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Patchouli Oil Alleviates Heat Stress-Induced Barrier Dysfunction in Bovine Ruminal Epithelial Cells: Involvement of the MAPK Signaling Pathway

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Simple Summary

Global warming exacerbates heat stress, which severely restricts the sustainable development of beef cattle production. Patchouli oil (PO), a natural plant bioactive substance, possesses antioxidant, anti-inflammatory and mucosal barrier-protective properties, yet its protective mechanisms against heat stress damage in bovine ruminal epithelial cells (BRECs) remain undefined. In this study, a heat stress model was established in primary BRECs in order to determine whether PO alleviates heat stress-induced injury and whether the mitogen-activated protein kinase (MAPK) signaling pathway is involved. The results showed that 20 μg/mL PO improved cell viability, antioxidant enzyme activities and transepithelial electrical resistance, reduced cell apoptosis and alleviated heat stress-induced morphological damage. Heat stress abnormally increased the expression of components of the MAPK cascade, which was accompanied by increased apoptosis, inflammation and autophagic disturbance, and by disruption of tight junction proteins in BRECs. PO treatment suppressed this upregulation and reversed these adverse changes, thereby maintaining ruminal epithelial barrier integrity. This study provides theoretical support for developing PO as a natural feed additive to counteract heat stress in beef cattle farming.

Abstract

Heat stress has become a critical environmental stressor limiting the sustainable development of the beef cattle industry amid worsening global warming. Patchouli oil (PO), a natural plant bioactive extract, exerts antioxidant, anti-inflammatory and mucosal barrier-protective activities. However, the cellular mechanisms by which PO protects bovine ruminal epithelial cells (BRECs) against heat stress remain unclear. In this study, a heat stress injury model was established in primary BRECs, and the effects of PO on oxidative damage, apoptosis, inflammation, autophagy and barrier function were examined, with a focus on the mitogen-activated protein kinase (MAPK) signaling pathway. Exposure to 43 °C for 4 h significantly reduced cell viability and the activities of superoxide dismutase (SOD) and total antioxidant capacity (T-AOC) and increased the apoptosis rate ($p < 0.05$), and was therefore used as the heat stress condition. Treatment with 20 μg/mL PO improved cell viability, significantly enhanced the activities of SOD and catalase (CAT), increased intracellular lactate dehydrogenase (LDH) activity, reduced the apoptosis rate, restored transepithelial electrical resistance (TEER) values and alleviated heat stress-induced morphological damage ($p < 0.05$). Heat stress increased the mRNA and total protein expression

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of p38, ERK and JNK, and upregulated pro-apoptotic (Bax, Caspase-3), pro-inflammatory (IL-1β, IL-6, TNF-α) and autophagy-related (Beclin-1, LC3, P62) genes, while downregulating the anti-apoptotic factor Bcl-2 and the tight junction proteins ZO-1, Occludin and Claudin-1 ($p < 0.05$). PO treatment reversed these changes, and the expression of MAPK pathway components and of their downstream effectors in the HS+PO group was significantly lower than that in the heat stress group ($p < 0.05$). In conclusion, 20 μg/mL PO alleviated heat stress-induced apoptosis, inflammatory responses and autophagic disturbance and preserved tight junction integrity and barrier function in BRECs, an effect that was accompanied by reduced expression of MAPK pathway components. These findings provide a theoretical basis for the potential application of PO as a natural plant-derived feed additive in beef cattle production.

Keywords: heat stress; patchouli oil; beef cattle; ruminal epithelial cells; MAPK signaling pathway

1. Introduction

Heat stress is acknowledged as a major environmental constraint affecting the healthy development of the global beef cattle industry [1]. With the progression of global warming and the increasing intensification of livestock production systems, the impact of high-temperature environments on the physiological homeostasis of animals has become increasingly pronounced. Studies have shown that the sustained rise in global temperature and the expansion of farming density have made heat stress the most important stress factor in southern China [2]. As the core of digestion and metabolism in ruminants, the rumen plays a pivotal role in determining nutrient utilization efficiency and animal productive performance. The rumen epithelium not only mediates the absorption and transport of key nutrients such as volatile fatty acids and ammonia nitrogen, but also acts as a physical barrier that effectively prevents the translocation of pathogens and endotoxins from the rumen lumen into the bloodstream, thereby contributing critically to the maintenance of immune and metabolic homeostasis.

Under heat stress conditions, redistribution of blood flow leads to gastrointestinal ischemia and hypoxia. The splanchnic circulation becomes ischemic and intestinal permeability increases. The rumen epithelial structure is damaged, characterized by papillary atrophy, hyperkeratosis and desquamation of the stratum corneum, widening of intercellular spaces, and disruption of tight junction complexes [3]. Concurrently, a burst of reactive oxygen species (ROS) occurs within epithelial cells, the antioxidant enzyme system becomes decompensated, pro-apoptotic signals are activated, and paracellular permeability is markedly elevated. Heat stress induces oxidative stress and increases ROS levels [4]. These pathological changes constitute the core mechanisms of rumen barrier dysfunction, and in severe cases can trigger local inflammation and systemic endotoxemia, ultimately suppressing feed intake, reducing feed conversion efficiency, and impairing animal growth performance.

At the molecular level, heat stress rapidly initiates the MAPK cascade [5]. Studies have shown that oxidative stress and the MAPK pathway play important roles in stress-induced cellular and organ damage, and that heat stress increases MAPK activity in the small intestine, causing severe small intestinal injury, enhancing oxidative stress, and activating the MAPK signaling pathway [6]. As a key signaling hub linking extracellular stimuli to intracellular responses, members of the MAPK family—including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK—are activated under heat stress conditions [7]. It has been demonstrated that ERK1/2 MAPK mediates the downregulation of intestinal tight junction proteins under heat stress [8]. These kinases

subsequently regulate the expression of downstream apoptosis-related proteins (Bax/Bcl-2, Caspase-3), autophagy-related genes (Beclin-1, LC3, P62), and inflammatory factors (IL-1β, IL-6, TNF-α), thereby directly or indirectly affecting the distribution and expression levels of tight junction proteins (ZO-1, Occludin, Claudin-1). This cascade constitutes a key pathological mechanism mediating epithelial barrier dysfunction [9]. Therefore, targeting the MAPK signaling pathway to identify natural bioactive compounds that can effectively alleviate heat stress-induced rumen epithelial barrier damage has become a potential strategy to enhance stress resilience in ruminants.

Patchouli oil (PO) is derived from Pogostemon cablin, a plant traditionally used both as a medicine and a food ingredient. Its main active constituents are patchoulol and pogostone. Modern pharmacological studies have shown that patchouli oil possesses multiple biological functions, including scavenging free radicals, inhibiting lipid peroxidation, regulating gastrointestinal motility, promoting digestive secretion, exerting anti-inflammatory effects, and protecting the mucosal barrier. It has also shown good application potential in the fields of antibiotic replacement and heat stress management [10]. Previous studies from the corresponding author's laboratory showed that dietary supplementation with patchouli oil significantly improved rumen fermentation patterns, enhanced nutrient digestibility and utilization, and increased growth performance in heat-stressed beef cattle: treatment with 50 mg/kg patchouli oil increased average daily gain and reduced serum diamine oxidase activity and malondialdehyde content. Patchouli oil also upregulated the relative mRNA expression of occludin in the rumen epithelium while downregulating the relative mRNA expression of JNK, p38 MAPK, caspase-3 and Beclin-1, indicating that dietary patchouli oil may improve growth performance and repair damaged rumen epithelium in heat-stressed beef cattle by downregulating the MAPK signaling pathway [11]. In addition, another study from the same laboratory found that patchouli oil increased the mean optical density and mRNA expression of ZO-1 and occludin in rumen tissue. Transcriptomic analysis of the rumen epithelium revealed that patchouli oil upregulated the expression levels of genes involved in tight junction and DNA replication/repair pathways while downregulating pro-apoptotic genes [12]. These results suggest that patchouli oil may exert its anti-heat stress effects by preserving rumen epithelial barrier function. However, whether patchouli oil acts directly on rumen epithelial cells to repair heat stress-induced barrier damage, its specific mechanism of action, and the role of the MAPK signaling pathway in this process have not been reported, and systematic evidence at the cellular and molecular level is still lacking.

Accordingly, bovine rumen epithelial cells (BRECs) were used as the experimental material to establish an in vitro heat stress injury model. The effects of PO on cell viability, antioxidant capacity, apoptosis, cell morphology and barrier permeability under heat stress were systematically investigated. In addition, the regulatory effects of PO on the expression of tight junction proteins, apoptosis-related factors, key kinases of the MAPK pathway and downstream genes associated with inflammation and autophagy in BRECs were examined. It was hypothesized that PO would attenuate heat stress-induced barrier damage in BRECs by counteracting the heat stress-induced increase in MAPK pathway expression and the associated apoptotic, inflammatory and autophagic responses, thereby preserving tight junction integrity. This study aimed to elucidate the cellular and molecular mechanisms by which PO alleviates heat-induced damage to the rumen epithelial barrier, thereby providing a theoretical basis for its application as a functional feed additive to mitigate heat stress in ruminant production.

2. Methods

2.1. Experimental Materials

PO (purity ≥98%, patchoulol content ≥26%) was purchased from Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China). The rumen tissue was collected from healthy adult Simmental crossbreed beef cattle (approximately 650 kg body weight) at Jiangxi Niuniu Industrial Co., Ltd. (Nanchang, China). Within 15 min after slaughter, approximately 5 cm × 5 cm tissue samples from the posterior ventral blind sac of the rumen were rapidly excised, immersed in PBS buffer containing 2× penicillin-streptomycin and 2× gentamicin (Thermo Fisher Scientific, Waltham, MA, USA), and transported to the laboratory under ice-cold conditions. Rumen tissue was collected from three donor animals in total, and each cell isolation was performed with tissue from a single animal; the three independent cell preparations used as biological replicates were therefore each derived from a different donor animal, and cells of passages 4–8 from the same isolation were used within a given experiment to avoid inter-animal variability.

2.2. Primary Isolation, Culture, Purification, and Identification of Bovine Rumen Epithelial Cells

Primary BRECs were isolated, cultured, purified and characterized by methodological adaptations of the traditional sterile primary cell isolation protocol. Rumen tissue was repeatedly rinsed with sterile PBS containing 5× antibiotics (penicillin-streptomycin, gentamicin) to remove surface residues. After the external muscular and connective tissues had been removed, the rumen epithelial layer was minced into fragments of approximately 1 mm^3 on an ultra-clean workbench. The minced tissue was digested with 0.1% type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 30 min, followed by removal of the supernatant. Then, 0.25% trypsin was added, and digestion was carried out at 37 °C for another 30 min. The cell suspension was collected, and this digestion procedure was repeated three times [13]. Digestion was terminated with DMEM/F-12 containing 15% FBS. The suspension was filtered through a 200-mesh sieve, centrifuged at 1500 rpm for 5 min, and washed twice with PBS. The cell pellet was resuspended in complete culture medium (DMEM/F-12 + 15% FBS + 1% penicillin-streptomycin + 1% amphotericin B + 0.2% epidermal growth factor) and seeded into T25 culture flasks, then incubated at 37 °C in a 5% CO_2 incubator. Purification was performed by differential adhesion. Because fibroblasts attach to the culture surface more rapidly than epithelial cells, the culture medium containing the non-adherent cell population was transferred to a new flask 6 h after seeding, whereas the firmly adherent, fibroblast-enriched fraction was discarded. The transferred population was identified as epithelial by CK-18 immunofluorescence (Figure 1C–E), which was positive in essentially all cells, and no spindle-shaped fibroblast-like cells were observed after purification. The medium was changed every 2–3 days, and cells were passaged when they reached 80–90% confluence. Cells from passages 4–8 were used for the subsequent experiments.

For identification, BRECs were seeded onto sterile coverslips placed in 6-well plates and cultured to approximately 70% confluence. Subsequently, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 5% BSA for 30 min. The cells were then incubated with anti-CK-18 primary antibody (1:200, ab668, Abcam, Cambridge, UK) overnight at 4 °C. After washing with PBS, cells were incubated with Alexa Fluor 488-conjugated secondary antibody (1:500, ab150113, Abcam) for 1 h at room temperature in the dark. Nuclei were counterstained with DAPI for 10 min, and images were acquired with a laser scanning confocal microscope (Zeiss LSM 880, Carl Zeiss, Oberkochen, Germany) [14].

The MTT assay was used to evaluate the viability of BRECs. Cells at passage 4 were seeded into 96-well plates at 0.5–1 × 10^4 cells/mL (100 µL per well, i.e., 0.5–1 × 10^3 cells/well), with six replicate wells per time point. The MTT assay was performed daily at fixed times to

measure OD_{490} for 8 consecutive days, and a growth curve was plotted. The cryopreservation medium was FBS:DMSO = 9:1, and cells were stored in liquid nitrogen. After thawing, viability was assessed by trypan blue exclusion.

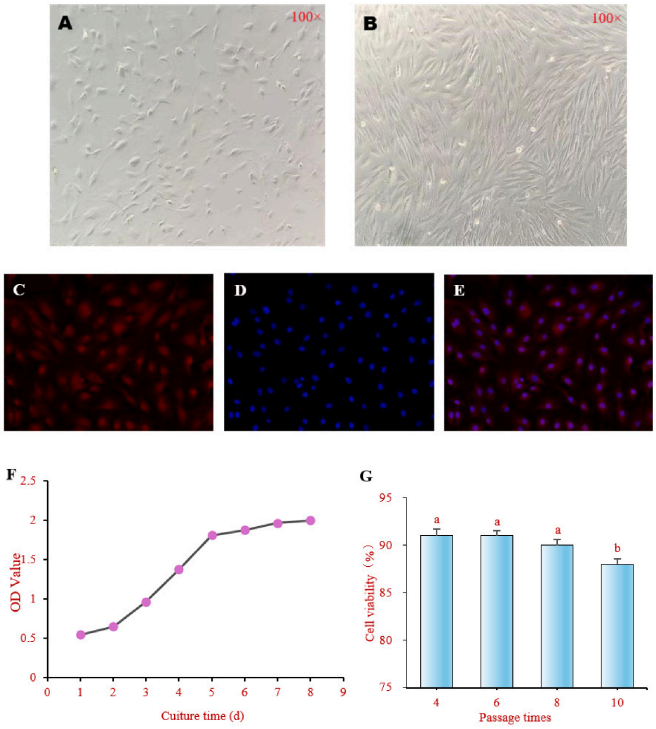

Figure 1. The morphology, identification and cell viability of primary rumen epithelial cells obtained through isolation and culture. Cells cultured for 3 days (**A**) and 6 days (**B**) after purification by the differential adherent method (100×); rumen epithelial cells stained with CK-18 (**C**), DAPI (**D**) and CK-18 + DAPI (**E**); growth curve of rumen epithelial cells (**F**); viability of frozen-thawed rumen epithelial cells at passages 4, 6, 8 and 10 (**G**). Data are expressed as means ± SEM (n = 6 wells; three independent biological replicates). Different letters indicate significant differences ($p < 0.05$).

2.3. Establishment of the Heat Stress Model

BRECs were precultured in 6-well plates (0.5–1 × 10^4 cells per well) with 2 mL of complete DMEM/F-12 culture medium under 5% CO_2 for 48 h. After the confluence exceeded 80%, cells were randomly divided into a control group (37 °C) and heat stress groups (42 °C and 43 °C) and treated for 1, 2, 4, 6 or 8 h. The following parameters were assessed in this study: relative cell viability, apoptosis rate, and the intracellular activities of SOD and T-AOC. The optimal heat stress conditions, including treatment temperature and duration, were determined on the basis of these comprehensive results. In the present study, the term "heat stress" is used to denote the in vitro thermal challenge applied to cultured BRECs, which corresponds to a cellular heat shock/thermal stress treatment rather than to heat stress at the whole-animal level.

2.4. Screening for the Optimal Treatment Concentration of Patchouli Oil

Cells were divided into two major groups: normothermic (37 °C) and heat-stressed (43 °C). In each group, a concentration gradient of PO (0, 10, 20, 30, 40 and 50 μg/mL) was tested. In this screening experiment, cells were pre-incubated with PO for 12 h before the thermal challenge, which allowed the compound to equilibrate within the cells; the normothermic group was then maintained at 37 °C, whereas the heat stress group was exposed to 43 °C for 4 h followed by recovery at 37 °C for 8 h in medium containing the same concentration of PO. The measured parameters included relative cell viability and the activ-

ities of SOD, CAT and LDH in cell lysates. The optimal concentration under normothermic conditions and the optimal protective concentration under heat stress were thus determined. At 37 °C, 10 and 20 μg/mL PO did not affect viability, whereas concentrations of 30 μg/mL and above reduced it; under heat stress, only 20 μg/mL PO significantly increased CAT and LDH activities, whereas 50 μg/mL further decreased cell viability. Because 10 μg/mL was insufficient to counteract heat stress-induced damage, 20 μg/mL was selected as the protective concentration under heat stress, whereas 10 μg/mL was used for the normothermic PO control.

2.5. Experimental Groups and Treatment

According to the optimized heat stress model and PO intervention conditions, cultured BRECs were divided into four groups (three wells per group) as follows. Control group (CG): cells were cultured in standard complete medium at 37 °C for 12 h. Patchouli oil group (PO): cells were cultured in complete medium containing 10 μg/mL PO at 37 °C for 12 h. Heat stress group (HS): cells were cultured in standard complete medium and exposed to 43 °C for 4 h, after which the medium was replaced with fresh medium and the cells were allowed to recover at 37 °C for 8 h. Heat stress + patchouli oil group (HS+PO): cells were cultured in complete medium containing 20 μg/mL PO and simultaneously exposed to 43 °C for 4 h, after which the medium was replaced with fresh medium containing 20 μg/mL PO and the cells were allowed to recover at 37 °C for 8 h. PO was present during both the thermal challenge and the recovery period in order to mimic the continuous exposure of rumen epithelial cells to a feed-derived compound, whereas the 12 h pre-incubation in Section 2.4 was used only for concentration screening. An equal volume of the vehicle (90% ethanol; final concentration <0.1%, v/v) was added to the CG and HS groups. After the treatments, the following parameters were measured: cell viability, apoptosis rate, antioxidant parameters (SOD, CAT, GSH-Px, MDA and T-AOC) and LDH activity, TEER, cell morphology by scanning electron microscopy (SEM), mRNA expression of target genes by quantitative real-time PCR (qRT-PCR), and protein expression of MAPK pathway components, tight junction proteins and apoptosis-related proteins by immunofluorescence.

2.6. Cell Viability Assay

Cell viability was assessed by using the MTT method. Cells were seeded in 96-well plates at 0.5–1 × 10^4 cells/mL (100 μL per well). After treatment according to the groups, 20 μL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated for an additional 4 h at 37 °C. The supernatant was aspirated, and 150 μL of DMSO was added to each well. After shaking for 10 min, the OD_{490} was measured with a microplate reader (Multiskan FC, Thermo Fisher Scientific, Waltham, MA, USA). Blank wells (medium and MTT without cells) were included on each plate, and their absorbance was subtracted when the relative viability was calculated.

$$\text{Relative cell viability (\%)} = [\text{OD(experimental group)} - \text{OD(blank group)}]/[\text{OD(control group)} - \text{OD(blank group)}] \times 100\%$$

2.7. Apoptosis Detection

Apoptosis was detected with the Annexin V-FITC/PI kit according to the manufacturer's instructions. Cells were collected, washed with PBS, and resuspended in 500 μL of binding buffer (1–5 × 10^5 cells). Then, 5 μL of Annexin V-FITC and 5 μL of PI were added, and the cells were incubated for 15 min at room temperature in the dark. The samples were analyzed by flow cytometry (BD Accuri C6, BD Biosciences, San Jose, CA, USA); at least 10,000 events were acquired per sample, and the percentages of early apoptotic (Annexin

V+/PI−) and late apoptotic (Annexin V+/PI+) cells were calculated with FlowJo software (v10.8, FlowJo, LLC, Ashland, OR, USA).

2.8. Determination of Antioxidant Parameters

Cells were collected, washed with PBS, and lysed with an ultrasonic cell disruptor on ice. After centrifugation, the supernatant was collected. The activities of SOD, CAT, LDH and GSH-Px, as well as the MDA content and T-AOC, were measured in cell lysates with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturers' protocols. In the main experiment, these parameters were additionally measured in the culture supernatant (Section 3.4). Protein concentration was determined by using the BCA method and used to normalize the results.

2.9. Transepithelial Electrical Resistance (TEER) Measurement

Cells were seeded into 24-well Transwell inserts (0.4 μm pore size, 0.33 cm^2 membrane area; Corning, Corning, NY, USA) at 0.5–1 × 10^4 cells/mL in 100 μL of medium per insert. The medium was changed daily, and TEER was measured with a Millicell ERS-2 volt-ohmmeter (Millipore, Billerica, MA, USA) after the plates had been equilibrated to room temperature in a laminar flow cabinet. For every measurement, the resistance of a cell-free insert (blank) was subtracted from the resistance of the sample, and the corrected value was multiplied by the membrane area to obtain TEER in Ω·cm^2. After stable TEER values had been achieved (approximately 7 days), the cells were treated according to the groups, and the final TEER values were measured within 24 h after treatment [15].

2.10. Scanning Electron Microscopy

After the different treatments, cells grown on coverslips were fixed with 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated through a graded ethanol series, critical-point dried, sputter-coated with gold, and observed with a scanning electron microscope (Hitachi S-3000N, Tokyo, Japan) to examine the surface ultrastructure [16].

2.11. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted with TRIzol reagent (TaKaRa, Dalian, China) and reverse-transcribed into cDNA with a PrimeScript RT reagent kit (TaKaRa). The qPCR reaction system (20 μL) consisted of 10 μL of 2× SYBR Green Mix, 0.4 μL of each forward and reverse primer, 2 μL of cDNA, and 7.2 μL of ddH_2O. The reaction conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s (annealing and extension). Gene-specific primers were designed with PrimerSelect based on bovine sequences retrieved from the NCBI database and were synthesized by BGI Genomics (Shenzhen, China); the primer sequences are shown in Table 1. The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method [17], with β-actin (ACTB) as the internal reference gene. The pro-inflammatory cytokines IL-1β, IL-6 and TNF-α were quantified at the mRNA level only, with the primers listed in Table 1; no protein-based detection of these cytokines was performed.

Table 1. Real-time quantitative PCR primer sequences.

Genes	Accession Number	Sequences of Primer (5′-3′)
ACTB (β-actin)	NM_173979.3	F: CCCTGGAGAAGAGCTACGAG R: CAGGAAGGAAGGCTGGAAGA
BCL2	XM_027526256.1	F: ATGACCGAGTACCTGAACCG R: GCCATACAGCTCCACAAAGG
BAX	XM_027515208.1	F: CCCGAGTTGATCAGGACCAT R: GTGGGTGTCCCAAAGTAGGA
CASP3 (Caspase-3)	XM_010820245.3	F: GAACGTCCCTTTCTGCCATC R: TGCAAAGGATGGATGAGGGT
CLDN1 (Claudin-1)	XM_027538401.1	F: GGCAGATCCAGTGCAAAGTC R: ATGCCAATCACCATCAAGGC
TJP1 (ZO-1)	XM_019983400.1	F: GCAGCCTGCATACAGATACG R: GGTAAGGCTGCTTGTCATCG
OCLN (Occludin)	XM_027520117.1	F: AGATGCACGTTCGACCAATG R: ATTACTCCGGGAGGAGAGGT
MAPK14 (p38)	XM_024983652.1	F: CTTTGACCCAGATGCCGAAG R: GTCGTCAGGATCGTGGTACT
MAPK1/MAPK3 (ERK1/2)	XM_027526589.1	F: TTCAGATTCTGCTGCGCTTC R: AGACATCCCTCATGGCTTCC
MAPK8/MAPK9 (JNK)	NM_001192974.2	F: CGCTACTACAGAGCACCTGA R: GCACCCAACTGACCAAATGT
MAP1LC3B (LC3)	XM_027513856.1	F: GTCCGACTTATCCGAGAGCA R: TGAGCTGTAAGCGCCTTCTT
SQSTM1 (p62)	XM_027548457.1	F: CTCCGGAAGCTGAAACATGG R: ACTGGGATCTTCCGATGGAC
BECN1 (Beclin-1)	NM_001033627.2	F: ACTGGACACGAGCTTCAAGA R: GGCTGTGGCAAGTAATGGAG
IL1B (IL-1β)	XM_027555462.1	F: GTCCACGTGGGCTGAATAAC R: ACTAATGCAGGGAAGGCAGT
IL6 (IL-6)	NM_173923.2	F: TGAGTGTGAAAGCAGCAAGG R: AAGACCAGCAGTGGTTCTGA
TNF (TNF-α)	XM_027524120.1	F: GAGATCAACCTGCCGGACTA R: ACTGAGGTGGGAGAAGATGC

F: forward primer; R: reverse primer. Gene symbols follow the NCBI nomenclature and the protein names used in the text are given in parentheses: *ACTB*, *β-actin* (used as the internal reference gene); *BCL2*, B-cell lymphoma-2 (*Bcl-2*); *BAX*, Bcl-2-associated X protein; *CASP3*, cysteine-aspartic protease-3 (*Caspase-3*); *CLDN1*, *Claudin-1*; *TJP1*, tight junction protein-1 (*ZO-1*); *OCLN*, *Occludin*; *MAPK14*, p38 mitogen-activated protein kinase; *MAPK1/MAPK3*, extracellular signal-regulated kinase 1/2 (*ERK1/2*); *MAPK8/MAPK9*, c-Jun N-terminal kinase (*JNK*); *MAP1LC3B*, microtubule-associated protein 1 light chain 3 β (*LC3*); *SQSTM1*, sequestosome-1 (*p62*); *BECN1*, *Beclin*-1; *IL1B*, interleukin-1β; *IL6*, interleukin-6; *TNF*, tumor necrosis factor-α.

2.12. Detection of Protein Expression by Immunofluorescence Staining

Cells grown on coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5% BSA. They were then incubated overnight at 4 °C with primary antibodies against p38 (ab31828, Abcam), ERK (ab17942, Abcam), JNK (ab179461, Abcam), phosphorylated p38 (ab4822, Abcam), phosphorylated ERK (ab201015, Abcam), phosphorylated JNK (ab124956, Abcam), Claudin-1 (ab15098, Abcam), ZO-1 (ab221547, Abcam), Occludin (ab216327, Abcam), Bcl-2 (ab182858, Abcam), Bax (ab32503, Abcam) and Caspase-3 (ab184787, Abcam), all at a 1:200 dilution. After washing, the cells were incubated with the corresponding Alexa Fluor 488/555/647-conjugated secondary antibodies (1:500, Invitrogen, Waltham, MA, USA) for 1 h at room temperature in the dark. Nuclei were counterstained with DAPI. Images were acquired with a confocal laser scanning microscope (Zeiss LSM 880) using identical laser power, gain and pinhole settings for all groups, and the mean optical density of at least five randomly selected fields per

well was analyzed with ImageJ software (v1.53t, NIH, Bethesda, MD, USA). In the present experiments, the phosphorylated kinases were included in the antibody panel, but only total p38, ERK and JNK were quantified and are reported below; the immunofluorescence data for p38, ERK and JNK therefore reflect total protein abundance rather than kinase activation. IL-1β, IL-6 and TNF-α were not examined by immunofluorescence and were quantified only at the mRNA level by qRT-PCR (Section 2.11, Table 1); no antibody details or protein detection procedures are therefore required for these cytokines.

2.13. Statistical Analysis

Data were analyzed with SPSS 26.0 software (SPSS Inc., Chicago, IL, USA). Each treatment comprised three wells per group, except for the growth curve measurements, for which six wells were used per time point. Every data set was obtained from three independent biological replicates, each performed with a separate cell preparation from a different donor animal, and the replicate wells within an experiment were treated as technical replicates. The n given in the figures, tables and text therefore refers to the number of technical replicate wells (six or three per group, as indicated in each legend), the values shown are the means ± SEM of these wells, and the three biological replicates gave consistent results. Normality of the data was assessed with the Shapiro–Wilk test and homogeneity of variance with Levene's test, and both assumptions were met. In the screening experiments (temperature × duration and temperature × concentration), each treatment combination was treated as an independent level and analyzed by one-way analysis of variance (ANOVA), because the purpose was to identify a single optimal condition rather than to partition interaction effects. In the main experiment, differences among the four groups were analyzed by one-way ANOVA followed by Tukey's post hoc test for all pairwise comparisons. Because multiple outcomes were measured, the significance level was set at $p < 0.05$ for each endpoint, and the results should be interpreted with this in mind. GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for graphical rendering. Results are presented as the mean ± standard error of the mean (SEM).

3. Results

3.1. Isolation, Identification and Viability of Primary Bovine Rumen Epithelial Cells

As shown in Figure 1A,B, after purification by differential adhesion, the cells exhibited uniform morphology, a transparent cytoplasm and clear outlines. CK-18 immunofluorescence staining showed that the cytoplasm of all cells was positively stained with green fluorescence, and nuclei were counterstained blue with DAPI, confirming that the harvested cells were epithelial cells and that the purified population was essentially free of fibroblasts (Figure 1C–E). Furthermore, Figure 1F shows that the growth curve displayed a typical "S" shape. Cell viability decreased significantly from passage 10 onward ($p < 0.05$, Figure 1G). Therefore, cells from passages 4 to 8 were used in the subsequent experiments.

3.2. Determination of the Heat Stress Model

Cell viability was assessed under conditions that ensured heat stress-induced damage while preserving recovery capacity. As shown in Figure 2A, treatment at 43 °C for 4 h significantly reduced cell viability ($p < 0.05$). Compared with the control group, the activities of SOD and T-AOC were significantly decreased ($p < 0.05$, Figure 2B), whereas the apoptosis rate was significantly increased ($p < 0.05$, Figure 2C,D). Therefore, treatment at 43 °C for 4 h was selected as the optimal condition for establishing the BREC heat stress model.

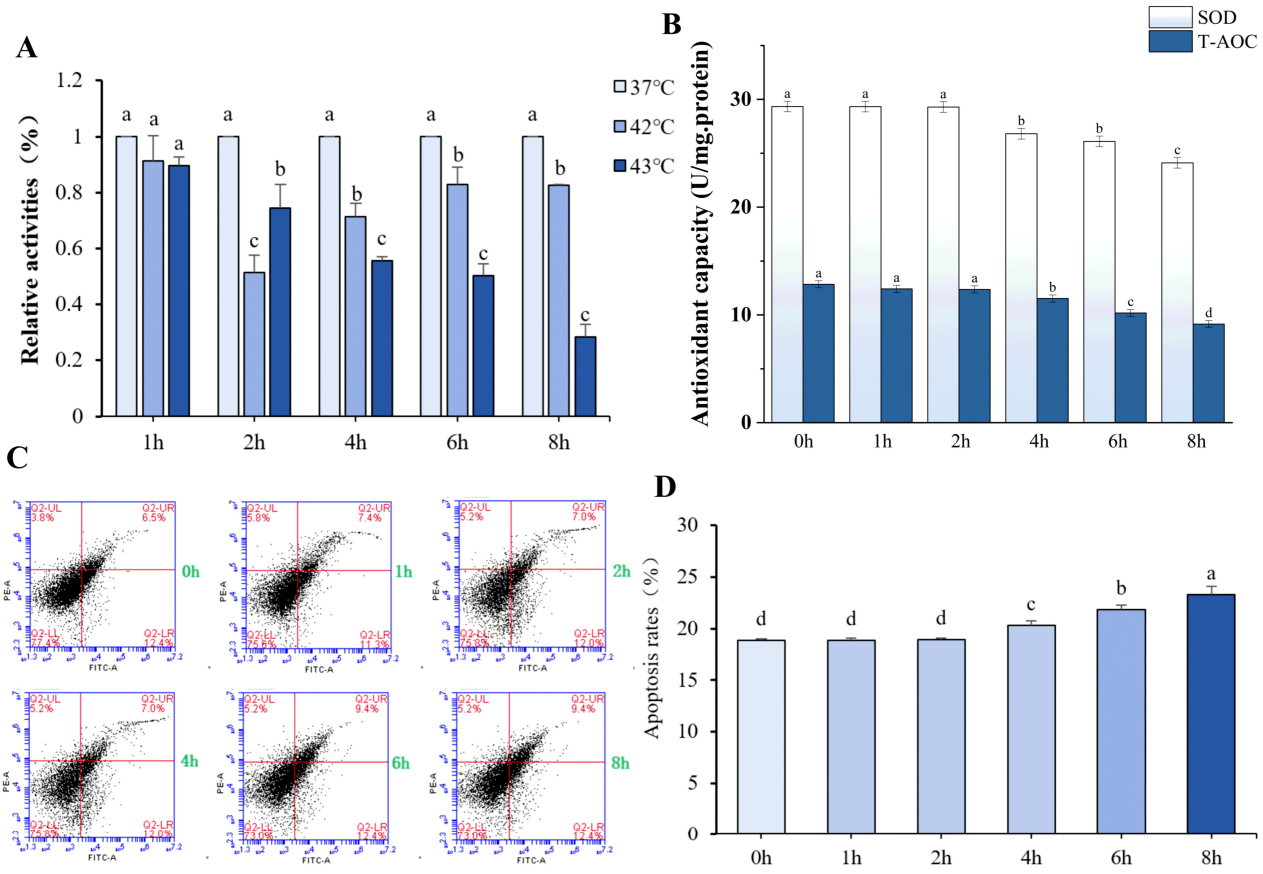

Figure 2. Effect of heat stress on rumen epithelial cells. Relative cell viability after treatment at 42 °C and 43 °C for different durations (**A**); intracellular SOD and T-AOC activities after treatment at 43 °C for 0, 1, 2, 4, 6 and 8 h (**B**); representative flow cytometry plots of apoptosis after treatment at 43 °C for 0, 1, 2, 4, 6 and 8 h (**C**); apoptosis rate in same groups (**D**). Data are expressed as means ± SEM (n = 3 wells; three independent biological replicates). Different letters indicate significant differences ($p < 0.05$).

3.3. Determination of the Optimal Treatment Concentration of Patchouli Oil

As shown in Figure 3A, compared with the control group, the addition of 10 or 20 μg/mL PO at 37 °C had no significant effect on the relative viability of BRECs ($p > 0.05$), whereas concentrations of 30 μg/mL and above reduced viability in a concentration-dependent manner ($p < 0.05$). Under heat stress (Figure 3B), compared with the HS group, the addition of 50 μg/mL PO significantly reduced relative cell viability ($p < 0.05$), whereas 20 μg/mL PO significantly increased the activities of SOD, CAT and LDH ($p < 0.05$, Figure 3C) and lowered the apoptosis rate ($p < 0.05$, Figure 3E,F). The apoptosis rate of the HS+PO group remained slightly higher than that of the CG group, whereas it was significantly lower than that of the HS group. On the basis of these results, 10 μg/mL PO at 37 °C and 20 μg/mL PO under 43 °C heat stress were selected as the treatment conditions for the subsequent experiments, the selection being based on the viability, antioxidant and apoptosis data rather than on viability alone.

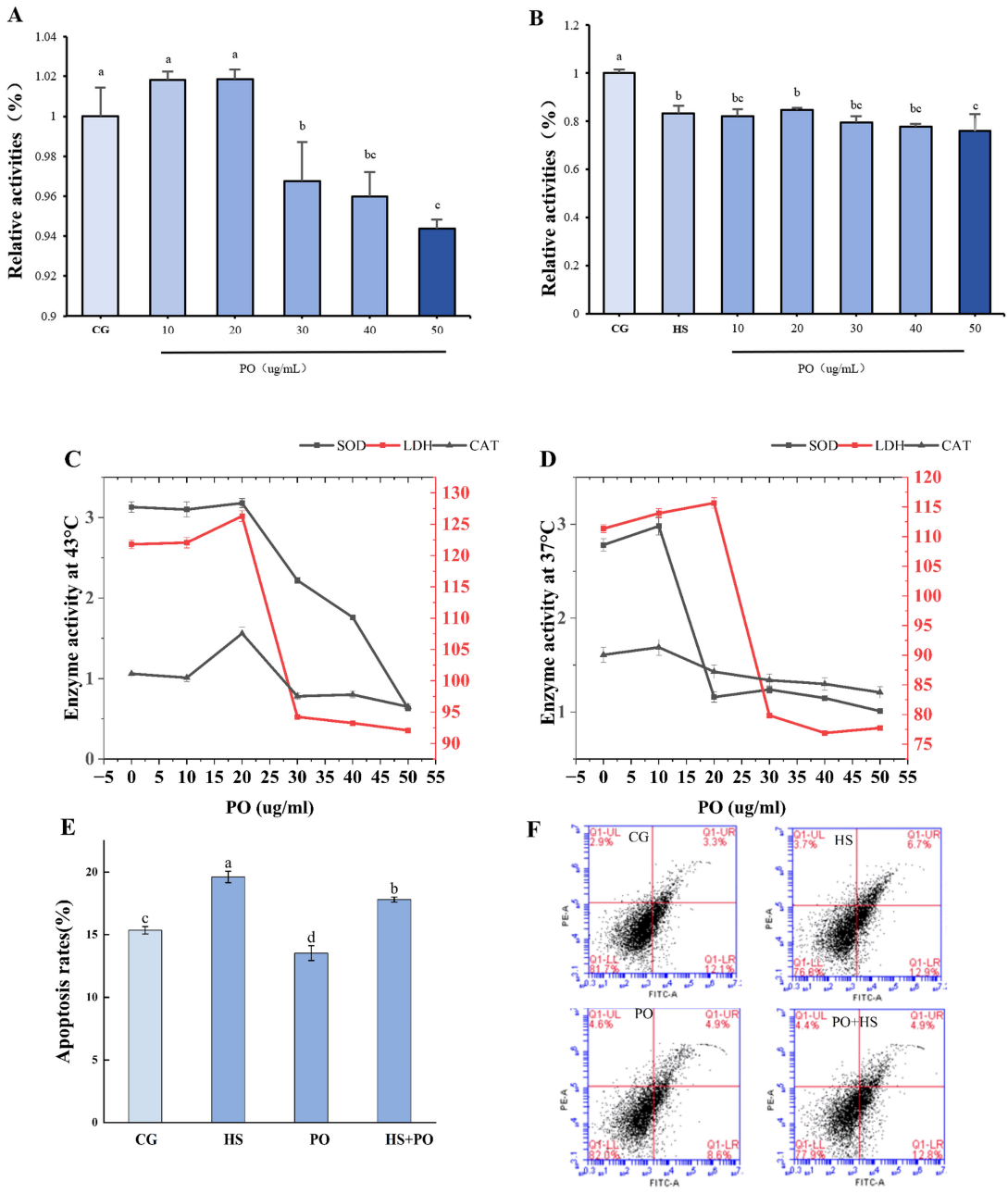

Figure 3. Determination of the optimal concentration of patchouli oil (PO) in rumen epithelial cells. Relative cell viability of BRECs treated with different concentrations of PO (0–50 μg/mL) at 37 °C (**A**) and under heat stress at 43 °C (**B**); activities of SOD, CAT and LDH in cell lysates under heat stress at 43 °C (**C**) and at 37 °C (**D**); apoptosis rate in the control (CG), PO, heat stress (HS) and HS+PO groups (**E**); representative flow cytometry plots of apoptosis in the same groups (**F**). Data are expressed as means ± SEM (n = 3 wells; three independent biological replicates). Different letters indicate significant differences ($p < 0.05$).

3.4. Effects of Patchouli Oil on Antioxidant Parameters in Culture Supernatant

To complement the oxidative stress data obtained in the concentration-screening experiment, the activities of SOD, CAT, LDH and GSH-Px, the T-AOC and the MDA content were also measured in the culture supernatant of the four treatment groups (Table 2). Relative to the CG group, the HS group showed higher SOD, CAT, LDH, GSH-Px and T-AOC activities and a lower MDA content in the supernatant ($p < 0.05$). Because these enzymes are normally far more abundant in the cytosol than in the extracellular space, the

increase in supernatant enzyme activity is most consistent with leakage of intracellular enzymes across a damaged plasma membrane rather than with enhanced antioxidant capacity, an interpretation supported by the concomitant rise in supernatant LDH, a classical marker of membrane integrity. Patchouli oil co-treatment significantly reduced the leakage of SOD, CAT, LDH and GSH-Px and lowered the supernatant T-AOC relative to the HS group ($p < 0.05$), whereas supernatant MDA was higher than in the HS group, possibly reflecting reduced consumption of MDA in the medium. These data indicate that patchouli oil preserves plasma membrane integrity under heat stress. The lower supernatant MDA concentration in the HS group should, however, be interpreted with caution, and measurement of MDA in cell lysates would be required in order to draw firm conclusions about lipid peroxidation.

Table 2. Effect of patchouli oil on antioxidant parameters in culture supernatant of heat-stressed bovine ruminal epithelial cells.

Parameter	CG	HS	PO	HS+PO	SEM	*p* Value
SOD (U/mg protein)	12.82 ^{bc}	14.42 ^{a}	12.37 ^{c}	13.21 ^{b}	0.24	0.004
CAT (U/mg protein)	0.67 ^{c}	1.73 ^{a}	0.47 ^{d}	1.22 ^{b}	0.09	0.013
LDH (U/L)	116.91 ^{c}	202.90 ^{a}	98.49 ^{d}	142.03 ^{b}	11.93	0.042
GSH-Px (U/mg protein)	57.47 ^{c}	91.39 ^{a}	50.48 ^{d}	65.47 ^{b}	3.63	0.035
MDA (nmol/mg protein)	1.89 ^{a}	0.70 ^{c}	2.04 ^{a}	1.44 ^{b}	0.12	<0.001
T-AOC (U/mg protein)	0.11 ^{b}	0.13 ^{a}	0.09 ^{c}	0.11 ^{b}	0.00	0.001

Data are expressed as means ± SEM (n = 3 wells; three independent biological replicates). Values with different superscript letters within a row differ significantly ($p < 0.05$). CG: control group; HS: heat stress group; PO: patchouli oil group (10 μg/mL, 37 °C); HS+PO: heat stress + patchouli oil group (20 μg/mL). SOD: superoxide dismutase; CAT: catalase; LDH: lactate dehydrogenase; GSH-Px: glutathione peroxidase; MDA: malondialdehyde; T-AOC: total antioxidant capacity.

3.5. Patchouli Oil Maintains Rumen Epithelial Barrier Integrity

Heat stress treatment significantly reduced the TEER value ($p < 0.05$, Figure 4A), indicating increased paracellular permeability. Scanning electron microscopy revealed that heat stress treatment led to cell shrinkage, a rough surface and loss of intercellular contacts (Figure 4B). However, 20 μg/mL patchouli oil significantly alleviated the heat stress-induced decrease in TEER values ($p < 0.05$, Figure 4A) and restored normal cell morphology, with cells appearing flatter, exhibiting a smoother surface and maintaining intact intercellular boundaries (Figure 4B).

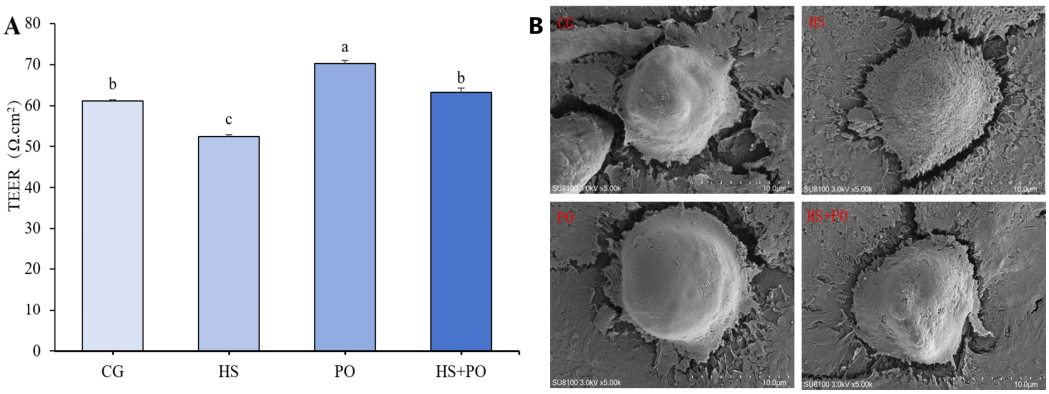

Figure 4. Protective effect of patchouli oil on rumen epithelial barrier integrity under heat stress. Transepithelial electrical resistance (TEER) values in control, PO, heat stress (HS), and HS+PO groups (**A**); scanning electron microscopy images of cell surface morphology in each group (scale bar = 10 μm, magnification 5000×) (**B**). Data are expressed as means ± SEM (n = 3 wells; three independent biological replicates). Different letters indicate significant differences ($p < 0.05$).

3.6. Patchouli Oil Modulates the Expression of MAPK Pathway Components Under Heat Stress

Compared with the CG group, heat stress significantly increased the total protein expression of p38, ERK and JNK, as shown by the immunofluorescence images (Figure 5A) and by the corresponding mean optical density values (Figure 5B), and increased their mRNA levels (Figure 5C) ($p < 0.05$). Compared with the HS group, the protein (Figure 5A,B) and mRNA (Figure 5C) expression levels of p38, ERK and JNK in the HS+PO group were significantly decreased ($p < 0.05$) and were comparable to those of the CG group. Among the three kinases, *ERK* showed the most pronounced change in both the mean optical density and the mRNA level (Figure 5B,C). These data therefore describe the amounts of total p38, ERK and JNK protein and mRNA and do not constitute a measure of kinase activation, because the phosphorylated forms of these kinases were not quantified.

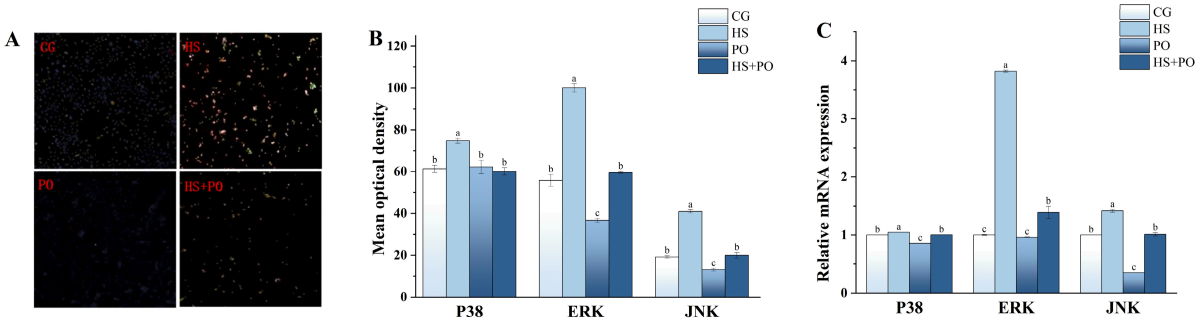

Figure 5. Modulation of the MAPK signaling pathway by patchouli oil in heat-stressed rumen epithelial cells. Immunofluorescence images of p38 (red), ERK (green) and JNK (yellow) in each group (**A**); mean optical density of p38, ERK and JNK (**B**); relative mRNA expression of *p38*, *ERK* and *JNK* determined by qRT-PCR (**C**). Data are expressed as means ± SEM (n = 3 wells; three independent biological replicates). Different letters indicate significant differences ($p < 0.05$).

3.7. Patchouli Oil Regulates the Expression of Genes and Proteins Related to Autophagy, Inflammation and Barrier Function

Heat stress significantly increased the fluorescence intensity of the pro-apoptotic proteins Bax and Caspase-3 and decreased that of the anti-apoptotic protein Bcl-2, as shown by the immunofluorescence images (Figure 6A) and confirmed by the mean optical density values ($p < 0.05$, Figure 6B), and it also markedly reduced the immunofluorescence intensity of the tight junction proteins Claudin-1, ZO-1 and Occludin ($p < 0.05$, Figure 6A). In contrast, patchouli oil treatment reversed these effects, reducing Bax and Caspase-3 expression ($p < 0.05$, Figure 6A,B) and restoring Bcl-2, Claudin-1, ZO-1 and Occludin to near-control levels ($p < 0.05$, Figure 6A). In addition, heat stress significantly upregulated the mRNA levels of the pro-apoptotic genes Bax and Caspase-3, the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, and the autophagy markers Beclin-1, P62 and LC3 ($p < 0.05$, Figure 6C). Conversely, the mRNA expression of anti-apoptotic Bcl-2 and of the tight junction genes Claudin-1, ZO-1 and Occludin was significantly downregulated by heat stress ($p < 0.05$, Figure 6C). Treatment with patchouli oil significantly reversed these changes, downregulating the heat stress-induced increases in apoptotic, inflammatory and autophagy-related genes and upregulating the expression of tight junction genes ($p < 0.05$, Figure 6C). It should be noted that the autophagy-related genes were quantified at the mRNA level only, so that these data indicate an altered autophagic program rather than a change in autophagic flux (see Section 4). The pro-inflammatory cytokines IL-1β, IL-6 and TNF-α were quantified only at the mRNA level by qRT-PCR (Figure 6C); their values in Figure 6 therefore represent relative mRNA expression rather than protein abundance.

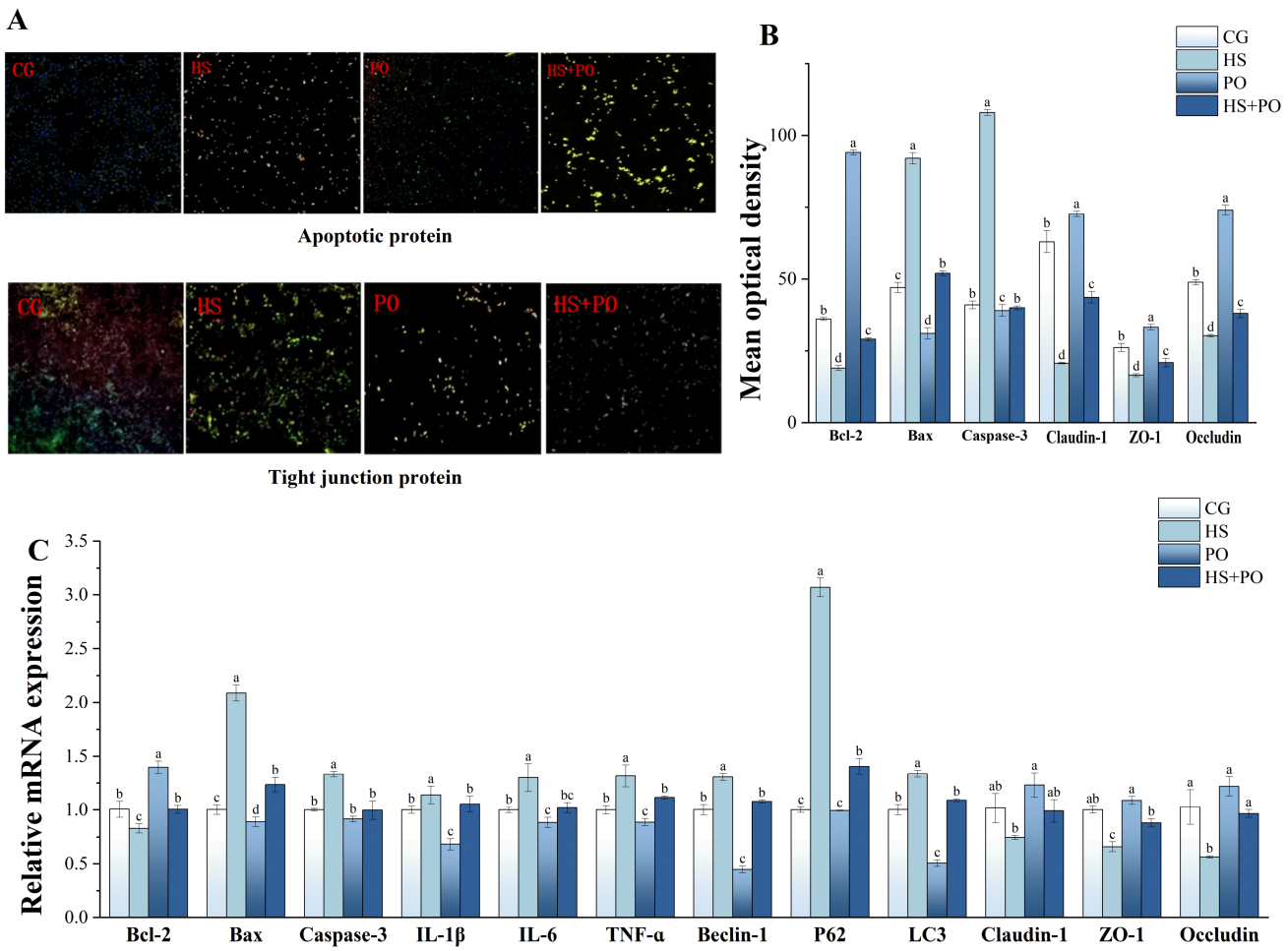

Figure 6. Regulation of apoptosis, inflammation, autophagy and tight junction proteins by patchouli oil in heat-stressed rumen epithelial cells. Immunofluorescence images of Bcl-2 (red), Bax (green) and Caspase-3 (yellow) (upper panels) and of Claudin-1 (red), ZO-1 (green) and Occludin (yellow) (lower panels) in each group (**A**); mean optical density of Bcl-2, Bax, Caspase-3, Claudin-1, ZO-1 and Occludin (**B**); relative mRNA expression of *Bcl-2*, *Bax*, *Caspase-3*, *IL-1β*, *IL-6*, *TNF-α*, *Beclin-1*, *P62*, *LC3*, *Claudin-1*, *ZO-1* and *Occludin* determined by qRT-PCR (**C**). Data are expressed as means ± SEM (n = 3 wells; three independent biological replicates). Different letters indicate significant differences ($p < 0.05$). The values for IL-1β, IL-6 and TNF-α in (**C**) are relative mRNA expression levels measured by qRT-PCR; these cytokines were not detected with antibodies, and no protein data are presented for them.

4. Discussion

This study aimed to establish an in vitro model with which to investigate the cyto-protective effects of patchouli oil on heat stress-induced injury in primary bovine rumen epithelial cells and to elucidate the underlying molecular mechanisms. The main findings indicate that patchouli oil protects rumen epithelial cells from heat stress-induced barrier dysfunction, and that this protection is accompanied by modulation of the MAPK signaling pathway and of its downstream effects on apoptosis, inflammation, autophagy and tight junction integrity. Establishing a reliable in vitro model was a critical first step. Consistent with previous reports in various cell types, exposure of rumen epithelial cells to 43 °C for 4 h induced a clear state of cellular stress, characterized by reduced viability, oxidative stress and increased apoptosis [18]. The decline in antioxidant enzyme activities (SOD, T-AOC) indicates disruption of redox balance, a hallmark of heat stress [19]. This model provides a useful platform for screening and testing potential protective agents such as patchouli oil.

The MAPK signaling pathway is a well-established mediator of cellular responses to environmental stressors [20]. The present results are consistent with those of Yu et al. [21], who reported that heat stress activates the MAPK signaling pathway in the rat small intestine. The present study further shows that heat stress increased the expression of all three major MAPK branches (ERK, JNK and p38) in rumen epithelial cells at both the mRNA and the total protein level, and that patchouli oil treatment attenuated these increases, which indicates that patchouli oil reduces the expression of these kinases. Because only total rather than phosphorylated p38, ERK and JNK were quantified, these findings report changes in the amount of MAPK pathway components and cannot be taken as a measure of kinase activity, or as evidence for activation or inhibition of the pathway; whether patchouli oil acts upstream of, or at the level of, these kinases to attenuate stress signaling therefore remains a hypothesis. Notably, patchouli alcohol, the main active component of patchouli oil, has been reported to act by inhibiting the MAPK signaling cascade [7]. It should be emphasized, however, that total rather than phosphorylated p38, ERK and JNK were quantified in the present experiments; the data therefore demonstrate altered expression of MAPK pathway components rather than a direct measure of kinase activity, and the causal role of reduced MAPK pathway expression in the protective effect of patchouli oil remains to be established by inhibitor or knockdown experiments.

Patchouli oil is a complex essential oil whose composition varies with plant origin, harvest time and extraction procedure, and constituents other than patchoulol and pogostone (for example other sesquiterpenes, alcohols and aldehydes) may also contribute to the effects observed here. In the present study, a reference-grade patchouli oil (purity ≥98%, patchoulol content ≥26%) was used, and a single batch was applied throughout to ensure internal consistency; however, a complete chromatographic (GC-MS) profile of the oil was not obtained, and the contribution of the individual constituents, as well as possible synergy or antagonism among them, cannot be resolved with the present design. Both patchoulol and pogostone have documented antioxidant, anti-inflammatory and barrier-protective activities; experiments with the purified compounds, alone and in combination, together with full GC-MS characterization of the oil, are therefore planned in order to identify the constituents responsible for the protection observed here.

Activation of the MAPK pathway is known to orchestrate downstream cellular programs, including apoptosis, inflammation and autophagy, which collectively contribute to barrier disruption. For example, the p38 MAPK pathway has been shown to regulate heat stress-induced ROS accumulation and apoptotic cell death [21]. In the present study, the heat stress-induced upregulation of the pro-apoptotic factors Bax and Caspase-3 and the concomitant downregulation of the anti-apoptotic factor Bcl-2 were reversed by patchouli oil, which suggests that its anti-apoptotic effect may be associated with the reduced expression of MAPK pathway components. This is consistent with previous findings in bovine ovarian granulosa cells, in which heat stress induced apoptosis via the BAX/BCL-2 pathway [22]. Similarly, the reduction in pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and in the key autophagy markers (Beclin-1, LC3, P62) in the HS+PO group further underscores the broad association between reduced MAPK pathway expression and the protective effects of patchouli oil. Notably, a 2024 study reported that niacin supplementation in heat-stressed beef cattle significantly reduced plasma IL-1β, IL-6 and TNF-α concentrations while upregulating the expression of the tight junction proteins ZO-1 and Occludin, which is in line with the present observations for patchouli oil [3]. Moreover, the anti-inflammatory activity of patchouli alcohol has been demonstrated to reduce the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 by inhibiting the NF-κB and MAPK signaling pathways [23]. Recent nutritional interventions in ruminants have similarly shown that improvement of antioxidant capacity and immune function, together with reinforcement

of the intestinal barrier, can alleviate stress-induced injury: dietary cecropin supplementation improved growth performance, serum antioxidant status and rumen fermentation in goats [24], and a probiotic strain attenuated high-concentrate diet-induced hepatic injury along the gut–liver axis by strengthening intestinal barrier function and reducing oxidative stress [25]. These findings support the concept that the epithelial barrier and the systemic antioxidant and immune status are closely coupled, and they are consistent with the barrier-protective effect of patchouli oil observed here.

The present study links MAPK-associated signaling to the disruption of tight junctions, which are core structural determinants of epithelial barrier function. Tight junction integrity, reflected by the expression of ZO-1, Occludin and Claudin-1, was severely compromised by heat stress, leading to decreased TEER values and to morphological changes visible by SEM. This is consistent with previous reports on intestinal epithelial barrier function under heat stress, which indicate that heat stress reduces the mRNA expression of ZO-1, Occludin and Claudin [26]. Gan et al. found that patchouli oil significantly upregulated the mRNA expression of ZO-1 and Occludin in a rat model of 5-fluorouracil-induced intestinal mucositis while decreasing the expression of Caspase-8, Caspase-3 and Bax and increasing Bcl-2 expression, indicating that patchouli oil not only repairs tight junction proteins but also participates in barrier protection through anti-apoptotic mechanisms [10]. Patchouli alcohol has also been reported to enhance the expression of ZO-1, ZO-2, Claudin-1 and Occludin in colonic tissue. Although these studies were performed in intestinal rather than ruminal epithelium, the tight junction complex is structurally conserved along the gastrointestinal tract, and rumen epithelial cells express the same core components (ZO-1, Occludin and Claudin-1); the present data therefore extend these observations to the ruminal epithelium. The restoration of these tight junction proteins and of barrier function by patchouli oil provides a link between modulation of the MAPK-associated response and the maintenance of epithelial integrity. Notably, the recovery of Claudin-1, ZO-1 and Occludin expression in the HS+PO group suggests that the action of patchouli oil is not merely general cytoprotection but includes a specific effect on barrier restoration.

Regarding autophagy, the present study found that heat stress upregulated the expression of the autophagy markers Beclin-1, LC3 and P62. This is consistent with the observations of Kassis et al. [18], who reported increased levels of Beclin-1 and LC3-II/LC3-I in cells subjected to heat shock at 42 °C. Zhuo et al. found that patchouli alcohol significantly inhibited CUMS-induced increases in LC3-II and p62 levels and restored autophagic flux by activating the mTOR signaling pathway [27]. Fei et al. further demonstrated that patchouli alcohol inhibits autophagy by regulating the ERK and NF-κB signaling pathways, providing an explanation at the MAPK pathway level [28]. Li et al. confirmed that heat stress simultaneously induces apoptosis and autophagy via the p38 MAPK/MK2/Hsp27-78 pathway and that inhibition of the p38 pathway significantly reduced LC3-II expression, indicating that p38 MAPK is a key node integrating the regulation of autophagy and apoptosis [29]. Autophagy occupies an ambiguous position in the heat stress response, since a moderate autophagic response removes damaged proteins and organelles and favors survival, whereas excessive or sustained autophagy contributes to cell death; the balance between autophagy and apoptosis therefore determines cell fate. Patchouli oil treatment reduced the heat stress-induced overexpression of autophagy-related genes, which suggests that it may shift this balance towards survival by attenuating excessive autophagic signaling. This interpretation must, however, remain provisional: Beclin-1, LC3 and P62 were quantified at the mRNA level only, and static markers cannot distinguish increased autophagosome formation from impaired autophagic degradation. The present data therefore indicate an altered autophagic program rather than a change in autophagic flux, and confirmation of the effect of patchouli oil on autophagy requires LC3-II/LC3-I immunoblotting, GFP-

LC3 punctum counting and flux assays with lysosomal inhibitors such as bafilomycin A1 or chloroquine.

In summary, the present results support a model in which heat stress increases the expression of MAPK pathway components in rumen epithelial cells, and in which this change is accompanied by pro-apoptotic, pro-inflammatory and autophagic signals that ultimately disrupt the tight junction complex and impair barrier function. Patchouli oil attenuated this pathological process, and the preservation of barrier integrity was accompanied by reduced expression of MAPK pathway components. Among the three MAPK branches, ERK showed the most pronounced change in response to heat stress and patchouli oil treatment, particularly at the protein level, which suggests that it may represent a central node in this response. ERK1/2 is rapidly and transiently activated by a wide range of growth factor and stress stimuli, and ERK1/2 has been reported to mediate the downregulation of intestinal tight junction proteins under heat stress [8], which is consistent with the concurrent loss and recovery of ZO-1, Occludin and Claudin-1 observed here. This finding is also consistent with the review by Enomoto et al. [20], which points out that the MAPK signaling pathway plays a key role in the heat stress response and that different MAPK branches have distinct biological functions. Nevertheless, because the three branches were not selectively inhibited in this study, the predominance of ERK remains a hypothesis that requires testing with MEK, p38 and JNK inhibitors.

Several limitations of this study should be acknowledged. First, only total p38, ERK and JNK were quantified, and the phosphorylated forms, although included in the immunofluorescence antibody panel, were not systematically analyzed; the present data therefore report the amounts of these proteins rather than their phosphorylation status, and do not establish that MAPK activity was altered. The results should accordingly be interpreted as changes in the expression of MAPK pathway components, not as evidence of pathway activation or inhibition, and the causal role of MAPK signaling in the protective effect of patchouli oil requires direct testing with pharmacological inhibitors (for example SB203580, PD98059 or SP600125) or with siRNA-mediated knockdown. Second, autophagy was assessed only by static mRNA markers, without measurement of autophagic flux. Third, patchouli oil was applied as a complex extract from a single batch, and the contribution of individual constituents and their possible synergy remain to be determined. Fourth, the concentration–response and time–course screening experiments did not allow the interaction effects of temperature, duration and concentration to be tested formally, and the timing of patchouli oil administration differed between the screening experiment (12 h pre-incubation) and the main experiment (continuous exposure during heat stress and recovery). Finally, the present findings were obtained in primary BRECs cultured in vitro at 20 μg/mL; they cannot be extrapolated directly to dietary supplementation in cattle, in which rumen microbial metabolism, bioavailability, palatability and in vivo dose–response relationships must be established before patchouli oil can be recommended as a feed additive.

5. Conclusions

In this study, an in vitro heat stress model was established using primary bovine rumen epithelial cells. The results indicate that patchouli oil alleviates heat stress-induced barrier dysfunction, and that this protection is accompanied by reduced apoptosis, inflammation and autophagy-related gene expression, by maintained expression of key tight junction proteins and by reduced expression of MAPK pathway components. These findings provide a cellular and molecular basis for considering patchouli oil as a potential feed additive to mitigate heat stress and maintain rumen health in ruminant production. Further studies are needed to identify the active constituents of patchouli oil (patchoulol and pogostone) in this process, to test directly whether the reduced expression of MAPK pathway components

is causally involved in the protection, and to evaluate the efficacy of dietary patchouli oil in vivo. In particular, the effective concentration in culture (20 μg/mL) cannot be translated directly into a dietary dose, and the present in vitro findings should be regarded as a basis for, rather than a demonstration of, the practical use of patchouli oil as a feed additive for heat-stressed cattle.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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Conflicts of Interest: The authors declare no conflicts of interest. The patchouli oil used in this study was a commercially available reference product purchased from Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China); the supplier had no role in the design of the study, in the collection, analysis or interpretation of the data, or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

MAPK	Mitogen-activated protein kinase
BRECs	Bovine ruminal epithelial cells
PO	Patchouli oil
SOD	Superoxide dismutase
T-AOC	Total antioxidant capacity
ROS	Reactive oxygen species
ERK	Extracellular signal-regulated kinase
JNK	C-Jun N-terminal kinase
CAT	Catalase
GSH-Px	Glutathione peroxidase
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
TEER	Transepithelial electrical resistance
qRT-PCR	Quantitative real-time PCR
SEM	Scanning electron microscopy
ANOVA	Analysis of variance
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin
DAPI	4′,6-Diamidino-2-phenylindole
ZO-1	Zonula occludens-1

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