Original Research Article

# Treatment of immune dysfunction in intrauterine growth restriction piglets via supplementation with dimethylglycine sodium salt during the suckling period

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## ARTICLE INFO

## ABSTRACT

This study aimed to investigate the mechanism of small intestinal immune dysfunction in intrauterine growth restriction (IUGR) newborn piglets and relieve this dysfunction via dimethylglycine sodium salt (DMG-Na) supplementation during the suckling period. Thirty sows (Duroc × [Landrace × Yorkshire]) were selected, and 1 male newborn piglet with normal birth weight (NBW) and 1 male newborn piglet with IUGR were obtained from each sow. Among them, 10 NBW and 10 IUGR newborns were euthanized without suckling. The other 20 NBW newborns were allocated to the group named NCON, which means NBW newborns fed a basic milk diet (BMD) ($n = 10$), and the group named ND, which means NBW newborns fed BMD supplemented with 0.1% DMG-Na ($n = 10$); the other 20 IUGR newborns were assigned to the group named ICON, which means IUGR newborns fed BMD ($n = 10$), and the group named ID, which means IUGR newborns fed BMD supplemented with 0.1% DMG-Na ($n = 10$). The newborns were fed BMD from 7 to 21 d of age and euthanized at 21 d of age to collect serum and small intestinal samples. The growth performance, small intestinal histological morphology and sub-organelle ultrastructure, serum immunoglobulin, small intestinal digestive enzyme activity, inflammatory cytokine level, and jejunum mRNA and protein expression of the toll-like receptor 4 (TLR4)/nucleotide-binding oligomerization domain protein (NOD)/nuclear factor-κB (NF-κB) network deteriorated in the ICON group compared to that in the NCON group. The small intestinal histological morphology and sub-organelle ultrastructure, serum immunoglobulin, small intestinal digestive enzyme activity, and inflammatory cytokine level improved ($P < 0.05$) in the ID group compared to those in the ICON group. The jejunum mRNA and protein expression of the TLR4/NOD/NF-κB network improved ($P < 0.05$) in the ID group compared to that in the ICON group. In conclusion, the activity of the TLR4/NOD/NF-κB pathway was inhibited in the IUGR newborns, which in turn led to their jejunum immune dysfunction and reduced their performance. By ingesting DMG-Na, the IUGR newborns activated the TLR4/NOD/NF-κB pathway, thereby improving their unfavorable body state during the suckling period.

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## 1. Introduction

Intrauterine growth restriction (IUGR) is a condition that produces fetuses that are small for their gestational age, with weights below the 10th percentile or the population mean minus 2 standard deviations on a population-based nomogram. IUGR is an important problem in mammals (Sacchi et al., 2020). It has a permanent stunting effect on postnatal growth and impairs long-term health and the efficiency of nutrient utilization. The small intestine is

crucial for the digestion, absorption, and metabolism of dietary nutrients, and it is also the main immune organ of the body. Intestinal immune dysfunction, resulting in infection, inflammation, or both, is closely associated with intestinal disease (Rhoades et al., 2021). This point is important because the small intestine is involved in the first steps of postnatal immune system maturation, the body's protection against food allergens and environmental microorganisms, and nutrient assimilation. After birth, altered small intestinal functions may lead to diseases, and a slow gastrointestinal growth, in turn, may contribute to slow postnatal growth rates in neonates with IUGR. Furthermore, the altered immune function of the small intestine may compromise the health of IUGR newborns throughout the postnatal stage and later on in adulthood (Tuganbaev et al., 2020). However, little is known about the effect of IUGR on immune dysfunction of the small intestine of suckling piglets.

Transmembrane toll-like receptors (TLRs) and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are the most important protein families of pattern recognition receptors, which participate in mediating the small intestinal immune response (Sahoo, 2020). Toll-like receptor 4 (TLR4) is an important TLR member and plays a crucial role in innate immunity and inflammation by discriminating pathogens. When the body is stimulated (Zhang et al., 2020), TLR4 is beneficial in the activation of nuclear factor-κB (NF-κB), which is an important transcription factor that modulates various genes involved in immunity and inflammation processes (Yu et al., 2020). A previous study found that the destroyed immune barrier in IUGR mice was associated with an imbalance in the TLR4-NF-κB pathway (Zarate et al., 2021). In addition, the activation of NF-κB regulates the generation of pro-inflammatory cytokines that finally lead to tissue damage (Yu et al., 2020).

Dimethylglycine sodium salt (DMG-Na) is similar to choline and betaine and can improve the body's immune status by acting as an important precursor for the synthesis of glutathione (Cools et al., 2010). DMG-Na can relieve intestine structural damage, thus improving small intestinal barrier function in IUGR weaned piglets (Bai et al., 2021). DMG-Na can improve the growth performance and immune status of weaned piglets (Bai et al., 2021). DMG-Na not only improves the utilization of oxygen to protect the body from excess free radicals but also enhances the immune response of mice and IPEC-J2 cells (Bai et al., 2018, 2019). However, there are few studies on the mechanism of dietary DMG-Na in a basic milk diet in relieving the IUGR-induced intestinal immune dysfunction of suckling piglets. In the current study, immune dysfunction of the jejunum in IUGR newborns was associated with suppressed TLR4/NOD/NF-κB activity. We aimed to provide novel insight into the beneficial effects of DMG-Na added to the basic milk diet on immune dysfunction of the jejunum in IUGR newborns via the TLR4/NOD/NF-κB pathway during the suckling period.

## 2. Materials and methods

### 2.1. Animal ethic statement

This trial was performed according to the Chinese Guidelines for Experimental Protocols of Animal Care and Animal Welfare and was authorized by the Nanjing Agricultural University Institutional Animal Care and Use Committee.

### 2.2. Experimental design and sampling

This study was performed at Yangzhou Fangling Agricultural and Pastoral Co., Ltd (Jiangsu, China). A total of 30 male newborn piglets of normal birth weight (NBW) (1.53 ± 0.04 kg) and 30 male IUGR newborn piglets (0.76 ± 0.06 kg) were selected from 30 sows (Duroc × [Landrace × Yorkshire]) according to a previously described method (Wang et al., 2005). All sows had a similar birth order (3rd) and were fed the same gestation diet, which met the National Research Council's (NRC, 2012) nutrient requirements. In brief, one male NBW newborn piglet and one male IUGR newborn piglet were obtained from each litter of 10 sows, and a total of 10 NBW and 10 IUGR newborn piglets were euthanized at birth without suckling. Additionally, one male NBW newborn piglet and one male IUGR newborn piglet were obtained from each litter of another 20 sows. The other 20 NBW newborns were allocated to the group named NCON ($n = 10$), which means NBW newborns fed a basic milk diet (BMD), and the group named ND ($n = 10$), which means NBW newborns fed BMD supplemented with 0.1% DMG-Na; the other 20 IUGR newborns were assigned to the group named ICON ($n = 10$), which means IUGR newborns fed BMD, and the group named ID ($n = 10$), which means IUGR newborns fed BMD supplemented with 0.1% DMG-Na. The piglets were fed their BMD from 7 to 21 d of age. DMG-Na was obtained from Qilu Sheng Hua Pharmaceutical Co., Ltd., Shandong, China, and the structure of DMG-Na is provided in Fig. S1.

All the piglets were fed warm milk collected from their corresponding sows every 2 to 3 h from 05:00 (approximately 9 to 10 times daily) during the suckling period. Each sow was assigned an experienced staff member to collect milk and calculate the body weights of the piglets. Before the trial, the corresponding staff visited the sow daily and touched it gently to allow the sow to adapt to the presence of staff and reduce unnecessary stress responses. The collected milk was immediately moved to a constant-temperature water tank. The supplied and remaining volumes of milk in each group were measured with a plastic volumetric cylinder, and the values were used to calculate the absolute intake (intake = supplied - remaining) at each feeding time from d 7 to 21. The body weight gain (BWG), feed intake (FI), and G:F were calculated from d 7 to 21. We tried to imitate the natural feeding conditions for piglets and fed them individually in bottles for 14 d. All the piglets were fed ad libitum each time until they were full, as assessed by the experienced workers. During the experiment, the piglets did not show any stress reactions, and there was no foraging after feeding. The piglets in the 4 groups (NCON, ND, ICON, and ID groups) were raised in plastic houses (1.5 m × 0.7 m × 0.7 m; environmentally controlled; at an ambient temperature of 33 °C) and had free access to water.

### 2.3. Sample collection

Colostrum within 24 h post-parturition, d 14 milk, and d 21 milk taken from 6 mammary glands (2nd, 3rd, and 4th pairs) were collected. Milk samples were collected after an intramuscular injection of 15 IU oxytocin and used for the colostrum and milk composition analysis. Milk composition, which comprised solids-not-fat, protein, lactose, and fat, was analyzed using an automated milk analyzer (Table S1).

A total of 10 NBW newborn piglets and 10 IUGR newborn piglets were selected. Blood samples were collected from the precaval vein, and the piglet was stunned by electric shock and euthanized by jugular exsanguination within 2 h after birth without suckling. Small intestine samples (duodenum, jejunum, and ileum) were collected in sterile containers, sealed, and immediately transferred to an anaerobic chamber. On d 21, 40 suckling piglets from the NCON, ND, ICON, and ID groups (10 piglets per group) were weighed prior to euthanasia, and blood samples were collected from the precaval vein. The piglets were anesthetized via electrical stunning and euthanized by exsanguination. Small intestine samples (duodenum, jejunum, and ileum) were then obtained. The serum samples were separated by centrifugation at 2,500 × *g* for

15 min at 4 °C and then stored at −80 °C until analysis. The small intestine tissue was removed from the abdominal cavity immediately after the animal died and was divided into the duodenum (approximately the first 10 cm segment after the stomach), jejunum (approximately half of the small intestine below the duodenum), and ileum (the left part of the small intestine). After that, they were subjected to analysis as indicated below.

### 2.4. Histological morphology study

Small intestine samples were fixed in 1% (vol/vol) glutaraldehyde solution and stored in the same solution until processing. After fixation for 5 min in 2% (wt/vol) osmium tetroxide, the samples were processed conventionally for transmission electron microscopy visualization and examined under a Philips 420 transmission electron microscope at 80 kV (Dong et al., 2016).

Small intestine samples fixed in 4% buffered formaldehyde were dehydrated using a graded series of xylene and ethanol and then embedded in paraffin for histological processing. Sample sections (8 μm in thickness) were mounted on slides, deparaffinized using xylene, and rehydrated with graded dilutions of ethanol. The slides were stained with hematoxylin and eosin (H&E). Ten slides for each sample (middle region of the samples) were prepared, and the images were acquired using an optical binocular microscope. Values of villus length (L), crypt depth, villus width (W), muscle thickness, and mucosa thickness were measured five times per slide from different villi and crypts. The villus area (S) was calculated using the following formula (Dong et al., 2016):

$$S = \pi \times \left(\frac{W}{2}\right)\sqrt{\left(\frac{W}{2}\right)^2 + L^2}$$

Small intestine samples fixed in 4% buffered formaldehyde were dehydrated, embedded in paraffin, mounted, dewaxed, and rehydrated as described above. The slides were then stained with H&E and periodic acid-Schiff to observe intraepithelial lymphocytes (IELs) and goblet cells (GCs), respectively, using corresponding kits, according to the manufacturer's instructions (Wuhan Servicebio Biotechnology Ltd., Wuhan, China). The IELs and GCs were observed using a Nikon ECLIPSE 80i optical microscope (Tokyo, Japan). Ten intact intestinal villi were selected from each section to count the number of IELs and GCs among 100 enterocytes.

### 2.5. Serum immunoglobulin concentration

The serum D-lactic acid (D-LA, H263), diamine oxidase (DAO, A088-1-1), immunoglobulin A (IgA, E027-1-1), immunoglobulin G (IgG, E026-1-1), and immunoglobulin M (IgM, E025-1-1) concentrations were measured using corresponding assay kits, according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

### 2.6. Digestive enzyme activity

Small intestine samples were homogenized in 0.9% sodium chloride buffer on ice and then centrifuged at 2,500 × g for 10 min at 4 °C. The supernatant was used to measure the levels of small intestinal amylase (C016-2-1), lipase (A054-1-1), and trypsin (A080-2-2) using corresponding assay kits (Nanjing Jiancheng Bioengineering Institute).

Additional small intestine samples were homogenized in 0.9% sodium chloride buffer on ice and then centrifuged at 2,800 × g for 15 min at 4 °C. The supernatant was used to measure the levels of small intestinal sucrase (A082-2-1), maltase (A082-3-1), and lactase (A082-1-1) using corresponding assay kits (Nanjing Jiancheng Bioengineering Institute).

### 2.7. Mucosal damage level

Small intestinal mucin 2 (MUC2, H470) and trefoil factor 3 (TFF3, H560-1) concentrations were measured using corresponding assay kits, according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute).

The number of apoptotic and necrotic cells was measured using an Alexa Fluor 488 Annexin V/Dead Cell Apoptosis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Briefly, the small intestine samples were homogenized with a glass homogenizer, and the cells were washed twice with cooled phosphate-buffered saline (pH = 7.4) and resuspended (2% suspension) in 1× annexin-binding buffer. Thereafter, the cell density was determined and the sample was diluted in 1× annexin binding buffer to $1 \times 10^6$ cells/mL. A sufficient volume of the cell suspension was stained with Annexin V-fluorescein isothiocyanate and propidium iodide (1:9 dilution) staining solution in the dark at room temperature for 15 min. After incubation, the forward scatter of cells was determined, and Annexin V fluorescence intensity was measured in fluorescence channel 1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACSCaliber flow cytometer (BD Biosciences).

### 2.8. Inflammatory cytokine concentrations

Small intestine samples were homogenized in 0.9% sodium chloride buffer on ice and then centrifuged at 2,800 × g for 15 min at 4 °C. The supernatant was used to measure the concentrations of small intestinal secretory immunoglobulin A (sIgA, H108-2), interleukin 1β (IL-1β, H002), interleukin 6 (IL-6, H007-1-2), interleukin 8 (IL-8, H008), and tumor necrosis factor α (TNF-α, H052-1) concentrations using corresponding assay kits, according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute).

### 2.9. Quantitative reverse transcription PCR

Quantitative reverse transcription PCR (RT-qPCR) was performed as described previously (Bergqvist et al., 2007). Total RNA was extracted from jejunum samples using TRIzol Reagent (TaKaRa, Dalian, China) and then reverse-transcribed using a commercial kit (Perfect Real Time, SYBR PrimeScript, TaKaRa) according to the manufacturer's instructions. The mRNA expression levels of specific genes were quantified via real-time PCR using SYBR Premix Ex Taq II (Tli RNaseH Plus) and an ABI 7300 Fast Real-Time PCR detection system (Applied Biosystems, Foster City, CA). The SYBR Green PCR reaction mixture consisted of 10 μL SYBR Premix Ex Taq (2×), 0.4 μL of the forward and reverse primers, 0.4 μL of ROX reference dye (50×), 6.8 μL of ddH2O, and 2 μL of cDNA template. Each sample was amplified in triplicate. The fold-expression of each gene was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), in which Actb was used as an internal standard. The primer sequences used are shown in Table S2.

### 2.10. Western blotting

Antibodies against related proteins [TLR4 (# 14358 S), MyD88 (# 4283 S), IκBα (# 4812 S), NF-κB p65 (# 8242 S), caspase-3 (# 14220 S), PCNA (# 13110 S), claudin (# 13255 S), and occludin (# 13255 S)] were purchased from Cell Signaling Technology (Danvers, MA, USA). The protein content of the samples was measured using a BCA Protein Assay Kit (Beyotime, Jiangsu, China).

**Table 1**
Supplementation with DMG-Na improves the growth performance (d 7 to 21) of IUGR piglets during the suckling period[1].

| Item | Treatments | | | | P-value | | |
|------|-----------|------|------|------|---------|---------|---------|
| | NCON[2] | ND[3] | ICON[4] | ID[5] | $P_G$ | $P_D$ | $P_{G \times D}$ |
| BWG, kg | 4.23 ± 0.29 | 5.42 ± 0.27 | 3.69 ± 0.21 | 4.80 ± 0.25 | 0.004 | <0.001 | 0.794 |
| FI, kg | 7.42 ± 0.30 | 6.78 ± 0.28 | 7.24 ± 0.26 | 6.67 ± 0.30 | 0.405 | 0.006 | 0.837 |
| G:F | 0.57 ± 0.02 | 0.80 ± 0.03 | 0.51 ± 0.02 | 0.72 ± 0.03 | 0.001 | <0.001 | 0.516 |
| IBW, kg | 3.48 ± 0.26 | 3.48 ± 0.33 | 2.23 ± 0.21 | 2.23 ± 0.18 | <0.001 | 1.000 | 1.000 |
| FBW, kg | 7.71 ± 0.58 | 8.90 ± 0.52 | 5.92 ± 0.60 | 7.03 ± 0.57 | 0.001 | 0.008 | 0.906 |

DMG-Na = dimethylglycine sodium salt; NBW = normal birth weight; IUGR = intrauterine growth restriction; BWG = body weight gain; FI = feed intake; G:F = weight gain to feed intake ratio; IBW = initial body weight; FBW = final body weight.
[1] Values are expressed as mean ± standard deviation ($n = 10$ suckling piglets).
[2] NCON = NBW newborn piglets fed a basic milk diet.
[3] ND = NBW newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na.
[4] ICON = IUGR newborn piglets fed a basic milk diet.
[5] ID = IUGR newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na.

For Western blotting analysis, 50 μg of protein from each jejunum sample was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the separated proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with blocking buffer (5% nonfat dry milk) for 12 h at 4 °C and probed with appropriate primary and secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G, Cell Signaling Technology; 1:10,000 dilution in 1% milk). Signals were detected using enhanced chemiluminescence (ECL-Kit, Beyotime), followed by autoradiography. Photographs of the membranes were taken using the Luminescent Image Analyzer LAS-4000 system (Fujifilm Co.) and quantified with ImageJ 1.42q software (NIH, Bethesda, MD, USA).

### 2.11. Statistical analysis

Body weight data modeling of repeated records (10 piglets per group) during the suckling period was conducted using a mixed model with the group (G), DMG-Na (D), and G × D as fixed effects and piglets as random effects. If the P-value of the interaction G × D was less than 0.05, the body weight among the different groups was determined using one-way ANOVA.

The data on small intestinal digestive enzyme activity, mucosal damage level, and inflammatory cytokine concentrations among trial A (NBW and IUGR), trial B (NBW, IUGR, NCON, and ICON), and trial C (NCON, ND, ICON, and ID) were analyzed separately. For trial A, the corresponding data were obtained using a mixed model with the group (GA), intestine (IA), and GA × IA as fixed effects and piglets as random effects. If the P-value of the interaction GA × IA was less than 0.05, a paired t-test was conducted among the different groups in each segment of the small intestine. For trial B, the corresponding data were obtained using a mixed model with the group (GB); time (T); intestine (IB); and GB × T, GB × IB, T × IB, and GB × T × IB as fixed effects and piglets as random effects. For trial C, the corresponding data were obtained using a mixed model with the group (GC); DMG-Na (D); intestine (IC); and GC × D, GC × IC, D × IC, and GC × D × IC as fixed effects and piglets as random effects. If the P-value of the interaction GB × T × IB or GC × D × IC was less than 0.05, a one-way ANOVA was conducted among the different groups in each segment of the small intestine.

The data on serum immunoglobulin concentration and jejunum mRNA and protein expression among trials A, B, and C were also analyzed separately. For trial A, as the newborn piglets of the NBW and IUGR groups were paired within litters, the difference between these 2 groups was analyzed using a paired t-test. For trial B, the corresponding data were obtained using a mixed model with the group (GB), time (T), and GB × T as fixed effects and piglets as

random effects. For trial C, the corresponding data were obtained using a mixed model with the group (GC), DMG-Na (D), and GC × D as fixed effects and piglets as random effects. If the P-value of the interaction GB × T or GC × D was less than 0.05, a one-way ANOVA was conducted among the different groups.

All data were analyzed using Statistical Analysis System software (version 9.1; SAS Institute, Inc., Cary, NC, USA). P-values less than 0.05 were considered significantly different. The data are expressed as mean ± standard deviation.

## 3. Results

### 3.1. Growth performance

The interaction effects (groups × DMG-Na) were not significant ($P > 0.05$) for any of the tested growth performance traits (BWG, FI, G:F, IBW, or FBW; Table 1). The BWG, FI, G:F, and FBW values were better in the ND and ID groups than in the NCON and ICON groups during the suckling period.

### 3.2. Histological morphology study

In trial A, the interaction effect (groups × intestines) was significant for villus width ($P = 0.001$), villus area ($P < 0.001$), muscle thickness ($P < 0.001$), and GCs ($P < 0.001$). The small intestinal villus width, villus area, muscle thickness, and GC values in the IUGR group decreased ($P < 0.05$) when compared to those in the NBW group. In trial B, the interaction effect (groups × time × intestines) was significant for muscle thickness ($P = 0.004$) and GCs ($P < 0.001$). The small intestinal muscle thickness and GC values were higher ($P < 0.05$) in the NCON and ICON groups than in the NBW and IUGR groups, respectively. In addition, the small intestinal muscle thickness and GC values were lower ($P < 0.05$) in the ICON group than in the NCON group. In trial C, the interaction effect (groups × DMG-Na × intestines) was significant for muscle thickness ($P < 0.001$) and GCs ($P < 0.001$). The small intestinal muscle thickness and GC values were higher ($P < 0.05$) in the ND and ID groups than in the NCON and ICON groups, respectively (Figs. 1A and 2).

The small intestinal villi in the IUGR group were shorter and of different lengths than those in the NBW group. The small intestinal microvilli in the IUGR group were shorter and less abundant than those in the NBW group. Additionally, autophagosomes and mitochondrial swelling were observed in the IUGR group but not in the NBW group. Over time, this phenomenon naturally existed in the ICON group when compared to the NCON group. However, supplementation with DMG-Na improved the shorter and less abundant microvilli and the autophagosomes and mitochondrial
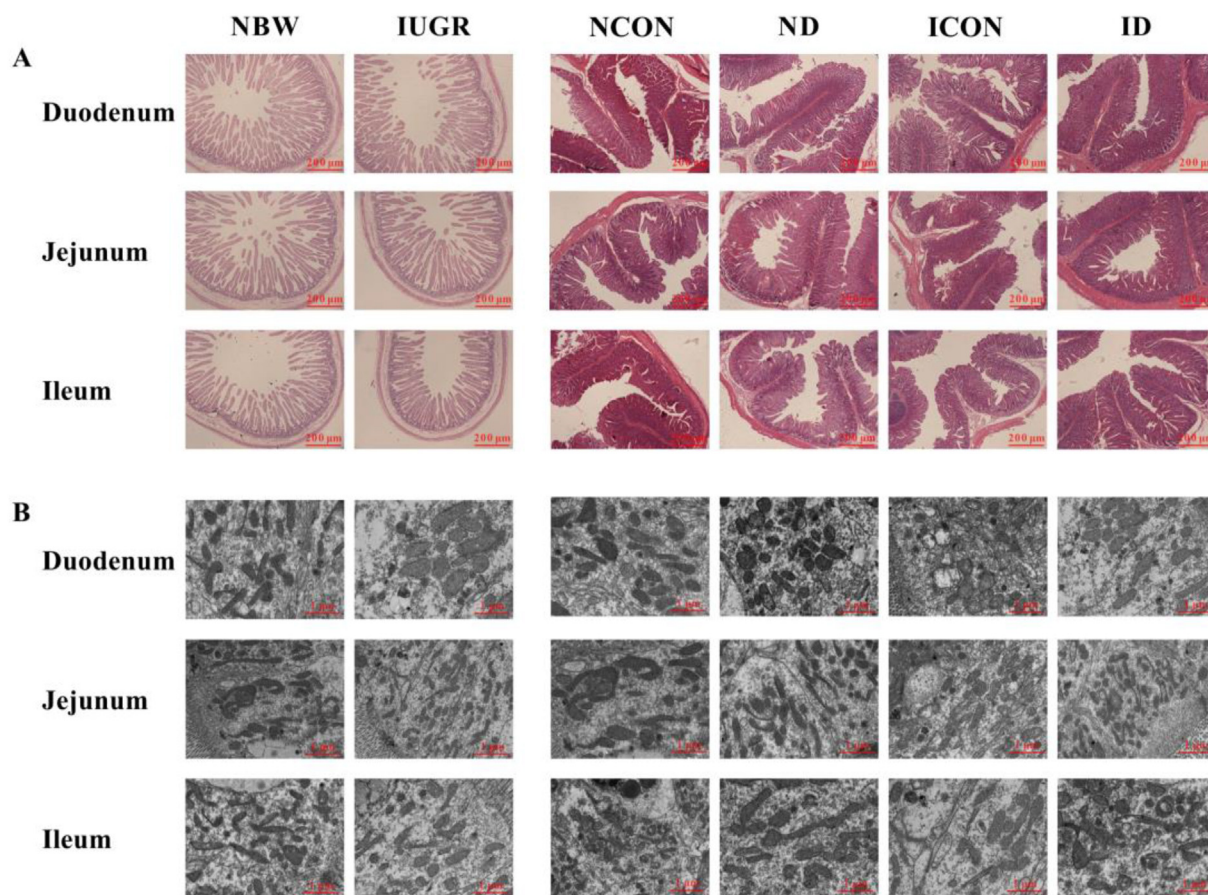
**Fig. 1.** Supplementation with DMG-Na improves histological morphology of the small intestine in IUGR piglets during the suckling period. (A) Hematoxylin and eosin staining of the small intestine; scale bars represent 200 μm. (B) Mitochondrial swelling of the small intestine; scale bars represent 2 μm. DMG-Na = dimethylglycine sodium salt. NBW = normal birth weight; IUGR = intrauterine growth restriction; NCON = NBW newborn piglets fed a basic milk diet; ND = NBW newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na; ICON = IUGR newborn piglets fed a basic milk diet; ID = IUGR newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na.

swelling of the small intestine of the ICON group when compared to those of the ID group (Fig. 1B).

### 3.3. Serum immunoglobulin concentration

In trial A, the serum D-LA, DAO, IgA, IgG, and IgM values in the IUGR group were lower ($P < 0.05$) than those in the NBW group. In trial B, the interaction effect (groups × time) was significant for D-LA ($P = 0.025$), DAO ($P = 0.001$), IgA ($P = 0.002$), IgG ($P = 0.002$), and IgM ($P = 0.010$). The serum D-LA, DAO, IgA, IgG, and IgM values were higher ($P < 0.05$) in the NCON and ICON groups than in the NBW and IUGR groups, respectively. In addition, the serum D-LA, IgA, IgG, and IgM values were lower ($P < 0.05$) in the ICON group than in the NCON group. In trial C, the interaction effect (groups × DMG-Na) was significant for D-LA ($P = 0.043$), IgA ($P < 0.001$), IgG ($P < 0.001$), and IgM ($P = 0.009$). The serum D-LA, IgA, IgG, and IgM values increased ($P < 0.05$) in the ND and ID groups compared to those in the NCON and ICON groups, respectively (Fig. 3).

### 3.4. Digestive enzyme activity

In trial A, the interaction effect (groups × intestines) was significant for amylase ($P = 0.033$). The small intestinal amylase values in the IUGR group were lower ($P < 0.05$) than those in the NBW group. In trial B, the interaction effect (groups × time × intestines)

was significant for lipase ($P = 0.001$). The small intestinal lipase values were higher ($P < 0.05$) in the NCON and ICON groups than in the NBW and IUGR groups, respectively. Additionally, the small intestinal lipase values were lower ($P < 0.05$) in the ICON group than in the NCON group. In trial C, the interaction effects (groups × DMG-Na × intestines) were not significant ($P > 0.05$) for any of the tested digestive enzyme traits (Fig. 4).

### 3.5. Mucosal damage level

The interaction effects in trial A (groups × intestines), trial B (groups × time × intestines), and trial C (groups × DMG-Na × intestines) were not significant ($P > 0.05$) for any of the tested mucosal damage traits (Fig. 5).

### 3.6. Inflammatory cytokine concentrations

In trial A, the interaction effect (groups × intestines) was not significant ($P > 0.05$) for any of the tested small intestinal inflammatory cytokine levels. In trial B, the interaction effect (groups × time × intestines) was significant for sIgA ($P < 0.001$). The small intestinal sIgA levels were higher ($P < 0.05$) in the NCON and ICON groups than in the NBW and IUGR groups, respectively. Additionally, the small intestinal sIgA levels were lower ($P < 0.05$) in the ICON group than in the NCON group. In trial C, the interaction effects (groups × DMG-Na × intestines) were significant for IL–1β
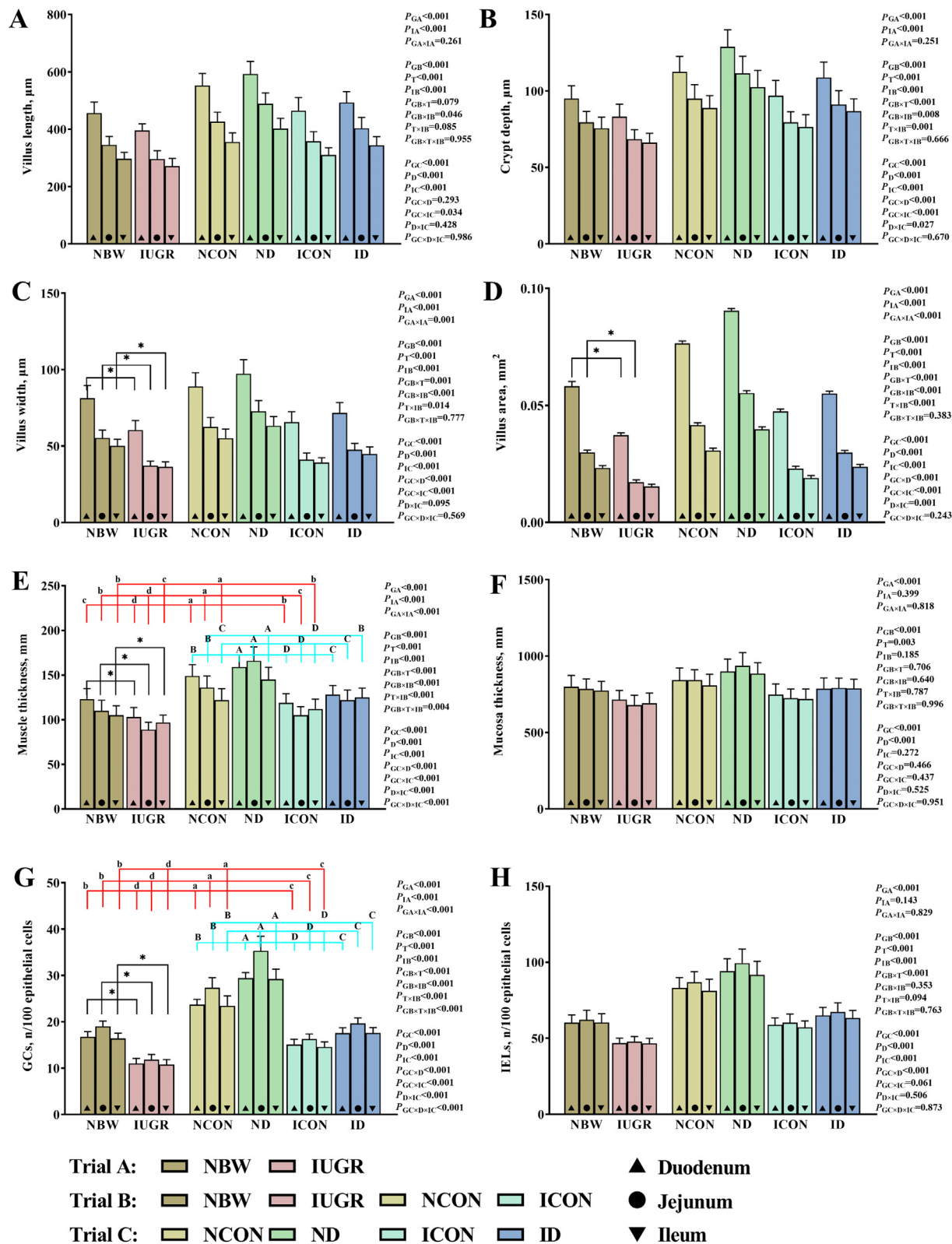
**Fig. 2.** Supplementation with DMG-Na improves the histological morphology values of the small intestine in IUGR piglets during the suckling period. (A) Villus length, (B) crypt depth, (C) villus width, (D) villus area, (E) muscle thickness, (F) mucosa thickness, (G) GCs, and (H) IELs values of the small intestine. Data are expressed as the mean ± standard deviation ($n = 10$ suckling piglets). Asterisks (*) for trial A (NBW, IUGR groups in black color), different lower-case letter (a, b, c, d) for trial B (NBW, IUGR, NCON, ICON groups in red color), and different upper-case letters (A, B, C, D) for trial C (NCON, ND, ICON, ID group in blue color) represent significant differences ($P < 0.05$). DMG-Na = dimethylglycine sodium salt; GCs = goblet cells; IELs = intraepithelial lymphocytes. NBW = normal birth weight; IUGR = intrauterine growth restriction; NCON = NBW newborn piglets fed a basic milk diet; ND = NBW newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na; ICON = IUGR newborn piglets fed a basic milk diet; ID = IUGR newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na.
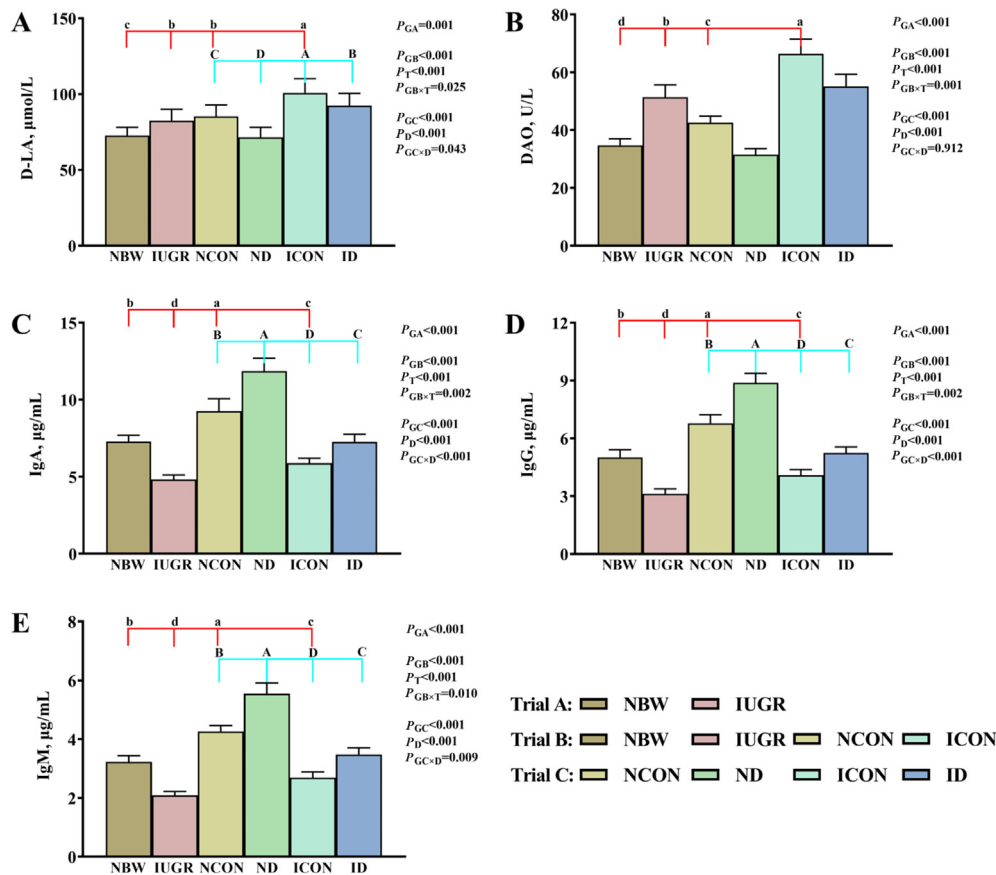
**Fig. 3.** Supplementation with DMG-Na improves the serum immunoglobulin values of IUGR piglets during the suckling period. (A) D-LA, (B) DAO, (C) IgA, (D) IgG, and (E) IgM values of the serum. Data are expressed as the mean ± standard deviation ($n$ = 10 suckling piglets). Different superscripts of a, b, c, d (Trial B: NBW, IUGR, NCON, ICON group in red color) and A, B, C, D (Trial C: NCON, ND, ICON, ID group in blue color) represent significant differences ($P < 0.05$). DMG-Na = dimethylglycine sodium salt; D-LA = D-lactic acid; DAO = diamine oxidase; IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M. NBW = normal birth weight; IUGR = intrauterine growth restriction; NCON = NBW newborn piglets fed a basic milk diet; ND = NBW newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na; ICON = IUGR newborn piglets fed a basic milk diet; ID = IUGR newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na.

($P$ = 0.050), IL-6 ($P$ = 0.034), and IL-8 ($P$ = 0.041). The small intestinal IL-1β, IL-6, and IL-8 levels were lower ($P < 0.05$) in the ND and ID groups than in the NCON and ICON groups, respectively (Fig. 6).

### 3.7. Quantitative reverse transcription PCR

In trial A, the expression of genes in the jejunum in the IUGR group was downregulated ($P < 0.05$) compared to that in the NBW group. In trial B, the interaction effects (group × time) were significant for *TLR4* ($P$ = 0.019), *TRAF6* ($P$ = 0.010), *NOD1* ($P$ = 0.025), *NOD2* ($P$ = 0.003), *NF-κB* ($P$ = 0.002), *Tollip* ($P$ = 0.003), *ERBB2IP* ($P$ = 0.002), *CENTB1* ($P$ = 0.001), *IgG* ($P$ = 0.001), *FcRn* ($P$ = 0.005), *CD4* ($P$ = 0.007), *CD8* ($P$ = 0.004), *MHC1* ($P$ = 0.009), *IL1B* ($P$ = 0.008), *IL8* ($P$ = 0.041), *IL6* ($P$ = 0.007), *TNF* ($P < 0.001$), and *OCLN* ($P$ = 0.005). The expression of these selected genes in the jejunum was higher ($P < 0.05$) in the NCON and ICON groups than in the NBW and IUGR groups, respectively. Additionally, the expression of the selected genes in the jejunum was lower ($P < 0.05$) in the ICON group than in the NCON group. In trial C, the interaction effects (groups × DMG-Na) were not significant ($P > 0.05$) for any of the tested gene expression levels (Fig. 7).

### 3.8. Western blotting

In trial A, jejunal protein expression in the IUGR group was downregulated ($P < 0.05$) compared to that in the NBW group. In trial B, the interaction effects (groups × time) were significant for C_NF-κB ($P$ = 0.004), PCNA ($P$ = 0.008), CLDN ($P$ = 0.006), and OCLN ($P$ = 0.008). The levels of selected proteins in the jejunum were higher ($P < 0.05$) in the NCON and ICON groups than in the NBW and IUGR groups, respectively. Furthermore, the levels of selected proteins in the jejunum were lower ($P < 0.05$) in the ICON group than in the NCON group. In trial C, the interaction effects (groups × DMG-Na) were not significant ($P > 0.05$) for any of the tested protein levels (Fig. 8).

## 4. Discussion

IUGR has received more attention in animal husbandry because of the irreversible mucosal damage, delayed postnatal growth, and intestinal immune dysfunction that it causes (Dong et al., 2016; Guerby and Bujold, 2020). Intestinal immune dysfunction, resulting in infection and/or inflammation, is closely associated with
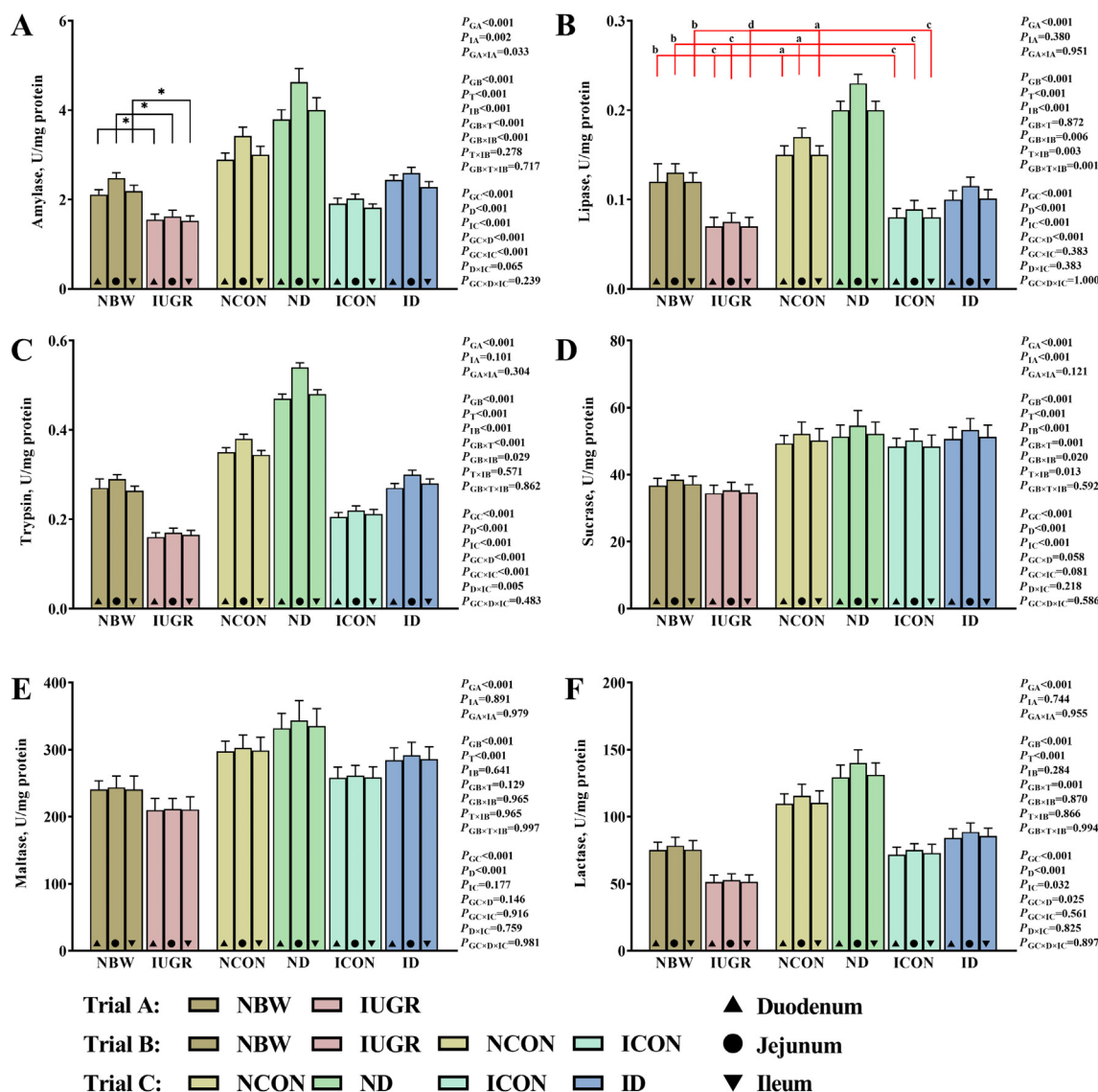
**Fig. 4.** Supplementation with DMG-Na improves the digestive enzyme values of the small intestine in IUGR piglets during the suckling period. (A) Amylase, (B) lipase, (C) trypsin, (D) sucrase, (E) maltase, and (F) lactase values of the small intestine. Data are expressed as the mean ± standard deviation ($n$ = 10 suckling piglets). Asterisks (*) for trial A (NBW, IUGR groups in black color) and different lower-case letter (a, b, c, d) for trial B (NBW, IUGR, NCON, ICON groups in red color) represent significant differences ($P$ < 0.05). DMG-Na = dimethylglycine sodium salt. NBW = normal birth weight; IUGR = intrauterine growth restriction; NCON = NBW newborn piglets fed a basic milk diet; ND = NBW newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na; ICON = IUGR newborn piglets fed a basic milk diet; ID = IUGR newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na.

intestinal disease (Rhoades et al., 2021); thus, it is important to study the mechanism of intestinal immune dysfunction induced by IUGR. Several studies have used IUGR newborn piglets as a model for poor performance (Dong et al., 2016; Zhang et al., 2019b; Sovio et al., 2020), and their findings agree with our results, which demonstrated that the IUGR group had lower growth performance than the NBW group. As expected, supplementation with DMG-Na in the basic milk diet improved the growth performance of the IUGR suckling piglets, which may be explained by the benefits of DMG-Na on intestinal function. A previous study by our team demonstrated dietary supplementation with DMG-Na in basic diets could improve the growth performance of weaned IUGR piglets (Bai et al., 2021). In addition, gastric gavage with DMG-Na at 12 mg/0.3 mL sterile saline solution significantly increased the body weights of mice at 28 d compared to those of control mice (Bai et al.,

2016). However, the effects of DMG-Na on improving the growth performance of IUGR newborns during the suckling period require further study.

The small intestine is crucial for nutrient digestion, absorption, and metabolism. IUGR leads to intestinal dysfunction in newborns and makes them prone to feeding intolerance and digestive diseases during the suckling period. The movement of substances across the cell membrane depends on diffusion or active transport, which are regulated by the intestinal structure. From the results of this study, we found that immune function most significantly varied in the jejunum between the NBW and IUGR groups. Thus, we hypothesized that the structural damage of the jejunum barrier in the IUGR newborns might be related to alterations in immune function. Consistent with our results, other studies have indicated that IUGR leads to intestinal villus atrophy, mucosal damage, and
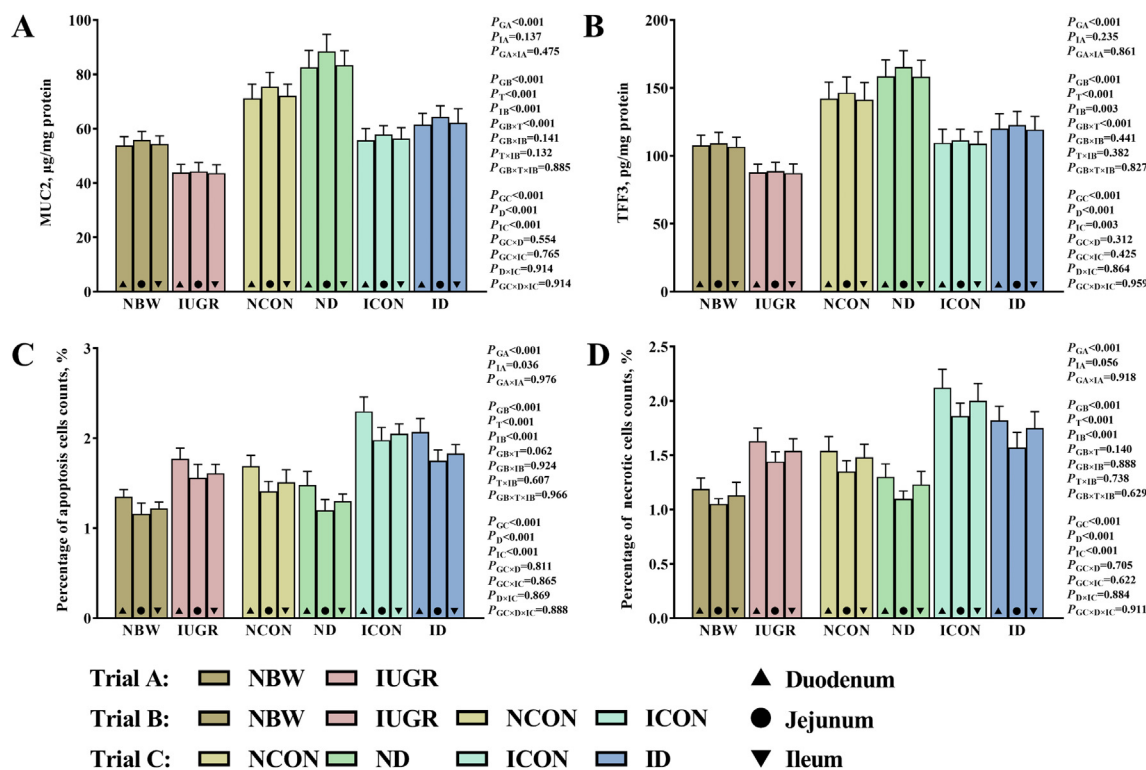
**Fig. 5.** Supplementation with DMG-Na improves the mucosal damage values of the small intestine in IUGR piglets during the suckling period. (A) MUC2, (B) TFF3, (C) percentage of apoptosis cells, and (D) percentage of necrotic cells values of the small intestine. Data are expressed as the mean $\pm$ standard deviation ($n = 10$ suckling piglets). DMG-Na = dimethylglycine sodium salt; MUC2 = mucin 2; TFF3 = trefoil factor 3. NBW = normal birth weight; IUGR =intrauterine growth restriction; NCON = NBW newborn piglets fed a basic milk diet; ND = NBW newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na; ICON = IUGR newborn piglets fed a basic milk diet; ID = IUGR newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na.

intestinal dysfunction, causing diarrhea and reductions in feed utilization in newborn piglets (Dong et al., 2016). Our study also showed that autophagosomes and mitochondrial swelling appeared in the small intestine in the IUGR group. Previous studies suggested that DMG-Na acts as an antioxidant, protecting the small intestine from oxidative damage and maintaining its normal histological morphology in weaned IUGR piglets and indomethacin-injured mice (Bai et al., 2019, 2021). Another study found that DMG-Na protected IPEC-J2 cells from oxidative damage (Bai et al., 2018), which might be one reason for the results observed in the histological morphology in the present study.

IUGR is a serious complication of the mammalian newborn, which limits its development and impairs the intestinal barrier during suckling, especially considering that the intestinal barrier protects the small intestine from harmful substances (Chopyk and Grakoui, 2020). DAO, an intracellular enzyme, is mainly located in the intestinal villus cells of mammalian species. D-LA is the product of anaerobic glucose metabolism and is mainly generated by bacteria in the small intestine. As the intestinal mucosal barrier becomes impaired, DAO and D-LA are released into the blood circulation. Therefore, serum DAO and D-LA concentrations can act as markers of intestinal barrier function (Camara-Lemarroy et al., 2021). IgA, IgG, and IgM concentrations are important because they reflect the immune status and its capacity to fight various infections. IUGR leads to abnormalities in intestinal structure and barrier function in newborn piglets (Dong et al., 2016). In the present work, significantly increased levels of serum DAO and D-LA and significantly decreased concentrations of serum IgA, IgG, and

IgM were observed in the IUGR group compared to those in the NBW group. This indicated a poor intestinal barrier function and immune status in the IUGR newborns in comparison with the NBW newborns. This could be alleviated by DMG-Na supplementation during the suckling period, which might be explained by the benefits of DMG-Na in enhancing small intestinal immune function in mice and piglets (Bai et al., 2019, 2021).

Digestive enzymes, cytokines, and immunoglobulins of the small intestine are related to its growth and immune function and play an important role in preventing bacterial invasion. Consistent with our results, previous studies revealed that intestinal growth in IUGR newborn piglets is blocked, causing digestive enzyme secretion abnormalities, which may be closely related to intestinal epithelial cell apoptosis and proliferation imbalances that would seriously affect the digestion and utilization of food (D'Inca et al., 2011; Dong et al., 2016). After adding 0.1% DMG-Na to the milk diet in our study, the results showed that DMG-Na may have improved the digestion and absorption of nutrients in the IUGR group because of its capacity to protect the intestine from immune disorders.

Mitochondrial swelling in the IUGR group suggests a destroyed intestinal barrier in the small intestine. The functional integrity of the intestinal barrier relies on intercellular tight junctions, the mucus layer, epithelial cells, and host innate and adaptive immune responses (Talbot et al., 2020). Secretory products produced by intestinal goblet cells (GCs) are major structural constituents of the mucus layer, which is the front line of host innate defenses (Talbot et al., 2020). GCs are specialized for the production and secretion of
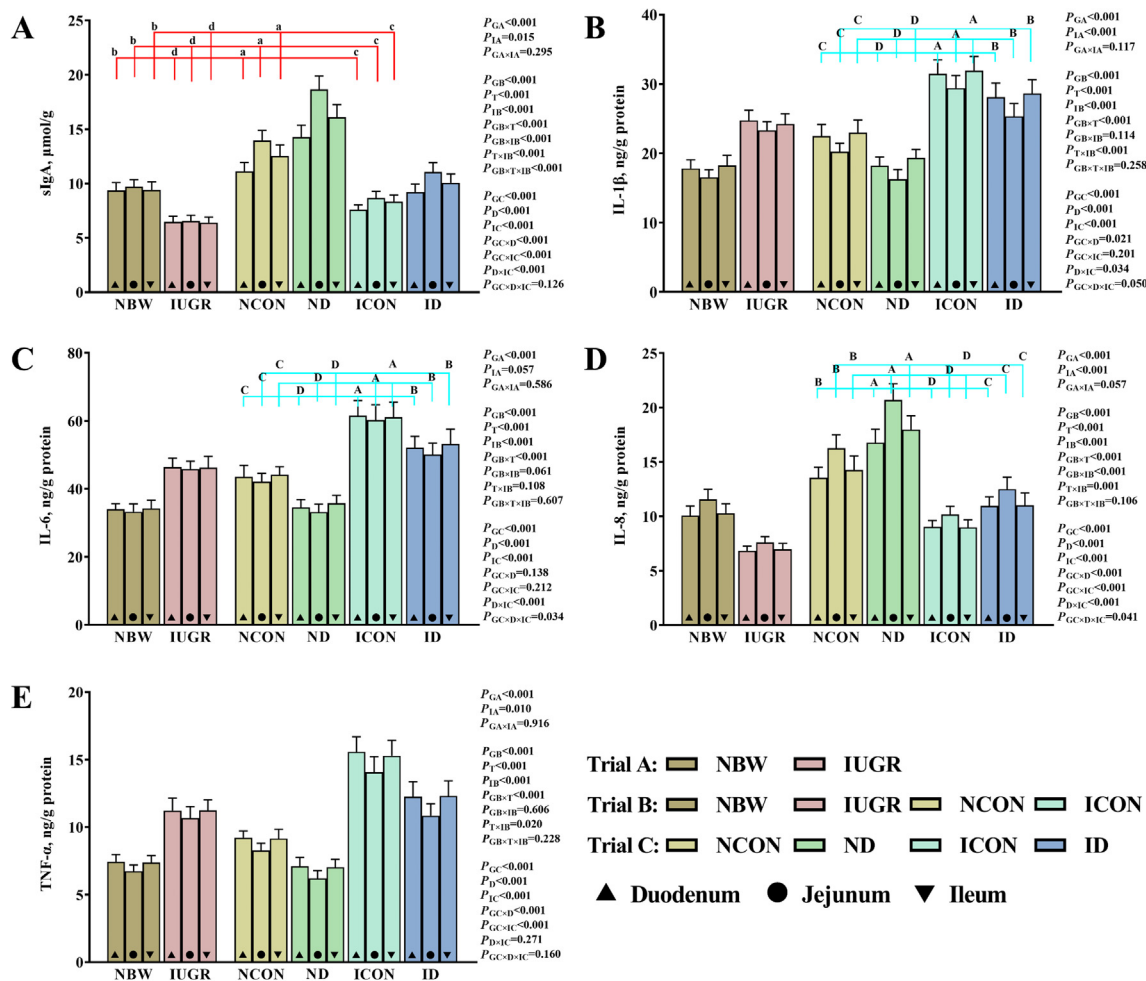
**Fig. 6.** Supplementation with DMG-Na improves the inflammatory cytokine levels in the small intestine of IUGR piglets during the suckling period. (A) sIgA, (B) IL-1β, (C) IL-6, (D) IL-8, and (E) TNF-α values of the small intestine. Data are expressed as the mean ± standard deviation (n = 10 suckling piglets). Different lower-case letter (a, b, c, d) for trial B (NBW, IUGR, NCON, ICON groups in red color), and different upper-case letters (A, B, C, D) for trial C (NCON, ND, ICON, ID group in blue color) represent significant differences (P < 0.05). DMG-Na = dimethylglycine sodium salt; sIgA = secretory immunoglobulin A; IL-1β = interleukin 1β; IL-6 = interleukin 6; IL-8 = interleukin 8; TNFα = tumor necrosis factor α. NBW = normal birth weight; IUGR = intrauterine growth restriction; NCON = NBW newborn piglets fed a basic milk diet; ND = NBW newborn piglets fed a basic milk diet supplemented. with 0.1% DMG-Na; ICON = IUGR newborn piglets fed a basic milk diet; ID = IUGR newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na.

mucin and bioactive molecules, such as trefoil factor peptides (TFFs). MUC2 was the first human secretory mucin to be identified and is synthesized and secreted by GCs in the small intestine. TFF3 is the second most abundant product expressed and secreted by GCs in the intestine. In the current study, the number of GCs in the small intestine and the concentrations of MUC2 and TFF3 in the jejunum were lower in the IUGR group than in the NBW group. These results agreed with previous findings that IUGR decreased intestinal MUC2 and GC numbers in weanling piglets (Zhang et al., 2019a). IELs located in the intestinal epithelium are primarily T cells showing immunoregulatory activity. IELs play a role in the immune system and balance the protective immunity and integrity of the epithelial barrier (Cheroutre et al., 2011). The IUGR group also exerted a higher level of apoptosis than the NBW group, which impaired the small intestinal barrier and function. Consistent with our results, a study indicated that IUGR newborn patients have a reduced intestinal barrier and are prone to suffering oxidative damage (Biri et al., 2007). After adding 0.1% DMG-Na to the milk diet, our results suggested that DMG-Na was beneficial in ameliorating MUC2 and TFF3 concentrations and the apoptosis level in the small intestine, thereby relieving intestinal histomorphological

damage. The administration of DMG-Na enhanced intestinal GC and IEL numbers and improved the concentrations of MUC2 and TFF3, indicating that DMG-Na might strengthen the intestinal barrier by regulating the mucus layer.

Disruption of the intestinal barrier increases intestinal permeability, enhances pro-inflammatory molecule release, and further activates mucosal immune and inflammatory responses, finally resulting in intestinal damage (Suzuki, 2013). IgA can be converted to sIgA by combining with secreted fragments produced by epithelial cells; sIgA then binds to its corresponding antigen to protect the intestine from damage. Excessive intestinal epithelial cell apoptosis damages the normal intestinal structure and thereby results in the release of pro-inflammatory cytokines. Our results showed that IUGR newborns had increased levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and decreased levels of anti-inflammatory molecules (IL-8 and sIgA). This finding is in accordance with that of a previous study, where Huang et al. (2019) demonstrated that IUGR newborn piglets increased the concentrations of TNF-α and IL-6, which indicated that IUGR newborn piglets were prone to inflammatory injury. Milk diets containing 0.1% DMG-Na could decrease the concentration of pro-
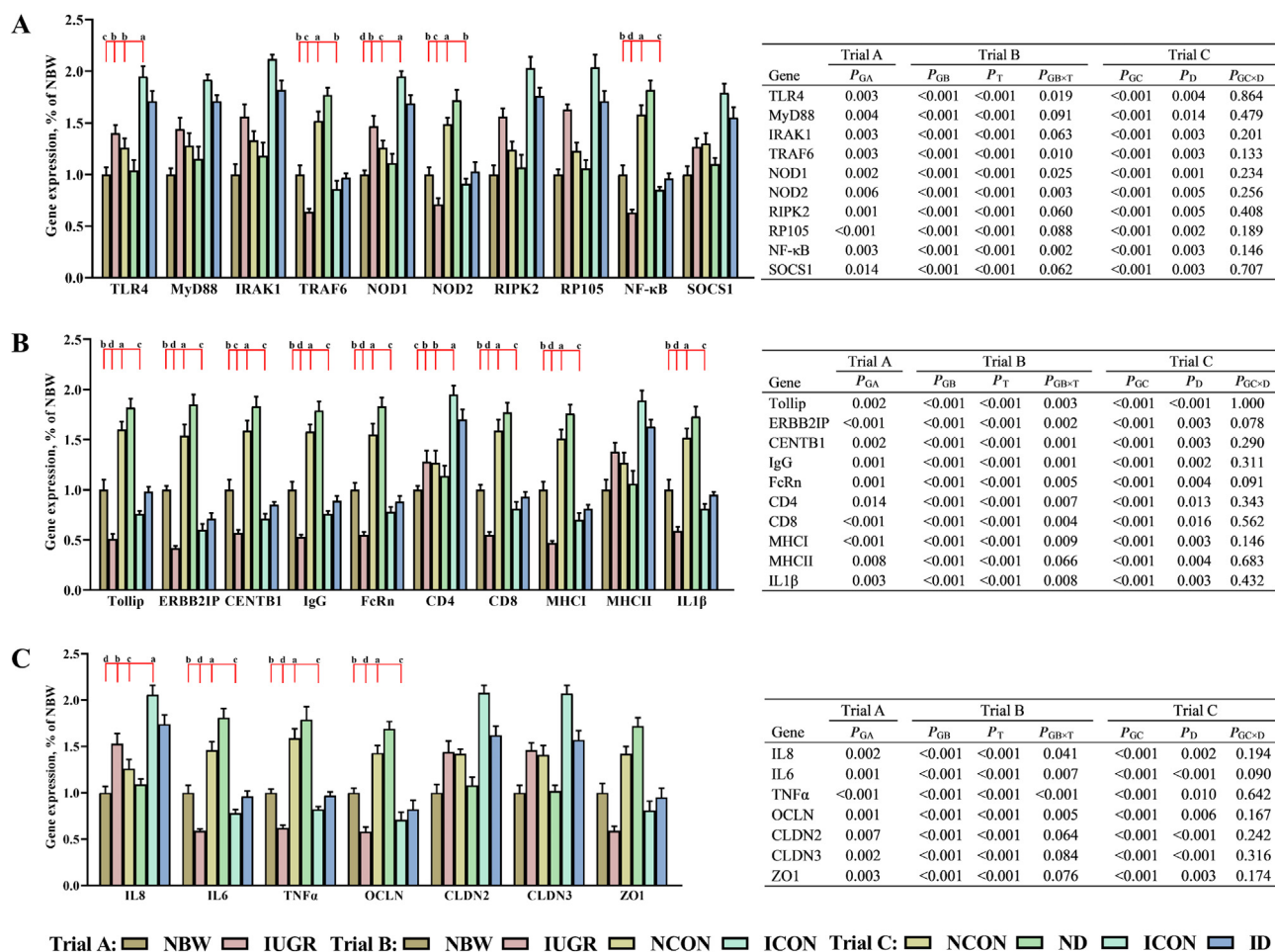
**Fig. 7.** (A) and (B) Toll like receptor and (C) cell cytokines and cell adhesion gene expression of the jejunum. Supplementation with DMG-Na improves gene expression in the jejunum of IUGR piglets during the suckling period. Data are expressed as the mean ± standard deviation ($n$ = 10 suckling piglets). Different superscripts of a, b, c, d (Trial B: NBW, IUGR, NCON, ICON group in red color) represent significant differences ($P$ < 0.05). DMG-Na = dimethylglycine sodium salt; $TLR4$ = toll-like receptor 4; $MyD88$ = myeloid differentiation factor 88; $IRAK1$ = IL1 receptor-associated kinase 1; $TRAF6$ = TNF receptor-associated factor 6; $NOD$ = nucleotide-binding oligomerization domain protein; $RIPK2$ = receptor-interacting serine/threonine-protein kinase 2; $RP105$ = radioprotective 105; $NF$-$\kappa B$ = nuclear factor-$\kappa$B; $SOCS1$ = suppressor of cytokine signaling 1; $Tollip$ = toll-interacting protein; $ERBB2IP$ = Erbb2 interacting protein; $CENTB1$ = centaurin $\beta$1; $IgG$ = immunoglobin G; $FcRn$ = Fc receptor for IgG; $CD4$ = cluster of differentiation 4; $CD8$ = cluster of differentiation 8; $MHC$ = major histocompatibility complex; $IL1\beta$ = interleukin 1$\beta$; $IL8$ = interleukin 8; $IL6$ = interleukin 6; $TNF\alpha$ = tumor necrosis factor $\alpha$; $OCLN$ = occluding; $CLDN2$ = claudin-2; $CLDN3$ = claudin-3; $ZO1$ = zonula occludens-1. NBW = normal birth weight; IUGR = intrauterine growth restriction; NCON = NBW newborn piglets fed a basic milk diet; ND = NBW newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na; ICON = IUGR newborn piglets fed a basic milk diet; ID = IUGR newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na.

inflammatory cytokines in IUGR newborns. Our results showed that DMG-Na, based on its anti-inflammatory activity, could ameliorate the intestinal immune status of newborns in the IUGR group by reducing the concentrations of pro-inflammatory cytokines.

TLR4, the best-characterized TLR member (Li and Wu, 2021), can recruit its adapter proteins MyD88 and IRAK1 on stimulation of the small intestine. This complex subsequently interacts with TRAF6 and co-activates the I$\kappa$B kinase complex (IKK$\alpha$ and IKK$\beta$). Phosphorylation of the I$\kappa$B kinase complex finally activates NF-$\kappa$B, which translocates to the nucleus. In addition, intracellular NLRs (NOD1 and NOD2) can participate in the activation of NF-$\kappa$B through their ligand RIPK2. The activated NF-$\kappa$B, in turn, increases the release of pro-inflammatory cytokines (IL-1$\beta$, IL-6, and TNF-$\alpha$) and finally damages intestinal function. In the present study, gene expression of the negative regulators of TLR4 (Tollip, RP105, and SOCS1) and NOD signaling (ERBB2IP and CENTB1) was downregulated compared with that of the NBW newborns. FcRn can mediate mucosal epithelial cells to transport IgG by binding tightly to the Fc portion of IgG, thus allowing the body to resist pathogens.

CD4 and CD8 can bind to MHC-II and MHC-I, respectively, activate T cells, and recognize exogenous antigens, finally initiating an immune response and exerting immunoregulatory functions. PCNA is an intranuclear polypeptide whose expression and synthesis are used as markers of cell proliferation. In the present study, the IUGR suckling piglets showed increased caspase-3 and decreased PCNA concentrations in the jejunum compared to the concentrations in the NBW suckling piglets. The results also indicated that IUGR could increase the intestinal inflammatory response and decrease the immune status. ZO1, which correlates with paracellular permeability, together with the $OCLN$ and $CLDN$ gene families, is a key regulator of intestinal permeability (Liang and Sheikh, 2020). The current study revealed that 0.1% DMG-Na added to milk diets could reduce the activity of the TLR4/NOD/NF-$\kappa$B pathway and increase the level of cytoplasmic NF-$\kappa$B in the jejunum of newborns during the suckling period. Thus, we conclude that dietary DMG-Na supplementation can alleviate intestinal dysfunction via the TLR4/NOD/NF-$\kappa$B signaling pathway in IUGR newborns during the suckling period. To our knowledge, this is the first study to show

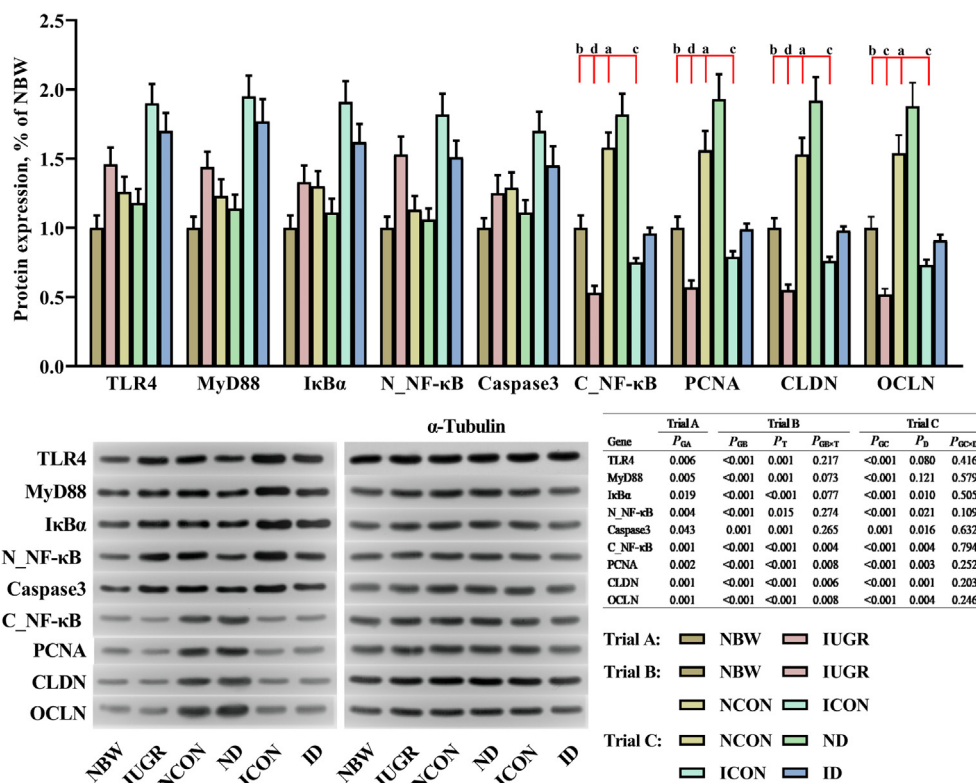| Gene | Trial A | Trial B | | | Trial C | | |
|---|---|---|---|---|---|---|---|
| | $P_{GA}$ | $P_{GB}$ | $P_T$ | $P_{GB \times T}$ | $P_{GC}$ | $P_D$ | $P_{GC \times D}$ |
| TLR4 | 0.006 | <0.001 | 0.001 | 0.217 | <0.001 | 0.080 | 0.416 |
| MyD88 | 0.005 | <0.001 | 0.001 | 0.073 | <0.001 | 0.121 | 0.579 |
| IκBα | 0.019 | <0.001 | <0.001 | 0.077 | <0.001 | 0.010 | 0.505 |
| N_NF-κB | 0.004 | <0.001 | 0.015 | 0.274 | <0.001 | 0.021 | 0.109 |
| Caspase3 | 0.043 | 0.001 | 0.001 | 0.265 | 0.001 | 0.016 | 0.632 |
| C_NF-κB | 0.001 | <0.001 | 0.001 | 0.004 | <0.001 | 0.004 | 0.794 |
| PCNA | 0.002 | <0.001 | <0.001 | 0.008 | <0.001 | 0.003 | 0.252 |
| CLDN | 0.001 | <0.001 | <0.001 | 0.006 | <0.001 | 0.001 | 0.203 |
| OCLN | 0.001 | <0.001 | <0.001 | 0.008 | <0.001 | 0.004 | 0.246 |

**Fig. 8.** Supplementation with DMG-Na improves protein expression in the jejunum of IUGR piglets during the suckling period. Data are expressed as the mean ± standard deviation ($n$ = 3 suckling piglets). Different superscripts of a, b, c, d (Trial B: NBW, IUGR, NCON, ICON group in red color) represent significant differences ($P < 0.05$). DMG-Na = dimethylglycine sodium salt; TLR4 = toll-like receptor 4; MyD88 = myeloid differentiation factor 88; IκBα = nuclear factor kappa-B alpha; N_NF-κB = nuclear factor-κB; C_NF-κB = cytoplasm factor-κB; PCNA = proliferating cell nuclear antigen; OCLN = occluding; CLDN = claudin. NBW = normal birth weight; IUGR = intrauterine growth restriction; NCON = NBW newborn piglets fed a basic milk diet; ND = NBW newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na; ICON = IUGR newborn piglets fed a basic milk diet; ID = IUGR newborn piglets fed a basic milk diet supplemented with 0.1% DMG-Na.

the beneficial effects of DMG-Na on jejunum immune disorders in IUGR newborns via the TLR4/NOD/NF-κB pathway, and further study is still needed on the specific mechanism.

## 5. Conclusions

In conclusion, the present study demonstrated that DMG-Na could effectively improve small intestinal dysfunction in IUGR newborns during the suckling period. We concluded that DMG-Na could directly enhance immune function, indirectly improve small intestinal metabolic abnormalities, and inhibit abnormal expression of stress-related factors via the TLR4/NOD/NF-κB network. This suggests that DMG-Na can serve as a health-promoting substance that could be used in the prevention of newborn intestinal disorders.

## Author contributions

**Kaiwen Bai:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing — original draft, Writing — review & editing. **Luyi Jiang:** Investigation. **Tian Wang:** Resources, Writing — review & editing, Supervision, Funding acquisition. **Wei Wang:** Resources, Writing — review & editing.

## Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

## Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.08.007.

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