Evaluation of Arginine and Tryptophan *In Ovo* Feeding on Production Performances, Glycogen Status, Intestinal Health and Immunity Related Gene Expression in Fast Growing Broilers

Nibedita Nayak (✉ dmibeditavet@gmail.com)

ICAR Central Coastal Agricultural Research Institute <https://orcid.org/0000-0001-5643-3831>

R. Asha Rajini

Madras Veterinary College

Amiya Ranjan Sahu

ICAR Central Coastal Agricultural Research Institute

J. John Kirubaharan

Madras Veterinary College

S. Ezhilvalavan

Madras Veterinary College

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Abstract

The aim of this study was to evaluate the impact of *in ovo* feeding of essential amino acids (arginine and tryptophan) on post-hatch growth performances, organ indices, glycogen status, intestinal health, immune response and immunity related gene expression in fast-growing broilers. Total 700 fertile eggs (Cobb-400) divided into five groups and *in ovo* injection of arginine and/or tryptophan was administered on 18d of incubation through amnion to a depth of about 8 mm, sealed with paraffin wax and seted for hatching. Arginine feeding had significantly ($P<0.01$) improved hatch weight, chick/egg weight ratio and placement weight. Body weight gain was observed up to 21d of age in arginine fed group, while till 14d in combination group. Arginine had improved eviscerated and ready-to-cook weight significantly ($P<0.01$), but tryptophan increases breast yield per cent. Liver glycogen was significantly ($P<0.01$) higher in arginine fed groups. On 4d, villi height was significantly ($P<0.05$) increased in duodenum and ileum in all amino acid fed groups. On 35d, villi height was improved in duodenum and jejunum in tryptophan and combination groups. Higher HI-titre was observed on 4d in Arginine group; however, combination group had better HI-titre on 21 and 35d due to humoral immunity. IL-8 gene had high expression in tryptophan group, while IFN- γ expressed more in combination group. Hence, arginine and tryptophan *in ovo* feeding had improved production performances, glycogen status, intestinal health and immune response after proper metabolism over the feeding in day old chicks.

Introduction

In ovo feeding plays a critical role in improving growth performances, feed conversion ratio, gut health and immunity in poultry. It is the novel way to achieve optimum broiler performance by minimizing feed costs and thereby pressure on food ecosystem (Bhanja and Mandal 2005). It is the administration of exogenous nutrients into the amnion of the late-term avian embryo only (Uni and Ferket 2003). The carbohydrate free medium consisting of finite proportion of energy helps in development of avian embryo i.e. closed nutrition and nutrients for growth and development invested by the broiler breeder hen to support embryonic growth and hatching (Burley and Vadehra 1989). Because of the oral consumption of amniotic fluid (mainly water as well as albumen protein) by the avian embryo prior to the pipping of air cell around 18d of incubation in chicken, *in ovo* feeding can improve the energy status and gut development of the perinatal chick by supplementing the amnion with nutrients (Moran 2007). Early nutritional feeding programme like *in ovo* feeding will stimulate early gut development leading to better absorption and quality chicks. The embryonic developmental period as well as the first week after hatching represents a larger proportion (45%) of the whole life span in fast growing broilers. Thus, pre-hatch as well as the transitional post-hatch period must be achieved efficiently for survival and subsequent growth of fast-growing broilers. Improvement in initial hatch weight and immunity reduces number of mortality and morbidity which in turn reduces production of bio-waste. Therefore, different nutrients need to be examined for accelerating hatchability, hatch weight and immunity using *in ovo* technology. Both the amino acids (arginine as well as tryptophan) have beneficial effect on avian systems of immune response (Nayak et al. 2017; Odutaya et al. 2020). Arginine is one of the most

versatile amino acids in the animal cells (Wu and Morris 1998). Ball et al. (2007) studied that, birds have the absolute requirement of arginine which is due to lack of endogenous arginine synthesis and high protein deposition. Arginine promotes the faster growth of broiler line being a secretagogue increasing the release of growth hormone (Edmonds and Baker 1987). The candidate nutrient tryptophan has great impact through *in ovo* feeding which boosts body weights, immune system and overall performance of chick (Nayak et al. 2018). However, there is no finding regarding the regulatory effects of tryptophan and the combination of arginine and tryptophan through *in ovo* feeding method. Hence the study was undertaken to evaluate the potential effects of *in ovo* arginine and tryptophan feeding on hatchability, production performances and immune response in broilers.

Materials And Methods

In ovo injection

Total 700 fertile broiler eggs were obtained from 34wk old Cobb 400 breeders and incubated in forced draft automatic chicken incubator. Infertile eggs were discarded after candling on 18d of incubation and fertile eggs were randomly assigned to each treatment. The surface of the egg shell was disinfected with ethanol (99.90%) and then a pin point hole was created just under the air cell opposite to the head spot by a sterile egg shell driller. Five treatment groups *viz.*, non-injected control, injected control (0.5ml of 0.9% normal saline), arginine (0.5ml of 0.5% arginine), tryptophan (0.5ml of 0.5% tryptophan) and combination (0.25ml of 0.5% each of arginine and tryptophan) were prepared. The treatment solutions were warmed up to room temperature and injected into the amnion of 18d old embryo using 25mm needle (Uni and Ferket 2003). The pinhole site was immediately sealed with sterile paraffin wax and eggs were kept in the incubator. On 19d of incubation, the eggs were transferred to a hatcher and placed in well-marked pedigree hatching boxes.

Housing and management practices

Total 540 chicks (108 birds per treatment with 6 replicates 18 in each replicate) were randomly selected and per cent hatchability was calculated for each treatment. Individual chick weight was recorded at hatching. Chicks hatched from various treatment groups were distributed randomly and placed in experimental brooding cages in a well-lit ventilated open sided house. On 15d chicks were transferred to growing cages and reared up to 35d. *Ad libitum* feed and water was provided with similar nutrient composition based on Vencobb standard guide. Throughout the experiment standard management practices were followed in test and control groups.

Hatchability and production performances

The hatchability percentage calculated based on fertile egg set basis. Each chick was individually weighed at farm after transporting from hatchery and the transit loss was calculated based on the hatch weight and placement weight. Body weight gain was estimated by weighing individual birds at weekly interval up to 35d. The per cent livability was worked out based on mortality of birds.

Organ indices

At the end of 35d, 12 birds close to mean body weight from each replicate were randomly selected for carcass characteristics. Live, eviscerated, giblets and ready-to-cook weights were measured and given in percentage based on live weight. Cut-up parts *viz.*, breast, back, thigh, drumstick, neck and wing were individually weighed and figured in percentage based on eviscerated weight.

Glycogen status

Six chicks of 4d and 21d old age from each experimental treatment were randomly selected. The weight of the chick was determined and liver tissue was collected from chicks and stored at -20°C for glycogen estimation. Glycogen content in the liver sample was estimated by Anthrone method (Seifter et al. 1949).

Intestinal morphometry

Twelve birds were randomly used on the 4 and 35d to study the intestinal length and histomorphometry. The collected intestines were hand pressed to remove the feed ingredients and then flushed with PBS (0.1M, pH 7.4) to remove all the remaining contents. Approximately two cms from each segment of duodenum, jejunum and ileum were cut, washed with PBS (0.1M, pH 7.4) and placed separately in a 10% formalin solution for further processing. Measurements such as villus height from tip of villi to crypt, mid villus width, crypt depth from the base of villi to the submucosae, ratio of villus height to crypt depth were taken.

Immunity parameter

Immunity parameters *viz.*, immune organ weight, gene expression, Haemagglutination (HA) and Haemagglutination inhibition (HI) titre were studied. Six birds from each replicate were sacrificed on 4, 21 and 35d. Thymus and splenocytes were collected for expression of immunity related IFN- γ and IL-8 genes. RNA was extracted from cells of spleen and intestine using TRIZOL® reagent as per the manufacture's instruction with minor modifications at RNA pellet washing step wherein the pellet was washed twice with 75% ethanol by centrifuging at 7500g for 5 mins and RNA pellet was air dried for 5mins. The RNA pellet was redissolved in DNase-RNase free water. The concentration of RNA was estimated at 260/280A° in a spectrophotometer (Biophotometer Plus, Eppendorf). The synthesis of cDNA was carried out using Fermentas cDNA synthesis kit. For each gene, 10 μ l cDNA was synthesized using negative sense gene specific primers. After cDNA synthesis, 1 μ l of cDNA from respective genes was pooled and serially diluted, quantified and used for putting standard curve in relative quantitative PCR assay. A concentration of ~200ng/ μ l was used for real time PCR. The primers (**Table 1**) for the amplification and quantification of cellular/cytokine genes by quantitative real-time PCR was carried out with minor modifications as prescribed by Li et al. (2007) for β -actin, IFN- γ and IL-8 genes. The annealing temperature for IFN- γ was modified to 58°C (Laurent et al. 2001) in a real-time thermocycler (Mastercycler® Eprealplex, model#22331, Eppendorf, Germany). The standard curve method was used for expression of cellular/cytokine genes.

The HA and HI was performed for New Castle disease virus for which two milliliters of blood was collected from six birds from each treatment on the 4, 21 and 35 d. The blood was kept undisturbed for separation of serum.

Statistical Analysis

Data was analyzed based on ANOVA using Version 20.0 of SPSS software. Differences between treatments were detected by the Duncan's multiple range tests following ANOVA, and values were considered statistically different at $P < 0.05$.

Results And Discussion

Hatchability

The effect of *in ovo* feeding of arginine, tryptophan and their combination on the 18 d of incubation through amnion in broiler breeder chicken egg on hatchability, hatch weight and chick to egg weight ratio is presented (Table 2). *In ovo* feeding with arginine, tryptophan and their combination had no significant effect on hatchability. However, a numerically higher percentage of hatchability was observed in the *in ovo* fed arginine and tryptophan (96.42% and 95.71%) groups as compared to positive and negative control groups (95.71% and 94.28%), respectively. Similar findings were observed by Ohta et al. (2004), Bhanja and Mandal (2005), Bhanja et al. (2012), Chamani et al. (2012) and Shafey et al. (2014). However, Foye et al. (2006) had recorded better hatchability at 0.7% arginine compared to 0.2% arginine *in ovo* fed turkey poults. *In ovo* feeding with arginine significantly ($P < 0.01$) improved hatch weight (45.18 ± 0.24) and was comparable with injected control (44.50 ± 0.26), while the effect of tryptophan was comparable with non-injected control. The per cent difference in hatch weight between the treatments relative to arginine is furnished in graph (Figure 1) which had the best hatchability and was taken as 100%. The hatch weight of chicks showed a highly significant ($P \leq 0.01$) difference between treatments. Arginine fed groups had highest hatch weight as compared to injected control groups. This agreed with findings of Al-Murrani (1982), Ohta et al. (2004), Bhanja and Mandal (2005), Foye et al. (2006), Kulandaivel (2007) and Shafey et al. (2014); but Ohta et al. (1999 and 2001) and Ohta and Kid (2001) found no significant difference in hatch weight due to *in ovo* amino acids feeding. Chick to egg weight ratio also showed significant ($P < 0.05$) difference ranging from 72.01 ± 0.49 to 74.62 ± 0.39 . *In ovo* feeding with arginine significantly improved egg to chick weight ratio than other *in ovo* fed groups and un-injected and injected control. Chick to egg weight ratio also followed a similar trend as hatch weight. This effect was also noticed by Ohta et al. (1999, 2001 and 2004), Bhanja and Mandal (2005) and Shafey et al. (2014). Whereas Ohta et al. (2001 and 2004) did not find significant difference in hatching body weight relative to egg weight.

Placement weight and transit weight loss

Effect of *in ovo* feeding of arginine, tryptophan and their combination on placement weight and transit weight loss is presented (Table 2). Body weight of chicks (first day at farm) specified as placement weight showed a highly significant difference between treatments, with arginine *in ovo* fed chicks having

significant ($P<0.01$) higher body weight. All other treatment had comparatively similar body weight; however, lowest placement weight was observed in the combination group and non-injected control and those were comparable. No significant difference was observed between treatments in transit weight loss however, numerically arginine fed groups had lower transit weight loss compared to all other treatment groups. Placement weight was significantly higher in arginine *in ovo* fed group and no relevant literature was available for comparison except Coles et al. (2001) who observed no significant difference in treatment with respect to placement weight. This could be related to transit weight loss although no significant difference was observed in transit weight loss in this study. It has been noticed that amino acid fed groups had numerically lower transit loss compared to others. Kanagaraju (2014) had observed significantly lower transit weight loss in lysine and threonine fed groups, which averaged to 4.99% as compared to control.

Effect Of In Ovo Feeding On Production Performance

Body weight gain

Mean body weight gain of broilers (g) as influenced by *in ovo* feeding of arginine, tryptophan and their combination at day old and at weekly intervals up to five weeks of age has been presented (**Table 3**). The body weight gain from 0-3, 3-5 and 0-5 weeks shown in graphical presentation (Figure 2). The results indicated a highly significant difference ($P<0.01$) between treatments at 1st wk and significant difference ($P<0.05$) in 2nd and 3rd wk. However, the weight advantage was sustained up to 21d only between treatments due to *in ovo* feeding. *In ovo* feeding with arginine produced significantly ($P<0.01$) heavier birds in 1st, 2nd and 3rd wk continuously as compared to injected control and also with the combination group during the 2nd wk alone. *In ovo* tryptophan added group and the negative control (un-injected control) had lower body weight from 1st to 3rd wk continuously. Birds fed with *in ovo* arginine had significantly ($P<0.05$) higher body weight gain in 2nd wk and highly significant ($P<0.01$) higher gain in 3rd wk, and the same result was obtained in 2nd wk among combination group.

The highly significant ($P\leq 0.01$) and a significant difference ($P\leq 0.05$) in body weight were observed in 1st wk and later in 2nd and 3rd wk of age respectively. Throughout the three wk of study, arginine injected *in ovo* group gave the best body weight. This increase in body weight was sustained up to 3rd wk. Among the literature reviewed two types of results were noticed of which work done by Coles et al. (2001), Bhanja and Mandal (2005), Foye et al. (2006), Abdukalykova et al. (2008), Chamani et al. (2012), Kanagaraju (2014) and Odutaya et al. (2020) had observed that the amino acids had improved body weight only up to 1st and 2nd wk; while Al-Murrani (1982) and Bhanja et al. (2012) working on broilers and Al-Daraji et al. (2012), who studied on quails, observed increased body weight in amino acid fed birds right up to the end of the study mainly 56 and 42 d of age respectively. Our result seems to fall between these two categories, where body weight increased up to 21d and later the effect of *in ovo* feeding with arginine, waned into insignificance. Arginine *in ovo* fed groups showed best body weight gain comprising significant ($P<0.05$) result during 2nd wk and highly significant ($P<0.01$) during 3rd wk; and it is in

agreement with the findings of Bhanja et al. (2012) and Shafey et al. (2014). Shafey et al. (2014) had provided three groups of amino acid combination and they observed that the combination with arginine, glutamine, glycine and proline gave the best result and they attributed this increased body weight gain to arginine. It could be assumed that *in ovo* treatment of arginine had probably supported chick weight up to 21d of age as a result of increasing amino acids which could have enhanced protein synthesis after hatching.

Feed consumption

Feed consumption in broilers was not influenced by *in ovo* feeding and had no significant difference between treatments; however, a numerically better feed consumption was observed in *in ovo* amino acid groups. There was no significant difference in feed efficiency, but it has been noticed that cumulative feed intake was numerically higher in tryptophan and in the combination group. Bhanja et al. (2004 and 2012), Saki et al. (2013) and Al-Daraji et al. (2012) found no significant treatment effect on feed intake with regards to amino acid *in ovo* feeding but they observed difference with respect to feed conversion ratio, while Bhanja and Mandal (2005), Gaafar et al. (2013), Shafey et al. (2014), Eslami et al. (2014) and Kanagaraju (2014) observed similar results with no significant difference for feed efficiency. However, the results of high feed intake were observed in the group of birds fed with tryptophan. Emadi et al. (2011) had found a complementary effect with increased feed intake due to the precursor function of tryptophan for neurotransmitter serotonin that found in brain increases feed intake (Tackman et al. 1990; Muller and Martin 1992; Shea-Moore et al. 1996; Peganova and Eder 2003).

Livability

The livability up to five weeks of age was lowest in control, while in the amino acid *in ovo* fed groups; it ranged from 99.07 to 100%. No significant difference was observed in livability percentage between all the treatment groups. However, it may be noted that arginine and tryptophan *in ovo* fed groups had no mortality. Uni and Ferket (2004) have stated *in ovo* feeding of nutrients improved livability and reduce mortality at any age period. Similar report was obtained by Dos Santos et al. (2010), Saki et al. (2013) and Kanagaraju (2014).

Carcass characteristics and cut-up parts of broilers

The effect of *in ovo* arginine and tryptophan nutrition on carcass characteristics and cut-up parts of broilers (**Table 4 and Table 5**) and the per cent breast with relation to eviscerated carcass yield (Figure 3) are presented. A highly significant ($P<0.01$) difference was observed between treatment groups for eviscerated and ready-to-cook weight. Arginine fed group stood out as significantly ($P<0.01$) the best, both in eviscerated and ready-to-cook weights. There was no significant difference between treatments in cut up parts except breast yield where the treatment groups differ significantly ($P<0.05$). All the *in ovo* fed groups gave significantly ($P<0.01$) higher breast yield with tryptophan and combination group. A clear increase in per cent yield of breast relative to carcass yield could be observed in tryptophan fed followed by the combination group.

Many literatures have observed better carcass yield in broilers towards general amino acid *in ovo* feeding rather than specifically with arginine and tryptophan (Bhanja et al. 2004; Hajihosaini and Mottaghtalab 2004; Kanagaraju 2014). However, a more specific study with *in ovo* nutrition by Al-Daraji (2012) on Japanese quails, agreed with our findings where arginine had improved carcass weight. No literature could be traced for tryptophan *in ovo* feeding with regards to carcass weight in broiler. The tryptophan and combination group gave higher breast yield than the arginine fed groups which is agree with Foye et al. (2006).

Glycogen status

A highly significant difference ($P<0.01$) was observed on 4th day and 21st day between treatments. *In ovo* fed arginine group had significantly higher ($P<0.01$) glycogen level which was comparable with combination and injected control, but differed significantly from tryptophan and un-injected control (**Table 6**). Foye et al. (2006) observed *in ovo* fed with arginine, β -hydroxy- β -methylbutyrate (HMB) and β -hydroxy- β -methylbutyrate (HMB) + Arginine (ARG) in poult had increased total hepatic glycogen content at day of hatch in comparison to controls. This was explained by the fact that avian embryo develops in virtually carbohydrate-free environment and must rely upon gluconeogenesis for the production of glucose, the primary fuel for growth and development. *In ovo* fed poult had a higher gluconeogenic activity at hatch than the control as indicated by enhanced hepatic glucose-6-phosphatase (G_6P) activity. Hence, *in ovo* feeding may increase the late term embryo's capacity to convert gluconeogenic precursors (amino acids and protein) into glucose, for storage in liver and muscle as glycogen and may directly enhance hepatic glycogen accretion.

Intestinal morphometry

The results indicated that there existed no significant differences between different treatments for the parameters like length (cm) of intestine, duodenum, jejunum and ileum during 35th day, but significant ($P<0.05$) difference was observed among treatments on 4th day. *In ovo* fed combination group presented highest total intestinal length (69.25 ± 1.59) followed by tryptophan (66.19 ± 2.06) and arginine (64.45 ± 2.49). Uni and Ferket (2004) observed at day one the gastro intestinal tract of *in ovo* fed chick were functionally at a similar stage of development as a conventional two-day old chick offered feed immediately after hatch. Dror et al. (1997) had also observed maximal relative size of digestive organs in chicks occurs at 3-7 day of age. This agreed with our findings where the increase in intestinal length was observed during 4th day and not later. The absorption of tryptophan, arginine and their combination had increased intestinal length most probably due to increase in ileum length. There was significant ($P<0.05$) difference in villi height in duodenum and ileum on 4th day; whereas on 35th day a highly significant ($P<0.01$) difference was observed in villus height and mid villus width in duodenum and jejunum; but not in ileum (**Table 7 and Table 8**). Murakami et al. (2012) had provided dietary arginine to broilers and observed duodenal villus: crypt ratio increased in the first week. The same was observed in the *in ovo* fed arginine group on 4th day where the villi height increased and crypt depth had decreased but the villus: crypt ratio was not significant. Sklan (2001) who first worked on early chick feeding had stated the ability of intestinal tissue to digest and absorb nutrients had caused an increase in villus growth.

Immune response

There was no significant difference between treatments for thymus weight; numerically the combination group (0.113 ± 0.005) gave better weight followed by tryptophan (0.095 ± 0.004) and arginine (0.090 ± 0.004) group. This observation was in line with the findings of Bhanja et al. (2004) who have reported that *in ovo* feeding of amino acids have no significant effects on weight of immune organs. The thymus is very sensitive to periods of food deprivation, which caused a rapid decline in CD4+ T cells resulting in lower IgG production. The CD4+ cells act as the source for IL-6, a pro-inflammatory cytokine, which induces the final maturation of B cells into antibody-secreting plasma cells causing the proliferation and differentiation of immunoglobins. Kidd et al. (2001 and 2002) also reported that commercial broilers fed arginine levels above NRC (1994) recommendations did not increase the weight of immune organs. Abdulkalykova and Ruiz-Feria (2006) and Kwak et al. (1999) fed broilers with graded level of arginine and found no significant difference in thymus weight. It may be that arginine supplementation resulted in significant dose related increase in immune organs. In broilers, growth of the thymus was affected to a great extent by arginine deficiency (Kwak et al. 1999).

The expression profile of IL-8 gene did not change in arginine and combination groups. IL-8 did not express itself in all the amino acid treatment groups except tryptophan to a certain extent. With regards to IFN- γ gene, combination group showed better immune response than all other treatments. *In ovo* fed arginine was found to be more immune-competent than tryptophan. The relative expression of cell mediated immunity related genes (IL-8 and IFN- γ) have been presented (Figure 4).

The IL-8 gene is pro inflammatory and responsive for influx of heterophils and initiation of wound healing cascade (Henderson et al. 1999). The static expression of IL8 gene justifies the fact that F-strain of RDVF vaccine is lentogenic and do not produce inflammation. IL-8 gene was expressed more in control followed by tryptophan group. With regards to IFN- γ gene, combination group showed better immune-response than all other treatments. *In ovo* fed arginine was found to be more immunocompetent than *in ovo* fed tryptophan which is in agreement with Emadi et al. (2011) who observed increased level of IFN- γ in serum when chicks challenged with infectious bursal disease are fed with different level of dietary tryptophan but, Bhanja et al. (2014) observed no difference in the expression of IFN- γ gene in amino acid (lysine and arginine) injected chicks when compared to un-injected control group. In this study it is concluded that the combination group of arginine and tryptophan enhanced the expression of immune genes in broiler chicken.

Effect of *in ovo* feeding of arginine, tryptophan and their combination on graphical representation of HI titre against ND virus is presented (Figure 5). In HI assay high antibody titre was found on 4d in all the treatments especially in arginine *in ovo* fed group which showed significantly ($P < 0.05$) higher antibody titre than other treatments, later it subsided after vaccination with RDVF on 10d. This may be due to the presence of higher concentration of maternal antibodies in the serum samples, which neutralized the vaccinated antigen and this exists up to 35d. This shows that there is no free antigen to induce humoral mediated immunity (HMI) which leads to no increase in antibody titre after vaccination. So, it is advisable

to wait for complete disappearance of maternal antibodies prior to vaccination. The positive effect of arginine on T-cell production, proliferation and maturation owes to its immunoregulatory effects (Daly et al. 1990; Ochoa et al. 2001). It also stimulates the proliferation of CD4+ and CD8+ cells (Abdukalykova et al., 2008). Mellor and Munn (2003) reported that tryptophan enhances T-cell response during infection and stimulates T-cell proliferation.

Conclusion

Nutritional and metabolic exposure of embryo and chick may have long term programming effects into maturity. *In ovo* feeding of arginine had improved chick performance, body weight and body weight gain and also provided the bird with higher immunomodulation although body weight and body weight gain were not sustained beyond 21d. Tryptophan on the other hand had not shown any significant effect with respect to hatch weight and body weight. However, it has effect on immunomodulation and increase in breast muscle fibre that improved breast per cent with relation to eviscerated carcass weight.

Declarations

Author contributions

Nibedita Nayak: Investigation, *in ovo* injection, scientific intervention in field trial, production data recording, biochemical and immunological reactions, carcass study, writing original draft, review and editing. **R. Asha Rajini:** Concept of work, project administration, draft review and editing. **Amiya Ranjan Sahu:** *In ovo* injection, carcass study, statistical data analysis and draft preparation. **J. John Kirubakaran:** Immunological study, gene expression and analysis, and draft editing. **S. Ezhilvalavan:** Resources, hatchery management, *in ovo* injection and draft editing.

Code availability

Not applicable.

Data availability

All the data are available from the sources cited in the “Materials and methods” and from the authors upon request.

Declarations Conflict of interest

The authors declare no competing interests.

Consent to participate

The corresponding author is willing to participate in reviewing the manuscripts submitted to this journal.

Consent for publication

The manuscript has not been submitted to any other journal. The publication of the manuscript in this journal will be highly appreciated.

Compliance with ethical standards

The samplings from experimental birds were done in accordance with the ethical standards approved by Institute Animal Ethical Committee.

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Tables

Table 1 Details of primer sequences used in qRT-PCR.

Gene	Primer sequences (5'-3') (F- Forward and R- Reverse)	Annealing Temperature (°C)	Product Size (bp)
β-actin	F- GAGAAATTGTGCGTGACATCA R- CCTGAACCTCTCATTGCCA	58	152
IFN-γ	F- GAACGACTTGAGAATCCAGC R- GAGCACAGGAGGTCATAAGA	58	146
IL-8	F- GCTCTGTCGCAAGGTAGGAC R- CCAGCACACCTCTCTTCCA	58	184

Table 2 Effect of *in ovo* feeding of arginine, tryptophan and their combination on hatchability, hatch weight, chick/egg weight ratio, placement weight and transit weight loss (Mean±S.E).

Variables (g)	Treatments				
	Control	Injected control	Arginine (Arg)	Tryptophan (Trp)	Combination (Arg+Trp)
Hatchability (%) ^{NS} (n=140)	95.71	94.28	96.42	95.71	93.57
Hatch egg weight ^{NS} (n=140)	60.54±0.004	60.53±0.011	60.55±0.004	60.54±0.008	60.53±0.004
Hatch weight ^{**} (n=108)	43.96 ^{bc} ±0.29	44.50 ^{ab} ±0.26	45.18 ^a ±0.24	44.35 ^{bc} ±0.22	43.59 ^c ±0.30
Chick/Egg weight ratio ^{**} (n=108)	72.6 ^{bc} ±0.48	73.5 ^{ab} ±0.43	74.62 ^a ±0.39	73.25 ^{bc} ±0.37	72.0 ^c ±0.49
Placement weight ^{**} (n=108)	40.26 ^c ±0.28	40.9 ^{bc} ±0.25	42.24 ^a ±0.23	41.10 ^b ±0.21	40.26 ^c ±0.28
Transit weight loss ^{NS} (n=108)	3.69±0.39	3.58±0.12	2.94±0.10	3.25±0.15	3.32±0.11

^{NS}-Not significant, *-Significant (P<0.05), **-Highly significant (P<0.01), Mean values within each row having common superscripts don't differ significantly, n=Number of observations

Table 3 Effect of *in ovo* feeding of arginine, tryptophan and their combination on weekly body weight gain (g) (Mean±S.E).

Week	Treatments				
	Control	Injected control	Arginine (Arg)	Tryptophan (Trp)	Combination (Arg+Trp)
First ^{NS} (n=96)	43.33±0.94	45.04±0.88	44.68±0.90	42.40±0.81	43.08±0.64
Second* (n=96)	164.35 ^b ±4.04	170.30 ^b ±3.49	181.37 ^a ±2.95	169.84 ^b ±3.43	173.26 ^{ab} ±3.82
Third** (n=94)	308.36 ^b ±7.89	305.28 ^b ±5.36	334.15 ^a ±5.03	310.94 ^b ±6.07	312.90 ^b ±6.15
Fourth ^{NS} (n=84)	457.69±6.72	462.76±7.59	468.13±5.94	448.53±10.31	468.92±6.88
Fifth ^{NS} (n=82)	317.24±10.30	304.68±9.43	309.84±9.93	307.90±10.59	313.62±9.26

^{NS}- Not significant,*- Significant (P<0.05), **- Highly significant (P<0.01), Means within each row having common superscripts don't differ significantly, n= Number of observations

Table 4 Effect of *in ovo* feeding of arginine, tryptophan and their combination on carcass traits (Mean±S.E) (n=12).

Variables (%)	Treatments				
	Control	Injected control	Arginine (Arg)	Tryptophan (Trp)	Combination (Arg+Trp)
New York dressed weight ^{NS}	91.85±0.18	91.77±0.24	91.62±0.23	92.24±0.21	91.73±0.23
Eviscerated weight**	70.27 ^b ±0.45	70.19 ^b ±0.71	72.97 ^a ±0.28	70.59 ^b ±0.43	71.56 ^b ±0.50
Ready to cook weight**	76.58 ^b ±0.62	76.47 ^b ±0.75	79.72 ^a ±0.45	76.73 ^b ±0.34	77.48 ^b ±0.66
Giblets ^{NS}	6.30±0.24	6.28±0.25	6.74±0.45	6.14±0.25	6.37±0.35

^{NS}-Not significant, **-Highly significant (P<0.01), Means within each row having common superscripts don't differ significantly, n=Number of observation

Table 5 Effect of *in ovo* feeding of arginine, tryptophan and their combination on cut up parts (Mean±S.E) (n=12).

Cut up parts (%)	Treatments				
	Control	Injected control	Arginine (Arg)	Tryptophan (Trp)	Combination (Arg+Trp)
Breast*	30.50 ^b ±0.68	31.14 ^b ±0.35	31.52 ^{ab} ±1.15	33.09 ^a ±0.40	32.75 ^a ±0.35
Back ^{NS}	22.43±0.65	22.12±0.50	22.18±0.44	21.80±0.32	21.67±0.32
Thigh ^{NS}	13.97±0.51	14.44±0.25	14.19±0.30	14.15±0.26	14.0±0.28
Drumstick ^{NS}	14.81±0.82	14.24±0.98	14.11±0.59	14.71±0.30	15.36±0.47
Wing ^{NS}	11.02±0.57	11.74±0.16	10.47±0.46	10.82±0.31	10.87±0.43
Neck ^{NS}	3.63±0.51	3.44±0.19	3.05±0.23	3.31±0.17	3.11±0.11

^{NS}-Not significant, *-Significant (P<0.05), Mean values within each row bearing common superscripts do not differ significantly, n=Number of observations

Table 6 Effect of *in ovo* feeding of arginine, tryptophan and their combination on glycogen (µg) status (Mean±S.E) (n=12).

Treatment	4 th day**	21 st day**
Control	04.61 ^b ±0.97	3.35 ^b ±0.60
Injected control	13.66 ^a ±0.70	3.17 ^b ±0.93
Arginine	18.52 ^a ±1.92	7.54 ^a ±0.20
Tryptophan	06.73 ^b ±0.42	3.37 ^b ±0.52
Combination (Arg+Trp)	11.54 ^a ±2.39	4.76 ^b ±1.13
F value	12.194	6.043

** - Highly significant (P < 0.01)

Mean values within each column bearing common superscripts do not differ significantly

n = Number of observations

Table 7 Effect of *in ovo* feeding of arginine, tryptophan and their combination on intestinal morphometry (µm) on 4th day (Mean±S.E) (n=12).

Treatment	Duodenum			
	Villus height**	Mid villi width ^{NS}	Crypt depth**	Villus / Crypt ratio**
Control	835.55 ^b ±26.41	108.31±6.44	88.20 ^a ±11.30	10.20 ^c ±1.14
Injected control	873.89 ^{ab} ±25.13	97.73±4.49	65.41 ^b ±7.84	23.35 ^a ±2.13
Arginine	938.28 ^a ±18.15	107.26±4.92	41.78 ^c ±3.69	14.17 ^{bc} ±1.37
Tryptophan	906.13 ^a ±23.24	104.67±5.01	56.19 ^{bc} ±3.33	16.48 ^b ±1.31
Combination (Arg+Trp)	907.59 ^a ±14.16	102.28±3.92	69.71 ^{ab} ±5.43	13.40 ^{bc} ±1.02
	Jejunum			
	Villus height ^{NS}	Mid villi width ^{NS}	Crypt depth ^{NS}	Villus / Crypt ratio ^{NS}
Control	504.48±9.49	71.23±1.83	65.78±7.56	8.12±0.74
Injected control	589.54±63.46	75.17±7.36	59.00±5.01	10.75±2.03
Arginine	556.47±25.17	68.82±3.00	64.35±6.70	9.02±0.82
Tryptophan	534.09±38.45	76.73±5.90	71.52±2.99	7.45±0.45
Combination (Arg + Trp)	513.04±37.82	82.18±8.36	70.34±5.38	7.43±0.61
	Ileum			
	Villus height*	Mid villi width ^{NS}	Crypt depth ^{NS}	Villus / Crypt ratio ^{NS}
Control	439.08 ^b ±20.28	68.87±6.21	49.10±3.53	6.85±0.53
Injected control	431.79 ^b ±30.03	67.82±3.22	49.62±5.30	8.58±0.93
Arginine	526.02 ^a ±39.52	65.52±3.46	61.23±5.94	7.67±0.29
Tryptophan	531.02 ^a ±23.20	63.14±12.59	63.36±3.22	7.19±0.49
Combination (Arg + Trp)	531.79 ^a ±30.03	68.89±5.38	65.10±6.16	6.74±0.26

NS- Not significant, *- Significant (P<0.05), **- Highly significant (P<0.01)

Means within each column having common superscripts don't differ significantly

n = Number of observations

Table 8 Effect of *in ovo* feeding of arginine, tryptophan and their combination on intestinal morphometry (μm) on 35th day (Mean $\pm$ S.E) (n=12).

Treatments	Duodenum			
	Villus height**	Mid villi width**	Crypt depth ^{NS}	Villus/Crypt ratio ^{NS}
Control	1529.06 ^{ab} ±79.40	148.52 ^c ±14.29	81.20±17.70	25.58±6.56
Injected control	1488.58 ^b ±65.21	163.88 ^{bc} ±21.98	103.84±7.20	14.86±1.58
Arginine	1469.37 ^b ±183.95	194.57 ^{abc} ±11.54	88.28±9.33	16.60±1.12
Tryptophan	1796.24 ^{ab} ±25.59	227.27 ^b ±15.17	97.69±8.49	19.14±1.75
Combination (Arg+Trp)	1845.86 ^a ±119.87	205.16 ^{ab} ±22.14	112.19±10.21	16.89±1.35
	Jejunum			
	Villus height**	Mid villi width**	Crypt depth ^{NS}	Villus/Crypt ratio ^{NS}
Control	1412.10 ^{bc} ±124.93	133.70 ^b ±16.62	116.92±15.14	13.41±2.28
Injected control	1212.34 ^c ±137.22	136.91 ^b ±14.01	128.52±8.93	9.50±0.95
Arginine	1161.97 ^c ±106.41	158.12 ^b ±14.63	97.44±5.05	11.83±0.59
Tryptophan	1624.71 ^{ab} ±52.61	162.66 ^{ab} ±11.30	112.19±5.30	14.70±1.07
Combination (Arg + Trp)	1777.52 ^a ±125.02	202.13 ^a ±14.78	130.24±14.92	14.93±2.32
	Ileum			
	Villus height ^{NS}	Mid villi width ^{NS}	Crypt depth ^{NS}	Villus/Crypt ratio ^{NS}
Control	862.03±23.70	135.44±19.59	126.70±15.90	7.45±1.08
Injected control	1234.01±230.42	120.85±18.74	95.25±6.62	13.74±3.17
Arginine	1310.34±182.59	138.58±15.73	103.26±9.76	13.71±2.89
Tryptophan	880.68±38.61	127.23±7.54	86.40±6.77	10.45 ±0.82
Combination (Arg + Trp)	891.27±73.00	139.26±12.52	108.58±9.80	8.84±1.52

NS- Not significant, *-significant (P<0.05), **- Highly significant (P<0.01)

Means within each column bearing common superscripts don't differ significantly

n = Number of observations

Figures

Figure 1

Per cent variation in hatch weight relative to arginine treatment.

Figure 2

Effect of *in ovo* feeding of arginine, tryptophan and their combination on body weight gain in three periods (0-3, 3-5 and 0-5weeks).

Figure 3

Effect of *in ovo* feeding of arginine, tryptophan and their combination on the per cent of breast yield in relation to eviscerated weight.

Figure 4

Effect of *in ovo* feeding of arginine, tryptophan and their combination on expression of IL-8 and IFN- γ gene in splenocytes.

Figure 5

Effect of *in ovo* feeding of arginine, tryptophan and their combination on graphical representation of HI titre against ND virus.