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The Effects of Tea Polyphenols to Feed on the Immunity, Antioxidant Capacity, and Gut Microbiota of Weaned Lambs

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Simple Summary: Weaning stress induces oxidative stress, which exerts a detrimental influence on the growth and intestinal health of lambs. Antibiotics are effective in alleviating weaning stress. However, the concerns associated with antibiotic resistance and residual side effects have motivated researchers to seek alternative strategies to address weaning stress. Tea polyphenols, as natural plant extracts, have certain biological activities such as antioxidant, anti-tumor, antibacterial, and anti-inflammatory. In this study, adding 4-6 g/Kg of tea polyphenols can effectively maintain gut microbiota homeostasis and have anti-inflammatory and antioxidant effects similar to antibiotics. The potential mechanism might be regulating the production of NO by modulating *iNOS* mRNA expression and synthesis, thus regulating the antioxidant capacity. It also enhances intestinal epithelial immune defense and reduces inflammatory damage by inhibiting the *TLR4/MyD88/NFκB* signaling pathway and cytokine-related gene expression. Therefore, tea polyphenols could be used as a substitute for antibiotics to ensure the safety of livestock products and achieve sustainable development of modern animal husbandry.

Abstract: In this present study, we aim to investigate the effects of adding tea polyphenols to feed on the immunity, antioxidant capacity, and gut microbiota of weaned lambs. Thirty weaned lambs (2 month old, average initial weight 9.32 ± 1.72 kg) were randomly divided into five groups with six lambs in each group. The goat kids were randomly divided into four groups: a control group (CON) fed the basal diet, and four other groups supplemented with 2, 4, 6 g/kg tea polyphenols and 50 mg/kg chlortetracycline in the basal diet (denoted as T1, T2, T3 and CTC groups, respectively). The results indicate that adding 4-6 g/kg tea polyphenols can raise the expression levels of antioxidant enzymes and their genes in lambs' intestines. It also increases the expression of *Nrf2*, *iNOS*, and *IL-10*, while reducing the levels and gene expression of cytokines (IL-1β, IL-6, IFN-γ, and TNF-α) (P<0.05). At the same time, it reduced the expression levels of signaling pathways *TLR4*, *MyD88*, and *NFκB* (P<0.05) activated intestinal protective mechanisms, and enhanced the immune defense of intestinal epithelium. Compared with other groups, feeding tea polyphenols significantly increased the acetic acid content in the cecum of lambs (P<0.05), effectively promoting intestinal health. Tea polyphenols significantly increase the Shannon and Simpson indices, boost the abundance of *Verrucomicrobiota*, and reduce that of *Proteobacteria* and *Firmicutes* (P<0.05). The relative abundance of *Candidatus_Soleaferrea*, *Christensenellaceae R-7 group*, and *Prevotella* in the tea polyphenol group is significantly higher than in the chlortetracycline group (P<0.05). Overall, these results indicate that tea polyphenols can effectively maintain the homeostasis of the gut microbiota and have anti-inflammatory and antioxidant effects similar to antibiotics.

Keywords: tea polyphenols; weaned lamb; immune function; Antioxidant function; Cecal short-chain fatty acids; Cecal microbes

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1. Introduction

The intestine, as a key organ for nutrient digestion and absorption, is the largest immune organ in the body. Therefore, effective gastric and intestinal function is crucial for animal health, growth, and production performance [1]. However, when lambs are weaned, factors such as underdeveloped intestines, changes in dietary structure, mother infant separation, and external environmental changes can cause nutritional and psychological stress. It brings a series of stress reactions to the growth, physiology and immunity of young animals, resulting in problems such as low weight, high mortality and increased incidence rate [2]. Some studies have shown that weaning may lead to oxidative stress, thereby damaging the intestinal barrier [3], causing disruption of intestinal immune function [4,5], imbalance of microbial communities [6], and diarrhea [7]. Therefore, how to alleviate the negative effects of weaning stress is an important prerequisite for producing healthy livestock products. Antibiotics have been widely used to reduce weaning stress response [8], but the side effects of drug resistance and residue have led to a global ban on mass feeding. Therefore, developing natural plant extracts with anti-inflammatory and antioxidant properties to replace antibiotics in agricultural production has become a global research focus.

Tea is one of the most widely consumed beverages globally, containing various bioactive compounds beneficial to health [9]. Tea polyphenols are a general term for polyphenolic substances in tea. According to chemical structure, tea polyphenols can be classified into catechins, flavonoids, flavonols, phenolic acids, peptides, anthocyanins, and other small amounts of polyphenols (such as epigallocatechin gallate, flavonoids, and tannins) [10]. It can be digested and broken down into catechins and flavonoids by the gastrointestinal tract and then absorbed by the intestine. These decomposition products can maintain stable biological activity and exert certain antioxidant capacity, which is achieved through hydrogen atom transfer through hydroxyl structures or single electron transfer through electron structures similar to free radicals, thereby inhibiting free radical reactions, reducing cell or tissue damage, and preventing intestinal diseases [11,12]. According to reports, polyphenols and their derivatives can promote the expression of antioxidant enzymes by upregulating the genes of antioxidant response elements (ARE) [13], restore the body's redox homeostasis, and prevent systemic or local inflammation. In addition, it can counteract inflammatory stimuli by stimulating the activation of pathways such as Nrf2, *NFκB*, and downstream iNOS and COX-2 [14]. This is attributed to the phenolic hydroxyl group. As a natural plant, tea polyphenols have been shown to regulate the balance of gut microbiota and promote gut health [15,16].

We previously research indicated that adding 4 - 6 g/kg of tea polyphenols or antibiotics to the diet significantly increased the average daily weight gain (ADG) and average daily feed intake (ADFI) compared with the control group. We found that feeding tea polyphenols at concentrations of 4 and 6 g/Kg could increase the activity of antioxidant enzymes in the intestines of weaned lambs, enhance the immune capacity of blood samples, promote the integrity of the intestinal barrier, and reduce intestinal damage [17]. However, there is currently no evidence to explain the mechanism by which tea polyphenols alleviate symptoms. Therefore, this article explores the mechanism of the effects of tea polyphenols on the antioxidant and immune abilities of weaned lambs, as well as their effects on short chain fatty acids and cecal microbiota, aiming to provide a reference for the intestinal health of young ruminants.

2. Materials and Methods

The experimental protocol applied in this study followed the guidelines of the Animal Care and Use Committee of Guangdong Ocean University.

2.1. Materials

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The tea polyphenols used in this experiment were provided by Xi'an Best Biotechnology Co., Ltd. (Shaanxi, China). Tea polyphenols were acquired in the form of brown powders with a special odor, in which the content of tea polyphenols is 98.1%.

2.2. Lambs and Experimental Protocol

The design of this study was described in an earlier paper [17]. Briefly, thirty Leizhou lambs with similar weight (9.32 ± 1.72kg， about 2 months old) and good health were selected as experimental animals. All lambs were artificially weaned at 2 months of age and randomly divided into 5 treatment groups with 6 replicates in each group. The pre trial period was 7 days, and the main trial period lasted for 45 days. The control group (named CON) was fed with basic feed, while the experimental group was fed with 2, 4, and 6 g/kg tea polyphenols and 50 mg/kg chlortetracycline (named T1, T2, T3, and CTC), respectively. Tea polyphenols and chlortetracycline fed every day were evenly mixed into the concentrated feed. During the experiment, lambs were fed concentrate first and then roughage, and enough clean drinking water was provided every day during the experiment.

Before the experiment begins, the lamb houses, feeding pens, metabolic cages, water troughs, and feed troughs were thoroughly cleaned and sterilized. After vaccination, deworming, and numbering, six lambs were placed in each pen and fed according to the designed diet, two times a day in the morning and at afternoon. During the feeding period, the feeding methods, experimental environment, and management mode of each group were the same. All experiments were designed by one-way random experiment. Daily teosinte was added into pellet feed by TMR, and the formulation of the basal diet (Table 1) was in accordance with the nutritional requirements of the Feeding standard of Goat, China (NY/T861-2004).

2.3. Sample Collection

On the 45th day of the experiment, blood samples were collected from the jugular vein. The blood samples were centrifuged at 1500× *g* for 10 min at 4 ℃, and the serum was collected and stored at -80 ℃ for further analysis.

The lambs were fasted for 12 h prior to slaughter at the end of the trial. After euthanasia through electrocution and bloodletting, the lamb was quickly dissected to collect tissue samples from the duodenum, jejunum, and ileum. The samples were washed three times with pre-cooled phosphate buffer and immediately transferred to liquid nitrogen. At the same time, the contents of the cecum were collected and frozen with liquid nitrogen for analysis of microbial communities and detection of short chain fatty acid concentrations.

2.4. Determination of Serum Antioxidant Indicators

The serum GSH-Px, CAT, T-SOD, MDA, and T-AOC levels were measured using ELISA assay kit, and the detection method was strictly in accordance with the kit instructions (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

Table 1. Composition and level of basal diet (dry matter basis).

Ingredients(%)	Content	Nutrient level	Content
Pennisetum × sinese	50.00	DM (%)	90.80
Corn	29.00	CP	14.69
Soybean meal	10.00	EE	2.84
Wheat bran	7.50	ADF	26.23
NaCl	0.50	NDF	39.90
$CaHPO_4$	0.50	Ca	0.54
Limestone	0.50	P	1.10
Premix1	2.00	CA	7.90
Total	100	ME[2] (MJ/Kg)	10.43

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[1] The premix provided the following per kg of diets: VA 17500 IU, VD 6200 IU, VE 50 IU, Cu 20mg, Fe 75mg, Se 0.4mg, Mn 80mg, Co 0.3mg, I 1.2mg, Zn 40mg. [2] DM, dry matter; ME, metabolic energy; ADF, acid detergent fiber; NDF, neutral detergent fiber. ME is the calculated value.

2.5. Determination of Intestinal Immune Indicators

After the frozen intestinal mucosa samples were thawed on ice, intestinal mucosa samples (0.5 g) were weighed and added to pre-cooled saline at a mass-to-volume ratio of 1:9 (g/mL) and ultrasonically pulverized to prepare tissue homogenates. After centrifugation at 3,000× *g* at 4 ℃ for 15min, the supernatant was collected and stored in a −80 ℃ refrigerator and the immune indicators of the intestine (IL-1β, IL-6, IL-10, IFN-γ, TNF-α, iNOS, and NO) were determined using ELISA kits according to the instructions of the kit (Jiangsu Meimian Industrial Co. Ltd., Jiangsu, China).

2.6. RT-qPCR Analysis

The total RNA extraction, reverse transcription to cDNA, and real-time quantitative polymerase chain reaction analysis employed the methods described by Xu et al. [17]. Primers were designed according to the mRNA sequence of goat target genes on the NCBI official website (Table 2), and then passed on to Shenggong Biotechnology Co., Ltd. (Shanghai, China) for synthesis. According to the instructions of ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China), the relative mRNA expression of genes was determined using a real-time fluorescence quantitative PCR instrument and the $2^{-\Delta\Delta Ct}$ method was used for calculation.

2.7. Determination of Volatile Fatty Acids

Mix the intestinal contents and ultrapure water evenly in a ratio of 1:9, centrifuge at 12000× g for 10 minutes. Add the supernatant to a solution of phosphoric acid and ethanol, and continue centrifugation at 12000× g for 10 minutes. After filtration through a 0.22 μ filter membrane, short chain fatty acids were determined using a gas chromatograph (Trace 1310 and ISQLT, Thermo, USA), and the measurement step is in accord with Guo et al. [7].

2.8. DNA Extraction

The total DNA was extracted from the cecal contents according to Yang et al. [18]. After estimating the concentration and quality of DNA with 1% agarose gel, the sample was diluted to 1 ng/μL with sterile water.

2.9. 16S rRNA Based Sequencing Analysis of Cecal Contents

Following the method of Yang et al. [18], primers were designed based on conservative regions, and sequencing adapters were added to the ends of the primers for PCR amplification. The products were purified, quantified, and homogenized to form a sequencing library. The library that has been constructed and passed quality inspection is sequenced using Illumina NovaSeq 6000. The raw image data files obtained from high-throughput sequencing are converted into raw sequenced reads through base calling analysis, and the results are stored in FASTQ file format, which includes sequence information and quality information.

Table 2. Real-time PCR primer sequences.

Gene	Primer sequences (5′-3′)	GenBank accession No.	Length (bp)
CAT	F:CACTCAGGTGCGGGATTTCT R:CTGGATGCGGGAGCCATATT	XM_004016396.5	163
INOS	F:ACGGGGACGGTAAAGACATC R:CCGGGGTCCTATGGTCAAAC	XM_013971952.2	210
GPX1	F:CAGTTTGGGCATCAGGAAAACG R:GCCTTCTCGCCATTCACCTC	XM_004018462.5	128

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SOD1	F:CCATCCACTTCGAGGCAAAG R:GCACTGGTACAGCCTTGTGTA	NM_001285550.1	124
Nrf2	F:TCTGCTGTCAAGGGACATGGA R:CGCCGGTCTCTTCATCTAGT	NM_001314327.1	212
NFκB	F:GAAGAGAAGGCGCTCACCAT R:ATCACAGCCAAGTGGAGTGG	XM_018066509.1	107
MYD88	F:ACTCATTGAGAAGAGGTGCCG R:CTTGATGGGGATCAGTCGCT	XM_013973392.2	139
TNF-α	F:TGCACTTCGGGGTAATCGG R:CGCTGATGTTGGCTACAACG	NM_001024860.1	144
TLR4	F:GGGTGCGGAATGAACTGGTA R:CTGGGACACCACGACAATCA	NM_001285574.1	158
IL-1β	F:AATGAGCCGAGAAGTGGTGT R:CAGTGTCGGCGTATCACCTT	XM_013967700.2	136
IL-10	F:TACCCACTCTGGGGTCTTGT R:CTGCCAAGCTCATTCACACG	XM_005690416.3	121
IFN-γ	F:AGATCCAGCGCAAAGCCATA R:TCTCCGGCCTCGAAAGAGAT	NM_001285682.1	110
GAPDH	F:GATGCCCCCATGTTTGTGATG R:CGTGGACAGTGGTCATAAGTC	XM_005680968.3	160
IL-6	F:ATCTGGGTTCAATCAGGCGAT R:TGCGTTCTTTACCCACTCGT	NM_001285640.1	247

2.10. Statistical Analysis

Firstly, the raw reads obtained from sequencing were filtered using Trimmomatic v0.33 software. Then, cutadapt 1.9.1 software was used to identify and remove primer sequences, resulting in clean reads without primer sequences. Finally, the divisive amplicon denoising algorithm 2 (DADA2) method in QIIME2 2020.6 was used for denoising [19]. After concatenating the double ended sequences and removing the chimeric sequences, the non-chimeric reads is obtained, which is further divided into feature (OTUs, ASVs), diversity analysis, difference analysis, correlation analysis, and functional prediction analysis.

All the experimental data was collated using Excel 2019 to establish a database, and one-way ANOVA was performed using SPSS software (version 26.0). Analyze inter group differences through Tukey's multiple comparison test and the column chart was made by using GraphPad Prism 8. The statistical significance was set at $P<0.05$.

3. Results

3.1. Effects of Tea Polyphenols on Serum Antioxidant Capacity of Weaned Lambs

The effect of dietary tea polyphenols on the serum antioxidant capacity of weaned lambs is shown in Table 3. Compared with the CON group, the activity of CAT in the serum of weaned lambs in T2, T3, and CTC groups was significantly increased ($P<0.05$), while the content of MDA in the serum of lambs in T2 and T3 groups was significantly decreased ($P<0.05$). In addition, the activities of GSH-Px, T-SOD, and T-AOC in the serum of lambs in the T2 and CTC groups were significantly higher than those in the other groups ($P<0.05$), while the differences in the other groups were not significant ($P>0.05$). The results indicated that dietary supplementation with tea polyphenols exhibited a similar effect to that of chlortetracycline in enhancing the serum antioxidant capacity of weaned lambs, and the optimal dosage appeared to be between 4-6 g/kg of tea polyphenols.

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Table 3. Effects of tea polyphenols on serum antioxidant capacity of weaned lambs.

Items	Groups					SEM	*P*-Value
	CON	T1	T2	T3	CTC		
MDA (nmol/mgprot)	2.18^{a}	2.00^{a}	1.07^{b}	0.82^{b}	1.89^{a}	0.162	0.002
GSH-Px (U/gprot)	65.29^{c}	65.12^{c}	97.52^{b}	66.01^{c}	106.28^{a}	4.908	<0.001
CAT (U/mgprot)	2.31^{c}	2.59^{bc}	2.94^{a}	2.77^{ab}	2.64^{b}	0.066	0.005
T-SOD (U/mgprot)	117.17^{b}	117.43^{b}	164.15^{a}	128.24^{b}	164.81^{a}	5.948	<0.001
T-AOC (mmol/gprot)	0.56^{cd}	0.51^{d}	0.87^{a}	0.61^{c}	0.79^{b}	0.037	<0.001

[1] MDA: malondialdehyde; GSH-pX: glutathione peroxidase; CAT: catalase; T-SOD: total superoxide dismutase; T-AOC: total antioxidant capacity. [2] SEM: standard error of the mean. $^{a–d}$ Values in the same row with different letters are significantly different (P<0.05). Results are presented as the mean ± SEM (n=6).

3.2. *Effects of Tea Polyphenols on the Expression of Antioxidant Genes in the Intestines of Weaned Lambs*

To further evaluate the antioxidant capacity of dietary tea polyphenols, we compared the expression of antioxidant genes in the intestinal tract of weaned lambs in CON, T2, T3, and CTC groups. As evident from Figure 1, compared to the CON group, the relative expression levels of *SOD*, *GPX*, *CAT*, and *Nrf2* genes were notably elevated in the intestines of weaned lambs treated with tea polyphenols and antibiotics (P<0.05). In the duodenum, there was no significant difference in the relative expression level of *Nrf2* gene between T3 and CTC groups (P>0.05), while the CAT gene in T2 group was significantly reduced compared with other groups (P<0.05). Simultaneously, compared with the tea polyphenol and CON groups, the *GPX* and *CAT* activities in the CTC group were significantly increased (P<0.05). In the jejunum, compared to the other groups, the activities of *GPX*, *CAT*, and *Nrf2* genes were notably increased in the CTC group (P<0.05). However, the *CAT* gene activity in the T2 group surpassed than T3 group (P<0.05). In the ileum, compared to the other groups , the *GPX* gene content was significantly elevated in the CTC group (P<0.05), whereas the *Nrf2* gene content in the T3 group was notably reduced compared to both the CTC and T2 groups (P<0.05).

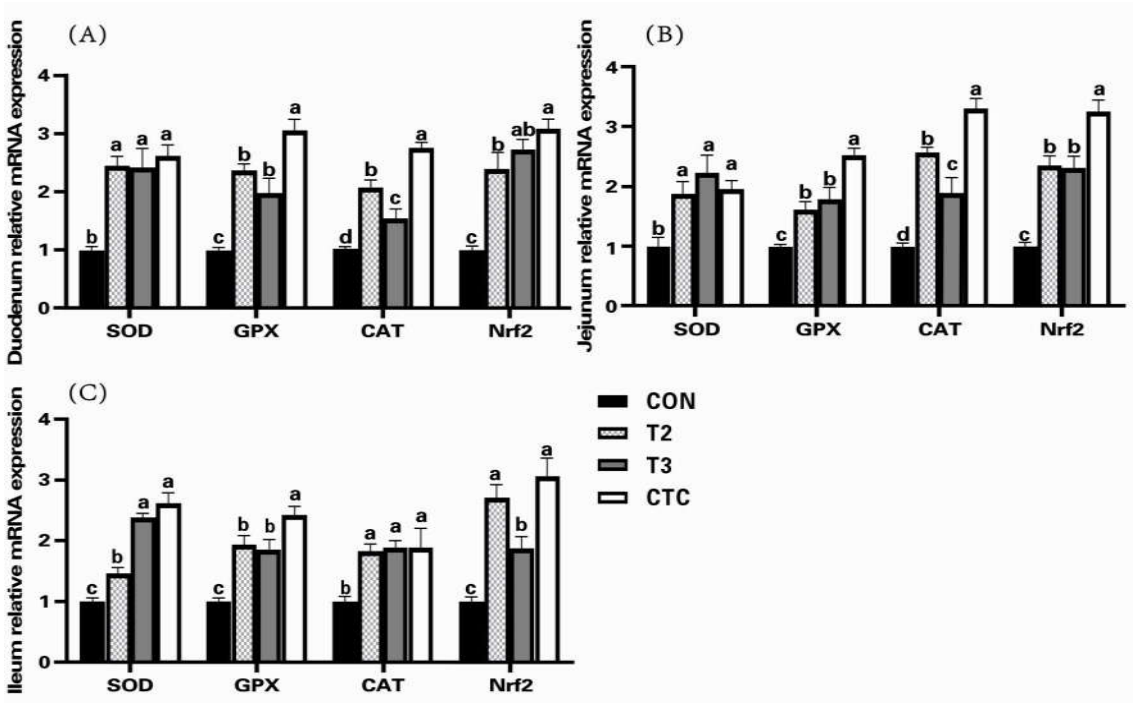

Figure 1. Effects of tea polyphenols on the expression of antioxidant genes in the intestines of weaned lambs. **(A–C)**: The gene relative expression of *SOD*, *GPX*, *CAT*, and *Nrf2* in the duodenum, jejunum, and ileum, respectively. The results are expressed as the mean ± SEM (n = 6). The mean values of $^{a–c}$ were significantly different (P < 0.05).

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3.3. Effects of Tea Polyphenols on Intestinal Immune in Weaned Lambs

To assess the anti-inflammatory properties of tea polyphenols, we measured cytokines indicative of inflammation in animals and investigated whether the supplementation of tea polyphenols could enhance the immune function of weaned lambs' intestines. The findings are presented in Table 4. Overall, the incorporation of tea polyphenols and aureomycin effectively decreased the concentrations of IL-1β, IL-6, TNF-α, and IFN-γ in the duodenum, jejunum, and ileum, while simultaneously elevated the concentrations of NO and IL-10 (P<0.05). In the duodenum, dietary supplementation with tea polyphenols and chlortetracycline notably decreased the levels of IL-1β, IL-6, and TNF-α, and significantly increased the levels of IL-10 and NO compared to CON (P<0.05). Notably, T2 performed as well as CTC in terms of IL-1β expression. Additionally, T3 and CTC exhibited significantly lower IFN-γ levels compared to other groups, whereas their iNOS levels were notably higher (P<0.05). Compared with the duodenum, similar expression patterns of these cytokines were observed in the jejunum and ileum, and CTC has the best anti-inflammatory effect. However, the difference is that there is no significant difference in the levels of IL-6 between T3 and CTC in the jejunum, while IL-6 and TNF-α in the ileum (P>0.05).

3.4. Effects of Tea Polyphenols on the Expression of Cytokine Genes in the Intestines of Weaned Lambs

Similarly, considering that tea polyphenols at doses of 4 and 6 g/kg have the best immune effects on lambs, selected CON, T2, T3, and CTC groups to further compare the expression of intestinal cytokine genes. As shown in Figure 2, compared to the CON group, supplementation with 4 g/kg and 6 g/kg of tea polyphenols, along with the antibiotic group, significantly reduced the gene expression levels of cytokines *IL-1β*, *IL-6*, *IFN-γ*, and *TNF-α* in the duodenum, jejunum, and ileum of weaned lambs (P<0.05), while markedly increasing *IL-10* levels (P< 0.05). In the duodenum, the CTC group had the best immune response (P<0.05), but the gene expression of *IFN-γ* in the T2 group was significantly lower than CTC group (P<0.05). In the jejunum, there was no significant difference in the gene expression of *IL-1β*, *IL-6*, and *IFN-γ* between T2, T3, and CTC groups (P>0.05), but *TNF-α* showed a gradually decreasing trend (P<0.05). In the ileum, the gene expression of *TNF-α* and *IFN-γ* in the CTC group was significantly lower than other groups, but the *IL-10* gene content was significantly higher (P<0.05).

Table 4. Effects of tea polyphenols on intestinal immune in weaned lambs.

Items	Groups					SEM	*P*-Value
	CON	T1	T2	T3	CTC		
Duodenum							
IL-1β (pg/mL)	75.10^{a}	59.79^{b}	54.73^{c}	49.77^{d}	43.75^{e}	2.054	<0.001
IL-6 (pg/mL）	141.35^{a}	126.30^{b}	122.91^{b}	104.18^{c}	81.18^{d}	3.937	<0.001
IL-10 (pg/mL)	43.64^{d}	49.91^{c}	52.24^{c}	58.08^{b}	65.22^{a}	1.429	<0.001
TNF-α (pg/mL)	266.69^{a}	213.88^{b}	212.14^{b}	200.75^{b}	154.36^{c}	6.923	<0.001
NO (μmol/L)	30.00^{d}	33.59^{c}	34.78^{c}	37.57^{b}	43.17^{a}	0.869	<0.001
iNOS (pg/mL)	75.78^{d}	79.98^{cd}	82.96^{c}	103.65^{b}	110.26^{a}	2.668	<0.001
IFN-γ (pg/mL)	569.04^{a}	560.0216^{a}	528.01^{a}	427.94^{b}	350.79^{c}	16.936	<0.001
Jejunum							
IL-1β (pg/mL)	66.60^{a}	62.55^{a}	56.39^{b}	48,84^{c}	39.63^{d}	1.858	<0.001
IL-6 (pg/mL）	138.85^{a}	122.39^{b}	117.66^{b}	94.67^{c}	86.69^{c}	3.678	<0.001
IL-10 (pg/mL)	42.19^{d}	48.13^{c}	53.50^{b}	56.03^{b}	63.60^{a}	1.450	<0.001
TNF-α (pg/mL)	284.46^{a}	236.28^{b}	227.80^{b}	202.40^{c}	167.86^{d}	7.363	<0.001
NO (μmol/L)	29.25^{d}	33.94^{c}	34.77^{c}	39.68^{b}	43.31^{a}	0.945	<0.001
iNOS (pg/mL)	75.44^{d}	79.73^{cd}	84.99^{c}	95.46^{b}	106.99^{a}	2.229	<0.001
IFN-γ (pg/mL)	610.97^{a}	543.99^{b}	489.79^{c}	422.83^{d}	367.66^{e}	16.964	<0.001
Ileum							
IL-1β (pg/mL)	74.59^{a}	64.91^{b}	56.55^{c}	52.49^{d}	41.83^{e}	2.104	<0.001

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IL-6 (pg/mL）	131.52^{a}	123.40^{a}	120.88^{a}	98.14^{b}	88.67^{b}	3.446	<0.001
IL-10 (pg/mL)	45.29^{c}	47.88^{c}	49.79^{c}	56.67^{b}	65.39^{a}	1.459	<0.001
TNF-α (pg/mL)	262.52^{a}	250.28^{ab}	229.75^{b}	184.37^{c}	167.63^{c}	7.312	<0.001
NO (μmol/L)	31.11^{c}	32.42^{c}	35.22^{b}	36.84^{b}	43.06^{a}	0.832	<0.001
iNOS (pg/mL)	68.669^{e}	78.74^{d}	91.25^{c}	103.74^{b}	111.23^{a}	2.990	<0.001
IFN-γ (pg/mL)	633.22^{a}	507.41^{b}	455.66^{c}	431.73^{c}	341.65^{d}	18.064	<0.001

[1] IL-1β: interleukin-1β; IL-10: interleukin-10; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-γ. [2] SEM: standard error of the mean. $^{a–e}$ Values in the same row with different letters are significantly different ($P< 0.05$). Results are presented as the mean ± SEM (n = 6).

3.5. Effects of Tea Polyphenols on the Expression of the TLR4/NFκB Pathway-Related Genes and iNOS Gene Expression in the Intestines of Weaned Lambs

According to Figure 3, overall, compared to the CON group, the gene content of NFκB, Myd88, and TLR4 in weaned lambs fed with tea polyphenols and antibiotics significantly decreased (P<0.05), while the expression level of iNOS gene significantly increased (P<0.05). In the duodenum, the content of NFκB gene in T3 and CTC groups was significantly lower than other groups (P<0.05), while Myd88 and TLR4 genes in the jejunum (P<0.05). Compared with the CON group, the expression levels of NFκB gene in T2, T3, and CTC groups were significantly decreased (P<0.05), while the gene level of iNOS showed a stepwise increase (P<0.05); In the ileum, the Myd88 gene content in the three groups was significantly lower than CON group, and the iNOS level was significantly increased. The expression levels of NFκB gene in the T3 and CTC groups were significantly lower than other groups (P<0.05), and TLR4 was the lowest in the T3 group (P<0.05). The differences between the other groups were not significant (P>0.05).

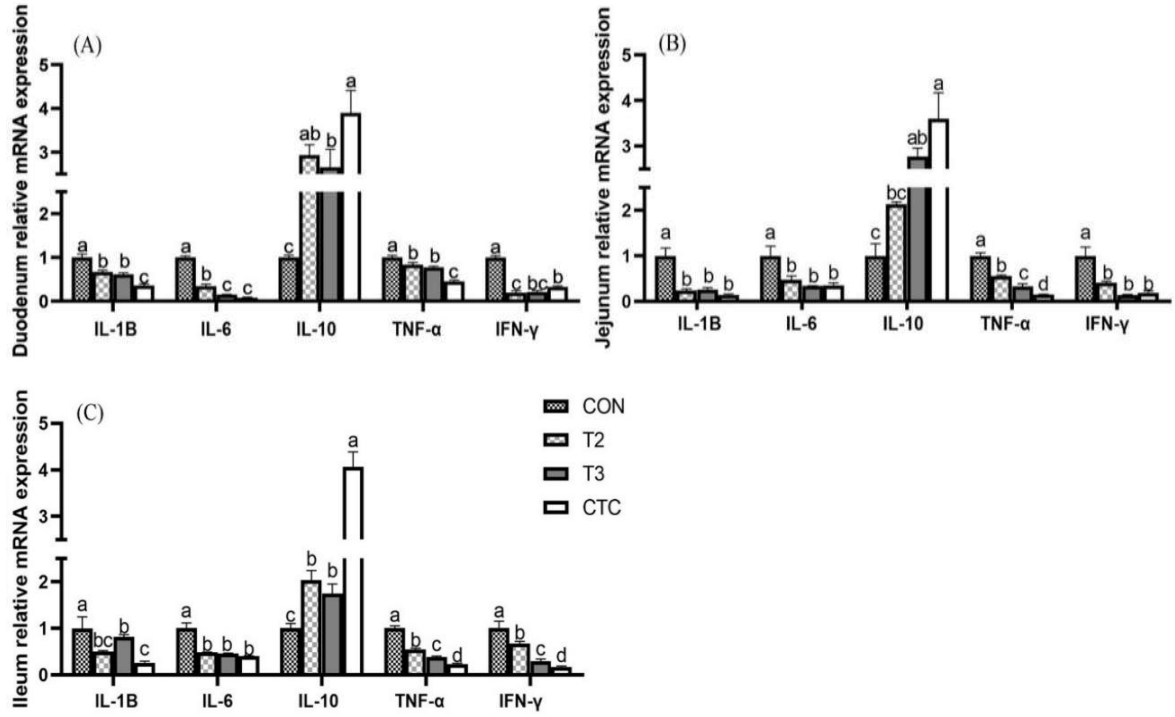

Figure 2. Effects of tea polyphenols on the expression of cytokine genes in the intestines of weaned lambs. **(A–C)**: The gene relative expression of *IL-1β*, *IL-6*, *IL-10*, *IFN-γ*, and *TNF-α* in the duodenum, jejunum, and ileum, respectively. The results are expressed as the mean ± SEM (n = 6). The mean values of $^{a–c}$ were significantly different ($P< 0.05$).

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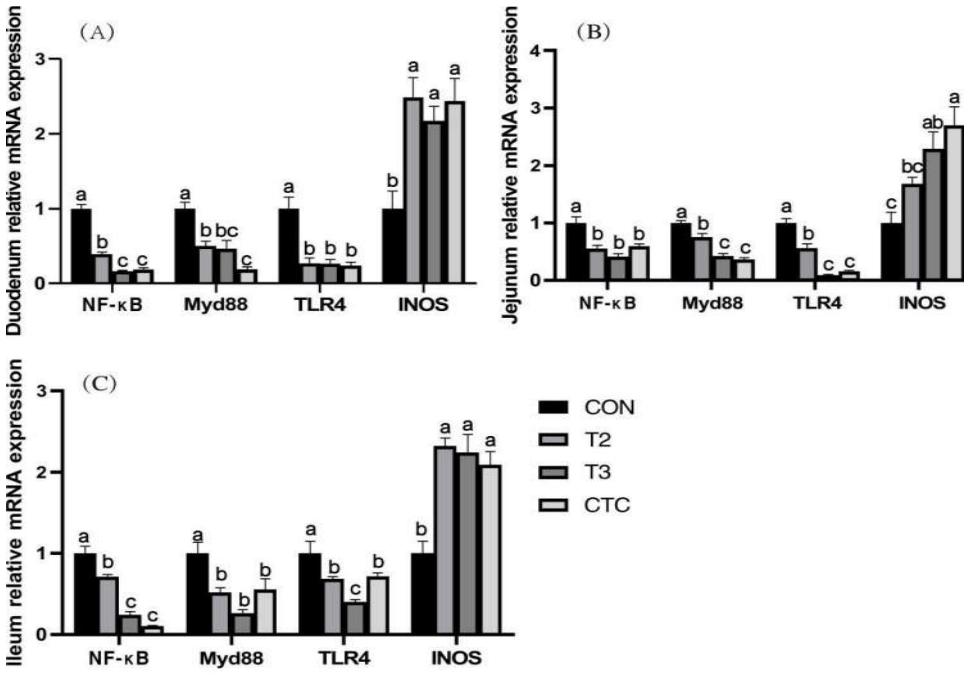

Figure 3. Effects of tea polyphenols on the expression of the *TLR4/NFκB* pathway-related genes and *iNOS* gene expression in the intestines of weaned lambs. **(A–C):** The gene relative expression of *NFκB, Myd88, TLR4* and *iNOS* in the duodenum, jejunum, and ileum, respectively. The results are expressed as the mean ± SEM (n = 6). The mean values of $^{a-c}$ were significantly different ($P < 0.05$).

3.6. Effects of Tea Polyphenols on Volatile Fatty Acids in the Cecum of Weaned Lambs

The content of short chain fatty acids, such as acetic acid, propionic acid, isobutyric acid, and butyric acid, can affect intestinal barrier function and body metabolism. As shown in Table 5, the content of acetic acid in the cecum of T2 group was significantly higher than other groups ($P<0.05$), while different concentrations of tea polyphenols and chlortetracycline had no significant effect on the content of propionic acid, isobutyric acid, butyric acid, and isovaleric acid ($P>0.05$).

Table 5. Effects of tea polyphenols on intestinal volatile fatty acids in weaned lambs.

Items	Groups					SEM	*P*-Value
	CON	T1	T2	T3	CTC		
Acetic Acid	448.80^{b}	660.50^{b}	1281.85^{a}	612.88^{b}	715.56^{b}	87.844	0.005
Propionic Acid	420.67	394.61	703.58	648.02	412.95	47.695	0.071
Isobutyric Acid	151.50	163.23	158.032	158.31	168.62	5.492	0.927
Butyric Acid	234.52	345.35	358.19	321.52	259.99	28.82	0.647
Isovaleric Acid	154.089	161.87	155.287	163.34	168.53	4.827	0.910

[1] CON: basal diet; T1: basal diet + 2 g/kg tea polyphenols; T2: basal diet + 4 g/kg tea polyphenols; T3: basal diet + 6 g/kg tea polyphenols; CTC: basal diet + 50 mg/kg chlortetracycline. [2] SEM: standard error of the mean. $^{a-d}$ Values in the same row with different letters are significantly different ($P<0.05$). Results are presented as the mean ± SEM (n=6).

3.7. Effects of Tea Polyphenols on the Composition of Cecal Bacteria in Weaned Lambs

By sequencing the bacterial 16S rDNA V3 + V4 region, the microbiota of the cecal contents in the five groups of weaned lambs were examined. High-throughput sequencing was conducted on three random cecum samples from each group, and the CON, T1, T2, T3, and CTC groups generated an average of 58886, 55578, 57978, 67672, and 52331 non-chimeric reads (n=3), respectively. These

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sequences were assigned to 31 phyla, 65 classes, 145 orders, 238 families, 430 genera, and 527 species based on a 97 % similarity definition of the operational taxonomic unit (OTU).

The Venn diagram can display the shared and unique microorganisms among different samples. The unique OTU of the CTC group is 880, which is between the number of OTUs in the three treatment groups. In the three treatment groups given 2-6 g/kg tea polyphenols, the number of unique OTUs was 565, 804, and 936 respectively, showing an increasing trend. This is consistent with the overall trend of OTU. These five groups had a total of 17 common OTUs, suggesting that different concentrations of tea polyphenols and chlortetracycline altered the existing microbial species in the intestinal of weaned lambs (Figure 4A).

When the number of valid sequences reaches 20,000, the microbial dilution curves of each group of samples tend to level off, suggesting that the detection is sufficient to cover all species and the sequencing quantity will not increase further (Figure 4C). Consequently, it implies that the microbial data is reliable. Figure 4D, the rank abundance curve, is a graphical representation of the feature abundance of each sample. The wider and flatter the curve is along the horizontal axis, the richer and more uniformly distributed the species composition will be. The abscissal span increases with the increase of tea polyphenol concentration, and this trend showed that the species abundance increases with the increase of dosage.

The Chao-1 index, Ace index, Shannon index, and Simpson index can be employed to measure the richness and diversity of microbial communities. The larger the Chao and ACE indices, the higher the richness of microbial communities. The larger the Shannon and Simpson indices, the higher the diversity of microbial communities. The alpha diversity analysis of gut microbiota is presented in Table 6. The Chao-1 index and Ace index of gut microbiota in each group did not exhibit significant changes (P>0.05), suggesting that there was no remarkable alteration in the abundance of gut microbiota. However, the Simpson index of the cecum in the CTC group was significantly lower than that of other groups (P<0.05). Meanwhile, the Shannon index of the CTC group was significantly lower than that of the CON and T3 groups (P<0.05).

Table 6. Effects of tea polyphenols on α-diversity index of caecum microorganisms in weaned lambs.

Items	Groups					SEM	*P*-Value
	CON	T1	T2	T3	CTC		
ACE	1123.65	1145.74	1390.47	1258.08	1075.16	52.046	0.343
Chao-1	1112.59	1135.93	1384.40	1249.713	1067.87	52.204	0.339
Simpson	0.988^{a}	0.986^{a}	0.992^{a}	0.991^{a}	0.963^{b}	0.003	0.015
Shannon	7.68^{a}	7.69^{ab}	8.27^{ab}	8.17^{a}	7.09^{b}	0.140	0.021
Coverage				＞99 %			

[1] SEM: standard error of the mean. $^{a–c}$ Values in the same row with different letters are significantly different (P<0.05). Results are presented as the mean ± SEM (n=6). The following table is the same.

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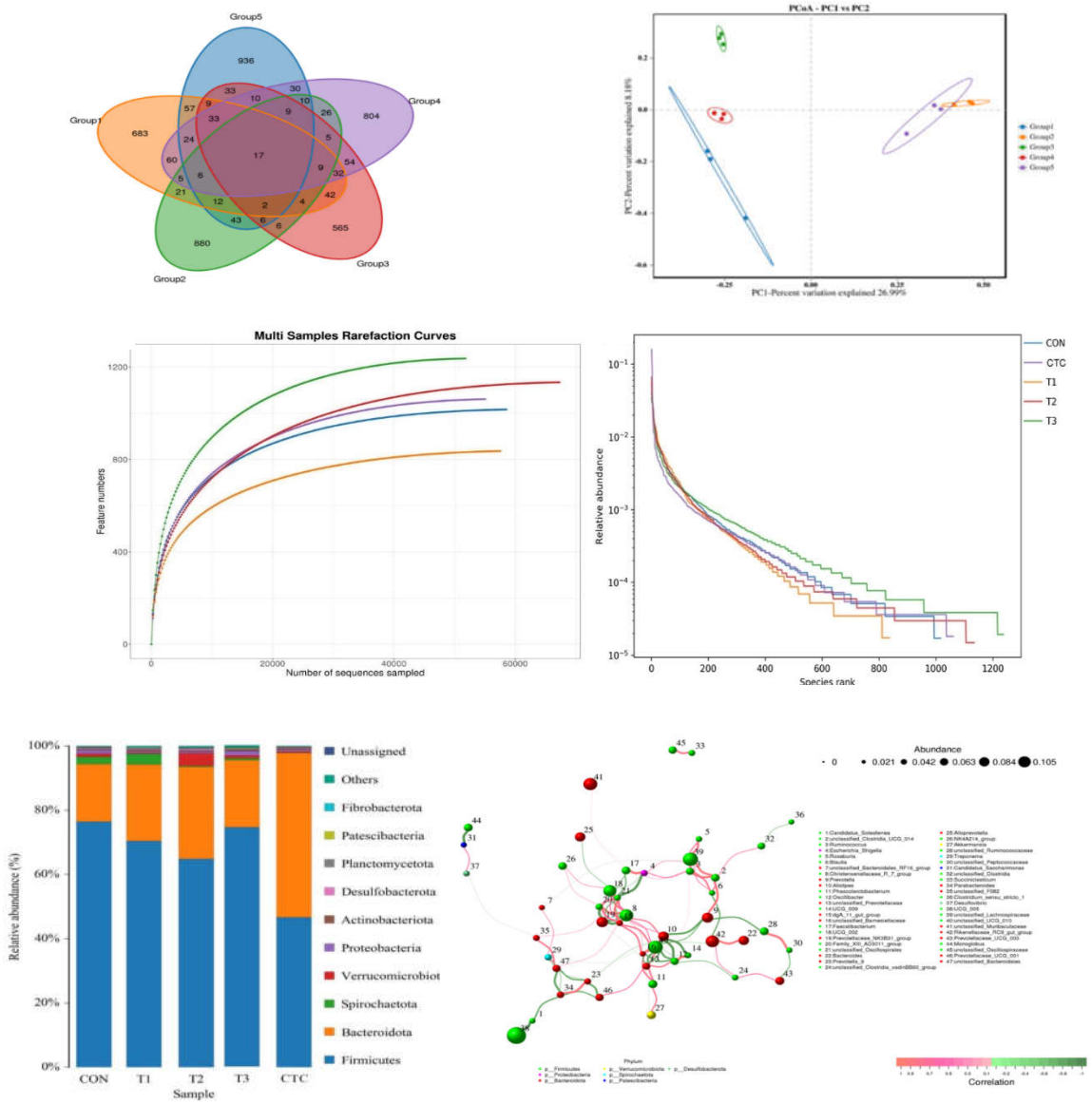

Figure 4. Effects of tea polyphenols on the composition of cecal bacteria in weaned lambs (n=3). (**A**): Venn diagram, Group1: CON, Group2: CTC, Group3: T1, Group4: T2, Group5: T3. The grouping settings are consistent with B. (**B**): principal co-ordinates (PCoA) analysis of OUTs. (**C-D**): Rarefaction curve (left) and rank abundance curve (right) of cecal microorganisms. (**E**): Histograms showing the abundance ot microbiota in the cecum at the phylum. (**F**): Network diagram of species at genus level

Principal coordinate 1 explains 26.99 % of the variation among microbial colonies in all cecal contents, while principal coordinate 2 explains 8.18 % (Figure 4B). From the figure, it can be seen that the CTC group and T3 group samples are the closest, indicating that there is not much difference in the microbial structure composition between them. The samples of the T1 and T2 groups were clearly separated from the control group, which indicates a significant difference in the composition of their microbial structures. Thus, tea polyphenols and chlortetracycline affect the composition of cecal microbiota in weaned lambs.

As shown in Figure 4E, tea polyphenols were found to alter the microbial community structure of the cecum in weaned lambs. At the phylum level, Firmicutes and Bacteroidetes accounted for over 80% of the total microbiota, which may play a key role in mataining the microbial environment. In this study (Table 7), compared with the CON group, the Firmicutes in the cecum of lambs in the T2 and CTC groups were significantly reduced (P<0.05), while the Bacteroidetes in the CTC group were

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significantly increased (P<0.05). The Verrucomicrobota in the cecum of lambs in the T2 group was significantly higher than that in the other groups (P<0.05). However, the Proteobacteria levels in the cecum of lambs in the high-dose T3 group were significantly higher than those in the T1, T2, and CTC groups (P<0.05). The differences among the other groups were not significant (P>0.05).

We further analyzed the top 10 genera with the highest species correlation (Figure 4F), and the results was shown in Table 8. Compared with the CON group, the abundance of Blautia in the cecal microbiota of lambs in the T2 and T3 groups was significantly increased (P<0.05), and the abundance of Prevotella in the T2 group was also significantly increased (P<0.05). However, the Candidatus_Soleaferrea and Christensennellaceae R-7 group in the cecum of lambs in the CTC group were significantly lower than other groups (P<0.05). The differences among the other groups were not significant (P>0.05).

4. Discussion

4.1. Effects of Tea Polyphenols on Antioxidant Capacity and iNOS in Weaned Lambs

When the body's antioxidant system cannot clear the excess free radicals caused by weaning stress, the redox homeostasis will be disrupted, resulting in the production of excessive reactive oxygen species (ROS) and damage to lipids, cell membranes, proteins, and nucleic acids, ultimately leading the occurrence of oxidative stress [20,21]. In the intestine, it manifests as loss of intestinal function, impaired intestinal barrier function, and induction of intestinal inflammatory response [22,23]. Chen et al. [24] found that tea polyphenols fed to squabs can improve the antioxidant enzyme activity in the gut, activate *Nrf2-ARE* antioxidant pathway, induce the expression of *Nrf2* and downstream antioxidant enzyme related genes (*SOD1, SOD2, CAT* and *HO-1*), and improve the antioxidant capacity of squabs 'body and intestines. Malondialdehyde (MDA), as the main product of lipid peroxidation, can monitor the state of lipid oxidation. Wang et al. [25] found that adding natural green tea polyphenols to the diet can alleviate liver function damage in D-galactose-induced oxidative aging model mice, increase GSH-Px and SOD content, reduce MDA expression, decrease oxidative stress, and maintain a balance between redox and inflammation in the body. These experimental results are analogous to those of this study in that the addition of 4 to 6 grams per kilogram of tea polyphenols and antibiotics was found to enhance the antioxidant activity in the intestines of weaned lambs, significantly reduce the MDA content, and boost the body's antioxidant capacity.

Table 7. Effects of tea polyphenols on cecal flora structure of weaned lambs (phylum level).

Items	Groups					SEM	P-Value
	CON	T1	T2	T3	CTC		
Firmicutes	69.12^{a}	61.83^{ab}	50.29^{b}	65.36^{a}	48.03^{b}	2.587	0.006
Bacteroidota	28.22^{b}	34.27^{ab}	36.74^{ab}	26.04^{b}	42.87^{a}	1.952	0.014
Spirochaetota	0.98	0.99	1.05	0.68	0.27	0.191	0.735
Verrucomicrobiota	1.89^{b}	1.49^{b}	3.68^{a}	0.57^{b}	0.73^{b}	0.348	0.006
Proteobacteria	0.932^{ab}	0.785^{b}	0.57^{b}	1.57^{a}	0.43^{b}	0.128	0.014
Actinobacteriota	0.51	0.29	0.27	0.26	0.435	0.059	0.64

Table 8. Effects of tea polyphenols on cecal flora structure of weaned lambs (genus level).

Items	Groups					SEM	*P*-value
	CON	T1	T2	T3	CTC		
Alistipes	2.00	3.71	2.92	1.99	1.22	0.377	0.284
Blautia	0.17^{b}	0.21^{b}	0.72^{a}	0.68^{a}	0.30^{ab}	0.078	0.019
Candidatus_Soleaferrea	0.41^{a}	0.47^{a}	0.65^{a}	0.42^{a}	0.18^{b}	0.045	0.002
Christensenellaceae R-7 group	5.83^{a}	7.31^{a}	4.13^{a}	5.10^{a}	2.69^{b}	0.531	0.035
unclassified_Bacteroidales_RF16_group	0.25	0.05	0.67	0.48	0.33	0.087	0.199

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Ruminococcus	1.10	1.12	1.19	1.36	0.28	0.187	0.452
Escherichia_Shigella	0.06	0.14	0.7	1.22	0.03	0.254	0.557
unclassified_Clostridia_UCG_014	1.52	0.58	1.42	1.29	0.87	0.171	0.414
Roseburia	0.38	0.52	0.36	0.91	0.418	0.109	0.136
Prevotella	1.11^{b}	0.30^{b}	5.85^{a}	3.20^{ab}	2.49^{b}	0.611	0.01

Nrf2, an important nuclear transcription factor, can effectively regulate the activity of antioxidant enzymes in animal bodies by interacting with the antioxidant response element ARE protein. Most *Nrf2*/*ARE* signaling pathways not only inhibit reactive oxygen species (such as ROS and RNS), but also induce the expression of a series of downstream protective target genes after activation (such as antioxidant genes, phase II detoxifying enzyme genes, molecular chaperone genes, etc.) [26]. Zhou et al. [27] found that adding tea polyphenols supplement to corn contaminated with low levels of fungal toxins as a diet for laying hens can not only alleviate the adverse reactions caused by toxin contamination, but also increase the gene expression levels of *SOD3*, *Nrf2*, and *GSTs* in the liver, thereby enhancing the antioxidant capacity of laying hens. In addition, it has been reported that overexpression of iNOS can protect liver damage by regulating oxidative stress.Tao et al. [28] demonstrated that tea polyphenol pretreatment weakened the down regulation of iNOS in I/R-induced liver tissue of mice. This is similar to the results of this study, which showed an increase in NO and iNOS in lambs in T2 and T3 groups. In addition, the stimulating effect of tea polyphenols on intestinal NO content was consistent with *iNOS* mRNA expression in this experiment. But in most other studies, Kudingcha can reduce the levels of NO and iNOS in oxidative damaged mice, and catechins can also reduce the NO content in RAW 264.7 macrophages, inhibiting the production of ROS [29,30]. This may be due to the increased levels of NO and iNOS in the intestine in this experiment, which also stimulated an increase in gene expression levels. In addition, some researchers suggest that elevated iNOS expression aids animals in adapting to hypoxic or stressful conditions [31]. Similarly, cell experiments show that exposure of endothelial cells to NO or peroxynitrite donors can lead to an adaptive increase in glutathione (GSH) synthesis and the expression of *Nrf2* genes [32]. These findings suggest that active compounds in tea polyphenols regulate NO production by modulating *iNOS* mRNA expression and synthesis, thereby modulating the antioxidant capacity of weaned lambs.

4.2. Effects of Tea Polyphenols on the Expression of Cytokines and TLR4/NFκB Pathway Related Genes in Lamb Intestinal Cells

The immune function of the intestinal mucosa is mediated by immune cells and cytokines. Oxidative stress in animals not only causes direct cellular damage but also activates *NFκB* in the liver, leading to the release of pro-inflammatory cytokines. NFκB, a key transcription factor, plays an important role in immune and inflammatory responses, as it can stimulate the expression of different pro-inflammatory cytokines, including genes encoding cytokines and chemokines [33]. In addition, *NFκB* is activated by various stimuli such as oxidative stress, cytokines, bacterial or viral antigens, and oxidized low-density lipoprotein, leading to its secretion in large quantities. It participates in regulating various genes that are important for cellular responses, including inflammation, innate immunity, growth, and cell death [34]. Interleukin-1 (IL), as a key immune regulatory cytokine, plays a crucial role in the body's defense system. The *TLR4*/*NFκB* signaling pathway, as an upstream pathway for inflammatory factors such as IL-1β, IL-6, IFN-γ, and TNF-α, is one of the important pathways in inflammatory response. TLR4, as an important recognition receptor on the cell membrane surface, can bind to the adaptor protein MyD88 upon activation, thereby regulating the expression levels of downstream genes and other transcription factors in the NF - κ B signaling pathway [35,36]. Research has shown that tea polyphenols alleviate TBBPA induced gill inflammation in carp by inhibiting the expression of the TLR4/*NFκB* signaling pathway, reducing the expression levels of cytokines IL-1β, IL-6, and TNF-α mRNA, thereby alleviating inflammation [37]. In addition, Zhao et al. [38] discovered that theaflavins isolated from Pu'er tea can boost the

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expression of *TLR2*, *TLR4*, and *MyD88*, thus activatining downstream *NFκB* and initiates inflammatory responses, thereby enhancing the innate immunity and anti-inflammatory effects of RAW264.7 macrophages. These results are similar to those of our study that lambs experience weaning stress, and the addition of 4-6 g/kg tea polyphenols significantly reduced the expression of cytokine mRNA and *TLR4*/*NFκB* pathway related genes. Therefore, tea polyphenols may enhance intestinal epithelial immune defense and reduce inflammatory damage by inhibiting the *TLR4*/*MyD88*/*NFκB* signaling pathway and cytokine related gene expression.

4.3. Effects of Tea Polyphenols on Volatile Fatty Acids in the Intestinal Tract of Weaned Lambs

Short chain fatty acids (SCFAs) have received widespread attention due to their positive effects on health. SCFAs are defined as fatty acids with fewer than six carbon atoms, primarily including acetate, propionate, and butyrate, which collectively constitute over 95% of total SCFAs with a typical ratio of 60:20:20 [39]. Among them, acetic acid produced by colonic bacteria is transported to the liver, where it is used as an energy source and a substrate for synthesizing cholesterol and long-chain fatty acids; propionate influences food intake and glucose homeostasis, serving as a substrate for hepatic gluconeogenesis; butyrate supports intestinal barrier function and reduce the occurrence of intestinal inflammation; The production of other SCFAs (formic acid, valeric acid, and hexanoic acid) is relatively low [40–42]. Some studies have shown that feeding Liubao tea extract to diabetes mice can reverse the decrease of SCFAs content in mouse feces, increasing propionate and butyrate concentrations, modulating microbiota composition, enhancing the abundance of beneficial bacteria, improving the intestinal barrier, promoting epithelial cell growth, and reducing systemic lipopolysaccharides (LPS) and inflammation [43]. In this experiment, lambs fed 4 g/kg tea polyphenols exhibited significantly higher levels of acetic acid in their cecum compared to the other groups. This phenomenon may be due to the fact that the vast majority of volatile fatty acids are produced by the rumen in ruminants, and then absorbed and utilized by rumen epithelial cells or transported to the liver for metabolism. The volatile fatty acids in monogastric animals are mainly produced by microorganisms in the colon and cecum, and most of them are absorbed in the intestinal epithelium [44,45]. Therefore, there was no significant change in butyric acid and propionic acid in this experiment. Zhuang et al. [46] reported similar findings, showing that fermented tea residue increased fecal acetate levels in heat-stressed fattening cattle, while there was no significant change in propionate and butyrate levels. The study also demonstrated that fermented tea residue promotes microbial fermentation in the large intestine, maintains health, and mitigates the adverse effects of heat stress, aligning with the results of this experiment. These findings highlight the potential of tea polyphenols to modulate SCFA levels, thereby contributing to gut health and systemic well-being.

4.4. Eeffect of Tea Polyphenols on the Gut Microbiota of Weaned Lambs

The gut microbiota plays a crucial role in digestion, immune system, metabolism, and overall health. Therefore, maintaining the dynamic balance of gut microbiota is essential for maintaining overall health. The microbiota in the gut system consists of a complex community of anaerobic bacteria, fungi, archaea, protozoa, and viruses, distributed throughout the entire gastrointestinal tract [47]. And when the microbiota is imbalanced, it can lead to the destruction of the intestinal barrier, increasing the susceptibility of the body to certain diseases. Only high levels of microbial diversity and richness can help maintain its resistance and stability after being stressed [48]. Zhao et al. [49] found that adding 300 mg/kg tea polyphenols to sturgeon feed significantly increased Chao1 and Shannon index values, enhancing microbial diversity and richness while reducing the *Firmicutes-to-Bacteroidetes* (F/B) ratio. *Bacteroidetes*, a dominant intestinal phylum, interact with T cells to promote IL-10 secretion, providing protection against colitis. Conversely, *Firmicutes* are associated with impaired intestinal barrier integrity and lipopolysaccharide (LPS) leakage. Therefore, a high F/B ration is considered an important biomarker of gut microbiota dysbiosis. *Proteobacteria*, another phylum, includes pathogens such as *Helicobacter pylori* and *Escherichia coli*, which are linked to intestinal damage and inflammation [50]. In this study, dietary supplementation of 4 g/kg tea

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polyphenols significantly increased the Shannon and Simpson indices of gut microbiota in weaned lambs, thereby enhancing biodiversity and increasing the abundance of *Bacteroidetes* and *Verrucomicrobiota*, while reducing the abundance of *Proteobacteria* and *Firmicutes*. Li et al. [51] found that antibiotics can cause disruption of the gut microbiota in mice, and oral tea polyphenols can significantly alleviate the antibiotic induced decrease in gut microbiota abundance and diversity, and increase the relative abundance of probiotics such as *Blautia*, *Roseburia*, and *Prevotella*. This is similar to the results of this study, that is the abundance of *Blautia* and *Prevotella* in the cecal microbiota of lambs at 4-6 g/kg was significantly increased. However, the *Candidatus_Soleaferrea* and *Christensennellaceae R-7 group* in the cecum of lambs in the CTC group were significantly lower than other groups, indicating that long-term use of antibiotics can reduce beneficial bacteria in the intestine. Among them, *Blautia* produces butyric and acetic acids, contributing to anti-inflammatory effects by preventing pathogen colonization and upregulating T cells [52,53]. *Prevotella* participates in glucose metabolism and inhibits the action of *Bifidobacterium* [54,55]. The *Christensenellaceae R-7 group* plays a crucial role in the degradation of carbohydrates and amino acids into acetate and ammonia, and its abundance is positively correlated with the improvement of lamb growth performance and meat quality [56,57]. Overall, the research results indicate that long-term use of antibiotics can lead to a decrease in beneficial bacteria in the intestines of weaned lambs, while tea polyphenols can alleviate intestinal ecological imbalances by promoting beneficial bacteria and inhibiting harmful bacteria. This helps to enhance intestinal health, improve intestinal barrier integrity, prevent colitis, and provides a feasible antibiotic alternative for managing weaning stress in lambs.

5. Conclusions

In summary, our results show that dietary supplementation of 4-6 g/kg tea polyphenols can boost the antioxidant and immune capabilities of weaned lambs' intestines. The potential mechanism might be regulating the production of *NO* by modulating *iNOS* mRNA expression and synthesis, thus regulating the antioxidant capacity. It also enhances intestinal epithelial immune defense and reduces inflammatory damage by inhibiting the *TLR4/MyD88/NFκB* signaling pathway and cytokine-related gene expression. Additionally, tea polyphenols can alleviate intestinal dysbiosis in weaned lambs by promoting beneficial bacteria and inhibiting harmful ones, thereby enhancing intestinal health and preventing colitis. These findings provide theoretical support for the development of tea polyphenols as feed additives to improve intestinal health in livestock.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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