RESEARCH

Betaine addition to the diet alleviates intestinal injury in growing rabbits during the summer heat through the AAT/mTOR pathway

Zimei Li[1†], Junning Pu[1†], Xiang Chen[1], Yanbin Chen[1], Xiaoyan Peng[1], Jingyi Cai[1], Gang Jia[1], Hua Zhao[1] and Gang Tian[1*]

Abstract

Background The aim of this experiment was to investigate the effect of different levels of betaine (Bet) inclusion in the diet on the intestinal health of growing rabbits under summer heat. A total of 100 weaned Qixing meat rabbits aged 35 d with body weight of 748.61 ± 38.59 g were randomly divided into 5 treatment groups: control group (basal diet) and Bet groups (basal diet + 0.75, 1.0, 1.5 or 2.0 g/kg Bet). The average daily temperature in the rabbitry during the experiment was 30.48 °C and the relative humidity was 69.44%.

Results Dietary addition of Bet had no significant effect on growth performance and health status of growing rabbits ($P > 0.05$), but it increased ileal secretory immunoglobulin A content compared to the control under summer heat ($P < 0.05$). Addition of 0.75 g/kg Bet up-regulated jejunal *IL-4*, down-regulated ileal *TNF-α* expression ($P < 0.05$). The addition of 1.0 g/kg Bet increased the villi height (VH) in the jejunum ($P < 0.05$). Serum glucose levels were reduced, and the expression of *SLC6A20* was up-regulated in jejunum and ileum of rabbits fed with 1.5 g/kg Bet ($P < 0.05$). When added at 2.0 g/kg, Bet reduced serum HSP70 content, increased jejunal VH, and up-regulated duodenal *SLC7A6*, *SLC38A2*, *mTOR* and *4EBP-2* expression ($P < 0.05$). Correlation analysis revealed that intestinal *mTOR* expression was significantly and positively correlated with *SLC7A6*, *SLC38A2*, *SLC36A1* and *IL-4* expression ($P < 0.05$).

Conclusions Dietary addition of Bet can up-regulate the expression of anti-inflammatory factors through the AAT/mTOR pathway, improve the intestinal immune function, alleviate intestinal damage in growing rabbits caused by summer heat, and improve intestinal health.

Keywords AAT/mTOR, Betaine, Growing rabbits, Intestinal injury, Summer heat

[†]Zimei Li and Junning Pu contributed equally to this work.

*Correspondence:
Gang Tian
13555@sicau.edu.cn
[1] Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

Background

As global temperatures rise, hot and humid environments are becoming one of the main problems faced by animal husbandry in many parts of the world. Sustained summer heat challenges can put animals under prolonged stress, leading to increased morbidity, decreased growth performance, impaired immune function, and serious economic losses [1–3]. As an animal lacking functional sweat glands, rabbits mainly rely on respiration and blood flow through their ears to dissipate heat. When the

breeding environment is too hot in summer, it is difficult for rabbits to release excess heat from their bodies, and rabbits are prone to stress and even death [4]. As one of the most important immune organs in the animal body, the intestine is vital for the growth and development of the animal [5]. After weaning, the intestinal development of newborn rabbits is still incomplete and susceptible to high temperature, which will block further development, trigger oxidative stress, produce inflammatory response, and damage intestinal health, resulting in decreased growth performance and increased morbidity and mortality of rabbits [6–8]. Therefore, effective approaches to alleviate the adverse effects of summer heat on rabbits need to be explored.

Betaine (Bet), as a methyl donor feed additive for animals which has significant effects on promoting animal growth, relieving stress and maintaining intestinal health [9, 10]. Addition of 1.0 g/kg Bet to the broiler diets can improve intestinal morphology, reduces intestinal permeability and improves the immune function of the organism [11]. Dietary 0.1% Bet significantly increased the serum immunoglobulin content of broilers under high temperature environment [12]. Dietary supplementation of 1.0 g/kg Bet also down-regulated the expression of inflammatory factor *IL-1β* in the intestinal tract of broilers, which alleviated the inflammatory response induced by high temperature [13]. Bet as a glycine derivative can be transported by amino acid transporters (AAT), and mTOR is a central regulator responsible for integrating various cellular signals, especially those from AAT [14]. Bet is transported by amino acid transporter A2 (SNAT2) [15], which also activates the downstream signaling factors P70 ribosomal protein S6 kinase 1 (P70S6k1) and eukaryotic translation initiation factor 4E binding protein-2 (4EBP-2) to maintain intestinal health [14, 16]. SNAT2 is composed of solute carrier (SLC) gene family [17], of which SLC38A2 and SLC7A5 have been reported to regulate protein synthesis as well as cell growth and proliferation by activating the mTOR signaling pathway [18]. Dietary Bet can also reduce oxidative stress and inflammatory response through mTOR signaling pathway in Hu sheep [19]. Therefore, we speculate that Bet may enhance body immunity and maintain intestinal health in growing rabbits through AAT/mTOR pathway. However, it has not been reported that Bet can improve the intestinal injury induced by summer heat in rabbits by upregulating the gene expression of mTOR and its downstream signaling factors through SNAT2 or other AATs that transport Bet in the rabbit intestine. Thus, this study aims to investigate the effects of dietary supplementation with different levels of Bet on the intestinal health of growing rabbits under the summer heat and to reveal the potential alleviating effect of Bet on the intestinal injury of growing rabbits and its possible mechanism.

Materials and methods

All experimental animal procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University (approval number: SICAU20220703).

Experimental design

A single-factor experimental design was used in this study, and 100 weaned Qixing meat rabbits (German white rabbit×Sichuan white rabbit), 35 days old with body weight of 748.61 ± 38.59 g, half male and half female, were purchased from Hongzhan Family Farm (Jintang County, Chengdu City, Sichuan Province, China). Rabbits were randomly divided into 5 treatment groups, which were supplemented with 0 (control group), 0.75, 1.0, 1.5 and 2.0 g/kg Bet (Yixing Tianshi Feed Co., Ltd., Jiangsu, China) in the basal diet, respectively. Each treatment had 20 replicates and one rabbit in each replicate. The animals were kept in single cages (galvanised wire cages) throughout the 21 d experiment [20].

The experimental basal diet was based on alfalfa, corn, wheat bran and soybean meal, and was formulated according to the nutritional requirements of growing and fattening rabbits [20]. The experimental diets were supplemented with 0.75, 1.0, 1.5 and 2.0 g/kg Bet in the form of equivalent replacement of rice husk powder in the basal diet. All diets were made into pellet feeds with a diameter of 2.5 mm and a length of 6 to 12 mm, packaged in moisture-proof feed bags, and stored in a dry and ventilated place before use. Basal diet composition and nutrient levels are shown in Table 1.

The experiments were carried out in July and August, and the enclosures were thoroughly disinfected before the start of the experiment. The rabbits were then randomly allocated according to treatment to galvanised wire mesh cages (50 cm×50 cm×40 cm, length×width×height) with water troughs and nipple drinkers. Temperature and humidity meter were hung on the walls around the rabbitry (at a height of 1.5 m above the floor) during the experiment to record the temperature and humidity at 06:00 and 14:00 each day [21]. The rabbits were fed twice a day (08:00 and 20:00) to allow ad libitum access to food and water. The rabbitry was cleaned daily to keep it ventilated and hygienic and a detailed record was kept of the health status of the rabbits.

Sample collection

At 06:00 h on the 22nd day of the experiment, 6 rabbits (3 males and 3 females), which were close to the average body weight of the group, were selected from each treatment group. Approximately 5 mL of blood was

Li *et al. Journal of Animal Science and Biotechnology*   (2024) 15:41

Page 3 of 15

Table 1 Composition and nutrient levels of diets (dry matter basis, %)

Ingredients	Inclusion, %	Nutrient	Level[2]
Alfalfa meal	20.00	DE, MJ/kg	10.20 (10.39)
Corn	13.10	CP	16.10 (15.90)
Soybean meal	10.90	CF	16.00 (16.23)
Corn germ meal	10.00	NDF	33.20 (44.50)
Wheat bran	20.00	ADF	18.51 (21.68)
Soybean hull	12.25	ADL	3.66 (4.15)
Rice bran	4.42	Ca	0.60
Rice hull power	5.82	TP	0.52
Soybean oil	1.31	SAA	0.52
$CaCO_3$	0.55	AFD Lys	0.52
NaCl	0.50	AFD Met	0.21
L-Lys HCl ($\geq$ 98%)	0.03	AFD Thr	0.43
L-Met ($\geq$ 99%)	0.03		
L-Thr ($\geq$ 98.5%)	0.09		
Premix[1]	1.00		
Total	100.00		

[1] Premix provided the following per kg of the diet: vitamin premix 250 mg (include 6,000 IU of VA, 1,200 IU of VD_3, 50 IU of VE, 2.4 mg of VK_3, 12.5 μg of VB_{12}, 240 μg of biotin, 1.8 mg of pyridoxine, 3.6 mg of riboflavin, 35 mg of niacin, 12.5 mg of pantothenic acid), 35 mg Zn ($ZnSO_4 \cdot H_2O$), 30 mg Fe ($FeSO_4 \cdot 7H_2O$), 8 mg Mn ($MnSO_4 \cdot H_2O$), 6 mg Cu ($CuSO_4 \cdot 5H_2O$), 0.4 mg I (KI), 0.05 mg Se (Na_2SeO_3), 0.3 mg Co ($CoCl_2 \cdot 6H_2O$), 100 mg Choline (choline chloride, 50%)

[2] Nutrient levels are measured values in parentheses, and the rest are calculated values

collected from the heart with butterfly blood sampling needle and injected into anticoagulant-free blood collection tubes. After standing for 1 h at room temperature, the blood was centrifuged at $3,000 \times g$ for 15 min and the supernatant was divided into centrifuge tubes and stored at -20 °C until further analysis.

After blood collection, the rabbits were electricity stunned (50 V, pulsed direct current, 60 Hz for 5 s) and killed by cervical dislocation. The duodenum, jejunum, and ileum were separated by dissecting rabbits, and 2–3 cm of tissue samples from the middle of the jejunal and ileal segments were collected. The intestinal samples were rinsed with precooled 0.9% saline and fixed in 4% paraformaldehyde fixative at room temperature in preparation of histological analysis. The jejunum was then gently squeezed with forceps and the extruded mucus was divided into lyophilized tubes and snap frozen in liquid nitrogen and stored at -80 °C in the

refrigerator for testing. The remaining intestinal tissues were cut longitudinally, rinsed with pre-cooled 0.9% saline, blotted on filter paper, collected near the middle of each intestinal segment, packed in frozen tubes, snap frozen in liquid nitrogen and stored at -80 °C in the refrigerator before testing.

Indicator measurement and analysis
Temperature and humidity index
The temperature and humidity of the rabbitry were used to calculate temperature and humidity index (THI) of the rabbitry according to the following formula [22]:

$$THI = T - [(0.31 - 0.31RH) \times (T - 14.4)]$$

T refers to temperature (°C), RH refers to relative humidity (%). When THI < 27.8 (no heat stress), THI = 27.8 to 28.9 (moderate heat stress), THI = 28.9 to 30.0 (severe heat stress), THI > 30.0 (very severe heat stress).

Growth performance
The feed intake of rabbits was recorded daily during the experiment, and on d 1 and 21 of the experiment, all rabbits were fasting weighed and body weight (BW) was recorded. Average daily gain (ADG) and average daily feed intake (ADFI) were calculated for rabbits in each treatment group for d 1–21, and then the feed to gain ratio (F/G) was calculated [23].

Health status
The health status (mental state, feeding and diarrhoea, etc.) of the rabbits was monitored daily during the experiment, and their morbidity, mortality and health risk index were calculated [23]:

Morbidity (%) = (number of rabbits with disease in the experiment/total number of rabbits at the start of the experiment) × 100%;

Mortality rate (%) = (number of rabbits that died in the experiment/total number of rabbits at the beginning of the experiment) × 100%;

Health risk index (%) = (the sum of the number of rabbits that became ill and the number of rabbits that died in the experiment/ the total number of rabbits at the start of the experiment) × 100%.

When calculating the morbidity, mortality, and health risk index for each group of rabbits during the experimental period, the morbidity or death of the rabbits was counted only once.

Intestinal morphology
The jejunal and ileal tissue fixed in 4% paraformaldehyde were dehydrated, trimmed, dipped in wax, embedded and sectioned, and then stained with hematoxylin-eosin (HE). Samples were observed with a light microscope (40× imaging), and a randomly selected morphologically intact target area was photographed and the villi height

(VH) and corresponding crypt depth (CD) were determined to calculate the villi to crypt ratio (V/C).

Indicators of intestinal immunity

Tissue homogenates were prepared by accurately weighing 0.1 g of duodenal, jejunal and ileal tissue samples with saline at a mass-to-body ratio of 1:9, before centrifugation at 3,000 r/min for 15 min at 4 °C, and extraction of the supernatant. The contents of sIgA in each intestinal segment and HSP70 in serum were determined by ELISA kits (Enzyme immunoassay, Jiangsu, China). Glucose (GLU) levels as well as glutamic oxaloacetic transaminase (AST) and glutamic alanine transaminase (ALT) activities in serum were determined using the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Samples were analysed according to the manufacturer's instructions.

Gene expression

The expression of intestinal inflammation-related genes *TNF-α*, *IL-2* and *IL-4*, intestinal Bet transporter-related genes *SLC9A3*, *SLC7A6*, *SLC36A1*, *SLC6A20* and *SLC38A2*, and mTOR pathway-related genes *mTOR*, *P70S6k1* and *4EBP-2* was determined using real-time fluorescence quantitation (RT-PCR).

Total RNA was extracted from each intestinal tissue sample using Trizol reagent and the integrity of the extracted RNA was evaluated at an absorbance ratio of 260/280 nm (1.8–2.0 is the ideal ratio). Genomic DNA was removed using the PrimeScript RT kit before reverse transcription was carried out according to the manufacturer's instructions. Real-time PCR analysis was performed using a SYBR®Primix Ex Taq^{TM} II kit (TaKaRa, Shiga, Japan) and a fluorescent quantitative PCR instrument (Applied Biosystems 7900HT, Foster city, California, USA). The relative gene expression was calculated by the $2^{-\Delta\Delta ct}$ method using β-action as the internal reference gene. The primer sequences are shown in Table 2 and the primer synthesis was performed at Shanghai Biotechnology Co., Ltd.

Statistical analysis

The data were analysed by one-way ANOVA using SAS 9.4 (V9.4, SAS Institute Inc., Cary, NC, USA) statistical software and combined with Tukey's method for multiple comparisons, and regression analysis was performed using SAS 9.4 software. The weighted least square method was used to analyse the mortality, morbidity and health risk index of rabbits at the unit of treatment, and the Pearson correlation coefficient between some gene expressions was obtained by correlation analysis. All

Table 2 Primer sequences for quantitative real-time polymerase chain reaction

Gene	Primer sequences (5′→3′)	Product length, bp	Accession number
β-action	F: CAGAAACGAGACGAGATT GGC R: CAATCAAAGTCCTCGGCC ACA	189	NM_001101683.1
IL-2	F: TGCCCAAGAAGGTCA CAGAA R: CCCCCATGAGAGTTTTTG CC	106	NM_001163180.1
IL-4	F: GCGACATCATCCTACCCG AA R: TCGGTTGTGTTCTTGGGG AC	115	NM_001163177.1
mTOR	F: CTTGAGTCCGAAGAGGAT GG R: ATGAGGGTGGCAAGA AAGTG	187	XM_008262137.2
P70S6k1	F: TGTGACGACAAGTGGGGA AG R: TGGCCGTTTGGAGATCAT GG	100	NM_001101690.1
SLC6A20	F: GGATGGCTGACTCAA GAACC R: CACACACTGAGGCGT AGAGC	226	XM_008269584.2
SLC7A6	F: TGCCCCTCTTCAGTGATACC R: GCAAAGCTTCTGGGT GACTC	161	XM_017342318.1
SLC9A3	F: ATCGGCTCATTGAGGTGA AC R: TCCCATTGGTGATTGGTGAT	198	NM_001082107.1
SLC36A1	F: ACTACTCGGAGGGCA TGAGT R: TGAAGATGGGAGCGT TGCTT	135	XM_008254846.2
SLC38A2	F: TGTGCAGCCAAAGATTTC AG R: GACGGGAGGATGAAG ATCAA	158	XM_002712695.3
TNF-α	F: AGCCCACGTAGTAGCAAA CC R: TGAGTGAGGAGCACG TAGGA	192	XM_008262537.2
4EBP-2	F: GTTGGATCGTCGCAATTC TCC R: ATTAAGGTGCCAGGGCTA GTG	87	XM_002718436.3

IL-2 Interleukin-2, *IL-4* Interleukin-4, *mTOR* Mammalian target of rapamycin, *P70S6k1* P70 ribosomal protein S6 kinase 1, *SLC7A6* Solute carrier family 7 member 6, *SLC9A3* Solute carrier family 9 member 3, *SLC6A20* Solute carrier family 6 member 20, *SLC36A1* Solute carrier family 36 member 1, *SLC38A2* Solute carrier family 38 member 2, *TNF-α* Tumor necrosis factor-alpha, *4EBP-2* Eukaryotic initiation factor 4E-binding protein-2

data results are expressed as mean and standard error of mean (SEM), with $P < 0.05$ as a significant difference and $0.05 \leq P < 0.10$ as a trend.

Table 3 Changes of the THI of the rabbitry during the experimental period

Items	Temperature, °C		Humidity, %		THI
	6:00	**14:00**	**6:00**	**14:00**	
1st week	27.45	35.18	71.21	54.96	29.81
2nd week	26.97	33.47	81.07	66.86	29.29
3rd week	26.24	33.62	81.89	60.68	29.06

THI Temperature humidity index

the rabbits had been under severe heat stress (Table 3). Compared with the control group, the dietary addition of Bet had no significant effect on BW, ADG, ADFI and F/G in growing rabbits from 1 to 21 d (Table 4). Dietary supplementation with different levels of Bet had no significant effects on morbidity, mortality and health risk index of growing rabbits compared with the control group without Bet (Table 5).

Table 4 Effects of dietary betaine on growth performance of growing rabbits from 1 to 21 d under the summer heat

Items	Betaine, g/kg					SEM	*P*-value		
	0	**0.75**	**1.00**	**1.50**	**2.00**		**ANOVA**	**Linear**	**Quadratic**
1 d BW, g	749.16	748.74	750.11	750.00	745.47	4.10	0.997	0.825	0.941
21 d BW, g	1,261.37	1,260.74	1,260.21	1,284.95	1,251.26	9.00	0.822	0.967	0.892
ADG, g/d	24.39	24.38	24.29	25.47	24.09	0.36	0.780	0.869	0.903
ADFI, g/d	69.46	69.56	73.42	72.56	69.43	0.63	0.106	0.598	0.173
F/G	2.90	2.90	3.05	2.87	2.95	0.04	0.633	0.822	0.874

BW Body weight, *ADG* Average daily gain, *ADFI* Average daily feed intake, *F/G* Feed to gain ratio

Table 5 Effects of dietary betaine on health status of growing rabbits under the summer heat

Items	Betaine, g/kg					SEM	*P*-value	
	0	**0.75**	**1.00**	**1.50**	**2.00**		**ANOVA**	**Linear**
Morbidity, %	13.33	10.00	10.00	6.67	6.67	-	0.894	0.323
Mortality, %	10.00	10.00	6.67	3.33	3.33	-	0.710	0.166
Health risk index, %	23.33	20.00	16.67	10.00	10.00	-	0.529	0.084

Table 6 Effects of betaine on serum stress indexes of growing rabbits under the summer heat

Items	Betaine, g/kg					SEM	*P*-value		
	0	**0.75**	**1.00**	**1.50**	**2.00**		**ANOVA**	**Linear**	**Quadratic**
ALT, U/L	19.51	15.88	18.83	16.64	16.29	1.05	0.766	0.465	0.760
AST, U/L	3.29[a]	3.09[ab]	2.11[ab]	1.97[b]	2.10[ab]	0.19	0.059	0.007	0.017
HSP70, ng/mL	74.34[a]	71.24[a]	74.18[a]	69.88[a]	62.70[b]	1.21	0.006	0.002	0.002
GLU, mmol/L	7.09[a]	6.45[abc]	6.28[bc]	5.80[c]	6.74[ab]	2.32	0.012	0.140	0.006

ALT Alanine aminotransferase, *AST* Aspartate aminotransferase, *HSP70* Heat shock protein 70, *GLU* Glucose

[a–c] Different superscript lowercase letters within the same row indicate significant differences ($P < 0.05$)

Results

Effects of betaine on growth performance and health status of growing rabbits in summer heat

During the experiment, the average daily temperature of the rabbitry was 30.48 °C, the relative humidity was 69.44%, and the average THI was 29.38, indicating that

Effects of betaine on serum stress indexes of growing rabbits in summer heat

Compared with the control group without Bet, the content of GLU in serum of growing rabbits was decreased by adding 1.0 g/kg Bet ($P < 0.05$, Table 6). When Bet

Li *et al. Journal of Animal Science and Biotechnology*   (2024) 15:41

Page 6 of 15

Table 7 Effects of betaine on intestinal morphology of growing rabbits under the summer heat

Items	Betaine, g/kg					SEM	P-value		
	0	0.75	1.00	1.50	2.00		ANOVA	Linear	Quadratic
Jejunum									
VH, μm	340[b]	350[b]	790[a]	530[ab]	700[a]	56.59	0.014	0.021	0.051
CD, μm	130[b]	260[b]	590[a]	110[b]	120[b]	56.91	0.013	0.673	0.068
V/C	2.60	1.64	2.76	4.76	5.75	0.05	0.058	0.007	0.013
Ileum									
VH, μm	350	380	410	500	460	25.37	0.360	0.052	0.150
CD, μm	100	110	100	100	100	2.56	0.804	0.461	0.767
V/C	3.35	3.47	4.00	5.07	4.53	0.23	0.079	0.011	0.040

VH Villus height, *CD* Crypt depth, *V/C* Villus height to crypt depth ratio

[a,b] Different superscript lowercase letters within the same row indicate significant differences ($P < 0.05$)

was added to 2.0 g/kg, the content of GLU and HSP70 in serum was decreased ($P < 0.05$, Table 6). Dietary Bet tended to reduce the activity of AST in serum of growing rabbits ($P = 0.059$), but had no effect on the activity of ALT ($P > 0.05$) (Table 6).

Effect of betaine on intestinal morphology of growing rabbits in summer heat

As shown in Table 7, compared with the control group, supplementation of 1.0 and 2.0 g/kg Bet increased jejunal VH in growing rabbits ($P < 0.05$), and supplementation of 1.0 g/kg Bet also increased jejunal CD ($P < 0.05$). At the same time, dietary Bet tended to increase V/C in the jejunum and ileum of growing rabbits ($P = 0.058$, $P = 0.079$), but had no effect on VH and CD in the ileum. The jejunal VH and V/C and ileal V/C of growing rabbits increased linearly with increasing levels of Bet in the diet ($P < 0.05$).

Effects of betaine on intestinal sIgA levels of growing rabbits in summer heat

Compared with the control group without Bet supplementation, 0.75 and 1.0 g/kg Bet supplementation increased duodenal sIgA levels ($P < 0.05$, Table 8). The highest jejunal sIgA levels were found with the addition of 1.5 g/kg Bet, but jejunal sIgA levels were lower when Bet was added at 0.75 g/kg ($P < 0.05$, Table 8). Dietary supplementation with different levels of Bet increased the

ileal sIgA level of growing rabbits ($P < 0.05$, Table 8), and the ileal sIgA level was the highest when the supplementation amount was 2.0 g/kg compared with the control group. With increasing Bet levels in the diet, the duodenal sIgA levels of growing rabbits showed a secondary increase followed by a decrease ($P < 0.05$), and ileal sIgA levels showed a linear increase ($P < 0.05$) (Table 8).

Effect of betaine on the expression of intestinal inflammation-related genes in growing rabbits in summer heat

There was no effect of diet addition of Bet on duodenal *TNF-α*, *IL-2* and *IL-4* expression compared with the control group (Fig. 1A). Dietary Bet supplementation at 0.75 g/kg increased the expression of *IL-4* in the jejunum ($P < 0.05$), while supplementation of different levels of Bet had no effect on the expression of *TNF-α* and *IL-2* in the jejunum (Fig. 1B). Adding 0.75 g/kg Bet down-regulated the expression of *TNF-α* in the ileum ($P < 0.05$), but different levels of Bet had no effect on the expression of *IL-2* and *IL-4* in the ileum (Fig. 1C).

Effect of betaine on the expression of genes related to betaine transporters in intestines of growing rabbits in summer heat

Compared with the group without Bet, the addition of different levels of Bet to the diet up-regulated the

Table 8 Effect of betaine on the content of sIgA in intestine of growing rabbits under the summer heat

Items	Betaine, g/kg					SEM	P-value		
	0	0.75	1.00	1.50	2.00		ANOVA	Linear	Quadratic
Duodenum, μg/mL	66.28[c]	80.45[a]	75.32[ab]	69.80[bc]	64.28[c]	1.57	< 0.001	0.156	0.002
Jejunum, μg/mL	74.30[ab]	69.25[b]	72.41[b]	81.33[a]	74.65[ab]	1.26	0.029	0.156	0.362
Ileum, μg/mL	72.46[c]	96.27[b]	89.00[b]	90.24[b]	124.79[a]	3.68	< 0.001	< 0.001	< 0.001

[a–c] Different superscript lowercase letters within the same row indicate significant differences ($P < 0.05$)
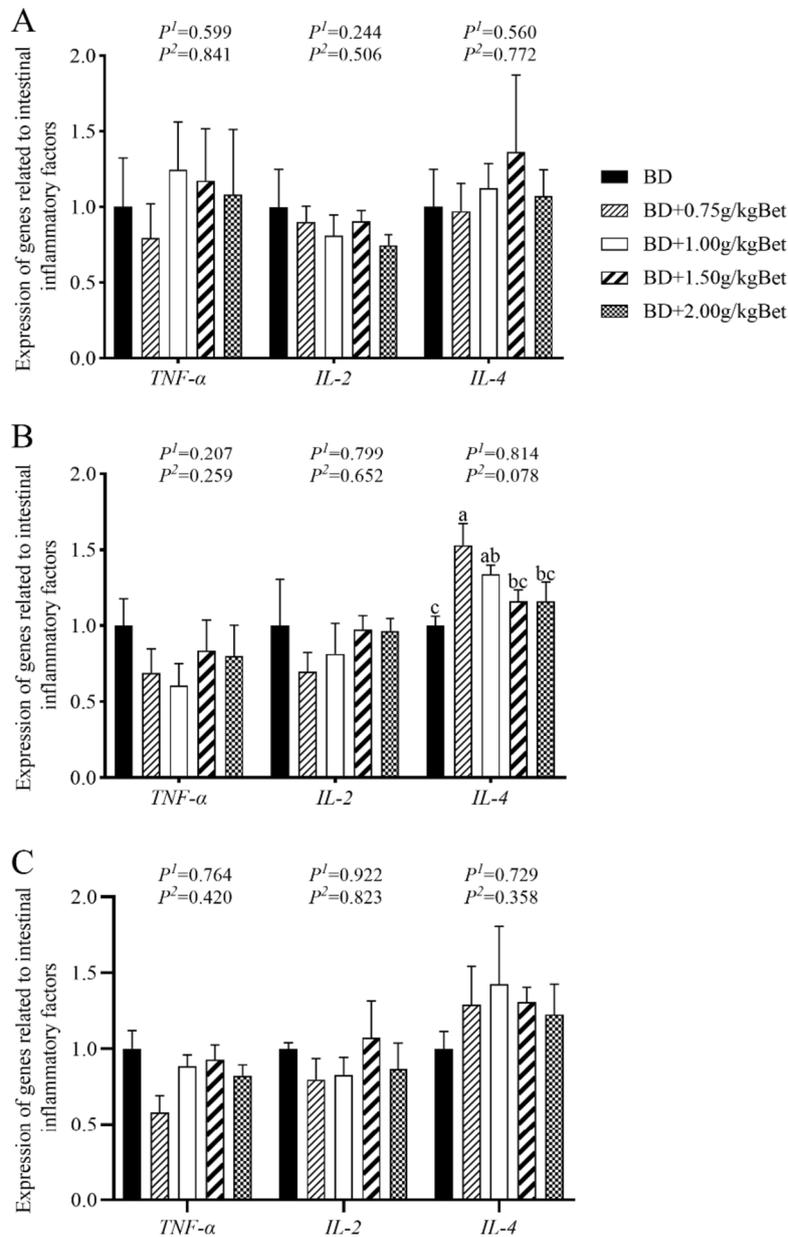
Fig. 1 Effects of betaine on intestinal immune function of growing rabbits under the summer heat. **A** Duodenum; **B** Jejunum; **C** Ileum. *TNF-α* Tumor necrosis factor-alpha, *IL-2* Interleukin-2, *IL-4* Interleukin-4. [a–c] Different superscript lowercase letters indicate significant differences ($P < 0.05$). P^1, linear; P^2, quadratic

expression of *SLC7A6* (except for the addition of 0.75 g/kg Bet) and *SLC38A2* in the duodenum of growing rabbit ($P < 0.05$, Fig. 2A), Bet added at 1.5 g/kg up-regulated the expression of *SLC7A6* in the jejunum ($P < 0.05$, Fig. 2B), and 0.75, 1.0 and 1.5 g/kg Bet up-regulated ileal *SLC6A20* expression ($P < 0.05$, Fig. 2C). However, the addition of different levels of Bet had no effect on the expression of *SLC9A3* and *SLC36A1*, as well as duodenal *SLC6A20*, jejunal *SLC38A2* and *SLC6A20*, and ileal *SLC7A6* and *SLC38A2* in each gut segment (Fig. 2). With the increase of dietary Bet level, the expression of *SLC7A6* and *SLC38A2* in duodenum, *SLC7A6* in jejunum and *SLC6A20* in ileum of growing rabbits increased linearly ($P < 0.05$, Fig. 2).
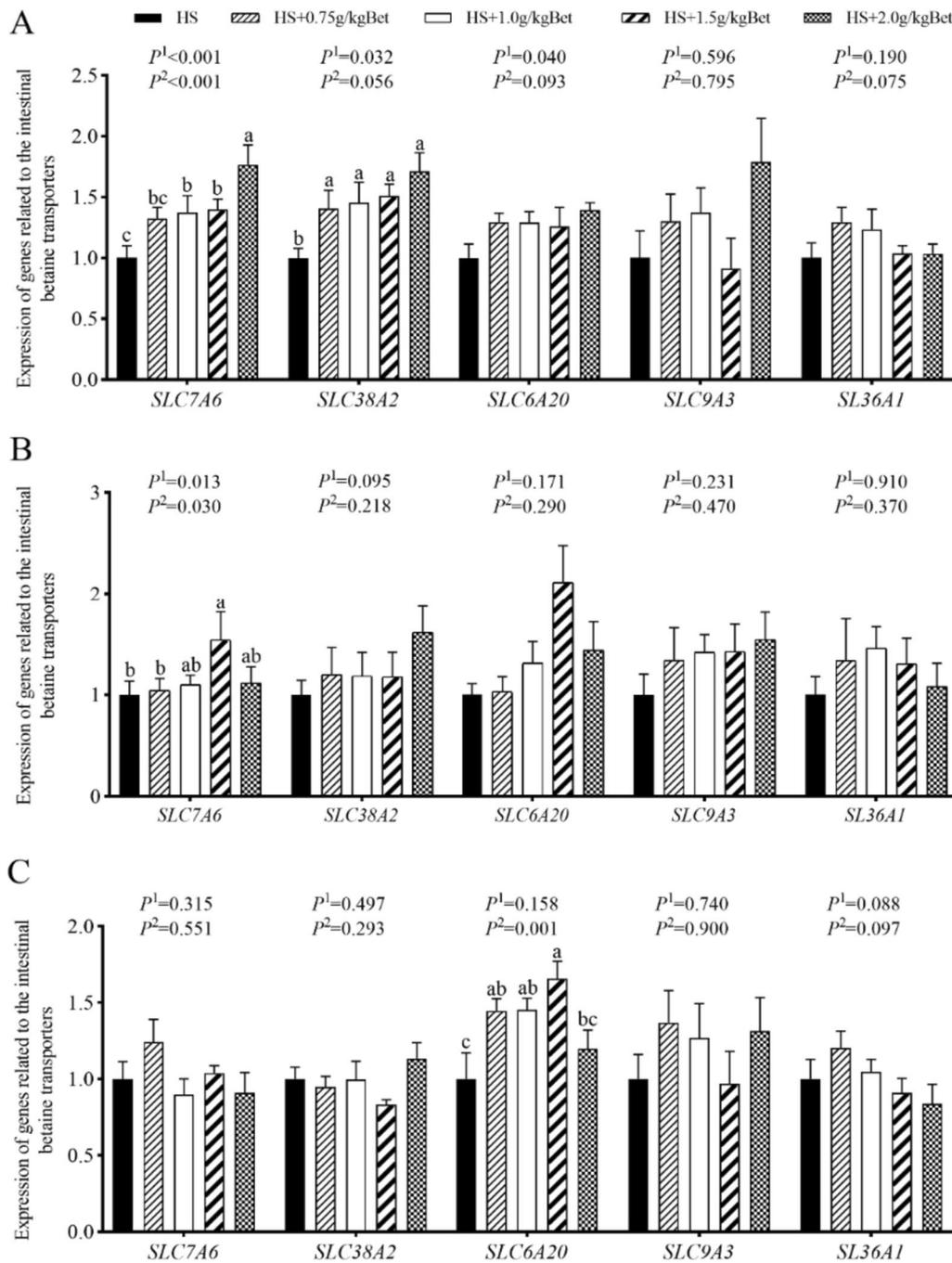
Fig. 2 Effects of betaine on expression of betaine transporter genes in intestine of growing rabbits under the summer heat. **A** Duodenum; **B** Jejunum; **C** Ileum. *SLC7A6* Solute carrier family 7 member 6, *SLC38A2* Solute carrier family 38 member 2, *SLC6A20* Solute carrier family 6 member 20, *SLC9A3* Solute carrier family 9 member 3, *SLC36A1* Solute carrier family 36 member 1. [a-c] Different superscript lowercase letters indicate significant differences ($P < 0.05$). P^1, linear; P^2, quadratic

Effect of betaine on the expression of mTOR pathway related genes in intestines of growing rabbits in summer heat

Compared with the control group without Bet, diets supplemented with 0.75 and 2.0 g/kg Bet increased the gene expression of *mTOR* and *4EBP-2* in the duodenum of growing rabbits ($P < 0.05$, Fig. 3A), and diets supplemented with 0.75 g/kg Bet increased the gene expression of *4EBP-2* in the ileum ($P < 0.05$, Fig. 3C). However, dietary Bet supplementation at different levels had no
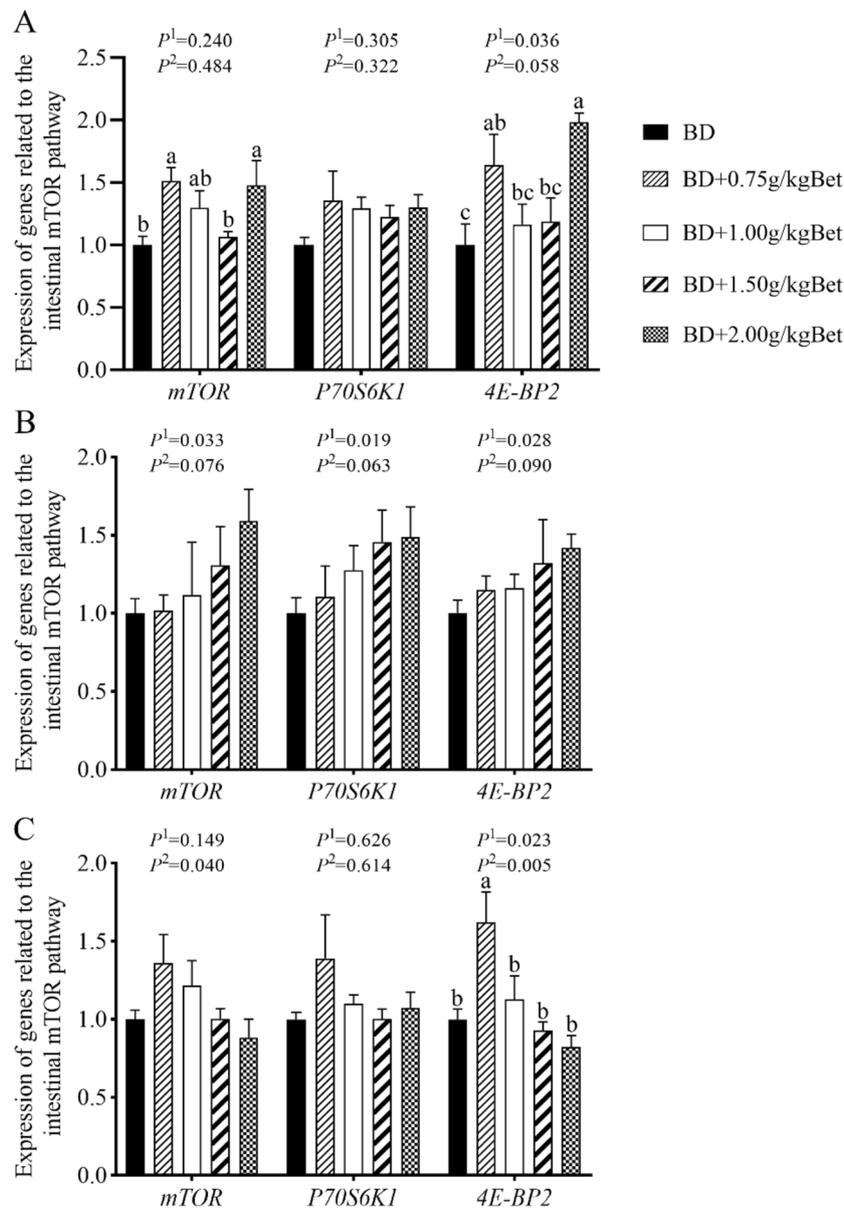
Fig. 3 Effects of betaine on the expression of mTOR pathway genes in intestines of growing rabbits under the summer heat. **A** Duodenum; **B** Jejunum; **C** Ileum. *mTOR* Mammalian target of rapamycin, *P70S6k1* P70 ribosomal protein S6 kinase 1, *4EBP-2* Eukaryotic initiation factor 4E-binding protein-2. [a–c] Different superscript lowercase letters indicate significant differences ($P < 0.05$). P^1, linear; P^2, quadratic

effects on the gene expression of *P70S6k1* in duodenum, *mTOR*, *P70S6k1* and *4EBP-2* in jejunum, and *mTOR* and *P70S6k1* in ileum (Fig. 3). With the increase of dietary Bet level, the expression of *4EBP-2* in duodenum, *mTOR*, *P70S6k1* and *4EBP-2* in jejunum increased linearly, and the expression of *4EBP-2* in ileum decreased linearly ($P < 0.05$, Fig. 3).

Correlation analysis of mTOR and AAT-related gene expression

As shown in Table 9, dietary Bet supplementation in growing rabbits regulated the gene expression of *mTOR* by affecting the expression of genes related to intestinal Bet transporters. There was a positive correlation between *mTOR* gene expression and *SLC7A6*, *SLC38A2*, *SLC9A3* and *SLC36A1* gene expression in duodenum of

Li *et al. Journal of Animal Science and Biotechnology*   (2024) 15:41

Page 10 of 15

Table 9 Correlation analysis of mTOR and inflammatory factor gene expression in intestines of growing rabbits

Items	Duodenum			Jejunum			Ileum		
	TNF-α	IL-2	IL-4	TNF-α	IL-2	IL-4	TNF-α	IL-2	IL-4
r	−0.096	0.042	0.302*	0.076	0.073	0.286	0.222	−0.10	0.354*
P	0.486	0.762	0.025	0.569	0.586	0.052	0.114	0.948	0.012

TNF-α Tumor necrosis factor-alpha, *IL-2* Interleukin-2, *IL-4* Interleukin-4, *r* stands for Pearson correlation coefficient, *P* stands for significance (two-tailed)

* Indicates significant correlation, $P < 0.05$

growing rabbits ($P < 0.05$), and there was a trend for a positive correlation with duodenal *SLC6A20* gene expression ($P = 0.059$). The jejunal *mTOR* gene showed a positive correlation with jejunal *SLC7A6*, *SLC38A2*, *SLC6A20* and *SLC36A1* gene expression ($P < 0.05$), and also with jejunal *SLC9A3* gene expression ($P = 0.075$). There was a positive correlation between the expression of ileal *mTOR* gene and ileal *SLC7A6*, *SLC38A2*, *SLC6A20*, *SLC9A3* and *SLC36A1* genes ($P < 0.05$).

Correlation analysis of mTOR and inflammation-related gene expression

Dietary Bet can regulate the expression of inflammatory factor-related genes by affecting the expression of the *mTOR* gene in the intestine of growing rabbits. There was a positive correlation between the expression of *mTOR* and *IL-4* genes in the duodenum of growing rabbits ($P < 0.05$, Table 10). The expression of the *mTOR* gene in the jejunum was positively correlated with that of the *IL-4* gene in the jejunum ($P = 0.052$), and the expression of the *mTOR* gene in ileum was positively correlated with that of the *IL-4* gene in ileum ($P < 0.05$, Table 10). However, the expression of the *mTOR* gene in duodenum, jejunum and ileum had no effect on the expression of the *TNF-α* and *IL-2* genes respectively (Table 10).

Discussion

During the summer, we observed that the THI of the rabbitry was 29.38. This value suggests that the rabbits were exposed to severe heat stress throughout the experiment. However, prolonged high temperature stimulation reduced the growth performance of rabbits, but supplementation of 1.0 or 1.5 g/kg Bet in the diet significantly improved the growth performance of rabbits [24]. Interestingly, we found that dietary betaine supplementation did not improve the growth performance of rabbits under summer heat. Experiments were performed between d 1 and 21 after weaning, which is a critical stage of rabbit gut development, so the improvement of growth performance of rabbits by Bet was not significant. Summer heat can increase morbidity and mortality, and increase the health risk index of rabbits [25]. Morbidity, mortality and health risk index are important indicators of the

health status of growing rabbits [23]. It was found that HS caused 138% higher mortality in growing rabbits than in adult rabbits, indicating that HS is the most harmful to the health status of growing rabbits [25]. The results of this experiment showed that the same rate of reduction in morbidity, mortality, and health risk index (49.9%, 66.7%, and 133.3% respectively) was observed when Bet was added at 1.5 or 2.0 g/kg. This is the first time that dietary supplementation with Bet has been shown to improve the health status of growing rabbits in the summer heat to some extent. It may be because Bet as a methyl donor, can promote the conversion of excitatory amino acid Hcy to SAM, increasing the expression of inhibitory neurotransmitter GABA, inhibiting the excitability of the nervous system, reducing the respiratory rate, and enhancing the heat tolerance of rabbits, thereby reducing the occurrence of heat exhaustion [26].

Glucose is co-mediated by cellular uptake and utilisation through the glucose transporter protein family (GLUT) and the sodium-glucose cotransporter protein family (SGLT) [27]. It was found that in high temperature environments, animals can increase blood glucose level and accelerate GLU consumption in vivo by upregulating SGLT-1 and GLUT-2 expression [28, 29]. The present experimental study found that Bet reduced blood glucose levels and slowed down the energy loss from the growing rabbits under the summer heat, which is consistent with previous work [24, 30] on New Zealand white rabbits. However, these experiments found that blood glucose concentrations increased instead when the additive amount of Bet reached 2.0 g/kg. This may be due to the fact that Bet supplementation leads to an increase in serum SAM levels and promotes the production of succinate and pyruvate, precursors of gluconeogenesis, thus increasing blood glucose concentrations [31].

The levels of AST and ALT in the serum may reflect normal or abnormal liver function. It was found that summer heat causes damage to the body's liver and a large release of AST and ALT from liver cells into the blood, leading to a significant increase in serum levels of AST and ALT [32]. The results of the present experimental study showed that Bet reduced serum levels of AST and ALT under the summer heat, which is consistent

Table 10 Correlation analysis of mTOR and betaine transporter protein relative gene expression in intestines of growing rabbits

Items	Duodenum					Jejunum					Ileum				
	SLC7A6	SLC38A2	SLC6A20	SLC9A3	SLC36A1	SLC7A6	SLC38A2	SLC6A20	SLC9A3	SLC36A1	SLC7A6	SLC38A2	SLC6A20	SLC9A3	SLC36A1
r	0.652**	0.680**	0.258	0.329*	0.630**	0.576**	0.729**	0.572**	0.249	0.338*	0.544**	0.643**	0.345*	0.483**	0.661**
P	<0.001	<0.001	0.059	0.024	<0.001	<0.001	<0.001	<0.001	0.075	0.012	<0.001	<0.001	0.012	0.001	<0.001

SLC7A6 Solute carrier family 7 member 6, *SLC38A2* Solute carrier family 38 member 2, *SLC6A20* Solute carrier family 6 member 20, *SLC9A3* Solute carrier family 9 member 3, *SLC36A1* Solute carrier family 36 member 1, *r* stands for Pearson correlation coefficient, *P* stands for significance (two-tailed)

** Represents highly significant correlation (*P* < 0.01)

* Indicates significant correlation (*P* < 0.05)

with previous studies on New Zealand white rabbits [33]. This may be due to the presence of Bet transport carriers in the liver, which promote the accumulation of Bet in hepatocytes, allowing it to exert antioxidant effects and alleviate liver damage [34]. It is shown that Bet can maintain the integrity of liver function in growing rabbits to some extent.

Prolonged exposure of animals to high temperatures enhances the expression of HSP in vivo [5], with HSP70 being the most extensive in the regulatory mechanisms of summer heat [35]. Numerous studies have found that the addition of different doses of Bet to the diets of goats [36], broilers [13], and beef cattle [37] under summer heat conditions reduced the serum levels of HSP70 to varying degrees, consistent with the results obtained in the present experiment. This may be due to the fact that Bet has a function similar to that of a heat shock protein molecular chaperone, which prevents stress-induced protein denaturation, thus stabilising protein folding and alleviating heat stress in growing rabbits to some extent [38].

VH and CD of the intestine are important for maintaining the normal morphology of the small intestine, normal nutrient absorption, and preventing bacterial translocation from the intestine [39], and small intestinal V/C reflects the developmental status of the intestine and its ability to digest and absorb nutrients [40, 41]. The results of this experiment showed that the addition of Bet to the diet increased jejunal VH and V/C and ileal V/C in growing rabbits under summer heat. These results are similar to previous studies in poultry [42] and rats [43]. Bet can maintain the stability of the intestinal morphological structure by preventing cellular water loss [44]. However, this experiment found that Bet significantly increased jejunal CD in growing rabbits under summer heat, contrary to the results of previous studies in poultry [45] and rats [43]. The increase in CD indicates poorer enterocyte maturation, possibly due to Bet promoting the production of new enterocytes, but due to the lack of data relating to the effect of Bet on the morphology of the intestine in growing rabbits under summer heat, this needs to be further investigated.

The sIgA is the main antibody of intestinal mucosal immunity and plays an important role in the clearance of pathogens and harmful substances, maintenance of intestinal environmental homeostasis and intestinal mucosal immunity. TNF-α is mainly produced by macrophages and monocytes, has pro-inflammatory properties and plays an important role in apoptosis, cell proliferation and immune response [46]. IL-4, as an anti-inflammatory factor, has a role in downregulating inflammatory response and antagonising inflammatory mediators [47]. In this study, Bet not only increased the level of sIgA in the small intestinal mucosa, but also up-regulated the gene expression of *IL-4* and down-regulated the gene expression of *TNF-α*, enhancing the immune function of the intestine. Alhotan et al. found that sIgA levels in the jejunum of broiler chickens were also significantly increased compared to the control group when supplemented with 1.0 g/kg Bet in high temperature-stressed broilers [13]. Sun et al. supplemented 3.2 g/kg Bet in grass carp and found that Bet could down-regulate the expression of the *TNF-α* gene and reduce the incidence of enteritis in the fish [48]. We also showed that Bet could improve intestinal immune function by increasing the content of intestinal immunoglobulins and regulating the expression of inflammatory factors to resist the damage caused by summer heat in growing rabbits.

The amino acid transporters SNAT2, IMINO, y+LAT2 and PAT1 that can transport Bet exist in the intestinal tract of animals [49–51], and are encoded by *SLC* gene family *SLC38A2*, *SLC6A20*, *SLC7A6* and *SLC36A1*, respectively. SLC38A2 can be used as a positive regulator of mTOR to participate in protein synthesis and amino acid uptake [18, 52]. After mTOR is activated, it can down-regulate the gene expression of pro-inflammatory factors by activating the phosphorylation state of downstream signalling factors 4EBP-2 and P70S6k1, and alleviate intestinal inflammation [53]. The results of this study indicate that dietary Bet supplementation can significantly up-regulate the gene expression of *SLC7A6*, *SLC6A20*, *SLC38A2*, *mTOR*, *4EBP-2*, and *P70S6k1* in the intestine of growing rabbits under summer heat. Correlation analysis showed that the expression of *mTOR* gene in the intestine of growing rabbits was significantly positively correlated with the expression of Bet transporters *SLC7A6*, *SLC38A2*, *SLC6A20* and *SLC36A1* genes in the intestine, and was also significantly positively correlated with the anti-inflammatory factor *IL-4* in the intestine. These results indicate that Bet can up-regulate the gene expression of tight junction proteins and anti-inflammatory factors in the intestine of growing rabbits through AAT/mTOR pathway, reduce intestinal inflammation induced by summer heat, and maintain intestinal health. This is the first time a study has confirmed this previous hypothesis [54]. However, this study found that *mTOR* gene expression had no significant effect on the gene expression of pro-inflammatory factors *TNF-α* and *IL-2*, suggesting that *mTOR* mainly plays an anti-inflammatory role by up-regulating the expression of anti-inflammatory factors.

Conclusion

Dietary Bet supplementation in growing rabbits under summer heat improved intestinal morphology, and also up-regulate the expression of anti-inflammatory factors through the AAT/mTOR pathway, improved intestinal immune function, improved intestinal health, and alleviated the intestinal damage caused by summer heat, with the best results achieved when the amount of Bet was 2.0 g/kg. These results provide a theoretical basis for future research on the effects of dietary betaine addition on different physiological stages of rabbits.

Abbreviations

AAT	Amino acid transporters
ADFI	Average daily feed intake
ADG	Average daily gain
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BD	Basal diet
Bet	Betaine
BW	Body weight
CD	Crypt depth
F/G	Feed to gain ratio
GLU	Glucose
HSP70	Heat shock protein 70
IL-2	Interleukin-2
IL-4	Interleukin-4
mTOR	Mammalian target of rapamycin
P70S6k1	P70 ribosomal protein S6 kinase 1
sIgA	Secretory immunoglobulin A
SLC7A6	Solute carrier family 7, member 6
SLC38A2	Solute carrier family 38, member 2
SLC6A20	Solute carrier family 6, member 20
SLC9A3	Solute carrier family 9, member 3
SLC36A1	Solute carrier family 36, member 1
THI	Temperature humidity index
TNF-α	Tumor necrosis factor-alpha
VH	Villus height
V/C	Villus height to crypt depth ratio
4EBP-2	Eukaryotic initiation factor 4E-binding protein-2

Availability of data and materials
All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
All experimental animal procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University (approval number: SICAU20220703). The guidelines for the Care and Use of Laboratory Animals of the National Research Council were complied with.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

References

1. Zeferino CP, Komiyama CM, Fernandes S, Sartori JR, Teixeira PSS, Moura ASAMT. Carcass and meat quality traits of rabbits under heat stress. Animal. 2013;7:518–23. https://doi.org/10.1017/S1751731112001838.
2. Ismail RFSA, Hassan MAE, Moustafa M, Al-Shehri M, Alazragi RS, Khojah H, et al. The influence of a nanoemulsion of cardamom essential oil on the growth performance, feed utilization, carcass characteristics, and health status of growing rabbits under a high ambient temperature. Animals (Basel). 2023;13:2990. https://doi.org/10.3390/ani13182990.
3. El-Raghi AA, Hassan MAE, Hashem NM, Abdelnour SA. Struggling thermal stress impacts on growth performance and health status of newly weaned rabbits using nanoemulsion of origanum majorana considering the economic efficiency of supplementation. Animals (Basel). 2023;13:1772. https://doi.org/10.3390/ani13111772.
4. Marai IFM, Habeeb AAM, Gad AE. Rabbits' productive, reproductive and physiological performance traits as affected by heat stress: a review. Livest Prod Sci. 2002;78:71–90. https://doi.org/10.1016/S0301-6226(02)00091-X.
5. Lian P, Braber S, Garssen J, Wichers HJ, Folkerts G, Fink-Gremmels J, et al. Beyond heat stress: intestinal integrity disruption and mechanism-based intervention strategies. Nutrients. 2020;12:734. https://doi.org/10.3390/nu12030734.
6. Abdelnour SA, Al-Gabri NA, Hashem NM, Gonzalez-Bulnes A. Supplementation with proline improves haemato-biochemical and reproductive indicators in male rabbits affected by environmental heat-stress. Animals (Basel). 2021;11:373. https://doi.org/10.3390/ani11020373.
7. Abdelnour SA, Swelum AA, Salama A, Al-Ghadi MQ, Qattan SYA, Abd El-Hack ME, et al. The beneficial impacts of dietary phycocyanin supplementation on growing rabbits under high ambient temperature. Ital J Anim Sci. 2020;19:1046–56. https://doi.org/10.1080/1828051X.2020.1815598.
8. Saghir SAM, Al Hroob AM, Majrashi KA, Jaber FA, Abduh MS, Al-Gabri N, et al. Effects of alginates on the growth, haematological, immunity, antioxidant and pro-inflammatory responses of rabbits under high temperature. Res Vet Sci. 2023;155:36–43. https://doi.org/10.1016/j.rvsc.2023.01.002.
9. Zaki A, Jiang S, Zaghloul S, El-Rayes TK, Saleh AA, Azzam MM, et al. Betaine as an alternative feed additive to choline and its effect on performance, blood parameters, and egg quality in laying hens rations. Poult Sci. 2023;102:102710. https://doi.org/10.1016/j.psj.2023.102710.
10. Shah AM, Ma J, Wang Z, Zou H, Hu R, Peng Q. Betaine supplementation improves the production performance, rumen fermentation, and antioxidant profile of dairy cows in heat stress. Animals (Basel). 2020;10:634. https://doi.org/10.3390/ani10040634.
11. Liu W-C, Guo Y, An L-L, Zhao Z-H. Protective effects of dietary betaine on intestinal barrier function and cecal microbial community in indigenous broiler chickens exposed to high temperature environment. Environ Sci Pollut Res Int. 2021;28:10860–71. https://doi.org/10.1007/s11356-020-11326-6.
12. Akhavan-Salamat H, Ghasemi HA. Alleviation of chronic heat stress in broilers by dietary supplementation of betaine and turmeric rhizome powder: dynamics of performance, leukocyte profile, humoral immunity, and antioxidant status. Trop Anim Health Prod. 2016;48:181–8. https://doi.org/10.1007/s11250-015-0941-1.
13. Alhotan RA, Al Sulaiman AR, Alharthi AS, Abudabos AM. Protective influence of betaine on intestinal health by regulating inflammation and improving barrier function in broilers under heat stress. Poult Sci. 2021;100:101337. https://doi.org/10.1016/j.psj.2021.101337.

14. Bond P. Regulation of mTORC1 by growth factors, energy status, amino acids and mechanical stimuli at a glance. J Int Soc Sports Nutr. 2016;13:8. https://doi.org/10.1186/s12970-016-0118-y.

15. Fu L, Zhang L, Liu L, Yang H, Zhou P, Song F, et al. Effect of heat stress on bovine mammary cellular metabolites and gene transcription related to amino acid metabolism, amino acid transportation and mammalian target of rapamycin (mTOR) signaling. Animals (Basel). 2021;11:3153. https://doi.org/10.3390/ani11113153.

16. Fritsch SD, Weichhart T. Metabolic and immunologic control of intestinal cell function by mTOR. Int Immunol. 2020;32:455–65. https://doi.org/10.1093/intimm/dxaa015.

17. He Y, Zhao S, Luo C, Zhang N, Wang J. Research progress on animal amino acid transporter. China Anim Husb Vet Med. 2020;47:744–53. https://doi.org/10.16431/j.cnki.1671-7236.2020.03.012.

18. Rosario FJ, Kanai Y, Powell TL, Jansson T. Mammalian target of rapamycin signalling modulates amino acid uptake by regulating transporter cell surface abundance in primary human trophoblast cells. J Physiol. 2013;591:609–25. https://doi.org/10.1113/jphysiol.2012.238014.

19. Cai Y, Deng M, Zhang Q, Liu Z, Wang L, Sheng W, et al. Effects of dietary betaine supplementation on biochemical parameters of blood and testicular oxidative stress in Hu sheep. Theriogenology. 2021;164:65–73. https://doi.org/10.1016/j.theriogenology.2021.01.006.

20. De Blas C, Mateos GG. Nutrition of the rabbit. 3rd ed. Wallingford: CAB International; 2020.

21. El-Ratel IT, Tag El-Din TE-DH, Bedier MM. Beneficial effects of curcumin as a native or nanoparticles form on productive efficiency, liver and kidney functions, antioxidative status and immunity of heat-stressed growing rabbits. J Anim Physiol Anim Nutr (Berl). 2020;104:1778–87. https://doi.org/10.1111/jpn.13420.

22. El-Ratel IT, Attia KAH, El-Raghi AA, Fouda SF. Relief of the negative effects of heat stress on semen quality, reproductive efficiency and oxidative capacity of rabbit bucks using different natural antioxidants. Anim Biosci. 2021;34:844–54. https://doi.org/10.5713/ajas.20.0258.

23. Arveux P, Bourdillon A, Briens C, Corrent E, Duperray J, Gidenne T, et al. Impact of replacing starch by digestible fibre, at two levels of lignocellulose, on digestion, growth and digestive health of the rabbit. Anim Sci. 2004;78:389–98. https://doi.org/10.1017/S1357729800058793.

24. Hassan RA, Ebeid TA, El-Lateif AIA, Ismail NB. Effect of dietary betaine supplementation on growth, carcass and immunity of New Zealand White rabbits under high ambient temperature. Livest Sci. 2011;135:103–9. https://doi.org/10.1016/j.livsci.2010.06.132.

25. Ondruska L, Rafay J, Okab AB, Ayoub MA, Supuka P. Influence of elevated ambient temperature upon some physiological measurements of New Zealand White rabbits. Vet Med (Praha). 2011;56:180–6. https://doi.org/10.3906/vet-1006-365.

26. Obeid R. The metabolic burden of methyl donor deficiency with focus on the betaine homocysteine methyltransferase pathway. Nutrients. 2013;5:3481–95. https://doi.org/10.3390/nu5093481.

27. Abbas Z, Sammad A, Hu L, Fang H, Xu Q, Wang Y. Glucose metabolism and dynamics of facilitative glucose transporters (GLUTs) under the influence of heat stress in dairy cattle. Metabolites. 2020;10:312. https://doi.org/10.3390/metabo10080312.

28. Garriga C, Hunter RR, Amat C, Planas JM, Mitchell MA, Moretó M. Heat stress increases apical glucose transport in the chicken jejunum. Am J Physiol Regul Integr Comp Physiol. 2006;290:R195–201. https://doi.org/10.1152/ajpregu.00393.2005.

29. Pearce SC, Mani V, Boddicker RL, Johnson JS, Weber TE, Ross JW, et al. Heat stress reduces intestinal barrier integrity and favors intestinal glucose transport in growing pigs. PLoS One. 2013;8:e70215. https://doi.org/10.1371/journal.pone.0070215.

30. Daader AH, Al-Sagheer AA, Gabr HA, El-Moniem EA. Alleviation of heat-stress-related physiological perturbations in growing rabbits using natural antioxidants. Span J Agric Res. 2018;16:e0610. https://doi.org/10.5424/sjar/2018163-13184.

31. Yeo EJ, Wagner C. Tissue distribution of glycine N-methyltransferase, a major folate-binding protein of liver. Proc Natl Acad Sci U S A. 1994;91:210–4. https://doi.org/10.1073/pnas.91.1.210.

32. Wen C, Leng Z, Chen Y, Ding L, Wang T, Zhou Y. Betaine alleviates heat stress-induced hepatic and mitochondrial oxidative damage in broilers. J Poult Sci. 2021;58:103–9. https://doi.org/10.2141/jpsa.0200003.

33. El-Azeem AEA, Al-Sagheer AA, Daader AH, Bassiony SM. Effect of dietary supplementation with betaine, thyme oil and their mixtures on productive performance of growing rabbits. ZJAR. 2019;46:815–28. https://doi.org/10.21608/ZJAR.2019.40971.

34. Kettunen H, Peuranen S, Tiihonen K. Betaine aids in the osmoregulation of duodenal epithelium of broiler chicks, and affects the movement of water across the small intestinal epithelium in vitro. Comp Biochem Physiol A Mol Integr Physiol. 2001;129:595–603. https://doi.org/10.1016/s1095-6433(01)00298-7.

35. Kang D, Shim K. Early heat exposure effect on the heat shock proteins in broilers under acute heat stress. Poult Sci. 2021;100:100964. https://doi.org/10.1016/j.psj.2020.12.061.

36. Dangi SS, Dangi SK, Chouhan VS, Verma MR, Kumar P, Singh G, et al. Modulatory effect of betaine on expression dynamics of HSPs during heat stress acclimation in goat (*Capra hircus*). Gene. 2016;575:543–50. https://doi.org/10.1016/j.gene.2015.09.031.

37. Craig SAS. Betaine in human nutrition. Am J Clin Nutr. 2004;80:539–49. https://doi.org/10.1093/ajcn/80.3.539.

38. Willingham BD, Ragland TJ, Ormsbee MJ. Betaine supplementation may improve heat tolerance: potential mechanisms in humans. Nutrients. 2020;12:2939. https://doi.org/10.3390/nu12102939.

39. Quinteiro-Filho WM, Ribeiro A, Ferraz-de-Paula V, Pinheiro ML, Sakai M, Sá LRM, et al. Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. Poult Sci. 2010;89:1905–14. https://doi.org/10.3382/ps.2010-00812.

40. Farahat M, Ibrahim D, Kishawy ATY, Abdallah HM, Hernandez-Santana A, Attia G. Effect of cereal type and plant extract addition on the growth performance, intestinal morphology, caecal microflora, and gut barriers gene expression of broiler chickens. Animal. 2021;15:100056. https://doi.org/10.1016/j.animal.2020.100056.

41. Kiela PR, Ghishan FK. Physiology of intestinal absorption and secretion. Best Pract Res Clin Gastroenterol. 2016;30:145–59. https://doi.org/10.1016/j.bpg.2016.02.007.

42. Santos TTD, Dassi SC, Franco CRC, Costa CRVd, Lee SA, Fisher da Silva AV. Influence of fibre and betaine on development of the gastrointestinal tract of broilers between hatch and 14 d of age. Anim Nutr. 2019;5:163–73. https://doi.org/10.1016/j.aninu.2018.06.005.

43. Wang H, Li S, Fang S, Yang X, Feng J. Betaine improves intestinal functions by enhancing digestive enzymes, ameliorating intestinal morphology, and enriching intestinal microbiota in high-salt stressed rats. Nutrients. 2018;10:907. https://doi.org/10.3390/nu10070907.

44. Ratriyanto A, Mosenthin R. Osmoregulatory function of betaine in alleviating heat stress in poultry. J Anim Physiol Anim Nutr (Berl). 2018;102:1634–50. https://doi.org/10.1111/jpn.12990.

45. Norouzian H, Alirezaei M, Dezfoulian O, Taati M. The effects of post-hatch feeding with betaine on the intestinal development of broiler chickens. Rev Bras Cienc Avic. 2018;20:403–12. https://doi.org/10.1590/1806-9061-2017-0468.

46. Song Y, Chen R, Yang M, Liu Q, Zhou Y, Zhuang S. Dietary betaine supplementation improves growth performance, digestive function, intestinal integrity, immunity, and antioxidant capacity of yellow-feathered broilers. Ital J Anim Sci. 2021;20:1575–86. https://doi.org/10.1080/1828051X.2021.1986681.

47. Turner JR. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol. 2009;9:799–809. https://doi.org/10.1038/nri2653.

48. Sun H, Jiang WD, Wu P, Liu Y, Feng L. Betaine supplementations enhance the intestinal immunity of on-growing grass carp (*Ctenopharyngodon idella*): partly related to TOR and NF-κB signaling pathways. Aquaculture. 2019;518:734846. https://doi.org/10.1016/j.aquaculture.2019.734846.

49. Li G, Li J, Tan B, Wang J, Kong X, Guan G, et al. Characterization and regulation of the amino acid transporter SNAT2 in the small intestine of piglets. PLoS One. 2015;10:e0128207. https://doi.org/10.1371/journal.pone.0128207.

50. Corbett HE, Dubé CD, Slow S, Lever M, Trasler JM, Baltz JM. Uptake of betaine into mouse cumulus-oocyte complexes via the SLC7A6 isoform of y+L transporter. Biol Reprod. 2014;90:81. https://doi.org/10.1095/biolreprod.113.116939.

51. Yin L, Li J, Wang H, Yi Z, Wang L, Zhang S, et al. Effects of vitamin B6 on the growth performance, intestinal morphology, and gene expression in weaned piglets that are fed a low-protein diet1. J Anim Sci. 2020;98:skaa022. https://doi.org/10.1093/jas/skaa022.

52. Menchini RJ, Chaudhry FA. Multifaceted regulation of the system A transporter Slc38a2 suggests nanoscale regulation of amino acid metabolism

and cellular signaling. Neuropharmacology. 2019;161:107789. https://doi.org/10.1016/j.neuropharm.2019.107789.

53. Shao Y, Wolf PG, Guo S, Guo Y, Gaskins HR, Zhang B. Zinc enhances intestinal epithelial barrier function through the PI3K/AKT/mTOR signaling pathway in Caco-2 cells. J Nutr Biochem. 2017;43:18–26. https://doi.org/10.1016/j.jnutbio.2017.01.013.

54. Pinilla J, Aledo JC, Cwiklinski E, Hyde R, Taylor PM, Hundal HS. SNAT2 transceptor signalling via mTOR: a role in cell growth and proliferation? Front Biosci. 2011;3:1289–99. https://doi.org/10.2741/e332.