**EFFECTS OF *BACILLUS SUBTILIS* SUPPLEMENTATION IN OVO OR WATER ON HATCHABILITY AND GROWTH PERFORMANCES OF BROILERS**

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**ABSTRACT**

  The present study aimed to determine the best method as well as appropriate concentration of *Bacillus subtilis* (*B.subtilis*) that could be advised to maximize the hatching and growth performances of Cobb500broilers. A total of 480 Cobb500 broiler hatching eggs were assigned to four experimental groups (T1, T2, T3 and T4) as: T1 allocated into three subgroups; T1A:eggspenetrated then sealed without injection (control negative); T1B**:**eggs injected with 0.1 ml of physiological saline ( 0.9 NaCl) as control positive as well asT1C (control feeding) eggs didn't receive any treatment. T2 and T3werethe groups injected by *B.subtilis* and T4 were eggs incubated for water treatment after hatch. On the 18thday of incubation, 100 μL of 107 and 1010 CFU of *B. subtilis***/**egg or saline was injected into the air cell of T2, T3 and the T1B eggs; respectively. Hatched chicks were re-assigned into: T1 (control group), T2, T3 and T4 had been divided into T4W1 and T4W2 groups to receive 107 CFU/ml and 1010 CFU/ml *B.subtilis* in water. All chicks of the experimental groups were managed for 35 days under optimum environmental conditions. Results revealed that, in ovo inoculation of *B.subtilis* of different concentrations had no significant effect on hatchability parameters except T3 showed the lowest hatching weight as well as the highest sticky embryo percentages. Broilers that supplemented in ovo via 107*B.subtilis*/egg had better weekly body weight, higher RGR through the experimental period. Irrespective of the method of *B. subtilis* supplementation to broiler,107concentration of *B.subtilis* showed the highest significant marketing weight (p<0.05).It was concluded that in ovo inoculation didn't statistically affect hatching performance but had beneficial improvement of growth performance of Cobb500 broilers.

**Keywords:** *Bacillus subtilis*, Broilers, Growth performance, Hatchability, Inovo, Water supplementation.

1. **INTRODUCTION**

Broilers represent developing and promising sector of white meat productivity. Different growth promoters including probiotics have been developed as a safe and natural non-antibiotic growth promoter **(Huyghebaert et al., 2011;** **Souza et al., 2018;** [**Ramlucken**](https://www.sciencedirect.com/science/article/pii/S0032579119578786#!) **et al., 2020).** Recent studies reported that supplementation of probiotics to poultry in adequate amounts had a beneficial impact on the poultry health via improving gut hemostasis**(Fuller 1989; Meng et al., 2010; Yu et al., 2020).** Several selected tested strains of bacteria have been used for probiotics preparation; however, *Bacillus* species is the superior probiotic feed additive for the commercial poultry. *Bacillus* bacteria are heat resistant, tolerate to the processing of pelleted food and can be added as spores to poultry food**(Moeller et al., 2009; Nicholson, 2002).**

 **Gao et al., 2017,** reported that feeding of 200mg/kg *B**. subtilis* to the broilers got better growth performance and feed efficiency. [**Ciurescu**](https://www.ncbi.nlm.nih.gov/pubmed/?term=Ciurescu%20G%5BAuthor%5D&cauthor=true&cauthor_uid=33142513) **et al., 2020,** recommendedusing5.0 × 1011 CFU spores g−1 feed had the tremendous potential effect on broiler growth performance. Whereas, the dietary supplementation of probiotic needs more time for colonization in the poultry intestine, so recent studies were attended to allow early colonization of probiotics via in ovo inoculation. **Triplett et al., 2017** reported that in ovo injection of *B.subtilis* through amniotic fluid adversely affects the hatching potentiality of broiler embryos. However, in ovo inoculation of *B.subtilis* in Saini chicken eggs through air cell improved hatchability and decreased embryonic mortality percentages, as well as improve subsequent growth performance **(Rizk et al., 2018)**.

 Therefore; the main objective of this study was to evaluate the effects of different concentrations of *B**.subtilis* as in ovo or water supplementation on hatching quality and growth performance parameters of Cobb500 broilers as a trail to determine the ideal method for probiotic delivery.

1. **MATERIAL AND METHODS**

 The present study was planned and carried out at the Department of Animal Husbandry and Animal Wealth Development, Faculty of Veterinary Medicine, Alexandria University. During the period from September 2020 to December 2020.

All experimental procedures and management conditions used in this study were approved by the Institutional Animal Care and Use Committee (AU-IACUC), Faculty of Veterinary Medicine, Alexandria University, Egypt.

**2.1. Hatching eggs (Source, sorting, disinfecting and candling)**

A total of 480Cobb500 broiler fertile hatching eggs were taken from 32 weeks old broiler breeder flock located at Rasheed Behaira. Eggs were checked for cracked and misshaped shells and were sprayed byD-904 **("BioSentry® 904 Disinfectant," 2016.)**with a dilution of 4ml/L**.** Incubator was fumigated by mixing of 40 ml formalin 40% and 20 g potassium permanganate (KMnO4) / three cubic meters for nearly 20 minutes. Incubation temperature adjusted to 37.5˚C and relative humidity 65%. Eggs were turned automatically every three hours. Eggs were candled twice at the7th andthe14th day of incubation to identify and remove the clear, infertile, early and mid-dead embryos.

* 1. **Experimental groups**

 **Table 1:The experimental groups of eggs after sorting, disinfection and candling**

|  |  |  |
| --- | --- | --- |
| **T1** | **T1A**(Control negative) | 30 eggs, where a pilot hole was made in air cell then sealed with paraffin oil without injection, |
| **T1B**(Control positive) | 30 eggs injected with 0.1 ml of sterile physiological saline |
| **T1C**(Control for feeding trail). | 50 fertile eggs of non in ovo or water treatments with *B. subtilis* |
| **T2** | 78 fertile eggs inoculated with 107 CFU *B. subtilis* /egg |
| **T3** | 87 eggs inoculated with 1010 CFU/ *B. subtilis* egg. |
| **T4** | 167 fertile eggswere used for water treatment after hatching. |

 **Table 2:**  **The experimental groups of the newly hatched chicks afterlabeling and transferring to brooding house**:

|  |  |
| --- | --- |
| **T1 (n=95)** | Chicks hatched from control negative; control positive, control waterand control feeding subgroups, |
| **T2 (n=64)** | Chicks hatched from eggs inoculated by 107 CFU *B. subtilis*/egg, |
| **T3 (n=69)** | Hatched chicks in Ovo inoculation of eggs by 1010 CFU *B. subtilis*/egg, |
| **TW1 (n=68)**  | Chicks treated in water by 107CFU *B. subtilis*/ml |
| **TW2 (n=71)** | Chicks treated in water by 1010 CFU *B. subtilis*/ml |

* 1. **Probiotic preparation**

CLOSTAT® is a commercial probiotic containing dried probiotic *B. subtilis* PB6 was utilized for in Ovo inoculation and water supplementation. CLOSTAT® product was analyzed before usage to determine the colony- forming units per gram. Counting of B. subtilis culture revealed that 10µl contained 200 colonies, and 5µl contained 100 colonies. *B. subtilis* culture was serially diluted and culture concentrations were verified before application.

* 1. **Injection procedure**

Environmental temperature of hatchery laboratory kept warm (around30 °C) to avoid the temperature shock of the embryos. All equipments used for in ovo inoculation were sterilized. Probiotics cultures for in ovo inoculation were prepared immediately before injection. Eggs handling time outside the incubator didn't exceed 8 minutes.

Two concentration treatments were prepared; the first used 0.25g of *Bacillus subtilis* dried powder dissolved in 5 liters of sterile physiological saline (1×1010CFU/0.1ml)**(Arreguin-Nava et al., 2019)**, while the second was prepared by dissolving 0.25g in 5 liters of sterile physiological saline then 50µl is dissolved in 50 ml (1×1010CFU/0.1ml) **(Schmidt et al., 2010).** On day 18th of incubation eggs were candled to determine air cell position (site of injection). Air cell site was sanitized with 70% isopropyl alcohol and once dried; a pilot hole was made in the center of air cell and injection of the solution using 16-gauge needle with a robber stopper to prevent the needle from piercing air cell. Needles were disinfected between injections. Then the hole sealed using paraffin oil and eggs were soon got back to the hatcher**.**

* 1. **Hatching**

On the 18th day of incubation, the eggs were transferred to the hatcher (37 °C, 75% relative humidity). The hatched chicks counted and weighted. Unhatched eggs were opened to determine the early, mid and late embryonic mortality. Mortality percentages were recorded as early, mid and late, from 3rd to 7th, 8th to 14th and 15th to 21st days; respectively.

The scientific hatchability was calculated by dividing the number of viable hatched chicks by the number of fertile eggs. While the commercial hatchability was calculated by dividing the number of viable hatched chicks by the total number of eggs set.

* 1. **Brooding management:**

 Experimental brooding house was prepared before chicks’ arrival. Chick groups were brooded at 35oC and separated by brooder guards. Temperature was gradually reduced by 3ºCweekly till reach 24**º**Cat the fifth week. The chicks were supplied with commercial standard basal diet ration contains all needed vitamins and amino acids according to the NRC recommendation (Table 3). Birds had access to feed and water ad libitum during the experimental period. For the first 48 hours, continuous lighting was provided and then decreased to 23 hours light and 1hour dark. Relative humidity was maintained between 65% and 75%. Chicks of each group were wing banded according to their treatment. Chicks were vaccinated against New castle disease (ND) on day 8th and 18th and against infectious bursal disease (IBD) at 14th day. Birds were weighted at hatch and every week until five weeks of age. Weekly body weight and relative growth rate percentage were recorded on a weekly basis **(Brody 1945)**:

Relative growth rate =$\frac{(w2-w1)}{{1}/{2} (w1+w2)}×100$

Where:

W1 = the weight at any week

W2 = the weight at next week

**Table 3: Ingredients and Nutrient Composition (% DM) of Broiler Starter and Finisher Rations**

|  |  |  |
| --- | --- | --- |
| **Finisher** | **Starter** | **Ingredient (%)** |
| 66.5 | 53.5 | Yellow corn |
| 24 | 34.38 | Soybean meal (48%) |
| 3.6 | 5.82 | Corn gluten |
| 2.82 | 3.00 | Corn oil |
| 0.3 | 0.3 | Salt |
| 2.48 | 2.7 | DicalciumphosphateA |
| 0.3 | 0.3 | PremixB |
| 100% | 100% | Total |
| Nutrients |
| 3096.31 | 2976.83 | ME (kcal/kg) |
| 18.07 | 22.97 | Crude protein |
| 0.9 | 1.08 | Calcium |
| 0.45 | 0.52 | Available phosphorus |
| 0.51 | 0.52 | Methionine |
| 1.13 | 1.29 | Lysine |

A Dicalcium phosphate, 18% granular phosphate and 23% calcium.

B Supplied per kg of diet: vitamin A 12,000 IU, vitamin D3 3,000 IU, vitamin E 40 mg, vitamin K3 3 mg, vitamin B1 2 mg, vitamin B2 6 mg, vitamin B6 5 mg, vitamin B12 0.02 mg, niacin 45 mg, biotin 0.075 mg, folic acid 2 mg, pantothenic acid 12 mg, manganese 100 mg, zinc 600 mg, iron 30 mg, copper 10 mg, iodine 1 mg, selenium 0.2 mg, cobalt 0.1 mg. C DL-Methionine, Met AMINO® (DL-2- amino-4-(methyl-thio)-butane acid, DL-methionine, α- amino-Y-methyl-oily acid) by Feed Grade 99% (EU). D LLysine HCL 99% (Feed Grade) L-Lysine: 78.0% Min (Indonesia).

**Statistical Analysis:**

All data were tested for normality and equality of variance. Data were normal distributed.

All data were analyzed by one-way ANOVA (SAS version 9.1.3, 2004)

When treatment was significant (P<0.05), the test of Duncan for multiple comparisons was used to test the significance of the differences between treatment means.

Statistical model: **Xij= µ + Ti +eij**

 Where:

Xij = Value of ith observation of the ith treatment

µ = Overall mean

Ti = Effect of ith treatment (5 treatments)

eij= Random error

1. **RESULTS**
	1. **Hatching performance**

**3.1.1. Hatchability percentage**

 Impact of inovo inoculation of *B.subtilis* on hatchability percentages of incubated eggs are shortened in Table 4. Non-significant differences were observed either for commercial or scientific hatchability percentages for a control and treated groups.

T1c had non- significant higher commercial hatchability percentage (80%) followed by those ofT1Aand T4(both of 79%) then by those ofT3 (77%). While, the highest scientific hatchability percentages were in T1A (86%) thenT4 and T1c and (both 85.7% and 85%).

 **3.1.2. Embryonic mortality percentage**

 T1A showed the highest early embryonic mortality (7%) followed by those of T4 (4.7%) and T2 (3.0%). T1C showed non-significant high mid embryonic mortality (3%). Meanwhile; T2 exhibited high non-significant late embryonic mortality (5%).

**3.1.3. Hatch weight**

 There was no significant change in hatching weight of inoculated eggs compared to control groups except for T3 which displayed the lowest significant (p<0.05) hatch weight (46.98 gm).

**Table 4. (Mean ±SD) of hatchability and mortality percentages of embryos due to treatment by *Bacillus subtilis***

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| TreatmentParameters | T1 | T2 | T3 | T4 |
| T1A | T1B | T1C |  |
| Hatchability | Commercial | 79.0± 0.04 | 75.7±0.169 | 80.0±00 | 71.0±0.08 | 77.0±0.12 | 79.0±0.072 |
| Scientific | 86.0± 0.08 | 80.7±0.157 | 85.0±00 | 82.0± 0.12 | 79.0± 0.15 | 85.7±0.070 |
| Mortality | Early | 7.0± 0.06 | 0.00± 0.00 | 0.00± 0.00 | 3.0±0.05 | 1.0±0.01 | 4.7± 0.050 |
| Mid | 2.0± 0.02 | 0.00± 0.00 | 3.0±0.00 | 2.0± 0.04 | 1.0± 0.02 | 1.0±0.017 |
| Late | 1.0± 0.02 | 0.00± 0.00 | 0.00± 0.00 | 5.0±0.06 | 0.00± 0.00 | 1.0± 0.017 |
| Hatch weight  | 53.58±4.26a | 48.46±6.16bc | 51.45+4.11ab | 51.38±3.71ab | 46.98±3.63c | 50.57±4.77ab |

Means within the same raw with different superscripts are significantly different (p<0.05) **.**T1A, control negative, T1B; control positive,T1C; control feeding, T2;inovo supplemented group with 1×107CFU*B.subtilis/*egg, T3;inovo supplemented group with 1×1010CFU*B.subtilis*/egg; T4; non inoculated eggs

 **3.1.4. Unhatched embryos**

Table (5) revealed that T3 resulted in high significant sticky embryo percentage (9.0%), followed by those of T2 (6.0%) and T1B (5.7%) and both percentages did not differ significantly from each other. Piped failed and deformed embryos revealed non-significant differences, although T4 had the highest percentage of pipe failed (3.7%) and T3 had the highest percentage of embryo deformity (5.0%).

**Table 5. (Mean ±SD of sticky, pip failed and deformed embryo percentages due to *Bacillus subtilis* supplementation**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| TreatmentParameters | T1 | T2 | T3 | T4 |
| T1A | T1B | T1C |  |
| Sticky | 1.0±0.02b | 5.7± 0.023ab | 0.00± 0.00 | 6.0±0.02ab | 9.0± 0.08a | 2.7± 0.023ab |
| Pip failed | 1.0± 0.023 | 2.3± 0.021 | 0.00± 0.00 | 0.0± 0.00 | 1.0± 0.02 | 3.7± 0.040 |
| Deformity | 1.0± 0.012 | 3.3± 0.035 | 0.00± 0.00 | 1.0± 0.02 | 5.0± 0.04 | 1.0± 0.017 |

Means within the same raw with different superscripts are significantly different (p<0.05**) .T1A**, control negative, T1B; control positive, T1C; control feeding, T2; inovo supplemented group with 1×107CFU*B.subtilis/*egg, T3; inovo supplemented group with 1×1010CFU*B.subtilis*/egg ;**T4**; non inoculated eggs.

* 1. **Broiler growth performance**

**3.2.1 Body weight**

 The effects of *B. subtilis* as in ovo or watery supplementation on the growth performance of Cobb broilers were recorded in Table 6.At the first three weeks of age T2 and T4W2 had the highest significant (p<0.05) weekly body weights (211.97, 555.59, 1085.61 gm) and (206.28, 555.98, 1058.58). Alongside the average body weight of T4W1elevated at the 4th week to the highest significant group (1934.35 g) (p<0.05). T2and T4W1had the maximum significant body weight at the fifth week (p<0.05) (2218.72 and 2138.91 gm).

**Table 6. (Mean±SD) of body weight development of Cobb500 broilers as influenced by *Bacillus subtilis* supplementation.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | T1 | T2 | T3 | T4W1 | T4W2 |
| Body weight (g) |
| Week1 | 190.90±35.50cd | 211.97±30.82a | 195.57±31.24bc | 182.49±38.26d | 206.28±25.26ab |
| Week2 | 508.30±69.01a | 555.59±57.84a | 537.76±64.96a | 508.66±80.81b | 555.98±58.81a |
| Week3 | 989.78±107.23d | 1085.61±99.28a | 1040.84±111.86bc | 1004.75±127.66cd | 1058.58±107.25ab |
| Week4 | 1759.65±199.42c | 1867.54±219.75ab | 1844.06±239.73b | 1934.35±237.74a | 1810.22±244.30bc |
| Week5 | 1993.63±209.52c | 2218.72±276.03a | 2108.22±263.64b | 2138.91±279.43ab | 2044.47±265.64bc |
| Sample size | 70 | 59 | 59 | 62 | 67 |

Means within the same raw with different superscripts are significantly different (p<0.05)

RGR= relative growth rate. T1;control group including (control negative, control positive and control feeding) , T2 in ovo supplemented group with 1×107CFU *B. subtilis*/egg, T3; in ovo supplemented group with 1×1010 CFU *B. subtilis*/egg; T4W1,drinking water supplied group with 1×107 CFU *B .subtilis*/ml, T4W2; drinking water supplied group with 1×1010 CFU *B. subtilis*/ml.

**3.2.2 Relative Growth Rate (RGR%)**

 T4W1 exhibited the highest significant (p<0.05%) RGR% for all week periods except W4-W5 period. RGR of the T4W1 were 95.0, 66.0, 63.3, as well as 168.4 for overall RGR. T4W2 had the lowest significant (p<0.05) overall RGR% (163.0%).

**Table 5. Mean±SD of Relative Growth Rate of Cobb500 broilers as influenced by Bacillus subtilis supplementation.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | T1 | T2 | T3 | T4W1 | T4W2 |
| RGR |
| W1-W2 | 91.4± 0.080bc | 89.9±0.061c | 93.7±0.0687ab | 95.0±0.071a | 91.8±0.060bc |
| W2-W3 | 64.50±0.085ab | 64.7±0.058ab | 63.8±0.067a | 66.0±0.062a | 62.3±0.058b |
| W3-W4 | 55.8±0.097b | 52.6±0.077cd | 55.3±0.092bc | 63.3±0.080a | 51.9±0.085d |
| W4-W5 | 12.5±0.067b | 17.1±0.042a | 13.4±0.035b | 10.0±0.041c | 12.2±0.047b |
| overall | 164.8±0.070bc | 164.7±0.061bc | 165.8±0.055b | 168.4±0.064a | 163.0±0.055c |
| Number | 70 | 59 | 59 | 62 | 67 |

Means within the same raw with different superscripts are significantly different (p<0.05)

RGR**=** relative growth rate.T1;control group including (control negative, control positive and control feeding) ,T2inovo supplemented group with 1×107CFU*B.subtilis/*egg, T3;inovo supplemented group with 1×1010CFU*B.subtilis*/egg;T4W1,drinking water supplied group with 1×107CFU*B.subtilis/*ml,T4W2; drinking water supplied group with 1×1010CFU*B.subtilis*/ml.

**3.2.3 Relative weights of carcass and internal organs:**

T4W1 showed the highest values of liver and trunk weights (2.6% and 21.3%).Also, this group as well as T4W2 had the greatest abdominal fat (1.3%), breast weight percentages (31.9%) **(Table 8).** T3 had the highest non-significant drumstick weight percentage (11.4%).

**Table 6. Mean±SD of slaughter weight, and relative weights of internal organs and, abdominal fat, breast, wing, drumstick and trunk of Cobb500 broilers as influenced by Bacillus subtilis supplementation.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Relative Wt. | T1 | T2 | T3 | T4W1 | T4W2 |
| Slaughter | 99.1±0.006a | 99.0± 0.005a | 98.4 ±0.007ab | 98.6 ±0.006ab | 98.0±0.006b |
| Liver | 2.3±0.001ab | 2.2 ±0.003b | 2.2 ±0.003b | 2.6 ±0.004a | 2.2± 0.003b |
| Spleen | 0.1± 0.001 | 0.1± 0.000 | 0.1± 0.000 | 0.1 ±0.001 | 0.1 ±0.001 |
| Heart | 0.5± 0.001 | 0.5± 0.001 | 0.5±0.001 | 0.5 ±0.001 | 0.5±0.000 |
| Gizzard | 1.7±0.002a | 1.4± 0.002b | 1.6 ±0.001a | 1.5± 0.001ab | 1.7 ±0.001a |
| Proventriculus | 0.4±0.001 | 0.4 ±0.001 | 0.4 ±0.001 | 0.4 ±0.001 | 0.4 ±0.001 |
| Intestine | 5.3±0.005 | 5.6 ±0.012 | 5.0 ±0.003 | 5.3 ±0.010 | 4.8±0.004 |
| Intestinal fat | 1.0±0.003ab | 1.3 ±0.005a | 0.8± 0.002b | 1.3 ±0.002a | 1.3± 0.003a |
| Breast | 31.6±0.028 | 33.1 ±0.026 | 30.4 ±0.022 | 31.9± 0.018 | 31.9± 0.030 |
| Wing | 8.0±0.007 | 7.1± 0.004 | 7.9 ±0.010 | 7.8± 0.009 | 7.7 ±0.007 |
| Drumstick | 10.3±0.007ab | 9.8± 0.008b | 11.4 ±0.013a | 10.7± 0.011ab | 10.2 ±0.013ab |
| Trunk | 21.0±0.015 | 20.0 ±0.013 | 20.2± 0.012 | 21.3± 0.006 | 20.4 ±0.010 |

Means within the same raw with different superscripts are significantly different (p<0.05)

RGR= relative growth rate. T1;control group including (control negative, control positive and control feeding) , T2 in ovo supplemented group with 1×107CFU *B. subtilis*/egg, T3; in ovo supplemented group with 1×1010 CFU *B. subtilis*/egg; T4W1,drinking water supplied group with 1×107 CFU *B .subtilis*/ml, T4W2; drinking water supplied group with 1×1010 CFU *B. subtilis*/ml.

1. **DISCUSSION**

 Recent researches declared that *B. subtilis* strain is the probiotic of selection in the poultry ([**Hmani**](https://www.researchgate.net/profile/Houda-Hmani) **et al., 2017; Park et al., 2017;Triplett et al., 2017**).*B.subtilis* in poultry had been applied as feed additive by different concentrations for newly hatched chicks **(Gao et al., 2017;Ciurescu et al., 2020)** to be suggested that using *B.subtilis* as feed additive could improve growth performance of broiler chickens. However**, Majidi-Mosleh et al., 2017;**[**Castañeda**](https://www.sciencedirect.com/science/article/pii/S0032579121001590#!) **et al., 2021**recommended applying *B.subtilis* via in ovo infusion into the fertile hatching eggs in late incubation stage to take full advantage of probiotic sporulation in the gut of chicks. So, this study investigated the impact of *B. subtilis* as in ovo injection or watery supplementation on hatching and growth performances of broiler chickens.

 The present study demonstrated that hatching quality of broiler eggs didn't influenced by in ovo injection of *B.subtilis* in air cell at 18th day of incubation. Studied hatching traits in T2 and T3didn't show significant difference comparable to T1 except for hatching weight. The in ovo injection of *B. subtilis* significantly decreased as the dose of *B.subtilis* increased the null effect of in ovo supplementation of *B. subtilis* on hatching performance agreed with [**Oladokun**](https://www.sciencedirect.com/science/article/pii/S0032579120308269#!) **et al., 2021.**However, **Triplett et al., 2017** reported negative impact of inovo injection of *B. subtilis* on broiler hatchability and hatching weight when injected in amniotic fluid at late stage of incubation. Likewise, **Cartman et al., 2008** referred the reason of decrease in hatching performance of chicken eggs to the competition for nutrients needed for Bacillus sporulation and hatching process. However, **Castañeda et al., 2021** conveyed that the effect of in ovo *B. subtilis* on hatchability of broiler eggs is serotype specific.

 Results of the current study declared that, the chicks of light hatching weight that induced by in ovo injection of *B. subtilis* in T2 can improve their growing performance after hatch. Irrespectively to the supplementation method, 107 concentration of *B.subtilis* in ovo or in water improved the marketing body weight of broiler chickens. Also, this concentration promoted the RGR% as supplemented in water.

 On the other hand, the positive impacts of the in ovo application of *B. subtilis* in T2 disagreed with **Ghasemi et al., 2010; Majidi-Mosleh et al., 2017**who reported that inovo infusion of *B. subtilis* in broiler eggs had no beneficial effect on growth performance. This Inconsistency may be due to the difference of inoculation site or *B. subtilis* serotype. However, the positive response of growth performance to107*B. subtilis* in water was supported by the findings of **Cartman et al., 2008; Gu et al., 2015;** [**Dumitru et al., 2019**](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7647910/#bib15)whosuggested that *B. subtilis* improved nutrient digestibility and promotes intestinal health.

The present study indicated that the evaluated carcass traits, including relative weights of organs and cuts didn’t affected significantly neither by in ovo injection nor via water supplementation of *B.subtilis* in different concentrations in comparison to T1. These findings are consistent with [**Zhang et al., 2012**](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7647910/#bib55)results for non-significant effect of inclusion of 108 CFU/kg *B. subtilis* in broiler food on relative weights of liver and bursa of fabricius*.*

1. **CONCLUSIONS**

 The in ovo treatment of *B. subtilis* into the air cell of 18th day fertile broiler hatching eggs by different concentrations couldn’t improve hatching performance.

Regardless the route of administration, the concentration of 107 CFU of *B. subtilis*/ egg or /ml could be used as safe and natural growth promoter to enhance broiler growth performance. These results could concrete the way for the need for further future studies to determine the better concentration and route of *B. subtilis* supplementation to fortify the broilers performances**.**

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