Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

# Agar oligosaccharides mitigate lipopolysaccharide-induced apoptosis and inflammatory damage in rumen epithelial cells via the tlr4-myd88-nf-κb signaling pathway

**Aihong Lao**
Guangdong Ocean University
**Xiaolin He**
Guangdong Ocean University
**Jian Ma**
Guangdong Ocean University
**Chunmei Du**
Guangdong Ocean University
**Fuquan Yin**
yinfuquan01@163.com
Guangdong Ocean University
**Yanli Lu**
Guangdong Ocean University

**Research Article**

**Keywords:** agar oligosaccharides, lipopolysaccharides, rumen epithelial cells, apoptosis, anti-inflammatory

**Posted Date:** July 24th, 2026

**DOI:** https://doi.org/10.21203/rs.3.rs-9764720/v1

**License:** This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

**Additional Declarations:** No competing interests reported.

# Agar oligosaccharides mitigate lipopolysaccharide-induced apoptosis and inflammatory damage in rumen epithelial cells via the tlr4-myd88-nf-κb signaling pathway

Aihong Lao[1,#], Xiaolin He[1,#], Jian Ma[1], Chunmei Du[1], Fuquan Yin[1,*], Yanli Lu[1,*]

[1]College of Coastal Agricultural Sciences, Guangdong Ocean University, Zhanjiang 524088, China

*Corresponding author. Tel:+86-15913509615, +86-13702692976; Fax: +86-759-2233926; EM: yinfuquan01@163.com, luyanli73@sina.com

[#]Equal contribution: These authors contributed equally to this work.

Co-authors' email addresses：Aihong Lao, 1483284711@qq.com; Xiaolin He, 13590036152@163.com; Jian Ma, Crazyma0411@163.com; Chunmei Du, duchunmeim@163.com

ORCID: Aihong Lao, https://orcid.org/0009-0001-5404-2236, Xiaolin He, https://orcid.org/0009-0007-9745-7742, Jian Ma, https://orcid.org/0000-0002-6979-5070, Fuquan Yin, https://orcid.org/0009-0009-4934-4289, Yanli Lu, https://orcid.org/0009-0004-9431-7646

Abstract

Agar oligosaccharide (AOS) is an oligosaccharide with a degree of polymerization (DP) of agarose, which is degraded by hydrolysis, chemical degradation, and other methods, and has an oligosaccharide with a DP of less than 20. It has anti-inflammatory and anti-apoptotic effects, but its intervention effect and mechanism on rumen epithelial damage are still unclear. Therefore, this study intends to study and analyze the regulatory effect of AOS on LPS-induced rumen epithelial cell damage in sheep through in vitro LPS-induced rumen epithelial cell injury model, so as to provide a reference idea for AOS as a green feed additive to alleviate ruminant Subacute rumen acidosis (SARA) or other metabolic diseases. First, the ovine rumen epithelial cells were divided into a control group and four AOS concentration groups (1, 15, 50, 100 μg/mL), and three independent biological replicates were set up in each group to stimulate the cells for 6, 12, 18, and 24 h respectively to determine the optimal AOS treatment time and concentration. The results showed that the content of pro-inflammatory cytokines and the relative expression of pro-apoptotic genes in rumen epithelial cells cultured with 15 μg/mL AOS for 18 h were significantly reduced ($p$<0.05), and the relative expression of cell viability and inhibition of apoptosis genes were significantly increased ($p$<0.05), and there were no adverse effects on the cells under the intervention of the concentration of this treatment and the time of the treatment. Second, establish a cell injury model. The cells were divided into a control group and four Lipopolysaccharide (LPS) concentration groups (1, 15, 50, 100 μg/mL), with three independent biological replicates in each group, and the cells were stimulated for 6, 12, and 24 hours. The results showed that after culturing rumen epithelial cells with 50 μg/mL LPS for 24 hours, reactive oxygen species (ROS), pro-inflammatory cytokine levels, and the relative expression of pro-apoptotic genes were significantly increased ($p$<0.05), while cell viability and the relative expression of anti-apoptotic genes were significantly decreased ($p$<0.05). Therefore, this condition was determined as the injury model. Third, based on the above experimental results, the cells were divided into a control group (CON group), LPS group, and AOS+LPS group (A+L group) to further explore the effect of AOS on LPS-induced rumen epithelial cell injury. The results showed that 50 μg/mL LPS incubation for 24 h could cause suitable damage to the cells. Cell morphology was improved and cell viability was significantly higher in the A+L group ($p$<0.05). The A+L group significantly decreased the reactive oxygen species (ROS) fluorescence intensity and interleukin-6 (IL-6) , IL-8, IL-1β and tumor necrosis factor-α (TNF-α) contents and increased the immunoglobulin A (IgA) , IgM, and IgG contents ($p$<0.05). The apoptosis rate were significantly reduced ($p$<0.05). In addition, the A+L group significantly inhibited the expression of genes and proteins related to the Toll-like Receptor 4-Myeloid Differentiationfactor 88-Nuclear Factor-κB (TLR4-MyD88-NF-κB) signaling pathway. In summary, AOS attenuated LPS-induced apoptosis and inflammatory injury in rumen epithelial cells in vitro via the TLR4-MyD88-NF-κB signaling pathway.

Keywords: agar oligosaccharides; lipopolysaccharides; rumen epithelial cells; apoptosis; anti-inflammatory

Abbreviations: AOS, agar oligosaccharide; SARA, subacute rumen acidosis; LPS , lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor-α; DON, deoxynivalenol; ROS, reactive oxygen species

## 1. Introduction

The rumen is a vital digestive organ in ruminants. After food is ingested, it is digested and fermented by rumen microorganisms, which produce volatile fatty acids (VFAs) in the rumen. This organ plays a crucial role in the growth and development of animals, as well as in disease prevention and control. More than 75% of the VFAs generated in the rumen are absorbed by the rumen epithelium and serve as the primary energy source for the body. The rumen epithelium is essential for the absorption and digestion of nutrients, transportation, energy metabolism, and the regulation of rumen pH in ruminants [1-2]. Subacute rumen acidosis (SARA) is a common metabolic disease in ruminants during lactation, and LPS is the major inflammatory component in the SARA-induced inflammatory response [3]. Studies have shown that lipopolysaccharide (LPS) produced by SARA not only transfers to the bloodstream through the damaged rumen epithelium, leading to a systemic inflammatory response, but may also indirectly affect milk fat synthesis through the release of pro-inflammatory cytokines into the bloodstream by localized macrophages, thus affecting milk production and economic efficiency of ruminants [3-4].

Marine active substances are recognized as green and safe marine biological resources with development and application prospects. Agar polysaccharide is a structurally simple linear macromolecular polysaccharide composed of 3,6-endoether-α-L-galactose and β-D-galactose residues alternately linked by α-(1,3) and β-(1,4) glycosidic bonds [5]. Despite many advantages, the high molecular weight, low solubility, and insoluble nature of agar polysaccharides have imposed a limitation on the application of agar polysaccharides. Agar oligosaccharide (AOS) is one of the important AOS is one of the important marine biological resources derived from the degradation of agar, which well solves the disadvantages of agar such as low viscosity, difficult dissolving in water and low utilization rate, and plays a role in biological properties such as antioxidant [6], anti-inflammatory [7], anti-tumor [8] and probiotic [9], which is promising for the research. Wang et al. [10] investigated the effect of AOS on the dysfunction of the intestinal tract of mice induced by deoxynivalenol (DON), and found that AOS can significantly reduce the protein expression level of the cytokine interleukin-1β (IL-1β), thereby alleviating DON-induced intestinal inflammation. Yun et al. [11] through constructing an in vitro model of colon cancer cells, found that AOS can increase the gene expression levels of cysteine-containing aspartate protease-3 (caspase-3), caspase-9, and pro-apoptotic protein BAX, while decreasing the gene expression levels of anti-apoptotic proteins BCL-2 and BCL-XL to promote apoptosis of colon cancer cells. This may be because AOS activates the execution phase of apoptosis, causing colon cancer cells in the body to undergo apoptosis, thereby achieving an anti-colon cancer effect. In addition, studies have shown that AOS can reduce inflammation by inhibiting the activation of the Toll-like Receptor 4-Nuclear Factor-κB/ Mitogen-activated Protein Kinase (TLR4- NF-κB/ MAPK) signaling pathway [12]. In addition, Yang et al. [13] found that AOS can reduce Reactive oxygen species (ROS) production and inflammatory damage by activating the Nrf2 signaling pathway. However, the research of AOS is still mainly focused on the field of beauty care, food preservation and medicine, and little research has been done in the field of ruminant animal health and farming. Therefore, in this study, we took rumen epithelial cells as the research object, firstly, we explored the intervention effect of AOS on rumen epithelial cells, and then we established an inflammatory injury model to analyze the effect of AOS to alleviate LPS-induced rumen epithelial cell injury, and at the same time, we elucidated the potential molecular mechanism behind, so as to provide a reference for the promotion of the development of animal husbandry as well as the in-depth development and utilization of AOS.

## 2. Materials and methods

### 2.1 Cell Line Culture for Experiments

Sheep rumen epithelial cells for this experiment were purchased from iCell Bioscience Inc. (Shanghai, China) and were identified as sheep rumen epithelial cells by mitochondrial sequencing and immunofluorescence.

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/Nutrient Mixture F-12 medium (ZETA LIFE, Menlo Park, CA, USA) containing 10% fetal bovine serum (FBS, ZETA LIFE, Menlo Park, CA, USA) and 2% penicillin-streptomycin double antibody solution (SERANA, Shanghai, China). Inoculate the recovered rumen epithelial cells into a T25 cell culture flask, mix gently, and then place in a 5% $CO_2$, 37 ℃ incubator for cultivation, and the cells were washed with PBS buffer (Gibco, Shanghai, China) and the basal medium was changed every 24 h. After 2-3 days, when the cell density reached 90% or more, the cells were processed according to the experimental protocols and the relevant indices were detected.

Notably, the $CD45^+$ cell depletion step was omitted in this study to retain a small number of B lymphocytes [14-15], a decision considered necessary for validating the inflammatory response.

### 2.2 Sources of AOS and Toxins for Experiments

The refined AOS (molecular structure shown in Figure. 1) required for this test was provided by Qingdao Hehai Biotechnology Co., Ltd. (S230608AA1-3K, Shandong, China), which was a white-like to light yellow powder with a molecular weight of 1.51 kDa determined by high-performance gel permeation chromatography. Preparation method of AOS working solution: 5 mg of AOS powder was weighed in 5 mL of cell basal medium with an analytical balance, mixed thoroughly, filtered with a 0.22 μm filter head, and prepared into a 1 mg/mL stock solution. According to the method of Wang et al. [16], the stock solution was then diluted to working concentrations of 1 μg/mL, 15 μg/mL, 50 μg/mL, and 100 μg/mL according to the protocol, and stored in a 4 ℃ freezer for 2 weeks.

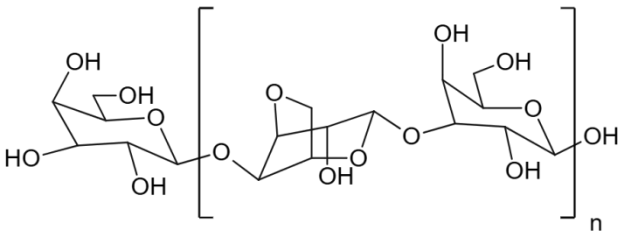



Figure. 1 Agar oligosaccharide molecular structure.

The LPS required for this trial was purchased from Sigma-Aldrich Corporation (No. L2880, Saint Louis, USA), derived from E. coli O55:B5. Preparation method of LPS working solution: Add 10 mL of PBS to 10 mg LPS powder, fully invert and mix well, prepare a mixture with a final concentration of 1 mg/mL, and then divide the mixture into ten 1.5-mL enzyme-free centrifuge tubes (1 mL/tube), and store at -20 ℃ in the dark for later use. To perform the test, thaw out from -20 ℃ in advance. According to the method of Chu et al. [17], dilute 1 mg/mL LPS stock solution to the working concentration of 1 μg/mL, 15

μg/mL, 50 μg/mL and 100 μg/mL according to the test needs, and store it in a refrigerator at 4 ℃ protected from light for 2 weeks.

2.3 Experimental Design

2.3.1 Effect of Agar-oligosaccharides on Viability, Cytokines and Apoptosis of Rumen Epithelial Cells

The rumen epithelial cells were divided into 5 groups during treatment: control and 4 AOS concentration groups (1, 15, 50, 100 μg/mL). After stimulating the cells for 6, 12, 18, and 24 h, respectively, the cell viability was detected by CCK-8 assay and screened for the optimal action time of AOS. The optimal time of AOS action and concentration were then screened for subsequent experiments by detecting IL-6, IL-8, IL-1β, TNF-α and ROS content with ELISA kits and determining the relative mRNA expression of BAX and BCL-2 with RT-qPCR.

2.3.2 Construction of a Model of LPS-induced Rumen Epithelial Cell Injury in Vitro

The rumen epithelial cells were divided into 5 groups during treatment: control group and 4 LPS concentration groups (1, 15, 50, 100 μg/mL). After stimulating the cells for 6, 12, and 24 h, respectively, the cell viability was detected and screened for the appropriate treatment time of LPS by CCK-8 assay. The optimal LPS treatment time and concentration were then screened for subsequent experiments by detecting the levels of IL-6, IL-8, IL-1β, TNF-α and ROS with ELISA kits and measuring the relative mRNA expression of BAX and BCL-2 with RT-qPCR.

2.3.3 Effect of Agar-oligosaccharides on LPS-induced Apoptosis and Inflammatory Response in Ruminal Epithelial Cells

According to the experimental results of 2.3.1 and 2.3.2, the cells were randomly divided into three groups: (1) Blank control group (CON group): Cells were cultured with prepared cell culture medium for 18–24 hours; (2) Injury model group (LPS group): Cells were cultured with prepared cell culture medium for 18 hours, then treated with LPS at a concentration of 50 μg/mL for 24 hours; (3) Protective treatment group (AOS LPS group): Cells were cultured with AOS at a concentration of 15 μg/mL for 18 hours, then treated with LPS at a concentration of 50 μg/mL for 24 hours. Cells were collected at the end of treatment and tested for cell survival, apoptosis and inflammation-related indexes and TLR4-MyD88-NF-KB key pathway-related indexes in each group.

2.4 Cell Viability Assay (CCK-8)

Cells were treated with different times of intervention using media containing different final concentrations of AOS or LPS, and after completion of the treatment, the drug-containing medium was removed, serum-free medium containing 10% Cell Counting Kit-8 (CCK-8, ZETA LIFE, Menlo Park, CA, USA) solution was added according to the CCK-8, and the optical density(OD) value was measured at 450 nm after after placing in a 5% $CO_2$, 37 ℃ cell incubator and incubating in the dark for 2 h. The experiment was performed across three independent biological replicates, each consisting of six technical replicates. Data were analyzed using the mean values of the technical replicates within each biological replicate. Cell survival (%) Calculation formula:

Cell survival (%) = [OD value (AOS/LPS addition group) - OD value (blank group)] / [OD value (control group) - OD value (blank group)] × 100

2.5 Cytokine and Immunoglobulin Assays (ELISA)

Cells were treated with different AOS or LPS final concentration media, and the cell supernatant was collected at the end of the culture, and all the indexes were strictly tested according to the procedure of the ELISA kit (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China). The experiment was conducted in three independent biological replicates, each consisting of three technical replicates. Data were analyzed using the average of the technical replicates for each biological replicate.

2.6 ROS Detection

The concentration of ROS was detected by ELISA kit (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China) to investigate the effect of AOS on rumen epithelial cells and to establish an in vitro injury model, the procedure was the same as 2.5. The experiment was conducted in three independent biological replicates, and the average was chosen for analysis.

The fluorescence intensity of ROS was detected using the fluorescent probe DCFH-DA (Beyotime Biotech Inc., Shanghai, China) to investigate the effect of AOS on LPS-induced rumen epithelial cell damage.

2.7 Apoptosis Rate Assay

The apoptosis rate was detected by Annexin V-FITC/PI (MultiSciences Biotech Co., Ltd., Hangzhou, China) method and the procedure was strictly followed [18]. The experiment was conducted in three independent biological replicates, and the average was chosen for analysis.

2.8 Extraction of Total RNA from Cells and Synthesis of cDNA

Total RNA was extracted by column method according to the instructions of Vazyme Total RNA Extraction Kit (Vazyme, Nanjing, China). After extraction, the total RNA was reverse transcribed into cDNA using the Biosharp Reverse Transcription Kit (Biosharp, Shanghai, China). The entire experiment was conducted on ice.

2.9 RT-qPCR for Detection of Gene Expression

Specific primers were synthesized by Invitrogen Biotech Co. Ltd.(Shanghai, China) and the specific primer sequences synthesized are shown in Table 1. The reaction was performed using the TB Green chimeric fluorescence method (Takara, China). The experiment was conducted in three independent biological replicates, each consisting of five technical replicates. Data were analyzed using the average of the technical replicates within each biological replicate. Finally, the results were calculated using the $2^{-\Delta\Delta CT}$ method [19].

234 Table 1 Real-time PCR primers

| Gene | Primer sequences(5′-3′) | Login number | Product size （bp） |
|---|---|---|---|
| *GAPDH* | F: GATAGGCCCACCCAAAAGGG<br>R: GGATGCGGCTGCGCTT | XM_060411595.1 | 142 |
| *BAX* | F: AAACTGGTGCTCAAGGCCC<br>R: TCTTCAGCGACTCAGCCAAG | XM_027978594.3 | 148 |
| *BCL-2* | F: GGGGTCATGTGTGTGGAGAG<br>R: ACAAAGGCATCCCAGCCTC | XM_012103831.5 | 134 |
| *TLR4* | F: CCTTGCGTACAGGTTGTTCC<br>R: GTCCAGCATCTCGGTTGACA | NM_001135930.1 | 99 |
| *MyD88* | F: TCATTGAGAAGAGGTGCCGTC<br>R: TGGCTTGTACTTGATGGGGA | NM_001166183.1 | 146 |
| *IRAK1* | F: CTCATCTACCAAGCCCAGCC<br>R: CAGCAGAACGAAGAGGAGGG | XM_060408110.1 | 72 |
| *NF-κB* | F: CTCCTGGAGCCTCAAACCTG<br>R: TCTACAGGGAAAACTGAATCTTTCT | XM_042238744.1 | 121 |
| *GAPDH* | F: GATAGGCCCACCCAAAAGGG<br>R: GGATGCGGCTGCGCTT | XM_060411595.1 | 142 |
| *ZO-1* | F: GGACAAAGAGAAGGGTGAGACC<br>R: GCTCCACTGGCTTCAGGAAC | XM_060401409.1 | 83 |
| Claudin-1 | F: CCCGGTCAATGCCAGGTATG<br>R: TTGTTTTCCGGGGACAGGAG | NM_001185016.1 | 113 |

235

### 2.10 Western Blot for Protein Expression

Cells were inoculated in 6-well plates and collected after the appropriate time and concentration. BCA protein concentration assay kit (Beyotime Biotech Inc., Shanghai, China) was used to do protein quantification of the supernatant taken out, which was performed in strict accordance with the instructions of the kit. Based on the results of BCA protein quantification, take 80 μg of protein sample, add an appropriate amount of 5× protein loading buffer, mix thoroughly, and denature in 100 ℃ boiling water for 10 minutes. SDS-PAGE protein separation gel was prepared according to the size of the target protein, and electrophoresis was carried out after adding the sample. After membrane transfer, rabbit primary antibodies to MyD88, IκBα, p65, p-IκBα, p-p65 (1:1000) and β-actin mouse primary antibody (1:2000) were incubated overnight at 4 ℃, and then horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G-coupled secondary antibody (1:4000) and goat anti-mouse immunoglobulin G-coupled secondary antibody (1:4000) for 2 h. Finally, the bands were developed according to the instructions of the ECL development kit (Beyotime Biotech Inc., Shanghai, China) and analyzed for gray value using the gray value software Gelpro 32. Information regarding the antibodies used in the Western blot analysis is provided in Table 2.

Table 2 Antibody information

| Antibody | Dilution ratio | Manufacturers | Catalog number |
|---|---|---|---|
| β-Actin | 1:2000 | Superior resistance | UM4001 |
| TLR4 | 1:1000 | Proteintech | 19811-1-AP |
| IRAK1 | 1:1000 | Proteintech | 10478-2-AP |
| IκBα | 1:1000 | CST | #9242 |
| MyD88 | 1:1000 | CST | #4283 |
| p65 | 1:1000 | CST | #8242 |
| p-IκBα | 1:1000 | CST | #2859 |
| p-p65 | 1:1000 | CST | #3033 |
| Sheep anti-rabbit IgG-HRP | 1:4000 | Affinity | S0001 |
| Sheep anti-mouse IgG-HRP | 1:4000 | Affinity | S0002 |

### 2.11 Experimental Data Analysis and Processing

One-way ANOVA and two-way ANOVA models in SPSS Statistics 27.0 software were used for one-way and two-way ANOVA, and Duncan's method and LSD method were used for multiple comparisons and significance analyses, and the measurement data were expressed as “mean ± standard deviation “ and $p<0.05$ represents significant difference. Resulting graphs were all plotted with GraphPad Prism 10.0.

## 3. Results

### 3.1 Effect of Different Agar-oligosaccharide Treatment Concentrations and Time on The Survival of Rumen Epithelial Cells

The results of the CCK-8 method to measure the survival rate of rumen epithelial cells with different AOS treatment concentrations and times are shown in Table 3. As can be seen from Table 3, the cell survival rate tended to decrease with the increase of AOS addition concentration when the incubation time was 6 h ($p<0.05$). When the incubation time was 12, 18 and 24 h, the cell survival rate showed a trend of increasing and then decreasing with the concentration of AOS added ($p<0.05$). At 12, 24 h, the cell viability was significantly higher ($p<0.05$) in the groups treated with AOS at 0, 1, and 15 μg/mL than in the group treated with AOS at 100 μg/mL. When the incubation time was 18 h, the cell survival rate of each AOS-added concentration group was significantly higher than that of the incubation time of 6 h ($p<0.05$). The cell survival rate of the incubation time 18 h group was significantly higher than that of the incubation time 6 and 12 h group at the AOS-added concentrations of 1, 15 and 50 μg/mL ($p<0.05$). In addition, when AOS was added at concentrations of 50 and 100 μg/mL, cell viability was inhibited to varying degrees ($p<0.05$), indicating that high concentrations of AOS may have toxic effects on cells. Therefore, according to the experimental results, the culture time of 18 h can be selected as the condition for subsequent experiments.

280 Table 3 Effect of AOS on rumen epithelial cell survival

| Incubation time (h) | Concentration of AOS（μg/mL） | | | | | *P*-value |
|---|---|---|---|---|---|---|
| | 0 | 1 | 15 | 50 | 100 | |
| 6 | 1.00±0.03$^{a}$ | 0.90±0.04$^{bB}$ | 0.84±0.05$^{bC}$ | 0.75±0.03$^{cC}$ | 0.75±0.03$^{cBC}$ | <0.05 |
| 12 | 1.00±0.07$^{a}$ | 1.01±0.03$^{aA}$ | 0.96±0.05$^{aBC}$ | 0.93±0.06$^{aB}$ | 0.85±0.05$^{bA}$ | <0.05 |
| 18 | 1.00±0.05$^{b}$ | 1.07±0.11$^{abA}$ | 1.19±0.13$^{aA}$ | 1.00±0.03$^{bA}$ | 0.79±0.03$^{cB}$ | <0.05 |
| 24 | 1.00±0.05$^{ab}$ | 1.09±0.04$^{aA}$ | 1.06±0.11$^{aAB}$ | 0.91±0.06$^{bB}$ | 0.71±0.03$^{cC}$ | <0.05 |
| *P*-value | 1.00 | <0.05 | <0.05 | <0.05 | <0.05 | |
| *P*-value of two-way ANOVA | | | | | | |
| Incubation time | | | | <0.05 | | |
| Concentration of AOS | | | | <0.05 | | |
| Incubation time × Concentration of AOS | | | | <0.05 | | |

281 When the incubation time is the same, data with different AOS concentrations in the same row are labeled
282 with lowercase letters (a,b,c) to indicate significant differences ($P$<0.05); when the AOS concentrations
283 are the same, data with different incubation times in the same column are labeled with uppercase letters
284 (A,B,C) to indicate significant differences ($P$<0.05).

### 3.2 Effects of Agar-oligosaccharides on ROS, Cytokine Content and Expression of Relevant Apoptotic Genes in Rumen Epithelial Cells

According to the above results, after culturing the different AOS at various concentrations for 18 hours, the cell supernatants were collected to detect different inflammatory factors (IL-1β, IL-6, IL-8, and TNF-α) and ROS levels., and the results are shown in Figure. 2A-E. The contents of IL-1β, ROS and TNF-α in all groups were less than those in the AOS-added 0 μg/mL group, and significant differences were observed in the IL-1β and TNF-α groups ($p<0.05$). At AOS-added concentrations of 1 and 15 μg/mL, the contents of IL-1β, IL-6 and ROS were less than those in the remaining three groups ($p>0.05$) and were significantly lower than those in the group with an AOS-added concentration of 100 μg/mL ($p<0.05$). The contents of IL-8 and TNF-α were significantly lower than those in the other four groups at an AOS-added concentration of 15 μg/mL ($p<0.05$). Therefore, based on the experimental results, the optimal action concentration of AOS can be preliminarily determined to be 15 μg/mL.

To further determine the optimal action concentration of AOS was 15 μg/mL and no toxic effects were observed in rumen epithelial cells at this concentration. In this experiment, the expression of apoptosis-related genes in rumen epithelial cells by AOS was determined by fluorescence quantitative PCR, and the results are shown in Figure. 2F-H. The relative mRNA expression of BCL-2 and BCL-2/BAX in the AOS group was higher than that in the control group ($p<0.05$), and the relative mRNA expression of BAX in the AOS group was lower than that in the control group ($p<0.05$).

From the above results, it can be seen that the concentration of AOS addition of 15 μg/mL and the treatment time of 18 h were chosen as the subsequent experimental conditions in this study.

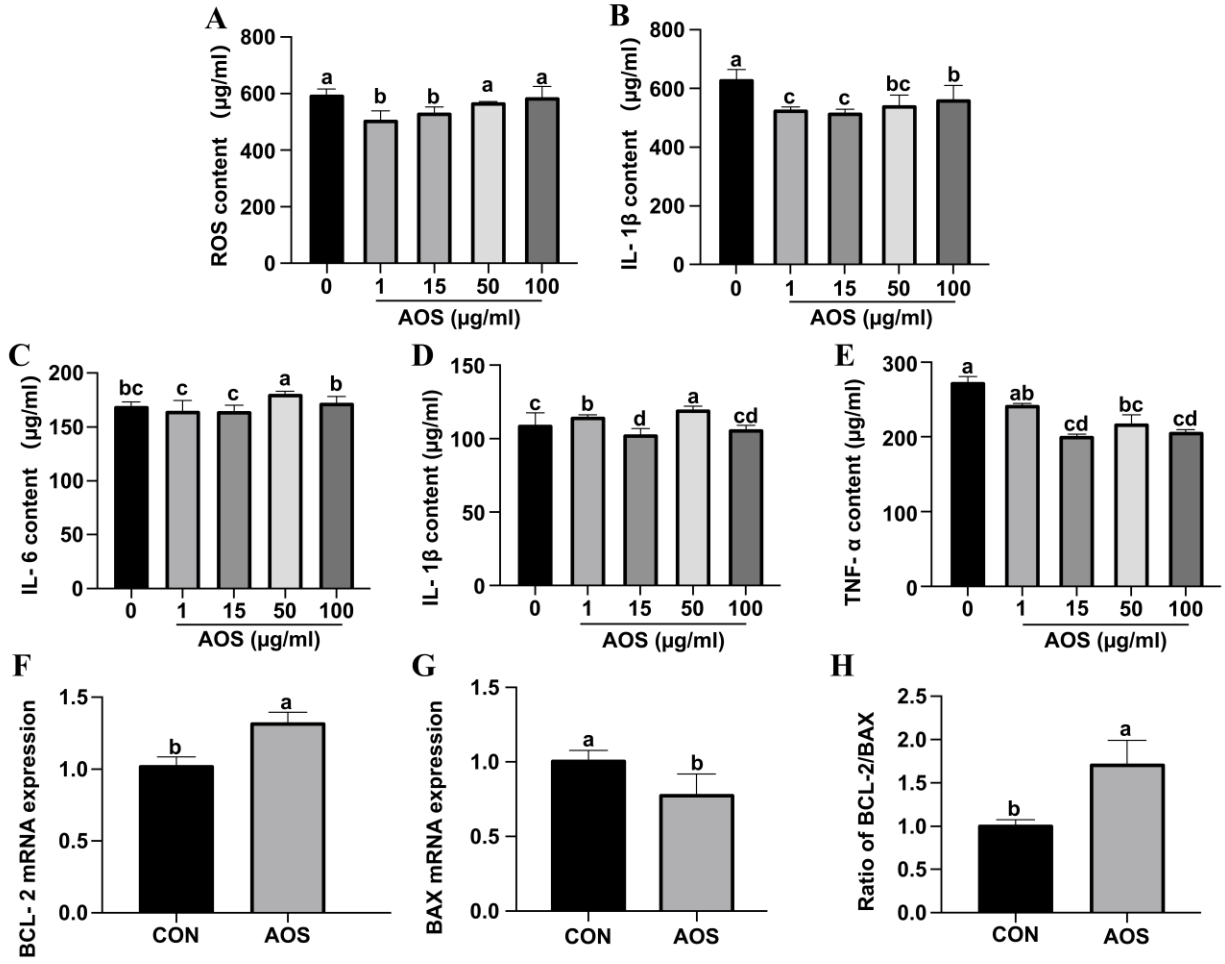



Figure. 2 The effects of different AOS treatment concentrations on ROS, cytokine content, and related apoptosis gene expression in sheep rumen epithelial cells. (A) ROS content; (B) IL-8 content; (C) IL-6 content; (D) ; (E) TNF-α content; (F) relative mRNA expression of BCL-2; (G) relative mRNA expression of BAX; and (H) ratio of BCL-2 to BAX. No letter or the same letter in the graphs indicated that the differences were not significant ($P$>0.05), and different letters indicated that (a,b,c) the differences were significant ($P$<0.05).

315 3.3 Effect of Different LPS Treatment Concentrations and Time on The Survival of Rumen Epithelial
316 Cells
317
318 The results of the effects of different LPS treatment concentrations (1, 15, 50, 100 μg/mL) and time (6,
319 12, 24 h) on rumen epithelial cell survival are shown in Table 4. When the incubation time was 12 h, the
320 rumen epithelial cell survival rate showed an increasing and then decreasing trend ($p$<0.05) with the
321 increase of LPS addition concentration. When the incubation time was 24 h, rumen epithelial cell survival
322 decreased with increasing LPS addition concentration ($p$<0.05). At the incubation time of 6 h and 12 h,
323 the cell survival rate was maintained at a high level, and at the incubation time of 24 h, all concentration
324 groups inhibited cell growth to different degrees. Therefore, the induction time of LPS on rumen
325 epithelial cells of 24 h was selected as appropriate.
326
327 Table 4 Effect of LPS on rumen epithelial cell survival

| Incubation time (h) | Concentration of LPS（μg/mL） | | | | | *P*-value |
|---|---|---|---|---|---|---|
| | 0 | 1 | 15 | 50 | 100 | |
| 6 | 1.00±0.01 | 1.05±0.05 | 0.97±0.06 | 0.96±0.08$^{A}$ | 0.90±0.11$^{A}$ | 0.10 |
| 12 | 1.00±0.03$^{ab}$ | 1.04±0.07$^{a}$ | 0.93±0.05$^{bc}$ | 0.88±0.05$^{cA}$ | 0.72±0.03$^{dB}$ | <0.05 |
| 24 | 1.00±0.07$^{a}$ | 0.97±0.06$^{a}$ | 0.87±0.07$^{b}$ | 0.76±0.03$^{cB}$ | 0.55±0.02$^{dC}$ | <0.05 |
| *P*-value | 0.10 | 0.16 | 0.11 | <0.05 | <0.05 | |
| *P*-value of two-way ANOVA | | | | | | |
| Incubation time | | | | <0.05 | | |
| Concentration of LPS | | | | <0.05 | | |
| Incubation time × Concentration of LPS | | | | <0.05 | | |

328 When the incubation time is the same, data with different LPS concentrations in the same row are labeled
329 with lowercase letters (a,b,c) to indicate significant differences ($P$<0.05); data with different incubation
330 times in the same column are labeled with uppercase letters (A,B,C) to indicate significant differences
331 ($P$<0.05) when the LPS concentrations are the same.

### 3.4 Effect of LPS on ROS, Cytokine Content and Expression of Relevant Apoptotic Genes in Rumen Epithelial Cells

In this experiment, after inducing rumen epithelial cells with different concentrations of LPS for 24 h, the ROS and related inflammatory factor contents of rumen epithelial cells were measured to determine whether the treatment of different concentrations of LPS groups for 24 h could induce inflammatory responses in rumen epithelial cells, and the results were shown in Figure. 3. Compared with the groups of LPS-added concentrations of 1 and 15 μg/mL, the group of LPS-added concentrations of 50 μg/mL had a ROS content was significantly higher ($p<0.05$) (Figure. 3A). From the results of Figure. 3D, the IL-1β content in the group with LPS spiked at a concentration of 50 μg/mL was significantly higher than that in the group with LPS spiked at a concentration of 0 μg/ml ($p<0.05$), while the differences in the other groups were not significant ($p>0.05$). In addition, the IL-6 and TNF-α contents in the LPS-added concentration of 50 and 100 μg/mL groups were significantly higher ($p<0.05$) than the remaining three groups (Figure. 3C, E). From this, it was tentatively concluded that the induction of rumen epithelial cells by 50 and 100 μg/mL of LPS for 24 h activated them to undergo an inflammatory response.

Based on the above results, 50 and 100 μg/mL LPS were further induced into rumen epithelial cells for 24 h, and the expression of apoptosis-related genes was detected as follows. From Figure. 3F-H, the relative mRNA expression of BCL-2 and the BCL-2/BAX ratio in the 50 and 100 μg/mL LPS-added concentration groups were significantly lower than those in the control group ($p<0.05$) (Figure. 3F, G), while the relative mRNA expression of BAX was significantly higher than that in the control group ($p<0.05$) (Figure. 3H).

From the above results, it can be seen that in this study, the LPS addition concentration of 50 μg/mL and the treatment time of 24 h were chosen as the treatment conditions for the establishment of the inflammatory injury model.

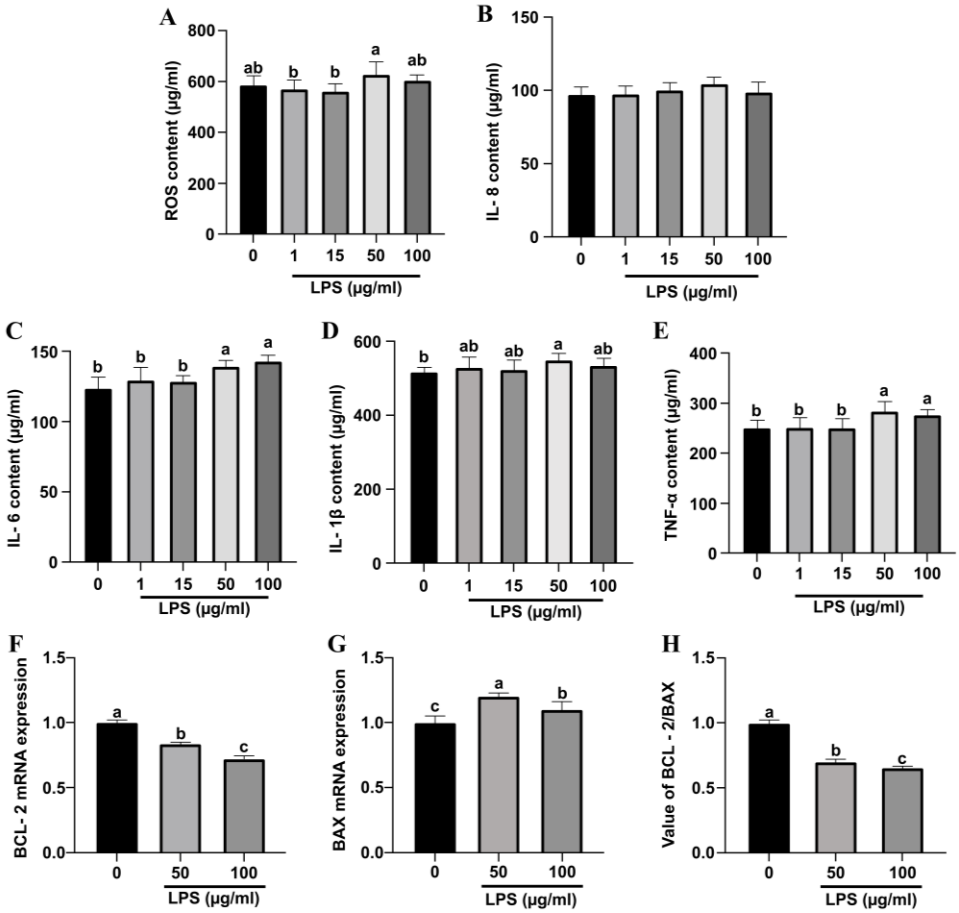
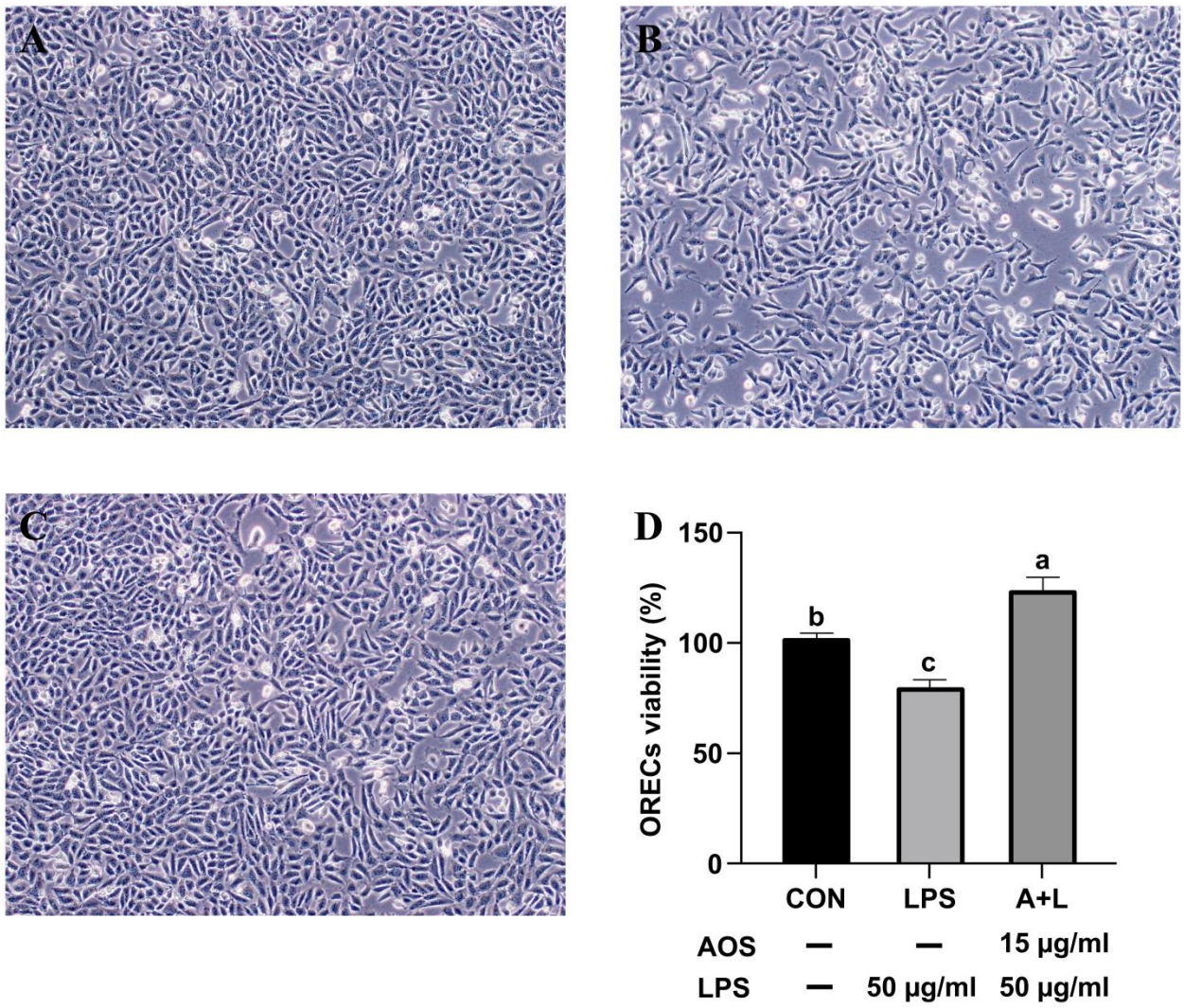



Figure. 3 Effects of different LPS concentrations on the content of ROS, cytokines and apoptosis-related genes in rumen epithelial cells. (A) ROS content; (B) IL-8 content; (C) IL-6 content; (D) IL-1β content; (E) TNF-α content; (F) relative mRNA expression of BCL-2; (G) relative mRNA expression of BAX; and (H) ratio of BCL-2 to BAX. No letter or the same letter in the graphs indicated that the differences were not significant ($P$>0.05), and different letters indicated that (a,b,c) the differences were significant ($P$<0.05).

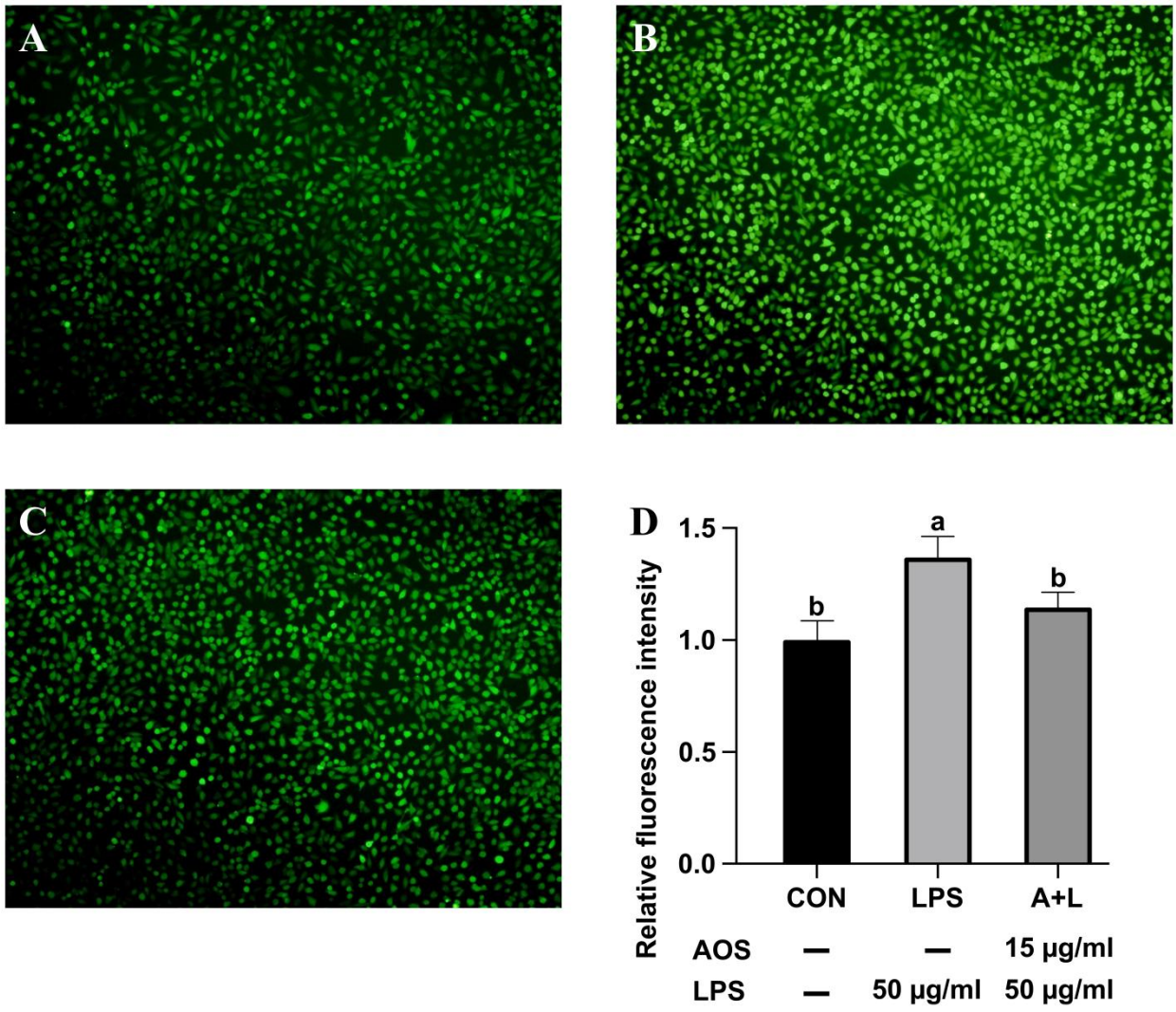
365 3.5 Effect of Agar-oligosaccharides on The Morphology and Survival of Rumen Epithelial Cells Induced
366 by LPS
367
368 To detect the effect of AOS on the morphological structure and survival rate of rumen epithelial cells
369 induced by LPS, the cell morphology was observed under an inverted microscope after AOS treatment,
370 and the survival rate was determined by the CCK-8 method. The results showed that the cells in the CON
371 group (Figure. 4A) were evenly arranged in the shape of normal paving stones. After 24 h of 50 ug/mL
372 LPS culture, the morphology of the cells in the LPS group (Figure. 4B) became significantly longer and
373 strung, the number of adherent cells decreased, most of the cells died and floated, and the cells vacuolated
374 severely. The cell stretching phenomenon in the A+L group (Figure. 4C) was reduced, and the
375 vacuolization of cells was significantly less pronounced than in the LPS group. Cell viability determined
376 by CCK-8 assay As shown by the results of Figure. 4D, the cell viability in the LPS group was
377 significantly lower than the other two groups ($p$<0.05). Cell viability was significantly higher in the A+L
378 group compared to the CON and LPS groups ($p$<0.05).
379




380
381 Figure. 4 Effect of AOS on LPS-induced rumen epithelial cell morphology and survival(Scale = 20
382 μm). (A-C) Cell morphology of rumen epithelial cells in CON, LPS and A+L groups. (D) Rumen

383 epithelial cell survival rate. No letter or the same letter in the graphs indicated that the differences were
384 not significant ($P>0.05$), and different letters indicated that (a,b,c) the differences were significant
385 ($P<0.05$).

386 3.6 Effect of Agar-oligosaccharides on The Fluorescence Intensity of ROS Induced by LPS in Rumen
387 Epithelial Cells
388
389 To investigate the effect of AOS on the ROS content of LPS-induced rumen epithelial cells, the cells were
390 treated according to the experimental design and then observed under an inverted microscope, and the
391 results, as shown in Figure. 5, showed that the ROS fluorescence intensity of the cells in the LPS group
392 was increased when compared with those in the CON group and the A+L group, and the difference was
393 statistically significant ($p$<0.05).
394




395
396 Figure. 5 Effect of AOS on LPS-induced fluorescence intensity of reactive oxygen species in rumen
397 epithelial cells(Scale = 20 μm). (A-C) ROS fluorescence images of rumen epithelial cells in CON, LPS
398 and A+L groups (D) ROS fluorescence intensity. No letter or the same letter in the graphs indicated that
399 the differences were not significant ($P$>0.05), and different letters indicated that (a,b,c) the differences
400 were significant ($P$<0.05).

3.7 Effect of Agar-oligosaccharides on Cytokines and Immunoglobulins in LPS-induced Injury of Ruminal Epithelial Cells

The results of ELISA detection of inflammatory factors and immunoglobulins in rumen epithelial cells are shown in Figure. 6. Figure. 6A-D shows the results of cell inflammatory factor content. The results showed that the contents of IL-6, IL-1β and TNF-α in the LPS group were significantly higher than those in the CON group and the A+L group ($p$<0.05), and the IL-8 content in the A+L group was significantly lower than that in the CON group and the LPS group ($p$<0.05). Figure. 6E-G showed that there was no significant difference in the contents of immunoglobulin A (IgA), IgM and IgG between the CON group and the LPS group ($p$>0.05), but they were significantly lower than those in the A+L group ($p$<0.05).

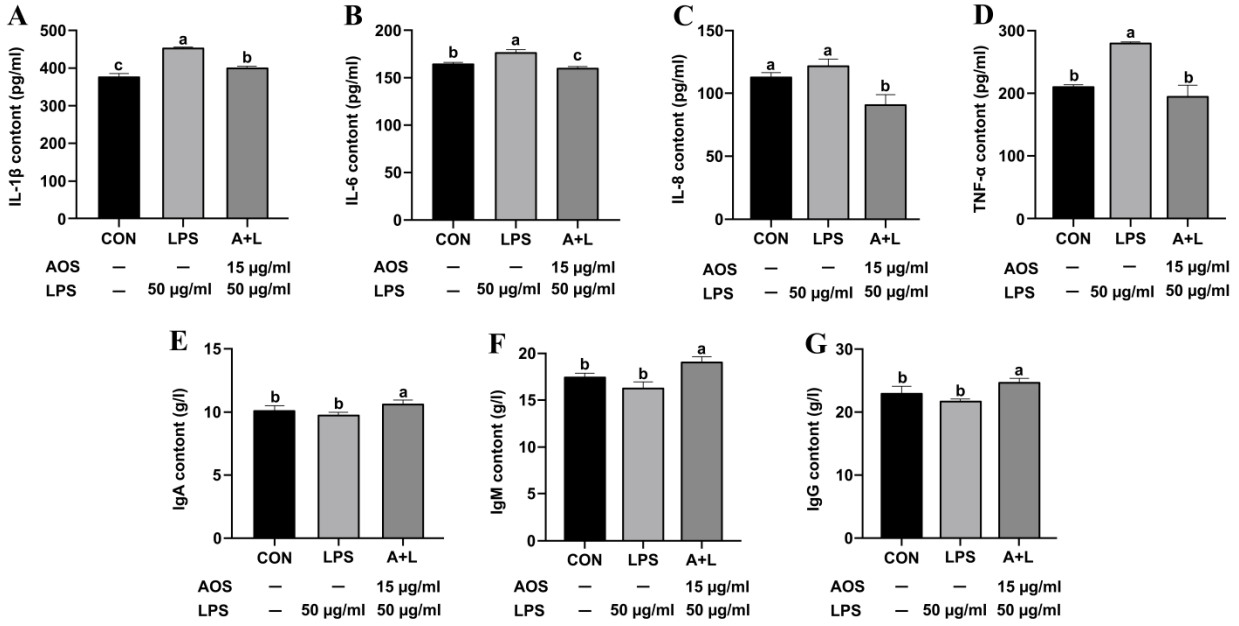



Figure. 6 Effect of AOS on inflammatory factors and immunoglobulins induced by LPS in rumen epithelial cells. (A) IL-1β content; (B) IL-6 content; (C) IL-8 content; (D) TNF-α content; (E) IgA content; (F) IgM content; (G) IgG content. No letter or the same letter in the graphs indicated that the differences were not significant ($P$>0.05), and different letters indicated that (a,b,c) the differences were significant ($P$<0.05).

3.8 Effect of Agar-oligosaccharides on LPS-induced Apoptosis in Ruminal Epithelial Cells

The results of the flow cytometry assay are shown in Figure. 7D, the apoptosis rate was significantly higher in LPS group compared with CON group ($p$<0.05), and the apoptosis rate was significantly lower in A+L group compared with LPS group ($p$<0.05).

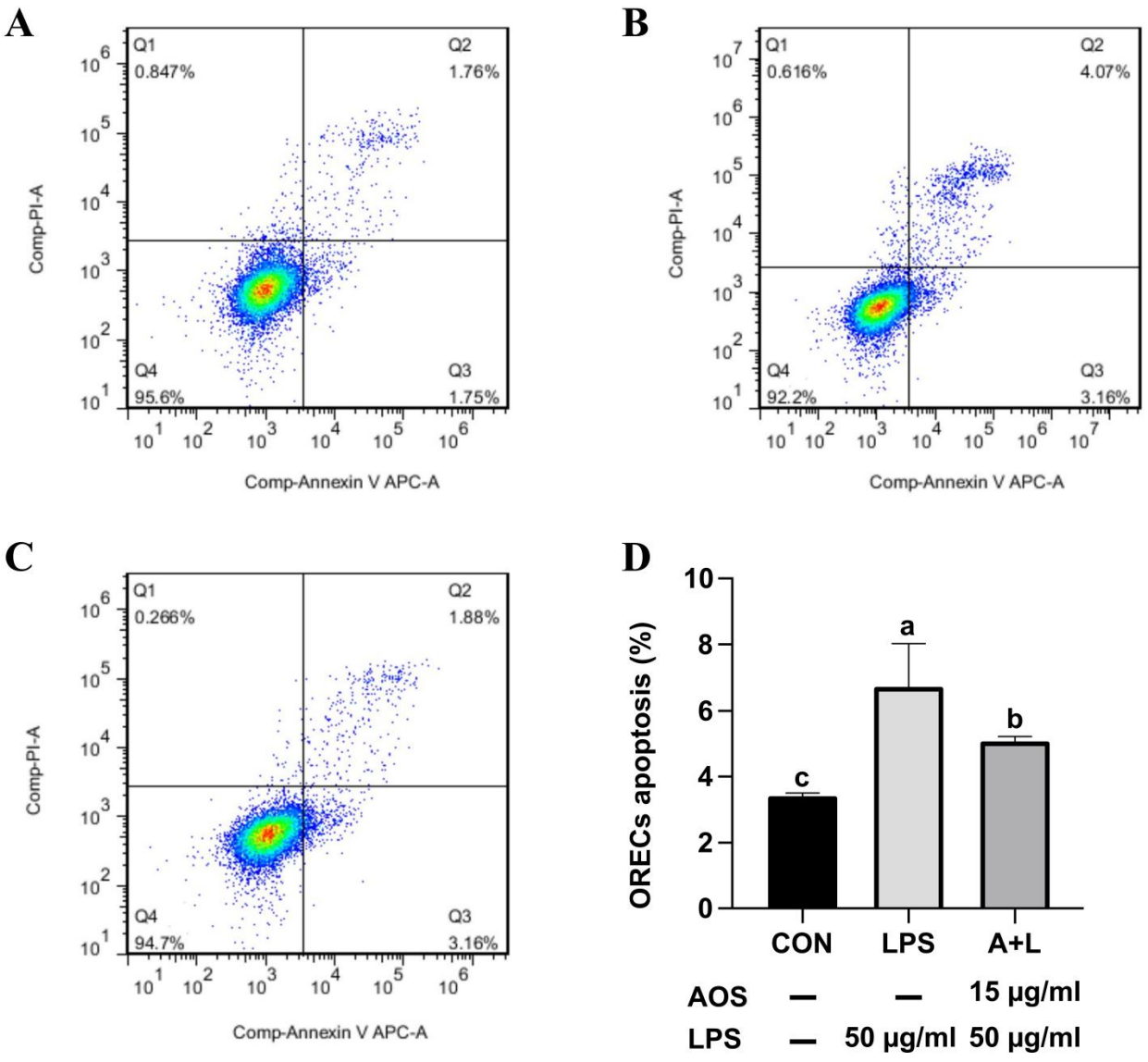



Figure. 7 Effect of AOS on LPS-induced apoptosis of rumen epithelial cells. (A) Apoptotic index plots of rumen epithelial cells in CON groups; (B) Apoptotic index plots of rumen epithelial cells in LPS groups; (C) Apoptotic index plots of rumen epithelial cells in A+L groups; (D) Comparison chart of rumen epithelial cell apoptosis rates in each group. No letter or the same letter in the graphs indicated that the differences were not significant ($P$>0.05), and different letters indicated that (a,b,c) the differences were significant ($P$<0.05).

### 3.9 Effect of Agar-oligosaccharides on LPS-induced Gene Expression of TLR4-MyD88-NF-κB Signaling Pathway

The RT-qPCR results are shown in Figure. 8. The relative mRNA expression of TLR4 was significantly higher in the LPS group compared with the CON group and the A+L group ($p<0.05$). The relative mRNA expression of MyD88 and IRAK1 in the LPS group was significantly higher than that in the A+L group ($p<0.05$), but neither of them was significantly different from the CON group ($p>0.05$). Compared with the A+L group, the relative mRNA expression of NF-κB was significantly higher in the CON and LPS groups ($p<0.05$), and the relative mRNA expression of NF-κB in the LPS group was significantly higher than that in the CON group ($p<0.05$).

### 3.10 Effect of Agar-oligosaccharides on LPS-induced Expression of Proteins Related to the TLR4-MyD88-NF-κB Signaling Pathway

After the rumen epithelial cells were treated for a specified time and with different AOS and LPS concentrations, the expression of the relevant proteins of the TLR4-MyD88-NF-κB signaling pathway was detected by protein immunoblotting, and the results were shown in Figure. 8. As shown in Figure. 9B-D, F and H, the LPS group significantly up-regulated the protein expression of TLR4, MyD88, IRAK1, p-IκBα and p-p65 ($p<0.05$), and the A+L group pretreated with AOS significantly decreased the protein expression of TLR4, MyD88, IRAK1, p-IκBα and p-p65 ($p<0.05$), but all of them were significantly higher than the CON group ($p<0.05$). As shown in Figure. 9E and G, there was no significant difference in the protein expression of IκBα and p65 between the groups ($p>0.05$).

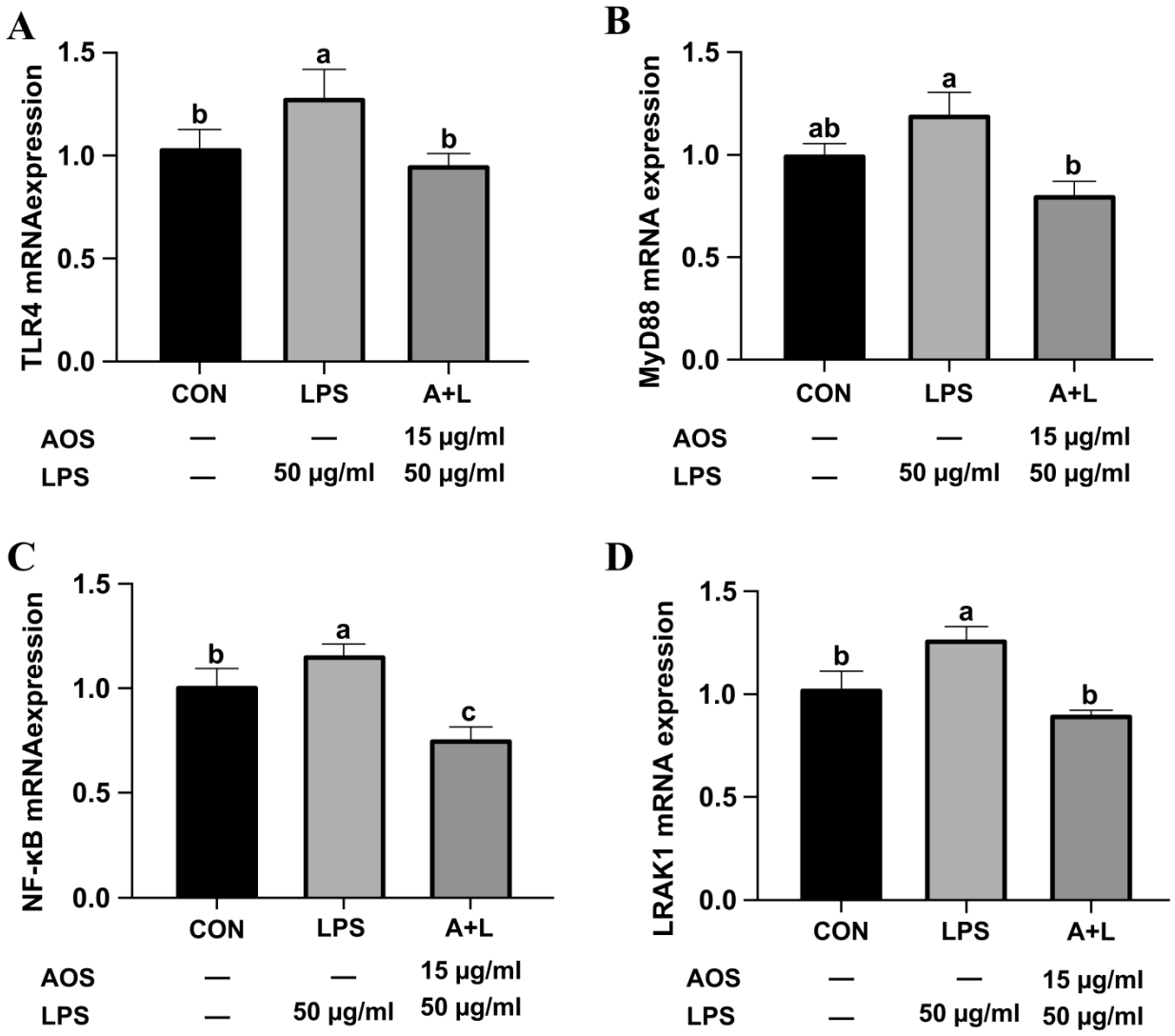



Figure. 8 Effect of AOS on LPS-induced expression of TLR4-MyD88-NF-κB pathway genes in rumen epithelial cells. (A) TLR4 relative mRNA expression; (B) MyD88 relative mRNA expression; (C) NF-κB relative mRNA expression; (D) IRAK1 relative mRNA expression. No letter or the same letter in the graphs indicated that the differences were not significant ($P$>0.05), and different letters indicated that (a,b,c) the differences were significant ($P$<0.05).

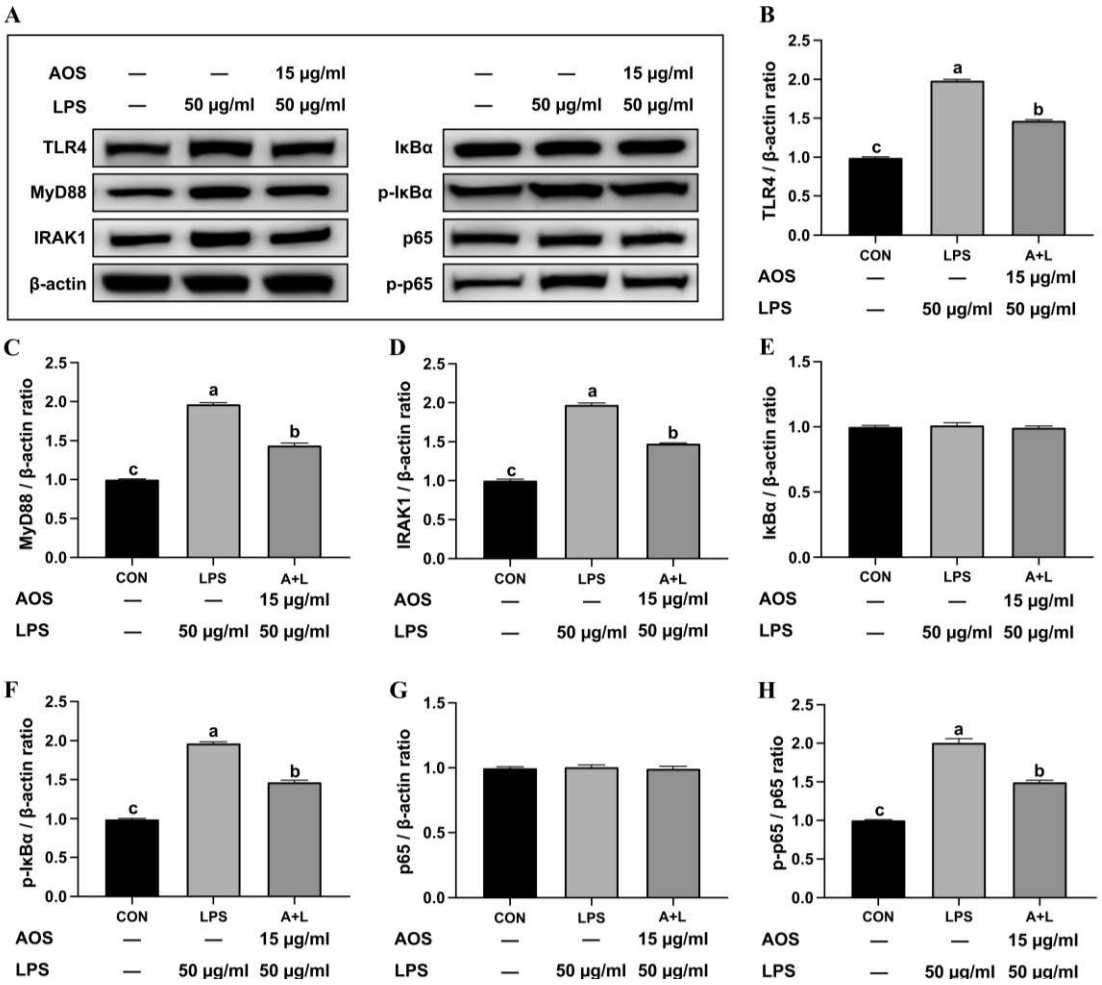



Figure. 9 Effect of AOS on LPS-induced expression of TLR4-MyD88-NF-κB pathway-related proteins in rumen epithelial cells. (A) Western blot results; (B) TLR4 gray scale analysis results; (C) MyD88 gray scale analysis results; (D) IRAK1 gray scale analysis results; (E) IκBα gray scale analysis results; (F) p-IκBα gray scale analysis results; (G) p65 gray scale analysis results; (H) p-p65 gray scale analysis results. No letter or the same letter in the graphs indicated that the differences were not significant ($P$>0.05), and different letters indicated that (a,b,c) the differences were significant ($P$<0.05).

## 4. Discussion

AOS is a product obtained by further degradation of agar extracted from red algae by hydrolysis method and chemical degradation, which has a variety of biological functions. Currently, the studies of AOS in animal organisms mainly focus on mice [20] and male *Drosophila* [21], among others, and the studies on ruminants have not been reported for the time being. Therefore, this experiment was conducted to investigate the effects of different times and different AOS concentrations on rumen epithelial cell viability. The results of this experiment showed that the rumen epithelial cell viability of some groups started to be higher than that of the group with 0 μg/mL AOS addition concentration when the incubation time reached 12 h and above, and reached the peak value when the incubation time was 18 h, and the viability started to drop after 18 h. Overall, AOS administration significantly increased the survival rate of rumen epithelial cells in a dose-dependent manner, and the cell viability of rumen epithelial cells showed a trend of first increasing and then decreasing with its concentration and culture time. When the concentration of AOS exceeded 50 μg/mL, AOS may have a toxic effect on the cells, leading to a decrease in cell viability.Premarathna et al. [22], in their study on the immunomodulatory effects of red algal extracts (xylan, carrageenan), found that the cellular activity increased in a concentration-dependent manner, which is in agreement with the results of the present experiment. Therefore, the present experiment was conducted to further investigate the effects of different added concentrations of AOS on rumen epithelial cells under the condition of incubation time of 18 h. ROS is a major regulator of cellular homeostasis and cellular dysfunction, and it plays an important signaling function in the cell [23]. When the oxidative and antioxidant balance system of the organism is disrupted, a large amount of ROS is released leading to the disruption of cellular lipids, which further affects cellular function. In addition, when tissue injury occurs, a large amount of ROS is released from mitochondria, leading to abnormal mitochondrial function, which mediates the production of inflammatory factors, such as IL-6, IL-1β, and TNF-α, and triggers inflammatory responses [23-24]. In this experiment, the intracellular ROS content was significantly lower than the other groups when AOS was added at concentrations of 1 and 15 μg/mL. This suggests that AOS has the ability to resist oxidative stress and may also further mediate the level of inflammatory factors. In addition, the intracellular ROS, IL-1β, IL-6, IL-8, and TNF-α levels were up-regulated at AOS concentrations of 50 and 100 μg/mL compared to 15 μg/mL, which may be due to the toxic effects of AOS on cells at these concentrations, leading to an inflammatory response in cells. Therefore, this experiment was continued to detect the effects on the content of some cytokines in rumen epithelial cells at different AOS addition concentrations. The results showed that when AOS was added at a concentration of 15 μg/mL, the rumen epithelial cell contents of IL-6, IL-8, IL-1β, and TNF-α were lower than those of the other four groups. This suggests that AOS has an inhibitory effect on cytokines and exhibits a potential anti-inflammatory effect, and based on this result, an AOS addition concentration of 15 μg/mL can be initially identified as the optimal concentration. BCL-2 and BAX proteins, both belonging to the BCL-2 family, are important regulators of apoptosis, and play an important role in maintaining the dynamic homeostasis and integrity of the rumen epithelium. Studies have shown that BAX is an interacting protein of BCL-2, which acts antagonistically with BCL-2 and promotes cell death [25]. The results of this experiment indicate that in the rumen epithelial cells of the group with 15 μg/mL AOS, the relative mRNA expression of BCL-2 and the BCL-2/BAX ratio were significantly upregulated, while the relative mRNA expression of the pro-apoptotic member BAX was significantly lower compared to the control group. This is inconsistent with the results of Yun et al. [11], which may be due to differences in the source and preparation method of agar oligosaccharides. Furthermore, the results show

that 15 μg/mL of AOS can inhibit apoptosis of rumen epithelial cells, potentially through a mechanism where AOS reduces ROS levels, thereby inhibiting the activation of early apoptosis pathways and ultimately suppressing cell apoptosis [26]. In conclusion, the concentration of AOS at 15 μg/mL and the incubation time of 18 h decreased the content of ROS and inflammatory factors, and inhibited apoptosis, so it was used as the subsequent experimental conditions.

LPS is a unique component of the cell wall of Gram-negative bacteria with inflammation-activating capacity, consisting mainly of lipids and polysaccharides [27]. So far, LPS has been widely and intensively studied in both pro-inflammatory and non-pro-inflammatory aspects, especially as a model of inflammation modeling, and these models have a key role in the in-depth study and prevention of certain inflammatory diseases, such as neuritis [28], inflammatory bowel disease [29] and SARA [30]. In this experiment, the results of screening the optimal LPS treatment time by CCK-8 method found that when the LPS treatment time was 24 h, the cell survival rate of each treatment concentration group showed different degrees of significant reduction, so the LPS treatment time was determined to be 24 h as a modeling condition for the subsequent experiments. The ROS and the related inflammatory factors (e.g., IL-6, IL-1β, TNF-α) content is a key indicator for successful modeling of inflammatory injury. In this experiment, the IL-6 and TNF-α contents in the 50 and 100 μg/mL LPS groups were significantly higher than those in the other three concentration groups. This suggests that after stimulating rumen epithelial cells for 24 h with LPS, intracellular ROS acted as signaling molecules and activated some of the inflammatory signaling pathways within the immune cells, thereby increasing the levels of inflammatory factors and causing an inflammatory response. In this study, we continued to investigate the effects of LPS addition concentrations of 50 and 100 μg/mL on the expression of apoptotic genes in rumen epithelial cells, and the results showed that both 50 μg/mL and 100 μg/mL LPS addition concentrations decreased the relative mRNA expression of BCL-2 and down-regulated the ratio of BCL-2/BAX, and increased the relative mRNA expression of BAX, which led to cellular apoptosis, but the cell survival rate was only 55% under the treatment condition of 100 μg/mL LPS addition concentration, which was unfavorable for subsequent experiments. Therefore, in this study, we chose 50 μg/mL LPS concentration and 24 h as the treatment conditions for establishing the inflammatory injury model.

In this experiment, we continued to analyze the effect of AOS on LPS-induced rumen epithelial cell damage based on the optimal AOS addition concentration and modeling conditions derived from previous studies. Most bioactive substances can improve cell bioactivity, such as tea polyphenols, inulin and ginseng polysaccharides, but the effects of marine bioactive substances on cell viability have rarely been reported. From the results of this experiment, it can be seen that the cells in the LPS group underwent morphological changes such as pulling, lengthening, etc., and the cell viability was significantly lower than that in the CON and A+L groups, and the ROS fluorescence intensity in the LPS group was significantly higher than that in the CON and A+L groups. This is due to the fact that LPS inhibits the proliferation of rumen epithelial cells, causing damage to intracellular mitochondria and elevated levels of ROS, which leads to the arrest of their cell cycle in the S-phase, and then causes apoptosis [31]. In general, ROS levels are usually associated with cellular damage and cytotoxicity, and mitochondria are considered to be the main site of ROS production, which plays a key role in executing metabolic cycles, maintaining ionic homeostasis, inducing autophagy, and eliciting immune responses against infections, and one of the main ways in which it activates immune signaling is through ROS production [32-33] The cell survival in the A+L group was significantly higher than that in the CON group and LPS group, which

shows that AOS has a certain mitigating effect on LPS-induced cell damage, and at the same time can significantly reduce the fluorescence intensity of ROS in rumen epithelial cells. This may be due to the fact that AOS can improve the structure and function of mitochondria in cells, making them more effective in scavenging free radicals in vivo, thus enhancing rumen epithelial cell activity.

SARA is closely related to the inflammatory response. Studies have shown that SARA leads to increased levels of pro-inflammatory factors such as IL-6, IL-1β and TNF-α in the rumen of ruminants [30,34] Therefore, inhibition of the production of inflammatory factors in rumen epithelial cells can be effective in the treatment of SARA. Studies have reported that oligosaccharides and monosaccharides produced by agar degradation have anti-inflammatory and immunomodulatory effects [35]. Wang et al. [36] demonstrated that AOS could alleviate inflammatory injury by modulating the expression level of inflammatory cytokine IL-1β. Higashimura et al. [37] studied that indicated that AOS could attenuate the inflammatory response by inhibiting the expression level of LPS-induced TNF-α. The results of the present study showed that AOS pretreatment inhibited the release of inflammatory factors IL-6, IL-8, IL-1β, and TNF-α and promoted the release of immunoglobulins IgA, IgM, and IgG, which suggests that AOS attenuates the damage of LPS to rumen epithelial cells and suppresses cellular inflammatory responses. In addition, Fu et al. [38] showed that pre-treatment with fucoidan could reduce the release of IL-1β and IL-6, but the results were not significant and actually increased the release of TNF-α. Yang et al. [39] also indicated that TNF-α levels only significantly decreased when chitosan oligosaccharide was added at concentrations above 250 μg/mL. This suggests that pre-treatment with a lower concentration of AOS can achieve a better anti-inflammatory effect.

LPS, a commonly used factor for modeling, damages rumen epithelial cells to varying degrees after its entry into the rumen, resulting in apoptosis. Apoptosis is usually caused by various internal or external stimuli (e.g., DNA damage, viral infection, or multiple immune responses), which results in the activation of apoptosis-specific pathways and further leads to changes in apoptosis-related genes and protein expression (including caspases and BCL-2 family protein members) [40-41]. In the present study, it was found that the apoptotic pathway was activated after LPS-induced injury, resulting in a significantly higher apoptosis rate, while the apoptosis rate was reduced after AOS pretreatment, which showed that AOS played a protective role for rumen epithelial cells.

TLR4 is a member of the pattern recognition receptor family, which participates in the innate immune response of the organism mainly by recognizing antigens and related damage factors, in addition, TLR4 is an upstream factor in the activation of the LPS-induced Myeloid differentiation primary response gene 88 (MyD88)/NF-κB p65 pathway [42-43]. MyD88 is an adaptor protein, and when the activation of the organism's immune response by LPS is recognized by TLR4, the organism initiates downstream signaling cascade responses, such as the MyD88-dependent pathway, which, when activated, recruits TNF receptor associated factor 6 (TRAF6) and promotes autophosphorylation of Interleukin-1 receptor-associated kinase (IRAK) , followed by activation of the translational Transforming Growth Factor-β-Activated Kinase 1 (TAK1) complex and phosphorylation of inhibitor of NF-κB (IκB) , which then activates transcription factors, such as NF-κB and MAPK, and promotes the production and secretion of a variety of inflammatory cytokines and chemokines [42,46]. NF-κB consists of NF -κB 1 (p50), NF-κB 2 (p52), ReIA (p65), ReIB, and c-ReI, and when the organism is stimulated by inflammation, the classical pathway of NF-κB is usually activated, leading to the phosphorylation and degradation of IκB, which

releases the NF-κB/Rel complex, resulting in the translocation of the complex to the nucleus, and eventually initiating gene expression [47-49]. Therefore, phosphorylation of p65 and IκBα is usually used as a marker of NF-κB activation [18]. The results of this study showed that the relative mRNA expression of TLR4 and NF-κB and the protein expression of TLR4, MyD88, IRAK1, p-IκBα, and p-p65 in rumen epithelial cells cultured with 50 μg/mL LPS for 18 h were significantly higher than that in the control group, which was consistent with the results of Mao et al. [50].

## 5. Conclusions

In conclusion, rumen epithelial cells that were damaged by LPS were pretreated with AOS at a concentration of 15 μg/mL for 18 hours. This pretreatment resulted in improved cell morphology, increased cell viability, enhanced immunoglobulin content, and higher expression levels of genes that inhibit apoptosis. Additionally, the levels of ROS, the apoptosis rate, and the expression levels of inflammatory factors and pro-apoptosis genes were reduced. These effects alleviated the LPS-induced apoptosis and inflammatory damage in the rumen epithelial cells. This study serves as an important reference for promoting the development of animal husbandry and highlights the potential future application of AOS as a beneficial additive in ruminants. However, the relative mRNA expression of TLR4, MyD88, IRAK1 and NF-κB and the protein expression of TLR4, MyD88, IRAK1, p-IκBα and p-p65 could be significantly reduced after AOS pretreatment, suggesting that AOS may attenuate LPS-induced damage to the rumen epithelial cells through the TLR4-MyD88-NF-κB signaling pathway.

## Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: All authors have approved the manuscript and agree with its submission.

Availability of data and materials: The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Competing interests: The authors declare no conflicts of interest.

Authors' contributions:A.L. and X.H. contributed to conceptualization, investigation, methodology, project administration, writing-original draft, writing-review & editing, and visualization. J.M. contributed to validation and writing-original draft. C.D. contributed to investigation, writing-original draft, and visualization. F.Y. and Y.L. contributed to conceptualization, data curation, formal analysis, resources, funding acquisition, project administration, and writing-review & editing. All authors reviewed and approved the final manuscript.

Acknowledgements:This research was funded by The Key Area Project of The 'Hundreds, Thousands, and Tens of Thousands' Service Program, Funded Project "Development of a High-Reproduction, High-Growth New Breed of Leizhou Goat Based on Provincial Breeding Farms and Integration of a Healthy Farming System"; by the Guangdong Feed Industry Technology System, under grant number 2024CXTD14; by the Guangdong Ocean University Graduate Education Innovation Program Funded

Project "Leizhou Goat Science and Technology Academy", under grant number 202406; and by the Guangdong Provincial Department of Science and Technology 'Hundreds, Thousands, Tens of Thousands Project' Rural Science and Technology Commissioner Program, Funded Project "Healthy Livestock and Poultry Farming and Integrated Crop-Livestock System", under grant number KTP20240547.

Authors' information (optional): Not applicable

References

1. Pokhrel B, Jiang H. Postnatal Growth and Development of the Rumen: Integrating Physiological and Molecular Insights. Biology (Basel). 2024; 13.
2. Na SW, Guan LL. Understanding the role of rumen epithelial host-microbe interactions in cattle feed efficiency. Anim Nutr. 2022;10:41-53.
3. Zhang H, Shi H, Zhou S, Li X, Wang Y, Liu J. Short-term effects of subacute ruminal acidsis on ferroptosis and iron metabolism in the livers of lactating sheep fed a high-grain diet. J Dairy Sci. 2025;108:4365-4380.
4. Zhang H, Shi H, Xie W, Meng M, Wang Y, Ma N, Chang G, Shen X. Subacute ruminal acidosis induces pyroptosis via the mitophagy-mediated NLRP3 inflammasome activation in the livers of dairy cows fed a high-grain diet. J Dairy Sci. 2024;107:4092-4107.
5. Araki C. Structure of the agarose constituent of agar-agar. Bulletin of the Chemical Society of Japan. 1956;29:543-544.
6. Wang L, Fu X, Hyun J, Xu J, Gao X, Jeon Y J. In Vitro and In Vivo Protective Effects of Agaro-Oligosaccharides against Hydrogen Peroxide-Stimulated Oxidative Stress. Polymers (Basel). 2023;15.
7. Praiboon J, Chantorn S, Krangkratok W, Choosuwan P, La-Ongkham O. Evaluating the Prebiotic Properties of Agar Oligosaccharides Obtained from the Red Alga Gracilaria fisheri via Enzymatic Hydrolysis. Plants (Basel). 2023;12.
8. Higashimura Y, Naito Y, Takagi T, Uchiyama K, Mizushima K, Ushiroda C, et al. Protective effect of agaro-oligosaccharides on gut dysbiosis and colon tumorigenesis in high-fat diet-fed mice. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2016;310:G367-G375.
9. Fan S, Zhang Q, She J, Dai X. Agar oligosaccharides improve the intestinal health of induced-aging mice by maintaining intestinal homeostasis via balancing the ISCs proliferation and differentiation. Eur J Nutr 2024;64:9.
10. Wang Q, Wang Y, Wang Y, Zhang L, Gao C, Wang B, et al. Agaro-oligosaccharides mitigate deoxynivalenol-induced intestinal inflammation by regulating gut microbiota and enhancing intestinal barrier function in mice. Food Funct. 2024;15:3380-3394.
11. Yun EJ, Yu S, Kim YA, Liu JJ, Kang NJ, Jin YS, Kim KH, et al. In vitro prebiotic and anti-colon cancer activities of agar-derived sugars from red seaweeds. Mar Drugs. 2021;19.
12. Wang X, Yang Z, Xu X, Jiang H, Cai C, Yu G. Odd-numbered agaro-oligosaccharides alleviate type 2 diabetes mellitus and related colonic microbiota dysbiosis in mice. Carbohydr Polym. 2020;240:116261.
13. Wang X, Yang Z, Xu X, Jiang H, Cai C, Yu G. Hepatoprotective Effect of Neoagarooligosaccharide via Activation of Nrf2 and Enhanced Antioxidant Efficacy. Biol Pharm Bull. 2020;43:619-628.
14. Jin X, Zhang M, Wang YH, Wei F, Wen JY, Yang YF. In vitro isolation, culture, cryopreservation and resuscitation of sheep ruminal epithelial cells. J China Agric Univ. 2019;24(5):57-66.

15. Kelly P, Barry-Reidy A, Brewer A, Meade KG, O'Farrelly C. Improved filtration method to isolate pure populations of primary bovine endometrial epithelial and stromal cells for immunological studies. Vet Res Commun. 2020;44(1):29-39.
16. Wang L, Fu X, Hyun J, Xu J, Gao X, Jeon Y.J. In Vitro and In Vivo Protective Effects of Agaro-Oligosaccharides against Hydrogen Peroxide-Stimulated Oxidative Stress. Polymers (Basel) 2023;15.
17. Chu C, Ru H, Chen Y, Xu J, Wang C, Jin Y. Gallic acid attenuates LPS-induced inflammation in Caco-2 cells by suppressing the activation of the NF-κB/MAPK signaling pathway. Acta Biochim Biophys Sin (Shanghai). 2024;56:905-915.
18. Qiu X, Yin F, Du C, Ma J, Gan S. Alginate Oligosaccharide Alleviates Lipopolysaccharide-Induced Apoptosis and Inflammatory Response of Rumen Epithelial Cells through NF-κB Signaling Pathway. Animals (Basel). 2024;14.
19. Wu SY, Yang WY, Cheng CC, Hsiao MC, Tsai SL, Lin HK, et al. Low Molecular Weight Fucoidan Prevents Radiation-Induced Fibrosis and Secondary Tumors in a Zebrafish Model. Cancers (Basel). 2020;12.
20. Zheng Y, Li M, Jiang Z, Zhu Y, Ni H, Zheng M. Inhibition of Agar-oligosaccharides on the Lipopolysaccharide-induced Inflammatory Reaction in Macrophages RAW264.7. J. Chin. Inst. Food Sci. Technol. 2022;22:72-82.
21. Ma C, Yang K, Wang Y, Dai X. Anti-aging Effect of Agar Oligosaccharide on Male Drosophila melanogaster and its Preliminary Mechanism. Mar Drugs 2019;17.
22. Premarathna AD, Ahmed TAE, Rjabovs V, Hammami R, Critchley AT, Tuvikene R, et al. Immunomodulation by xylan and carrageenan-type polysaccharides from red seaweeds: Anti-inflammatory wound healing cytoprotective and anticoagulant activities. Int J Biol Macromol. 2024;260:129433.
23. Forrester SJ, Kikuchi DS, Hernandes MS, Xu Q, Griendling KK. Reactive Oxygen Species in Metabolic and Inflammatory Signaling. Circ Res. 2018;122:877-902.
24. Palma FR, Gantner BN, Sakiyama MJ, Kayzuka C, Shukla S, Lacchini R, et al. ROS production by mitochondria: function or dysfunction? Oncogene. 2024;43:295-303.
25. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog Bax that accelerates programmed cell death. Cell. 1993;74:609-619.
26. Jeong CH, Joo SH. Downregulation of Reactive Oxygen Species in Apoptosis. J Cancer Prev. 2016;21:13-20.
27. Zhang X, Tian X, Wang Y, Yan Y, Wang Y, Su M. et al. Application of lipopolysaccharide in establishing inflammatory models. Int J Biol Macromol. 2024;279:135371.
28. da Silva AAF, Fiadeiro MB, Bernardino LI, Fonseca CSP, Baltazar GMF, Cristóvão ACB. "Lipopolysaccharide-induced animal models for neuroinflammation - An overview.". J Neuroimmunol. 2024;387:578273.
29. Lee JY, Kim JH, Choi JM, Noh BW, Kim HY, Cho EJ. Anti-Inflammatory Effects of Artemisia argyi H. Fermented by Lactobacillus plantarum in the LPS-Induced RAW 264.7 Cells and DSS-Induced Colitis Model. Foods. 2024;13.
30. Tan P, Yang J, Yi F, Mei L, Wang Y, Zhao C. et al. Strontium Attenuates LPS-Induced Inflammation via TLR4/MyD88/NF-κB Pathway in Bovine Ruminal Epithelial Cells. Biol Trace Elem Res. 2024;202:3988-3998.
31. Zhou J, Wang Y, Liu Z, Feng Y, Chen F, Tai G. Activation-Induced Apoptosis of Mouse Macrophage RAW264.7 by Lipopolysaccharide. Chin. J. Biol. 2009;22:136-138.

32. Mukherjee A, Ghosh KK, Chakrabortty S, Gulyás B, Padmanabhan P, Ball W.B. Mitochondrial Reactive Oxygen Species in Infection and Immunity. Biomolecules. 2024;14.
33. Faas MM, de Vos P. Mitochondrial function in immune cells in health and disease. Biochim Biophys Acta Mol Basis Dis. 2020;1866:165845.
34. Fan S, Zheng M, Ren A, Mao H, Long D, Yang L. Effects of High-Concentrate-Induced SARA on Antioxidant Capacity Immune Levels and Rumen Microbiota and Function in Goats. Animals (Basel). 2024;14.
35. Wang C, Long J, Li X, Chen M, Liu T, Ruan R, et al. Preparation of agar polysaccharides and biological activities and relationships of agar-derived oligosaccharides and monosaccharides: A review. Int J Biol Macromol. 2025;295:139552.
36. Wang C, Long J, Li X, Chen M, Liu T, Ruan R. et al. Agaro-oligosaccharides mitigate deoxynivalenol-induced intestinal inflammation by regulating gut microbiota and enhancing intestinal barrier function in mice. Food Funct. 2024;15:3380-3394.
37. Higashimura Y, Naito Y, Takagi T, Mizushima K, Hirai Y, Harusato A. et al. Oligosaccharides from agar inhibit murine intestinal inflammation through the induction of heme oxygenase-1 expression. J Gastroenterol. 2013;48:897-909.
38. Fu X, Huang X, Tan H, Huang X, Nie S. Regulatory Effect of Fucoidan Hydrolysates on Lipopolysaccharide-Induced Inflammation and Intestinal Barrier Dysfunction in Caco-2 and RAW264.7 Cells Co-Cultures. Foods. 2024;13.
39. Yang Y, Tong Q, Luo H, Ruo H, Zhong Y. Chitooligosaccharides attenuate lipopolysaccharide-induced inflammation and apoptosis of intestinal epithelial cells: Possible involvement of TLR4/NF-κB pathway. Indian J Pharm Educ Res. 2016;50:109-115.
40. Obeagu EI, Ubosi NI, Obeagu GU, Egba SI, Bluth MH. Understanding apoptosis in sickle cell anemia patients: Mechanisms and implications. Medicine (Baltimore). 2024;103:e36898.
41. Savitskaya MA, Onishchenko GE. Mechanisms of Apoptosis. Biochemistry (Mosc). 2015;80:1393-1405.
42. Long L, Dai X, Yao T, Zhang X, Jiang G, Cheng X. et al. Mefunidone alleviates silica-induced inflammation and fibrosis by inhibiting the TLR4-NF-κB/MAPK pathway and attenuating pyroptosis in murine macrophages. Biomed Pharmacother. 2024;178:117216.
43. Fu YJ, Xu B, Huang SW, Luo X, Deng XL, Luo S. et al. Baicalin prevents LPS-induced activation of TLR4/NF-κB p65 pathway and inflammation in mice via inhibiting the expression of CD14. Acta Pharmacol Sin. 2021;42:88-96.
44. Xu J, Lu C, Liu Z, Zhang P, Guo H, Wang T. Schizandrin B protects LPS-induced sepsis via TLR4/NF-κB/MyD88 signaling pathway. Am J. Transl Res 2018;10:1155-1163.
45. Zhang G, Ghosh S. Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through Toll-like receptors. J Endotoxin Res. 2000;6:453-457.
46. Hu N, Wang C, Dai X, Zhou M, Gong L, Yu L. et al. Phillygenin inhibits LPS-induced activation and inflammation of LX2 cells by TLR4/MyD88/NF-κB signaling pathway. J Ethnopharmacol. 2020;248:112361.
47. Laurindo LF, Santos AROD, Carvalho ACA, Bechara MD, Guiguer EL, Goulart RA. et al. Phytochemicals and Regulation of NF-kB in Inflammatory Bowel Diseases: An Overview of In Vitro and In Vivo Effects. Metabolites. 2023;13.

771 48. Kuzmich NN, Sivak KV, Chubarev VN, Porozov YB, Savateeva-Lyubimova TN, Peri F. TLR4
772 Signaling Pathway Modulators as Potential Therapeutics in Inflammation and Sepsis. Vaccines
773 (Basel). 2017;5.
774 49. Zinatizadeh MR, Schock B, Chalbatani GM, Zarandi PK, Jalali SA, Miri SR. The Nuclear Factor
775 Kappa B (NF-kB) signaling in cancer development and immune diseases. Genes & diseases.
776 2021;8:287-297.
777 50. Mao N, Yu Y, Lu X, Yang Y, Liu Z, Wang D. Preventive effects of matrine on LPS-induced
778 inflammation in RAW 264.7 cells and intestinal damage in mice through the TLR4/NF-κB/MAPK
779 pathway. Int Immunopharmacol. 2024;143:113432.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterial.zip