



## Maternal dietary linoleic acid altered intestinal barrier function in domestic pigeons (*Columba livia*)

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### Abstract

Linoleic acid (LA) is predominantly essential for poultry. Poultry lacking LA show retarded growth and reduced disease resistance. Intestinal barrier function plays an important role in pigeon squab growth, whereas research on the effects of LA on intestinal health in altrices is scant. Considering that squabs are fed by their parents, the study aimed to explore the effects of maternal dietary LA on intestinal morphology, tight junction proteins, immune cytokines and microbial flora in squabs. A completely randomised design with a control group, 1 % LA supplementation group, 2 % LA supplementation group and 4 % LA supplementation group was used. Six squabs from each treatment were randomly sampled at 21 d post-hatching. The results indicated that LA supplementation improved intestinal morphology, as reflected by increased villus height, villus area and the ratio of villi to crypts. Also, 1 % LA supplementation elevated the density of goblet cells in the intestine and strengthened tight junctions by up-regulating claudin-3 and occludin gene expression but down-regulating claudin-2 gene expression. Moreover, 1 % LA supplementation reduced the secretion of proinflammatory cytokines and partly increased anti-inflammatory cytokines. The intestinal microbial diversity in the 1 % LA supplementation group was higher than that in the other groups. As beneficial bacteria, *Butyrivibrio* was the biomarker of 1 % LA supplementation. However, excessive (4 %) LA supplementation led to adverse impacts on intestinal immunity and microbiota. In conclusion, maternal dietary LA might alter intestinal barrier function in pigeon squabs in a dose-dependent manner. Supplementation with 1 % LA was suggested in parental pigeons.

**Key words:** Intestinal barrier: Intestine immunity: Linoleic acid: Pigeon squabs

The meat of pigeon squabs can be compared with that of broilers in terms of nutritive value and acceptability. Pigeon squabs have been considered profitable sources of poultry meat from *Columba* species as they grow more rapidly with minimum input<sup>(1)</sup>. Squabs have a high growth rate, which is attributed to special crop milk feeding. Several fatty acids available in crop milk are necessary for normal growth<sup>(2)</sup>. Therein, linoleic acid (LA) is predominantly essential for poultry and its higher derivative arachidonate is the most important<sup>(3–5)</sup>.

The symptoms of LA deficiency in poultry manifest in various aspects, such as retarded growth, increased water consumption, enlarged liver with increased lipid content and reduced resistance to disease<sup>(5)</sup>. In addition, a study stated that the LA-enriched diet enhanced growth in hens immunised with *Mycobacterium butyricum* after immunisation<sup>(6)</sup>. The maternal LA isomer regulated hepatic lipid metabolism via the adenosine 5'-monophosphate-activated protein kinase signalling pathway

in chick embryos<sup>(7)</sup>. Additionally, our group previously found that parental dietary LA supplementation altered lipid metabolism and antioxidant status in pigeon squabs in a dose-dependent manner<sup>(8)</sup>. Because of its high concentration in crop milk, LA is considered to be an important active substrate to maintain pigeon squab growth and health. However, using the pigeon squab model, we previously reported that supplemental LA had negligible effects on growth and development in pigeon squabs, but parental dietary LA at a concentration of 1 % could have beneficial effects on maintaining squab health, as reflected by the improved antioxidant capacity and lipid metabolism<sup>(8)</sup>. The present study continued to characterise the effects of LA on maintaining squab health from an intestinal perspective.

Studies *in vivo* and *in vitro* have focused on the effects of LA on intestinal health and immune function over the years<sup>(9–13)</sup>. Li *et al.*<sup>(9)</sup> indicated that in Caco2 cells, LA up-regulated both

**Abbreviations:** CLDN2, claudin-2; CLDN3, claudin-3; GC, goblet cell; LA, linoleic acid; OCLN, occludin; sIgA, secretory IgA; VCR, villus height to crypt depth ratio.

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the mRNA and protein levels of ApoA-IV which is produced predominantly in the intestine and functions in anti-inflammation<sup>(14)</sup>. In Ht-29 cells, the LA isomer inhibits cell proliferation and stimulates apoptosis<sup>(10)</sup>. Moreover, in humans, duodenal ulcers were associated with a deficiency of dietary LA intake<sup>(11)</sup>. In IL-10-deficient mice, Su1947-Hya, an LA metabolite, ameliorated colonic inflammation<sup>(12)</sup>. A study by Rodrigues *et al.*<sup>(13)</sup> also indicated that LA might affect the course of inflammation. However, research related to the effects of LA on intestinal health in poultry was scant.

The *Columba* pigeons typically regurgitate milk from the crop by both parents for the nourishment of the young. As squabs grow, crop milk is mixed with grains soaked in the crop and gradually totally replaced by grains<sup>(15)</sup>. This course is a gradual 'weaning' process<sup>(16)</sup>. Weaning is generally regarded as a stressful event that could lead to intestinal impairment<sup>(17)</sup>. Thus, the maintenance of intestinal function and immunity is particularly important. Considering the effects of LA on intestinal health *in vitro* and in mammals, we hypothesised that moderate LA could also strengthen the intestinal function and immunity of pigeon squabs. Therefore, the purpose of the present study was to explore the effects of maternal dietary LA at three levels on intestinal barrier function in squabs by determining intestinal morphology, gene expression of tight junction proteins, immune cytokines and microbial flora.

## Materials and methods

All experimental protocols involving animals were approved by the Animal Care and Welfare Committee of the Animal Science College and Scientific Ethical Committee of Zhejiang University (no. ZJU2013105002) (Hangzhou, China).

### Animals, diets and experimental design

A total of 240 paired parent White King pigeons, 60 weeks of age, were obtained from a commercial pigeon farm (Wenzhou, China). An artificial aviary with a perch and a nest was provided for each pair. Parent pigeons were randomly allocated to four treatments, which consisted of six replications of ten pairs of pigeons each. The four treatments were as follows: control group, 1% LA supplementation group (LA1%), 2% LA supplementation group (LA2%) and 4% LA supplementation group (LA4%). LA (> 99.0% purity) was obtained from a commercial supplier (Hebei Bawei Biotechnology Co., Ltd). All experimental diets were isonitrogenous and isoenergetic. The LA source was substituted for colseeds oil of the same weight to equalise the total fat level among diets<sup>(18)</sup>. The ingredients, nutrient levels and analysed LA content of the experimental diets for parent pigeons are shown in Table 1. The fatty acid composition of each diet analysed according to Xie *et al.*<sup>(19)</sup> is shown in online Supplementary Table S1. The fatty acid contents of crop milk are shown in online Supplementary Table S2 in which the LA levels (% total fatty acids) in crop milk from the four treatment groups were 18.20, 23.56, 28.62 and 31.52%, respectively. The pigeons were given water *ad libitum* and were fed twice daily (07.00 and 15.00 hours) throughout the experiment.

**Table 1.** Ingredient compositions and nutrient levels of experimental diets for parental pigeons\* (on as-fed basis)

| Items                                    | Control | LA1 %  | LA2 %  | LA4 %  |
|------------------------------------------|---------|--------|--------|--------|
| <b>Ingredients of a whole-grain form</b> |         |        |        |        |
| feed (%)                                 |         |        |        |        |
| Maize                                    | 56.27   | 56.27  | 56.27  | 56.27  |
| Pea                                      | 28.12   | 28.12  | 28.12  | 28.12  |
| Wheat                                    | 5.63    | 5.63   | 5.63   | 5.63   |
| Sorghum                                  | 5.63    | 5.63   | 5.63   | 5.63   |
| Colseeds oil                             | 4.35    | 3.26   | 2.17   | 0      |
| Linoleic acid                            | 0       | 1.09   | 2.18   | 4.35   |
| Total                                    | 100.00  | 100.00 | 100.00 | 100.00 |
| <b>Calculated nutrients† (%)</b>         |         |        |        |        |
| Metabolisable energy (MJ/kg)‡            | 13.84   | 13.84  | 13.84  | 13.84  |
| Crude protein                            | 12.63   | 12.63  | 12.63  | 12.63  |
| Analysed linoleic acid                   | 1.82    | 2.75   | 3.68   | 5.53   |
| <b>Ingredients of grit meal (%)</b>      |         |        |        |        |
| Limestone                                | 52.93   | 52.93  | 52.93  | 52.93  |
| Shell meal                               | 28.10   | 28.10  | 28.10  | 28.10  |
| Yellow mud                               | 14.05   | 14.05  | 14.05  | 14.05  |
| Salt                                     | 1.41    | 1.41   | 1.41   | 1.41   |
| Ferrous sulphate (monohydrate)           | 0.23    | 0.23   | 0.23   | 0.23   |
| Premix§                                  | 3.28    | 3.28   | 3.28   | 3.28   |
| Total                                    | 100.00  | 100.00 | 100.00 | 100.00 |
| <b>Calculated nutrients (%)</b>          |         |        |        |        |
| Ca                                       | 27.79   | 27.79  | 27.79  | 27.79  |
| P                                        | 0.01    | 0.01   | 0.01   | 0.01   |
| Na                                       | 0.03    | 0.03   | 0.03   | 0.03   |
| Cl                                       | 0.59    | 0.59   | 0.59   | 0.59   |
| Fe                                       | 0.30    | 0.30   | 0.30   | 0.30   |

Control, control group; LA1 %, 1% linoleic acid addition group; LA2 %, 2% linoleic acid addition group; LA4 %, 4% linoleic acid addition group.

\* All feeds were fed in a whole-grain form at 07.00 and 15.00 hours each day, and grit meal was offered to the birds on a continuous basis.

† Nutrient values were calculated from tables of feed composition and nutritive values in China (twenty-eighth edition, 2017).

‡ Metabolisable energy values determined in pigeons were calculated from those reported for chickens in accordance with a previous study (Hullar *et al.*<sup>(68)</sup>), which observed that the metabolisable energy values of feed in pigeons did not differ significantly from those in chickens.

§ The premix provided the following per kg of diet: vitamin A 5000 IU, vitamin E 50 IU, vitamin D<sub>3</sub> 2000 IU, copper sulphate 15 mg, manganese sulphate 45 mg, zinc sulphate 90 mg.

Each pair of parent pigeons laid two eggs in a nest. Eggs were picked out by hand, and fake eggs were put into nests to meet parents' broodiness. The picked eggs were transferred to an incubator for an 18-d artificial incubation. On the day of hatching, 480 artificially hatched pigeon squabs with similar body weight were selected from this pigeon farm. These squabs were randomly pair-matched and assigned to the nests of the selected parent pigeons to replace the fake eggs. Each parent pair adopted a pair of squabs. Parent pigeons were fed experimental diets continually for the next 21 d. Squabs were fed crop milk that was secreted by parent pigeons. The ambient temperature was 18–26°C. The relative humidity was 60–70%. The photoperiod was 12 h light–12 h dark throughout the entire experimental period.

### Sample collection

On day 21 post-hatch, six squabs per treatment (one for each replication) were selected for sampling. The squabs were fasted for 12 h before weighing and slaughter. In addition, they were sedated before cervical dislocation. Ileal contents were sampled into sterile plastic tubes with a sterile spatula, immediately frozen into liquid N<sub>2</sub> and then stored at –80°C for subsequent analyses.

**Table 2.** Primers used for quantitative real-time PCR analysis of gene expression in domestic pigeons

| Gene  | Primer  | Sequence (5' → 3')  | Amplicon size (bp) | Accession number |
|-------|---------|---------------------|--------------------|------------------|
| CLDN2 | CLDN2-F | GTGCAGATGGGAACAAGGT | 119                | XM_021283269.1   |
|       | CLDN2-R | GAGCCAAGGAAGCTACGG  |                    |                  |
| CLDN3 | CLDN3-F | ACCTCATCCCCGTCTCCT  | 109                | XM_005515008.2   |
|       | CLDN3-R | CAGCCCACGTAGAGCGA   |                    |                  |
| OCLN  | OCLN-F  | CAGGACGTGGCAGAGGA   | 105                | XM_005509325.2   |
|       | OCLN-R  | GTGGAAGAGCTTGTTCGGT |                    |                  |
| GAPDH | GAPDH-F | CCCAATGTCTCTGTTGTGG | 116                | NM_001282835.1   |
|       | GAPDH-R | TATGCCAGGATGCCCTT   |                    |                  |

CLDN2, claudin-2; F, forward; R, reverse; CLDN3, claudin-3; OCLN, occludin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Three segments of the small intestine (duodenum, jejunum and ileum) were sampled, flushed with 0.9% saline to remove all the contents and processed for morphological examination. The collected segments were the loop of the duodenum (duodenum), the tract before Meckel's diverticulum (jejunum) and the final segment of the small intestine (ileum). The mucosa of each intestinal segment was carefully scraped off with a glass slide, frozen in liquid N<sub>2</sub> rapidly and then stored at -80°C for subsequent analyses.

#### Secretory IgA and cytokine analysis

The homogenates of intestinal mucosa were prepared with PBS for secretory IgA (sIgA) and cytokine analyses. **The sIgA concentration and the concentrations of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10 and IL-4 were determined by absorbance changes at a wavelength of 450 nm with commercial ELISA kits (Shanghai Mlbio Institute) according to the manufacturer's protocol.** The final sIgA and cytokine concentrations were expressed as units per mg of protein.

#### RNA extraction and quantitative PCR analysis

Total RNA of the intestinal mucosa was extracted using the TRIzol procedure (Invitrogen) according to the instructions of the manufacturer. The extracted RNA was quantified by a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Inc.). The RNA integrity was verified by native RNA electrophoresis on a 1.0% agarose gel. Complementary DNA was synthesised from 2  $\mu$ g of total RNA by M-MLV RT (Takara) with oligo dT-adaptor primers at 42°C for 60 min following the protocol of the manufacturer. The abundance of mRNA was assayed on a StepOne Plus Real-time PCR system (ABI 7500, Applied Biosystems). The specific primers used are indicated in Table 2. SYBR Green Real-time PCR Master Mix (Toyobo Co., Ltd) was used for PCR, consisting of an initial DNA denaturation of 95°C for 60 s, followed by forty cycles of 95°C for 15 s and 60°C for 60 s. Glyceraldehyde-3-phosphate dehydrogenase was considered an appropriate endogenous reference. The average gene expression relative to the endogenous reference for each sample was calculated according to the 2<sup>- $\Delta\Delta$ Ct</sup> method<sup>(20)</sup>. The calibrator for each gene in the study was the average  $\Delta$ Ct value of the control group.

#### Intestinal morphological examination

Approximately 1-cm intestinal samples of each segment were collected and fixed in 10% neutral-buffered formalin solution.

Each sample was dehydrated, cleared and embedded in paraffin. Serial sections (5  $\mu$ m) were placed on glass slides and submitted to two different stainings. Haematoxylin and eosin staining was used to identify villus height, villus area, crypt depth and enterocytes. Periodic acid-Schiff staining was used for the identification of goblet cells (GC). The examination was performed with light microscopy (Nikon Corp.) using Image-Pro Plus 6.0 (Media Cybernetics, Inc.). The density of enterocytes was expressed as the number per mm of epithelial layer length. The density of GC was expressed as the number per 100 enterocytes according to Tamura *et al.*<sup>(21)</sup>.

#### 16S rDNA high-throughput sequencing

Total microbial genomic DNA was extracted from the ileal contents of squabs from each treatment using the E.Z.N.A.® Stool DNA Kit (Omega Bio-Tek). The concentration and quality of the extracted DNA were assessed with 2% agarose gel electrophoresis and a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Inc.). The V3-V4 hypervariable region of the 16S rRNA genes was amplified by specific degenerate primers (338F: ACTCTACGGGAGGCAGCAG, 806R: GGACTACHVGGGTWCTAAT). The PCR programme was conducted with the following conditions: initial denaturation step, 98°C, 30 s; denaturation, 35 cycles, 98°C, 10 s; annealing, 54°C, 30 s; elongation, 72°C, 45 s and final extension, 72°C, 10 min. The PCR amplification products were detected by 2% agarose gel electrophoresis. The target fragments were recovered using AMPure XT Beads (Beckman Coulter Genomics). Qubit was used to quantify the purified PCR products, and the qualified library concentration was more than 2 nM. Sequencing was performed with V3 chemistry, and 2  $\times$  300 bp double-end reads were generated using MiSeq Reagent Kit V3 with the MiSeq Illumina platform (Illumina) at Hangzhou Personal Biotechnology Co., Ltd.

#### Statistical analyses

The data obtained from sIgA and cytokine analysis, quantitative PCR analyses and intestinal morphological examination were subjected to one-way ANOVA using SPSS 24.0 (SPSS Inc.) for Windows. Treatment means were separated by the Tukey least significant difference *post hoc* test at the  $P < 0.05$  statistical level. When significant differences were found, orthogonal polynomial contrasts were further used to examine the linear and quadratic effects of different inclusion levels of dietary LA. Plotting was performed with GraphPad Prism 7.0 (GraphPad Software Inc.).

The raw sequence data obtained from the Illumina MiSeq platform were quality-filtered and demultiplexed using Quantitative Insights into Microbial Ecology, version 1.8.0-dev (<http://qiime.org/index.html>). After trimming and assembling, the final clean data were obtained and assigned into bacterial operational taxonomic units using the UCLUST function in Quantitative Insights into Microbial Ecology ([http://qiime.org/scripts/pick\\_otus](http://qiime.org/scripts/pick_otus)) according to a 97% similarity threshold. The data were categorised with the Ribosomal Database Project, version 2.2 (<http://sourceforge.net/projects/rdp-classifier/>). The  $\alpha$ -diversity measures, including the observed species, Chao 1 and Shannon indices, were calculated using MicrobiomeAnalyst (<https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/faces/home.xhtml>). A Venn diagram was generated in Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>). Principal coordinate analysis was conducted using Galaxy (<http://huttenhower.sph.harvard.edu/galaxy/>) according to unweighted Unifrac representing  $\beta$ -diversity values. The Kolmogorov–Smirnov programme was used to verify the normality of the data. Data with a normal distribution were counted by one-way ANOVA, and multiple comparisons were conducted by Tukey *post hoc* tests. Non-normally distributed data were analysed by the Kruskal–Wallis test and Duncan's multiple range test.  $P < 0.05$  indicates a significant difference.

## Results

### Intestinal morphometric traits

Haematoxylin and eosin staining of the intestines (duodenum, jejunum and ileum) of the four groups showed the effects of maternal dietary LA on the intestinal morphometric traits in squabs (Fig. 1). In the duodenum, the villus height, villus area and villus height to crypt depth ratio (VCR) responded to increasing supplementation of LA in a linear ( $P < 0.001$ ,  $P < 0.001$  and  $P = 0.01$ , respectively) and quadratic (all  $P < 0.001$ ) manner; their maximal response was observed in LA1%. In the jejunum, the villus height and villus area emerged as linear ( $P = 0.001$  and  $0.01$ , respectively) and quadratic (both  $P < 0.001$ ) trends with their maximum observed in LA1% as LA supplementation was increased. The crypt depth showed a linear ( $P < 0.001$ ) increase as the level of LA supplementation increased. In the ileum, villus height and the VCR responded to increasing supplementation with LA in a linear ( $P = 0.001$  and  $0.002$ ) and quadratic (both  $P < 0.001$ ) manner, and their maximum values were observed in LA1%. The villus area displayed a quadratic ( $P < 0.001$ ) trend with the maximum in LA1% as LA supplementation was increased. In addition, the VCR in the jejunum and crypt depth in the duodenum and ileum were not significantly different among the four groups. There was no significant difference ( $P > 0.05$ ) in enterocyte density among the four experimental groups.

### Tight junction protein gene expression

Tight junction proteins include the claudin family and occludin<sup>(22)</sup>. Recently, claudin-1, claudin-2, claudin-3, claudin-5, claudin-16 and occludin have been reported in poultry<sup>(23–25)</sup>. Among these genes, the mRNA expression of claudin-2 (*CLDN2*),

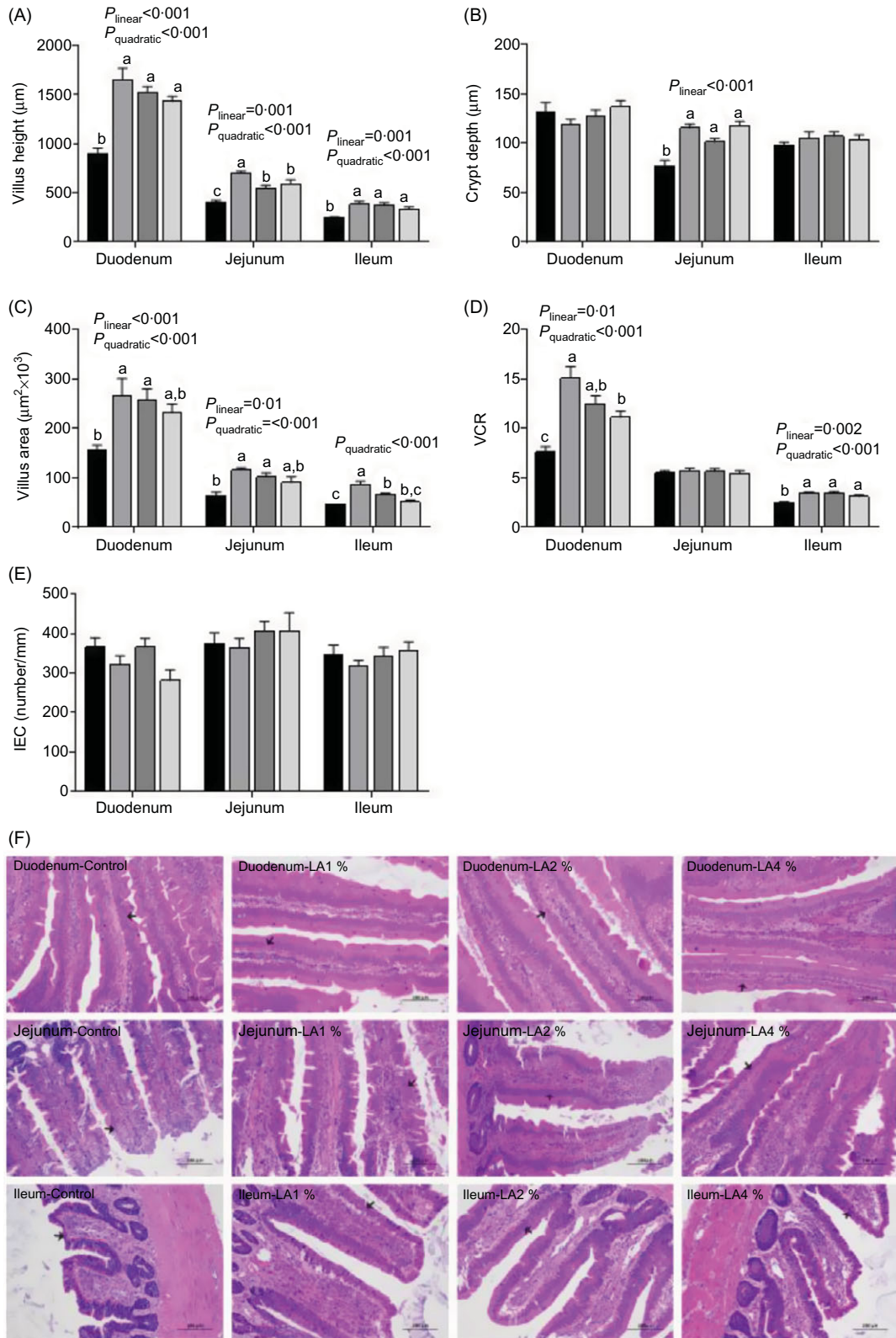
claudin-3 (*CLDN3*) and occludin (*OCLN*) is usually used to evaluate tight junction functions and barrier permeability<sup>(26)</sup>. In the present study, the effects of maternal dietary LA on the mRNA expression of these three tight junction proteins are shown in Fig. 2. In the duodenum, the mRNA expression of *CLDN3* exhibited a quadratic ( $P = 0.03$ ) trend, with the maximum observed in LA1% as LA supplementation was increased. *CLDN2* mRNA expression responded to increasing supplementation with LA in a linear ( $P = 0.02$ ) and quadratic ( $P < 0.001$ ) manner, with the minimum in LA1%. Although maternal dietary LA had no significant effect ( $P > 0.05$ ) on the mRNA expression of *OCLN*, a quadratic ( $P = 0.01$ ) trend was observed, with the maximum response in LA2%. In the jejunum, the mRNA expression of *CLDN2* exhibited a quadratic ( $P < 0.001$ ) trend, with the minimum in LA1% as the supplementation of LA was increased. However, *CLDN3* and *OCLN* in the jejunum had no significant differences ( $P > 0.05$ ) among the four treatments. In the ileum, *CLDN2* mRNA expression displayed linear ( $P = 0.001$ ) and quadratic ( $P = 0.004$ ) trends, with the maximum observed in LA4% as LA supplementation was increased. Moreover, *CLDN3* and *OCLN* mRNA expression responded to increasing supplementation of LA in a linear (both  $P = 0.003$ ) and quadratic ( $P = 0.003$ ;  $P < 0.001$ ) manner; their maximal responses were observed in LA1%.

### Goblet cell density

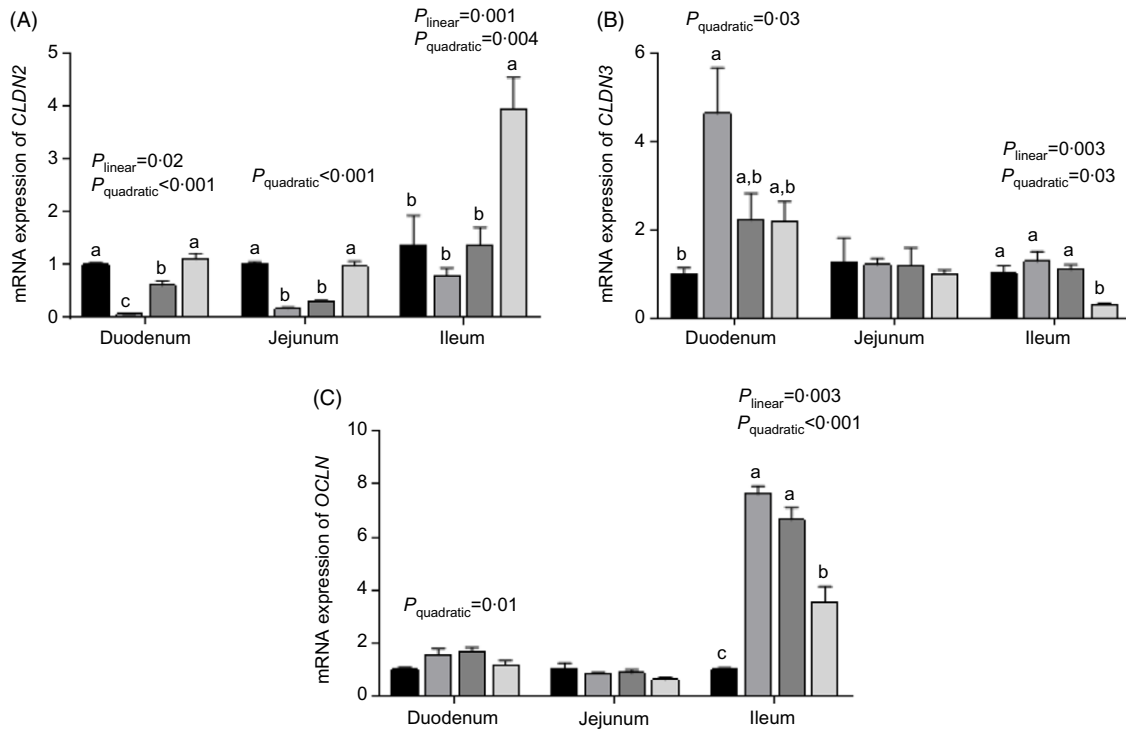
Periodic acid–Schiff staining of the intestines (duodenum, jejunum and ileum) of the four groups showed the effects of maternal dietary LA on the density of GC (Fig. 3). The GC density in the duodenum and jejunum responded linearly to increasing supplementation with LA ( $P = 0.006$ ;  $P < 0.001$ ), with the maximum observed in the LA1% and control group, respectively. The GC density in the ileum displayed linear ( $P = 0.02$ ) and quadratic ( $P = 0.009$ ) trends, with the maximum density in LA1%.

### Secretory IgA and cytokine analysis

The effects of maternal dietary LA on the concentrations of sIgA and cytokines are shown in Fig. 4. In the duodenum, sIgA and IL-10 concentrations responded quadratically to increasing LA supplementation ( $P = 0.009$  and  $0.001$ ), with their maximum observed in LA1%. The IL-6 concentration exhibited linear ( $P < 0.001$ ) and quadratic ( $P = 0.05$ ) trends, with the minimum concentration in LA1% as LA supplementation was increased. TNF- $\alpha$  and IL-1 $\beta$  concentrations increased linearly (both  $P < 0.001$ ) with increasing LA supplementation, whereas the IL-4 concentration decreased linearly ( $P = 0.002$ ). In the jejunum, the TNF- $\alpha$  concentration exhibited linear ( $P < 0.001$ ) and quadratic ( $P = 0.01$ ) trends, with the minimum in LA1% as LA supplementation was increased. The IL-10 concentration exhibited linear ( $P = 0.009$ ) and quadratic ( $P = 0.003$ ) trends, with the maximum in LA1% as LA supplementation was increased. IL-6 concentrations responded quadratically to increasing LA supplementation ( $P = 0.005$ ), with the minimum observed in LA1%. The IL-4 concentration responded quadratically to increasing LA supplementation ( $P = 0.05$ ), with their maximum observed in LA1%. There was no significant difference ( $P > 0.05$ ) in the sIgA concentration in the jejunum among the four experimental groups. In the ileum, TNF- $\alpha$  and IL-1 $\beta$  concentrations increased



**Fig. 1.** Effects of maternal dietary linoleic acid (LA) on intestinal morphometric trait in domestic pigeon squabs. (A) Villus height, (B) crypt depth, (C) villus area, (D) ratio of villus height to crypt depth (VCR), (E) density of enterocytes (intestinal epithelial cells; IEC), (F) haematoxylin and eosin staining of intestine (duodenum, jejunum and ileum) in the four treatment groups. The arrow points to enterocyte. Bar = 100  $\mu\text{m}$ . Control = control group; LA1% = 1% linoleic acid supplementation group; LA2% = 2% linoleic acid supplementation group; LA4% = 4% linoleic acid supplementation group. Values are means with their standard errors of six squabs,  $n$  6. <sup>a,b,c</sup> Means with unlike letters are significantly different (Tukey test,  $P < 0.05$ ). (A–E) ■, Control; ▒, LA1%; ▓, LA2%; □, LA4%.



**Fig. 2.** Effects of maternal dietary linoleic acid on mRNA expression of tight junction proteins in domestic pigeon squabs. (A) mRNA expression of claudin-2 (*CLDN2*), (B) mRNA expression of claudin-3 (*CLDN3*), (C) mRNA expression of occludin (*OCLN*). Control = control group; LA1 % = 1 % linoleic acid supplementation group; LA2 % = 2 % linoleic acid supplementation group; LA4 % = 4 % linoleic acid supplementation group. Values are means with their standard errors of six squabs, *n* 6. <sup>a,b,c</sup> Means with unlike letters are significantly different (Tukey test, *P* < 0.05). (A–C) ■, Control; ■, LA1 %; ■, LA2 %; □, LA4 %.

linearly ( $P = 0.002$  and  $0.04$ , respectively) with increasing LA supplementation, whereas the IL-10 concentration decreased linearly ( $P = 0.03$ ). The IL-4 concentration responded to increasing LA supplementation quadratically ( $P = 0.004$ ), with their maximum observed in LA1 %. The IL-6 concentration responded quadratically to increasing LA supplementation ( $P < 0.001$ ), with the minimum observed in LA1 %. There was no significant difference ( $P > 0.05$ ) in the sIgA concentration in the ileum among the four experimental groups.

### Ileal microbiota analysis

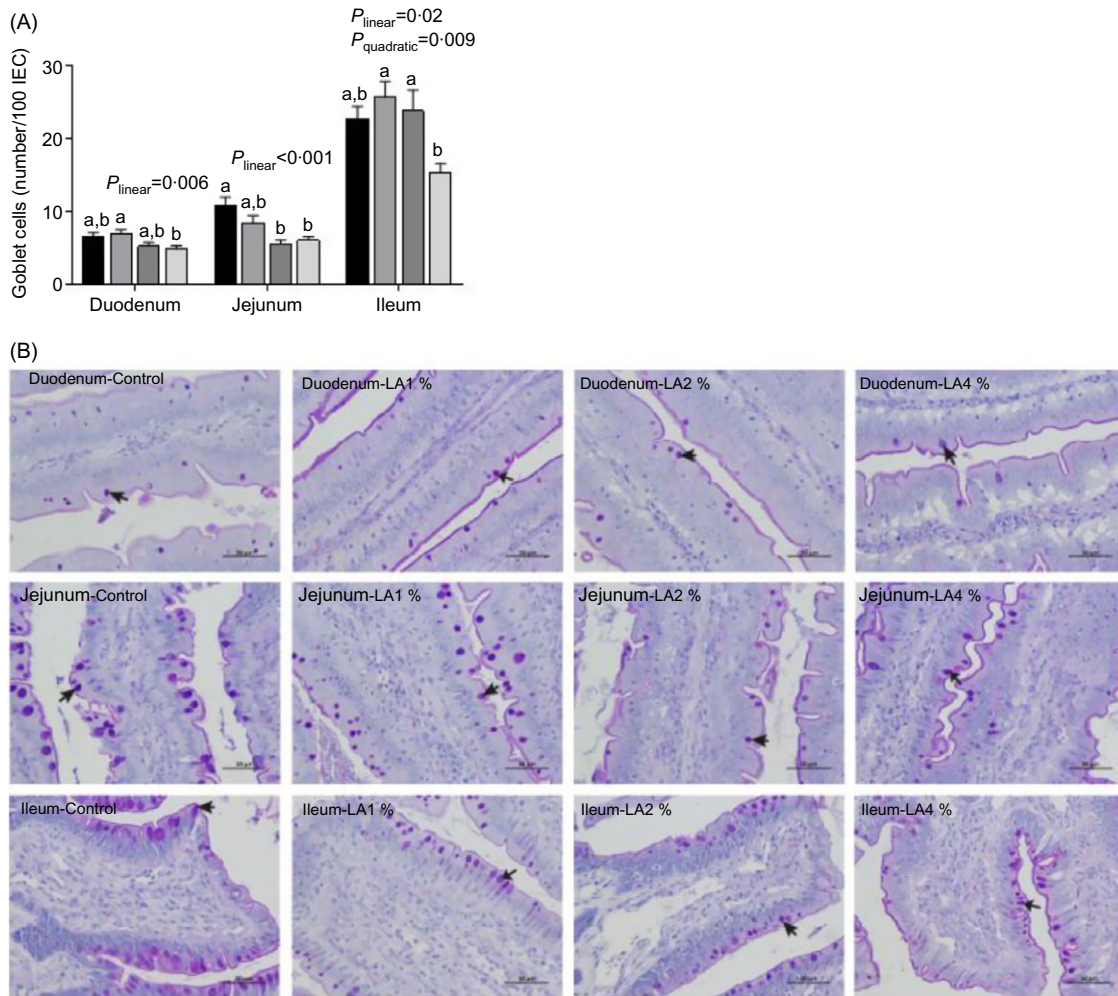
Ileal microbiota analyses of twenty-four samples were performed. An average of 33 218 raw sequences per sample was obtained. After trimming and assembling, an average of 29 573 valid sequences per sample remained. Furthermore, an average of 421 operational taxonomic units based on 97 % sequence similarity was identified from these sequences.

The effects of maternal dietary LA on the  $\alpha$ -diversity of ileal microbiota in domestic pigeon squabs are shown in Fig. 5. Rarefaction curves for the observed species (Fig. 5(A)) approached a plateau, indicating the availability of sufficient reads to reflect each microbiome community. The total operational taxonomic unit number of ileal contents was maximum in LA1 %, as was the unique operational taxonomic unit number (Fig. 5(B)). No significant difference was observed in the Chao1  $\alpha$ -diversity index among the four groups (Fig. 5(C)). The Shannon  $\alpha$ -diversity index was significantly increased in the LA1% and LA2 % compared with the control group, whereas there was no significant difference between the

control group and LA4 % (Fig. 5(D)). The effects of maternal dietary LA on the  $\beta$ -diversity of ileal microbiota in domestic pigeon squabs are shown in Fig. 6. As shown in Fig. 6(A), the microbial communities from each treatment were mainly divided into four groups. Clustering analysis at the phylum level is shown in Fig. 6(B). *Firmicutes* and *Proteobacteria* were the predominant phyla in the ileal microbiota, followed by *Actinobacteria*, *Cyanobacteria* and *Bacteroides*. All samples at the phylum level were not clustered into four groups. However, clustering analysis at the genus level (Fig. 6(C)) showed that samples at the genus level were clearly clustered into four groups, although one sample in LA4 % was clustered with LA2 %. *Lactobacillus* was the predominant genus in most samples. A cladogram representative of the dominant microflora, which were called biomarkers identified from phyla to genera, is shown in Fig. 7. It was observed that *Lactobacillus* was the biomarker of the control group and *Butyrivibrio* was the biomarker of LA1 % at the genus level. In addition, the biomarkers of LA2 % were *Aeriscardovia*, *Deinococcus*, *Streptococcus*, *Rhodopseudomonas*, *Methylobacterium*, *Sphingomonas*, *Ralstonia* and *Pseudomonas*. The biomarkers of LA4 % were *Chlorophyta*, *Enterococcus* and *Escherichia*.

### Discussion

The intestine plays a crucial role in nutrient digestion and absorption<sup>(27)</sup>. Intestinal functions are usually reflected by intestinal morphology, such as villus height, villus area and crypt depth<sup>(28)</sup>. In the present study, maternal dietary LA supplementation resulted in a

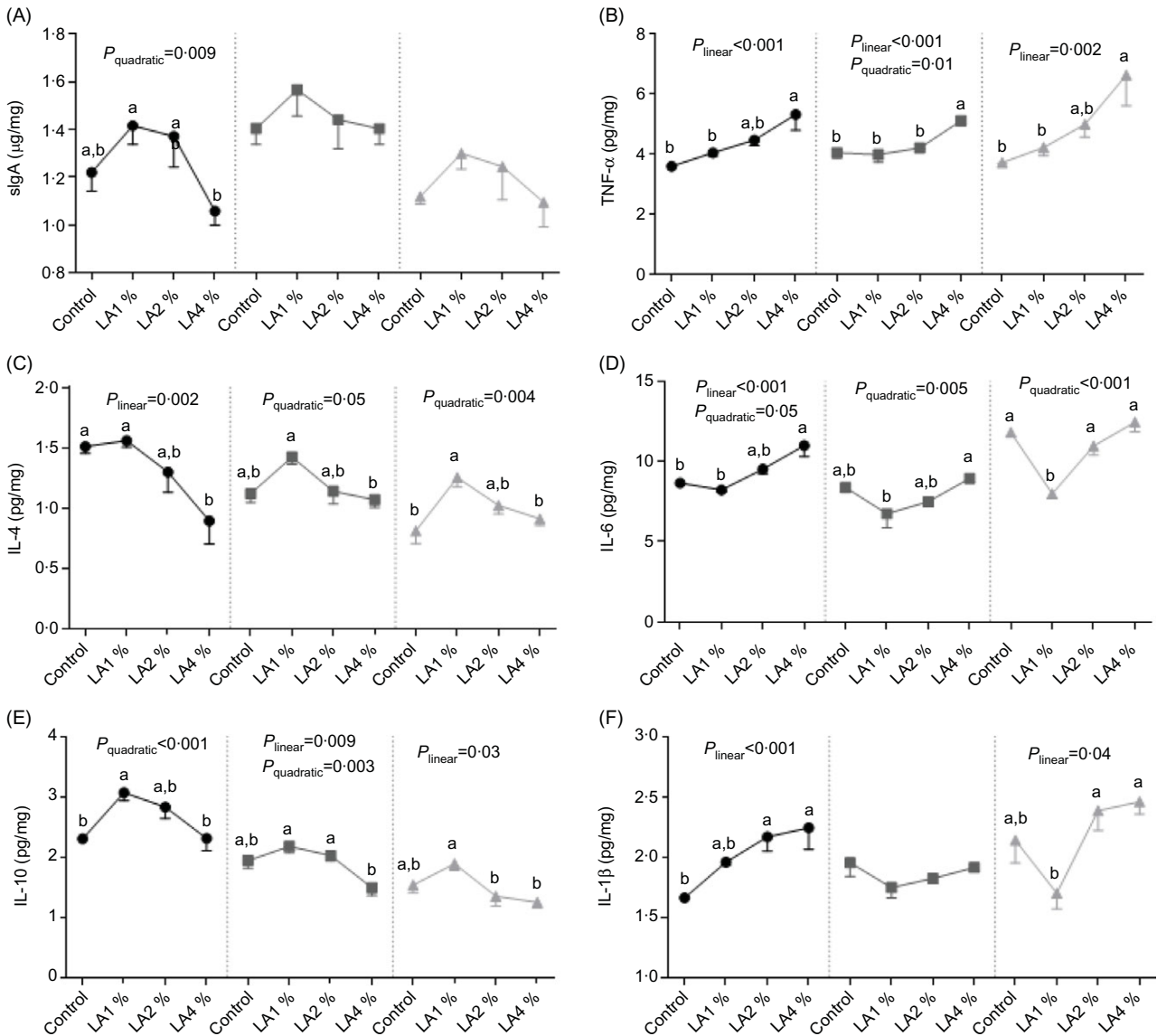


**Fig. 3.** Effects of maternal dietary linoleic acid on the density of intestinal goblet cells (GC) in domestic pigeon squabs. (A) Density of GC (per 100 intestinal epithelial cells; IEC), (B) periodic acid–Schiff staining of intestine (duodenum, jejunum and ileum) in the four treatment groups. The arrow points to GC. Bar = 50  $\mu$ m. Control = control group; LA1 % = 1 % linoleic acid supplementation group; LA2 % = 2 % linoleic acid supplementation group; LA4 % = 4 % linoleic acid supplementation group. Values are means with their standard errors of six squabs,  $n$  6. <sup>a,b</sup> Means with unlike letters are significantly different (Tukey test,  $P$  < 0.05). (A) ■, Control; ▒, LA1 %; ▓, LA2 %; □, LA4 %.

higher villus height and villus area compared with those of the control group regardless of intestinal site. In addition, the highest was observed in LA1 %. A previous study indicated that the increased villus area allowed animals to absorb more nutrients into the body<sup>(29)</sup>. The increase in villus height might be linked with the improved health status of animals<sup>(30)</sup>. Therefore, our results suggested that maternal dietary LA, especially at the 1 % level, could improve the intestinal morphology of squabs, indicating that their intestinal development was strengthened. A study by Thomson *et al.*<sup>(31)</sup> indicated that LA-deficient rats had significantly lower villus heights in the ileum than control rats, proving the importance of dietary LA. The higher the VCR, the higher the maturity and functional capacity of enterocytes<sup>(32)</sup>. In the present study, the VCR in both the duodenum and ileum was significantly increased in squabs by maternal dietary LA supplementation. The VCR in the jejunum was not significantly changed by LA supplementation because both villus height and crypt depth in the jejunum were significantly increased compared with the control. Taken together, LA enhanced the development of the duodenum and ileum more

obviously, and the effects of LA on the jejunum in pigeon squabs might be complicated. Because information about the relationship between LA and intestinal morphology in poultry is not available, further studies are needed to explore the mechanisms.

The intestinal epithelium serves as a front-line protective barrier between the host and the external environment against enteric pathogens, food antigens and physiochemical stresses<sup>(33,34)</sup>. Enterocytes that are closely connected with one another are the main components of the intestinal epithelium<sup>(35)</sup>. In our study, the density of enterocytes in the epithelial layer had no significant effect in the three intestinal tracts of squabs by maternal LA supplementation, meaning that the density of enterocytes was not affected by increased villus height. This result further confirmed that LA promoted villus growth. GC in the intestine play an indispensable role in mucosal protection mainly by secreting mucus that, together with water, Fe and peptides, forms a viscous gel reticular mucus layer overlying the epithelial cell surface<sup>(36)</sup>. The density of GC indicates the capability of mucus secretion<sup>(37)</sup>. In the present study, the maximum density of GC in the duodenum and ileum was observed in

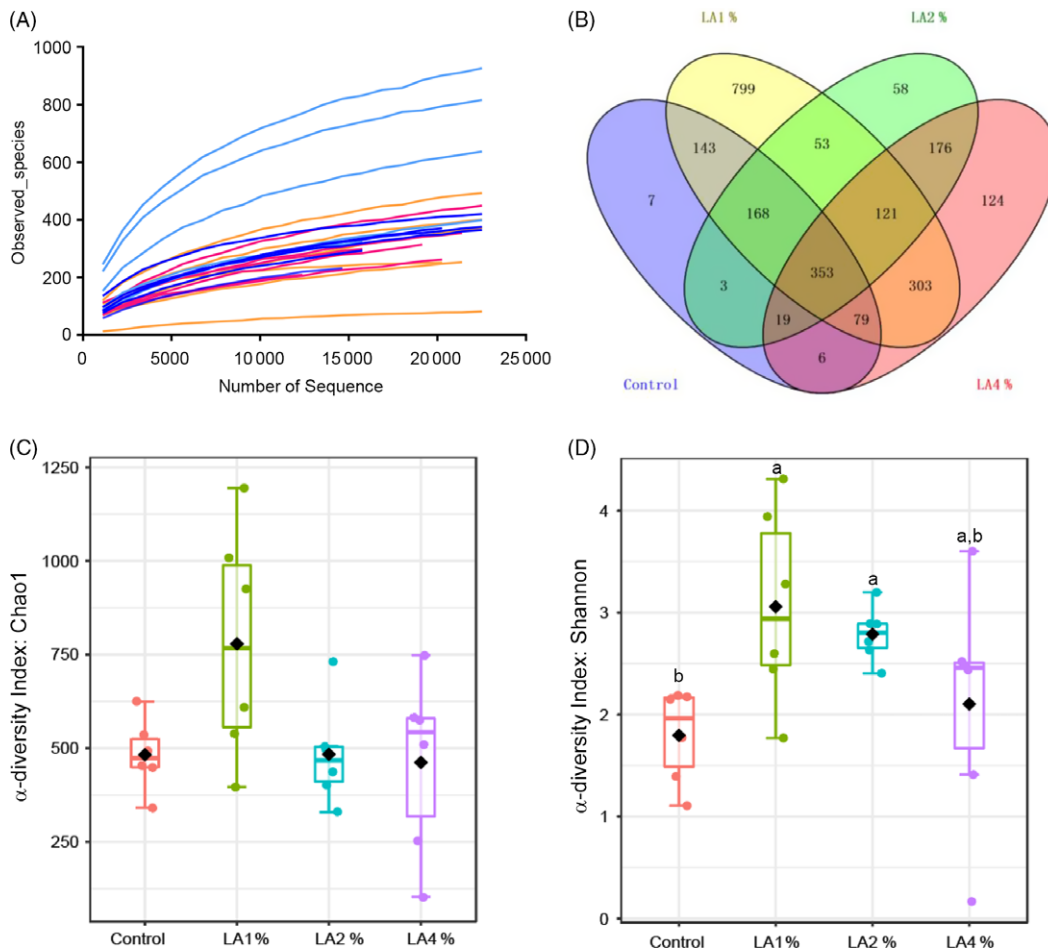


**Fig. 4.** Effects of maternal dietary linoleic acid on intestinal secretory IgA (slgA) and cytokine concentrations in domestic pigeon squabs. (A) slgA, (B) TNF- $\alpha$ , (C) IL-4, (D) IL-6, (E) IL-10, (F) IL-1 $\beta$ . Control = control group; LA1% = 1% linoleic acid supplementation group; LA2% = 2% linoleic acid supplementation group; LA4% = 4% linoleic acid supplementation group. Values are means with their standard errors of six squabs,  $n$  6. <sup>a,b</sup> Means with unlike letters are significantly different (Tukey test,  $P < 0.05$ ). (A–F) ●, Duodenum; ■, jejunum; ▲, ileum.

LA1%. The maximum GC density in the jejunum was found in the control, and the GC density of the jejunum in LA4% was significantly lower than that in the control. These results suggested that moderate maternal LA (1%) supplementation might be beneficial to GC formation and distribution in the duodenum and ileum, thus enhancing mucosal protection, whereas excessive LA (4%) supplementation might have the opposite effect in the jejunum. We conjectured that LA might alter the mucus layer of the intestine in pigeon squabs in a site-dependent and dose-dependent manner. Unfortunately, research regarding the influence of LA on the intestinal mucus layer was limited, and we have been unable to find any other study with which to confirm this result. Further studies are still needed to be conducted to verify this conjecture. Overall, GC density increased with distal progression along the intestine in squabs, which was similar to chickens<sup>(38)</sup>.

Tight junctions are present between individual enterocytes<sup>(39)</sup>. Tight junctions create a major barrier in the intercellular space, which regulates the movement of water and solutes across epithelia<sup>(40)</sup>. The protein composition of tight junctions includes claudins and occludins<sup>(22)</sup>. Claudins perform different functions and can roughly be divided into two types: those involved in barrier formation and those important in channel formation<sup>(41)</sup>. CLDN2 expression was associated with increased tight junction permeability and decreased tightness of the epithelial barrier<sup>(42)</sup>. In inflammatory bowel disease, CLDN2 expression correlated positively with inflammatory activity<sup>(43)</sup>. In the present study, the lowest mRNA expression of *CLDN2* was observed in LA1% regardless of the intestinal site, whereas *CLDN2* mRNA expression in the ileum in LA4% was significantly higher than that in the control group. The results indicated that a moderate maternal dietary level of LA (1%) could



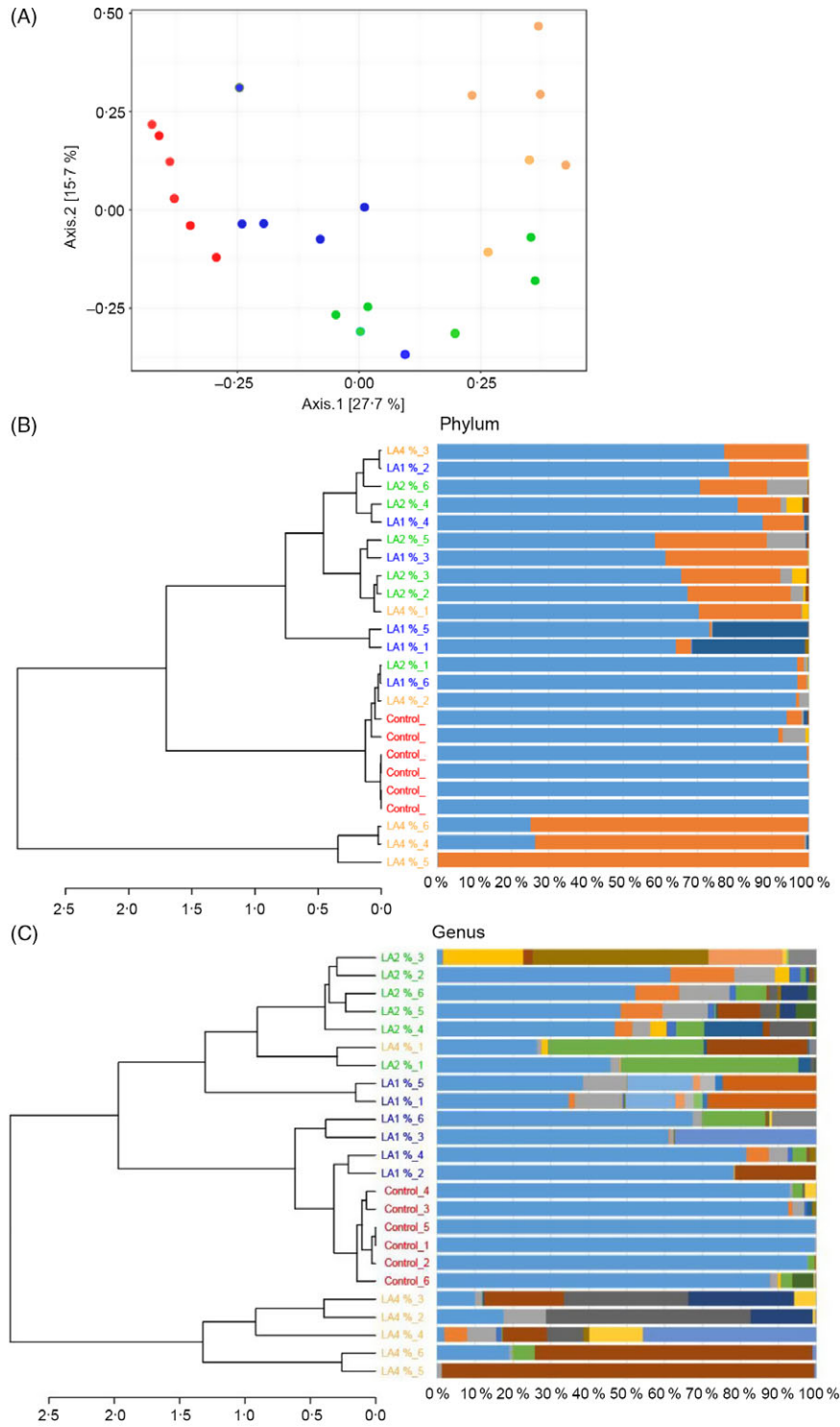


**Fig. 5.** Effects of maternal dietary linoleic acid on the  $\alpha$ -diversity of ileal microbiota in domestic pigeon squabs among groups. (A) Rarefaction curves, (B) operational taxonomic unit (OTU) distribution, (C)  $\alpha$ -diversity index: Chao1, (D)  $\alpha$ -diversity index: Shannon. Control = control group; LA1% = 1% linoleic acid supplementation group; LA2% = 2% linoleic acid supplementation group; LA4% = 4% linoleic acid supplementation group. Values are means with their standard errors of six squabs,  $n = 6$ . <sup>a,b</sup> Means with unlike letters are significantly different (Kruskal–Wallis test and Duncan’s test,  $P < 0.05$ ). (A) —, Control; —, LA1%; —, LA2%; —, LA4%. (C and D) —, Control; —, LA1%; —, LA2%; —, LA4%.

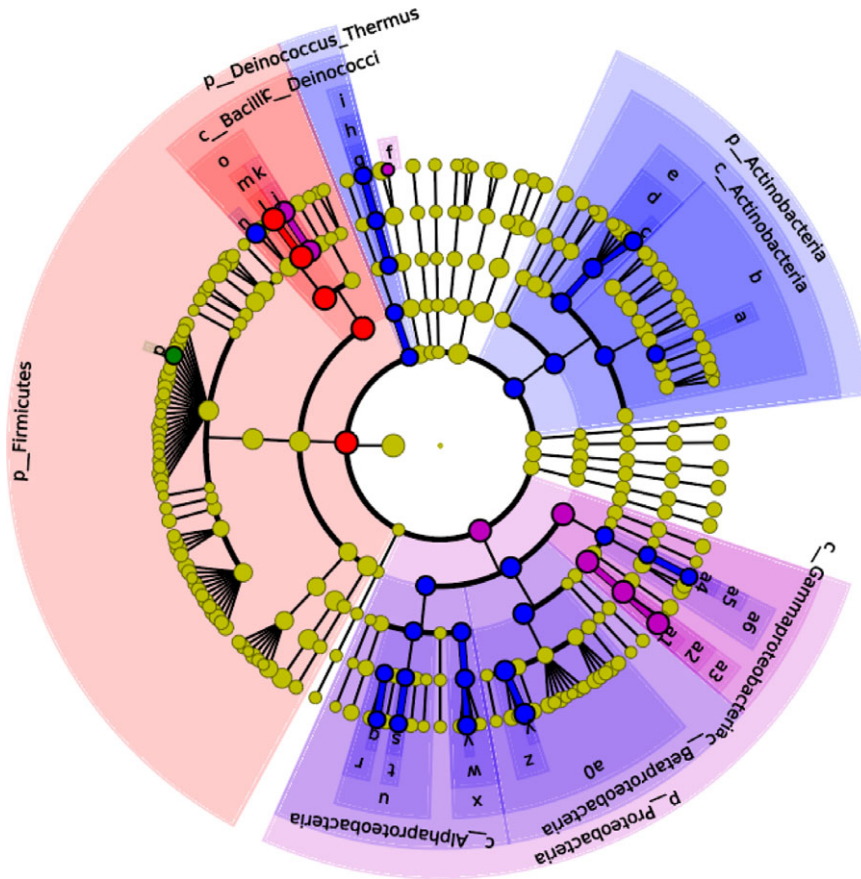
strengthen tight junctions by decreasing their permeability, while excessive LA supplementation might weaken tight junctions. A similar result was also observed in juvenile grass carp (*Ctenopharyngodon idella*)<sup>(44)</sup>. CLDN3 is known to be a ‘tightening’ claudin<sup>(45)</sup>. CLDN3 expression decreased in association with increased permeability in hens<sup>(46)</sup>. In the present study, the highest mRNA expression of *CLDN3* in the duodenum and ileum was observed in LA1%, whereas *CLDN3* mRNA expression in the ileum in LA4% was significantly lower than that in the control group. These results suggested that 1% LA supplementation in maternal diets could decrease permeability in squabs, but high-dose LA supplementation had adverse effects. The indication of the results was consistent with that of CLDN2. OCLN plays an important role in the assembly and maintenance of tight junctions<sup>(47)</sup>. In our study, *OCLN* mRNA expression was up-regulated in the ileum by supplementation with three LA levels. The result was inconsistent with that of CLDN2 and CLDN3, suggesting that different tight junction proteins had different expression patterns.

The intestine is not only a nutrient digestion and absorption centre but also an important place for immune function<sup>(48)</sup>. SIgA is a vital immune effector molecule on the intestinal mucosal

surface, which is the first line of defense against the adhesion of pathogenic bacteria in the intestinal mucosa<sup>(49)</sup>. In the present study, the sIgA in the duodenum was maximum in LA1%, which indicated that maternal dietary LA supplementation at 1% might enhance the ‘immune exclusion’ ability by stimulating the secretion of sIgA to some extent. Cytokines are important mediators and regulators of the host against foreign antigens, and their main function is to coordinate the functional activities of immune system cells<sup>(50)</sup>. IL-1 $\beta$ , TNF- $\alpha$  and IL-6 generated by macrophages are proinflammatory cytokines that exert multiple roles in both physiological and pathological conditions<sup>(51,52)</sup>. IL-4 and IL-10 are classified as anti-inflammatory cytokines because they prevent the production of proinflammatory cytokines such as IL-2 and IL-12 by stimulated monocytes/macrophages<sup>(53)</sup>. In our study, the IL-6 in the intestine was minimised by supplementation with 1% maternal dietary LA, and the IL-4 and IL-10 were maximised in this group, suggesting that 1% maternal dietary LA supplementation could partly reduce inflammation. The decreased IL-6 was consistent with the CLDN2 expression results since a previous study showed that IL-6 markedly induced CLDN2 expression, which is associated with increased tight



**Fig. 6.** Effects of maternal dietary linoleic acid on the  $\beta$ -diversity of ileal microbiota in domestic pigeon squabs among groups ( $n$  6). (A) Principal coordinates analysis, (B) clustering analysis at phylum level, (C) clustering analysis at genus level. Control = control group; LA1 % = 1 % linoleic acid supplementation group; LA2 % = 2 % linoleic acid supplementation group; LA4 % = 4 % linoleic acid supplementation group. (A) ●, Control; ●, LA1 %; ●, LA2 %; ●, LA4 %. (B) ■, p\_Firmicutes; ■, p\_Proteobacteria; ■, p\_Actinobacteria; ■, p\_Cyanobacteria; ■, p\_unclassified; ■, p\_Acidobacteria; ■, p\_Bacteroidetes; ■, p\_Deinococcus\_Thermus; ■, p\_Tenericutes; ■, p\_Verrucomicrobia. (C) ■, g\_Lactobacillus; ■, g\_Ralstonia; ■, others; ■, g\_Streptophyta; ■, g\_Sphingomons; ■, g\_Candidatus\_Arthromitus; ■, g\_Streptococcus; ■, g\_Escherichia; ■, g\_Enterococcus; ■, g\_Acinetobacter; ■, g\_Veillonella; ■, g\_Aeriscardovia; ■, g\_Lachnospiraceae\_unclassified; ■, g\_Ruminococcaceae\_unclassified; ■, g\_Butyrvibrio; ■, g\_Clostridium\_sensu\_stricto; ■, g\_Enterobacteriaceae\_unclassified; ■, g\_Lactococcus; ■, g\_Oscillibacter; ■, g\_Porphyromonadaceae\_unclassified; ■, g\_Turcibacter.



**Fig. 7.** Effects of maternal dietary linoleic acid on the microbial biomarkers of ileum in domestic pigeon squabs (*n* 6). Control = control group; LA1 % = 1 % linoleic acid supplementation group; LA2 % = 2 % linoleic acid supplementation group; LA4 % = 4 % linoleic acid supplementation group. Linear discriminant analysis effect size analysis shows differentially abundant genera as biomarkers determined using the Kruskal–Wallis test ( $P < 0.05$ ) with the logarithmic linear discriminant analysis score  $> 2.0$ . ■ Control; ■ LA1%; ■ LA2%; ■ LA4%; ■ a: f\_Microbacteriaceae; ■ b: o\_Actinomycetales; ■ c: g\_Aeriscardovia; ■ d: f\_Bifidobacteriaceae; ■ e: o\_Bifidobacteriales; ■ f: g\_Chlorophyta; ■ g: g\_Deinococcus; ■ h: f\_Deinococcaceae; ■ i: o\_Deinococcales; ■ j: g\_Enterococcus; ■ k: f\_Enterococcaceae; ■ l: g\_Lactobacillus; ■ m: f\_Lactobacillaceae; ■ n: g\_Streptococcus; ■ o: o\_Lactobacillales; ■ p: g\_Butyrvibrio; ■ q: g\_Rhodospseudomonas; ■ r: f\_Bradyrhizobiaceae; ■ s: g\_Methylobacterium; ■ t: f\_Methylobacteriaceae; ■ u: o\_Rhizobiales; ■ v: g\_Sphingomonas; ■ w: f\_Sphingomonadaceae; ■ x: o\_Sphingomonadales; ■ y: g\_Ralstonia; ■ z: f\_Burkholderiaceae; ■ a0: o\_Burkholderiales; ■ a1: g\_Escherichia; ■ a2: f\_Enterobacteriaceae; ■ a3: o\_Enterobacteriales; ■ a4: g\_Pseudomonas; ■ a5: f\_Pseudomonadaceae; ■ a6: o\_Pseudomonadales.

junction permeability, undermining the integrity of the intestinal barrier<sup>(42)</sup>. Overall, appropriate LA supplementation consolidated intestinal function. However, excessive (4%) LA supplementation might lead to adverse impacts. In the present study, IL-1 $\beta$  and IL-6 in the duodenum as well as TNF- $\alpha$  in the intestine of squabs in the LA4 % were significantly higher than those in the control group, whereas IL-4 in the duodenum was lower. A previous study showed that a higher than normal ratio of proinflammatory and anti-inflammatory factors promoted pathological changes in tissues<sup>(54)</sup>. Moreover, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 are expanded in inflammatory bowel disease<sup>(52)</sup>. These results suggested that 4% maternal dietary LA supplementation might enhance intestinal pathology.

The intestine contains an enormous number of micro-organisms primarily consisting of bacteria, which play a crucial role in animal health<sup>(55)</sup>. Dietary composition not only affects the intestinal epithelial barrier but also significantly influences the microbial community<sup>(56)</sup>. In our study, maternal dietary LA at 1 and 2 % increased the diversity of intestinal microbiota in squabs. It is

widely believed that the higher the diversity, the better the intestine health, because diversity is one of the key determinants of colonisation resistance against invading pathogens<sup>(57)</sup>. The results indicated that squabs had greater resistance in LA1 and LA2%. In addition, *Butyrvibrio* was the biomarker of LA1 %, which means that the relative abundance of *Butyrvibrio* was significantly higher than that of the other groups. The bacterial genus can produce butyrate which as a SCFA can lower the pH of the intestinal lumen and regulate the microbial composition especially to stimulate the growth of beneficial bacteria<sup>(58,59)</sup>. The results further revealed that the maintenance of intestinal health was strengthened in squabs by supplementing maternal dietary LA at 1%. However, the changes in the microbial community in LA2% were complicated. On the one hand, the biomarkers of LA2% included beneficial bacteria. Therein, *Aeriscardovia* belongs to *Bifidobacteria* which can protect against enteropathogenic infection through acetate production<sup>(60)</sup>. *Streptococcus* was observed to be negatively related to inflammation in previous research<sup>(61)</sup>. On the other hand, the

biomarkers of LA2 % also included opportunistic pathogens such as *Methylobacterium*, *Ralstonia* and *Pseudomonas*<sup>(62–64)</sup>. Thus, it was vital to maintain the intestinal lumen environment and the balance between these bacteria in LA2 %. Unfortunately, pathogenic *Escherichia* was observed in the biomarkers of LA4 %. Previous studies showed that inflammation caused by Crohn's disease and colitis was characterised by an increased abundance of *Escherichia* in the intestinal microbiota<sup>(65–67)</sup>. As mentioned above, 4% maternal dietary LA supplementation promotes inflammation. Thus, excessive (4%) LA supplementation might result in the breakdown of homeostasis between the mucosal immune system and the intestinal microbiota.

In conclusion, maternal dietary LA at all levels could improve intestinal morphology in squabs. Therein, appropriate dosage (1%) supplementation might enhance mucosal protection and epithelium barriers in squabs by promoting GC formation and distribution in the intestine, and strengthening tight junctions between enterocytes. Moreover, moderate supplementation consolidated intestinal immunity and the luminal microbial environment by partially reducing inflammation and promoting the growth of beneficial bacteria. However, unfortunately, excessive (4%) LA supplementation might lead to adverse impacts on immunity and microbiota. We conjectured that LA might alter intestinal barrier function in pigeon squabs in a dose-dependent manner. The reason might partly rely on that moderate LA satisfied squabs, whereas excessive addition impacted the balance between *n-6* and *n-3* PUFA, which need further study to verify.

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### Supplementary material

For supplementary materials referred to in this article, please visit <https://doi.org/10.1017/S0007114520004973>

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