

Novel human milk-derived peptide β -casein 65 repairs intestinal injury via modulating gut microbiota

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ABSTRACT

Necrotizing enterocolitis (NEC) remains a leading cause of morbidity and mortality in preterm infants, and effective pharmacologic options are scarce. Studies have demonstrated that human milk peptides can improve NEC, β -casein 65 is a novel peptide derived from preterm human milk, yet its specific function and mechanism remain unclear. Here we evaluated the therapeutic potential role of β -casein 65 in a mouse model of NEC. β -casein 65 improved survival and mitigated abdominal distension and hematochezia in experimental NEC models. Concomitantly, β -casein 65 significantly reduced pro-inflammatory cytokines (TNF- α and IL-6 by approximately 56% and 71%, respectively) and increased intestinal barrier proteins compared with the NEC group. Using 16S rRNA sequencing and untargeted metabolomics, β -casein 65 altered gut microbiota profiling by lowering pathogenic *Proteobacteria* and promoting beneficial bacteria like *Bifidobacterium* and *Akkermansia*. Metabolomics analysis showed notable changes in immune-regulating metabolites, especially enriched in the IgA immune network. This finding not only provides a mechanistic understanding but also highlights β -casein 65 as a promising candidate for the development of preventative therapeutics for this devastating neonatal disease.

1. Introduction

Necrotizing enterocolitis (NEC) is a life-threatening inflammatory gastrointestinal disorder primarily affecting preterm infants, with an incidence of approximately 7–13% in very low birth weight (VLBW) infants and a case-fatality rate of 20–30% (Gitau, Ochieng, Limbe, Kathomi, & Orwa, 2023; Rich & Dolgin, 2017). Survivors often experience short bowel syndrome, malnutrition, recurrent infections, and neurodevelopmental impairment (Jones & Hall, 2020). Despite advances in neonatal intensive care, the multifactorial pathogenesis of NEC, spanning epithelial barrier dysfunction, microbial dysbiosis, and dysregulated mucosal immunity, has hindered the development of specific pharmacologic preventions or treatments, and the role and timing of surgery remain debated (Wei, Meng, Li, Dang, & Wu, 2023). Therefore, there is a pressing need for new therapeutic strategies to prevent and treat this condition.

Breastfeeding is consistently associated with a lower risk of NEC (Roberts, Younge, & Greenberg, 2024). Meta-analyses and trials indicate

that human milk, compared with formula, reduces NEC, supporting human milk-derived bioactive components as a plausible protective factor against NEC in premature newborns (Altobelli, Angeletti, Verratti, & Petrocelli, 2020; Fu, Sun, Wang, & Zhu, 2023). For instance, lactoferrin and human milk oligosaccharides in breast milk have been shown to effectively prevent the occurrence of NEC (Pammi & Abrams, 2019; Sodhi et al., 2023).

Milk-derived peptides are now recognized as bioactive modulators rather than inert digestion products (Erdem Büyükkiraz & Kesmen, 2022; Liang et al., 2022). Our previous peptidomics studies identified 41 peptides that are significantly regulated and demonstrate abundant casein-derived peptides in human milk, with composition differing between preterm and term infants (Wang et al., 2019). Mounting evidence suggests that the therapeutic benefits of breast milk are partly attributed to its rich repository of bioactive peptides, which are encrypted within milk proteins and released upon digestion (Beverly, Woonnimani, Scottoline, Lueangsakulthai, & Dallas, 2021; Enjapoori, Kukuljan, Dwyer, & Sharp, 2019; Yao et al., 2025). These peptides have

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demonstrated a remarkable capacity to modulate intestinal immunity, enhance barrier integrity, and shape the gut microbiota composition. Since β -casein 65 is a hydrolysate of β -casein, a major milk protein, we reasoned that it might also have these broad gut-protective functions. Notably, β -casein 65 is identified and abundant in preterm milk, along with strong stability, high lipophilicity, and efficient cellular permeability, suggesting potential for direct interaction with bacterial membranes or host epithelial cells. However, it is not known whether β -casein 65 can directly protect against NEC in vivo.

NEC pathobiology is closely linked to early-life host-microbe interactions. Microbial dysbiosis, characterized by an increased relative abundance of Proteobacteria and alterations in microbial metabolites, mainly occurs before clinical disease (Kaplina et al., 2023), and these metabolites shape the neonatal intestinal immune system through Toll-like receptor (TLR) activation (Beharry et al., 2023). We therefore hypothesized that whether the peptide β -casein 65 mitigates experimental NEC by restoring barrier function and modulating the gut microbiota-metabolites milieu, presenting a novel approach to identifying targets for the prevention and treatment of NEC.

2. Materials and methods

2.1. Chemicals

SYBR Green was obtained from Vazyme Biotech (Nanjing, China). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (NY, USA). Lipopolysaccharide (LPS) was obtained from Merck (Beijing, China). The BeyoRT First Strand cDNA Synthesis Kit was obtained from Vazyme Biotech (Nanjing, China). The tissue or cell total protein extraction kit was obtained from Servicebio (Wuhan, China). Tumor Necrosis Factor- α (TNF- α) assay kit and Interleukin-6 (IL-6) assay kit were obtained from Enzyme-linked Biotechnology (Shanghai, China). Anti-ZO-1 rabbit, anti-Occludin rabbit, anti-Claudin-1 rabbit, and rabbit anti-GAPDH, HRP-conjugated goat anti-rabbit IgG (H + L), Cy3-conjugated goat anti-rabbit IgG (H + L) antibodies, and DAPI staining solution were obtained from Servicebio (Wuhan, China). The primers were synthesized by Realgene (Nanning, China).

2.2. Peptide

β -casein 65 (LNPTHQIYPVTQPLAPVHNPIIS) was chemically synthesized by Science Peptide Biological Technology Co., Ltd. (Shanghai, China). Its purity exceeded 95%, as confirmed by reverse-phase high-performance liquid chromatography. We prepared the β -casein 65 stock solution in deionized water and stored it at -20°C . Using the ProtParam tool (<https://web.expasy.org/protparam/>), we calculated the properties of peptide β -casein 65.

2.3. Animal experiments

For the experiments, 5- to 6-day-old neonatal mice (wild-type male C57BL/6 J) were obtained from Specific Pathogen-Free sources. Nanjing Medical University's Animal Research and Care Committee approved the animal procedures (IACUC-2303007). All experiments involving animals were conducted in accordance with the Human & Animal Welfare policy.

Five to six days later, pups were randomly assigned to the following groups using a random number table: (1) Control group, (2) NEC model group, (3) NEC + β -casein 65 treatment group. The NEC experimental procedure was performed as described. Pups were separated from their mother and housed in a temperature-controlled (37°C) neonatal incubator to maintain homeostasis. In summary, pups received $40\text{ }\mu\text{L/g}$ of body weight of formula via gavage [Similac Advance infant formula (Abbott Nutrition); Esbilac dog milk substitute, 2:1] four times daily using a 24-French angiocatheter, along with hypoxia exposure three

times a day (5% O_2 , 95% N_2) for 5 min. Peptide-supplemented (10 mg/kg) formulas were prepared and administered to the designated group.

We recorded body weight over 4 days. On day 5 of the model, mouse pups were euthanized by decapitation with surgical scissors. The intestines of all mice were scored for NEC pathology. To prevent sampling bias, we often select a fixed point on the intestinal samples, primarily from the terminal ileum 1 cm section, which is preserved at -80°C .

2.4. Histopathology analysis

Intestines were fixed overnight at room temperature in 4% paraformaldehyde, dehydrated with xylene and ethanol, and then embedded in paraffin. Small intestine specimens embedded in paraffin were then sliced into $5\text{ }\mu\text{m}$ sections. Sections underwent deparaffinization and rehydration. Subsequently, the sections were stained at room temperature with Mayer's hematoxylin for 30 s and 1% eosin Y solution for 10 to 30 s. After mounting and dehydration, the slices were examined using a light microscope (Zeiss, Germany). Histopathological scoring was performed by two individuals who were blinded to the experimental procedure.

2.5. Measurement of inflammatory factors in intestinal tissue

The intestinal tissue samples were weighed, homogenised in physiological saline solution (w/v, 1:9) in an ice bath, and then centrifuged to collect the supernatant. The concentrations of proinflammatory cytokines TNF- α and IL-6 were measured using the corresponding enzyme-linked immunosorbent assay kits.

2.6. qRT-PCR analysis

50–100 mg of intestinal tissue was excised, and total RNA was extracted using the Trizol method. We extracted the total RNA according to the manufacturer's instructions. Using a PrimeScript RT reagent kit and gDNA eraser (Takara, Japan), complementary DNA (cDNA) synthesis was performed. qRT-PCR with SYBR Premix Ex Taq was used to analyse the cDNA (Takara, Japan). GAPDH was used to normalise the data. The primer list is shown in Table 1.

2.7. Immunofluorescence

Tissue sections were incubated overnight at 4°C with the indicated primary antibodies, including anti-ZO-1 rabbit (1:500), anti-Occludin rabbit (1:500), and anti-Claudin-1 rabbit (1500). The sections were then incubated with Cy3-conjugated goat anti-rabbit IgG (H + L) secondary antibody (1200) for 60 min at room temperature in the dark. Cell nuclei were stained with DAPI. Images were captured using a laser scanning confocal microscope.

2.8. Immunohistochemistry

Five-micrometre paraffin-embedded sections of the ileum were prepared. After 20 min of blocking at room temperature with 5% bovine serum albumin, we incubated the sections overnight at 4°C with

Table 1
Primer sequences

Target	Primer sequence (5' ~ 3') (F: Forward; R: Reverse)
mouse Occludin	F: CAGCCTCGGTACAGCAGCAAT
	R: AGCTGTGCATGGCCTCTTGT
mouse ZO-1	F: CGGAACATATGACCATCGCCTAC
	R: CTCGGGATGTTGTCTGGAGTC
mouse Claudin-1	F: AGCTGTGCATGGCCTCTTGT
	R: CCAATGTCAATGGCAACACCCT
mouse GAPDH	F: CAAGAAGGTGGTGAAGCAGGC
	R: CATACCAGGAATGAGCTTGAC

primary antibodies against toll-like receptor 4 (TLR4). After 60 min, the sections were incubated with secondary antibodies. Following three washes with PBS, the chromogen 3-diaminobenzidine was applied to each area to stain it. Mayer's hematoxylin was used for nuclear counterstaining. Stained sections were examined with light microscopy (Zeiss, Germany).

2.9. Western blot

50–100 mg of the intestinal tissue was excised, and the entire proteins were extracted using a Tissue or Cell Total Protein Extraction Kit. The samples underwent Western blot analysis with the specified primary antibodies: rabbit anti-Ecadherin (11000) and rabbit anti- β -actin (1200). The Bio-Rad gel imaging system was used to photograph the gels, and band intensities were quantified using ImageJ software.

2.10. 16S rRNA sequence analysis

16S rRNA sequence analysis was conducted on the colon fecal samples ($n = 18$). Microbial DNA was extracted using the OMEGA Soil DNA Kit (M5635–02, Omega-Tek, Norcross, GA, USA). For amplifying the microbial V3–V4 region via PCR, the primers 5'-CCTACGGGNGGCWGCAG and 3'-GACTACHVGGGTATCTAATCC were used. Subsequently, amplification and sequencing methods were performed as described in the literature (Drewes et al., 2017).

2.11. Metabolomics analysis

100 μ L of the sample was taken and mixed with 400 μ L of extraction solution (MeOH: ACN, 1:1 (v/v)). The extraction solution contained deuterated internal standards. The mixture was vortexed for 30 s, sonicated for 10 min in a 4 °C water bath, and incubated for one hour at –40 °C to precipitate proteins. Next, the samples were centrifuged at 12000 rpm (RCF = 13,800 ($\times g$), $R = 8.6$ cm) for 15 min at 4 °C. The supernatant was transferred to a new glass vial for analysis. The quality control (QC) sample was prepared by mixing an equal aliquot of the supernatant from the samples. Polar metabolites were separated on a Waters ACQUITY UPLC BEH Amide column (2.1 \times 100 mm, 1.7 μ m) using mobile phase A (25 mmol/L ammonium acetate/ammonium hydroxide, pH 9.75) and B (acetonitrile). Non-polar metabolites were analyzed using a Phenomenex Kinetex C18 column (2.1 \times 100 mm, 2.6 μ m) with mobile phase A (0.01% acetic acid) and B (IPA: ACN, 1:1). Both analyses used a Vanquish UHPLC system coupled to an Orbitrap Exploris 120 mass spectrometer. Samples (2 μ L) were injected at 4 °C. MS operated in information-dependent acquisition (IDA) mode: full MS at 60,000 resolution and MS/MS at 15,000 resolution. ESI settings: sheath gas 50 Arb, aux gas 15 Arb, capillary 320 °C, spray voltage ± 3.8 kV (positive) or –3.4 kV (negative), SNCE 20/30/40 eV. Data processing involved raw data (converted to mzXML via ProteoWizard), which underwent peak detection, extraction, alignment, and integration using an in-house R pipeline based on XCMS. Metabolites were identified using BiotreeDB (V3.0).

2.12. Statistical analysis

Statistical analyses were conducted in R (version 4.0.1; R Foundation for Statistical Computing, Vienna, Austria). All experiments were independently repeated at least three times, and data are shown as mean \pm standard deviation (SD) unless stated otherwise. For comparisons involving more than two groups, we first checked the data's distributional assumptions (normality and homogeneity of variances) using the Shapiro-Wilk test and Levene's test, respectively. When these assumptions were satisfied, differences between groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for pairwise comparisons. If the assumptions of normality or homoscedasticity were violated, the Kruskal–Wallis test was used instead,

followed by Dunn's test with suitable multiple-comparison correction.

For metabolic pathway enrichment analysis, raw p -values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) procedure. Pathways with FDR < 0.05 were considered statistically significant. Unless otherwise noted, a two-sided p < 0.05 was regarded as indicating statistical significance. Statistically significant differences in the figures are marked with asterisks (*).

3. Results

3.1. Characteristics of β -casein 65

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis revealed significantly higher levels of β -casein 65 in preterm compared to term human milk (p < 0.05; Fig. S1). This 22-amino acid peptide (sequence: LNPHTHQIYPVTQPLAPVHNPIIS; MW: 2436.80 Da) exhibited physicochemical properties typical of bioactive molecules. ProtParam analysis showed the following parameters: theoretical pI = 6.92, instability index = 51.03 (indicating instability), aliphatic index = 101.82, GRAVY = –0.232, and a predicted mammalian reticulocyte half-life of 5.5 h (Fig. 1A). Helical wheel projection displayed clear amphipathicity with separated hydrophobic and hydrophilic sectors (Fig. 1B), suggesting membrane-disruptive potential against bacterial pathogens. Tertiary structure prediction using PEP-FOLD 3.5 confirmed a helical shape consistent with known antimicrobial peptide motifs (Fig. 1C).

3.2. Polypeptide β -casein 65 gavage alleviated NEC in mice

To evaluate the protective effects of β -casein 65 against NEC, neonatal mice were subjected to established NEC induction methods, including hypoxia, hypothermia, and formula feeding (Fig. 2A). Starting on postnatal day 3 (P3), the experimental groups received daily oral gavage of β -casein 65 (10 mg/kg). In contrast, NEC controls received phosphate-buffered saline (PBS). Treatment continued until postnatal day 4 (P4), which corresponds to 96 h after induction. Histopathological analysis showed significantly higher median NEC scores in controls compared to β -casein 65-treated mice (Fig. 2B). The β -casein 65 group exhibited significantly greater weight gain (Fig. 2C) and improved survival rates versus NEC controls (Fig. 2D). Macroscopic examination revealed characteristic NEC pathology in controls, while β -casein 65 markedly reduced these changes, including a significant decrease in segmental pneumatosis intestinalis (Fig. 2E). Overall, β -casein 65 supplementation lessened NEC progression by preserving intestinal barrier integrity and decreasing inflammation.

3.3. Polypeptide β -casein 65 reduced histological damage and inflammation of the intestine in NEC mice

Hematoxylin and eosin (H&E) staining confirmed that β -casein 65 administration significantly preserved intestinal mucosal integrity in NEC-induced mice compared to untreated NEC controls (Fig. 3A). Immunohistochemical analysis showed a substantial reduction in TLR4 overexpression in the ileal epithelium after β -casein 65 treatment (Fig. 3B and D). Compared to the NEC model group, β -casein 65 treatment suppressed systemic TNF- α and IL-6 levels to 0.44-fold and 0.29-fold of the levels in the NEC model group, respectively (Fig. 3C). Overall, these findings suggest that β -casein 65 alleviates NEC-related intestinal injury and inflammation by modulating TLR4 signaling and cytokine responses.

3.4. Polypeptide β -casein 65 mitigates intestinal epithelial barrier damage

Immunofluorescence analysis showed that β -casein 65 promoted the restoration of tight junction (TJ) protein expression compared to NEC controls (Fig. 4A). Western blot analysis confirmed a significant increase

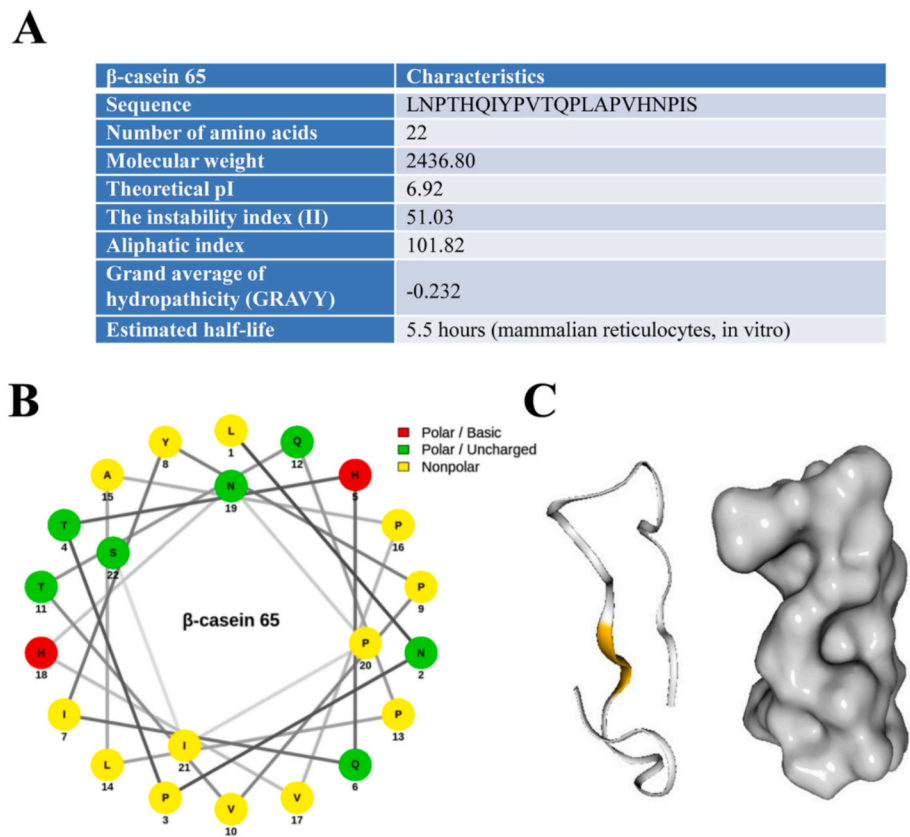


Fig. 1. Characteristics of β -casein 65. (A) Results from the ProtParam online analysis of β -casein 65; (B) Results from the HelicalWheel software analysis of β -casein 65. Polar and basic residues are highlighted in red; polar and uncharged residues are highlighted in green; and nonpolar residues are highlighted in yellow. (C) Predicted structure of β -casein 65. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in TJ protein levels (Fig. 4B). At the transcriptional level, NEC induction greatly reduced TJ gene expression, but β -casein 65 treatment reversed this reduction, significantly restoring mRNA levels (Fig. 4C). These results suggest that β -casein 65 helps protect the intestinal barrier by regulating TJ expression at multiple levels.

3.5. β -Casein 65 influences the composition of the gut microbiota in NEC mice

16S rRNA sequencing demonstrated that β -casein 65 partially improved NEC-related gut dysbiosis. Across groups, 179 operational taxonomic units (OTUs) were consistent. In contrast, the control, NEC, and β -casein 65 groups had 74, 93, and 80 unique OTUs, respectively (Fig. 5A). Principal component analysis (PCA) clearly distinguished β -casein 65-treated mice from NEC and healthy controls (Fig. 5B). Taxonomically, β -casein 65 treatment decreased Proteobacteria levels while increasing Firmicutes and Bacteroidota compared to NEC mice (Fig. 5C). At the genus level, treatment reduced *Escherichia-Shigella* and *Clostridium*, while promoting *Lactobacillus* and *Bacteroides* (Fig. S1). Additionally, we identified significantly different genera among all groups through linear discriminant analysis and effect size analysis (Fig. 5D). Linear discriminant analysis (LDA > 3.0) highlighted *Parabacteroides* and *Akkermansia* as key taxa in the treatment group, contrasting with *Escherichia-dominated* NEC microbiota. These results indicate that β -casein 65 promotes beneficial bacteria growth, alters gut microbiota composition, and helps restore microbial balance.

3.6. Effects of β -casein 65 on intestinal metabolites in NEC mice

Untargeted HPLC-MS analysis of intestinal contents identified 59,596 metabolic features across positive and negative ion modes.

Orthogonal partial least squares-discriminant analysis (OPLS-DA) showed clear separation of metabolic profiles between NEC controls and β -casein 65-treated groups (Negative mode: $R^2Y = 0.988$, $Q^2 = 0.758$; Positive mode: $R^2Y = 0.987$, $Q^2 = 0.707$). Model robustness was confirmed through permutation testing (Q^2 intercept < -0.04; Fig. 6A-D). Comparative analysis revealed 841 metabolites with differential abundance (805 upregulated, 36 downregulated; $|\log_2FC| > 1$; Fig. 6E-F), with 55 significant metabolites shown after normalization (Fig. S2). Enrichment analysis pointed to a substantial role of the intestinal immune network for IgA production pathway. Key enriched mediators included prostaglandin E₂, 12-hydroxyeicosatetraenoic acid, arachidonic acid, and linoleic acid (Fig. 6G). These findings suggest that β -casein 65 alleviates NEC through targeted lipid reprogramming that modulates IgA-related immunometabolism networks.

3.7. Correlation analysis of differential bacterial genera, metabolites, and proteins

Sankey analysis revealed links between 14 metabolites from 12 different genera and the expression of tight junction (TJ) proteins or inflammatory mediators (Fig. S3). Prostaglandins (PGH₂, PGE₂, PGD₂) showed strong negative associations with TNF- α and positive associations with Claudin-1. Simultaneously, retinoic acid derivatives were linked to ZO-1 (Fig. 7A). Notably, argininosuccinic acid was positively associated with both barrier integrity and reduced inflammation. Pearson correlation analysis also identified *Arthrobacter*, *Bifidobacterium*, and *Parabacteroides*—the main genera in β -casein 65-treated mice—as key mediators, positively correlated with TJ proteins and negatively correlated with TNF- α (Fig. 7B). Overall, these findings demonstrate that β -casein 65 promotes a protective microbiota-metabolite network that strengthens the intestinal barrier and reduces inflammation.

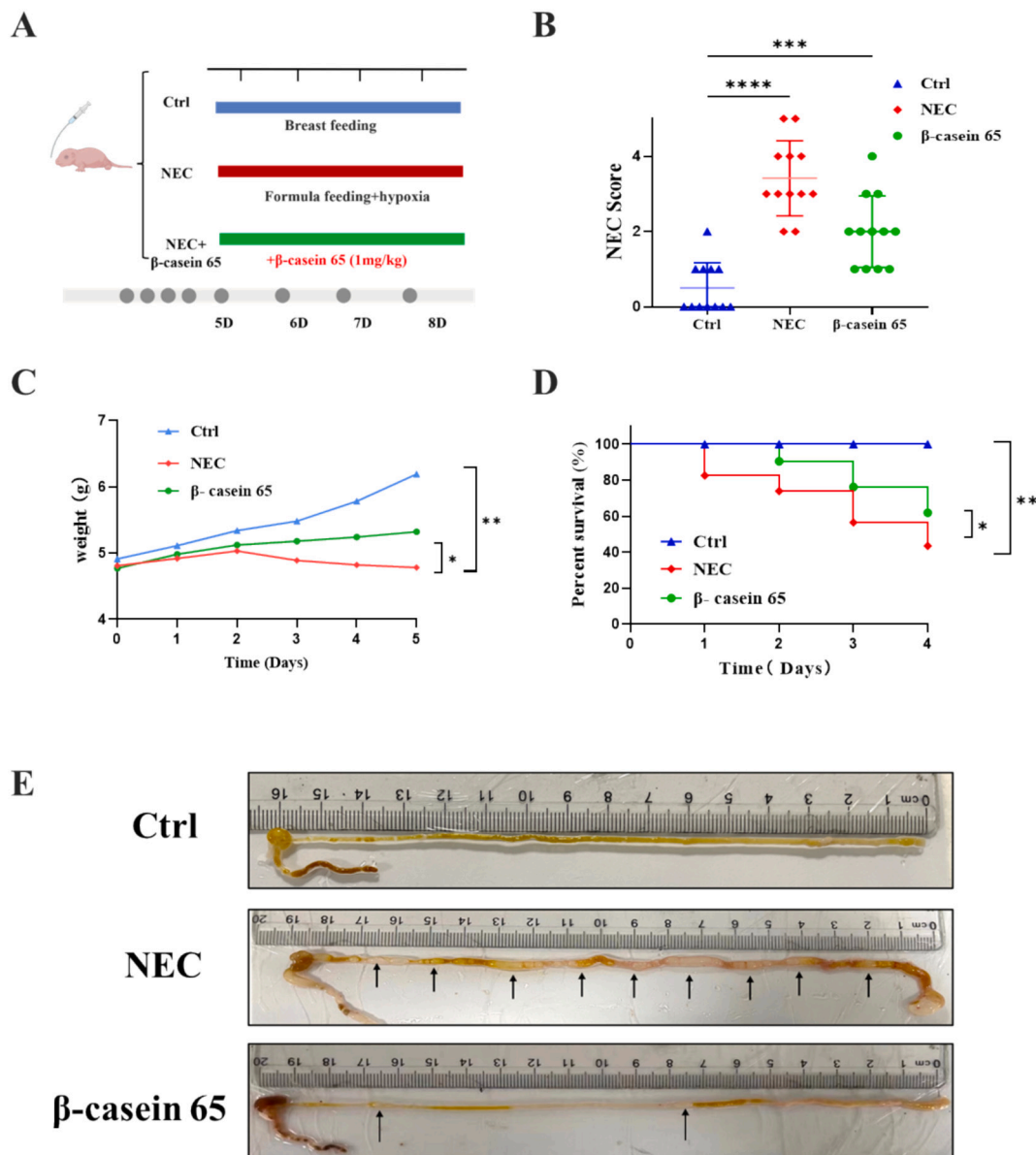


Fig. 2. Polypeptide β -casein 65 gavage alleviated necrotizing enterocolitis in mice. (A) Experimental design for administering β -casein 65 to the NEC mouse model. (B) Quantification of the intestinal NEC severity scores across three groups of mice. (C) Weight of neonatal mice within 96 h after treatment. (D) Survival of neonatal mice within 96 h after treatment. (E) Intestinal morphology of newborn mice that were either mother-fed in the Ctrl group, induced to develop NEC in the NEC group, or administered with β -casein 65 in the NEC + β -casein 65 group. * $p < 0.05$ and ** $p < 0.01$ compared to the Model group.

4. Discussion

NEC is a critical gastrointestinal crisis in preterm infants, with alarmingly high mortality rates despite treatment improvements (Duess et al., 2023). Although its exact cause remains unclear, increasing evidence suggests that gut dysbiosis, intestinal barrier failure, and immune dysregulation are central to disease progression (Golubkova & Hunter, 2023). Breastfeeding reduces NEC risk by 50–70%, mainly due to bioactive elements in human milk, such as immunoglobulins, lactoferrin, and functional peptides (Lapidaire, Lucas, Clayden, Clark, & Fewtrell, 2022). In this study, we demonstrated that β -casein 65, a novel peptide derived from human milk, increased survival rates and mitigated weight loss in a mouse model of NEC, while preserving intestinal epithelial integrity and suppressing inflammatory responses. Further analyses showed that β -casein 65 decreased intestinal epithelial damage, inhibited pro-inflammatory cytokine release and promoted tight junction (TJ) protein expression. Mechanistically, β -casein 65 modulated the

gut microbiota and metabolome, leading to coordinated metabolite changes within the IgA-centred intestinal immune network that collectively strengthened the mucosal barrier. Taken together, these findings present β -casein 65 as a potential peptide-based therapy for NEC and lay the groundwork for the development of human milk-derived peptide interventions.

NEC pathogenesis prominently involves exaggerated Toll-like receptor 4 (TLR4) signaling in preterm infants following hypoxic stress and formula feeding (Liu et al., 2022). Overactivation of TLR4 triggers a harmful cascade that damages the intestinal epithelial barrier and increases pro-inflammatory cytokine production (Niño, Sodhi, & Hackam, 2016). In our research, the observed reduction of TLR4 expression and downstream inflammatory cytokines like TNF- α and IL-6 with β -casein 65 aligns with this paradigm and suggests a plausible anti-inflammatory mechanism. Importantly, TNF- α , a key cytokine in enteritis, promotes cell death, activates macrophages and T cells, and attracts neutrophils, thereby worsening inflammation (Fischer & Maier, 2015). Similarly, IL-

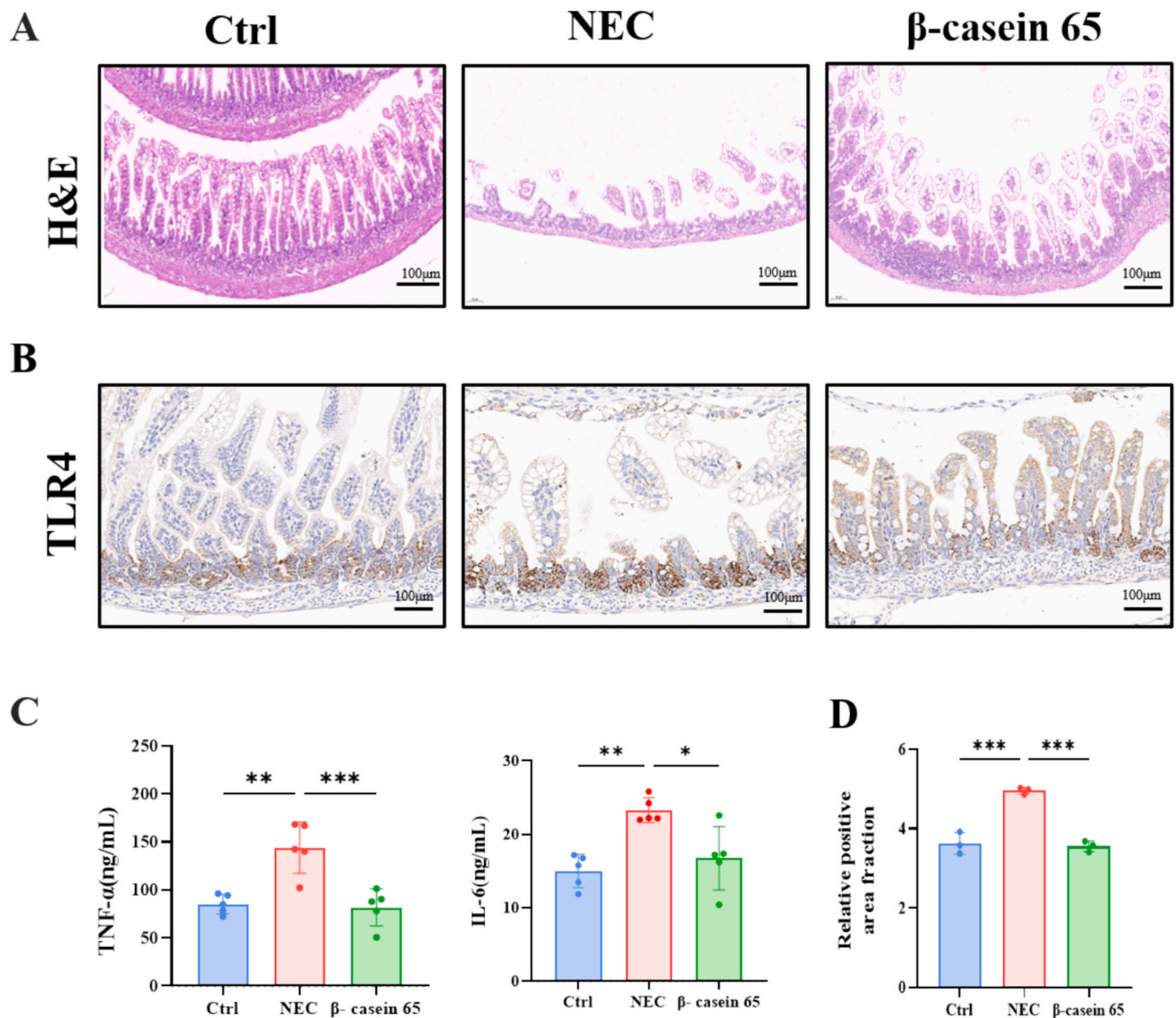


Fig. 3. β -casein 65 reduces histological damage and inflammation in the intestines of NEC mice. (A) Histological intestinal tissue stained with HE at a magnification of $\times 50$. (B and D) TLR4 immunohistochemical images of the intestine. Scale bar = 100 μ m. (C) Levels of TNF- α and IL-6 in the intestinal tissue were measured. Values are presented as means \pm SD ($n = 5$ for each group). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the Model group.

6 damages the integrity of intestinal tight junctions (TJs) and boosts inflammation, which can potentially lead to colitis-associated cancer through NF- κ B pathway activation (Yang et al., 2014). Therefore, decreasing TLR4 overexpression and reducing the cytokine storm are essential therapeutic strategies to prevent NEC (Mi et al., 2023).

The intestinal epithelial barrier functions as the primary defense against luminal pathogens and antigens. It consists of tight junction (TJ) proteins, such as zonula occludens-1 (ZO-1), claudin-1, and occludin (Garcia-Hernandez, Quiros, & Nusrat, 2017). NEC causes the breakdown of these TJ proteins, which increases paracellular permeability and promotes bacterial translocation, leading to systemic inflammation (Ares et al., 2019; Moore et al., 2016). Our findings show that β -casein 65 significantly boosts the expression of these crucial junctional proteins and helps restore barrier integrity, indicating its direct protective effects against intestinal barrier damage in experimental NEC models.

The gut microbiota is essential for supporting epithelial cell growth and maintaining the integrity of the intestinal barrier (Di Vincenzo, Del Gaudio, Petito, Lopetuso, & Scaldaferri, 2024). Additionally,

microbiome-derived metabolites act as signaling molecules that influence host metabolism. Consequently, we examined the mechanisms behind the protective effects of β -casein 65. Our experimental NEC model successfully replicated the typical gut microbiota characteristics of preterm infants, namely reduced diversity and dominance of *Proteobacteria*, which aligns with the dysbiotic features observed clinically (Brunse et al., 2022). This study further confirmed that intervention with β -casein 65 can reverse this pathological state. The effects of inhibiting *Proteobacteria* and enriching *Firmicutes* and *Bacteroidetes* are similar to the beneficial microbial shifts observed in clinical studies that supplement specific prebiotics or human milk oligosaccharides, unlike most probiotic strategies, which typically promote *Lactobacillus*. β -casein 65 notably increased beneficial bacteria like *Bifidobacterium*, *Parabacteroides*, and *Akkermansia*. Among these, *Bifidobacterium* enhances barrier function by producing acetate and upregulating claudin-4 (Gavzy et al., 2023), a mechanism that corroborates our observed increase in tight junction protein expression. These beneficial bacteria negatively correlate with pro-inflammatory TNF- α and positively with

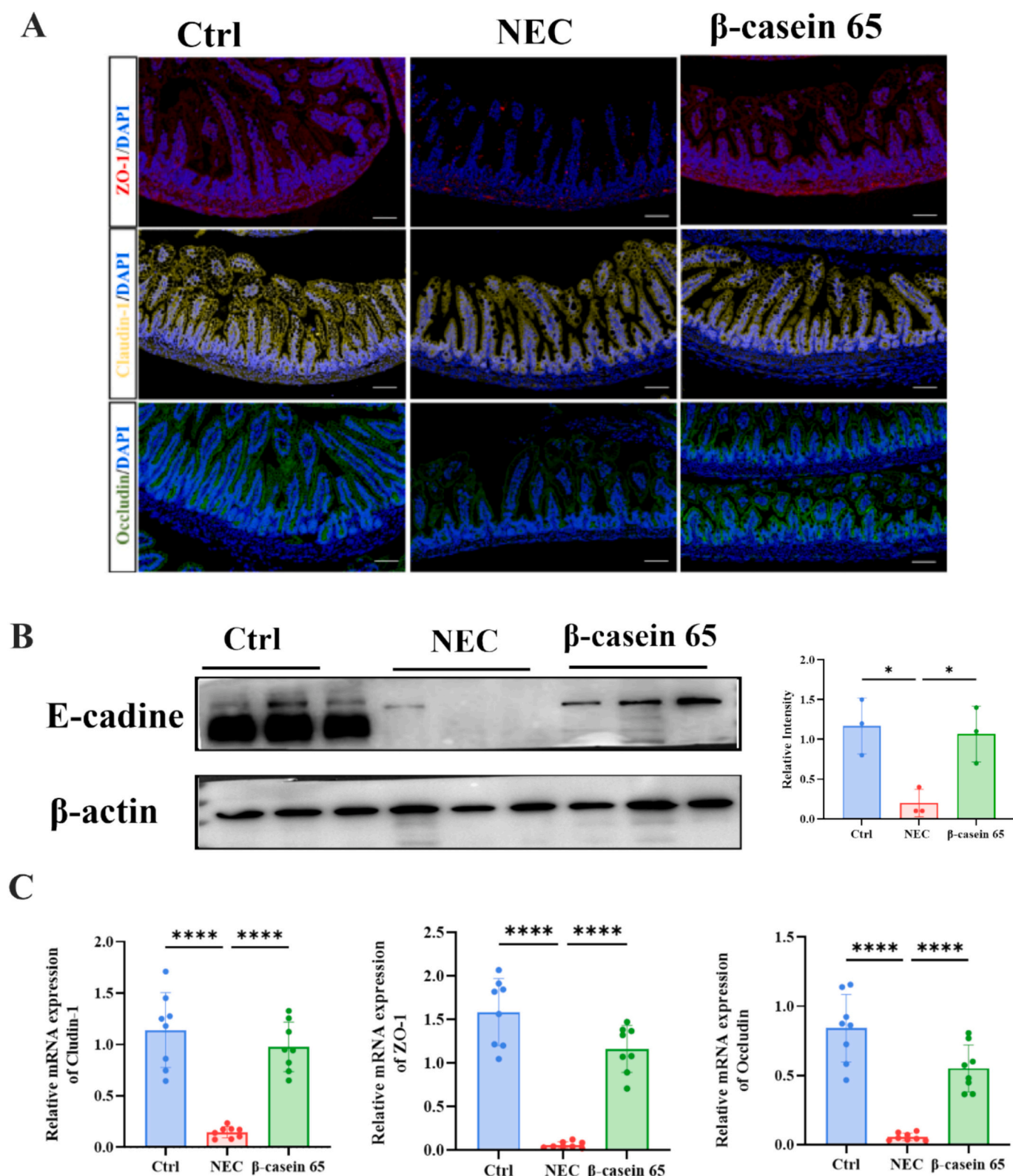


Fig. 4. β -casein 65 Mitigates Intestinal Epithelial Barrier Damage. (A) Epifluorescence images of colonic tissue TJ-proteins stained with anti-ZO-1 antibody (red), anti-Claudin-1 antibody (yellow), anti-Occludin antibody (green), and 4,6-diamidino-2-phenylindole (blue). (B) Western blot of E-cadherin in colonic tissue. Three slices were obtained from each group, and in each slice, three different areas were analyzed. (C) The mRNA expression levels of TJ proteins in the colon tissues were examined. Values are shown as means \pm SD ($n = 7-9$ for each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared with the Model group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

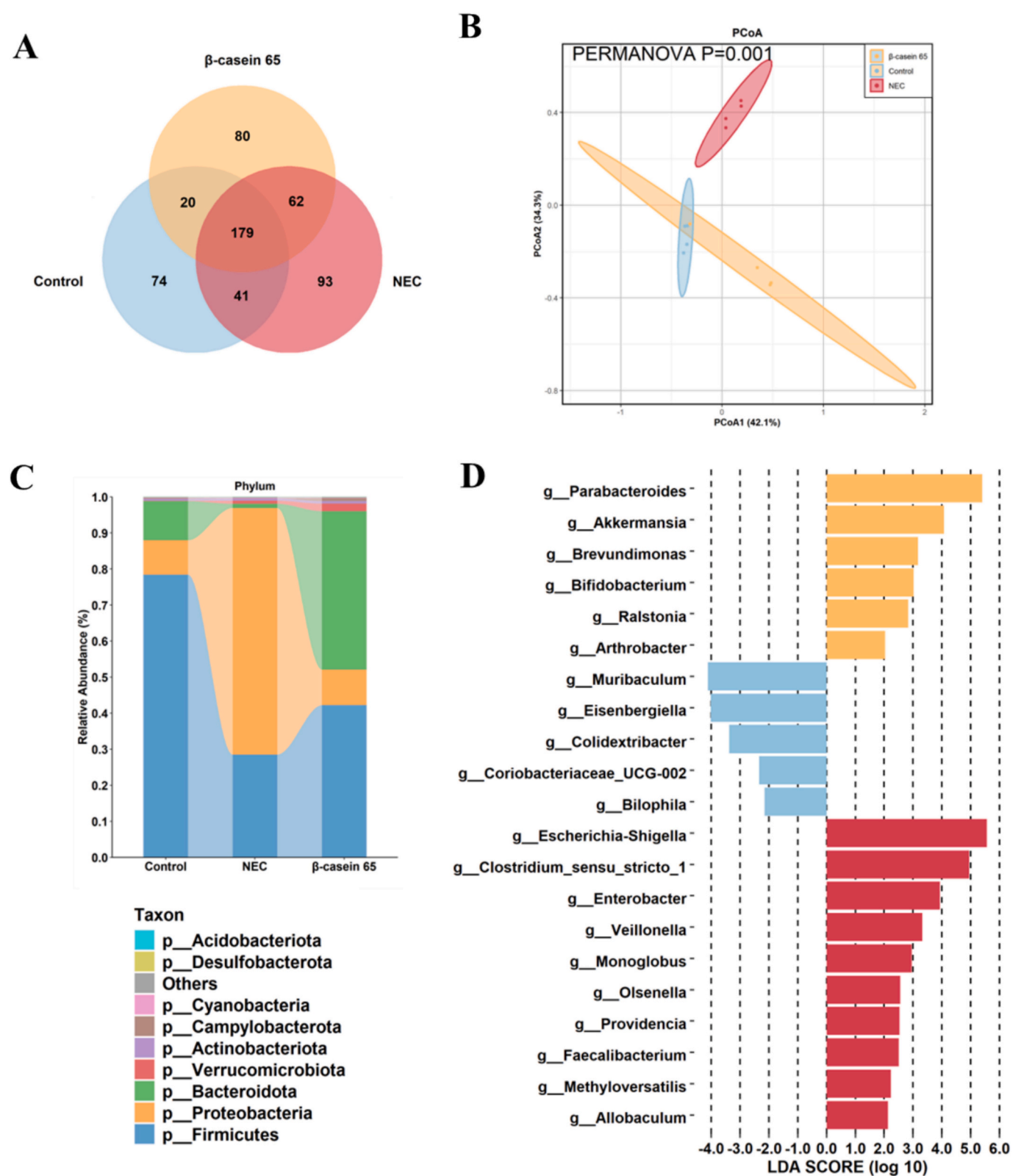


Fig. 5. β-casein 65 affects the composition of gut microbiota in mice. (A) Venn diagram of OTUs. (B) PCoA plot of gut microbiota. (C) Relative abundance of gut microbiota at the phylum level. (D) Differential microbiome based on LDA scores. Values are expressed as means ± SD (n = 4 for each group).

tight junction proteins, opposite to pro-inflammatory genera like *Escherichia-Shigella* and *Clostridium sensu stricto 1* (Chen et al., 2025). This result not only supports the established theory of “specific bacterial genera regulating inflammation and barrier function” but also identifies the key beneficial bacterial species influenced by β-casein 65.

Integrated microbiome-metabolome analysis revealed β-casein 65 influences host-microbe co-metabolism. Several metabolites increased in the IgA production pathway of the intestinal immune network, consistent with previous research on microbial ecology and mucosal immunity (Wen et al., 2022). In particular, our data support a dual

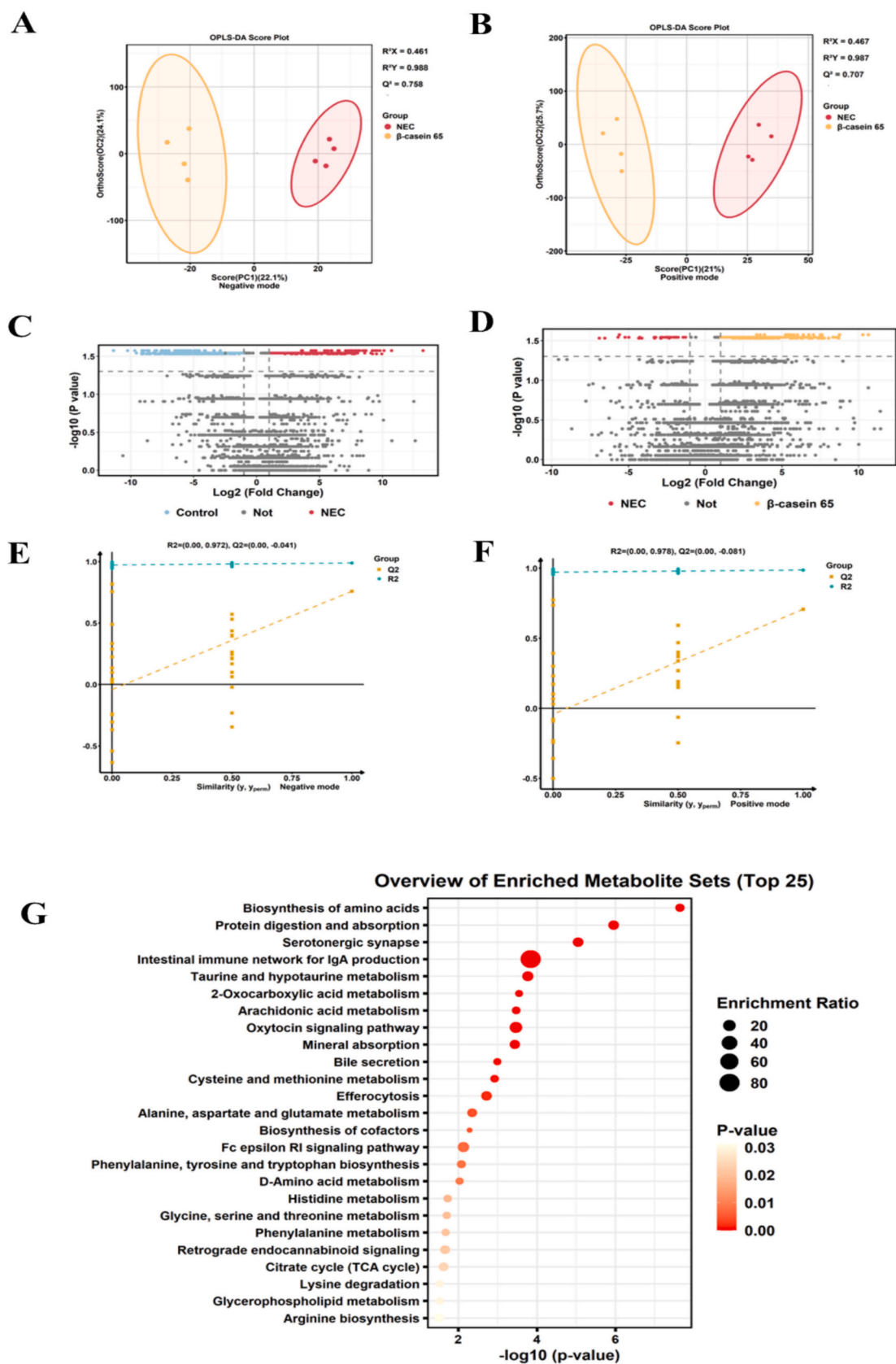


Fig. 6. β -casein 65 Modulates the Intestinal Microbiota Structure in NEC Mice. (A and B) OPLS-DA plots between the NEC and β -casein 65 groups in positive and negative modes. (C and D) Volcano plots showing differential metabolites. (E and F) Permutation tests of the NEC and β -casein 65 groups in positive and negative modes. (G) KEGG pathway enrichment analysis.

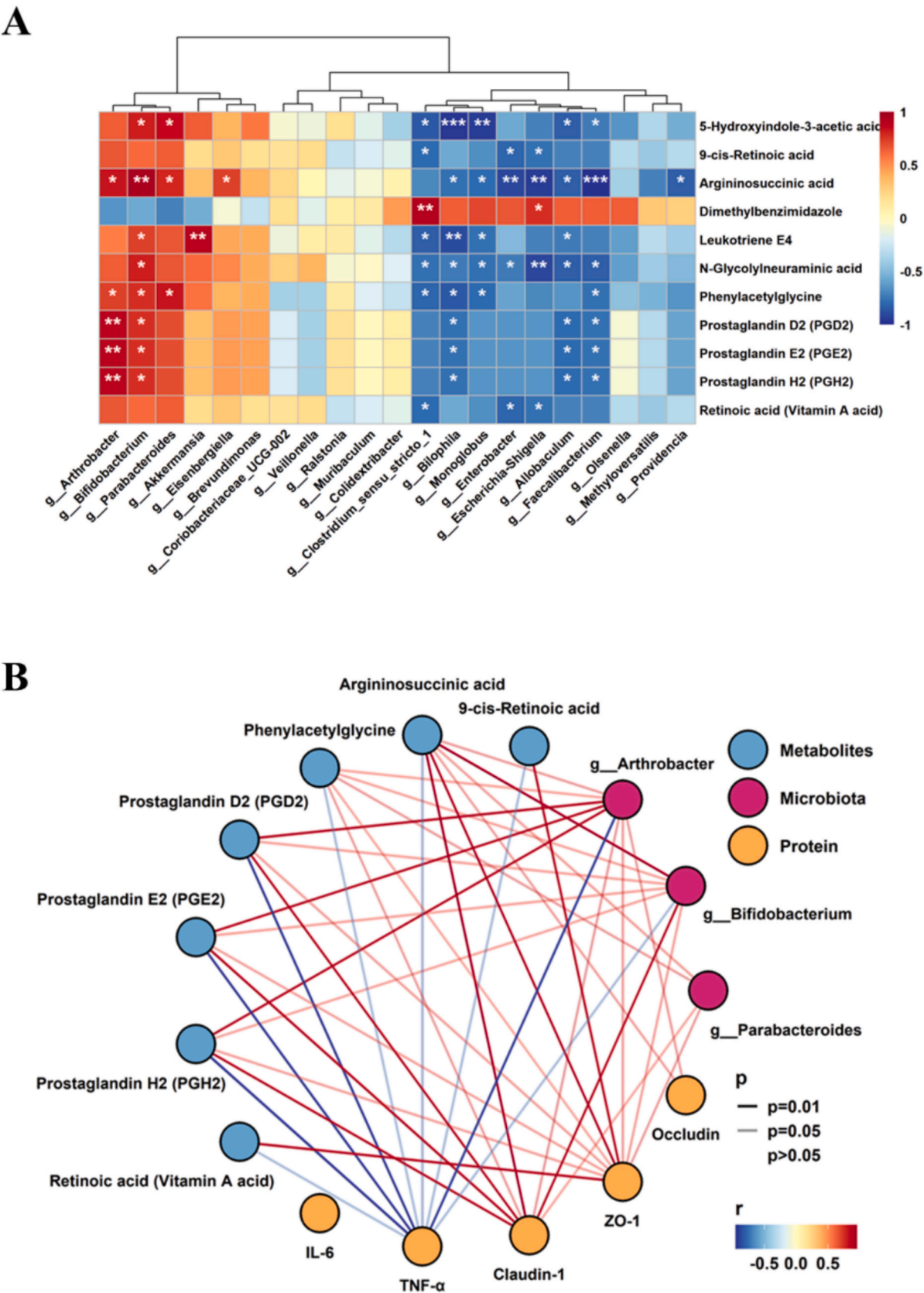


Fig. 7. Correlation Analysis among Differential Genes. (A) Heatmap showing different microorganisms and metabolites. (B) Correlation among gut microbiota, genes, metabolites, proteins, and metabolites.

restorative role of arginine-related pathways in this process: inhibition of NF- κ B-mediated inflammation and promotion of epithelial repair, which is in line with earlier reports on the protective effects of arginine in intestinal injury (Beutheu, Ghoulzali, Galas, Déchelotte, & Coëffier, 2013). Also, arachidonic acid derivatives like prostaglandins E₂ and D₂ regulate Th17/Treg differentiation, supporting their known role in maintaining intestinal immune balance (Monk et al., 2014). In summary, this study not only confirms the vital role of the gut microbiota-metabolite-immune axis in NEC but also, more importantly, demonstrates that β -casein 65 can work in a coordinated manner to strengthen the barrier and reduce inflammation by regulating specific beneficial bacterial populations and their associated metabolic processes within this axis. This study is the first to clarify the role of the preterm human milk-derived peptide β -casein 65 in preventing and treating NEC through a multi-target mechanism. Its natural origin suggests good biocompatibility and safety, offering a clear rationale and a potential molecule for developing microecological agents or nutritional supplements based on breast milk bioactive components to prevent NEC in high-risk preterm infants. Compared to traditional single-target anti-inflammatory drugs, β -casein 65 provides protective effects by synergistically regulating the “gut microbiota-metabolite-immune barrier” axis (Alshammari et al., 2025), showing pleiotropic and systemic regulatory benefits that may be better suited for complex multifactorial diseases like NEC.

However, this study has certain limitations. Only a single dose of β -casein 65 was tested, and a dose-response relationship has not yet been established. Additionally, animal models cannot fully replicate the complexity of NEC in human preterm infants. To address this, our research group plans to validate its efficacy further using human-derived organoid systems and conduct systematic dose-response studies. Furthermore, the long-term safety of β -casein 65, its stability in the complex human intestinal environment, and its interactions with other nutrients or therapeutic drugs remain critical issues to be resolved before clinical translation.

In conclusion, β -casein 65 exerts a protective effect in a neonatal mouse model of NEC by attenuating TLR4-linked inflammation signaling, lowering pro-inflammatory cytokines, and reinforcing the epithelial barrier, thereby improving disease severity. These effects are accompanied by microbiota remodeling, including a reduction in *Proteobacteria*, enrichment of beneficial taxa, and shifts in metabolite pathways toward those within the intestinal immune network for IgA production, thereby supporting host-microbe homeostasis. As a small human milk-derived peptide with amphipathic helical features that is amenable to chemical synthesis and optimization, and consistent with the recognized bioactivity of casein-derived peptides, β -casein 65 emerges as a promising candidate for NEC prevention or adjunctive therapy. Future studies are needed to define its dose-response relationship and safety profile, to validate efficacy in more clinically relevant large-animal models and to explore potential combinations with probiotics or other microbiota-targeted interventions.

Authors contribution

Min Zhang: Conceived and designed the research, performed the experiments, revised the manuscript, analyzed data, and wrote the original draft. Fan Zhang, Liping Xu: Analyzed data, conducted experiments, and developed methodology. Juyi Zhao, Yanjie Chen, Xiangyun Yan, Xiaohui Chen: Assisted with sample collection and experimental procedures. Jun Zhang: Supervised the research and revised the manuscript. Shushu Li: Provided project supervision and edited the manuscript. Shuping Han: Conceptualized the research, managed the project, supervised the work, and edited the manuscript.

CRediT authorship contribution statement

Min Zhang: Writing – original draft, Methodology, Data curation,

Conceptualization. **Liping Xu:** Data curation. **Fan Zhang:** Writing – original draft, Methodology, Formal analysis. **Juyi Zhao:** Methodology, Formal analysis. **Yanjie Chen:** Methodology, Data curation. **Xiaotong Chen:** Methodology, Data curation. **Xiangyun Yan:** Methodology, Funding acquisition, Formal analysis. **Xiaohui Chen:** Methodology, Formal analysis. **Shushu Li:** Writing – review & editing, Project administration, Funding acquisition. **Jun Zhang:** Writing – review & editing, Funding acquisition. **Shuping Han:** Writing – review & editing, Project administration, Funding acquisition.

Ethics statement

The experiment was approved by the Animal Ethics Committee of Nanjing Medical University (permission number: IACUC-2303007). All experiments involving animals were conducted in accordance with the Human & Animal Welfare policy.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2026.107203>.

Data availability

Data will be made available on request.

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