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In *ovo* feeding technology: embryonic development, hatchability and hatching quality of broiler chicks

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Abstract: This paper reviewed the effects of *in ovo* feeding application on incubation performance and chick quality in broilers. Effectiveness of incubation and chick quality in commercial chicken broiler production have a decisive role in production cost as well as efficiency and are a prerequisite for sustainable production. To date, several studies have been done to improve incubation performance and chick quality, and significant gains have been achieved in this area in the last fifty years. The average hatchability and survivability in the sector have reached 85%–90% and 90%–95% respectively, and with new research, theoretical limits have been closely approached. *In ovo* feeding, one of the newest and technical applications in the incubation sector which successfully implements different methods and technologies in the area of performance and quality improvement has not yet been commercialized. Although *in ovo* feeding effects on chick weight and hatch properties have been evaluated and scientifically positive in some researches, sufficient progress for the spread of the application in the field has not been registered. Therefore, it is useful to continue studies on this subject, testing two or three interactions together with other supportive applications, and to investigate possible synergistic effects.

Key words: *In ovo* feeding, incubation, hatchability, chick quality, broiler

1. Introduction

The world chicken broiler industry is one of the rare sectors that has shown a continuous incremental trend in production with 114 million tonnes in 2018 compared to 80 million tonnes in 2008.[1] Additionally, between 2008 and 2017, global per capita consumption of poultry meat (predominantly chicken) had a superior growth rate (+16%) a contrast to beef and veal with a decrease of 5% in the same period [1]. This growth rate is due to a wide range of factors such as vertical integration and advanced feeding technologies that make the broiler sector more efficient, cost-effective, and productive than other industries. Besides the factors affecting the demand for animal-derived foods are population growth, increased living standards, and national income growth [2].

However, the success of hatchery enterprises due to the growing demand for quality chicks by commercial farmers directly impacts the development of chicken broiler industry. Therefore, the continuous production of healthy chicks under ideal environment controlled conditions is a requirement. Usually, incubation performance and chick quality in industrial chicken meat production are directly associated with the cost of chicks as well as the yield.

Generally, the biological, physiological, and biochemical requirements of the newly developed genotypes as a result of ongoing breeding studies have diverged considerably compared to traditional genetic materials. Thus, in today's hatchery sector, it is common that rather than standard procedures, different incubation conditions are provided depending on the diversity and requirements of genetic material. The embryonic period in fast-growing and high metabolic rate genotypes such as broilers is known to own critical importance to hatch and posthatch performance and has decisive effects on the production period of commercial genotypes.

This condition has accelerated research in different embryo manipulation methods including epigenetic adaptation, *in ovo* feeding, *in ovo* vaccination, and *in ovo* lighting. As a result of the adaptation of different techniques and methods, and innovation in incubation technology, hatchability performance can exceed 90% and one of the main objectives in these processes is to improve chick

[1] Food Agriculture Organization (2018). FAOSTAT [online]. Website http://www.fao.org/faostat/en/#data/QL [accessed 23 July 2020].

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quality. However, optimization of incubation performance and chick quality is highly known to be affected by farm-related factors such as breeder nutrition, egg size, and breeder age and, the hatchery associated elements like incubation conditions, egg storage conditions, and time.

Today, although research is focused on optimizing both elements of production and hatchery operations, chick loss still exists due to embryonic mortalities and deformations. It is established that embryonic and perinatal phases are vital in obtaining quality chicks and reducing post-hatch challenges. Thus, even progress from the last quarter of embryonic development to the hatching time is of remarkable benefit to ensure quality chicks and, *in ovo* feeding is described as an alternative to improving incubation and posthatch performance in broiler production [3].

This review aims to provide a brief account of the development of *in ovo* feeding in chicken broilers and to evaluate its several applications in nutritional supplementation and the successive analogous findings reported in the literature.

2. *In ovo* feeding

In ovo feeding is an embryo management technique developed as a perinatal nutrient supplementation via the egg yolk, directly into the embryonic body, to air cell and amniotic fluid [3–5]. As patented by Uni and Ferket [6], this application was designed to ensure adequate nutrition of the embryo during the incubation period, relieve the posthatch physiological limitations, and modulate the enteric development with the suspension of nutrients mixed with a suitable solution or a natural compound for a smooth change from embryonic nutrition that consists of egg yolk fatty acids to an external diet comprising of carbohydrates and proteins to improve the chick`s nutritional status. However, the injected compounds cause the variation in the original benefits of *in ovo* feeding.

Inoculation of compounds into chicken eggs (*in ovo* injection) became more successful with vaccines and up to date, the technique is being used for commercial vaccination (*in ovo* vaccination) in hatcheries. The technique is applied by (i) drilling or puncturing a small diameter hole in the eggshell, (ii) a needle descending to a controlled depth from the injection tube deposit an ideal compound indefinite amount, and (iii) after retracting the needle, the hole is sealed with paraffin or any appropriate material [6–9]. This system is known for its economic and procedural benefits which include inoculated; compounds are in standard volumes and concentrations without traumatizing the developing embryo, reducing chick stress via handling, improving incubation management through automation, and reducing some production costs. Therefore, the method replaces hand vaccination of chicks that negatively impacts chicken welfare and production in addition to being potent, secure, cheap, and convenient for mitigating broiler diseases [10–14].

Earlier studies showed that *in ovo* vaccination ensures protection to chickens against Marek's disease four times or more than chick vaccination [15]. In hatcheries, *in ovo* injection had been deliberately developed for vaccination process in broilers, but in the last 20 years, it has been technically used for nutrient supplementation of the fast-growing embryos and, accepted as *in ovo* feeding [6,16]. It is noted that factors such as the chicken type, egg size, injection time and site, incubation system and regime, and the material composition determine the amount and concentration of the injected compounds [11]. Several studies have indicated that the time and site of injection greatly affect hatchability [17,18], the effectiveness of inoculated materials, and the embryonic mortality rates [19].

2.1. Injection site

In the final stage of embryonic development, it is well documented that the embryo and embryonic membranes composed of air cells, allantois, and egg yolk sac have a continuous variation in both the amount and positioning within the eggshell. Thus, appropriate nutrient compounds can be inoculated to five different compartments of the egg, that is; the air cell, allantoic sac, amniotic fluid, the embryonic body, and egg yolk [19,20]. However, the effectiveness of *in ovo* feeding is higher in the amniotic sac where the inoculated substances are easily absorbed by the embryo when it consumes the amniotic fluid and this makes them available for the intestinal cells to boost gut development [6].

Siwek et al. [21] noted that the bioactive components (probiotics, prebiotics, and synbiotics) are administered into the air chamber to enhance their transit via the incompletely vascularized primitive allantochorion. Furthermore, in the author`s review, it was clarified that the latter process is referred to *in ovo* stimulation, and defined as *in ovo* feeding when the bioactive substances are injected into the amniotic fluid in a similar procedure as patented by Uni and Ferket. [6]. Similarly, various studies that have *in ovo* stimulated embryos with bioactive materials into the air cell have concluded that it enhances gut function and morphology [22], upregulates metabolic gene expression in broiler muscles [23], and has the potential to enhance the chicken`s ability to mitigate heat stress [24]. Another study reported that *in ovo* Dickkopf-related protein 1 application into the yolk sac modulates the establishment of feather follicles and growth of feathers during incubation [25].

Also, 2 trials were carried out to find the effects of *in ovo* propolis feeding and injection sites on hatching characteristics. During the process, eggs from two

different lines of slow-growing broiler breeders were injected with propolis solution via air cell or amniotic sac on the 19th day of incubation (experiment 1) and the 18th day of incubation (experiment 2). In both experiments, the results showed that *in ovo* injection of propolis and injection sites did not affect hatching parameters such as hatchability, chick weight at hatch, and chick survival [26].

2.2. Injection time

In the last quarter of incubation, the embryonic development is nearly complete that shock tolerance at the actual injection site is high without a significant negative effect on vital body functions or hatchability rate but *in ovo* vaccination is highly recommended on day 18 of embryonic development to ensure ideal protection [10]. Williams [27] noted that based on the stage of embryonic development, *in ovo* injection time is between the start of drawing of the yolk sac into the abdomen and head positioning under the wing until the external pipping thus, from day 17 to 19 day of incubation but, *in ovo* vaccination on the 17th day of incubation is indicated to decrease hatchability by approximately 1%–2% when compared to 18th day of embryonic development. The above scientific theories have been principally adopted for *in ovo* feeding of various substances. For example, on the 18th day of incubation, *in ovo* vitamin D3 or 25- hydroxycholecalciferol increases chick hatch weight compared with diluent treatment (42.3 g and 42.0 g versus 40.2 g), however, it has no significant effect on hatchability (83.3% and 90.3% versus 90.6%) [28], and *in ovo* glycerol inoculation increases liver glycogen in hatched chicks with no noted effect on hatchability and incubation time [29,30]. Moreover, at the 12th day of incubation, *in ovo* stimulation with bioactive compounds improves their passage from the air cell to the allantochorion that enhances the early establishment of gut microflora and immune system, positively affects growth performance [20–22,31], and regulates broiler transcriptome [32–34] but synbiotics downregulates metabolic gene expression in the liver causing epigenetic effects [35].

3. Compounds applied during *in ovo* feeding

It is known that the concentration and the amount of nutrients within an egg are associated with the nutrient levels needed for metabolism by the fast-growing embryo. *In ovo* feeding is a biotechnological tool that improves the embryo`s nutrient status, increases enteric capacity and hatchability, and stabilizes the bird`s resistance to infections [36]. According to Uni and Ferket [6], *in ovo* feeding is carried out with substances that stimulate the development and metabolism of digestive system cells such as enterocytes, goblet cells, and intestinal lymphocytes. These enteric modulators include glutamine or glutamate, arginine, carnitine, creatine, vitamin A, D or E, betaine, choline, and lecithin. Moreover, the basic nutrients like proteins, carbohydrates, and lipids, etc., offer their biological functions to the embryos. It is a common fact that genetic selection for fast growth rate and increased breast muscle gain increases the energy and protein requirements of the developing embryo, and creates an imbalance between embryonic nutritional requirements and egg nutrient composition. The above factors retard ideal embryonic growth and development. It is established that chick mortality within 1–2 weeks after hatching is 2% to 3% and, the majority of the remaining chicks encounter a range of conditions such as weakness, low feed intake, impaired growth, increased susceptibility to disease, mortality and inferior meat production. These negative outcomes are related to three main elements [37]:

- The egg nutrient composition required for tissue development and nutrient reserves in the embryonic tissues;
- The potential for the metabolism of extraneous carbohydrates and protein diets by the digestive system;
- The chick`s potential to depend on the yolk sac associated nutrients in the first 1–2 weeks after hatch but, the mentioned chick quality limitations can be overcome by *in ovo* nutritional supplementation.

Like other animals, chickens also need a strong defense system to fight against deleterious microbes and, this has always been achieved with control over feed quality and environmental conditions. However, a stressful environment increases the bird`s susceptibility to harmful microorganisms that suppress the animal`s reproduction and production potential, thus the utilization of substances that boost their immune system [38]. It is well understood that the development of the immune system in chickens begins during embryogenesis but is affected by environmental conditions and genetics and, the antioxidant and prooxidant mechanism supports normal embryo development, survival and, is responsible for nearly all physiological processes in the embryonic body. The oxidant and prooxidant balance is safeguarded by antioxidant enzymes such as superoxide dismutase, water-soluble antioxidants like ascorbic acid and, fat-soluble antioxidants including vitamin E, carotenoids localized in the embryos and newly hatched chicks and, they join biological reactions with free radicals or pioneering metabolites to prohibit the oxidation of biological molecules in the embryonic body by transforming them into less reactive molecules [38,39].

However, it is documented that as the incubation period progresses, lipid peroxidation of mostly polyunsaturated fatty acids containing tissues and susceptibility to attacks by free radicals increases with oxygen demand and break down of lipids for energy. Apart from oxygen and energy demands, factors including environmental pollution,

toxins, chemicals, drug, and uncontrollable external factors such as ionizing radiation damages the oxidants and antioxidant balance leading to oxidative stress and, thus high levels of free radicals [reactive oxygen and nitrogen species (ROS and RNS)] [40] and, antioxidant structures safeguard embryonic tissue antioxidants during embryogenesis [41]. Many of these antioxidants originate from egg yolk to embryo in the last stage of embryonic development and, before hatch, the embryonic liver stores most of the antioxidants which enter the circulatory system during the neonatal period, thus arresting the unfavorable effects of lipid peroxidation, free radicals and toxic metabolites on animals [41].

Interestingly, supplementation of breeder diets with natural antioxidants such as vitamin E, selenium, and vitamin C increases the egg antioxidants, and it positively affects embryo development, hatchability, chick weight, and immune system, survivability, animal welfare, and behavior parameters of newly hatched chicks and posthatched chickens [38,40–42]. Also, it enhances the performance of the breeders [38,40], and their addition in the commercial chicken feeds improves the egg quality and production the immune status in layers [40,43,44], and meat quality [45,46]. However, over the past years, some studies on *in ovo* injection of antioxidants or vitamins have produced positive results over embryonic and chick performances and, these antioxidants are heightened below.

3.1. Vitamins

Ascorbic acid is a well-known water-soluble vitamin containing various biochemical functions that maintain chicken embryonic development. During incubation, the concentration of ascorbic acid in chicken embryo starts to increase from the 6th day of incubation, reaches 5.6 nmol/mg in tissues on the 10th day of embryonic development, becomes relatively high between 8th and 18th days of embryogenesis and, gradually drops to 32% before hatch but the plasma ascorbic acid concentration reaches a peak on 12th day, gradually drops and, rises before internal pipping [47]. It was determined that the up and downregulation of embryonic ascorbic acid concentration is paramount for normal embryonic development and, ascorbic acid enhances heat stress control in the last stage of embryonic development, reduces embryonic mortality rates, increase hatchability percentage and hatch body weight [48]. Contrary, Zhang et al. [49] tested the effect of *in ovo* L-ascorbic acid in varying doses of 0.5, 1.5, 4.5, or 13.5 mg dissolved in 100 mL sterile saliva on the 17th day of embryonic development on hatch properties. They noted that there was no statistical difference between treatment groups and uninjected control groups for dead embryos, dead chicks, hatchability after 500 h of incubation and, the bodyweight at hatch to the set egg weight was higher but not significant. Zhang et al. [50] confirmed the nonsignificant effect of varying doses of *in ovo* vitamin C on chick body weight at hatch. Broiler eggs were injected 3, 6, 12, and 36 mg/egg vitamin C into the amnion on the 17th day of incubation. They reported that bodyweights at hatch were 45.1, 45.5, 44.7, and 44.7 g, respectively versus 45.3 and 44.3 g, for saline-treated and noninjected groups. Also, an experiment by Khaligh et al. [51] revealed that *in ovo* vitamin C (6 mg/egg) into amnion cavity on day 17 of embryonic development has no significant effect on hatchability (95%) and chick hatch weight (37.2 g) versus (95% and 37.4 g) from uninjected eggs of broiler chickens. Effect of *in ovo* inoculation of ascorbic acid on antioxidant capacity and immunity of local Chinese yellow broiler was investigated by El-Senousey et al. [52]. In this study, 90 Chinese yellow broiler eggs were administered with 3 mg/egg of ascorbic acid into the yolk sac on day 18 of incubation. The authors observed that *in ovo* vitamin C feeding enhances antioxidant capacity by upregulating mRNA expression of plasma glutathione peroxidase and superoxide dismutase in the spleen of the hatched chicks and downregulating the expression of malondialdehyde in the broiler chicks. Furthermore, it improves the immune status of hatched chicks by downregulating the mRNA content of immune-related genes in the spleen which is a better sign of immune response. To determine the effects of *in ovo* feeding of vitamin C on antioxidation and immune status of broiler chickens. Zhu et al. [53] injected 3 mg vitamin C via the blunt end of Arbor Acres broiler at 15 day of embryonic development. The results showed that the treatment increased hatchability compared with the group injected with normal saline (77.4% versus 64.3%). Similarly, Zhu et al. [54] injected vitamin C into the yolk sac of Arbor Acres broiler breeder eggs of average weight 63 g on day 11 of incubation to examine its impact on posthatch parameters, immune status, and DNA methylation associated gene expression. They found that eggs treated with vitamin C had a better hatchability rate compared with a normal saline-treated group (93.0% versus 74,1%), and the level of vitamin C at day 1 posthatch was highly significant.

Vitamin E is a major fat-soluble antioxidant known to damage lipid peroxidation passways and to shield organelles, subcellular or noncellular membranes against attacks from clear free radicals. Araujo et al. [55] investigated the impact of *in ovo* vitamin E feeding on hatchability, chick quality, and oxidative condition of broilers. Cobb broiler eggs were hand injected with varying doses of 0.0, 27.5, 38.5, 49.5, and 60.4 IU on the 18th day of embryonic development. It was shown that vitamin E injection significantly improved chick body weight, chick length, neonatal chick quality score, and

higher chick weight to egg weight ratios compared to the uninjected control group. Bhanja et al. [56] examined the effect of *in ovo* vitamin injection with doses of 100 UI vitamin A, 0.5 IU vitamin E, 50 mg vitamin C, 100 ng B1 vitamin B1, and 100 ng vitamin B6 dissolved in 0.5 mL sterile water in broiler eggs on 14th day of the incubation on embryonic performances. They noted that eggs injected with vitamin E had an increased number of dead embryos of 32.1% before pipping and reduced hatchability of 54.7% compared with other treatments and uninjected group. An experiment by Salary et al. [57] studied the effect of *in ovo* vitamin E on chicken performance and posthatch immunological parameters while injecting 15–20 mg vitamin E per fertilized egg with a 25 mm needle on the 14th day of embryo development. *In ovo* vitamin E treatment was found to significantly increase hatchability compared to *in ovo* injection with 0.5 mL sterile physiology serum and uninjected control group but, there was no significant difference for chick weight at hatch between the trials represented by 44.22 and 45.04 g, respectively. Ebrahimi et al. [58] evaluated the effect of *in ovo* injection of antioxidants in three different doses of 0.25, 0.50, and 0.75 mL vitamin E at 7th day of embryonic development in Cobb 500 eggs that were stored for 13 days before putting them in the incubator on hatchability and chick quality. They identified that hatchability was significantly decreased in eggs injected with antioxidants compared with the uninjected control group. However, no significant effect on hatchability was observed with *in ovo* vitamin E inoculation of the same doses into the amniotic sac of broiler embryos on day 17.5 of embryonic development [59] and injecting vitamin E into PB-2 broiler breeder eggs on the 18th day of incubation [60].

3.2. Carbohydrates

In ovo injected carbohydrates have shown positive effects such as increasing body and breast muscle weight by 7% in hatched chicks, limits break down of muscle proteins for energy production when glycogen reserves and albumin are depleted towards hatching and, increases stored glycogen in the liver by 4% compared with no treatment group [61]. Zhai et al. [62] evaluated *in ovo* injections with different volumes of carbohydrate solution on the 19th day of incubation. They identified that the volume used was positively proportional to the chick body weight at hatch. However, as the volume of injected carbohydrates increased, hatchability decreased. Salmanzadeh [63] confirmed that the bodyweight of Cobb 500 chicks that hatched from eggs injected with glucose solution on the 7th day of incubation was higher than in the group injected with deionized and an uninjected control group but hatchability was reduced to 68% as compared to 86% of the control group.

Smirnov et al. [64] revealed the positive effects of *in ovo* feeding on the development of intestinal epithelium. In this study, *in ovo* carbohydrate injection on the 18th day of incubation had a tropic effect on the jejunum and increased the villus surface area by 27% at hatch. Furthermore, they stated that carbohydrate absorption has an anabolic effect on the proliferation of intestinal epithelial cells so that insulin raises blood levels and, *in ovo* carbohydrate application positively affected goblet cell ratio and mucin with increased insulin by 50% after injection compared to the control group.

In a trial by Zhang et al. [65], *in ovo* injection of 25 mg glucose via the broad end of Arbor Acre broiler eggs on 17.5 day of incubation did not affect the hatchability, hatching time, somatic features, and the glycogen and glucose levels in the liver and breast muscle. However, when it was combined with 6 mg creatine monohydrate, a synergistic effect that increased; (i) bodyweight on 19.5 day of incubation, (ii) residual yolk sac at hatch, (iii) glycogen and glucose content in the liver, and (iv) creatine and phosphocreatine in the breast muscle on embryonic day 19.5. They concluded that the above results are indicators for enhancement of embryo development that leads to improved chick growth and performance in the posthatch period. Kanagaraju et al. [66] investigated the effect of *in ovo* feeding of glucose on the hatch and posthatch performance and, intestinal histomorphometry of broiler. In the study, embryos were administered with 0.5 mL of 25% glucose into the amniotic fluid at day 18 of embryonic development. The results indicated that hatchability and chick weight at hatch were significantly increased by the treatment. Effect of *in ovo* dextrose feeding on hatchability and broiler chick performance at hatch was examined by Nazem et al. [67]. Broiler eggs from Ross 308 at 42 weeks old were treated with 0.70 mL sterile solution containing either 10% or 20% dextrose into the yolk sac on the 14th day of incubation. The findings showed a significant decrease in hatchability (89.58% and 83.33% versus 95.83%), chick weight at hatch (37.04 g versus 37.22 g), and increased glucose vacuole diameter (19.23 and 19.59 versus 16.91 μm) in the injected groups compared with the noninjected group.

An experiment assessed how broiler chicks respond to *in ovo* dextrin feeding by injecting embryos with varying concentrations of dextrin (0%, 20%, or 40%) into the amniotic fluid on the 18th day of incubation. The authors revealed that the dosage rate was inversely proportional to hatchability (97.33%, 87.87%, and 82.58%), and there was no significant effect on body weight at hatch (44.93, 44.78, and 44.71 g), respectively. However, 40% dextrin increased the liver and breast glycogen content (mg/total liver or breast) (2.32 and 2.39), respectively versus (1.28 and 1.12, 0% dextrin; 1.76 and 1.77, 20% dextrin) [68].

When Kop Bozbay et al. [69] inoculated various carbohydrates (either 0.25 mg of glucose, sucrose, and starch) dissolved in 100 mL isotonic solution respectively into the Ross 308 breeders via the amniotic sac on the 18th day of incubation, they concluded that *in ovo* feeding of carbohydrates affected the entire or part of broiler chicken digestive system at the different direction and stability. However, it enhanced the chicken gastrointestinal tract establishment and chick weight.

3.3. Amino acids and proteins

Effect of *in ovo* injection of amino acid of 0.5 mL on day zero and 7th day of incubation in the air cell and egg yolk sac of Cobb eggs was assessed by Ohta et al. [70]. It was shown that hatchability of eggs injected with amino acids on day zero of incubation was reduced to 13% compared to 87% of uninjected eggs. However, eggs injected with amino acids in the yolk sac on the 7th day of incubation had the same hatchability percentage of 67% as the noninjected eggs and the chick body weight increased with preincubational egg weight. Kadam et al. [71] studied the effects of *in ovo* injection of threonine in different quantities of 10, 20, 30, or 40 mg dissolved in 0.5 mL sterile saline in egg yolk from the narrow end of an egg on the 14th day of incubation on the early growth of broiler chicks. They reported that the proportion of chicks relative to egg weight was 2% higher in the group injected with 30 mg of threonine compared to the untreated control group. Moreover, there was no significant difference between the injected groups and uninjected control group in terms of egg weight, hatchability, and chick weight.

Impact of needle length either 13 mm or 19 mm and of gauge 27 gauge for *in ovo* injection of amino acid solution in Cobb broiler eggs on the 7th day of incubation over hatchability and chick weight was tested by Ohta and Kidd [72]. It was observed that hatchability decreased in eggs injected with amino acid using a 19 mm needle compared to a 13 mm needle and, the bodyweight relative to before incubation egg weight of the chicks that hatched from eggs injected with amino acid using a 13 mm needle was increased. Ohta et al. [73] concluded that to increase hatch and posthatch weights, the *in ovo* injected and the available fertilized egg amino acid patterns should be the same because individual amino acid utilization is improved. Similarly, Bhanja et al. [74] experimented the effect of the injection site at the wide or narrow end of an egg and needle length of 11 and 24 mm for *in ovo* amino acid on the 7th and 14th incubation days over the embryonic performance of broilers. It was shown that using an 11 mm needle at the wide end with amino acids and the narrow end with a 24 mm needle for *in ovo* amino acid injection had lower hatchability than 86% of the control group. Likewise, the bodyweight of chicks that hatched from *in ovo* amino acid injected eggs on the 14th day of incubation was 2% heavier than the ones from the control group.

Effect of *in ovo* injection of various essential and nonessential amino acid mixtures of Lysine + Arginine, Lysine + Methionine + Cysteine, Threonine + Glycine + Serine, Isoleucine + Leucine + Valine and Glycine + Proline with an 11 mm needle at the narrow end in fertilized broiler breeder eggs on the 14th day of incubation was tested by Bhanja and Mandal [75]. It was determined that a combination of amino acids significantly increased chick weight and chick per egg weight ratio. Shafey et al. [76] assessed the effect of various *in ovo* amino acid application comprising 23.72 mg of lysine, glutamine, glycine, and proline (AA1), 23.60 mg of arginine, glutamine, glycine, and proline (AA2) or 28.76 mg of lysine, arginine, glutamine, glycine, and proline (AA3) on day 15 of incubation in the fertile eggs from Ross broiler breeder flock of 38 weeks over incubation performances. They identified that *in ovo* injection of AA2 increased the incubation time and reduced the percentage of hatched chicks in 468 h of incubation than other treatments. However, in 480 h of incubation, this group had more hatched chicks and increased hatch weight. It was concluded that *in ovo* AA2 application significantly increased the weight of chicks at hatch as a percentage of egg weight without affecting hatch properties. Similarly, a study by Nazem et al. [67] indicated that hatch chick weight (38.33 g versus 37.22 g) was increased with *in ovo* feeding of 0.70 mL solution of 10% amino acid composition into the yolk sac of Ross 308 broiler embryos on day 14 of incubation, and significantly increased the glycogen content (vacuole diameter) (23.04 versus 16.91 μm). However, the treatment significantly decreased hatchability (72.92% versus 95.84%) in contrast to the uninjected group.

In another study, the impacts of feeding an essential amino acid L-arginine (Arg) *in ovo* into the amniotic sac of broiler embryos (0.6 mL of 0.5, 1 or 2% Arg/egg) at 17.5 day of embryonic development on hatchability, growth, and posthatch performance was tested by Gao et al. [77]. The authors discovered that only 2% of the Arg treated group had a lower hatchability percentage (81.25%) than the noninjected group (88.13%) thus, the appropriate *in ovo* Arg concentration should not exceed 1%. Still, there was a significant difference between dose concentrations for chick weight though they were higher than the noninjected group. Furthermore, L-arginine (Arg) was inoculated into the amniotic sac of broiler embryos (0.6 mL of 1.0% Arg/ egg) at 17.5-day embryonic development to assess its impact on energy metabolism after hatch. It was demonstrated that Arg treatment increased glycogen and glucose content in the liver (4.32 and 4.64 mg/g) versus (2.91 and 3.59 mg/g uninjected; 3.00 and 3.74 diluent treated), and in the pectoral muscles (1.02 and

1.41 mg/g) versus (0.76 and 1.34 mg/g uninjected; 0.79 and 1.24 mg/g diluent treated), respectively of the hatched chicks. Also, the Arg application increased the plasma glucose and insulin level at hatch (10.22 mmo1/L and 12.89 μIU/mL) versus (8.63 mmo1/L and 10.33 μIU/mL uninjected; 9.00 mmo1/L and 9.90 μIU/mL diluent), respectively. Additionally, the authors observed elevated hepatic glucose - 6 – phosphate (0.14 versus 0.11 and 0.11 U/mg), but decreased hexokinase activities in the pectoral muscles (73.02 versus 78.15 U/g protein diluent injected) in the Arg treated group at hatch. With the above findings, they concluded that *in ovo* arginine inoculation modulates energy metabolism early in broilers [78]. On the other hand, when Omidi et al. [79] administered varying doses of arginine (0.5% or 1% L Arg) into the amniotic fluid of broiler embryos at day 14 of incubation, they noted that there was no significant effect no hatchability, body weight and weight of the organs for the immune system establishment. Effects of *in ovo* administration of L-Arg in varying concentrations (100 μg, 1000 μg or 2500 μg/μL/egg) into the amniotic sac of Ross broilers at days 8, 14, or 18 of incubation on hatchability, survival, and hatch bodyweight were investigated by Subramaniyan et al. [80]. They concluded that *in ovo* L-Arg feeding is more effective on the 14th day of embryonic development due to higher survival, hatchability rate, and chick hatch weight compared with days 8 and 18 but should be given in low concentration (100 μg).

In ovo feeding of leucine [*β*-Hydroxy-*β*-methylbutyrate (HMB)] at 7th day of embryonic development into the air chamber (L1) or day 18 of incubation into the amniotic sac (L2) of Arbor Acre broiler eggs was evaluated in terms of its effect on hatchability, chick quality, and posthatch performances [81]. The authors found that L1 and L2 increased hatchability (89.67% and 88.67% versus 85.33%) though an increment of 4.43% was observed for L1 compared with the noninjected group, and enhanced chick body weight at hatch (44.82 g and 44.33 g versus 42.11 g). However, no significant difference was shown for breast muscle yield. Moreover, in the study by Ghanaatparast-Rashti et al. [68], chick embryos were treated with varying concentrations of HMB (0, 0.5, or 1%) into the amniotic fluid on the 18th day of incubation. It was revealed that the dosage rate does not affect hatchability (89.41, 88.70, and 89.67%), body weight at hatch (44.82, 45.05, and 44.55 g) respectively but 1% HMB increased the liver glycogen content (mg/total liver) (2.02) versus 1.56 of 0% and 1.80 of 0.5%, and the breast glycogen level (mg/total breast) (2.18) versus 1.32 of 0% and 1.79 of 1%.

Ebrahimi et al. [82] evaluated the effects of *in ovo* L-lysine feeding into the amniotic sac of Ross 308 broiler breeder eggs on the 14th day of incubation on hatchability and chick performance. The embryos were fed doses of 10, 20, 30, 30, or 50 mg lysine dissolved in 1 mL sterile water. The results showed that only 20 mg lysine increased hatchability and decreased embryonic mortality number after inoculation compared with the noninjected group (73% and 5 versus 47% and 9) but there was no significant difference for chick weight at hatch between the treated groups and the controls (non- and sterile water injected).

A nonessential amino acid glutamine (Gln) in different concentrations (10, 20, 30, 40, or 50 mg) in 0.5 mL was injected into the albumen of broiler eggs on the 7th day of embryonic development to determine its effect on hatchability and chick weight [83]. The authors noted that there was no significant effect of the treatments on chick weight at hatch but, hatchability of all the *in ovo* Gln applied group was lower than 89.58% of the noninjected group.

In ovo administration of 1.5 mg/embryo of N-acetyl-L- glutamate (NAG) into the amniotic sac at day 17.5 of embryonic development led to a decrease in both hatchability and healthy chick percentage compared to saline-injected group (80.34% and 92.62% versus 84.62% and 94.32%) but showed no significant effect on chick weight at hatch [84].

In a study, Kop Bozbay et al. [85] injected varying concentrations of β-Alanine (0.75 and 1.5%) into the amnion of the Ross 308 breeder embryos to investigate its effect on hatching characteristics. The authors determined that 1.5% of β-Alanine inoculation had no negative effect on chick weight, quality, and mortality rates but 0.75% β-Alanine enhanced hatching parameters. Furthermore, Kop Bozbay and Ocak [86] demonstrated that inoculation of a blend of branded chain amino acids (0.2% BCAA; 2 L-leucine; 1 L-valine; 1 L-isoleucine) significantly improved the gizzard weight of chicks when injected via the albumen compared to other egg sites including the yolk sac and amnion. However, the authors found no interaction effect between BCAA and injection sites for growth performance, muscle weights, digestive tract weight and length, and other internal organs like the heart.

3.4. Minerals

For many years, extensive studies have been carried out on the incidence and prevalence of leg or bone disorders in broilers that are highly linked to various metabolic problems. These skeletal disorders have increased primarily due to the genetic selection for rapid growth rate and increased weight gain [87]. The rapid growth rate of chickens is associated with increased bone accumulation on the periosteum surface that increases cortical bone porosity and then leads to weak biomechanical properties of the bone [88]. It is known that microminerals play an important role in bone formation and strength, and as coenzymes in metabolic paths associated with the formation of the skeletal system. However, Yair and Uni

[89] stated that the concentration of microminerals such as copper, zinc, and manganese significantly reduces on the 17th day of embryonic development in an egg. Effect of the commercial dilute *in ovo* injection containing additional microminerals of Zn, Mn, and Cu into Ross 708 eggs using a commercial multi-egg injector on the 17th day of embryonic development on hatchability and chick quality variables were evaluated by Oliveira et al. [90]. Noninjected eggs (T1) and eggs injected with a dilute only (T2) were set as control groups. Eggs injected with a dilution containing 0.181, 0.087, and 0.010 mg/mL of Zn, Mn, and Cu, respectively (T3), or 0.544, 0.260, and 0.030 mg/mL of Zn, Mn, and Cu, respectively (T4). The percentage hatchability of eggs in T4 was found to be significantly lower than the uninjected control group. However, embryos taken from eggs treated with T4 contained a significantly higher proportion of bone ash than other treatments and, dilute injection containing high micromineral concentration like T4 was suggested to have the ability to improve bone mineralization. Scott et al. [91] revealed that *in ovo* administration of 50 and 100 mg/kg copper sulfate ($CuSO_4$) into the air cell of Ross broiler eggs at day 1 incubation does not promote embryo development but upregulates metabolic rate. Similarly, Scott et al. [92] found that inoculation of 50 mg/kg ($CuSO_4$) had no effect on embryo development, and the effect on hatch weight and hatchability was not significant compared with the noninjected group.

In recent years, nanobiotechnology has accelerated the utilization of essential mineral nanoparticles (NPs) in chicken nutrition [93]. These compounds occur in different forms such as ashes and still, they can be synthesized from several methods including; biological (produced from biomolecules of natural plants, algae, fungi, yeast, etc.) [93–95], physical such as laser ablation, and chemical like colloidal formation [93,96]. Studies have shown that the addition of metallic NPs in chicken diets has positive benefits on the performance measurements associated with reproduction and production, the immunity response, and the health of chickens [97–99]. Also, several trials on the effects of *in ovo* feeding of essential mineral NPs in chickens have produced viable results. For example, the effects of zinc, copper, and selenium NPs via *in ovo* on hatchability and chick quality were assessed by Joshua et al. [100]. The methods for the synthesis of the nanoform of zinc (Zn), copper (Cu), and selenium (Se) were chemical methods with a stabilizing agent as starch, electrochemical, and water solution-phase, respectively. Vencobb 400 broiler eggs were injected with concentrations of 25%, 50%, 75%, or 100% of Zn, Cu, or Se into the amniotic sac on the 18th day of incubation. It was revealed that in all the nano mineral treated groups, hatchability was lower than 96.7% of the normal saline-injected group but within the nanomineral treated, 25 and 50% nano-Zn had better hatchability rates (both 96.3%). On the other hand, 75% of Se had the highest chick weight (48.0 g) and relative chick weight to egg weight percentage (79.4%) of all the treatments. They concluded that *in ovo* feeding of the above nanominerals does not affect hatchability and chick weight. Scott et al. [91,92] revealed that *in ovo* feeding 50 and 100 mg/ kg Cu NPs into the air cell of Ross broiler eggs on 1 day of incubation does not enhance embryo development but improves metabolic rate, and the impact on hatch weight and hatchability was not significant compared with the noninjected group. When Ahmadzadeh et al. [101] inoculated chicken eggs with biogenic (from Enterobacter aerogenes) and chemically synthesized ionic nanohydroxyapatite (Bio-HA and Ch-HA, respectively) into the yolk sac on the 7th day of incubation the results indicated that compared to noninjected group, *in ovo* 100 and 50 mg/mL Bio-HA feeding increased the chick body weight at hatch by 3.55 and 1.32%, respectively. A total of 50 mg/ml Ch-HA group had the highest hatchability (80%) compared with the two Bio-HAs and 100 mg/mL Ch-HA but the percentage of unhatched eggs due to infection was lower in 100 (0%) and 50 mg/mL (10) Bio-HA. The authors linked this factor to the antimicrobial role of zinc and magnesium that were only contained in Bio-HA even though both compounds contained elements; carbon, oxygen, phosphorus, and calcium. The above factor may further be the reason for the reported increase in bone mineral density of chicks from Bio-HA groups. A study by Goel et al. [102] explored the impact of 15 µg silver (Ag) NPs of 3.5 nm through *in ovo* at 7 and day 18 of incubation into the extraembryonic cavity and amniotic or yolk sac respectively on hatchability measurements. Compared with the noninjected group, chick weight was increased by 1 g in all the treated groups but chick weight to egg weight ratio was similar in all the trials, and hatchability was decreased in all the injected groups, a difference of 7.5% and 1.7% on day 7 and 18 of incubation, respectively. Some studies have again combined mineral NPs with amino acids and registered positive impacts on hatchability and chick quality. For example, Subramaniyan et al. [103] conjugated 100 µg L-Arg + 1000 µg biologically (BOL) Ag NPs and 100 µg L-Arg + 100 µg chemically (C) + Ag NPs and they were administered via the air chamber of broiler eggs at day 8, 14 or 18 of incubation. It was indicated that at all the injection stages, hatchability and chick weight at hatch were enhanced by the treatments but inoculation on day 14 is desired because of the highest figures. They emphasized that *in ovo* feeding of NPs is a new method of nanonutrition.

3.5. Probiotics

Probiotics are growth stimulating and inhibiting microorganisms for gut microflora and disease causative

flora, respectively and, are developed as alternatives to antibiotics (curative medicine and growth promoter). The latter have restricted application in chicken nutrition due to concerns of their residues in meat, the build-up of bacterial resistance, and the disproportion of normal gut flora [104]. Investigations on the use of probiotics through chicken broiler feeds have shown their adequacy in improving intestinal function, reducing pathogenic flora, stimulating immunity, and posthatch performance [105–107]. Such benefits have been further enhanced by the injection of probiotics in chicken embryos. Pender et al. [108] treated broiler chicken embryos with probiotic (primalac W/S) on day 18 of incubation to study its effect on hatchability, early posthatch performance, and intestinal immune-related gene expression of broiler chicks. They found that *in ovo* administration of probiotics has a significant effect on body weight and body weight gain from day 1 to day 4 posthatch, up and downregulates gene expression in the ileum and cecal tonsils but has no effect on hatchability. Beck et al. [109] inoculated probiotics (*Lactobacillus animalis* and *Enterococcus faecium*) separately or in combination to study the effects on hatch and posthatch parameters. They suggested that administering a combination of *Lactobacillus animalis* and *Enterococcus faecium* via *in ovo* improves hatch performance and development of the digestive system. Skjot-Rasmussen et at. [110] injected probiotic Enterococcus faecium M74 strain (1.4 × 107 CFU/ egg) via the yolk sac and intestinal tract. After isolation and typing using pulsed-field gel electrophoresis (PFGE), the M74 PFGE profiles were high on day 1 (88%) and day 7 (67%) old chicks. They indicated that M74 strain is feasible for gut establishment via *in ovo* application and that the M74 multiplies in the chicken digestive system after hatching. Moreover, it was concluded that *in ovo* injection of probiotics bacteria (*Bacillus subtilis)* into the amniotic fluid of broiler embryos on day 18 of embryonic development has a significant effect on ileal MUC2 gene expression at hatch and 3 days posthatch, decreases and increases Escherichia coli and lactic acid bacteria population, respectively during the first week posthatch [111]. In another study, different doses (1×10^5, 1×10^6, and 1×10^7 CFU/egg) of a multistrain lactobacillus mixture (*Lactobacillus salivarius, Lactobacillus reuteri, Lactobacillus crispatus,* and *Lactobacillus johnsonii*) obtained from the gut content of healthy broiler chicken was delivered into the chicken embryo at 18th day of incubation, and their impacts on the cytokine gene expression were evaluated. The findings showed that *in ovo* administration of lactobacilli upregulated both the splenic expression of cytokines such as interferon (IFN)-alpha, beta and gamma, interleukin (IL)-8 and IL-12 on day 5 after hatch and IL-3 in the bursa on days 5 and 10 after hatching. However, expression of IL-6 on day 5 after hatch and IL-2 and IL-8 on day 10 posthatch in the cecal tonsils were downregulated by lactobacilli inoculation. The study demonstrated that injecting chicken eggs with lactobacilli does not impact hatchability. The authors concluded that *in ovo* lactobacilli treatment can regulate cytokine expression in various tissues of chickens [112]. El-Moneim et al. [113] injected probiotics (G3; 1×10^9 and G4; 1×10^7 CFU/egg *Bifidobacterium bifidium*, and G4; 1×10^9 and G6; 1×10^7 CFU/egg *Bifidobacterium longum*) into the yolk sac of broiler breeder chicken embryos at day 17 of incubation and the study aimed to assess their effects on growth performance and biochemical parameters of broilers. The authors demonstrated that *in ovo* probiotics administration has a significant effect on hatchability though both G3 and G6 had the highest value (100%) compared to the other treatments. Also, bodyweight gain at hatch was significantly increased by probiotic treatment but weights of G4 (41.64 g) and G6 (41.56 g) were higher than the rest of the groups.

3.6. Prebiotics

Like probiotics, the banning and restricted use of antibiotics in chicken production has enhanced applications of feed additives such as prebiotics as possible replacements to maintain production. Extensive investigation on chicken dietary prebiotics have found significant benefits on growth performances including enhanced feed conversion rate and increased body weight gain [114], mitigation of stress [115], and other studies have concluded positive impacts on gut function and establishment [116] and enhancement of the immune system [105]. Over the past decades, it has become clear that the above parameters can be achieved with *in ovo* prebiotics administration. A study by Berrocoso et al. [117] examined the effects of *in ovo* prebiotics (Raffinose) on growth performance, the relative weight of different gut organs and body parts, and ileum mucosa morphology. In the process, Cobb 500 breeder embryos were injected raffinose (RFO) in varying doses of 1.5, 3.0, and 4.5 mg in 0.2 mL of an aqueous solution into the air chamber on day 12 of embryonic development. The authors found no significant effect of the treatment on chick body weight at day one, but an increase in the RFO dose significantly affected the chick ileum mucosa morphology as regards to linear ($p < 0.001$) and quadratic ($p < 0.001$) for the height of the villus and villus height to crypt depth ratio at days 20 and 21 of incubation. However, at hatch, a linear increasing tendency was observed for crypt depth ($p = 0.051$). Inoculation of 2 different prebiotics [DN; Dinovo beta-glucan extracted from Laminaria spp and BI; Bi^2 tos a galactooligosaccharide (GOS)] separately, and in varying doses of 0.18, 0.88, 3.5, and 7.0 mg/egg in Ross 308 breeder eggs on day 12 of embryonic development demonstrated that *in ovo* prebiotics impacts hatchability, and *Bifidobacterium* and *Lactobacillus* count [31]. The authors indicated that both the tested prebiotics (DN and BI) injected in doses of 0.18, 0.88 mg, and 3.5 mg only with

BI increases hatchability (DN; 90.4, 89.2% and BI; 91.0, 92.6%, and 89.7%) more than (88.7%) of the control group injected with physiological saline. However, a higher prebiotic dose of 7.0 mg significantly decreases hatchability (DN; 56.5% and BI; 71.4% versus 88.7%). Also, the number of both *Bifidobacterium* and *Lactobacillus* in BI treated embryos were significantly increased irrespective of the dose. Furthermore, DN inoculation showed no statistical difference for *Bifidobacterium* count, and in regards to the number of *Lactobacillus*, only doses of 0.18 and 0.88 mg/embryo had significant differences compared with the group injected with physiological saline. Dankowiakowska et al. [118] injected prebiotics either (P1, 1.760 mg inulin or PB, 0.528 mg of commercial prebiotics Bi^2 tos a nondigestive trans-galactooligosaccharide (GOS) obtained from milk lactose digested with Bifidobacterium bifidum NCIMB41171) on day 12 of incubation into the air space of 2 groups of eggs from a 32-week-old parent stock (Ross 308). This resulted in a lower hatchability percentage P1 (89.58%) and PB (91.82%) versus (97. 72%) of the group treated with physiological saline. Also, Stefaniak et al. [119] investigated the effect of *in ovo* application of prebiotics (1.760 mg inulin and 0.528 mg Bi^2 tos) into the air space of broiler eggs on day 12 of incubation on yolk sac IgG (Y) concentration. They collected the yolk sac content of the treated embryos on the 18th day incubation and at hatch, and they diluted the samples with phosphorus buffered saline (PBS) at pH 7.3 in a ratio of 1:4. After radial immunodiffusion of the samples that were stored at –22 °C, the findings indicated no significant difference for IgG (Y) value between day 18 of incubation and at hatch, thus no viable impact on the establishment of the immune system during this stage.

3.7. Organic acids

Organic acids are part of the several chicken feed additives required for metabolic processes of the body. In recent years, they are extensively tested because of their potential to replace antibiotics. Their supplementation in chicken feeds has been noted to enhance the performance of the gastrointestinal system, reduce metabolites of toxic bacteria, pathogenic agents, and some diseases in the digestive system [120], but their addition in breeder rations has no significant effect on hatchability and chick quality parameters [121]. Moreover, injecting organic acids into the chicken eggs is also shown as a mode of application that achieves feasible benefits. A study determined the effects of *in ovo* inoculation of folic acids on growth and blood parameters in broilers. They injected folic acid in varying doses of 40, 80, and 120 µg into the air cell of chicken eggs on the 7th day of incubation. It was shown that folic acid treatment has no significant difference in hatchability but, the dose rate was proportional to blood glucose content, and a significant increase in serum folic acid level was noted at day 1 posthatch compared to uninjected and sham or water injected groups [122]. The effects of *in ovo* application of 0.5 mL of essential oils and organic acids (Biacid) into the amniotic fluid of Ross breeder embryos on the 18th day of embryonic development was investigated by Toosi et al. [121]. The authors found that hatchability (76.7 versus 76.5%), chick weight at hatch (44.1 versus 44.3 g), and embryo mortality (11.1 versus 11.7%) were not significant to the embryos administered with distilled water.

4. Conclusion

Generally, *in ovo* feeding method can ensure epigenetic effect and improve chick quality and growth performance while administering appropriate solutions or nutrient compounds at an appropriate time and in appropriate doses. Thus, errors or inadequacies due to feeding of breeders and adverse effects of inappropriate environmental conditions can be partially compensated. However, some losses may occur in hatchability due to some errors in the application of the method such as cracking of the eggshell and injuring the embryo. The use of the inovoject system developed for *in ovo* vaccine application if used for *in ovo* feeding purposes can increase the speed and success of the application. Also, determining the most appropriate time for *in ovo* injection in terms of embryonic age, the injection site and, the most appropriate injection dose while establishing embryo nutrient needs for today's modern genotypes will increase the success and spread of the method. Finally, efficient incubation and hatchability of chickens are an integral part of the industry and, increasing this efficiency can certainly benefit the poultry industry as poultry meat and egg demand in 2050 have been projected to increase by 121% and 65%, respectively with a global human population of 9.6 billion [123]. Interestingly, the poultry industry relies highly on chickens, and based on the positive research findings of *in ovo* feeding, efficiency in poultry can be increased with continuous study of the application.

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