

Effects of dietary probiotic (*Pediococcus acidilactici*) supplementation on productive performance, egg quality, and body composition in laying hens fed diets varying in energy density

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ABSTRACT This study was conducted to determine the effect of probiotic *Pediococcus acidilactici* (PA) strain MA18/5M supplementation of diets with different dietary energy levels on productive performance, egg quality, and body composition in Hy-Line Brown hens during a 16-week period from 32 to 47 wk of age. The experimental treatments with a 2 × 2 factorial design received a 2 wheat–corn–soybean diet: a moderately low energy density diet with 2,650 kcal ME/kg (M-LED) and a low energy density diet based on the M-LED diet with 2,550 kcal ME/kg (LED), each diet without and with probiotic supplementation (M-LED, LED, M-LEDp, and LEDp, respectively). Reduced dietary energy levels had a particularly negative effect on egg weight (61.7 vs. 63.3 g; −2.6%, $P < 0.001$), egg mass output (1.67 vs. 1.71 kg; −2.4%, $P = 0.015$), and FCR (2.01 vs. 1.97 kg feed/kg egg; +2%, $P = 0.028$). In hens administered the LED diet, deteriorated productive performance was accompanied by greater body weight loss ($P < 0.001$) and

reduced abdominal fat content ($P < 0.033$) as compared with the M-LED group. Dietary probiotic inclusion increased egg weight ($P = 0.015$), including relative eggshell weight ($P = 0.008$) and eggshell thickness ($P = 0.002$) and significantly improved FCR ($P = 0.010$). No interactions between the PA-based probiotic and dietary energy levels were found in any of the tested parameters. Adding the probiotic on top of the M-LED diet improved layers performance but resulted in non-bioequivalence for the egg weight, egg mass output, and FCR compared with this group without probiotic. Probiotic supplementation of the LEDp diet improved all performance parameters except for egg weight. As a result, the laying rate, egg mass output, daily feed intake, and FCR in the LEDp treatment were bioequivalent to those noted in the M-LED group without the probiotic. The results of a bioequivalence test suggest that a low energy diet fed to laying hens promoted a probiotic response to improve energy utilization by birds.

Key words: body composition, egg quality, laying hen, *Pediococcus acidilactici*, performance

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INTRODUCTION

Probiotics do not lead to the development or spread of resistant pathogenic bacteria in animals, thus offering immense potential as an alternative to antibiotic growth promoters in the poultry industry (Griggs and Jacob, 2005; Nava et al., 2005; Kabir, 2009). However, the efficacy of probiotics is inconsistent because of the differences in their microbial composition (e.g., single-strain or multi-strain probiotics), livability in the

gastrointestinal tract, supplemental dose, method and frequency of application, diet composition, bird age, as well as interactions with environmental stress factors (Balevi et al., 2001; Kalavathy et al., 2003, 2006; Zhang et al., 2012).

It has also been suggested that the efficacy of probiotics can be affected by dietary energy levels and nutrient density. According to the above concept, a high nutrient density diet provides more nutrients for the growth and proliferation of live microorganisms, thus increasing the efficacy of probiotics. Probiotics (*Enterococcus faecium*, *Bacillus subtilis* with *Clostridium butyricum*) and essential oils added to high-energy and high nutrient density diets exerted a more beneficial effect on performance, nutrient digestibility, and gut balance in pigs (Meng et al., 2010; Yan et al., 2010; Yan and Kim, 2013) and laying hens (Zhang and Kim, 2013), as compared with

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low energy and low nutrient density diets. Therefore, we hypothesized that the effect of the probiotic *Pediococcus acidilactici* (PA) could be different in layers fed diets varying in energy and nutrient density.

To our knowledge, most studies investigating the efficacy of probiotics have involved treatments with identical dietary nutrient concentrations. Our previous experiment revealed that the addition of PA strain MA18/5M at 100 mg/kg (1×10^9 CFU/kg feed) to a commercial diet (2,700 kcal ME/kg and 17.5% CP) improved hen performance and eggshell quality during the early laying period (Mikulski et al., 2012). The present study aimed to further expand our knowledge and investigate the effects of these probiotic bacteria on productive performance, body composition, and egg quality in laying hens fed diets varying in energy density.

MATERIALS AND METHODS

Ethics Statement

The experiment was conducted at the Animal Research Laboratory (Department of Poultry Science, University of Warmia and Mazury, Olsztyn, Poland) in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes (OJEU, 2010). The protocol for this study was approved by the Local Ethics Committee (University of Warmia and Mazury, Olsztyn, Poland).

Animals and Husbandry Conditions

A total of 200 31-week-old Hy-Line Brown hens with approximately the same BW were obtained from a local commercial flock and were distributed in a completely randomized design in a factorial arrangement. The birds had been previously vaccinated against infectious bronchitis, the Newcastle disease virus, and egg drop syndrome. Each treatment consisted of 50 laying hens kept in individual Big Dutchman double-sided, three-tier battery cages (40 × 35 × 60 cm, with a floor slope of 12°). The treatment groups were distributed alternately between the upper, middle, and lower tiers, with 1 bird of each group per cage, to minimize the cage-level effect.

Each cage was equipped with an individual nipple drinker. A continuous, metal feed-trough was divided by replicate to ensure that the hens were not able to consume feed assigned to the adjoining replicate. The feed-trough was manually filled on a daily basis from clearly marked bags. A wire egg collector was installed in the front of each cage to prevent eggs from separate replicates from being mixed. The house was provided with artificial programmable lights and climate, a gas heating system, and forced ventilation. All hens were housed in a windowless and environmentally controlled room with the room temperature kept at 20°C to 22°C, and the light cycle set at 16 h of continuous light (incandescent lighting, 10 lux) and 8 h of dark period. The experiment began at 31 wk of age and lasted for 16 wk, that is four 4-week periods.

Experimental Treatments and Diets

A completely randomized 2 × 2 factorial arrangement with moderately low energy density (M-LED) and low energy density (LED) diet (2,650 and 2,550 kcal ME/kg), each without (M-LED and LED, respectively) and with the addition of a probiotic (M-LEDp and LEDp, respectively), was used to evaluate productive performance, egg quality parameters, and body composition in laying hens (32–47 wk of age).

The experimental diets were formulated to contain 2,650 and 2,550 kcal of apparent metabolizable energy (AME)/kg, and equal amounts of CP, lysine, methionine + cysteine, threonine, calcium (Ca), and phosphorus per kilocalorie of ME. Soybean oil was supplemented as an additional source of energy and to adjust the ME content of diets. According to the Recommended Allowances and Nutritive Value of Feedstuffs (Smulikowska and Rutkowski, 2005), approximately 2,650 kcal ME/kg and 165 g CP/kg feed are provided to hens of this age group, with predicted intake of up to 120 g/D. The composition of the diets and their calculated or analyzed nutrient content are shown in Table 1. The M-LED and LED diets contained 41 and 46% of wheat, 20 and 20% of maize, 10 and 7.8% of soybean meal, 3 and 0.7% of soybean oil, respectively. All raw materials were ground in a disc mill (Skold A/S, Denmark) at 2.5 mm disc distance, mixed without any heat treatment, and stored in sacks in a cool place. The Bactocell PA 10 probiotic (Lallemand S.A.S., Blagnac, France) used in the study was formulated with a specific live culture of lactic acid bacteria PA strain MA 18/5M which was guaranteed to contain at least 1.0×10^{10} CFU/g. Experimental diets were produced in a local feed mill under the direct supervision of a representative of the Department of Poultry Science, University of Warmia and Mazury. The diets in mash form and water were provided *ad libitum* throughout the 16-week study.

Parameters Recorded and Methods Applied

Laying Performance The birds were weighed at the beginning (32 wk of age) and at the end of the trial (47 wk of age). Mortality rates were monitored every day. Eggs were collected daily, and egg production was expressed on a hen-day basis (% hen-day) for 4-week intervals. Individual egg weights were recorded by weighing individually 2 eggs on a cage basis (hen) every 2 wk (a total of approximately 800 eggs per group during the experiment) and were used to calculate average egg weight for 4-week intervals. Total egg mass was calculated by multiplying average egg weight by egg production.

Feed intake was measured on a cage basis every 4 wk, at 35, 39, 43, and 47 wk of age. Daily feed intake (DFI) per bird was calculated on a cage-hen total feed consumption basis for the entire experimental period and for the number of days in the period. Daily ME intake was calculated using the recorded DFI and energy content of feed. The FCR (kg of feed/kg of eggs) for each period was

Table 1. Ingredient composition and nutrients content of basal diets (g/kg, as-fed basis).

Items	Moderately low energy density (M-LED)	Low energy density (LED)
Ingredients		
Wheat	411.6	465.4
Maize	200.0	200.0
Soybean meal (48% CP)	104.7	78.1
Sunflower meal (36% CP)	100.0	100.0
Rapeseed meal	40.0	40.0
Soybean oil	30.1	7.0
Limestone	93.3	90.0
Monocalcium phosphate	11.3	10.6
Salt	3.7	3.5
Choline chloride	1.0	1.0
L-Lysine 99 HCL	0.7	1.0
DL-Methionine	1.1	0.9
Vitamin-mineral premix ¹	2.5	2.5
Calculated nutrient content		
AME, kcal/kg ^{2, 3}	2,650 (2,631)	2,550 (2,534)
Crude protein	166.6	160.3
Digestible Lysine	6.38	6.12
Digestible Met + Cys	6.57	6.30
Digestible Threonine	5.35	5.09
Linoleic acid	24.5	12.0
Calcium	38.2	36.8
Phosphorus total	6.73	6.58
Available phosphorus	3.90	3.80
Analyzed nutrient content²		
Dry matter	894.3	889.3
Crude protein	168.0	163.8
Crude fat	39.8	21.6
Starch	391.9	410.1
Sugars	38.2	36.6
Calcium	38.6	36.9
Phosphorus total	7.22	6.89

AME (kcal/kg) = ((0.155 × %CP) + (0.343 × %Fat) + (0.167 × %Starch) + (0.130 × %Sugars)) × 239.

¹Supplied the following per kilogram of feed: 8 000 IU vit A, 2 500 IU vit D₃, 20 mg vit E, 1.0 mg vit K₃, 1.5 mg vit B₁, 4 mg vit B₂, 1.0 mg vit B₆, 0.02 mg vit B₁₂, 0.1 mg biotin, 6.0 mg pantothenic acid, 65.0 mg Mn from manganese oxide, 52 mg zinc from zinc oxide, 45.0 mg I from ethylene diamine dihydroiodide, 0.15 mg Se from sodium selenite, 6 mg Cu.

²Calculated from the Polish Feedstuff Analysis Tables (Smulikowska and Rutkowski, 2005).

³The value in parentheses was estimated using the equation of Fisher and McNab (1987).

calculated on a cage basis from egg production, egg weight, and feed consumption. To evaluate the effect of reduced dietary energy density and PA supplementation on laying performance, the concept of bioequivalence was also applied. Bioequivalence is defined by EFSA (2018) as follows: if 2 products are said to be bioequivalent, it means that they would be expected to be, for all relevant effects, the same.

Egg Quality Egg and eggshell quality (egg specific gravity, eggshell weight, eggshell thickness, eggshell strength, yolk weight, yolk index, yolk color, albumen weight, and albumen Haugh unit score) was evaluated at 35 wk of age and then at 3 four-week intervals. To this end, 15 eggs laid between 09:00 and 12:00 h were randomly picked from each group at 35, 39, 43, and 47 wk of age (a total of 60 eggs per group during the experiment). Eggs were weighed individually, and the specific gravity of eggs was measured using a densitometer (Axis Hydro AD, Gdansk, Poland). After the eggs had been broken on the EQM plate measurement stand (Egg Quality Microprocessor, Technical Services & Supplies Ltd., Dunnington, York, UK), the height of albumen and yolk was measured with an electronic gauge EQM system. The average of 2 measurements of thick-albumen height (one near to the yolk and the other at the end of dense albumen) together with egg weight were used to compute the Haugh unit

score for each individual egg according to the Haugh (1937) formula. A Vernier caliper was used to measure yolk diameter. The yolk index was calculated as the ratio of yolk height to yolk width. Yolk color intensity was evaluated and scored according to the DSM yolk color fan (1, light yellow; 15, orange). The yolk was then separated from the albumen using a Teflon spoon. Before yolk weight determination, the chalaza was removed with a spatula, and each yolk was rolled on a blotting paper towel to remove adhering albumen. Albumen weight was calculated by subtracting the weights of yolk and eggshell from whole egg weight. To determine eggshell weight, eggshells were cleaned of any adhering albumen, the membrane was removed; eggshells were dried at room temperature and expressed as a percentage of the whole egg. Eggshell thickness was measured at 3 different locations (middle, broad, and narrow ends) using a digital micrometer gauge ($\pm 1 \mu\text{m}$, Mitutoyo QuantuMike, Poland Ltd., Wroclaw, Poland), and the mean value was taken as thickness. Egg internal and external quality analyses were completed within 24 h of egg collection.

Body Composition of Hens At the end of the trial, 7 birds from each treatment were randomly selected, weighed, and killed by electrocution at the Department's slaughterhouse 12 h after feed withdrawal. After killing (without carcass bleeding), the birds were scalded at

61 to 65°C for 60 s, hand plucked, and whole carcasses (head, feet, blood, total viscera, etc.) were air-chilled at 4°C. After chilling, carcasses were weighed 24 h *post-mortem*. Abdominal fat was then removed through the cloaca and weighed. After pretreatment, the carcasses (with abdominal fat) were tightly bagged and frozen at -20°C. At a later date, the frozen carcasses were removed from the freezer, placed into a cooler to slowly thaw, and cut into appropriately sized portions. Portions from each carcass were ground using a large Hobart meat chopper, model 4822 (Hobart Corp., Columbus, OH 43123), and the ground portion was passed through a grinder 3 times to ensure proper mixing. On the completion of grinding with sieves of different diameters, the device was thoroughly washed and dried. Ground carcass samples (about 150 g) were freeze-dried before further analyses.

Chemical Analyses

Samples of feed and freeze-dried carcasses were analyzed in duplicate for the content of dry matter (method 934.01), crude protein ($N \times 6.25$; method 976.05), ether extract (method 920.39), ash (method 942.05), and starch in feed (method 996.11) as described by AOAC (2005). Reducing sugars were extracted from feed samples with 40% ethanol for 1 h, and their content was determined with the Luff-Schoorl method (PN-R-64784, 1994). The content of Ca and total P in feed was determined in duplicate by optical emission spectrometry with excitation in the inductively coupled argon plasma in the Optima 2,000 DV camera (Perkin Elmer) after prior drying in the microwave system (Anton Paar, Austria). The results of body chemistry analysis were expressed as the wet whole-body composition of hens, whereas abdominal fat content was determined relative to live body weight.

Calculation and Statistical Analysis

The ME requirements of layers were calculated according to the NRC (1981) formula: ME per hen daily = $W^{0.75} (173 - 1.95 T) + 5.5 dW + 2.07 EE$; where, W = body weight (kg), T = ambient temperature (°C), dW = change in body weight (g/D), and EE = egg mass (g/D).

The experiment had a completely randomized 2 × 2 factorial design, and a two-way ANOVA was performed to assess the main effects of dietary energy density (M-LED and LED), without and with probiotic supplementation, as well as the interaction between probiotic supplementation and dietary energy density. In the case of performance parameters, the effects of the experimental period and its interactions with diet type (M-LED and LED) and probiotic supplementation were determined using two-way repeated measures ANOVA. The timing of laying performance assays was used as the repeated measures factor (4 levels corresponding to every 4-week period, i.e., wk 32 to 35, 36 to 39, 40 to 43, 44 to 47). For performance, a single cage (each laying

hen; n = 50) was considered as a replicate experimental unit. Egg quality was analyzed statistically by age, that is separately for 35, 39, 43, and 47 wk, and after the data had been pooled for all 4-week periods. The latter results, that is egg quality for the entire experimental period, are presented in the manuscript. In bioequivalence calculation, the treatment receiving a moderately low energy diet without PA supplementation (M-LED) was chosen as a reference to which the remaining treatments were compared. Bioequivalence was demonstrated if the 90% confidence intervals of the difference between 2 treatments lay in the range of the equivalence interval. Equivalence interval was defined as ± 3% of the Least Square Means of M-LED as issued from above described ANOVA. All calculations were performed using the GLM procedures of the STATISTICA software system ver. 10.0 (StatSoft Inc., 2011). All data were presented as means with pooled standard error of the mean estimates, and differences were considered statistically significant at $P < 0.05$, whereas $0.05 < P < 0.10$ was considered a tendency.

RESULTS

Diet Composition

As shown in Table 1, the experimental diets were formulated to contain 2,650 and 2,550 kcal of AME/kg, and equal amounts of CP, lysine, methionine + cysteine, threonine, Ca, and phosphorus per kilocalorie of ME. The AME content of experimental diets, calculated based on ingredient composition (2,650 and 2,550 kcal ME/kg), slightly exceeded the values estimated from the analyzed chemical composition (2,631 and 2,534 kcal ME/kg). Nutrient concentrations in experimental diets were also close to the values adopted in the experimental design model.

Laying Performance

Mortality was low and not related to the dietary treatments. Over the experimental period, one hen died in each of LED groups (soon after the beginning of the trial), and one hen was culled from group M-LEDp after 8 wk because of eating of its own eggs.

Compared with initial BW, the final BW of hens decreased in all treatment groups (Table 2). Laying hens fed LED diets were significantly ($P < 0.001$) lighter than hens fed M-LED diets. In laying hens fed LED diets, BW loss was significantly higher (6.1%; $P < 0.001$) than in birds fed M-LED diets (1.8%).

No significant interactions between dietary energy level and probiotic supplementation were found in any of the tested parameters. Dietary energy levels had no significant effect on feed intake or egg production. As expected, diet had a significant effect ($P < 0.001$) on ME intake because hens fed moderately low ME diets (M-LED) consumed more calories than those fed low ME (LED) diets (314 vs. 304 kcal/hen/D). Reduced dietary energy levels significantly decreased egg weight (by 1.6 g, $P < 0.001$) and egg

Table 2. Effects of energy density of diets and dietary probiotic supplementation on the laying performance of hens during a 16-week feeding period.

Item	n	Laying rate (%) ¹	Egg weight (g) ¹	Egg mass output (kg/hen) ¹	ADFI (g/hen) ¹	Daily ME intake (kcal/hen) ¹	FCR (g feed/g eggs) ¹	Initial BW (kg)	Final BW (kg)	BW change (%) ²
Diet (D)										
M-LED	99	96.56	63.32 ^a	1.709 ^a	118.4	314 ^a	1.970 ^b	1.940	1.908 ^a	1.77 ^b
LED	98	96.85	61.72 ^b	1.672 ^b	119.2	304 ^b	2.009 ^a	1.942	1.835 ^b	6.08 ^a
SEM		0.316	0.325	0.011	0.651	1.693	0.013	0.015	0.015	0.560
P-value		0.507	<0.001	0.015	0.380	<0.001	0.028	0.910	<0.001	<0.001
Probiotic (P)										
without	99	96.27 ^x	62.13 ^b	1.675 ^b	119.2	310	2.012 ^a	1.943	1.869	4.13
with	98	97.14 ^y	62.91 ^a	1.706 ^a	118.5	308	1.966 ^b	1.940	1.875	3.70
SEM		0.316	0.325	0.011	0.651	1.693	0.013	0.015	0.015	0.560
P-value		0.053	0.015	0.038	0.466	0.478	0.010	0.891	0.518	0.575
Treatment ³										
M-LED	50	96.25	62.91	1.693	118.3	313	1.982	1.936	1.908	1.55
M-LED _P	49	96.86	63.74	1.724	118.5	314	1.958	1.944	1.909	2.01
LED	49	96.28	61.35	1.656	120.0	306	2.043	1.949	1.829	6.76
LED _P	49	97.42	62.08	1.688	118.4	302	1.975	1.936	1.841	5.39
SEM		0.446	0.460	0.015	0.920	2.394	0.018	0.021	0.021	0.788
P-value (D × P)		0.550	0.817	0.997	0.324	0.332	0.220	0.605	0.806	0.256
Age period (A)										
P-value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001			
A × D interaction		0.401	0.602	0.302	0.016	0.047	0.002			
A × P interaction		0.175	0.113	0.473	0.430	0.436	0.820			
A × D × P interaction		0.106	0.952	0.422	0.701	0.710	0.974			

^{a,b}Means within the same column with different superscripts differ significantly ($P < 0.05$).

^{x,y}Means within the same column with different superscripts show a near significant trend ($0.05 < P \leq 0.10$).

¹Data were determined using a two-way repeated measures ANOVA. The timing of laying performance assays was used as the repeated measures factor (4 levels corresponding to every 4-week period, i.e., wk 32 to 35, 36 to 39, 40 to 43, 44 to 47).

²Body weight changes in birds in different dietary treatments were all negative, indicating body weight loss.

³Treatments = M-LED and M-LED_P, groups fed moderately low energy density diets without and with the addition of probiotic, respectively; LED and LED_P, groups fed low energy density diets without and with the addition of probiotic, respectively.

Table 3. Bioequivalence of performance parameters in laying hens fed probiotic-supplemented diets with different energy densities compared with the M-LED group.

Item	Laying rate (%)	Egg weight (g)	Egg mass output (kg/hen)	ADFI (g/hen)	FCR (g/g)
Treatment ¹					
M-LED	96.25	62.91	1.693	118.3	1.982
M-LEDp	96.86	63.74	1.724	118.5	1.958
LED	96.28	61.35	1.656	120.0	2.043
LEDp	97.42	62.08	1.688	118.4	1.975
Bioequivalence 3% ²					
LED vs. M-LED	BE	Not BE -	Not BE -	Not BE +	Not BE -
M-LEDp vs. M-LED	BE	Not BE +	Not BE +	BE	Not BE +
LEDp vs. M-LED	BE	Not BE -	BE	BE	BE

Abbreviations: BE, bioequivalence; Not BE -, non-bioequivalence with reduced performance; Not BE +, non-bioequivalence with improved performance; FCR, feed conversion ratio.

¹Treatments = M-LED and M-LEDp, groups fed moderately low energy density diets without and with the addition of the probiotic, respectively; LED and LEDp, groups fed low energy density diets without and with the addition of the probiotic, respectively.

²The treatment receiving a moderately low energy diet without probiotic supplementation (M-LED) was chosen as a reference to which the remaining treatments were compared.

mass output (by approx. 0.04 kg, $P = 0.015$) and contributed to an increase in FCR ($P = 0.028$).

Over the entire experimental period, probiotic supplementation increased egg weight ($P = 0.015$) and egg mass output ($P = 0.038$), significantly improved feed conversion efficiency ($P = 0.010$; 1.97 vs. 2.01 kg feed/kg eggs), and tended to increase laying intensity ($P = 0.053$). The BW of hens and feed intake were not affected by dietary probiotic supplementation ($P > 0.05$).

Linear progress was observed in the laying rate, egg weight, ADFI, and FCR at consecutive age intervals ($P < 0.001$, Table 2). Dietary energy level \times hens' age interaction ($P = 0.016$) for ADFI was because it was similar in wk 32 to 39 and slightly higher in LED hens in wk 40 to 47 (Supplementary Figure 1A). The opposite trend was noted for daily ME intake ($P < 0.047$) because it was significantly lower in LED hens in wk 32 to 39, and

it was similar in M-LED and LED hens in wk 40 to 47 (Supplementary Figure 1B). A significant ($P < 0.002$) interaction between dietary energy density and hens' age for FCR resulted from the fact that it was similar in M-LED and LED hens in wk 32 to 39, and it was significantly higher in LED treatments in wk 40 to 43, whereas the effect of dietary energy on FCR was reduced in wk 44 to 47 (Supplementary Figure 1C).

There were no 3-way interactions between hens' age \times dietary energy density \times probiotic supplementation in any of the measured variables of laying performance. In the first 4 wk of the experiment, PA supplementation increased egg production ($P = 0.018$) and egg mass output ($P = 0.023$), but not egg weight. In week 5 to 8 of the experiment, PA had a beneficial influence on egg mass output ($P = 0.047$) and FCR ($P = 0.082$). In week 9 to 12, probiotic supplementation tended to

Table 4. Abdominal fat content and body chemistry composition in feathered hens fed 2 dietary energy concentrations without and with probiotic supplementation.

Item	n	Body composition (%)				Abdominal fat (% BW)
		Dry matter	Protein	Crude fat	Ash	
Diet (D)						
M-LED	14	40.43	16.20	20.22	3.05 ^x	4.20 ^a
LED	14	39.55	16.53	19.30	2.86 ^y	3.52 ^b
SEM		0.499	0.172	0.642	0.078	0.220
P-value		0.227	0.200	0.341	0.089	0.033
Probiotic (P)						
Without	14	40.04	16.39	19.94	2.92	3.94
With	14	39.94	16.34	19.59	2.99	3.78
SEM		0.499	0.172	0.642	0.078	0.220
P-value		0.893	0.848	0.713	0.534	0.602
Treatment ¹						
M-LED	7	40.67	16.25	20.43	3.04	4.47
M-LEDp	7	40.18	16.16	20.01	3.07	3.93
LED	7	39.40	16.53	19.44	2.81	3.41
LEDp	7	39.70	16.52	19.17	2.91	3.63
SEM		0.705	0.243	0.939	0.110	0.312
P-value (D \times P)		0.584	0.861	0.937	0.757	0.224

^{a,b}Means within the same column with different superscripts differ significantly ($P \leq 0.05$).

^{x,y}Means within the same column with different superscripts show a near significant trend ($0.05 < P \leq 0.10$).

¹Treatments = M-LED and M-LEDp, groups fed moderately-low energy density diets without and with the addition of the probiotic, respectively; LED and LEDp, groups fed low energy density diets without and with the addition of the probiotic, respectively.

Table 5. Effects of energy density levels and dietary probiotic supplementation on egg quality in laying hens during a 16-wk feeding period.

Item	n	Egg specific gravity (g·cm ⁻³)	Eggshell strength (N)	Eggshell thickness (mm·10 ⁻²)	Yolk color	Yolk index	Albumen Haugh units	Relative yolk weight (%)	Relative eggshell weight (%)	Relative albumen weight (%)
Diet (D)										
M-LED	120	1.085 ^x	44.40	0.361	3.77 ^s	0.477	88.88	25.34	10.59 [*]	64.07
LED	120	1.084 ^y	43.98	0.358	3.65 ^y	0.479	89.46	25.49	10.42 ^y	64.09
SEM		0.001	0.531	0.001	0.045	0.002	0.454	0.142	0.069	0.172
P-value		0.090	0.574	0.114	0.079	0.454	0.369	0.452	0.082	0.938
Probiotic (P)										
Without	120	1.084	43.88	0.357 ^b	3.66	0.476	89.06	25.29	10.38 ^b	64.33 ^a
With	120	1.085	44.50	0.363 ^a	3.76	0.479	89.27	25.53	10.64 ^a	63.83 ^b
SEM		0.001	0.531	0.001	0.045	0.002	0.454	0.142	0.069	0.172
P-value		0.536	0.406	0.002	0.104	0.314	0.737	0.242	0.008	0.043
Treatment ¹										
M-LED	60	1.085	43.85	0.357	3.70	0.477	88.80	25.10	10.45	64.45
M-LEDp	60	1.085	44.95	0.365	3.83	0.477	88.96	25.57	10.74	63.69
LED	60	1.084	43.90	0.356	3.62	0.476	89.33	25.49	10.31	64.20
LEDp	60	1.085	44.05	0.360	3.69	0.481	89.59	25.48	10.54	63.98
SEM		0.001	0.751	0.002	0.064	0.003	0.642	0.200	0.098	0.243
P-value (D x P)		0.469	0.527	0.272	0.648	0.271	0.936	0.235	0.739	0.266

^{a, b}Means within the same column with different superscripts differ significantly ($P < 0.05$).

^{x, y}Means within the same column with different superscripts show a near significant trend ($0.05 < P \leq 0.10$).

¹Treatments = M-LED and M-LEDp, groups fed moderately low energy density diets without and with the addition of the probiotic, respectively; LED and LEDp, groups fed low energy density diets without and with the addition of the probiotic, respectively.

increase egg weight ($P = 0.082$), and in wk 13 to 16 of the experiment, PA significantly increased egg weight (by 1.15 g, $P = 0.030$) and FCR ($P = 0.007$).

Table 3 summarizes the results of a bioequivalence test of the performance parameters of hens. Reduced energy content of LED diets resulted in nonbioequivalence with M-LED diets for all performance parameters except laying intensity. The moderately low energy diet with the probiotic (M-LEDp) was bioequivalent to the M-LED group for laying rate and feed intake. The remaining parameters (egg weight, egg mass output, and FCR), despite being improved by the probiotic, were non-bioequivalent to the M-LED group. Probiotic supplementation of the LEDp diet improved all performance parameters except for egg weight. As a result, the laying rate, egg mass output, DFI, and FCR in the LEDp treatment were bioequivalent to those noted in the M-LED group without the probiotic.

Abdominal Fat Content and Body Composition in Hens

The wet chemistry analysis of hen body composition revealed no differences in whole body dry matter, protein, or fat content (Table 4). Abdominal fat deposition decreased significantly ($P < 0.033$), whereas whole-body ash tended to decrease by 7% ($P = 0.089$) in LED hens compared with M-LED hens. No interactions between dietary energy levels and probiotic supplementation were found in any of the tested parameters of body composition. Dietary probiotic supplementation had no effect on abdominal fat content or the body composition of laying hens ($P > 0.05$).

External and Internal Quality of Eggs

Yolk color intensity, egg specific gravity, and eggshell percentage tended to decrease ($P = 0.079$, $P = 0.082$, and $P = 0.090$, respectively) as the dietary energy level decreased from 2,650 to 2,550 kcal/kg (Table 5). Layers fed probiotic-supplemented diets had greater eggshell thickness ($P < 0.002$) and higher relative eggshell weight ($P < 0.008$) compared with those fed diets without probiotic supplementation. The desirable changes in eggshell thickness and weight were accompanied by a decrease in albumen percentage ($P = 0.043$), without changes in yolk percentage.

DISCUSSION

Effect of Dietary Energy Levels

It is often accepted that laying hens have retained the ability to adjust feed intake to dietary energy, and a decrease in dietary energy content leads to an increase in feed intake (Grobas et al., 1999a; Harms et al., 2000). Therefore, it was surprising that in our study, a decrease in dietary energy content from 2,650 to 2,550 kcal of AME/kg (3.8%) had no effect on compensatory feed intake. On the other hand, average

ME intake in LED and M-LED groups (304 and 314 kcal/hen/D) was lower than the calculated requirement (335 kcal/hen/day) based on the [NRC recommendations \(1994\)](#), even though the birds consumed almost 8 g feed/hen per day on average above the level recommended by breeder's manual ([Hy-Line, 2018](#)) for their age group.

The results from previous experiments on the effects of the level of dietary energy on feed consumption in laying hens are conflicting. For instance, [Zhang and Kim \(2013\)](#) reported that hens fed a diet with 2,700 kcal ME/kg consumed 9.0% more feed than hens fed the control diet with 2,800 kcal ME/kg. [Pérez-Bonilla et al. \(2012\)](#) found that on average, a 10% decrease in the AMEn content of the diet from 2,950 to 2,650 kcal/kg resulted in an increase in feed intake of 4 to 5%, whereas a change in dietary energy content from 2,750 to 2,650 kcal ME/kg did not affect feed consumption. Similarly, in other studies, feed intake was not affected by changes in dietary energy content from 3,002 to 2,747 kcal ME/kg ([Keshavarz and Nakajima, 1995](#)), from 2,951 to 2,831 kcal ME/kg ([Wu et al., 2005](#)), and from 2,900 to 2,810 kcal ME/kg ([Jalal et al., 2006](#)). In contrast, [Valkonen et al. \(2008\)](#) demonstrated that feed intake increased with increasing dietary energy levels from 2,390 to 2,629 kcal/kg feed. These conflicting results may be attributed to differences in environmental conditions, diet composition, egg production, hens' genotype, and age ([Ribeiro et al., 2014](#); [Classen, 2017](#)). In a recently published review, [Classen \(2017\)](#) demonstrated that the nature of the feed intake response to dietary energy is neither uniform nor predictable in laying hens.

According to several studies, considerably reduced dietary ME content substantially decreases the BW and BW gain of hens, as well as abdominal fat deposition ([Scragg et al., 1987](#); [March and MacMillian, 1990](#); [Zimmerman, 1997](#); [Bozkurt et al., 2012](#)) and egg weight ([Whitehead et al., 1993, 1995](#); [Grobas et al., 1999a, b, 2001](#)). In this context, our results do not differ from those presented by other authors. In the current experiment, a comparison of ME intake with the theoretical ME requirements (NRC, 1981) or Hy-Line recommendations ([Hy-Line, 2018](#)) revealed that ME intake was insufficient in all treatments. The hens were compensating energy supply shortage by using body reserves, as confirmed by a decrease in the BW of hens in all groups. Our results are also consistent with previous studies which revealed that dietary energy levels had no influence on egg production and egg quality parameters or affected them only slightly ([Leeson et al., 2001](#); [Guangbing et al., 2007](#); [Yu et al., 2008](#); [Hussein et al., 2010](#)). However, [Mathlouthi et al. \(2002\)](#) reported increased laying rate at an energy content of 2,753 kcal of ME/kg of feed compared with 2,653 kcal of ME/kg of feed. In turn, [Çiftci et al. \(2003\)](#) found that decreasing the energy content of feed from 2,751 to 2,641 kcal of ME/kg increased the laying rate from 86.4 to 88.3%.

One of the most important quality parameters for buying retail eggs is yolk color because consumers associate this trait with nutritional value and vitamin content

([Galobart et al., 2004](#); [Zhang et al., 2011](#)). In the current study, the small amount of corn used in diets (20%) affected the pigment content leading to dramatically pale yolks (below 4 on the DSM-YC fan) in all layers. However, the diets with higher energy density slightly increased egg specific gravity, eggshell relative weight, and egg yolk color intensity, most probably because of a higher content of Ca and pigments in the diets, the latter from soybean meal and soybean oil. The above and other changes in egg quality correspond with previous findings ([Grobas et al., 2001](#); [Hassan et al., 2013](#)).

Although few data have been published regarding the effects of dietary energy on hen body composition, it appears that body fat content may be a sensitive indicator of dietary energy status. In general, it is accepted that lower dietary energy levels lead to a reduction in total body fat deposition by decreasing the activity levels of a number of enzymes linked to hepatic lipogenesis ([Tanaka et al., 1983](#)). Similarly to [Ricard et al. \(1983\)](#), our results indicate that considerable changes in abdominal fat may occur without profound changes in total body fat. In the current experiment, decreasing dietary energy levels had no significant influence on the body composition of hens, whereas abdominal fat deposition decreased significantly. Considering the actual ME difference between LED and M-LED groups (3.8%), it should be noted that this difference can be explained by an improved FCR (a difference of ca. 2%) and by a 1% difference in body fat content (20.22 vs. 19.3%) in hens fed M-LED diets. Therefore, an analysis of the body composition of hens helps understand energy allocation between feed efficiency and body reserves.

Interaction Between Dietary Energy Density and Probiotic Supplementation

An interaction between dietary energy levels and probiotic supplementation was previously observed in pigs, where the positive effect of probiotic supplementation on nutrient digestibility and growth performance was enhanced by the high nutrient density diet ([Meng et al., 2010](#); [Yan and Kim, 2013](#)). However, in broiler chickens, an interaction between a probiotic (a mixture of *B. subtilis* and *C. butyricum*) and nutrient density was observed only for BW gain during the first wk of life, where the positive effect of probiotic supplementation on growth performance was reduced by the high nutrient density (3,153 kcal) diet treatment ([Chen et al., 2013](#)). In a study by [Lan et al. \(2016\)](#), interactive effects on average DFI in weaning pig were observed, where a probiotic (a mixture of Bacilli and Clostridium strains) improved DFI more considerably in low nutrient density diets (3,700 vs. 3,850 kcal ME/kg). On this basis, the authors concluded that the intake of probiotics is affected by dietary nutrient density. In this experiment, no interactions between the PA-based probiotic and dietary energy density levels were found in any of the tested parameters. Our results corroborate the findings of [Zhang and Kim \(2013\)](#), who observed no interactions between dietary

energy content (2,700 and 2,800 kcal ME/kg) and probiotic (*E. faecium*) supplementation for egg production, feed intake, or egg quality.

Effect of Probiotic Supplementation

In this experiment, beneficial effects of PA were directly associated with improved performance (egg weight by approx. 1% and FCR by 2%) and eggshell quality (eggshell thickness and relative weight). The average time required by PA to establish a significant effect was 4 wk (data not shown), which is consistent with the findings of [Abdelqader et al. \(2013\)](#) who demonstrated that the time required for *B. subtilis* to exert a significant influence on egg weight and eggshell quality was 3 to 6 wk depending on the dose.

Our results corroborate previous research which revealed that supplemental probiotics containing bacteria such as Lactobacilli, *B. subtilis* and PA, added to laying hen diets, exerted beneficial effects on productive performance and egg quality ([Nahashon et al., 1994a,b](#); [Abdulrahim et al., 1996](#); [Quarantelli et al., 2008](#); [Kalavathy et al., 2009](#); [Mikulski et al., 2012](#); [Zhang et al., 2012](#); [Zhang and Kim, 2013](#)). The use of PA MA18/5M in poultry diets has been documented in the literature over the last 20 yr. [Barbe et al. \(2018\)](#) performed a multi-analysis of 25 published studies and concluded that PA-based probiotics had a beneficial effect on the growth performance of both broiler chickens and laying hens. Significantly higher body weight gains (+5.7%, $P < 0.05$) and improved FCR (by 6.3%, $P < 0.05$) was reported in broilers, whereas an improvement in the laying rate (+2.8%, $P < 0.05$) and feed efficiency (by 2.8%, $P < 0.05$) was noted in layers. Those beneficial effects may be related to the properties of probiotics that is lactic acid and enzyme production, competitive exclusion of pathogens, and an improvement in intestinal epithelial barrier integrity and nutrient retention ([Montes and Pugh, 1993](#); [Leeson and Summer, 1997](#)).

The improvement in egg weight and eggshell quality, noted in our study, may be attributed to the enhancement of Ca absorption and retention associated with probiotic supplementation ([Nahashon et al., 1994b, 1996](#); [Mohan et al., 1995](#); [Haddadin et al., 1996](#)), as confirmed by increased Ca digestibility ([Mikulski et al., 2012](#)) and elevated serum Ca concentrations ([Panda et al., 2008](#)).

We also assumed that PA supplementation could have a positive influence on dietary energy utilization in laying hens, which was earlier observed in broilers ([Stella et al., 2009](#); [Alkhalif et al., 2010](#); [Taheri et al., 2010](#); [Habibi et al., 2013](#)). Therefore, the low energy diet was selected based on the specified top-down approach which could demonstrate the effect of PA on ME utilization. Interestingly, despite similar daily ME intake in the groups without and with probiotic supplementation, PA-supplemented hens were characterized by higher egg laying rates and egg weight and improved feed conversion efficiency. A combination of these factors may suggest that PA supplementation allows the birds

to better utilize dietary ME. In addition, the concept of bioequivalence applied in our study indicates that a low energy diet fed to laying hens promoted a probiotic response to improve energy utilization by birds. As a result, the inclusion of PA in the LED diet contributed to achieving productive performance bioequivalent to that noted in the M-LED treatment without the probiotic.

The price of low energy and low nutrient density diets can be substantially lower than that of high nutrient density diets, which can increase returns to the producer if the former are also effective in maintaining long-term egg production and egg weight ([DePersio et al., 2015](#)). Our study demonstrated that the inclusion of the PA-based probiotic in the energy-reduced (and protein-reduced) diet could be a viable strategy for reducing feed costs. If probiotic supplementation of energy-reduced (and protein-reduced) diets could enable birds to achieve similar performance to that noted in birds fed standard control diets, low-cost rations could be formulated ([Upadhaya et al., 2019](#)). Moreover, the nitrogen content of manure and ammonia concentrations in poultry buildings, which are related to dietary protein levels, have become a growing concern for environment sustainability. Therefore, effective probiotics can contribute to lowering the nitrogen supply to birds without hampering their performance and exert a positive impact on the farm environment.

In conclusion, the dietary supplementation with the PA-based probiotic had a positive effect on the productive performance of laying hens and egg quality by increasing egg weight, egg mass output, and eggshell thickness during a 16-wk laying period. No interactions between PA supplementation and dietary energy were found. The results of this study also suggest that a LED diet fed to laying hens promoted a probiotic response to improve energy utilization by birds.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2019.11.046>.

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