The interactive effect of dietary n-6: n-3 fatty acid ratio and vitamin E level on tissue lipid peroxidation, DNA damage in intestinal epithelial cells, and gut morphology in chickens of different ages

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ABSTRACT Feeding chickens diets high in n-3 fatty acids (**FA**) increases their incorporation into tissue lipids, but leads to oxidative stress in cells. This study investigated the effect of the dietary polyunsaturated FA ratio (**PUFA n-6: n-3**) and vitamin E (**vE**) level on DNA damage and morphological changes in the gut epithelium of chickens. One-day-old female broiler chicks (n = 176) were divided into 4 groups fed for 43 d diets with a high (**HR**) or low (**LR**) PUFA n-6: n-3 ratio and supplemented with 50 or 300 mg vE kg^{-1}. Performance was calculated for periods of d 1 to 9, d 9 to 16, d 9 to 35, and d 9 to 42, while organs were sampled at d 9, d 17, d 36, and d 43. At d 17 and d 43, DNA damage of epithelial cells in the duodenum and jejunum was measured and duodenal and jejunal morphology was analyzed. HR diets improved FCR for the periods of d 1 to 9, d 9 to 16 and d 9 to 42, whereas the increased vE level improved FCR for the period of d 9 to 16. In the jejunum DNA damage was greater in chickens fed LR than HR diets at d 17 ($P < 0.001$) and the increased vE level promoted DNA damage in both intestinal segments ($P < 0.02$) in younger birds. The morphology of the duodenum was marginally affected by the diets, whereas LR diets in the jejunum reduced villus surface area at d 17 ($P = 0.022$), and mucosa thickness ($P = 0.029$) and villus height ($P = 0.035$) at d 43. The results indicated that feeding birds LR diets and vE levels significantly exceeding the recommendation induced DNA damage in epithelial cells, but this effect varied depending on the intestinal segment and the age of birds.

Key words: DNA damage, lipid oxidation, comet assay, gut integrity, vitamin E

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INTRODUCTION

Studies on beneficial activities of n-3 long-chain polyunsaturated fatty acids (**LC-PUFA**) in key biological processes have been conducted for at least 3 decades. A simple dietary intervention with PUFA n-3 may affect chicken immunity (Swiatkiewicz et al., 2015; Konieczka et al., 2017a), and enriches poultry products with these fatty acids (**FA**) to the level providing health benefits for humans (Givens, 2015; Konieczka et al., 2017b,c). Therefore, studies in broiler chickens have been mostly focused on the functional impact of different LC-PUFA sources and their dietary level on lipid metabolism in birds. Regardless of the LC-PUFA source, elevated levels of FA unsaturation, particularly of the n-3 family, leads to accelerated lipid oxidation. This is important, since it has been shown that broiler chickens are particularly exposed to oxidative stress due to the genetic selection (Sihvo et al., 2014). Thus, the activity of vitamin E (**vE**), the major lipid-soluble chain-breaking antioxidant, appears to be of great significance. Recent investigations have primarily focused on the use of vE as a stabilizing agent for meat lipids (Cherian et al., 1996; Lauridsen et al., 1997; Rymer and Givens, 2010) in chickens fed diets containing FA with elevated unsaturation levels. However, little attention has been paid to the interactions between FA, vE and the gastrointestinal tract of birds.

The duodenum and upper jejunum are the major sites of lipid digestion and absorption in chickens (Tancharoenrat et al., 2014), and the intestinal epithelium is highly sensitive to oxidative damage. It has been reported that lipids are the most susceptible nutrients to oxidative reactions, leading to the increased generation of free radicals (Lykkesfeldt and Svendsen, 2007), and consequently may contribute to oxidative damage

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of macromolecules such as DNA. DNA damage, in response to a diet, has been measured more frequently in colonocytes due to its importance in colon cancer development (Bancroft et al., 2003; Toden et al., 2007; Taciak et al., 2015), but it has also been analyzed in chicken jejunal epithelial cells, as an indicator of gut health status (Czerwinski et al., 2015). Nonetheless, at present, the effect of nutritional factor interactions on gut integrity, including DNA damage, is poorly understood in chickens. To the best of our knowledge, this is the first study investigating different dietary PUFA n-6: n-3 ratios and vE levels in broiler diets with particular emphasis on tissue lipid peroxidation and gut integrity.

MATERIALS AND METHODS

Animal Ethics

Experimental procedures were approved by the Third Local Animal Experimentation Ethics Committee at the Warsaw University of Life Sciences-SGGW, Poland, and were in accordance with the principles of the European Union and Polish Law on Animal Protection.

Birds, Diets and Housing

A total of 176 1-day-old female broiler chickens (Ross 308) were obtained from a local commercial hatchery. The birds were divided into 4 groups of 44 birds each and fed wheat, soybean meal-, and corn-based diets with a high or low PUFA n-6: n-3 ratio (**HR** or **LR**, respectively) and supplemented with vE, 50 mg kg^{-1} (basal) or 300 mg kg^{-1} (increased). All diets were isonitrogenous and isocaloric. Birds were fed starter diets for d 1 to 14 (crumbles for the first 3 d and pellets thereafter), grower diets for d 15 to 35, and finisher diets for d 36 to 43. Composition of the experimental diets is presented in Table 1. The birds were reared in heated battery brooders between d 1 to 8. On d 9, after 4 h of feed deprivation, the birds were individually weighed, and 36 chickens, with a body weight close to the group average, were selected per treatment, and placed in individual cages. Each chicken was considered a

Table 1. Composition and calculated nutrient contents (g kg^{-1}), and selected fatty acids profile (% of total fatty acids) of starter, grower, and finisher diets with high (HR) and low (LR) PUFA n-6: n-3 ratio.

Indices	Starter, from d 1 to 14		Grower, from d 15 to 35		Finisher, from d 36 to slaughter	
	HR	LR	HR	LR	HR	LR
Ingredients						
Wheat	280.3	280.3	280.15	280.15	302.8	302.8
Soybean meal	359	359	346	346	318	318
Corn	300	300	300	300	300	300
Fish oil	–	0.5	–	0.5	–	0.5
Corn oil	23	–	36	–	45	–
Linseed oil	–	22.5	–	35.5	–	44.5
Limestone	13.5	13.5	13.52	13.52	13.61	13.61
Mono-Ca-phosphate	13.6	13.6	12.85	12.85	11.7	11.7
NaCl	2	2	2	2	2	2
$NaHCO_3$	1	1	1	1	1	1
L-Lys (78%)	1	1	1.48	1.48	–	–
DL-Met (98%)	1.59	1.59	1.76	1.76	0.89	0.89
L-Thr (98%)	0.01	0.01	0.24	0.24	–	–
Vitamin-mineral premix[1,2,3]	5.0[1]	5.0[1]	5.0[1]	5.0[1]	5.0[2]	5.0[2]
Nutrient content						
Crude protein (N × 6.25)	215.7	215.7	210	210	200.4	200.4
ME, (MJ/kg)	11.88	11.88	12.25	12.25	12.63	12.63
Crude fat	48.87	48.87	61.4	61.4	70.15	70.15
Analyzed fatty acids composition						
SFA[4]	15.29	13.04	17.38	13.13	12.8	10.46
MUFA[5]	24.64	22.48	25.8	22.36	24.89	20.33
PUFA[6]	60.07	64.48	56.82	64.52	62.31	69.21
PUFA n-6[7]	58.68	32.32	55.92	25.55	61.11	23.6
PUFA n-3[8]	1.39	32.16	0.91	38.96	1.2	45.61
PUFA n-6: n-3	43.1	1.0	62.2	0.7	51.1	0.5

[1–2]Provided per kilogram diet [1/2]: (IU) vit. A (*trans*-retinyl acetate), 12,500/10,000; vit. D_3 (cholecalciferol), 5,000/4,000; (mg) vit. B_1, 3/2; vit. B_2, 8/5; biotin, 0.2/0.1; vit. B_6, 5/3; vit. B_{12}, 0.02/0.01; vit. K_3, 3/2; nicotinic acid, 55/35; folic acid, 2/1.5; pantothenic acid, 13/13; choline, 350/225; Mn, 120/120; Zn, 100/100; Se, 0.3/0.3; Cu, 16/16; Fe, 50/50; J, 1.3/1.3; Co, 0.3/0.3; Ca, 1.1/1.4 g; endo-1,4-β-xylanase (EC 3.2.1.8), -/70 U; coccidiostat (narasin and nicarbazin), 80/- mg.

[3]Diets were analyzed for vitamin E concentration and added premixes for HR and LR diets of basal or increased vitamin E level provided either 50 or 300 mg L-α-tocopheryl acetate kg^{-1} in total of diet, respectively.

[4]SFA, saturated fatty acids (C14:0 + C16:0 + C18:0 + C20:0 + C22:0 + C24:0).

[5]MUFA, monounsaturated fatty acids (C16:1 + C17:1 + C18:1 + C20:1 + C21:1 + C22:1).

[6]PUFA, polyunsaturated fatty acids (C18:2n-6 + C18:3n-3 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C20:5n-3 + C22:4n-6 + C22:5n-3 + C22:6n-3).

[7]PUFA n-6 (C18:2n-6 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C22:4n-6).

[8]PUFA n-3 (C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3).

replication, and feed intake was measured individually. The remaining 32 chickens were sacrificed by cervical dislocation for organ sampling. Birds were kept in a room with a temperature maintained at 35°C during the first 8 d of life and at 22°C thereafter; 18 h light and 6 h dark cycle was applied. Climatic conditions were constantly monitored and followed the breed recommendations. Feed and water were offered ad libitum.

Sampling Procedures

Feed intake and body weight were measured, and birds' performance was calculated for d 1 to 9, d 9 to 16, d 9 to 35 and d 9 to 42. Chickens (8 birds per each dietary treatment) were sacrificed by cervical dislocation at d 9, 17, 36, and 43, and the liver, gizzard, and heart were excised and weighed.

Intestinal tissue samples were taken at d 17 and 43 from randomly selected chickens in each group. For epithelial cell isolation and DNA damage measurements, a 10-cm segment of the middle duodenum and jejunum (starting 11 cm anterior to Meckel's diverticulum) was taken from 6 birds per treatment, washed with 0.9% NaCl to remove the digesta and then rinsed with ice-cold solution containing 96 mM NaCl, 27 mM Na-citrate, 1.5 mM KCl, 0.8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 25 mM $NaHCO_3$, 1 mM dithiothreitol, and 2.5 mM L-glutamine, and purged with carbogen gas. Intestinal samples were kept in this solution on ice until the end of the sampling procedure.

For the evaluation of intestinal morphology, tissue samples were taken from 8 birds per treatment, washed with 0.9% NaCl and placed in Bouin's fixative.

The samples of the liver, abdominal fat as well as breast and thigh meat for oxidative stress marker analysis were taken from birds slaughtered only at d 43 (8 birds per treatment), and then immediately frozen and stored at –30°C. Breast muscle samples for FA determination were also collected only on d 43, and then vacuum-packed and stored at –30°C.

Fatty Acid Analysis

Prior to the analysis, experimental diet samples were finely ground and breast muscle samples were thawed at 4°C for 12 h and then homogenized. FA were extracted, saponified and methylated, as described earlier (Czauderna et al., 2007), and analyzed using a gas chromatograph (Shimadzu GC-MS-QP2010 Plus EI) equipped with a BPX70 fused silica capillary column (120 m × 0.25 mm, i.d. × 0.25 μm film thickness), a quadrupole mass selective detector (Model GCMS-QP2010 Plus EI) and an injection port. Helium was the carrier gas operated at a constant pressure and initial flow rate of 1 mL min^{-1}.

Malondialdehyde Assay

The concentration of malondialdehyde (**MDA**), as a marker of lipid peroxidation, was analyzed in the liver, meat (breast and thigh), and abdominal fat samples according to a previously established procedure (Konieczka et al., 2014) using 2-thiobarbituric acid for derivatization and ultra-fast-performance liquid chromatographic system (Shimadzu, Kyoto, Japan) for separation. The system was equipped with an Accucore C18-column (particle size: 2.6 μm; Hydro-RP, 150 × 3.0 mm; Thermo-Scientific, Waltham, MA, USA), a guard column containing Accucore C18 pellicular packing material (particle size: 2.6 μm: 10 × 4 mm: Thermo-Scientific, Waltham, MA, USA) and diode array detector. The derivatized MDA was detected at 530 nm.

Epithelial Cell Isolation and Comet Assay

Epithelial cells were isolated from the duodenum and jejunum, using EDTA treatment, collagenase digestion and mechanical disruption as described previously (Taciak et al., 2015). Isolated cells were used for the analysis of DNA damage by the alkaline comet assay performed using Trevigen CometSlide™ according to the manufacturer's protocol (Trevigen, Gaithersburg, MD, USA). The slides were stained with ethidium bromide (2 μg mL^{-1}) and evaluated at 40X objective using an Olympus BX51 fluorescent microscope (Olympus Corp., Tokyo, Japan) equipped with a 510 to 550 nm excitation filter and a 590 nm barrier filter. For each sample, 180 randomly selected comets were analyzed (90 from each of 2 replicate slides) using Komet 6.0 image analysis software (Andor Technology, Belfast, UK). DNA damage was expressed as tail DNA (the percentage of DNA in the comet tail relative to the total nuclear DNA, Figure 1).

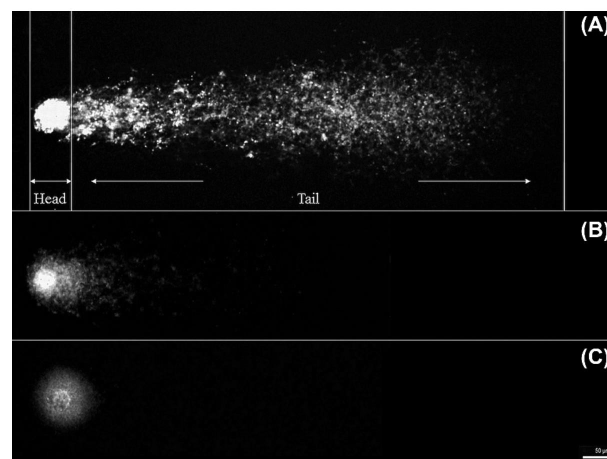

Figure 1. Representative comet images showing different degrees of DNA damage in the intestinal epithelial cells of broiler chickens. The comet head represents intact DNA (tightly coiled in the nucleus), whereas the comet tail indicates damaged DNA, which has migrated out from the nucleus during electrophoresis. A, B, and C show 90%, 50%, and 5% of DNA in the comet tail, respectively.

Intestinal Histomorphometry

Fixed tissue samples were dehydrated, embedded in paraffin wax, and cut on a microtome into transverse sections (7 μm). Then, the sections were mounted on slides, and stained with hematoxylin and eosin. Images were analyzed using light microscopy (Olympus BX51 microscope; Olympus Corp., Tokyo, Japan) and Cell^D Imaging Software (Olympus Soft Imaging Solutions, Münster, Germany). Measurements were taken only from sections where the section plan ran a vertical orientation (averages represent at least 2 slides with a minimum of 20 well-oriented indices). The following parameters were measured: 1) villus height (**VH**), measured from the tip of the villus to the villus-crypt junction, 2) crypt depth (**CD**), measured from the crypt mouth to the base, 3) thickness of the tunica mucosa (**MT**), measured from the tip of the villus to the bottom of the lamina muscularis mucosae, 4) villus width (**VW**) taken at the midline of the villus, 5) villus perimeter, calculated as the ($2\pi \times$ (average villi width/2) $\times$ VH), 6) villus surface area (**VA**), calculated as villus perimeter $\times$ VH, 7) VH: CD ratio.

Statistical Analysis

This experiment was run as a completely randomized design. All data were analyzed as a 2 $\times$ 2 factorial arrangement, considering the dietary PUFA n-6: n-3 ratio and vE level as the main factors. For the performance response a total of 44, 36, 28, and 20 birds per treatment for the feeding periods of d 1 to 9, d 9 to 16, d 9 to 35, and d 9 to 42 respectively, was considered. DNA damage was analyzed including 6 replication per treatment, while other indices were analyzed considering 8 replication per treatment.

The following statistical model was applied:

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk},$$

where: y_{ijk} is an individual observation; μ is overall mean; α_i is the effect of PUFA n-6: n-3 ratio (i = 1, 2); β_j is the effect of vE level (j = 1, 2); $(\alpha\beta)_{ij}$ is the effect of interaction; and e_{ijk} is residual error.

Multifactorial analysis of variance was performed using STATGRAPHICS® Centurion XVI 16.1.03 (StatPoint Technologies, Inc., Warrenton, VA, USA). Multiple comparisons were made using the least significant difference test. The effects were considered significant at $P < 0.05$.

RESULTS

Performance and Organ Weights

Final body weight (**FBW**), feed intake (**FI**), feed conversion ratio (**FCR**), and the relative weights of the liver, gizzard, and heart are listed in Table 2 and Table 3. Both the dietary PUFA n-6: n-3 ratio and the

Table 2. Main effects of dietary factors on final body weight (FBW, g), feed intake (FI, g), and feed conversion ratio (FCR, g feed/g body weight gain) of individual chickens calculated for different feeding periods.[1]

Main effect		d 1 to 9[2]			d 9 to 16			d 9 to 35			d 9 to 42		
Dietary PUFA n-6: n-3 ratio (D)[3]	Vitamin E level (E)[4]	FBW	FI	FCR	FBW	FI	FCR	FBW	FI	FCR	FBW	FI	FCR
High	Basal	207.6 ± 10.3	196.4 ± 9.4	0.95 ± 0.02	454.8 ± 26.4	297.3 ± 17.4	1.20 ± 0.09	2286.2 ± 117.8	2931.8 ± 107.9	1.42 ± 0.05	2843.8 ± 183.0	3885.0 ± 315.7	1.91 ± 0.11
	Increased	213.3 ± 7.3	205.6 ± 5.8	0.97 ± 0.03	500.6 ± 30.0	314.3 ± 21.2	1.13 ± 0.04	2254.2 ± 83.8	2781.1 ± 108.6	1.36 ± 0.04	2950.4 ± 160.1	4020.4 ± 186.5	1.91 ± 0.08
Low	Basal	196.1 ± 4.6	203.3 ± 5.6	1.04 ± 0.02	455.7 ± 25.5	328.6 ± 15.7	1.27 ± 0.05	2238.1 ± 138.2	2808.6 ± 226.0	1.38 ± 0.05	2792.2 ± 245.6	3920.6 ± 252.6	1.97 ± 0.08
	Increased	202.8 ± 3.6	210.1 ± 8.8	1.04 ± 0.05	468.9 ± 30.4	316.7 ± 30.0	1.23 ± 0.09	2256.7 ± 107.2	2871.8 ± 243.9	1.40 ± 0.09	2915.2 ± 104.3	4156.4 ± 139.6	1.99 ± 0.07
Probability (P-value)													
D		0.001	0.06	0.001	0.13	0.037	0.002	0.59	0.81	0.89	0.50	0.31	0.027
E		0.027	0.010	0.45	0.75	0.75	0.024	0.87	0.52	0.41	0.08	0.033	0.63
D × E		0.84	0.66	0.39	0.07	0.07	0.52	0.55	0.12	0.09	0.90	0.55	0.68

[1]Values are means ± SEM.
[2]Chickens were kept in battery cages, thus FBW, FI, and FCR calculated for this period are the mean values of 44 birds per treatment.
[3]High, diets with corn oil; Low, diets with mixture of fish oil and linseed oil.
[4]Basal, 50 mg kg^{-1} L-α-tocopheryl acetate; Increased, 300 mg kg^{-1} L-α-tocopheryl acetate.

Table 3. Main effects of dietary treatments on the relative weight of organs of 9-, 17-, 36-, and 43-day-old chickens, g 100 g^{-1} live body weight.[1]

Main effect		d 9			d 17			d 36			d 43		
Dietary PUFA n-6: n-3 ratio (D)[2]	Vitamin E level (E)[3]	Liver	Gizzard	Heart	Liver	Gizzard	Heart	Liver	Gizzard	Heart	Liver	Gizzard	Heart
High	Basal	8.21 ± 1.13	8.36 ± 0.47	1.48 ± 0.09	3.30 ± 0.14	2.82 ± 0.27	0.72 ± 0.08	2.14 ± 0.31	1.54 ± 0.18	0.41 ± 0.05	2.05 ± 0.16	1.40 ± 0.15	0.40 ± 0.07
	Increased	8.62 ± 1.25	8.99 ± 1.36	1.44 ± 0.27	3.27 ± 0.16	2.83 ± 0.19	0.72 ± 0.06	2.24 ± 0.29	1.59 ± 0.05	0.41 ± 0.05	2.15 ± 0.20	1.40 ± 0.16	0.41 ± 0.05
Low	Basal	8.23 ± 0.85	7.54 ± 0.66	1.39 ± 0.18	3.60 ± 0.17	2.97 ± 0.30	0.72 ± 0.05	2.17 ± 0.27	1.87 ± 0.42	0.47 ± 0.11	1.90 ± 0.13	1.50 ± 0.27	0.37 ± 0.02
	Increased	8.79 ± 1.32	7.77 ± 0.78	1.57 ± 0.10	3.47 ± 0.17	2.57 ± 0.23	0.77 ± 0.08	2.38 ± 0.31	1.83 ± 0.41	0.45 ± 0.04	2.48 ± 0.14	1.44 ± 0.27	0.39 ± 0.02
Probability (*P*-value)													
D		0.82	0.003	0.69	0.001	0.52	0.24	0.16	0.023	0.05	0.12	0.37	0.18
E		0.24	0.18	0.30	0.18	0.041	0.33	0.16	0.96	0.60	0.001	0.70	0.34
D × E		0.84	0.52	0.08	0.43	0.028	0.28	0.60	0.75	0.59	0.001	0.74	0.99

[1]Values are means ± SEM.
[2]High, diets with corn oil; Low, diets with mixture of fish oil and linseed oil.
[3]Basal, 50 mg kg^{-1} L-α-tocopheryl acetate; Increased, 300 mg kg^{-1} L-α-tocopheryl acetate.

vE level affected bird performance and the relative organ weights. At d 9, FBW was higher in chickens fed HR than LR diets ($P < 0.001$), and in those fed diets with the increased vE level ($P = 0.027$); however, FBW was not affected by diets in older chickens. LR diets increased FI ($P = 0.037$) at d 16, while the higher vE level increased it at d 9 ($P < 0.01$) and d 42 ($P = 0.033$). FCR was better in chickens fed HR diets for all periods of chicken growth, except for d 9 to 35. FCR was also higher for the 9- to 16-d period in chickens ($P = 0.024$) fed diets with increased vE. The gizzard was lighter in 9-day-old chicks ($P < 0.003$), but heavier in 36-day-old birds ($P = 0.023$) fed LR diets than in those fed HR diets; it was lighter in 17-day-old birds fed diets with the increased vE level ($P = 0.041$) compared with those fed the basal vE diet. At d 17, birds fed LR diets had a heavier liver than those fed HR diets ($P < 0.001$). The increased vE level in the diet also caused a heavier liver at d 43 ($P < 0.001$). The interaction between the dietary PUFA n-6: n-3 ratio and vE level affected gizzard weight at d 17 ($P = 0.028$) and liver weight at d 43 ($P < 0.001$). Thus, birds fed HR diets with the increased vE level had a heavier gizzard compared to those fed the basal vE level, whereas the opposite was true in birds fed HR diets. Birds fed diets with increased vE had a heavier liver in general, while the magnitude of this increase was lower in birds fed HR diets (< 5% increase) compared to those fed LR diets (> 30% increase).

FA profile

The profile of selected FA in the lipids of chicken breast meat is shown in Table 4. Differences in the dietary PUFA n-6: n-3 ratio, but not vE supplementation were clearly reflected in the level of arachidonic acid (**AA**), α-linolenic acid (**ALA**), EPA and DHA incorporation in breast lipids. LR diets led to higher levels of ALA, EPA, and DHA ($P < 0.001$), while the opposite diets increased the incorporation of AA ($P < 0.001$) in the lipids. The SFA level was not affected, but the MUFA level was higher when birds were fed HR diets ($P < 0.001$). The PUFA level was higher in chickens fed LR diets ($P < 0.003$), and these diets promoted incorporation of PUFA n-3 in meat lipids ($P < 0.001$). The level of PUFA n-6 was higher ($P < 0.001$) when fed HR diets, which in consequence was manifested in a 33-fold higher PUFA n-6: n-3 ratio in chickens fed HR diets ($P < 0.001$). Supplementation of vE did not affect the level of FA incorporated into the lipids of chicken breast meat.

Tissue MDA concentration

The concentration of oxidative stress marker in different chicken tissues is shown in Table 5. The concentration of MDA in the abdominal fat and liver was affected by the dietary PUFA n-6: n-3 ratio. Feeding

Table 4. Main effects of dietary PUFA n-6: n-3 ratio and vitamin E level on the selected fatty acid profile of breast meat of 43-day-old chickens, % of total fatty acid content.[1]

Main effect												
Dietary PUFA n-6: n-3 ratio (D)[2]	Vitamin E level (E)[3]	C18:2n-6 (LA)	C20:4n-6 (AA)	C18:3n-3 (ALA)	C20:5n-3 (EPA)	C22:6n-3 (DHA)	SFA	MUFA	PUFA	PUFA n-6	PUFA n-3	PUFA n-6: n-3 ratio
High	Basal	28.8 ± 10.24	4.1 ± 1.5	0.82 ± 0.27	trace[4]	0.14 ± 0.06	35.4 ± 6.1	27.6 ± 4.2	37.1 ± 8.5	4.6 ± 1.6	1.5 ± 0.9	3.39 ± 0.7
	Increased	31.6 ± 13.88	3.3 ± 1.3	0.79 ± 0.33	trace[4]	0.17 ± 0.08	32.9 ± 6.1	28.4 ± 7.0	38.7 ± 12.6	3.8 ± 1.4	1.2 ± 0.3	3.12 ± 0.7
Low	Basal	42.6 ± 1.61	0.9 ± 0.3	8.77 ± 2.64	0.46 ± 0.13	0.34 ± 0.12	26.7 ± 5.2	17.6 ± 4.6	55.7 ± 8.7	1.1 ± 0.3	10.4 ± 2.4	0.12 ± 0.06
	Increased	27.0 ± 8.21	0.7 ± 0.2	9.45 ± 2.26	0.33 ± 0.15	0.27 ± 0.09	36.0 ± 3.3	23.0 ± 4.0	41.0 ± 6.3	0.9 ± 0.2	10.6 ± 2.1	0.09 ± 0.04
Probability (*P*-value)												
D		0.25	0.001	0.001	0.001	0.001	0.14	0.001	0.003	0.001	0.001	0.001
E		0.11	0.16	0.61	0.06	0.67	0.08	0.09	0.06	0.17	0.96	0.40
D × E		0.024	0.43	0.57	0.12	0.17	0.004	0.22	0.019	0.46	0.68	0.51

[1]Values are means ± SEM.

SFA, saturated fatty acids (C14:0 + C16:0 + C18:0 + C20:0 + C22:0 + C24:0); MUFA, monounsaturated fatty acids (C16:1 + C17:1 + C18:1 + C20:1 + C21:1 + C22:1); PUFA—polyunsaturated fatty acids (C18:2n-6 + C18:3n-3 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C20:5n-3 + C22:4n-6 + C22:5n-3 + C22:6n-3); PUFA n-6 (C18:2n-6 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C22:4n-6); PUFA n-3 (C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3).

[2]High, diets with corn oil; Low, diets with mixture of fish oil and linseed oil.

[3]Basal, 50 mg kg^{-1} L-α-tocopheryl acetate; Increased, 300 mg kg^{-1} L-α-tocopheryl acetate.

[4]Less than 0.006% of total fatty acid.

LR diets resulted in an approximate 18-fold higher concentration of this marker in the abdominal fat, while in the liver it was increased by 20% ($P < 0.001$). The increased vE level reduced MDA concentration in the liver ($P < 0.001$), breast meat ($P < 0.001$) and thigh meat ($P = 0.012$) compared to the basal level, but not in the abdominal fat. The highest concentration of MDA was found in the abdominal fat, followed by the liver and meat. A significant interaction was found between the dietary PUFA n-6: n-3 ratio and the vE level in the liver ($P < 0.001$). The increased level of vE marginally reduced MDA concentration in the liver when birds were fed HR diets, but it reduced liver MDA concentration approximately 2-fold when chickens were fed LR diets.

DNA damage

The extent of DNA damage in the intestinal epithelial cells varied with respect to the dietary PUFA n-6: n-3 ratio, vE level, bird age and intestinal segments (Table 6). In the jejunum DNA damage was greater in 17-day-old chickens fed LR diets ($P < 0.001$), and in those fed diets with the increased level of vE ($P = 0.016$). Higher level of vE caused a greater DNA damage in the duodenum in these birds. DNA damage was also numerically greater in older chickens, and higher in the jejunum than in the duodenum.

Morphology of duodenum and jejunum

The effect of experimental factors on morphological parameters of the duodenum and jejunum in 17- and 43-day-old birds is shown in Table 7 and Table 8. Feeding LR diets decreased CD in the duodenum ($P = 0.017$), but increased the VH: CD ratio ($P = 0.017$) in 17-day-old birds, whereas all indices in older birds did not differ. In the jejunum, VA ($P = 0.022$) of younger birds, as well as MT ($P = 0.029$) and VH ($P = 0.035$) of older birds were decreased by LR diets. Dietary levels of vE and interactions between experimental factors did not affect morphological indices of the duodenum and jejunum.

DISCUSSION

Lipids are one of the most unstable feed components, as they are highly susceptible to oxidation, leading to damage of various tissues. Therefore, in the recent study we investigated the minute changes occurring in the intestinal mucosa, which is potentially the first target for free radicals formed during oxidative reactions.

Feeding chickens with experimental diets resulted in a slightly poorer FCR than that expected for female Ross 308 broilers up to d 16 (Aviagen, 2014). This difference disappeared as the birds got older. More specifically, LR diets tended to compromise the performance, while the opposite was true for diets with the increased

Table 5. Main effects of dietary PUFA n-6: n-3 ratio and vitamin E level on malondialdehyde (MDA) concentration (mg kg^{-1}) in different tissues of 43-day-old chickens.[1]

Main effect Dietary PUFA n-6: n-3 ratio (D)[2]	Vitamin E level (E)[3]	Tissue Abdominal fat	Liver	Breast meat	Thigh meat
High	Basal	0.22 ± 0.03	0.83 ± 0.05	0.17 ± 0.02	0.17 ± 0.02
	Increased	0.17 ± 0.02	0.67 ± 0.03	0.10 ± 0.02	0.13 ± 0.03
Low	Basal	3.70 ± 0.84	1.10 ± 0.03	0.19 ± 0.02	0.16 ± 0.03
	Increased	3.17 ± 0.88	0.68 ± 0.01	0.11 ± 0.02	0.11 ± 0.01
Probability (*P*-value)					
D		0.001	0.001	0.30	0.43
E		0.18	0.001	0.001	0.012
D × E		0.27	0.001	0.95	0.96

[1]Values are means ± SEM.
[2]High, diets with corn oil; Low, diets with mixture of fish oil and linseed oil.
[3]Basal, 50 mg kg^{-1} L-α-tocopheryl acetate; Increased, 300 mg kg^{-1} L-α-tocopheryl acetate.

Table 6. Main effects of dietary PUFA n-6: n-3 ratio and vitamin E level on the extent of DNA damage, expressed as % DNA in the comet tail.[1]

Main effect Dietary PUFA n-6: n-3 ratio (D)[2]	Vitamin E level (E)[3]	17-day-old Duodenum	17-day-old Jejunum	43-day-old Duodenum	43-day-old Jejunum
High	Basal	10.41 ± 6.0	9.85 ± 2.6	29.53 ± 10.2	38.12 ± 11.9
	Increased	18.67 ± 8.8	15.41 ± 5.8	29.80 ± 10.6	36.43 ± 15.7
Low	Basal	12.33 ± 5.4	23.45 ± 15.1	26.15 ± 4.6	54.71 ± 22.9
	Increased	26.50 ± 11.1	42.38 ± 16.1	37.0 ± 11.2	46.55 ± 12.8
Probability (*P*-value)					
D		0.15	0.001	0.63	0.06
E		0.003	0.016	0.17	0.47
D × E		0.38	0.18	0.19	0.63

[1]Values are means ± SEM.
[2]High, diets with corn oil; Low, diets with mixture of fish oil and linseed oil.
[3]Basal, 50 mg kg^{-1} L-α-tocopheryl acetate; Increased, 300 mg kg^{-1} L-α-tocopheryl acetate.

Table 7. Main effects of dietary PUFA n-6: n-3 ratio and vitamin E level on morphological indices of duodenal and jejunal mucosa in 17-day-old chickens.[1]

Main effect Dietary PUFA n-6: n-3 ratio (D)[2]	Vitamin E level (E)[3]	Mucosa thickness	Villi height	Villi width	Crypt depth	Villi area	Villi height to crypt depth
				Duodenum			
High	Basal	1054 ± 75	983 ± 66	70 ± 4.70	84 ± 6.29	220 ± 21.3	12.0 ± 1.07
	Increased	1032 ± 41	957 ± 46	76 ± 3.20	90 ± 6.73	232 ± 20.7	10.8 ± 0.71
Low	Basal	1012 ± 44	949 ± 43	71 ± 6.16	83 ± 7.61	217 ± 25.3	12.3 ± 0.98
	Increased	1033 ± 47	963 ± 52	73 ± 5.34	78 ± 7.95	224 ± 15.5	12.9 ± 1.52
Probability (*P*-value)							
D		0.39	0.53	0.64	0.017	0.58	0.017
E		0.99	0.78	0.05	0.99	0.30	0.48
D × E		0.36	0.38	0.38	0.05	0.77	0.05
				Jejunum			
High	Basal	410 ± 70	360 ± 64	55 ± 4.22	63 ± 5.32	65 ± 16.4	5.9 ± 0.83
	Increased	384 ± 39	340 ± 33	50 ± 4.93	58 ± 4.10	56 ± 7.5	6.0 ± 0.61
Low	Basal	383 ± 31	335 ± 32	46 ± 3.75	61 ± 4.58	50 ± 6.8	5.7 ± 0.57
	Increased	404 ± 54	355 ± 51	46 ± 5.45	60 ± 4.20	53 ± 7.4	6.1 ± 0.89
Probability (*P*-value)							
D		0.85	0.77	0.001	0.77	0.022	0.91
E		0.88	0.99	0.20	0.08	0.40	0.33
D × E		0.21	0.24	0.10	0.28	0.12	0.51

[1]Values are means ± SEM.
[2]High, diets with corn oil; Low, diets with mixture of fish oil and linseed oil.
[3]Basal, 50 mg kg^{-1}; Increased, 300 mg kg^{-1} L-α-tocopheryl acetate.

Table 8. Main effects of dietary PUFA n-6: n-3 ratio and vitamin E level on morphological indices of duodenal and jejunal mucosa in 43-day-old chickens.[1]

Main effect Dietary PUFA n-6: n-3 ratio (D)[2]	Vitamin E level (E)[3]	Mucosa thickness	Villi height	Villi width	Crypt depth	Villi area	Villi height to crypt depth
				Duodenum			
High	Basal	990 ± 101	889 ± 108	47 ± 4.10	102 ± 6.55	136 ± 25	9.0 ± 1.4
	Increased	990 ± 88	886 ± 79	48 ± 2.43	117 ± 10.06	136 ± 12	7.8 ± 1.3
Low	Basal	949 ± 124	850 ± 153	48 ± 6.47	111 ± 15.92	131 ± 41	8.1 ± 2.6
	Increased	930 ± 184	832 ± 184	45 ± 1.42	107 ± 15.08	118 ± 26	7.4 ± 2.1
Probability (*P*-value)							
D		0.29	0.36	0.34	0.83	0.25	0.34
E		0.84	0.83	0.62	0.20	0.50	0.17
D × E		0.85	0.88	0.09	0.040	0.52	0.72
				Jejunum			
High	Basal	621 ± 79	554 ± 73	43 ± 3.29	82 ± 10.64	77 ± 15.3	6.9 ± 1.0
	Increased	568 ± 114	506 ± 106	44 ± 1.59	75 ± 12.17	71 ± 16.1	7.0 ± 1.4
Low	Basal	499 ± 78	444 ± 73	44 ± 1.93	75 ± 6.90	64 ± 10.8	6.1 ± 0.9
	Increased	537 ± 93	478 ± 88	43 ± 1.36	77 ± 3.71	66 ± 13.1	6.4 ± 1.1
Probability (*P*-value)							
D		0.029	0.035	0.61	0.38	0.07	0.09
E		0.82	0.82	0.98	0.41	0.68	0.61
D × E		0.18	0.20	0.19	0.19	0.41	0.82

[1]Values are means ± SEM.
[2]High, diets with corn oil; Low, diets with mixture of fish oil and linseed oil.
[3]Basal, 50 mg kg^{-1}; Increased, 300 mg kg^{-1} L-α-tocopheryl acetate.

vE level. Since the effect of dietary FA and vE dose appeared to be interactive, as chicken growth proceeded, this could partially explain different response in performance at the early growth stages. Overall, the results are consistent with other studies reporting that feeding chickens with diets of different PUFA n-6: n-3 ratios and vE levels does not usually negatively impact bird performance (Kim et al., 2006; Qi et al., 2010). However, our data showed that the relative weights of internal organs differed between the dietary treatments. At d 42, the relative weight of the liver was increased in birds fed diets with a high vE level, which could be a consequence of the pro-oxidative effect of vE when it was above the level normally found in broiler feed. The liver plays a key role in the metabolism and distribution of vE, and it is also the first organ of its accumulation. It was shown in rats (Bjorneboe et al., 1990) that 45 min after intravenous injection of α-tocopherol, more than 50% of the injected dose was recovered in the liver. The liver product of α-tocopherol oxidation (α-tocopheryl quinone) was eventually excreted in the bile, thus the hyperactivity of hepatocytes could have led to liver enlargement. Similarly, it was documented in our previous study (Konieczka et al., 2017a) that feeding diets with the increased vE level (300 mg kg^{-1}) over a period of 42 d caused the enlargement of the bursa, suggesting a pro-oxidative activity of high vE doses in broilers.

In the present study, the dietary PUFA n-6 to n-3 ratio affected the PUFA profile of breast meat, whereas the dietary vE level did not, which was consistent with other studies (Rymer and Givens, 2010; Ribeiro et al., 2013; Konieczka et al., 2015, 2017b). The induction of oxidative stress was a natural consequence of the increased incorporation of n-3 FA into the tissue lipids of birds fed LR diets, and manifested in elevated MDA concentrations in the abdominal fat and liver of chickens fed LR diets. However, recent findings showed that MDA concentration in the liver, breast and thigh meat, but not in the abdominal fat was decreased in chickens fed diets with higher vE levels. This confirmed the effectiveness of vE in the downregulation of oxidative stress in different tissues, but might suggest that the increased level of vE (300 mg kg^{-1}) was still not sufficient to inhibit lipid peroxidation in the abdominal fat due to the high content of PUFA prone to oxidation.

Previously, it was reported in chickens that membrane lipids of intestinal epithelial cells were the major components modified by dietary FA (Aziza et al., 2014). Consequently, gut physiology depends on the dietary LC-PUFA and their oxidative potential. It has been demonstrated that secondary lipid oxidation products, which are initially formed from the peroxidative cleavage of PUFA, directly affect gut tissues, leading to cell damage (Dibner et al., 1996; Zhang et al., 2012; Awad et al., 2014). This results in serious side effects for the whole organism, because oxidants cause DNA damage. In our study, it was shown that dietary n-3 FA increased DNA damage in gut epithelial cells. Moreover, it was found that their effect depended on the intestinal segment. For instance, DNA damage in epithelial cells was greater in the jejunum than in the duodenum. There are 2 likely reasons for that. Firstly, the jejunum, compared to the duodenum, is the predominant site of fat digestion and FA absorption in birds. Therefore,

oxidized lipids can exert more deleterious effect on DNA of epithelial cells in this segment. Secondly, the passage of the digesta through the duodenum is significantly shorter than through the jejunum (Tancharoenrat et al., 2014), thus the time of contact between oxidized lipids and the epithelium is shorter.

Overall, n-3 FA increased DNA damage in epithelial cells probably by inducing apoptosis. This phenomenon was documented in rats fed a high n-3 FA diet, in which EPA and DHA induced apoptosis and increased the count of differentiating cells in the colonic mucosa (Calviello et al., 1999). The effect of the dietary FA ratio on DNA damage in intestinal epithelial cells may also result from cellular protein oxidation. Lipid peroxidation may initiate protein carbonylation (Estévez, 2014) by chemical modifications of side chains of susceptible amino acids. As proteins (histones) play a key role in DNA packaging and chromatin organization (Rothbart and Strahl, 2014), oxidation may lead to changes in their properties, and in consequence, to DNA fragmentation. Interestingly, in our study, diets with the increased vE level had a more deleterious effect on DNA in younger than older birds. It is known that vE protects cell membranes by promoting bilayer integrity. Vitamin E is mainly hydrolyzed and absorbed by the gut epithelium (Khan et al., 2012) and is readily incorporated into mucosal cells. Thus, vE was expected to exert the greatest protective effect on this structure. In a previous study (Villaverde et al., 2008), it has been suggested that vE, before it is absorbed, may play a protective role against oxidized lipids in the chicken gastrointestinal tract. However, our results suggest that the dose of 300 mg kg^{-1} is above the physiological requirements to maintain the redox balance in the intestinal mucosa and might have induced oxidative stress. The pro-oxidative activity of vE on cellular DNA may involve the formation of MDA and 4-hydroxynonenal. The former is toxic, and because it binds DNA, mutagenic adducts may be formed; on the other hand, 4-hydroxynonenal triggers apoptosis and consequently leads to cell death (Nair et al., 1999; Corpet, 2011). It is also possible that a high dose of vE caused DNA damage through histone oxidation, but confirmation of this hypothesis would require further research.

Our study indicated that feeding birds with LR diets had a minor but undesirable influence on the gut structure, and in consequence, on bird performance during the early growth period. The gut epithelium is organized into villus-crypt units to provide maximal mucosal surface area for nutrient absorption. Therefore, nutrient uptake and absorptive efficiency depend on the integrity of the gut mucosa (Zeitz et al., 2015). Overall, longer villi provide more mucosal surface, while deeper crypts indicate more rapid enterocyte turnover. Thus, the high VH: CD ratio indicates a well-differentiated gut mucosa with a maximum absorptive surface (Choct, 2009). In our study, LR diets reduced CD in the duodenum and VA in the jejunum in 17-day-old chickens, and decreased jejunal MT and VH in 43-day-old birds. This effect of LR diets on gut integrity is consistent with the findings of other researchers (Aziza et al., 2014), and is possibly caused by the negative activity of n-3 FA on epithelial cell proliferation, differentiation and apoptosis (Calviello et al., 1999; Perin et al., 1999). Nevertheless, our findings suggest that the response of chickens to a dietary challenge in regard to gut integrity is different in younger than older birds. Interestingly, we have not observed any significant effects of the vE level on gut morphology, which may suggest that it does not play a role in gut development. Since dietary vE did not compromise gut mucosa absorptive surface, the reported improvement in performance indices may have been a consequence of better nutrient uptake obtained by feeding diets with the increased vE level.

In conclusion, feeding broilers with diets low in the PUFA n-6: n-3 ratio might have negatively affected bird performance, but predominantly in younger birds. In contrast, diets high in vE had a positive effect on birds' performance indices but this effect varied in different growth periods. Dietary n-3 FA, despite its effective incorporation into meat lipids, increased oxidative processes in different tissues, but their intensity might have been reduced, to some extent, by vE supplementation. The effect of the PUFA n-6: n-3 ratio and vE level on the extent of DNA damage in epithelial cells appeared to be depended on the chicken growth stage and intestinal segment. Our study provides direct evidence that diets with a low PUFA n-6: n-3 ratio, supplemented with vE levels markedly exceeding the recommendation, increase DNA damage, whereas diets low in PUFA n-6: n-3 ratios, regardless of the vE level, may also negatively affect gut morphology.

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