Maternal Dietary Linoleic Acid Altered Intestinal Barrier Function in Domestic Pigeons (*Columba Livia*)

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Research

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Abstract

Background

Linoleic acid (LA) is predominantly essential for poultry. Deficiency of LA in poultry were manifested in various aspects such as retarded growth and reduced resistance to disease. The effects of LA on intestinal health in vitro and in mammals has been studied, whereas research related to the effects of LA on intestine health in poultry was scanty. Intestinal health and immune function play an important role in pigeon squab growth. Considering squabs are fed by their parents, the purpose of this study was to explore the effects of maternal dietary LA on intestinal barrier function in squabs by determining intestinal morphology, gene expression of tight junction protein, immune cytokines, and microbial flora.

Results

A completely randomized design with a control group, 1% dietary LA supplementation group, 2% dietary LA supplementation group, and 4% dietary LA supplementation group was used. Six squabs from each treatment were randomly sampled at 21 posthatch. Results indicated that LA supplementation improved intestinal morphology as reflected by increased villus height, villus area and the ratio of villus to crypt, and the promotion at dosage of 1% was most significant. Besides, 1% LA supplementation elevated distribution density of goblet cell in intestine, and strengthened tight junction between enterocytes by up-regulating claudin3 and occludin gene expression, but down-regulating claudin2 gene expression. Moreover, 1% LA supplementation reduced secretion of pro-inflammation cytokines and increased anti-inflammation cytokines partly. The diversity index Chao1 of intestinal microbiota in 1% LA supplementation group was higher than other groups. And *Butyrivibrio* as beneficial bacteria was the biomarker of LA1%. However, excessive (4%) LA supplementation led to adverse impact on intestinal immunity and microbiota.

Conclusions

Maternal dietary LA in three levels all could improve intestinal morphology in squabs. Therein, appropriate dosage (1%) supplementation might enhance mucosal protection and epithelium barrier in squabs, and furthermore consolidated intestine immunity and luminal microbial environment. However, excessive (4%) LA supplementation might lead to adverse impact on immunity and microbiota. Maternal dietary LA might alter intestinal barrier function in pigeon squabs in a dose-dependent manner.

Background

Meat of pigeon squabs can well be compared with that of broilers in terms of nutritive value and acceptability. Pigeon squabs have been considered profitable sources of poultry meat from *Columbia* species as they grow more rapidly with minimum input.[1] Squabs have a high growth rate which is attributed to the special crop milk feeding. Several fatty acids available in the crop milk are necessary for

normal growth.[2] Therein, linoleic acid (**LA**) is predominantly essential for poultry and its higher derivative arachidonate is the most important. [3–5]

The symptoms of LA deficiency in poultry were manifested in various aspects such as retarded growth, increased water consumption, enlarged liver with increased lipid content and reduced resistance to disease.[5] In addition, study stated that the LA-enriched diet enhanced growth in hens immunized with *M. butyricum* after immunization [6]. And maternal LA isomer regulated hepatic lipid metabolism via the AMPK signaling pathway in chick embryos [7]. Also, our group previously found parental dietary LA supplementation altered lipid metabolism and antioxidant status in pigeon squabs in dose-dependent manner [8].

Researches in vivo and in vitro have focused on the effects of LA on intestinal health and immune function over the years. Li et al. indicated that in Caco2 cells, LA up-regulated both mRNA and protein level of ApoA-IV which was produced dominantly in intestine and functioned anti-inflammation.[9, 10] And in Ht-29 cells, LA isomer inhibits cell proliferation and stimulates apoptosis [11]. Moreover, In human, duodenal ulcer was associated with deficiency of dietary LA intake [12]. And in Interleukin-10-deficient mice, a LA metabolite ameliorated colonic inflammation [13]. Study by Rodrigues et al. also indicated that LA might affect the course of inflammation [14]. However, research related to the effects of LA on intestine health in poultry was scanty.

Consider that the crop milk produced by parent pigeons and regurgitated to squabs is gradually mixed with increasing grain particles, intestinal health and immune function play an important role in squab growth [15, 16]. Therefore, the purpose of this study was to explore the effects of maternal dietary LA on intestinal barrier function in squabs by determining intestinal morphology, gene expression of tight junction protein, immune cytokines, and microbial flora.

Materials And Methods

Birds and conditions

A total of 240-pair parent White King pigeons, 60 wk 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 6 replications of 10-pair pigeons each. The four treatments are: control group, 1% LA supplementation group (**LA1%**), 2% LA supplementation group (**LA2%**), 4% LA supplementation group (**LA4%**). The LA (≥99.0% purity) was obtained from a commercial supplier (Hebei bawei biotechnology co., LTD, Handan, China). All experimental diets were isonitrogenous and isocaloric. The LA source was substituted for colleseed oil by the same weight to equalize the total fat level among diets [17]. The ingredients, nutrient levels and analyzed LA content of experimental diets for parent pigeons were shown in Table 1. The fatty acid composition of each diet analyzed according to Xie et al. [18] were shown in Additional file 1. The

pigeons were given to water *ad libitum* and were fed twice daily (7:00 a.m. and 3:00 p.m.) throughout the experiment.

Table 1

Ingredient compositions and nutrient levels of experimental diets¹ for maternal pigeons.² (on as-fed basis)

Items	Control	LA1%	LA2%	LA4%
Ingredients of a whole-grain form feed (%)				
Corn	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
Colleseed 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
Calculated nutrients ³ (%)				
Metabolizable energy (MJ/kg) ⁴	13.84	13.84	13.84	13.84
Crude protein	12.63	12.63	12.63	12.63
Analyzed linoleic acid	1.82	2.75	3.68	5.53
Ingredients of grit meal (%)				
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 sulfate (monohydrate)	0.23	0.23	0.23	0.23
Premix ⁵	3.28	3.28	3.28	3.28
Total	100.00	100.00	100.00	100.00
Calculated nutrients ² (%)				
Calcium	27.79	27.79	27.79	27.79
Phosphorus	0.01	0.01	0.01	0.01
Sodium	0.03	0.03	0.03	0.03
Chlorine	0.59	0.59	0.59	0.59

Items	Control	LA1%	LA2%	LA4%
Ferrum	0.30	0.30	0.30	0.30
¹ Control = control group; LA1% = 1% linoleic acid addition group; LA2% = 2% linoleic acid addition group; LA4% = 4% linoleic acid addition group.				
² All feeds were fed in a whole-grain form at 07:00 and 15:00 a day, and grit meal was offered to the birds on a continuous basis.				
³ Nutrient values were calculated from tables of feed composition and nutritive values in China (twenty-eighth edition, 2017).				
⁴ Metabolizable energy values determined in pigeons were calculated from those reported for chickens in accordance with a previous study (Hullar et al., 1999), which observed that the metabolizable energy values of feed in pigeons did not differ significantly from those in chickens.				
⁵ The premix provided the following per kg of diet: Vitamin A 5000 IU, Vitamin E 50 IU, Vitamin D ₃ 2000 IU, copper sulfate 15 mg, manganese sulfate 45 mg, zinc sulfate 90 mg.				

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

Sample collection

On the day 21 posthatch, six squabs per treatment (one for each replication) were selected for sampling. The squabs were fasted for 12 h before weighing and slaughter. And they were sedated before cervical dislocation. Ileal contents were sampled into sterile plastic tubes with a sterile spatula, immediately frozen in liquid nitrogen, then stored at -80 °C for subsequent analyses. Three segments of the small intestine (duodenum, jejunum and ileum) were sampled, flushed with 0.9% saline to remove all the content, and processed for morphological examination. The collected segments were the loop of the duodenum (duodenum), the tract before Meckel's diverticulum (jejunum), the final segment of small intestine (ileum). The mucosa of each intestinal segment was scraped off with a glass slide carefully, frozen in liquid nitrogen rapidly, and then stored at -80 °C for subsequent analyses.

Secretory IgA and cytokine analysis

The homogenates of intestinal mucosa were prepared with phosphate buffered saline for secretory IgA (slgA) and cytokine analyses. The slgA concentration and the concentrations of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), 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, Shanghai, China) according to the manufacture's protocol. The final slgA and cytokine concentrations were expressed as units per milligram of protein.

RNA extraction and quantitative PCR analysis

Total RNA of intestinal mucosa was extracted using TRIzol procedure (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. The extracted RNA was quantified by NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). The RNA integrity was verified by native RNA electrophoresis on 1.0% agarose gel. The complementary DNA was synthesized from 2- μ g total RNA by M-MLV reverse transcriptase (Takara, Dalian, China) with oligo dT-adaptor primer 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, Foster City, CA). The specific primers used were indicated in Table 2. The SYBR Green Realtime PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) was used for PCR reaction, consisting of an initial DNA denaturation of 95 °C for 60 s, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. The GAPDH was considered as an appropriate endogenous reference. The average gene expression relative to the endogenous reference for each sample was calculated according to the $2^{-\Delta\Delta C_t}$ method [19]. The calibrator for each gene in study was the average ΔC_t value of control group.

Table 2

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

Gene ¹	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	GTGGAAGAGCTTGTTGCGT		
GAPDH	GAPDH-F	CCCAATGTCTCTGTTGTGG	116	NM_001282835.1
	GAPDH-R	TATGCCAGGATGCCCTT		

¹CLDN2 = Claudin2; CLDN3 = Claudin 3; OCLN = Occludin; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

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 µm) were placed on glass slides and submitted to two different staining. The hematoxylin and eosin staining was for the identification of villus height, villus area, crypt depth, and enterocyte. The periodic-acid Schiff staining was for the identification of goblet cell (**GC**). The examination was performed with light microscopy (Nikon Corp., Tokyo, Japan) using the Image-pro plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). The density of enterocyte was expressed as number per millimeter of epithelial layer length. The density of GC was expressed as number per 100 enterocytes according to Tamura et al. [20].

16S rDNA high-throughput sequencing

The 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, USA). The concentration and quality of the extracted DNA were assessed with 2% agarose gel electrophoresis and a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). The V3-V4 hyper-variable region of the 16S rRNA genes was amplified by specific degenerate primers (338F: ACTCCTACGGGAGGCAGCAG, 806R: GGACTACHVGGGTWTCTAAT). The PCR program was conducted following the 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, Danvers, MA, USA). Qubit was used to quantify the purified PCR products, and the qualified library concentration should be more than 2 nM. Sequencing was performed with V3 chemistry and generated 2 × 300 bp double-end reads using MiSeq Reagent Kit V3 with the MiSeq Illumina platform (Illumina, Santa Clara, CA, USA) at Hangzhou Personal Biotechnology Co., Ltd. (Hangzhou, China).

Statistical analyses

The data obtained from sIgA and cytokine analysis, quantitative PCR analyses, and intestinal morphological examination were subjected to one-way analysis of variance in SPSS 24.0 (SPSS Inc., Chicago, USA) for Windows. The significance level was set at $P < 0.05$. When significant differences were found, Tukey post-hoc tests were performed. Plotting was placed with GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA).

The raw sequence data obtained from the Illumina MiSeq platform were quality-filtered and demultiplexed using Quantitative Insights into Microbial Ecology (**QIIME**), 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 (**OTUs**) using the UCLUST function in QIIME (http://qiime.org/scripts/pick_otus) according to a 97% similarity threshold. The data were categorized

with the Ribosomal Database Project (**RDP**), 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>). Venn diagram was performed in Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>). Principal coordinate analysis (**PCoA**) was conducted using Galaxy (<http://huttenhower.sph.harvard.edu/galaxy/>) according to Unweighted Unifrac representing Beta diversity values. Kolmogorov-Smirnov program is used to verify the normality of data. Data in line with normal distribution were counted by one-way ANOVA, and multiple comparisons were conducted by Tukey post-hoc tests. Nonnormal distribution data were analyzed by Kruskal-Wallis test and Duncan's multiple range tests. $P < 0.05$ means significant difference.

Results

Intestinal morphometric trait

Hematoxylin and eosin staining of intestine (duodenum, jejunum, and ileum) in the four treatment groups has shown the effects of maternal dietary LA on the intestinal morphometric trait in squabs (Fig. 1). In duodenum, the villus height, villus area and ratio of villus height to crypt depth (**VCR**) responded to increasing supplementation of LA in linear ($P < 0.01$, $P = 0.01$ and $P < 0.01$, respectively) and quadratic (all $P < 0.01$) manner; their maximal response were observed in LA1%. In jejunum, the villus height and villus area emerged linear ($P < 0.01$ and $P = 0.01$, respectively) and quadratic (both $P < 0.01$) trends with their maximum observed in LA1% as LA supplementation was increased. While the crypt depth showed a linear ($P < 0.01$) increase with the level of LA supplementation increasing. In ileum, the villus height and VCR responded to increasing supplementation of LA in linear (both $P < 0.01$) and quadratic (both $P < 0.01$) manner, and their maximum were observed in LA1%. The villus area displayed a quadratic trend with the maximum in LA1% as LA supplementation was increased. In addition, the VCR in jejunum, crypt depth in duodenum and ileum had no significance among four groups. There was no significance ($P > 0.05$) in enterocyte density among four experimental groups.

Tight junction protein gene expression

Effects of maternal dietary LA on the mRNA expression of three tight junction proteins have shown in Fig. 2. In duodenum, the mRNA expression of Claudin 3 (**CLDN3**) emerged a quadratic ($P = 0.03$) trend with the maximum observed in LA1% as the LA supplementation was increased. Claudin 2 (**CLDN2**) mRNA expression responded to increasing supplementation of LA in linear ($P = 0.02$) and quadratic (both $P < 0.01$) manner with the minimum in LA1%. Although maternal dietary LA had no significant effect ($P > 0.05$) on mRNA expression of Occludin (**OCLN**), a quadratic ($P = 0.01$) trend was observed with the maximum response in LA2%. In jejunum, the mRNA expression of CLDN2 emerged a quadratic ($P < 0.01$) trend with the minimum in LA1% as the supplementation of LA was increased. However, CLDN3 and OCLN in jejunum had no significance ($P > 0.05$) among four treatments. In ileum, CLDN2 mRNA

expression displayed linear ($P < 0.01$) and quadratic ($P < 0.01$) trends with the maximum observed in LA4% as the LA supplementation was increased. Moreover, CLDN3 and OCLN mRNA expression responded to increasing supplementation of LA in linear (both $P < 0.01$) and quadratic (both $P < 0.01$) manner; their maximal response were observed in LA1%.

Goblet cell density

Periodic-acid Schiff staining of intestine (duodenum, jejunum, and ileum) in the four treatment groups has shown effects of maternal dietary LA on the density of GC (Fig. 3). The GC density in duodenum and jejunum responded to increasing supplementation of LA linearly (both $P < 0.01$), with the maximum observed in LA1% and Control, respectively. The GC density in ileum displayed linear ($P = 0.02$) and quadratic ($P < 0.01$) trends with the maximum density in LA1%.

SlgA and cytokine analysis

Effects of maternal dietary LA on the concentrations of slgA and cytokines have shown in Fig. 4. In duodenum, slgA and IL-10 concentrations responded to increasing LA supplementation quadratically (both $P < 0.01$) with their maximum observed in LA1%. IL-6 concentration emerged linear ($P < 0.01$) and quadratic ($P < 0.05$) trends with the minimum in LA1% as the LA supplementation was increased. TNF- α and IL-1 β concentrations increased linearly (both $P < 0.01$) with LA supplementation increasing, whereas IL-4 concentration decreased linearly ($P < 0.01$). In jejunum, TNF- α concentration emerged linear ($P < 0.01$) and quadratic ($P = 0.01$) trend with the minimum in LA1% as the LA supplementation was increased. While IL-10 concentration emerged linear ($P = 0.01$) and quadratic ($P < 0.01$) trend with the maximum in LA1% as the LA supplementation was increased. IL-6 concentrations responded to increasing LA supplementation quadratically ($P < 0.01$) with the minimum observed in LA1%. While IL-4 concentration responded to increasing LA supplementation quadratically ($P < 0.05$) with their maximum observed in LA1%. There was no significance ($P > 0.05$) in slgA concentrations in jejunum among four experimental groups. In ileum, TNF- α and IL-1 β concentrations increased linearly ($P < 0.01$ and $P = 0.04$, respectively) with LA supplementation increasing, whereas IL-10 concentration decreased linearly ($P = 0.03$). IL-4 concentration responded to increasing LA supplementation quadratically ($P < 0.01$) with their maximum observed in LA1%. While IL-6 concentration responded to increasing LA supplementation quadratically ($P < 0.01$) with their minimum observed in LA1%. There was no significance ($P > 0.05$) in slgA concentration in ileum among four experimental groups.

Ileal microbiota analysis

The ileal microbiota analyses of 24 samples were performed. An average of 33218 raw sequences per sample was obtained. After trimming and assembling, an average of 29573 valid sequences per sample

was remained. Further, an average of 421 OTUs based on a 97% sequence similarity was identified from these sequences.

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. 5a) approached a plateau, indicating the availability of sufficient reads to reflect each microbiome community. The total OTU number of ileal contents was maximum in LA1%, and so was the unique OTU number (Fig. 5b). No significant difference was observed in alpha diversity index Chao1 among four groups (Fig. 5c). The alpha diversity index Shannon was significant increased in LA1% and LA2% compared with control group, whereas there was no significance between control group and LA4% (Fig. 5d). 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. 6a, the microbial communities from each treatment were mainly divided into 4 groups. Clustering analysis in phylum level was shown in Fig. 6b. *Firmicutes* and *Proteobacteria* were predominant phyla in ileal microbiota, followed by *Actinobacteria*, *Cyanobacteria* and *Bacteroides*. All samples in phylum level were not clustered into four groups. However, clustering analysis in genes level (Fig. 6c) showed that samples in genes level were clearly clustered into four groups, though one sample in LA4% was clustered with LA2%. *Lactobacillus* was predominant genes in most samples. A cladogram representative of the dominant microflora called biomarkers identified from phyla to genera was shown in Fig. 7. It was observed that *Lactobacillus* was the biomarker of control group and *Butyrivibrio* was the biomarker of LA1% in genes level. Besides, 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 played crucial role in nutrient digestion and absorption [21]. And intestinal functions were usually reflected by intestinal morphology such as villus height, villus area and crypt depth [22]. In the present study, maternal dietary LA supplementation resulted in higher villus height and villus area compared with those of control group whatever intestinal site. And the highest was observed in LA1%. Previous study indicated that the increased villus area allowed animals to absorb more nutrients into the body [23]. And the increase in villi height might be linked with improved health status of animals [24]. therefore, our results suggested that maternal dietary LA especially at 1% level could improve the intestinal morphology of squabs, indicating that their intestinal development was strengthened. Study by Thomson et al. indicated that LA-deficiency rats had significantly lower villus height in ileum than control, proving the importance of dietary LA [25]. The higher the VCR, the higher the maturity and functional capacity of enterocyte [26]. In the current study, the VCR both in duodenum and ileum were significantly increased in squabs by maternal dietary LA supplementation. While VCR in jejunum had no significant change by LA supplementation because the crypt depth in jejunum was significant increased compared with control. Taken together, LA enhanced the development of duodenum and ileum more obviously and the effects of LA on jejunum in pigeon squabs might be complicated. In view of 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 served as the front protective barrier between the host and the external environment against enteric pathogens, food antigens, and physiochemical stresses [27, 28]. Enterocytes which were closely connected with one another were the main components of intestinal epithelium [29]. In our study, the density of enterocytes in epithelial layer had no significant effect in three intestinal tracts of squabs by maternal LA supplementation, meaning the density of enterocytes was not affected by increased villus height. This result further confirmed that LA promoted villi growth. GC in intestine played an indispensable part in mucosal protection mainly by secreting mucus that together with water, ions and peptides formed a viscous gel reticular mucus layer overlying epithelial cell surface [30]. And the density of GC indicated the capability of mucus secretion [31]. In the present study, the maximum density of GC in duodenum and ileum were observed in LA1%. While the maximum in jejunum was in control, and the GC density of jejunum in LA4% was significantly lower than that in control. These results suggested that moderate maternal LA (1%) supplementation might be beneficial to GC formation and distribution in duodenum and ileum thus enhancing mucosal protection, whereas excessive LA (4%) supplementation might have the opposite effect in jejunum. We conjectured that LA might alter mucus layer of intestine in pigeon squabs in a site-dependent and dose-dependent manner. As a whole, GC density increased with distal progression along the intestine in squabs, which was similar with chicken [32].

Between the individual enterocyte presented tight junctions [33]. The tight junctions acted as a regulatory barrier in the intercellular space and maintain a palisade between the apical and basolateral domains of the plasma membrane [34]. The protein composition of tight junctions included claudins and occludins [33]. Claudins performed different functions and could roughly be divided into two types, those involved in barrier formation, and those important in channel formation [35]. Therein, CLDN2 expression was associated with increased tight junction permeability, and decreased the tightness of the epithelial barrier [36]. In inflammatory bowel disease, CLDN2 expression correlated positively with inflammatory activity [37]. In the current study, lowest mRNA expression of CLDN2 were observed in LA1% whatever intestinal site, whereas CLDN2 mRNA expression of ileum in LA4% was significantly higher than control group. The results indicated that moderate maternal dietary level (1%) of LA could strengthen tight junction by decreasing its permeability. While excessive LA supplementation might weaken tight junction. CLDN3 was known to be a “tightening” claudin [38]. And CLDN3 expression decreased in association with increased permeability in hens [39]. In the present study, highest mRNA expression of CLDN3 in duodenum and ileum were observed in LA1%, whereas CLDN3 mRNA expression of ileum in LA4% was significantly lower than control group, suggesting similar effects with the results of CLDN2 mRNA expression. OCLN played an important role in the assembly and maintenance of tight junctions [40]. In our study, OCLN mRNA expression was up-regulated in ileum by supplementation of three LA levels. The result was inconsistent with that of CLDN2 and CLDN3, suggesting that different tight junction proteins had different expression pattern. And LA might regulate tight junction of intestine in pigeon squabs in a dose-dependent manner and in an intestinal site-specific pattern.

Intestine is not only the nutrients digestion and absorption centers, but also an important place to play immune function [41]. 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 [42]. In present study, the sIgA in 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 host against foreign antigens, and their main function is to coordinate the functional activities of immune system cells [43]. IL-1 β , TNF- α , and IL-6 generated by macrophages were pro-inflammatory cytokines that exert multiple roles in both physiological and pathological conditions [44, 45]. 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/macrophage [46]. In our study, the IL-6 in intestine was minimum by supplementation with 1% maternal dietary LA, and the IL-4 and IL-10 were maximum in this group, suggesting that 1% maternal dietary LA supplementation could reduce inflammation partly. And the decreased IL-6 was consistent with the result of CLDN2 expression since previous study has shown that IL-6 markedly induced CLDN2 expression which is associated with increased tight junction permeability, undermining the integrity of the intestinal barrier [47]. Overall speaking, appropriate LA supplementation consolidated intestinal function. However, excessive (4%) LA supplementation might lead to adverse impact. In the present study, IL-1 β and IL-6 in duodenum as well as TNF- α in intestine of squabs in LA4% were significantly higher than control group, whereas IL-4 in duodenum was lower. A previous study displayed that higher than normal ratio of pro-inflammatory and anti-inflammatory factors promoted pathological changes in tissues [48]. Moreover, IL-1 β , TNF- α , and IL-6 were expanded in the inflammatory bowel disease [45]. These results suggested that 4% maternal dietary LA supplementation might enhance the intestinal pathology.

Intestine contains a enormous amount of microorganisms primarily consisting of bacteria, which plays a crucial part in animal health [49]. Dietary composition not only affects the intestinal epithelial barrier, but also significantly influences the microbial community [50]. 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 the diversity is one of the key determinants of colonization resistance against invading pathogens [51]. The result indicated that squabs had greater resistance in LA1% and LA2%. In addition, *Butyrivibrio* was the biomarker of LA1%, which means the relative abundance of *Butyrivibrio* was significantly higher compared with other groups. The bacteria genes can produce butyrate which as a short-chain fatty acid can lower the pH of intestinal lumen and regulate the microbial composition especially stimulate the growth of beneficial bacteria [52, 53]. The result further revealed the maintaining of intestine health was strengthened in squabs by supplementing maternal dietary LA at 1%. However, the changes of microbial community in LA2% was complicated. On the one hand, the biomarkers of LA2% included beneficial bacteria. Therein, *Aeriscardovia* belongs to *Bifidobacteria* which can protect from enteropathogenic infection through acetate production [54]. And *Streptococcus* was observed to be negatively related with inflammation in previous research [55]. While on the other hand, the biomarkers of LA2% also included opportunistic pathogens such as

Methylobacterium, *Ralstonia* and *Pseudomonas* [56–58]. 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 characterized by the increased abundance of *Escherichia* in intestinal microbiota [59–61]. And as mentioned above, 4% maternal dietary LA supplementation promote inflammation. Thus, excessive (4%) LA supplementation might result in breakdown of homeostasis between mucosal immune system and the intestinal microbiota.

Conclusion

In conclusion, maternal dietary LA in three levels all could improve intestinal morphology in squabs. Therein, appropriate dosage (1%) supplementation might enhance mucosal protection and epithelium barrier in squabs by promoting GC formation and distribution in intestine, and strengthening tight junction between enterocytes. Moreover, moderate supplementation consolidated intestine immunity and luminal microbial environment by reducing inflammation partly, and promoting the growth of beneficial bacteria. However, unfortunately, excessive (4%) LA supplementation might lead to adverse impact 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 ω -6 and ω -3 polyunsaturated fatty acids, which need further study to verify.

Abbreviations

LA: linoleic acid

LA1%: 1% dietary LA supplementation group

LA2%: 2% dietary LA supplementation group

LA4%: 4% dietary LA supplementation group

slgA: secretory IgA

TNF- α : tumor necrosis factor- α

IL: interleukin

GC: goblet cell

QIIME: Quantitative Insights into Microbial Ecology

OTU: operational taxonomic unit

RDP: Ribosomal Database Project

PCoA: Principal coordinate analysis

VCR: ratio of villus height to crypt depth

CLDN3: Claudin 3

CLDN2: Claudin 2

OCLN: Occludin

Declarations

Ethics approval and consent to participate

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

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

The article was mainly conceived and designed by XD and XZ. QX performed the experiments and analyzed the data. The manuscript was mainly written by QX. XD and XZ revised it critically for important content. All authors have read and approved the final manuscript.

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Figures

Figure 1

Effects of maternal dietary 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 enterocyte, f Hematoxylin and eosin staining of intestine (duodenum, jejunum, and ileum) in the four treatment groups. The arrow points to enterocyte. Bar = 100 μ 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 $\pm$ SEM of 6 squabs, n=6. a,b,c Means sharing no common superscripts differ significantly (Tukey test, $P < 0.05$).

Figure 2

Effects of maternal dietary linoleic acid on mRNA expression of tight junction proteins in domestic pigeon squabs. a mRNA expression of Claudin2 (CLDN2), b mRNA expression of Claudin3 (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 $\pm$ SEM of 6 squabs, n=6. a,b,c Means sharing no common superscripts differ significantly (Tukey test, $P < 0.05$).

Figure 3

Effects of maternal dietary linoleic acid on the density of intestinal goblet cell (GC) in domestic pigeon squabs. a Density of GC, b Periodic-acid Schiff staining of intestine (duodenum, jejunum, and ileum) in the four treatment groups. The arrow points to GC. Bar = 50 μ 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 $\pm$ SEM of 6 squabs, n=6. a,b Means sharing no common superscripts differ significantly (Tukey test, $P < 0.05$).

Figure 4

Effects of maternal dietary linoleic acid on intestinal sIgA and cytokine concentrations in domestic pigeon squabs. a sIgA, b TNF-α, c IL-4, d IL-6, e IL-10, f IL-1β. Control = control group; LA1% = 1% linoleic acid supplementation group; LA2% = 2% linoleic acid supplementation group; LA4% = 4% linoleic acid supplementation group. Values are means ± SEM of 6 squabs, n=6. a,b Means sharing no common superscripts differ significantly (Tukey test, $P < 0.05$).

Figure 5

Effects of maternal dietary linoleic acid on the alpha diversity of ileal microbiota in domestic pigeon squabs among groups. a Rarefaction curves, b 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 $\pm$ SEM of 6 squabs, n=6. a,b Means sharing no common superscripts differ significantly (Kruskal-Wallis test and Duncan's test, $P < 0.05$).

Figure 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 in phylum level, c Clustering analysis in genes level. Control = control group; LA1% = 1% linoleic acid supplementation group; LA2% = 2% linoleic acid supplementation group; LA4% = 4% linoleic acid supplementation group.

Figure 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 Kruskal-Wallis test ($P < 0.05$) with the logarithmic linear discriminant analysis score > 2.0 .

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