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Effects of toll like receptor 4 overexpression on blood parameters, body composition, and gastrointestinal microbial diversity in sheep

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Abstract: Genetic modifications can improve the health status and disease resistance of farm animals. In the previous study, genetically modified (GM) sheep overexpressing toll like receptor 4 (*TLR4*) were produced. The GM sheep had stronger ability to eliminate invasive microbes compared to the wild-type (WT) sheep. Physiological status and immune homeostasis, blood parameters, body composition, and gastrointestinal microbial diversity were evaluated in this study to elucidate the effects of *TLR4* overexpression. In a set of 10 GM rams, the *TLR4* mRNA and protein expression levels in the peripheral blood mononuclear cells, muscle, kidney, and spleen were higher than those of the WT (n = 10; $P < 0.05$). No significant differences in the parameters of red blood cells, white blood cells, platelets and in the composition of circulating T lymphocyte subsets were observed between the GM and WT sheep ($P > 0.05$). Of the biochemical parameters, only total protein levels differed (were higher) in the GM sheep than in the WT sheep ($P < 0.05$) while no significant differences were observed for the other indices ($P > 0.05$). GM and WT sheep showed similar offal weights and muscle fatty acid and amino acid compositions ($P > 0.05$). Furthermore, *TLR4* overexpression did not affect the community structure of the gastrointestinal tract microbiota. Eleven KEGG pathways associated with the *TLR4* gene, physiology and biochemistry, growth and metabolism, fatty acids, and amino acids did not demonstrate significant differences between the GM and WT sheep ($P > 0.05$). In conclusion, *TLR4* overexpression had no principal or adverse effects on the physiological development and health of sheep, except for the general mRNA and protein expression levels.

Keywords: genetically modified sheep; *TLR4* gene; microbiomes; immune homeostasis; biosafety assessment

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A genetically modified (GM) animal is one whose genetic material has been altered using genetic engineering techniques. Since the 1970s, genetic modifications that is the use to change the genes of an organism have been applied in agriculture, biomedicine and industry (Lee et al. 2020). In agriculture, this technology can increase farm animal productivity and produce exogenous proteins as GM animal bioreactors. In addition, genetic modification of immune genes has been used to improve the health status and disease resistance of farm animals (Lee et al. 2020). Recombinant human β-defensin-3 in the milk of transgenic goats supported resistance to mastitis through enhanced antibacterial activity (Liu et al. 2013). The presence of human lysozyme in the milk of transgenic pigs potentially benefits piglets by enhancing their immune function and defending against pathogen infection (Lu et al. 2015). Inactivation of the CD163 receptor in pigs confers complete resistance to viral infections, such as that caused by porcine reproductive and respiratory syndrome virus (Yang et al. 2018).

The mammalian toll like receptor 4 (TLR4), a key component of innate immune signaling, is the first line of defense against gram-negative bacterial infections (e.g., *Escherichia coli*, *Proteus vulgaris*, *Shigella dysenteriae*, and *Brucella melitensis*) by recognising lipopolysaccharides/endotoxins. After pathogen invasion, the activation of TLR4 signaling contributes to the elimination of microorganisms, while ubiquitination-mediated degradation prevents immune imbalance caused by the overactivation of TLR4 signaling (Chuang and Ulevitch 2004). Mice overexpressing *TLR4* showed a significant survival advantage during *Salmonella* infection, whereas those lacking TLR4 were susceptible (Roy et al. 2006; Cawthraw et al. 2011). In previous studies, we produced GM sheep overexpressing *TLR4*, which enhanced the clearance of invasive microbes (*Salmonella* and *Escherichia coli*) by inducing bacterial internalisation, secretion of inflammatory cytokines, and activation of oxidative stress and autophagy activity (Wang et al. 2018; Wang et al. 2020).

Despite the potential assets of genetic modification technology, it may still lead to physiological disorders or growth abnormalities in animals, as reported for contactin 5 deficiency in mice (Smirnov et al. 2018) and human factor VIII expression in rabbits (Chrenek et al. 2007). GM pigs expressing bovine growth hormone had a significantly increased incidence of gastric ulcers and cardiomegaly (Solomon et al. 1994). Therefore, before GM animals are used in agriculture, it is necessary to evaluate the effects of genetic modification on the physiological development and health status of animals, along with the environmental risk estimate.

Yao et al. (2017) demonstrated that GM sheep overexpressing *TLR4* exhibited normal growth performance and reproductive capability, and Bai et al. (2015) reported that the consumption of meat from the GM sheep had no adverse effect on Sprague-Dawley rats compared with that of wild-type (WT) sheep. In the present study, we further evaluated the blood parameters, body composition, and gastrointestinal microbial diversity in GM sheep. The present study aimed to elucidate the effects of *TLR4* overexpression on the physiological development and health status of sheep and to provide a scientific basis for further biosafety assessment. In addition, the results of this study will contribute to the knowledge of physiological development and biosafety in animals with increased expression of important immune response genes.

MATERIAL AND METHODS

Ethics statement

All the sheep were managed under normal husbandry conditions. All experimental animal protocols were approved and performed in accordance with the requirements of the Animal Care and Use Committee of the Northeast Agricultural University (approval ID: 14-067).

Animals

For this study, twenty 1–1.5-year-old rams (GM rams: n = 10; WT rams: n = 10) were selected and housed at the breeding base of Northeast Agricultural University. GM rams overexpressing *TLR4* were produced as described previously (Bai et al. 2015; Yao et al. 2017; Wang et al. 2018; Wang et al. 2020). In brief, the GM sheep were produced by transferring the linearised vector into the pronuclei of fertilised eggs by microinjection. Generally, the transformed exogenous *TLR4* genes were detected by southern blotting and reverse

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transcription–quantitative real-time PCR (RT-qPCR). WT rams of the same breed and age were randomly selected as the control group. All the rams were raised in the same rearing environment.

Sample collection

Peripheral blood was aseptically collected from the jugular vein of the sheep into tubes containing heparin or ethylenediaminetetraacetic acid (EDTA). Carcass segmentation and various body composition analyses were conducted after slaughter according to the China National Standard GB 9961-1988. The animals were slaughtered and dressed by professional butchers, and faecal samples were collected from the intestines of each animal. The rumen of each sheep was cut open and the rumen fluid was strained through four layers of cheesecloth. Two-ml aliquots of rumen fluid were collected in microcentrifuge tubes. Tissues, including the spleen, muscle, and kidneys, were collected. All tissue samples, rumen fluid, and faeces were stored at −80 °C until analysis.

Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cell (PBMC) isolation is based on the density differences between PBMCs and other peripheral blood components, as described previously (Bai et al. 2015; Yao et al. 2017; Wang et al. 2018; Wang et al. 2020). In brief, peripheral blood containing heparin was diluted with an equal volume of Hank's balanced salt solution (Gibco, Grand Island, NY, USA), after which 5 ml of the diluted blood was carefully layered on top of 5 ml PBMC separation medium (TBD, Tianjin, China). After centrifugation at 500 × *g* for 20 min at 20–25 °C, the cloudy-looking phase containing PBMCs was collected and subsequently used for RNA isolation, RT-qPCR and western blot analysis.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from PBMCs using TRIzol reagent (Takara, Dalian, China). Then, total RNA concentration was determined by calculating absorbance ratios at 230, 260, and 280 nm using a NanoDrop spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA integrity was assessed using 1.2% (w/v) agarose gel electrophoresis, and samples with good RNA quality were selected for reverse transcription. cDNA was synthesised from 1 µg of total RNA using PrimeScript RT reagent with the gDNA Erase kit (Takara, Dalian, China) in accordance with the manufacturer's instructions. The products were stored at −20 °C until use.

RT-qPCR

The relative expression of *TLR4* was quantified using an ABI 7500 real-time PCR detection system (Applied Biosystems, Foster City, CA, USA) and FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics GmbH, Mannheim, Germany). Each reaction mixture was 10 µl and contained 5 µl SYBR Green Master, 0.4 µl PCR forward and reverse primers (10 µM), 3.6 µl dH_2O, and 1 µl cDNA template. The PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 minute. Melting curves were generated to determine the specificity of the amplified products. Quantification was normalised to the expression of reference gene actin beta. The following primers were used: TLR4 F: 5'-CTTGCGTACAGGTTGTTCCTAAC-3' and R: 5'-GAAGTTATGGCTGCCTAAATGTC-3' (NM_001135930.1); and actin beta F: 5'-AGATGTGGATCAGCAAGCAG-3 and R: 5'-CCAATCTCATCTCGTTTTCTG-3' (NM_001009784.3).

Western blotting

PBMCs, spleen, muscle, and kidney were lysed using radioimmunoprecipitation assay buffer (Beyotime, Beijing, China). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Beyotime, Beijing, China). Then, 40 µg of the total protein sample was treated with 5× sodium dodecyl sulfate (SDS) loading buffer at 95 °C for 10 min and separated using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred onto a nitrocellulose membrane. The membrane was blocked in 5% skim milk for 2 h at 37 °C. Considering the limitation of the amount of protein obtained from each individual, the membrane from SDS-PAGE gel of the

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same plate was cut open according to the size of the target protein, then independently incubated at 4 °C overnight with rabbit anti-sheep TLR4 antibody (1 : 1 000; Affinity Biosciences, Cincinnati, OH, USA) or rabbit anti-sheep ACTB antibody (1 : 1 000; Cell Signalling Technology, Danvers, MA, USA). After washing three times with Tris-buffered saline with Tween 20 (TBST), the membrane was incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody (1 : 500; Beyotime, Beijing, China) at 20–25 °C for 1 hour. Following three additional washes with TBST, the membrane was developed using an enhanced chemiluminescence kit (Beyotime, Beijing, China). The grey value of each band was calculated using ImageJ software (v1.45; National Institutes of Health, Bethesda, MD, USA). The relative expression of proteins is expressed as the ratio of the target protein band intensity to that of actin beta.

Blood analysis

Within 45 min after sample collection, the peripheral blood in a tube containing EDTA was used for haematological analysis by In-Clinic Automated Hematology Analyzer (IDEXX, Westbrook, MA, USA), and the peripheral blood in a plain tube was used for biochemical analysis by a Synchron CXH5 (Beckman Instruments, Brea, CA, USA).

T lymphocyte subsets analysis

PBMCs were isolated from peripheral blood, placed in tubes containing heparin, and lysed with red blood cell (RBC) lysis buffer (Solarbio, Beijing, China). Then, the percentages of $CD4^+$, $CD8^+$, and both $CD4^+$ $CD25^+$ cells in ovine PBMCs were measured with mouse anti-sheep CD25-FITC, CD4-PE and CD8-FITC antibodies (Abcam, Dusseldorf, Germany). The percentages of $CD4^+$ and $CD8^+$ lymphocytes in the total population were determined using double staining with CD4-PE and CD8-FITC. The percentage of $CD4^+CD25^+$ cells in the total population was determined using double staining with CD4-PE and CD25-FITC. PBMCs were incubated with labelled antibodies for 30 min at 4 °C in the dark, and then fixed with 4% paraformaldehyde PBS for 10 minutes. Cells-bound antibodies were washed twice with centrifugation and resuspended in PBS for cell counting by flow cytometry. Flow cytometry was performed using a FACSCalibur (BD Biosciences, San Jose, CA, USA), and the data were analysed using FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Amino acid and fatty acid composition analysis

After mincing, the muscle's composition of amino acids and fatty acids was analysed according to China National Standard (GB 5009.124-2016, GB 5009.168-2016). Fatty acid profiles were determined by capillary gas chromatography using an HP6890 (FID detector) and an SPTM2380 column. Amino acids were analysed using the Technicon Sequential Multisample Amino Acid Analyzer (Technicon Instruments Corporation, Dublin, Ireland).

Metagenomic sequencing analysis

Total genomic DNA in rumen fluid and faecal samples was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Idar-Oberstein, Germany). The integrity and concentration of genomic DNA were evaluated using 1.2% agarose gel electrophoresis and a NanoPhotometer® microvolume spectrophotometer (Implen, Westlake, CA, USA), respectively. Libraries were constructed using genomic DNA extracted from each sample and purified using QIAquick PCR purification kit (Qiagen, Idar-Oberstein, Germany). Each library was quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and then sequenced on a Hiseq 2 500 platform (Illumina, San Diego, CA, USA). In this study, after DNA extraction of all rumen fluid and faecal samples, some samples did not meet the requirements of subsequent metagenomic sequencing (such as, integrity and DNA concentration). In order to ensure that DNA from both rumen fluid and faeces of the same animal met sequencing requirements, five out of 10 animals were chosen for subsequent metagenomic sequencing analysis. Bioinformatics analysis of metagenomic sequences was performed as described by Qin et al. (2012). In brief, following previously reported pipelines and parameters, fastp was used for quality control and Bowtie2 was used to remove host-derived reads. Contigs were assembled using SOAPdenovo

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assembler (v2.21). Fragments shorter than 500 bp in the scaftigs generated by the assembly were filtered for statistical analysis. MetaGeneMark (v2.10) was used to predict the open-reading frames on scaftigs, and CD-HIT used to cluster and remove the redundant transcripts/redundancy removal software (v4.6.1; http://www.bioinformatics.org/cd-hit) was used to obtain the unique initial gene catalogue. Clean data for each sample were mapped to the initial gene catalogue using Bowtie2 to obtain the number of reads and statistical abundance of each gene in each sample (Bowtie2 parameters for paired-end reads: -p8 --very-sensitive-local -k 100 --score-min L,0,1.2). Unigenes were subjected to Basic Local Alignment Search Tool (BLAST) analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The parameter setting used was blastp, *e*-value < 0.000 01. The abundance of any taxonomic group in a sample was estimated from the sum of gene abundances.

Statistical analysis

Statistical analysis was performed using SPSS software (v18.0; SPSS Inc. Chicago, IL, USA) and the STAMP software package (v2.1.3). The independent samples *t*-test was used to evaluate the different variables between the GM and WT sheep. The data are presented as the mean value ± standard error of the mean (means ± S.E.M). Statistical significance was set at $P < 0.05$.

RESULTS

TLR4 expression levels of GM and WT sheep

qPCR and western blot revealed *TLR4* mRNA and protein expression in the PBMCs, muscle, kidney, and spleen of GM and WT sheep. As shown in Figure 1, GM sheep exhibited significantly higher *TLR4*

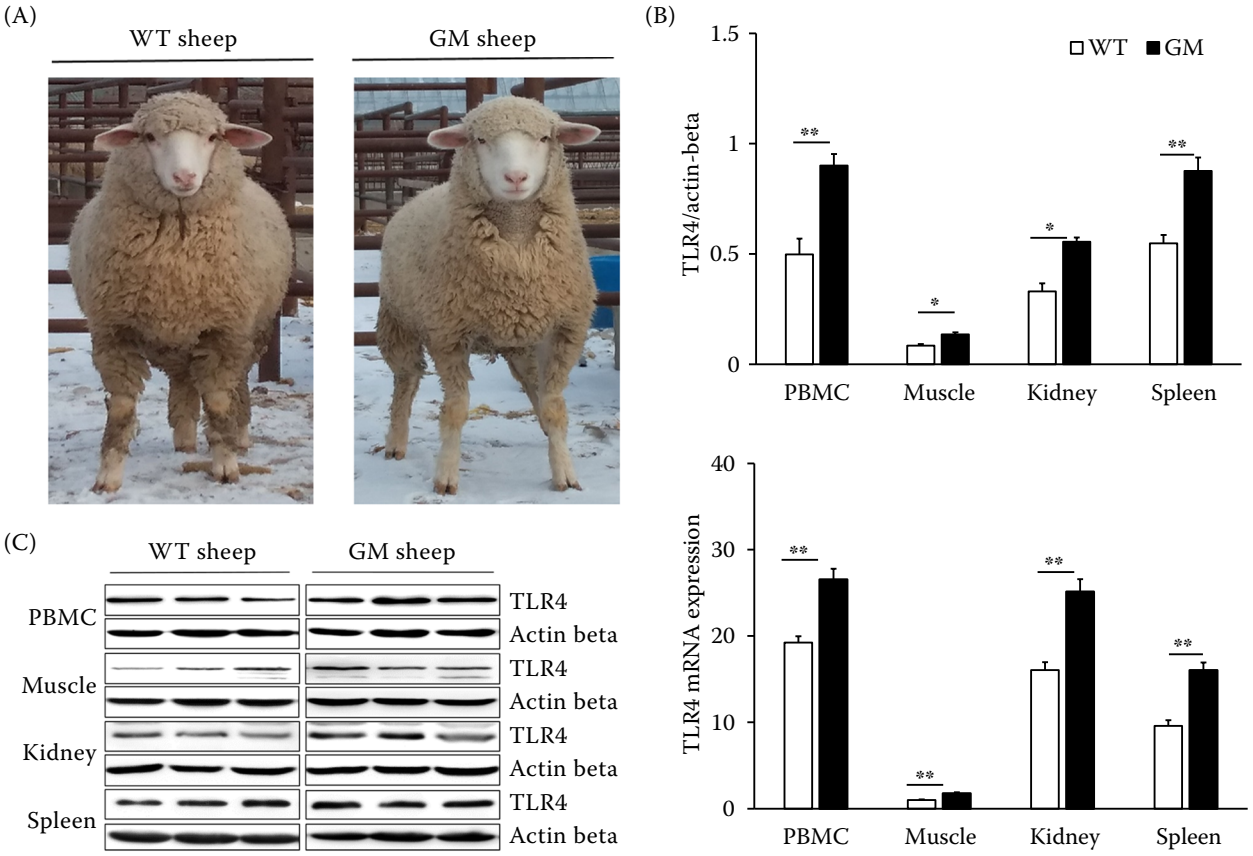

Figure 1. TLR4 expression in genetically modified (GM, $n = 10$) and wild-type (WT, $n = 10$) sheepp
(A) Photos of GM and WT sheep. (B) The *TLR4* mRNA expression in the peripheral blood mononuclear cells (PBMCs), muscle, kidney, and spleen of GM and WT sheep using qPCR. (C) The TLR4 protein expression in the PBMCs, muscle, kidney, and spleen of GM and WT sheep using western blot
Three samples from each group are shown. Results are presented as means ± SEM; *$P < 0.05$

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mRNA and protein expression levels than WT sheep in PBMCs, muscle, kidney, and spleen ($P < 0.05$).

Haematological and biochemical parameters of GM sheep

The hematological parameters are listed in Table 1. No significant differences in all parameters of RBCs, white blood cells (WBCs), and platelets were observed between GM and WT sheep. Furthermore, the composition of circulating T lymphocyte subsets was analysed in this study. Similarly, the percentages of $CD4^+$, $CD8^+$, and regulatory T cells were not significantly different between GM and WT sheep (Figure 2).

Table 1. The hematological parameters of genetically modified (GM, $n = 10$) and wild-type (WT, $n = 10$) sheep

Item	GM sheep	WT sheep	*P*-value
Parameters of red blood cells			
RBC (10^{12}/l)	11.35 ± 0.40	11.96 ± 0.71	0.47
HCT (%)	30.91 ± 1.20	32.63 ± 1.98	0.47
HGB (g/dl)	11.03 ± 0.39	11.33 ± 0.54	0.66
MCV (fl)	27.24 ± 0.66	27.33 ± 0.43	0.92
MCH (pg)	9.73 ± 0.14	9.51 ± 0.12	0.28
MCHC (g/dl)	35.70 ± 0.53	34.87 ± 0.50	0.28
Parameters of white blood cells			
WBC (10^9/l)	9.70 ± 0.87	9.20 ± 0.89	0.69
NEU (10^9/l)	3.45 ± 0.44	3.40 ± 0.57	0.64
NEU (%)	35.10 ± 1.80	35.63 ± 3.10	0.89
LYM (10^9/l)	5.03 ± 0.50	4.72 ± 0.40	0.29
LYM (%)	51.94 ± 2.78	52.46 ± 3.21	0.89
MONO (10^9/l)	1.07 ± 0.20	0.83 ± 1.10	0.29
MONO (%)	11.46 ± 2.46	9.17 ± 1.11	0.41
EOS (10^9/l)	0.12 ± 0.03	0.19 ± 0.05	0.27
EOS (%)	1.26 ± 0.26	2.07 ± 0.49	0.17
BASO (10^9/l)	0.02 ± 0.02	0.06 ± 0.04	0.31
BASO (%)	0.24 ± 0.23	0.67 ± 0.36	0.33
Parameters of platelets			
PLT (K/μl)	583.00 ± 46.00	553.00 ± 40.00	0.64
MPV (fl)	8.32 ± 0.22	8.41 ± 0.36	0.84

BASO = basophil; EOS = eosinophil; HCT = haematocrit; HGB = hemoglobin; LYM = lymphocyte; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; MONO = monocyte; MPV = mean platelet volume; NEU = neutrophils; PLT = platelet; RBC = red blood cell; WBC = white blood cell count

Results are presented as means ± SEM

The biochemical parameters of the serum are listed in Table 2. In GM sheep, total protein was significantly higher than WT sheep ($P < 0.05$). No significant differences in other biochemical parameters (including liver function, blood lipid, blood glucose, and main ions) were observed between GM and WT sheep (Table 2; $P > 0.05$).

Body compositions of GM sheep

To determine whether *TLR4* overexpression influenced ovine body composition, we measured offal weights and muscle proximate composition, including moisture, protein, and fat content. As shown in Table 3, there were no significant differences in any of these parameters between GM and WT sheep ($P > 0.05$).

In addition, the composition of amino and fatty acids in the muscle was analysed. Similarly, no significant differences were observed between GM and WT sheep (Table 4; $P > 0.05$).

Taxonomic distribution of rumen and faecal microbiota in GM sheep

To investigate microbial composition at the species level, we performed metagenomic sequencing

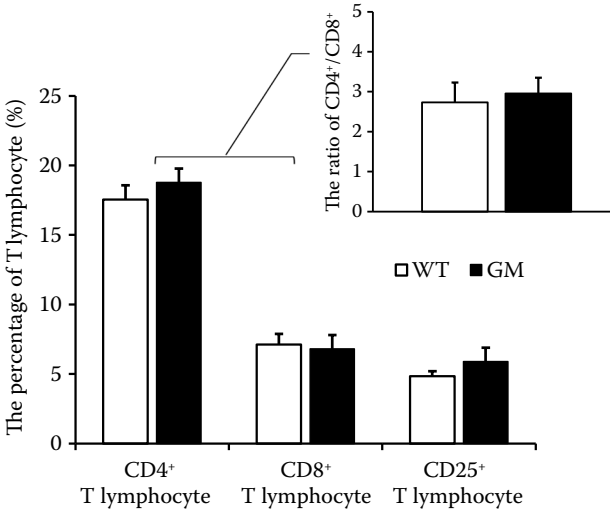

Figure 2. The percentages of T lymphocyte subsets in peripheral blood of genetically modified (GM, $n = 10$) and wild-type (WT, $n = 10$) sheep

Results are presented as means ± SEM

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Table 2. The biochemical parameters of the serum in genetically modified (GM, n = 10) and wild-type (WT, n = 10) sheep

Item	GM sheep	WT sheep	P-value
Liver function related parameters			
Cre (μmol/l)	68.67 ± 3.46	65.27 ± 6.39	0.62
CK (IU/l)	105.53 ± 11.35	112.20 ± 11.13	0.79
LDH (IU/l)	562.59 ± 50.37	500.96 ± 41.67	0.35
Blood lipid related parameters			
Tp (g/l)	65.44 ± 3.09	60.89 ± 2.54	0.04
Alb (g/l)	20.90 ± 2.90	20.01 ± 2.54	0.94
HDL (mmol/l)	1.13 ± 0.07	1.23 ± 0.07	0.42
LDL (mmol/l)	3.00 ± 0.63	4.33 ± 0.48	0.06
Blood glucose related parameters			
Glu (mg/dl)	47.05 ± 3.65	43.87 ± 6.05	0.17
TCh (mmol/l)	0.66 ± 0.62	0.70 ± 0.90	0.77
TG (mmol/l)	0.32 ± 0.06	0.48 ± 0.07	0.12
Parameters of main ions			
K (mmol/l)	8.96 ± 0.65	7.21 ± 0.89	0.06
Na (mmol/l)	142.58 ± 10.64	136.36 ± 11.35	0.79
Cl (mmol/l)	95.34 ± 8.34	86.24 ± 7.29	0.75
Ca (mmol/l)	1.05 ± 0.96	1.21 ± 1.04	0.24

Alb = albumin; Ca = calcium; CK = creatine kinase; Cl = chlorine; Cre = creatinine; Glu = glucose; HDL = high density lipoprotein; K = kalium; LDH = lactic dehydrogenase; LDL = low density lipoprotein; Na = sodium; TCh = total cholesterol; TG = triglyceride; Tp = total protein
Results are presented as means ± SEM

Table 3. The offal weights and muscle proximate composition in genetically modified (GM, n = 10) and wild-type (WT, n = 10) sheep

Item	GM sheep	WT sheep	P-value
Offal weights (kg)			
Head	4.17 ± 0.20	3.60 ± 0.22	0.06
Heart	0.39 ± 0.05	0.37 ± 0.02	0.60
Liver	0.99 ± 0.04	0.92 ± 0.05	0.29
Lung	0.74 ± 0.06	0.61 ± 0.04	0.09
Spleen	0.09 ± 0.01	0.15 ± 0.05	0.26
Stomach and intestine	13.44 ± 0.85	12.82 ± 0.56	0.57
Kidney	0.14 ± 0.00	0.13 ± 0.01	0.44
Muscle proximate composition (%)			
Moisture	77.55 ± 0.24	76.94 ± 0.48	0.61
Fat	9.94 ± 1.16	10.47 ± 0.78	0.96
Protein	16.83 ± 1.88	17.02 ± 0.95	0.83

Results are presented as means ± SEM

Table 4. The amino and fatty acid composition in the muscle of genetically modified (GM, n = 10) and wild-type (WT, n = 10) sheep

Item	GM sheep	WT sheep	P-value
Amino acid composition (%)			
Aspartic acid	1.92 ± 0.03	1.87 ± 0.03	0.21
Threonine	0.99 ± 0.02	0.95 ± 0.01	0.10
Serine	0.85 ± 0.01	0.84 ± 0.01	0.36
Glutamic acid	3.26 ± 0.05	3.21 ± 0.04	0.41
Glycine	0.93 ± 0.02	0.92 ± 0.01	0.73
Alanine	1.14 ± 0.02	1.12 ± 0.02	0.43
Cysteine	0.24 ± 0.03	0.23 ± 0.02	0.91
Valine	0.98 ± 0.02	0.95 ± 0.01	0.17
Isoleucine	0.96 ± 0.02	0.85 ± 0.01	0.50
Leucine	1.82 ± 0.03	1.79 ± 0.02	0.51
Tyrosine	0.67 ± 0.02	0.65 ± 0.01	0.37
Phenylalanine	0.93 ± 0.02	0.90 ± 0.02	0.22
Histidine	0.74 ± 0.02	0.72 ± 0.02	0.54
Arginine	1.32 ± 0.03	1.31 ± 0.02	0.90
Proline	0.77 ± 0.01	0.76 ± 0.01	0.62
Lysine	1.87 ± 0.03	1.93 ± 0.03	0.36
Total amino acid	20.00 ± 0.32	19.61 ± 0.26	0.35
Fatty acid composition (%)			
Linolenic acid (C18: 3)	1.54 ± 0.20	1.52 ± 0.19	0.95
Linoleic acid (C18: 2)	5.93 ± 0.48	5.82 ± 0.35	0.86
Stearic acid (C18: 0)	28.27 ± 1.01	29.27 ± 0.31	0.42
Oleic acid (C18: 1)	44.37 ± 0.28	43.59 ± 0.67	0.26
Palmitic acid (C16: 0)	17.72 ± 0.27	17.72 ± 0.18	0.99
Myristic acid (C14: 0)	2.20 ± 0.15	2.08 ± 0.09	0.57

Tryptophan and methionine were not determined. Aspartic acid includes also asparagine, and glutamic acid includes also glutamine
Results are presented as means ± SEM

of rumen fluid and faecal samples from GM and WT sheep. All samples were sequenced on an Illumina HiSeq platform, generating a total of 339.36 Gb of raw data and an average of 16.97 Gb per sample. After quality control, an average of 12.54 Gb clean data and 83 594 531 reads per sample were retained [Tables S1 and S2 in electronic supplementary material (ESM; for the ESM see the electronic version)]. Metagenomic assembly produced a total of 3 063 026 contigs with an average length of 1 221 bp and an average N50 length of 1 372 bp and predicted 2 338 465 gene catalogues. Rarefaction curve analysis of all samples approached saturation, suggesting that mostly non-redundant genes

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of the gastrointestinal tract (GT) microbiota were detected in different samples (Figure S1 in ESM).

The phylum and genus distributions in rumen fluid and fecal samples were consistent between GM and WT sheep (Figure 3 and Tables S3 and S4 in ESM). In rumen fluid samples, the dominant bacterial phylum was *Bacteroidetes* (GM: 74.58 ± 2.19%, WT: 76.55 ± 2.78%), followed by *Firmicutes* (GM: 6.71 ± 0.76%, WT: 6.14 ± 0.60%). The phylum distribution of faecal samples was similar to that of rumen fluid samples, with *Bacteroidetes* (GM: 59.08 ± 5.5%, WT: 56.04 ± 2.89%) being the most

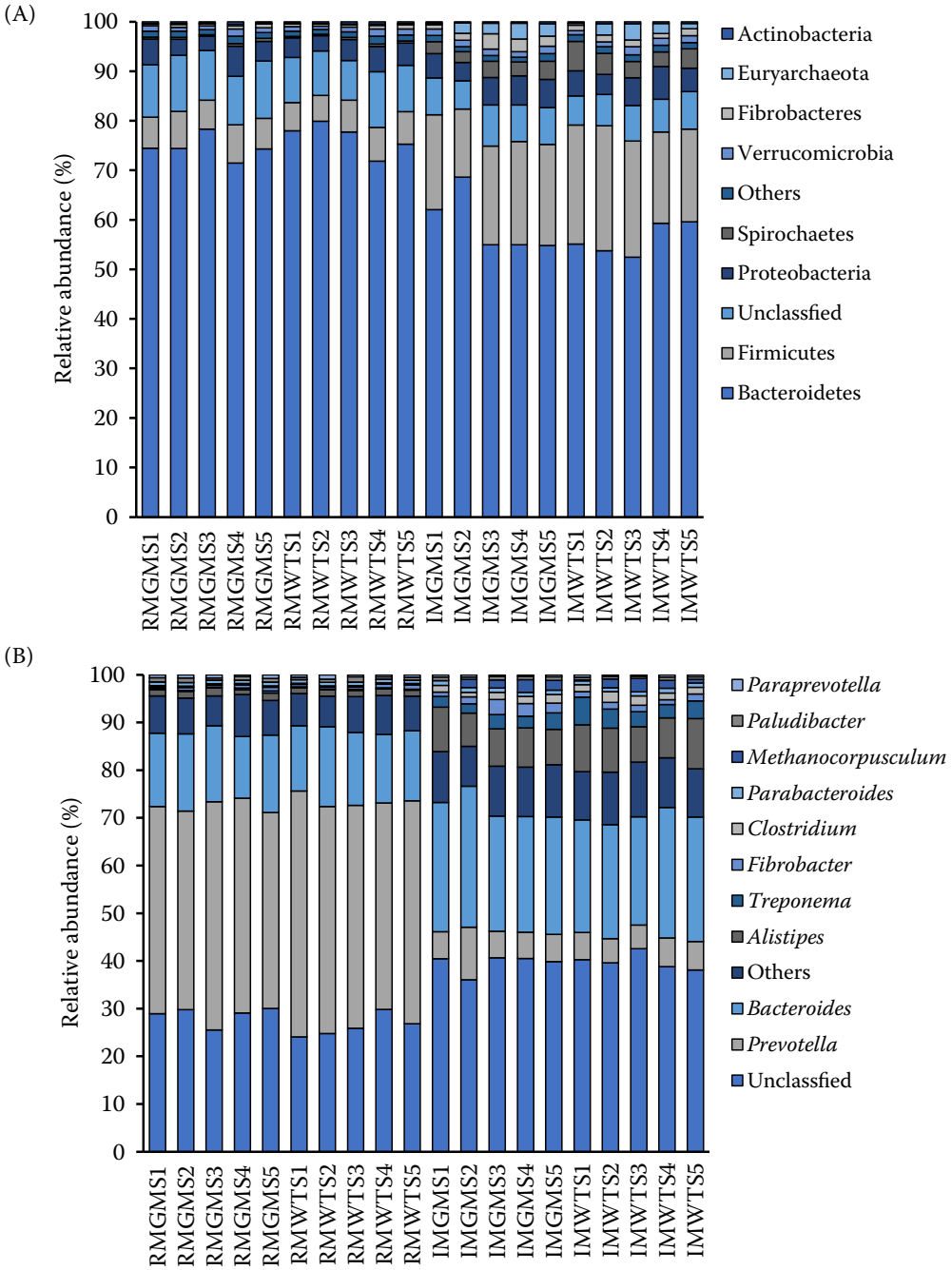

Figure 3. Taxonomic distribution of rumen and fecal microbiotas in genetically modified and wild-type sheep
(A) Microbiota at the phylum level. (B) Microbiota at the genus level
IMGMS = intestinal microbiota of genetically modified sheep, n = 5; IMWTS = intestinal microbiota of wild-type sheep, n = 5; RMGMS = rumen microbiota of genetically modified sheep, n = 5; RMWTS = rumen microbiota of wild-type sheep, n = 5

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abundant phylum, followed by *Firmicutes* (GM: 18.82 ± 2.58%, WT: 21.97 ± 2.82%). At the genus level, *Prevotella* (GM: 49.5 ± 1.30%, WT: 46.9 ± 1.20%), *Bacteroides* (GM: 15.22 ± 0.50%, WT: 14.87 ± 0.60%), and *Alistipe*s (GM: 1.38 ± 0.02%, WT: 1.27 ± 0.1%) were the three most abundant in rumen fluid samples. In the faecal samples, the three most highly represented genera were also *Bacteroides* (GM: 25.32 ± 1.07%, WT: 24.22 ± 0.83%), *Alistipes* (GM: 7.77 ± 0.39%, WT: 8.88 ± 0.54%) and *Prevotella* (GM: 6.55 ± 1.07%, WT: 5.42 ± 0.22%). Furthermore, principal component analysis (Figure 4) also revealed no significant difference between GM and WT sheep in the taxonomic distribution in rumen fluid ($P = 0.35$) and faecal samples ($P = 0.56$). Thus, *TLR4* overexpression did not affect the bacterial community structure of the GT.

Functional capacity profiling of rumen and faecal microbiota in GM sheep

To investigate whether TLR4 overexpression affects the functional capacity of the rumen and gut microbiome, we aligned the predicted gene catalogues and performed functional analysis using the KEGG database. The results of regression analysis showed that the predicted genes had a linear relationship between GM and WT sheep in the rumen ($R_2 = 1$; Figure 5A) and faecal ($R_2 = 1$; Figure 5B) samples. After removing redundant sequences, 794 741 catalogued genes were assigned to KEGG pathways. The predicted genes from the rumen and gut microbiomes were annotated to 193 and 199 KEGG pathways, respectively (Table S5 in ESM). We further investigated the KEGG pathways in the rumen and gut microbiomes and selected a total of 11 KEGG pathways associated with the *TLR4* gene, physiology and biochemistry, growth and metabolism, fatty acids, and amino acids, all of which were not statistically different between GM and WT sheep ($P > 0.05$; Figure 5C).

DISCUSSION

In the present study, we characterised GM sheep with intentionally increased *TLR4* expression in PBMCs, muscle, spleen, and kidney, albeit at varying levels. Usually, TLRs that sense pathogen-associated molecular patterns could lead to protective immune responses against invading pathogens; however, inappropriate activation of TLR pathways may lead to the initiation of excessive or aberrant innate immune responses. Abnormal immune responses contribute to autoimmune diseases wherein symptoms develop after the onset of an excessive or

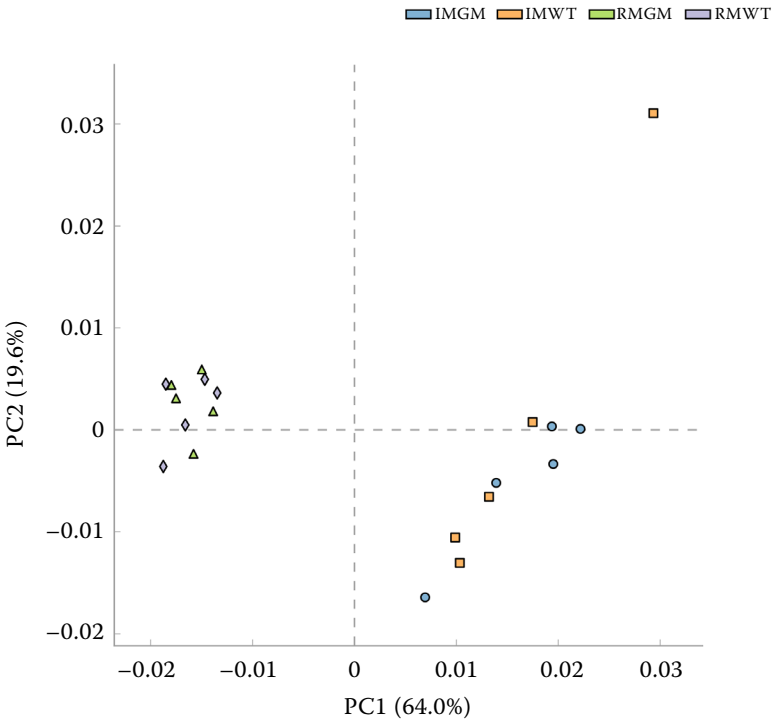

Figure 4. PCA cluster analysis of rumen and faecal microbiota in genetically modified and wild-type sheep
IMGMS = intestinal microbiota of genetically modified sheep, $n = 5$; IMWTS = intestinal microbiota of wild-type sheep, $n = 5$; RMGMS = rumen microbiota of genetically modified sheep, $n = 5$; RMWTS = rumen microbiota of wild-type sheep, $n = 5$

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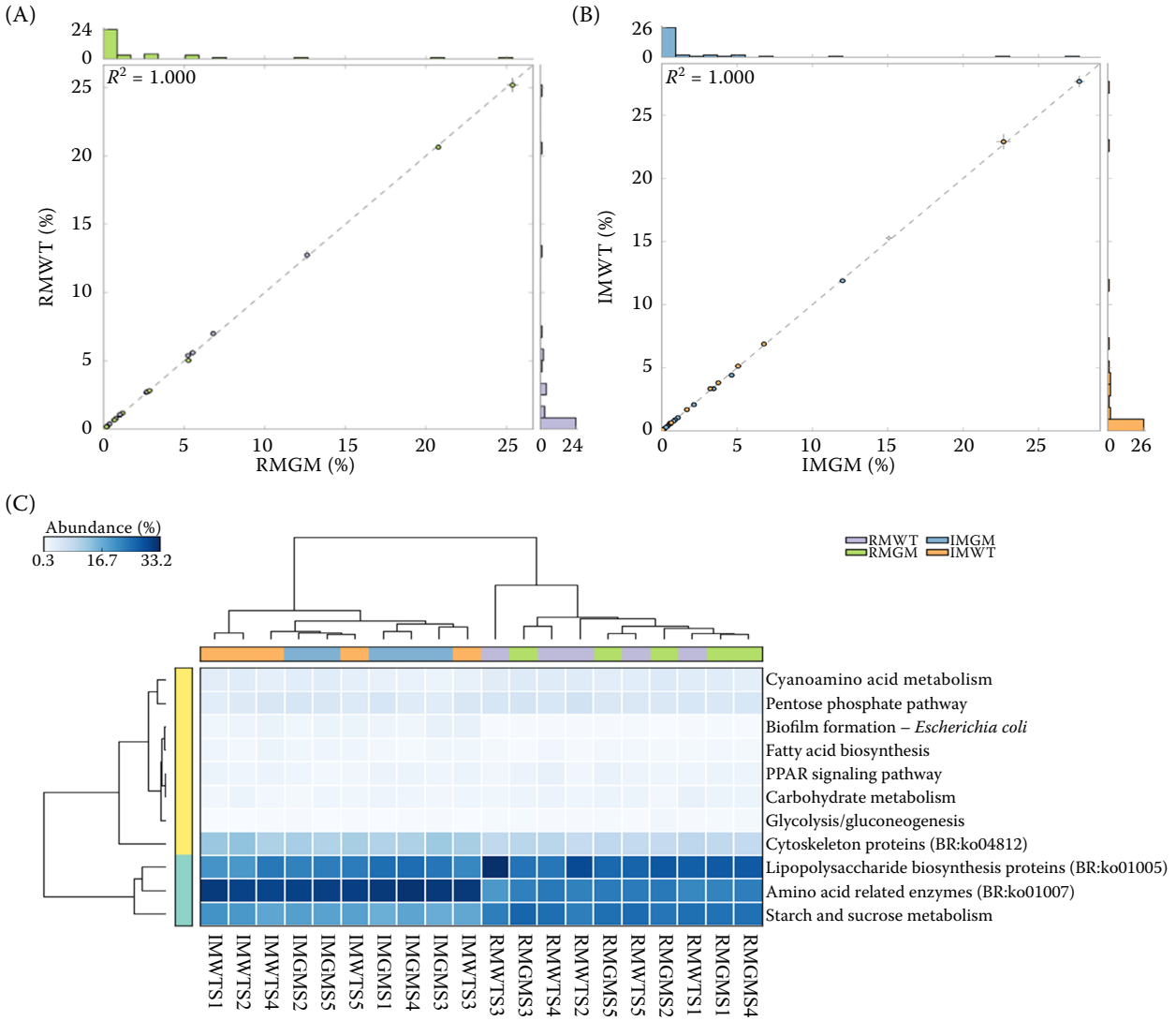

Figure 5. The KEGG function capacity of fecal and rumen microbiota in genetically modified and wild-type sheep sheep
(A) Regression analysis of the predicted KEGG genes in rumen microbiota of GM and WT sheep. (B) Regression analysis of the predicted KEGG genes in faecal microbiota of GM and WT sheep. (C) Heatmap of 11 KOs pathways in rumen fluid and faecal samples of GM and WT sheep

IMGMS = intestinal microbiota of genetically modified sheep, n = 5; IMWTS = intestinal microbiota of wild-type sheep, n = 5; RMGMS = rumen microbiota of genetically modified sheep, n = 5; RMWTS = rumen microbiota of wild-type sheep, n = 5

The x-axis shows the sample IDs, the y-axis represents the KOs

aberrant immune response. In mice, genetic duplication of *TLR7* increased B cell recognition of nucleolar antigens, inducing a spontaneous systemic lupus erythematosus-like syndrome (Pisitkun et al. 2006). Therefore, whether *TLR4* overexpression could alter the normal physiological development and health status of sheep needs to be evaluated.

Physiological and biochemical parameters, which can provide valuable information about metabolic and immunological conditions, are the most straightforward parameters for the management of animal health status (Peng et al. 2018). In peripheral blood, WBCs are part of the innate immune system, which is the primary defence mechanism of the host against invading pathogens and is believed to be evolutionarily conserved in the immune system (Smith 2013). *TLR4* is expressed in three types of WBCs, including lymphocytes, monocytes/macrophages, and granulocytes. It affects the innate immune response mediated by monocytes/macrophages and the adap-

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tive immune response mediated by lymphocytes. Meanwhile, RBCs carry oxygen and nutrients to living cells, and platelets form a plug in damaged blood vessels to prevent blood loss (Smith 2013). RBCs and platelets have also been found to have similar functions as WBCs in affecting immune function and inducing inflammatory responses (Jayachandran et al. 2007; Karsten et al. 2018). Mice lacking the *TLR4* gene have lower circulating and reticulated platelet counts (Jayachandran et al. 2007). However, in this study, *TLR4* overexpression did not result in defects in platelet count. Overall, WBC, RBC, and platelet counts were not significantly altered between the groups, in agreement with the results obtained in GM goats (Freitas et al. 2012) and pigs (Ekser et al. 2012). We hypothesised that an improved immune response might be achieved by increasing the activation of *TLR4* in the immune cells of GM sheep without accompanying deleterious effects.

Fatty acids and amino acids are structural units of cells. A recent study indicated that they could provide a basis for categorising diseases by regulating the key metabolic pathways of the immune response, including immune tolerance (Kelly and Pearce 2020). Deficiency in fatty acids and amino acids is known to impair immune function and increase the susceptibility of animals and humans to infectious diseases (Kampman-van de Hoek et al. 2016). The exogenous addition of specific amino acids (e.g., arginine, glutamine, and cysteine) or short-chain fatty acids could improve the immune status of newborn animals, thus reducing the morbidity and mortality from infectious pathogens (Kampman-van de Hoek et al. 2016). TLR4 signalling links innate immunity to lipid and amino acid metabolism, the dysregulation of which precedes autoimmunity in humans (Luu et al. 2019). In the absence of TLR4, the capacity of fatty acids to induce inflammatory signalling is decreased in adipose cells and macrophages (Shi et al. 2006). In this study, all parameters of fatty acids and amino acids were within the normal range, similar to the results obtained in other reports (Cloete et al. 2012). No significant differences were observed for any of the parameters between the GM and WT sheep. Therefore, in the present study, *TLR4* overexpression had no effect on the composition of fatty acids and amino acids in GM sheep.

The GT microbiome regulates the interactions between the immune system and homeostasis, and an imbalance in microbiota-immunity interactions could result in various immune-mediated diseases in genetically susceptible hosts (Zheng et al. 2020). Major variations in the GT microbiome are believed to be due to differences in diet as well as in morphology, physiology and behaviour. The authors observed significant differences due to these factors, as measured by taxonomic composition and KEGG orthologs. Host genetic variants predispose individuals to microbiome dysbiosis, an important factor in metabolic and immune diseases. *TLR4* deficiency in mice affected the composition of the intestinal microbiota under homeostatic conditions and increased the risk of colitis compared to WT mice (Ubeda et al. 2012). In our study, the phyla Bacteroidetes and Firmicutes and the genera *Prevotella*, *Bacteroides*, and *Alistipes* were prevalent in the rumen fluid and faeces of sheep, which is consistent with the findings of other study (Lei et al. 2019). There was no significant difference in the community composition of the rumen or faecal microbiome between the GM and WT sheep. Furthermore, the bacterial KEGG pathways associated with the *TLR4* gene activity, host physiology and biochemistry, growth and metabolism, fatty acid and amino acid contents also did not show significant differences between GM and WT sheep.

CONCLUSION

In conclusion, GM sheep overexpressing *TLR4* did not demonstrate obvious physiological and biochemical abnormalities. *TLR4* overexpression did not affect the composition of fatty acids and amino acids in the muscles of GM sheep. Furthermore, there were no significant differences between GM and WT sheep in terms of the taxonomic distribution and functional capacity of the gastrointestinal tract microbiome. Overall, *TLR4* overexpression had no obvious negative effects on the physiological development and health of sheep.

Conflict of interest

The authors declare no conflict of interest.

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