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RESEARCH ARTICLE

In ovo feeding technique of probiotics in broiler chickens: Achievements, Prospective and Challenges

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Abstract

In ovo feeding refers to a method of delivering nutrients or supplements directly to the developing embryo inside the egg before it hatches. This method is commonly used in the poultry industry to improve the health and performance of the chicks after they hatch. One specific application of in ovo feeding is the use of probiotics. Probiotics are beneficial bacteria that can help improve gut health and overall immune function. By delivering probiotics directly to the developing embryo in ovo feeding can help establish a healthy gut microbiota early on leading to improved health and performance in the hatchlings. Probiotics can compete with harmful bacteria in the gut preventing them from colonizing and causing infections. This can reduce the need for antibiotics as the chicks are better equipped to fight off infections naturally. This can lead to better growth rates and feed conversion efficiency in the chicks. In ovo feeding probiotics is a relatively new and evolving technique in the poultry industry. Research is ongoing to optimize the delivery methods doses and types of probiotics used. Overall, in ovo feeding probiotics is a promising approach to improve the health and performance of poultry. By supporting the gut microbiota early on in the development of the chicks the benefits can be seen throughout their lifespan. Continued research and advancements in in ovo feeding techniques will likely lead to further improvements in the future.

Keywords: Broilers; Biotechnology; Nanotechnology: In ovo feeding strategies

1. Introduction

Different probiotic strains may have different effects on the gut microbiota and performance of the chicks so it is important to choose the right combination of probiotics for the desired outcomes. There are several potential benefits of in ovo feeding probiotics. Firstly, it can help enhance the immune system of the chicks making them more resistant to diseases. Additionally, in ovo feeding probiotics can improve nutrient absorption and digestion. Probiotics help break down and ferment nutrients in the gut making them more bioavailable and easier to absorb. In ovo feeding of probiotics in chickens has been shown to improve gut health, increase weight gain, and reduce mortality rates. It is a promising intervention for improving poultry production. Improved farm performance, lower antibiotic use, less condemnation at the slaughter, and higher product quality are all positively correlated with better first-day-old chicken quality (Yoho *et al.,* 2008). It was also noted that the immune system was affected,

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which may explain why infectious disease susceptibility was decreased and animal welfare standards were raised (Das *et al.,* 2021). For chicken embryos, pre-hatch nutrition is just as crucial as post-hatch nutrition since incubation period impacts embryonic growth, hatchability, and post-hatch performance (Das *et al.,* 2021).

In ovo technique (IOT) has been chosen as the first method of vaccination against Marek's disease (Sharma and Burmester, 1982). In addition to in ovo vaccination (IOV), this technology is being used to determine the sex of the embryo (Schijns *et al.,* 2014), delivering growth-promoting substances and nutrients at the embryonic stage (Uni *et al.,* 2005), improving the performance and gut health of poultry (Jha *et al.,* 2019b), and start epigenetic changes that improve the health and production status of the bird at the post-hatch period (Bednarczyk *et al.,* 2021). Slawinska *et al.* (2019) illustrated that in ovo injection is used to give bioactive substances to early-stage embryos because they can promote the proliferation of bacteria and have a good impact on intestinal development and health. It is commen to use antibiotics in chicken feed, namely in commercial poultry farms around the world, to enhance feed conversion ratio and meat output and to prevent pathogenic bacteria in the gut (Gaskins *et al.,* 2002; Diarra *et al.,* 2014).

However, using antibiotics excessively or carelessly in chicken and animal feed may result in the growth of antibiotic-resistant bacteria or drug residues in the tissue and may also lessen the efficiency of antibiotic therapy (Kim *et al.,* 2019; Vinueza *et al.,* 2019). Nevertheless, it is already illegal to add antibiotics to feed to stimulate growth in Europe, the United States, and a few other nations, including China, where this practise was outlawed in 2020 (Al-Khalaifah *et al.,* 2018).

There are significant efforts being made to find probiotic substitutes for antibiotics; at the moment, the majority of probiotics used in chicken farming are *Lactobacillus* , *Bifidobacterium* , *Bacillus* , or *Enterococcus*(Al-Khalaifah *et al.,* 2018; Seal *et al.,* 2018). Probiotics are live microorganisms that when administered in adequate amounts confer a health benefit on the host (WHO, 2002).

Probiotics are fed to animals to stabilise healthy germs, inhibit the buildup of gastrointestinal harmful bacteria, and ultimately to support animal health (Patterson and Burkholder, 2003). By supplementing probiotics, broiler growth performance and feed efficiency have improved (Murshed and Abudabos, 2015; Abudabos *et al.,* 2017). Short-chain fatty acid levels are dramatically increased when *Lactobacillus* spp.are added to feed, and broiler chickens' gut microbiota changes in a healthy way (Shokryazdan *et al.,* 2017; Peng *et al.,* 2016). Bacillus subtilis dietary supplementation benefits broilers' productivity, immune system activation, and increase of antioxidant capacity (Bai *et al.,* 2018).

Many bifidobacteria strains, including *Bifidobacterium longum*, *B. bifidum*, *B. pseudolongum*, *B. animalis*, and *B. infantis*, have been used as probiotics in both animals and people because they can restore a healthy gut microflora following antibiotic treatment (Abd El-Moneim *et al.,* 2019b; Dankowiakowska *et al.,* 2013; Gibson and Wang, 1994). Bacteriocins (bifidin and bifidocin B), lactic acid, and acetic acid, which are produced by bifidobacteria, are thought to suppress the growth of a number of gram-positive and gram-negative bacteria *in vitro* (Gibson and Wang, 1994; Shah and Dave, 2002). In general, probiotics are typically given to animals by feed and water, and they improve the natural gut bacteria balance in broiler chickens, which benefits their health and performance (Dankowiakowska *et al.,* 2013; Chotinsky *et al.,* 2003). Pre-hatch colonisation of the embryonic gut with advantageous bacteria can therefore assist the chicks in better managing stress during hatching, improving their growth, enhancing feed utilization, improving nutrient digestibility and absorption, reducing mortality, and reducing the burden of pathogenic diseases (Banerjee *et al.,* 2017; Chichlowski *et al.,* 2007; Abd El-Moneim *et al.,* 2019a). One of the most prevalent bacterial diseases in poultry is avian pathogenic *Escherichia coli* (*E. coli*), also known as extra-intestinal pathogenic *E. coli* (Kabir, 2010; Kathayat et al., 2021). Egg yolk sac infection, omphalitis, respiratory tract infection, swollen head syndrome, septicemia, polyserositis, coligranuloma, enteritis, cellulitis, and salphingitis are just a few of the localised and systemic infections caused by avian pathogenic *E. coli* that affect poultry (Kabir, 2010; Kathayat *et al.*, 2021; Johnson *et al.*, 2008).

Nevertheles, limiting antibiotic use on farms, might sharply raise morbidity and mortality, jeopardising security, food safety, and production efficiency (Hao *et al.,* 2014). Therefore, there is an urgent need to create novel, potent antibiotic substitutes that can improve poultry health and productivity, address the issue of antibiotic resistance, encourage antibiotic stewardship, and protect public health.

According to Bermudez-Brito *et al.* (2012) probiotics have antibacterial properties, encourage growth, uphold intestinal health, and fortify the immune system; as a result, they can replace antibiotics in controlling bacterial infections and boosting output. Various mechanisms of action, including (i) enhancement of epithelial barrier functions, (ii) competitive exclusion of pathogenic microorganisms, (iii) production of antimicrobial substances, and (iv) modulation of the host immune system, are how probiotics exert their antibacterial effects (Bermudez-Brito *et al.,* 2012; Servin *et al.,* 2004). The purpose of the current review is to summarise the key findings of some recent studies evaluating the mechanism of action of probiotics fed to birds in utero as well as the effects of these probiotics on the health and functionality of the bird's gastrointestinal system, with particular focus on nutrient digestibility, gut microbiota, immune system, oxidative status, and growth performance of broiler chickens.

2. In ovo technology and its application in poultry industry

As a result of the harmful effects of antibiotic use, which have sparked a global movement to forbid their use outside of legal frameworks, it was necessary to look for biological substitutes that can assist in containing the spread of diseases because of the epidemics and diseases that have spread in the poultry industry. Antibiotic residues have accumulated in animal feed as a result of the use of subtherapeutic doses of antimicrobial agents, including antibiotics, to fight or eradicate harmful bacteria and promote animal growth and feed efficiency, and drug-resistant microbes have emerged in the feed supply chain. Previous research has demonstrated a variety of probiotic from the biotechnology prospective effects via a variety of pathways, including pathogen protection, improved immunomodulation, altered gut microbiota, enhanced gut epithelium barrier function, and production of short-chain fatty acids (SCFAs).

Probiotics are now being used more frequently than antibiotics in the production of poultry due to concerns about public health. A novel class of dietary supplements and feed additives known as probiotics contains bacterial, fungal, and yeast cultures derived from various sources. Overall, probiotics are thought to improve the health and happiness of humans, animals, and birds in a range of environments. It has been shown that adding probiotics to the diets of cattle and poultry improves the animals' development, feed conversion efficiency, immune responses, and capacity to handle enteric illnesses. The modification of the gut microbiota, competitive adhesion to the mucosa and epithelium, strengthening of the gut epithelial barrier, and modulation of the immune system to give the host an advantage are a few significant mechanisms underlying the antagonistic effects of probiotics on various microorganisms. Due to the importance of using the inovo technique after its use in vaccination before hatching during the stages of embryonic development, some researchers have tended to use this technique to deliver bioactive nutrients to the chick embryo to provide the bird lifelong traits including improved performance, immunity, and a healthy gut flora.

The direct injection of bioactive compounds into the growing embryo in order to produce superior life-long effects while taking into account the dynamic physiology of the chicken embryo is known as in ovo technology (Oladokun and Adewole, 2020). It is based on the straightforward idea of providing the chick embryo with bioactive supplements to create lifetime characteristics in the bird, such as enhanced performance, immunity, and a healthy gut microbiome (Siwek *et al.*, 2018). The particular kind of external replenishment has an impact on how well both the embryo and its neonate grow. For equivalent growth outcomes (Slawinska *et al.*, 2016). The chicken business has a chance to create viable substitutes for antibiotic growth promoters (AGP) thanks to a recently developed field called in ovo technology, which involves delivering bioactives directly to the developing embryo (Oladokun and Adewole, 2020). In ovo vaccination has been demonstrated to trigger an early immune response in newborn chicks in comparison to post-hatch immunization (Negash *et al.*, 2004). Early immunological programming in fetuses is the major objective of in ovo technology, which is essentially a biotechnological intervention. In addition to promoting healthy immune responses in birds, in ovo technology may be used to lessen the perinatal nutritional deficiencies of the bird. According to Noy and Uni (2010), these deficiencies are frequently caused by the switch from embryonic yolk nutrition to exogenous feeding, a lengthy hatchery window (24–36 hours), and laborious hatchery logistics like sorting, sexing, vaccinations, beak trimming, comb dubbing, and chick transport. Additionally, this technique offers the opportunity to encourage the growth of the embryonic gastrointestinal tract (GIT), gut-associated lymphoid tissue, and colonisation of the embryonic gut with a healthy microbiota (Siwek *et al.*, 2018). Numerous variables, such as the timing of the injection, the location of the injection, the quantity of bioactives, the cleanliness of the hatchery, and others, have an impact on the effectiveness of in ovo injection (Bednarczyk *et al.*, 2016). It has been established that embryonic day 12 is the ideal period to provide prebiotics and synbiotics by in ovo technique (Villaluenga *et al.*, 2004; Bednarczyk *et al.*, 2016). The embryonic GIT is located in the highly vascularized chorioallantoic membrane at this period, making the air cell the best place for inoculation of these bioactives because the injected bioactive substance may flow there without difficulty (Oladokun and Adewole, 2020). Probiotics that are administered by in ovo method can potentially act as pioneer colonisers by changing the environment in the gut, which affects the gut microbiota (Pedroso *et al.*, 2016). Because the size and structure of the egg change at different times, the sites of injection alter between the two methods (Siwek *et al.*, 2018). The embryonic GIT, which is located in the highly vascularized chorioallantoic membrane at this period, can easily be reached from the air cell, making it the best place for inoculation of these bioactives (Oladokun and Adewole, 2020). This injection method has been demonstrated by an increased presence of bifidobacteria by (Tako et al., 2004; Villaluenga et al., 2004) to stimulate the growth of beneficial microflora in the embryonic gastrointestinal tract (GIT). According to numerous studies (Pilarski *et al.*, 2005; Bednarczyk *et al.*, 2011; Bednarczyk *et al.*, 2016; Maiorano *et al.*, 2012), this approach is successful and has no detrimental effects on hatchability. Furthermore, Dankowiakowska *et al.* (2019) found that using in ovo technique have no negative effect on hatchability. When compared to inoculation on the seventh day of incubation, in ovo delivery of silver nanoparticles at embryonic day 18 through the air cell has no detrimental effects on hatchability (Goel *et al.*, 2017).

3. Probiotic Mode of action

Probiotics work by introducing beneficial bacteria into the gut, which can help to improve digestion, boost the immune system, and reduce inflammation.

The primary probiotic modes of action include enhancement of the epithelial barrier, increased intestinal mucosal adhesion, simultaneous suppression of pathogen adhesion, competitive exclusion of pathogenic microorganisms, synthesis of anti-microorganism chemicals, and immune system regulation (Bermudez-Brito *et al.*, 2012). According to Juntunen *et al.* (2001) and Schiffrin *et al.* (1997), adhesion to intestinal mucosa is necessary for colonisation and is crucial for the interaction between probiotic strains and the host. Probiotics must adhere to the intestinal mucosa in order to exert their antimicrobial effects and modulate the immune system (Schiffrin *et al.*, 1997; Perdigon *et al.*, 2002; Hirano *et al.*, 2003). According to observations, the host activates its first line of chemical defence in response to an attack by pathogenic bacteria by producing more antimicrobial proteins (AMPs), including defensins, cathelicidins, C-type lectins, and ribonucleases (Ayabe *et al.*, 2000; Gallo *et al.*, 2012). Many AMPs are enzymes that attack bacterial cell walls and/or alter the bacterial membrane non-enzymatically to kill bacteria (Bermudez-Brito *et al.*, 2012). Defensins, which are tiny peptides or proteins, can also be released from epithelial cells by probiotic strains (Bermudez-Brito *et al.*, 2012). These tiny peptides and proteins help to maintain the integrity of the intestinal barrier (Furrie *et al.*, 2005). Hassan et al. (2012) investigated that bacteriocin-mediated death frequently involves the development of pores in target cells and/or the suppression of cell wall production. According to Liévin *et al.* (2000), two bifidobacterium strains were found to have a potent killing effect on a number of dangerous bacteria, including Salmonella enterica serovar. typhimurium SL1344 and E. coli C1845. The intestinal barrier is a crucial defence system that keeps the epithelium intact and shields the organism from its surroundings. The intestinal barrier's defences include the mucous layer, antimicrobial peptides, secretory IgA, and the epithelial junction adhesion complex (Ohland *et al.*, 2010). If this barrier function is impaired, microorganisms and food antigens can enter the submucosa and trigger inflammatory responses, which can lead to intestinal diseases such inflammatory bowel disease (Hooper *et al.*, 2001; Sartor *et al.*, 2006).

Some probiotic strains have antagonistic activity against the adherence of gastrointestinal pathogens, which results in specific adhesiveness qualities that may impede the colonisation of pathogenic bacteria (Servin *et al.*, 2004). Bacteria can also alter their environment to make it unfavourable to rivals in order to obtain a competitive advantage (Bermudez-Brito *et al.*, 2012). One instance of this kind of environmental alteration is the creation of antibacterial compounds like lactic and acetic acid (Schiffrin *et al.*, 2002). Organic acids, especially acetic acid and lactic acid, which have a potent inhibitory impact against Gram-negative bacteria, are regarded to be the main antimicrobial compounds responsible for the inhibitory activity of probiotics against pathogens (Alakomi *et al.*, 2005; Makras *et al.*, 2006).

The bacterial cell receives the organic acid in its undissociated state, which then breaks down in the cytoplasm of the cell (Bermudez-Brito *et al.*, 2012). The pathogen may eventually experience a drop in internal pH or a buildup of ionised organic acid inside cells (Ouwehand *et al.*, 1998; Russell *et al.*, 1998). As an addition to green feed, probiotics have been shown to: stabilise gut flora, lower the spread of infectious agents, compete with pathogenic bacteria for nutrients and attachment sites in the intestine, provide the host with metabolic energy through the production of volatile fatty acids, and enhance host production and immune response (Estrada *et al.,* 2001; Abd El-Moneim *et al.,* 2019b). Van Immerseel et al. (2004) said that the short chain fatty acids (SCFA) are viewed as a possible antibiotic substitute. Un-dissociated fatty acids are absorbed by the bacterial cell, where they are ionised and cause a shift in the intracellular pH that results in bacterial death (Khan and Iqbal, 2016). Probiotics abundance in the digestive tract is associated to these advantages (Abd El-Moneim *et al.,* 2019b; Banerjee *et al.*, 2017).

Due to the nearly endless possibilities for probiotic effects on the poultry industry, more research is required. However, more studies in more standardised settings are still needed to assess probiotics and the precise mechanism of action of probiotic anti-stress attributes.

4. Effect of probiotic on poultry performance

In-ovo feeding with probiotics can help improve the gut microbiota of chicks and enhance their immune system, leading to improved growth, health, and overall performance. Numerous studies on the effectiveness of broiler chicken production have concentrated on the use of probiotics to improve the health of broiler hens; both live and inactivated probiotics are successful (Yousaf *et al.,* 2022). Lactobacillus strain may strengthen the immune systems of both types of chickens Hajati and Rezaei (2010). Harmful microflora declines as the community of unpleasant stomach bacteria increases (Fathabad *et al.,* 2011). After supplementing with probiotics, the non-pathogenic bacteria from the probiotics compete with the pathogenic bacteria in the gut for nutrients, colonise the intestine, leaving no space for harmful bacteria to occupy or establish, and secrete digestive enzymes (Galactosidase, amylase, etc.), which aids in the increased absorption of nutrients and enhances the growth performance of animals (Jadhav *et al.,* 2015). Using the in ovo approach on viable eggs that were inoculated into the yolk sac on day 17 of embryogenesis significantly increased live body weight and weight gain when compared to the control group (Abdel-Moneim *et al.,* 2019a). These outcomes supported the findings of Kabir *et al.* (2004) and Sen *et al.* (2012), who discovered that pigeons given probiotics gained more live weight. Additionally, it was discovered by Lan *et al.* (2003) and Huang *et al.* (2004) that utilising activated and inactivated probiotics at particular concentrations has a favourable effect on growth performance measures. Abdel-Moneim *et al.* (2019a) theorised that the enhancement in the effect of bifidobacteria to the ability of the probiotic to increase digestive enzyme activities and decrease ammonia production (Wang and Gu, 2010; Sugiharto, 2016), elevate nutrient digestibility and its availability for absorption (Siddiqui *et al.*, 2017), increase the surface area of villi for nutrient absorption and act as a dietary antimicrobial agent (Yazhini *et al.,* 2018). Stęczny *et al.* (2021) used tow probiotic products; they reported that there was a less noticeable impact on body weight on day 42, they hypothesised that this was because experimental chickens had a higher feed intake (better appetite) than control chickens. These findings are at odds with those reported in (Biesiada-Drzazga *et al.*, 2011; Kokoszynski *et al.,* 2013). A probiotic supplement including Lactobacillus, Bifidobacterium, and Clostridium bacteria, on the other hand, significantly boosted the body weight gains of broiler chickens (Song *et al.,* 2014). Similar findings were made by Apata *et al.* (2008), who found that Lactobacillus bulgaricus probiotics helped people gain weight. Improved feed conversion was observed in broiler chickens receiving a probiotic containing Pseudomonas putido and Pantoea agglomeran, according to Jouybari *et al.* (2009). Additionally, Opalinski *et al.* (2007) revealed significant decreases in feed intake and feed convertion but discovered no positive effects of probiotics containing Bacillus subtilis on body weight of ross chickens from 1 to 21 days of age.

According to Adli *et al.* (2023), addition of probiotics induced a rise in body weight gain (P< 0.001) and the inclusion of probiotics had no effect on the feed conversion ratio or feed intake . Probiotics containing Lactobacillus fermentum and Saccharomyces cerevisiae were used by Bai *et al.* (2013). They found that from 1 to 21 days of age, male Cobb broilers' average daily gains (ADGs) and feed efficiency significantly improved, but that there were no significant differences in the growth performance of the broilers between 22 and 42 days of age. Probiotics have been researched for their potential to improve growth performance in commercial poultry production since the phase-out of AGP in chicken feed (Bai *et al.*, 2013). The AGPs work by limiting the production and excretion of catabolic mediators by gut inflammatory cells, which in turn lowers intestinal microbiota (Niewold, 2007). Furthermore, according to Adamski *et al.* (2004) over a 49-day rearing span, Hybro broiler chickens experienced higher mortality rates (7.4% and 8.6%, respectively). However, Stęczny *et al.* (2021) investigated that mortality during the 42-day rearing period was 2.37% in the control group and 2.23% in the experimental group, they hypothesised that the lower mortality rate in the experiment was likely caused by the probiotics' positive effects on intestinal microflora and immune stimulation. Similary, Broiler chickens supplemented with a combination of probiotics and prebiotics of bacterial origin had reduced mortality rates than non-supplemented birds (Pelicia *et al.*, 2004). Neverthels, probiotic use results in an increase in body weight gain and feed converion ratio, though this improvement in feed converion ratio may not always occur, they said that a higher average daily feed intake (ADFI) and a better feed conversion ratio (feed converion ratio) are frequently linked to improvements in body weight growth (body weight gain) (Jha *et al.*, 2020).

By modifying the gut environment, strengthening the gut barrier, and excluding pathogens through competitive exclusion as well as immune system stimulation, probiotics help to foster development (Jha *et al.*, 2020). According to Awad *et al.* (2010), broiler chicks were used to test the effectiveness of *L. salivarius* and *L. reuteri*, the finisher stage broiler chicks' body weight increased as a consequence of probiotics. Additional research revealed that multistrain Lactobacillus supplements could be used as probiotics in industrial poultry production because they foster development (Gheorghe *et al.*, 2018; Lokapirnasari *et al.*, 2020). Ipek *et al.* (2016) used 720 Cobb 500 broiler chicks and divided them to four treatment groups and fed diets containing control, three groups of various probiotics and prebiotics (PPS), including live Saccharomyces cerevisiae strains, and one group of each of the other, they found that the body weight gain was considerably higher in all three groups of birds supplemented with PPS than in the control group. According to Zhen *et al.* (2018), supplementing with Bacillus coagulans caused Salmonella enteritidis-challenged Cobb broilers to produce more body weight gain and feed converion ratio from days 15 to 21 than non-supplemented birds did. On the other hand (Gadde *et al.*, 2017; Martínez *et al.*, 2017) showed no effect of probiotics supplementation on broiler growth performance. Similary, Olnood *et al.* (2015) found that probiotic supplementation did not significantly enhance body weight gain, and feed converion ratio when 294-day-old Cobb broiler chickens were used to study the effects of four *Lactobacillus* strains (*L. johnsonii, L. crispatus, L. salivarius, and an unidentified Lactobacillus sp*) on the gut microbial profile and growth performance. Likewise, Fathi *et al.* (2017) paper showed no positive effects of probiotic supplementation on grill growth performance under high-heat conditions.

Abd El-Hack *et al.* (2017) employed Bacillus subtilis to examine the effects of a graded probiotic dose on performance, egg quality, blood metabolites, and nitrogen and phosphorus excretion in the dung. There were eight treatment groups, each with 216 Hi-sex chickens, brown laying hens were randomly allocated, and they received two levels of *B. subtilis* probiotics. When comparing the probiotic-supplemented birds to the control group, it was found that the ADFI, egg shape index, and yolk colour had improved. The inclusion of B. subtilis probiotic enhanced egg weight, egg bulk, and overall feed efficiency. De Oliveira *et al.* (2014) were successful in isolating Enterococcus faecium and Bacillus subtilis from seven other commercial probiotic products, they found that in ovo probiotics didn't affect on hatchability, considering that the hatchability jeopardization is one of the factors impairing the commercialization of this technology for delivery of bioactives. Despite *B. subtilis* had a numerically greater hatch percentage (96.11 vs. 81.67%), E. faecium and B. subtilis study had no effect on hatchability rate (De Oliveira *et al.*, 2014). In the same line, *Pediococcus acidilactici*, *E. faecium*, and *B. subtilis* were all delivered in ovo at 10^7 CFU, didn't affect significantly on hatchability (Majidi-Mosleh *et al.*, 2017). On the other hand, Triplett *et al.* (2018) discovered that injecting B. subtilis (10^3-10^6 CFU/ 50 mL diluent) into the amnion sac on day 18 (using a commercial inovoject device) negatively impacted hatchability. This was due to the B. subtilis species' energy-draining sporulating activity. Beck *et al.* (2019) successfully inoculated *Lactobacillus animalis* and *E. faecium* combinations into the amnion at day 18 of incubation without any adverse effects on hatchability, indicating that other factors (such as probiotic strain, volume, and dosage) besides injection method may be able to affect hatchability. In addition, probiotic treatments improved the feed conversion ratios on day 14 after hatching.

The overall birds' body weight, body weight gain, and feed conversion ratio increased when 1-day-old broiler chicks were fed either the single-strain Bacillus licheniformis (3.2×10^9 cfu/g) or the multi-strain (Bacillus coagulans (2×10^9 cfu/g) and Bacillus licheniformis (8×10^9 cfu/g)) of probiotic strains (Elleithy *et al.*, 2023). The production and metabolism of proteins, short-chain fatty acids, and vitamins by Bacillus coagulans may be responsible for this considerable improvement (Le Blanc *et al.*, 2017). Bacillus coagulans may assist the birds digest proteins and carbs once it has been active and germinated (Maathuis *et al.*, 2010). Additionally, supplying broiler feed with different strains of Bacillus dramatically increased body weight gain and the feed conversion ratio (Ahmat *et al.*, 2021; Wealleans *et al.*, 2017). Al-Khalaifa *et al.* (2019) conducted an experiment on 2 broiler cycles, the 1st during winter and the 2nd during summer on a 1-day-old Cobb 500 broiler chicks for each cycle, they used probiotics (Bacillus coagulans at 1 g/kg dried culture and Lactobacillus at 1 g/kg dried culture of 12 commercial strains, the results showed that there was no effect of the different probiotics and prebiotics on the production performance of broilers. In ovo Bifidobacterium bifidum at 10^9 and 10^7 CFU/egg and Bifidobacterium longum, at 10^9 and 10^7 CFU/egg respectively significantly improved body weight gain and feed converion ratio compared to the control groups (Abd El-Moneim *et al.*, 2020a).

5. Effect of probiotic on cacass criteria

Probiotic supplementation in ovo can enhance the carcass quality of broilers by improving feed efficiency and reducing mortality rates.

According to Stęczny *et al.* (2021) probiotic-supplemented broiler chicken groups did not differ in terms of mean body weight, carcass weight, or dressing percentage. In the same line, Milczarek *et al.* (2015) found a comparable high dressing percentage (73.6%) in Ross 308 chickens that were 42 days old.

The percentage of dissected carcass components did not vary noticeably between the carcasses from the 42-day-old Ross 308 chickens that fed on the multicomponent probiotics (Stęczny *et al.*, 2021). Comparing the carcasses of experimental and control birds, Stęczny *et al.* (2021) found that the carcasses from experimental birds of both sexes had greater percentages of skin with subcutaneous fat, wings, and the rest of the carcass and lower percentages (P> 0.05) of neck, breast muscle, leg muscle, and abdominal fat.

Ashayerizadeh *et al.* (2009) found that 42-day-old broiler hens supplemented with Primalac probiotic (900 g/ton of the compound feed) had significantly higher percentages of breast and legs (culinary cuts) and lower percentages of abdominal fat in their body weight.

Pelicia *et al.* (2004) reported a similar proportion of breast and leg meat as well as a non-significantly higher content of abdominal fat was found in the carcasses of 84-day-old free-range chickens supplemented with a mixture of probiotics and prebiotics of bacterial or yeast. On the other hand, Beck *et al.* (2019) reported that in ovo probiotic treatments improved the intestine (jejunum and ileum) weights on day 14 following hatching. Additionally, the intestinal morphology consequently immunological response was enhanced when *L. salivarius* and *Pediococcus parvulus* were combined (Neveling *et al.*, 2019).

The body weight gain and feed conversion ratio were essentially improved by probiotic supplementation, either with or without Aflatoxin B1(Suganthi *et al.*, 2011; Yunus *et al.*, 2011; findings, El-Sayed *et al.*, 2022). Additionally, supplementing chicken diets with probiotics, whether they contained or weren't infected with AFB1, markedly enhanced the weight of dressing % and reduced fat in comparison to the control group (El-Sayed *et al.*, 2022), these results are consistent with those of other studies (Aravind *et al.*, 2003; Indresh *et al.*, 2013). Comparing to the control treatment, Abdel-Moneim *et al.* (2019a) found that in ovo injection of bifidobacteria dramatically raised the ileal villus height (VH) and VH: crypt depth (CD) ratio, higher VH is related to higher digestive enzyme activity as well as increased small intestine segment absorption surface area. A longer enterocyte lifespan, less cell replacement, quicker repair of damaged enterocytes, and better performance are all correlated with shallower crypts and longer villi (Abdel-Moneim *et al.*, 2019b; Awad *et al.*, 2009).

The breast weight percentage increased by 8.5% compared to the control, while the bursa weight percentage increased by 26% and 14%, respectively, when using either the single-strain Bacillus licheniformis probiotic (3.2×10^9 cfu/g) or the multi-strain Bacillus coagulans (2×10^9 cfu/g) and Bacillus licheniformis (8×10^9 cfu/g) probiotic strains (Elleithy *et al.*, 2023). However, there were no significant (P > 0.05) differences found in immune organ weight percentages or carcass features (dressing yield, relative weights of the breast, thigh, drum, liver, and gizzard) between the treatment group and the control group were found (Elleithy *et al.*, 2023). Similar results were reported by Ahmat *et al.* (2021). In the same line, other earlier investigations, carcass characteristics did not improve when Bacillus probiotics were added compared to the control group (Upadhaya *et al.*, 2019 a, b; Bahrampour *et al.*, 2020). Other studies had reported that bacilli-based probiotics had no significant effect on the weight of immune organs (Ghahri *et al.*, 2013; Yun *et al.*, 2017). By raising villus height and the ratio of villus height to crypt depth in the gut of broilers, Bacillus increases the capacity of the small intestine for nutrient absorption and digestion (Sen *et al.*, 2012). This result was consistent with the large difference in intestinal villi length that we observed in the duodenum, jejunum, and ilium. Additionally, there was a significant difference in the duodenal crypt depth (Elleithy *et al.*, 2023). In terms of early nutrition, there were no differences in the proportion of carcass between the control and probiotic-supplemented groups (Nam *et al.*, 2022). Similary, Qorbanpour *et al.* (2018)

observed that dietary supplementation of probiotics did not affect the weights of the carcass, breast, and thigh of chickens. However, Mehr *et al.* (2007) reported that high levels of probiotic supplementation resulted in higher carcass weights and a higher percentage of breast weight. With the exception of the heart, where the weight proportion did not alter between the treatments groups, the majority of internal organ weight percentages were slightly greater in the positive control group (Nam *et al.,* 2022). In comparison to the negative control, the probiotic-treated groups displayed a rising trend in the percentage weight of the liver and spleen (Nam *et al.,* 2022). These results mostly agreed with studies showing that adding Bacillus subtilis to broilers' diets considerably increased the relative weight of the spleen but not the liver (Zhang *et al.,* 2013; Stefaniak *et al.,* 2020).Since no differences in the percentage weights of the liver, spleen, gizzard, and heart were seen between broilers given control or a probiotic-supplemented diet in earlier trials, it is unclear how probiotics affect internal organ weight (Stęczny *et al.,* 2020; Malik *et al.,* 2018)

There were no differences in the immune organs (thymus , spleen and Bursa of fabricius on the 1st day (P > 0.05) after the in ovo administration of probiotic, on the 14th and 21st days, the thymus and spleen organ indices in the probiotic group were considerably greater than those of the two control groups (P< 0.05), furthermore, at the 14th day revealed that, in comparison to the two control groups, both probiotic groups significantly (P< 0.05) increased the Fabricius bursa (Duan *et al.,* 2021).

Between the 14th and the 21st day after the in ovo injection, the probiotic group's villus height of the duodenum, jejunum, and ileum layers increased significantly (P< 0.05) in comparison to the non-injected and saline groups. Additionally, the probiotic group's crypt depth in the intestine was found to be significantly (P< 0.05) lower than that of the non-injected and saline groups, and its ileum villi (Duan *et al.,* 2021). By increasing the absorptive surface area, which is crucial for the administration of alternative growth stimulators, morphological changes in the small intestine, such as raising villus height and the villus height / crypt depth ratio, might improve the performance of chicks (Calik *et al.,* 2015). There was a significant effect on thymus (P = 0.027), the thymus weight in the group fed Lactobacillus was significantly higher than that in the control group, this increase in the thymus weight may indicate an increase in the T-immune cells in the thymus, on the other hand, there was no significant effect of the experimental diets observed on tissue weight for abdominal fat, heart, spleen, liver, bursa, breast, and leg and thigh (Al-Khalaifa *et al.,* 2019).

Supplementation of probiotic in broiler diet from day 1 resulted in statistical different in the weights of livers, hearts, and abdominal fat (Haščík *et al.,* 2014). Also, impact of Probiotics to broiler feed exhibited substantial advantages in the carcass percentage at selling age (Soliman *et al.,* 2003). The average values of internal organs as a percentage of body weight (kidneys and liver) increased significantly in the groups supplemented by Lactobacillus acidophilus, to broiler feed at 1.0 g/kg compared to the control group (Kumar *et al.,* 2003). Nevertheless, supplementation of probiotics to diets raised the weights of the liver, large and small intestines, and the empty gizzard (Çelik *et al.,* 2007). In addition, employing Lactobacillus strains, considerably improved the dressing and carcass qualities of broiler chickens as well as the weights of the liver and liver dressing (Fayed and Tony, 2008). Carcass weight of broilers had improved positively by using *P. acidilactici* in broiler (Chafai, 2006). On the other hand, probiotics had no discernible impact on absolute dressing weights, dressing percentage, heart, gizzard, or pancreas (Abo-Mahara *et al.,* 2010). Supplementation of B. subtilis at a level of 2.08 $\times 10^8$ cfu/g diet resulted in a considerable decrease in the liver percentage (Molnár *et al.,* 2011). Feeding broilers on Pediococcus species significantly (P<0.01) improved the weight of both slaughter and carcass of broilers (*Brzóska et al.*, 2010).

Also, the dietary probiotic additions had no impact on the quantity of the abdominal fats/or fat yield, as well as the relative weight of the proventriculus, liver, and gizzard (Ibrir *et al.*, 2008).

Awad *et al.* (2009) revealed a comparable, but not significant, decrease in carcass % in broilers fed a B. subtilis-supplemented diet compared to control broilers. The probiotic additions to the feed of broiler chicks had no appreciable impact on carcass quality (Khani and Hosseini, 2008; Mohamed and Bahnas, 2009; Taklimi *et al.*, 2010).

6. Effect of probiotic on blood biochemical parameters

Studies have shown that in ovo administration of probiotics can improve blood biochemical parameters, such as Short-chain fatty acid (SCFA), antioxidants, glucose, cholesterol, and triglyceride levels, in broilers. However, further research is needed to determine the optimal dosage and timing of probiotic administration. The SCFA production and immunomodulation are both improved by probiotic-induced alterations in the microbial populations of the gastrointestinal tract (Anwar *et al.*, 2016). SCFAs interact with a variety of receptors, but they also stimulate B-immune cells to produce IgA, block the NF-B transcription factor, and lower chemokine and cytokine production (Delgado *et al.*, 2020). According to Abdel-Moneim *et al.* (2019b), probiotic medication had no effect on serum glucose levels when compared to control levels, however it did boost thyroid hormone activityProbiotics may also increase the activity of the corticotrophin releasing factor (CRF), which in turn stimulates the release of thyrotropin and, consequently, the production of T4 (Klieverik *et al.*, 2009). Abdel-Moneim *et al.* (2019b) reported that GSH concentration was unaffected by in ovo therapy, whereas serum MDA content was dramatically reduced and SOD activity significantly increased, they proposed that probiotics physiologically strengthen birds' natural antioxidant defences. These results are in close agreement with the findings of many other researchers (Abd El-Moneim *et al.*, 2019a; Abudabos *et al.*, 2016). According to Popović *et al.* (2015), birds in ovo treated with bifidobacterial strains showed noticeably stimulated immunological responses, notably elevated serum immunoglobulin levels (IgA, IgM, IgY, and total Igs), they said that increase in blood Ig levels demonstrates how the gut microbiota encourages the production of natural antibodies in birds. This increase in blood Ig levels demonstrates how the gut microbiota encourages the production of natural antibodies in birds (Popović *et al.*, 2015).

Numerous studies have demonstrated that taking probiotics aids in immunological development and boosts the production of natural antibodies that are crucial for pathogen defence (Ochsenbein *et al.*, 1999; Abd El-Moneim, 2017). Macpherson *et al.* (2000) investigated that the interactions between the host cells and the helpful bacteria in the colon clearly demonstrate that commensal microorganisms are in close touch with the cells of the gut-associated immune system. Additionally, Sohail *et al.* (2015) discovered that incorporating probiotics into the diet of fowl had either negative or neutral impacts on the gut flora.

Recent studies show that specific non-pathogenic intestinal microbiota species interact with the immune system and epithelium to modify tissue physiology and the body's capacity to fight infection (Jha *et al.*, 2020). In the lymphoid tissue associated with the gut, the dendritic and epithelial cells of the intestine serve as mucosal sentinel cells (Jha *et al.*, 2020). Through immune activation, antigen presentation, and the production of antimicrobial agents, this activation results in the overexpression or repression of genes that control the inflammatory response as well as cytoprotective effects (Kemgang *et al.*, 2014).

The in ovo feeding method involves injecting supplements into incubating eggs to control the growth of healthy birds and enhanced gut health, boosting chicken performance from preto post-hatch to adult age (Jha *et al.*, 2019b). According to Pender *et al.* (2017) evaluation of the effects of in ovo inoculation, both strains of *S. faecalis* and *L. acidophilus* were found to act as immunomodulators, as demonstrated by the effect on the expression of many immune-related genes inside the ileum and cecal tonsils. Despite the fact that probiotic administration induced favourable immunological responses, Sadeghi *et al.* (2015) found that environmental factors significantly influenced the strain's efficiency. Sadeghi *et al.* (2015) study the effects of *B. subtilis* on antibody titers against Newcastle and infectious bursal viruses, the findings revealed that B. subtilis had no appreciable effects on the immune parameters of chickens in non-contaminated environments but showed excellent efficacy in environments contaminated with pathogens. Contrary, Bai *et al.* (2017) evaluated B. subtilis' impacts on intestinal immunological features showed beneficial effects on the intestinal T-cell immune system.

According to Adli *et al.* (2023) data, using probiotics can help lower cholesterol characteristics, however the effect is statistically negligible. Microorganisms like Lactobacillus and Bifidobacterium work together to produce short-chain fatty acids, which lowers the level of blood cholesterol, additionally, the increast in total protein and albumin are reliable signs that domestic rabbits have stronger immune systems and immunoglobulins (Helal *et al.*, 2021).

For instance, probiotics contribute to the mechanisms that lower triglyceride levels after lowering blood cholesterol levels (Sjofjan *et al.*, 2021). According to Fathi *et al.* (2017), the reduction of remnant lipoprotein and the shift of lipids from plasma to target organs, in this case the liver, are also associated with reduced triglyceride levels. The production of digestive vitamins, enzymes, and antibacterial agents (such as bacteriocins, organic acids, hydrogen peroxide, lactoperoxidase system, lactone components, diacetyl, and acetaldehyde) by probiotics can also lower blood cholesterol levels, boost immunity, prevent the growth of infectious bacteria, remove carcinogens, and remove carcinogens (Nandi *et al.*, 2017). Probiotics in ovo did not significantly affect the avian immune response, according to De Oliveira *et al.* (2014). Andreatt-Filho et al. (2006) confirmed the immunomodulatory effects of probiotics. Alizadeh *et al.* (2020) used molecular techniques to demonstrate that in ovo-delivered L. spp. can decrease the expression of cytokine genes in the cecal tonsils. In comparison to the control birds, the probiotic therapy had significantly higher specific activity of -galactosidase and -galactosidase.

Even though the changes weren't statistically significant, utilising the multi-strain revealed expressed greater serum albumin concentrations than the control (Elleithy *et al.*, 2023). Heat-inactivated probiotics had no significant effects on total protein, albumin, or lipid contents in the blood Zhu *et al.* (2020). The antibacterial compounds released by Bacillus that prevent the growth of infections and improve the utilisation of food protein were thought to be the cause of the higher blood albumin levels in birds receiving bacilli supplements (Ahmat *et al.*, 2021). Broilers given probiotics showed an increase in the level of total protein and albumin when compared to controls (Khabirov *et al.*, 2020).

Broilers fed diets supplemented with biological additives showed increased blood total plasma protein, globulin, haemoglobin, and albumin levels, according to Abdel-Azeem (2002). The addition of B. licheniformis, and B. subtilis to broiler diets throughout growth, according to Abaza *et al.* (2008), had no appreciable impact on the levels of total protein, cholesterol, globulin, and albumin in the blood. According to other studies (Sherif, 2009; Rabie *et al.,* 2010), adding probiotic to broiler diet had little to no effect on blood plasma parameters such globulin, total protein, albumin, It is well known that excessive concentration of Alanine aminotransferase (ALT), in the serum indicates the development of the organ dysfunction and disease progression; liver pathology being the major reason for its increase (Khabirov, *et al.,* 2020). Aspartate aminotransferase (AST) is an enzyme involved in protein metabolism it performs a number of important functions, one of which is the participation in the construction of cell membranes and the synthesis of amino acids (Khabirov *et al.,* 2020). AST, ALT usually appear in serum when there is damage on the liver and muscle tissues caused by excessive stress (Scholl *et al.,* 2006; Ozyurt *et al.,* 2006).

The hypocholesterolemic effect of probiotics is attributed to their ability to bind cholesterol in the small intestine (Ahmat *et al.,* 2021; Manafi *et al.,* 2017). Cholesterol is included in developing cell membranes and bile formation, and is a precursor of vitamin D and many hormones, serum cholesterol levels in probiotic were lower than in the control group (Elleithy *et al.,* 2023). Broiler supplemented with either the single-strain or the multi-strain of Bacillus-based probiotic raised exhibited significantly ($P \leq 0.05$) improved, Alanine aminotransferase ALT, aspartate aminotransferase AST, and alkaline phosphatase levels than the control (Elleithy et al., 2023)

Kumar *et al.* (2003) used probiotic (L. acidophilus) at a feeding rate of 1.0 g/kg in Broiler chicks diet, they revealed that, at 45 days of age, treated birds had lower mean AST and ALT rates, triglyceride levels, and low-density lipoprotein (LDL) than the control one, groups that supplemented with B. subtilis resulted in linear decreases in ALT, AST, urea, and creatinine levels while increasing blood levels of albumin and total protein. However, there was no quadratic or linear relationship between blood levels of globulin, and uric acid and B. subtilis intake in the diet (Al-Otaibi *et al.,* 2023). Probiotic supplementation enhanced average albumin and total protein levels and decreased average cholesterol, ALT, AST, and total lipids values, according to Abd El-Gawad et al. (2004).

Additionally, plasma cholesterol and total lipids values fell, while AST and ALT enzymes were unaffected (Tolba *et al.,* 2004). El-Yamny and Fadel. (2004) found that yeast supplementation at dosages of 0.2, 0.4, and 0.6% enhanced blood total protein, A/G ratio, and albumin; however, AST and ALT were unchanged, at the same time, cholesterol and lipid levels were considerably reduced.

Using probiotic on one-day old male Japanese quail chicks in drinking water at 3%, 6%, 9% and 12 % resulted in significantly increased AST enzyme level compared to control group ($p<0.05$), ALT enzyme activity in all treatment groups was higher than control group but the changes weren`t significance (Vahdatpour and Babazadeh, 2016). Enhanced liver functions were identified in sera of probiotic treatments as reduced ALT and AST concentrations, which indicated the significant efficacy of bacilli in the protection of treated birds from hepatocellular damage compared to controls (Hussein *et al.,* 2020).

Serum Triglycrides decreased due to the addition of the combined Bacillus coagulans and Bacillus licheniformis probiotic compared to the control; however, the decrease was not statistically significant (Elleithy *et al.,* 2023). These result consistent with (Zhu *et al.,* 2020). By reducing the blood cholesterol levels in hens, the probiotic dietary supplements had a beneficial impact on the health of the host animal (Kim *et al.,* 2003; Kurtoglu *et al.,* 2004; Hajjaj *et al.,* 2005). It has been demonstrated that the probiotic strains L. acidophilus and E. faecium M74 reduce plasma cholesterol levels (De Roos and Katan, 2000).

Numerous probiotics, including *Rhodobacter* spp., *Lactobacillus* spp., Bacillus spp., and A. oryzae, have been demonstrated to lower blood serum cholesterol in broiler chickens (Yoon *et al.*, 2004; Capcarova *et al.*, 2010). After two weeks post-treatment, laying hens fed a diet containing liquid cultures of Bifidobacterium bifidum (B) or Lactobacillus acidophilus (L), or a combination of both, 50 g of L + 50 g of B/kg feed diet, experienced a significant reduction in total lipid and cholesterol levels. Broilers fed diets containing Lactobacillus at the sixth week of life exhibited lower cholesterol concentrations in their carcasses and livers, according to Kalavathy *et al.* (2003).

On the other hand, Broiler Chicks fed a commercial diet containing fermented probiotic product from B. subtilis at 0.5, 1.0, and 2.0% for 21 days between 7 and 28 days of age did not affect the content of plasma cholesterol, phospholipids, and triglycerides, according to Santoso *et al.* (2001).The specificity of the probiotic effect on serum cholesterol, according to Kurtoglu *et al.* (2004), did not manifest until days 30 and 60. Broiler chicks fed diets supplemented with or without 1 g/kg of S. cerevisiae showed little difference in the amount of total protein, globulin, or albumin El-Ghamry *et al.* (2002). Broiler Supplemented with either the single-strain or the multi-strain of Bacillus-based probiotic raised exhibited significantly ($P \leq 0.05$) improved cholesterol levels than the control (Elleithy *et al.*, 2023) Adding multi-Bacillus strains reduced serum uric acid levels compared to the control, however, no significant differences were found between the groups in the uric acid, uric acid is the nitrogenous excretory product of protein metabolism in birds and the measurement of its serum levels is one of the renal functiontests (Elleithy *et al.*, 2023). This result indicated that bacilli probiotics reduced the pressure on birds' kidneys by reducing the serum nonprotein nitrogen as uric acid (Ahmat *et al.*, 2021).

In the 1st cycle immunoglobulin (IgA) titers in broilers fed on diets supplemented with B. coagulans were higher than those observed in birds fed on the control and the Lactobacillus diets, but not significantly, however, there was no significant effect of the different dietary treatments on IgM titers, in the 2nd cycle there was no significant effect of the different dietary treatments on total antibody titers in the broilers(Al-Khalaifa *et al.*,2019).On the contrary, a study was conducted to determine the effects of dietary probiotics on natural IgM levels binding keyhole limpet hemocyanin (KLH) in indigenous chicken (IC) in Kenya, one hundred and fifty 2-month-old chickens were randomly divided into 5 treatments of 25 birds each, and 5 mL of the probiotic, dietary probiotic supplementations did not significantly affect the levels of IgM binding the KLH (Khobondo *et al.*, 2015). Comparing to the control, in probiotic supplemented groups, T3 and T4 hormons activity increased linearly (Abdel-Moneim *et al.*, 2020). Comparatively to the untreated control, the serum malondialdehyde (MDA) contents were reduced by all tested B. subtilis dietary amounts. The glutathione (GSH) activities were linearly increased in groups BS5 and BS at the same time (Abdel-Moneim et al., 2020).

7. Effect of probiotic on intestinal microflora

Studies have shown that probiotics in ovo can improve intestinal microflora balance and promote immune system development in chicks.

In the early 1980s, in ovo immunisation against Marek's disease was successfully proven to be a reliable method of preventing infection from virus exposure during development (Ricks *et al.*, 1999; Bublot *et al.*, 2004). As a result, numerous studies on the injectable form of biological substances, such as amino acids, vitamins, minerals, hormones, immunostimulants, probiotics, prebiotics, and other bioactive chemicals, have been conducted as a result of the success of in-ovo immunization (Roto *et al.*, 2016).

Exposure to pathogenic bacteria during the perinatal period affects the growth capacity and immune responses of newly hatched chicks by impairing the development of immune organs, the GIT, and skeletal muscles (Gensollen *et al.*, 2016).

Probiotics and bioactive substances are injected in ovo with the intention of promoting early colonisation of the embryonic gut with the beneficial microbiota (Shehata *et al., 2021*).

The success of the birds' health and growth during their anticipated 45-day production phase is significantly influenced by the GIT. According to Sohail *et al.* (2015), the health and function of the host depend on the microbial populations in the gut. For instance, live B. subtilis strains of microorganisms or probiotics have been provided to chickens to enhance the integrity of their gastrointestinal tracts, the secretion of IgAs from their duodenum, and their feed conversion ratio (Amerah *et al.,* 2013).

In general, only organisms that have been proven through in vitro studies to be nonpathogenic to the future host and acidity tolerant should be taken into consideration for in ovo delivery (Bajagai *et al.,* 2016). Birds' overall health, growth performance, nutritional digestion, and host metabolism are significantly influenced by their diverse gut flora (Yadav and Jha, 2019). Age, particularly in the early stages of life, genotype, farming conditions/environment, and diet/feed additives all have an impact on the makeup of chicken gut microbiota (Diaz *et al.*, 2018).

Probiotics can change dysbiosis and improve the balance of the gut microbiota in healthy hosts by decreasing the proliferation of pathogenic species and increasing the number of beneficial bacteria (Bajagai *et al.,* 2016; Yadav and Jha, 2019a), as a result, they can have an impact on the hosts' health, performance, and risk of disease. According to Yitbareket *et al.* (2015), the most popular probiotic species come from the genera Lactobacillus, Streptococcus, Bacillus, Bifidobacterium, Enterococcus, Aspergillus, Candida, and Saccharomyces and preferentially benefit the host's health by actively excluding harmful bacteria and modifying the immune system in the gut (Bajagai *et al.,* 2016). Numerous papers have discovered benefits of probiotics supplementation on the digestive tract's microbial fermentation, enzyme activity, and gut microbiota in broiler chickens (Nakphaichit *et al.,* 2011; Martínez *et al.,* 2016; Ahmed *et al.,* 2014; Yitbarek *et al.,* 2015; Asghar *et al.,* 2016; Wang *et al.,* 2017).

According to Abdel-Moneim *et al.* (2019a), the in ovo injection process had a significant impact on the microbial environment in chickens' guts, this was accompanied by a significant increase in Bifidobacterium spp. and lactic acid bacteria counts as well as a decrease in the population of total coliform bacteria in the ileal digesta. Earlier studies by Mountzouris *et al.* (2010); Higgins *et al.* (2008); Vicente et al. (2008), investigated that various probiotics may have comparable potential effects to modify and reinforce the composition of the ileal microbiota of chickens by enhancing beneficial microbes and reducing harmful microorganisms. A better epithelial barrier, improved good bacteria attachment to the intestinal mucosa, and concurrent suppression of pathogen adhesion are further advantages (Broom *et al.*, 2018). De Oliveira *et al.* (2014) found that the chosen probiotics had the maximum number (P> 0.05) of recovered bacteria in the gizzard and ceca, further justifying their choice at 48 hours after hatching. Probiotics were found to boost the intestinal population of lactic acid bacteria on day 3 after hatching, while decreasing the population of Escherichia coli, according to De Oliveira *et al.* (2014).

In a recent study by Skjt-Rasmussen *et al.* (2019), the viability of the E. faecium (M74) strain for probiotic injection on day 18 via amniotic fluid was confirmed. Both visual colony examination and DNA genotypic fingerprinting revealed high recoveries of enterococci in the yolk sac, cecal tonsils, and the rest of the intestinal tract. Inoculating *Lactobacillus* spp. (*Lactobacillus acidophilus, L. fermentum,* and *L. salivarius*) through the air cell at embryonic day

18 did not successfully ward off Salmonella Enteritidis infection, as Yamawaki *et al.* (2013) showed. Arreguin *et al.* (2019) recently established that the severity of virulent E. coli cross-infection in broiler chickens can be reduced by the in ovo distribution of vegetative Bacillus spp. strains (included in Norum). A multi-bacterial species probiotic that included two Lactobacillus strains, one Bifidobacterium strain, one Enterococcus strain, and one Pediococcus strain was evaluated by Mountzouris *et al.* (2007), they said that probiotics altered the composition and activities of the cecal microbiota of broiler chickens. The role of L. reuteri in freshly hatched broiler chicks was examined by Nakphaichit *et al.* (2011), the findings revealed that the probiotic group's ileum samples at day 42 had five times more total bacteria than the control group's samples did.

In a different study, L. *johnsonii*, *L. crispatus*, *L. salivarius*, and one unidentified Lactobacillus spp. were examined, along with 294 day-old Cobb broiler chickens. The findings showed that adding probiotic Lactobacillus spp. to feed increased the number of lactic acid bacteria and Lactobacilli in the ceca and the total number of anaerobic bacteria in the ileum and ceca. Additionally, when compared to the control treatments, the amount of Enterobacteria in the ileum was generally reduced by all four probiotics (Olnood *et al.,* 2015). Martínez *et al.* (2016) used Propionibacterium acidipropionici's probiotic at a concentration of 10^6 cfu/mL in the drinking water, this supplementation demonstrated lactic acid bacteria and bifidobacteria growing normally, however Bacteroides colonisation was delayed, at the end, this resulted in an increase in lactic acid production, a decrease in butyric acid synthesis, and an increase in mucus secretion, which improved defence against infections. The ileal and cecal microbiota of broilers supplemented with B. licheniformis and B. subtilis did not significantly change (Zaghari *et al.,* 2020). When Ross 308 broiler chickens were given a mash meal supplemented with Lactobacillus acidophilus, L. casei, Enterococcus faecium, and Bifidobacterium thermophilus, a non-significant effect on the total aerobic and Salmonella count in the stomach was also discovered (Cengiz *et al.*, 2015).

Neveling *et al.* (2019) found that Salmonella was prevented from colonising the gastrointestinal tracts of broilers by a mixture of Lactobacillus crispatus, Lactobacillus salivarius, Lactobacillus gallinarum, Lactobacillus johnsonii, Enterococcus faecalis, and Bacillus amyloliquefaciens. Salmonella enteritidis exposure caused a decrease in the amount of coliform and salmonella in the cecum while an increase in lactobacilli and bifidobacterium was observed in broilers fed a diet containing Bacillus coagulans (Zhen *et al.*, 2018). Rotolo *et al.* (2014) stated that it has been proven through the use of microbiological instruments that bacteria make up the majority of probiotics and collaborate closely with other microorganisms in the rabbit caecum. The bacteria enhance the morphology of intestinal barrier-cell systems in rabbits and penetrate into the bloodstream to attract epithelial cells (Rotolo *et al.,* 2014). According to bifidobacteria's positive effects subsequently decreased the incidence of infectious and enteric illnesses, particularly Salmonella enteritidis and E. coli. (Chotinsky *et al.,* 2003; Hashemzadeh *et al.*, 2010; Abudabos *et al.*, 2016; Vicente *et al.*, 2008), and increased the producers profits (Okanović *et al.*, 2014).

Broiler chickens' growth and ileal development were increased by in ovo stimulation by Bifidobacterium bifidum and Bifidobacterium longum through inyolk sac injection on day 17 of incubation (Abd El-Moneim *et al.*, 2020). In addition, compared to the control, in ovo inoculation of *Bifidobacterium* spp. enhanced the numbers of ileal Bifidobacterium spp.and lactic acid bacteria. Additionally, with in ovo treatments, the overall coliform and bacterial levels dropped (Shang *et al.,* 2018). On the other hand, in ovo inoculation with Bacillus spp.

significantly reduced the total number of Gram-negative bacteria at the day of hatching and day 7 of age compared to the control group (Shehata *et al.,* 2021). Due to the in ovo injection of probiotics, the Firmicutes phylum showed a large rise whereas the Proteobacteria phylum significantly decreased (Shehata *et al.,* 2021). Bacillus species appear to have this effect by changing the microbiota's populations and community structures (Arreguin *et al.,* 2019). Additionally, early gut microbiota stimulation with probiotic, prebiotic, or their combination treatment can improve the health and productivity of freshly hatched chicks (Abd El-Moneim *et al.,* 2020; Madej *et al.,* 2015; Slawinska *et al.,* 2016). Furthermore, the in ovo inclusion of these compounds enhanced the development of the GIT, gut microbiota, and lymphoid organs (Arreguin *et al.,* 2019; El-Kholy *et al.,* 2019; De Oliveira *et al.,* 2014).On day 17 of incubation, the use of various probiotics (2 $\times 10^8$ CFU/egg of *Bifidobacterium bifidum, B. animalis, B. longum, or B. infantis*) for in ovo stimulation led to a significant increase in the count of ileal lactic acid bacteria and Bifidobacterium spp. compared to the control group, while the total coliform and total bacterial counts were significantly decreased(Abdel-Moneim *et al.,* 2019a).

Ross 708 fertilized eggs were utilised in the in ovo procedure by Li *et al.* (2021) at embryonic day 18, heart, liver, and spleen samples were taken on days 0, 14, 28, and 42, while yolk sac samples were taken on days 0 and 14 due to the fact that, in most birds, after day 14, the yolk is typically absorbed through the yolk stalk duct. They found that in ovo administration of various probiotic species did not significantly affect the incidence of avian pathogenic Echrechia coli (APEC-like) strains in broiler chickens,

In the summer cycle, supplementation of probiotic didn’t affect the count of both lactic acid bacteria (LAB) and E. coli at 3-week-old broilers, at 5-weeks-old of the same cycle, the bacterial count of E. coli increased even with control, whereas Salmonella growth was inhibited, in the winter cycle, there was no Salmonella sp. recorded using the experimental treatments, and the growth of E. coli was significantly reduced (Al-Khalaifa *et al.,* 2019). In ovo inoculation of probiotics was found to be effective at lowering Salmonella colonisation from day 1 to day 7 (Hashemzadeh *et al.,* 2010). The population of lactic acid bacteria in the jejunum of broiler chickens was not affected by in ovo injection at the 1st day ($P > 0.05$), but a difference was observed at the 3rd day post-hatch ($P < 0.05$) (Majidi-Mosleh *et al.,* 2017). On the 3rd day after infusion, pediococcus acidilactici and Bacillus subtilis had the highest lactic acid bacteria populations of all the in ovo infused groups (Majidi-Mosleh *et al.,* 2017). Compared to the control group, inovo injection of probiotic had no effect ($p>0.05$) on E. coli population in one-day-old chicks but reduced it on day three of age (Majidi-Mosleh *et al.,* 2017). Lourenco *et al.* (2012) indicated that oral feeding Bacillus subtilis decreased significantly Salmonella population in broiler gut. Pediococcus acidilactici prevents growth and development of intestinal small bacteria such as Shigella, clostridium and E. coli. Therefore, Pediococcus acidilactici increases the resistance of birds to pathogenic bacteria (Lee *et al.,* 2007).

8. Conclusion

The performance of broilers, including growth rate, feed conversion ratio, and immunological response, can be enhanced by in ovo probiotics, according to the available research. To fully comprehend the advantages that could be gained from using in ovo probiotics, however, more research is required.

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The authors disclosed no conflict of interest

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