>.
- Lou, Y., Q. Gao, M. Fan, et al. 2023. "Ferulic Acid Ameliorates Hyperuricemia by Regulating Xanthine Oxidase." *International Journal of Biological Macromolecules* 253: 126542. <https://doi.org/10.1016/j.ijbiomac.2023.126542>.
- Lu, Y., H. Liu, D. Yang, et al. 2021. "Affinity Mass Spectrometry-Based Fragment Screening Identified a New Negative Allosteric Modulator of the Adenosine A<sub>2A</sub> Receptor Targeting the Sodium Ion Pocket." *ACS Chemical Biology* 16, no. 6: 991–1002. <https://doi.org/10.1021/acscchembio.0c00899>.
- Mehmood, A., J. Li, A. U. Rehman, et al. 2022. "Xanthine Oxidase Inhibitory Study of Eight Structurally Diverse Phenolic Compounds." *Frontiers in Nutrition* 9: 966557. <https://doi.org/10.3389/fnut.2022.966557>.
- Mehmood, A., L. Zhao, C. Wang, I. Hossen, and M. Nadeem. 2020. "Stevia Residue Extract Alone and Combination With Allopurinol Attenuate Hyperuricemia in Fructose–PO-Induced Hyperuricemic Mice." *Journal of Food Biochemistry* 44, no. 1: e13087. <https://doi.org/10.1111/jfbc.13087>.
- Miconai, A., F. Wien, L. Kernya, et al. 2015. "Accurate Secondary Structure Prediction and Fold Recognition for Circular Dichroism Spectroscopy." *Proceedings of the National Academy of Sciences of the United States of America* 112, no. 24: E3095–E3103. <https://doi.org/10.1073/pnas.1500851112>.
- Nguyen, A. H., A. T. Do, N. N. Linh, M. Q. Pham, S. T. Ngo, and T. K. Le. 2026. "Searching for Potential Xanthine Oxidase Inhibitors From Vietnamese Herb Plants via Computational Studies." *Computational Biology and Chemistry* 120, no. Pt 1: 108763. <https://doi.org/10.1016/j.compbiolchem.2025.108763>.
- Paul, S., C. A. Geng, T. H. Yang, Y. P. Yang, and J. J. Chen. 2019. "Phytochemical and Health-Beneficial Progress of Turnip (*Brassica rapa*)." *Journal of Food Science* 84, no. 1: 19–30. <https://doi.org/10.1111/1750-3841.14417>.
- Ren, C.-X., M.-Y. Gao, N. Li, et al. 2024. "Identification and Mechanism Elucidation of Medicative Diet for Food Therapy XQCSY in NAFLD Prevention: An Integrative in Silico Study." *Food & Medicine Homology* 1: 9420015. <https://doi.org/10.26599/FMH.2024.9420015>.
- Sahoo, G. G., A. Nayak, A. Gadnayak, et al. 2025. "Computer-Aided Screening of Phytoconstituents From Plumeria Species Targeting Xanthine Oxidase Inhibition: A Combination of Pharmacokinetics, Molecular Docking and Molecular Dynamics Approaches." *Computational Biology and Chemistry* 119: 108557. <https://doi.org/10.1016/j.compbiolchem.2025.108557>.
- Sousa, C., D. M. Pereira, P. Valentão, et al. 2009. "Pieris Brassicae Inhibits Xanthine Oxidase." *Journal of Agricultural and Food Chemistry* 57, no. 6: 2288–2294. <https://doi.org/10.1021/jf803831v>.
- Ullah, Z., Z. Yan, M. Zhang, et al. 2025. "A Comprehensive Review on Targeting Hyperuricemia With Edible Bioactive Polysaccharides: Advances in Structure, Xanthine Oxidase Inhibition, Uric Acid-Lowering Strategies, Mechanisms, and Current Applications." *Food Reviews International* 42: 1112–1150.
- Ullah, Z., P. Yue, G. Mao, et al. 2024. "A Comprehensive Review on Recent Xanthine Oxidase Inhibitors of Dietary Based Bioactive Substances for the Treatment of Hyperuricemia and Gout: Molecular Mechanisms and Perspective." *International Journal of Biological Macromolecules* 278: 134832. <https://doi.org/10.1016/j.ijbiomac.2024.134832>.
- Wan, X., C. Xu, Y. Lin, et al. 2016. "Uric Acid Regulates Hepatic Steatosis and Insulin Resistance Through the NLRP3 Inflammasome-Dependent Mechanism." *Journal of Hepatology* 64, no. 4: 925–932. <https://doi.org/10.1016/j.jhep.2015.11.022>.
- Wang, J., Y. Chen, H. Zhong, et al. 2022. "The Gut Microbiota as a Target to Control Hyperuricemia Pathogenesis: Potential Mechanisms and Therapeutic Strategies." *Critical Reviews in Food Science and Nutrition* 62, no. 14: 3979–3989. <https://doi.org/10.1080/10408398.2021.1874287>.

- Wei, X., Y. Yuan, U. K. Nath, Q. Xu, and X. Zhang. 2025. *Vegetable Crops*. Springer, 795–815.
- White, W. B., K. G. Saag, M. A. Becker, et al. 2018. “Cardiovascular Safety of Febuxostat or Allopurinol in Patients With Gout.” *New England Journal of Medicine* 378, no. 13: 1200–1210. <https://doi.org/10.1056/NEJMoa1710895>.
- Wu, H., J. Li, X. Wu, et al. 2026. “Unlocking Hypoglycemic Effect of Black Highland Barley by Targeting  $\alpha$ -Glucosidase Inhibitors Based on UF-HPLC-MS/MS.” *Talanta* 309: 130012. <https://doi.org/10.1016/j.talanta.2026.130012>.
- Wu, M., Y. Ma, X. Chen, N. Liang, S. Qu, and H. Chen. 2021. “Hyperuricemia Causes Kidney Damage by Promoting Autophagy and NLRP3-Mediated Inflammation in Rats With Urate Oxidase Deficiency.” *Disease Models & Mechanisms* 14, no. 3: dmm048041. <https://doi.org/10.1242/dmm.048041>.
- Yao, Y., M. Zhang, J. Chen, et al. 2025. “Novel Mechanistic Insights Into Luteolin and Its Analogues as Potent Inhibitors of Xanthine Oxidase Activity: Inhibition Thermodynamics, Kinetics and Docking Simulation.” *Food Bioscience* 71: 107087. <https://doi.org/10.1016/j.fbio.2025.107087>.
- Zaabat, N., A. Bousetla, L. Khattabi, et al. 2025. “Phytochemical Analysis, In Vitro Biological Activities, and In Silico Prediction of Hydromethanolic Root Extract of *Brassica rapa* L. From Setif.” *Chemistry & Biodiversity* 22, no. 12: e01464. <https://doi.org/10.1002/cbdv.202501464>.
- Zhang, T., S. Bian, Y. Gu, et al. 2020. “Sugar-Containing Carbonated Beverages Consumption Is Associated With Hyperuricemia in General Adults: A Cross-Sectional Study.” *Nutrition, Metabolism and Cardiovascular Diseases* 30, no. 10: 1645–1652. <https://doi.org/10.1016/j.numecd.2020.05.022>.
- Zhang, X.-D., B. Liu, Y. F. Zou, et al. 2025. “An Efficient Strategy for the Rapid Exploration of Lipase Inhibitors Derived From the *Lindera* Aggregate Leaves by Utilizing Hybrid Lipase-Catalytic Zeolitic Imidazolate Framework Reactor Combined With HPLC-Q-TOF-MS/MS and Molecular Docking Techniques.” *Food & Medicine Homology* 2, no. 4: 9420097. <https://doi.org/10.26599/FMH.2026.9420097>.
- Zhang, Y., C. Ban, D. Su, Y. Liu, S. Zhou, and J. Fan. 2024. “Xanthine Oxidase Inhibitors: Virtual Screening and Mechanism of Inhibition Studies.” *International Journal of Biological Macromolecules* 281: 136281. <https://doi.org/10.1016/j.ijbiomac.2024.136281>.
- Zhong, J., H. Cai, Z. Zhang, et al. 2024. “Serum Uric Acid and Prognosis of Ischemic Stroke: Cohort Study, Meta-Analysis and Mendelian Randomization Study.” *European Stroke Journal* 9, no. 1: 235–243. <https://doi.org/10.1177/23969873231209620>.
- Zhu, H., Y. Yang, Z. Li, X. Wang, and H. Qian. 2022. “An Integrated Network Pharmacology and Metabolomics Approach to Reveal the Immunomodulatory Mechanism of *Brassica rapa* L. (Tibetan Turnip) in Fatigue Mice.” *Food & Function* 13, no. 21: 11097–11110. <https://doi.org/10.1039/d2fo02308c>.
- Zhu, Y., Y. Hu, T. Huang, et al. 2014. “High Uric Acid Directly Inhibits Insulin Signalling and Induces Insulin Resistance.” *Biochemical and Biophysical Research Communications* 447, no. 4: 707–714. <https://doi.org/10.1016/j.bbrc.2014.04.080>.

## Supporting Information

Additional supporting information can be found online in the Supporting Information section.

**Supplementary Material:** fff270362-sup-0001-SuppMat.docx



编号: SCIE-H-20260908-18

检索报告

根据委托人陈宣伯委托,通过网络检索,陈宣伯发表的 1 篇论文被《科学引文索引》扩展版(SCI-Expanded)数据库收录。数据库具体检索结果如下:

标题: Integrated Analysis of CRY1 Gene Expression and InDel Polymorphism Reveals Associations with Ovarian Morphological Traits in Chinese Holstein Dairy Cattle

作者: Chen, XB(Chen, Xuanbo)[1]; Jiang, EH(Jiang, Enhui)[1]; Yang, YT(Yang, Yuta)[1]; Zhang, HT(Zhang, Haotian)[1]; Liu, ZY(Liu, Zhaoyu)[1]; Areb, E(Areb, Ebadu)[1]; Wang, YS(Wang, Yongsheng)[2]; Lan, XY(Lan, Xianyong)[1]

期刊: BIOMOLECULES 卷: 16 期: 8 文献号: 1201 文献类型: Article

DOI: 10.3390/biom16081201

出版年: AUG 17 2026

在线发表时间:AUG 2026

已索引:2026-08-30

通讯作者地址: [Lan, Xianyong] (corresponding author), Northwest A&F Univ, Coll Anim Sci & Technol, Key Lab Anim Genet Breeding & Reprod Shaanxi Prov, Yangling 712100, Peoples R China

[Wang, Yongsheng] (corresponding author), Northwest A&F Univ, Coll Vet Med, Yangling 712100, Peoples R China  
地址:

1. Northwest A&F Univ, Coll Anim Sci & Technol, Key Lab Anim Genet Breeding & Reprod Shaanxi Prov, Yangling 712100, Peoples R China

2. Northwest A&F Univ, Coll Vet Med, Yangling 712100, Peoples R China

出版商:MDPI

Web of Science 类别: Biochemistry & Molecular Biology

语种: English

入藏号: WOS:001859864700001

eISSN: 2218-273X

Web of Science 核心合集中的 "被引频次":0

第一作者陈宣伯, 第一署各单位西北农林科技大学动物科技学院。

通讯作者蓝贤勇, 署各单位西北农林科技大学动物科技学院。

通讯作者王勇胜, 署各单位西北农林科技大学动物医学院。

2026 年公布的影响因子: 5.6, JCR 分区情况:

| JCR® 类别                          | 类别中的排序 | JCR 分区 |
|----------------------------------|--------|--------|
| BIOCHEMISTRY & MOLECULAR BIOLOGY | 72/328 | Q1     |

2025 年公布的中科院分区 (升级版):

|    | 学科名称                                         | 分区 | Top 期刊 |
|----|----------------------------------------------|----|--------|
| 大类 | 生物学                                          | 2  | 否      |
| 小类 | BIOCHEMISTRY & MOLECULAR BIOLOGY<br>生化与分子生物学 | 3  | -      |

查证检索:

西北农林科技大学图书馆  
2026 年 9 月 8 日

## Article

# Integrated Analysis of *CRY1* Gene Expression and InDel Polymorphism Reveals Associations with Ovarian Morphological Traits in Chinese Holstein Dairy Cattle

Xuanbo Chen <sup>1,†</sup>, Enhui Jiang <sup>1,†</sup>, Yuta Yang <sup>1</sup>, Haotian Zhang <sup>1</sup>, Zhaoyu Liu <sup>1</sup>, Ebadu Areb <sup>1</sup>, Yongsheng Wang <sup>2,\*</sup> and Xianyong Lan <sup>1,\*</sup>

<sup>1</sup> Key Laboratory of Animal Genetics, Breeding and Reproduction of Shaanxi Province, College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China; 2023010827@nwafu.edu.cn (X.C.); jiangenhui666@nwafu.edu.cn (E.J.); yydahsy@163.com (Y.Y.); zht0501@nwafu.edu.cn (H.Z.); liuzhaoyu19939469829@nwafu.edu.cn (Z.L.); ebaduareb3@nwafu.edu.cn (E.A.)

<sup>2</sup> College of Veterinary Medicine, Northwest A&F University, Yangling 712100, China

\* Correspondence: wangyongsheng01@nwsuaf.edu.cn (Y.W.); lanxianyong79@nwsuaf.edu.cn (X.L.)

<sup>†</sup> These authors contributed equally to this work.

## Abstract

Clock genes, such as cryptochrome 1 (*CRY1*), exhibit rhythmic expression in the reproductive organs. This gene is a key component of the circadian clock and is involved in various physiological processes, including reproduction, suggesting that it may be linked to ovarian activity via neuroendocrine pathways. However, the association between *CRY1* variants and ovarian traits in dairy cows is unclear. In this study we used qRT-PCR to assay the mRNA expression of this gene, and found that *CRY1* expression was highest in oocytes in several tissues; subsequently, we found that *CRY1* expression was highest in the ovary in Chinese Holstein dairy cattle, followed by muscle and spleen tissues ( $p < 0.01$ ). Next, we identified a six bp insertion/deletion (indel) locus within the *CRY1* gene in Chinese Holstein dairy cattle. Only two genotypes were detected: II ( $n = 854$ , 83.7%) and ID ( $n = 166$ , 16.3%). Association analysis revealed that the identified indel was significantly associated with dominant follicle and corpus albicans diameters ( $p < 0.05$ ) during metestrus. Specifically, individuals with the ID genotype exhibited smaller dominant follicles and corpus albicans than those with the II genotype. Our preliminary findings suggest that *CRY1* is associated with specific ovarian morphological traits and may serve as a basis for further investigation of its potential role in bovine reproductive function.

**Keywords:** *CRY1*; circadian rhythm; Chinese Holstein; gene expression; InDel polymorphism; ovarian traits; molecular marker

Academic Editor: Jürg Bähler

Received: 26 June 2026

Revised: 11 August 2026

Accepted: 12 August 2026

Published: 17 August 2026

**Copyright:** © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

## 1. Introduction

Simultaneously improving milk production and fertility is challenging in dairy production. Although milk production has greatly improved [1], the reproductive efficiency of cows has decreased in the past few decades [2]. Consequently, improving fertility has become an urgent priority in recent years. The determinants of dairy cow fertility are numerous and complex [2]. As a crucial component of the female reproductive system, the ovary not only generates gametes but also functions as an endocrine gland that provides



fertility-associated steroid hormones [2]. Therefore, it has long been a subject of exploration in the field of female reproduction [3]. Cows with larger ovaries tend to have a higher number of follicles and oocyte masses [4].

The *CRY1* protein regulates cell metabolism and proliferation and is involved in tumorigenesis. It plays an important role at several levels in mammals by controlling circadian rhythms [5]. Disruption of the circadian clock can cause diseases of a wide range of systems and organs [6,7], such as pulmonary diseases [8], thyroid cancer [9], and infertility [10]. The clock can be affected by many factors [11]; among them, genetic factors are the key to the molecular structure of the circadian clock, and clock genes and proteins form an intricate regulatory network for the clock [12,13]. Cryptochromes play a pivotal role in circadian clock maintenance [14] and other physiological processes. The Cryptochrome 1 (*CRY1*) gene regulates cell metabolism and proliferation [15] and is involved in tumorigenesis [16]. For example, circadian behaviors are disrupted in mice that are homozygous null for *CRY1* and *CRY2* [17]. Female mice deficient in *CRY1* or *CRY2* exhibit irregular and unstable estrous cycles and infertility in middle age [18], indicating that cryptochrome genes may affect female reproductive functions. Furthermore, other evidence suggests a profound bidirectional link between circadian rhythms and reproductive functions [19,20]. The circadian timing system modulates diverse physiological processes via intricate hormonal and neural pathways; consequently, dysregulation of clock gene expression can precipitate a spectrum of reproductive impairments [21] and metabolic shifts [22]. In livestock, this molecular oscillatory machinery is intimately integrated with the hypothalamic–pituitary–ovarian (HPO) axis, which orchestrates the rhythmic secretion of gonadotropins essential for follicular recruitment and ovulation [23]. Given the intensive metabolic demands placed on high-yield dairy cows [24], *CRY1* may function as a pivotal metabolic–reproductive sensor bridging systemic energy homeostasis with localized ovarian function.

Studies have shown that clock genes have the potential to influence the fertility of female livestock, which may help solve the problem of fertility decline in cows [25]. According to Huang et al. (2022) [26], cryptochrome genes affect the litter size of sheep, which preliminarily confirmed the association between clock genes and fertility. A recent study investigating the function of *CRY1* using single-nucleotide polymorphisms within the gene demonstrated the practicability of employing variants to further investigate clock genes. It has been reported that clock genes such as *BMAL1*, *CLOCK*, and *CRY1* exhibit rhythmic expression in the ovary [27], indicating that there is an association between clock genes and ovary function. However, the precise relationship between the ovary and clock genes is unclear. In this study, we investigated the polymorphisms of the bovine *CRY1* gene to clarify the association between the *CRY1* gene and the ovary. To investigate genetic variants and associations with ovarian traits, data were collected from 1020 Chinese Holstein dairy cattle at different stages of the estrous cycle. This study provides a preliminary potential marker for breeding improvement programs.

However, the relationship between the ovary and clock genes remains unclear. Therefore, the aim of this study was to conduct a preliminary investigation into tissue-specific *CRY1* expression, as well as to identify functional InDel polymorphisms and evaluate their nominal associations with ovarian morphological traits in Chinese Holstein cattle. This investigation would generate hypotheses and provide foundational data to determine whether *CRY1* represents a potential candidate for further exploration of its role in bovine reproductive function.

## 2. Materials and Methods

### 2.1. Ethics Approval

All experimental procedures involving animals were conducted in strict accordance with the relevant guidelines and regulations and were approved by the Institutional Animal Care and Use Committee of Northwest A&F University (IACUC-NWAFU)) (protocol code DK20220201 and date of approval 1 February 2022).

### 2.2. Sample Collection and Morphological Classification of Ovarian Tissues Across Estrous Cycle Stages

Ovary samples were obtained from 1020 Chinese Holstein dairy cows at a commercial abattoir in Xi'an, China. All animals were clinically healthy at the time of slaughter, as confirmed by veterinary inspection, with no detectable reproductive tract diseases. The inclusion criteria were as follows: (1) age of 5–6 years and parity of 2–4; (2) non-pregnant status, confirmed by uterine examination and absence of embryos; (3) no history of reproductive disorders; (4) origin from conventional dairy farms with similar management practices, including tie-stall housing, comparable diet formulations, and standardized milking routines; and (5) slaughter at the same abattoir under standard commercial conditions. The primary reasons for culling were low milk production and reproductive failure, defined as failure to conceive after multiple inseminations. All 1020 animals were multiparous cows, and no bulls were included in the study.

Ovarian tissues were classified into different estrous cycle stages based on corpus luteum morphology and follicular characteristics, as described previously [3,28]. Briefly, estrus was defined as the presence of a large dominant follicle and the absence of an active corpus luteum (CL). Proestrus was characterized by the absence of a functional CL and the presence of developing follicles in the ovaries. Metestrus was defined as the presence of early-stage CL, including type I (cone-shaped) and type II (crater-shaped) CL. Diestrus was characterized by the presence of mature CL, including type III (mushroom-shaped) and type IV CL. The corpus albicans, representing regressed CL from previous cycles, was recorded but not used as a primary criterion for estrous cycle classification. In addition, CL color and vascularization (low, moderate, or high) were recorded to assist with morphological evaluation. Each ovary was measured for length, width, height, and weight, and the corpus lutea were evaluated for diameter and number [29]. The distribution of samples across estrous cycle stages was as follows: estrus ( $n = 115$ ), diestrus ( $n = 498$ ), metestrus ( $n = 268$ ), proestrus ( $n = 126$ ), and unclassified ( $n = 13$ , excluded).

The differing denominators for various traits are due to the biological characteristics of the structures. For instance, not every ovary contains a dominant follicle (DF) or a corpus albicans (CA) at a given stage of the estrous cycle. Only ovaries in which a DF or CA was present and measurable were included in the analysis for those specific traits. Similarly, the corpus luteum (CL) was not always identifiable or measurable in every ovary, particularly during the estrus and proestrus stages where a functional CL is absent. This biological variation explains the differences in sample sizes ( $n$ ) reported for each trait. For example, during the metestrus stage, the number of ovaries with a measurable dominant follicle ( $n = 93$ ) is smaller than the total number of ovaries in that stage ( $n = 268$ ) because a DF is not always present at this specific phase.

For genotype frequency comparison across breeds, DNA samples from Charolais ( $n = 54$ ) and Simmental ( $n = 40$ ) cattle were obtained from previous collaborative studies. Blood samples were collected from the tail vein into EDTA-anticoagulated tubes with informed owner consent. Genomic DNA was extracted from whole blood using the salt-extraction method [30].



### 2.3. DNA Extraction and Total RNA Isolation

After collection, the ovary samples were packed in sample sacks, marked with the number, and stored at  $-80^{\circ}\text{C}$ . In addition, the CL, the dominant follicle (defined as a follicle with a diameter  $\geq 8$  mm, corresponding to the physiological stage of follicle deviation [31,32]), and the corpus albicans (CA) were identified and quantified. The maximum diameter of each structure was measured as a morphological indicator of ovarian activity. After aspirating the liquor inside the larger follicles, we randomly cut the medulla part from the ovaries for DNA extraction (but avoided cutting the CL and corpus albicans). Genomic DNA extraction was performed following the standard procedures described by Aljanabi and Martínez (1997) [30]. Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify and assess of the purity of the extracted DNA samples. DNA samples were diluted to  $20\text{ ng}/\mu\text{L}$  and preserved at  $-40^{\circ}\text{C}$ .

For RNA extraction, additional tissues (spleen, lung, kidney, muscle, brain, and ovary) were collected from six healthy multiparous Holstein cows (5–6 years old) at the same abattoir. Tissues were collected within 30 min post-slaughter, immediately frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Total RNA was extracted using RNAex Pro Reagent (Accurate Biology, Hunan, China) according to the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) [33]. Only samples with A260/A280 ratios between 1.8 and 2.0 were used for subsequent analyses. Genomic DNA was removed using DNase I (Thermo Fisher Scientific, Waltham, MA, USA) treatment according to the manufacturer's protocol. No-reverse-transcriptase (NRT) controls and no-template controls (NTC) were included in all qPCR runs to confirm the absence of genomic DNA contamination and primer dimers, respectively.

### 2.4. cDNA Synthesis and Reverse Transcriptase Quantitative Real-Time PCR (RT-qPCR)

Reverse transcription was performed using the Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (YEASEN, Shanghai, China) with  $1\text{ }\mu\text{g}$  of total RNA as the template, according to the manufacturer's protocol. cDNA from the spleen, lung, kidney, muscle, brain, and ovary was used to analyze mRNA expression. The primer for RT-qPCR was designed to span an exon–exon junction and its efficiency was determined using a standard curve generated from a 5-fold serial dilution of pooled cDNA; efficiencies ranged from 90% to 110% for all primers, with  $R^2 > 0.99$ . The RT-qPCR mixture ( $20\text{ }\mu\text{L}$ ) contained  $10\text{ }\mu\text{L}$  of  $2\times$  SYBR Premix Ex Taq (Takara Biotech, Shiga, Japan),  $2\text{ }\mu\text{L}$  of cDNA (1:100 dilution),  $7.2\text{ }\mu\text{L}$  distilled water, and  $0.4\text{ }\mu\text{L}$  of forward and reverse primers. To increase the efficiency, each cDNA sample was replicated three times. A melt curve analysis was performed to verify the specificity of amplification (ramping from  $60^{\circ}\text{C}$  to  $95^{\circ}\text{C}$  at  $0.5^{\circ}\text{C}$  increments). Ct values showed a standard deviation  $< 0.25$  across technical replicates. Relative gene expression levels were calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method and normalized to the reference gene (*GAPDH*). To improve accuracy, three replicates were performed for each tissue sample [34].

### 2.5. Primer Design and Polymorphism Detection

Five InDel variants (rs133189221, rs442912813, rs526350742, rs454970402, and rs800313145) over 5 bp in length were selected from the Ensembl database (<https://asia.ensembl.org/index.html>, accessed on 15 July 2025) for the *CRY1* gene (Table 1). A primer was designed for each variant based on the sequence of the bovine *CRY1* gene (ID: 535947, NC\_037332.1:70260216-70356120) using Primer Premier 5 software, and synthesized by Sangon Biotech (Shanghai, China).

**Table 1.** PCR primer pair sequences of the bovine *CRY1* gene.

| Loci/Genes    | Primer Sequences (5' to 3') | Location | Sizes (bp) | Variant ID  |
|---------------|-----------------------------|----------|------------|-------------|
| P1-D6-bp      | F: CGTATGTGTTCTAACTCCTTCCTT | Intron 1 | 254/248    | rs133189221 |
|               | R: ATCTAGACAGAAAA-GACCCCACT |          |            |             |
| P2-D5-bp      | F: AAGCAGATGACACCTCCCTT     | Intron 1 | 231/226    | rs442912813 |
|               | R: TGGCTGTAGTCATCATCTGCA    |          |            |             |
| P3-I8-bp      | F: CCTCTTGGCTTTGCTGTTGT     | Intron 1 | 225/233    | rs526350742 |
|               | R: GCTCCAATACTTCAGCCACC     |          |            |             |
| P4-D6-bp      | F: TCCAATTTTACCCCATGTGCC    | Intron 1 | 169/163    | rs454970402 |
|               | R: CAGTGTTCTTGCCTGGAGAA     |          |            |             |
| P5-D5-bp      | F: CTTGTTACTTTGACAGCGAGGC   | Intron 2 | 250/245    | rs800313145 |
|               | R: TGTACACACTCTTGGGCTCAT    |          |            |             |
| CRY1-RT-qPCR  | F: CAGGAAGAGGACACGCAGAG     | Exon     | 77         | -           |
|               | R: ACCAAAGCTCTCCTCCTTACC    |          |            |             |
| GAPDH-RT-qPCR | F: CACTCACTCTTCTACCTT       | Exon     | 91         | -           |
|               | R: GCCAAATTCATTGTCGTA       |          |            |             |

Note: “D” means Deletion, “I” means Insertion. In the “Sizes (bp)” column, the length in front of the “/” represents the non-mutated target fragment, and the length after the “/” represents the mutated fragment (insertion or deletion).

## 2.6. Polymerase Chain Reaction Amplification and Genotyping of *CRY1*

Amplification was performed using a touchdown polymerase chain reaction (PCR) [35]. The touchdown PCR profile consisted of an initial 5 min denaturation at 95 °C, followed by 12 cycles in which the annealing temperature dropped from 68 °C to 50 °C (decreasing 3 °C every two cycles). Each cycle included denaturation at 95 °C (30 s), annealing (30 s), and extension at 72 °C (30 s). The program was concluded after 30 additional cycles using a fixed annealing temperature of 50 °C. The PCR products were electrophoresed on 3.0% agarose gels to identify the genotypes of each individual sample. Genotypes were determined using a DNA marker according to electrophoretic band patterns. A single band migrating farther from the sample well was classified as the DD genotype (Deletion/Deletion), whereas a single band closer to the well was classified as the II genotype (Insertion/Insertion). Samples displaying two bands were classified as having the ID genotype (Insertion/Deletion). Notably, the DD genotype was not observed in this population. The PCR products were sent to Tsingke Biotechnology Company (Beijing, China) for Sanger sequencing.

## 2.7. Tissue Expression Profiling and Relative Expression Analysis of *CRY1*

Variant information at position 5:70,335,655 was extracted from previously generated whole-genome re-sequencing data from our laboratory. The distribution and allele frequencies of the different genotypes at this locus were calculated. The mRNA expression profile of *CRY1* was analyzed using the publicly available CattleGTEx dataset (Gene\_TPM\_Matrix.txt.gz) [36]. TPM expression data for *CRY1* (ENSBTAG00000010149) were extracted from all available tissues. In total, expression data from 94 tissues comprising 26,414 samples were collected. For subsequent analyses, only tissues with a sample size  $\geq 5$  was retained. The expression levels were normalized to the median<sub>log<sub>2</sub>(TPM + 1)</sub>, and the tissues were ranked accordingly. The top 20 tissues with the highest median expression levels were selected for the visualization. For reproductive-related tissues, three categories, including the female reproductive system, male reproductive system,



and embryo, were selected based on the system classification. The expression levels of *CRY1* in these tissues were ranked using  $\text{median\_log}_2(\text{TPM} + 1)$  and presented accordingly.

### 2.8. Statistical Analysis

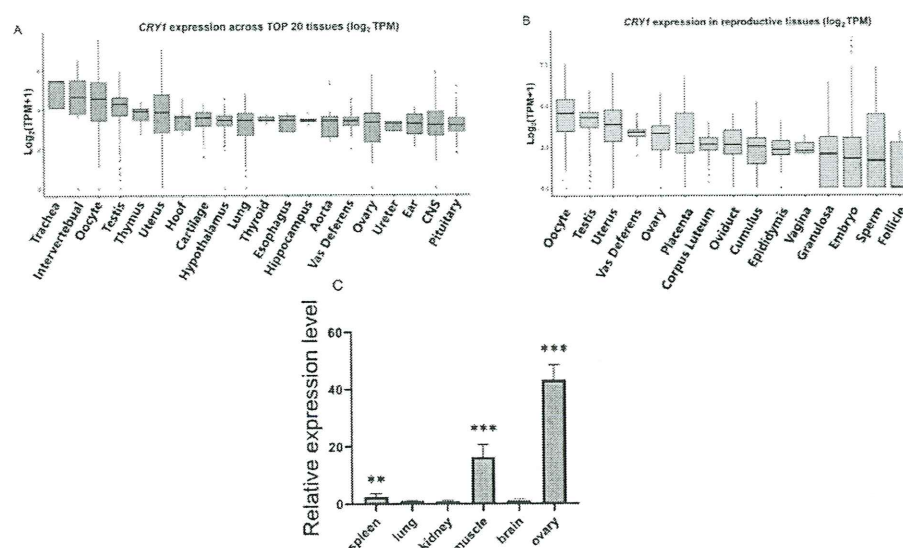
CattleGTEx public expression data ( $n = 26,414$  samples, 94 tissues) was used to generate the tissue expression profile of *CRY1*. Also, analysis of expected heterozygosity ( $H_e$ ), observed homozygosity ( $H_o$ ), effective allele number ( $N_e$ ), polymorphism information content (PIC), and Hardy–Weinberg equilibrium (HWE) for Chinese Holstein cows ( $n = 1.020$ ) was performed using the SHEsis online platform (<http://analysis.bio-x.cn>). The association between the variants and ovarian traits was determined using an independent-samples *t*-test with SPSS software (version 25.0). Only variants possessing at least two genotypes with a minimum of three individuals per genotype ( $n > 3$ ) were included in the statistical analysis. Similarly, a comparison of allele frequency was carried out for Charolais ( $n = 54$ ) and Simmental ( $n = 40$ ) breeds.

## 3. Results

### 3.1. Tissue Distribution and Reproductive Expression Profile of *CRY1* in Cattle

*CRY1* expression was analyzed across 94 tissues comprising 26,414 samples. The top 20 tissues with the highest expression levels are presented in Figure 1A. *CRY1* was highly expressed in the trachea, intervertebral tissue, oocyte, testis, thymus, uterus, hoof, cartilage, hypothalamus, lung, thyroid, esophagus, hippocampus, aorta, vas deferens, ovary, ureter, ear, central nervous system (CNS), and pituitary. Further analysis focused on reproductive-related tissues by selecting three categories from the system classification: female reproductive system, male reproductive system, and embryo. *CRY1* expression was highest in oocytes, followed by the testis, uterus, vas deferens, ovary, placenta, corpus luteum, oviduct, cumulus, epididymis, vagina, granulosa, embryo, sperm, and follicle (Figure 1B).

Subsequently, RT-qPCR analysis was performed using Holstein cattle tissues. *CRY1* expression was highest in the ovary, followed by muscle and spleen tissues ( $p < 0.01$ , Figure 1C).



**Figure 1.** Expression profile of the *CRY1* gene across multiple tissues. (A) Boxplot showing *CRY1* expression profile across the top 20 tissues based on  $\log_2(\text{TPM} + 1)$  values. Higher expression was





**Table 3.** *CRY1* gene P1-D6-bp loci allelic frequencies in other cattle breeds.

| Cattle Breeds    | Number of Samples | Reference Allele Frequency (I) | Alternate Allele Frequency (D) |
|------------------|-------------------|--------------------------------|--------------------------------|
| Charolais cattle | 54                | 1.000                          | 0                              |
| Simmental cattle | 40                | 0.950                          | 0.050                          |

### 3.4. Associations Between Genotypes and Ovarian Traits

Ovarian trait data and genotyping results revealed an association between P1-D6-bp (Table 4 and Table A1). In the metestrus cycle, P1-D6-bp was significantly associated with the diameter of the dominant follicle and corpus albicans ( $p < 0.05$ ). Individuals with the II genotype exhibited dominant follicles and corpus albicans with larger diameters than those with the ID genotype. However, P1-D6-bp showed no association with ovarian traits at the other three estrous cycle stages.

**Table 4.** Association between the P1-D6-bp variation within the *CRY1* gene and the ovarian traits (metestrus) of Chinese Holstein dairy cattle (LSM  $\pm$  SE).

| Quantitative Traits |               | Genotypes                |                         | <i>p</i> -Values |
|---------------------|---------------|--------------------------|-------------------------|------------------|
|                     |               | II                       | ID                      |                  |
| Ovary               | length (mm)   | 42.5 $\pm$ 0.6 (n = 223) | 40.0 $\pm$ 1.3 (n = 45) | 0.086            |
|                     | width (mm)    | 22.5 $\pm$ 0.5 (n = 223) | 23.6 $\pm$ 1.0 (n = 45) | 0.328            |
|                     | height (mm)   | 28.5 $\pm$ 0.4 (n = 222) | 28.3 $\pm$ 1.0 (n = 45) | 0.906            |
|                     | weight (g)    | 12.3 $\pm$ 0.3 (n = 223) | 12.0 $\pm$ 0.6 (n = 45) | 0.749            |
| Corpus luteum       | number        | 1.3 $\pm$ 0.4 (n = 218)  | 1.2 $\pm$ 0.1 (n = 45)  | 0.076            |
|                     | diameter (mm) | 15.4 $\pm$ 0.5 (n = 214) | 17.8 $\pm$ 1.8 (n = 44) | 0.109            |
| Dominant follicle   | number        | 1.4 $\pm$ 0.1 (n = 78)   | 1.4 $\pm$ 0.2 (n = 15)  | 0.906            |
|                     | diameter (mm) | 12.1 $\pm$ 0.6 (n = 78)  | 8.6 $\pm$ 1.4 (n = 15)  | <b>0.027</b>     |
| Corpus albicans     | number        | 1.5 $\pm$ 0.1 (n = 62)   | 1.8 $\pm$ 0.3 (n = 13)  | 0.160            |
|                     | diameter (mm) | 4.6 $\pm$ 0.3 (n = 60)   | 2.8 $\pm$ 0.3 (n = 12)  | <b>0.022</b>     |

Note: Bold font indicates a significant difference ( $p < 0.05$ ). Genotypes are omitted when the number of individuals was less than three.

## 4. Discussion

### 4.1. *CRY1* Tissue Expression

The circadian clock is thought to modulate fertility by coordinating reproductive processes, such as the estrus cycle and ovulation [19,20]. As a core component of the molecular oscillatory machinery, *CRY1* contributes to the fundamental structure of the circadian timing system [37]. In this study, we observed the expression pattern of *CRY1* in multiple tissues, which is consistent with its role as a core component of the mammalian circadian rhythm system [38]. Previous studies have demonstrated that core clock genes exhibit broad yet tissue-specific expression characteristics in mammals, suggesting the pivotal role of peripheral clocks in regulating tissue-specific physiological metabolism [39–41].

Although the central role of *CRY1* in the mammalian SCN is well-established, its expression across different animal taxa displays remarkable diversity and adaptability. In mammalian peripheral tissues, the liver exhibits the highest amplitude of *CRY1* expression, which is independently modulated by feeding rhythms, whereas *CRY1* is abundant but arrhythmic in the reproductive system [42,43]. In birds, *CRY1* is co-expressed in the retina and pineal gland, where it contributes to photoperiodic integration; European robin *CRY1* was long considered a magnetoreceptor, although this view has recently been challenged by structural evidence [44]. In reptiles and amphibians, *CRY1* expression has been linked to temperature-dependent rhythms and metamorphic development, respectively

[45]. In fish, genomic duplication has given rise to functionally divergent paralogs; in seasonally breeding species, *CRY1* expression in the hypothalamus–pituitary–gonadal axis correlates positively with GnRH, acting as a transducer of photoperiodic signals [46], whereas in cave-dwelling and deep-sea fish, prolonged absence of light has led to reduced expression and sequence degeneration [47]. Among insects, *Drosophila* carries a single *Cry* gene that functions primarily as a photoreceptor for light resetting, while honeybees and silkworms possess *CRY1* and *CRY2*, with *CRY1* mediating light-dependent behavioral regulation [48,49]. Collectively, these studies indicate that *CRY1* not only plays a central role in time sensing but has also acquired diverse species-specific non-circadian functions in reproduction, development, and navigation. Of particular interest is the abundant expression of *CRY1* in reproductive tissues across multiple species, suggesting a conserved but incompletely understood role in reproductive regulation.

Our observation that *CRY1* is highly expressed in oocytes and reproductive tissues was further validated by RT-qPCR in Chinese Holstein dairy cattle, which confirmed its enrichment in the ovary and was consistent with transcriptomic data. This expression pattern aligns with previous reports that circadian genes are actively expressed throughout the hypothalamic–pituitary–gonadal (HPG) axis and within reproductive organs, directly participating in gametogenesis, ovulation cycles, and early embryonic development [19,50,51]. Together, these findings support the hypothesis that genetic variants in *CRY1* may influence reproductive efficiency. Nevertheless, the specific effects of such polymorphisms on ovarian traits remain to be systematically characterized.

#### 4.2. *CRY1* in Ovarian Physiology

According to Wiggins and Legge (2016) [52], *CRY1* expression is significant across various follicular stages, including primordial, primary, and antral follicles, as well as the CL. These findings suggest that *CRY1* may be associated with ovarian function through its potential involvement in follicle development. Follicular growth is a complex process modulated by hormones, whereas follicles themselves serve as endocrine units. Previous studies have suggested that the sensitivity of ovarian follicular cells to gonadotropins may be associated with the activity of clock genes [53]. Consequently, endocrine factors could potentially contribute to the observed association between circadian genes and ovarian traits.

The potential association between *CRY1* and ovarian activity may be discussed from neuroendocrine and genetic perspectives. It is established that estradiol can modulate the expression of light-responsive genes and transcription factors in the SCN [54], where the presence of estrogen receptors has been confirmed [55–57]. Environmental factors, such as changing photoperiodism, are known to influence the rhythmic expression of clock genes, including *CRY1* [58]. Previous studies have suggested potential associations between ovarian steroids, the SCN, and circadian rhythms, which may be related to ovarian function. In this context, the P1-D6-bp mutation may be associated with an altered response within this neuroendocrine axis, potentially contributing to the observed variations in follicle and corpus albicans size. However, as environmental light conditions were not monitored in this study, the direct impact of photoperiodism remains a subject for further investigation. Disruption of *CRY1* function could therefore alter the timing and amplitude of hormone production, affecting follicular development and luteal function. However, direct evidence linking *CRY1* variants to altered steroidogenesis in cattle is currently lacking and warrants further investigation.

#### 4.3. Functional Implications of the P1-D6-bp InDel

The evaluated 6 bp InDel (rs133189221) is located in intron 1 of the bovine *CRY1* gene. While intronic variants are often considered non-functional, they can affect gene



expression through several mechanisms. First, intronic sequences may contain enhancer or silencer elements that regulate transcription. The P1-D6-bp InDel could disrupt transcription factor binding sites, thereby altering *CRY1* expression levels. Second, intronic variants can affect splicing regulation by creating or destroying splice enhancers or silencers [59]. Third, intronic regions may influence chromatin accessibility and three-dimensional genome organization, affecting long-range regulatory interactions [60]. The P1-D6-bp mutation might associate with an interrupted regulatory process, which could potentially contribute to the decreased diameter of dominant follicles and corpus albicans observed in this study.

*CRY1* allele I was found to be the dominant allele across all tested populations, reaching fixation (frequency = 1.000) in Charolais cattle and maintaining an exceptionally high frequency (0.919) in Chinese Holsteins. This high degree of conservation suggests that allele I may have been favored during long-term evolutionary processes or through intensive artificial selection for production traits in these breeds. Chinese Holstein dairy cattle exhibited a much higher frequency of the II genotype (0.837) than the ID genotype (0.163). The low genetic diversity of this population was evidenced by a PIC value of less than 0.25.

A notable deviation from Hardy–Weinberg equilibrium was detected at this locus ( $p < 0.05$ ). In contemporary dairy cattle, such deviations are frequently observed and are typically driven by a combination of factors, including intensive selection pressure, non-random mating practices associated with widespread AI sire usage [61,62], and underlying population substructure (e.g., Wahlund effect). For the Chinese Holstein population examined here, the significant HWE imbalance is probably a consequence of cumulative selection for production traits over generations, which can reduce effective population size and cause linked variation to drift. In addition, the heavy reliance on elite sires for artificial insemination inevitably introduces non-random mating, further pushing genotype frequencies away from HWE predictions.

However, due to the lack of genome-wide marker data and detailed pedigree information, these factors could not be formally evaluated in the present study. Our analysis of 1020 Chinese Holstein dairy cattle revealed that the P1-D6-bp variant was significantly associated with the diameter of dominant follicles and the corpus albicans during the metestrus phase. Specifically, individuals carrying the deletion mutation exhibited smaller dominant follicles and corpus albicans. In healthy adult cows, larger follicles usually have a higher probability of being selected as dominant follicles during the follicular wave. Although this process is co-regulated by multiple factors, such as the luteinizing hormone surge [63], these results suggest a potential role for *CRY1* in ovarian dynamics. Furthermore, follicle size correlates with serum estradiol concentrations [64]. Following ovulation, the follicle transforms into the CL, which eventually undergoes luteolysis to form the corpus albicans [40]. While the corpus albicans is non-endocrine, its size reflects the regression status of the preceding CL [40]. Thus, the observed association suggests that *CRY1* may participate in follicular development and subsequent post-ovulatory structural changes. However, the relatively small sample size of the ID genotype subgroup, the lack of hormone and reproductive performance data and the lack of functional analysis may be considered as limitations of the current study.

#### 4.4. Study Contribution and Implication

Despite these limitations, our findings provide valuable preliminary insights and generate testable hypotheses regarding the role of *CRY1* in bovine fertility. Building upon previous reports, our study demonstrates a statistical link between *CRY1* variants and ovarian morphological traits. These results suggest that *CRY1* may have potential as a biological marker and as a candidate gene for improving bovine fertility. However, as an

association study, these results establish correlation rather than direct causality; therefore, further large-scale functional studies are required to fully bridge the gap between CRY1 genotypes and localized ovarian physiology. The identified InDel, though requiring further validation, represents a potential candidate marker for future investigation, not an immediate tool for breeding programs. Marker-assisted selection (MAS) using variants in candidate genes offers a complementary approach to traditional phenotypic selection, particularly for traits with low heritability such as fertility. The high frequency of the II genotype in Holstein populations suggests that allele I may have been indirectly selected through its linkage with favorable production traits or may be maintained due to its potential beneficial effects on ovarian function.

## 5. Conclusions

A deletion mutation (P1-D6-bp) within the CRY1 gene showed a nominal association with the diameter of the dominant follicle and corpus albicans at metestrus in Chinese Holstein dairy cattle. These preliminary findings suggest that CRY1 may be associated with ovarian traits in cattle and could be a potential subject for further research into its role in reproductive function. However, these findings require validation in larger independent populations with appropriate correction for multiple testing. The current study may help to generate hypotheses for understanding the function of CRY1 and preliminarily explores its potential as a candidate marker for improving cattle fertility. Crucially, the biological basis for the estrus stage-specific observations, the influence of fixed effects such as season, age, parity, and source of ovary, and the lack of pedigree information are significant limitations that must be addressed in future investigations before any practical applications can be considered.

**Author Contributions:** Conceptualization, X.L. and Y.W.; methodology, X.C., E.J., Y.Y. and H.Z.; formal analysis, X.C., Z.L., X.L. and Y.Y.; investigation, X.C., E.J., H.Z., Y.Y., Z.L., X.L. and E.A.; data curation, X.C., E.J., Y.Y. and X.L.; writing—original draft preparation, X.C. and Y.Y.; writing—review and editing, X.C., Y.Y., H.Z., Z.L. and E.A.; supervision, X.L. and Y.W.; project administration, X.L. and Y.W.; funding acquisition, X.L. and Y.W. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by Key R&D Program of Ningxia Hui Autonomous Region, grant number 2024BBF01005; the National Innovation Project for Undergraduate of Northwest A&F University, grant number S202010712004; and the University Innovation Project for Undergraduate of Northwest A&F University, grant number 04009.

**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Northwest A&F University (IACUC-NWAFU) (protocol code DK20220201 and date of approval 1 February 2022).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author upon reasonable request.

**Acknowledgments:** We acknowledge the support of Jie Li from the College of Animal Science and Technology, Northwest A&F University, and Zhihan Niu from the College of Veterinary Medicine, Northwest A&F University, who collected ovary samples. We want to thank Zhou Yang and his team for providing samples and analyses of Charolais cattle and Simmental cattle in this work. Also, we wish to thank Chen Ningbo and his team for providing samples and analyses of other cattle breeds in this work.

**Conflicts of Interest:** The authors declare that they have no conflicts of interest.



## Abbreviations

The following abbreviations are used in this manuscript:

|             |                                  |
|-------------|----------------------------------|
| Indel       | Insertion–deletion               |
| <i>CRY1</i> | Cryptochrome gene 1              |
| PCR         | Polymerase chain reaction        |
| He          | Expected heterozygosity          |
| Ho          | Observed homozygosity            |
| Ne          | Effective allele number          |
| PIC         | Polymorphism information content |
| HWE         | Hardy–Weinberg equilibrium       |
| SCN         | Suprachiasmatic nucleus          |
| PCOS        | Polycystic ovary syndrome        |
| II          | Insertion/Insertion              |
| ID          | Insertion/Deletion               |
| DD          | Deletion/Deletion                |

## Appendix A

**Table A1.** Association between the 6 bp variation within the *CRY1* gene and the ovarian traits (diestrus, proestrus, and estrus) of Chinese Holstein dairy cattle (LSM  $\pm$  SE) ( $p > 0.05$ ).

| Quantitative Traits                    |               | Genotypes            |                     | p-Values |
|----------------------------------------|---------------|----------------------|---------------------|----------|
|                                        |               | II                   | ID                  |          |
| Diestrus (Stage of the estrous cycle)  |               |                      |                     |          |
| Ovary                                  | length (mm)   | 41.2 ± 0.5 (n = 424) | 42.4 ± 0.9 (n = 73) | 0.281    |
|                                        | width (mm)    | 23.6 ± 0.3 (n = 424) | 23.5 ± 0.9 (n = 73) | 0.868    |
|                                        | height (mm)   | 25.5 ± 0.4 (n = 424) | 25.2 ± 0.7 (n = 73) | 0.714    |
|                                        | weight (g)    | 11.3 ± 0.3 (n = 392) | 11.0 ± 0.6 (n = 73) | 0.760    |
| Corpus luteum                          | Number        | 1.7 ± 0.1 (n = 425)  | 1.8 ± 0.2 (n = 74)  | 0.221    |
|                                        | diameter (mm) | 10.4 ± 0.4 (n = 419) | 10.0 ± 0.9 (n = 74) | 0.619    |
| dominant follicle                      | Number        | 1.3 ± 0.1 (n = 115)  | 1.2 ± 0.1 (n = 25)  | 0.713    |
|                                        | diameter (mm) | 9.9 ± 0.5 (n = 115)  | 11.5 ± 1.3 (n = 24) | 0.226    |
| Corpus albicans                        | Number        | 1.6 ± 0.2 (n = 110)  | 1.6 ± 0.2 (n = 19)  | 0.926    |
|                                        | diameter (mm) | 4.0 ± 0.3 (n = 108)  | 4.1 ± 0.7 (n = 19)  | 0.971    |
| Ovary                                  | length (mm)   | 38.6 ± 1.0 (n = 105) | 40.2 ± 1.7 (n = 21) | 0.481    |
|                                        | width (mm)    | 18.1 ± 0.4 (n = 105) | 16.1 ± 1.3 (n = 21) | 0.091    |
|                                        | height (mm)   | 21.7 ± 0.8 (n = 105) | 21.0 ± 2.0 (n = 21) | 0.723    |
|                                        | weight (g)    | 7.0 ± 0.2 (n = 106)  | 6.7 ± 0.6 (n = 21)  | 0.520    |
| Proestrus (Stage of the estrous cycle) |               |                      |                     |          |
| Corpus albicans                        | number        | 1.4 ± 0.1 (n = 53)   | 1.7 ± 0.3 (n = 7)   | 0.236    |
|                                        | diameter (mm) | 4.5 ± 0.4 (n = 53)   | 4.4 ± 0.6 (n = 7)   | 0.962    |
| Estrus (Stage of the estrous cycle)    |               |                      |                     |          |
| Ovary                                  | length (mm)   | 37.8 ± 0.8 (n = 93)  | 38.6 ± 1.8 (n = 22) | 0.680    |
|                                        | width (mm)    | 18.8 ± 0.6 (n = 93)  | 19.3 ± 1.1 (n = 22) | 0.734    |
|                                        | height (mm)   | 21.9 ± 0.6 (n = 93)  | 22.6 ± 1.4 (n = 22) | 0.600    |
|                                        | weight (g)    | 7.8 ± 0.3 (n = 91)   | 8.2 ± 0.5 (n = 22)  | 0.456    |
| dominant follicle                      | number        | 1.3 ± 0.1 (n = 93)   | 1.3 ± 0.1 (n = 22)  | 0.971    |
|                                        | diameter (mm) | 11.8 ± 0.6 (n = 93)  | 11.5 ± 1.3 (n = 21) | 0.801    |
| Corpus albicans                        | number        | 1.3 ± 0.1 (n = 21)   | 1.5 ± 0.3 (n = 6)   | 0.570    |
|                                        | diameter (mm) | 6.1 ± 1.0 (n = 21)   | 3.8 ± 0.6 (n = 6)   | 0.214    |

Note: Genotypes are omitted if the number of individuals is less than three.

## References

1. Bach, A.; Terré, M.; Vidal, M. Symposium review: Decomposing efficiency of milk production and maximizing profit. *J. Dairy Sci.* **2020**, *103*, 5709–5725.
2. Gupta, D.; Sarkar, A.; Pal, Y.; Suthar, V.; Chawade, A.; Kushwaha, S.K. Bovine reproductive tract and microbiome dynamics: Current knowledge, challenges, and its potential to enhance fertility in dairy cows. *Front. Microbiomes* **2024**, *3*, 1473076.
3. Zhao, J.; Li, J.; Jiang, F.; Song, E.; Lan, X.; Zhao, H. Fertility-Associated Polymorphism within Bovine ITGβ5 and Its Significant Correlations with Ovarian and Luteal Traits. *Animals* **2021**, *11*, 1579.
4. Kouamo, J.; Dawaye, S.M.; Zoli, A.P.; Bah, G.S. Evaluation of bovine (*Bos indicus*) ovarian potential for in vitro embryo production in the Adamawa plateau (Cameroon). *Open Vet. J.* **2014**, *4*, 128–136.
5. Kennaway, D.J. The role of circadian rhythmicity in reproduction. *Hum. Reprod. Update* **2005**, *11*, 91–101.
6. Roenneberg, T.; Mellow, M. The Circadian Clock and Human Health. *Curr. Biol.* **2016**, *26*, R432–R443.
7. Xie, Y.; Tang, Q.; Chen, G.; Xie, M.; Yu, S.; Zhao, J.; Chen, L. New Insights Into the Circadian Rhythm and Its Related Diseases. *Front. Physiol.* **2019**, *10*, 682.
8. Gao, L.; Wang, K.; Cheng, M.; Zeng, Z.; Wang, T.; Wen, F.; Chen, J. Circadian clock dysfunction of epithelial cells in pulmonary diseases. *Int. J. Biochem. Cell Biol.* **2021**, *141*, 106110.
9. Malaguarnera, R.; Ledda, C.; Filippello, A.; Frasca, F.; Francavilla, V.C.; Ramaci, T.; Parisi, M.C.; Rapisarda, V.; Piro, S. Thyroid Cancer and Circadian Clock Disruption. *Cancers* **2020**, *12*, 3109.
10. Sciarra, F.; Franceschini, E.; Campolo, F.; Gianfrilli, D.; Pallotti, F.; Paoli, D.; Isidori, A.M.; Venneri, M.A. Disruption of Circadian Rhythms: A Crucial Factor in the Etiology of Infertility. *Int. J. Mol. Sci.* **2020**, *21*, 3943.
11. Bae, S.A.; Fang, M.Z.; Rustgi, V.; Zarbl, H.; Androulakis, I.P. At the Interface of Lifestyle, Behavior, and Circadian Rhythms: Metabolic Implications. *Front. Nutr.* **2019**, *6*, 132.
12. Akhtar, R.A.; Reddy, A.B.; Maywood, E.S.; Clayton, J.D.; King, V.M.; Smith, A.G.; Gant, T.W.; Hastings, M.H.; Kyriacou, C.P. Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Curr. Biol.* **2002**, *12*, 540–550.
13. Hastings, M.H.; Reddy, A.B.; McMahon, D.G.; Maywood, E.S. Analysis of circadian mechanisms in the suprachiasmatic nucleus by transgenesis and biolistic transfection. *Methods Enzymol.* **2005**, *393*, 579–592.
14. Patke, A.; Murphy, P.J.; Onat, O.E.; Krieger, A.C.; Özçelik, T.; Campbell, S.S.; Young, M.W. Mutation of the Human Circadian Clock Gene *CRY1* in Familial Delayed Sleep Phase Disorder. *Cell* **2017**, *169*, 203–215.e13.
15. Habashy, D.M.; Eissa, D.S.; Aboelez, M.M. Cryptochrome-1 Gene Expression is a Reliable Prognostic Indicator in Egyptian Patients with Chronic Lymphocytic Leukemia: A Prospective Cohort Study. *Turk. J. Haematol.* **2018**, *35*, 168–174.
16. Shafi, A.A.; McNair, C.M.; McCann, J.J.; Alshalalfa, M.; Shostak, A.; Severson, T.M.; Zhu, Y.; Bergman, A.; Gordon, N.; Mandigo, A.C.; et al. The circadian cryptochrome, *CRY1*, is a pro-tumorigenic factor that rhythmically modulates DNA repair. *Nat. Commun.* **2021**, *12*, 401.
17. Putker, M.; Wong, D.C.S.; Seinkmane, E.; Rzechorzek, N.M.; Zeng, A.; Hoyle, N.P.; Chesham, J.E.; Edwards, M.D.; Feeney, K.A.; Fischer, R.; et al. CRYPTOCHROMES confer robustness, not rhythmicity, to circadian timekeeping. *EMBO J.* **2021**, *40*, e106745.
18. Takasu, N.N.; Nakamura, T.J.; Tokuda, I.T.; Todo, T.; Block, G.D.; Nakamura, W. Recovery from Age-Related Infertility under Environmental Light-Dark Cycles Adjusted to the Intrinsic Circadian Period. *Cell Rep.* **2015**, *12*, 1407–1413.
19. Boden, M.J.; Kennaway, D.J. Circadian rhythms and reproduction. *Reproduction* **2006**, *132*, 379–392.
20. Kennaway, D.J.; Boden, M.J.; Varcoe, T.J. Circadian rhythms and fertility. *Mol. Cell. Endocrinol.* **2012**, *349*, 56–61.
21. Miller, B.H.; Olson, S.L.; Turek, F.W.; Levine, J.E.; Horton, T.H.; Takahashi, J.S. Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy. *Curr. Biol.* **2004**, *14*, 1367–1373.
22. Turek, F.W.; Joshu, C.; Kohsaka, A.; Lin, E.; Ivanova, G.; McDearmon, E.; Laposky, A.; Losee-Olson, S.; Easton, A.; Jensen, D.R.; et al. 2005 Obesity and metabolic syndrome in circadian Clock mutant mice. *Science* **2005**, *308*, 1043–1045.
23. Erickson, G.F.; Shimasaki, S. The role of the oocyte in folliculogenesis. *Trends Endocrinol. Metab.* **2000**, *11*, 193–198.
24. Casey, T.M.; Plaut, K. Circadian clocks and their integration with metabolic and reproductive systems: Our current understanding and its application to the management of dairy cows. *J. Anim. Sci.* **2022**, *100*, skac233.
25. Dillon, P.; Berry, D.P.; Evans, R.D.; Buckley, F.; Horan, B. Consequences of genetic selection for increased milk production in European seasonal pasture based systems of milk production. *Livest. Sci.* **2006**, *99*, 141–158.
26. Huang, Y.; Su, P.; Akhatayeva, Z.; Pan, C.; Zhang, Q.; Lan, X. Novel InDel variations of the *Cry2* gene are associated with litter size in Australian White sheep. *Theriogenology* **2022**, *179*, 155–161.



27. Karman, B.N.; Tischkau, S.A. Circadian clock gene expression in the ovary: Effects of luteinizing hormone. *Biol. Reprod.* **2006**, *75*, 624–632.
28. Gao, J.M.; Wang, Y.D.; Fan, Y.X.; Wu, X.Q.; Guo, Y.; Gao, L.Y.; Wan, S.X. Relationship between situation of the bovine ovarian corpus luteum and the numbers of isolated preantral follicles from them. *Chin. J. Vet. Sci.* **2001**, *21*, 181–183.
29. Li, J.; Zhang, S.; Shen, C.; Niu, Z.; Yang, H.; Zhang, K.; Liu, Z.; Wang, Y.; Lan, X. Indel mutations within the bovine HSD17B3 gene are significantly associated with ovary morphological traits and mature follicle number. *J. Steroid Biochem. Mol. Biol.* **2021**, *209*, 105833.
30. Aljanabi, S.M.; Martinez, I. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Res.* **1997**, *25*, 4692–4693.
31. Ginther, O.J.; Beg, M.A.; Donadeu, F.X.; Bergfelt, D.R. Mechanism of follicle deviation in monovular farm species. *Anim. Reprod. Sci.* **2003**, *78*, 239–257.
32. Rodgers, R.J.; Irving-Rodgers, H.F. Morphological classification of bovine ovarian follicles. *Reproduction* **2010**, *139*, 309–318.
33. Jiang, E.; Zhang, C.; He, Z.; Zhang, Y.; Yang, Y.; Pan, C.; Jiang, F.; Song, E.; Zhang, S.; Lan, X. A novel A-to-G mutation in circBDP1 alters adipocyte proliferation and differentiation and affects bovine carcass traits. *J. Zhejiang Univ. Sci. B* **2026**, Early View.
34. Wang, J.; Li, Q.; Wang, Z.; Zhang, S.; Bekana, K.; Zhang, Q.; Zheng, J.; Xu, H.; Lan, X.; Pan, C. LncRSFD Alternative Splicing Modulates the Proliferation and Differentiation of Adipocytes Through Sponging miRNAs. *Anim. Res. One Health* **2025**, Early View.
35. Zhang, K.J.; Mi, F.; Li, X.L.; Wang, Z.Y.; Jiang, F.G.; Song, E.L.; Guo, P.; Lan, X.Y. Detection of genetic variation in bovine CRY1 gene and its associations with carcass traits. *Anim. Biotechnol.* **2023**, *34*, 3387–3394.
36. Liu, S.; Gao, Y.; Canela-Xandri, O.; Wang, S.; Yu, Y.; Cai, W.; Li, B.; Xiang, R.; Chamberlain, A.J.; Pairo-Castineira, E.; et al. A multi-tissue atlas of regulatory variants in cattle. *Nat. Genet.* **2022**, *54*, 1438–1447.
37. Takahashi, J.S. Transcriptional architecture of the mammalian circadian clock. *Nat. Rev. Genet.* **2017**, *18*, 164–179.
38. Zhang, R.; Lahens, N.F.; Ballance, H.I.; Hughes, M.E.; Hogenesch, J.B. A circadian gene expression atlas in mammals: Implications for biology and medicine. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 16219–16224.
39. Sellix, M.T. Circadian clock function in the mammalian ovary. *J. Biol. Rhythms* **2015**, *30*, 7–19.
40. Sellix, M.T.; Menaker, M. Circadian clocks in the ovary. *Trends Endocrinol. Metab.* **2010**, *21*, 628–636.
41. Monaco, C.F.; Davis, J.S. Mechanisms of angioregression of the corpus luteum. *Front. Physiol.* **2023**, *14*, 1254943.
42. Xu, Y.Q.; Zhang, D.; Jin, T.; Cai, D.J.; Wu, Q.; Lu, Y.; Liu, J.; Klaassen, C.D. Diurnal variation of hepatic antioxidant gene expression in mice. *PLoS ONE* **2012**, *7*, e44237. <https://doi.org/10.1371/journal.pone.0044237>.
43. Tsuchiya, Y.; Minami, I.; Kadotani, H.; Todo, T.; Nishida, E. Circadian clock-controlled diurnal oscillation of Ras/ERK signaling in mouse liver. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* **2013**, *89*, 59–65. <https://doi.org/10.2183/pjab.89.59>.
44. Wickramaratne, A.C.; Rasmussen, E.S.; Chelliah, Y.; Schuhmann, F.; Solov'yov, I.A.; Mouritsen, H.; Green, C.B.; Zoltowski, B.D.; Takahashi, J.S. Structure of European robin cryptochrome 1 reveals a role in circadian rhythms, not magnetoreception. *iScience* **2025**, *28*, 114015.
45. Currie, S.P.; Doherty, G.H.; Sillar, K.T. Deep-brain photoreception links luminance detection to motor output in *Xenopus* frog tadpoles. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 6053–6058. <https://doi.org/10.1073/pnas.1515516113>.
46. Ikegami, T.; Takeuchi, Y.; Hur, S.P.; Takemura, A. Impacts of moonlight on fish reproduction. *Mar. Genom.* **2014**, *14*, 59–66. <https://doi.org/10.1016/j.margen.2013.11.007>.
47. Sun, Y.; Liu, C.; Huang, M.; Huang, J.; Liu, C.; Zhang, J.; Postlethwait, J.H.; Wang, H. The molecular evolution of circadian clock genes in spotted gar (*Lepisosteus oculatus*). *Genes* **2019**, *10*, 622. <https://doi.org/10.3390/genes10080622>.
48. Jackson, F.R.; You, S.; Crowe, L.B. Regulation of rhythmic behaviors by astrocytes. *Wiley Interdiscip. Rev. Dev. Biol.* **2020**, *9*, e372. <https://doi.org/10.1002/wdev.372>.
49. Yuan, X.N.; Luo, C.; Zhao, Q.F.; Zhong, S.Y.; Hang, Q.; Dai, T.M.; Pan, Z.H.; Sima, Y.H.; Qiu, J.F.; Xu, S.Q. The clock gene *Cryptochrome 1* is involved in the photoresponse of embryonic hatching behavior in *Bombyx mori*. *Arch. Insect Biochem. Physiol.* **2023**, *114*, e22046. <https://doi.org/10.1002/arch.22046>.
50. Dolatshad, H.; Campbell, E.A.; O'Hara, L.; Maywood, E.S.; Hastings, M.H.; Johnson, M.H. Developmental and reproductive performance in circadian mutant mice. *Hum. Reprod.* **2006**, *21*, 68–79.
51. Sellix, M.T.; Yoshikawa, T.; Menaker, M. A circadian egg timer gates ovulation. *Curr. Biol.* **2010**, *20*, R266–R267.
52. Wiggins, G.; Legge, M. Cyclic Variation of Cellular Clock Proteins in the Mouse Estrous Ovary. *J. Reprod. Infertil.* **2016**, *17*, 192–198.

53. Sellix, M.T. Clocks underneath: The role of peripheral clocks in the timing of female reproductive physiology. *Front. Endocrinol.* **2013**, *4*, 91.
54. Abizaid, A.; Mezei, G.; Horvath, T.L. Estradiol enhances light-induced expression of transcription factors in the SCN. *Brain Res.* **2004**, *1010*, 35–44.
55. Shughrue, P.J.; Lane, M.V.; Merchenthaler, I. Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *J. Comp. Neurol.* **1997**, *388*, 507–525.
56. Su, J.D.; Qiu, J.; Zhong, Y.P.; Chen, Y.Z. Expression of estrogen receptor -alpha and -beta immunoreactivity in the cultured neonatal suprachiasmatic nucleus: With special attention to GABAergic neurons. *Neuroreport* **2001**, *12*, 1955–1959. <https://doi.org/10.1097/00001756-200107030-00036>.
57. Shima, N.; Yamaguchi, Y.; Yuri, K. Distribution of estrogen receptor beta mRNA-containing cells in ovariectomized and estrogen-treated female rat brain. *Anat. Sci. Int.* **2003**, *78*, 85–97. <https://doi.org/10.1046/j.0022-7722.2003.00042.x>.
58. Zhao, S.Q.; Zhang, Y.; Gao, Y.; Yang, X.; Yang, Z.; Yang, Z.J. The in vitro effects of melatonin and Cry gene on the secretion of estradiol from camel ovarian granulosa cells. *Domest. Anim. Endocrinol.* **2021**, *74*, 106497.
59. Narasimamurthy, R.; Hatori, M.; Nayak, S.K.; Liu, F.; Panda, S.; Verma, I.M. Circadian clock protein cryptochrome regulates the expression of proinflammatory cytokines. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 12662–12667.
60. Ren, X.P.; Liu, M.M.; Chen, L.; Chen, T.Y.; Zhang, Y.H.; Li, P.L.; Liu, L.; Yuan, Z.Y. Serum Nuclear Factor-kB and Transforming Growth Factor-beta1 and Those Relationship with Insulin Resistance in Polycystic Ovary Syndrome. *Reprod. Contracept.* **2013**, *33*, 529–533, 545.
61. Evans, J.A.; Pan, H.; Liu, A.C.; Welsh, D.K. *CRY1*<sup>-/-</sup> circadian rhythmicity depends on SCN intercellular coupling. *J. Biol. Rhythms.* **2012**, *27*, 443–452.
62. Partch, C.L.; Green, C.B.; Takahashi, J.S. Molecular architecture of the mammalian circadian clock. *Trends Cell Biol.* **2014**, *24*, 90–99.
63. Atkins, J.A.; Smith, M.F.; Wells, K.J.; Geary, T.W. Factors affecting preovulatory follicle diameter and ovulation rate after gonadotropin-releasing hormone in postpartum beef cows. Part II: Anestrous cows. *J. Anim. Sci.* **2010**, *88*, 2311–2320.
64. Schams, D.; Berisha, B. Regulation of corpus luteum function in bovine—an overview. *Reprod. Domest. Anim.* **2004**, *39*, 241–251.

**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.



检索报告 (网络在线)

根据委托人陈宣伯委托, 通过网络检索, 陈宣伯发表的 1 篇论文于 2024 年 11 月 25 日网络在线发表。数据库具体检索结果如下:

标题: Exploring the distribution of polymorphism across diverse breeds Worldwide in the bovine NR5A2 gene and its correlation with number of mature follicles and corpus albicans  
作者: Enhui Jiang<sup>a\*</sup>, Xuanbo Chen<sup>a\*</sup>, Taotao Yan<sup>a</sup>, Yi Bi<sup>a,b</sup>, Juanshan Zheng<sup>a</sup>, Haiyu Zhao<sup>d</sup>, Yongsheng Wang<sup>e</sup>, Xiangchen Li<sup>c</sup> and Xianyong Lan<sup>a</sup>

期刊: AnimAl Biotechnology

Published online: Available online 25 November 2024

地址: <sup>a</sup>Shaanxi Key laboratory of molecular Biology for Agriculture, college of Animal Science and technology, northwest A&F University, yangling, Shaanxi, china; <sup>b</sup>institute of Biological and chemical Systems, Karlsruhe institute of technology, eggenstein-leopoldshafen, Karlsruhe, germany; <sup>c</sup>college of Animal Science and technology, Zhejiang A&F University, hangzhou, Zhejiang, china; <sup>d</sup>School of lifescience, lanzhou University, lanzhou, gansu, china; <sup>e</sup>college of veterinary medicine, northwest A&F University, yangling, Shaanxi, china

通讯作者地址: Xiangchen Li (通讯作者) college of Animal Science and technology, Zhejiang A&F University, hangzhou, Zhejiang, china; Xianyong Lan (通讯作者) Shaanxi Key laboratory of molecular Biology for Agriculture, college of Animal Science and technology, northwest A&F University, yangling, Shaanxi, china

DOI: 10. 1080/10495398. 2024. 2429692 ISSN: 1049-5398 eISSN: 1532-2378

第一作者蒋恩惠, 第一署各单位西北农林科技大学动物科技学院农业分子生物学陕西重点实验室。经查原文, 陈宣伯与蒋恩惠同等贡献, 署各单位西北农林科技大学动物科技学院农业分子生物学陕西重点实验室。

通讯作者李向臣, 署各单位浙江农林大学动物科学与技术学院。

通讯作者蓝贤勇, 署各单位西北农林科技大学动物科技学院农业分子生物学陕西重点实验室。

期刊《AnimAl Biotechnology》(ISSN: 1049-5398) 是 2026 年 SCI-Expanded 数据库收录期刊。

2026 年公布的影响因子: 2.0, JCR 分区情况:

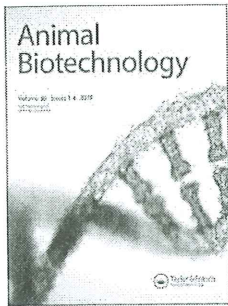
| JCR® 类别                              | 类别中的排序  | JCR 分区 |
|--------------------------------------|---------|--------|
| AGRICULTURE, DAIRY & ANIMAL SCIENCE  | 33/87   | Q2     |
| BIOTECHNOLOGY & APPLIED MICROBIOLOGY | 137/180 | Q4     |

2025 年公布的中科院分区 (升级版):

|    | 学科名称                                            | 分区 | Top 期刊 |
|----|-------------------------------------------------|----|--------|
| 大类 | 农林科学                                            | 3  | 否      |
| 小类 | AGRICULTURE, DAIRY & ANIMAL SCIENCE 奶制品与动物科学    | 4  | -      |
| 小类 | BIOTECHNOLOGY & APPLIED MICROBIOLOGY 生物工程与应用微生物 | 4  | -      |

查证检索:

西北农林科技大学图书馆  
2026 年 9 月 8 日



# Exploring the distribution of polymorphism across diverse breeds Worldwide in the bovine *NR5A2* gene and its correlation with number of mature follicles and corpus albicans

Enhui Jiang, Xuanbo Chen, Taotao Yan, Yi Bi, Juanshan Zheng, Haiyu Zhao, Yongsheng Wang, Xiangchen Li & Xianyong Lan

To cite this article: Enhui Jiang, Xuanbo Chen, Taotao Yan, Yi Bi, Juanshan Zheng, Haiyu Zhao, Yongsheng Wang, Xiangchen Li & Xianyong Lan (2024) Exploring the distribution of polymorphism across diverse breeds Worldwide in the bovine *NR5A2* gene and its correlation with number of mature follicles and corpus albicans, Animal Biotechnology, 35:1, 2429692, DOI: [10.1080/10495398.2024.2429692](https://doi.org/10.1080/10495398.2024.2429692)

To link to this article: <https://doi.org/10.1080/10495398.2024.2429692>



© 2024 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group



View supplementary material [↗](#)



Published online: 25 Nov 2024.



Submit your article to this journal [↗](#)



Article views: 61



View related articles [↗](#)



View Crossmark data [↗](#)



# Exploring the distribution of polymorphism across diverse breeds Worldwide in the bovine *NR5A2* gene and its correlation with number of mature follicles and corpus albicans

Enhui Jiang<sup>a\*</sup>, Xuanbo Chen<sup>a\*</sup>, Taotao Yan<sup>a</sup>, Yi Bi<sup>a,b</sup>, Juanshan Zheng<sup>a</sup>, Haiyu Zhao<sup>d</sup>, Yongsheng Wang<sup>e</sup>, Xiangchen Li<sup>c</sup> and Xianyong Lan<sup>a</sup>

<sup>a</sup>Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi, China; <sup>b</sup>Institute of Biological and Chemical Systems, Karlsruhe Institute of Technology, Eggenstein-Leopoldshafen, Karlsruhe, Germany; <sup>c</sup>College of Animal Science and Technology, Zhejiang A&F University, Hangzhou, Zhejiang, China; <sup>d</sup>School of life science, Lanzhou University, Lanzhou, Gansu, China; <sup>e</sup>College of veterinary medicine, Northwest A&F University, Yangling, Shaanxi, China

## ABSTRACT

The *Nuclear receptor subfamily 5 group A member 2 (NR5A2)* gene plays a pivotal role in ovarian development, ovulation, and reproductive traits. There is a lack of studies on its impact on ovarian traits and reproductive traits in cattle. This study aimed to explore *NR5A2* gene polymorphisms associations with reproductive traits and investigate the distribution of *NR5A2* gene polymorphisms across diverse bovine breeds worldwide. We identified a novel 17-bp deletion within the *NR5A2* gene specifically in Chinese Holstein cows ( $n=1033$ ) leading to the observation of two genotypes DD and ID. Subsequent association analysis revealed a significant correlation between the 'ID' genotype at this locus and a larger number of corpus albicans ( $p=0.042$ ) in diestrus, as well as a higher number of mature follicles ( $p=0.038$ ) in estrus. In addition, we also found that the distribution of this deletion exhibits strong regionality across different cattle breeds globally. These findings indicate that the 17-bp deletion mutation within the *NR5A2* gene is significantly associated with an increased corpus luteum diameter and a greater number of mature follicles, suggesting its potential utility as a valuable DNA marker for enhancing cow fertility.

## ARTICLE HISTORY

Received 23 August 2024  
Accepted 7 November 2024

## KEYWORDS

Cow; *NR5A2* gene; deletion; ovary; reproduction

## 1. Introduction

In recent decades, there has been a notable emphasis on milk production which has overshadowed the importance of fertility in dairy cow breeding, resulting in a gradual decline in reproductive efficiency.<sup>1</sup> Until the late twentieth century, dairy selection indices predominantly prioritized milk fat and protein production, often neglecting other critical traits.<sup>2</sup> However, low fertility significantly impacts dairy herd regeneration and cows' ability to conceive seasonally. Failure to maintain a one-year calving interval can result in substantial economic losses, often outweighing those incurred due to decreased milk production.<sup>3</sup> The fertility of dairy cows plays a pivotal role in determining the economic sustainability of dairy farming. As a result, modern selection indices globally no longer

rely solely on milk fat and protein production; milk production traits now typically account for approximately 50% of the overall index.<sup>2</sup> Therefore, improving the reproductive performance of dairy cows through selective breeding is crucial for the dairy industry.

In the process of breed selection, molecular marker-assisted selection (MAS) has garnered widespread adoption due to its efficiency and precision.<sup>4</sup> The initial step in utilizing the MAS method involves the identification of candidate genes, particularly those with essential functions in the female ovary. In this context, the *nuclear receptor subfamily 5 group A member 2 (NR5A2)* gene emerges as a significant candidate gene that has been extensively studied in various female organisms. *NR5A2*, also known as liver receptor homolog-1 (LRH-1), belongs to the nuclear

**CONTACT** Xianyong Lan ✉ [lanxianyong79@126.com](mailto:lanxianyong79@126.com); [lanxianyong79@nwsuaf.edu.cn](mailto:lanxianyong79@nwsuaf.edu.cn) College of Animal Science and Technology, Zhejiang A&F University, Hangzhou 311300, Zhejiang, China; Xiangchen Li ✉ [xcli863@zafu.edu.cn](mailto:xcli863@zafu.edu.cn) College of Animal Science and Technology, Northwest A&F University, Yangling 712100, Shaanxi, China.

\*These authors contributed equally to this work.

 Supplemental data for this article can be accessed online at <https://doi.org/10.1080/10495398.2024.2429692>.

© 2024 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group  
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The terms on which this article has been published allow the posting of the Accepted Manuscript in a repository by the author(s) or with their consent.



receptor NR5A subfamily.<sup>5,6</sup> It encodes a protein that belongs to the subfamily of orphan nuclear receptors, possessing a DNA-binding zinc finger.<sup>7,8</sup> NR5A2 is expressed in various endoderm-derived organs including the liver, pancreas, and intestine.<sup>9</sup> NR5A2 is expressed in granulosa cells<sup>10</sup> where it modulates their proliferation. Research has shown that the depletion of NR5A2 retards the proliferation of granulosa cells in the ovary, arresting them in the S phase, inducing anovulation in mice.<sup>11–13</sup> Moreover, the NR5A2 gene plays crucial roles in sex hormone secretion. In the process of gonadotropin synthesis, NR5A2 promotes the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the anterior pituitary gland and gonadotropic cell lines by regulating the expression of gonadotropin-related genes in rats.<sup>14,15</sup> NR5A2 knockout mice (NR5A2<sup>+/-</sup>) exhibit significantly lower fertility.<sup>16</sup> Collectively, these findings suggest that the NR5A2 gene is instrumental in regulating reproductive traits in females. Therefore, exploring the indel mutations of NR5A2 could be beneficial for breeding cows with superior reproductive performance.

The ovary, a key indicator of fertility, performs various functions encompassing ovulation, reproductive hormone secretion, and regulation of the female estrus cycle.<sup>17,18</sup> In dairy cattle breeding, ovarian functions directly influence reproduction traits with reproductive failure constituting a primary criterion for culling individuals.<sup>19</sup> Therefore, it's imperative to investigate the factors influencing ovarian function and traits. However, the ovaries exhibit distinct morphological characteristics during the various estrus stages (proestrus, estrus, postestrus, and diestrus): during proestrus, follicles initiate their development; during estrus, these follicles undergo rapid development; in the postestrus stage, follicles rupture to complete ovulation, marking the beginning of the formation of a new corpus luteum; and during diestrus, the corpus luteum enlarges, reaching its maximum size.<sup>20</sup> Therefore, in this study, we collected ovaries from cows at different estrus cycles for research (Figure 1).

Marker-assisted selection (MAS), particularly through the utilization of insertion/deletion (indel) variations, has recently emerged as a widespread practice in livestock breeding, encompassing pigs, chickens, goats, and cattle.<sup>21–26</sup> MAS holds promise in enhancing ovarian functions in cows, thereby optimizing their reproductive potential.

The polymorphism of NR5A2 has been applied to the research of various animal diseases and traits. In human, it has often been found that polymorphisms

in the NR5A2 gene are significantly associated with a range of conditions, including pancreatic cancer, bone density issues and spontaneous abortion.<sup>27–29</sup> In cattle, through genome-wide association analysis, it was found that NR5A2 may be associated with bull reproductive traits.<sup>30</sup> In sheep, multiple single nucleotide polymorphisms in the NR5A2 gene are significantly associated with litter size. One of these SNPs, T>G, can regulate upstream transcription factors to promote the expression of the NR5A2 gene, thereby affecting litter size in sheep.<sup>5,31</sup> However, there remains a paucity of studies regarding their associations with ovary traits in cows. Therefore, this study was conducted to detect possible novel indels within the NR5A2 gene and evaluate their effects on reproductive traits in dairy cows, to support some theoretical basis for the fertility breeding of cows.

## 2. Materials and methods

Experimental protocols were approved by the Faculty Animal Policy and Welfare Committee of Northwest A&F University under contract (protocol no. NWAUFU-314020038).

### 2.1. Bioinformatic analysis

Nucleotide sequences from *Bos taurus* (NM\_001206816.1), *Ovis aries* (NM\_001246280.1), *Capra hircus* (XM\_018060819.1), *Sus scrofa* (NM\_001267893.1), *Homo sapiens* (NM\_001276464.2) and *Gallus gallus* (NM\_205078.1) were obtained from the NCBI database. The Blast database (blastn) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to detect nucleotide sequence similarity, and the MEGA7 MUSCLE program was used to compare multiple nucleotide sequences and perform evolutionary analysis.

### 2.2. Samples and related data collection

In this study, ovary samples were obtained from 1033 Chinese Holstein cows in the same breeding farm. Based on the external behavior of the cows and the morphology of the corpus luteum in the ovary, we were able to determine the estrus period in which each cow was located, including interestrus, proestrus, estrus, and postestrus (Figure 1).<sup>32,33</sup> Subsequently, these samples were used to extract genomic DNA for further experiments.

In details, ovary samples were obtained from Chinese Holstein cows of the same age (5–6 years old). The ovary morphological features were measured by



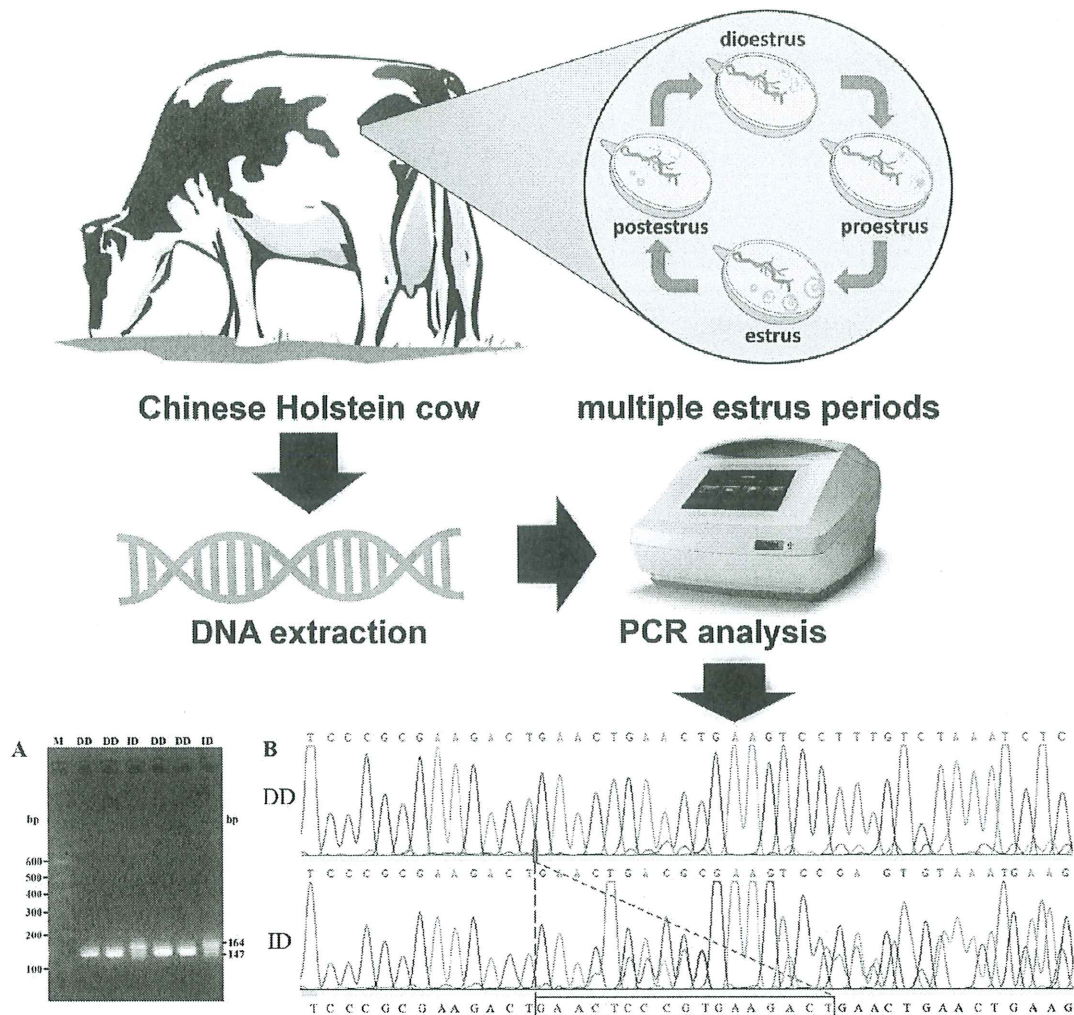


Figure 1. Agarose gel electrophoresis of Chinese Holstein cow *NR5a2* gene loci P7. (A) Agarose gel electrophoresis of bovine *NR5a2* gene P8 locus. (B) DNA sequencing diagram of the 17-bp deletion of *NR5a2* gene (ID and DD genotypes).

the same standard, including the length: the length of the long axis of the ovary, width: the length of the short axis of the ovary, height: the thickness of the ovary, weight of ovary; the number of corpus albicans, corpus luteum, mature follicles; the diameter of corpus luteum, corpus albicans, and mature follicles. In this process, sterile electronic scales and a vernier caliper were used to measure weight, length, width, and height of the ovaries (Figure 2).<sup>32,33</sup>

### 2.3. Genomic DNA isolation and DNA pool construction

According to Aljanabi's method, whole genome DNA was extracted from blood tissues.<sup>34,35</sup> Next, DNA was quantified by Nanodrop 1000 and diluted to 50 ng/μL. In addition, 50 DNA samples were selected

randomly to construct a genomic DNA pool to explore genetic variation in the candidate gene.<sup>32,36</sup> The purpose of the DNA mixing pool was to detect whether these mutations were polymorphic in Chinese Holstein cows.<sup>37</sup>

### 2.4. Primer design and PCR amplification

A total of eight indels of the *NR5A2* gene were derived from the NCBI (<https://www.ncbi.nlm.nih.gov/>) and Ensembl (<http://www.ensembl.org/index.html>) databases. The specific forward and reverse primers were designed using the Primer Premier 5.0 software (Premier, Canada). Primers for each mutation site were designed to maximize the amplification of the target fragment to make sure that each pair of primers was the most appropriate for each

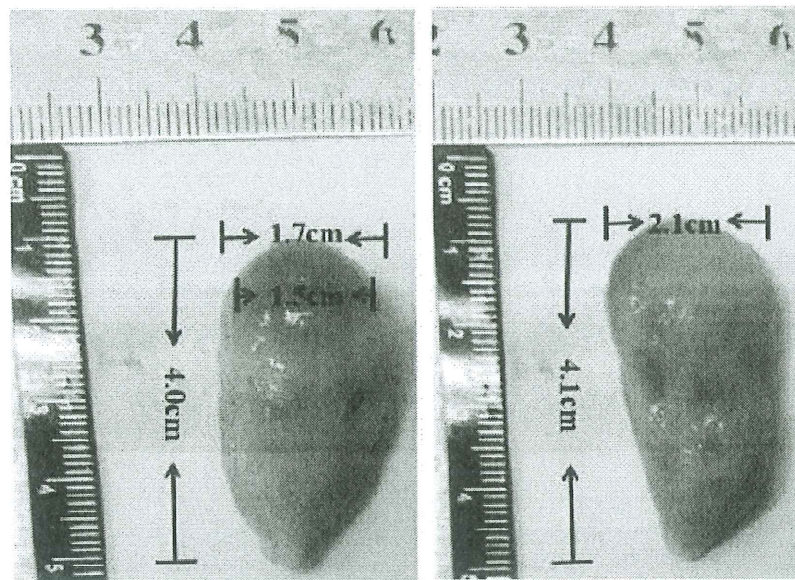


Figure 2. Diagram of measured ovaries of Chinese Holstein cow.

Table 1. Primers used for detecting *NR5A2* indel variants.

| Loci | Primer sequences (5'-3')                              | Product Size (bp) | Region   | Variant ID  |
|------|-------------------------------------------------------|-------------------|----------|-------------|
| P1   | F: CTTCTGATGGGCTTTTCGAGT<br>R: GTGCTTTGGAGAAACCGGAA   | 205/191           | Intron 1 | rs433404285 |
| P2   | F: AACGCCATGTTTCCGACCT<br>R: TGGGGCGCTCTTTTAGTCTT     | 167/157           | Intron 4 | rs721454859 |
| P3   | F: AAGCTCTTATTGCGTGAGTGTC<br>R: CGGAAGTAAGGGTGTGGTTCT | 288/271           | Intron 4 | rs482206194 |
| P4   | F: GCCGTGTAGAGGGGAATCAG<br>R: CGGACTAATCGGGTCTCTGC    | 295/280           | Intron 5 | rs450027422 |
| P5   | F: TAGGGTACGTGGAGAGCTGTT<br>R: AAGAGCTGCCACGATGGATT   | 260/248           | Intron 6 | rs717775415 |
| P6   | F: CGTTGCTCTTGGCCGTT<br>R: CAAAGACTGAACCTACCGTCC      | 237/230           | Intron 6 | rs718234858 |
| P7   | F: GCAAGTTGTACAGGCTCATTCTA<br>R: CCACTTCGCATCCGGAGAA  | 297/280           | Intron 6 | rs470625485 |
| P8   | F: TTTTACATGCAGTTCCCGCCAAA<br>R: TCCCGCGAAGACTGAACTG  | 164/147           | Intron 6 | rs135422978 |

variation (Table 1). All primer pairs were synthesized by Tsingke Biotech (Xian, Shaanxi, China). In this study, a touch-down polymerase chain reaction (TD-PCR) program was performed. The reaction mixture contained genomic DNA, primers, Master Mix (Vazyme, Shaanxi, China). Eventually, fragments of PCR products were identified by electrophoresis; then homozygous and heterozygous variants were sequenced directly by Tsingke Biotech (Xian, Shaanxi, China).<sup>38,39</sup> TD-PCR was performed in 12.5  $\mu$ L reaction volume, containing 1.5  $\mu$ L (10 ng/ $\mu$ L) of genomic DNA, 0.5  $\mu$ L of each primer, 6.0  $\mu$ L 2 $\times$  Taq Master Mix, 4.0  $\mu$ L ddH<sub>2</sub>O. The thermal cycling program was 5 min at 95°C for pre-denaturation, then 18 cycles at 94°C for 30 s, then annealing for 30 s at

68°C (with a decrease of 1°C per cycle), 15 s at 72°C, and a final extension at 72°C for 10 min. Finally, 4.0  $\mu$ L PCR products were directly assayed by electrophoresis on 3.0% agarose gels stained with nucleic acid dye.

## 2.5. Potential transcription factor binding site prediction

We used JASPAR (<http://jaspar.genereg.net/collection/core/>) and Alibaba2 ([gene-regulation.com/pub/programs/alibaba2/index.html](http://gene-regulation.com/pub/programs/alibaba2/index.html)) online databases to predict the transcription factors (TFs) that bind to mutations.

## 2.6. Worldwide bovine breeds' sample information

Our data were retrieved from the BGVD database (accessed on July 3rd, 2022, via <http://animal.omics.pro/code/index.php/BosVar>), encompassing 54 bovine breeds and 432 samples. The comprehensive sample set comprised individuals from diverse geographical backgrounds: 108 from West Europe, 28 from Northeast Asia, 9 from the Middle East, 9 from Tibet, 47 from North-Central China, 21 from Northwest China, 33 from South China, 24 from Indo-Pakistan, 83 from Central-South Europe, and 70 from Africa.

This database provided signatures of selection specific to eight distinct groups, six of which are designated as the 'core' cattle groups and acknowledged as ancestral lineages. These included Indian indicine (IN), Chinese



Table 2. Genetic parameters of the 17-bp deletion within the *NR5A2* gene in Chinese Holstein cow.

| Sample size | Genotypes frequencies |                  |            | Alleles frequencies |       | <i>p</i> value (HWE)  | Population indexes |       |       |       |
|-------------|-----------------------|------------------|------------|---------------------|-------|-----------------------|--------------------|-------|-------|-------|
|             | DD                    | ID               | II         | D                   | I     |                       | Ho                 | He    | Ne    | PIC   |
| 1033        | 0.587<br>(n=606)      | 0.413<br>(n=427) | 0<br>(n=0) | 0.793               | 0.207 | $5.6 \times 10^{-17}$ | 0.672              | 0.328 | 1.488 | 0.274 |

Note: HWE: Hardy-Weinberg equilibrium; Ho: observed homozygosity; He: heterozygosity; Ne: effective allele numbers; PIC: polymorphism information content.

indicine (CN), East Asian taurine (EA), Eurasian taurine (EUA), European taurine (EUR), and African taurine (AFR). Furthermore, this database boasts the identification of approximately 60.4 million autosomal SNPs and around 6.8 million autosomal INDELs. It offered a comprehensive display of the minor allele frequencies (MAF) of these genetic variations across the entire cattle population, along with allele frequencies specific to individual breeds and the designated 'core' cattle groups.

### 2.7. Statistical analyses

The genotypic and allelic frequencies, the population genetic parameters (HWE, Hardy-Weinberg equilibrium; He, heterozygosity; Ho, homozygosity; Ne, effective allele number, polymorphism information content, PIC, the polymorphism information content) of the mutation site was calculated according to<sup>39</sup> The independent sample *t*-test used in the SPSS software was to analyze the correlation between genotypes and traits (version 25.0, IBM, USA).

## 3. Results

### 3.1. Identification of a novel 17-bp deletion within the bovine *NR5A2* gene

DNA genotyping revealed a novel 17-bp deletion (5'-GAAGTACCGCGAAGACT-3') in the intron 7 of the bovine *NR5A2* gene, located on chromosome 16 at position 78947846-78947862 (Figure 1B). Using agarose gel electrophoresis, we distinguished two genotypes: DD and ID. The DD genotype exhibited an amplified fragment size of 147bp, while the ID genotype displayed a dual fragment size of 164bp and 147bp (Figure 1A). Notably, the deletion sequence significantly differed from that reported in the Ensembl database (GAAGACGCTGGTTTGTC; rs470625485), indicating a potential novel variant within the bovine *NR5A2* gene.

### 3.2. Genetic parameters of the 17-bp deletion within the *NR5A2* gene

The detailed genotype and allele frequency distribution, including He, Ho, Ne, PIC, and other relevant param-

Table 3. Association of the 17-bp deletion with ovarian morphological traits in multiple estrus.

| Estrus periods | Traits                        | Genotypes (Mean $\pm$ SE)  |                            | <i>p</i> -values |
|----------------|-------------------------------|----------------------------|----------------------------|------------------|
|                |                               | ID                         | DD                         |                  |
| Diestrus       | Number of Corpus albicans     | 1.68 $\pm$ 0.19<br>(n=31)  | 1.24 $\pm$ 0.09<br>(n=33)  | 0.042            |
|                | Corpus albicans diameter (mm) | 4.35 $\pm$ 0.48<br>(n=31)  | 4.64 $\pm$ 0.72<br>(n=33)  | 0.749            |
| Estrus         | Number of mature follicles    | 1.44 $\pm$ 0.15<br>(n=36)  | 1.10 $\pm$ 0.05<br>(n=39)  | 0.038            |
|                | Mature follicle diameter (mm) | 14.17 $\pm$ 0.61<br>(n=36) | 14.03 $\pm$ 0.77<br>(n=39) | 0.888            |

eters for this mutation, were presented in Table 2. In the Chinese Holstein cow population, only two genotypes, 'DD' and 'ID', were observed. The frequency of the 'DD' genotype (0.587) was higher than that of the 'ID' genotype (0.413). The frequency of the 'D' allele (0.793) was also higher than that of the 'I' allele (0.207). Since the *p*-value was  $5.6 \times 10^{-17}$  ( $p < 0.05$ ), this deletion variation deviates significantly from HWE. The PIC value indicates that this variant exhibited medium genetic diversity in the population of interest.

### 3.4. Association analysis between the 17-bp deletion and ovarian morphological characteristics

To explore the influence of the 17-bp deletion within the *NR5A2* gene on fertility, an independent-sample *t*-test analysis was used to detect associations between the deletion and ovarian morphological characteristics during various stages, including the diestrus, proestrus, estrus, and postestrus (Table 3; Tables S1–S4). We found that the 17-bp deletion was significantly associated with the number of Corpus albicans in diestrus ( $p=0.042$ ) and the number of mature follicles in estrus ( $p=0.038$ ) (Table 3). In addition, individuals carrying the 'DD' genotype exhibited superior traits compared to those with the 'ID' genotype. Although we tested the association between the 17-bp deletion and other ovarian morphological characteristics, such as ovarian length, ovarian width, and ovarian height, no significant correlation was observed ( $p > 0.05$ ) (Tables S1–S4.).

3.5. Prediction of potential transcription binding factors

To explore whether this deletion affects the binding of transcription factors to the *NR5A2* gene, the JASPAR online database was used. Specifically, the database predicted that a subsequence within the 17-bp deletion, namely ACTACCGCGAA, might bind to the E2F transcription factor 1 (E2F1) [MA0024.2] (Figure 3).

3.6. Analysis of *NR5A2* gene distribution in 54 bovine breeds and 6 core bovine breeds Worldwide

To investigate the global distribution of the 17-bp deletion, we utilized the BGVD database (accessible at <http://animal.omics.pro/code/index.php/BosVar>) to analyze 54 bovine breeds, as depicted in Figure 4.

Notably, the deletion's distribution appeared to be influenced by geographical location, with distinct patterns emerging between southern and northern regions (divided with a dashed line). Specifically, the 'I' allele was relatively rare in Indian indicine (0.174), Chinese indicine (0.132), and Africa taurine (0.100) breeds. Conversely, it was more prevalent in East Asian taurine (0.608), European taurine (0.803), and Eurasian taurine (0.737) breeds (Figure 5).

4. Discussion

In recent decades, the emphasis on milk production in dairy cow breeding has led to a decline in fertility rates.<sup>1</sup> Low fertility often indicated by failure to conceive can collapse milk productivity for dairy cows and result in significant economic losses for the dairy industry. Therefore, it is crucial to improve the

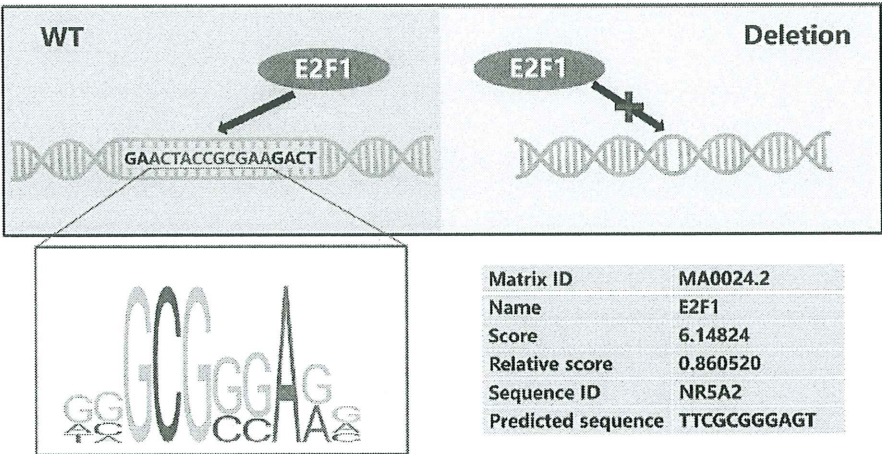


Figure 3. Predicted combination of transcription factors (TFs) and sequence of the *NR5A2* gene.

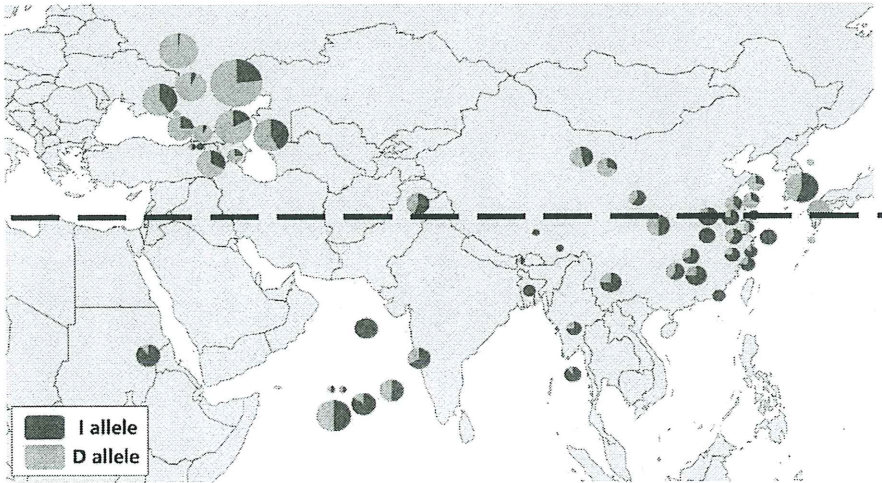


Figure 4. Allele frequency distribution of P7-<sub>17bp</sub> of *NR5A2* in world-wide 54 cattle breeds.



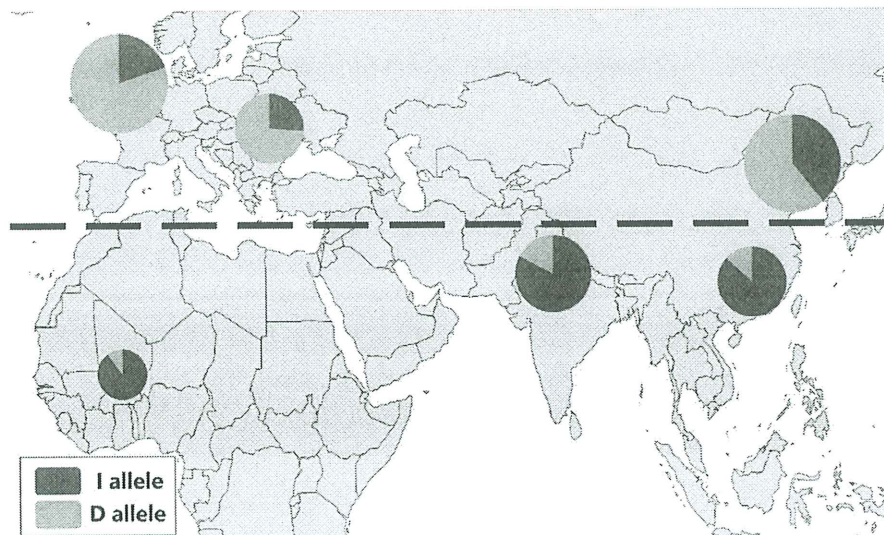


Figure 5. Allele frequency distribution of P7-<sub>17bp</sub> of NR5A2 in worldwide 6 core cattle breeds.

fertility of dairy cows. To achieve this, various reproductive traits, such as the estrous cycle,<sup>40</sup> litter size,<sup>24,41</sup> and ovarian status,<sup>32,42,43</sup> can be leveraged in breeding processes. Exploring polymorphisms within candidate genes associated with ovarian development and reproduction is imperative, and genetic selection based on these polymorphisms can yield significant benefits. Marker-assisted selection (MAS) method is a feasible approach to explore mutations in reproductive-related candidate genes, including the *NR5A2* gene (reference for MAS method).

This study explored a novel deletion mutation within the bovine *NR5A2* gene in a large population, including 1,033 Chinese Holstein cows. In the Chinese Holstein cow population, the 17-bp deletion showed moderate polymorphism. In addition, the population deviated from Hardy-Weinberg equilibrium, suggesting it was not at dynamic equilibrium probably due to artificial selection, migration, and genetic drift.<sup>44</sup> The 'D' allele corresponded to high frequencies in the Chinese Holstein cow population (0.793), which could be identified as a proto-gene. In addition, no individual with the genotype 'II' was found in this population. Furthermore, based on the distribution of this polymorphism across some global cattle breeds, the genotype frequency of II is generally low, and some breeds completely lack individuals with this genotype. This suggests that this breed may have undergone genetic drift, founder effects, and selective pressure leading to the absence of the II genotype.<sup>45,46</sup> Ovarian morphology characteristics serve as a reproductive indicator to select the best individuals. We conducted independent-sample t-test analyses to examine the

association between the deletion and ovarian morphological traits. Our results indicated that the 17-bp deletion significantly affected the number of mature follicles in estrus and the corpus albicans in diestrus.

During the estrus cycle, follicles rapidly grow, develop, mature, and lead to ovulation during estrus, followed by the formation of the corpus luteum. The corpus luteum then gradually shrinks after ovulation and transforms into a white connective tissue scar known as the corpus albicans. During this process, individuals with ID genotype exhibit an increase in the number of mature follicles during estrus, indirectly leading to a tendency of increased corpus luteum in later stages, which in turn diestrus and increases the number of corpus albicans. This suggests that individuals with ID genotype have more active ovulation activities compared to those with DD genotype. This suggests that the *NR5A2* gene is involved in ovarian development given the significant relationship between its 17-bp deletion polymorphism and ovarian morphological traits.

In this study, we observed that ovarian morphological traits of cows were affected by the 17-bp deletion mutation, which might regulate the *NR5A2* gene function. *NR5A2* gene has been reported to affect fertility in several species such as mice,<sup>47</sup> rats,<sup>48</sup> goats,<sup>24,49</sup> and sheep.<sup>5,31</sup> Numerous studies have indicated that the *NR5A2* gene plays a pivotal role in follicular development and hormone secretion. In humans, large-scale meta-analyses have highlighted associations between the *NR5A2* gene and menarche timing.<sup>50</sup> In geese, RNA-seq and ATAC-seq analyses revealed that the *NR5A2* gene influences early goose

ovarian development.<sup>51</sup> *NR5A2* was highly expressed in mouse ovarian tissue particularly in ovarian granulosa cells.<sup>52</sup> During follicular formation, the *NR5A2* gene is expressed in the progranulosa cells of the primary follicle, granulosa cells of the primary follicle, and in the anaphase of follicle formation. *In vitro* studies have demonstrated its role in the differentiation of pluripotent and mesenchymal stem cells into steroid cells.<sup>13,53</sup> Various investigations have highlighted the functions of *NR5A2* in the ovary of laboratory animal models. For instance, the *NR5A2* depletion in granulosa cells resulted in ovulation failure<sup>11</sup> and decreased granulosa cell proliferation in mice.<sup>9</sup> Additionally, granulosa-specific *NR5A2* knockout females exhibited impaired cumulus expansion and ovulation.<sup>11,54</sup> Transgenic mouse models with depletion of *NR5A2* in granulosa cells of antral follicles generate similar outcomes.<sup>16</sup> Notably, specialized *NR5A2* knockout mouse models capable of reproduction, ovulation, and fertilization were found to be sterile. In these models, the corpus luteum exhibited reduced size and impaired function. The expression of ovarian steroid factors, such as STAR and CYP11A1, was significantly reduced, underscoring the necessity of the *NR5A2* gene for corpus luteum function.<sup>55</sup>

Furthermore, JASPAR prediction suggests that the 17-bp deletion may disrupt the binding of the gene transcription factor E2F1. E2F1 is significant in the reproductive physiology of female animals, particularly in ovarian function. In bovine ovarian granulosa cells, E2F1 has been found to enhance cell proliferation and suppress estradiol production by inhibiting FGF9.<sup>56</sup> In human ovaries, LARS2/E2F1/Mfn-2 exhibits regulatory functions in mitochondrial dysfunction and endoplasmic reticulum stress within ovarian granulosa cells.<sup>57</sup> In porcine ovarian granulosa cells, miR-17-5p regulates cell proliferation and estradiol synthesis by targeting the E2F1 gene.<sup>58,59</sup> Collectively, these studies suggest that this deletion might influence bovine ovarian traits by altering the interaction between E2F1 and *NR5A2*.

Although our study elucidated the influence of the 17-bp deletion mutation on ovarian morphological and reproductive traits in dairy cows, the specific mechanism of the 17-bp deletion mutation affecting ovarian morphological and reproductive traits of dairy cows remains to be further explored.

## 5. Conclusion

In conclusion, the novel 17-bp deletion mutation within the bovine *NR5A2* gene was significantly associated with increased corpus luteum diameter, number of mature

follicles and decreased age at first calving. All these outcomes suggest that it can be used as a candidate molecular marker for breeding high-fertility dairy cows.

## Acknowledgments

We appreciate Ph.D. Yongsheng Wang and his team for collecting the ovary samples and recording corresponding trait data. We are very thankful for the assistance of Jie Li, Shaoli Zhang, Yangyang Bai, Dongyun Xin, Tingting Liu, Yunyun Luo, Han Yang and Min Wang during genomic DNA extraction process. We also appreciate Ph.D. Hong Chen and his team for providing the platform for experiments.

## Authors' contributions

Enhui Jiang: Formal analysis, Software, Investigation, Writing - original draft. Xuanbo Chen: Investigation, Writing-review & editing; Project administration; Taotao Yan: Investigation, Writing-review & editing, Project administration, Yi Bi: Writing-review & editing. Juanshan Zheng: Formal analysis, Investigation. Haiyu Zhao: Software, Investigation. Yongsheng Wang: Writing-review & editing. Xiangchen Li: Conceptualization, Project administration. Xianyong Lan: Conceptualization, Validation, Funding acquisition, Supervision.

## Disclosure statement

The authors declare no conflict of interest.

## Funding

This work was supported by the Science Fund for Distinguished Young Scholars of Shaanxi Province (No. 2024JC-JCQN-30), the Shaanxi Provincial Innovation Leadership Program in Sciences and Technologies for Young and Middle-aged Scientists(No.2023SR205)and the Project of Undergraduate Science.

## Data availability statement

Data supporting the results of this study can be obtained from corresponding authors upon reasonable request.

## References

1. Ryotaro M. Physiological characteristics and effects on fertility of the first follicular wave dominant follicle in cattle. *J. Reprod. Dev.* 2019;65(4):289–295.
2. Cole, VanRaden, VanRaden PM. Symposium review: Possibilities in an age of genomics: The future of selection indices. *J Dairy Sci.* 2018;101(4):3686–3701.
3. Silviu-Ionuț B, Alina B. Ovarian cysts, an anovulatory condition in dairy cattle. *J Vet Med Sci.* 2020;82(10):1515–1522.
4. Cao C, Zhou Q, Kang Y, et al. A repertoire of single nucleotide polymorphisms (SNPs) of major fecundity



- BMPIR1B gene among 75 sheep breeds worldwide. *Theriogenology*. 2024;219:59–64.
5. Li Y, Zhang J, Qian Y, et al. A T>G mutation in the NR5A2 gene is associated with litter size in hu sheep through upregulation of promoter activity by transcription factor MTF-1. *Front Genet*. 2019;10:1011.
  6. Meinsohn MC, Hughes CH, Estienne A, et al. A role for orphan nuclear receptor liver receptor homolog-1 (LRH-1, NR5A2) in primordial follicle activation. *Sci Rep*. 2021;11(1):1079.
  7. Hughes CH, Rogus A, Inskeep EK, Pate JL. NR5A2 and potential regulatory miRNAs in the bovine CL during early pregnancy. *Reproduction*. 2021;161(2):173–182.
  8. Svenja M, Thomas B. Nuclear-mitochondrial crosstalk: On the role of the nuclear receptor liver receptor homolog-1 (NR5A2) in the regulation of mitochondrial metabolism, cell survival, and cancer. *IUBMB Life*. 2021;73(3):592–610.
  9. Marie C, Fanny M, et al. The orphan nuclear receptor liver homolog receptor-1 (Nr5a2) regulates ovarian granulosa cell proliferation. *J. Endocr. Soc*. 2017;2(1):24–41.
  10. Elisabeth F, Johan A, Kristina S. LRH-1: an orphan nuclear receptor involved in development, metabolism, and steroidogenesis. *Trends Cell Biol*. 2004;14(5):250–260.
  11. Duggavathi R, Volle D, Matakis C, et al. Liver receptor homolog 1 is essential for ovulation. *Genes Dev*. 2008;22(14):1871–1876.
  12. Han G, Marc C, Annemie M, et al. Reversible infertility in a liver receptor homologue-1 (LRH-1)-knockdown mouse model. *Reprod Fertil Dev*. 2014;26(2):293–306.
  13. Marie C, Fanny M, Kalyne B, Raj D, Kristina S, Bruce D. The orphan nuclear receptor liver homolog receptor-1 (Nr5a2) regulates ovarian granulosa cell proliferation. *J Endocr Soc*. 2018;2(1):24–41.
  14. Stan D, Bryn M, Angie L, et al. Nuclear receptor LRH-1 induces the reproductive neuropeptide kisspeptin in the hypothalamus. *Mol. Endocrinol*. 2013;27(4):598–605.
  15. Zheng W, Jimenez L, Rubin B, Halvorson L. Anterior pituitary gene expression with reproductive aging in the female rat. *Biol Reprod*. 2007;76(6):1091–1102.
  16. Labelle-Dumais C, Paré J, Bélanger L, Farookhi R, Dufort D. Impaired progesterone production in Nr5a2<sup>-/-</sup> mice leads to a reduction in female reproductive function. *Biol Reprod*. 2007;77(2):217–225.
  17. Hu WP, Liu MQ, Tian ZL, et al. Polymorphism, expression and structure analysis of key genes in the ovarian steroidogenesis pathway in sheep (*Ovis aries*). *Vet Med Sci*. 2021;7(4):1303–1315.
  18. Lan D, Xiong X, Huang C, Mipam T, Li J. Toward understanding the genetic basis of yak ovary reproduction: a characterization and comparative analyses of estrus ovary transcriptomes in yak and cattle. *PLoS One*. 2016;11(4):e0152675.
  19. Yimer N, Haron AW, Yusoff R. Determination of ovarian cysts in cattle with poor reproductive performance using ultrasound and plasma progesterone profile. *Vet Med Open J*. 2018;3(1):1–9.
  20. Li J, Zhang S, Shen C, et al. Indel mutation within the bovine HSD17B3 gene are significantly associated with ovary morphological traits and mature number. *J Steroid Biochem Mol Biol*. 2021;209:105833.
  21. Abdullah IO, Mahmuda BB, David RN, et al. Association of single nucleotide polymorphism in NLRC3, NLRC5, HIP1, and LRP8 genes with fecal egg counts in goats naturally infected with *Haemonchus contortus*. *Trop Anim Health Prod*. 2020;52(4):1583–1598.
  22. Bozena H, Katerina V, Rene K, et al. Associations between IGF1, IGFBP2 and TGFβ3 genes polymorphisms and growth performance of broiler chicken lines. *Animals*. 2020;10(5):800.
  23. Varinder SR, Aneet K, Atish K, Vikas V. Marker-assisted selection vis-à-vis bull fertility: coming full circle-a review. *Mol Biol Rep*. 2020;47(11):9123–9133.
  24. Wang K, Liu X, Qi T, et al. Whole-genome sequencing to identify candidate genes for litter size and to uncover the variant function in goats (*Capra hircus*). *Genomics*. 2021;113(1 Pt 1):142–150.
  25. Yang A, Chen B, Ran M, Yang G, Zeng C. The application of genomic selection in pig cross breeding. *Yi Chuan*. 2020;42(2):145–152.
  26. Gamarra D, Aldai N, Arakawa A, Pancorbo MM, Taniguchi M. Effect of a genetic polymorphism in SREBP1 on fatty acid composition and related gene expression in subcutaneous fat tissue of beef cattle breeds. *Anim Sci J*. 2021;92(1):e13521.
  27. Flandez M, Cendrowski J, Cañamero M, et al. Nr5a2 heterozygosity sensitises to, and cooperates with, inflammation in KRas(G12V)-driven pancreatic tumorigenesis. *Gut*. 2014;63(4):647–655.
  28. Kaluarachchi D, Momany A, Busch T, et al. Polymorphisms in NR5A2, gene encoding liver receptor homolog-1 are associated with preterm birth. *Pediatr Res*. 2016;79(5):776–780.
  29. Zhao Y, Liu W, Hua M, Shi R, Wang H, Yang W. Relationship between CATSPERB, NR5A2 gene polymorphisms and peak bone mineral density in college students in China. *Iran J Public Health*. 2014;43(8):1060–1069.
  30. Yin H, Zhou C, Shi S, et al. Weighted single-step genome-wide association study of semen traits in holstein bulls of China. *Front Genet*. 2019;10:1053.
  31. Li Y, Zhang J, Qian Y, et al. Molecular characterization, expression, polymorphism of NR5A2 and its relationship with litter size in Hu sheep. *Genet Mol Res*. 2015;14(4):12765–12775.
  32. Li J, Shen C, Zhang K, et al. Polymorphic variants of bovine ADCY5 gene identified in GWAS analysis were significantly associated with ovarian morphological related traits. *Gene*. 2021;766:145158.
  33. Zhao J, Li J, Jiang F, Song E, Lan X, Zhao H. Fecundity-associated polymorphism within bovine ITGβ5 and its significant correlations with ovarian and luteal traits. *Animals*. 2021;11(6):1579.
  34. Aljanabi SMM, Aljanabi SMS, et al. Rapid and inexpensive plasmid DNA extraction method. *DIRASAT*. 1997;24(2):400–403.
  35. Wang XY, Yang Q, Wang K, et al. Two strongly linked single nucleotide polymorphisms (Q320P and V397I) in GDF9 gene are associated with litter size in cashmere goats. *Theriogenology*. 2019;125:115–121.
  36. Kang Z, Jiang E, Wang K, et al. Goat membrane associated ring-CH-type finger 1 (MARCH1) mRNA expression and association with litter size. *Theriogenology*. 2019;128:8–16.

37. Xu H, Li H, Wang Z, et al. A deletion downstream of the CHCHD7 gene is associated with growth traits in sheep. *Animals (Basel)*. 2020;10(9):1472.
38. Wang XY, Yang Q, Wang K, et al. A novel 12-bp indel polymorphism within the GDF9 gene is significantly associated with litter size and growth traits in goats. *Anim Genet*. 2017;48(6):735–736.
39. Wang Z, Zhang X, Jiang E, et al. InDels within caprine IGF2BP1 intron 2 and the 3'-untranslated regions are associated with goat growth traits. *Anim Genet*. 2020;51(1):117–121.
40. Hankele A, Rehm K, Berard J, Schuler G, Bigler L, Ulbrich S. Progesterone profiling in plasma during the estrous cycle in cattle using an LC-MS based approach. *Theriogenology*. 2020;142:376–383.
41. Hui Y, Zhang Y, Wang K, et al. Goat DNMT3B: An indel mutation detection, association analysis with litter size and mRNA expression in gonads. *Theriogenology*. 2020;147:108–115.
42. Lan K, Shen C, Li J, et al. A novel indel within the bovine SEPT7 gene is associated with ovary length. *Anim Biotechnol*. 2021;34(1):8–14.
43. Zheng J, Deng T, Jiang E, et al. Genetic variations of bovine PCOS-related DENND1A gene identified in GWAS significantly affect female reproductive traits. *Gene*. 2021;802:145867.
44. Jiang E, Kang Z, Wang X, et al. Detection of insertions/deletions (InDels) within the goat Runx2 gene and their association with litter size and growth traits. *Anim Biotechnol*. 2021;32(2):169–177.
45. Calò C, Onali F, Robledo R, Flore L, Massidda M, Francalacci P. Worldwide variation of the COL14A1 gene is shaped by genetic drift rather than selective pressure. *Mol Genet Genomic Med*. 2021;9(4):e1629.
46. Martin C, Sheppard E, Ali H, et al. Genomic landscapes of divergence among island bird populations: Evidence of parallel adaptation but at different loci? *Mol Ecol*. 2024;33(12):e17365.
47. Bertolin K, Meinsohn M, Suzuki J, et al. Ovary-specific depletion of the nuclear receptor NR5A2 compromises expansion of the cumulus oophorous but not fertilization by intracytoplasmic sperm injection. *Biol Reprod*. 2017;96(6):1231–1243.
48. Zhang X, Xu Y, Fu L, et al. Identification of mRNAs related to endometrium function regulated by lncRNA CD36-005 in rat endometrial stromal cells. *Reprod Biol Endocrinol*. 2018;16(1):96.
49. Sahmi F, Nicola E, Zamberlam G, Gonçalves P, Vanselow J, Price C. Factors regulating the bovine, caprine, rat and human ovarian aromatase promoters in a bovine granulosa cell model. *Gen Comp Endocrinol*. 2014;200:10–17.
50. Triin L, Viktorija K, Duncan P, et al. Large-scale meta-analysis highlights the hypothalamic–pituitary–gonadal axis in the genetic regulation of menstrual cycle length. *Human Mol Genet*. 2018;27(24):4323–4332.
51. Hu S, Yang S, Lu Y, et al. Dynamics of the transcriptome and accessible chromatin landscapes during early goose ovarian development. *Front Cell Dev Biol*. 2020;8:196.
52. Margaret M, Joyce J, John M, James A, David J, Carole R. Expression of LRH-1 and SF-1 in the mouse ovary: localization in different cell types correlates with differing function. *Mol. Cell Endocrinol*. 2003; 207(1–2):39–45.
53. Takashi Y, Yoshitaka I, Kaoru M, Akihiro U, Takanobu T. Differentiation of mesenchymal stem cells into gonad and adrenal steroidogenic cells. *World J. Stem Cells*. 2014;6(2):203–212.
54. Pancharatnam J, Yayoi I, Soazik P, et al. Cell-specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function. *Mol. Endocrinol*. 2004;18(7): 1610–1619.
55. Zhang C, Large M, Duggavathi R, et al. Liver receptor homolog-1 is essential for pregnancy. *Nat Med*. 2013;19(8):1061–1066.
56. Morrell B, Perego M, Maylem E, Zhang L, Schütz L, Spicer L. Regulation of the transcription factor E2F1 mRNA in ovarian granulosa cells of cattle. *J Anim Sci*. 2020;98(1):skz376.
57. Feng S, Wan S, Liu S, et al. LARS2 regulates apoptosis via ros-mediated mitochondrial dysfunction and endoplasmic reticulum stress in ovarian granulosa cells. *Oxid Med Cell Longev*. 2022;2022:5501346–5501318.
58. Zhang S, Wang L, Wang L, Chen Y, Li F. MiR-17-5p affects porcine granulosa cell growth and oestradiol synthesis by targeting E2F1 gene. *Reprod Domest Anim*. 2019;54(11):1459–1469.
59. Marie C, Olivia E, et al. The orphan nuclear receptors steroidogenic factor-1 and liver receptor homolog-1: structure, regulation, and essential roles in mammalian reproduction. *Physiol Rev*. 2019;99(2):1249–1279.



# 证书

陈宣伯 同学：

在参与中国科学技术协会“大学生科技社团支持计划”项目执行工作中，在基础科研训练、科学沙龙交流、外出参观实习等方面表现突出、成绩优异。特授予你“优秀社员”荣誉称号，以资鼓励。

西北农林科技大学科学技术协会

共青团西北农林科技大学委员会

2025年12月  
委员会

誠樸勇毅





# CULSC第十届全国大学生生命科学竞赛（创新创业类）

## 获奖证书

获奖项目：牛 FBLN1 基因 Indel 和 CNV 挖掘及其对屠宰性状的遗传效应研究

获奖学生：顾洪焯 张驰原 陈宣伯 张浩天

指导老师：蓝贤勇

获奖单位：西北农林科技大学

获奖类型：一等奖

证书编号：CULSC2025CY0234



全国大学生生命科学竞赛委员会  
二〇二五年七月



# 中华人民共和国国家版权局 计算机软件著作权登记证书

证书号： 软著登字第14742448号

软件名称： 肉牛生长发育相关性状收集管理系统  
V1.0

著作权人： 西北农林科技大学

权利取得方式： 原始取得

权利范围： 全部权利

登记号： 2025SR0086250

根据《计算机软件保护条例》和《计算机软件著作权登记办法》的规定，经中国版权保护中心审核，对以上事项予以登记。



2025年01月14日



# 开 发 者 证 明

软件名称：肉牛生长发育相关性状收集管理系统 V1.0

著作权人：西北农林科技大学

开发完成日期：2025 年 01 月 04 日

登记号：2025SR0086250

登记日期：2025 年 01 月 14 日

陈宣伯；蓝贤勇；何卓远；张浩天；宋清川；陈蕾；潘传英；  
陈小蕾；赵海谕；杜书增为以上软件著作权开发者。

特此说明！

深圳市康弘知识产权有限公司合肥分公司

2025 年 1 月 20 日





# 中华人民共和国国家版权局 计算机软件著作权登记证书

证书号： 软著登字第14149396号

软件名称： 肉牛育种管理系统  
V1.0

著作权人： 西北农林科技大学

权利取得方式： 原始取得

权利范围： 全部权利

登记号： 2024SR1745523

根据《计算机软件保护条例》和《计算机软件著作权登记办法》的规定，经中国版权保护中心审核，对以上事项予以登记。



2024年11月11日



# 开 发 者 证 明

软件名称：肉牛育种管理系统 V1.0

著作权人：西北农林科技大学

开发完成日期：2024 年 6 月 12 日

登记号：2024SR1745523

登记日期：2024 年 11 月 11 日

张驰原；蓝贤勇；朱琦慧；陈宣伯；顾洪烨；潘传英；宋恩亮；姜富贵；杜书增为以上软件著作权开发者。

特此说明！

深圳市康弘知识产权代理有限公司合肥分公司

2024 年 12 月 10 日

