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Effects of Dietary Chinese Herbal Formula on Immune Capacity, Gastrointestinal Barrier Function and Nutrient Utilization in Weaned Hu Lambs

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Simple Summary

Weaning is one of the most stressful periods in a lamb's early life, which causes oxidative damage, immune suppression and gastrointestinal barrier disruption, leaving lambs vulnerable to poor health and slow growth. Dietary Chinese herbal formulas have shown some benefits for the health of young animals, although their effects on gastrointestinal barrier function and nutrient absorption in weaned lambs are not understood. Therefore, this study investigated the effects of different Jianpi herbal formulas on immunity, gastrointestinal barrier function and nutrient absorption in weaned Hu lambs over a 60-day trial. Lambs fed the diet containing 0.5% of Jianpi herbal formula I showed improved antioxidant capacity and immune function, a healthier rumen with better-developed papillae, and increased expression of gastrointestinal barrier and nutrient transporters. These results indicate that dietary supplementation with 0.5% Jianpi herbal formula can enhance antioxidant capacity, immunity, gastrointestinal barrier integrity, and nutrient absorption in weaned lambs, providing a safe and effective nutritional intervention strategy to alleviate weaning-stress-induced injury in lambs.

Abstract

Weaning stress is a major challenge in lamb production, triggering oxidative damage, immune suppression, and gastrointestinal barrier disruption. Therefore, safe and effective nutritional strategies are urgently needed to alleviate weaning-stress-induced injuries. This study aimed to investigate the influences of dietary Jianpi Herbal Formula (JHF) on immunity, gastrointestinal barrier function and nutrient absorption in weaned Hu lambs. One hundred weaned Hu lambs (male, 14.55 ± 0.21 kg) were randomly allotted to five groups (four replicates per group, five lambs per replicate; 20 per treatment) for a 60-day trial, receiving basal diet (control), or basal diet supplemented with 0.3% and 0.5% of two JHFs (JHF I and JHF II). Compared with the control group, dietary supplementation with 0.3% and 0.5% JHF I increased serum immunoglobulin G level by 25.1% and 24.5% ($p < 0.05$) and glutathione peroxidase activity by 16.3% and 10.6% ($p < 0.05$), while decreasing malondialdehyde content by 26.5% and 28.7% ($p < 0.05$) and diamine oxidase activity by 20.7% and 21.0% ($p < 0.05$), respectively. Dietary 0.5% JHF I also increased papilla width by 26.8% and the thickness of the stratum granulosum and total epithelium in the Saccus dorsalis

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by 16.5% and 10.8% ($p < 0.05$), respectively, and decreased stratum corneum thickness in both ruminal sacs ($p < 0.05$). Furthermore, 0.5% JHF I upregulated mRNA expression of *Occludin* and *MCT1* in both ruminal sacs, *Claudin-1*, *DRA*, and *NHE3* in the Saccus ventralis, *ZO-1* in the ileum, and *SGLT1* and *GLUT2* in the jejunum ($p < 0.05$). It also increased duodenal maltase and jejunal sucrase activities and elevated protein expression of Occludin and MCT1 in the Saccus dorsalis, ZO-1 in the ileum, and GLUT2 in the jejunum ($p < 0.05$). In conclusion, dietary supplementation with 0.5% JHF I effectively enhanced antioxidant capacity and immune function, increased intestinal digestive enzyme activities, improved ruminal epithelial morphology, protected gastrointestinal barrier integrity, and upregulated nutrient transporter expression, thereby alleviating weaning stress in Hu lambs.

Keywords: antioxidant capacity; gastrointestinal epithelial barrier; herbal formula; immunity; nutrient absorption; weaned lambs

1. Introduction

Hu sheep, as a distinctive Chinese meat-type breed, occupy a significant position in intensive meat sheep production due to their early sexual maturity, high fecundity, and strong adaptability [1]. To enhance farming efficiency, lambs are typically separated from their dams and fully transitioned to solid feed at 30–60 days of age [2]. Although this practice accelerates recovery of maternal body condition, shortens the reproductive interval, and improves overall production efficiency [3], early weaning can lead to reduced feed intake and behavioral abnormalities in lambs, severely compromising their growth performance and health [4,5]. Studies have demonstrated that weaning stress induces activation of the hypothalamic–pituitary–adrenal axis, elevates circulating levels of stress hormones including cortisol, and disrupts metabolic homeostasis [6]. Concurrently, weaning can impair immune function, increase susceptibility to pathogens, and upregulate the expression of pro-inflammatory cytokines (e.g., TNF-α, IL-1β), inducing chronic low-grade inflammation [7]. The rumen serves as the key organ for ruminant nutrient digestion and absorption. Weaning stress impairs ruminal epithelial development, presenting with abnormal ruminal papilla morphology and disruption of epithelial tight-junction structures (e.g., Occludin, Claudin-1), leading to compromised barrier function [8]. More importantly, these stress-triggered epithelial injuries, combined with cortisol-mediated metabolic disturbance and reshaped ruminal fermentation profiles, may further suppress nutrient transport and absorption. Early-weaned ruminants have been reported to display reduced expression of ruminal epithelial volatile fatty acid (VFA) transporters and small intestinal glucose transporters, which directly impeds the absorption efficiency of VFAs and glucose and results in growth retardation [5,9].

Nutritional modulation is a primary strategy for alleviating weaning stress. Traditional Chinese medicine formulations (TCMFs) as feed additives, owing to their multi-component, multi-target characteristics and minimal side effects, have been widely utilized in the prevention and treatment of digestive disorders in livestock [10]. Modern pharmacological research indicates that Jianpi (spleen-invigorating) herbal formulas and the active compounds in their constituent herbs (e.g., polysaccharides, flavonoids, organic acids) can promote gastrointestinal health in animals through various pathways [11]. Studies have found they can increase the apparent digestibility of dry matter, crude protein, and fiber by promoting digestive enzyme secretion, improving intestinal motility, and modulating the ruminal microbiota and fermentation patterns [12]. In addition, TCMF can improve production performance by promoting the secretion of intestinal digestive enzymes, altering the diversity of gut microbiota, regulating the content of short-chain fatty acids in the

intestine, enhancing intestinal health, and improving nutrient digestibility [13]. Concerning gastrointestinal barrier function, active components in TCMF can upregulate tight junction protein expression and restore epithelial barrier integrity by inhibiting inflammatory pathways like NF-κB [14].

The Jianpi Herbal Formula (JHF), composed of herbs such as *Dioscoreae Rhizoma*, *Crataegi Fructus*, *Atractylodis Macrocephalae Rhizoma*, *Aucklandiae Radix*, *Raphani Semen*, *Citri Reticulatae Pericarpium Viride*, *Glycyrrhizae Radix et Rhizoma and Astragali Radix*, is designed to invigorate the spleen, strengthen the stomach, promote qi flow, and alleviate pain. The selection of these herbs was based on their well-documented bioactive constituents, including polysaccharides from *Dioscoreae Rhizoma* and *Astragali Radix* [15], flavonoids (e.g., hyperoside, quercetin) from *Crataegi Fructus*, and glycyrrhizic acid from *Glycyrrhizae Radix et Rhizoma*, which have been reported to exhibit antioxidant, anti-inflammatory, and intestinal barrier-protective properties in ruminants [16,17]. It is primarily used to relieve indigestion and anorexia in weaned lambs resulting from spleen and stomach weakness. Preliminary research has shown that dietary supplementation with JHF can significantly improve the growth performance, rumen fermentation parameters, and the diversity and richness of the ruminal microbiota in weaned Hu lambs. However, the mechanisms by which JHF improves gastrointestinal barrier function and nutrient absorption, particularly at the molecular level, remain to be elucidated. The rumen plays a central role in nutrient absorption and metabolism in ruminants, and its proper function is closely linked to the alleviation of weaning stress [18,19]. Furthermore, the ruminal epithelium serves as a critical barrier for the absorption of VFAs and glucose; its morphological integrity, tight junction barrier function, and the expression levels of nutrient transporters are closely associated with nutrient utilization efficiency and the body's resistance to external stressors [20]. However, direct evidence regarding whether JHF alleviates weaning stress by simultaneously improving ruminal epithelial morphology, enhancing tight junction barriers, and regulating the expression of VFA and glucose transporters in weaned lambs remains scarce. Elucidating the regulatory role of this formula on these specific pathways is key to a deeper understanding of its mechanism in mitigating weaning stress.

We hypothesized that dietary supplementation with JHF would enhance antioxidant capacity and immune function, improve ruminal epithelial morphology and barrier integrity, and upregulate nutrient transporter expression, thereby alleviating weaning stress in weaned Hu lambs. Therefore, this study aims to investigate the regulatory effects of JHF on antioxidant capacity and immune performance, ruminal epithelial morphology, tight junction barrier function, and nutrient transport function in weaned Hu lambs. The findings will help fill the knowledge gap regarding the mechanism by which JHF alleviates weaning stress and provide a new theoretical basis for the application of TCMF in the nutritional management of weaned ruminants.

2. Materials and Methods

2.1. Animal Care

Animal procedures were approved by the Animal Care and Use Committee of Jiangxi Agricultural University and complied with the current laws relating to animal protection (Ethics Approval Code: No. JXAULL-2026-05-02, approved on 11 May 2026). Throughout the study, the lambs were maintained under species-appropriate housing conditions, with free access to feed and fresh water. The experimental unit was the pen (four pens per treatment, five lambs per pen). Animal handling was performed by trained personnel to minimize disturbances, and gentle handling was provided during sampling procedures to reduce stress.

2.2. Animals and Experimental Design

A total of 100 healthy male 2-month-old weaned Hu lambs with similar body weight (14.55 ± 0.21 kg) and genetic background were randomly allocated into five groups. The lambs were weaned at 60 ± 2 days of age and transferred to the experimental facility immediately after weaning. Random allocation was performed using a random number table, and the allocation was stratified by initial body weight to ensure balance across groups. Each experimental group comprised four pens, each housing five lambs. The sample size was constrained by the availability of animals and housing facilities, with a final allocation of five lambs per pen and four pens per group. The five groups included the basal diet (control), the basal diet + 0.3% JHF I, the basal diet + 0.5% JHF I, the basal diet + 0.3% JHF II, and the basal diet + 0.5% JHF II (supplemented in the concentrate, on an air-dried basis). The two formulas were provided by Jiangxi Zhongchengren Pharmaceutical Co., Ltd. (Nanchang, China). JHF I was composed of eight dried Chinese herbs, including Dioscoreae Rhizoma (Common Yam Rhizome), Crataegi Fructus (Hawthorn Fruit), Atractylodis Macrocephalae Rhizoma (Largehead Atractylodes Rhizome), Aucklandiae Radix (Costus Root), Raphani Semen (Radish Seed), Citri Reticulatae Pericarpium Viride (Immature Tangerine Peel), Glycyrrhizae Radix et Rhizoma (Licorice Root and Rhizome) and Astragali Radix (Astragalus Root), at a mass ratio of 1:1:1:1:1:1:1:1. In comparison, JHF II was composed of six dried Chinese herbs, including Crataegi Fructus (Hawthorn Fruit), Aucklandiae Radix (Costus Root), Raphani Semen (Radish Seed), Citri Reticulatae Pericarpium Viride (Immature Tangerine Peel), Astragali Radix (Astragalus Root), and Glycyrrhizae Radix et Rhizoma (Licorice Root and Rhizome), at a mass ratio of 2:2:1:1:1:1. The herbal materials were authenticated and voucher specimens were deposited at the Jiangxi Provincial Key Laboratory of Animal Nutrition (Nanchang, China). The herbs were dried at 45 °C, ground to a particle size of 0.5 mm, and mixed uniformly. The formulas were prepared as a fine powder and stored at 4 °C in sealed containers until use. The detailed composition and main active constituents of JHF I and JHF II are presented in Table 1. The total experimental period lasted 70 days, consisting of a 10-day adaptation period and a subsequent 60-day experimental period. The feeding trial was conducted from November 2022 to January 2023 at Jiangxi Chunhui Sheep Industry Co., Ltd. (Yichun, Jiangxi, China). Throughout the feeding trial, lambs were fed twice daily at 08:00 and 16:00, with concentrate offered before roughage. All lambs had ad libitum access to feed and fresh water. Feed intake was recorded on a per-pen basis, and the actual refusal percentage averaged 9.8% ± 1.2%. Feed refusals were collected and weighed daily, and the daily feed supply was adjusted to ensure approximately 10% refusals. Pens were cleaned and disinfected weekly. The basal diet followed the Chinese Feeding Standard of Meat-producing Sheep and Goats (NY/T 816-2004), and consisted of a 4:6 concentrate-to-roughage ratio. The ingredients and nutrient composition of the basal diet are presented in Table 2. No a-priori statistical power analysis was performed before the start of this feeding trial due to the constraints of animal resources and housing capacity.

The supplement was added on top of the basal diet, replacing an equivalent amount of concentrate, resulting in final whole-ration inclusion levels of 0.12% and 0.20% for 0.3% and 0.5% JHF I, respectively. Because the supplement was mixed into the concentrate and feed refusals were recorded per pen, the actual consumed dose was calculated based on the total feed intake per pen, and the average daily intake of JHF was estimated to be 0.28% and 0.47% for the 0.3% and 0.5% JHF I groups, respectively. Based on the results of growth performance in our preliminary experiment [18] the 0.5% JHF I group exhibited the most favorable regulatory effects among all JHF-supplemented groups and was therefore selected as the optimal treatment level. Accordingly, subsequent in-depth investigations on rumen histomorphology, gastrointestinal barrier function, and nutrient

transporter expression were conducted by comparing the control group and the 0.5% JHF I group.

Table 1. Composition and main active constituents of JHF I and JHF II (air-dried basis).

Latin Name	Main Active Constituent	Used Part	Content (%)
JHF I			
Dioscoreae Rhizoma	Dioscin (≥0.5 mg/g)	Dried rhizome	12.50
Crataegi Fructus	Hyperoside (≥1.0 mg/g)	Dried fruit	12.50
Atractylodis Macrocephalae Rhizoma	Atractylenolide I (≥0.5 mg/g)	Dried rhizome	12.50
Aucklandiae Radix	Costunolide (≥5.0 mg/g)	Dried root	12.50
Raphani Semen	Sinapine thiocyanate (≥1.0 mg/g)	Dried seed	12.50
Citri Reticulatae Pericarpium Viride	Hesperidin (≥30 mg/g)	Dried peel	12.50
Glycyrrhizae Radix et Rhizoma	Glycyrrhizic acid (≥20 mg/g)	Dried root and rhizome	12.50
Astragali Radix	Astragaloside IV (≥0.4 mg/g)	Dried root	12.50
Total			100.00
JHF II			
Crataegi Fructus	Hyperoside (≥1.0 mg/g)	Dried fruit	25.00
Aucklandiae Radix	Costunolide (≥5.0 mg/g)	Dried root	25.00
Raphani Semen	Sinapine thiocyanate (≥1.0 mg/g)	Dried seed	12.50
Citri Reticulatae Pericarpium Viride	Hesperidin (≥30 mg/g)	Dried peel	12.50
Astragali Radix	Astragaloside IV (≥0.4 mg/g)	Dried root	12.50
Glycyrrhizae Radix et Rhizoma	Glycyrrhizic acid (≥20 mg/g)	Dried root and rhizome	12.50
Total			100.00

Main active constituents and their content standards refer to the Chinese Pharmacopoeia (2020 edition). These are reference standards for single-herb identification, not experimentally measured concentrations of bioactive compounds in the final mixed JHF compound formulations. Quantitative phytochemical analysis was not performed on the finished JHF mixtures in the present trial. JHF I, Jianpi Herbal Formula I (mass ratio of eight herbs = 1:1:1:1:1:1:1:1); JHF II, Jianpi Herbal Formula II (mass ratio of six herbs = 2:2:1:1:1:1).

Table 2. Composition and nutrient level of basic diet (air-dried basis).

Ingredients	Content (%)	Nutrient Levels [2]	Content (%)
Peanut vine	60.00	Dry matter	86.53
Corn	23.40	Nemf (MJ/kg)	6.33
Wheat bran	3.20	Crude protein	12.74
Soybean meal	6.80	Neutral detergent fiber	47.67
corn germ meal	4.00	Acid detergent fiber	34.52
$NaHCO_3$	0.40	Crude ash	8.79
limestone	0.60	Calcium	0.99
$CaHPO_4$	0.60	Phosphorus	0.40
NaCl	0.20		
Premix [1]	0.80		
Total	100.00		

[1] The premix provided the following per kg of the diet: VA 2640IU, VD302IU, E17mg, Fe60mg, Cu10mg, Zn36mg, Mn48mg, Co0.12mg, I0.3mg, Se0.24mg. [2] Dry matter, crude protein, neutral detergent fiber, acid detergent fiber and crude ash are measured values, while combined net energy (Nemf), calcium, and phosphorus are calculated values.

2.3. Serum Biochemical Parameters

On experimental day 60, eight lambs were randomly selected from each group (one healthy lamb with body weights approximating the pen average). Blood samples were collected from the jugular vein before morning feeding, allowed to clot for 5 min, centrifuged at 3000 rpm, aliquoted, and stored at −80 °C until analysis. Serum concentrations of Total Protein (TP), Albumin (ALB), Globulin (GLB), Glucose (GLU), Urea (UREA), Total Cholesterol (TC), Triglyceride (TG), High-density Lipoprotein (HDL), and Low-density

Lipoprotein (LDL) were measured using a fully automatic biochemical analyzer (Mindray BS-420, Beijing, China). The analyzer was calibrated daily using a multi-analyzer calibration standard (Mindray, Cat. No. 105-005546-00), and quality control was performed using two levels of control sera (Mindray, Cat. No. 105-005547-00). The intra- and inter-assay coefficients of variation were <5% and <8%, respectively.

2.4. Serum Antioxidant, Intestinal Barrier, and Immune Parameters

Serum activities of SOD, GSH-Px, and T-AOC, as well as MDA content, were measured using the same fully automatic biochemical analyzer (Mindray BS-420, Beijing, China) with commercial assay kits according to the manufacturer's instructions. Serum levels of diamine oxidase (DAO), D-lactic acid (D-LC) [21], immunoglobulin IgA, IgG, and IgM were determined using ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The ELISA kit catalog numbers were: DAO (Cat. No. A088-3-1, UV microplate method), D-LC (Cat. No. A019-3-1, colorimetric method), IgA (Cat. No. H108), IgG (Cat. No. H109), and IgM (Cat. No. H110). The detection ranges were 0.5–100 pg/mL for DAO, 0.2–50 pg/mL for D-LC, 0.1–10 g/L for IgA, 1–50 g/L for IgG, and 0.1–5 g/L for IgM. The intra- and inter-assay coefficients of variation were <10% and <12%, respectively.

2.5. Rumen Histomorphological Analysis

On day 60 of the experimental period, four lambs were randomly selected from the control group and the 0.5% JHF I group (one lamb per pen, ensuring sampling across all four pens for each group; eight lambs in total). Lambs were euthanized by exsanguination after deep anesthesia with sodium pentobarbital (30 mg/kg BW, intravenous injection), in accordance with the guidelines of the Animal Care and Use Committee of Jiangxi Agricultural University. After slaughter, tissue samples were collected according to standard procedures as previously described [22]. Specifically, 2 × 2 cm^2 tissue samples were excised from the Saccus dorsalis and Saccus ventralis of the rumen. Ruminal papilla morphology was assessed as previously described [23]. Briefly, 1 cm^2 ruminal tissue was excised from the Saccus dorsalis, the number of papillae was recorded, and the longitudinal length and transverse width of papillae were measured. Papilla surface area was calculated as S = length × width. Subsequently, a portion of the rumen tissue was fixed in 4% paraformaldehyde (GenStar, Beijing, China), dehydrated through a graded ethanol series (GenStar, Beijing, China), embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for microscopic examination and image analysis. For each animal, five sections per ruminal sac were examined, and three fields per section were randomly selected for measurement. All histomorphometric measurements were performed by two independent observers blinded to the treatment groups, and the average values were used for analysis. The thickness of the stratum corneum (SC), stratum granulosum (SG), stratum spinosum and basale (SSB), and total epithelium (TE) was measured using ImageJ software (version 1.53, National Institutes of Health, USA). Another portion of the rumen tissue was rinsed with phosphate-buffered saline (PBS) (pH 7.4, Solarbio, Beijing, China), fixed in 2.5% glutaraldehyde (GenStar, Beijing, China), dehydrated, dried, mounted on conductive carbon film, sputter-coated with gold, and observed under a scanning electron microscope (Hitachi SU8100, Tokyo, Japan) at an accelerating voltage of 3.0 kV and a working distance of 10 mm for ultrastructural examination and image acquisition. For each animal, five fields per ruminal sac were examined at ×100 and ×500 magnification. Fresh tissue samples for subsequent molecular analyses were immediately snap-frozen in liquid nitrogen and stored at −80 °C until use.

2.6. Detection of Intestinal Digestive Enzyme Activities

Immediately after dissection, the small intestinal tissues were separated and the contents were removed. Tissue samples were collected from three segments. The duodenum was sampled 10 cm distal to the pylorus, the jejunum from the mid-small intestine, and the ileum 10 cm proximal to the ileocecal junction. The samples were rinsed with sterile ice-cold PBS buffer (pH 7.4, Solarbio, Beijing, China) and then divided into two parts for different treatments. One part was used for the determination of small intestinal digestive enzyme activities. Tissue samples from each segment of the small intestine were accurately weighed and homogenized in ice-cold normal saline (mass volume ratio 1:9, g/mL). The activities of sucrase, maltase, and lactase were determined using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. The kit catalog numbers were: sucrase (Cat. No. A082-2-1), maltase (Cat. No. A082-3-1), and lactase (Cat. No. A082-1-1). Enzyme activities were expressed as U/mg protein, and protein concentration was determined using a BCA protein assay kit (GenStar, Beijing, China) [24]. All assays were performed at 37 °C in triplicate. The other part was cut into small tissue pieces, aliquoted into cryotubes, and immediately frozen in liquid nitrogen for subsequent PCR and Western blot analyses.

2.7. RT-PCR Analysis

Approximately 0.1 g of gastrointestinal tissue was placed into a 1.5 mL centrifuge tube. Grinding beads, 1 mL of TransZol Up, and 0.2 mL of RNA Extraction Agent were added, and the mixture was homogenized using a low-temperature tissue grinder until no obvious tissue fragments remained. Total RNA was extracted from the tissues following the instructions of the TransZol Up Plus RNA Kit (TransGen Biotech, Beijing, China). RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the A260/A280 ratio was between 1.9 and 2.1 for all samples. Reverse transcription was performed using the Evo M-MLV Reverse Transcription Premix Kit (Cat. No. AG11706, Accurate Biology, Changsha, China) according to the manufacturer's protocol: 37 °C for 15 min, 85 °C for 5 s, and hold at 4 °C, and the reaction was carried out in a PCR instrument (Bio-Rad Inc., Hercules, CA, USA). The reverse-transcribed cDNA was used for quantitative real-time PCR (qPCR) analysis using PerfectStart Green qPCR SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's recommended protocol (Cat. No. AQ601). The qPCR reaction was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Inc., Hercules, CA, USA, Model No. 1855195) following the recommended reaction conditions. The amplification efficiency for each primer pair was between 95% and 105%, and melting curve analysis showed a single peak for each gene. No-template controls were included in each run. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with *GAPDH* as the reference gene and the control group as the calibrator [25]. Primer sequences for target genes were designed using PrimerSelect software (version 5.0, DNASTAR, Madison, WI, USA) based on sequences available in the NCBI database (Table 3). The designed primers were synthesized by Sangon Biotech (Shanghai, China).

Table 3. Primers used for real-time polymerase chain reaction.

Gene	Accession No	Primer, 5′–3′	Size (bp)
GAPDH	NM_001190390.1	F: AGAGTGAGTGTCGCTGTTGAAGT R: TCCGTTGTGGATCTGACCTG	60
CLDN1	NM_001185016.1	F: GAAGATGCGGATGGCTGTCT R: TACCATGCTGTGGCAACCAA	60

Table 3. *Cont.*

Gene	Accession No	Primer, 5′–3′	Size (bp)
ZO-1	XM_060401397.1	F: ACGATGCTGTGCCCTAAAGT R: AGAAATCGTGCTGATGTGCC	59
OCLN	XM_012096797.5	F: AACTGGGCTGAACACTCCAA R: CCTGATTGCCCAGGATGTGA	59
PAT1	XM_042236259.2	F: GCCACCATCCTGCTGAATCT R: GGCGTTGGCAAAGTACACAG	60
AE2	XM_042249261.1	F: CACCGAGCGGGAGATGAAAT R: TGGGCATCTCGTTGTACTCG	59
DRA	NM_001184899.1	F: GCAGCACCCTGGCTAATGTT R: ATTCGAAGTGGACGAAAGCCA	60
MCT1	XM_060412883.1	F: GCCACCAGTTTTAGGTCGTCT R: GCATCGGTGACTTCTTTTGGC	60
MCT4	XM_042252144.1	F: TTACCTGGTTTGGGCTCTGCT R: ATAAGGGCACCTGTCCAGTCATA	60
NHE1	XM_004005085.5	F: GCTACATGGCCTACCTGTCG R: TGATGGTCGTGTGGGACTTG	60
NHE3	XM_027980208.2	F: ATCAGGGCAAATCGGACACA R: GGGAGAACGGATGAAAGCCA	60
SGLT1	NM_001009404.1	F: GGTCTTTCATCCTGACTGGGTT R: GGGTGTAGCACTCTTTCTTGACG	60
GLUT2	XM_004003162.6	F: ACACTCAGGGGTGCTATCGG R: GGTATCTGGGGCTTTCTGGAC	60

The full names and corresponding abbreviations of the genes are as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), claudin-1 (CLDN1), zonula occludens-1 (ZO-1), occludin (OCLN), putative anion transporter 1 (PAT1), anion exchanger 2 (AE2), downregulated in adenoma (DRA), monocarboxylate transporter 1 (MCT1), monocarboxylate transporter 4 (MCT4), Na^+/H^+ exchanger 1 (NHE1), Na^+/H^+ exchanger 3 (NHE3), sodium-glucose linked transporter 1 (SGLT1), and glucose transporter 2 (GLUT2).

2.8. Western Blot Analysis

Proteins extracted from ruminal and small intestinal tissues were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA; pore size 0.45 μm) using a Hoefer TE22 transfer tank with Tris-Glycine-SDS-methanol transfer buffer. The membranes were blocked with 5% non-fat milk (Solarbio, Beijing, China) in TBST for 30 min at room temperature, then incubated overnight at 4 °C with diluted rabbit-derived primary antibodies. The primary antibodies used were: MCT1 (Servicebio, Wuhan, China; Cat. No. GB114599; 1:500 dilution), Occludin (Servicebio; Cat. No. GB111401; 1:1000 dilution), ZO-1 (Servicebio; Cat. No. GB111402; 1:500 dilution), and GLUT2 (Boster Biological Technology, Wuhan, China; Cat. No. PB9436; 1:1000 dilution). All antibodies have been validated for use in sheep according to the manufacturers' datasheets and previous publications [26–28]. After washing to remove unbound primary antibodies, the membranes were incubated with HRP-labeled goat anti-rabbit IgG secondary antibody (Servicebio, Wuhan, China; Cat. No. GB23303; 1:10,000 dilution). Following a final wash, the membranes were dried by blotting with absorbent paper, incubated in enhanced chemiluminescence (ECL) detection solution (Thermo Fisher Scientific, Waltham, MA, USA), and visualized using a ChemiDoc Western blot imaging system (Bio-Rad Inc., Hercules, CA, USA). Images were captured and saved for analysis [29]. β-actin (Servicebio; Cat. No. GB15003; 1:5000 dilu-

tion) was used as the loading control. The band intensities were quantified using ImageJ software (version 1.53, National Institutes of Health, USA), and relative protein expression levels were calculated. The protein loading amount was 30 μg per lane, and all experiments were repeated three times.

2.9. Statistical Analysis

All statistical analyses were performed using SPSS Statistics 26.0 (IBM Corp., Armonk, NY, USA). Data were first subjected to descriptive statistics and expressed as mean ± standard error of the mean (SEM). The experimental unit was the pen for growth performance data and the individual lamb for other measurements. Prior to formal analysis, normality of residuals was assessed using the Shapiro–Wilk test, and homogeneity of variances among groups was assessed using Levene's test. One-way analysis of variance (ANOVA) was conducted to test for significant differences among the five dietary treatment groups. When a significant overall F-test was observed (indicated by a $p < 0.05$), post-hoc multiple comparisons between groups were performed using Duncan's multiple range test. For the subsequent in-depth investigations comparing the control group and the 0.5% JHF I group, the independent samples *t*-test was used to assess the statistical significance of differences, with the same normality assessment applied as described above. For all analyses, a $p > 0.05$ was considered not statistically significant, while a $p < 0.05$ was considered statistically significant. A limitation of this study is that lambs were housed in pens, and the clustering of animals within pens was not accounted for in the statistical models, which may affect the independence of observations. Another limitation is that only a single reference gene (GAPDH) was used for RT-qPCR normalization. Without full stability validation, potential normalization bias cannot be excluded.

3. Results

3.1. JHF Supplementation Improved Serum Antioxidant Capacity, Immune Parameters, and Intestinal Permeability Markers

The indicators related to serum biochemistry, immunity, and antioxidant capacity are shown in Table 4. Compared with the control group, in terms of serum biochemistry, the 0.3% JHF II group showed a significantly increased serum LDL level ($p = 0.027$). Regarding immunity, IgA levels were significantly increased in the 0.3% JHF I and 0.5% JHF II groups ($p = 0.024$), and IgG levels were significantly increased in the 0.3% JHF I and 0.5% JHF I groups ($p = 0.018$). However, the 0.5% JHF I group did not show significant differences in IgA, SOD, T-AOC, or D-lactic acid compared with the control group ($p > 0.05$). DAO activity was significantly decreased in the 0.3% JHF I, 0.5% JHF I, and 0.3% JHF II groups ($p = 0.001$), and D-lactic acid content was significantly decreased in the 0.5% JHF II group ($p = 0.030$). In terms of antioxidant performance, serum SOD and T-AOC activities were significantly increased in the 0.3% JHF I and 0.5% JHF II groups ($p = 0.010$ and $p = 0.040$, respectively); serum GSH-Px activity was significantly increased in the 0.3% JHF I, 0.5% JHF I, and 0.5% JHF II groups ($p = 0.030$); and serum MDA content was significantly decreased in the 0.3% JHF I and 0.5% JHF I groups ($p = 0.030$). No significant differences were observed in TP, ALB, GLB, GLU, TC, TG, HDL, UREA, or IgM among all groups ($p > 0.05$).

Table 4. Serum biochemical, immunological and antioxidant parameters of weaned Hu lambs supplemented with JHF.

Items [1]	Control	JHF I		JHF II		SEM	*p*-Value
		0.3%	0.5%	0.3%	0.5%		
Serum biochemical indices							
TP (g/L)	66.72	70.84	66.72	67.16	68.85	0.97	0.658
ALB (g/L)	23.64	24.37	23.83	24.66	24.43	0.36	0.910
GLB (g/L)	43.08	46.46	42.90	42.50	44.42	1.09	0.817
GLU (mmol/L)	3.81	3.54	3.97	4.13	3.91	0.10	0.508
TC (mmol/L)	1.28	1.22	1.29	1.33	1.23	0.03	0.865
TG (mmol/L)	0.33	0.22	0.35	0.34	0.28	0.02	0.103
HDL (mmol/L)	0.73	0.70	0.78	0.66	0.67	0.02	0.447
LDL (mmol/L)	0.41 ^{b}	0.40 ^{b}	0.37 ^{b}	0.55 ^{a}	0.44 ^{b}	0.02	0.027
UREA (mmol/L)	7.54	7.36	6.47	6.67	7.33	0.29	0.757
Immunity indices							
IgA (g/L)	1.72 ^{b}	1.89 ^{a}	1.81 ^{ab}	1.76 ^{b}	1.89 ^{a}	0.03	0.024
IgG (g/L)	17.44 ^{b}	21.82 ^{a}	21.71 ^{a}	19.63 ^{ab}	20.18 ^{ab}	0.51	0.018
IgM (g/L)	1.61	1.62	1.67	1.67	1.62	0.03	0.963
Intestinal permeability indices							
DAO (pg/mL)	323.89 ^{a}	256.71 ^{b}	255.88 ^{b}	237.63 ^{b}	299.04 ^{a}	7.83	0.001
D-LC (pg/mL)	491.42 ^{a}	476.50 ^{ab}	471.98 ^{ab}	397.51 ^{bc}	382.41 ^{c}	14.82	0.030
Antioxidant indices							
SOD (U/mL)	74.64 ^{c}	86.62 ^{a}	79.31 ^{bc}	80.11 ^{bc}	83.24 ^{ab}	1.23	0.010
GSH-PX (U/mL)	385.81 ^{b}	448.7 ^{a}	426.53 ^{a}	414.59 ^{ab}	440.19 ^{a}	7.16	0.030
T-AOC (U/mL)	8.20 ^{c}	9.32 ^{ab}	8.51 ^{bc}	8.95 ^{abc}	9.52 ^{a}	0.16	0.040
MDA (nmol/mL)	3.66 ^{a}	2.69 ^{b}	2.61 ^{b}	3.01 ^{ab}	3.19 ^{ab}	0.12	0.030

[1] Means within a row with different superscripts differ significantly ($p < 0.05$). The same annotations apply to all subsequent tables (Tables 5–7).

3.2. JHF Supplementation Increased Ruminal Papilla Width and Surface Area in the Saccus Dorsalis

As shown in Table 5, the JHF group exhibited significantly increased papilla width (by 26.8%) in the Saccus dorsalis compared to the control group ($p = 0.020$). However, papilla surface area did not differ significantly between the JHF and control groups (67.33 vs. 61.33 mm^2/cm^2; $p = 0.510$). Other parameters, including papilla height and papillary density, showed no significant differences between the JHF group and the control group ($p > 0.05$).

Table 5. Ruminal papilla morphology of weaned Hu lambs supplemented with JHF.

Items [1]		Control	JHF	SEM	*p*-Value
papilla height (mm)	SD	1.33	1.47	0.08	0.110
	SV	1.83	1.93	0.15	0.500
papilla width (mm)	SD	0.56	0.71	0.04	0.020
	SV	0.90	1.01	0.11	0.300
papillary density (n/cm^2)	SD	0.75	0.99	0.08	0.010
	SV	1.71	2.04	0.33	0.320
papilla surface area (mm^2/cm^2)	SD	67.33	61.33	8.24	0.510
	SV	58.33	57.00	8.99	0.890

[1] SD: Saccus dorsalis, SV: Saccus ventralis. Control: basal diet; JHF: 0.5% JHF I (the optimal supplementation level identified in this study). The same definitions apply to all subsequent tables (Tables 6 and 7).

3.3. JHF Supplementation Thickened the Stratum Granulosum and Reduced Stratum Corneum Thickness in the Ruminal Epithelium

As shown in Table 6 and Figure 1, compared with the control group, the JHF group exhibited significantly increased thickness of the stratum granulosum (by 16.5%; $p = 0.010$) and a tendency toward increased total epithelium thickness (by 10.8%; $p = 0.050$) in the Saccus dorsalis, while the thickness of the stratum corneum tended to decrease ($p = 0.050$).

Table 6. Ruminal epithelial thickness of weaned Hu lambs supplemented with JHF.

Thickness, μm		Control	JHF	SEM	*p*-Value
stratum corneum	SD	25.02	22.84	1.09	0.050
	SV	24.89	19.71	1.92	0.010
stratum granulosum	SD	26.85	31.28	1.59	0.010
	SV	33.53	33.94	1.75	0.820
stratum spinosum and basale	SD	65.24	69.21	4.31	0.360
	SV	77.59	79.58	4.37	0.650
total epithelia	SD	115.56	128.08	6.20	0.050
	SV	134.12	141.80	4.75	0.110

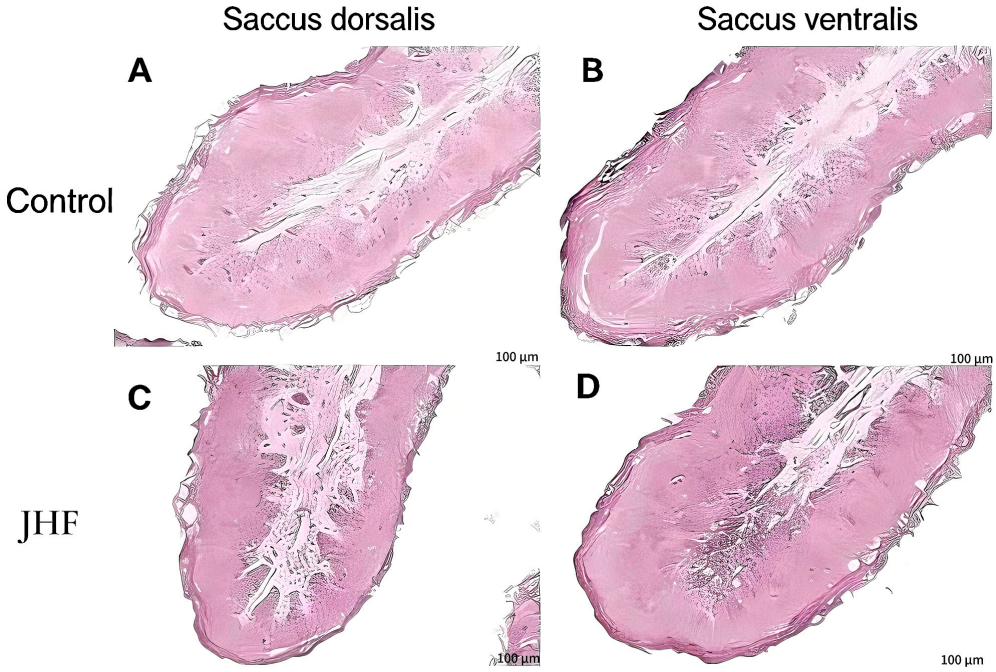


Figure 1. Histomorphology of the ruminal epithelium in weaned Hu lambs supplemented with JHF. Representative hematoxylin and eosin (H&E)-stained sections of the Saccus dorsalis (SD) and Saccus ventralis (SV). (**A**) SD, control group; (**B**) SV, control group; (**C**) SD, JHF group; (**D**) SV, JHF group. Control lambs received a basal diet; JHF lambs received the basal diet supplemented with 0.5% JHF I (the optimal level identified in this study). The same definitions apply to all subsequent figures. Scale bar = 100 μm.

3.4. JHF Supplementation Alleviated Ruminal Epithelial Surface Damage and Hyperkeratosis

As shown in Figure 2, scanning electron microscopy revealed extensive damage, shedding, and hyperkeratosis on the ruminal epithelial surface in the control group (Figure 2A,C). In contrast, the ruminal epithelial surface in the JHF group (Figure 2B,D) appeared relatively smooth, with no obvious damage or hyperkeratosis. These observations were qualitative and based on representative images; the selection of fields was performed randomly, and no quantitative pathological scoring was applied across animals.

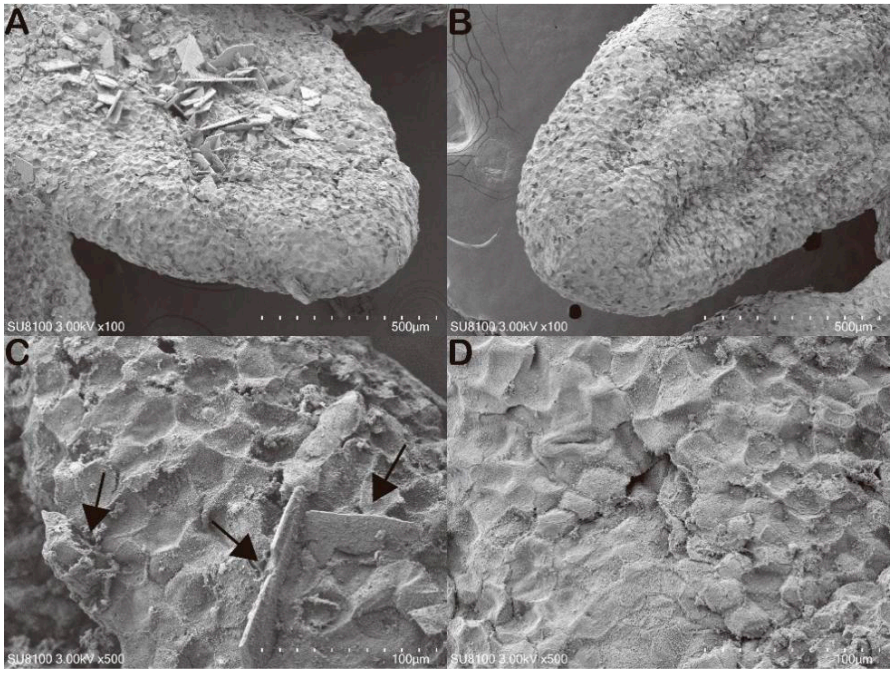


Figure 2. Scanning electron microscopy of the ruminal epithelial surface in weaned Hu lambs supplemented with JHF. (**A**,**C**) control group; (**B**,**D**) JHF group. Black arrows indicate obvious damage sites on the ruminal epithelial surface.

3.5. JHF Supplementation Upregulated Ruminal mRNA Expression of Tight Junction Proteins and VFA Transporters

As shown in Figure 3, compared with the control group, the JHF group significantly upregulated the mRNA expression levels of *Occludin* and *MCT1* in the Saccus dorsalis ($p < 0.05$), and the mRNA expression levels of *Claudin-1*, *Occludin*, *MCT1*, *DRA*, and *NHE3* in the Saccus ventralis ($p < 0.05$). No significant differences were observed for *MCT4*, *AE2*, *PAT1*, or *NHE1* in either ruminal sac ($p > 0.05$).

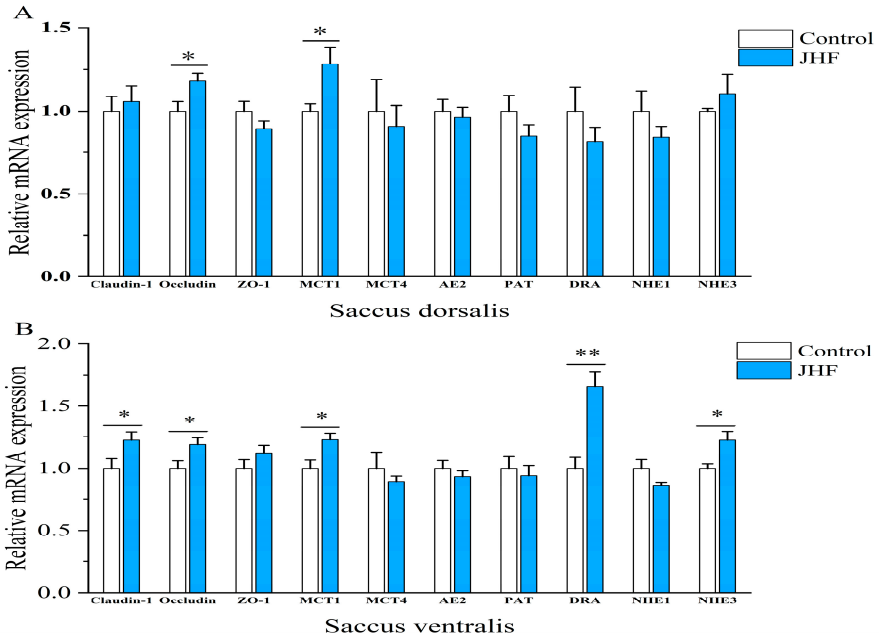


Figure 3. The mRNA expression of tight junction proteins and VFA transporters in the rumen of weaned Hu lambs supplemented with JHF. (**A**) Saccus dorsalis; (**B**) Saccus ventralis. * $p < 0.05$, ** $p < 0.01$.

3.6. JHF Supplementation Elevated Protein Expression of Occludin and MCT1 in the Ruminal Epithelium

As shown in Figure 4, compared with the control group, the JHF group significantly increased protein expression levels of Occludin and MCT1 in the Saccus dorsalis ($p < 0.05$), and Occludin in the Saccus ventralis ($p < 0.05$). MCT1 protein expression in the Saccus ventralis did not differ significantly between groups ($p > 0.05$).

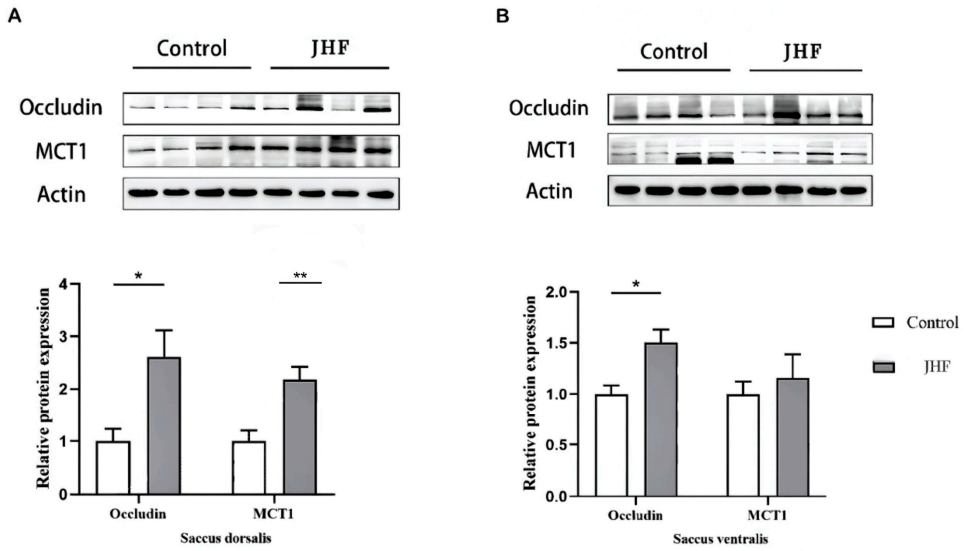


Figure 4. Protein expression of Occludin and MCT1 in the ruminal epithelium of weaned Hu lambs supplemented with JHF. (**A**) Saccus dorsalis; (**B**) Saccus ventralis. * $p < 0.05$, ** $p < 0.01$.

3.7. JHF Supplementation Increased Duodenal Maltase and Jejunal Sucrase Activities in the Small Intestine

As shown in Table 7, compared with the control group, the JHF group exhibited significantly increased activities of duodenal maltase (by 47.2%; $p = 0.040$) and jejunal sucrase (by 83.5%; $p = 0.001$). Jejunal maltase, duodenal sucrase, ileal sucrase, and lactase activities in all segments did not differ significantly between groups ($p > 0.05$).

Table 7. Intestinal digestive enzyme activities of weaned Hu lambs supplemented with JHF.

Items, U/mg Protein		Control	JHF	SEM	*p*-Value
Sucrose enzyme	Duodenum	12.59	14.79	3.24	0.520
	Jejunum	10.16	18.64	1.57	0.001
	Ileum	17.13	14.26	5.32	0.610
Maltase	Duodenum	5.93	8.73	1.05	0.040
	Jejunum	7.62	6.11	2.24	0.530
	Ileum	4.53	6.10	1.26	0.260
Lactase	Duodenum	1.38	1.20	0.14	0.250
	Jejunum	0.79	0.54	0.24	0.340
	Ileum	0.97	1.40	0.31	0.220

3.8. JHF Supplementation Upregulated mRNA Expression of ZO-1 in the Ileum and SGLT1/GLUT2 in the Jejunum

As shown in Figure 5, compared with the control group, the JHF group significantly upregulated the mRNA expression of *ZO-1* in the ileum ($p < 0.05$), as well as significantly increased mRNA levels of *SGLT1* and *GLUT2* in the jejunum ($p < 0.05$). In the duodenum, *ZO-1* mRNA expression was significantly decreased in the JHF group compared with the

control group ($p < 0.05$), while no significant differences were observed for *Claudin-1*, *Occludin*, *SGLT1*, or *GLUT2* in the duodenum or ileum ($p > 0.05$).

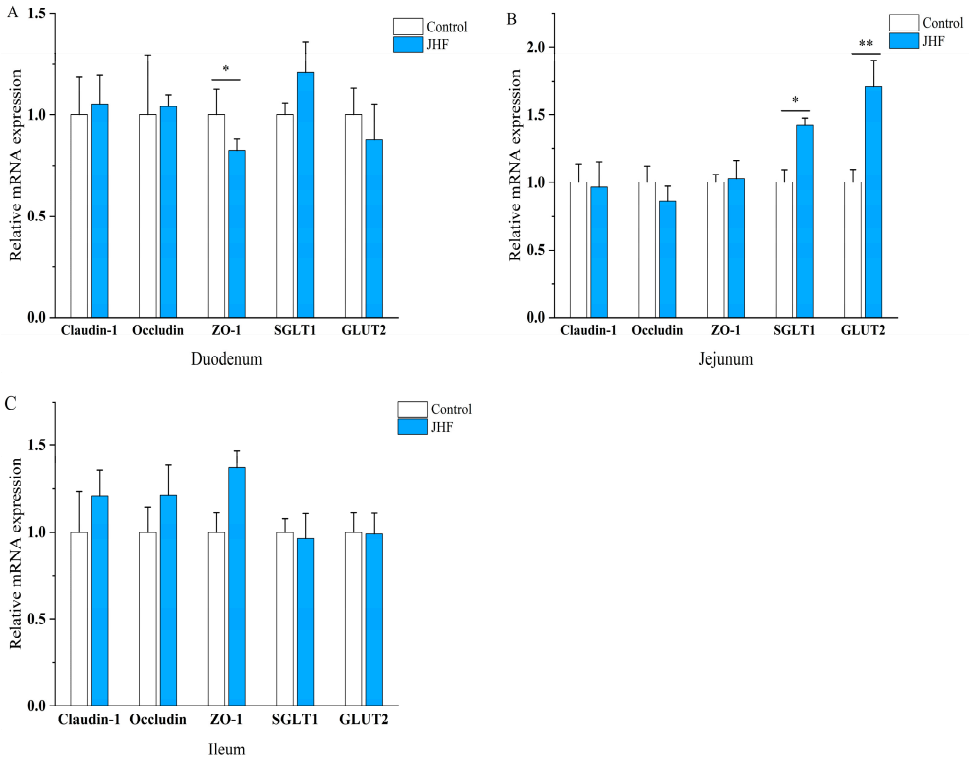


Figure 5. The mRNA expression of tight junction proteins and glucose transporters in the small intestine of weaned Hu lambs supplemented with JHF. (**A**) Duodenum; (**B**) jejunum; and (**C**) ileum. * $p < 0.05$, ** $p < 0.01$.

3.9. JHF Supplementation Enhanced Jejunal GLUT2 Protein Expression

As shown in Figure 6, compared with the control group, the JHF group significantly increased the protein expression level of GLUT2 in the jejunum ($p < 0.05$). No significant differences were observed in GLUT2 protein expression in the ileum ($p > 0.05$).

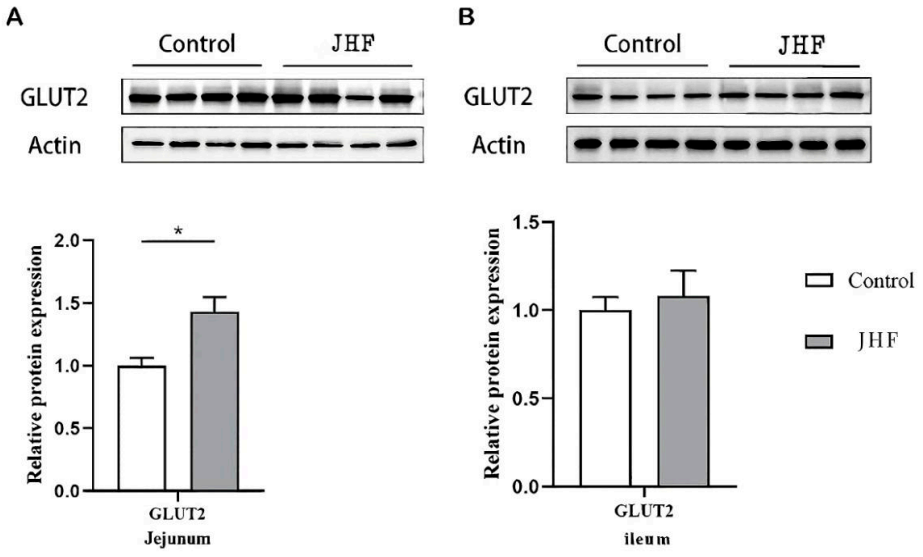


Figure 6. Protein expression of GLUT2 in the jejunum and ileum of weaned Hu lambs supplemented with JHF. (**A**) Jejunum and (**B**) ileum. * $p < 0.05$.

4. Discussion

The physiological impact of weaning stress on lambs is systemic, often manifesting as immunosuppression, oxidative damage, and impaired intestinal barrier function. These changes are sensitively reflected in blood biochemical and immune parameters [4]. Studies have also demonstrated that the weaning transition represents one of the most critical periods for redox imbalance across farm species, during which excessive reactive oxygen species (ROS) are generated, disrupting cellular structure and function [30]. Common strategies to alleviate weaning stress include supplementing antioxidants like vitamin E and selenium, or using immunostimulants; however, these approaches often focus on modulating single pathways [31]. This study demonstrated that supplementation with 0.5% JHF I was associated with improvements in blood homeostasis in weaned Hu lambs. Specifically, 0.5% JHF I notably increased serum immunoglobulin (IgG) levels and key antioxidant enzyme activities (GSH-Px), while reducing oxidative stress markers (MDA) and indicators associated with intestinal permeability (DAO). However, the 0.5% JHF I group did not show significant differences in IgA, SOD, T-AOC, or D-lactic acid compared with the control group. These results suggest that this formula was associated with improved indicators of immune enhancement, antioxidant defense, and intestinal barrier protection.

Compounds such as flavonoids (e.g., hyperoside, quercetin) found in *Crataegi Fructus* within this formula are potent natural antioxidants, confirmed to possess strong free radical scavenging capabilities and the ability to upregulate the expression of endogenous antioxidant enzymes [32], thereby effectively rebuilding the antioxidant barrier in weaned lambs. In recent years, plant extracts and TCMF have gained attention due to their multi-target and holistic regulatory characteristics [13]. Studies have shown that dietary supplementation with plant-derived extracts such as polysaccharides, polyphenols, essential oils, and alkaloids can enhance antioxidant defense, modulate immune and inflammatory responses, repair the intestinal barrier, and reshape the gut microbiota through multi-target mechanisms [11]. These effects have also been observed in ruminants, where plant-derived additives improved milk production and quality [33] and modulated growth performance, antioxidant status, and meat quality [34]. Intestinal barrier integrity is vulnerable during weaning; its compromise can lead to the translocation of intestinal endotoxins and harmful substances into the bloodstream, triggering systemic inflammation and metabolic disorders. Weaning stress is known to disrupt intestinal morphology and barrier function, and nutritional interventions targeting intestinal health have been extensively explored in young animals [7,8]. Polysaccharides from *Dioscoreae Rhizoma* can strengthen the intestinal mucosal barrier by promoting mucus secretion and maintaining epithelial cell integrity [35]. In parallel, a recent study in Karakul sheep demonstrated that dietary supplementation with licorice crude extract, rich in flavonoids and polysaccharides, enhanced serum antioxidant capacity and humoral immunity, and improved gastrointestinal enzyme activities, suggesting a beneficial role of licorice-derived bioactive compounds in maintaining gastrointestinal health in ruminants [36]. *Aucklandiae Radix*, rich in bioactive metabolites such as costunolide and dehydrocostus lactone, possesses anti-inflammatory and antimicrobial properties that also contribute to the regulation of gastrointestinal health [37]. Compared with traditional strategies intervening in single aspects like oxidation or immunity, this formula integrates multiple plant components with well-defined bioactivities. Potential complementary or interactive effects among these herbal constituents remain untested in the present study, and it is hypothesized that multi-pathway effects may contribute to improved physiological resilience of weaned lambs against weaning stress. However, direct comparisons with single-pathway interventions were not performed in this study, and the potential advantages of this formula require further verification.

Beyond systemic blood parameters, the protective effects of JHF on the gastrointestinal tract itself were further investigated. A healthy ruminal epithelial barrier is crucial for ruminant health, serving not only as the primary site for VFA absorption but also as the first line of defense against pathogens and toxins [38,39]. Weaning stress can severely impair ruminal epithelial development. A previous transcriptomic study in a calf model revealed that the introduction of solid feed profoundly influences rumen wall morphological and functional development at the molecular level, including pathways related to epithelial cell proliferation and barrier formation [40]. Existing nutritional interventions, such as supplementing encapsulated butyrate with zinc, have been evaluated in lambs abruptly transitioned to grain-based diets, with demonstrated potential to improve growth performance and gastrointestinal health [41]. This study elucidates the protective and promoting effects of JHF I on the ruminal epithelial barrier from three levels: tissue morphology, ultrastructure, and molecular expression. Results showed that this formula significantly increased papilla width in the dorsal ruminal sac, reduced abnormal thickening of the stratum corneum, and upregulated the gene expression of tight junction proteins *Occludin* and *Claudin-1*. These improvements were observed in a limited number of animals (four lambs per group), which constrains the strength of mechanistic conclusions. These improvements at the morphological and molecular levels corroborate the scanning electron microscopy observations of a smoother epithelial surface with less damage in the JHF group. The underlying mechanism may involve multiple pathways. On one hand, the formula may optimize ruminal fermentation patterns, increasing fermentation products like propionate that stimulate papilla growth [42]. On the other hand, components such as polysaccharides from Dioscoreae Rhizoma and glycyrrhizic acid from Glycyrrhizae Radix et Rhizoma may alleviate epithelial inflammation and promote tight junction assembly and stability by modulating inflammatory signaling pathways such as NF-κB [43]. However, rumen fermentation parameters and inflammatory signaling pathways were not directly measured in this study, and these mechanisms should be considered as possible explanations rather than demonstrated mechanisms.

This study not only confirms the structural protection of the ruminal physical barrier by this herbal formula but also mechanistically links it to tight junction function at the molecular level, representing a deeper investigation compared to studies focusing solely on morphological indicators. In contrast to traditional methods primarily providing single nutritional substrates, a recent study demonstrated that a Chinese herbal extract (CHE) alleviated weaning stress and improved growth performance in lambs by enhancing immune function, antioxidant capacity, and rumen microbial composition [11]. These parallel findings indicate that multi-herb formulas operate through an integrated mechanism of "improving the microbial environment—anti-inflammation—repairing junctions," although microbial and inflammatory parameters were not measured in the present study. Therefore, this integrated mechanism should be considered a hypothesis derived from previous studies rather than a verified pathway in the current experiment.

In addition to maintaining ruminal barrier integrity, the formula's impact on digestive and absorptive functions was evaluated. The successful transition of lambs from milk to solid feed as their primary nutrient source post-weaning relies on efficient gastrointestinal digestion and absorption capacity. Insufficient small intestinal digestive enzyme activity and downregulated expression of nutrient transporters are key intrinsic factors contributing to post-weaning growth retardation [44]. Conventional solutions include supplementing diets with digestive enzymes or highly digestible ingredients to compensate for endogenous enzyme deficiencies, but these approaches do not fundamentally enhance the host's own absorptive capacity [45]. The results of this study demonstrate that JHF I was associated with enhanced nutrient digestion and absorption chain in weaned Hu lambs: on the di-

gestive side, it significantly increased duodenal maltase and jejunal sucrase activities; on the absorptive side, it simultaneously upregulated the expression of key VFA transporters (*MCT1*, *DRA*, *NHE3*) in the rumen and primary glucose transporters (*SGLT1*, *GLUT2*) in the jejunum. This potentially complementary effect of "promoting digestion" and "enhancing absorption" is valuable. Increased disaccharidase activity facilitates the more complete release of monosaccharides, providing substrates for subsequent absorption [46]. The upregulation of key transporters may contribute to the capacity of epithelial cells to take up VFAs and glucose. For example, MCT1 is a crucial protein for transporting VFAs, particularly butyrate, and its expression is finely regulated by nutritional and cellular signaling pathways [47]. However, direct measurements of nutrient flux or absorption were not performed in this study, and the observed expression changes should be interpreted as indicators of potential absorptive capacity rather than functional proof. Phytochemicals within this formula may mimic or modulate these signaling pathways, which remains a hypothesis for future investigation. Strategies relying solely on exogenous enzyme supplementation or high-glucose diets provide substrates or enzymes directly. In contrast, the herbal formula investigated in this study focuses on enhancing the host's intrinsic digestive and absorptive functions. However, direct comparative evidence is lacking. This highlights the potential of TCMF in restoring and enhancing the body's own functional capacity through multi-target synergy, representing a more fundamental approach to nutritional regulation. This embodies the modern scientific connotation of the "spleen-invigorating" theory in Traditional Chinese Medicine, specifically the concept of "transporting and transforming food essence," i.e., improving nutrient conversion and utilization by regulating the body's own functions. This traditional theoretical interpretation should be distinguished from experimentally demonstrated biological mechanisms. It provides a novel perspective and robust experimental evidence for developing functional feed additives to promote growth in young ruminants.

It is important to acknowledge key statistical limitations of the present study. Only four independent pens were available per treatment. For individual-animal measurements, one lamb was sampled from each pen, yet statistical models could not incorporate pen as a random effect due to the small pen number, creating pseudoreplication risk. Intra-pen correlations among lambs may lead to inflated type-I error; hence statistically significant differences observed for serum, tissue histology, gene- and protein-expression outcomes should be viewed as preliminary associations rather than definitive treatment effects. Conclusions drawn from pen-level outcomes are also constrained by the low number of experimental units. Further trials with a greater number of pens are needed to robustly validate the biological effects of JHF I supplementation in weaned Hu lambs. In addition, although each raw herbal material was authenticated according to Chinese Pharmacopoeia criteria, quantitative phytochemical profiling was not conducted on the final compound JHF mixtures. Therefore, the actual concentrations of bioactive constituents within the experimental diets remain unknown, which represents an important limitation of the present work.

5. Conclusions

In conclusion, dietary supplementation with JHF was associated with modulations in antioxidant status, immune function, intestinal digestive enzyme activities, ruminal epithelial morphology, and nutrient transporter expression in weaned Hu lambs. Under the current experimental conditions and among the doses evaluated in this trial, 0.5% JHF I showed the greatest efficacy in increasing serum IgG and GSH-Px while decreasing MDA and DAO, and in improving duodenal maltase and jejunal sucrase activities, ruminal papilla width, and the expression of tight junction proteins and nutrient transporters. However, these findings should be interpreted with caution given the limited number of

pens per treatment, the limited number of animals used for tissue analyses, the single endpoint assessment, and the selection of the 0.5% JHF I treatment based on observed results. Without a pre-weaning baseline trajectory, this study demonstrates differences in selected endpoints in weaned lambs rather than confirmed relief of weaning stress. The most responsive treatment within the present experimental setting was 0.5% JHF I, and it should not be regarded as a generally optimal supplementation level for broader application. Further studies with larger sample sizes and direct measurements of nutrient flux and inflammatory or microbial parameters are needed to confirm the mechanisms by which JHF may alleviate weaning stress in lambs.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

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Conflicts of Interest: Minling Zong and Xiaobin Yang are employees of Jiangxi Zhongchengren Pharmaceutical Co., Ltd., which provided the raw materials for JHF I and JHF II. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results. The remaining authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

DAO	diamine oxidase
DRA	downregulated in adenoma
GLUT2	glucose transporter 2
GSH-Px	glutathione peroxidase
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
JHF	Jianpi Herbal Formula
MCT1	monocarboxylate transporter 1
MDA	malondialdehyde
NHE3	Na^+/H^+ exchanger 3
SGLT1	sodium-glucose linked transporter 1
SOD	superoxide dismutase

T-AOC	total antioxidant capacity
TCMF	traditional Chinese medicine formulation
VFA	volatile fatty acid
ZO-1	zonula occludens-1

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