Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X 2022 / v.24 / n.3 / 001-006

http://dx.doi.org/10.1590/1806-9061-2021-1524

Original Article

Effect of Dietary Tetramethylpyrazine on Egg Production, Nutrient Retention and Cecal Bacterial Diversity in Aged Laying Hens

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■Keywords

Alpha diversity; beta diversity; crude protein retention; energy retention; egg laying rate; egg mass.

Submitted: 27/September/2021
Approved: 25/November/2021

ABSTRACT

This study aimed to investigate the effect of tetramethylpyrazine (TMP) supplementation on egg production, nutrient retention and cecal microbiota diversity using 288 commercial Hy-Line brown hens as of wk 75 to 86. Four treatments consisted of TMP addition at 0 (control, basal diet), 100, 150 and 200 mg/kg of diet. The results showed that diets supplemented with TMP addition improved egg-laying rate as of wk 77 compared to the control, which led to an increase (p<0.001) of egg mass by 97-225 g/hen throughout the whole trial, and a linear increase (p=0.003) of egg mass to the incremental TMP doses was found. At wk 86, the apparent digestibilities of dry matter and crude protein were enhanced (p<0.05), exhibiting consistent linear increases ($p\leq$0.033) with the TMP doses. However, TMP did not cause alpha and beta diversity of cecal microbiota. The results suggest that TMP can be an additive to improve egg production and nutrient digestibility of aged laying hens.

INTRODUCTION

Gradually decreased egg production of aged laying hens accompanying a decline of profitability is the main reason for hens facing molting or being culled earlier than a normal physiological age (Aerni *et al.*, 2005; Gongruttananun *et al.*, 2017). In practice, the egg production period of hens, even the same genetic strain, always experience a tremendous variation from about 70 to 100 wk of age among layer enterprises, especially in developing countries, coupled with the feeding cost from pullet period, which causes a considerable profit difference (Altahat *et al.*, 2012; Sirajuddin *et al.*, 2019). However, this is also an opportunity for the improvement in feeding management. Indeed, some researchers have recently focused on improving egg production from aged laying hens via dietary manipulations including supplements (Ghasemian & Jahanian, 2016; Saleh *et al.*, 2019).

Tetramethylpyrazine is an alkaloid extracted from herbal Chuanxiong (*Ligusticum wallichii*) and also a bacterial metabolite rich in vinegar, nattō and fermented cocoa beans (Zhao *et al.*, 2016; Xiao *et al.*, 2018; Chagas Junior *et al.*, 2021). This compound has been credited effective against oxidation, inflammation and some pathogens in rodents and livestock (Zhao *et al.*, 2016; Liu *et al.*, 2018a; Ding *et al.*, 2019; Wang *et al.*, 2019). Also, TMP improved nutrient digestion, serological markers and native immunity of broilers (Shi *et al.*, 2022). As known, the rapid descent of egg-laying rate of aged hens is an integrated result by deteriorating physiological functions mainly in defense and consequent reproduction. Given the beneficial effects of TMP on body defense in rodents, rabbits and broilers, theoretically, it can also improve overall health and production performance of aged animals, but scientific literature is unavailable in this area. Additionally, dietary supplements also interact with gut microbiota, leading to an alteration to some extent of overall health and digestion. Whether TMP affects gut microbiota diversity is unclear in both the clinic and the animal.

The present study aims to test the hypothesis that TMP can beneficially influence egg production and gut microbiota of aged hens to counteract the rapid egg-laying decline by detecting egg-laying rate, egg mass, nutrient retention and cecal bacterial diversity.

MATERIALS AND METHODS

Ethics statement

The experimental protocol of the present study was approved by the Institutional Committee for Animal Use and Ethics of Henan University of Science and Technology (No. 2018022).

Tetramethylpyrazine and treatments

The TMP (2,3,5,6-Tetramethylpyrazine, purity: ≥ 98%) was obtained from Sigma-Aldrich (Shanghai, China). Treatments included a control (NC, basal diet) and TMP addition at 100 (100-TMP), 150 (150-TMP) and 200 (200-TMP) mg/kg of diet. Analyzed levels of 100-, 150- and 200-TMP were 48, 103 and 144 mg/kg, respectively. Nutrition levels of the basal diet (Table 1) were referred to the Hy-Line management manual and local practice with minor modulation. All diets were isocaloric and isonitrogenous and fed as mash on an air-dry basis. Titanium dioxide was used as an external marker to determine the apparent nutrient retention.

Animals and samples

A total of 288 commercial Hy-Line brown hens at 74 wks of age with similar egg production and body weights were randomly assigned to four dietary groups. Each treatment contained 6 replicates of 12 hens each in four continuous cages of three-layers and all replicates were uniformly distributed in a chicken house to minimize the environmental effect (Deng *et al.*, 2020a). All hens were supplied with feed at 108 g/hen/day and *ad libitum* water according to the management manual of Hy-Line brown strain in the house with auto-ventilation, lighting system at 16L/8D and the temperature at approximately 22°C. The feeding trial lasted from 75 to 86 wks of age. Feed intake, egg laying rate, egg mass and mortality per replicate were recorded daily.

At the last day of 78, 82 and 86 wks, feces per replicate were collected for the analysis of nutrient retention (Shi *et al.*, 2022). At the 86th wk, 8 hens (2 hens/cage) per replicate were randomly selected and suffocated by carbon dioxide, the cecal content was collected, pooled by replicate and stored (Deng *et al.*, 2020a) for the analysis of microbiota diversity.

Chemical and microbial analysis

The contents in dietary and excreta samples were determined according to AOAC methods (1990) for dry matter, P, Ca, crude protein, nitrogen, crude fat, gross energy and titanium dioxide (Liu *et al.*, 2018b). The contents of TMP in the products and diets were determined according to the Chinese Standard of Light Industrial Manufacturing (QB/T 2748-2012; Liu *et al.*, 2019).

For microbial analysis, the cecal content per hen was equally pooled by replicate and mixed. DNA extraction and PCR amplification of microbial samples were conducted as described by Wang *et al.* (2021) and Shi *et al.* (2021a). Briefly, the forward and reverse primer sequences containing illumina overhang

Table 1 – Composition and nutrient level of basal diet (air-dry basis, %).

Ingredient	Content	Nutrient	Content
Corn	68.0	Calculated level[2]	
Soybean meal	14.4	Metabolizable energy, Mcal/kg [2]	2.80
Corn gluten	3.5	Non-phytate phosphorus	0.34
DL-methionine (98.5%)	0.2	Methionine	0.44
L-lysine (98%)	0.2	Lysine	0.78
Salt	0.4	Sulfur-containing amino acids	0.67
Soybean oil	1.5	Analyzed level	
Di-calcium phosphate	1.0	Crude protein	14.83
Limestone	9.8	Calcium	3.75
Premix[1]	0.5	Phosphorus	0.51
Titanium dioxide	0.5		

[1]Provides (per kg of diet): vitamin A (retinyl acetate), 8,000 IU; cholecalciferol, 1,600 IU; vitamin E (DL-α-tocopheryl acetate), 5 IU; vitamin K, 0.5 mg; riboflavin, 2.5 mg; D-pantothenic acid, 2.2 mg; niacin, 20 mg; pyridoxine, 3.0 mg; biotin, 0.10 mg; folic acid, 0.25 mg; vitamin B12, 0.004 mg; choline, 500 mg; manganese, 60 mg; iodine, 0.35 mg; iron, 60 mg; copper, 8 mg; zinc, 80 mg; and selenium, 0.30 mg.

[2]Calculated by Chinese Feed Database (2014, 25th ed).

adapter and locus-specific sequence for 16S rRNA amplicon were 5'-TCGTCGGCAG CGTCAGATGT GTATAAGAGA CAGCCTACGG GNGGCWGCAG-3' and 5'-GTCTCGTGGG CTCGGAGATG TGTATAAGAG ACAGGACTAC HVGGGTATCT AATCC-3', respectively.

The paired-end sequencing was performed for 300 bp and the V3-V4 hypervariable region of 16S rRNA gene was targeted. The processing for phylogeny and taxonomy was performed using QIIME plugins and a sampling depth of 40,000 sequences per sample was chosen. For taxonomy, a Nave Bayes classifier pretrained on the Greengenes 13_8 99% operational taxonomic unit (OTU) was applied. The classified sequences were then analyzed for alpha and beta diversity.

Statistics

Data were represented as mean and SEM using one-way ANOVA and means (n = 6) were separated by Tukey's-b test using IBM SPSS (version 23) for egg production and nutrient digestibility. The response trends of experimental parameters including egg mass and nutrient retention to TMP doses at 100, 150 and 200 g/kg were analyzed using linear and quadratic polynomial contrasts. Kruskal-Wallis pairwise test was performed for alpha diversity and permutational multivariate analysis of variance for beta diversity analysis in QIIME2. Levels of significance were set at *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$.

RESULTS

Tetramethylpyrazine and egg production

Mortalities of hens throughout the trial were < 5%, not statistically significant between treatments and feed intake was also unaltered by the dietary manipulation (data not shown). A significant effect of TMP was found on the egg-laying rate at 77 (p=0.014), 79 (p=0.003), 82 (p=0.001), 83 ($p<0.001$), 84-85 ($p\leq0.007$), 86 ($p<0.001$) wk of age (Fig. 1), but there were no statistical differences from TMP doses. Accordingly, in contrast with the control, feed conversion ratio was also decreased ($p<0.01$) by 8.6% during 82-86 wk (data not shown).

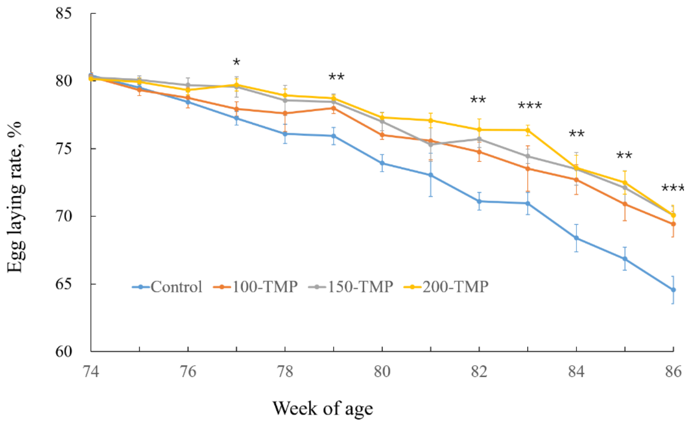

Figure 1 – Effect of tetramethylpyrazine (TMP) on the egg-laying rate of Hy-Line hens. Control, 100-, 150-, and 200-TMP, containing TMP at 0, 100, 150 and 200 mg/kg of diet, respectively. * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$), treatment effect by Turkey's-b test.

Accumulated egg mass of hens was also affected by the dietary treatment during 75-78 (p=0.021), 75-82 ($p<0.001$) and 75-86 ($p<0.001$) wk of age (Table 2). During the first four-week, there were no significant TMP dose effects on the egg mass, but as of wks 75-82 and 75-86, the doses of TMP at 150 and 200 mg/kg showed more pronounced effects ($p<0.05$) than the dose at 100 mg/kg, which led to linear effects ($p\leq0.003$) and a weak quadratic dose response (p=0.045). Compared to the control, at wk 86, the egg mass differences from the graded doses of TMP reached up to 97-225 g/hen.

Tetramethylpyrazine and nutrient retention

At 78 wk of age, a significant treatment effect ($p<0.001$) was found on nitrogen, showing that the nitrogen retention of TMP at 150 and 200 mg/kg were higher ($p<0.05$) than the control, but there were no differences among the three TMP doses (Table 3). At wk 82, the retention of DM, nitrogen and energy were influenced ($p\leq0.010$) by dietary manipulation, and the increased effect by TMP at 200 mg/kg were more pronounced ($p<0.005$) on energy than the dose at 100 mg/kg. At 86 wk, compared to the control, the three doses of TMP increased ($p<0.05$) the retention of DM and nitrogen. Linearly increases were found on DM retention ($p\leq0.033$) at all the test time points.

Table 2 – Effect of dietary tetramethylpyrazine (TMP) on egg mass of Hy-Line hens (g/hen).

Item	Control	TMP, mg/kg of diet			SEM	*p*-value		
		100	150	200		T	L	Q
75-78 wk	1369^{b}	1388^{ab}	1404^{a}	1404^{a}	8.39	0.021	0.210	0.471
75-82 wk	2570^{c}	2624^{b}	2684^{a}	2687^{a}	11.44	<0.001	0.001	0.045
75-86 wk	3542^{c}	3639^{b}	3747^{a}	3767^{a}	26.13	<0.001	0.003	0.183

$^{a-c}$ Means in a row with different superscripts are significantly different ($p<0.05$).

T = treatment effect by Turkey's-b test; L and Q = linear and quadratic, dose trend effect of TMP at 100, 150 and 200 mg/kg by polynomial contrasts.

Table 3 – Effect of dietary tetramethylpyrazine (TMP) on nutrient retention of Hy-Line hens (%).

Item	Control	TMP, mg/kg of diet			SEM	*p*-value		
		100	150	200		T	L	Q
78 wk								
DM	69.5	69.4	70.2	72.4	0.976	0.159	0.019	0.499
Nitrogen	59.9^{b}	65.2^{ab}	66.1^{a}	69.1^{a}	1.107	<0.001	0.056	0.509
Energy	11.68	11.96	11.96	12.15	0.125	0.129	0.330	0.535
82 wk								
DM	59.2^{b}	64.3^{ab}	67.9^{a}	69.0^{a}	0.837	0.001	0.001	0.090
Nitrogen	66.4^{b}	67.5^{ab}	68.2^{ab}	72.1^{a}	1.272	0.001	0.074	0.566
Energy	11.74^{b}	11.75^{b}	12.07^{ab}	12.27^{a}	0.111	0.010	0.002	0.629
86 wk								
DM	57.7^{c}	65.5^{b}	67.5^{a}	68.9^{a}	1.317	0.001	0.033	0.539
Nitrogen	60.1^{c}	64.9^{b}	66.0^{ab}	69.1^{a}	0.105	<0.001	0.001	0.623
Energy	11.32	10.82	11.17	11.79	0.347	0.296	0.060	0.743

$^{a\text{-}c}$ Means in a row with different superscripts are significantly different (p<0.05).

DM = dry matter; T = treatment effect by Turkey's-b test; L and Q = linear and quadratic, dose trend effect of TMP at 100, 150 and 200 mg/kg by polynomial contrasts.

Tetramethylpyrazine and cecal microbiota diversity

As shown in Figure 2, at the level of phyla, compositions of bacterial communities varied among sediments and were dominated by *Firmicutes* (81.2%), *Bacteroidetes* (14.0%), *Proteobacteria* (2.3%), and *Actinobacteria* (1.1%) among the treatments. At the genus level, *Subdoligranulum*, *Bacteroides* (13.6%), *Lactobacillus* (10.9%), *Faecalibacterium* (10.3%), and *Clostridium* (7.9%) were the dominant bacteria communities (Figure 3). There were no statistical differences for alpha diversity by testing observed OUTs and Shannon diversity index (Figure 4) and beta diversity by weighted UniFrac distance and Bray-Curtis distance analysis (Figure 5). The cecal microbial information indicates that dietary TMP is not enough to cause bacterial variation in both species and richness in the aged laying hens in the present study.

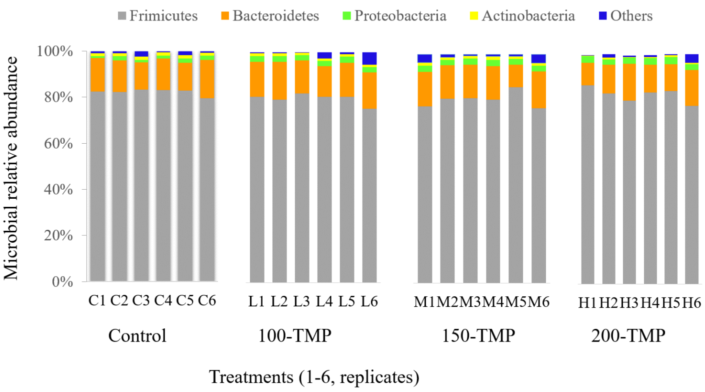

Figure 2 – Stacked bar graph of tetramethylpyrazine (TMP) on comparisons of microbial relative abundance at phylum level among treatments of Hy-Line laying hens at 86 wk of age. Control, 100-, 150-, and 200-TMP, containing TMP at 0, 100, 150 and 200 mg/kg of diet, respectively.

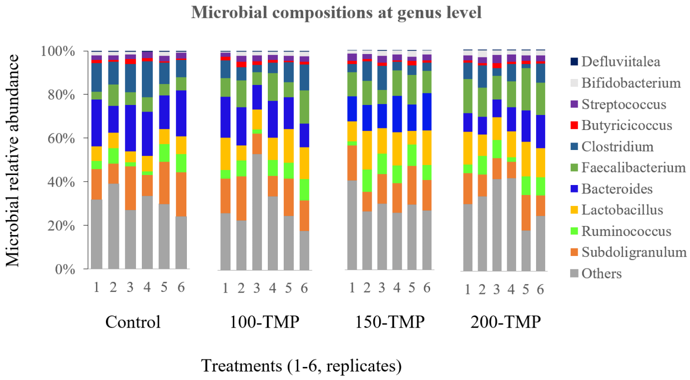

Figure 3 – Effect of tetramethylpyrazine (TMP) on comparisons of microbial relative abundance at genus level among treatments of Hy-Line laying hens at 86 wk of age. Control, 100-, 150-, and 200-TMP, containing TMP at 0, 100, 150 and 200 mg/kg of diet, respectively.

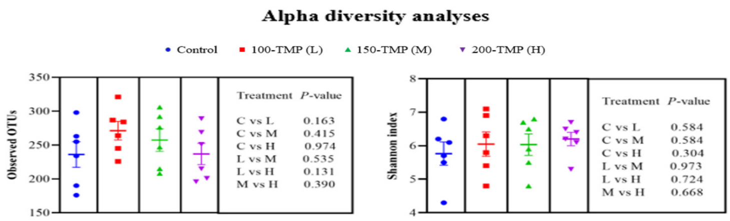

Figure 4 – Effect of tetramethylpyrazine (TMP) on alpha diversity of cecal microbiota of Hy-Line laying hens at 86 wk of age. 100-, 150-, and 200-TMP, containing TMP at 100, 150 and 200 mg/kg of diet.

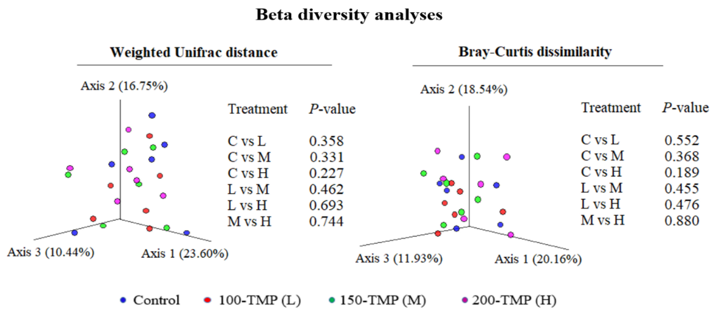

Figure 5 – Effect of tetramethylpyrazine (TMP) on beta diversity of cecal microbiota of Hy-Line laying hens at 86 wk of age. 100-, 150-, and 200-TMP, containing TMP at 100, 150 and 200 mg/kg of diet.

DISCUSSION

The finding in the present study that supplemental TMP improved egg-laying rate and egg mass is indicative of the fact that TMP beneficially regulates the egg production of aged hens. Literature is unavailable for the TMP effect on egg production, but previous studies demonstrated that dietary TMP improved growth of broilers and rabbits (Ding *et al.*, 2019; Liu *et al.*, 2019; Deng *et al.*, 2020b; Shi *et al.*, 2022). Importantly, the increase in egg mass by TMP up to 225 g/hen during the 12-week trial period implies that the dietary manipulation by using some bioactive ingredients can be a practicable strategy to avoid premature ovarian failure and to prolong the egg-laying period of hens as much as possible in some farms suffered from abnormal culling. In most cases, the efficacy of nutritional regulation on the delay of egg production decline is obscure, but this is of no less importance when a profit gap exists for the same chicken strain among enterprises. Additionally, the linear increase of egg mass with the graded TMP doses implies that 200 mg/kg of diet is not the limitation for the beneficial effect of TMP on aged laying hens, but further studies are needed.

The TMP or ligustrazine as a bioactive component in conventional herbs and fermented foods has been received increasing attention for counteracting oxidation, inflammation and vascular disease in humans (Zhao *et al.*, 2016; Zheng *et al.*, 2018; Jiao *et al.*, 2019; Wang *et al.*, 2019) and animals (Liu *et al.*, 2018a, 2019; Ding *et al.*, 2019). In the body, via oxidative metabolism, TMP is converted into polar, water soluble metabolites and excreted rapidly by renal metabolism (Huang *et al.*, 1994). Its action mechanisms are involved in several molecular signal pathways (Zheng *et al.*, 2018). However, information is scarce about its possible interactions with the gut environment in terms of nutritional physiology. The present study first reported that TMP improved the retention of dry matter, nitrogen and energy, indicating its active participation in the gut scenario, whether this should be attributed to its health benefits literature mentioned needs further study.

In ecology, alpha diversity is variation of microbes in a single sample including species richness and species diversity whereas beta diversity is difference in microbial composition between samples. OTUs is an operational definition used to classify groups of closely related individuals. Shannon index relates both OTUs richness and evenness. UniFrac distance takes into account the phylogentic relatedness of OUTs whereas Bray-Curtis dissimilarity only consider the abundance. The further investigation into gut microbiota information showed that TMP did not cause alpha and beta diversity, i.e. no major changes occurred in gut microbiota across treatments. This might be due to rapid degradation of TMP in the gut (Huang *et al.*, 1994), or the reason that the diet with a minor alteration especially from supplements is not enough to cause the alpha or beta diversity of gut microbiota (Li *et al.*, 2016; Wang *et al.*, 2021) although the gastrointestinal tract of the chicken harbors very complex and diverse microbial communities. Gut microbiota with a high diversity, however, is credited to link to a healthy state (Consortium, 2012; Lozupone *et al.*, 2012), and the finding in the present study seems to be unrelated between TMP and gut flora, which deserves further study.

Worth noting is that TMP is also a bacterial metabolite (Weisskopf *et al.*, 2021), and thereby it is curious whether the gut microbiota and TMP are a two-way process in which gut microbes transform diet ingredients into TMP having improved bioavailability and health effects, whereas TMP, exogenous and gut microbiota derived metabolites, can support growth of beneficial bacteria and inhibit pathogens. This curiosity was partially supported by several studies that concluded that dietary TMP inhibited *Clostridium perfringens, Escherichia coli, Salmonella, Campylobacter* and Gram-negative bacteria in growing broilers and rabbits (Liu *et al.*, 2018b, 2019; Ding *et al.*, 2019; Shi *et al.*, 2020). Volatile compounds including pyrazines, the parent body of TMP, in fermented alcoholic beverages profoundly regulated gut microbiota and serum metabolism in mice (Ji *et al.*, 2021). Therefore, the paradox between the literature and the present study about TMP relationship with gut microbiota needs further study.

In conclusion, TMP supplemented at 100, 150 and 200 mg/kg improved egg-laying rate from 77 wk of age, which led to an increase in egg mass by 97-225 g/hen during 75 to 86 wk of age. Apparent nutrient digestibility was also improved at different test time points, reflecting nitrogen at 78 wk, DM, nitrogen and energy at 82 wk, DM and nitrogen at 86 wk. With the increase in TMP doses, significant linear responses were found on egg mass at 75-86 wk, DM digestibility at 78, 82 and 86 wk, energy at 82 wk, and nitrogen at 86 wk. However, TMP did not influence the alpha and beta diversity of cecal microbiota of hens. The results suggest that TMP can be used in feeds to improve egg production of aged laying hens.

ACKNOWLEDGMENTS

The authors thank the laboratory staff of Animal Nutrition Group, Henan University of Science and Technology, and all colleagues and students for the support in practical work.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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