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Effect of tetramethylpyrazine on growth performance, *Campylobacter jejuni* carriage and endogenous antimicrobial peptides in rabbits

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Abstract: The present study aimed to investigate the effect of tetramethylpyrazine (TMP) supplementation on growth performance, *Campylobacter jejuni* (*C. jejuni*) carriage and antimicrobial peptides in the epithelial tissue of caecum and skin in rabbits. Five treatments included control and *C. jejuni* challenge with the addition of TMP at 0, 50, 100 or 150 mg/kg of diet. The trial lasted for 35 days and *C. jejuni* challenge occurred on first day of feeding trial. The results showed that *C. jejuni* challenge worsened ($P < 0.05$) feed intake, body weight gain and feed efficiency, whereas TMP supplementation partially compensated ($P < 0.05$) growth performance. *C. jejuni* populations in the caecal content and on the skin were decreased ($P < 0.05$) in the treatments containing TMP. The mRNA levels of antimicrobial peptides, including defensin neutrophil peptide 4, macrophage cationic peptide 2, galectin 3 and cathelicidin were also decreased ($P < 0.05$) by *C. jejuni* challenge while they were increased ($P < 0.05$) with supplemental TMP. Linear and quadratic trends ($P \leq 0.012$) of the three doses of TMP were found in growth performance, linear trends ($P \leq 0.049$) in *C. jejuni* carriage, and linear and quadratic trends ($P \leq 0.012$) in galectin 3. The results suggest that TMP can partially protect from *C. jejuni* infection by decreasing *C. jejuni* carriage and activating epithelial antimicrobial peptides.

Keywords: body weight gain; caecum; feed intake; gene expression; skin

Campylobacter jejuni (*C. jejuni*) infection is one of the most widespread infectious diseases in both developed and developing countries (Kaakoush et al. 2015). The main natural reservoir of the bacterium is poultry, and humans can contract the disease from eating contaminated food (Laukova et al. 2017). Another source of campylobacteriosis is contact with infected animals, which often carry *C. jejuni* asymptomatically. Most livestock species including rabbits are also susceptible groups. An investigation in Italy found that a total of 36 out of 39 farmed rabbits and all the 13 farms resulted positive for *Campylobacter* species including *C. jejuni* (Revez et al. 2008). Furthermore, the incidence and prevalence of campylobacteriosis are being exacerbated by the ban of antibiotics for non-therapeutic reasons for farmed animals in some countries (Rukambile et al. 2019). Additionally, with the increasing trend of health awareness, production of rabbit meat has been growing worldwide due to its low levels of fat, cholesterol and sodium, but high levels of protein (Dalle Zotte and Szendro 2011; Cullere and Dalle Zotte 2018).

Tetramethylpyrazine (TMP), also known as ligustrazine, a chemical compound abundant in natto and fermented cocoa beans, has been found in recent studies to be effective against oxidation, inflammation and bacteria (Xu et al. 2011; Donkor et al. 2016; Wang et al. 2019b). TMP reduced toxicity and liver oxidative stress in chickens with tibial dyschondroplasia, inhibited inflammatory

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cytokine expression in hepatic stellate cells, and attenuated pro-inflammatory mediators in rats (Kim et al. 2014; Mehmood et al. 2018). Moreover, TMP showed antimicrobial activity against *Salmonella, Escherichia coli, Clostridium perfringens* and Gram-negative bacteria in the intestine of broilers (Liu et al. 2018, 2019). In addition, Vieira et al. (2007) found that the pyrazine compounds reduced populations of *Staphylococcus aureus* and *Staphylococcus agalactiae*. Furthermore, TMP regulated the activity of critical proteins or peptides, such as hepcidin, nuclear factor kappa-light-chain-enhancer of activated B cells and glutathione in animals (Kim et al. 2014; Liu et al. 2018; Zhang et al. 2019).

The effect of TMP on protecting against *Campylobacter* infection and antimicrobial peptides in farm animals remains untested, however. The present study aimed to investigate the effect of TMP supplementation on growth performance, *C. jejuni* carriage and antimicrobial peptides in growing Rex rabbits challenged with *C. jejuni*.

MATERIAL AND METHODS

Tetramethylpyrazine, treatment and diet. TMP (2,3,5,6-tetramethylpyrazine, purity: ≥ 99%) was purchased from the Deruifeng commercial company (Jinan, China). Treatments included a control (basal) diet, *C. jejuni* challenge plus TMP at 0, 50, 100 or 150 mg/kg of diet. The analysed TMP levels for the last three treatments were 53, 98 and 149 mg/kg, respectively. Nutrition levels of basal diet were recommended by the China Agricultural Standard for Farm Rex Rabbits (NY/T2765-2015). All diets were isonitrogenous and isocaloric and fed as pellets (cold formed; diameter × length, 3.5 × 8.0 mm). No antibiotics were offered to rabbits via either feed or water throughout the trial. The formulation and nutrition levels of basal diet are listed in Table 1.

Animal management. The trial protocol was approved by the Institutional Committee for Animal Use and Ethics of the College of Animal Science of the Henan University of Science and Technology (Luoyang, Henan, China).

One hundred and fifty male Rex rabbits (weaned at 30 days of age) at approximately 35 days of age with the initial body weight of 753 ± 1.43 g (mean ± SEM) were randomly assigned to 5 treatments containing 6 replicates per treatment and 5 rabbits per replicate. Before a feeding trial, rectal swabs from all rabbits were negative for *C. jejuni*, and then all rabbits were individually raised in stainless steel cages (length × width × height, 35 × 45 × 40 cm) and had free access to diets and water (Wang et al. 2019a). The feeding trial lasted for 35 days. Rabbits and feeds in each replicate were weighed at 35 and 70 days of age and average daily feed intake (ADFI), average daily body weight gain (ADG) and feed efficiency (gain/feed) were calculated. All rabbits were monitored for general health at least twice a day.

Table 1. Ingredient and nutrition levels in the basal diet[1] (as fed basis)

Ingredient	Content (%)	Calculated composition	Content (%)
Corn	20.0	crude protein	17.08
Soybean meal	8.0	digestible energy (MJ/kg)	10.84
Brewers dried grain	12.0	crude fibre	13.78
Corn DDGS	8.0	lysine	0.70
Alfalfa meal	33.0	methionine + cysteine	0.54
Wheat bran	15.0	Ca	1.00
Dicalcium phosphate	1.5	P	0.58
Premix[2]	2.5	non-phytate P	0.35

DDGS = distillers dried grains with solubles

[1]calculated by Chinese Feed Database (Version 25, 2014)

[2]premix provided the following per kg of diets: vitamin A 12 000 IU, vitamin D 2000 IU, vitamin E 30 IU, Cu 12 mg, Fe 64 mg, Mn 56 mg, Zn 60 mg, I 1.2 mg, Se 0.4 mg, Co 0.4 mg, NaCl 6.4 g

C. jejuni challenge. The strain of *C. jejuni* (ATCC 33291) was obtained from China Microbiological Culture Collection Center (Beijing, China). The strain was grown at 40°C for 48 h in Bolton broth (HB0273-2; Qingdao Hopebio Co., Ltd., China) in an incubator containing 5% O_2, 10% CO_2 and 85% N_2. On the first day of feeding trial, each rabbit in *C. jejuni* challenged treatments was orally administrated 1 ml of 10^8 count of colony forming units (CFU) of *C. jejuni*, while rabbits in the control received the same liquid without *C. jejuni* strain.

Sample collection. At the end of feeding trial, all rabbits were weighed, euthanized by CO_2, and then dissected. Caecal content (approximately 5 g) was collected and stored at –40°C for *C. jejuni* analysis. The digesta were prepared as described by Liu et

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al. (2010). Liver (approximately 6 g), whole spleen, and neck skin (2 × 2 cm) located at the third and fourth cervical vertebra were collected and stored in halves, one at –40°C for bacterial enumeration, the other in RNAlater solution (TaKaRa, China) for the analysis of gene expression. Distal part (2 cm) of the dissected caecum was collected and flushed with phosphate-buffered saline (PBS, 0–4°C, 0.03 mol/l, pH 7.2–7.4), and then it was immersed in RNAlater solution for gene quantification.

Bacterial enumeration. Each caecal content or tissue aforementioned was homogenized, weighed, and diluted at 1 : 10 (wt/vol) with PBS and mixed thoroughly (Fei et al. 2018). The suspension of each sample was serially diluted between 10^{-1} to 10^{-7} dilutions, and 100 µl of each diluted sample (10–100 CFU) was spread onto a duplicate Bolton broth medium containing 1.8% agar at microaerobic condition, 42 ± 1°C for 24 h. The amount of bacteria was expressed as a logarithmic ($\log_{10}$) transformation per gram of sample.

TMP and gene quantification. The concentrations of TMP in the products and diets were determined according to the Chinese Standard of Light Industrial Manufacturing (QB/T 2748-2012). For qRT-PCR analysis, reagents, primer synthesis (Table 2) and cDNA sequencing were provided by TaKaRa. The transcriptional profiles of target genes were expressed as the relative expression to a housekeeping gene ($2^{-\Delta\Delta Ct}$; Livak and Schmittgen 2001). The qPCR reactions were set at 10 µl with 5 µl of SYBR® Green PCR Master Mix (Thermo Fisher Scientific, USA), 1 µl of primer, 4 µl of 10× diluted cDNA. All qPCRs were run in triplicates in the same thermal cycles (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min) on the ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). No amplification signal was detected in water or no-RT RNA samples.

Statistical analysis. Data are presented as mean and standard error of the mean (SEM) using SPSS software (IBM SPSS, Version 23). Differences between mean values of normally distributed data were assessed by one-way ANOVA (Tukey's *b* test) at $P < 0.05$ level of significance, and Tamhane's T2 test for parameters with heterogeneity variance. The average mean of 5 rabbits per replicate was the statistical unit for growth performance, *C. jejuni* colonization and antimicrobial peptides. The trend of TMP doses at 50, 100 and 150 mg/kg for each experimental parameter was analysed using contrasts of linear and quadratic polynomials.

RESULTS AND DISCUSSION

Growth performance. In the present study, there was no mortality for all replicates throughout the feeding trial, but ADFI, ADG and gain/feed were worsened ($P < 0.05$) by *C. jejuni* challenge without TMP (Table 3), indicating that rabbits suffered from subclinical campylobacteriosis. It has been noted that livestock is a natural source and reservoir for *C. jejuni,* in general, asymptomatic and chronic carriage post infection, but it is very likely to infect the carcass in a slaughtering process and subsequently humans (Bednarski et al. 2011). The decreased growth performance by *C. jejuni* challenge in the present study was supported by the finding that *C. jejuni* colonization increased the intracellular calcium level, impaired nutrient absorption and expression of nutrient transporter genes in the chicken intestinal epithelium (Awad et al. 2014, 2015b). Additionally, a decrease in the intestinal epithelial barrier function by *C. jejuni*

Table 2. Information on primers for quantitative real-time PCR

Items	GenBank Accession No.	Primers (5'-3')		Length (bp)
		forward	reverse	
NP4	NM_001082299.1	CTCAGGGATGGCTGATGACG	TCCCCAAACCCACAACTGAA	162
MCP2	M28073.1	TAGGTGAGAGATGCAGCACG	GTCTTTGCAGAACGTGCCAG	177
LGALS3	NM_001082338.1	CTGCCTGTGCCTTATGACCT	TGTTCTCATTGAAGCGGGGG	160
CAMP	NM_001082305.1	ATGGAGACCCATAAGCACGGA	ATGCTCAGGAGGCGGTAGAG	179
GAPDH	NM_001082253.1	AGACACGATGGTGAAGGTCG	TGCCGTGGGTGGAATCATAC	164

CAMP = cathelicidin antimicrobial peptide, *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase, *LGALS3* = galectin 3, *MCP2* = macrophage cationic peptide 2, *NP4* = defensin neutrophil peptide 4

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colonization also contributed to the worsened growth performance (Awad et al. 2015a).

Importantly, ADFI, ADG and gain/feed were reversely influenced ($P < 0.05$) by TMP supplementation. Some parameters reached the level of control treatment, and linear and quadratic trends ($P \leq 0.012$) of TMP doses were found. Those findings indicate that TMP is capable of promoting the growth of rabbits challenged with *C. jejuni*. Previous studies showed that TMP added at 150 mg/kg did not influence ADFI and feed efficiency, but increased ADG of broilers, while using a necrotic enteritis or salmonellosis model the effect of TMP on ADFI, ADG and feed efficiency was more pronounced (Liu et al. 2018, 2019). As known, TMP is one of the components of some herbal medicines, and more focuses were on its medical values in humans (Chen et al. 2017; Michel et al. 2017). Information about TMP as an additive to replace antibiotics is very limited. Anyway, more studies are needed to certify its function and safety as a growth-promoting additive in farm animals.

C. jejuni carriage. It is known that *C. jejuni* is capable of systemic invasion in the rabbit, and develops a diarrhoea symptom (Shang et al. 2016). Importantly, in the present study, the treatments containing TMP showed lower ($P < 0.05$) *C. jejuni* carriage, compared to the challenge group (Table 3), but did not reach ($P < 0.05$) the levels of control treatment, indicating that TMP partially inhibited *C. jejuni* proliferation. Furthermore, the dose effect of TMP on reducing *C. jejuni* colonization in the caecum exhibited a linear trend ($P = 0.049$), and on the skin a similar trend ($P = 0.003$) was found. With the increase in TMP dose, *C. jejuni* counts in the liver and spleen were linearly ($P < 0.001$) and quadratically ($P \leq 0.026$) reduced, also implying the effectiveness of TMP on blocking *C. jejuni* transmission. Liu et al. (2018) reported that the populations of intestinal *Clostridium perfringens, Escherichia coli, Salmonella* and Gram-negative bacteria were decreased by TMP, and enterotoxicity, anti-oxidation and anti-inflammation were also beneficially modified by TMP through modulating endotoxins, lipid and protein oxidation, glutathione turnover and inflammatory factors in broilers. Additionally, dietary TMP decreased *Salmonella* transmission from intestinal to mesenteric lymphoid follicles, liver, spleen and skin (Liu et al. 2019).

Epithelial antimicrobial peptides. In the caecum, the mRNA levels of antimicrobial peptides, including defensin neutrophil peptide 4 (*NP4*), macrophage cationic peptide 2 (*MCP2*), galectin 3 (*LGALS3*) and cathelicidin antimicrobial peptide (*CAMP*), were downregulated ($P < 0.05$) in the treatment with *C. jejuni* challenge (Table 4), and were upregulated ($P < 0.05$) with the addition of TMP at 50, 100 or 150 mg/kg, but they did not reach ($P < 0.05$) the levels of control treatment. Quadratic trends of TMP doses were found in *NP4* and *MCP2* ($P \leq 0.021$), linear and quadratic trends in *LGALS3* ($P \leq 0.021$), and a linear trend in *CAMP* ($P < 0.001$). Similar effects of experimental factors on those genes were found on the skin, but TMP at 100 and 150 mg/kg upregulated ($P < 0.05$) mRNA expression of *CAMP*

Table 3. Effect of tetramethylpyrazine supplementation on the growth performance and *Campylobacter jejuni* carriage of rabbits

Items	Control	*C. jejuni* challenge + TMP (mg/kg)				SEM	*P*-value	
		0	100	150	200		linear	quadratic
Growth performance								
ADFI (g)	66.54^{ab}	63.16^{d}	65.41^{bc}	65.22^{c}	66.96^{a}	0.314	0.001	0.010
ADG (g)	17.34^{a}	13.80^{d}	15.73^{c}	16.94^{ab}	16.74^{b}	0.127	< 0.001	0.001
Gain/feed	0.261^{a}	0.219^{d}	0.241^{c}	0.260^{a}	0.250^{b}	0.002	0.012	< 0.001
***C. jejuni* carriage** (CFU/g)								
Caecal content	0.66^{c}	7.33^{a}	4.89^{b}	4.82^{b}	4.57^{b}	0.106	0.049	0.526
Skin	0.20^{d}	2.16^{a}	1.76^{b}	1.64^{bc}	1.50^{c}	0.048	0.003	0.929
Liver	0.05^{d}	2.04^{a}	0.82^{b}	0.81^{b}	0.69^{c}	0.019	< 0.001	0.026
Spleen	0.06^{d}	1.40^{a}	0.85^{b}	0.67^{c}	0.66^{c}	0.020	< 0.001	0.001

ADFI = average daily feed intake, ADG = average daily gain, *C. jejuni* = *Campylobacter jejuni* challenged at first day of feeding trial, SEM = standard error of the mean, TMP = tetramethylpyrazine

$^{a–d}$means in a row not sharing a superscript were significantly different ($P < 0.05$)

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Table 4. Effect of tetramethylpyrazine supplementation on epithelial antimicrobial peptides of rabbits challenged with *Campylobacter jejuni*

Items	Control	*C. jejuni* challenge + TMP (mg/kg)			SEM	*P*-value	
		0	100	150	200		linear	quadratic
Caecum (mRNA, $2^{-\Delta\Delta Ct}$)								
NP4	3.00[a]	0.96[d]	1.49[c]	2.02[b]	1.42[c]	0.061	0.405	< 0.001
MCP2	4.42[a]	2.92[c]	3.19[b]	3.47[b]	3.09[bc]	0.101	0.536	0.021
LGALS3	6.76[a]	2.92[d]	3.66[c]	4.75[b]	5.04[b]	0.123	< 0.001	0.012
CAMP	2.28[a]	0.16[e]	1.50[d]	1.73[c]	2.06[b]	0.046	< 0.001	0.421
Skin (mRNA, $2^{-\Delta\Delta Ct}$)								
NP4	4.06[a]	1.02[c]	1.81[b]	1.84[b]	1.94[b]	0.087	0.260	0.688
MCP2	4.58[a]	2.35[c]	3.19[b]	3.17[b]	3.18[b]	0.100	0.929	0.907
LGALS3	3.22[a]	1.47[c]	1.69[c]	2.00[b]	2.11[b]	0.082	0.005	0.392
CAMP	1.91[a]	0.79[c]	1.56[b]	1.63[ab]	1.92[a]	0.086	0.010	0.303

CAMP = cathelicidin antimicrobial peptide, *LGALS3* = galectin 3, *MCP2* = macrophage cationic peptide 2, *NP4* = defensin neutrophil peptide 4, SEM = standard error of the mean, *C. jejuni* = *Campylobacter jejuni* challenged at first day of feeding trial, TMP = tetramethylpyrazine

[a–e]means in a row not sharing a superscript were significantly different ($P < 0.05$)

gene to the level of the control, and linear trends were observed in *LGALS3* and *CAMP* ($P \leq 0.010$).

Defensins, small cysteine-rich cationic proteins, are host defence peptides which are active against bacteria, fungi and viruses. Most defensins function by binding to the microbial cell membrane, and, once embedded, forming pore-like membrane defects that allow the efflux of essential ions and nutrients (Fruitwala et al. 2019; Hendrikx and Schnabl 2019). In the present study, defensin *NP4* was influenced by the TMP addition, indicating that *NP4* can be activated by TMP and collaboratively fight *C. jejuni*. Literature about a dietary factor influencing body defensins is unavailable. Besides their anti-microbial activity, accumulating data indicate that defensins have extended functions in human physiology (Suarez-Carmona et al. 2015). Therefore, how the dietary phytochemicals cooperate with body antimicrobial peptides affecting physiology is interesting for further studies.

Cationic peptides possess antimicrobial activity against many species, including bacteria, fungi and viruses (Hancock et al. 1995). A study showed that *MCP1* and *MCP2* enhanced the ability of rabbit alveolar macrophages to ingest *Staphylococcus aureus, Klebsiella pneumoniae, Bordetella bronchiseptica* and *Candida albicans in vitro* (Fleischmann et al. 1985). Similarly, *LGALS3* inhibited the growth of *Cryptococcus neoformans* and *Paracoccidioides brasiliensis* (Almeida et al. 2017). In addition, galectin-3 directed antimicrobial guanylate binding proteins to vacuoles furnished with bacterial secretion systems as a part of the coordinated host defence program (Feeley et al. 2017; Ayyappan et al. 2019). Cathelicidin *CAMP* is related to innate immune system and defensins. Studies reported that *CAMP* possessed effective antibacterial activities against multi-drug resistant *Acinetobacter baumanii* and *Klebsiella pneumoniae*. In the present study, the mRNA levels of *MCP2, LGALS3* and *CAMP* were upregulated by dietary TMP, implying that the activities of these genes can be induced by TMP, and then they may perform an independent or coordinated action with TMP against *C. jejuni*. Paradoxically, these genes showed lower activities in the presence of *C. jejuni* than in its absence. Furthermore, some more potent antimicrobial peptides can have a lytic activity on mammalian cells (Hancock et al. 1995). Obviously, the activation of antimicrobial peptides has its two sides for the host, which deserves further study.

CONCLUSION

Inclusion of TMP in the rabbit diet attenuated the negative effect of *C. jejuni* on ADFI, ADG and gain/feed, reduced *C. jejuni* carriage in the caecal content, liver, spleen and skin epithelium, and upregulated mRNA profiles of endogenous antimicrobial peptides, including *NP4*, *LGALS4*, *CAMP* and *MCP2* in the epithelial tissues of caecum and skin. The results

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illustrate that dietary TMP can promote growth by inhibiting colonization and transfer of *C. jejuni* as well as activating endogenous antimicrobial peptides.

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