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The Order of Limiting Amino Acids in a Wheat–Sorghum-Based Reduced-Protein Diet for Laying Hens

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Abstract: Understanding the order of limiting amino acids (AA) in reduced-protein (RP) diets for laying hens will facilitate precise feed formulation and ensure that AA requirements are met cost-effectively. The order of the first three limiting AAs—lysine (Lys), methionine (Met), and threonine (Thr)—has been well established in RP laying hen diets. This study aimed to determine the priority order of eight additional limiting AAs (critically important AAs) when formulating wheat–sorghum-based RP diets for laying hens: tryptophan (Trp), valine (Val), isoleucine (Ile), arginine (Arg), leucine (Leu), histidine (His), phenylalanine (Phe), and glycine$_{equivalent}$ (Gly). A total of 330 Hy-Line Brown laying hens were randomly assigned to 11 dietary treatments (30 replicates of individual birds per treatment) from 20 to 39 weeks of age (WOA). Treatments were a standard-protein (17.24% CP) diet as the control (SP); a reduced-protein (15.00% CP) diet with sufficient levels of Lys, Met, and Thr but insufficient levels of the eight experimental essential AA (RP); a reduced-protein diet with sufficient levels of all essential AAs (RP-EAA); and eight subsequent dietary treatments of the RP-EAA diet with one of the experimental essential AAs removed: Trp (RP-EAA-Trp), Val (RP-EAA-Val), Ile (RP-EAA-Ile), Arg (RP-EAA-Arg), Leu (RP-EAA-Leu), His (RP-EAA-His), Phe (RP-EAA-Phe), and Gly (RP-EAA-Gly). Eggs were collected and weighed daily, and feed intake and feed conversion ratio (FCR) were calculated weekly. External and internal egg quality was measured at 29 and 39 WOA. Nutrient digestibility, serum uric acid concentration, caecal microbiota composition, and tibia parameters were measured at 40 WOA. Overall, hens fed the RP-EAA-Val, RP-EAA-Ile, and RP diets presented significantly lower egg mass compared to hens fed the SP, RP-EAA-His, and RP-EAA-Gly diets ($p < 0.001$). Hens fed the RP diet and RP-EAA-Val diet had a higher FCR compared to those offered the RP-EAA, RP-EAA-Leu, RP-EAA-Phe, and RP-EAA-Gly diets ($p = 0.046$). Lower protein intake and excretion were observed in hens offered the RP diets compared to hens fed the SP diet ($p = 0.001$ and 0.018, respectively). Based on the egg mass, Ile may be considered the fourth and Val the fifth limiting AA, after Lys, Met, and Thr, in laying hens fed wheat–sorghum-based RP diets during peak lay. However, if ranked based on FCR, Val may be considered the fourth limiting AA, followed by Trp, Ile, Arg, and His as the co-fifth limiting AAs. Leu, Phe, and Gly may be considered as non-essential AAs for laying hens fed RP diets.

Keywords: amino acid; egg production; egg quality; low protein; microbiota; nutrition; poultry

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1. Introduction

Protein is a pivotal dietary component, considered to be one of the most expensive nutrients in commercial poultry rations [1]. The global increase in urbanisation and human population has reduced the availability of agricultural land, and climate change has negatively impacted sustainable crop production [2]. Consequently, accessibility of feed ingredients for poultry diets has decreased, and the costs of these ingredients have increased substantially, making meeting commercial poultry feed demands challenging. Refinement of dietary amino acid (AA) profiles might allow for reductions in dietary crude protein (CP) levels, reducing dependency on protein-rich feed ingredients such as soybean meal [3], and thus reducing feed costs, the use of arable land to produce oilseed meals, and nitrogen excretion into the environment [4,5]. Biologically, birds are unable to sufficiently synthesise several AAs (the essential AAs), meaning dietary protein from feed ingredients is still required, while in reduced-protein (RP) diets, the inclusion rates of protein sources such as soybean meal are reduced, and proportions of crystalline AAs and other cereal grains (e.g., wheat) are increased, to ensure that nutrient demands are being met [3,6]. Thus, supplementing poultry diets with crystalline or synthetic AAs can reduce reliance on soybean meal [7,8]. In recent years, feed-grade AAs have become more commercialised and available at competitive prices in the market, allowing for their inclusion in poultry feed formulations [9].

A reduction of 45 g/kg dietary CP was found to significantly increase ileal AA digestibility coefficients by 6.18% in broiler chickens raised under tropical conditions [10]. Additionally, reducing dietary CP levels in wheat–soybean meal diets by 30 g/kg and in maize–soybean meal-based diets by 45 g/kg increased ileal digestibility by 9.10% and 5.82%, respectively [11,12]. In contrast, a high concentration of protein-bound AAs from high-CP corn-based diets increases undigested protein reaching the hindgut, which can result in more nitrogen excretion to the environment and proliferation of gut-specific pathogens, including *Clostridium perfringens*, *Escherichia coli*, and *Salmonella* spp. This affects gut health integrity and production performance [13–16]. Supplementation of AAs could reduce the dietary requirement of soybean meal in wheat-based diets by up to 50% and CP by 20%, without affecting performance, compared to standard-protein diets. Furthermore, nitrogen excretion can be reduced by 3 to 10% per 10 g/kg lower dietary CP [3,17].

Determining the optimal AA profiles and order of limiting AAs (the critically important AAs) is required when replacing bound CP in soybean meal with crystalline AAs in RP diets, to ensure requirements for growth and production are being met [18]. Numerous studies have presented positive effects when feeding poultry RP corn and soybean meal-based diets supplemented with crystalline AA, but a lack of consensus about which supplemental AA are the most crucial is apparent [19–21]. The order of limiting AAs varies between the diets, dictated by the AA profiles of the major feed ingredients [22]. Crystalline AAs are absorbed rapidly, and do not need to be digested. In a recent review, Selle, et al. [8] indicated that inclusion rates of crystalline AAs could be increased from 7.23 to 38.49 g/kg, and soybean meal inclusion rate could be reduced by 66% (113 versus 334 g/kg) when formulating reduced-CP diets. Further research is warranted into the implications of this in the laying hen industry.

There are ten essential AAs required in poultry diets for optimal production performance: lysine (Lys), methionine (Met), tryptophan (Trp), threonine (Thr), arginine (Arg), isoleucine (Ile), leucine (Leu), histidine (His), phenylalanine (Phe), and valine (Val). Lysine, Met, and Thr are the first three limiting essential AAs for both broilers and layers [23–25]. Glycine (Gly) and serine (Ser) are non-essential limiting AAs [26]. The use of synthetic Lys, Met, and Thr in commercial poultry diets is a common practice worldwide. In most cases, other essential AAs will not be deficient, due to the high levels in soybean meal in the diet. However, as the level of soybean meal is reduced in RP diets, these other AAs may become limiting [3,27]. There is evidence that synthetic AA supplementation can be used as a tool to overcome this. For example, Kidd, et al. [3] observed that the inclusion of crystalline Met, Lys, Thr, Val, Ile, and Arg into broiler chicken diets permitted the dietary levels of

soybean meal and CP to be reduced by up to 50% and 20%, respectively. Moreover, Vieira, et al. [28] found that egg production and feed efficiency were similar in laying hens fed a corn–soybean meal RP diet (140 g/kg CP) supplemented with Lys, Met, Thr, Trp, Ile, and Val compared to those fed a standard-protein diet with 160 g/kg CP.

After Met, Lys, and Thr, the next limiting AAs in broiler chickens are Val, Ile, and Arg, in wheat- and sorghum-based soybean meal and canola meal diets [8,23,29,30]. However, information on the order of limiting AAs in laying hens is lacking, particularly in wheat–sorghum soybean meal-based diets, and is likely different from broilers. Early work by Bray [31] suggested that Ile could be the fourth limiting AA in low-protein corn–soybean meal-based diets for laying hens. In contrast, Dong, et al. [32] found that supplementation of Ile in RP diets did not affect egg production, egg quality, or immunological parameters in laying hens. This may reflect the greater egg production and different body development rates in modern-strain layers compared to breeds used decades ago. Vieira, et al. [28] indicated that other AAs, such as Trp, Val and Ile, are just as important as Met, Lys, and Thr for laying hen performance. Thus, priority AAs in formulating RP diets for laying hens still need to be explored. It is evident that the dietary AA requirements of laying hens should be revised, as current recommendations by NRC [33] do not reflect the needs of modern birds. Increasing commercial availability of limiting AA other than Met, Lys, and Thr might allow for more pronounced reductions in dietary CP levels and reduce reliance on soybean meal inclusion. Therefore, this study was focused on how hen laying performance and egg quality were compromised by the individual deficiency of eight essential AAs other than Met, Lys, and Thr in the RP diet compared to the conventional standard-protein diet to determine the limiting order of these essential AAs in the practical Australian wheat–sorghum-based RP diet. Moreover, nutrient digestibility, bone parameters, and the caecal microbiome were further taken into account to determine if deficiency of specific AAs may have a negative effect during the production cycle. It is hypothesised that the limiting orders of essential AAs in the RP wheat-based diets may be different, and deficiency of essential AAs in these diets would reduce laying hen performance, nutrient digestibility, and health conditions. The findings of this study may help to facilitate precise feed formulations of reduced-protein diets for laying hens that may consequently promote sustainable layer hen production.

2. Materials and Methods

2.1. Birds and Animal Husbandry

This study was performed at the Laureldale Research Station at the University of the New England, Armidale, New South Wales, Australia and according to the Australian Code of Practice, the requirements of bird care and use are met for scientific purposes. (ARA21-096) [34]. A total of 330 Hy-Line Brown (*Gallus gallus domesticus*) pullets at 14 weeks of age (WOA) were obtained from a local commercial layer farm in Tamworth, New South Wales, Australia. All birds were from the same flock and were reared under standard conditions, as per Hy-Line Brown specifications [35], before they were transferred to the research facility. Upon arrival, the birds were randomly distributed into 330 cages (a single bird per cage, 45 cm height × 50 cm depth × 30 cm width) in a curtain-sided shed. Birds were fed a common commercial diet from 14 to 20 WOA (Barastoc—Premium Top Layer Mash: 16.5% CP, 2.5% crude fat, 6% crude fibre, copper 8.0 mg/kg, selenium 0.3 mg/kg, 3.6% calcium, and 0.3% salt, Melbourne, VIC, Australia). They were fed the experimental diet treatments from weeks 20 to 40. Birds had access to feed and water *ad libitum* throughout the study duration by one feed trough and one nipple drinker per bird, respectively. White LED (IP65 Dimmable LED Bulb, B-E27:10W, 5K; Eco Industrial Supplies, Zhenjiang, China) poultry-specific bulbs were used for lighting, and the lighting schedule was maintained as 16L: 8D hours (lights on at 5 a.m. and off at 9 p.m.) using an automatic timer. Inside shed ambient temperature and relative humidity were recorded twice daily (morning and afternoon) at bird height with a thermometer/hygrometer (Temp Alert, FCC RoHS, 2011/65/EU, FCC: R17HE910, S4GEM35XB, WI, USA). Hens were rehomed upon completion of the study.

2.2. Experimental Design and Dietary Treatment

At 20 WOA, birds were weighed individually and randomly allocated to eleven dietary treatments (30 replicates/treatment). Average hen starting weights did not differ significantly between the dietary treatments ($p > 0.05$). The first three dietary treatments were a standard-protein diet (SP, 17.24% CP); a reduced-protein diet (15.00% CP) with sufficient levels of Lys, Met, and Thr but insufficient levels of other essential AAs (RP); and a reduced-protein diet (15.00% CP) with sufficient levels of all essential AAs (RP-EAA). Eight subsequent dietary treatments were formulated by the deletion method as described by Fernandez, et al. [19], whereby each specific AA was removed from the RP-EAA diet: tryptophan (RP-EAA-Trp), valine (RP-EAA-Val), isoleucine (RP-EAA-Ile), arginine (RP-EAA-Arg), leucine (RP-EAA-Leu), histidine (RP-EAA-His), phenylalanine (RP-EAA-Phe), and glycine$_{equivalent}$ (RP-EAA-Gly) (Table 1). The CP level in the SP diet was per Hy-Line Brown nutritional recommendations [35], while the CP levels in the RP diets were selected followed the recommendations of previous studies on RP diets [28,36]. The levels of essential AAs were selected based on Hy-Line Brown nutritional recommendations [35]. Feed was provided in mash form. The nutritional composition of the major ingredients, including dry matter (DM), gross energy (GE), CP, crude fat, and ash content, was analysed prior to formulating the diets, and these values used for diet formulation (Table 2).

Table 1. Description of dietary treatments.

Treatment Number	Treatment Code	Treatment Description
1	SP	A standard-protein diet with sufficient levels of all essential amino acids
2	RP	A reduced-protein diet with sufficient levels of Lys, Met, and Thr but deficient in Trp, Arg, Ile, Val, Leu, His, Phe, and Gly
3	RP-EAA	A reduced-protein diet with sufficient levels of all essential amino acids, including Lys, Met, Thr, Trp, Arg, Ile, Val, Leu, His, Phe, and Gly
4	RP-EAA-Trp	Treatment 3 deficient in Trp
5	RP-EAA-Val	Treatment 3 deficient in Val
6	RP-EAA-Ile	Treatment 3 deficient in Ile
7	RP-EAA-Arg	Treatment 3 deficient in Arg
8	RP-EAA-Leu	Treatment 3 deficient in Leu
9	RP-EAA-His	Treatment 3 deficient in His
10	RP-EAA-Phe	Treatment 3 deficient in Phe
11	RP-EAA-Glycine$_{equivalent}$	Treatment 3 deficient in Gly

Table 2. Nutrient composition of the standard- and reduced-protein diets (as-is basis).

Ingredients (%, Otherwise as Indicated)	SP	RP
Wheat	38.44	49.52
Barley	4.00	4.00
Sorghum	20.00	20.00
Soybean meal	13.77	2.68
Canola meal	10.00	9.66
Canola oil	2.59	0.80
Limestone (fine)	4.93	4.94
Limestone (coarse)	4.93	4.94
Wheat	38.44	49.52
Barley	4.00	4.00
Sorghum	20.00	20.00

Table 2. *Cont.*

Monocalcium phosphate	0.549	0.623
Salt	0.232	0.159
Sodium bicarbonate	0.200	0.300
Choline Cl 60%	0.000	0.000
L-lysine HCl 78.5%	0.078	0.410
DL-methionine 99%	0.163	0.245
L-threonine 99%	0.016	0.159
L-tryptophan 99%	-	0.018
L-valine 99%	-	0.137
L-isoleucine 99%	-	0.175
L-arginine 99%	-	0.237
L-leucine 99%	-	0.287
L-histidine 99%	-	0.101
L-phenylalanine 99%	-	0.194
L-glycine 99%	-	0.276
Xylanase [1]	0.010	0.010
Phytase [2]	0.006	0.006
Pigment jabiru red	0.004	0.004
Pigment jabiru yellow	0.003	0.003
Vitamin–mineral premix [3]	0.100	0.100
Calculated nutrient composition (%, otherwise as indicated)		
AMEn [4], kcal/kg	2740	2740
Crude protein	17.24	15.00
Crude fat	4.60	2.90
Crude fibre	3.19	3.00
SID [5] arginine	0.893	0.780
SID lysine	0.740	0.740
SID methionine	0.400	0.435
SID cysteine	0.269	0.233
SID methionine + cysteine	0.670	0.670
SID tryptophan	0.192	0.160
SID histidine	0.370	0.370
SID phenylalanine	0.706	0.706
SID leucine	1.152	1.152
SID isoleucine	0.590	0.590
SID threonine	0.520	0.520
SID valine	0.692	0.650
SID glycine equivalent	0.971	0.971
Calcium	4.10	4.10
Available phosphorus	0.40	0.40
Sodium	0.17	0.17
Potassium	0.64	0.46
Chloride	0.22	0.24
Choline, mg/kg	1557	1333
Linoleic acid	1.55	1.12

SP: standard-protein diet (17.24% CP); RP: reduced-protein diet (15.00% CP plus adequate levels of all essential AAs). Crystalline AAs were purchased from Hard Eight Nutrition LLC 7511 Eastgate Rd Henderson, Nevada, United States. [1] Xylanase: Axtra XB TPT 201, Danisco Animal Nutrition (IFF); [2] Phytase: Quantum blue 5G, 60 g/MT, AB Vista; [3] vitamin–mineral premix (per kilogram): vitamin A (10 MIU); vitamin D (3 MIU); vitamin E (20 g); vitamin K (3 g); thiamine (2 g); riboflavin (6 g); cyanocobalamin (0.02 g); nicotinic acid (35 g); pantothenic acid (12 g); folic acid (1 g); biotin (0.1 g); pyridoxine (5 g); copper (8 g as copper sulphate pentahydrate); cobalt (0.2 g as cobalt sulphate 21%); molybdenum (0.5 g as sodium molybdate); iodine (1 g as potassium iodide 68%); selenium (0.3 g as selenium 2%); iron (60 g as iron sulphate 30%); zinc (60 g as zinc sulphate 35%); manganese (90 g as manganous oxide 60%); antioxidant (20 g). [4] AMEn: N-corrected apparent metabolisable energy. [5] SID: standardised ileal digestibility where coefficients of digestible AA for raw ingredients were determined by near-infrared spectroscopy (Foss NIR 6500, Hilleroed, Denmark) standardised with Evonik AMINONIR Advanced calibration.

The compositions of the final dietary nutrients were also analysed and presented in Table 3. Generally, analysed nutrient contents were close to the calculated values, indicating the dietary formulation objectives were achieved. Additionally, the RP diet deficient in all essential AAs except Lys, Met, and Thr and the RP-EAA diet sufficient in all essential AAs but with lower CP levels relative to the SP diet were obtained as expected (Tables 2 and 3).

Table 3. Analysed nutrient values of experimental diets (%, as-is basis) [1].

Nutrient Composition	SP	RP	RP-EAA	RP-EAA-Trp	RP-EAA-Val	RP-EAA-Ile	RP-EAA-Arg	RP-EAA-Leu	RP-EAA-His	RP-EAA-Phe	RP-EAA-Gly
Dry matter	91.46	91.34	91.66	91.76	91.25	91.06	91.08	91.34	91.35	91.33	91.26
Gross energy, kcal/kg	3712	3523	3638	3589	3584	3569	3571	3536	3571	3583	3509
Crude protein	18.15	14.37	15.82	15.66	15.84	15.25	15.44	15.30	15.20	14.90	14.77
Ash	15.07	15.04	13.64	14.70	14.27	14.53	14.52	14.77	14.40	13.75	15.73
Calcium	5.00	5.27	4.81	4.37	4.60	4.33	4.84	4.45	4.22	4.48	5.53
Total phosphorus	0.60	0.58	0.63	0.57	0.58	0.57	0.61	0.57	0.58	0.54	0.58
Aspartic acid	1.073	0.645	0.671	0.688	0.687	0.691	0.723	0.723	0.750	0.696	0.692
Serine	0.836	0.483	0.517	0.525	0.572	0.555	0.560	0.560	0.550	0.548	0.527
Glutamic acid	2.897	2.323	2.506	2.501	2.547	2.512	2.607	2.607	2.656	2.543	2.512
Glycine	0.723	0.406	0.671	0.684	0.778	0.721	0.715	0.715	0.686	0.701	0.463
Histidine	0.447	0.212	0.342	0.345	0.383	0.353	0.350	0.350	0.253	0.349	0.329
Arginine	0.922	0.536	0.742	0.824	0.888	0.806	0.560	0.859	0.754	0.838	0.828
Threonine	0.637	0.433	0.510	0.514	0.557	0.528	0.525	0.530	0.502	0.540	0.499
Alanine	0.649	0.465	0.496	0.512	0.533	0.522	0.528	0.537	0.546	0.521	0.508
Proline	1.265	0.768	0.885	0.809	0.937	0.914	0.916	0.914	0.937	0.960	0.944
Cysteine	0.621	0.528	0.317	0.430	0.611	0.575	0.571	0.563	0.612	0.623	0.569
Tyrosine	0.578	0.268	0.213	0.328	0.359	0.313	0.325	0.314	0.318	0.344	0.321
Valine	0.575	0.307	0.515	0.473	0.359	0.500	0.499	0.499	0.456	0.484	0.499
Methionine	0.460	0.315	0.565	0.324	0.413	0.432	0.448	0.349	0.397	0.376	0.328
Lysine	0.708	0.746	0.795	0.740	0.853	0.823	0.864	0.844	0.911	0.858	0.790
Isoleucine	0.491	0.298	0.387	0.487	0.516	0.366	0.507	0.504	0.482	0.527	0.493
Leucine	1.197	0.698	0.844	1.072	1.119	1.125	1.094	0.852	1.054	1.088	1.058
Phenylalanine	0.847	0.396	0.669	0.707	0.726	0.706	0.648	0.668	0.623	0.504	0.699

[1] Values of all the AAs presented are total AAs (measured on an as-is basis). SP: standard-protein diet (17.24% CP); RP: reduced-protein diet (15.00% CP) plus sufficient levels of Lys, Met, and Thr); RP-EAA: reduced protein (15% CP) plus sufficient levels of all essential AA); RP-EAA-Trp: RP-EAA minus Trp; RP-EAA-Val: RP-EAA minus Val; RP-EAA-Ile: RP-EAA minus Ile; RP-EAA-Arg: RP-EAA minus Arg; RP-EAA-Leu: RP-EAA minus Leu; RP-EAA-His: RP-EAA minus His; RP-EAA-Phe: RP-EAA minus Phe; RP-EAA-Gly: RP-EAA minus $\text{Glycine}_{\text{equivalent}}$.

2.3. Data Collection

Data were collected over a duration of 20 weeks. Hens were weighed at the beginning (week 19), at the middle (week 29), and at the end of the study period (week 39). Eggs were collected and weighed daily. Feed intake (per hen per day in grams) was recorded weekly. Egg quality (both external and internal) was measured at 29 and 39 WOA. The apparent total GE and CP digestibility of the dietary treatments was determined at 38 WOA. Hens of approximately similar body weight were selected (8 hens/treatment, 88 hens in total) to collect excreta samples by the total excreta collection method. An individual tray wrapped with aluminium foil paper was placed directly under the cage. Loose feathers and feed residues were removed from the tray, and then the collected excreta were transferred into polypropylene transparent zipper poly bags. This was conducted once daily between 9 a.m. to 1 p.m., over 3 consecutive days (72 h). The collected samples were then transported to the laboratory and mixed thoroughly before taking subsamples in 70 mL plastic containers, which were then stored at 4 °C. Approximately 5 g of fresh excreta sample was weighed into a preweighed crucible and dried in a forced air oven (Qualtex, Solidstat Temperature Control Oven, Model No. OM24SE3, Morningside, QLD, Australia) at 105 °C for around 48 h (to constant weight), to remove the moisture for determination of DM. The remaining excreta subsample was preserved at −20 °C for further analysis. Feed consumption of the individual hens was also recorded across the 3 days of the excreta collection period.

A subgroup of hens (8 hens/treatment, 88 hens in total) were euthanised at 40 WOA by decapitation after electrical stunning. Blood samples were collected in silica-coated vacutainers (Becton, Dickinson UK. Limited, Plymouth, UK) containing serum separator polymer gel from the jugular vein during decapitation of the hens to measure serum uric acid levels. Right tibia samples (8 hens/treatment, 88 hens in total) were collected for evaluation of bone parameters. Bones were excised and defleshed manually by hand using a scalpel and scissors following collection, and then transferred to the laboratory in cool box. The fresh wet bones were weighed using a Discover Precision balance (FX-3000i, A & D Company Ltd., Tokyo, Japan) and then air-dried in a fume hood for 2 days, reweighed, and stored at 5 °C. Hen caecal digesta samples (8 hens/treatment, 88 hens in total) were collected to determine the relative total bacterial population. Caecal contents were collected into 2 mL Eppendorf tubes and then snap-frozen in liquid nitrogen. They were then stored at −20 °C for further processing. The percentage of dirty eggs was also determined at 40 WOA to establish the correlation between the eggshell cleanliness and caecal microbial load. Daily hen mortality across the study was also recorded. Only one mortality in treatment RP-EAA-Arg was observed over the entire study.

2.4. Laying Performance

Hen laying performance, including egg weight (g), egg mass (g/day/hen), hen-day egg production (%), feed intake (g), and feed conversion ratio (FCR) were calculated on a weekly basis from 20–29, 30–39, and 20–39 WOA.

$$\text{Hen-dayeggproduction}(\%) = \frac{\text{Totalnumberofeggs}}{\text{Totalnumberofhens} \times 7\ (\text{days})} \times 100$$

$$\text{Egg mass}\ (\text{g/day/hen}) = \text{Hen-day egg production}\ (\%)\ \times\ \text{Average egg weight}\ (\text{g})$$

$$\text{FCR}\ (\text{per kg egg mass}) = \frac{\text{kg of feed consumed}}{\text{kg of egg produced}}$$

2.5. Egg Quality

External and internal egg quality traits and egg proportions were assessed at 29 and 39 WOA. Approximately 15 eggs per treatment (165 eggs in total) were collected in the morning. Measurements were performed by one experimenter within 4 h of egg collection, except for eggshell weight and thickness (i.e., waited until dried). All significantly deformed

eggs (4 and 9 deformed eggs at week 29 and 39, respectively) were discarded and excluded from the analysis. Eggs were analysed for both external (eggshell thickness, breaking strength and reflectivity, and egg length, breadth, and shape index) and internal quality (albumen height, yolk height, yolk diameter, yolk index, yolk colour, and Haugh unit). Egg length (dimension between the poles) and breadth (dimension at the equator) were measured using a Kincrome 0–150 mm Digital Vernier calliper (Kincrome, Scoresby, Victoria, Australia). Then, egg shape index was determined as the egg length divided by egg breadth. Eggshell reflectivity was determined with a shell reflectivity meter (Technical Services and Supplies, Dunnington, York, UK), while eggshell breaking strength and egg internal quality traits were measured using a digital egg tester (DET6500, Nabel Co., Ltd., Kyoto, Japan). Egg yolks were transferred onto preweighed filter papers (diameter 90 mm, CAT No.1541-090, Whatman, Buckinghamshire HP7 9NA, UK) and weighed. For eggshell weight and thickness, the eggshell residue was washed in slow-running tap water to remove the albumin from the eggshell surface, and then the eggshell was air-dried thoroughly for at least 48 h. Eggshell weight was taken using an Adventurer TM Precision analytical balance (Model AX423, Ohaus®, Newark, NJ, USA). Eggshell thickness, including the shell membrane, was measured on two pieces of shell collected from different places on the egg using a custom-built gauge (Mitutoyo Dial Comparator Gauge, Model 2109-10, Kawasaki, Japan), and the average value was used for the analysis. To measure egg proportions, egg albumen weight was calculated by subtracting yolk weight and shell weight from the intact egg weight. Percentages of albumen, yolk, and eggshell were obtained by dividing weights of these egg components by the total egg weight.

2.6. Gross Energy and Protein Digestibility

The frozen excreta samples were placed in a freeze dryer (Christ Alpha 1–4 LD plus, Osterode am Harz, Germany) for drying, and then dried samples were ground into fine particles using an ultra-centrifugal mill (Retsch ZM 200, Fisher Scientific, Hampton, NH, USA) with a 0.5 mm screen. Feed samples were also ground using the same technique. Protein concentration in both the excreta and feed samples was measured as described by Dumas [37] following the Dumas combustion method using a nitrogen analyser (LECO Corporation, St. Joseph, MI, USA), where EDTA was used as the calibration standard. The GE concentration in the excreta and feed samples was determined using a Parr Adiabatic Oxygen Bomb calorimeter (Parr Instrument Co., Moline, IL, USA) with benzoic acid as the calibration standard. Mixed feed samples were also oven-dried at 105 °C for approximately 24 h (to constant weight), as described above, for determination of diet DM, so GE and CP digestibility could be presented on a DM basis. Apparent GE and CP digestibility on a DM basis was calculated using the following equations described by Kong and Adeola [38].

$$\text{Apparent protein digestibility (\%)} = (\text{CP}_{\text{retained}}/\text{CP}_{\text{intake}}) \times 100$$

$$\text{Apparent energy digestibility (\%)} = (\text{GE}_{\text{retained}}/\text{GE}_{\text{intake}}) \times 100$$

$$\text{CP}_{\text{intake}}\ (\text{g/day}) = \text{CP}_{\text{feed}}\ (\%) \times \text{FI}\ (\text{g/day/hen})$$

$$\text{GE}_{\text{intake}}\ (\text{kcal/day}) = \text{GE}_{\text{feed}}\ (\text{kcal/g}) \times \text{FI}\ (\text{g/day/hen})$$

$$\text{CP}_{\text{retained}}\ (\text{g/day}) = \text{CP}_{\text{intake}} - \text{CP}_{\text{excreta}}\ (\%) \times \text{excreta volume}\ (\text{g/day/hen})$$

$$\text{GE}_{\text{retained}}\ (\text{kcal/day}) = \text{GE}_{\text{intake}} - \text{GE}_{\text{excreta}}\ (\text{kcal/g}) \times \text{excreta volume}\ (\text{g/day/hen})$$

where CP, GE and FI are crude protein, gross energy, and feed intake, respectively.

2.7. Serum Uric Acid

Blood samples were immediately transferred to the laboratory after collection and kept at 4 °C for 5 h. For collection of the serum, tubes were centrifuged at 3000× *g* at 4 °C for 10 min and the supernatant was poured into 2 mL micro-centrifuge tubes. These were stored at −20 °C until further analysis. Serum uric acid concentration was measured in

duplicate using a Thermo Indiko™ Plus auto-analyser (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instruction [39].

2.8. Bone Parameters

The length (between tip of the proximal end and tip of the distal end) and width (at the midpoint) of the air-dried tibias was measured using a Kincrome 0–150 mm Digital Vernier calliper (Kincrome, Scoresby, VIC, Australia). The bone Seedor index was calculated as described by Seedor, et al. [40]: Seedor index = weight of air-dried bone (mg)/length of air-dried bone (mm). The air-dried tibias were tested for breaking strength using Instron® electromechanical universal testing machine (Instron® Mechanical Testing Systems, 825 University Ave., Norwood, MA, USA). The breaking strength was tested with 3-point flexure test setup at 300 KN load cell and 50 mm at 0.2 mm/second speed, with 20 data points per second. The universal materials testing software Bluehill (ver.2, Instron®Mechanical Testing Systems, 825 University Ave., Norwood, MA, USA) was used for recording the data. The mechanical force at the midpoint of the bone was applied from a 2 cm distance between two fixed points (50 mm) supporting the bone. The test was conducted on all bones in a single day. Tibia samples were then weighed into crucibles, ashed in a muffle furnace (Carbolite, Sheffield, UK) set to run at 350 °C for 1 h followed by increase to 600 °C for 16 h, and then reweighed. The ash (%) was determined as ash weight divided by oven-dried bone weight, multiplied by 100.

2.9. Caecal Microbiome Profile

Total caecal bacteria population was determined by quantitative real-time PCR (Rotor-gene 6500 RT-PCR machine, Corbett, Sydney, Australia), as described by Kheravii, et al. [41]. The specific primers (16S rRNA) were used to quantify targeted bacterial populations, including *Bacillus* spp., *Bacteroides* spp., *Bifidobacterium* spp., *Enterobacteriaceae*, *Lactobacillus* spp., *Ruminococcus* spp., and total bacteria (Table 4). The quantification of the bacterial population was expressed as $\log_{10}$ genomic DNA copies per gram of caecal digesta. On the day of euthanasia, all eggs from the sampled hens were also collected and graded as clean or dirty eggs using Australian standards [42]. Eggshell cleanliness was correlated with total caeca bacterial load.

Table 4. Sequence of primers used for the qPCR analysis of selected bacterial populations in caecal digesta samples as described by Kheravii, et al. [41].

Target Group or Organism	Primer Sequence (5′–3′)	Annealing Temperature (°C)	Reference
Bacillus spp.	F-GCA ACG AGC GCA ACC CTT GA R-TCA TCC CCA CCT TCC TCC GGT	63	[43]
Bacteroides spp.	F-GAG AGG AAG GTC CCC CAC R-CGC TAC TTG GCT GGT TCA G	63	[44]
Bifidobacterium spp.	F-GCG TCC GCT GTG GGC R-CTT CTC CGG CAT GGT GTT G	63	[45]
Enterobacteriaceae	F-CAT TGA CGT TAC CCG CAG AAG AAG C R-CTC TAC GAG ACT CAA GCT TGC	63	[46]
Lactobacillus spp.	F-CAC CGC TAC ACA TGG AG R-AGC AGT AGG GAA TCT TCC A	63	[47]
Ruminococcus spp.	F-GGC GGC YTR CTG GGC TTT R-CCA GGT GGA TWA CTT ATT GTG TTA A	63	[48]
Total bacteria	F-CGG YCC AGA CTC CTA CGG G R-TTA CCG CGG CTG CTG GCA C	63	[49]

2.10. Statistical Analysis

Data were organised and validated in Microsoft Excel spreadsheet and analysed using IBM SPSS statistical software (Version: 28.0.1.0, IBM Corp., Armonk, NY, USA) with α-level fixed at 0.05. However, a trend effect was considered at $p = 0.05$ or ≤ 0.10. Prior to statistical

analyses, data were tested for normal distribution using Kolmogorov–Smirnov test and approximately equal variances between the dietary treatments. Data were subjected to ANOVA with univariate general linear models (GLM) fitted to each variable, and treatment as the fixed effect, to determine the mean differences between the dietary treatment groups. Tukey's post hoc test was applied where significant differences or trends were present, to recognise pairwise variances between the dietary treatments.

3. Results

3.1. Housing Environment, Hen Weight, and Mortality

The average ambient temperature (°C) and relative humidity (%) inside the shed are illustrated in Figure 1. The mean air temperature and relative humidity for the study duration were 14.9 °C (ranging from 5 to 25 °C) and 67.9% (ranging from 43.5% to 97.8%), respectively. The maximum air temperature ranged from 8 to 27 °C (average 18.7 °C), while the minimum temperature ranged from 1 to 23 °C (average 11.5 °C).

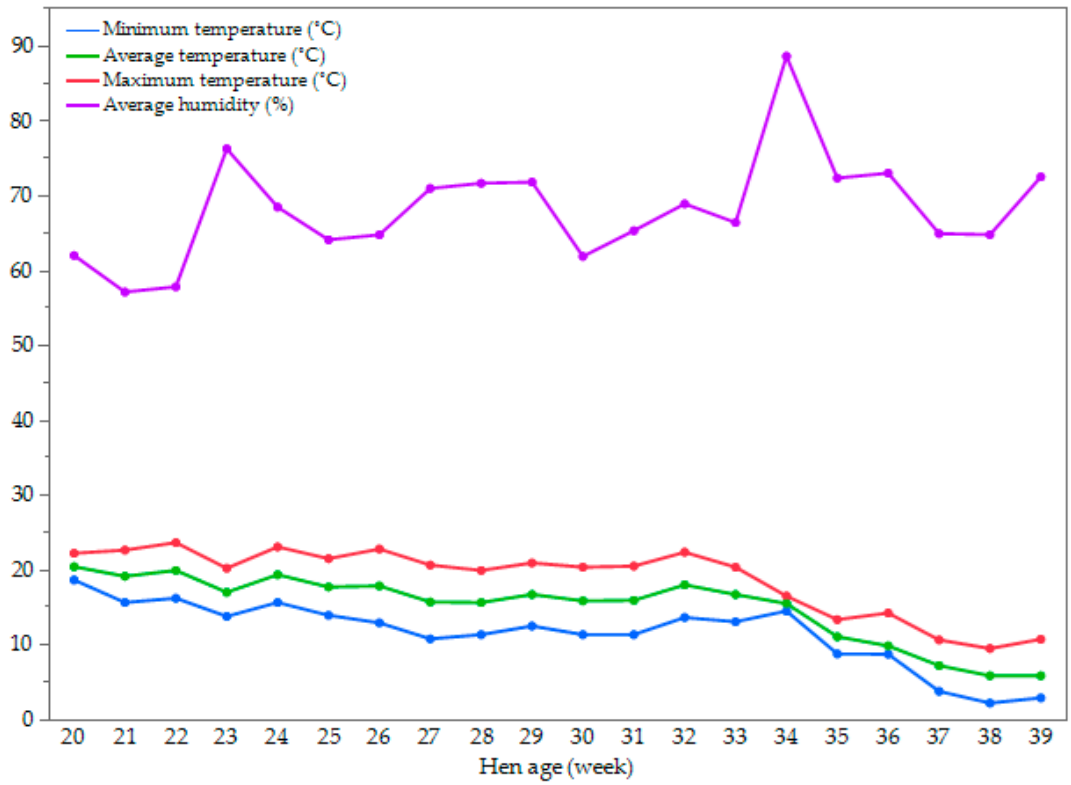

Figure 1. In-house temperature and relative humidity from weeks 20 to 39.

Hen weight was not different between the dietary treatment groups at 20, 29, or 39 WOA ($p > 0.05$; Table S1). Hen mortality for the Arg-deficient RP diet was 3.33%, and no mortality was recorded for any other treatments throughout the study period (Table S1).

3.2. Laying Performance and Egg Quality

Hen laying performance for the study duration (20 to 39 WOA) on a weekly basis is shown in Figure 2. Moreover, the laying performance from 20 to 29 WOA and 30 to 39 WOA separately, as well as for the entire duration from 20 to 39 WOA are presented in Table 5. The results showed that dietary treatments had a trend effect on hen-day egg production during 20–29 WOA ($p = 0.051$), where hens fed the RP-EAA, RP-EAA-Ile, and RP-EAA-Val diets had lower egg production compared to the SP diet. At 30–39 WOA, dietary treatments did not affect the hen-day egg production ($p > 0.05$). Over the entire study, hen-day egg production tended to be increased in hens fed the SP diet and diets

deficient in Gly, Arg, Leu, and His compared to those offered the RP, EAA-Val, and EAA-Ile diets (p = 0.098, Table 5).

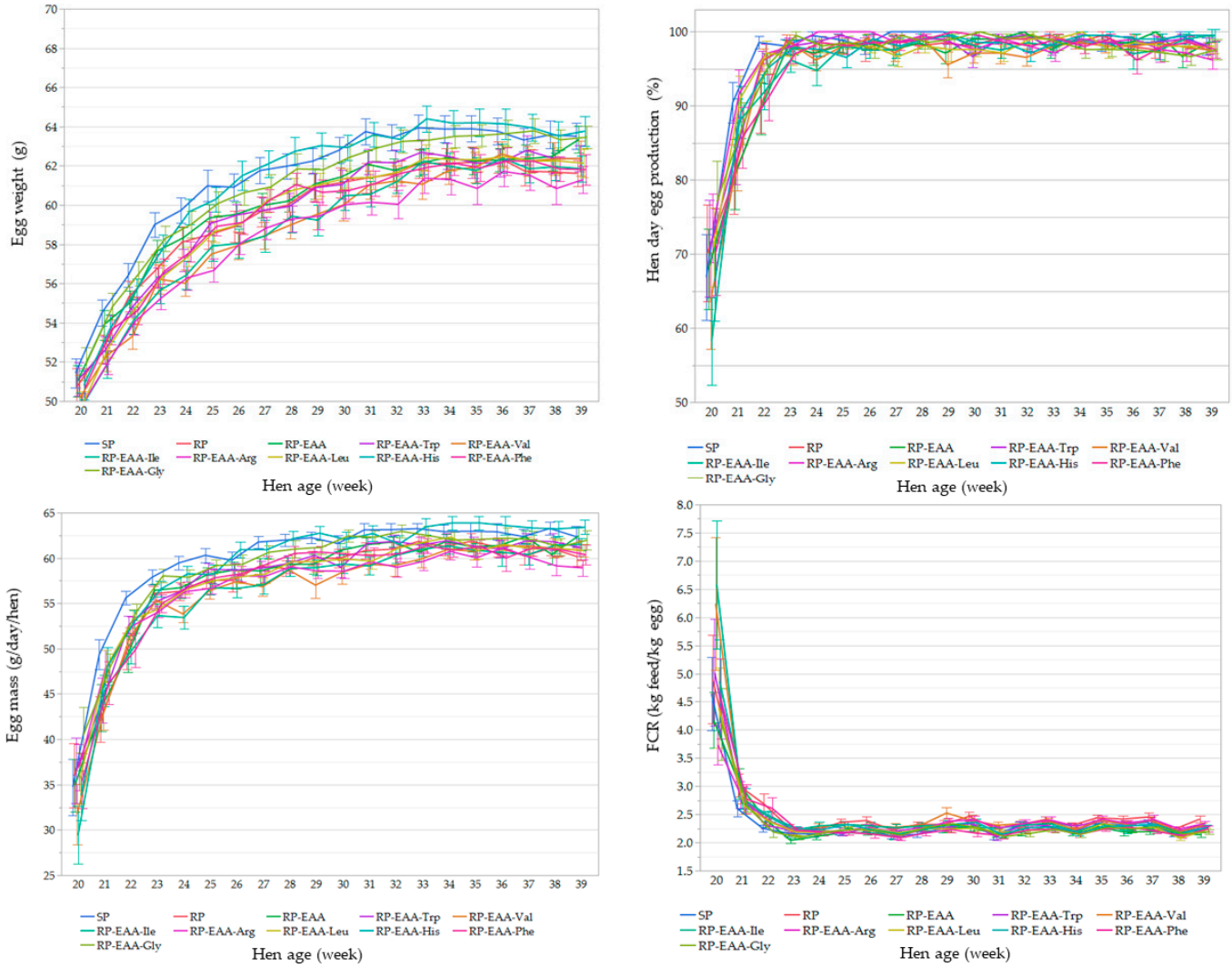

Figure 2. Average (LSM ± SEM) egg weight, hen day egg production, egg mass, and FCR of the dietary treatments from weeks 20 to 39. SP: standard-protein diet (17.24% CP); RP: reduced-protein diet (15.00% CP) plus sufficient levels of Lys, Met, and Thr); RP-EAA: reduced-protein (15% CP) plus sufficient levels of all essential AA); RP-EAA-Trp: RP-EAA minus Trp; RP-EAA-Val: RP-EAA minus Val; RP-EAA-Ile: RP-EAA minus Ile; RP-EAA-Arg: RP-EAA minus Arg; RP-EAA-Leu: RP-EAA minus Leu; RP-EAA-His: RP-EAA minus His; RP-EAA-Phe: RP-EAA minus Phe; RP-EAA-Gly: RP-EAA minus Glycine$_{equivalent}$.

Table 5. Laying performance of hens offered the dietary treatments from weeks 20 to 39.

Hen Age (Week)	Nutrient Composition	SP	RP	RP-EAA	RP-EAA-Trp	RP-EAA-Val	RP-EAA-Ile	RP-EAA-Arg	RP-EAA-Leu	RP-EAA-His	RP-EAA-Phe	RP-EAA-Gly	SEM	*p*-Value
20–29	Hen-day egg production (%)	95.51	93.34	92.76	94.84	93.21	92.52	95.8	94.52	93.99	94.14	95.25	0.82	0.051
	Egg weight (g)	59.02 ^{a}	57.25 ^{bcd}	57.78 ^{abc}	57.32 ^{bcd}	56.13 ^{d}	56.20 ^{cd}	56.06 ^{d}	57.17 ^{bcd}	58.93 ^{a}	57.49 ^{abcd}	58.63 ^{ab}	0.57	<0.001
	Egg mass (g)	56.65 ^{b}	53.76 ^{cde}	53.80 ^{cde}	54.61 ^{bc}	52.55 ^{de}	52.30 ^{e}	53.92 ^{cde}	54.27 ^{bcd}	55.64 ^{abc}	54.34 ^{bcd}	56.06 ^{ab}	0.67	<0.0001
	Feed intake (g)	129 ^{abc}	131 ^{ab}	122 ^{d}	126 ^{bcd}	127 ^{abcd}	126 ^{bcd}	126 ^{bcd}	125 ^{bcd}	132 ^{a}	124 ^{cd}	128 ^{abc}	2.13	0.039
	FCR (kg feed/kg egg)	2.456	2.616	2.451	2.515	2.696	2.740	2.452	2.478	2.574	2.490	2.430	0.08	0.089
30–39	Hen-day egg production (%)	98.74	98.60	98.70	98.47	97.72	98.27	97.95	98.33	98.91	98.28	98.14	0.38	0.551
	Egg weight (g)	63.51 ^{ab}	61.81 ^{bcd}	62.28 ^{abcd}	62.22 ^{abcd}	61.55 ^{cd}	61.62 ^{cd}	60.91 ^{d}	62.06 ^{abcd}	63.80 ^{a}	61.76 ^{bcd}	63.31 ^{abc}	0.64	0.032
	Egg mass (g)	62.70 ^{ab}	60.95 ^{bcd}	61.45 ^{abcd}	61.27 ^{abcd}	60.14 ^{d}	60.54 ^{cd}	59.68 ^{d}	61.00 ^{bcd}	63.11 ^{a}	60.72 ^{cd}	62.16 ^{abc}	0.69	0.012
	Feed intake (g)	144 ^{ab}	144 ^{a}	135 ^{d}	140 ^{abcd}	137 ^{abcd}	140 ^{bcd}	140 ^{abcd}	136 ^{cd}	142 ^{abc}	133 ^{d}	137 ^{cd}	2.38	0.018
	FCR (kg feed/kg egg)	2.300 ^{abcd}	2.377 ^{a}	2.201 ^{d}	2.296 ^{abcd}	2.334 ^{abc}	2.271 ^{abcd}	2.347 ^{ab}	2.228 ^{cd}	2.252 ^{bcd}	2.203 ^{d}	2.209 ^{d}	0.04	0.007
20–39	Hen-day egg production (%)	95.4	92.8	94.7	94.6	93.3	93.4	95.8	95.6	95.2	94.8	96.2	0.25	0.098
	Egg weight (g)	60.2 ^{abc}	57.7 ^{d}	59.1 ^{abcd}	58.5 ^{cd}	57.7 ^{d}	57.6 ^{d}	57.8 ^{d}	59.1 ^{abcd}	60.5 ^{ab}	58.7 ^{bcd}	60.7 ^{a}	0.21	0.001
	Egg mass (g)	57.5 ^{ab}	53.7 ^{d}	56.0 ^{abcd}	55.4 ^{bcd}	54.0 ^{cd}	53.9 ^{d}	55.4 ^{bcd}	56.5 ^{abc}	57.7 ^{ab}	55.7 ^{bcd}	58.4 ^{a}	0.28	<0.001
	Feed intake (g)	128	127	123	125	125	124	125	125	131	122	128	0.7	0.281
	FCR (kg feed/kg egg)	2.238 ^{bc}	2.378 ^{a}	2.203 ^{c}	2.262 ^{abc}	2.334 ^{ab}	2.305 ^{abc}	2.263 ^{abc}	2.212 ^{c}	2.278 ^{abc}	2.205 ^{c}	2.193 ^{c}	0.013	0.046

$^{a–e}$ Means within columns with different suffixes are significantly different at $p < 0.05$ and considered a trend at $p = 0.05 \leq 0.10$. SP: standard-protein diet (17.24% CP); RP: reduced-protein diet (15.00% CP) plus sufficient levels of Lys, Met, and Thr); RP-EAA: reduced-protein (15% CP) plus sufficient levels of all essential AA); RP-EAA-Trp: RP-EAA minus Trp; RP-EAA-Val: RP-EAA minus Val; RP-EAA-Ile: RP-EAA minus Ile; RP-EAA-Arg: RP-EAA minus Arg; RP-EAA-Leu: RP-EAA minus Leu; RP-EAA-His: RP-EAA minus His; RP-EAA-Phe: RP-EAA minus Phe; RP-EAA-Gly: RP-EAA minus $\text{Glycine}_{\text{equivalent}}$.

Egg weight was significantly reduced in hens offered the RP-EAA-Arg diet, followed by the RP-EAA-Val, RP-EAA-Ile, RP-EAA-Leu, RP-EAA-Trp, and RP diet compared to hens offered the SP diet during 20 to 29 WOA ($p < 0.001$). Egg weight of hens fed the RP-EAA, RP-EAA-His, and RP-EAA-Gly diets were not different from the SP diet during the same period ($p > 0.05$). From 30 to 39 WOA, hens offered the RP-EAA-Arg diet presented the lowest egg weight, followed by RP-EAA-Val and RP-EAA-Ile groups compared to the SP group ($p = 0.032$), whereas no differences were observed for egg weight of the hens offered the RP, RP-EAA, and RP diets deficient in Trp, Leu, His, Phe, and Gly ($p > 0.05$). Over the entire study from 20 to 39 WOA, hens fed the RP-EAA-Ile diet had the lowest egg weight, followed by the RP-EAA-Val and RP groups as compared to the SP, RP-EAA-His, and RP-EAA-Gly groups ($p < 0.001$). However, no differences in egg weight were observed between the RP-EAA, RP-EAA-Trp, RP-EAA-Arg, RP-EAA-Leu, and RP-EAA-Phe treatments in comparison to the SP treatment ($p > 0.05$, Table 5).

Egg mass was lower in hens fed the RP-EAA-Ile diet, followed by the RP-EAA-Val, RP, RP-EAA, and RP-EAA-Arg groups compared to the SP group from 20 to 29 WOA ($p < 0.0001$). However, birds fed the RP-EAA-Gly diet presented the highest egg mass compared to the RP-EAA diet, but the RP diets deficient in Trp, Leu, His, and Phe diets had similar effects ($p > 0.05$). During 30 to 39 WOA, the lowest egg mass was observed in birds fed the RP-EAA-Arg diet, followed by RP-EAA-Val, RP-EAA-Ile, RP, RP-EAA-Leu, and RP-EAA-Phe groups as compared to the SP group ($p = 0.012$), and the differences were identical for the RP diets deficient in Trp, His and Gly compared to the RP-EAA and/or SP diet ($p > 0.05$). Overall, egg mass was significantly decreased in birds fed the RP-EAA-Ile diet, followed by the RP-EAA-Val and RP groups, compared to the SP group ($p < 0.001$), while the effects of other essential AA deficiencies in RP diets were similar compared to the RP-EAA and SP diets ($p > 0.05$, Table 5).

Hens fed the RP-EAA-Gly, SP, RP, and RP-EAA-His diets had higher feed intake during 20–29 WOA compared to those fed the RP-EAA diet ($p = 0.039$). The remaining AA-deficient treatments had similar effects on feed intake compared to the SP and RP-EAA treatments during 20 to 29 WOA ($p > 0.05$). Hens fed the RP-EAA-His, RP, and SP diets had higher feed intake compared to those offered the RP-EAA-Phe, RP-EAA, RP-EAA-Leu and RP-EAA-Gly diets ($p = 0.018$). No differences were found for the RP diets deficient in Trp, Val, Ile, Arg in comparison with either SP or RP-EAA diets during 30–39 WOA ($p > 0.05$). Overall, feed intake of the hens did not differ between the dietary treatments from 20 to 39 WOA ($p > 0.05$, Table 5).

The dietary treatments did not affect the FCR during 20 to 29 WOA ($p > 0.05$). Differences in FCR were evident between the treatment groups at 30 to 39 WOA ($p = 0.007$), showing that hens fed the RP diet had the highest FCR, followed by RP-EAA-Arg and RP-EAA-Val groups, compared to hens offered the RP-EAA diet. The lowest FCR was observed in hens fed the RP-EAA-Phe and RP-EAA-Gly diets compared to those fed the RP-EAA diet. Hens fed the RP diets deficient in the remaining AA had similar FCR compared to those offered either the SP or RP-EAA diet ($p > 0.05$). Over the entire study, FCR was higher in hens fed the RP diets followed by the RP-EAA-Val group as compared to those offered the RP-EAA diet, while the lowest FCR was observed in hens fed the RP-EAA-Leu, RP-EAA-Phe, and RP-EAA-Gly diets compared to the RP-EAA group ($p = 0.046$). The FCR was not affected by the removal of Trp, Ile, Arg, and His in the RP diets when compared to the SP and/or RP-EAA diets ($p > 0.05$). Internal and external egg quality parameters as well as egg proportions were not affected by the dietary treatments at 29 and 39 WOA (all $p > 0.05$; Tables S2–S4).

3.3. Apparent Protein and Gross Energy Digestibility, and Serum Uric Acid Levels

Apparent CP and GE digestibility are presented in Table 6. The result showed that the dietary treatment groups had no impact on protein or energy digestibility ($p > 0.05$). However, hens offered the RP diets presented lower protein intake compared to hens fed the SP diet, except for the diet deficient in Val, which was statistically similar to the SP

diet ($p = 0.001$). Excretion of protein in hens offered the RP diets deficient in Arg, Leu, and Gly was significantly lower compared to the SP diet ($p = 0.018$). There was no statistical difference in protein intake or excretion between the different RP diets ($p > 0.05$). Moreover, no differences were observed between the treatment groups for energy intake, energy excretion, and for both retained protein and energy (all $p > 0.05$). The dietary treatments did not affect serum uric acid levels ($p > 0.05$) at week 40, as shown in Table 6.

Table 6. Apparent protein digestibility, gross energy digestibility, and serum uric acid levels of hens offered the dietary treatments at week 40 (as per dry matter basis).

Treatment	Protein Intake (g/day)	Protein Excreted (g/day)	Retained Protein (g/day)	Protein Digestibility (%)	Energy Intake (Kcal/day)	Energy Excreted (Kcal/day)	Retained Energy (Kcal/day)	Energy Digestibility (%)	Serum Uric Acid Level ((mg/dl))
SP	27.74 ^{a}	13.05 ^{a}	14.68	51.72	567.25	109.8	457.45	80.05	5.58
RP	20.97 ^{b}	10.33 ^{ab}	10.64	49.99	514.19	102.48	411.7	79.82	4.41
RP-EAA	21.28 ^{b}	10.87 ^{ab}	10.4	49.05	489.38	95.24	394.14	80.65	5.20
RP-EAA-Trp	21.75 ^{b}	10.52 ^{ab}	11.23	51.53	498.58	96.07	402.51	80.65	4.63
RP-EAA-Val	22.62 ^{ab}	10.04 ^{ab}	12.58	54.72	511.92	93.84	418.07	81.37	4.94
RP-EAA-Ile	21.63 ^{b}	11.38 ^{ab}	10.25	46.93	506.36	102.27	404.09	79.65	5.31
RP-EAA-Arg	20.58 ^{b}	9.7 ^{b}	10.87	52.57	476.09	86.83	389.26	81.72	4.67
RP-EAA-Leu	19.64 ^{b}	9.61 ^{b}	10.03	50.79	454.01	89.07	364.94	80.25	4.98
RP-EAA-His	20.8 ^{b}	10.74 ^{ab}	10.11	48.64	490.05	96.26	393.78	80.42	5.00
RP-EAA-Phe	20.4 ^{b}	10.19 ^{ab}	10.25	48.56	491.73	94	397.72	80.46	4.37
RP-EAA-Gly	20.9 ^{b}	9.7 ^{b}	11.28	53.44	498.73	97.36	401.37	80.46	4.88
SEM	1.18	0.64	1.13	3.18	26.66	6.01	24.82	1.16	0.32
p-value	0.001	0.018	0.155	0.854	0.367	0.347	0.603	0.984	0.234

^{a,b} Means within columns with different suffixes are significantly different at $p < 0.05$ and considered a trend at $p = 0.05 \leq 0.10$. SP: standard-protein diet (17.24% CP); RP: reduced-protein diet (15.00% CP) plus sufficient levels of Lys, Met, and Thr; RP-EAA: reduced protein (15% CP) plus sufficient levels of all essential AAs; RP-EAA-Trp: RP-EAA minus Trp; RP-EAA-Val: RP-EAA minus Val; RP-EAA-Ile: RP-EAA minus Ile; RP-EAA-Arg: RP-EAA minus Arg; RP-EAA-Leu: RP-EAA minus Leu; RP-EAA-His: RP-EAA minus His; RP-EAA-Phe: RP-EAA minus Phe; RP-EAA-Gly: RP-EAA minus $Glycine_{equivalent}$.

3.4. Bone Parameters

The experimental diets had no effect on the bone parameters, including tibia fresh weight, air-dry weight, Seedor index, bone breaking strength, ash/mineral content, and ash percentages (all $p > 0.05$; Table 7).

Table 7. Tibia morphological parameters of hens offered the dietary treatments at week 40.

Treatments	Fresh Weight (g)	Air-Dry Weight (g)	Length (mm)	Width (mm)	Seedor Index	Bone Breaking Strength (Kgf)	Ash Content (g)	Ash (%)
SP	12.38	9.73	125.04	8.70	0.070	158.76	3.23	37.15
RP	12.01	9.55	124.27	8.63	0.069	134.64	3.29	38.64
RP-EAA	11.90	9.25	123.39	8.78	0.067	150.54	3.20	38.70
RP-EAA-Trp	12.10	9.45	124.84	8.70	0.068	138.54	3.21	38.02
RP-EAA-Val	12.16	9.53	125.40	8.67	0.068	126.26	3.20	37.66
RP-EAA-Ile	12.25	9.71	123.55	8.72	0.070	146.58	3.35	38.70
RP-EAA-Arg	27.83	9.72	124.54	9.04	0.070	127.01	3.17	36.58
RP-EAA-Leu	12.17	9.66	123.45	8.69	0.071	134.71	3.28	37.65
RP-EAA-His	12.60	10.02	126.48	8.76	0.071	134.43	3.40	37.87
RP-EAA-Phe	12.48	9.78	123.73	8.80	0.071	151.79	3.24	37.03
RP-EAA-Gly	11.98	9.45	124.09	8.77	0.069	134.96	3.14	36.67
SEM	4.709	0.230	1.18	0.172	0.002	16.89	0.120	1.004
p-value	0.448	0.626	0.761	0.943	0.741	0.947	0.926	0.804

SP: standard-protein diet (17.24% CP); RP: reduced-protein diet (15.00% CP) plus sufficient levels of Lys, Met, and Thr; RP-EAA: reduced protein (15% CP) plus sufficient levels of all essential AAs; RP-EAA-Trp: RP-EAA minus Trp; RP-EAA-Val: RP-EAA minus Val; RP-EAA-Ile: RP-EAA minus Ile; RP-EAA-Arg: RP-EAA minus Arg; RP-EAA-Leu: RP-EAA minus Leu; RP-EAA-His: RP-EAA minus His; RP-EAA-Phe: RP-EAA minus Phe; RP-EAA-Gly: RP-EAA minus $Glycine_{equivalent}$.

3.5. Caecal Microbiome

There was a trend for increasing *Bacteroides* spp. count in hens fed the RP diets compared to those fed the SP diet ($p = 0.067$, Table 8). Specifically, hens fed the RP, RP-EAA-Val, RP-EAA-Ile, RP-EAA-Phe, and RP-EAA-Gly diets had higher caecal *Bacteroides* count compared to those fed the SP and other diets, whereas the number of *Ruminococcus* spp. tended to be increased in hens offered the RP-EAA, RP-EAA-Arg, RP-EAA-Leu, RP-EAA-Phe, and RP-EAA-Gly diets compared to those offered the SP diet ($p = 0.082$, Table 8). However, the numbers of the other microbiota including *Lactobacillus* spp., *Bacillus* spp., *Bifidobacterium* spp., *Enterobacteriaceae*, and total bacteria were not significantly different between the dietary treatments at week 40 (all $p > 0.05$; Table 8). The eggshell cleanness score also was not significantly correlated with the caecal total bacterial count at week 40 ($p = 0.249$, $R^2 = 0.125$).

Table 8. Caecal microbial quantification of the hens fed the dietary treatments at 40 weeks of age (log10 copies/g).

Treatment	*Lactobacillus*	*Ruminococcus*	*Bacteroides*	*Bacillus*	*Bifidobacteria*	*Enterobacteria*	Total Bacteria
SP	8.98	9.45	10.88	7.84	9.65	7.27	12.59
RP	8.70	9.34	11.05	7.58	9.76	7.61	12.48
RP-EAA	8.65	9.53	10.95	7.85	9.85	7.40	12.52
RP-EAA-Trp	8.67	9.41	11.01	7.84	9.80	8.07	12.55
RP-EAA-Val	8.86	9.42	11.04	7.68	9.79	7.18	12.51
RP-EAA-Ile	8.69	9.40	11.09	7.77	9.83	7.30	12.52
RP-EAA-Arg	8.72	9.59	10.96	7.86	9.91	7.32	12.57
RP-EAA-Leu	8.71	9.50	10.94	7.77	9.79	7.14	12.59
RP-EAA-His	8.69	9.48	10.95	7.84	9.83	7.60	12.53
RP-EAA-Phe	8.68	9.52	11.10	7.93	9.78	7.52	12.56
RP-EAA-Gly	8.81	9.50	11.06	7.76	9.80	7.88	12.56
SEM	0.11	0.05	0.05	0.14	0.06	0.59	0.05
p-value	0.744	0.082	0.067	0.908	0.373	0.991	0.903

SP: standard-protein diet (17.24% CP); RP: reduced-protein diet (15.00% CP) plus sufficient levels of Lys, Met, and Thr; RP-EAA: reduced protein (15% CP) plus sufficient levels of all essential AAs; RP-EAA-Trp: RP-EAA minus Trp; RP-EAA-Val: RP-EAA minus Val; RP-EAA-Ile: RP-EAA minus Ile; RP-EAA-Arg: RP-EAA minus Arg; RP-EAA-Leu: RP-EAA minus Leu; RP-EAA-His: RP-EAA minus His; RP-EAA-Phe: RP-EAA minus Phe; RP-EAA-Gly: RP-EAA minus $Glycine_{equivalent}$.

4. Discussion

The precise formulation of RP diets must ensure a balanced AA profile, with the inclusion of crystalline or synthetic AAs being crucial to achieve this [50]. Incorporating synthetic Met, Lys, and Thr is a common practice during the formulation of RP diets for laying hens, to reduce feed cost without compromising laying performance. Determining the next limiting AA for laying hens is necessary to minimise diet costs further and reduce nitrogen excretion. This study aimed to determine the order of limiting AAs after Met, Lys, and Thr in wheat–sorghum soybean meal-based RP diet for laying hens. The study showed that egg weight, egg mass, FCR, protein intake and protein excretion were compromised in hens fed the RP diet; however, the diets deficient in Val and Ile had most adverse effects compared to the SP and RP-EAA diets.

In this study, hens fed the RP diets (15.00% CP) with sufficient levels of all essential AAs had similar laying performance compared to hens offered the SP diet (17.24% CP), while hens fed the RP diets deficient in certain AAs or all essential AAs after Met, Lys, and Thr had relatively lower laying performance compared to those fed the SP diet. This suggests that RP diets could affect laying performance in absence of adequate supplemental essential AAs. These results are consistent with previous research findings showing reduced egg weight [36,51], egg mass [16,52], and egg production [53,54], and increased FCR [52,53,55] in birds fed RP diets where the levels of essential AAs or other nutrients might be deficient. In this study, a tendency of decreasing hen-day egg production in the RP diet corresponds

with those reported in other studies, and might be attributed to AA deficiency and/or an imbalance in AA ratio resulting from the reduction in body protein reserves [53,56]. In addition, AAs have functional properties, improving feed efficiency by increasing metabolic activity. In this study, hens fed the RP diet exhibited lower feed intake compared to those fed the SP diet, irrespective of AA deficiency, which might be partly due to the deficiency of total sulfur AAs in these diets [57,58].

Isoleucine appears to be the fourth, and Val the fifth, limiting AA for laying hens fed the wheat–sorghum soybean meal diet, based on egg mass in this study. Also, hens fed the Ile- and Val-deficient RP diets had lower egg weight and egg mass compared with the SP diet in this study. These findings are consistent with Harms and Ivey [59], who reported that supplementation of either Ile or Val to corn–soybean meal-based RP diets (13.8% CP) with sufficient levels of Met, Lys, Thr, Trp, and Arg increased egg mass in Hy-Line Brown laying hens from 31 to 38 WOA. Isoleucine, Val, and Leu are branched-chain AAs that act as precursors of other essential AA and proteins. Branched-chain AAs can regulate fatty acid metabolism in the liver [60], which plays a vital role in hepatic lipoprotein production, an important limiting factor for egg formation [61]. It has been presumed that the Leu requirement can be met by various sources of protein in diets [25], but it appears that supplementation of Ile and Val in RP diets is necessary to maintain egg production, egg weight, and egg mass [28,62]. Moreover, an antagonism effect between Ile, Val, and Leu may appear at higher concentrations in higher-protein diets compared to the RP diets [63,64].

The importance of Ile was highlighted in earlier studies. For instance, it has been indicated that the deficiency of Ile in the corn–soybean meal RP diet compromised egg weight and egg mass in Hy-Line Brown laying hens during 35 to 43 WOA [65]. Others have reported that adding Ile to the RP diet reduced CP requirements (2%) without affecting laying hen performance during 20 to 46 WOA [66]. In contrast, Dong, et al. [32] found that Ile supplementation from 0.1 to 0.4% to a corn–soybean meal RP diet (14% CP) with sufficient levels of Met, Lys, Thr, Trp, and Val did not affect laying performance in Lohmann Brown laying hens during 28 to 40 WOA. The lack of response to Ile supplementation in this scenario might be due to the insufficient levels of Arg and Phe in the diet, as Trp, Arg, and Phe were shown to be of equal importance (the sixth co-limiting AA) in the RP diets in the current study.

Arginine stimulates luteinising hormone secretion, which impacts ovarian follicle development and ovulation [67]. This supports the present findings, in that Arg deficiency negatively impacts egg weight and egg mass, reaffirming the importance of considering the Arg requirement in RP diet formulations. Tryptophan and Phe are aromatic AAs, reducing stress by the synthesis of neurotransmitters such as serotonin and dopamine [68,69], thus influencing hen behaviour. Adequate supplementation of these AA in RP diets is required to maintain hens' reproductive organs' growth and development [70,71]. On the other hand, the findings of this study suggested that Leu, His, and Gly may be considered non-essential AA as the egg mass of hens fed these diets was similar to those fed the RP-EAA and SP diets. The main function of His in poultry nutrition is to synthesise carnosine, which substantially increases the antioxidant capacity of muscles, having minimal impact on laying performance [72,73]. The lack of effect of Gly in the present study supports previous studies that supplementation of Gly did not affect egg production and egg mass [74,75]. However, El-Atty, et al. [76] found some positive effects of Gly supplementation on egg weight and egg mass in Mandara laying hens during 28 to 40 WOA that might be due to breed differences from the current study.

In this study, if the AA order is ranked based on FCR, Val may be considered the fourth limiting, and Trp, Ile, Arg and His may be considered as co-fifth limiting AA in wheat-sorghum soybean meal RP diets for laying hens. This ordering is different from Da Silva, et al. [55], who demonstrated Trp as the 4th, and Val and Ile as co-fifth, limiting AA in corn–soybean meal RP diets, based on the laying performance, including FCR. The discrepancy between the studies might be due to the differences in diet composition and study duration. Our research findings support the prediction of Lelis, et al. [77], who indicated that Val

may be the next limiting AA after Met, Lys, Trp, and Thr in a RP diet (14.8% CP) for laying hens, based on the requirements of digestible Val: Lys ratios in commercial corn–soybean meal layer hen diets. The necessity of Val in layer hen diets is evident in other studies showing that increasing Val level significantly decreases FCR [78–80]. Moreover, Wen, et al. [80] stated that Val requirement in laying hens fed a corn–peanut-based diet was highest to optimise egg mass, followed by egg production, and lowest to optimise FCR. This study provides new information that Trp may be less important than Val, and that Val requirements for FCR may be higher than the requirement for egg mass in wheat–sorghum soybean meal-based RP diets for laying hens. Leucine, Phe, and Gly may be considered as non-essential AAs for laying hens due to not having effects on FCR compared to the SP and RP-EAA diets, as shown by the results of this study. The differences in the effects of Ile and Val on egg mass and FCR in the present study might be due to the moderate differences between the RP and SP diets. However, determining the orders of essential AAs based on FCR may be more meaningful than the egg mass, as FCR reflects the economic efficiency to the industry [25].

The present study demonstrated that deficiency of essential AA did not affect internal and external egg quality or egg components, which is supported by earlier studies [28,32,81]. Moreover, the findings of this study revealed that RP diets with 20 g/kg lower CP level compared to SP diets can be achieved without substantial negative effects on egg quality of the hens, which is consistent with previous findings [28,53,56].

This study revealed that protein intake and excretion in hens fed the RP diets were lower than the SP diet, thus improving environmental benefits. This is in agreement with other studies presenting that lowering levels of intact protein in the diet substantially reduced N-excretion [16,51,82]. The lower protein intake in hens fed the RP diets in this study might be due to the similar feed intake but lower CP levels in the RP diets compared to the SP diet. Additionally, the effect of Arg, Leu, and Gly deficiency in the RP diets was more pronounced in lowering protein excretion, which might be attributed to the lower protein intake but similar protein retainment in hens offered the respective diets compared to those offered the SP diet in this study. However, AA deficiency may not affect apparent protein and energy digestibility, as shown by the results of this study and others; Dao, et al. [51]. No significant difference in serum uric acid level was observed between the dietary treatments in this study. Uric acid is the metabolic end product of protein metabolism, and the relative values of serum uric acid levels and dietary CP are thought to be inversely correlated, as documented in previous broiler studies [5,83,84]. In the current study, similar serum uric acid levels were observed in hens fed the SP and RP diets, corresponding to the findings of other laying hen studies [51,82]. As the RP diets in the current and aforementioned studies had adequate levels of Met, Lys, and Thr supplementation, the findings may indicate the critical roles of Met, Lys, and Thr in protein utilisation in laying hens [84,85].

Tibia morphology, breaking strength, and ash/mineral content are indicators of bone quality. In the current study, neither dietary protein level nor deficiency of essential AA exerted any effect on tibial characteristics, which supports earlier studies [82,86,87]. Therefore, it can be postulated that lowering CP levels might not affect bone quality in laying hens.

Proteins are one of the important gut-active nutrients that can be modulated by gut microbiota in the intestinal tract [88]. Reducing dietary CP levels is thought to increase gut microbial utilisation of AAs, as the presence of undigested protein will be decreased in the hindgut [83,89]. Moreover, numerous studies in laying hens and broiler chickens have shown that when birds are fed RP diets supplemented with crystalline AAs, the populations of beneficial gut bacteria increase while the numbers of pathogenic bacteria decrease [85,90–92]. The present study partially supported these previous studies, in that hens fed the RP diet had relatively higher *Bacteroides* spp. counts compared to those fed the SP diet. Moreover, the RP diets deficient in Val, Ile, Phe, and Gly showed a trend

of increasing *Bacteroides* spp. count, which may indicate that these AAs are required to maintain gut integrity in laying hens.

5. Conclusions

Based on the RP diets used in this study, it could be concluded that Val and Ile are the most important limiting AAs, after Lys, Met, and Thr, whilst Leu and Gly are the least important in hens fed wheat–sorghum soybean meal-based RP diets. Thus, along with Lys, Met, and Thr, Val and Ile should be considered first, while Leu and Gly should be considered last when formulating RP diets based on wheat, sorghum, and soybean meal for laying hens. Additionally, the current findings illustrated that reducing dietary CP levels by two percentage point with supplementation of crystalline AAs is effective to maintain laying hens performance and egg quality. The findings from this study may facilitate precise feed formulations for laying hens, and increase adoption of RP diets, reducing industry reliance on soybean meal. The decrease in prices of crystalline AAs is crucial to extend the adoption of RP diets in the future. In this respect, economic analysis indicating the points at which the next limiting AA may be included into diets may be beneficial for the industry.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app132312934/s1, Table S1: Hen weight and mortality under offered dietary treatments at different age points of the study; Table S2. Internal egg quality of hens offered the dietary treatments at weeks 29 and 39; Table S3. External egg quality of hens offered the dietary treatments at weeks 29 and 39; and Table S4. Egg proportion of hens offered the dietary treatments at weeks 29 and 39.

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