Poultry Science

In ovo probiotic supplementation supports hatchability and improves hatchling quality in broilers

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ABSTRACT In modern broilers, the period of embryonic development constitutes a greater proportion of a broiler's productive life. Hence, optimum embryonic development can exert a significant influence not only on chick hatchability and hatchling quality but also on overall broiler growth and performance. Further healthy and active hatchlings are correlated with improved posthatch performance. In this regard, probiotics are good candidates to mediate early-life programming. Therefore, we evaluated the effect of In ovo probiotic spray application on broiler hatchability and hatchling quality. The experiment was set out as a completely randomized study with 2 independent trials. In each trial, 540 eggs (Ross 308) were either sprayed with phosphate buffered saline (**PBS**; control) or probiotics [~9 log CFU/egg of *Lactobacillus rhamnosus* NRRL B-442(LR) or *Lactobacillus paracasei* DUP 13076 (**LP**)] during incubation. On day 18, eggs were transferred to the hatcher and set up for hatching. Starting on day 19, eggs were observed for hatching to determine the spread of hatch and hatchability. Hatched chicks were then assessed for quality using the Tona and Pasgar score and morphometric measurements including hatchling weight, yolk-free-body-mass and hatchling length were measured. Further, chicks were reared in floor pens for 3 wk to assess posthatch growth. Overall, In ovo probiotic supplementation improved hatchability and hatchling quality. Specifically, the spray application of LP improved hatchability by ~ 5% without affecting the spread of hatch. Further, both LR and LP significantly improved Pasgar and Tona score, indicating an improvement in hatchling quality. Also, LP and LR significantly improved hatchling weight, yolk-free-body-mass, and posthatch growth in chicks. LR significantly improved hatchling weight and hatchling length ($P < 0.05$). Moreover, this increase in posthatch growth was positively correlated with hatchling weight in the probiotic groups. Overall, our study demonstrates that In ovo probiotic application exerts a positive effect on hatchability, hatchling quality, and subsequent posthatch growth.

Key words: in ovo, probiotic application, broiler, hatchability, hatchling quality

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INTRODUCTION

Since the 1950s, the slaughter age of broilers has been reduced by approximately 40% (Givisiez et al., 2020). As the slaughter age decreases, the period of embryonic development constitutes a greater proportion of a broiler's productive life (de Oliveira et al., 2008). In fact, in modern broilers, the incubation period takes up one-third of the broiler's life span (Uni and Ferket, 2004; Cox and Dalloul, 2015). Suboptimal embryonic development causes reduction in hatchability and poor chick quality, which can result in significant economic loss to the poultry industry. Towards this, over $500 million in losses were reported due to reduced hatchability in broilers and turkeys (Schaal and Cherian, 2007). Beyond hatchability, chick quality is critical to subsequent broiler growth and profitability of poultry producers (Van de Ven et al., 2012). Hence, the essential objective in hatcheries is to maximize hatchability with a great number of high-quality and saleable chicks desired for their high viability and slaughter yield (Decuypere and Bruggeman, 2007). Hatchling survivability is critical since current commercial practices involve transportation of the newly hatched chicks to grow-out farms usually with no access to feed for up to 72 h posthatch (Souza da Silva et al., 2021). Data show that approximately 2 to 5% of chicks are lost during transit and in the first week posthatch due to limited energy reserves (Uni and Ferket, 2004; Yerpes et al., 2021). The importance of supporting embryonic development, hatchability, and chick quality is further highlighted by the

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implications for stunted growth, inefficient feed utilization, poor meat yield, and associated economic costs for low quality chicks (Yeboah et al., 2019; Molenaar et al., 2023; Orellana et al., 2023).

To solve this problem, several strategies have been investigated. Optimal egg storage and incubation parameters have been well studied and are currently in practice at commercial hatcheries (American Poultry Association, 2022). Besides, preincubation, SPIDES (short periods of incubation during egg storage) and provision of LED light are also used to improve hatchability (Pas Reform, 2014; Huth and Archer, 2015; Poultry World, 2015). Further, HatchCare system was developed to create an incubation condition with optimal temperature, and provide light, water and feed to hatchlings immediately after hatch. It shows that the HatchCare system can increase hatchability and deliver good chick quality (HatchTech, 2023). Besides, van de Ven evaluated Patio system, which combines hatching and brooding phase. Results indicated that the hatching system had minor effects on hatchling physiology and did not affect posthatch growth and livability (Van de Ven et al., 2011).

In addition to these strategies, supplementing nutrients to the developing embryos could serve to improve embryonic development and energy reserves. Towards this, In ovo injection of nutrients is one of the most investigated approaches. In ovo injection of carbohydrates, amino acids, minerals, vitamins, growth hormones and other compounds were found to improve hatchling weight, liver glycogen, breast muscle and intestine development. Further, this growth-promoting effect was found to be sustained in the posthatch period through improved body weight gain, gut microbiome modulation, increased feed utilization efficiency, and enhancement of overall health (Tako et al., 2004; Uni et al., 2005; Jha et al., 2019; Neves et al., 2020; Subramaniyan et al., 2020). Although primarily used as in-feed additives, probiotics are ideal candidates for In ovo supplementation to modulate the gut microbiota and mediate early-life programming in broiler chickens (Shehata et al., 2021).

In ovo inoculation of probiotics in the late-term embryo was found to increase villus height and crypt depth, improve gut microbial composition, reduce FCR, decrease *Enterobacteriaceae* and Gram-negative bacteria population in the gut and increase gluconeogenesis in chicks and adult birds (Teague et al., 2017; Arreguin-Nava et al., 2020; EI-Moneim et al., 2020; Rodrigues et al., 2020; Wilson et al., 2020; Das et al., 2021). However, in the above-mentioned studies, probiotic supplementation was performed by In ovo injection into the air sac, amnion, or yolk sac to late-term embryos. These invasive procedures have been associated with a reduction in hatchability varying from 0.8 to 10% (de Oliveira et al., 2014; Oke et al., 2021; Geng et al., 2022), Further, in these studies, In ovo injection were performed manually and hence highly dependent on the expertise of the person. Additionally, although In ovo technology has been developed for over 2 decades, its commercial application has been limited due to the need for specialized inoculation equipment, time, and capital investment (Ravindran and Abdollahi, 2021). Our study employed a noninvasive spray application approach to deliver probiotics (Amalaradjou, 2022). Besides being user-friendly, spraying is a commonly used method to disinfect hatching eggs prior to setting (Sheldon and Brake, 1991; Buhr et al., 1994; Bourassa et al., 2002; Copur et al., 2011). Hence, the spray application could be easier to be integrated with routine poultry management practices. Further, recent research from our lab demonstrates the ability of probiotic spray application to promote embryo growth and muscle development in broiler and layer embryos (Amalaradjou, 2022; Muyyarikkandy et al., 2023a, b). Since optimum embryonic growth is critical to hatchability, based on our previous findings, we hypothesize that In ovo probiotic application would support hatchability and improve hatchling quality in broilers thereby subsequently promoting posthatch performance.

MATERIALS AND METHODS

Probiotic Culture Preparation

Lactobacillus rhamnosus NRRL B-442 (**LR**) was obtained from the USDA Agriculture Research Service NRRL culture collection (Peoria, IL). *Lactobacillus paracasei* DUP 13076 (**LP**) was kindly provided by Dr. Bhunia, Molecular Food Microbiology Lab, Purdue University, West Lafayette, IN. LR and LP were selected based on preliminary screening and published literature (Amalaradjou, 2022; Muyyarikkandy et al., 2023a, b). The cultures were grown in de Mann, Rogosa, Sharpe broth (**MRS**; Fisher Scientific, Waltham, MA) at 37°C for 16 to 18 h. Overnight cultures were centrifuged (3,500 g, 10 min, 4°C), and washed twice with sterile phosphate buffered saline (**PBS**, pH 7.0). Bacterial counts of LR and LP were determined following serial dilution and plating on MRS agar and incubated at 37°C for 24 to 48 h (Muyyarikkandy and Amalaradjou, 2017).

Experimental Design and Egg Incubation

Overall experimental design is depicted in Figure 1. All trials were conducted at the UConn poultry research unit with approval from the UConn Institutional Animal Care and Use Committee. Ross 308 hatching eggs (n = 600/trial) from 40 to 42-wk-old birds were kindly provided by Aviagen (Huntsville, AL). These eggs did not receive any disinfection treatments and were shipped as clean nest eggs. On receipt, damaged eggs were discarded, and the rest were stored at 12.8°C for no more than 24 h (Christensen et al., 2002). Prior to incubation, all settable eggs were weighed (starting egg weight), numbered, and randomly assigned to the 3 treatment groups (180 eggs/group). Group 1: Eggs sprayed with PBS (vehicle control), Group 2: Eggs sprayed with LP, Group 3: Eggs sprayed with LR. Preliminary trials were conducted to compare PBS sprayed eggs and unsprayed eggs, and results demonstrated that PBS spray did not show any

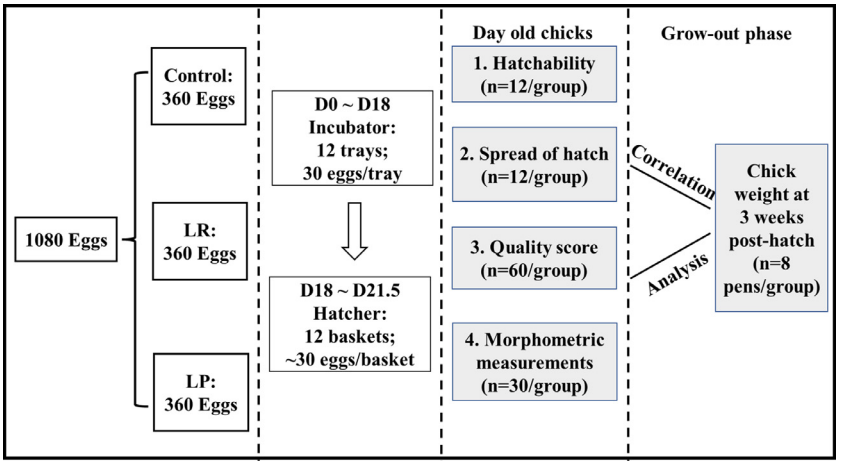

Figure 1. Schematic representation of the experimental design.

significant effect on embryo growth and hatchability (data not shown). Eggs were individually sprayed with different probiotic cultures (~ 9 log CFU/egg) or sterile PBS (Control) on days 0, 3, 7, 10, 14, and 18 of incubation using an atomizer as previously described (Amalaradjou, 2022; Muyyarikkandy et al., 2023a, b). These time points for spraying were chosen to help maintain significant probiotic populations on the egg surface. The eggs were incubated in the GQF 1502 incubator with an automatic egg turner (GQF Manufacturing Co., Savannah, GA) at 37.8°C and 55 to 60% relative humidity from day 0 to day 18. Eggs were candled on day 10 to check for fertility and early embryonic mortality.

Hatching, Spread of Hatch and Hatchability

On day 18, eggs were transferred to a GQF 1550 digital hatcher and incubated at 37.8°C and 65 to -70% relative humidity until hatch (Aviagen, 2020). Throughout the study, eggs in different groups were placed in separate incubators/hatchers to avoid cross-contamination (Archer and Cartwright, 2017). From day 19, the number of pipped chicks and hatched chicks were recorded every 12 h until day 21.5 to calculate the spread of hatch (Abioja et al., 2022; Sözcü et al., 2022). Hatchability was calculated according to the formula: [no. of hatchlings / total no. of fertile eggs] * 100. Each hatching tray or basket was considered as an experimental unit. We conducted 2 independent trials with 6 replicates per group in each trial (Gucbilmez et al., 2013).

Chick Quality

At hatch (day 21.5), 10 chicks were randomly picked from each hatching basket (60 chicks/treatment/trial) for chick quality evaluation using the Tona and Pasgar scoring systems. Pasgar score is a 10-point scoring system evaluating chicks on their reflex (activity/alertness) and navel, legs, beak, and belly appearance. One point is subtracted from total of 10 for each observed abnormality such as 1) low alertness 2) suboptimal navel condition 3) red or swollen hocks 4) abnormal beak 5) hard belly or tense skin (Boerjan, 2006). Individual attribute evaluation scores are then expressed as percentage of observed abnormalities or defects. Lower abnormality percentage indicates better the chick quality (Van de Ven et al., 2012). On the other hand, Tona score is a 100-point scoring system assessing chick quality on their activity, down and appearance, retracted yolk, eyes, legs, navel area, remaining membrane, and remaining yolk (Figure 2). Total Tona score is the sum of all individual scores. Higher Tona score indicates a better chick quality (Tona et al., 2003).

Hatchling Morphometry

Following quality analysis, chicks were sexed (feather and vent sexing; Aviagen, 2017) and 5 male chicks from each hatching basket were chosen for morphometric measurements (30 chicks/treatment/trial). The chicks were weighed to record the hatchling weight and euthanized by CO_2 inhalation for additional morphometric measurements. At necropsy, hatchling length was measured from the tip of the beak to the tip of the middle toe (excluding the nail) as previously described (Meijerhof, 2006). Yolk-free-body-mass (**YFBM**) was measured as the weight of the hatchling after removal of the residual yolk sac. Following this, organ weights including heart, liver, gizzard, and intestine were measured. All organ weights were expressed as percentage of hatchling weight (relative organ weight; Sahan et al., 2014).

Grow-Out Study

Since literature demonstrates correlation between chick quality and posthatch performance (Wolanski et al., 2006; Molenaar et al., 2008; Willemsen et al., 2008), a grow-out trial was also performed. In each independent trial, following quality assessments, the remaining male chicks from each hatching basket were transferred to a floor pen (12 birds/pen, 4 pens/group/trial). Birds were floor reared with ad libitum water and feed. The chicks

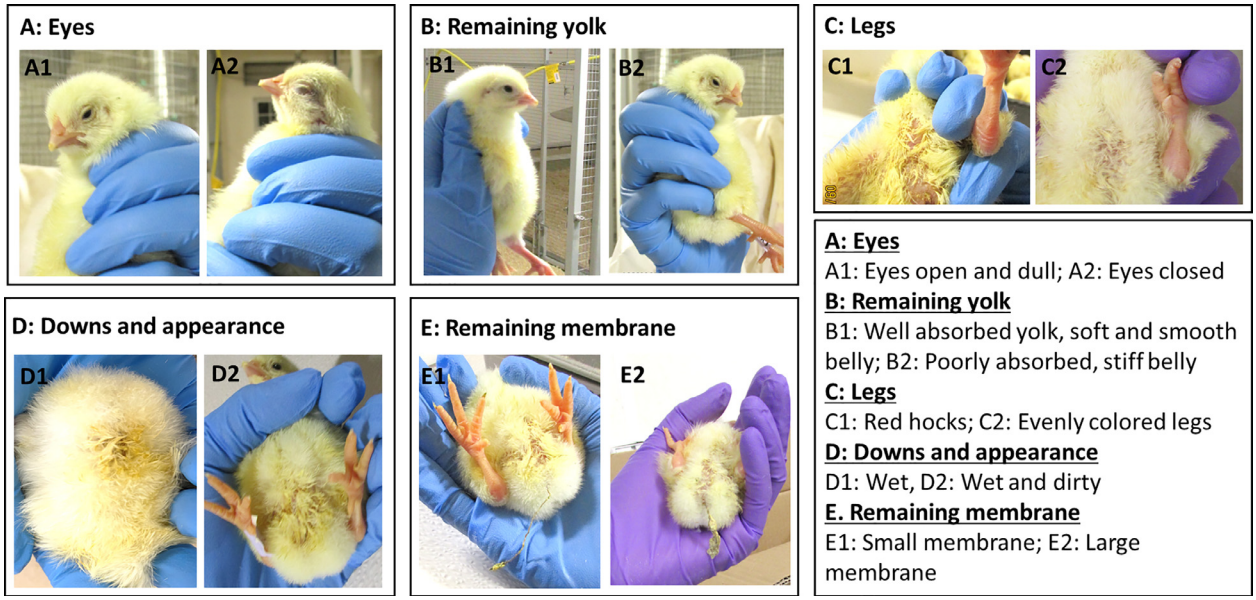

Figure 2. Representative images of individual scoring attributes as applied in the Tona and Pasgar scoring systems.

were fed with commercial starter diet from day 1 to day 13 (ME - 3,000kcal/kg; CP - 23%), and then fed with commercial grower feed (ME - 3,100 kcal/kg; CP - 20%) from day 14 to day 21 (Aviagen, 2014). The treatment regimen was the same as during incubation with LP and LR groups receiving daily in-feed supplemented of the respective probiotic strain (9 log CFU/kg feed) from day 1 to day 21, while control group received none. Standard management practices including recommendations for lighting and heating were followed as per the Aviagen management guide (Aviagen, 2018). Briefly, the grower house temperature was maintained at 32°C from day 1 to day 7 and gradually reduced to 25°C by day 21. Similarly, an automated lighting schedule was set up to provide 22 h of light from day 1 to day 7 followed by 20 h of light from day 8 to day 21. Chicks were weighed at the end of the 21-d grow-out period to assess posthatch growth.

Correlation and Statistical Analysis

The experiment was set up as a completely randomized design with 2 independent trials. For the hatchability calculation, hatching tray/basket was considered as the experimental unit (n = 12/group). For chick quality scoring (n = 120/group) and morphometric measurements (n = 60/group), each chick served as the experimental unit. For posthatch chick weight measurements, each pen served as the experimental unit (8 pens/group). Data are expressed as mean ± standard error. All statistical analyses were performed using R software (version 3.4.0). The normality of the data was checked using Shapiro-Wilk test. Treatments comparisons were performed using the least significant differences test (LSD) with $P \leq 0.05$ considered as being statistically significant. In addition, correlation between chick quality score (Tona, Pasgar), quality parameters (hatchling weight) and growth performance (chick weight at week 3) were analyzed with hatching basket or pen as the replicate. When the data were normally distributed, the correlation analysis was performed using the Pearson correlation; otherwise, Spearman correlation was used (Willemsen et al., 2008). The correlation was considered significantly different at $P \leq 0.05$.

RESULTS AND DISCUSSION

Although probiotics are widely used as feed supplements in poultry production, their application has been primarily limited to in-feed or in-water supplementation in grow-out birds (Salim et al., 2013). However, poultry researchers have now realized that future gains in production potential of these birds will come from advancements made on embryogenesis during incubation (Collin et al., 2007; de Oliveira et al., 2008). Consequently, any approach that supports or limits growth and development during incubation is expected to have a significant effect on overall growth, health, and performance of broiler chicken (Hulet, 2007). Therefore, In ovo probiotic supplementation could be a potential and viable approach to promote performance in broilers. In this regard, few studies have evaluated the effects of In ovo probiotic inoculation on hatchability and posthatch performance in broilers with varying results (de Oliveira et al., 2014; Pender et al., 2017; Beck et al., 2019; Castañeda et al., 2020; Leão et al., 2021).

Hatchability

Beyond posthatch performance, an important parameter that directly impacts overall productivity along the production pipeline is hatchability. Studies have shown that reduced hatchability can result in significant economic loss to the poultry industry (Schaal and Cherian, 2007). Higher hatchability means more chicks for the start of posthatch period. Hence, the essential objective in hatcheries is to maximize hatchability with a great

number of high-quality and saleable chicks (Decuypere and Bruggeman, 2007).

Along these lines, few performance studies have evaluated the effect of In ovo probiotic inoculations on hatchability with conflicting results. For instance, In ovo injection of *Lactobacillus animalis* and *Enterococcus faecium* to 18-day old broiler embryos was not shown to exert any significant effect on hatchability (Beck et al., 2019; Castañeda et al., 2020). Similarly, it was observed that In ovo injection of commercial probiotics Flora-Max-B11 or Primalac did not negatively affect hatchability (Pender et al., 2017; Teague et al., 2017). However, In ovo injection of *Bacillus subtilis* ATCC 6051 was seen to reduce the hatchability to 17.3% (Castañeda et al., 2021). Similarly, In ovo inoculation of *Lactobacillus* cocktails (3 mg/egg) was associated with ∼ 20% reduction in hatchability (de Oliveira *et al.*, 2014). In addition, a different dose (6 mg/egg) of the same *Lactobacillus* cocktails created a further decrease of hatchability by ∼ 50% (de Oliveira et al., 2014). Further, these studies supplemented probiotics via invasive inoculations and did not study hatchability as a primary outcome of the supplementation. As opposed to this, the primary focus of our study is to determine the effect of noninvasive sustained probiotic application on hatchability and hatchling quality as a means to support subsequent performance.

In the present study, hatchability in the control, LR and LP group was determined to be 79.32 ± 1.85%, 79.11 ± 2.06%, and 84.62 ± 2.04%, respectively (Table 1). Specifically, we observed a slight reduction in hatchability in the LR group by 0.21% when compared to the control ($P = 0.94$). Whereas hatchability in the LP group was higher than the control by 5.3% ($P = 0.060$). This is in line with our recent studies in layer embryos demonstrating a significant improvement in embryonic growth alongside an increase in hatchability in the probiotic treated embryos (83.50 ± 4.95%) when compared to the control (74.58 ± 2.95%; Muyyarikkandy et al., 2023b). Similarly, we observed that In ovo supplementation of probiotics to broiler embryos led to improved embryo growth and development that is critical to hatchability and hatchling quality. Towards this, in the current study, we observed that at hatch, the chick weight accounted for a significantly ($P = 0.017$) higher proportion of the starting egg weight in the probiotic groups (LP: 70.30 ± 0.65%; LR: 70.35 ± 0.65%) when compared to the control (68.12 ± 0.57%). Particularly, In ovo application of LP was associated with a 3.44% and 12% increase in embryo and breast weight in 18-day-old broiler embryos (Muyyarikkandy et al., 2023a). Further, following In ovo probiotic application, we observed a consistent increase in YFBM throughout the incubation period. Also, as observed with the hatchling weight, YFBM in the probiotic groups was found to account for a significantly ($P = 0.049$) higher proportion of the starting egg weight in the probiotic groups (LP: 64.40 ± 0.61%; LR: 63.62 ± 0.54%) when compared to the control (62.47 ± 0.51%). Since optimum embryonic growth and development is critical to hatchability and given our recent findings, we hypothesize that by promoting optimum embryonic growth and development, probiotics also support hatchability.

Spread of Hatch

In addition to hatchability, we also determined the spread of hatch. Starting on day 19, the number of pipped chicks and hatched chicks was recorded every 12 h until day 21.5 to calculate the spread of hatch. The hatchlings started pipping from day 19.5, and the percentage of total pipped eggs in control, LP, and LR was 80.79% ± 1.94%, 85.19% ± 2.49%, and 79.21% ± 2.77% at D21, respectively (Figure 3A). Overall, we did not observe any significant difference in pipping percentage between control and treatment groups on day 19.5, 20, 20.5, and 21 ($P > 0.05$). Across all groups, hatching started on day 20, and most chicks were hatched between day 20 and day 21. As observed with the hatching window, In ovo probiotic application did not result in any significant difference in time of hatch when compared to the control (Figure 3B). Taken together, these data show that probiotic treatment did not affect the pipping and hatching time, which prevents the chicks in the probiotic group from hatching too early or too late. This is significant since it is reported that chicks that

Table 1. Effects of probiotic supplementation on hatchability and chick morphometry.

Parameters	Control	LP	LR
Hatchability (%)	79.32 ± 1.85%	84.62 ± 2.04%	79.11 ± 2.06%
Relative change compared to control (%)		+5.30%	−0.21%
Hatchling weight (g)	42.16 ± 0.38[a]	43.66 ± 0.48[b]	43.85 ± 0.48[b]
Relative change compared to control (%)		+3.56%	+4%
YFBM (g)	38.48 ± 0.34[a]	39.80 ± 0.39[b]	39.41 ± 0.35[b]
Relative change compared to control (%)		+3.45%	+2.42%
Hatchling length (cm)	18.45 ± 0.16[a]	18.73 ± 0.12[ab]	18.94 ± 0.14[b]
Relative change compared to control (%)		+1.60%	+2.63%
Chick weight at week 3 posthatch	680.61 ± 8.47[a]	719.87 ± 11.07[b]	696.43 ± 9.48[ab]
Relative change compared to control (%)		+5.77%	+2.33%

Data are represented as mean ± SEM.

[a,b]Different superscripts within each row indicate significant difference between treatments at $P < 0.05$.Hatchling (%): calculated as a percentage of the total no. of fertile eggs set for incubation.YFBM: Yolk-free body mass is weight of hatchling after removal of the residual yolk sac.Hatchling length: measured from the tip of the beak to the tip of the middle toe (excluding the nail)

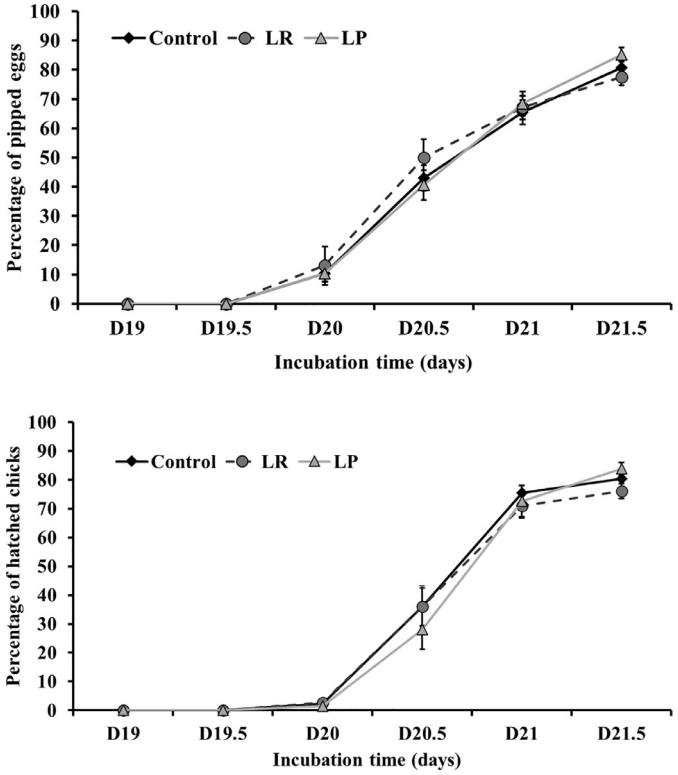

Figure 3. Effects of In ovo probiotic supplementation on spread of hatch represented as a percentage of pipped (A) and hatched chicks (B) over time[1]. Starting on day 19 of incubation, the number of pipped and hatched chicks were recorded every 12 h until day 21.5 to calculate the spread of hatch. [1]Data are represented as mean ±SEM.

hatch early or late can be associated with impaired post-hatch performance (Careghi et al., 2005; Willemsen et al., 2010). For instance, chicks that hatch early tend to stay in the hatcher for longer than most hatchlings. This in turn leads to an increased delay to first feed and a higher risk for dehydration (Kingston, 1979). Further, it is reported that extended holding in the hatcher can lead to a decrease in body weight (Casteel et al., 1994). Similarly, chicks that hatch late may not have enough time for navel closure and healing (Araújo et al., 2016). More importantly, early, and late hatching can negatively affect current industry practices that are based on a 21-d incubation period to ship out hatchlings to the grow-out farms (Aviagen, 2020).

Chick Quality

Besides hatchability, the hatchling quality is also of importance to the hatchery and broiler production pipeline (Sözcü and İpek, 2015). Good chick quality is the result of optimal incubation and hatching. In fact, quality of the day-old bird is employed as an indicator of broiler performance in the poultry industry (Tona et al. 2003). Further, higher chick quality scores were observed to be associated with an improved body weight gain and posthatch performance in broilers (Molenaar et al., 2008; Petek et al., 2010; da Silva et al., 2017). Chick quality is assessed using Tona or Pasgar scoring system and/or measuring morphometric attributes including hatchling/chick weight, yolk-free-body-mass, and chick length (İpek and Sözcü, 2013). Using the Tona score system chicks are scored between 0 and 100 with 100 representing a good quality chick (Tona et al., 2003;2005). On the other hand, in the Pasgar score, the chick is assessed on a 10-point scale where ten is a good quality chick. Points are subtracted for each abnormality recorded starting from a score of ten (Boerjan, 2006).

In the present study, In ovo probiotic application significantly improved chick quality as observed by higher Tona and Pasgar total scores in the probiotic groups when compared to the control ($P < 0.001$; Table 2). As seen in the table, the total Tona score in control, LP and LR is 82.40 ± 1.04, 97.13 ± 0.36, and 94.83 ± 0.76, respectively. Specifically, hatchlings in the LR and LP group were found to be significantly more active, alert, with open eyes, healed navel, good confirmation of the legs and well-developed toes. This is important since

Table 2. Effects of In ovo probiotic application on hatchling quality.

		Control	LP	LR
Tona	Total score*	82.40 ± 1.04[a]	97.13 ± 0.36[c]	94.83 ± 0.76[b]
	Activity score	3.80 ± 0.27[a]	6.00 ± 0.00[b]	5.75 ± 0.11[b]
	Down and appearance score	8.73 ± 0.22	9.05 ± 0.16	8.90 ± 0.14
	Eyes score	14.90 ± 0.25[a]	15.93 ± 0.07[b]	16.00 ± 0.00[b]
	Legs score	11.37 ± 0.47[a]	15.73 ± 0.16[b]	15.20 ± 0.26[b]
	Navel score	11.40 ± 0.17[a]	11.95 ± 0.05[b]	11.88 ± 0.12[b]
	Remaining membrane score	11.43 ± 0.20	11.73 ± 0.09	11.70 ± 0.12
	Remaining yolk score	13.07 ± 0.23[a]	14.73 ± 0.18[b]	14.60 ± 0.18[b]
	Retracted yolk score	7.70 ± 0.53[a]	12.00 ± 0.00[c]	10.80 ± 0.33[b]
Pasgar	Total score*	8.63 ± 0.09[a]	9.55 ± 0.06[b]	9.48 ± 0.08[b]
	Abnormal beak %	7.50% ± 2.18%	6.67% ± 2.56%	2.73% ± 1.35%
	Suboptimal navel condition %	14.55% ± 4.72%	15.00% ± 4.36%	13.64% ± 4.88%
	Hard belly %	30.00% ± 5.63%[a]	0.00% ± 0.00%[b]	6.36% ± 2.96%[b]
	Low alertness %	28.18% ± 5.60%[a]	5.00% ± 2.30%[b]	6.67% ± 3.10%[b]
	Red hocks %	40.83% ± 5.57%[a]	4.17% ± 1.93%[b]	0.00% ± 0.00%[b]

Data are represented as mean ± SE.

[a,b,c]Different superscripts within a row indicate a significant difference between groups ($P<0.05$)

*Total scores are the sum of individual scores. Higher total scores are indicative of better chick quality.Tona score: Individual attribute scores are represented as averages for each group. Higher the attribute score, higher the quality.Pasgar score: Individual attribute scores as represented as percentage of total abnormalities observed. A lower individual attribute score indicates better quality. A score of 0% indicates that no hatchlings presented with red hocks in that group.

alert and active chicks are prone to investigate their environment and seek out water and feed critical to their survival and performance (Tona et al., 2005). Further, they did not present any swelling or lesions on the hock and skin when compared to the control (Control: 11.37 ± 0.47, LP: 15.73 ± 0.16, LR: 15.20 ± 0.26; $P < 0.001$; Table 2). With the navel, in the probiotic groups, the navel appeared clean and completely sealed with significantly higher scores (Navel score − LP: 11.95 ± 0.05; LR: 11.88 ± 0.12; $P < 0.001$). With the control hatchlings, several birds had improperly sealed navel (Navel score − Control: 11.40 ± 0.17). This is critical since abnormalities in the navel region can have a negative effect on the survival and growth of the chick (Tona et al., 2005). Improperly sealed navel increases the risk for yolk sac infections which can result in chick mortality (Fasenko and O'Dea, 2008; Sözcü and İpek, 2015; Hjelm, 2018). Moreover, it is reported that the suboptimal navel condition is an indicator of impaired absorption of residual yolk sac, which can further impact intestinal villi growth (Kawalilak et al., 2010). Moreover, Fasenko and O'Dea (2008) reported that broiler chicks with suboptimal navel conditions were associated with lower body weights at market age when compared to chicks with fully a healed navel.

As seen with the Tona score, probiotic treated groups (LP: 9.55 ± 0.06, LR: 9.48 ± 0.08) had significantly higher total Pasgar scores compared to the control (Control: 8.63 ± 0.09, $P < 0.001$; Table 2). Additionally, similar to the Tona score, Pasgar scores also revealed improved reflex action and alertness in probiotic treated groups as seen by the significantly lower percentage of chicks with reduced alertness in the probiotic treated groups (Control: 28.18 ± 5.60%; LP: 5.00 ± 2.30%, LR: 6.67 ± 3.10; $P < 0.001$; Table 2). Further, as part of the Pasgar scoring system, we also evaluated the legs particularly the hocks for swelling and discoloration. Specifically, legs were evaluated by examining for red hocks and infected toes, which are reported to be associated with prolonged pushing against eggshell during hatching (Wilson, 2004). In concurrence with the Tona scores, LR and LP groups had a significantly lower percentage of red hocks when compared to the control [Control: 40.83 ± 5.57; LP: 4.17 ± 1.93; LR: 0% (no hatchlings observed with red hocks); $P < 0.001$; Table 2]. Further, hatchlings in the LP and LR groups had significantly reduced percentage of chicks with hard belly compared to control, which shows that the chicks in LP and LR have significantly softer, smoother, and more supple belly [Control: 30.00 ± 5.63%, LP: 0% (no chicks observed with hard belly); LR: 6.36 ± 2.96%; $P < 0.001$].

Although primarily evaluated for its growth promoting attributes, few studies have investigated the effect of In ovo nutrient administration on chick quality. For instance, Oke et al. (2021) reported that In ovo injection of black cumin extract at embryonic day 17.5 did not exert any significant effects on activity, appearance, eye, leg, retracted yolk, navel area, and remaining yolk. On the other hand, In ovo inoculation of *Moringa oleifera* leaf extract at day 18 was observed to significantly improve the navel area score. The authors hypothesized that the improved score could contribute to the yolk absorption and metabolism during the last 3 d of incubation (N'nanle et al., 2017). Along the same lines, it is reported that In ovo feeding of vitamin E at day 17.5 significantly increased the percentage of high-quality chicks (with Tona score higher than 91; Araújo et al., 2019).

Chick Morphometry

In addition to the Tona and Pasgar score, hatchling weight, YFBM and hatchling length were measured. Hatchling weight is the weight of day-old chick and is a widely used parameter in chick quality assessment (Wolanski et al., 2006; Molenaar et al., 2008). On the other hand, Yolk-free-body-mass is the chick body weight without residual yolk sac, which is considered as the actual body weight of hatchling. The heavier YFBM indicates better embryonic development (Meijerhof, 2009; Molenaar, 2011). This is related to our previous findings demonstrating a significant improvement in embryonic growth following In ovo probiotic application to broiler and layer embryos (Muyyarikkandy et al., 2023a, b). Hatchling length is the length from the tip of beak to middle toe, which shows a positive correlation with embryonic organ development and posthatch performance (Meijerhof, 2006; Mukhtar et al., 2013). In general, we observed that hatchling weight, length, and YFBM were significantly higher in the probiotic groups when compared to the control (Table 1; $P \leq 0.05$). With the hatchling weight, In ovo application of LR and LP resulted in a 4 and 3.56% increase in weight compared to the control, respectively. Similarly, we observed a 2.42 to 3.45% increase in YFBM in the chicks hatched from the probiotic treated eggs when compared to the control. This improvement in weight measurements was also associated with a 1.60 to 2.63% increase in hatchling length following In ovo probiotic application (Table 1). Overall, the improved Tona and Pasgar scores, and increase in hatchling weight, YFBM, and hatchling length in LP and LR group suggest that the probiotic spray significantly improved the chick quality and thereby could promote posthatch performance.

In this regard, some studies have evaluated the effect of In ovo probiotic inoculation on hatchling weight, YFBM, and hatchling length with varying results. For instance, In ovo supplementation of commercial probiotic products (Primalac or FloraMax-B11) did not result in a significant difference in hatchling weight when compared to the untreated control (Pender et al., 2017; Teague et al., 2017). Similarly, In ovo inoculation of *Bacillus subtilis* ATCC 8473, *B. subtilis* ATCC 9466, *Lactobacillus animalis*, or *Enterococcus faecium* were not associated with any effect on chick weight (Castañeda et al., 2021; Beck et al., 2019). However, In ovo injection of *Lactobacillus acidophilus* ATCC 314, *Bifidobacterium animalis* ATCC 27536 or *Bacillus subtilis* ATCC 6051 significantly reduced hatchling weight (Triplett et al., 2018; Castañeda et al., 2021). The author

speculated that In ovo injection of *L. acidophilus* or *B. animalis* may have slightly altered egg nutrients, which led to a lighter hatching weight. Contrary to these observations, we observed significant improvements in chick weight, YFBM and hatchling length. This could be due to the difference in the probiotic strains employed, route of administration and application regimen. Further, the improved hatchling quality could be associated with the improved embryonic growth (YFBM and crown-rump length) observed in our previous study (Muyyarikkandy et al., 2023a, b). Thus, supporting our hypothesis that In ovo probiotic application during incubation supports embryonic growth thereby improving hatchability and hatchling quality.

Correlating Chick Quality Scores, Hatchling Morphometry and Posthatch Growth

Beyond chick scores and hatchling morphometry, we also conducted a grow-out study to determine the effect of probiotic supplementation on posthatch growth. As seen in Table 1, probiotic supplementation was associated with higher weight at hatch and a corresponding heavier weight at week 3 ($P \leq 0.05$). At the end of the 3-wk grow-out study, body weights were determined to be 680.61 ± 8.47, 719.87 ± 11.07 and 696.43 ± 9.48 g in the Control, LP and LR groups, respectively. Supplementation of LR and LP resulted in a 2.33 to 5.77 % increase in body weight when compared to the control (Table 1). Further, since it is reported that chick quality scores could provide an indication of posthatch performance, we performed correlation analysis which is presented in Table 3. The correlation between the Tona and Pasgar score was positive and statistically significant in the control ($r = 0.88$, $P = 0.0084$), LP ($r = 0.9$, $P = 0.0055$), and LR ($r = 0.82$, $P = 0.012$) groups, which indicates that our evaluation for chick quality was consistent across the 2 scoring systems. Further, we observed that Pasgar score and Tona score were positively correlated with the hatchling weight in the probiotic-treated groups. For instance, correlation between the Pasgar scores and hatchling weight was weak in control ($r = 0.038$) while it was positive in LP ($r = 0.70$) and LR ($r = 0.56$) groups. Similarly, Tona et al. (2004) reported that chicks of higher quality had heavier body weights than those of lower quality, indicating a positive correlation between quality score and hatchling weight, which is in line with our results.

When considering the grow-out period, the correlation between Pasgar score and chick weight at week 3 was weak in control ($r = -0.062$), while it was positive in LP ($r = 0.67$) and LR ($r = 0.67$). Similarly, the correlation between Tona score and chick weight at week 3 was weak in control ($r = -0.018$), while it was positive and statistically significant in LP ($r = 0.79$, $P = 0.033$). Willemsen et al. (2008) found weak correlations between Tona score and chick weight from day 7 to day 42, which was consistent with the control group in our study. Moreover, the correlation between hatchling weight and chick weight at week 3 was positive in control ($r = 0.66$), LR ($r = 0.89$), and LP ($r = 0.49$), and it was significant in LP ($P = 0.012$), indicating the positive relationship between hatchling weight and chick weight at week 3 (Table 3). Similar findings indicating the positive correlation between hatchling weight and chick growth performance were also reported in other studies (Sklan et al., 2003; Willemsen et al., 2008). In effect, day-old chick weight has been suggested to be indicative of slaughter weight in broilers. This correlation is however not fully understood since some have found a relationship between the 2 traits and some have not (Tona et al., 2004; Molenaar et al., 2008; Willemsen et al., 2008).

Since chick weight is dependent on egg weight and includes the residual yolk sac, it may not be a good predictor of the development of the chick (du Preez, 2007). Hence, we also determined the correlation between YFBM and other chick quality measurements including hatchling weight, chick length and posthatch chick weight at 3 wk. However, our results indicate a weak negative correlation between the scoring systems and

Table 3. Correlations between quality scores and chick weights in different treatment groups.

		Scoring methods			
Groups	Methods	Pasgar	Tona	Hatchling weight	Weight at week 3
Control	Tona	0.88*	-	-	-
	Hatchling weight	0.038	−0.037	-	-
	Weight at week 3	-0.062	−0.018	0.66	-
	YFBM	−0.21	−0.44	0.057	0.27
LP	Tona	0.9*	-	-	-
	Hatchling weight	0.7**	0.64	-	-
	Weight at week 3	0.67**	0.79*	0.89*	-
	YFBM	0.77*	0.53	0.3	0
LR	Tona	0.82*	-	-	-
	Hatchling weight	0.56	0.38	-	-
	Weight at week 3	0.67**	0.68**	0.49	-
	YFBM	0.28	0.4	-0.31	0.077

Correlation between chick quality score (Tona, Pasgar), quality parameters (hatchling weight, YFBM) and posthatch growth (chick weight at week 3) were analyzed. When the data were normally distributed, the correlation analysis was performed using the Pearson correlation; otherwise, Spearman correlation was used.

*$P < 0.05$

**$P < 0.01$A correlation value of 0 indicates no observed correlation.

YFBM. Further, we also observed weak correlation between the YFBM and hatchling weight/chick weight at 3 wks post hatch. Similarly, Sözcü and İpek (2015) reported a weak negative correlation between YFBM and Tona/Pasgar score, indicating the complex relationship between hatchling quality parameters. They also reported that correlation between YFBM and weight at day 7 was weak, indicating a weak correlation between YFBM and posthatch body weight (Sözcü and İpek, 2015).

Overall, our data demonstrate that In ovo probiotic spray application was associated with an improvement in hatchability and hatchling quality. Particularly, hatchlings in LP and LR groups were found to be alert with better reflex activity, sealed navel. Also, LP and LR improved the hatchling weight, hatchling length, and yolk-free-body-mass, which are indicators of posthatch performance. Moreover, correlation analysis revealed that chicks with higher quality scores were associated with improved hatchling morphometry and posthatch growth. Further, the observed improvement in hatchability and hatchling quality could be due to enhanced embryonic growth and development as seen with our previous studies. Hence, the above-mentioned probiotics could be employed to promote embryonic growth, hatchability, and hatchling quality. Further, it could be used in conjunction with current posthatch approaches to improve overall growth and performance of broiler chickens.

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DISCLOSURES

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mary Anne Amalaradjou has patent #US11497197B2 issued to University of Connecticut. Corresponding author serves as an associate editor for Poultry Science.

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