



Optimising reduced-protein diets for an efficient and sustainable layer production

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by A.F. Moss and T.H. Dao

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Researcher/Author Contact Details

Name: Dr Amy F. Moss/Dr Thi Hiep Dao
Address: Faculty of Science, Agriculture, Business and Law
The University of New England
Homestead Building, Armidale, NSW 2351
Phone: 02 6773 5217
Email: amoss22@une.edu.au (Dr Amy F. Moss)
tdao2@une.edu.au (Dr Thi Hiep Dao)

In submitting this report, the researcher has agreed to Australian Eggs Limited publishing this material in its edited form.

Australian Eggs Limited Contact Details:

Australian Eggs Limited
A.B.N: 66 102 859 585
Suite 7.01, Level 7, 1 Pacific Highway
North Sydney NSW 2060

Phone: 02 9409 6999
Fax: 02 9954 3133
Email: research@australianeggs.org.au
Website: www.australianeggs.org.au

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Foreword

This project was conducted to optimise reduced protein diets for laying hens to enhance economic efficiency and environmental sustainability of layer hen production. Two studies were undertaken to achieve this objective. The first study investigated the optimal inclusion rates of exogenous enzymes, specifically phytase and a carbohydrase enzyme containing xylanase and β -glucanase, in reduced protein diets to improve nutrient digestibility in laying hens during the post-peak laying period from 35 to 57 weeks of age. The second study examined the effects of dietary supplementation with arginine, guanidinoacetic acid and citrulline on the productive performance of laying hens during the late laying phase from 60 to 75 weeks of age.

This project was funded from industry revenue, which is matched by funds provided by the Australian Government.

This report is an addition to Australian Eggs Limited's range of peer reviewed research publications and an output of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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About the Authors

Dr Amy F. Moss is the Senior Poultry Scientist at Poultry Hub Australia and a DECRA postdoctoral fellow at the University of New England (UNE), with a passion for improving the environmental and economic sustainability of the poultry industry and helping to solve industry challenges. Dr Moss leads laying hen nutrition projects that aim to deliver industry-focused outcomes, and has a strong track record with over 76 published papers since 2016. Originally from the south coast of New South Wales, her early interest in breeding quail led her to pursue a career in poultry science, culminating in a PhD in poultry nutrition at the University of Sydney. Following her PhD, Dr Moss relocated from Sydney to Armidale to commence postdoctoral research with Professor Mingan Choct, Professor Tamsyn Crowley and the UNE poultry group. Since then, she has successfully led and completed numerous research projects, earning a prestigious Discovery Early Career Researcher Award from the Australian Research Council and a 2024 NSW Young Tall Poppy Award in recognition of her contributions as an early career researcher.

Dr Thi Hiep Dao is a postdoctoral research fellow at the University of New England (UNE), specialising in precision feeding, poultry nutrition, food waste recycling, and the prevention of animal diseases. She earned her PhD from UNE in 2021 and was awarded the UNE Chancellor's Doctoral Research Medal for research excellence. Dr Dao has authored over 40 peer-reviewed publications, including those in top-ranking journals such as *Nature Scientific Reports*, and her internationally recognised research focuses on improving the efficiency and sustainability of poultry production. Her contributions have earned her a place as a finalist in the 2023 NSW Sustainability Awards, and her work has been featured as an Editor's Choice article in *Poultry Science*, the leading journal in the field. Through her research, Dr Dao continues to advance innovation in poultry nutrition and sustainability, delivering significant benefits to both industry and academia.

Mr Aamir Nawab is a PhD candidate at the University of New England who worked on this project as part of their PhD under the guidance of Drs Moss and Dao.

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Abbreviations

AA	Amino acid
AMEn	Apparent metabolisable energy
Arg	Arginine
AvP:Ca	Available phosphorus to calcium ratio
Cit	Citrulline
CP	Crude protein
DM	Dry matter
FCR	Feed conversion ratio
FI	Feed intake
FSH	Follicle-stimulating hormone
FTU	Phytase unit
GAA	Guanidinoacetic acid
GE	Gross energy
GnRH	Gonadotropin-releasing hormone
His	Histidine
Ile	Isoleucine
Leu	Leucine
LH	Luteinising hormone
Lys	Lysine
Met	Methionine
NO	Nitric oxide
NSP	Non-starch polysaccharides
PhyG	Enzyme Axtra® PHY Gold
RP	Reduced protein diet
RP-ARG	Reduced protein diet supplemented with arginine
RP-CIT	Reduced protein diet supplemented with citrulline
RP-GAA	Reduced protein diet supplemented with guanidinoacetic acid
SP	Standard protein diet
Thr	Threonine
Trp	Tryptophan
Val	Valine
XB	Enzyme Axtra® XB 201 TPT
XOS	Xylooligosaccharide

Executive Summary

Two studies were conducted to optimise reduced protein (RP) diets for laying hens to enhance economic efficiency and environmental sustainability of laying hen production.

The first study evaluated the effects of enzyme inclusion rates in RP diets to improve laying hen nutrient digestibility. A total of eight dietary treatments were each allocated 13 replicate cages containing two hens per cage ($n = 208$) from 35 to 57 weeks of age. Experimental diets comprised two protein levels (standard protein (SP) at 16.5% crude protein and RP at 14.5% crude protein), two phytase levels (600 and 1,200 FTU/kg Axtra® PHY Gold (PhyG), Danisco Animal Nutrition, IFF, UK) and two levels of a carbohydrase enzyme containing xylanase and β -glucanase (XB, Axtra® XB 201 TPT, Danisco Animal Nutrition, IFF, UK; 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase and 150 g/tonne provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase) examined via a $2 \times 2 \times 2$ factorial design. Egg weight and hen-day egg production were measured daily, and feed consumption and the feed conversion ratio were measured weekly. Egg quality was measured at 47 and 57 weeks of age. Excreta moisture content, apparent dry matter, energy and protein digestibility were determined by the total excreta collection method at 57 weeks of age. From 35 to 57 weeks of age, no significant interactions were observed for laying performance parameters. However, RP diets significantly decreased egg weight across all periods, 35–47 weeks ($P = 0.049$), 48–57 weeks ($P = 0.045$), and overall 35–57 weeks ($P = 0.044$); reduced egg mass from 35–47 weeks ($P = 0.035$) and tended to reduce egg mass from 35–57 weeks ($P = 0.069$). High XB inclusion (150 g/tonne) lowered hen day egg production during 48–57 weeks ($P = 0.015$). At 47 weeks, RP diets reduced yolk weight ($P = 0.021$) and increased egg shape index ($P = 0.048$), with no effect on shell strength or other external quality traits. A PhyG $\times$ XB interaction was detected for shell breaking strength ($P = 0.046$), where XB improved shell breaking strength at 600 FTU/kg PhyG but reduced it at 1200 FTU/kg. At 57 weeks, RP diets decreased albumen weight ($P = 0.016$), increased yolk proportion ($P = 0.005$), and reduced albumen proportion ($P = 0.006$). XB inclusion at 150 g/tonne reduced shell breaking strength ($P = 0.036$), and a protein $\times$ XB interaction was observed for yolk colour ($P = 0.038$), with XB reducing yolk colour score only in SP diets. RP diets also reduced excreta moisture ($P = 0.015$) and improved dry matter digestibility ($P = 0.020$). Notably, feeding RP diets increased apparent energy digestibility ($P = 0.003$), decreased protein excretion ($P < 0.001$) and increased apparent protein digestibility ($P = 0.013$).

The second study evaluated the effects of arginine (Arg), guanidinoacetic acid (GAA) and citrulline (Cit) supplementation in RP diets on the production performance, egg quality, nutrient digestibility, serum uric acid level and bone quality of aged laying hens. There were eight dietary treatments, including an SP diet, an RP diet, and RP diets supplemented with two levels (0.06 or 0.12%) of either Arg, Cit or GAA, resulting in the remaining six dietary treatments. Each treatment had 13 replicate cages of two hens per cage ($n = 208$). A significant Arg source $\times$ level interaction was observed for feed intake during 60–67 weeks ($P = 0.007$) and 60–75 weeks ($P = 0.014$), where increasing Cit supplementation increased feed intake, while Arg and GAA did not. A similar trend was noted during 68–75 weeks ($P = 0.055$). Additionally, Cit supplementation significantly increased egg weight ($P = 0.049$) and tended to increase egg mass ($P = 0.063$) compared to GAA from 60–67 weeks. From 68–75 weeks, Cit supplementation improved egg mass ($P = 0.014$) and feed intake ($P = 0.009$) compared to Arg and GAA. Over the entire 60–75 week period, Cit increased egg weight ($P = 0.048$) and egg mass ($P = 0.019$) compared to GAA. One-way ANOVA results showed that hens fed RP diet with Cit supplementation at 0.12% had significantly higher feed intake ($P < 0.001$) and tended to have higher egg mass ($P = 0.058$) than those fed SP and RP diets from 60–67 weeks. These improvements in egg mass and feed intake were consistent from 68–75 and 60–75 weeks ($P < 0.05$). At 75 weeks, Cit supplementation tended to increase yolk diameter ($P = 0.086$), and both Cit and Arg supplementation increased yolk weight

compared to GAA ($P = 0.018$). No differences were observed in egg quality between SP and RP diets. Reducing Arg supplemental level tended to reduce ileal energy digestibility ($P = 0.083$), and Arg supplementation to RP diet at 0.06% significantly increased energy digestibility compared to the SP and RP diets ($P = 0.037$). No treatment effects were observed on serum uric acid, although hens fed RP diets supplemented with 0.06% Cit tended to have lower levels than those on the SP diet ($P = 0.091$). Significant Arg source $\times$ level interactions were found for tibia ($P = 0.014$) and femur ($P = 0.006$) breaking strength. Increasing Cit supplemental level enhanced bone breaking strength, whereas Arg and GAA reduced it.

Thus, it could be concluded that reducing dietary crude protein from 16.5% to 14.5% decreased egg weight and tended to reduce egg mass without compromising feed efficiency from 35 to 57 weeks of age. Supplementing with 600 FTU/kg PhyG and 100 g/tonne XB was sufficient, while higher XB levels impaired egg production. The further reduction in dietary crude protein level from 15.8% to 13.8%, combined with Cit supplementation at 0.12%, was effective in enhancing egg mass, egg weight, yolk weight, and bone breaking strength in aged laying hens from 60 to 75 weeks of age. Environmentally, feeding RP diets reduced nitrogen excretion, excreta moisture and improved nutrient digestibility, supporting their use for both productive and sustainable layer hen production. Our work successfully demonstrated that Cit supplementation at 0.12% can enhance the economic feasibility of RP diets, further enabling their industry adoption and thus providing a clearer pathway for realisation of the demonstrated economic and environmental benefits.

Overall Conclusions

In two studies, reducing dietary crude protein by two percentage points in laying hen diets showed potential. In the first study, dietary protein reduction from 16.5% to 14.5% slightly reduced egg weight and egg mass but had no effect on feed efficiency or hen weight in laying hens aged 35 to 57 weeks. In contrast, the second study showed that reducing dietary protein level from 15.8% to 13.8% maintained performance, egg quality, and nutrient digestibility in hens aged 60 to 75 weeks. Supplementing reduced protein diets with standard doses of phytase (600 FTU/kg Axtra® PHY Gold, Danisco Animal Nutrition, IFF, UK) and carbohydrase enzyme containing xylanase and β -glucanase (100 g/tonne Axtra® XB 201 TPT, Danisco Animal Nutrition, IFF, UK) was adequate, while higher Axtra XB levels reduced egg production from 35 to 57 weeks of age. However, citrulline supplementation to reduced protein diet at 0.12% improved egg and yolk weights, egg mass, and bone strength from 60 to 75 weeks of age, with economic benefits justifying its use despite higher cost. Environmental advantages of feeding reduced protein diets, including reduced nitrogen excretion, lower excreta moisture, and improved nutrient digestibility, were demonstrated. Arginine supplementation to reduced protein diets at 0.06% also increased ileal energy digestibility at 75 weeks of age. Economically, a reduced protein diet supplemented with 0.06% Cit reduced feed cost per kg of eggs by AU\$ 0.076 (6.8%) compared to the standard protein diet from 60 to 75 weeks of age. With rising soybean meal prices and stable or declining prices of crystalline amino acids, reduced protein diets supplemented with citrulline offer a cost-effective and environmentally sustainable alternative to standard protein formulations. Our work highlights these benefits and offers solutions to increase the economic gain even further.

1 Introduction

1.1 General introduction

Reducing the dietary level of crude protein (CP) within poultry diets has been one focus of recent poultry nutrition research for the possible economic and environmental benefits (Greenhalgh et al. 2020); however, the extent of economic benefit is often poorly defined. Reducing the CP component of hen diets has had prior attention due to concern for the impact of nitrogen deposition into the environment (Summers 1993); in fact, reduced protein (RP) diets have been explored for nearly a century (Kidd et al. 2021). Despite this ongoing research focus, there are still challenges faced and many opportunities for gains within RP diets. Additionally, the increasing cost of poultry feed year on year – principally driven by the high cost of soybean meal – has generated renewed interest.

The modern laying hen industry aims to produce eggs at a high production rate, with consistent quality, while maintaining a minimum production cost. As feed represents the majority of production costs (Wen et al. 2018), RP diets present an opportunity to generate savings in egg production. Reduction in dietary CP level alongside supplementation of crystalline amino acids (AA) allows a more precise AA nutrition of the hens as the formulation is brought closer to the ideal AA balance, and may thereby allow better protein utilisation while still maintaining performance (Macelline et al. 2021). As birds are unable to endogenously synthesise essential AA (Jahan et al. 2023), it is crucial to meet the requirement of essential AA during the reduction of dietary CP levels by adding crystalline AA. The limiting order of AA is important in this respect, as AA is relatively expensive and thus priority must be given to those most crucial to performance. Previously, our research group demonstrated that after lysine (Lys), methionine (Met), and threonine (Thr), valine (Val) may be considered the fourth limiting AA, followed by tryptophan (Trp), isoleucine (Ile), arginine (Arg), and histidine (His) as the co-fifth limiting AA for feed conversion ratio (FCR) in hens fed wheat, sorghum and soybean meal-based RP diets (Jahan et al. 2023). However, optimising the inclusion of crystalline AA is only one part of the equation to maximise the benefits of RP diets for laying hens.

Phytate and non-starch polysaccharides (NSP) are antinutrient factors in cereal grains and soybean meals (Purohit et al. 2023). The increased inclusion of wheat in RP diets at the expense of soybean meal would alter enzyme substrates and affect enzyme efficacy (Moss et al. 2020). There is no doubt that the phytase enzyme enhances the digestion of AA in poultry, but most studies have focused on broiler chickens (Cowieson et al. 2017; Selle et al. 2006; Ravindran et al. 1999). In reference to the AA identified as limiting in RP diets for laying hens, phytase has been shown in a systematic review of 24 studies on broiler chickens (Cowieson et al. 2017) to improve the digestibility of Lys (3.4%), Met (1.3%), Thr (6%), Val (4.6%), Trp (3.4%), Ile (4.6%), Arg (2.6%), and His (3.9%). Thus, the improved digestibility in these AA may be of particular advantage to hens offered RP diets. Along with phytase, xylanase enzymes are commonly included in wheat-based poultry diets (Bedford & Cowieson 2020) to break down NSP components for beneficial effects on bird performance and health, including the intestinal microbiota (Morgan, 2023). Few studies have explored the effects of xylanase in hens, and the reports are conflicting (Nguyen et al. 2021; Lázaro et al. 2003; Safaa et al. 2009), possibly due to variation in the amount of substrate presented in diets. Therefore, elucidating the effect of xylanase inclusion in wheat-based diets for laying hens requires further study. As such, exploration of xylanase inclusion in RP diets for hens is crucial, as wheat inclusion level is often higher in RP diets compared to standard protein (SP) diets when wheat is a base grain. Thus, enzyme inclusion in RP diets for laying hens requires consideration, particularly for phytase and xylanase, as these are the most common enzymes utilised by the poultry industry and their substrate concentration would differ in SP versus RP diets.

Chickens are unable to endogenously synthesise Arg due to an incomplete urea cycle and the absence

of key enzymes required for Arg biosynthesis (Birmani et al. 2019). Consequently, chickens rely entirely on dietary sources to meet their Arg requirements. In chickens, dietary Arg supplementation has been shown to lower lipid peroxidation and superoxide emission, while improving antioxidant status and overall performance (Wang et al. 2015; Dao et al. 2022). Crystalline Arg, guanidinoacetic acid (GAA) and citrulline (Cit) are commercially available sources of Arg used in poultry nutrition (Dao et al. 2021a). According to Heuser et al. (2025), crystalline Arg and its metabolites have greater digestibility and bioavailability than protein-bound Arg. In birds, GAA is endogenously synthesised in the liver and kidneys from glycine and Arg, serving as a precursor for creatine synthesis (Portocarero & Braun 2021). Therefore, dietary supplementation with GAA can reduce the metabolic demand for Arg in creatine synthesis, effectively sparing Arg for protein synthesis and other physiological functions. In addition to its functional role, GAA presents economic and practical advantages as it is less expensive and more chemically stable than both Arg and creatine, making it a promising feed additive. Guanidinoacetic acid has also been associated with improved energy homeostasis, growth rate and feed efficiency in poultry (Dilger et al. 2013). Furthermore, previous studies have reported that dietary GAA supplementation enhanced tibia mineral density and breaking strength in broilers, further supporting its potential benefits in poultry diets (Khajali et al. 2020). Similarly, Cit, a metabolite of Arg, can spare Arg in poultry, despite not being directly incorporated into protein synthesis. Previous studies have shown that dietary Cit supplementation in laying hens with Cit could enhance Arg utilisation, promote nitric oxide (NO) synthesis, boost antioxidant defence and increase egg shape index. While the effects of various Arg sources including crystalline Arg, GAA and Cit have been well documented in broilers and broiler breeders, limited research has focused on their application in aged laying hens maintained on RP diets.

Therefore, this research project was conducted to determine two aims. Firstly, the optimal inclusion rates of phytase and a carbohydrase enzyme containing xylanase and β -glucanase in the RP diets for laying hens to improve nutrient digestibility during the post-peak laying period. Secondly, the effects of dietary supplementation with Arg, GAA, and Cit in the RP diets on laying hen performance, egg quality, nutrient digestibility, serum uric acid level, and bone quality during the late laying phase.

1.2 Objectives

This project was conducted to optimise reduced protein diets for laying hens, to enhance both economic efficiency and environmental sustainability. Two studies were undertaken to achieve this objective. The first study investigated the optimal inclusion rates of exogenous enzymes, specifically phytase and a carbohydrase enzyme containing xylanase and β -glucanase, in reduced protein diets to improve nutrient digestibility in laying hens during the post-peak laying period from 35 to 57 weeks of age. The second study examined the effects of dietary supplementation with arginine, guanidinoacetic acid, and citrulline in the reduced protein diets on the productive performance, egg quality, nutrient digestibility, serum uric acid level, and bone quality of laying hens during the late laying phase from 60 to 75 weeks of age.

2 Materials and methods

2.1 Study 1

2.1.1 Birds and animal husbandry

This study was performed at the Laureldale Research Station at the University of New England, Armidale, New South Wales, Australia. Animal ethics approval was granted by the University of New England Animal Ethics Committee (ARA23-045). The study was completed according to the Australian Code of Practice, and the requirements of bird care and use for scientific purposes were met (NHMRC 2013). In total, 208 Hy-Line Brown pullets at 15 weeks of age were obtained from a local commercial layer farm in Tamworth, New South Wales, Australia. All birds were from the same flock and were reared as per Hy-Line Brown management guideline (Hy-Line International 2016). Birds were housed in barn-laid floor pens with 21 birds/pen from 15 to 23 weeks of age. Following their relocation, the birds were housed in a layer cage facility within a curtain-sided shed, with two birds per cage (cage dimensions: 45 cm height × 50 cm depth × 30 cm width). They participated in a separate 5-week study from 24 to 28 weeks of age (Moss et al. 2024), which aimed to identify the optimal enrichment program for caged laying hens used in research and teaching environments. After this period, the birds remained in the same facility until the commencement of the experimental phase of the current study at 35 weeks of age. From arrival until the start of the trial, all birds were fed a standard commercial diet (Barastoc—Premium Top Layer Mash; 16.5% CP, 2.5% crude fat, 6% crude fibre, 8.0 mg/kg copper, 0.3 mg/kg selenium, 3.6% calcium, and 0.3% salt; Melbourne, Victoria, Australia). At 35 weeks of age, hens were individually weighed and randomly allocated to 104 cages (two birds per cage), ensuring that the mean body weights were similar across dietary treatments. All cages were equipped with three forms of enrichment: a perch, a scratch pad, and a hanging CD, in accordance with the methods described by Moss et al. (2024). Hens had access to feed and water *ad libitum* throughout the study duration by one feed trough and two nipple drinkers per cage. White LED (IP65 Dimmable LED Bulb, B-E27:10W, 5K) bulbs were used for lighting, and the lighting schedule was maintained as 16 hours of light and 8 hours of dark using an automatic timer. Inside the 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). The average hen house temperature and relative humidity by week are shown in Figure 1. Hens were rehomed upon completion of the study.

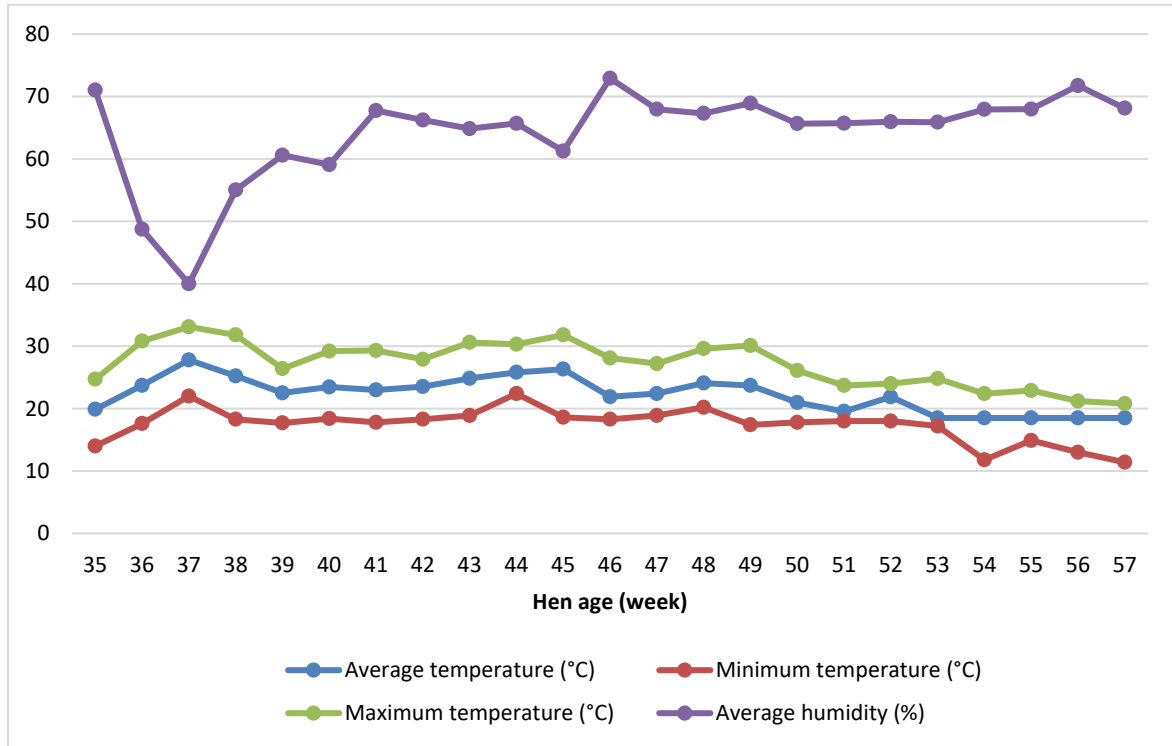


Figure 1 Temperature and relative humidity of the layer hen house in Study 1 from 35 to 57 weeks of age

2.1.2 Experimental design and diets

The enzymes used in this study include phytase (PhyG; Axtra® PHY Gold, Danisco Animal Nutrition, IFF, UK) and a carbohydrase enzyme containing xylanase and β -glucanase (XB; Axtra® XB 201 TPT, Danisco Animal Nutrition, IFF, UK). Two dietary protein levels (SP at 16.5% CP and RP at 14.5% CP), two PhyG levels (60 g/tonne and 120 g/tonne to provide 600 and 1,200 FTU/kg, respectively) and two XB levels (XB supplemented at 100 g/tonne to provide 1,220 U/kg xylanase and 152 U/kg β -glucanase, and XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg β -glucanase) were examined in a $2 \times 2 \times 2$ factorial arrangement. Thus, 8 dietary treatments were used in this study, with 13 replicate cages of two hens per cage per treatment. PhyG and XB levels were selected based on the published recommended levels of these enzymes (FEEDAP 2022a) and advice from the enzyme manufacturer (Danisco Animal Nutrition, IFF). This experiment was conducted in a layer cage facility over 23 weeks, from 35 to 57 weeks of age, to capture the period after peak lay once feed intake and egg mass had stabilised.

2.1.3 Experimental diets

Diets were fed as mash, and based on wheat, sorghum, canola meal, barley and soybean meal. The CP level in the SP diet was per Hy-Line Brown nutritional recommendations (Hy-Line International 2023), while the RP diet had a 20 to 30 g/kg lower CP level compared to the SP diet. The levels of essential AA were selected based on Hy-Line Brown nutritional recommendations (Hy-Line International 2023). Feed was provided in mash form. The nutritional profiles of the main feed ingredients, encompassing parameters such as dry matter (DM), apparent metabolisable energy (AMEn), CP, crude fat, crude fibre and total and digestible AA, as well as mineral and ash contents, were analysed using near-infrared reflectance spectroscopy (Foss NIR 6500, Hillerød, Denmark), standardised with Adisseo AMINONIR Advanced calibration. These results were then used for diet formulation. The diet composition and calculated nutrient values of the SP and basal RP diets are shown in Table 1. The enzymes PhyG and XB

were added on top of the RP diet at the expense of wheat to generate the remaining experimental diets; matrix values were not incorporated. The analysed nutrient values of the diets including DM, gross energy (GE), CP, ash, calcium, total phosphorus and AA contents were measured using standard methods (AOAC 2019) and presented in Table 2. Generally, analysed nutrient contents were close to the calculated values.

Table 1 Diet composition and calculated nutrient values of basal standard and reduced protein diets in Study 1 (as-fed basis)

Ingredients, g/kg	Standard protein diet	Reduced protein diet
Wheat	279.5	294.0
Sorghum	275	295
Soybean meal	162	88
Barley	100	125
Limestone grit	67	67
Canola meal	54	66
Fine limestone	30	30
Canola oil	22	17
Sodium bicarbonate	2.80	3.70
D,L-methionine	1.95	2.50
L-lysine HCl	1.05	3.10
L-threonine	0.10	1.00
Salt	1.70	1.00
Monocalcium phosphate	1.00	2.00
Choline Cl 60%	0.65	0.65
Xylanase ¹	0.10	0.10
Phytase ²	0.06	0.06
Layer vitamin-mineral premix ³	1.00	1.00
Pigment Jabiru red	0.04	0.04
Pigment Jabiru yellow	0.03	0.03
L-valine	-	0.95
L-isoleucine	-	1.05
L-arginine	-	0.70
Total	1000	1000
<i>Calculated nutrient (% , otherwise as indicated)</i>		
AME ⁴ , kcal/kg	2,725	2,725
Crude protein	16.5	14.5
Crude fat	4.10	3.70
Crude fibre	3.00	3.10
Ash	12.8	12.7
Total lysine	0.857	0.840
Total methionine	0.446	0.470
Total methionine + cysteine	0.738	0.734
Total threonine	0.623	0.610
Total isoleucine	0.656	0.642
Total leucine	1.342	1.150
Total tryptophan	0.225	0.190
Total arginine	0.973	0.840
Total histidine	0.393	0.330
Total valine	0.769	0.747
Digestible ⁵ lysine	0.735	0.735
Digestible methionine	0.421	0.450
Digestible methionine + cysteine	0.662	0.661

Digestible threonine	0.522	0.520
Digestible isoleucine	0.581	0.573
Digestible leucine	1.197	1.091
Digestible tryptophan	0.198	0.165
Digestible arginine	0.890	0.772
Digestible histidine	0.345	0.306
Digestible valine	0.660	0.647
Calcium	4.000	4.000
Available phosphorus	0.320	0.320
Total phosphorus	0.369	0.359
Phytate	0.891	0.842
Sodium	0.180	0.180
Chloride	0.181	0.181
Potassium	0.633	0.569
Choline, mg/kg	1,673	1,590
Linoleic acid	1.400	1.300
Dietary electrolyte balance ⁶ , mEq/kg	189	173

¹ Axta® XB 201 TPT, Danisco Animal Nutrition, IFF, UK.

² Axta® PHY Gold 10,000 FTU/g, Danisco Animal Nutrition, IFF, UK.

³ Vitamin-mineral premix provided the following per kilogram diet: vitamin A, 10000 IU; vitamin D, 3000 IU; vitamin E, 20 mg; vitamin K, 3 mg; nicotinic acid (niacin), 35 mg; pantothenic acid, 12 mg; folic acid, 1 mg; riboflavin (B2), 6 mg; cyanocobalamin (B12), 0.02 mg; biotin, 0.1 mg; pyridoxine (B6), 5 mg; thiamine (B1), 2 mg; copper, 8 mg as copper sulphate pentahydrate; cobalt, 0.2 mg as cobalt sulphate 21%; molybdenum, 0.5 mg as sodium molybdate; iodine, 1 mg as potassium iodide 68%; selenium, 0.3 mg as selenium 2%; iron, 60 mg as iron sulphate 30%; zinc, 60 mg as zinc sulphate 35%; manganese, 90 mg as manganous oxide 60%; antioxidant, 20 mg.

⁴ AMEn: N corrected apparent metabolisable energy.

⁵ Digestible amino acid coefficients of conventional feed ingredients were determined by Near-Infra Red spectroscopy (Foss NIR 6500, Denmark) standardised with Adisseo calibration.

⁶ Dietary electrolyte balance was calculated as $10,000 \times (\text{Na}^+ + \text{K}^+ - \text{Cl}^-)$.

Table 2 Analysed nutrient values of the experimental diets in Study 1 (as-fed basis)

Factors	Experimental diets							
	Standard	Standard	Standard	Standard	Reduced	Reduced	Reduced	Reduced
Protein level								
PhyG level (FTU/kg)	600	600	1200	1200	600	600	1200	1200
XB level (g/tonne)	100	150	100	150	100	150	100	150
<i>Nutrient composition (% , otherwise as indicated)</i>								
Dry matter	91.4	91.3	91.0	91.2	91.1	91.1	90.9	90.9
Gross energy (Kcal/kg)	3666	3656	3644	3707	3642	3664	3622	3658
Crude protein	16.0	16.1	16.3	16.5	14.2	14.1	14.5	14.6
Ash	13.2	13.2	12.8	12.1	12.3	12.1	12.6	12.2
Calcium	3.76	3.87	3.52	3.36	3.57	3.34	3.70	3.52
Total phosphorus	0.34	0.33	0.33	0.33	0.34	0.33	0.35	0.34
Aspartic acid	1.341	1.338	1.327	1.471	1.041	1.060	1.063	1.079
Serine	0.798	0.790	0.798	0.853	0.674	0.672	0.693	0.663
Glutamic Acid	3.260	3.267	3.223	3.551	2.918	2.989	2.984	3.037
Glycine	0.572	0.575	0.598	0.595	0.519	0.517	0.561	0.504
Histidine	0.380	0.376	0.391	0.402	0.335	0.343	0.365	0.335
Arginine	0.867	0.848	0.867	0.929	0.778	0.784	0.842	0.788
Threonine	0.563	0.565	0.575	0.606	0.586	0.573	0.629	0.584
Alanine	0.725	0.709	0.715	0.754	0.646	0.650	0.684	0.667
Proline	1.002	1.028	1.031	1.030	0.967	0.974	1.026	0.994
Tyrosine	0.424	0.447	0.446	0.467	0.399	0.400	0.442	0.417
Valine	0.509	0.513	0.546	0.605	0.580	0.589	0.662	0.629
Methionine	0.406	0.425	0.445	0.399	0.484	0.448	0.479	0.472
Lysine	0.822	0.819	0.820	0.899	0.834	0.832	0.856	0.874
Isoleucine	0.473	0.484	0.510	0.562	0.571	0.579	0.642	0.613
Leucine	1.173	1.175	1.205	1.256	1.061	1.076	1.169	1.119
Phenylalanine	0.726	0.754	0.762	0.786	0.679	0.681	0.734	0.697
Phytase activity (FTU/kg)	1009	1296	1909	1745	987	1102	1920	1960
Xylanase activity (U/kg)	3987	5811	4034	4902	4785	5748	3786	5181
B-Glucanase activity (U/kg)	241	285	251	291	231	288	240	253

Values of all the amino acids presented were total amino acids (measured on an as-is basis).

2.1.4 Data collection

Data were collected over 23 weeks. Hens were weighed at 35, 47 and 57 weeks of age. Average hen starting weights did not differ significantly between the dietary treatments ($P > 0.05$). Eggs were collected and weighed daily. Feed intake was recorded weekly. Egg mass and feed conversion ratio (FCR, kg of feed/kg of eggs produced) were then calculated from egg production, egg weight, and feed consumption. Egg quality was measured at 47 and 57 weeks of age. Excreta moisture content, apparent DM, energy and protein digestibility of the dietary treatments were determined at 57 weeks of age. Specifically, hens with body weights close to the average body weight of each treatment were selected (6 hens/treatment) to collect excreta samples by the total excreta collection method, along with feed consumption. Individual trays wrapped with aluminum foil were placed directly under the cages. Loose feathers and feed residues were removed from the trays, and then the collected excreta were transferred into polypropylene transparent zipper poly bags. This was conducted once daily between 9 am to 1 pm, over 3 consecutive days (72 hours). 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 until further analysis.

2.1.5 Egg quality

A total of 100 and 104 eggs (approximately 13 eggs/treatment) were collected shortly after laying at 47 and 57 weeks of age, respectively. Any significantly deformed eggs were excluded from the analysis. Egg length and width were measured using a Digital Vernier calliper (Kincrome®, 0–150 mm scale, Scoresby, Victoria, Australia) to calculate the egg shape index (width/length). Eggshell reflectivity was assessed using a shell reflectivity meter (Technical Services and Supplies, Dunnington, York, UK). Eggshell breaking strength and internal egg quality characteristics were assessed using a digital egg tester (DET6500®, Nabel Co., Ltd., Kyoto, Japan). The egg yolk was separated from egg albumin using Whatman filter papers (CAT No. 1541–090, Whatman®, Buckinghamshire HP7 9NA, Amersham, UK) and weighed. The eggshells were washed, air-dried for at least 72 hours, and weighed using a precision analytical balance (Adventurer™, Model AX423, Ohaus, NJ, USA), and then the eggshell thickness (including membrane) was measured using a custom-built gauge (Mitutoyo Dial Comparator Gauge, Model 2109-10, Kawasaki, Japan). 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 the weights of these egg components by the total egg weight. All measures were taken by trained personnel within 3 hours of egg collection.

2.1.6 Wet chemistry analyses

Approximately 5 g of fresh excreta sample taken at 57 weeks of age was weighed into a pre-weighed 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 hours (to constant weight), to remove the moisture for determination of DM. The remaining excreta sample was preserved at -20°C for further analysis. The excreta samples were then freeze-dried using a freeze dryer (Christ Alpha 1–4 LD plus, Osterode am Harz, Germany). Freeze-dried excreta samples and diet 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. The CP concentrations in both excreta and feed samples were measured as described by Dumas (1831) following the Dumas combustion method using a nitrogen analyser (LECO Corporation, St. Joseph, MI, USA), where EDTA was used as the calibration standard. Whereas the GE 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. Freeze-dried excreta and feed samples were also oven-dried at 105°C for approximately 24 hours (to constant weight) for the determination of DM content following a similar method as described for the fresh excreta samples. Apparent energy and protein digestibility were calculated as per DM basis using the following equations described by Kong and Adeola (2014).

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

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

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

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

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

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

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

2.1.7 Statistical analysis

Data were organised and validated in a Microsoft Excel spreadsheet and analysed using IBM SPSS statistical software (Version: 28.0.1.0, IBM Corp., Armonk, NY, USA). The data were analysed as a 2 x 2 x 2 factorial array of dietary treatments via univariate, general linear models procedure using the SPSS®IBM Statistics 20 software program (IBM Corporation, Somers, NY, USA). Prior to statistical analyses, data were tested for normal distribution using the Kolmogorov–Smirnov test and approximately equal variances were observed between the dietary treatments. The differences were considered significant at the 5% level of probability. Tukey’s post-hoc test was subsequently used to identify pairwise differences between the treatment means.

2.2 Study 2

2.2.1 Birds and animal husbandry

This study was implemented at the Laureldale Research Station at the University of New England, Armidale, New South Wales, Australia. Animal ethics approval was granted by the University of New England Animal Ethics Committee (ARA23-074). The study was completed according to the Australian Code of Practice, and the requirements of bird care and use for scientific purposes were met (NHMRC 2013). In total, 208 Hy-Line Brown laying hens were obtained from a local commercial layer farm in Tamworth, New South Wales, Australia. All birds were from the same flock and were reared in cages as per the Hy-Line Brown management guideline (Hy-Line International, 2016). Upon arrival at 58 weeks of age, the birds were randomly distributed into 104 cages (two birds per cage, cage dimensions: 45 cm height × 50 cm depth × 30 cm width) in a curtain-sided shed. Upon arrival, birds were fed a common commercial diet for two weeks until the study start (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, Victoria, Australia). Hens had *ad libitum* access to the feed and water throughout the study duration by one feed trough and two nipple drinkers per cage. All cages were equipped with three forms of enrichment: a perch, a scratch pad, and a hanging CD, in accordance with the methods described by Moss et al. (2024). White LED (IP65 Dimmable LED Bulb, B-E27:10W, 5K) bulbs were used for lighting, and the lighting schedule was maintained as 16 hours of light and 8 hours of dark using an automatic timer throughout the study. Inside the 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). The average hen house temperature and relative humidity by week are shown in Figure 2. Hens were rehomed upon completion of the study.

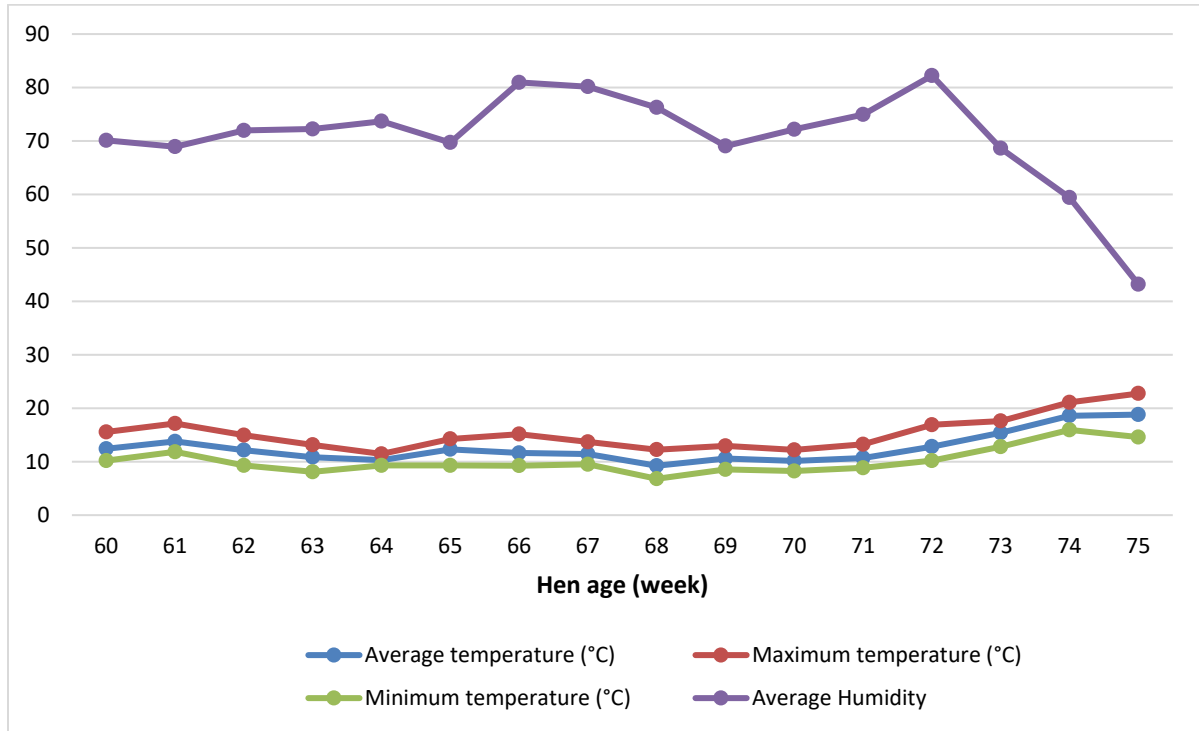


Figure 2 Temperature and relative humidity of the layer hen house in study 2 from 60 to 75 weeks of age

2.2.2 Experimental design and diets

There were eight dietary treatments including a standard protein (SP) diet, an RP diet and RP diet supplemented with two levels (0.06% or 0.12%) of either Arg, Cit or GAA, resulting in the remaining six dietary treatments. Each treatment had 13 replicate cages of two hens per cage per treatment ($n = 208$). This study was conducted in a layer cage facility over 16 weeks from 60 to 75 weeks of age. Average hen starting weights did not differ significantly between the dietary treatments ($P > 0.05$). The diets were fed as mash and based on wheat, soybean meal, sorghum, barley and canola meal. Arginine, GAA and Cit were added on top of the RP diet at the expense of wheat, and thus did not consider any Arg sparing effects. The SP diet with standard CP level (15.8%) and RP diet with 2 percentage points lower CP level compared to the SP diet (13.8%) were formulated according to Hy-Line Brown nutritional recommendations (Hy-Line International 2023). Dietary Arg, GAA and Cit supplemental levels were selected based on the manufacturer's recommended dosages and published reports (FEEDAP 2022b). Feed was provided as mash. Titanium dioxide was added to all diets at 0.5% as an internal marker for digestibility assay.

The nutritional profiles of the major feed ingredients, encompassing parameters such as dry matter (DM), apparent metabolisable energy (AMEn), CP, crude fat, crude fibre, AA, as well as mineral and ash contents, were analysed using near-infrared reflectance spectroscopy (Foss NIR 6500, Hillerød, Denmark) standardised with Evonik AMINONIR Advanced calibration. These results were then used for diet formulation. The diet composition and calculated nutrient values of the SP and basal RP diets are shown in Table 3. The analysed nutrient values of the final diets including DM, gross energy (GE), CP, ash, calcium, total phosphorus and AA were determined using standard methods (AOAC 2019) and are presented in Table 4. Generally, the analysed nutrient contents of the final diets were close to the calculated values.

Table 3 Diet composition and calculated nutrient values of basal standard and reduced protein diets in Study 2 (as-fed basis)

Ingredients, %	Standard protein diet	Reduced protein diet
Wheat	32.4	33.8
Sorghum	27.5	29.5
Soybean meal	15.6	9.4
Barley	10.0	12.5
Canola meal	2.3	2.5
Limestone grit	6.8	6.8
Fine limestone	3.0	3
Canola Oil	1.10	0.60
Titanium dioxide	0.5	0.5
Salt	0.15	0.090
Sodium bicarbonate	0.14	0.240
D,L-methionine	0.125	0.185
Monocalcium phosphate	0.1	0.2
Choline chloride 60%	0.09	0.115
L-lysine HCl	0.045	0.23
Xylanase ¹	0.01	0.01
Phytase ²	0.006	0.006
Layer vitamin-mineral premix ³	0.1	0.1
Pigment Jabiru red	0.004	0.004
Pigment Jabiru yellow	0.003	0.003
L-valine	0.00	0.03
L-isoleucine	0.00	0.04
L-threonine	0.00	0.05
Potassium carbonate	0.00	0.05
Total	1,000	1,000
<i>Calculated nutrient, % (otherwise as indicated)</i>		
AME ⁴ , kcal/kg	2,690	2,690
Crude protein	15.8	13.8
Crude fat	3.00	2.60
Crude fibre	2.80	2.80
Ash	13.1	13.0
Total lysine	0.753	0.734
Total methionine	0.365	0.392
Total methionine + cysteine	0.642	0.64
Total threonine	0.574	0.526
Total isoleucine	0.627	0.559
Total leucine	1.294	1.122
Total tryptophan	0.216	0.183
Total arginine	0.927	0.747
Total histidine	0.375	0.315
Total valine	0.731	0.653

Total glycine	0.614	0.516
Total phenylalanine	0.768	0.651
Digestible ⁵ lysine	0.670	0.670
Digestible methionine	0.359	0.386
Digestible methionine + cysteine	0.603	0.603
Digestible threonine	0.507	0.469
Digestible isoleucine	0.583	0.523
Digestible leucine	1.206	1.045
Digestible tryptophan	0.195	0.165
Digestible arginine	0.876	0.704
Digestible histidine	0.343	0.287
Digestible valine	0.659	0.590
Digestible glycine	0.555	0.464
Digestible phenylalanine	0.708	0.599
Calcium	4.000	4.000
Available phosphorus	0.332	0.32
Total phosphorus	0.352	0.343
Sodium	0.160	0.160
Chloride	0.161	0.161
Potassium	0.602	0.540
Choline, mg/kg	1,600	1,600
Linoleic acid	1.200	1.100
Dietary electrolyte balance ⁶ , mEq	178	162

¹ Axta® XB 201 TPT, Danisco Animal Nutrition, IFF, UK.

² Axta® PHY Gold 10,000 FTU/g, Danisco Animal Nutrition, IFF, UK.

³ Vitamin-mineral premix provided the following per kilogram diet: vitamin A, 10000 IU; vitamin D, 3000 IU; vitamin E, 20 mg; vitamin K, 3 mg; nicotinic acid (niacin), 35 mg; pantothenic acid, 12 mg; folic acid, 1 mg; riboflavin (B2), 6 mg; cyanocobalamin (B12), 0.02 mg; biotin, 0.1 mg; pyridoxine (B6), 5 mg; thiamine (B1), 2 mg; copper, 8 mg as copper sulphate pentahydrate; cobalt, 0.2 mg as cobalt sulphate 21%; molybdenum, 0.5 mg as sodium molybdate; iodine, 1 mg as potassium iodide 68%; selenium, 0.3 mg as selenium 2%; iron, 60 mg as iron sulphate 30%; zinc, 60 mg as zinc sulphate 35%; manganese, 90 mg as manganous oxide 60%; antioxidant, 20 mg.

⁴ AMEn: N corrected apparent metabolisable energy.

⁵ Digestible amino acid coefficients of conventional feed ingredients were determined by Near-Infra Red spectroscopy (Foss NIR 6500, Denmark) standardised with Adisseo calibration.

⁶ Dietary electrolyte balance was calculated as $10,000 \times (\text{Na}^+ + \text{K}^+ - \text{Cl}^-)$.

Table 4 Analysed nutrient content of the dietary treatments in Study 2 (as-fed basis, % otherwise as stated)¹

Nutrient composition	SP	RP	RP-Arg low	RP-Arg hi	RP-Cit low	RP-Cit hi	RP-GAA low	RP-GAA hi
Dry matter	90.4	90.3	90.7	90.6	90.6	90.6	90.7	91.1
Gross energy (Kcal/kg)	3,583	3,569	3,568	3,571	3,569	3,565	3,616	3,478
Crude protein	15.1	13.4	13.4	13.7	13.6	13.8	14.0	13.3
Ash content	12.6	12.2	12.9	12.3	12.6	12.6	11.6	14.5
Calcium	3.35	3.16	3.63	3.18	3.43	3.81	2.96	3.52
Total phosphorus	0.33	0.31	0.33	0.33	0.34	0.35	0.36	0.36
Arginine	7.90	5.31	7.67	8.20	6.70	7.06	6.84	6.73
Methionine	3.51	3.32	3.32	3.32	3.32	3.32	3.32	3.32
Lysine	6.47	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Threonine	4.61	3.58	3.58	3.58	3.58	3.58	3.58	3.58
Valine	5.03	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Isoleucine	5.24	4.19	4.19	4.19	4.19	4.19	4.19	4.19
Leucine	11.3	8.65	8.65	8.65	8.65	8.65	8.65	8.65
Phenylalanine	7.53	5.30	5.30	5.30	5.30	5.30	5.30	5.30
Histidine	3.10	2.17	2.17	2.17	2.17	2.17	2.17	2.17
Serine	6.93	5.05	5.05	5.05	5.05	5.05	5.05	5.05
Glycine	6.37	4.49	4.49	4.49	4.49	4.49	4.49	4.49
Alanine	6.19	4.73	4.73	4.73	4.73	4.73	4.73	4.73
Proline	10.2	8.09	8.09	8.09	8.09	8.09	8.09	8.09
Glutamic Acid	27.3	21.2	21.2	21.2	21.2	21.2	21.2	21.2
Tyrosine	4.02	2.97	2.97	2.97	2.97	2.97	2.97	2.97
Aspartic acid	10.3	6.96	6.96	6.96	6.96	6.96	6.96	6.96

Values of all the amino acids presented were total amino acids (measured on an as-is basis).

2.2.3 Data collection

Data were collected over 16 weeks. Eggs were collected and weighed daily. Feed intake was recorded weekly. Hens were weighed at 60 and 75 weeks of age. Egg mass and feed conversion ratio (FCR, kg of feed/kg of eggs produced) were then calculated from egg production, egg weight, and feed consumption. Egg quality was measured at 75 weeks of age. Additionally, eight hens per treatment were randomly selected, electrically stunned (MEFE CAT 44N, Mitchell Engineering Food Equipment, Clontarf, QLD, Australia), and euthanised by decapitation for collection of blood serum, ileal digesta and bone samples. The collected samples were subsequently analysed to determine serum uric acid level, ileal protein and energy digestibility and bone quality parameters.

2.2.4 Egg quality analysis

A total of 97 eggs (approximately 12 eggs per treatment) were collected shortly after laying at 75 weeks of age for egg quality analysis. Any significantly deformed eggs were excluded from the analysis. Egg length and width were measured using a digital Vernier calliper (Kincrome®, 0–150 mm scale, Scoresby, Victoria, Australia) to calculate the egg shape index (width/length). Eggshell reflectivity was assessed using a shell reflectivity meter (Technical Services and Supplies, Dunnington, York, UK). Eggshell breaking strength and internal egg quality characteristics were assessed using a digital egg tester (DET6500®, Nabel Co., Ltd., Kyoto, Japan). The egg yolk was separated from egg albumen using

Whatman filter papers (CAT No. 1541–090, Whatman®, Buckinghamshire HP7 9NA, Amersham, UK) and weighed. The eggshells were washed, air-dried for at least 72 hours, and weighed using a precision analytical balance (Adventurer™, Model AX423, Ohaus, NJ, USA), and then the eggshell thickness (including membrane) was measured using a custom-built gauge (Mitutoyo Dial Comparator Gauge, Model 2109-10, Kawasaki, Japan). To measure egg proportions, 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 the weights of these egg components by the total egg weight. All measures were performed by trained personnel within 3 hours of the egg collection.

2.2.5 Analysis of serum uric acid

At 75 weeks of age, blood samples were collected into Vacutainers (Becton, Dickinson U.K. Limited, Plymouth, UK) containing a silica coating and polymer gel. The samples were maintained at 4°C and centrifuged at 3,000 × g for 10 minutes at 4°C within 5 hours of collection to collect serum. The serum samples were then stored at -20°C until further analysis. Serum uric acid levels were determined in duplicate using an integrated chemistry analyser (Reference number: DF77 [URCA Uric Acid], Siemens Dimension Xpand Plus, Siemens Healthcare, Newark, NJ, US), according to the manufacturer's protocol.

2.2.6 Energy and protein digestibility

The whole ileum of each sampled hen, beginning at Meckel's diverticulum up to 1 cm anterior to the ileoceocolonic junction, was removed at 75 weeks of age. The intestinal content was collected into 70 mL-containers, stored at -20°C, freeze-dried (Christ Alpha 1-4 LD plus, Osterode am Harz, Germany), and ground using an ultra-centrifugal mill (Retsch ZM 200, Fisher Scientific, Hampton, NH, USA) with a 0.5 mm screen. Protein content in feed and ileal digesta samples were measured using the Dumas combustion method (Dumas 1831) in a nitrogen analyser (LECO Corporation, St Joseph, MI, US) with EDTA as a calibration standard. Gross energy levels in feed and ileal digesta samples were determined by a Parr adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL, USA) calibrated using benzoic acid as a standard. The titanium content in ground diets and ileal digesta samples were analysed in duplicates using an established method (Short et al. 1996). The samples were reanalysed if the variation between the duplicates was higher than 5%. The apparent ileal energy and protein digestibility coefficients were calculated using the following formula (Jasek et al. 2018).

Where E_d and E_{di} were gross energy of the diet and ileal digesta samples, respectively, and Ti_d and Ti_{di} were titanium dioxide content in the diet and ileal digesta samples, respectively, and CP indicated either feed or ileal digesta CP content. The DM of the feed samples and freeze-dried digesta samples were determined by drying the samples in a drying oven at 105°C for 24 hours for calculations of nutrient digestibility on a DM basis.

2.2.7 Bone quality parameters

Right leg bones including the tibia and femur were separated, cleaned, and air-dried in a fume hood for 48 hours. Then bone weight, length, width and breaking strength were measured according to Dao et al. (2022).

2.2.8 Statistical analysis

Data were analysed using R Commander (version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria). Prior to statistical analyses, data were tested for normal distribution t and homogeneity of variances between the dietary treatments. Depending on the results of these two tests, either one-way ANOVA or the non-parametric ANOVA (Kruskal–Wallis test) was employed to test statistical differences between the eight dietary treatments. Additionally, a two-way ANOVA was used to test the interaction between Arg sources (Arg, GAA or Cit) and level (low or high). Tukey's post-hoc test was subsequently used to identify pairwise differences between the treatment means. The differences were considered significant at the 5% probability level.

2.3 Economic analysis and economic modelling for optimal reduced-protein diets

2.3.1 Economic analysis

The feed cost (AU\$/kg) was calculated to detail the economic impact of the dietary treatments in both studies using the following equation.

Feed cost (AU\$/kg egg) = (Feed intake (kg) × Feed cost (AU\$/kg))/Egg mass (kg).

2.3.2 Economic modelling for optimal reduced-protein diets

One critical factor determining the implementation of any research outcome is the cost or return of investment of the proposed initiative; despite this, it is disappointing that economic returns are seldom calculated in scientific studies. Thus, to ensure the greatest benefit and adoptability of our work, we have modelled the economic impact of our main findings. From the data, we calculated the revenue from the sale of each dozen eggs produced, using the average egg grade (based on the average egg weight). This was compared to the feed cost to produce the eggs in those diets.

Formulation of RP diets typically requires increased inclusion of wheat, crystalline Lys, Met, and Thr, as well as the addition of crystalline Ile, Arg, and Val. This comes alongside a reduction in soybean meal and supplemental oil levels compared to conventional SP diets. As soybean meal is partially substituted with crystalline AA in RP diets, the economic viability of such formulations largely depends on the relative market prices of the AA and soybean meal. While the prices of Lys, Met, and Thr have remained relatively stable over the past five years, the costs of other AA such as Ile and Arg are decreasing due to increasing demand and advances in mass production. Therefore, it is pertinent to evaluate how the declining prices of Ile, Leu, and Arg could further enhance the economic efficiency of RP diets for laying hens. To assess this, SP and RP diets for Hy-Line Brown laying hens from 60 to 75 weeks of age were formulated, and diet costs were calculated under varying AA price scenarios. Specifically, two basal SP diets (2690 kcal AME/kg, 15.8% CP) and two basal RP diets (2690 kcal AME/kg, 13.8% CP) were developed using current market prices for feed ingredients. All formulations adhered to the nutritional guidelines provided by Hy-Line International (2023) and were nutritionally comparable to those used in Study 2, where RP diets were shown to support optimal laying hen performance. The first basal SP diet included 4% canola meal, while the corresponding RP diet contained 6% canola meal and included only crystalline Ile, due to least-cost formulation constraints. However, this limited AA inclusion may reduce the practical applicability of the RP diet. To address this, a second set of basal SP and RP diets was formulated with 12% canola meal. In this formulation, the RP diet incorporated crystalline Ile, Leu, and Arg. Subsequently, the prices of Ile and Arg were systematically reduced in 10% increments (i.e. 0%, -10%, -20%, ..., -70%) relative to their current market prices. Diet costs were recalculated at each pricing step. At -70% of the current price of Leu, Ile and Arg, the price of Ile was similar to the current price of Lys.

3 Results and discussion

3.1 Study 1

3.1.1 Results

3.1.1.1 Laying performance and hen weight

Tables 5, 6 and 7 show the laying performance from 35 to 47 weeks, 48 to 57 weeks, and over the entire duration of 35 to 57 weeks of age, respectively. Neither protein $\times$ PhyG $\times$ XB nor two-way interactions were observed for laying performance measurements including egg weight, hen day egg production, egg mass, feed intake and FCR over the experimental periods (Tables 5, 6 and 7). Birds fed the RP diet had a significantly decreased egg weight at 35 to 47 weeks of age ($P = 0.049$; Table 5), 48 to 57 weeks of age ($P = 0.045$; Table 6), and 35 to 57 weeks of age ($P = 0.044$; Table 7), respectively as shown by the main effect of protein level. Additionally, birds fed the RP diet had decreased egg mass ($P = 0.035$) as compared to those offered the SP diet from 35–47 weeks of age as shown by the main effect of protein level (Table 5). Higher XB inclusion significantly decreased ($P = 0.015$) hen day egg production during 48–57 weeks of age as shown by the main effect of XB level in Table 6. The mortality rate over the entire experimental period was less than 0.5%, and this was not related to dietary treatments.

Table 5 Laying performance of hens fed the dietary treatments in Study 1 from weeks 35 to 47

Effects			Egg weight (g)	Hen day egg production (%)	Egg mass (g)	Feed intake (g)	FCR (kg feed/kg egg)
Protein level	PhyG level (FTU/kg)	XB level (g/tonne)					
Two-way interaction (PhyG × XB)							
	600	100	66.7	98.8	65.9	122	1.839
		150	66.0	98.6	65.1	123	1.867
	1200	100	65.8	98.1	64.1	119	1.864
		150	66.6	98.5	65.6	122	1.863
Three-way interaction							
Standard	600	100	67.0	98.6	66.0	123	1.837
		150	66.4	98.5	65.4	123	1.856
	1200	100	67.0	98.4	65.9	121	1.832
		150	67.1	98.0	65.8	121	1.849
Reduced	600	100	66.4	99.1	65.8	121	1.841
		150	65.6	98.6	64.7	124	1.879
	1200	100	64.7	97.8	62.3	117	1.896
		150	66.1	99.0	65.4	123	1.879
Main effect							
Protein	Standard		66.9 ^b	98.4	65.8 ^b	122	1.843
	Reduced		65.7 ^a	98.7	64.5 ^a	121	1.873
PhyG	600		66.4	98.7	65.5	123	1.853
	1200		66.2	98.3	64.8	121	1.864
XB	100		66.3	98.5	65.0	120	1.852
	150		66.3	98.5	65.3	123	1.865
Pooled SEM			0.31	0.16	0.30	0.79	0.009
P-values							
Main effect	Protein		0.049	0.405	0.035	0.643	0.103
	PhyG		0.810	0.220	0.256	0.203	0.548
	XB		0.950	0.848	0.581	0.177	0.445
Interactions	Protein × PhyG		0.405	0.833	0.179	0.877	0.365
	Protein × XB		0.652	0.393	0.234	0.282	0.813
	PhyG × XB		0.254	0.326	0.052	0.606	0.447
	Protein × PhyG × XB		0.543	0.131	0.112	0.743	0.476

^{a,b} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Standard protein diet contains 16.5% crude protein. Reduced protein diet contains 14.5% crude protein.

PhyG = phytase Aextra® PHY Gold (Danisco Animal Nutrition, IFF, UK) providing 10,000 FTU/g. PhyG supplemented at 60 g/tonne and 120 g/tonne provide 600 and 1,200 FTU/kg respectively.

XB = Aextra® XB 201 TPT (Danisco Animal Nutrition, IFF, UK). XB supplemented at 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase. XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase.

Table 6 Laying performance of hens fed the dietary treatments in Study 1 from weeks 48 to 57

Effects			Egg weight (g)	Hen day egg production (%)	Egg mass (g)	Feed intake (g)	FCR (kg feed/kg egg)
Protein level	PhyG level (FTU/kg)	XB level (g/tonne)					
Three-way interaction							
Standard	600	100	67.2	98.9	65.5	124	1.899
		150	66.6	97.0	64.6	122	1.900
	1200	100	67.5	98.4	66.4	123	1.857
		150	67.0	97.1	65.1	125	1.903
Reduced	600	100	66.2	98.5	65.2	122	1.874
		150	66.0	97.4	64.3	122	1.898
	1200	100	64.8	97.5	63.2	122	1.906
		150	66.3	97.9	64.9	125	1.928
Main effect							
Protein	Standard		67.1 ^b	97.8	65.4	124	1.889
	Reduced		65.8 ^a	97.8	64.4	122	1.901
PhyG	600		66.5	97.9	64.9	122	1.893
	1200		66.4	97.7	64.9	124	1.898
XB	100		66.4	98.3 ^b	65.1	123	1.884
	150		66.5	97.4 ^a	64.7	124	1.907
Pooled SEM			0.31	0.20	0.35	0.75	0.011
P-values							
Main effect	Protein		0.045	0.976	0.156	0.483	0.611
	PhyG		0.896	0.588	0.998	0.418	0.827
	XB		0.922	0.015	0.624	0.552	0.308
Interactions	Protein × PhyG		0.477	0.931	0.332	0.935	0.261
	Protein × XB		0.327	0.122	0.261	0.701	0.9996
	PhyG × XB		0.499	0.160	0.430	0.248	0.618
	Protein × PhyG × XB		0.515	0.603	0.291	0.901	0.608

^{a,b} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Standard protein diet contains 16.5% crude protein. Reduced protein diet contains 14.5% crude protein.

PhyG = phytase Aextra® PHY Gold (Danisco Animal Nutrition, IFF, UK) providing 10,000 FTU/g. PhyG supplemented at 60 g/tonne and 120 g/tonne provide 600 and 1,200 FTU/kg respectively.

XB = Aextra® XB 201 TPT (Danisco Animal Nutrition, IFF, UK). XB supplemented at 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase. XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase.

Table 7 Laying performance of hens fed the dietary treatments in Study 1 from weeks 35 to 57

Effects			Egg weight (g)	Hen day egg production (%)	Egg mass (g)	Feed intake (g)	FCR (kg feed/kg egg)
Protein level	PhyG level (FTU/kg)	XB level (g/tonne)					
Two-way interaction							
Protein × XB							
Standard	-	100	67.2	98.7	66.0	122	1.861
		150	66.8	97.7	65.3	123	1.882
Reduced	-	100	65.5	98.3	64.1	121	1.890
		150	66.0	98.3	64.9	123	1.892
PhyG × XB							
	600	100	66.7	98.9	65.7	123	1.875
		150	66.1	98.0	64.8	122	1.878
	1200	100	66.0	98.0	64.4	120	1.877
		150	66.6	98.1	65.3	123	1.896
Three-way interaction							
	600	100	67.1	99.0	65.8	123	1.880
		150	66.5	97.8	65.1	122	1.875
	1200	100	67.2	98.4	66.1	122	1.843
		150	67.1	97.6	65.5	123	1.888
Reduced	600	100	66.3	98.9	65.5	122	1.869
		150	65.8	98.1	64.6	123	1.880
	1200	100	64.7	97.7	62.7	119	1.911
		150	66.2	98.5	65.2	124	1.904
Main effect							
Protein	Standard		67.0 ^b	98.2	65.6	122	1.871
	Reduced		65.7 ^a	98.3	64.5	122	1.891
PhyG	600		66.4	98.4	65.2	123	1.876
	1200		66.3	98.1	64.9	122	1.886
XB	100		66.3	98.5	65.0	122	1.876
	150		66.4	98.0	65.1	123	1.887
Pooled SEM			0.30	0.15	0.31	0.73	0.010
P-values							
Main effect	Protein		0.044	0.750	0.069	0.780	0.336
	PhyG		0.845	0.188	0.543	0.689	0.624
	XB		0.937	0.120	0.959	0.398	0.578
Interactions	Protein × PhyG		0.429	0.929	0.230	0.768	0.269
	Protein × XB		0.486	0.094	0.232	0.404	0.650
	PhyG × XB		0.341	0.094	0.152	0.251	0.687
	Protein × PhyG × XB		0.524	0.319	0.169	0.832	0.414

^{a,b} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Standard protein diet contains 16.5% crude protein. Reduced protein diet contains 14.5% crude protein.

PhyG = phytase Axta[®] PHY Gold (Danisco Animal Nutrition, IFF, UK) providing 10,000 FTU/g. PhyG supplemented at 60 g/tonne and 120 g/tonne provide 600 and 1,200 FTU/kg respectively.

XB = Axta[®] XB 201 TPT (Danisco Animal Nutrition, IFF, UK). XB supplemented at 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase. XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase.

Table 8 shows the hen body weight and weight gain at 35, 47 and 57 weeks of age; where dietary treatments (protein, PhyG and XB) had no significant effects on hen body weight and weight gain during the study period ($P > 0.05$; Table 8).

Table 8 Hen weight of the dietary treatments during the experimental period in Study 1

Effects			Hen weight (g)			Weight gain (g)		
Protein level	PhyG level (FTU/kg)	XB level (g/tonne)	Week 35	Week 47	Week 57	Weeks 35–47	Weeks 47–57	Weeks 35–57
Three-way interaction								
Standard	600	100	2,191	2,346	2,453	171	107	262
		150	2,217	2,407	2,519	190	113	302
	1200	100	2,186	2,348	2,425	162	115	239
		150	2,197	2,354	2,489	166	125	291
Reduced	600	100	2,214	2,386	2,505	172	119	291
		150	2,218	2,405	2,505	191	123	286
	1200	100	2,161	2,328	2,447	169	116	286
		150	2,182	2,385	2,491	203	106	309
Main effect								
Protein	Standard		2,198	2,364	2,472	172	115	273
	Reduced		2,194	2,375	2,487	184	116	293
PhyG	600		2,210	2,385	2,496	181	115	285
	1200		2,182	2,354	2,463	175	115	281
XB	100		2,188	2,352	2,458	169	114	269
	150		2,204	2,387	2,501	188	116	297
Pooled SEM			12.67	14.18	16.89	6.03	3.95	7.94
P-values								
Main effect	Protein		0.868	0.667	0.659	0.340	0.884	0.228
	PhyG		0.274	0.263	0.346	0.606	0.988	0.786
	XB		0.550	0.216	0.211	0.126	0.798	0.076
Interactions	Protein × PhyG		0.539	0.802	0.927	0.393	0.224	0.420
	Protein × XB		0.915	0.926	0.531	0.538	0.518	0.244
	PhyG × XB		0.989	0.868	0.763	0.992	0.775	0.527
	Protein × PhyG × XB		0.761	0.425	0.739	0.571	0.562	0.806

Standard protein diet contains 16.5% crude protein. Reduced protein diet contains 14.5% crude protein.

PhyG = phytase Aextra® PHY Gold (Danisco Animal Nutrition, IFF, UK) providing 10,000 FTU/g. PhyG supplemented at 60 g/tonne and 120 g/tonne provide 600 and 1,200 FTU/kg respectively.

XB = Aextra® XB 201 TPT (Danisco Animal Nutrition, IFF, UK). XB supplemented at 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase. XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase.

3.1.1.2 Internal and external egg quality

The external egg quality of hens fed dietary treatments at 47 weeks of age is shown in Table 9. Regarding the external egg quality, feeding RP diets had no significant effect at 47 weeks of age on shell breaking strength, shell thickness, egg width or shell reflectivity. However, feeding RP diets increased ($P = 0.048$) the egg shape index at 47 weeks of age as a main effect. There was also significant PhyG × XB interaction, where 150 g/tonne XB inclusion to 600 FTU/kg PhyG increased shell breaking strength but 150 g/tonne XB inclusion to 1200 FTU/kg PhyG decreased shell breaking strength ($P = 0.046$). There was also a significant protein × XB interaction for egg width, where XB inclusion to SP diets reduced egg width, but XB inclusion to RP diets increased egg width ($P = 0.041$). Finally, there was a significant protein × PhyG × XB interaction ($P = 0.039$) for egg length, where 150 g/tonne XB inclusion to SP diets with 600 FTU/kg PhyG had no effect, but 150 g/tonne XB inclusion reduced egg length in SP diets with 1200 FTU/kg PhyG. However, in RP diets, 150 g/tonne XB inclusion to diets with

600 FTU/kg PhyG reduced egg length, but 150 g/tonne XB inclusion to diets with 1200 FTU/kg PhyG increased egg length. The internal egg quality of hens fed dietary treatments at 47 weeks of age is shown in Table 10, where there was no significant effect of dietary treatments ($P > 0.05$). The egg proportions of hens fed dietary treatments at 47 weeks of age are shown in Table 11, where as a main effect, RP diets decreased yolk weight in comparison to those from hens offered SP diets ($P = 0.021$). There was also protein $\times$ PhyG interactions for albumen and shell proportions, where increasing PhyG dose from 600 to 1200 FTU/kg in SP diets increased albumen proportion, but increasing PhyG dose from 600 to 1200 FTU in RP diets decreased albumen proportion ($P = 0.032$). However, for shell proportion, increasing PhyG dose from 600 to 1200 FTU/kg in SP diets reduced shell proportion, but increasing PhyG dose from 600 to 1200 FTU/kg in RP diets did not influence shell proportion ($P = 0.044$). External egg quality at 57 week is shown in Table 12, where increasing XB inclusion level from 100 to 150 g/tonne significantly reduced shell breaking strength ($P = 0.036$) as shown by the main effect of XB level. There was no significant effect of dietary treatments on shell thickness, egg length, egg width, egg shape index or reflectivity at 57 weeks of age. Internal egg quality at 57 weeks of age is shown in Table 13, where there was a significant protein $\times$ XB interaction ($P = 0.038$). In more detail, increasing XB inclusion level from 100 to 150 g/tonne reduced yolk colour score in SP diets, but increasing XB inclusion level from 100 to 150 g/tonne did not influence yolk colour score in RP diets ($P = 0.038$). There were no significant effects of dietary treatments on albumin height, Haugh unit, yolk height, yolk diameter or yolk index at 57 weeks of age. The egg proportions of hens fed dietary treatments at 57 weeks of age is shown in Table 14. There was a significant main effect of dietary protein level for albumin weight ($P = 0.016$), yolk proportion ($P = 0.005$) and albumin proportion ($P = 0.006$), where reducing dietary protein level reduced albumin weight, increased yolk proportion and decreased albumin proportion at 57 weeks of age. There were no effects of dietary treatments on yolk weight, shell weight and shell proportion at 57 weeks of age.

Table 9 External egg quality of hens fed dietary treatments in Study 1 at week 47

Effects			Shell breaking strength (Kgf)	Shell thickness (mm)	Egg length (mm)	Egg width (mm)	Egg shape index	Reflectivity (%)
Protein level	PhyG level (FTU/kg)	XB level (g/tonne)						
Two-way interaction								
Protein × XB								
Standard	-	100	4.23	0.431	59.0	45.6 ^d	0.777	24.9
		150	4.03	0.432	58.4	45.2 ^b	0.778	24.9
Reduced	-	100	4.11	0.425	57.6	45.1 ^a	0.783	25.2
		150	4.08	0.432	57.5	45.4 ^c	0.789	26.5
PhyG × XB								
	600	100	4.07 ^b	0.430	58.3	45.4	0.780	25.2
		150	4.24 ^c	0.434	57.9	45.4	0.788	25.3
	1200	100	4.27 ^c	0.425	58.4	45.3	0.780	24.9
		150	3.86 ^a	0.430	58.0	45.2	0.779	26.3
Three-way interaction								
Standard	600	100	4.15	0.437	58.6 ^c	45.7	0.781	25.0
		150	4.37	0.435	58.6 ^c	45.2	0.778	24.4
	1200	100	4.32	0.425	59.4 ^d	45.6	0.773	24.9
		150	3.59	0.427	58.0 ^b	45.1	0.777	25.6
Reduced	600	100	3.99	0.424	58.0 ^b	45.2	0.779	25.5
		150	4.12	0.432	57.1 ^a	45.5	0.798	26.1
	1200	100	4.22	0.425	57.2 ^a	45.0	0.788	24.8
		150	4.04	0.432	58.0 ^b	45.2	0.780	26.9
Main effect								
Protein	Standard		4.14	0.431	58.7	45.4	0.777 ^a	24.9
	Reduced		4.09	0.429	57.6	45.2	0.786 ^b	25.8
PhyG	600		4.16	0.432	58.1	45.4	0.784	25.2
	1200		4.08	0.428	58.2	45.3	0.780	25.6
XB	100		4.17	0.428	58.3	45.4	0.780	25.0
	150		4.06	0.432	57.9	45.3	0.784	25.8
Pooled SEM			0.08	0.002	0.19	0.10	0.002	0.30
P-values								
Main effect	Protein		0.858	0.524	0.003	0.376	0.048	0.158
	PhyG		0.568	0.325	0.701	0.386	0.293	0.639
	XB		0.445	0.388	0.375	0.630	0.484	0.250
Interactions	Protein × PhyG		0.228	0.246	0.979	0.792	0.971	0.646
	Protein × XB		0.473	0.439	0.469	0.041	0.500	0.288
	PhyG × XB		0.046	0.877	0.888	0.787	0.249	0.267
	Protein × PhyG × XB		0.295	0.801	0.039	0.977	0.062	0.972

^{a,b,c,d} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Standard protein diet contains 16.5% crude protein. Reduced protein diet contains 14.5% crude protein.

PhyG = phytase Axtra® PHY Gold (Danisco Animal Nutrition, IFF, UK) providing 10,000 FTU/g. PhyG supplemented at 60 g/tonne and 120 g/tonne provide 600 and 1,200 FTU/kg respectively.

XB = Axtra® XB 201 TPT (Danisco Animal Nutrition, IFF, UK). XB supplemented at 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase. XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase.

Table 10 Internal egg quality of hens fed dietary treatments in Study 1 at week 47

Effects			Albumen height (mm)	Yolk colour	Haugh unit	Yolk height (mm)	Yolk diameter (mm)	Yolk index
Protein level	PhyG level (FTU/kg)	XB level (g/tonne)						
Two-way interaction								
Protein × PhyG								
Standard	600	-	9.50	11.1	94.8	23.2	35.6	0.666
	1200		8.87	11.5	91.1	22.6	38.1	0.616
Reduced	600	-	8.35	11.0	88.4	22.9	39.1	0.582
	1200		9.59	11.9	97.2	23.0	37.9	0.621
Protein × XB								
Standard	-	100	8.56	11.7	89.8	22.8	37.7	0.626
		150	9.91	10.9	96.8	23.1	35.9	0.659
Reduced	-	100	9.31	11.4	94.6	22.9	37.7	0.619
		150	8.53	11.4	90.8	22.9	39.4	0.582
Three-way interaction								
Standard	600	100	9.04	11.5	92.7	23.1	36.3	0.659
		150	9.97	10.8	97.0	23.4	35.0	0.674
	1200	100	8.12	11.8	86.6	22.6	38.9	0.596
		150	9.84	11.0	96.6	22.8	37.1	0.643
Reduced	600	100	8.96	11.0	90.7	22.8	38.4	0.602
		150	7.75	10.9	86.0	23.0	39.9	0.562
	1200	100	9.73	11.8	98.8	23.1	37.0	0.638
		150	9.45	12.0	95.5	22.9	38.9	0.603
Main effect								
Protein	Standard		9.19	11.3	93.1	23.0	36.9	0.641
	Reduced		8.92	11.4	92.7	22.9	38.5	0.601
PhyG	600		8.91	11.0	91.6	23.1	37.3	0.623
	1200		9.22	11.7	94.3	22.8	38.0	0.618
XB	100		8.93	11.5	92.2	22.9	37.7	0.623
	150		9.19	11.2	93.6	23.0	37.7	0.619
Pooled SEM			0.28	0.18	1.65	0.12	0.65	0.013
P-values								
Main effect	Protein		0.601	0.793	0.885	0.930	0.202	0.130
	PhyG		0.523	0.084	0.390	0.301	0.633	0.848
	XB		0.653	0.383	0.689	0.562	0.974	0.907
Interactions	Protein × PhyG		0.114	0.355	0.067	0.157	0.170	0.111
	Protein × XB		0.055	0.281	0.097	0.663	0.219	0.210
	PhyG × XB		0.432	0.918	0.597	0.569	0.979	0.736
	Protein × PhyG × XB		0.946	0.777	0.751	0.723	0.852	0.813

Standard protein diet contains 16.5% crude protein. Reduced protein diet contains 14.5% crude protein.

PhyG = phytase Axtra® PHY Gold (Danisco Animal Nutrition, IFF, UK) providing 10,000 FTU/g. PhyG supplemented at 60 g/tonne and 120 g/tonne provide 600 and 1,200 FTU/kg respectively.

XB = Axtra® XB 201 TPT (Danisco Animal Nutrition, IFF, UK). XB supplemented at 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase. XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase.

Table 11 Egg proportions of hens fed dietary treatments in Study 1 at week 47

Effects			Yolk weight (g)	Albumen weight (g)	Shell weight (g)	Yolk (%)	Albumen (%)	Shell (%)
Protein level	PhyG level (FTU/kg)	XB level (g/tonne)						
Two-way interaction								
Protein × PhyG								
Standard	600	-	17.55	44.13	6.69	25.69	64.43 ^a	9.86 ^c
	1200		17.03	44.98	6.46	25.06	65.58 ^c	9.42 ^a
Reduced	600	-	16.74	44.26	6.47	24.96	65.59 ^c	9.74 ^b
	1200		16.76	42.63	6.45	25.34	65.19 ^b	9.74 ^b
Protein × XB								
Standard	-	100	17.37	45.20	6.66	25.10	65.21	9.69
		150	17.23	43.75	6.49	25.76	64.75	9.62
Reduced	-	100	16.56	43.03	6.44	25.17	65.52	9.77
		150	16.94	43.86	6.49	25.14	65.18	9.72
Three-way interaction								
Standard	600	100	17.49	44.52	6.74	25.45	64.63	9.93
		150	17.62	43.72	6.64	25.96	64.24	9.79
	1200	100	17.25	45.89	6.59	24.76	65.78	9.46
		150	16.76	43.79	6.28	25.50	65.38	9.36
Reduced	600	100	16.82	43.98	6.46	25.43	65.29	9.75
		150	16.65	44.58	6.49	24.45	65.89	9.74
	1200	100	16.28	41.90	6.42	24.92	65.79	9.78
		150	17.22	43.25	6.49	25.73	64.58	9.70
Main effect								
Protein	Standard		17.30 ^b	44.54	6.58	25.40	65.00	9.66
	Reduced		16.75 ^a	43.44	6.46	25.16	65.35	9.74
PhyG	600		17.14	44.19	6.58	25.34	64.99	9.80
	1200		16.89	43.78	6.46	25.21	65.37	9.59
XB	100		16.97	44.16	6.55	25.14	65.36	9.73
	150		17.08	43.81	6.49	25.43	64.98	9.67
Pooled SEM			0.12	0.35	0.05	0.17	0.19	0.05
P-values								
Main effect	Protein		0.021	0.130	0.232	0.413	0.289	0.382
	PhyG		0.290	0.511	0.162	0.777	0.310	0.045
	XB		0.619	0.755	0.456	0.415	0.338	0.459
Interactions	Protein × PhyG		0.268	0.079	0.218	0.187	0.032	0.044
	Protein × XB		0.249	0.090	0.176	0.291	0.881	0.758
	PhyG × XB		0.569	0.845	0.654	0.138	0.217	0.912
	Protein × PhyG × XB		0.072	0.465	0.506	0.262	0.226	0.788

^{a,b,c} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Standard protein diet contains 16.5% crude protein. Reduced protein diet contains 14.5% crude protein.

PhyG = phytase Axtra® PHY Gold (Danisco Animal Nutrition, IFF, UK) providing 10,000 FTU/g. PhyG supplemented at 60 g/tonne and 120 g/tonne provide 600 and 1,200 FTU/kg respectively.

XB = Axtra® XB 201 TPT (Danisco Animal Nutrition, IFF, UK). XB supplemented at 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase. XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase.

Table 12 External egg quality of hens fed dietary treatments in Study 1 at week 57

Effects			Shell breaking strength (Kgf)	Shell thickness (mm)	Egg length (mm)	Egg width (mm)	Egg shape index	Reflectivity (%)
Protein level	PhyG level (FTU/kg)	XB level (g/tonne)						
Three-way interaction								
Standard	600	100	3.99	0.447	58.5	45.6	0.780	23.6
		150	3.80	0.452	58.7	45.3	0.772	24.2
	1200	100	4.37	0.453	58.8	45.2	0.768	25.3
		150	3.44	0.441	58.8	44.9	0.765	24.3
Reduced	600	100	4.07	0.449	58.7	45.2	0.771	25.6
		150	4.08	0.439	58.3	45.3	0.778	24.7
	1200	100	4.30	0.449	57.6	44.9	0.779	24.3
		150	4.13	0.437	58.0	44.9	0.774	25.1
Main effect								
Protein	Standard		3.89	0.448	58.7	45.2	0.771	24.4
	Reduced		4.15	0.443	58.2	45.1	0.776	25.0
PhyG	600		3.99	0.447	58.5	45.4	0.775	24.5
	1200		4.05	0.445	58.3	45.0	0.772	24.8
XB	100		4.18 ^b	0.449	58.4	45.2	0.775	24.7
	150		3.86 ^a	0.442	58.4	45.1	0.772	24.6
Pooled SEM			0.08	0.002	0.17	0.10	0.002	0.23
P-values								
Main effect	Protein		0.093	0.270	0.131	0.459	0.291	0.201
	PhyG		0.608	0.725	0.491	0.058	0.370	0.559
	XB		0.036	0.139	0.870	0.574	0.543	0.799
Interactions	Protein × PhyG		0.639	0.844	0.205	0.999	0.145	0.137
	Protein × XB		0.105	0.408	0.897	0.455	0.443	0.849
	PhyG × XB		0.122	0.328	0.681	0.994	0.657	0.937
	Protein × PhyG × XB		0.350	0.412	0.512	0.760	0.305	0.085

^{a,b} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Standard protein diet contains 16.5% crude protein. Reduced protein diet contains 14.5% crude protein.

PhyG = phytase Aextra® PHY Gold (Danisco Animal Nutrition, IFF, UK) providing 10,000 FTU/g. PhyG supplemented at 60 g/tonne and 120 g/tonne provide 600 and 1,200 FTU/kg respectively.

XB = Aextra® XB 201 TPT (Danisco Animal Nutrition, IFF, UK). XB supplemented at 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase. XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase.

Table 13 Internal egg quality of hens fed dietary treatments in Study 1 at week 57

Effects			Albumen height (mm)	Yolk colour	Haugh unit	Yolk height (mm)	Yolk diameter (mm)	Yolk index
Protein level	PhyG level (FTU/kg)	XB level (g/tonne)						
Two-way interaction (Protein × XB)								
Standard	-	100	9.07	11.67 ^c	92.0	22.9	41.9	0.546
		150	8.90	10.96 ^a	91.1	22.9	40.9	0.550
Reduced	-	100	9.26	11.41 ^b	94.0	23.2	40.7	0.574
		150	8.21	11.46 ^b	87.8	23.1	42.1	0.551
Three-way interaction								
Standard	600	100	8.79	13.1	89.6	23.0	42.3	0.545
		150	9.23	11.8	92.3	23.1	41.7	0.533
	1200	100	9.37	12.3	94.6	22.8	41.5	0.548
		150	8.59	11.6	90.1	22.7	40.1	0.565
Reduced	600	100	9.51	11.3	95.3	23.2	40.1	0.582
		150	8.73	12.6	90.7	23.2	42.2	0.554
	1200	100	9.01	12.3	92.6	23.2	41.2	0.566
		150	7.73	12.0	85.2	23.0	41.9	0.549
Main effect								
Protein	Standard		8.99	12.2	91.6	22.9	41.3	0.548
	Reduced		8.75	12.0	90.9	23.2	41.3	0.564
PhyG	600		9.07	12.2	92.0	23.1	41.5	0.554
	1200		8.66	12.0	90.5	22.9	41.2	0.558
XB	100		9.16	12.2	93.0	23.1	41.3	0.561
	150		8.54	12.0	89.4	23.0	41.4	0.550
Pooled SEM			0.19	0.17	1.12	0.09	0.40	0.007
P-values								
Main effect	Protein		0.537	0.654	0.791	0.145	0.972	0.336
	PhyG		0.309	0.718	0.554	0.272	0.639	0.793
	XB		0.108	0.512	0.116	0.808	0.864	0.537
Interactions	Protein × PhyG		0.336	0.290	0.217	0.727	0.330	0.365
	Protein × XB		0.264	0.038	0.260	0.913	0.143	0.400
	PhyG × XB		0.267	0.442	0.272	0.588	0.508	0.514
	Protein × PhyG × XB		0.642	0.111	0.632	0.853	0.858	0.765

^{a,b,c} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Standard protein diet contains 16.5% crude protein. Reduced protein diet contains 14.5% crude protein.

PhyG = phytase Aextra® PHY Gold (Danisco Animal Nutrition, IFF, UK) providing 10,000 FTU/g. PhyG supplemented at 60 g/tonne and 120 g/tonne provide 600 and 1,200 FTU/kg respectively.

XB = Aextra® XB 201 TPT (Danisco Animal Nutrition, IFF, UK). XB supplemented at 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase. XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase.

Table 14 Egg proportions of hens fed dietary treatments in Study 1 at week 57

Effects			Yolk weight (g)	Albumen weight (g)	Shell weight (g)	Yolk (%)	Albumen (%)	Shell (%)
Protein level	PhyG level (FTU/kg)	XB level (g/tonne)						
Three-way interaction								
Standard	600	100	17.30	43.38	6.83	25.69	64.18	10.17
		150	17.37	43.72	6.93	25.57	64.20	10.23
	1200	100	17.74	44.01	6.93	25.82	63.93	10.25
		150	17.18	42.92	6.68	25.77	64.20	10.16
Reduced	600	100	17.92	42.89	6.82	26.52	63.38	10.10
		150	17.87	42.20	6.75	26.79	63.10	10.11
	1200	100	17.35	41.04	6.78	26.67	62.96	10.42
		150	17.62	41.16	6.66	26.97	62.87	10.17
Main effect								
Protein	Standard		17.38	43.49 ^b	6.84	25.71 ^a	64.13 ^b	10.20
	Reduced		17.70	41.84 ^a	6.75	26.74 ^b	63.08 ^a	10.20
PhyG	600		17.62	43.04	6.84	26.15	63.70	10.15
	1200		17.46	42.24	6.76	26.32	63.48	10.25
XB	100		17.58	42.81	6.84	26.19	63.60	10.24
	150		17.51	42.50	6.76	26.27	63.59	10.17
Pooled SEM			0.12	0.34	0.05	0.19	0.19	0.06
P-values								
Main effect	Protein		0.213	0.016	0.325	0.005	0.006	0.979
	PhyG		0.534	0.246	0.413	0.659	0.563	0.414
	XB		0.810	0.632	0.379	0.780	0.951	0.540
Interactions	Protein × PhyG		0.309	0.339	0.972	0.997	0.795	0.442
	Protein × XB		0.485	0.963	0.894	0.617	0.671	0.663
	PhyG × XB		0.772	0.837	0.302	0.946	0.777	0.377
	Protein × PhyG × XB		0.343	0.416	0.424	0.978	0.968	0.814

^{a,b} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Standard protein diet contains 16.5% crude protein. Reduced protein diet contains 14.5% crude protein.

PhyG = phytase Aextra® PHY Gold (Danisco Animal Nutrition, IFF, UK) providing 10,000 FTU/g. PhyG supplemented at 60 g/tonne and 120 g/tonne provide 600 and 1,200 FTU/kg respectively.

XB = Aextra® XB 201 TPT (Danisco Animal Nutrition, IFF, UK). XB supplemented at 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase. XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase.

3.1.1.3 Excreta moisture content and apparent nutrient digestibility

The excreta moisture content and apparent total tract DM digestibility of hens fed dietary treatments at 57 weeks of age are shown in Table 15. There was a significant main effect of protein, where reducing dietary protein level reduced excreta moisture ($P = 0.015$) and increased apparent total tract DM digestibility ($P = 0.020$) at 57 weeks of age. There were also significant PhyG × XB interactions for DM intake ($P = 0.032$) and excreted DM ($P = 0.026$), where increasing XB inclusion level from 100 to 150 g/tonne in diets with 600 FTU/kg PhyG reduced the intake and excretion of DM, but increasing XB inclusion level from 100 to 150 g/tonne in diets with 1200 FTU/kg PhyG increased the intake and excretion of DM at 57 weeks of age.

Table 15 Excreta moisture content and total dry matter digestibility of hens fed dietary treatments in Study 1 at week 57

Effects			Excreta moisture (%)	Dry matter intake (g/day)	Dry matter excreted (g/day)	Dry matter retained (g/day)	Total dry matter digestibility (%)
Protein level	PhyG level (FTU/kg)	XB level (g/tonne)					
Two-way interaction (PhyG × XB)							
	600	100	76.5	115 ^c	30.3 ^c	84.3	73.6
		150	76.7	109 ^a	27.6 ^a	79.2	74.2
	1200	100	77.6	109 ^a	28.9 ^b	82.2	73.9
		150	77.0	113 ^b	30.6 ^c	81.9	72.8
Three-way interaction							
Standard	600	100	77.2	114	30.6	83.5	73.1
		150	77.8	106	27.7	78.5	74.0
	1200	100	77.6	110	30.4	80.0	72.5
		150	77.4	112	31.3	80.8	72.1
Reduced	600	100	75.9	115	29.9	85.1	74.0
		150	75.6	111	27.6	80.0	74.5
	1200	100	77.6	108	27.5	84.3	75.4
		150	76.6	113	29.9	83.0	73.5
Main effect							
Protein	Standard		77.5 ^b	111	30.0	80.7	72.9 ^a
	Reduced		76.4 ^a	112	28.7	83.1	74.3 ^b
PhyG	600		76.6	112	29.0	81.8	73.9
	1200		77.3	111	29.8	82.0	73.4
XB	100		77.1	112	29.6	83.2	73.8
	150		76.8	111	29.1	80.6	73.5
Pooled SEM			0.23	1.09	0.48	0.92	0.31
P-values							
Main effect	Protein		0.015	0.661	0.173	0.214	0.020
	PhyG		0.109	0.761	0.394	0.897	0.346
	XB		0.571	0.525	0.624	0.161	0.673
Interactions	Protein × PhyG		0.110	0.397	0.345	0.656	0.202
	Protein × XB		0.304	0.423	0.592	0.770	0.408
	PhyG × XB		0.365	0.032	0.026	0.203	0.124
	Protein × PhyG × XB		0.975	0.934	0.809	0.795	0.641

^{a,b,c} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Standard protein diet contains 16.5% crude protein. Reduced protein diet contains 14.5% crude protein.

PhyG = phytase Aextra® PHY Gold (Danisco Animal Nutrition, IFF, UK) providing 10,000 FTU/g. PhyG supplemented at 60 g/tonne and 120 g/tonne provide 600 and 1,200 FTU/kg respectively.

XB = Aextra® XB 201 TPT (Danisco Animal Nutrition, IFF, UK). XB supplemented at 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase. XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase.

The apparent total tract energy and protein digestibility of hens fed dietary treatments at 57 weeks of age is shown in Table 16. There was a main effect of protein level for apparent energy digestibility ($P = 0.003$), protein excreted ($P < 0.001$) and apparent protein digestibility ($P = 0.013$), where reducing dietary protein level increased apparent energy digestibility, reduced protein excreted and thus also increased apparent protein digestibility at 57 weeks of age. There were also PhyG × XB interactions, where increasing XB from 100 to 150 g/tonne in diets with 600 FTU/kg PhyG reduced energy and protein intake per day, but increasing XB from 100 to 150 g/tonne in diets with 1200 FTU/kg PhyG increased energy ($P = 0.017$) and protein ($P = 0.044$) intake per day.

Table 16 Total energy and protein digestibility of hens fed dietary treatments at week 57

Effects			Energy intake/day (Kcal/day)	Energy excreted (Kcal/day)	Energy retained (Kcal/day)	Total energy digestibility (%)	Protein intake/day (g/day)	Protein excreted (g/day)	Protein retained (g/day)	Total protein digestibility (%)
Protein level	PhyG level (FTU/kg)	XB level (g/tonne)								
Two-way interaction (PhyG × XB)										
	600	100	459 ^c	97.4	362	78.8	18.9 ^b	9.81	9.13	48.4
		150	435 ^a	93.8	338	78.8	17.7 ^a	9.31	8.37	47.5
	1200	100	437 ^a	94.7	349	78.6	18.8 ^b	9.40	9.43	50.1
		150	455 ^b	98.4	355	77.9	19.2 ^c	9.88	9.35	48.7
Three-way interaction										
Standard	600	100	458	100.4	357	78.0	20.0	11.1	8.81	44.2
		150	425	93.3	332	78.2	18.7	9.82	8.93	47.7
	1200	100	443	97.7	345	77.9	19.8	10.3	9.50	48.0
		150	456	99.2	353	77.4	20.3	11.0	9.33	45.9
Reduced	600	100	460	94.4	366	79.5	17.9	8.47	9.44	52.6
		150	447	94.4	343	79.4	16.6	8.80	7.82	47.3
	1200	100	430	91.8	353	79.3	17.8	8.48	9.36	52.1
		150	454	97.8	357	78.5	18.2	8.80	9.38	51.4
Main effect										
Protein	Standard		445	97.6	347	77.9 ^a	19.7	10.6 ^b	9.14	46.4 ^a
	Reduced		449	94.6	355	79.2 ^b	17.6	8.64 ^a	9.00	50.9 ^b
PhyG	600		447	95.7	350	78.8	18.3	9.56	8.75	47.9
	1200		446	96.5	352	78.3	19.0	9.64	9.39	49.4
XB	100		448	96.1	355	78.7	18.9	9.60	9.28	49.2
	150		446	96.1	346	78.4	18.5	9.59	8.86	48.1
Pooled SEM			4.41	1.30	3.84	0.23	0.25	0.22	0.20	0.91
P-values										
Main effect	Protein		0.802	0.240	0.317	0.003	< 0.001	< 0.001	0.723	0.013
	PhyG		0.882	0.767	0.764	0.271	0.078	0.825	0.114	0.419
	XB		0.788	0.983	0.244	0.431	0.297	0.984	0.303	0.503
Interactions	Protein × PhyG		0.294	0.836	0.829	0.885	0.956	0.841	0.812	0.815
	Protein × XB		0.394	0.282	0.956	0.734	0.888	0.364	0.337	0.294
	PhyG × XB		0.017	0.180	0.053	0.445	0.044	0.180	0.398	0.885
	Protein × PhyG × XB		0.821	0.805	0.816	0.982	0.975	0.175	0.231	0.148

^{a,b,c} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Standard protein diet contains 16.5% crude protein. Reduced protein diet contains 14.5% crude protein.

PhyG = phytase Aextra® PHY Gold (Danisco Animal Nutrition, IFF, UK) providing 10,000 FTU/g. PhyG supplemented at 60 g/tonne and 120 g/tonne provide 600 and 1,200 FTU/kg respectively.

XB = Aextra® XB 201 TPT (Danisco Animal Nutrition, IFF, UK). XB supplemented at 100 g/tonne provide 1,220 U/kg xylanase and 152 U/kg beta-glucanase. XB supplemented at 150 g/tonne to provide 1,830 U/kg xylanase and 228 U/kg beta-glucanase.

3.1.2 Discussion

In the present study, dietary CP levels were reduced through the inclusion of crystalline AA, resulting in a nearly 50% reduction in soybean meal content within the formulated diets. While this strategy effectively minimised reliance on high-protein feed ingredients, it was associated with a reduction in average egg weight and a tendency toward decreased overall egg mass throughout the trial period. Importantly, the lower protein formulation led to a reduction in nitrogenous excretion of over 18%, highlighting the potential environmental benefits of RP feeding strategies in commercial egg production. These findings are consistent with Dao et al. (2021a,b) who reported a decline in egg weight in hens fed RP diets compared to those receiving SP diets. Khajali et al. (2007) observed significantly lower egg mass and FCR in hens offered RP diets over a 40–44 week laying period. Several other studies have corroborated these outcomes, noting reduced egg weight, egg mass, and egg production performance in response to RP feeding regimens (Keshavarz 1984; Summers & Leeson 1994; Alagawany et al. 2020). The underlying mechanism may relate to AA availability during albumen formation. Penz and Jensen (1991) reported that the magnum, the primary site of albumen synthesis, incorporates protein at approximately twice the rate of other oviduct regions, including the infundibulum, isthmus, uterus, and vagina. As albumen deposition requires a substantial AA supply, insufficient provision under RP conditions may impair this process, contributing to the observed decline in egg weight.

Excessive XB activity can result in high concentrations of XOS (xylo-oligosaccharides) due to the over-degradation of both soluble and insoluble NSPs (Ali et al. 2025). The presence of too much XOS in the small intestine can disrupt fermentation patterns and microbial balance (Lyu et al. 2020). In addition to altering gut motility, this can also increase fermentation gas and impair nutrient absorption (Mutuyemungu et al. 2023). This explains why in trials exploring high doses of carbohydrase enzymes, performance may be reduced, as energy may be allocated from production (such as egg formation) to gut maintenance and microbe processing. While we explored the common XB dose rate in the present study, as highlighted, RP diets also have reduced NSP substrate concentrations due to reductions in soybean meal inclusions. Thus, it is possible that the XB level utilised was too high for RP diets. Previous research on laying hen diets with standard protein levels doesn't report negative effects, but responses are generally inconsistent to XB supplementation. For example, Mathlouthi et al. (2003) reported no improvements in egg production, egg weight, egg mass, and feed intake in Isa Brown hens aged 45 to 54 weeks supplemented with xylanase (560 IU/kg) or β -glucanase (2800 IU/kg). Similarly, Cufadar et al. (2010) found no significant effects on egg production and egg quality between 52 and 64 weeks of age in hens receiving wheat-based diets supplemented with xylanase at 100 or 200 mg/kg.

The variability in XB efficacy is likely due to variability in dietary substrate between studies, which may also partly be explained by age-related physiological differences, as hens have an extended production cycle compared to broiler chickens. Olukosi et al. (2007) noted that older birds may require different enzyme supplementation strategies due to changes in digestive capacity and nutrient metabolism over time. In contrast, Scheideler et al. (2005) found increased egg production when multi-enzyme preparations (xylanase, protease, and α -amylase) were provided to Babcock B-300 (with ME of 2890 kcal/kg) or Hy-Line W36 hens (with ME of 2805 kcal/kg) during the early laying phase (25 to 40 weeks of age). Mirzaie et al. (2012) demonstrated positive effects of xylanase supplementation (ranging from 840 to 1960 U/kg) on egg production, egg mass, and feed efficiency in Hy-Line W36 hens from 25 to 47 weeks of age. These divergent findings may be attributed to several interacting factors, including the age of the birds, the carbohydrase dosage and combination of enzymes used, and the specific composition and available substrate of the experimental diet.

Previous studies have documented the negative impact of low protein diets on bodyweight in laying hens (Sahota & Bhatti 2002; Alagawany et al. 2020). For instance, Novak et al. (2008) reported that

White Leghorn layers exhibited reduced body weight when fed diets containing lower CP levels (12%, 14%, and 16%) compared to diets with higher CP levels (17% and 18%), emphasising the importance of adequate dietary protein to support bodyweight maintenance during the laying phase. However, findings on this topic have not been entirely consistent. Moraes Sá et al. (2007) found no significant effect on body weight gain in brown hens fed a 15% CP diet from 34 to 50 weeks of age. Similarly, Leeson and Caston (1996) reported that a diet containing 14.4% CP had no adverse effects on feed intake or bodyweight from 40 to 70 weeks of age. In addition, Silversides et al. (2006) observed that the inclusion of phytase in xylanase-supplemented diets did not influence body weight in Isa Brown hens between 34 and 49 weeks of age. These results align with the current study, in which no significant effects of dietary protein level, PhyG, or XB supplementation were observed on bodyweight gain throughout the experimental period. While short- to mid-term feeding of RP diets may not significantly alter bodyweight, prolonged exposure to such diets, particularly those deficient in essential AA could impair physiological functions critical to production. These include egg formation, shell calcification, muscle maintenance, and immune response, all of which are dependent on adequate AA supply and energy balance. Therefore, sustained low protein intake, especially in the absence of sufficient AA supplementation, may eventually compromise bodyweight and overall productivity.

The results of the present study indicated that dietary treatments had no significant impact on external egg quality parameters, including eggshell thickness and shell strength, at both 47 and 57 weeks of age. These findings are consistent with previous reports. Kashani et al. (2014) found no significant differences in shell thickness or breaking strength in hens fed RP diets containing 13.9% CP at 59 and 63 weeks of age. Similarly, Oluwabiye et al. (2022a) observed no notable effects of varying protein levels (14% and 16% CP) on external egg quality at 36 weeks. Gumpha et al. (2019) also reported that shell thickness was unaffected by CP levels ranging from 13% to 17.5% at 44 weeks of age. However, in the current study, hens fed diets supplemented with 600 FTU/kg of PhyG and 150 g/tonne of XB showed improved shell breaking strength at 47 weeks, compared to those receiving 1200 FTU/kg PhyG with the same XB inclusion, which is likely due to an imbalanced AvP:Ca ratio as enzymes were included over-the-top in the present study. This is in line with findings by Lim et al. (2003) and Augspurger et al. (2007), who reported enhanced shell strength in hens supplemented with lower phytase levels (e.g. 300 FTU/kg) between 70 and 76 weeks of age. Kamińska (1997) also found improved shell strength in hens receiving 150 FTU/kg phytase compared to those fed 450 FTU/kg between 48 and 70 weeks. However, Yueming et al. (2018) observed that Isa Brown hens fed 600 FTU/kg phytase produced similar eggshells to those receiving 1200 FTU/kg from 21 to 57 weeks of age. Moderate phytase supplementation may optimise mineral availability by reducing the anti-nutritional effects of phytate, which are known to chelate minerals such as calcium, zinc, and magnesium, thereby limiting their bioavailability. In the present study, PhyG at 600 FTU/kg appeared sufficient to counteract phytate without disrupting mineral balance, as phytate was added over-the-top. Potentially the over-the-top inclusion (no matrix values ascribed) may have impacted available P to Ca ratios in the present study and the prior mentioned studies which demonstrate that high levels of phytase impacted egg quality.

The Haugh unit is widely recognised as a key indicator of internal egg quality. In the present study, no significant effects of dietary treatments were observed on internal egg quality or egg component proportions at both 47 and 57 weeks of age. These findings are consistent with those reported by Novak et al. (2006), who found that low protein diets did not significantly affect shell weight or other internal quality parameters in laying hens. Similarly, Khajali et al. (2007) reported comparable egg quality indices in pullets fed RP diets during the grower (14.6% CP) and peak production (16.3% CP) phases relative to those on SP diets (17.8% CP). In the current study, a high dose of PhyG in RP diets led to a reduction in albumen proportion at 47 weeks, whereas in SP diets, the same PhyG dose increased albumen proportion. This aligns with the findings of de Lima et al. (2010), who reported that supplementing a 13% CP diet with 600 FTU/kg phytase reduced albumen percentage during peak lay. Furthermore, the present study showed that shell proportion was unaffected by PhyG at 1200 FTU/kg in RP diets, yet decreased significantly in SP diets at the same enzyme inclusion level. This observation

is also in line with de Lima et al. (2010), who noted that elevated phytase levels in low-CP diets did not alter shell percentage, yolk weight, or yolk proportion during peak production. The altering effect of phytase inclusion to SP and RP diets may reflect the differing substrates in these diets, and perhaps also, the over-the-top inclusion of the enzymes in the present study.

Furthermore, the present study demonstrated that reducing dietary CP levels adversely affected albumen characteristics at 57 weeks of age. Specifically, hens fed RP diets exhibited lower albumen weight and percentage, and increased yolk proportion, compared to those on SP diets. These results agree with the findings of Novak et al. (2006), who reported a significant reduction in albumen weight and albumen percentage, accompanied by an increase in yolk proportion in hens receiving 13% CP diets between 44 and 63 weeks of age. Similarly, Mousavi et al. (2013) observed decreased albumen and increased yolk percentages in hens fed a 15.5% CP diet at 33 weeks of age. Penz and Jensen (1991) also reported that RP diets (13% CP) were associated with reduced albumen and elevated yolk proportions. These consistent findings suggest that, despite meeting essential AA requirements through supplementation, overall CP intake plays a vital role in supporting efficient albumen synthesis. Albumen production occurs predominantly in the magnum region of the oviduct and is particularly sensitive to protein availability during the initial 2 to 3 hours following ovulation (Macelline et al. 2021). A limited supply of intact protein may restrict the capacity for optimal albumen deposition during this critical window, ultimately reducing egg white volume and potentially overall egg size. Moreover, yolk deposition tends to be more stable and less influenced by short-term dietary fluctuations. As such, a decline in albumen mass without a corresponding reduction in yolk size results in a relatively higher yolk to albumen ratio. This shift in egg composition underlines the importance of adequate total protein intake, not just AA balance, for maintaining internal egg quality throughout the laying cycle.

This study demonstrated that dietary CP levels influenced nutrient utilisation, particularly at 57 weeks of age. Hens fed RP diets exhibited lower excreta moisture content and higher apparent DM digestibility compared to those on SP diets. These results are consistent with those of Heo et al. (2023), who observed improved apparent DM digestibility and reduced excreta moisture in pullets and hens fed 13% CP diets between 19 and 32 weeks of age. The improved moisture profile under RP feeding may be attributed to reduced nitrogen intake, which limits the excretion of nitrogenous waste products such as uric acid. With lower nitrogen excretion, birds require less water to eliminate waste, resulting in drier faeces. Conversely, high protein intake increases nitrogen turnover, potentially elevating moisture content in the excreta. In addition, excessive dietary protein can lead to undigested protein entering the hindgut, which may disrupt the gut microbiota, impair nutrient absorption, and reduce overall digestive efficiency (Aletor et al. 2000; Francesch & Brufau 2004; Corzo et al. 2005; Collett 2012). Therefore, RP diets may promote a more favourable digestive environment by minimising the burden of excess protein on the gastrointestinal tract. In the current study, supplementation with 600 FTU/kg of PhyG and 150 g/tonne of XB reduced DM intake and excretion compared to supplementation with 1200 FTU/kg PhyG and the same XB level. Again, the improved performance of the 600 FTU/kg phytase may reflect the over-the-top inclusion of the enzymes in the present study.

Reducing nitrogenous waste in poultry production is a key strategy for enhancing environmental sustainability (Kamiya et al. 2020). The present study demonstrated that feeding RP diets significantly lowered nitrogen excretion while improving apparent energy and protein digestibility at 57 weeks of age. These findings align with recent work by Liu et al. (2024), who reported that broilers fed diets containing 13.9% and 15.6% CP exhibited lower nitrogen excretion compared to those receiving a 16.3% CP diet. Similarly, Novak et al. (2008) found that administering a 13.5% CP diet to laying hens resulted in a 17.3% reduction in nitrogen content of the excreta. An earlier study by Summers (1993) also reported a substantial reduction (up to 40%) in nitrogen excretion when dietary CP levels were decreased from 17% to 11%. The reduction in nitrogen output with RP diets may be attributed not only to lower nitrogen intake, but also to positive shifts in gut microbial activity. A lower CP diet can limit the availability of undigested protein as a substrate for proteolytic microbes, thereby reducing protein

fermentation in the hindgut. This microbial shift may enhance carbohydrate and fibre digestion, and promote a more stable and beneficial gut microbiota. Consequently, a more balanced microbial ecosystem may improve nutrient absorption, lower the production of harmful metabolic by-products such as ammonia and hydrogen sulfide, and enhance overall gut health. These combined effects likely contribute to the improved energy and protein digestibility observed in hens fed RP diets.

3.2 Study 2

3.2.1 Results

3.2.1.1 Laying performance and hen weight

The mortality rate over the entire experimental period was less than 0.5%, and this was not related to dietary treatments. Tables 17, 18 and 19 show the laying performance from 60 to 67 weeks, 68 to 75 weeks, and over the entire duration of 60 to 75 weeks of age, respectively. An Arg source $\times$ level interaction was observed for feed intake from 60 to 67 weeks of age ($P = 0.007$; Table 17) and 60 to 75 weeks of age ($P = 0.014$; Table 19) where increasing dietary Cit supplemental level increased feed intake while Arg and GAA supplemental levels did not affect feed intake in laying hens. A similar trend was observed for feed intake from 68 to 75 weeks of age although a significant Arg source $\times$ level was not obtained ($P = 0.055$; Table 18). Dietary Cit supplementation also increased egg weight ($P = 0.049$) and tended to increase egg mass ($P = 0.063$) compared to the GAA supplementation from 60 to 67 weeks of age as shown by the main effect of Arg source (Table 17). From 68 to 75 weeks of age, hens fed diets supplemented with Cit had higher egg mass ($P = 0.014$) and feed intake ($P = 0.009$) compared to those fed diets supplemented with Arg and GAA as shown by the main effect of Arg source in Table 18. Additionally, hens fed the diets supplemented with Cit tended to have higher egg weight ($P = 0.053$) compared to hens fed diets supplemented with GAA and tended to have higher hen day egg production ($P = 0.080$) compared to those offered diets supplemented with Arg from 68 to 75 weeks of age as shown by the main effect of Arg source (Table 18). Over the entire study from 60 to 75 weeks of age, higher egg weight ($P = 0.048$) and egg mass ($P = 0.019$) were noted in hens offered the diets supplemented with Cit compared to hens offered the diets supplemented with GAA as shown by the main effect of Arg source (Table 19). The one-way ANOVA results showed that hens fed the RP diet supplemented with the high Cit level (RP-Cit hi) had higher feed intake ($P < 0.001$) and tended to have higher egg mass ($P = 0.058$) compared to hens fed the SP and RP diets from 60 to 67 weeks of age (Table 17). Likewise, higher egg mass and feed intake were observed in hens fed the RP-Cit hi diet compared to those offered the SP and RP diets from 68 to 75 and 60 to 75 weeks of age, as shown by the one-way ANOVA results (all $P < 0.05$; Tables 18 and 19). No differences in FCR were found between the dietary treatments over the entire study. Similarly, the hen weight at 75 weeks of age and weight gain from 60 to 75 weeks of age were not different between the dietary treatments (Table 20).

Table 17 Laying performance of hens fed the dietary treatments in Study 2 from weeks 60 to 67

Effects		Egg weight (g)	Hen day egg production (%)	Egg mass (g)	Feed intake (g)	FCR (kg feed/ kg egg)
<i>Two-way ANOVA results (2 × 3 factorial arrangement of treatments)</i>						
Treatment	RP-Arg low	64.3	95.5	61.3	136 ^{ab}	2.235
	RP-Arg hi	65.0	95.3	62.0	133 ^a	2.155
	RP-Cit low	65.1	94.7	61.7	134 ^a	2.184
	RP-Cit hi	66.2	97.7	65.0	142 ^b	2.203
	RP-GAA low	63.3	95.8	60.9	130 ^a	2.143
	RP-GAA hi	63.5	95.8	60.8	128 ^a	2.122
Source	Arg	64.7 ^{ab}	95.4	61.7	134 ^b	2.193
	Cit	65.7 ^b	96.3	63.4	138 ^b	2.194
	GAA	63.4 ^a	95.8	60.9	129 ^a	2.133
Level	Low	64.2	95.4	61.3	133	2.187
	High	64.9	96.2	62.5	134	2.159
SEM		0.37	0.41	0.44	0.91	0.015
P-values	Source	0.049	0.673	0.063	< 0.001	0.144
	Level	0.383	0.333	0.183	0.600	0.307
	Source × level	0.857	0.222	0.270	0.007	0.388
<i>One-way ANOVA results</i>						
Treatment	SP	63.5	95.2	60.2	133 ^a	2.211
	RP	63.3	94.8	60.0	129 ^a	2.157
	RP-Arg low	64.3	95.5	61.3	136 ^{ab}	2.235
	RP-Arg hi	65.0	95.3	62.0	133 ^a	2.155
	RP-Cit low	65.1	94.7	61.7	134 ^{ab}	2.184
	RP-Cit hi	66.2	97.7	65.0	142 ^b	2.203
	RP-GAA low	63.3	95.8	60.9	130 ^a	2.143
	RP-GAA hi	63.5	95.8	60.8	128 ^a	2.122
SEM		0.31	0.38	0.39	0.82	0.012
P-value		0.167	0.668	0.058	< 0.001	0.248

^{a,b} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

SP: A standard protein diet contains 15.8% crude protein.

RP: A reduced protein diet contains 13.8% crude protein.

RP-Arg low, RP-Cit low, RP-GAA low: RP diet supplemented with Arg, Cit and GAA at the low level (0.06% or 0.6 g/kg), respectively.

RP-Arg hi, RP-Cit hi, RP-GAA hi: RP diet supplemented with Arg, Cit and GAA at the high level (0.12% or 1.2 g/kg), respectively.

Table 18 Laying performance of hens fed the dietary treatments in Study 2 from weeks 68 to 75

Effects		Egg weight (g)	Hen day egg production (%)	Egg mass (g)	Feed intake (g)	FCR (kg feed/ kg egg)
<i>Two-way ANOVA results (2 × 3 factorial arrangement of treatments)</i>						
Treatment	RP-Arg low	64.4	94.0	60.5	127	2.117
	RP-Arg hi	64.9	93.8	61.0	125	2.070
	RP-Cit low	65.4	96.3	63.0	127	2.030
	RP-Cit hi	66.6	97.0	64.8	136	2.109
	RP-GAA low	63.9	94.0	60.1	125	2.087
	RP-GAA hi	63.5	95.4	60.8	125	2.075
Source	Arg	64.6	93.9	60.8 ^a	126 ^a	2.092
	Cit	66.0	96.6	63.9 ^b	132 ^b	2.071
	GAA	63.7	94.7	60.4 ^a	125 ^a	2.081
Level	Low	64.6	94.7	61.1	126	2.081
	High	65.0	95.3	62.1	128	2.084
SEM		0.39	0.50	0.52	0.98	0.016
P-values	Source	0.053	0.080	0.014	0.009	0.864
	Level	0.546	0.525	0.351	0.310	0.922
	Source × level	0.731	0.815	0.851	0.055	0.274
<i>One-way ANOVA results</i>						
Treatment	SP	63.5	92.9	58.8 ^a	125 ^a	2.130
	RP	63.2	92.9	58.8 ^a	123 ^a	2.113
	RP-Arg low	64.4	94.0	60.5 ^{ab}	127 ^{ab}	2.117
	RP-Arg hi	64.9	93.8	61.0 ^{ab}	125 ^a	2.070
	RP-Cit low	65.4	96.3	63.0 ^{ab}	127 ^{ab}	2.030
	RP-Cit hi	66.6	97.0	64.8 ^b	136 ^b	2.109
	RP-GAA low	63.9	94.0	60.1 ^{ab}	125 ^a	2.087
	RP-GAA hi	63.5	95.4	60.8 ^{ab}	125 ^a	2.075
SEM		0.33	0.49	0.48	0.86	0.013
P-value		0.141	0.339	0.030	0.008	0.688

^{a,b} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

SP: A standard protein diet contains 15.8% crude protein.

RP: A reduced protein diet contains 13.8% crude protein.

RP-Arg low, RP-Cit low, RP-GAA low: RP diet supplemented with Arg, Cit and GAA at the low level (0.06% or 0.6 g/kg), respectively.

RP-Arg hi, RP-Cit hi, RP-GAA hi: RP diet supplemented with Arg, Cit and GAA at the high level (0.12% or 1.2 g/kg), respectively.

Table 19 Laying performance of hens fed the dietary treatments in Study 2 from weeks 60 to 75

Effects		Egg weight (g)	Hen day egg production (%)	Egg mass (g)	Feed intake (g)	FCR (kg feed/ kg egg)
<i>Two-way ANOVA results (2 × 3 factorial arrangement of treatments)</i>						
Treatment	RP-Arg low	64.3	94.8	60.9	132 ^{ab}	2.176
	RP-Arg hi	64.9	94.5	61.5	129 ^a	2.113
	RP-Cit low	65.3	95.5	62.3	131 ^a	2.107
	RP-Cit hi	66.4	97.4	64.9	139 ^b	2.156
	RP-GAA low	63.6	94.9	60.5	127 ^a	2.115
	RP-GAA hi	63.5	95.6	60.8	127 ^a	2.099
Source	Arg	64.6 ^{ab}	94.6	61.2 ^{ab}	130 ^{ab}	2.143
	Cit	65.8 ^b	96.5	63.7 ^b	135 ^b	2.132
	GAA	63.5 ^a	95.3	60.7 ^a	127 ^a	2.107
Level	Low	64.4	95.0	61.2	130	2.134
	High	65.0	95.8	62.3	131	2.121
SEM		0.38	0.39	0.46	0.90	0.014
P-values	Source	0.048	0.168	0.019	< 0.001	0.542
	Level	0.456	0.361	0.234	0.400	0.631
	Source × level	0.790	0.557	0.543	0.014	0.275
<i>One-way ANOVA results</i>						
Treatment	SP	63.5	94.3	59.6 ^a	129 ^a	2.180
	RP	63.3	93.9	59.4 ^a	126 ^a	2.135
	RP-Arg low	64.3	94.8	60.9 ^{ab}	132 ^{ab}	2.176
	RP-Arg hi	64.9	94.5	61.5 ^{ab}	129 ^a	2.113
	RP-Cit low	65.3	95.5	62.3 ^{ab}	131 ^{ab}	2.107
	RP-Cit hi	66.4	97.4	64.9 ^b	139 ^b	2.156
	RP-GAA low	63.6	94.9	60.5 ^{ab}	127 ^a	2.115
	RP-GAA hi	63.5	95.6	60.8 ^{ab}	127 ^a	2.099
SEM		0.32	0.40	0.42	0.80	0.012
P-value		0.144	0.511	0.034	< 0.001	0.482

^{a,b} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

SP: A standard protein diet contains 15.8% crude protein.

RP: A reduced protein diet contains 13.8% crude protein.

RP-Arg low, RP-Cit low, RP-GAA low: RP diet supplemented with Arg, Cit and GAA at the low level (0.06% or 0.6 g/kg), respectively.

RP-Arg hi, RP-Cit hi, RP-GAA hi: RP diet supplemented with Arg, Cit and GAA at the high level (0.12% or 1.2 g/kg), respectively.

Table 20 Hen weight and weight gain of the dietary treatments in Study 2

Effects		Hen weight at week 60 (g)	Hen weight at week 75 (g)	Weight gain weeks 60–75 (g)
<i>Two-way ANOVA results (2 × 3 factorial arrangement of treatments)</i>				
Treatment	RP-Arg low	2,059	2,295	236
	RP-Arg hi	2,098	2,305	207
	RP-Cit low	2,077	2,254	200
	RP-Cit hi	2,080	2,344	264
	RP-GAA low	2,029	2,223	194
	RP-GAA hi	2,114	2,292	178
Source	Arg	2,079	2,300	221
	Cit	2,079	2,303	235
	GAA	2,072	2,258	186
Level	Low	2,055	2,258	211
	High	2,097	2,314	216
SEM		14.26	17.30	10.99
P-values	Source	0.975	0.509	0.174
	Level	0.143	0.115	0.837
	Source × level	0.511	0.617	0.184
<i>One-way ANOVA results</i>				
Treatment	SP	2,073	2,305	232
	RP	2,072	2,286	214
	RP-Arg low	2,059	2,295	236
	RP-Arg hi	2,098	2,305	207
	RP-Cit low	2,077	2,254	200
	RP-Cit hi	2,080	2,344	264
	RP-GAA low	2,029	2,223	194
	RP-GAA hi	2,114	2,292	178
SEM		13.00	15.24	8.96
P-value		0.863	0.680	0.314

SP: A standard protein diet contains 15.8% crude protein.

RP: A reduced protein diet contains 13.8% crude protein.

RP-Arg low, RP-Cit low, RP-GAA low: RP diet supplemented with Arg, Cit and GAA at the low level (0.06% or 0.6 g/kg), respectively.

RP-Arg hi, RP-Cit hi, RP-GAA hi: RP diet supplemented with Arg, Cit and GAA at the high level (0.12% or 1.2 g/kg), respectively.

3.2.1.2 Internal and external egg quality

The external egg quality, internal egg quality and egg proportions of hens fed the dietary treatments at 75 weeks of age are presented in Tables 21, 22 and 23, respectively. All external egg quality parameters, including shell breaking strength, shell thickness, egg length, egg width, egg shape index and shell reflectivity, were not different between the dietary treatments at 75 weeks of age (Table 21). Hens fed the diets added with Cit tended to have higher yolk diameter compared to hens fed the diets added with GAA at 75 weeks of age, as shown by the main effect of Arg source ($P = 0.086$; Table 22). Furthermore, higher yolk weights were observed in hens offered the diets supplemented with Cit and Arg compared to those offered the diets supplemented with GAA at 75 weeks of age, as shown by the main effect of Arg source ($P = 0.018$; Table 23). The one-way ANOVA results showed that hens fed the RP-Arg low diet had higher yolk weight compared to hens fed the RP-GAA low diet at 75 weeks of age ($P = 0.024$; Table 23). Whereas, all egg quality parameters were not different between the SP and RP treatment groups at 75 weeks of age.

Table 21 External egg quality of hens fed dietary treatments in Study 2 at week 75

Effects		Shell breaking strength (Kgf)	Shell thickness (mm)	Egg length (mm)	Egg width (mm)	Egg shape index	Reflectivity (%)
<i>Two-way ANOVA results (2 × 3 factorial arrangement of treatments)</i>							
Treatment	RP-Arg low	3.68	0.432	58.4	45.4	0.778	28.5
	RP-Arg hi	3.66	0.435	59.1	45.2	0.766	28.6
	RP-Cit low	3.16	0.422	58.5	45.0	0.770	27.8
	RP-Cit hi	3.72	0.428	58.0	45.0	0.777	27.5
	RP-GAA low	3.83	0.426	57.3	44.7	0.781	28.7
	RP-GAA hi	3.74	0.435	57.8	44.7	0.773	26.2
Source	Arg	3.67	0.434	58.8	45.3	0.772	28.5
	Cit	3.47	0.425	58.2	45.0	0.774	27.6
	GAA	3.78	0.431	57.6	44.7	0.777	27.4
Level	Low	3.58	0.427	58.1	45.1	0.777	28.4
	High	3.71	0.433	58.3	45.0	0.772	27.4
SEM		0.11	0.003	0.25	0.16	0.003	0.49
P-values	Source	0.477	0.431	0.165	0.338	0.849	0.648
	Level	0.521	0.284	0.589	0.812	0.516	0.350
	Source × level	0.412	0.931	0.630	0.903	0.515	0.512
<i>One-way ANOVA results</i>							
Treatment	SP	3.63	0.418	57.7	44.3	0.767	26.1
	RP	2.97	0.430	59.4	44.7	0.755	27.3
	RP-Arg low	3.68	0.432	58.4	45.4	0.778	28.5
	RP-Arg hi	3.66	0.435	59.1	45.2	0.766	28.6
	RP-Cit low	3.16	0.422	58.5	45.0	0.770	27.8
	RP-Cit hi	3.72	0.428	58.0	45.0	0.777	27.5
	RP-GAA low	3.83	0.426	57.3	44.7	0.781	28.7
	RP-GAA hi	3.74	0.435	57.8	44.7	0.773	26.2
SEM		0.10	0.003	0.22	0.14	0.003	0.41
P-value		0.338	0.681	0.284	0.495	0.521	0.594

SP: A standard protein diet contains 15.8% crude protein.

RP: A reduced protein diet contains 13.8% crude protein.

RP-Arg low, RP-Cit low, RP-GAA low: RP diet supplemented with Arg, Cit and GAA at the low level (0.06% or 0.6 g/kg), respectively.

RP-Arg hi, RP-Cit hi, RP-GAA hi: RP diet supplemented with Arg, Cit and GAA at the high level (0.12% or 1.2 g/kg), respectively.

Table 22 Internal egg quality of hens fed dietary treatments in Study 2 at week 75

Effects		Albumen height (mm)	Yolk colour	Haugh unit	Yolk height (mm)	Yolk diameter (mm)	Yolk index
<i>Two-way ANOVA results (2 × 3 factorial arrangement of treatments)</i>							
Treatment	RP-Arg low	7.92	12.0	86.0	21.9	44.5	0.496
	RP-Arg hi	7.67	12.9	83.7	21.5	42.4	0.520
	RP-Cit low	7.68	12.7	84.4	21.3	43.2	0.495
	RP-Cit hi	7.85	12.5	85.7	21.7	44.2	0.492
	RP-GAA low	8.99	12.3	93.0	21.1	42.1	0.503
	RP-GAA hi	7.86	12.5	85.8	21.0	42.3	0.489
Source	Arg	7.79	12.5	84.9	21.7	43.4	0.508
	Cit	7.78	12.6	85.2	21.5	43.8	0.494
	GAA	8.40	12.4	89.2	21.0	42.2	0.496
Level	Low	8.23	12.3	88.0	21.4	43.3	0.498
	High	7.80	12.6	85.1	21.4	43.0	0.499
SEM		0.22	0.17	1.47	0.14	0.31	0.004
P-values	Source	0.439	0.961	0.402	0.151	0.086	0.332
	Level	0.355	0.371	0.331	0.857	0.604	0.874
	Source × level	0.490	0.381	0.498	0.589	0.105	0.175
<i>One-way ANOVA results</i>							
Treatment	SP	8.49	12.9	90.7	20.9	43.1	0.487
	RP	7.78	12.5	85.1	21.6	43.0	0.502
	RP-Arg low	7.92	12.0	86.0	21.9	44.5	0.496
	RP-Arg hi	7.67	12.9	83.7	21.5	42.4	0.520
	RP-Cit low	7.68	12.7	84.4	21.3	43.2	0.495
	RP-Cit hi	7.85	12.5	85.7	21.7	44.2	0.492
	RP-GAA low	8.99	12.3	93.0	21.1	42.1	0.503
	RP-GAA hi	7.86	12.5	85.8	21.0	42.3	0.489
SEM		0.19	0.14	1.23	0.12	0.26	0.003
P-value		0.640	0.734	0.528	0.280	0.196	0.393

SP: A standard protein diet contains 15.8% crude protein.

RP: A reduced protein diet contains 13.8% crude protein.

RP-Arg low, RP-Cit low, RP-GAA low: RP diet supplemented with Arg, Cit and GAA at the low level (0.06% or 0.6 g/kg), respectively.

RP-Arg hi, RP-Cit hi, RP-GAA hi: RP diet supplemented with Arg, Cit and GAA at the high level (0.12% or 1.2 g/kg), respectively.

Table 23 Egg proportions of hens fed dietary treatments in Study 2 at week 75

Effects		Yolk weight (g)	Albumen weight (g)	Shell weight (g)	Yolk (%)	Albumen (%)	Shell (%)
<i>Two-way ANOVA results (2 × 3 factorial arrangement of treatments)</i>							
Treatment	RP-Arg low	18.2	42.2	6.40	27.3	63.1	9.61
	RP-Arg hi	17.1	43.6	6.51	26.1	64.3	9.62
	RP-Cit low	18.0	42.7	6.14	26.9	63.9	9.19
	RP-Cit hi	17.6	41.5	6.31	26.9	63.4	9.66
	RP-GAA low	16.4	41.6	6.17	25.5	64.8	9.63
	RP-GAA hi	16.7	41.4	6.35	26.0	64.2	9.85
Source	Arg	17.7 ^b	42.9	6.45	26.7	63.7	9.61
	Cit	17.7 ^b	42.0	6.23	26.9	63.6	9.46
	GAA	16.6 ^a	41.5	6.26	25.8	64.5	9.74
Level	Low	17.5	42.1	6.25	26.5	64.0	9.49
	High	17.1	42.1	6.38	26.3	63.9	9.71
SEM		0.20	0.43	0.07	0.25	0.25	0.07
P-values	Source	0.018	0.407	0.309	0.129	0.302	0.243
	Level	0.294	0.981	0.268	0.644	0.991	0.118
	Source × level	0.277	0.487	0.970	0.390	0.280	0.442
<i>One-way ANOVA results</i>							
Treatment	SP	16.5 ^{ab}	40.8	5.99	26.1	64.4	9.49
	RP	16.8 ^{ab}	42.7	6.19	25.6	65.0	9.41
	RP-Arg low	18.2 ^b	42.2	6.40	27.3	63.1	9.61
	RP-Arg hi	17.1 ^{ab}	43.6	6.51	26.1	64.3	9.62
	RP-Cit low	18.0 ^{ab}	42.7	6.14	26.9	63.9	9.19
	RP-Cit hi	17.6 ^{ab}	41.5	6.31	26.9	63.4	9.66
	RP-GAA low	16.4 ^a	41.6	6.17	25.5	64.8	9.63
	RP-GAA hi	16.7 ^{ab}	41.4	6.35	26.0	64.2	9.85
SEM		0.16	0.35	0.06	0.20	0.21	0.06
P-value		0.024	0.540	0.356	0.263	0.319	0.398

^{a,b} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

SP: A standard protein diet contains 15.8% crude protein.

RP: A reduced protein diet contains 13.8% crude protein.

RP-Arg low, RP-Cit low, RP-GAA low: RP diet supplemented with Arg, Cit and GAA at the low level (0.06% or 0.6 g/kg), respectively.

RP-Arg hi, RP-Cit hi, RP-GAA hi: RP diet supplemented with Arg, Cit and GAA at the high level (0.12% or 1.2 g/kg), respectively.

3.2.1.3 Serum uric level and ileal protein and energy digestibility

The results on ileal protein and energy digestibility and serum uric acid level of hens fed the dietary treatments at 75 weeks of age are given in Table 24. No significant Arg source × level interactions were obtained for the ileal protein and energy digestibility. Decreasing Arg level tended to decrease ileal energy digestibility at 75 weeks of age, as shown by the main effect of Arg level ($P = 0.083$). Furthermore, the one-way ANOVA results showed that Arg supplementation to the RP diet at the low level increased ileal energy digestibility compared to the RP and SP diets at 75 weeks of age ($P = 0.037$). Similarly, no significant Arg source × level interaction was obtained for the serum uric acid level. Likewise, Arg level and Arg source as the main effect did not affect the serum uric acid levels of the treatment groups. However, the one-way ANOVA results showed that hens fed the RP-Cit low diet tended to have lower serum uric acid levels compared to those fed the SP diet at 75 weeks of age ($P = 0.091$).

Table 24 Ileal protein and energy digestibility and serum uric level of hens fed the dietary treatments in Study 2 at week 75

Effects		Ileal protein digestibility coefficient	Ileal energy digestibility coefficient	Serum uric acid level (mg/dL)
<i>Two-way ANOVA results (2 × 3 factorial arrangement of treatments)</i>				
Treatment	RP-Arg low	0.828	0.761	5.04
	RP-Arg hi	0.802	0.737	5.07
	RP-Cit low	0.819	0.751	4.75
	RP-Cit hi	0.813	0.726	5.43
	RP-GAA low	0.815	0.746	5.19
	RP-GAA hi	0.819	0.723	5.00
Source	Arg	0.815	0.749	5.05
	Cit	0.816	0.737	5.09
	GAA	0.817	0.734	5.09
Level	Low	0.821	0.753	4.99
	High	0.811	0.729	5.17
SEM		0.004	0.007	0.09
P-values	Source	0.986	0.647	0.990
	Level	0.318	0.083	0.308
	Source × level	0.418	0.999	0.130
<i>One-way ANOVA results</i>				
Treatment	SP	0.794	0.698 ^a	5.77
	RP	0.798	0.698 ^a	5.19
	RP-Arg low	0.828	0.761 ^b	5.04
	RP-Arg hi	0.802	0.737 ^{ab}	5.07
	RP-Cit low	0.819	0.751 ^{ab}	4.75
	RP-Cit hi	0.813	0.726 ^{ab}	5.43
	RP-GAA low	0.815	0.746 ^{ab}	5.19
	RP-GAA hi	0.819	0.723 ^{ab}	5.00
SEM		0.004	0.006	0.09
P-value		0.367	0.037	0.091

SP: A standard protein diet contains 15.8% crude protein.

RP: A reduced protein diet contains 13.8% crude protein.

RP-Arg low, RP-Cit low, RP-GAA low: RP diet supplemented with Arg, Cit and GAA at the low level (0.06% or 0.6 g/kg), respectively.

RP-Arg hi, RP-Cit hi, RP-GAA hi: RP diet supplemented with Arg, Cit and GAA at the high level (0.12% or 1.2 g/kg), respectively.

3.2.1.4 Bone quality parameters

The bone quality parameters of hens fed the dietary treatments at 75 weeks of age are shown in Table 25. Arg source $\times$ level interactions were observed for tibia ($P = 0.014$) and femur ($P = 0.006$) breaking strength where increasing dietary Arg and GAA supplemental levels to the RP diets reduced tibia and femur breaking strength, while increasing dietary Cit supplemental level increased tibia and femur breaking strength in laying hens at 75 weeks of age. Additionally, hens supplemented with Arg sources at the low levels had higher fresh tibia weight ($P = 0.020$), higher femur length ($P = 0.038$) and tended to have higher fresh femur weight ($P = 0.057$) compared to hens supplemented with Arg sources at the high levels at 75 weeks of age, as shown by the main effect of Arg level. The one-way ANOVA results showed that hens offered the RP-Cit hi and RP-GAA low diets had higher tibia breaking strength compared to hens offered the RP diet at 75 weeks of age ($P = 0.003$). Similarly, hens fed the RP-Cit hi diet had increased femur breaking strength compared to those fed the RP diet at 75 weeks of age ($P = 0.019$). Hens offered the RP-GAA hi diet tended to have decreased tibia width ($P = 0.079$) and femur length ($P = 0.097$) compared to hens offered the SP diet at 75 weeks of age (Table 25).

Table 25 Bone quality parameters of hens fed dietary treatments in Study 2 at week 75

Effects		Tibia				Femur			
		Breaking strength (N)	Weight of fresh bone (g)	Length (mm)	Width (mm)	Breaking strength (N)	Weight of fresh bone (g)	Length (mm)	Width (mm)
<i>Two-way ANOVA results (2 × 3 factorial arrangement of treatments)</i>									
Treatment	RP-Arg low	228 ^b	13.1	126	7.83	269 ^{bc}	11.7	89.2	8.97
	RP-Arg hi	180 ^a	12.7	124	7.87	206 ^a	11.0	88.6	8.94
	RP-Cit low	196 ^a	12.8	125	7.85	217 ^a	11.3	89.7	8.84
	RP-Cit hi	233 ^{bc}	12.7	124	7.80	290 ^c	11.1	88.1	8.86
	RP-GAA low	261 ^c	13.7	125	7.81	286 ^c	11.6	89.2	8.94
	RP-GAA hi	207 ^{ab}	12.0	123	7.55	243 ^b	10.3	87.4	8.64
Source	Arg	204	12.9	125	7.85	237	11.3	88.9	8.95
	Cit	214	12.7	124	7.83	253	11.2	88.9	8.85
	GAA	234	12.9	124	7.68	265	11.0	88.3	8.79
Level	Low	228	13.2 ^b	125	7.83	257	11.5	89.4 ^b	8.92
	High	207	12.5 ^a	124	7.74	246	10.8	88.0 ^a	8.81
SEM		8.45	0.17	0.46	0.04	10.58	0.18	0.32	0.07
P-values	Source	0.211	0.900	0.647	0.185	0.380	0.650	0.687	0.648
	Level	0.296	0.020	0.122	0.270	0.880	0.057	0.038	0.462
	Source × level	0.014	0.073	0.899	0.305	0.006	0.435	0.747	0.622
<i>One-way ANOVA results</i>									
Treatment	SP	222 ^{ab}	13.4	126	8.07	265 ^{ab}	11.4	90.5	8.96
	RP	162 ^a	12.9	124	8.01	202 ^a	11.2	88.6	8.95
	RP-Arg low	228 ^{ab}	13.1	126	7.83	269 ^{ab}	11.7	89.2	8.97
	RP-Arg hi	180 ^{ab}	12.7	124	7.87	206 ^{ab}	11.0	88.6	8.94
	RP-Cit low	196 ^{ab}	12.8	125	7.85	217 ^{ab}	11.3	89.7	8.84
	RP-Cit hi	233 ^b	12.7	124	7.80	290 ^b	11.1	88.1	8.86
	RP-GAA low	261 ^b	13.7	125	7.81	286 ^{ab}	11.6	89.2	8.94
	RP-GAA hi	207 ^{ab}	12.0	123	7.55	243 ^{ab}	10.3	87.4	8.64
SEM		7.46	0.15	0.40	0.04	9.16	0.15	0.26	0.06
P-value		0.003	0.101	0.484	0.079	0.019	0.462	0.097	0.922

The means and pooled standard errors of tibia and femur breaking strength were calculated from raw untransformed data, while statistical analysis was performed using log-transformed data.

^{a,b} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

SP: A standard protein diet contains 15.8% crude protein.

RP: A reduced protein diet contains 13.8% crude protein.

RP-Arg low, RP-Cit low, RP-GAA low: RP diet supplemented with Arg, Cit and GAA at the low level (0.06% or 0.6 g/kg), respectively.

RP-Arg hi, RP-Cit hi, RP-GAA hi: RP diet supplemented with Arg, Cit and GAA at the high level (0.12% or 1.2 g/kg), respectively.

3.2.2 Discussion

Given that Arg, GAA, and Cit can all contribute to Arg-related physiological functions in poultry (Dao et al. 2021a), and considering that Cit supplementation has been shown to elevate plasma Arg concentrations and enhance nitric oxide (NO) synthesis more efficiently than Arg supplementation (Schwedhelm et al. 2008; Lassala et al. 2009; Wijnands et al. 2012), it was hypothesised that Cit may confer greater benefits when included in layer hen diets during the late laying phase. In the present study, neither low nor high levels of Arg or GAA supplementation in RP diets influenced the production performance of laying hens. These findings align with those of Khakran et al. (2018), who reported no significant effects on feed intake, egg mass and hen day egg production in response to incremental GAA levels (from 0 to 0.171%), although a reduction in egg weight was observed at the highest inclusion

level (0.171%) between 31 and 42 weeks of age. Conversely, Salah et al. (2020) revealed that dietary supplementation with 1.5 g/kg of GAA significantly increased egg mass in laying hens compared to hens fed the unsupplemented diet from 72 and 78 weeks of age. Similarly, Azizollahi et al. (2024) demonstrated that inclusion of GAA at 0.6 g/kg in the layer hen diet increased egg mass from 52 to 64 weeks of age. These inconsistent findings may reflect variability in the response of aged laying hens to GAA supplementation, potentially attributable to differences in dietary composition, hen age, and the dosage of GAA used across studies. In contrast, Cit supplementation at the high level in the RP diet resulted in increased feed intake and egg mass across the experimental period (60–75 weeks of age), without altering hen body weight, when compared to both the RP and SP treatments in this study. Notably, only Cit supplementation stimulated feed intake, while Arg and GAA had no effect on this parameter in the current study. Furthermore, Cit consistently enhanced egg mass and/or egg weight relative to Arg and GAA treatments. Collectively, these results indicate that dietary Cit supplementation exerted more favourable effects on laying performance than either Arg or GAA under the conditions of this study.

Arginine is a key AA involved in multiple metabolic pathways, notably protein synthesis, growth hormone stimulation, and NO production (Kwak et al. 1999; Newsholme et al. 2005; Szabó et al. 2014). Nitric oxide regulates pituitary nitric oxide synthase activity, which influences the secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH) and may enhance gonadotropin-releasing hormone (GnRH) release. This pathway supports follicular development and ovulation, potentially increasing egg production, egg weight, and egg mass (Uyanga et al. 2022). Moreover, Arg and NO are thought to play regulatory roles in modulating feed intake and appetite-related pathways in poultry (Tan et al. 2007; Yang & Denbow 2007; Wang et al. 2014). This may partly explain the higher feed intake observed in hens offered the RP diet with the high Cit supplemental level in this study. While Arg, GAA, and Cit can all serve as sources of Arg activity in poultry diets (Dao et al. 2021a), previous studies have demonstrated that Arg supplementation can improve egg production metrics, egg quality, and/or feed efficiency in laying hens (Yuan et al. 2016; Sun et al. 2022), laying ducks (Xia et al. 2017), and broiler breeder hens (Silva et al. 2012; Li et al. 2021). However, several mechanistic factors may explain the superior performance outcomes observed with Cit supplementation compared to Arg in this study. Firstly, Cit resists catabolism by intestinal arginase, thereby maintaining higher plasma levels post-ingestion, whereas Arg is rapidly degraded at the mucosal level (McCarty 2010). Moreover, dietary Arg undergoes substantial first-pass metabolism in the liver, leading to its conversion into urea and other nitrogenous by-products (Allerton et al. 2018), which limits its systemic availability. In contrast, Cit is absorbed primarily in the kidneys, where it is converted into Arg through the enzymatic actions of argininosuccinate synthetase and argininosuccinate lyase, subsequently entering systemic circulation as Arg (El-Hattab et al. 2012). Secondly, Cit may present a lower risk of gastrointestinal irritation or toxicity compared to Arg (Grimble 2007). Thirdly, due to Arg's involvement in diverse metabolic functions, including roles in the urea cycle, polyamine synthesis, creatine production, and NO generation, its utilisation efficiency may be compromised (Baker 2005). Conversely, dietary Cit may preferentially support NO synthesis via the Arg-Cit-Arg cycle, thereby enhancing Arg- and NO-mediated physiological responses more effectively than direct Arg supplementation (Ball et al. 2007; Fernandes & Murakami 2010).

Reducing CP levels in layer diets while supplementing with arginine precursors represents a viable strategy to lower feed costs without compromising hen performance. According to Badawi et al. (2019), a 1% reduction in dietary CP can yield a cost savings of approximately US\$5 per tonne of poultry feed. Additionally, lower CP diets have been associated with improved housing conditions through reduced nitrogen excretion and associated emissions (Chrystal et al. 2020). According to Chrystal et al. (2020), the development of RP diets holds significant potential for advancing sustainable poultry production by enabling more efficient use of resources. In the current study, the supplementation of AA to the RP diets enabled a reduction of approximately 40% in soybean meal level. Ji et al. (2014) reported that feeding low protein diets to Hy-Line laying hens had no detrimental effect on egg

production or FCR between 21 and 34 weeks of age. Similarly, Keshavarz and Austic (2004) found that egg production in laying hens receiving a 13% protein diet supplemented with essential AAs was comparable to that of hens fed a 16% protein diet from 36 to 48 weeks of age. In another study, Dao et al. (2021b) demonstrated that RP diet (13% CP) along with supplementation of Arg metabolites maintained egg production levels similar to those achieved with higher protein diets (17% CP) during 20 to 29 weeks of age. Novak et al. (2006) observed that older hens may exhibit a more pronounced decline in egg production when subjected to low protein diets, suggesting a delayed sensitivity to protein or AA deficiencies. However, in the present study, a moderate reduction in the dietary CP level by two percentage points balanced with essential AA was sufficient to sustain productive performance in aged laying hens from 60 to 75 weeks of age. Furthermore, high-level Cit supplementation in the RP diet significantly increased egg mass compared to both the RP and SP treatments. This result highlights the potential of Cit as a functional substitute for Arg in poultry diets and suggests its applicability for improving productivity in hens during the late laying phases.

The results of this study showed that Arg, GAA and Cit supplementation to the RP diets did not affect external and internal egg quality parameters. These findings align with those observed by Dao et al. (2021b), who reported that supplementation of Arg, GAA, or Cit to an Arg-deficient RP diet did not influence the shell breaking strength, shell reflectivity, albumen height, yolk color and Haugh unit of laying hens during 20 to 39 weeks of age. Furthermore, the internal and external egg quality parameters were also not different between the RP and SP treatments in this study. This observation is consistent with prior research, including work by Mousavi et al. (2013), which demonstrated that shell breaking strength, shell thickness, yolk color, albumen height and Haugh unit were comparable in hens offered diets containing CP level of 15.5%, 16.5% or 17.5% during 25 to 33 weeks of age. Similarly, other investigators have reported no significant differences in external and internal egg quality parameters when hens were fed diets with varying CP levels (Novak et al. 2006; Oluwabiya et al. 2022b). Nevertheless, a notable finding in the present study was that supplementation of Arg or Cit to the RP diets significantly increased yolk weight in comparison to the GAA-supplemented RP diets. This finding is in agreement with Al-Daraji (2011), who found that laying turkeys supplemented with 0.1% Arg had increased yolk weight compared to those supplemented with 0.05% Arg from 32 to 47 weeks of age. Supporting this, Xia et al. (2017) reported that diets for laying ducks with Arg concentrations of 1.26% increased egg yolk percentage in comparison to other Arg levels (0.66, 0.86, 1.06, and 1.46%) from 17 to 31 weeks of age. The increase in yolk weight observed with Arg and Cit supplementation may be attributed to NO-mediated hormonal regulation. Nitric oxide has been proposed to stimulate the secretion of GnRH, FSH and LH hormones, thereby promoting follicular development and yolk deposition. Additionally, egg yolk physiologically contains higher levels of Arg than albumen (Lieboldt et al., 2015), which may reflect the increased Arg availability and/or greater protein synthesis in the RP-Arg and RP-Cit groups relative to the RP-GAA groups in the current study. Previous research on GAA supplementation in layer hen diets has yielded variable outcomes. For instance, Salah et al. (2020) found that increasing dietary GAA level from 0.05% to 0.15% increased yolk index, shell thickness, and Haugh unit in hens aged 72 to 78 weeks. In contrast, Dao et al. (2021b) reported no effects of dietary GAA supplementation on egg quality traits in hens fed Arg-deficient RP diets from 20 to 39 weeks of age. Such discrepancies may stem from differences in hen age, hen breed, dietary CP level, and overall nutrient composition across studies.

In the present study, supplementation of the RP diet with a low level of Arg resulted in a significant increase in ileal energy digestibility compared to both the RP and SP diets at 75 weeks of age. This finding aligns with previous studies indicating that Arg can improve intestinal morphology and nutrient absorption in poultry, potentially through its involvement in polyamine synthesis and NO production (Khajali & Wideman 2010; Abdulkarimi et al. 2017). These results support the notion that optimal levels of dietary Arg can improve energy utilisation in aged laying hens, while excessive supplementation may have adverse effects on energy digestibility. The results of the current study also showed that hens fed

the RP diet supplemented with the low level of Cit exhibited a trend toward lower serum uric acid levels compared to those receiving the SP diet at 75 weeks of age. Serum uric acid level is considered a negative indicator of net protein utilisation, reflecting the balance between protein intake, assimilation, and catabolism (Robin et al. 1987). Previous research has shown that higher dietary CP levels are associated with elevated serum uric acid in laying hens, likely due to increased intake of AA in the form of intact protein, which characterises high protein diets compared to RP formulations (Hilliar et al. 2019; Dao et al. 2021b). Moreover, adequate levels of dietary Arg may promote protein synthesis, whereas excessive Arg can disrupt the Arg-to-lysine ratio, leading to elevated renal arginase activity and increased conversion of Arg into uric acid (Carvalho et al. 2015). Supporting this, Yuan et al. (2016) reported a rise in serum uric acid level in broilers fed an SP diet (17% CP) as dietary Arg levels increased from 1.27% to 1.66%. In the present study, the observed trend toward reduced serum uric acid levels in hens fed the RP diet supplemented with the low level of Cit suggests enhanced net protein utilisation in this group compared to those receiving the SP diet.

Arginine is known to contribute to bone development through its involvement in collagen synthesis and the formation of connective tissues (Corzo et al. 2003; Jahanian 2009). In the current study, dietary supplementation of Cit at a high level in the RP diet was associated with improved bone breaking strength in aged laying hens. In contrast, high-level supplementation of Arg or GAA did not confer similar benefits when compared to their respective low-level inclusions. Osteoporosis is a leading cause of mortality in caged laying hens (Mayeda & Ernst 2008). Therefore, dietary supplementation with Cit may enhance bone health and welfare, potentially reducing associated economic losses. The beneficial effect of Cit on bone strength may be attributed to its metabolic role as a precursor of Arg, which is subsequently converted to NO. Nitric oxide has been shown to inhibit osteoclast-mediated bone resorption and stimulate osteoblast proliferation, thereby promoting bone formation and mineralisation (Jin et al. 2021). Supporting this mechanism, Meesters et al. (2020) demonstrated that daily oral Cit supplementation at 5 g/kg bodyweight increased plasma concentrations of Cit and ornithine in wild mice after 14 days, which may reflect enhanced collagen synthesis via polyamine production. These findings are consistent with the observed improvements in bone integrity in our study. These findings suggest that Cit may offer specific advantages for skeletal health during the late laying phases. Such improvements in bone integrity may have important implications for enhancing hen welfare and potentially extending the productive lifespan of laying hens up to 100 weeks of age.

3.3 Economic analysis and economic modelling for optimal reduced-protein diets

3.3.1 Results

3.3.1.1 Economic analysis

The feed costs (AU\$; in 2024) per kilogram of eggs produced by dietary treatments in studies 1 and 2 are presented in Tables 26 and 27, respectively. No significant differences were observed for the feed cost per kilogram of eggs produced in Study 1. However, in Study 2, hens offered the RP and RP-Cit low diets had lower feed costs per kilogram of eggs produced compared to hens offered the SP diet from 60 to 75 weeks of age, as shown by the one-way ANOVA results ($P = 0.007$; Table 27). The diet cost of SP diets was \$348.94/tonne and RP diets was \$326.79/tonne, with the difference in diet cost owing greatly to the reduction of soybean meal included in RP diets.

Table 26 Economic analysis for Study 1 from 35 to 57 weeks of age

Effects		AU\$/kg of eggs
Treatment	1 (SP; 600 FTU phytase; 100 g/tonne XB)	1.057
	2 (SP; 600 FTU phytase; 150 g/tonne XB)	1.056
	3 (SP; 1200 FTU phytase; 100 g/tonne XB)	1.037
	4 (SP; 1200 FTU phytase; 150 g/tonne XB)	1.064
	5 (RP; 600 FTU phytase; 100 g/tonne XB)	1.030
	6 (RP; 600 FTU phytase; 150 g/tonne XB)	1.038
	7 (RP; 1200 FTU phytase; 100 g/tonne XB)	1.054
	8 (RP; 1200 FTU phytase; 150 g/tonne XB)	1.052
<i>Main effect</i>		
Protein	Standard	1.053
	Reduced	1.044
PhyG	Standard	1.045
	High	1.052
XB	Standard	1.044
	High	1.053
SEM		0.006
<i>P-values</i>		
Main effect	Protein	0.419
	PhyG	0.565
	XB	0.484
Interaction	Protein × PhyG	0.272
	Protein × XB	0.648
	PhyG × XB	0.681
	Protein × PhyG × XB	0.414

Table 27 Economic analysis for Study 2 from 60 to 75 weeks of age

Effects		AU\$/kg of eggs
<i>Two-way ANOVA results (2 × 3 factorial arrangement of treatments)</i>		
Treatment	RP-Arg low	1.112
	RP-Arg hi	1.092
	RP-Cit low	1.076
	RP-Cit hi	1.113
	RP-GAA low	1.086
	RP-GAA hi	1.094
Source	Arg	1.102
	Cit	1.096
	GAA	1.090
Level	Low	1.092
	High	1.099
SEM		0.007
P-values	Source	0.787
	Level	0.655
	Source × level	0.286
<i>One-way ANOVA results</i>		
Treatment	SP	1.155 ^b
	RP	1.079 ^a
	RP-Arg low	1.112 ^{ab}
	RP-Arg hi	1.092 ^{ab}
	RP-Cit low	1.076 ^a
	RP-Cit hi	1.113 ^{ab}
	RP-GAA low	1.086 ^{ab}
	RP-GAA hi	1.094 ^{ab}
SEM		0.006
P-value		0.007

^{a,b} Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

SP: A standard protein diet contains 15.8% crude protein.

RP: A reduced protein diet contains 13.8% crude protein.

RP-Arg low, RP-Cit low, RP-GAA low: RP diet supplemented with Arg, Cit and GAA at the low level (0.06% or 0.6 g/kg), respectively.

RP-Arg hi, RP-Cit hi, RP-GAA hi: RP diet supplemented with Arg, Cit and GAA at the high level (0.12% or 1.2 g/kg), respectively. The cost of inclusion for L-Arg was \$5700/tonne, Cit at \$7100/tonne and GAA at \$7200/tonne.

3.3.1.2 Economic modelling for optimal reduced-protein diets

As only Study 2 demonstrated a difference in feed cost, this was explored more closely via economic models. While the 0.06% level of citrulline showed the least feed cost per kilo of eggs produced, upon the determination of margin cost and marginal revenue, it is evident that it is still economically valid to utilise the higher citrulline level of 0.12%, as the marginal cost is still lower than the marginal revenue at this level. Furthermore, the cost of many AA supplements that are not already produced in mass are reducing over time, as production is become more large-scale. Thus, over time citrulline supplementation will likely become even more economical.

Table 28 Calculated marginal cost (A) and revenue (B) of citrulline inclusion**(A)**

Citrulline level (%)	Average quantity	Average cost	Change in quantity	Change in cost	Marginal cost
0	8.76	3.56	-	-	-
0.06	8.91	3.74	0.15	0.171	1.123
0.12	9.09	4.02	0.17	0.283	1.621

(B)

Citrulline level (%)	Average quantity	Average revenue	Change in quantity	Change in revenue	Marginal revenue
0	8.76	15.11	-	-	-
0.06	8.91	15.62	0.15	0.50	3.315
0.12	9.09	15.91	0.17	0.30	1.696

The effects of progressively reduced prices of Leu, Ile, and Arg on diet costs and soybean meal inclusion levels in simulated SP and RP diets are presented in Figures 3 and 4, respectively. The detailed ingredient compositions and calculated nutrient profiles of the simulated basal SP and RP diets are provided in Appendix Table 1. Results showed that, at 2024 market prices, RP diets were already less expensive than SP diets. As the prices of Leu, Ile, and Arg were reduced, the cost of RP diets declined further, though only marginally. This limited cost reduction is attributed to the relatively low inclusion rates of these AA in the diets (maximum 0.054%). Diets formulated with 12% canola meal were more cost-effective than those with lower canola meal inclusion. This is due to the interchangeable use of canola meal and soybean meal in the formulations, with canola meal being the less expensive protein source. Interestingly, while the cost of the SP diet containing 4% canola meal remained unaffected by AA price reductions, the cost of the SP diet with 12% canola meal decreased when the prices of Leu, Ile, and Arg were reduced by at least 60%. Specifically, Arg was incorporated into the SP diet with 12% canola meal to reduce soybean meal inclusion when its price dropped by at least 60%.

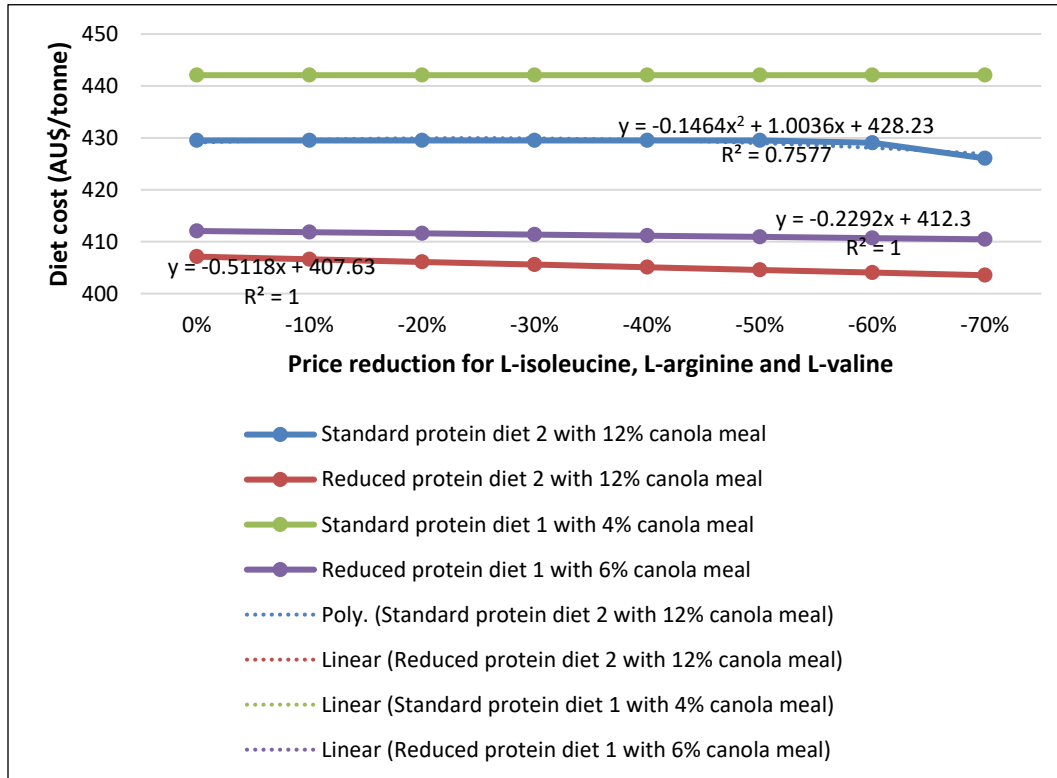


Figure 3 Costs of simulated standard and reduced protein diets according to step-wise reduced prices of leucine, isoleucine and arginine

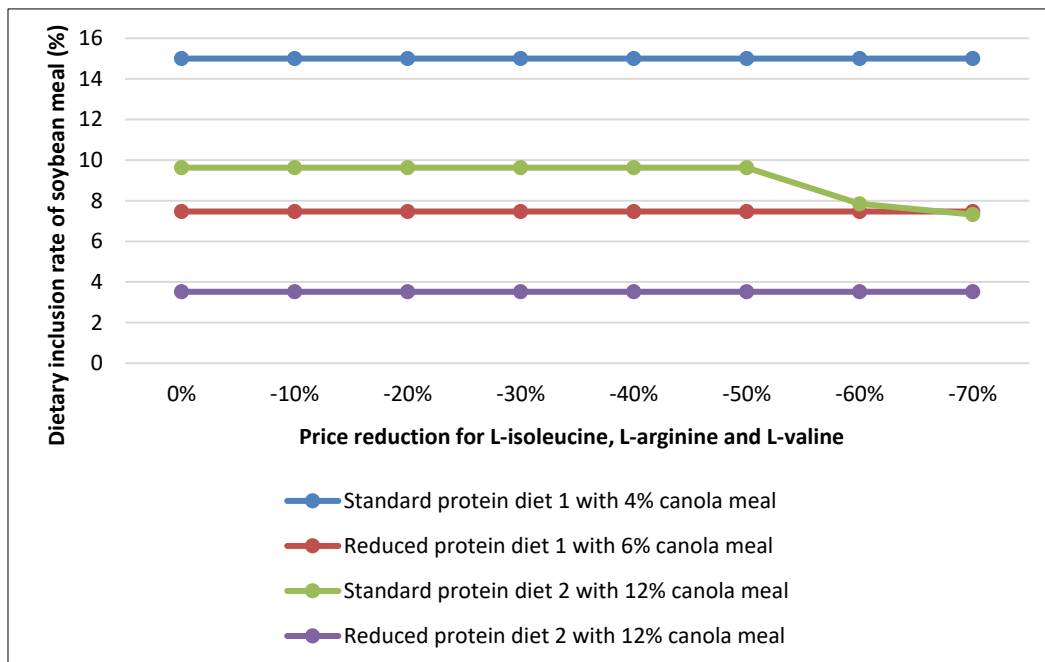


Figure 4 Inclusion rate of soybean meal in simulated standard and reduced protein diets according to step-wise reduced prices of leucine, isoleucine and arginine

3.3.2 Discussion

According to Chrystal et al. (2020), the successful development and implementation of RP diets represent a key strategy for achieving more sustainable poultry production through reduced resource use. Results from Study 2 demonstrated that the feed cost per kilogram of eggs produced was AU\$0.079 and AU\$0.076 lower for the RP and RP-Cit Low diets, respectively, compared to the SP diet. In addition, the price of soybean meal has risen substantially in recent years. For example, in 2022, the cost of soybean meal doubled from approximately AU\$500 to AU\$1000 per tonne. As a result, there has been growing interest in RP diets to reduce reliance on soybean meal, particularly in countries where soybean meal is not produced locally. Looking ahead, if the price of soybean meal continues to rise while the prices of crystalline AA such as Arg, Val, and Ile remain stable or decline, the cost advantage of RP diets over SP diets is expected to become even more pronounced. Moreover, the environmental benefits of RP diets such as reduced nitrogen excretion and lower ammonia emissions further support their adoption as a sustainable feeding strategy in poultry production.

It is important to note that Cit (which can provide Arg activity and was shown as an effective replacement in our second study) also follows a similar trend of reducing price and is a similar cost to Arg, and thus the projections regarding Arg may be applied to Cit if used in replacement of Arg. Therefore, the reducing cost of Leu, Ile and Arg will help to reduce the cost of RP diets slightly in the future, but this is limited due to the small inclusion rates. However, when the amount of canola meal in the diet increased, more synthetic AA could be incorporated, further reducing the diet cost. Thus, exploring RP diets with a focus on utilising more canola and increased synthetic AA inclusions could be an area for the future to reduce reliance on imported SBM, improve sustainability, and reduce diet cost.

The greatest cost-benefit observed regarding AA supplementation was from the replacement of Arg with Cit in RP diets, which generated an increase in egg mass and thus significantly reduced the cost of producing a kilo of eggs when compared to SP diets by 6.8%. Thus, by switching from the current SP diet to a RP diet supplemented with Cit, farmers may make significant economic gain. The economic figures used for this study were averages and thus the profitability should be explored in detail for each farm's specific situation.

4 Conclusions

In the first study, a reduction in dietary CP level from 16.5% to 14.5% led to a decrease in egg weight and a tendency toward reduced egg mass between 35 and 57 weeks of age. However, feed efficiency remained unaffected. The use of RP diets also resulted in lower albumen weight, an increased yolk proportion, and a decreased albumen proportion at 57 weeks of age. In contrast, the second study, which involved reducing dietary CP level from 15.8% to 13.8%, did not negatively impact any production parameters, egg quality traits, or nutrient digestibility in hens aged 60 to 75 weeks.

In terms of dietary enzyme supplementation, inclusion of PhyG at 600 FTU/kg and XB at 100 g/tonne proved sufficient in RP diets from 35 to 57 weeks. Elevating XB inclusion to 150 g/tonne did not offer additional benefits and was associated with reduced hen day egg production. Conversely, supplementing RP diets with 0.12% Cit in the second study significantly improved egg mass, egg and yolk weights, and tibia breaking strength in older laying hens from 60 to 75 weeks of age. These enhancements in bone strength may contribute to improved welfare and the potential to extend laying cycles toward 100 weeks of age.

Environmental benefits were also observed. In Study 1, lowering dietary protein content decreased nitrogen excretion by more than 18%, reduced excreta moisture, and enhanced the digestibility of DM, protein, and energy at 57 weeks of age. In Study 2, supplementing 0.06% Arg to the RP diet significantly improved ileal energy digestibility at 75 weeks of age compared to both RP and SP diets. Collectively, these results indicate that reducing dietary protein can reduce nitrogen excretion and moisture content in the excreta while improving nutrient utilisation, contributing to more sustainable poultry production.

Although dietary interventions in Study 1 had limited economic impact, findings from Study 2 demonstrated that inclusion of 0.06% Cit in the RP diets reduced feed cost per kilogram of eggs by AU\$ 0.076 (approximately 6.8%) relative to the SP control from 60 to 75 weeks of age. Moreover, while the 0.06% Cit inclusion was most cost-efficient, supplementation of Cit to the RP diet at 0.12% also remained economically justifiable, as the marginal cost of the higher Cit level was still lower than the associated marginal revenue. Therefore, transitioning from conventional SP diets to RP diets supplemented with Cit may yield meaningful economic returns for egg producers. Given the ongoing volatility in soybean meal markets, continued increases in soybean meal costs, alongside stable or declining prices of Cit and synthetic AA such as Arg, Val, and Ile, would further amplify the cost advantage of RP diets over SP diets in the future.

5 Implications and recommendations

By optimising RP diets for laying hens, this study may help to increase production efficiency while reducing carbon footprint and industry reliance on imported expensive soybean meal, leading to a more efficient and sustainable layer production. This is particularly important for Australia, which often experiences drought and must otherwise import more high protein feed ingredients for poultry diets. It appears that laying hens are more adaptive/tolerant to dietary protein reductions compared to broilers, given the high protein requirement of rapid-growing broiler strains. Thus, although soybean meal inclusion rates in layer diets may be lower than in broiler diets, the opportunity to successfully develop RP diets that meet the economic and environmental purposes may be higher in laying hens compared to broiler chickens. By reducing dietary protein level, the dietary inclusion rate of soybean meal was decreased by nearly 50% and 40% in the first and second studies in this project, respectively. This may help to reduce reliance on imported, expensive soybean meal, particularly in countries where soybean meal is not produced locally.

The reducing cost of leucine (Leu), Ile and Arg will help to reduce the cost of RP diets slightly in the future, but this is limited due to the small inclusion rates. However, when the amount of canola meal in the diet increased, more synthetic AA could be incorporated, further reducing the diet cost. Thus, exploring RP diets with a focus on utilising more canola and increased synthetic AA inclusions could be an area for the future to reduce reliance on imported SBM, improve sustainability and reduce diet cost. Additionally, further research is warranted to determine the optimal dosage of Cit for maximising production efficiency and supporting the health and welfare of laying hens during the late laying phase.

6 Appendix

Appendix Table 1 Diet composition and calculated nutrient values of simulated basal standard and reduced protein diets for economic modelling (as-is basis)

Ingredients, %	Standard protein diet 1 with 4% canola meal	Reduced protein diet 1 with 6% canola meal	Standard protein diet 2 with 12% canola meal	Reduced protein diet 2 with 12% canola meal
Wheat	30.0	36.0	30.0	33.0
Soybean meal	15.0	7.0	10.0	4.0
Sorghum	28.0	30.0	27.0	28.0
Canola meal	4.00	6.00	12.0	12.0
Barley	12.0	9.00	10.0	12.0
Limestone grit	6.80	6.80	6.80	6.80
Fine limestone	3.17	3.20	2.90	2.97
Canola oil	0.89	0.00	0.62	0.00
Salt	0.152	0.087	0.138	0.068
Sodium bicarbonate	0.100	0.200	0.100	0.212
Monocalcium phosphate	0.100	0.134	0.100	0.114
Choline Chloride 60%	0.118	0.146	0.137	0.160
L-lysine HCl	0.022	0.216	0.065	0.249
D,L-methionine	0.139	0.164	0.103	0.145
L-threonine	0.000	0.054	0.000	0.057
L-isoleucine	0.000	0.033	0.000	0.054
L-arginine	0.000	0.000	0.000	0.017
L-Valine	0.000	0.000	0.000	0.003
Layer vitamin-mineral premix	0.100	0.100	0.100	0.100
Axtra XB 201 TPT	0.010	0.010	0.010	0.010
Axtra PHY Gold 10,000 FTU/g	0.006	0.006	0.006	0.006
Pigment Jabiru red	0.004	0.004	0.004	0.004
Pigment Jabiru yellow	0.003	0.003	0.003	0.003
Total	100	100	100	100
Formula cost (AU\$/tonne)	442	412	430	407
<i>Calculated nutrient, % (otherwise as indicated)</i>				
Dry matter	90.2	89.9	90.1	89.9
AME, kcal/kg	2,690	2,690	2,690	2,690
Crude protein	15.8	13.8	15.8	13.8
Crude fat	2.67	1.92	2.58	2.02
Crude fibre	3.06	3.14	3.84	3.84
Ash	9.41	9.15	9.52	9.27
Digestible lysine	0.670	0.670	0.670	0.670
Digestible methionine	0.369	0.374	0.347	0.363
Digestible methionine + cysteine	0.600	0.600	0.600	0.600
Digestible threonine	0.496	0.470	0.493	0.470
Digestible isoleucine	0.605	0.530	0.578	0.530
Digestible leucine	1.177	1.015	1.139	0.979

Digestible tryptophan	0.184	0.156	0.182	0.154
Digestible arginine	0.878	0.702	0.843	0.690
Digestible histidine	0.333	0.284	0.334	0.274
Digestible valine	0.692	0.593	0.686	0.590
Digestible glycine	0.517	0.446	0.532	0.439
Digestible phenylalanine	0.655	0.545	0.626	0.503
Calcium	4.061	4.061	4.000	4.000
Available phosphorus	0.337	0.320	0.342	0.320
Sodium	0.160	0.160	0.160	0.160
Chloride	0.173	0.173	0.170	0.170
Choline, mg/kg	1,600	1,600	1,600	1,600
Linoleic acid	1.20	1.03	1.27	1.12

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8 Plain English Summary

Project Title:	Optimising reduced-protein diets for an efficient and sustainable layer production
Australian Eggs Limited Project No.	INNO020
Researchers Involved	A.F. Moss, T.H. Dao and A. Nawab
Organisations Involved	The University of New England, Armidale, NSW 2351
Phone	02 6773 5217
Fax	N/A
Email	amos22@une.edu.au tdao2@une.edu.au
Objectives	This project was conducted to optimise reduced protein diets for laying hens to enhance the economic efficiency and environmental sustainability of laying hen production.
Background	Recent advances in poultry nutrition have increasingly focused on the reduction of crude protein levels in layer diets, driven by potential benefits in both economic efficiency and environmental sustainability. One key environmental concern associated with high dietary crude protein is nitrogen excretion and its subsequent contribution to environmental nitrogen loading. Efforts to formulate reduced protein diets date back nearly a century, yet despite substantial investigation, there remain significant challenges and untapped opportunities in optimising these dietary strategies. Formulating reduced protein diets typically requires adjustments in ingredient composition and nutrient matrices, often involving increased inclusion of cereal grains at the expense of soybean meal. This shift alters the dietary balance of protein and starch, potentially influencing the efficacy of exogenous enzymes such as phytase and xylanase, which may perform differently depending on substrate availability. Moreover, specific amino acid and precursor supplementation has shown promise in improving laying performance under reduced protein feeding conditions. Arginine and guanidinoacetic acid, for instance, have been shown to enhance performance during the peak and late phases of egg production, respectively. Additionally, as arginine, guanidinoacetic acid, and citrulline all contribute to arginine activity within avian metabolism, citrulline supplementation may offer superior benefits due to its potential to elevate circulating arginine concentrations more effectively than direct arginine supplementation. This study was therefore designed to address key knowledge gaps in the optimisation of reduced protein diets for laying hens. Specifically, this study investigated the effects of varying enzyme inclusion levels, and supplementation of arginine, guanidinoacetic acid, and citrulline, on the productive performance of laying hens consuming reduced protein diets.

Research

Two studies were undertaken to optimise reduced protein diets for laying hens. The first study investigated the optimal inclusion rates of exogenous enzymes, specifically phytase and a carbohydrase enzyme containing xylanase and β -glucanase, in reduced protein diets to improve nutrient digestibility in laying hens during the post-peak laying period from 35 to 57 weeks of age. The second study examined the effects of dietary supplementation with arginine, guanidinoacetic acid and citrulline on the productive performance of laying hens during the late laying phase from 60 to 75 weeks of age.

Outcomes

Reducing dietary crude protein by two percentage points in laying hen diets showed promising results. In the first study, dietary protein reduction from 16.5% to 14.5% slightly reduced egg weight and egg mass but had no effect on feed efficiency in laying hens aged 35 to 57 weeks. In contrast, the findings of the second study showed that reducing dietary protein level from 15.8% to 13.8% maintained performance, egg quality, and nutrient digestibility in hens aged 60 to 75 weeks. Supplementing reduced protein diets with standard doses of phytase (600 FTU/kg Aextra® PHY Gold, Danisco Animal Nutrition, IFF, UK) and carbohydrase enzyme containing xylanase and β -glucanase (100 g/tonne Aextra® XB 201 TPT, Danisco Animal Nutrition, IFF, UK) was adequate, while higher Aextra XB levels reduced egg production from 35 to 57 weeks of age. However, citrulline supplementation to reduced protein diet at 0.12% improved egg and yolk weights, egg mass, and bone strength from 60 to 75 weeks of age, with economic benefits justifying its use despite higher cost.

Environmental advantages of feeding reduced protein diets, including reduced nitrogen excretion, lower excreta moisture, and improved nutrient digestibility, were demonstrated. Arginine supplementation to reduced protein diets at 0.06% also increased ileal energy digestibility at 75 weeks of age.

Economically, a reduced protein diet supplemented with 0.06% Cit reduced feed cost per kg of eggs by AU\$ 0.076 (6.8%) compared to the standard protein diet from 60 to 75 weeks of age. With rising soybean meal prices and stable or declining prices of crystalline amino acids, reduced protein diets offer a cost-effective and environmentally sustainable alternative to standard protein formulations.

Implications

By optimising reduced protein diets for laying hens, this project may help to increase production efficiency while reducing carbon footprint and industry reliance on imported expensive soybean meal, leading to a more efficient and sustainable layer production. This is particularly important for Australia, which often experiences drought and must otherwise import more high protein feed ingredients for poultry diets. It appears that laying hens are more adaptive to dietary protein reductions compared to broilers, given the high protein requirement of rapid-growing broiler strains. Thus, although soybean meal inclusion rates in layer diets may be lower than in broiler diets, the opportunity to successfully develop reduced protein diets that meet the economic and environmental purposes may be higher in laying hens compared to broiler chickens. By reducing dietary protein level, the dietary inclusion rate of soybean meal was decreased by nearly 50% and 40% in the first

	and second studies in this project, respectively. This may help to reduce reliance on imported, expensive soybean meal, particularly in countries where soybean meal is not produced locally. Furthermore, the reducing cost of leucine, isoleucine and arginine will help to reduce the cost of reduced protein diets slightly in the future, but this is limited due to the small inclusion rates. However, when the amount of canola meal in the diet increased, more amino acids could be incorporated, further reducing the diet cost. Thus, exploring reduced protein diets with a focus on utilising more canola and increased synthetic amino acid inclusions could be an area for the future to reduce reliance on imported SBM, improve sustainability and reduce diet cost.
Key Words	Reduced protein diet, laying hens, enzymes, amino acid, nutrient digestibility, laying performance, egg quality, economic efficiency, environmental sustainability
Publications	A minimum of two manuscripts are currently in preparation (the two in preparation include one for each study, but these may be split into multiple papers depending on their final size and the journal's preference). Further, now that the project is complete, we plan to present the outcomes at APSS 2026. Dr Moss has presented the outcomes of the first trial at the Australian Eggs Forum in November 2024 and the final results and economic analysis at the Australian Eggs Mini-Forum in May 2025.