Varying combinations of *Lactobacillus* species: impact on laying hens' performance, nitrogenous compounds in manure, serum profile, and uric acid in the liver

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ABSTRACT: This study was conducted to evaluate the effects of various combinations of *Lactobacillus* species (*L. rhamnosus, L. paracasei*, and *L. plantarum*) on closely associated variables of production of laying hens, nitrogenous compounds in manure, the serum concentration of specific chemicals, and liver uric acid (UA) concentrations at peak lay. White Leghorns W-36 (32-week-old) were randomly assigned to five treatments for 8 weeks. Treatments were T1, the Control, a commercial feed; T2, the Control + *L. paracasei* + *L. plantarum*; T3, the Control + *L. paracasei* + *L. rhamnosus*; T4, the Control + *L. plantarum* + *L. rhamnosus* and T5, the Control + *L. paracasei* + *L. plantarum* + *L. rhamnosus.* Each bacterial species was included at 3.33×10^{11}cfu/kg feed for a total of 6.66×10^{11} cfu/kg feed for T2–T4 and a total of 1.0×10^{12} cfu/kg feed for T5. Major effects among combinations of probiotics on production were not noted. The interaction of Probiotics by Week (Probiotics*Time) affected feed intake ($P = 0.0007$) and feed conversion ratio (FCR, $P = 0.0049$) due to fluctuation by week. Significant effects of time were also recorded for a gradual increase in body weight (BW, $P = 0.0007$); lowest and greatest feed intake at weeks 2 and 7, respectively ($P < 0.0001$); an increase in egg production ($P = 0.0007$) and maximum FCR at week 7 ($P < 0.0001$). Ammonia (NH_3) concentration, ammonium nitrogen (NH_4–N), total Kjeldahl nitrogen (TKN), and total nitrogen remained unaffected at $P < 0.05$. Although there were fluctuations, a trend emerged for the reduction of TKN. Combinations of probiotics did not affect NH_3, UA, total protein (TP), albumin (ALB), creatine kinase (CK), and UA in the liver. Temporal (Time as a fixed effect) effects were noted for all nitrogenous compounds present in manure. For ammonia, temporal effects were significant due to fluctuation over time. Week 0 had the lowest value followed by weeks 4 and 8. Week 6 had the greatest value. For ammonium nitrogen, week 8 had the lowest value followed by week 0 and 4 with the next highest value. Week 6 had the greatest value. For TKN, week 4 had the lowest value followed by weeks 6 and 8. Week 0 had the greatest value. For TN, weeks 4, 6, and 8 had similar and lowest values followed by week 0 having the greatest value. However, an overall reduction in NH_4-N, TKN, and TN was noted. Fluctuations in NH_3 ($P = 0.0033$) and CK ($P = 0.0085$) were noted for Time. There was also a trend ($P = 0.0706$) for the increase of UA in serum. Two or more species of probiotics with yeast should be investigated. If the combination is applicable for increasing production measurements and reducing nitrogenous and serum compounds, the most appropriate time to feed the probiotics from day 1 to the end of production should be investigated.

Key words: combinations of *Lactobacillus* species, manure, nitrogen-containing compounds, performance, serum chemistry, uric acid in the liver, white leghorn hens

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Received June 16, 2020.
Accepted February 16, 2021.

Transl. Anim. Sci. 2021.5:1-14
doi: 10.1093/tas/txab018

INTRODUCTION

Effect of Probiotics on Health of Animals

Antibiotics improve animal health by inhibiting pathogenic bacteria (Ferket, 2004). They improve nutrient usage and reduce N and P excretion (Cromwell, 1999). However, according to some investigators, humans may become resistant to antibiotics in many foods, including eggs (Mortier et al., 2005; Nisha, 2008; Cornejo et al., 2020). Lactic acid bacteria (noted as probiotics in this paper) are recommended for the replacement of antibiotics and are used to improve bird growth and performance (Harimurti and Hadisaputro, 2015). Probiotics maintain the health of birds by maintaining gut flora (Fuller, 1989). When fed to the host, probiotics enhance the immune system by spreading throughout the gut and by absorbing antigens released from dead organisms (Ahmad, 2006). For instance, earlier studies showed that *Bifidobacterium longum* or *Lactobacillus acidophilus* ($\sim 8 \times 10^{10}$ nonviable cells per day) when fed to mice, produced a significant response to serum antibody after 6 or 10 weeks (Takahashi et al., 1993). In another study, *Lactobacillus casei* GG, when fed to children, significantly improved IgA against acute diarrhea (Kaila et al., 1992).

Effect of Probiotics on Health and Performance of Layers

Specific to layers, the addition of various probiotic combinations (heat-inactivated *Lactobacillus salivarius, Clostridium butyricum, Bacillus subtilis*, and sodium butyrate) positively impacted performance, egg quality, and the immune system (Zhang et al., 2012). Also, supplementation of probiotics (*Lactobacillus sporogenes*) to laying hens, especially at peak production, significantly improved the same measurements in addition to shell quality (Panda et al., 2008).

Effect of Probiotics on Metabolism of Excess Nitrogen in Layers

Laying hens, like most animals, are fed more protein than needed. When protein is fed in excess, laying hens produce several nitrogenous compounds (Bittman and Mikkelsen, 2009). In layer houses, one nitrogenous compound, NH_3, reduces production and is the most common, noxious, water-soluble, and colorless gas (pungent and unpleasant to humans) that can adversely affect birds, humans, and the environment (Santoso et al., 1999; Almuhanna et al., 2011, Naseem and King, 2018). Therefore, it is important to find ways to increase production and reduce nitrogenous compounds; the use of probiotics has been recommended to produce these effects (Chen et al. 2012; Khan and Naz, 2013).

Probiotics also reduced the concentration of specific chemical compounds in the blood of poultry. They inhibited urease activity that is responsible for the production of ammonia (NH_3) (Yeo and Kim, 1997). Bird growth can be improved when NH_3 is reduced, thereby preventing damage to the surface of the intestinal mucosa (Yeo and Kim, 1997). Amer et al. (2004) reported negative effects of NH_3 at 100,000 µg/kg on egg production, body weight, and feed intake. Positive effects such as reduced serum NH_3, uric acid (produced by the liver and deposited in fecal matter), and creatine kinase (normal liver and muscle function) have been reported after feeding *B. subtilis* to ducks and *E. faecium* to weaned lambs (Antunović et al., 2005; Li et al., 2011).

Simultaneous Investigation of Many Variables Affected by Probiotics

As noted above, probiotics can reduce nitrogenous compounds in serum (caused by excess protein in diets) and manure (source of excess ammonia in layer houses). As well, they can improve performance and egg quality measurements by affecting digestion and retention of nutrients (Naseem and King, 2018).

We have conducted previous studies to determine the effect of *Lactobacillus species*, across many variables, at various quantities in water and feed of layers. Previously, we conducted two studies with three species (*L. paracasei* + *L. plantarum* + *L. rhamnosus*) of Lactobacillus in drinking water at (a) 1.092×10^7 cfu/L for 4 weeks and (b) 1.84×10^{10} cfu/L for 8 weeks. We investigated effects on gas concentration and emissions from manure as well as compounds in blood serum (Naseem and King, 2020a; b). Another study, supplying the same combinations of probiotics (1.0×10^{12} cfu/kg feed) in the feed of hens for 8 weeks, was conducted (Naseem et al., 2020). For these three studies, increasing combinations of three bacteria in two different modes of delivery did not significantly improve production performance or reduce NH_3.

While bacteria can be synergistic, thereby enhancing efficacy as evaluated in our previous studies, they can also be competitive, reducing efficacy. Thus, to further investigate ways to increase production and reduce nitrogenous compounds in serum, liver, and manure, combinations with two species and that with three species were evaluated.

MATERIALS AND METHODS

Two hundred and forty W-36 White Leghorns (32-week-old) were individually caged (18 in × 18 in × 21 in) and grown under 16 hours of light and 8 hours of darkness throughout the 8-weeks period. The study was conducted in a temperature-controlled room with an average maximum of 22.7°C ± 1.0 and minimum of 18.7°C ± 1.1 from September to November. The Institutional Animal Care and Use Committee approved the protocol for feeding, handling, housing, and care of birds (University of California, Davis, CA).

Powdered forms of probiotics (*Lactobacillus rhamnosus,* UAL r-06, human origin; *L. paracasei*, UAL pc-04, dairy origin; and *L. plantarum*, UALp-05, plant origin) were purchased from UAS Lab (Madison, WI). The inclusion rate was 3.33×10^{11} cfu of each species/kg feed. Feed (Control feed, see T1 below) was mixed every fourth day in the layer house using 1.66 g/kg feed of *L. rhamnosus*, 0.66 g/kg feed of *L. paracasei*, and 0.74 g/kg feed of *L. plantarum*. To ensure even distribution, probiotics were mixed in small batches of commercial feed (16% Hi-Energy Layer Crumble, BAR ALE, Williams, CA) manually and gradually mixed with larger batches. Diets met the nutrient requirement of laying hens (NRC, 1994). Water and diets were administered *ad libitum*.

Birds (240 layers) were randomly allocated into six replicates of eight birds to receive one of the following five treatments. To eliminate the effect of quantity of each species on variables evaluated, each *Lactobacillus species* in all combinations was held constant (Table 1).

Table 1. Nutrients in 16% Hi-Energy[1] layer crumble feed (Naseem et al. 2020)

Nutrients	Quantity
Crude protein	16%
Crude fat	2.5%
Crude fiber	6.5%
Lysine	0.8%
Methionine	0.3%
Ash	8.0%
Calcium (Min) 2.0%	3.0%
Phosphorus	0.5%
Sodium (Min) 0.2%	0.4%
Copper	106 mg/kg
Manganese	117 mg/kg
Zinc	106 mg/kg
Vitmain A	3 KIU
Vitamin D	1 KIU
Vitamin E	20 IU

[1] BAR ALE, Willliams, CA.

Ingredients: Corn (ground), soybean meal, ground grains, calcium carbonate, wheat millrun, monocalcium phosphate, corn starch, sodium chloride, L-lysine, diatomaceous earth, *saccharomyces cerevisiae* yeast and the media, DL-methionine dry, zinc amino acid complex, natural sources of yellow made of saponified extracts of marigold flowers, choline chloride, ferrous sulfate, manganese oxide, zinc oxide, niacin, selenium, vitamin E oil, vitamin E adsorbate, D-CA pantothenate, vitamin A 650, basic copper chloride, vitamin D3 500, biotin, riboflavin, vitamin K MSBC, pyridoxine hydrochloride, ethylenediamine dihydriodide, vitamin B12, thiamine mononitrate, folic acid.

$$\text{T1} = \text{Control, a commercial feed (Table 1)}$$

$$\text{T2} = \text{Control} + \textit{L. paracasei} + \textit{L. plantarum} \text{ at } 6.66 \times 10^{11} \text{cfu/kg feed in total}$$

$$\text{T3} = \text{Control} + \textit{L. paracasei} + \textit{L. rhamnosus} \text{ at } 6.66 \times 10^{11} \text{cfu/kg feed in total}$$

$$\text{T4} = \text{Control} + \textit{L. plantarum} + \textit{L. rhamnosus} \text{ at } 6.66 \times 10^{11} \text{cfu/kg feed in total}$$

$$\text{T5} = \text{Control} + \textit{L. paracasei} + \textit{L. plantarum} + \textit{L. rhamnosus} \text{ at } 1.0 \times 10^{12} \text{cfu/kg feed in total}$$

External Production Measurements

Daily egg production was recorded. Weekly (Time) body weight (BW) and feed intake were recorded; the feed conversion ratio (FCR: kg/dozen eggs) was computed.

Manure Collection and Sample Preparation

Manure was pooled from all replicates within the treatments (six replicates × eight birds) after 24 hours of collection at weeks 0, 4, 6, and 8. Manure was pooled to determine the effect of time by feeding different combinations of probiotics. Pooled samples were homogenized for 3 min in a food chopper (Rival FPRVMC3002, Jarden Corporation, China) before further analysis (3–4 subsamples).

Ammonia Concentration in Fresh Manure

Ammonia concentration was measured by storing 1 g of homogenized pooled manure in 10 mL of 1M KCl at 4°C. Foster (1995) and Verdouw et al. (1978) adopted a method to measure NH3 concentration in soil. This same method was used to measure NH_3 concentration in the manure of laying hens.

Ammonium Nitrogen, Total Kjeldahl Nitrogen, and Total Nitrogen

Samples (~50 g) of homogenized pooled manure were dried in an oven at 55°C for 48 hours. Dried manure samples were analyzed for NH_4-N, TKN, and TN at the UC Davis Analytical Laboratory as reported by Naseem and King (2020a).

Serum Profile

At weeks 0, 4, and 8, blood from the brachial veins of all layers within treatments was collected in vacuette-serum-tubes (Greiner Bio-One Serum Tubes: Clot Activator with Gel Separator, Greiner Bio-One 456073), pooled, and stored at 22.7°C. After an hour, it was centrifuged at 841 g for 10 min to separate serum (stored at −80°C before further analysis of NH_3), uric acid (UA), total protein (TP), albumin (ALB), and creatine kinase (CK). Analyses were conducted following the procedures of Naseem and King (2020a).

Statistical Analysis

The Wilk–Shapiro test was used to check the normality assumption for the residual errors. Mixed Model ANOVA (mixed procedure, version 9.4; SAS Institute Inc., Cary, NC) and the PDMIX800.SAS macro was used to analyze production and UA data whereas manure and serum data were examined by one-way ANOVA (Proc GLM procedure). The mixed models had a repeated measures structure, with fixed effects of diet, week, and diet*week and random effects for laying hens, laying hen*diet, and laying hens*week. When the week was insignificant, the analysis was rerun as a one-way ANOVA on the treatment effect. FCR values were not normal, therefore log-transformed data of FCR were inspected to check the validity of normality of residual errors due to outliers. Log transformed data are presented. Tukey–Kramer adjustment was used to compare the least square means where probability values were significant at $P < 0.05$. Trends were reported for $P < 0.1$.

RESULTS

Production—Treatment Effects

BW, feed intake, egg production, and FCR were measured after the supplementation of *Lactobacillus* species in different combinations in an 8-week study. The addition of probiotics in various combinations did not change BW (Table 2). As well, feeding combinations of Probiotics did not affect feed intake, egg production, or FCR (Table 2). Notable numerical differences observed for T3 were increased feed intake of hens by 14%–18% compared to all other treatments at week 3. Similar to feed intake, FCR was greatest for T3 at week 3 (T1 = 1.30 kg/dozen eggs and T3 = 1.56 kg/dozen eggs). This combination of Probiotics (*L. paracasei* + *L. rhamnosus*) increased the FCR of laying hens by 15%–20% in comparison to all other treatments at week 3.

Production—Time and Time (Week)*Treatment

With Time (Figure 1a), BW varied significantly ($P = 0.0007$) with a gradual increase throughout the study. Hens were significantly heavier at weeks 7 and 8 compared to weeks 0 and 1 (Figure 1a). Feed intake was also affected significantly ($P < 0.0001$) by Time. Numerically lowest and greatest feed intake values were recorded at weeks 2 and 7, respectively (Figure 1b). Time significantly increased ($P = 0.0007$) egg production (Figure 1c). Numerical minimum (6.55/hen/week) and maximum (6.83/hen/week) egg production was noted at weeks 1 and 4, respectively. Egg production was significantly lowered at week 1 compared to weeks 3–8. After week 2, no numerical difference was observed in egg production. Significant temporal differences in FCR ($P < 0.0001$) were also observed (Figure 1d). The FCR value, a numerical maximum at week 7, was

Table 2. Major effects of probiotics[1] on performance measurements[2] of 32- to 40-week-old white leghorn laying hens

Diets	Bodyweight, g[2]	Feed intake, g/laying hen/d[2]	Egg production, per hen per week[2]	Feed conversion ratio, kg/dozen eggs[2,3]
T1	1666.60 ± 16.31	107.38 ± 0.93	6.72 ± 0.07	1.34 ± 1.33, 1.36
T2	1679.88 ± 16.14	108.99 ± 0.93	6.73 ± 0.07	1.37 ± 1.36, 1.38
T3	1671.13 ± 16.19	109.94 ± 0.93	6.69 ± 0.07	1.38 ± 1.36, 1.39
T4	1644.71 ± 16.26	109.84 ± 0.93	6.78 ± 0.07	1.36 ± 1.35, 1.37
T5	1666.90 ± 16.19	108.58 ± 0.93	6.70 ± 0.07	1.36 ± 1.35, 1.37
P-value	0.6310	0.3044	0.9348	0.6788

[1] Combinations of *Lactobacillus* species in diets:

T1 = Control, commercial feed (16% Hi-Energy Layer Crumble, BAR ALE, Williams, CA).

T2 = Control + *L. paracasei* + *L. plantarum* at 6.66 × 10^{11} cfu/kg feed in total.

T3 = Control + *L. paracasei* + *L. rhamnosus* at 6.66 × 10^{11} cfu/kg feed in total.

T4 = Control + *L. plantarum* + *L. rhamnosus* at 6.66 × 10^{11} cfu/kg feed in total.

T5 = Control + *L. paracasei* + *L. plantarum* + *L. rhamnosus* at 1.0 × 10^{12} cfu/kg feed in total.

[2] Means are of six replications.

[3] For the feed conversion ratio, the first and second number of the standard error are the lower and upper limits, respectively.

7% greater than the numerical minimum at week 2 (Figure 1d).

No differences in the Treatment*Time (Week) interactions for BW and egg production were observed (Figure 2a and b). Feed intake ($P = 0.0007$) and FCR ($P = 0.0049$) exhibited significant Treatment*Time interactions (Figure 2c and d).

Manure

NH_3 and NH_4-N. Analysis of data for NH_3 and NH_4-N showed no differences across treatments (Table 3). Temporal NH_3 and NH_4-N concentrations fluctuated by week (Figure 3).

TKN and TN. Probiotic combinations did not affect TKN concentrations at $P < 0.05$; however, fluctuation in values existed, trending toward an overall decrease (Table 3). Temporal differences in concentrations of TKN were recorded (Figure 3). No difference was noted in TN concentration by feeding combinations of *Lactobacillus* species (Table 3). Beginning at week 4, TN concentrations exhibited temporal reductions. (Figure 3).

Analysis of Serum

Production of NH_3 in serum and TP, ALB, CK, and UA (serum and liver) were not affected by combinations of probiotics (Table 4 and Supplemental Figure). NH_3 displayed temporal differences in concentration (Figure 4). There was no temporal effect on UA concentrations at $P < 0.05$; but it trended higher at $P < 0.10$ over time (Figure 4b). Moreover, temporal effects were not noted for concentrations of TP and ALB (Figure 4c and d). A temporal increase in CK was noted (Figure 4e).

DISCUSSION

Evaluating many variables are necessary to assess the overall impact of three *Lactobacillus species* in various combination on laying hens.

Production Measurements

Different combinations of probiotics did not affect BW significantly; this finding was similar to our previous findings when 52-to 54-week-old White Leghorns were provided the three *Lactobacillus* species (*L. paracasei* + *L. plantarum* + *L. rhamnosus*) at 1.84 × 10^{10} cfu/L in drinking water for the next 8 weeks (Naseem and King, 2020b). Moreover, Naseem et al. (2020) reported insignificant effects of the three probiotics in the feed of younger laying hens (32–40 weeks old). However, a significant increase in BW of 27-week-old Hy-Line hens was reported when Protexin (0.01% of feed; the mixture of microorganisms produced by Novartis Limited, International, UK) and Closta (0.05% of feed; *B. subtilis* produced by Kemin Industries, Inc.) were fed for the following 12 weeks (Youssef et al., 2013). Insignificant interaction of Probiotic*Time on BW was also similar to results from our earlier work (Naseem and King, 2020b). Moreover, we reported insignificant effects on BW of 32-week-old laying hens when fed three species of probiotics at 1.0 × 10^{12} cfu/kg feed (Naseem et al. 2020). As previously mentioned, work that simultaneously examines many factors (bacterial species, breeds of birds, and duration/method of administration) is needed to explain differences in results from our present and previous work (Naseem and King, 2020a; b; Naseem et al., 2020).

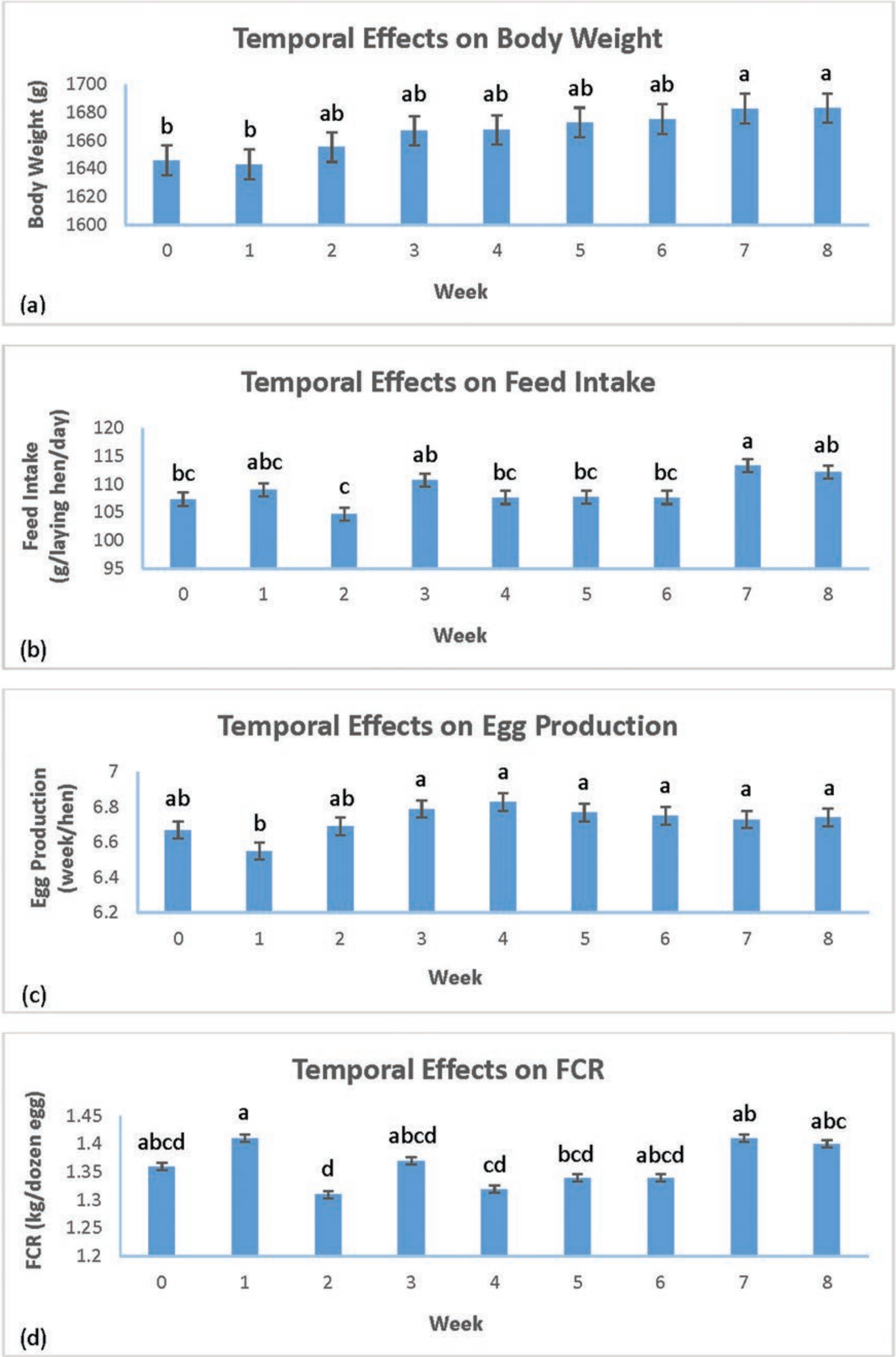

Figure 1. Temporal effects on performance measurements of 32- to 40-week-old white leghorn laying hens. Bars with the same superscripts are not significantly different at $P < 0.05$. FCR = Feed conversion ratio.

Insignificant effects on feed intake, egg production, and FCR in this work supported our previous findings (Naseem and King, 2020b; Naseem et al., 2020). In another 8-week study, investigators reported insignificant effects on feed intake and FCR by feeding *Saccharomyces spp. SB-6* (at 0, 2.0, 4.0, and 6.0 g of probiotic/kg of diet) to 32- to 40-week-old laying hens (Bidura et al., 2016). However, these investigators reported a significant increase in egg production. Furthermore, Haddadin et al. (1996) reported a significant increase in egg production, and FCR by feeding *L. acidophilus* (at 0.67 × 10^6, 2.0 × 10^6, and 4.0 × 10^6 cfu/ g feed) to 40-week-old Lohman-white laying hens until the age of 48 weeks. Khan et al. (2011) reported improvements in BW, feed intake, egg production, and FCR by

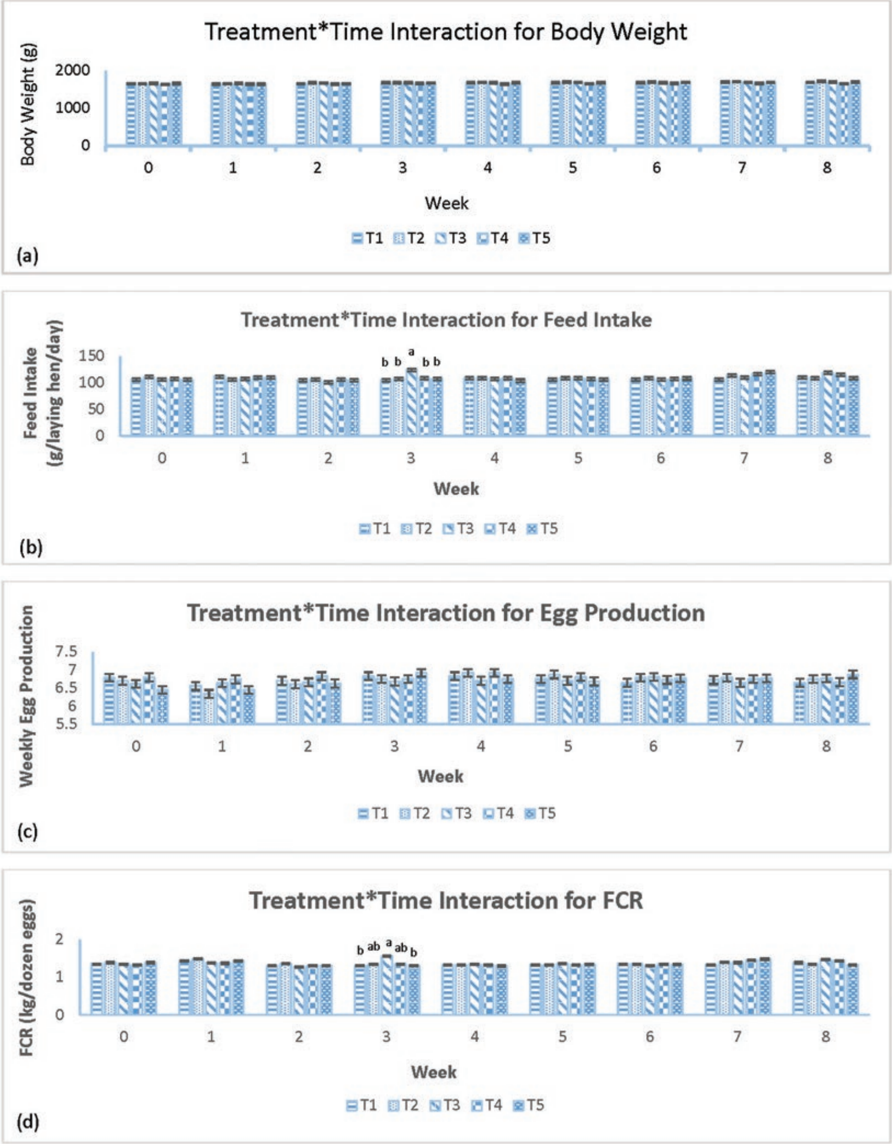

Figure 2. Dietary treatment*time interaction for performance measurements of 32- to 40-week-old white leghorn laying hens. Bars with no superscripts are not significantly different. T1 = Control, commercial feed (16% Hi-Energy Layer Crumble, BAR ALE, Williams, CA). T2 = Control + *L. paracasei* + *L. plantarum* at 6.66 × 10^{11} cfu/kg feed in total. T3 = Control + *L. paracasei* + *L. rhamnosus* at 6.66 × 10^{11} cfu/kg feed in total. T4 = Control + *L. plantarum* + *L. rhamnosus* at 6.66 × 10^{11} cfu/kg feed in total. T5 = Control + *L. paracasei* + *L. plantarum* + *L. rhamnosus* at 1.0 × 10^{12} cfu/kg feed in total. FCR = Feed conversion ratio.

feeding Protexin (2 × 10^9 cfu/g; *Lactobacillus plantarum*, *L. bulgaricus*, *L. acidophilus*, *L. rhamnosus*, *B. bifidum*, *S. thermophiles*, *E. faceium*, *A. oryzae*, and *C. pinpolopesi*) to Hy-Line W-98 hens at age 40–50 weeks. In our present study, T3 (*Lactobacillus paracacei and L. rhamnosus*) was most effective numerically for increases in feed intake and FCR. More research needs to be conducted to determine if higher concentrations of each species in combination would produce significant overall improvements.

In work reported above and in the scientific literature *Saccharomyces sp* (yeast probiotics) are often combined with bacterial probiotics (Bai et al. 2013; Egorova et al., 2016). The mode of action for yeast in improving digestibility in the GI tract of animals differs from that of bacteria (Candrawati et al., 2014; Bidura et al., 2016). According to Bidura et al. (2016), yeast as probiotics aid in nutrient digestibility (protein and minerals) and increase the population of microorganisms in the GI tract by producing growth factors, pro-vitamins, and other bacterial growth stimulants. Beneficial outcomes of *Lactobacillus* bacteria are often obtained when they survive/multiply in the GI tract. *Lactobacillus* species can attach to the intestinal epithelium and are resistant to bile and acidic conditions (Jin et al., 1996; Khan et al., 2011). By lowering pH, probiotics

Table 3. Nitrogenous compounds[1] in the manure of 32- to 40-week-old white leghorn laying hens

Means[2]				
Diets[3]	NH_3 (ppm)	NH_4–N (ppm)	TKN (%)	TN (%)
T1	44.86 ± 0.57	2568.33 ± 77.39	4.65 ± 0.02	4.96 ± 0.03
T2	44.38 ± 0.57	2466.25 ± 77.39	4.67 ± 0.02	4.98 ± 0.03
T3	44.58 ± 0.57	2340.63 ± 77.39	4.60 ± 0.02	4.95 ± 0.03
T4	43.05 ± 0.57	2296.25 ± 77.39	4.59 ± 0.02	4.89 ± 0.03
T5	44.12 ± 0.57	2383.75 ± 77.39	4.61± 0.02	4.97 ± 0.03
p-value	0.2615	0.1670	0.0943	0.2490

[1] NH_3, ammonia; NH_4-N, ammonium-nitrogen; TKN, total Kjeldahl nitrogen; TN, total nitrogen.

[2] Means are of 2–4 subsamples.

[3] Combination of *Lactobacillus* species in diets:

T1 = Control, commercial feed (16% Hi-Energy Layer Crumble, BAR ALE, Williams, CA).

T2 = Control + *L. paracasei* + *L. plantarum* at 6.66 × 10^{11} cfu/kg feed in total.

T3 = Control + *L. paracasei* + *L. rhamnosus* at 6.66 × 10^{11} cfu/kg feed in total.

T4 = Control + *L. plantarum* + *L. rhamnosus* at 6.66 × 10^{11} cfu/kg feed in total.

T5 = Control + *L. paracasei* + *L. plantarum* + *L. rhamnosus* at 1.0 × 10^{12} cfu/kg feed in total.

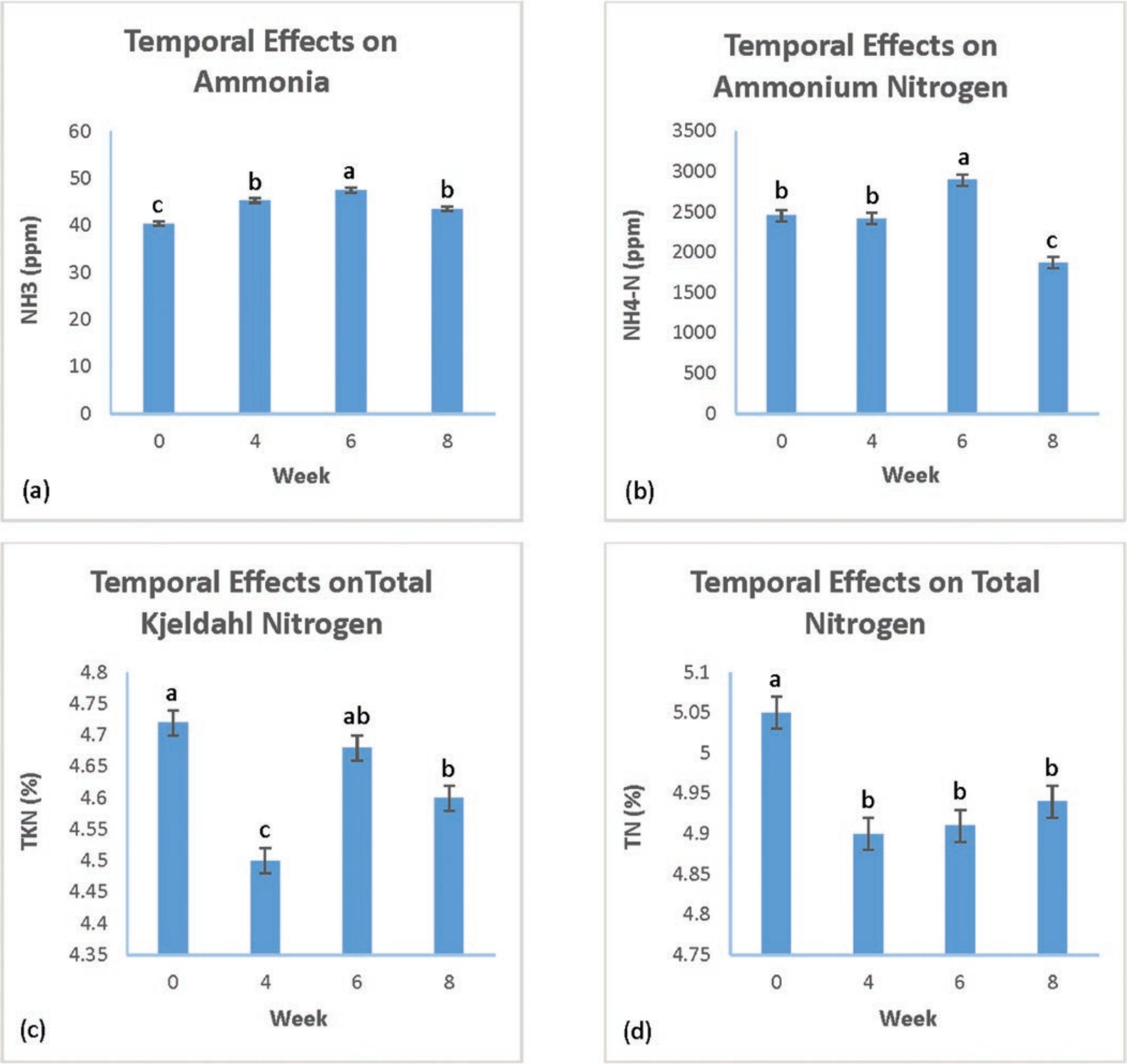

Figure 3. The temporal concentration of nitrogenous compounds in the manure of 32- to 40-week-old white leghorn laying hens. Bars with the same superscripts are not significantly different at $P < 0.05$.

Table 4. Effect of *Lactobacillus* species on serum components[1] of 32- to 40-week-old white leghorn laying hens

Means[2]						
Diets[3]	NH_3 (mg/dl)	UA (mg/dl)	TP (mg/dl)	ALB (g/dl)	CK (U/L)	UA (liver) (ng/mg tissue)
T1	$1.21 \times 10^{-4} \pm 4.80 \times 10^{-6}$	2.28 ± 0.25	5159 ± 42.8	2.06 ± 0.01	704.92 ± 85.45	255.75 ± 127.75
T2	$1.17 \times 10^{-4} \pm 4.80 \times 10^{-6}$	2.35 ± 0.25	5115 ± 42.8	2.04 ± 0.01	722.00 ± 85.45	647.01 ± 127.75
T3	$1.17 \times 10^{-4} \pm 4.80 \times 10^{-6}$	2.19 ± 0.25	5242 ± 42.8	2.08 ± 0.01	787.92 ± 85.45	155.72 ± 127.75
T4	$1.28 \times 10^{-4} \pm 4.80 \times 10^{-6}$	2.56 ± 0.25	5166 ± 42.8	2.06 ± 0.01	721.75 ± 85.45	328.50 ± 127.75
T5	$1.16 \times 10^{-4} \pm 4.80 \times 10^{-6}$	1.97 ± 0.25	5261 ± 42.8	2.08 ± 0.01	913.42 ± 85.45	420.34 ± 127.75
P-value	0.4533	0.5654	0.1797	0.3548	0.4527	0.2195

[1] NH_3, ammonia; UA, uric acid; TP, total protein; ALB, albumin; CK, creatine kinase.

[2] Means are of three subsamples within treatment for NH_3 and four subsamples within treatment for UA, TP, ALB, CK, and UA in the liver.

[3] Combinations of *Lactobacillus* species in diets:

T1 = Control, commercial feed (16% Hi-Energy Layer Crumble, BAR ALE, Williams, CA).

T2 = Control + *L. paracasei* + *L. plantarum* at 6.66×10^{11} cfu/kg feed in total.

T3 = Control + *L. paracasei* + *L. rhamnosus* at 6.66×10^{11} cfu/kg feed in total.

T4 = Control + *L. plantarum* + *L. rhamnosus* at 6.66×10^{11} cfu/kg feed in total.

T5 = Control + *L. paracasei* + *L. plantarum* + *L. rhamnosus* at 1.0×10^{12} cfu/kg feed in total.

also decrease pathogenic bacteria (*Salmonella spp*, *E. coli*, gram-negative bacteria), produce bacteriocins (lactocidin and lactocin), increase the quantity of short-chain fatty acids (organic acids such as acetic acid and propionic acid) as well as compete for nutrients (Jin et al., 1996; Jin et al., 1997; Gunal et al., 2006; Khan et al., 2011). They can improve performance and health by the fermentation of undigested carbohydrates over time (Khan et al., 2011). Results for egg production in this study did not indicate the capacity of various two-species combinations of bacteria to produce these outcomes. Due to numerical differences caused by T3, further research on the synergistic relationship between probiotics as yeast and this combination at the same and increased quantities of each species is warranted.

Manure

Although different combinations of probiotics supplied in feed did not change NH_3 concentration at the end of an 8-week study, some numerical differences were noted. Findings of this study supported the results of Naseem and King (2020a, b) and Naseem et al. (2020) where a combination of three lactic acid bacteria (1.092×10^7 cfu/L water, 1.84×10^{10} cfu/L water, and 1.0×10^{12} cfu/kg feed) was provided to laying hens at different ages. Results of our studies were not similar to the findings of Tang et al. (2018) who reported a decrease in NH_3 concentration after feeding *B. amyloliquefaciens* (1.0×10^7 cfu/kg and 2.0×10^7 cfu/kg) to 28- to 34-week-old layers. In addition, Mo et al. (2004) found a significant reduction in NH_3 concentration by feeding three types of yeast probiotics (*P. farinosa* SKM-1, *P. anomala* SKM-T, and *G. geotrichum* SJM-59) to 21-week-old Hy-line Brown layers for the following 8 weeks. These investigators outlined the assimilating ability of yeast and noted that they reduced NH_3 generation. Candrawati et al. (2014) noted a significant decrease in ammonia nitrogen (NH_3–N) in the manure of 2-week-old broilers when *Saccharomyces Spp.S-7* isolate was supplied in varying concentrations (0.20%, 0.40%, and 0.60%) and mixed with feed for the next 4 weeks. Bacterial probiotics caused a significant decrease in intestinal pH by producing lactic acid from *B. amyloliquefaciens* (Tang et al., 2018). A lower pH may be responsible for the inhibition of urease activity (Ahmed et al., 2014). According to Park et al. (2016), bacterial probiotics can increase nitrogen retention, decrease its excretion and ultimately improve nitrogen digestibility. The probiotic combinations used in this

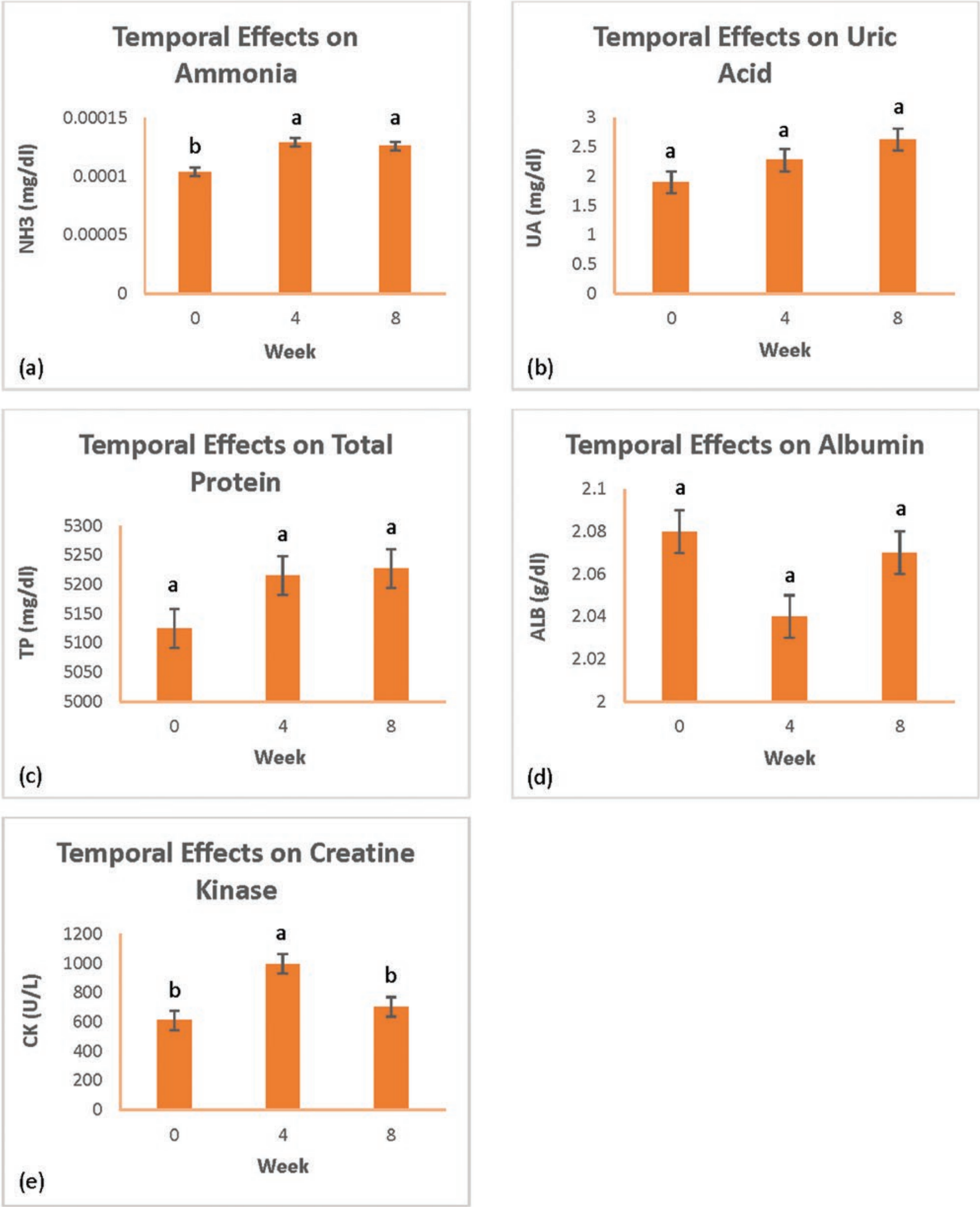

Figure 4. Temporal effects on serum components of 32- to 40-week-old white leghorn laying hens. Bars with the same superscripts are not significantly different at $P < 0.05$.

study were not as effective as yeast probiotics or other bacterial ones in improving nutrient digestibility and inhibiting urease activity.

Negligible effects on NH_4–N by feeding probiotics in different combinations supported our previous findings. We noted overall insignificant effects on NH_4–N when three species of *Lactobacillus* were fed to older (>1-year-old) hens in drinking water (Naseem and King, 2020a; b). The effect of time on NH_4-N in this study was in agreement with a significant decrease in NH_4–N concentration described earlier (Naseem et al., 2020).

The overall trend for reduction of TKN in this study agreed with our previous findings when 65- to 74 week-old-hens were fed three probiotics in drinking water for an additional 4 weeks (Naseem and King, 2020a). We reported contradictory results when probiotics were fed to laying hens in drinking water and feed, respectively for 8 weeks (Naseem and King, 2020b; Naseem et al., 2020). Results for

TKN for four studies, as delineated above, point to the contradictions in our findings and serve as an example of the confusion for observations among investigators.

Results from this study revealed no effects for combinations of probiotics on TN. We reported similar findings when probiotics were provided in drinking water of second-cycle hens (Naseem and King, 2020a, b). Moreover, results for TN in the manure of this work were similar to results of earlier research with no effect of the three combined probiotics for 32 to 40-week-old layers (Naseem et al., 2020). No effect on TN in this study was in agreement with the findings of Tanaka and Santoso (2000). These investigators reported an insignificant effect for feeding a probiotic (*B. subtilis* culture, 0.5%, 1.0%, and 2.0%) to 7- to 21-day-old broilers.

Low TN production in the manure of hens provided by T4 (*L. plantarum* + *L. rhamnosus*) at week 8 compared to week 0 indicated that feeding of this combination at a higher inclusion rate for a longer period of time might be effective in reducing TN concentration in poultry manure. A longer feeding rate may be possible; however, feeding probiotics at a higher inclusion rate is rarely reported in the literature. Another possibility for efficacious results is to feed probiotics for the first weeks or months after hatch to enhance complete colonization.

Serum

These results of NH_3 concentration by feeding *Lactobacillus* species in different combinations supported our earlier findings (Naseem and King, 2020a; Naseem et al., 2020) where no effects of *L. rhamnosus*, *L. paracasei*, and *L. plantarum* were observed when added to drinking water for older hens and in feed for those at peak lay. In contrast, we reported a reduction in serum NH_3 when hens (52- to 54-week-old) were fed probiotics (1.84×10^{10} cfu/L) in drinking water for the next 8 weeks (Naseem and King, 2020b). Samanya and Yamauchi (2002) also reported a lowered blood NH_3 by feeding *B. subtilis natto* (1×10^8–1×10^{10} microorganism/g) to adult male White Leghorn chickens for 3 days. Yeo and Kim (1997) noted that probiotics decreased NH_3 concentration by inhibiting urease-producing bacteria. Inhibition of these bacteria prevents UA, produced in the livers of chickens, from absorption in the intestines and conversion into NH_3 in the presence of urease. Feeding probiotics (*B. subtilis natto*, 1×10^8–1×10^{10} microorganism/g) improved villi height contributing to lowering serum NH_3 and promoting the relationship between serum NH_3 and intestinal NH_3 (Samanya and Yamauchi, 2002).

Negligible effects of feeding probiotics on UA agreed with findings of previous studies (Naseem and King, 2020a, b; Naseem et al., 2020). Hashemzadeh et al. (2013) reported a decrease in UA when one-day-old male broiler chicks were supplied *Lactobacillus rhamnosus* (7 log cfu/mL), *B. laterosporus* (7 log cfu/mL), and *Escherichia coli* Nissle 1917 (7 log cfu/mL) in drinking water for an additional 21 days. These investigators reported that probiotics were advantageous in kidney function. They explained that NH_3 produced in the body of chickens stimulates UA production. These findings are not consistent with our findings for serum NH_3 and serum UA.

Current insignificant findings for TP and ALB were in agreement with those from our previous work (Naseem and King, 2020a, b; Naseem et al., 2020). These results supported the insignificant findings of Hashemzadeh et al. (2013). Moreover, Endo et al. (1999) reported no effect of probiotics (*B. subtilis*, *B. natto*, *B. megaterium*, *B. thermophiles*, *L. acidophilus*, *L. plantarum*, *L. brevis*, *L. casei*, *S. faecalis*, *S. lactis*, *S. thermophiles*, *C. butyricum*, *S. cerevisiae*, and *C. utilis*, each at 10^{7-8} cfu/g of rice bran) on TP when White Leghorn cocks were fed from 5 to 9 weeks. In addition, our present findings agreed with Ashayerizadeh et al. (2009) when TP remained unaffected by feeding *Primalac* (1×10^8 cfu/g *L. casei*, *L. acidophilus*, *B. thermophilum*, and *E. faesium*) to one-day to 42-day-old broilers. In contrast, Shareef and Al-Dabbagh (2009) detected an increased level of TP in the serum of 1-day to 21-day-old-broilers fed 1.0%, 1.5%, and 2.0% probiotic yeast (3.44×10^8 cfu/g of *S. cerevisiae*). Moreover, Paryad and Mahmoudi (2008) reported higher TP and ALB by feeding yeast (1.5% of *S.*) to chicks for 1–42 days. These researchers illustrated the direct relation of TP and ALB with protein intake and its quality. As noted previously, bacterial probiotics and fungal probiotics, as different organisms, may be effective at different capacities or may develop synergistic relationships (Czerucka et al., 2007; Roto et al., 2015).

CK, an enzyme, creates phosphocreatine and adenosine diphosphate (ADP) by using adenosine triphosphate (ATP). Findings for CK in our present study supported previous findings. We reported similar effects on CK by feeding probiotics (*L. rhamnosus*, *L. paracasei*, and *L. plantarum*) to older and younger laying hens for 4 and 8 weeks, respectively (Naseem and King, 2020a, b; Naseem et al., 2020). In addition, feeding multispecies probiotics (2 × 10^9 cfu/g: containing *S. salivarius* sub sp. Thermophilus, *L. delbruckii* sub sp. *bulgaricus*, *L. acidophilus*, *L. plantarum*, *L. rhamnosus*, *B. bifidum*, *E. faecium*, *C. pintoloppesii*, and *A. oryzae*) to 1-day-old quail did not affect CK concentration over the next 6 weeks (Babazaheh et al., 2011). Our present results were contradicted by those from our previous work where second cycle laying hens were fed *Lactobacillus* species (1.84 × 10^{10} cfu/L water) in drinking water for 8 weeks and had a significant reduction in CK (Naseem and King, 2020b).

Current findings of insignificant effects on UA in liver supported results obtained when 52- to 54-week-old hens were fed three *Lactobacillus* species (1.8375 × 10^{10} cfu/L) in drinking water for the following 8 weeks (Naseem and King, 2020b). Similar findings were reported when we provided probiotics (1.0 × 10^{12} cfu/kg feed) to hens of the same age (Naseem et al., 2020).

CONCLUSION

In an 8-week study, the supplementation of *Lactobacillus* species in different combinations (3.33 × 10^{11} cfu of each species/kg feed and 1.0 × 10^{12} cfu/kg feed in total) did not affect production measurements, nitrogenous compounds in manure, and serum compounds. UA in the liver of hens was not affected. Interaction for treatment and time was significant for feed intake and FCR.

SUPPLEMENTARY DATA

Supplementary data are available at *Translational Animal Science* online.

ACKNOWLEDGMENTS

We appreciate the support from JS West and Companies (Modesto, CA) for the donation of laying hens and BAR ALE (Williams, CA) for providing the feed. Assistance was provided by Jacqueline Pisenti, Ph.D., in the Department of Animal Science (Hopkins Avian Facility UC Davis), Ketwee Saksrithai, Ph.D., and Linda Tuong. Research was partially funded by the CA Agricultural Experiment Station.

Conflicts of Interest: The authors declare no conflicts of interest.

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