Effects of probiotic supplementation and postmortem storage condition on the oxidative stability of *M. Pectoralis major* of laying hens

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ABSTRACT Dietary probiotic supplementation is a promising alternative to antibiotics in the poultry industry, and some studies have found its positive impacts on meat quality attributes. The objective of this study was to evaluate the effects of dietary treatments on oxidative stability of postmortem chicken fillet (*M. Pectoralis major*) muscles during 7 d of storage under either high oxygen (**HIOX**) or vacuum (**VAC**) conditions. A total of 264 Hy-Line Brown laying hens (11 birds per cage) were assigned to 2 different dietary treatments (regular diet or 300 ppm probiotic, Sporulin enhanced diet) before being sacrificed. At 17 wk, one bird per cage from 6 cages per treatment was randomly selected and slaughtered (n = 12). Chicken fillets from both sides were collected and randomly assigned to HIOX or VAC conditions. Within each fillet side, the muscle was divided into 3 portions, assigned to 1 or 7 d storage period, while the caudal portion was allocated as the 0 d samples for proximate composition and fatty acid profiling. Results revealed that the muscle crude compositions were not altered in laying hens that were fed probiotic supplementary diet compared to controls ($P > 0.05$). However, the muscle samples from probiotic fed laying hens had higher proportions of unsaturated fatty acid (74.76 vs. 71.65%, $P < 0.05$) and 2-thiobarbituric acid reactive substances (0.79 vs. 0.67 mg malondialdehyde/kg meat, $P < 0.05$) than the samples from the control. In addition, the probiotic fed laying hens, but not controls, had higher levels of conjugated dienes (3.32 vs. 2.63 μg/g meat) and peroxides (0.40 vs. 0.30 milliequivalent O_2/kg fat) in the muscle samples after 7 d of storage ($P < 0.05$). The results from the present study indicated that postmortem fillet muscles obtained from the laying hens fed with probiotic supplementation would be more prone to be oxidized, especially under more oxidizing conditions (HIOX and extended storage time).

Key words: atmospheric storage condition, fatty acid profile, laying hen *M. Pectoralis. major* muscle, oxidative stability, probiotic supplementation

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INTRODUCTION

There is an emerging interest in using dietary probiotic supplementation as a potential nutraceutical alternative to the utilization of antibiotics in the poultry industry. Probiotic supplementations have been reported to improve gut health and nutrient absorption in broilers by modulating their gut microbiota (Ghadban, 2002). Furthermore, some studies found positive impacts of dietary probiotic supplementation on meat quality attributes of chickens, such as improved water-holding capacity and tenderness (Aksu et al., 2005; Zhang et al., 2005). Other studies observed enhanced antioxidants and reduced oxidants in the serum and liver of animals that were fed probiotic supplementations before being sacrificed (Capcarova et al., 2010; Bai et al., 2016). The effect of probiotic feeding on oxidative stability of chicken muscles has been evaluated in some recent studies (Capcarova et al., 2010; Bai et al., 2016; Kim et al., 2017a; Kim et al., 2017b; Cramer et al., 2018). However, inconsistent results between studies have been reported. Some studies found that muscles from probiotics fed chickens had lower lipid oxidation products, such as malondialdehyde or higher antioxidant components (e.g., albumin, and bilirubin) compared to muscles from regular diet fed counterparts (Capcarova et al., 2010; Bai et al., 2016; Kim et al., 2017a), whereas no direct impacts were also reported in other studies (Kim et al., 2017b; Cramer et al., 2018). These discrepancies could be attributed to various intrinsic/extrinsic factors (e.g., probiotic strains,

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dosage, feeding condition, poultry type, post-harvesting processing condition, type of oxidation products, etc.), indicating that the impacts of dietary probiotic supplementation on the oxidative stability of chicken meat has not been fully understood.

It has been well accepted that oxidation will produce primary and secondary oxidation products (such as hydroperoxide, conjugated dienes, malondialdehyde), which are considered as quality markers of meat deterioration. The consequence of lipid or protein oxidation, or both in meat causes rancidity, off-flavor, discoloration, or other eating quality deteriorations (Kanner, 1994). During post-harvest processing, fresh meat will be exposed to different oxidative stressors, such as light, oxygen, postmortem storage duration, and holding temperature (Estévez, 2015). Further, oxidative susceptibility of the fresh muscle is largely dependent on the intrinsic properties of muscle. In particular, its fat composition, including fat content, phospholipid level and fatty acid profile, may affect the extent of oxidation responses. Given that unsaturated fatty acids (**UFA**) are more sensitive to reactive oxygen species (**ROS**) attack compared to saturated fatty acids (Falowo et al., 2014), it would be reasonable to assume that dietary probiotic supplementation may affect the fatty acid composition, resulting in various responses to oxidative damage.

Therefore, the objective of the present study was to evaluate the effects of dietary probiotic supplement on oxidative stability of postmortem chicken *M. Pectoralis major* muscles exposed to various oxidative conditions. The current study was a part of parallel projects, which investigated the effect of probiotic supplementation on the aggressive behavior and the skeletal health of laying hens (study in progress, data unpublished). The outcomes of the present study could advance the understanding of oxidative stability of skeletal muscle in chickens fed with probiotic supplement. Furthermore, it would provide some practical implications for the poultry industry to develop processing systems in order to better utilize probiotic supplementations for the improvement of production performance and meat quality.

MATERIALS AND METHODS

Animal Handling Approval

The following procedures were approved by the Purdue University Animal Care and Use Committee (**PACUC**# 1,607,001,454).

Animal Dietary Treatment

A total of 264 1-day-old Hy-Line Brown chicks (Hy-Line International; Darren, IN, USA) were used for the study. For the first 11 wk, the chicks were raised in a grower room, in which 24 cages were facilitated with perches, and 12 cages were used per treatment (11 birds/cage). At 11 wk, the chicks were relocated to a layer facility and housed in enriched cages with perches and nesting areas (L × W × H: 120 × 77 × 46 cm). The raising environment was set following the Hy-Line management guideline (Hy-Line, 2017). A regular diet with 20% CP, 1.0% Ca, and 0.45% nonphytate phosphorus was adopted as starter diet to feed female chicks till 3.9 wk of age followed by a grower diet from 4 to 15.9 wk with 18.6% CP, 1.0% Ca, and 0.40% nonphytate phosphorus. A regular pre-lay diet with 18.4% CP, 2.50% Ca, and 0.35% non-phytate phosphorus was fed from 16 to 17 wk of age. Chicks fed a regular diet were considered as the control, while chicks fed the regular diet mixed with 300 ppm Sporulin (1.0×10^6 cfu/g, Novus International Inc. St Louis, MO, USA) were set as the treatment group. Sporulin is a commercially available probiotic that contains 3 strains of *Bacillus subtilis*.

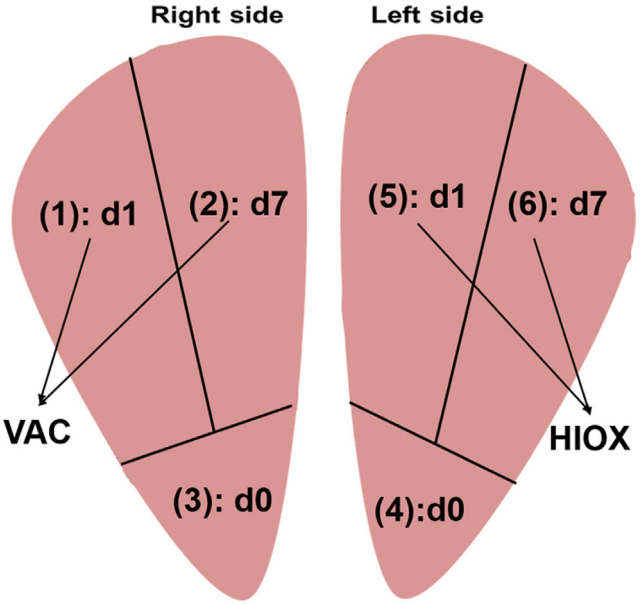

Figure 1. Schematic diagram of the sample assignments (n = 6 per group, per time point). HIOX: high-oxygen storage condition; and VAC: vacuum storage condition.

Animal Slaughter and Sample Processing

At 17 wk, one bird per cage from six cages per treatment was randomly selected and slaughtered (n = 12). As mentioned, hens were slaughtered by following the approved PACUC lab procedure instead of the industry slaughter process in order to obtain the blood, intestines and muscle samples for the current and parallel studies in a timely manner. In brief, the hens were sedated with sodium pentobarbital (30 mg/kg of body-weight) via the brachial vein, and were euthanized via cervical dislocation. Both side fillet muscles (*M. Pectoralis major*) were collected within 3 min postmortem. Different atmospheric condition (vacuum: **VAC** or high-oxygen: **HIOX**) was randomly assigned to one side of both fillet muscles. As illustrated in Fig. 1, each side of the *M. Pectoralis major* was cut into 3 portions and different

storage times (1 or 7 d) were randomly assigned to the portions, except for the 0 d sample (which was assigned to the caudal part of the muscle). Portions assigned to 0 d for proximate composition analysis and fatty acid profiling were snap-frozen using liquid nitrogen immediately after dividing. All the samples were placed in Nylon/polyethylene pouches and transferred to the Purdue Meat Science and Muscle Biology Laboratory (30 min driving) on ice in the ice chest containers. The VAC samples were placed in Nylon/polyethylene pouches (Bunzl processor; Riverside, MO, USA; the oxygen transmission rate of 1.09 mL/cm^2/24 h) and were vacuum-packaged. The HIOX samples were placed in the same sampling pouches, flushed with high oxygen gas for 7 s (90% oxygen and 10% nitrogen; Indiana Oxygen, Lafayette, IN, USA) and packaged using a modified atmosphere packaging machine (TC-420F, Promax, Ontario, CA, USA). The packaged samples were stored at 4°C for the designated time (1 or 7 d). Then, the samples were snap-frozen, powdered by a commercial blender (Waring Products, CT, USA) and stored at −80°C till further analyses.

Proximate Compositions

Proximate compositions of the muscle samples (0 d postmortem) were determined according to the Association of Official Analytical Chemistes method described in a previous study (Rohman et al., 2011).

Measurement of pH Value

To measure the pH value of the samples, 1.0 g from each sample was homogenized with 5 mL of distilled water and read using the benchtop pH meter (Sartorius Basic Meter PB-11, Sartorius AG, Germany). Each sample was measured in triplicate and the mean value was used for the statistical analysis.

Determination of Fatty Acid Profile

Lipid Extraction To determine the fatty acid profile, each muscle sample (0 d postmortem) was extracted based on a modified protocol of Bligh and Dyer (1959). Briefly, 1.0 g of each sample was homogenized with 7 mL of methanol for 30 s and then added 14 mL of chloroform for another 30 s. The homogenate was filtered through No. 1 Whatman filter paper (GE Healthcare, Co., Ltd., Chicago, IL, USA), followed by mixing with 8 mL of 0.88% potassium chloride. The mixture was shaken for 10 min and then centrifuged for 5 min (1000 g) at room temperature (25°C). The lower layer was collected and evaporated via a nitrogen evaporator (24 position N-EVAP, Organomation Inc., MO, USA) till ∼3 mL left. The remaining sample was transferred to a new tube, and the original tube was rinsed with 2 mL of 2:1 (v/v) chloroform/methanol, and the rinse was combined with the remaining sample, and stored at −20°C until methylation.

Fatty Acid Methylation The preparation of fatty acid methyl esters (**FAME**) concentration was performed using sodium methoxide in methanol (Hartman and Lago, 1973). The FAME was then reconstituted in chromatography grade hexane for gas chromatography (Varian CP 3900, Varian Analytical Instruments, Walnut Greek, CA, UAS). The gas chromatograph (GC) was equipped with a 105 m Rtx-2330 (Restek, Bellefonte, PA, USA) fused silica capillary GC column (0.22 mm I.D. and 0.20 μm of film thickness). Helium was used as the carrier gas at a flow rate of 40 mL/min. Injector and detector temperatures were 260°C. The injected volume was 1 μL at a 50:1 split injection. Column oven temperature was increased from 140 to 180°C at a rate of 8°C/min, from 180 to 260°C at a rate of 5°C/min and then held at 260°C for 15 min. Fatty acids were identified by comparison of retention time of a known standard (Supelco 37 components FAME Mix, Sigma-Aldrich, USA) and expressed as a ratio: the peak area of the detected fatty acid to the total peak area x 100%. The results of saturated fatty acids, UFA, mono- and poly-unsaturated fatty acids (**MUFA** and **PUFA**) were calculated based on the content of the whole set of FA profile (%).

Primary Lipid Oxidation

The extent of primary lipid oxidation level of the muscle samples stored at different postmortem times (1 and 7 d) and holding conditions (VAC and HIOX) was determined using the two different methods as described below.

Conjugated Dienes

The extent of conjugated dienes (**CD**) in the muscle samples was evaluated based on the method described by Ma et al. (2017), which was a modified version of the protocol used by Srinivasan et al. (1996). Briefly, the samples and distilled water were mixed and homogenized at a ratio of 1:10 (w/v); and then 0.5 mL of the aliquot homogenate was mixed with an extraction solution (isopropanol and hexane, 3:1 ratio) and centrifuged at 2000 g for 5 min. The supernatant was read at 233 nm in a 1-cm cuvette using a spectrophotometer (Epoch, BioTek Instrument Inc, VT, USA). The absorbance value of the extraction solution was subtracted as blank, and molar extinction coefficient of 2.52×10^4 $M^{-1}cm^{-1}$ was used to calculate the CD concentration (μmol/mg meat).

Peroxide Value

The peroxide value (**POV**) of the muscle samples was measured using the ferric thiocyanate method as described by Weber et al. (2008). Lipid was extracted based on the Bligh and Dyer (1959) method. Absorbance reading was carried out at 500 nm against the

blank that contained all reagents without lipid. The POV was expressed as milliequivalent O^2/kg fat.

Secondary Lipid Oxidation

The secondary lipid oxidation product was evaluated by 2-thiobarbituric acid reactive substances (**TBARS**) value. According to the protocol described by Kim et al. (2016), 5.0 g of the muscle sample was homogenized with 15 mL distilled water added 50 μL of butylated hydroxyl anisole solution (10% in 90% of ethanol) for preventing further oxidation. 1 mL of the homogenate was mixed with 2 mL of 20 mM thiobarbituric acid (**TBA**) solution. The mixture was incubated in a water bath at 80°C for 15 min, and then immediately cooled down in an ice bath for another 10 min, followed by centrifugation (2000 g, 10 min), and the supernatants were filtered through a filter paper (Whatman No. 4). Absorbance reading was performed at 538 nm (Epoch, Biotek Instruments Inc., VT, USA). The TBARS value was presented as mg MDA/kg meat.

Determinations of Protein Carbonyl Content

Carbonyl content of the samples was performed based on the 2,4-dinitrophenylhydrzine (**DNPH**) protocol as described by Levine et al. (1990). Each muscle sample was homogenized with 20 mM phosphate buffer (0.6 M NaCl, pH 6.5) at the ratio of 1:10 (w/v). Muscle proteins were precipitated using ice-cold 10% trichloroacetic acid (**TCA**) and collected by centrifugation at 2000 g for 30 min. The procedure was repeated twice before being mixed with DNPH solution (10 mM). The mixtures were shaken in the dark for 1 h before adding 0.5 mL of ice-cold 20% TCA and then centrifuged at 2000 g for 20 min. The precipitate was rinsed using an equal volume of ethanol/ethyl acetate solution for three times. Following the rinsing, the precipitate was collected by centrifugation (2000 g, 20 min), and the excessive solvent was removed by evaporation under a fume hood. The pellet was then mixed with guandine-hydrocholoride acid (6 M, 20 mM phosphate buffer, pH 6.5) and was shaken for 30 min under dark to allow the formation of protein hydrazones. Centrifugation (9500 g, 10 min) was performed to exclude the influence of insoluble proteins before reading the absorbance at 280 nm and 370 nm respectively. The carbonyl content was expressed as nmol/mg protein followed the formula: the absorption coefficient of 2.2×10^4 M^{-1} cm^{-1} for protein hydrazones.

Measurement of 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity

The 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging capacity (**DPPH**) was estimated following a modified protocol introduced by Blois (1958). In brief, 5.0 g of sample was homogenized with 15 mL distilled water, and 0.2 mL of the aliquot homogenate was mixed with 0.8 mL of extra distilled water and 1 mL of methanolic DPPH solution. The above mixture was vortexed and reacted for 30 min at room temperature under dark. Absorbance of the sample was performed at 517 nm; and DPPH radical scavenging activity was calculated according to the following equation: DPPH (%) = [1–(absorbance of sample/absorbance of control)] × 100.

Table 1. Proximate compositions of laying hens fed with regular or probiotic diet.

Treatments[1]	Fat content (%)	Ash content (%)	Protein content (%)	Moisture (%)
Control	1.23	2.26[a]	23.44	73.07
Probiotic	1.03	2.07[b]	23.54	73.36
SEM[2]	0.0903	0.0667	0.0937	0.1872
P-value	0.0969	0.0475	0.4383	0.4089

[a–b]Least squares means within a column for the 2 treatments lacking a common superscript differ ($P < 0.05$).
[1]Treatments include two different dietary treatments, where Control represents laying hens that fed with a regular diet; Probiotic represents laying hens fed with probiotic diet (300 ppm Sporulin).
[2]Standard error of means, n = 12 per treatment.

Statistical Analysis

A total of 3 different factors were considered to design the experiment: two dietary treatments (regular vs. probiotic), two storage time points (1 d and 7 d), and two storage conditions (VAC vs. HIOX), which comprised the 2 × 2 × 2 factorial arrangement in 6 randomized blocks. Dietary, storage time, and storage conditions were fixed as the main effects, laying hens and their interactions were set as the random effects. The obtained data were subjected to PROC MIXED procedure on SAS 9.4 software (SAS, 2012) for the statistical analysis. The impacts of main effects and their interactions on the results were analyzed, and the significant influence would be considered with the results beyond the confidence interval of 95% ($P < 0.05$).

RESULT AND DISCUSSION

Proximate Compositions and pH Values

Proximate compositions (fat, ash, protein, and moisture contents) of chicken fillets from laying hens fed either regular diet or probiotic supplementation are presented in Table 1. Different diet treatments did not affect proximate compositions of the fillet muscles ($P > 0.05$) except the ash content, which was lower in probiotic fed hens than regular diet fed controls ($P < 0.05$). A similar result was reported by Zhou et al. (2010) that there were no significant differences in moisture, crude fat, crude ash or crude protein contents of chicken fillet muscles between Guangxi Yellow chicken fed a regular diet or probiotic-enhanced (*Bacillus coagulans* ZJU0616) diet. Conversely, some

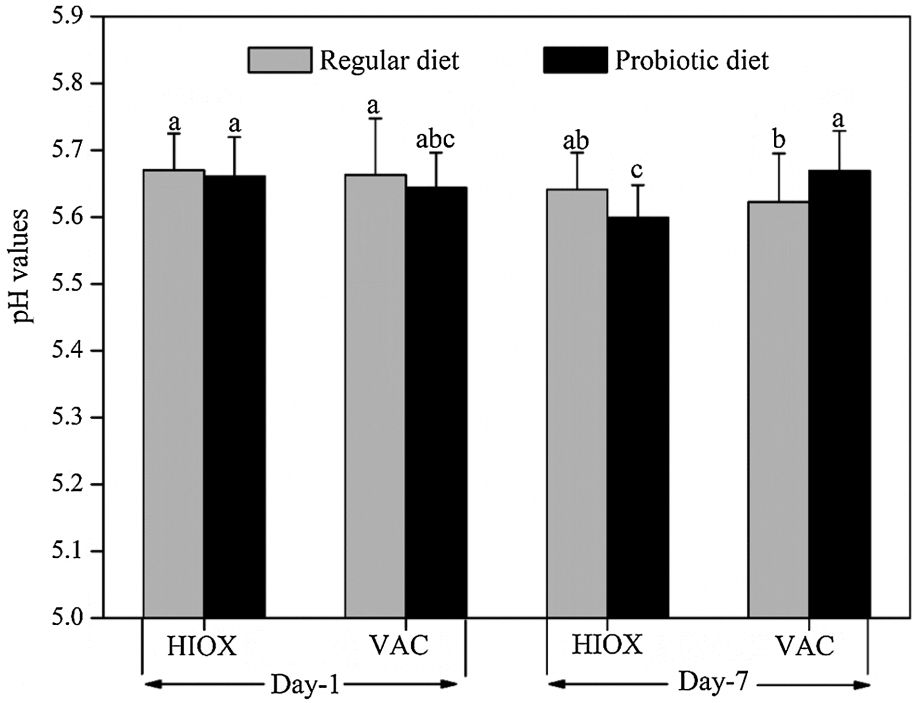

Figure 2. Changes in pH values in laying hen *M. Pectoralis major* muscles during the storage period under different atmospheric storage conditions. Results were displayed as means ± standard error. Notes: The results without identical letters (a∼c) differed significantly ($P < 0.05$). HIOX: high-oxygen storage condition; and VAC: vacuum storage condition.

other studies observed a lower fat and ash content of chicken breast muscles in broilers fed probiotic supplementation (Khaksefidi and Rahimi, 2005; Mahmood et al., 2005). Possibly, different probiotic strains used in the studies might be a potential reason for the discrepancy between those results, as consuming different strains of bacteria could differently alter gut microbiota and nutrient absorption, subsequently influencing muscle composition (Ghadban, 2002).

In terms of pH value changes in the fillet muscle during the storage, there were significant time and treatment effects ($P < 0.05$, Fig. 2, Table 2) and various interactions between storage time*package, packaging*diet, and storage time*packaging*diet ($P < 0.05$; Table 2). There was no difference in the 1 d pH value of the samples regardless of treatments ($P > 0.05$, Table 2). However, as storage time extended, a slight decrease in pH values was observed ($P < 0.05$, Table 2), irrespective of diet treatment ($P > 0.05$) and packaging condition ($P > 0.05$). A decrease in pH value with postmortem storage may be attributed to the processes of postmortem glycolysis, and protein degradation, where the former progress produces lactic acid, and the latter usually generates basic degradation products (Bendall, 1979). While there were significant interactions between treatments, the maximum numerical difference between the treatments was still less than 0.1 unit (Table 2). Therefore, the given magnitude of the pH difference would be unlikely considered as a major factor affecting chemical and redox stability attributes of the fillet muscle samples in the present experiment.

Fatty Acid Profile

The proportion of a total UFA in the regular diet fed laying hens was lower than the probiotic fed counterparts ($P < 0.05$). No differences in either total MUFA or PUFA contents were observed between treatments ($P > 0.05$). While the lipid content was not affected by the dietary treatments as discussed above, probiotic dietary supplementation had impacts on the FA composition ($P < 0.05$). As show in in Table 3, the probiotic supplementation significantly increased the relative proportions of C20:1n9. C20:3n6, C20:4n6 and C24:0 ($P < 0.05$), while the ratio of C16:0, C16:1, and C18:3n3 ($P < 0.05$) were reduced in the laying hen fillet muscles. This observation agrees in part with the findings from Liu et al. (2016), where they found significant decreases in C16:0, C22:4n6, and n-6: n-3 ratio and increases in C18:3n3, C20:5n3, C22:6n3, total PUFA, n-3 PUFA and PUFA: saturated fatty acid ratio in the meat from broilers fed with probiotics (*Lactobacillus johnsonii* BS15). The changes of FA compositions are likely related to gut microbial modulations, regulating the absorption of nutrients and energy metabolism in a certain manner, therefore leading to the differences of chemical (FA) compositions in muscle tissue (Ghadban, 2002). This result is consistent with a previous study performed by Endo and Nakano (1999), where they found that broilers fed with probiotic supplementations had higher UFA content in thigh muscle. Given that unsaturated fatty acid is well-known for its susceptibility to oxidation, it is reasonable to assume that unsaturated fatty acids, especially polyunsaturated fatty acids are

Table 2. Summary of the statistical results.

Treatments[1]		pH	Carbonyl	CD	POV	TBARS	DPPH
Storage time (T)	DAY 1	5.66	5.39	2.96	0.34	0.70	17.12
	DAY 7	5.63	6.83	3.20	0.36	0.76	12.82
	SEM[2]	0.0094	0.3613	0.0471	0.0326	0.0242	0.6769
	P-value	0.0063	0.0003	<.0001	NS	0.0214	<.0001
Atmospheric condition (A)	HIOX	5.64	6.37	2.98	0.38	0.78	15.64
	VAC	5.65	5.84	3.18	0.32	0.69	14.29
	SEM[2]	0.0094	0.3613	0.0471	0.0326	0.0242	0.6769
	P-value	NS	NS	0.0002	0.0706	0.0007	0.0539
Diet (D)	CON	5.65	6.26	3.19	0.35	0.67	14.86
	PRO	5.65	5.95	2.97	0.35	0.79	15.08
	SEM[2]	0.0094	0.3613	0.0471	0.0326	0.0242	0.6769
	P-value	NS	NS	<.0001	NS	<.0001	NS
T*A	SEM[2]	0.0133	0.5109	0.0666	0.0461	0.0343	0.9572
	P-value	0.0475	NS	<.0001	0.0247	NS	0.0009
T*D	SEM[2]	0.0133	0.5109	0.0666	0.0461	0.0343	0.9572
	P-value	NS	NS	<.0001	0.0108	0.0536	NS
A*D	SEM[2]	0.0133	0.5109	0.0666	0.0461	0.0343	0.9572
	P-value	0.0388	NS	<.0001	NS	NS	0.0314
T*A*D	SEM[2]	0.0188	0.7225	0.0942	0.0652	0.0485	1.3538
	P-value	0.0115	NS	<.0001	NS	NS	NS

[1]DAY 1: *M. Pectoralis major* of laying hens stored for 1 d; DAY 7: *M. Pectoralis major* of laying hens stored for 7 d; HIOX: *M. Pectoralis major* of laying hens stored under High-oxygen condition; VAC: *M. Pectoralis major* of laying hens stored under vacuum condition; CON: *M. Pectoralis major* from laying hens fed regular diet; PRO: *M. Pectoralis major* from laying hens fed probiotic supplementation.

CD represents Conjugated dienes (μmol/mg meat); POV represents Peroxide value (milliequivalent O^2/kg fat); TBARS value indicates 2-thiobarbituric acid reactive substances value (mg MDA/kg meat); DPPH indicates 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging capacity (%).

[2]SEM represents standard error of means (n = 12).

Table 3. Fatty acid profiles of breast muscles obtained from the regular or probiotic fed laying hens.

Fatty acid	Control[1]	Probiotic[1]	SEM[2]	*P* value
C12:0	0.01	0.02	0.0060	0.0809
C14:0	0.06	0.05	0.0126	0.2608
C16:0	12.70[a]	7.17[b]	0.4368	<0.0001
C16:1	1.28[a]	0.49[b]	0.0123	0.0014
C18:0	8.46[b]	10.45[a]	0.2570	0.0006
C18:1n9c	27.82	24.82	2.0842	0.1810
C18:2n6c	13.51	11.82	1.0102	0.1549
C20:0	0.06	0.05	0.0145	0.2757
C18:3n6	0.07	0.05	0.0201	0.3825
C20:1n9	0.33[b]	0.51[a]	0.0620	0.0347
C18:3n3	0.72[a]	0.20[b]	0.0981	0.0003
C20:2	0.19	0.28	0.0544	0.1411
C22:0	0.31	0.31	0.0138	0.8190
C20:3n6	0.72[b]	1.01[a]	0.0774	0.0137
C22:1n9	0.03	0.05	0.0127	0.2639
C20:3n3	0.02	0.22	0.0833	0.0585
C20:4n6	8.50[b]	11.08[a]	0.5033	0.0037
C24:0	0.03[b]	0.04[a]	0.0050	0.0301
C20:5n3	0.13	0.17	0.0522	0.5374
C24:1n9	0.03	0.05	0.0071	0.0671
C22:6n3	1.49	1.04	0.4744	0.3737
ΣSFA	28.35[a]	25.24[b]	1.0007	0.0266
ΣUFA	71.65[b]	74.76[a]	1.7948	0.0265
ΣMUFA	38.40	39.04	2.2412	0.7878
ΣPUFA	33.25	35.72	1.0000	0.2264

[a-b]Least squares means within a row for the 2 treatments lacking a common superscript differ ($P < 0.05$).

[1]Control: laying hens that fed with a regular diet; Probiotic: laying hens fed with probiotic diet (300 ppm Sporulin).

[2]SEM represents standard error of means (n = 12)

SFA: Saturated fatty acids; UFA: Unsaturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.

more susceptible to oxidation, hence it is reasonable to assume that the altered FA profile would lead to the different biochemical environment in the postmortem muscles, such as oxidation reactions (Ponnampalam et al., 2014), which are discussed in the following sections.

Primary Lipid Oxidation

Conjugated Dienes CD are primary lipid oxidation products that are formed from the formation of hydroperoxides from PUFA (Houhoula et al., 2002). As displayed in Table 2, significant main effects and three-way interactions (storage time*atmospheric condition*diet) on CD were found ($P < 0.01$). In general, as displayed in Fig. 3, CD content in the *M. Pectoralis major* muscles from regular diet fed chickens had no difference between the storage atmospheric condition at 1 d ($P > 0.05$), but only decreased at storage 7 d under VAC condition ($P < 0.05$). However, the muscle samples from probiotic fed chickens had a lower CD content under HIOX compared to VAC condition at 1 d postmortem ($P < 0.05$). Further, a significant increase of CD content was observed in the muscle samples from probiotic fed chickens during 7 d of storage under HIOX, while the CD content of probiotic samples under VAC did not change throughout the storage ($P > 0.05$). This observation could indicate that the oxidation susceptibility of the muscle samples from probiotics treated chickens would be an atmospheric condition-specific, where it would be more prone to oxidation under oxidizing conditions (e.g., HIOX). This

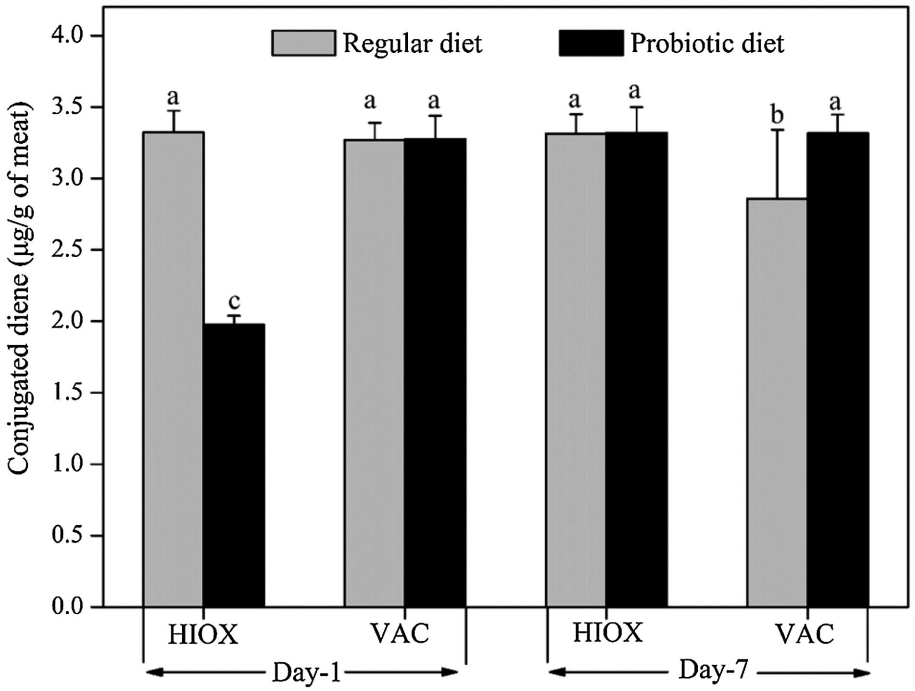

Figure 3. Changes in conjugated dienes in laying hen *M. Pectoralis major* muscles during the storage period under different atmospheric storage conditions. Note: The results were displayed as means ± standard error. The results without identical letters (a∼c) differed significantly ($P < 0.05$). HIOX: high-oxygen storage condition; and VAC: vacuum storage condition.

could be likely attributed to the higher UFA in the muscle, as reported by Ajuyah et al. (1993), where the higher ratio of UFA could possibly indicate the great potential of muscles being oxidized under oxidizing conditions. Moreover, the higher proportion of C20:4n6, which has the highest degree of unsaturation, was found in postmortem fillet muscle collected from the laying hens treated with probiotics compared to the control diet. This could likely imply a higher susceptibility of the muscles from probiotics supplemented laying hens being attacked by the ROS than the control, especially under the HIOX condition (Esterbauer et al., 1992). However, no clear explanation could be made for the lower CD content of probiotic muscle samples under the HIOX condition on storage d 1 with the given results. Probably, it might be related to the probiotic supplementation, which has the potential to alter the biochemical properties of early postmortem muscles of the chickens; and would in turn reduce its sensitivity to oxidizing condition at the early stage (Andersen et al., 2005).

Peroxide Value As an oxidation product, POV has been used as an indicator of the primary oxidation of lipids (Melton, 1983). As presented in Table 2, dietary treatment, storage period, or atmospheric condition alone did not have significant impacts on the extent of POV, although storage condition had a trend to increase POV in the muscle samples under HIOX compared to VAC ($P = 0.07$). However, POV was affected by the interactions between storage time*diet, storage time*storage atmospheric condition ($P < 0.05$). In particular, although not significant, numerically lower POV levels were observed in the muscle samples from the probiotic-fed group compared to the control at storage d 1 (Fig. 4a). Nevertheless, as storage time extended, a significant increase of POV level was observed in the muscle samples from probiotic treated laying hens ($P < 0.05$), while no difference was found in the samples from laying hens with regular diet. This could indicate that the fillet muscles from the probiotic fed laying hens were more prone to oxidation during postmortem storage compared to the muscle samples from the regular diet treated ones.

Regarding the interaction between storage time*storage atmospheric condition, the POV was not different between the packaging conditions at the initial storage ($P > 0.05$). At 7 d of storage, however, significantly higher POV levels were found in the muscles stored under HIOX compared to the samples stored in VAC ($P < 0.05$; Fig. 4b). This observation is expected, as high-oxygen atmospheric conditions would induce more oxidation and result in producing more oxidized products in postmortem muscle (Esterbauer et al., 1992). Together, with the given CD and POV results, it can be surmised that the fillet muscles from chickens fed probiotics would develop an increase in the extent of primary lipid oxidation with responding to the prolonged storage time and high-oxidation atmospheric conditions.

Secondary Lipid Oxidation—TBARS

Lipid oxidation of the muscle samples were significantly affected by the diet, storage time, and

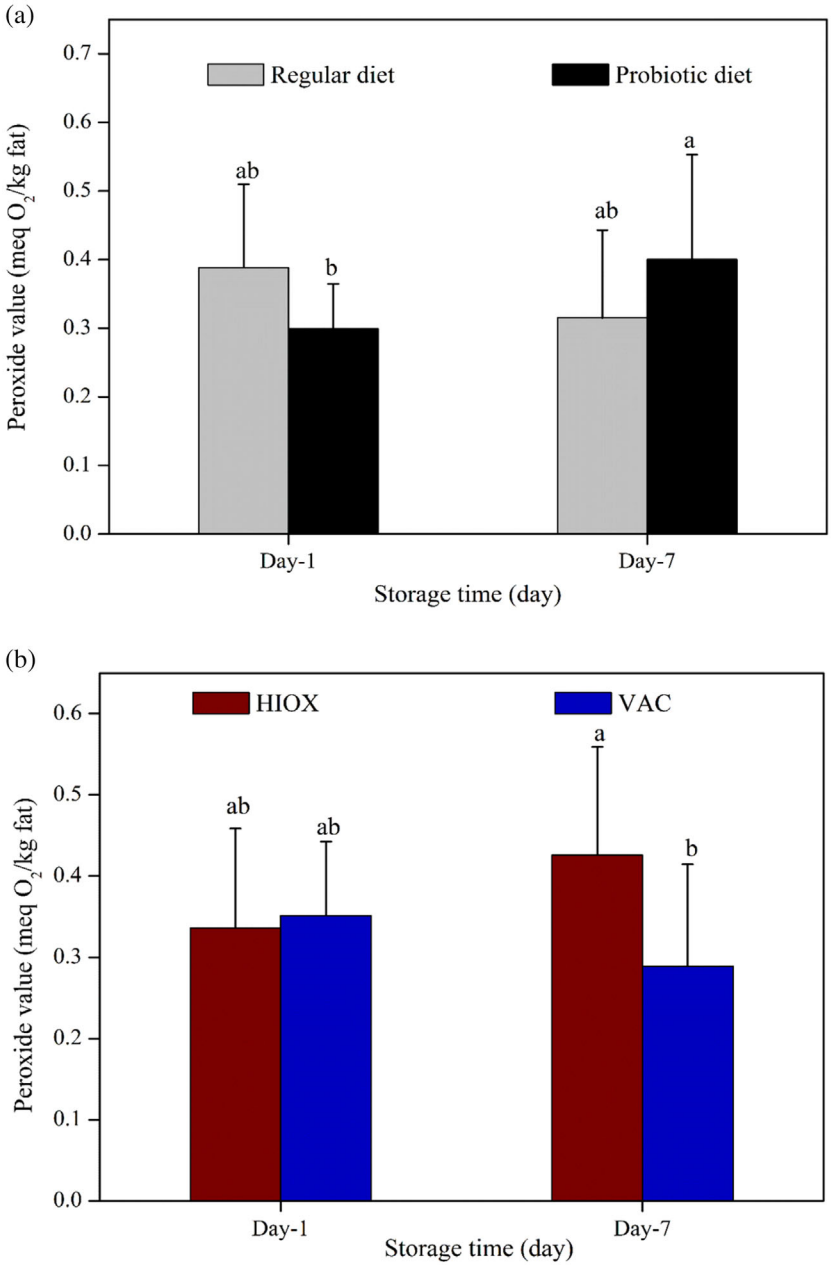

Figure 4. (a) Changes in peroxide values in laying hen *M. Pectoralis major* muscles fed regular diet or probiotic supplementary diet during the storage period. Note: The results were displayed as means ± standard error. The results without identical letters (a, b) differed significantly ($P < 0.05$). HIOX: high-oxygen storage condition; and VAC: vacuum storage condition. (b) Changes in peroxide values in laying hen *M. Pectoralis major* muscles under different atmospheric storage conditions. Note: The results were displayed as means ± standard error. The results without identical letters (a, b) differed significantly ($P < 0.05$). HIOX: high-oxygen storage condition; and VAC: vacuum storage condition.

atmospheric storage condition, respectively as revealed by TBARS values ($P < 0.05$, Table 2). No significant interactions between main effects were found, except for a strong trend of storage time*diet on TBARS value ($P = 0.054$). A significant diet impact on TBARS values of the muscle samples was found, where the samples from laying hens fed probiotic-enhanced diet had higher TBARS values than the regular diet fed counterparts at both storage 1 d and 7 d ($P < 0.05$; Fig. 5). Some previous works found positive or no impacts of probiotic supplement on postmortem lipid oxidation (Bai et al., 2016; Kim et al., 2017a). For example, Kim et al. (2016) found no differences of TBARS value among control and two other different probiotic treatments (250 ppm of Sporulin, containing 1.0×10^6 cfu/g of *Bacillus subtilis;* and 500 ppm of PoultryStar, which consists of 1.0×10^5 cfu/g of *Enterococcus* spp., *Pediococcus* spp., *Bifidobacterium* spp., and *Lactobacillus* spp.). As previously discussed, one of the possible reasons for the different findings may be related to either probiotic strains or the dosage applied (Kim et al., 2016).

The storage time*diet impact on TBARS value ($P = 0.054$) was in line with the results of the primary lipid oxidation in that fillet muscle samples of

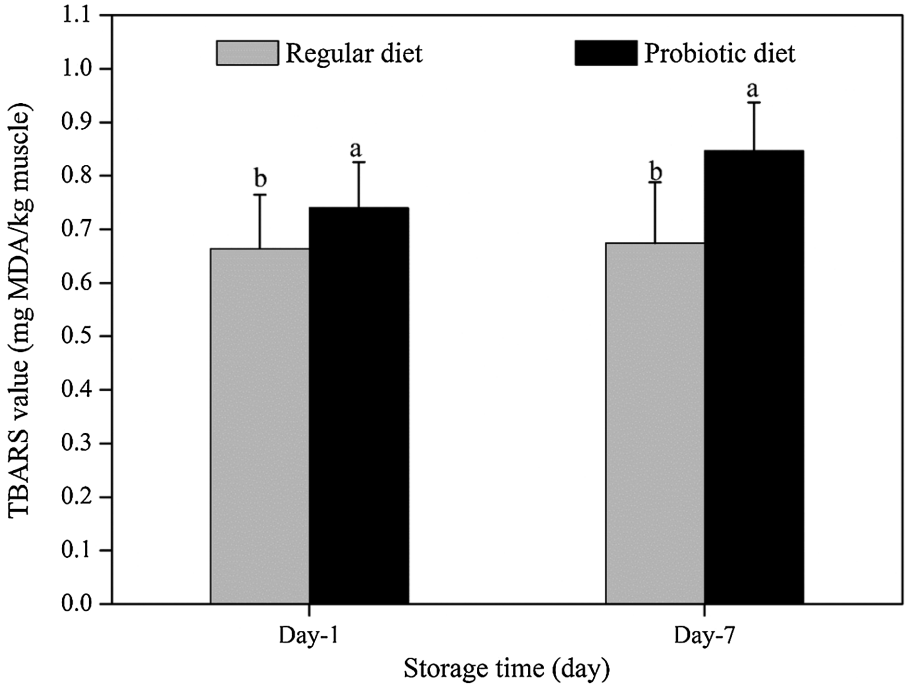

Figure 5. Changes in TBARS in laying hen *M. Pectoralis major* muscles fed regular diet or probiotic supplementary diet during the storage period. Note: The results were displayed as mean ± standard error. The results without identical letters (a, b) differed significantly ($P < 0.05$).

probiotic-fed laying hens would be more susceptible to lipid oxidation during storage. Further, considering the fact that the fat content of regular or probiotic-fed hens was not different (Table 1, $P > 0.05$), this observation would be likely attributed to the higher content of UFA in probiotic-fed laying hen muscles, implying greater susceptibility of oxidation (Melton, 1983). However, unlike the POV and CD, no atmosphere-specific lipid oxidation (TBARS) result was found in the muscle samples from probiotic-fed hens. Possibly, the contradicting results between the primary and secondary oxidation products may be attributed to the different biochemical pathways producing these oxidation products.

The atmospheric condition, however, significantly affected the TBARS values of the muscle samples, where the samples stored under HIOX had higher TBARS values compared to the VAC counterparts ($P < 0.05$), as expected. This result coincides with the findings from other similar studies that muscle samples stored in high oxygen atmospheric condition cause an increase in lipid oxidation (Ajuyah et al., 1993; Jakobsen and Bertelsen, 2000).

Protein Carbonyl Content

Carbonylation of a certain group of protein residues, such as lysine, arginine, proline and threonine, is generally considered as the process of protein oxidation (Levine et al., 1990). In the current study, the extended storage time increased the protein carbonyl content (Table 2, $P < 0.05$) of the muscle samples. However, no probiotic feeding, storage conditions or their interactions were found to be significant influencing the formation of protein carbonyl content ($P > 0.05$). These observations are in agreement with the finding reported by Kim et al. (2017b), where they found that postmortem storage significantly affected the extent of protein oxidation, but no other treatments (heat stress and/or probiotic supplementation) influenced the carbonyl contents of broiler thigh muscle samples (Kim et al., 2017b).

A coupling reaction between protein oxidation and lipid oxidation has been suggested, as lipid peroxidation would trigger the process of protein carbonylation (Gatellier et al., 2000). Further, carbonyl groups have a potential to be the substrate to promote the reaction between proteins and aldehydes, by-products of lipid oxidation (Gatellier et al., 2000). However, in the present study, the muscle samples from probiotics fed laying hens only showed the elevated lipid oxidation products, but no impact on protein oxidation, indicating no clear linkage between lipid and protein oxidation in the chicken fillet muscles.

2,2-diphenyl-1-picrylhydrazyl Radical Scavenging Capacity

DPPH is widely used to determine the antioxidative ability of food products (Bondet et al., 1997). The results of the present study revealed that there were significant storage time and storage condition effects, interactions of storage time*storage atmospheric condition and storage atmospheric condition*diet treatments on the DPPH levels in the fillet muscles from the

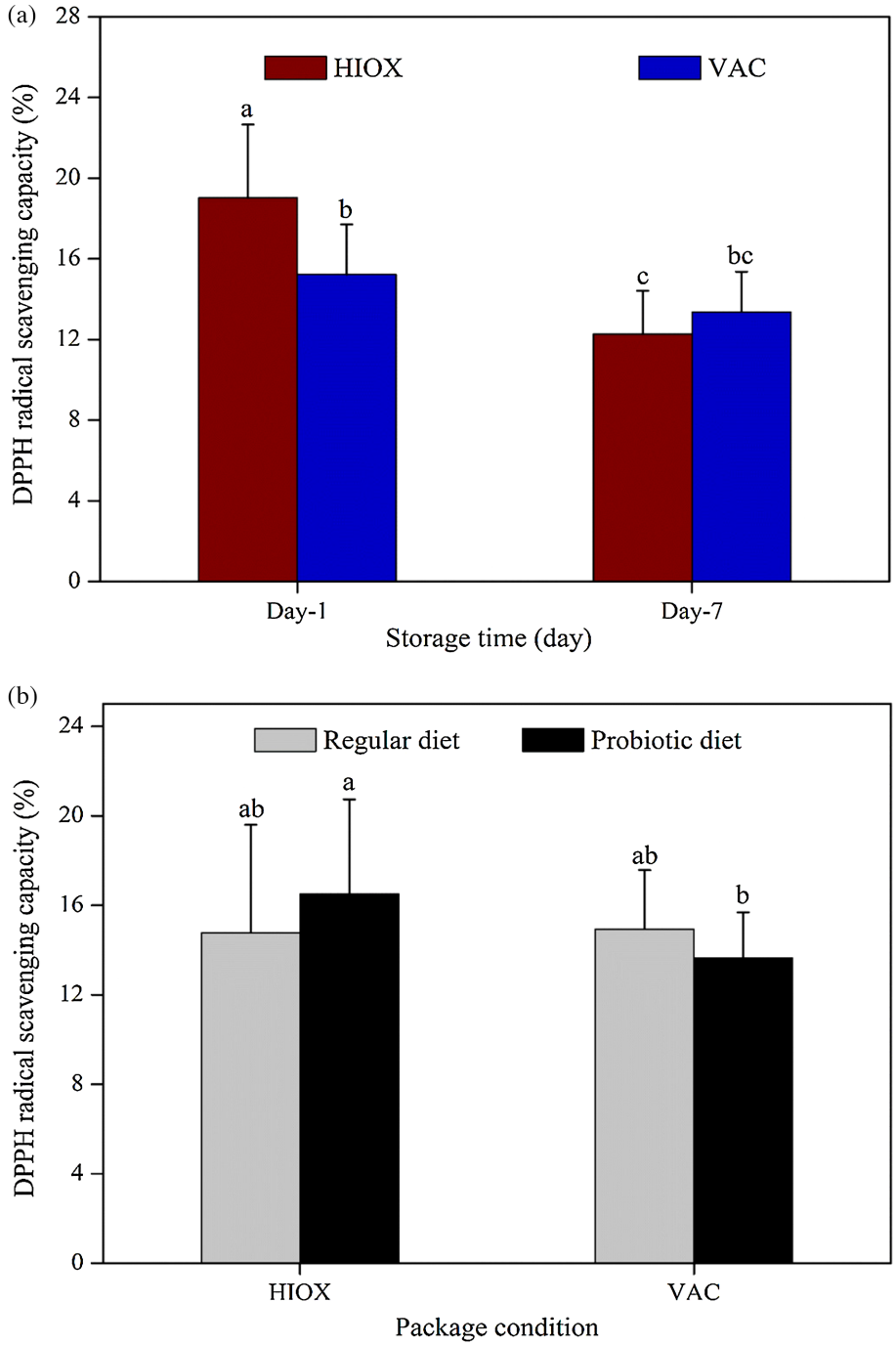

Figure 6. (a) Changes in DPPH radical scavenging capacity in laying hen *M. Pectoralis major* muscles under different atmospheric storage periods. Note: The results were displayed as mean ± standard error. The results without identical letters (a∼c) differed significantly ($P < 0.05$). HIOX: high-oxygen storage condition; and VAC: vacuum storage condition. (b) Changes in DPPH radical scavenging capacity in laying hen *M. Pectoralis major* muscles fed with regular diet or probiotic supplementary diet under different atmospheric storage conditions. Note: The results were displayed as mean ± standard error. The results without identical letters (a, b) differed significantly ($P < 0.05$). HIOX: high-oxygen storage condition; and VAC: vacuum storage condition.

laying hens (Table 2, $P < 0.05$). In respect of the storage time*storage atmospheric condition (Fig. 6a), the samples packaged in HIOX had a greater DPPH value compared to VAC at the initial storage ($P < 0.05$). However, after 7 d of storage, a significant decrease in DPPH was found in the samples stored in HIOX, while no change was observed in the samples under VAC ($P > 0.05$). It might be attributed to that oxidative environment in HIOX, which has potential to trigger more activation of antioxidant performance of the muscle system at the early postmortem stage, when the ROS defensive mechanism in postmortem muscle is still available, although limited (Wood et al., 2004). As molecular oxygen can induce the production of ROS (Cramer et al., 2018), oxygen stress could prompt the activity of the DPPH radical scavenging system as a subsequent response to

mitigate oxidative damage. However, no difference in DPPH between HIOX and VAC samples at the end of the storage would possibly imply that the activities of endogenous enzymes in the muscle might be impaired or become limited with extended storage (Ryu and Kim, 2005). This postulation would warrant further studies for confirmation.

The significant diet*storage condition also showed the atmosphere-specific response in DPPH of the muscle samples from the probiotics fed laying hens, where the samples stored under HIOX had a higher DPPH value compared to the VAC counterparts (Table 2, $P < 0.05$). On the other hand, no packaging impact on DPPH was found in the muscle samples from the regular diet treated laying hens (Fig. 6b, $P > 0.05$). It is well accepted that the endogenous skeletal muscle antioxidant defense system is composed of cytosolic- and lipid-based antioxidants as well as enzymatic antioxidants, all of which might be affected by the antemortem or postmortem handlings or both (Decker and Mei, 1996). Moreover, DeVore et al. (1983) reported that 0.25 ppm selenium supplementation to chicken diet increased glutathione peroxidase activity in breast and leg muscles, confirming the potential of dietary supplementation in altering the antioxidant systems in postmortem muscle. Therefore, considering the results obtained in the current study, it is reasonable to assume that the endogenous anti-oxidative system in the probiotic muscle samples was more sensitive to postmortem oxidative stressors (Cramer et al., 2018).

CONCLUSION

The results from the present study found that postmortem fillet muscles obtained from the laying hens fed with probiotic supplementation are more prone to be oxidized compared to the regular diet, especially under more oxidizing conditions (HIOX and extended storage time). Different fatty acid compositions could account for the different oxidative potential between control and probiotic muscle samples in part, as higher UFA was found in the probiotic-fed laying hen muscles. However, inconsistent results between primary and secondary lipid oxidation products may imply the complexity of postmortem oxidation affected by probiotic feeding. The findings in the current study could provide some insights for the poultry industry to develop practical post-harvest strategies for preventing any quality deterioration of poultry products caused by dietary probiotic supplementations. Further investigations to elucidate the impact of probiotic feeding on oxidative stability and antioxidation mechanisms of chicken muscles would be warranted.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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