Alterations on vitamin C synthesis and transportation and egg deposition induced by dietary vitamin C supplementation in Hy-Line Brown layer model

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Article history:
Received 12 November 2020
Received in revised form
6 January 2021
Accepted 12 January 2021
Available online 26 June 2021

Keywords:
Vitamin C
Sodium-dependent vitamin C transporter
L-Gulonolactone oxidase
Albumen
Yolk

In ovo feeding of vitamin C (VC) has positive effects on the growth performance, immune and antioxidant function in poultry, which indicates that increasing VC content in eggs may be of benefit. This study was to investigate the effects of dietary VC supplementation on VC synthesis and transportation and egg deposition. In Exp. 1, in order to select a suitable animal model, VC content was detected in different eggs from different layer species. Vitamin C content was lower in ISA Brown breeder eggs and Hy-Line Brown layer eggs (P < 0.05) then in Arbor Acres breeder eggs. In Exp. 2, a total of 24 Hy-Line Brown layers (42-week-old) were randomly divided into 3 treatments with 8 replicates and fed a basal diet with VC at 0, 200 and 400 mg/kg. Sodium-dependent VC transporter 1 and 2 (SVCT1 and SVCT2) expressions were higher in ileum than in duodenum and jejunum (P < 0.05). SVCT1 expression was higher but SVCT2 expression was lower in the magnum than in the ovary (P < 0.05). L-Gulonolactone oxidase (GLO) and SVCT1 expressions were higher but SVCT2 was lower in the kidney than in the liver (P < 0.05). Dietary VC supplementation at 400 mg/kg increased SVCT1 expression in duodenum, ovary and magnum, but decreased GLO and SVCT1 expression in liver (P < 0.05). Dietary VC supplementation at 200 and 400 mg/kg increased SVCT2 expression in duodenum, but decreased GLO and SVCT1 expression in kidney and SVCT2 expression in liver (P < 0.05). Dietary VC supplementation promoted VC absorption in duodenum and jejunum, but reduced endogenous VC synthesis in liver and kidney. Although dietary VC supplementation enhanced VC transportation in ovary and magnum, it did not increase VC deposition in produced eggs.

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1. Introduction

Embryonic nutrient supplementation is expected to become a great potential way to improve the performance in poultry (Peebles, 2018; Retes et al., 2018). Some researchers have indicated that in ovo feeding of vitamin C (VC) had positive effects on growth performance, immune and antioxidant function in poultry (El-Senousy et al., 2018; Zhang et al., 2019; Zhu et al., 2019, 2020). In humans, more and more studies have proved that the epigenetics diet (including VC), offered for pregnant mothers, led to beneficial health outcomes in newborn babies by nutri-epigenetics (Li et al., 2019). Unlike viviparous animals, birds lack a placental structure and embryonic development is completely separated from mothers, thus embryonic VC requirements theoretically rely...
on egg deposition from mother and self-synthesis by embryo. Vitamin C can be synthesized via glucuronate–xylulose cycle in the presence of L-gulonolactone oxidase (GLO) by developing embryo (Gan et al., 2018), but its synthesis amount may be insufficient (Nowaczewski et al., 2012), which is why new-hatched chicks usually need to be supplemented with VC in farm or hatchery. And increasing egg VC deposition is a way to improve embryonic VC supplementation.

In eggs, fat-soluble vitamins are merely deposited in yolk, while water-soluble vitamins are deposited in both yolk and albumen (Rehault-Godbert et al., 2019). It is possible for some vitamins to achieve higher enrichment in produced eggs by dietary supplementation. Water-soluble vitamins (including VC), the literatures are scare. According to nutrient requirements of poultry in NRC, VC is not a vitamin that must be added in vitamin premix of poultry, and whether dietary VC supplementation can achieve egg VC content in produced eggs still has not been reported.

Egg yolk and albumen are formed at the hen’s ovary and magnum, respectively (Estienne et al., 2020; Nys and Guyot, 2011). Consequently, exploring VC deposition in eggs would be inseparable from detecting the transportation function of ovary and magnum. The source of the hen’s VC pool is mainly from exogenous absorption by sodium-dependent VC transporter 1 and 2 (SVCT1 and SVCT2) in the intestine and endogenous synthesis by GLO in the liver and kidney (Hooper et al., 2002; Lindblad et al., 2013; Lykkesfeldt and Tveden-Nyborg, 2019; Mandl et al., 2009; Subramanian et al., 2017). As the oxidized form of VC, dehydroascorbic acid can be transported by glucose transporters, but plasma dehydroascorbic acid content was only from about <1% to 2% of plasma VC (Padayatty and Levine, 2016). Therefore, the transportation and distribution of VC in various tissues (including ovary and magnum) mainly depends on blood circulation and sodium-dependent VC transporters (SVCT).

In this study, we first selected a better animal model by comparing the egg parameters and VC contents of Arbor Acres breeder hens, ISA Brown breeder hens and Hy-Line Brown layers. Then, we measured the effects of dietary VC supplementation on VC synthesis and transportation and egg deposition. This study will prove whether dietary VC supplementation can achieve egg VC deposition and provide new insights for the approaches of embryonic VC supplementation in poultry.

2. Materials and methods

The use of animals and all experimental protocols were authorized by the Institutional Animal Care and Use Committee of Northwest A&F University (Yangling, Shaanxi, China).

2.1. Experiment 1

2.1.1. Experimental procedures

Experiment 1 was conducted to compare the egg parameters and VC contents among the eggs of Arbor Acres breeder hens, ISA Brown breeder hens and Hy-Line Brown layers. All 3 hen diets were corn-soybean meal without VC supplementation, but with different nutrient levels. On the same day, 15 fertilized eggs were selected from 52-week-old Arbor Acres breeder hens, 19 fertilized eggs were selected from 39-week-old ISA Brown breeder hens and 15 unfertilized eggs were selected from 47-wk-old Hy-Line Brown layers. Every egg was weighed. Fresh albumen and yolk were separated, weighed, and packed into 50-mL cryotube, respectively. After being placed at −80 °C for 24 h, the albumen and yolk samples were immediately put into a pre-cooled vacuum freeze dryer (Siemens, Berlin, Germany) for the preparation of lyophilized powders. Lyophilized powders of albumen and yolk were weighed and stored at −20 °C for further analysis.

2.1.2. Egg parameters

Egg parameters included egg weight, the ratio of yolk fresh weight to albumen fresh weight (YFW:AFW ratio), the ratio of yolk dry weight to albumen dry weight (YDW:ADW ratio), yolk moisture content (YMC) and albumen moisture content (AMC). The weight of lyophilized powders of albumen and yolk represented albumen and yolk dry weights, respectively. The formulas employed were as follows.

\[
\begin{align*}
YFW/AFW (%) & = \frac{YFW}{AFW} \\
YDW/ADW (%) & = \frac{YDW}{ADW} \\
YMC (%) & = \frac{[YFW - YDW]/YFW}{100} \\
AMC (%) & = \frac{[AFW - ADW]/AFW}{100}
\end{align*}
\]

2.1.3. Measurement of vitamin C content in the eggs

Five milliliters of pre-cooled physiological saline and 1 g of albumen or yolk lyophilized powder were added into a 10-mL centrifuge tube in sequence. The mixture was shaken violently and centrifuged at 664 × g for 15 min. Then, the VC content in the supernatant was measured by commercially available kits (A009, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on kit instructions. According to the kit's specification, the sample quality control was strictly implemented. The VC content in the eggs was calculated as follows.

Yolk VC content (YVCC, µg/g of yolk DM) = [the VC content in the supernatant (µg/mL) × 5 mL]/1 g of yolk DM

Albumen VC content (AVCC, µg/g of albumen DM) = [the VC content in the supernatant (µg/mL) × 5 mL]/1 g of albumen DM

2.2. Experiment 2

2.2.1. Experimental procedures

A total of 24 Hy-Line Brown layers (42-week-old) were randomly divided into 3 treatments with 8 replicates and fed a basal diet (Table 1) with 0, 200 and 400 mg/kg VC supplementation, respectively. One hundred and twenty grams of feed per layer was supplied daily to maintain the consistency of VC intake in the same treatment. Drinking water was adequate and the feeding time was at 09:00 and 16:00. The feeding trial lasted for 28 d and the trial time was named sequentially from d 1 to 28. The eggs produced on d 23 and 28 were collected for egg quality analysis and the preparation of lyophilized powder. In the end, all layers were fasted for 12 h. Then, blood samples were taken from the jugular vein, and all layers were euthanized by cervical dislocation. The tissue samples (liver, kidney, ovary and magnum) and the intestinal mucosa samples (duodenum, jejunum and ileum) were collected, snap-frozen in liquid N2 and then stored at −80 °C for further analysis.

2.2.2. Laying performance and egg quality

The daily produced eggs were weighed and counted. Egg production rate, egg weight and feed-to-egg ratio were calculated. The
eggs produced on d 23 were analyzed for egg quality, including yolk color, Haugh unit, albumen height, shell strength, and shell thickness.

2.2.3. Measurement of egg parameters and vitamin C content in serum and produced eggs

Egg parameters measurement and lyophilized powders preparation were the same as in Exp. 1. The blood samples were centrifuged at 664 × g for 10 min to obtain the serum samples and stored at −20 °C for further analysis. The VC content in lyophilized powders and serum was measured by commercially available kits, which was the same as in Exp. 1.

2.2.4. Quantitative real-time PCR

Total RNA was extracted from the liver, kidney, intestinal mucosa (duodenum, jejenum and ileum) and reproductive tract (ovary and magnum), following TRIzol Reagent protocol (AG21102, AG, Changsha, China). The concentration, purity and integrity of RNA were analyzed with a SYBR Green Premix Pro Taq HS qPCR Kit (AG11701, AG, China). The integrity and purity of RNA were measured by the 260/280 ratio. The concentration of RNA was measured by the 260/230 ratio. The samples were treated with DNase I (Roche, Mannheim, Germany) and purified with a MiniSpin Plus Kit (BD, Franklin Lakes, NJ, USA). Total RNA (1 μg) was reverse-transcribed into cDNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, Waltham, MA, USA).

Table 1
Composition and nutrient levels of basal diet in Exp. 2 (% as-fed basis).

| Item                  | Content   |
|-----------------------|-----------|
| Ingredients           |           |
| Corn                  | 59.76     |
| Soybean meal          | 23.00     |
| Distillers dried grains with solubles | 4.00     |
| Calcium carbonate     | 8.65      |
| Dicalcium phosphate   | 1.05      |
| Soybean oil           | 1.25      |
| Sodium chloride       | 0.25      |
| L-Lysine-H2SO4        | 0.13      |
| L-Threonine           | 0.31      |
| DL-Methionine         | 0.25      |
| Choline chloride      | 0.10      |
| Sand                  | 0.25      |
| Vitamin and mineral premix
  1 Provided the following per kilogram of diet: manganese 60 mg, copper 8 mg, zinc 80 mg, iodine 0.35 mg, selenium 0.3 mg, vitamin A 8,000 IU, vitamin D3 1,600 IU, vitamin E 30 mg, vitamin K3 1.5 mg, thiamin 4 mg, riboflavin 13 mg, pantothenic acid 15 mg, nicotinamide 20 mg, pyridoxine 6 mg, biotin 0.15 mg, folic acid 1.5 mg, cobalamin 0.02 mg. No vitamin C added.
| Total                 | 100.00    |
| Nutrient levels (calculated) |     |
| Metabolizable energy, kcal/kg | 2,700   |
| Crude protein         | 16.5      |
| Total phosphorus      | 0.51      |
| Non-phytate phosphorus| 0.32      |
| Calcium               | 3.50      |

2.3. Statistical analysis

Expressions of SVCT1 and SVCT2 between different tissues (ovary vs. magnum, and liver vs. kidney) and GLO expression (liver vs. kidney) were compared, and data were analyzed by independent sample t test. All other data were analyzed by one-way ANOVA.

Table 2
Primer sequence of target genes in Exp. 2.

| Gene   | Accession number | Primer sequences | Product Size, bp |
|--------|------------------|------------------|-----------------|
| β-actin| NM_205518.1 F: ATTGTCCACCGCAAATGCTTC R: AAATAGCATTGCACCTTGCCTC | 113 |
| SVCT1  | XM_004944768.3 F: GGCTGTCACGAGTCCAGGACG R: AGGTGAAGATGGTGCGATG | 173 |
| SVCT2  | XM_025142777.1 F: AGGCAAAACCTGGGGTGATCG R: GCGAGCATAGAAGGCTACT | 247 |
| GLO    | XM_015285218.2 F: GGGCAAGGGATTCAAGGT R: GATGTCAGGGGCGAGTG | 167 |

Using the SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was considered at P < 0.05 and trends at P < 0.1.

3. Results

3.1. Experiment 1

3.1.1. Comparison of egg parameters and vitamin C content among different layer species

As shown in Table 3, egg weight was the heaviest in Arbor Acres breeder hens and was the lightest in Hy-Line Brown layers (P < 0.05). Compared with ISA Brown breeder hens and Hy-Line Brown layers, Arbor Acres breeder hens had significantly higher YFW:AFW ratio, YDW:ADW ratio, AMC, YVCC and AVCC (P < 0.05). Compared with Arbor Acres breeder hens and ISA Brown breeder hens, Hy-Line Brown layers had significantly higher YMC (P < 0.05).

3.2. Experiment 2

3.2.1. Laying performance, egg quality, egg parameters, and vitamin C content in serum and produced eggs

As shown in Table 4, the limited feed supply was executed as 120 g of daily among 3 groups and dietary VC supplementation had no significant effects on egg production rate, egg weight and feed-to-egg ratio (P > 0.05). As shown in Table 4, dietary VC supplementation had no significant effects on yolk color, Haugh unit, albumen height, shell strength and shell thickness on d 23 (P > 0.05). As shown in Table 5, dietary VC supplementation had no significant effects on egg parameters (Egg weight, YFW:AFW ratio, YDW:ADW ratio, AMC, YVCC and AVCC) (P < 0.05).

3.2.2. Tissue mRNA expression profile of SVCT1, SVCT2 and GLO

As shown in Table 6, compared with duodenum and jejenum, SVCT1 and SVCT2 expressions were both significantly higher in ileum (P < 0.05). Compared with ovary, SVCT1 expression was significantly higher in magnum, while SVCT2 expression was significantly lower (P < 0.05). Compared with liver, GLO and SVCT1 expressions were both significantly higher in kidney, while SVCT2 expression was significantly lower (P < 0.05).

3.2.3. Vitamin C absorption: mRNA expressions of SVCT1 and SVCT2 in the intestine

Dietary supplementation of VC at 400 mg/kg increased SVCT1 expression in the duodenum (Fig. 1A, P < 0.05) and had an increasing trend in the jejenum (Fig. 1B, P = 0.067). Dietary 200 and 400 mg/kg VC supplementation increased SVCT2 expression in the
3.2.4. Vitamin C transport: mRNA expressions of SVCT1 and SVCT2 in the ovary and magnum

Dietary supplementation of VC at 400 mg/kg increased SVCT1 expression in the ovary (Fig. 2A) and magnum (Fig. 2B, P < 0.05). However, dietary VC supplementation had no significant effect on SVCT2 expression in the ovary (Fig. 2A) and magnum (Fig. 2B, P > 0.05).

3.2.5. Vitamin C synthesis: mRNA expressions of GLO, SVCT1 and SVCT2 in the liver and kidney

Dietary supplementation of VC at 400 mg/kg decreased GLO and SVCT1 expression in the liver (Fig. 3A, P < 0.05). Dietary supplementation of VC at 200 and 400 mg/kg decreased GLO and SVCT1 expression in the kidney (Fig. 3B) and SVCT2 expression in the liver (Fig. 3A, P < 0.05). However, dietary VC supplementation had no significant effect on SVCT2 expression in the kidney (Fig. 3B, P > 0.05).

4. Discussion

Arbor Acres breeder hens, ISA Brown breeder hens and Hy-Line Brown layers all belong to layer species and have similar reproductive tract structure for yolk and albumen synthesis. Nevertheless, different egg parameters among different layer species were indeed observed, which might be related to nutrition (different diets) and genetics (different layer species). Studies have shown that the animal with lower plasma or tissue VC content at baseline is more conducive to use as an animal model for demonstrating benefit or harm from dietary VC supplementation (Lykkesfeldt and Poulsen, 2010; Padayatty and Levine, 2009, 2014), and egg VC content at baseline should be regarded as an important indicator for the selection of the animal model. Therefore, ISA Brown breeder hens and Hy-Line Brown layers are both suitable as the animal model. In addition, due to the high price of breeder hens or breeder hens and Hy-line Brown layers are both suitable as the animal model.
eggs caused by African swine fever, the farms are reluctant to sell breeder hens, and it is difficult for us to purchase breeder hens. Finally, Hy-Line Brown layers were selected as the animal model in current study for studying the effects of dietary VC supplementation on VC synthesis and transportation and egg deposition.

Some previous studies indicated that the decline of production performance in old layers was inseparable with oxidative stress status (Molnar et al., 2016; Wang et al., 2011). Under the stress conditions, VC supplementation improved the production performance in old layers (72-week-old) (Ahmed et al., 2008) but had no effects on that in young layers (31-week-old) (Wang et al., 2016). Therefore, we speculated that the effects of VC supplementation were related to oxidative stress status in layers, and the anti-stress ability of young layers is better than that of old layers. Obviously, it is more likely to be effective to supplement VC for laying hens with poor anti-stress ability and/or stress ability. In this study, dietary VC supplementation had no significant impact on laying performance and egg quality in 42-wk-old layers (young layers), which supported the above speculation.

In order to explore egg VC deposition induced by dietary VC supplementation, VC absorption in the intestine, VC synthesis in the liver and kidney, and VC transport in the ovary and magnum were detected. GLO is the key enzyme for VC synthesis and gene mutations in GLO lead to VC synthesis ability loss in some species (like humans) (Linster and Van Schaftingen, 2007). However, Hy-line Brown layers have the ability of VC synthesis, which is much
higher in the kidney than in liver (Gan et al., 2018; Maurice et al., 2002). VC absorption, transportation and distribution are primarily regulated by SVCT (Lykkesfeldt and Tveden-Nyborg, 2019).

Tissue mRNA expression profile showed that ileum was the main site for VC absorption with higher SVCT expression. Higher SVCT1 expression and lower SVCT2 expression in the magnun than those in the ovary indicated that SVCT1 may be the main VC transporter and may be related to higher VC content in albumen. GLO expression was extremely higher in the kidney than that in the liver, indicating that kidney was main tissue for VC synthesis in layers, which was also supported by the results of GLO enzyme activity in the liver accounting for only 0.5% to 0.7% of that in the kidney (Gan et al., 2018) and higher SVCT1 expression in the kidney than that in the liver.

SVCT1 is the absorptive VC transporter with low affinity and high capacity, which is responsible for whole-body homeostasis of VC and is widely expressed in the tissues of endothelial system tissues (including intestine and kidney) and some other tissues (lung, liver and skin) (Savini et al., 2008). And SVCT2 is the tissue transporter with high affinity and low capacity, which is widely expressed in various tissues and is responsible for tissue transporter for VC (Savini et al., 2008). SVCT1 is the major transporter to VC in the intestine and higher SVCT1 expression showed that dietary VC supplementation promoted VC absorption in the duodenum and jejunum. Although dietary VC supplementation decreased SVCT2 expression in the ileum, SVCT2 was not a major transporter with low capacity in the intestine. In addition, lower GLO expression in the liver and kidney indicated that dietary VC supplementation enhanced the overall VC absorption of the intestine. Compared with VC in plant-derived feed ingredients, VC in the form of additives could contact the intestinal wall faster and earlier in intestinal lumen without digesting plant cells to release VC (Fenech et al., 2019), which may be why dietary VC supplementation promoted VC absorption in the duodenum and jejunum rather than that in the ileum.

Alterations on SVCT expression indicated that dietary VC supplementation enhanced VC absorption in the duodenum and jejunum, but surprisingly, no difference in serum VC content was observed in VC supplementation groups. Some research showed that the peak of plasma VC content reached a plateau even if the oral dose continued to increase, and the intravenous VC injection with the maximum tolerable oral dose produced extremely higher (30- to 70-fold) plasma VC content than that of oral (Padayatty et al., 2004). Thus, dietary VC supplementation is not feasible to achieve high plasma VC contents. Vitamin C was rapidly metabolized and excreted within 3 h after oral high-dose VC (the time was shorter after intravenous injection) for the stability of plasma VC content (Furuita et al., 2009). In this study, serum samples were collected from Hy-Line Brown layer after 12 h fasting and this is why no difference in serum VC content was observed.

Dietary VC supplementation significantly reduced GLO expression in the liver and kidney. Although dietary VC supplementation increased GLO enzyme activity in the liver based on Gan’s research, its activity was reduced in the kidney and VC synthesis was primarily in the kidney, as evidenced by GLO enzyme activity in the liver accounting for only 0.5% to 0.7% of that in that kidney (Gan et al., 2018; Hooper et al., 2000). Alterations on SVCT expression were consistent with GLO expression in the liver and kidney, proving that dietary VC supplementation caused the reduction of endogenous VC synthesis.

Yolk and albumen are formed in the ovary and magnum, respectively. In this study, VC content was higher in albumen than in yolk, which may be related with 40-fold higher SVCT1 expression in the magnun than that in the ovary. But its mechanism of VC secretion into albumen and yolk was unknown (Padayatty and Levine, 2016). The moisture content (53%) is lower and the fat content (28%) is higher in yolk, compared with moisture content (82%) and no fat in albumen (Uni et al., 2012). And in this study, the moisture content was 49% in yolk and 88% in albumen, respectively. Higher SVCT1 expression, higher moisture content and no fat may benefit VC deposition in albumen rather than in yolk. However, dietary VC supplementation had no effect on VC deposition in yolk and albumen, which was contrary with higher SVCT1 expression in
the ovary and magnum. Whether VC was taken orally or intravenously, the plasma VC content could be quickly returned stability by metabolism and excretion (Padayatty et al., 2004). Thus, it was difficult to keep a high dose of plasma VC content through VC supplementation (Lykkesfeldt and Tveden-Nyborg, 2019; Padayatty et al., 2004). The follicle maturation process is accompanied by the deposition of lipids (Schneider, 2016), which may not benefit VC deposition. And, it only takes 1.5 h to complete albumen formation in the magnum (Estienne et al., 2020), which gives a very short window time for VC deposition. In addition, limited increase in SVCT1 expression may make it difficult to activate the secretion of the ovary and magnum, so dietary VC supplementation failed to increase VC deposition in produced eggs.

Obviously, there are some limitations in this study. According to industry routine dosage (dietary supplementation of VC at 200 mg/kg), VC supplementations at 0, 200 and 400 mg/kg were selected. However, the higher-dose vitamin C group should be contained in the current study, which may be more convincing to demonstrate the effects of dietary VC supplementation on VC deposition in produced eggs. In addition, egg VC deposition is affected by VC transport and secretion in the ovary and magnum. However, there is no research basis for the mechanism of VC secretion, and VC secretion was not detected in this study.

5. Conclusion

In summary, based on egg VC content at baseline and the availability of animal materials, Hy-Line Brown layer was selected as an animal model. Dietary VC supplementation had no significant impacts on laying performance and egg quality in 42-week-old Hy-Line Brown layers. GLO expression indicated that the ability of VC synthesis was extremely stronger in the kidney than in the liver. And, a higher VC content in albumen than in yolk may be related to the higher SVCT1 expression in the magnum than in ovary. Lower GLO expression in the liver and kidney indicated that dietary VC supplementation enhanced the overall VC absorption of the intestine. Although SVCT expression indicated that the ileum was the main site for VC absorption, dietary VC supplementation enhanced intestinal VC absorption by promoting VC absorption in the duodenum and jejunum. And, dietary VC supplementation increased SVCT1 expression in the ovary and magnum, but had no significant effects on VC deposition in produced eggs (Fig. 4).

Author contributions

Yufei Zhu, Wei Guo, Jianfei Zhao and Xiaojun Yang conceived and designed the experiments; Yufei Zhu, Wei Guo, Jianfei Zhao, Kailong Qin and Jiakun Yan mainly performed the experiments; Yufei Zhu and Yanli Liu analyzed the data and wrote the manuscript; Wei Guo, Jianfei Zhao, Zhourzheng Ren, Xin Yang, Yanli Liu and Xiaojun Yang participated in the revision of the manuscript.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgements

This work was funded by the National Key Research and Development Program of China (2017YFD0500500, 20170502200), the National Natural Science Foundation of China (No. 31972529), the Program for Shaanxi Science & Technology (2017TSCXLY-04-04, 2018ZDCXLY-0201, 2018ZDMX-NY-051) and the Program for Yangling Agricultural High-tech Industries Demonstration Zone (2018CYX-10). Appreciation is extended to the staff of the Innovative Research Team of Animal Nutrition & Healthy of Northwest A&F University for their assistance during animal breeding, sample collection and sample analysis in the experiments.

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