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Impact of Early Threonine Supplementation on Gut Tissue Morphology, Liver Histology, and the Possible Changes in Leukocyte Numbers of Broilers

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Simple Summary: Broiler chickens travel long distances from the hatchery to the farms. This lag time may affect not just the later performance, but also the development of the immune system. Thus, the study aimed to examine the effects of some early nutritional practices such as- *in ovo* and hydrogel-supplemented feeding- on the morphology of the gut, histology of the liver and the possible changes in white blood cell counts, with or without adding an essential amino acid in an early stage of life. Our results confirmed that birds without a feed deprivation period had the best performance, and gut morphology, while threonine supplementation could partly compensate for the loss attributed to the post-hatch delay in feed access. There were no effects of the threonine supplementation on the rate of white blood cells and histological parameters of the liver. The use of Thr fortified Hydrogel® at the post-hatch period according to the performance results, thus it can be recom

Abstract: Slaughter weight and feed efficiency are highly dependent on the length of post-hatch feed deprivation, due to the short fattening time. Late feed access has negative consequences on the development of the intestine, while threonine might be a candidate to support epithelial tissue development. The present study aimed to examine the effects of different early nutrition methods, particularly early Thr supplementation on the performance, gut and liver histomorphology, and the number of different leukocyte types in broiler chicken. Control birds were fed immediately post-hatch, while others had access to solid feed with a 48-hour delay. In one group *in ovo* threonine, administration was applied at d17 of incubation, while other groups of birds received hydrogel with or without Thr fortification in the first 48h. Our results confirmed that the best performance was achieved in birds that had no delay in feed access. Early Thr supplementation could at least partly compensate for the loss attributed to the post-hatch delay in feed access. The higher ability of compensation can be explained by the better architecture of the gut tissue, while there were no significant effects of Thr supplementation on the rate of leukocytes and liver histology parameters.

Keywords: broilers; threonine; *in ovo* nutrition; hydrogel; compensation; performance; leukocytes; gut morphology

.1. Introduction

Fast-growing broilers must have a continuous and well-managed nutrient supply to realize the genetically determined slaughter weight at the target age. The nutritional status of the embryo is highly dependent on the nutritional status of the hen, as maternal nutrition can affect early

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development and the metabolism of the progeny through gene modification [1]. In addition, breed and dietary factors strongly affect egg quality and nutritional profile [2]. The hatching egg contains essential nutrients for embryo development, but the length of the hatching window as well as the time to the first feed post-hatch determines slaughter weight. It has been confirmed that in intensive broilers even 24-hour-delay in feed access post-hatch reduces the body weight by 2-3%, while a longer time of feed deprivation (48-72 hours) results in 5-8% less slaughter weight at 42 days of age (de Jong e al., 2017). During the early period of life, the embryo relies on the antibodies and nutrients of the albumen, however, the dynamic changes of the egg yolk from the continuous metabolism can influence the development of the chick [3]. In addition, other stressors such as limited nutrients in the final period of incubation [4] as well as wide hatching window (12-24h), and delayed first feeding [5] can also harm the immune functions and vitality of broilers. Thus, mortality in the first week is 1.5-2.2 times higher if the access to the first feed is delayed by 48-72 hours compared to birds provided solid feeds within 24-36 hours post-hatch [3].

To avoid these outcomes, different early feeding methods have been developed such as offering hydrogel enriched with various nutrients [6], ensuring immediate feed access post-hatch (Patio system; [7]), or providing nutrients *in ovo* [8]. Vitamins, probiotics, and amino acids can be supplemented as a complementary feed in a gel form by spraying the chicks in the incubator [6] so consuming the gel is carried out by pecking each other. Another option for early nutrition of hydrated gels is applying them in the chick box during transport. The benefits of jelly masses are avoiding dehydration by prompt water consumption [9] and providing probiotics to boost intestinal microbiota and compensate for environmental stressors. It has been well documented that immediate feed access resulted in higher final weight, better breast meat-to-carcass ratio [10] rapid intestinal development [11], greater duodenal villi surface, favorable gut microbiota, and prevention from pathogenic infections by boosting the immune system [12,13]. However, in practice, immediate post-hatch feeding is difficult to proceed. *In ovo* intervention was first applied in vaccination research in the late '80s [14], and the technique was used later to provide nutrients, particularly carbohydrates [9]. Over the years, several experiments were carried out to determine the optimal timing and site of injection by introducing exogenous substances to the air sac, yolk sac, and amniotic fluid [15,16,17]. It turned out, that by consumption of the amniotic fluid proteins, minerals, and hormones can be supplied to the late-term embryo, therefore *in ovo* nutrient provision is a reliable non-invasive method to boost immature digestive systems around the first days of life [16]. Improving chick immunity is also possible by adding vitamins, prebiotics, or amino acids before hatching [18, 19]. From the immunomodulatory point of view, amino acids such as methionine (Met), arginine (Arg), and threonine (Thr) contribute to lymphocyte proliferation and enroll monocytes and heterophils from the bone marrow [20, 21, 22].

According to Sirsat et al [23], modern broiler strains require reevaluation of their nutrient provisions, and specific early-phase nutrition must be applied, particularly, in the pre-hatch and the transitional post-hatch period. Specific feeding strategy that improves the digestive efficiency and/or resilience of birds increases the economics of poultry production. The individual amino acids have a prominent role in this respect. Threonine supplementation may improve the morphological characteristics of the intestine, thereby providing a better and larger absorption surface. This supports the supply of nutrients, which on one hand can result in better growth performance and on the other hand may improve feed efficiency. A better slaughter yield can also accompany a higher live weight if the proportion of valuable meat parts is improved.

Hence, the present study aimed to examine the effects of different early nutrition methods, particularly early Thr supplementation on the performance, gut histomorphology, liver histology, and differentiated leukocyte number of broiler chicken.

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2. Materials and Methods

2.1 Hatching protocol and treatment groups

The experiment was carried out at the Hungarian University of Agricultural and Life Sciences (MATE) Kaposvár Campus, Department of Farm Animal Nutrition by the Declaration of the Hungarian National Scientific Ethical Committee of Animal Experimentation for studies involving animals, protocol license number is SO/31/00444-2/2021.

A total of 1120 Ross 308 broiler eggs were involved in the study. Prior to hatch, the eggs were held in transport boxes at 17-18°C for 6 days without rotation or extra humidification due to the short storage time. A PLM B1350 two-staged incubator was used for the hatching process with 9 tray levels. Each level was equipped with a built-in measurement system for ventilation, humidity, and temperature between the levels. The hatching protocol was carried out according to the recommendations of the Aviagen Hatching Management Guide (2019): the dry bulb temperature and humidity were set at 37.9 ± 0.1°C and 65 ± 3%, respectively (Table 1). The eggs were candled on days 10 and 17 to exclude infertile eggs or dead embryos. The fertility of eggs was calculated after candling by the following formula:

Fertility rate % = number of fertile eggs/total number of eggs set [25].

Table 1. Temperature and CO_2 level during incubation.

Hatching day		°C	CO_2 concentration %
1	Incubation	37.9	0.60
2		37.9	0.60
3		37.9	0.60
4		37.9	0.60
5		37.9	0.60
6		37.9	0.60
7		37.8	0.60
8		37.8	0.60
9		37.6	0.60
10	Candling	37.6	0.60
11		37.5	0.35
12		37.5	0.35
13		37.4	0.35
14		37.3	0.35
15		37.3	0.35
16		37.2	0.35
17	Candling, in-ovo intervention, placing into the incubator	37.1	0.35
18		37.0/36.7	0.35/0.60
19		36.7	0.60
20		36.5	0.60
21		36.2	0.60
22		36.2/35.8	0.35

At the beginning of the study, the eggs were assigned to 7 experimental groups. In the two control treatments, chickens were fed immediately after hatch (Int_0 and IoS_0), while chickens emerged from the rest of the eggs and received solid feed only 48 hours post-hatch. Two groups of eggs were injected with 0.5 mL of physiological saline (0.9 g/mL concentration of NaCl), either from the immediate (IoS_0) or from the delayed fed groups (IoS_48). This intervention was needed to evaluate the possible stress caused by the needle puncture. Another group was treated with *in ovo*

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Thr using 0.5% concentration solution dissolved in physiological saline and the birds had 48 hours of feed deprivation post-hatch (IoT_48). There was no intervention in four groups of eggs, but the hatched chicks in those treatments had different feeding management. Chicken in group Int_0 and Int_48 had immediate or delayed feed access, Int_48G and Int_48GT had no solid feed in the first 48 hours but immediate access to Hydrogel® (Bábolna Feed Ltd.) without Threonine or with 5 g/kg Thr enrichment, respectively, in the transport boxes. The basal Hydrogel® composed of corn starch (30%), probiotic lactic acid bacteria (*Pediococcus acidilactici* (E1712) 1 x10^{10} CFU/g), and vitamin C (5000 mg/kg) and contained 5,4 MJ AMEn/kg. The rationale of the experimental treatments is shown in Table 2.

Table 2. Definition of treatment groups in the study.

Treatment code	Feed access	Early nutrition method	numbr of eggs
Int_0	Immediate	-	160
IoS_0		*in ovo*, saline	160
Int_48	48h delayed solid feed access	-	160
IoS_48		*in ovo*, saline	160
IoT_48		*in ovo* Thr	160
Int_G48		Hydrogel	160
Int_GT48		Hydrogel+Thr	160

2.2 In ovo intervention

The *in-ovo* injection was carried out following the protocol of Uni and Ferket [24] with a 2 mL syringe and a 21-gauge needle. The injection procedure was performed in a sterile cabinet (ScanLaf, LaboGen Inc., Denmark) to prevent any microbiological contamination. Before the intervention all eggs were cleaned with cotton wool dipped in an iodine solution. The eggs were then carefully drilled on the blunt side through the air chamber without reaching the shell membrane. Before the intervention, the position of the embryo was checked, and a 0.5 ml solution (either 0.9% (w/V) NaCl solution or 0.5% Thr solved in 0.9% (w/V) NaCl solution) was transferred to the amniotic fluid without injuring the embryo afterward. To avoid entry of pathogens, a sterile, plastic tape was applied, and the eggs were placed back into the incubator until day 21 of hatching.

2.3 Identification, feeding management, and housing

The birds were weighted with gram precision at hatching day right after harvesting, and wing tagged for individual identification. Birds in treatment Int_0 and IoS_0 were immediately placed in the barn where a mash starter feed was offered *ad libitum*. The rest of the birds were stored in a transporter paper box (25 birds/box) and kept at 32°C for 48 hours in a separate room. Birds assigned to Int_48, InS_48, and IoT_48 received no feed supplementation, while in Int_G48 and Int_GT48 groups the birds had access to Hydrogel with or without Thr fortification. On day 2 all birds were weighed with gram precision and placed in the barn where the starter feed was freely available.

The broilers were randomly placed into floor pens (16 birds/pen, 8 pen/treatment). Each pen represented an individual treatment group and was equipped with one feeder and one drinker. The housing management was set in compliance with Aviagen's (2019) recommendation for temperature, humidity, hours, and intensity of light.

A three-phase feeding program was applied as follows: between days 1 and 10 starter feed (mashed feed), between days 11 and 21 pelleted grower feed, and between days 22 and 35 pelleted finisher feed produced by the Department of Farm Animal Nutrition was offered to all birds. Each feed was formulated on a corn-soybean meal basis. Nutritional content (dry matter, crude protein, fat, ash, calcium, and phosphorus) was determined by the University Lab Center of MATE according to the recommendations of the Association of Official Analytical Chemists (AOAC, [25]).

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The birds were fed *ad libitum* from self-feeders during the trial. One feeder was presented per pen. Drinking water was also available *ad libitum*. The analyzed composition of the feed is presented in Table 3.

Table 3. Analyzed feed composition in the three feeding phases.

Ingredients	Starter (1–10)	Grower (11–21)	Finisher (22–35)
Corn (grain)	551	577	601
Corn gluten (60%)	32	32	32
Sunflower meal	53.5	53.5	75
Soybean meal (CP 44.2%)	262	230	175
Fat, vegetable	44.7	55	67.00
MCP	18.7	17.5	15
Limestone	15	13.5	12.2
NaCl	2.7	2.7	2.7
L-Lysin HCl	5.2	4.6	4.3
DL-Methionin	4.5	3.9	3.2
L-Treonin	2.6	2.3	1.8
Premix[1]	5.00	5.00	5.00
Total	**1000.00**	**1000.00**	**1000.00**
Nutrient content (g/kg)			
AMEn (MJ/kg)	12.5	12.9	13.4
DM %	90	91.3	91.1
Crude protein	204.2	190.7	174.9
Crude fat	71.87	82.3	94.4
Crude fiber	41.5	41.1	44.8
Lysine*	13.5	12,1	10,8
M + C*	10.8	9.9	9.0
Threonin*	9.7	8,8	7,8
Triptophan*	2.4	2.3	1.7
Ca	9.6	8.7	7.8
P $_{avaliable}$	4.7	4.5	3.9
Na	1.7	1.7	1.7

[1]Premix feed contents per kilogram: Zn: 22,032 mg, Cu: 3200 mg, Fe: 16,020 mg, Mn: 21,948 mg, I: 300 mg, Se: 70 mg, Co: 20 mg, Vit. A: 324,0000 IU, Vit. D3: 810,000 IU, Vit. E: 20,800 mg, Vit K3: 810 mg, Vit. B1: 810 mg, Vit. B2: 1890 mg, Vit. B3: 10,800 mg, Vit. B5: 3240 mg, Vit. B6: 1350 mg, Vit B12: 6.8 mg, Folic acid: 270 mg, Biotin: 32 mg.* calculated values

2.4 Experimental procedure

The live weight of the birds was measured at hatch (d1), on days 3, 10, 21, and 35. The average daily gain (ADG) was calculated individually, while feed intake (FI) was recorded per pen for the time intervals by measuring the offered and remaining feed for each phase. The feed conversion ratio (FCR) was also calculated per pen basis.

At hatch, 48 hours later, and at d21 of the experiment, 10 birds from each treatment were used to collect blood, liver, and intestinal samples. For that purpose, the birds were slaughtered following carbon dioxide stunning in compliance with the relevant legal regulations (Council Regulation 1099/2009/EC). In the case of day-old and 3-day-old chicks, capillary blood samples were collected from the dorsal metatarsal vein and direct blood smears were prepared immediately from 10 birds/treatment. Similarly, to day-old chickens, the three-week-old birds were sampled for bloodwork, but this time blood samples were collected from the jugular vein into 5 ml tubes containing EDTA for further evaluation. Blood smears were prepared immediately after the collection process.

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To determine gut morphology through villus height (VH), crypt depth (CD), and villus/crypt ratio (VH/CD) tissue samples were collected from *duodenum, ileum,* and *colon* (10 birds/treatment). Liver samples were also collected, and all samples were placed into a 10% neutral buffered formalin solution and processed in an Epredia™ Citadel 2000 Tissue Processor (Thermo Fischer Scientific, Waltham, MA, USA) to make paraffin-embedded blocks. Two-micrometer-thick paraffin sections were obtained in a rotary microtome unit (HistoCore BIOCUT, Leica Biosystems, Nussloch, Germany). These sections were de-waxed, rehydrated, and transferred to glass slides, and hematoxylin-eosin staining was performed according to standard histological techniques [26]. Each sample from each broiler was cross-sectioned three times, and three measurements were carried out on each section. Villus sizes were measured at 400X magnification [27]. The measurement method is demonstrated in Figure 1.

Histological injuries on the liver were classified as none, mild, moderate, or marked lesions according to their severity with scores from 0 to 3.

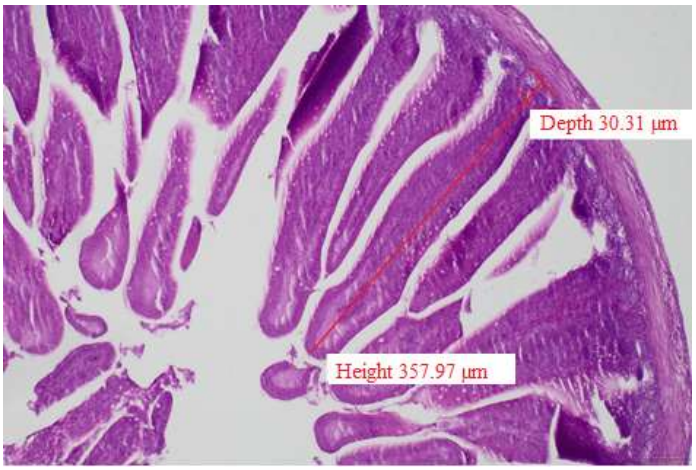

Figure 1. Transversal section of duodenum in a three-day-old chicken, H-E., 100X. Red lines represent the morphometric measurement of villus height and crypt depth

2.4. Statistical analysis

Data were analyzed according to a completely randomized block design with 7 treatments. A Shapiro–Wilk normality test was carried out on the base data. The outliers were checked and excluded from the statistical analysis. Outliers were defined as values being more than 2 times of standard deviation away from the mean, Levene's test was used to examine group homogeneity among treatment groups. A one-way ANOVA was applied to performance data and other results considering the effect of treatment as the main factor of variation in body weight at a different age, feed intake (FI), feed conversion ratio (FCR), and the number of white blood cells, as well as morphometry parameters. In case of statistically significant difference ($P<0.05$) Tukey's post-hoc test was applied to check the differences between treatment groups. Frequency distributions were calculated for the histological injuries of liver tissue samples along with a Kurksal-Wallis test to the ordinal scale of the injuries.

3. Results

3.1. Growth performance and feed efficiency

Hatchability (hatched chicken/total number of eggs) was in the range of 0.74-0.79 in eggs having no intervention (intact eggs), and 0.77-0.78 in groups applied physiological saline solution and 0.81 when *in ovo* threonine supplementation was applied at day 17 of the incubation. The live weight of birds in different experimental treatments at hatch and 48 hours later is shown in Figure 2. The ANOVA confirmed a significant difference in hatching weight, as birds in one of *in ovo* saline-treated

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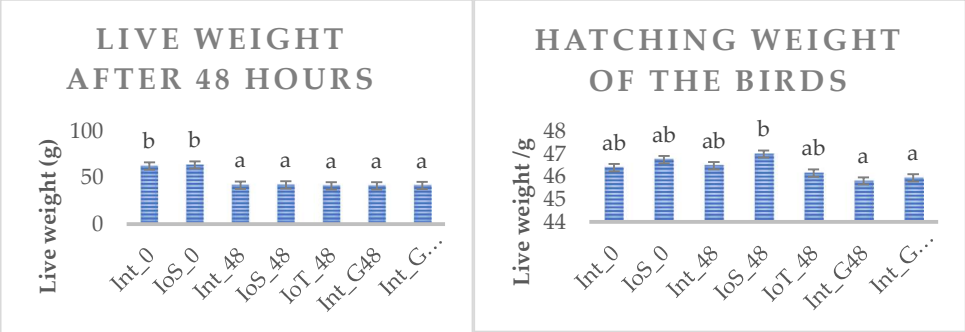
group assigned to IoS_48 group was heavier than all others (P<0.05). Live weight on the 3rd day of the trial was much higher in birds fed immediately post-hatch (Int_0 and IoS_0 vs. other groups).



Bars represent treatment means and SD[a, b] different letters indicate a significant difference (P<0.05)

Figure 2. Hatching weight and live weight at 48 hours post-hatch of broilers in different experimental treatment groups.

The effect of dietary treatments including early threonine supplementation on LW, FI, FCR, and ADG of broilers is shown in Tables 4., and 5. Birds with immediate feed access reached 240 g on average by day 10, while birds with a 48-hour delay in access to the first solid feed had more than 20% lower body weight (194 g on average). At the end of the starter phase, eggs supplemented with *in ovo* saline (IoS_0) had higher LW, than the intact group (Int_0), while treatments with 48 hours of delayed feeding did not differ from each other. LW at day 21 was the highest in birds fed immediately post-hatch and differed significantly from the different groups (Int_0 and IoS_0 vs others, P<0.05). The lowest LW was recorded in the Int_48 group, and from the delayed groups the *in ovo* Thr-treated birds had the highest body weight at the end of the grower phase. At the end of the trial, on day 35, the immediately-fed birds still had the highest LW. Compared to the immediate fed counterparts there was no statistically confirmed difference between the LW of the delayed and in-ovo Thr or Hydrogel ® supplemented birds. Group IoT_48 significantly did not differ either from the IoS_48 and Int_48GT or from the Int_48 which had the lowest final LW.

Table 4. Effect of dietary treatments on body weight at the end of different phases.

	Int_0	IoS_0	Int_48	IoS_48	IoT_48	Int_G48	Int_GT48	RMSE	P-value
d10	233**a**	245**b**	191**c**	193**c**	196**c**	194**c**	192**c**	25.8	**<0.0001**
d21	855**ab**	882**a**	766**c**	782**cd**	809**bd**	777**cd**	785**cd**	95.3	**<0.0001**
d35	2218**a**	2238**a**	2072**b**	2113**ab**	2086**b**	2096**ab**	2100**ab**	257.4	**<0.0001**

a,b means in the same row with different letters statistically differ (P<0.05).

The FI of the birds showed significant differences in all of the examined time intervals, however, the advantage of groups Int_0 and IoS_0 remained constant. Statistics confirmed a significant treatment effect on FCR in the starter, grower, and finisher phases. *In ovo* Thr-supplemented birds had more efficient feed utilization in the starter phase than birds who received their diet immediately post-hatch and were treated with *in ovo* saline (IoS_0, P=0.04). The average daily gain was significantly affected by dietary treatments in all phases and the whole trial. In the starter phase, the 48-hour delay in FI resulted in a lower growth rate (Int_0 and IoS_0 vs others, P<0.05). In the grower and finisher phases, the advantage of the immediate fed birds was obvious, while supplementation of *in ovo* Thr and the hydrogel with or without Thr enrichment could compensate at least partly the loss caused by the late feed access.

Table 5. Effect of dietary treatments on feed intake (FI) feed conversion ratio (FCR) and average daily gain (ADG) in different phases and the whole experiment.

	Int_0	IoS_0	Int_48	IoS_48	IoT_48	Int_G48	Int_GT48	RMSE	P-value
Feed intake (kg/day/pen)									

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d1-10	23.2**a**	24**a**	17.9**b**	17.7**b**	17.8**b**	17.1**b**	17.9**b**	1.72	**<0.0001**
d11-21	72.8**ab**	75.6**a**	66.8**c**	66.7**c**	69.1**bc**	67.3**c**	66.8**c**	1.36	**<0.0001**
d22-35	145.6**a**	144.4**a**	136.9**b**	138.6**ab**	137.6**b**	138.6**ab**	140.1**ab**	6.22	**0.04**
d1-35	85.35**a**	86.3**a**	78.3**b**	78.2**b**	79.3**b**	78.9**b**	79.1**b**	3.52	**<0.0001**
Feed conversion ratio (kg feed/ kg gain)									
d1-10	1.24**a**	1.21**ab**	1.24**a**	1.21**ab**	1.18**b**	1.15**b**	1.23**ab**	0.13	**0.04**
d11-21	1.28**ab**	1.3**a**	1.28**ab**	1.24**ab**	1.24**b**	1.26**ab**	1.23**b**	0.77	**0.001**
d22-35	1.49**b**	1.48**b**	1.45**ab**	1.45**a**	1.5**b**	1.46a	1.48**b**	0.13	**0.03**
d1-35	1.37**ab**	1.37**ab**	1.35**b**	1.32**bc**	1.36**ac**	1.34**c**	1.34c	0.13	**0.043**
Average daily gain (g/d)									
d1-10	18.6**b**	19.8**a**	14.4**c**	14.6**c**	15.0**b**	14.8**b**	14.5**b**	2.57	**<0.0001**
d11-21	56.5**ab**	57.9**a**	52.1**c**	53.5**bc**	55.6**ab**	53.0**bc**	53.9**bc**	7.1	**<0.0001**
d22-35	97.5	97.1	93.8	95.0	91.2	94.5	94.2	14.28	0.13
d1-35	62.0**a**	62.6**a**	57.8**b**	59**ab**	58.3**b**	58.5**ab**	58.7**ab**	7.19	**<0.0001**

a,b,c means in the same row with different letters statistically differ (P<0.05).

3.2. Intestinal morphometry

Tables 6, 7, and 8 present the gut morphology results affected by dietary treatments at hatch, 48 hours post-hatch, and 21 days of age, respectively.

The intestinal morphological results on villus height measured at hatch (Table 6) show a significant difference in the duodenum comparing IoS_0 vs Int_48 groups, in the ileum comparing Int_0 and IoS_0 vs. IoS_48 and Int_G48 groups, and between IoS_48 and IoT_48 vs. Int_GT48 in the colon region. Crypt depth was affected in the duodenum and the colon. IoS_48 group had the lowest crypt depth as it differed significantly from Int_0, IoS_0, Int_48, and IoT_48 groups in the duodenum. In the architecture of the colon, birds assigned to *in ovo* saline (IoS_0 and IoS_48) and Int_GT48 group showed the lowest crypt depth, while that of birds in Int_48 and IoT_48 treatments had the highest.

Table 6. Effect of dietary treatments on villus height, crypt depth, and villus height/crypt depth ratio in the duodenum, ileum, and colon (D, I, and colon, respectively) at hatch.

	Int_0	**IoS_0**	**Int_48**	**IoS_48**	**IoT_48**	**Int_G48**	**Int_GT48**	**P-value**
Villus height (μm)								
D	580.8**ab**	506.5**a**	673.2**b**	639.8**ab**	621.8**ab**	579.2**ab**	586.1**ab**	**0.032**
I	302.6**a**	295.4**a**	363.6**ab**	375.3**b**	358.7**ab**	400**b**	364.5**ab**	**0.0011**
C	318.3**ab**	377.8**a**	347.2**ab**	308.1**ab**	357.3**a**	310.5**ab**	258.2**b**	**0.0055**
Crypt depth (μm)								
D	111.3**a**	110.3**a**	109.5**a**	80.5**b**	115.4**a**	100.1**ab**	103.9**ab**	**0.001**
I	88.1	94.6	93.3	94.9	83.4	106.0	85.6	0.16
C	92.5**ab**	87.4**a**	104.5**b**	81.4**a**	110.5**b**	94.4**ab**	82**a**	**0.0004**
Villus height/Crypt depth ratio								
D	4.3**a**	4.6**a**	4.8**a**	8.2**b**	5.5**a**	5.1**a**	3.9**a**	**<0.001**
I	3.4**ab**	3**a**	3.8**ab**	4**ab**	3.8**ab**	3.8**ab**	4.4**b**	**0.0082**
C	4.0	4.2	3.5	4.0	5.5	3.4	3.3	0.28

a,b means in the same row with different letters statistically differ (P<0.05).

The villus height was affected by treatments in two examined intestinal sections on day 3 (48 hours post-hatch) (Table 7). It was the highest in the Int_0 group and the lowest in the IoS_0 group both in the duodenum and colon. Crypt depth was statistically the same in all groups. VH/CD ratio was affected only in the colon, showing the lowest rate in the Int_48 group significantly differing from that of birds in Int_0, IoS_0, Int_G48, and Int_GT48.

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Table 7. Effect of dietary treatments on villus height, crypt depth, and villus height/crypt depth ratio in the duodenum, ileum, and colon (D, I, and colon, respectively) 48 hours post-hatch.

	Int_0	IoS_0	Int_48	IoS_48	IoT_48	Int_G48	Int_GT48	P-value
				Villus height (μm)				
D	340.6**c**	214.9**a**	288.5**b**	240.8**b**	283.4**b**	321.6**bc**	250.0**ab**	**<0.001**
I	158.6	140.2	167.4	140.8	154.7	157.1	142.9	0.057
C	164.2**b**	126.4**a**	159.8**ab**	146.4**ab**	156.5**ab**	132.1**ab**	130.0**ab**	**0.0085**
				Crypt depth (μm)				
D	36.3	29.2	35.6	34.0	36.5	31.8	35.6	0.43
I	32.5	30.3	33.7	29.6	32.6	33.6	32.8	0.14
C	29.9	25.3	30.3	29.1	28.3	26.5	26.7	0.059
				Villus height/Crypt depth ratio				
D	7.7	8.1	8.1	7.6	7.8	9.2	7.2	0.28
I	4.8	4.5	4.8	4.8	4.8	4.7	4.6	0.96
C	5.7**b**	5.1**b**	3.7**a**	4.5**ab**	4.5**ab**	4.9**b**	4.9**b**	**0.006**

a,b,c means in the same row with different letters statistically differ (P<0.05)

Significant differences were found on day 21 regarding villus height in the duodenum and the ileum, but not in the colon (Table 8). A statistically confirmed difference was obtained in duodenal villus height between Int_48 and Int_G48 birds, while all others did not differ. The *ileal villi* were the longest in birds assigned to IoT_48 treatments, and differed from almost all other groups, except for birds in IoS_0. Crypt depth was not affected by dietary treatments. Villus height/crypt depth ratio was different between InT_0 and Int_G48, and between IoT and Int_GT48, in the duodenum and ileum, respectively.

Table 8. Effect of dietary treatments on villus height, crypt depth, and villus height/crypt depth ratio in the duodenum, ileum, and colon (D, I, and colon, respectively) at 21 days of age.

	Int_0	IoS_0	Int_48	IoS_48	IoT_48	Int_G48	Int_GT48	P-value
				Villus height (μm)				
D	768.9**ab**	748.8**ab**	830.9**a**	790.8**ab**	755.6**ab**	690.1**b**	765.8**ab**	0.04
I	210.6**a**	221.3**ab**	216.1**a**	199.3**a**	282.8**b**	218.7**a**	180.4**a**	0.0004
C	214.9	235.3	220.8	237.9	205.2	211.1	217.2	0.45
				Crypt depth (μm)				
D	86.1	103.5	106.8	89.5	97.4	104.5	113.2	0.09
I	61.8	59.2	55.3	58.4	72	66.8	60.7	0.08
C	61.9	64.3	54.3	56.1	58.8	56.1	55.2	0.26
				Villus height/Crypt depth ratio				
D	9.6**a**	7.8**ab**	8.1**ab**	9.1**ab**	8.2**ab**	6.6**b**	7.4**ab**	0.01
I	3.5**ab**	3.8**ab**	4**ab**	3.5**ab**	4.2**a**	3.3**ab**	3**b**	0.01
C	3.6	3.7	4.2	4.6	3.8	3.9	4.1	0.29

a,b means in the same row with different letters statistically differ (P<0.05)

3.3. Liver histology and blood cell results

Results of the liver histology demonstrate that most of the alterations regarding the heterophil granulocyte infiltration within the periportal regions occurred right after hatch, and remained constant by day 21. Interestingly it showed the worst results in the *in ovo* treated groups by Day 3. From day 3 another pathological change, mononuclear infiltration appeared in the liver, mostly in the periportal areas, but sometimes as multifocal lesions scattered in the parenchyma. This lymphohistiocytic invasion increased and gradually became predominant by day 21 in almost all groups. In addition, hepatocytes showed moderate to marked lipid accumulation in every section. This hepatic lipidosis gradually reduced from day 1 to day 21, and completely disappeared in most

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of the groups. Only in groups Int_G48 and Int_GT48, a mild lipidosis had been detected on day 21, and it remained as a faint vacuolization in group IoS_48 by this time.

Table 9. Effect of dietary treatment on heterophil granulocyte infiltration, vacuolization, mononuclear infiltration in the liver tissue, and lipid accumulation in liver cells at different time points of the experiment.

	Int_0	IoS_0	Int_48	IoS_48	IoT_48	Int_G48	Int_GT48	P-value*
Heterophil granulocyte infiltration								
day 1	0.66ab	0.75ab	1.0b	0.58a	1.0b	1.0b	1.0b	0.01
day 3	0.41ab	1.0b	0.25a	0.57ab	1.0b	1.0b	0.75ab	0.0007
day 21	1.1ab	0.3a	0.58a	1.41b	1.66b	1.19ab	1.6b	<0.001
Vacuolization								
day 1	0	0	0	0	0	0	0	-
day 3	0.16ab	0a	0a	0.42b	0a	0a	0a	0.017
day 21	0a	0a	0a	0.28b	0a	0a	0a	0.007
Mononuclear infiltration								
day 1	0a	0a	0a	0.25	0a	0a	0a	0.01
day 3	1.41b	0.33a	0a	0.71ab	0.5a	1ab	0a	0.008
day 21	1.77ab	2b	1.6ab	1.3ab	2.33b	1.33a	1.33a	<0.001
Lipid accumulation in liver cells								
day 1	1.0a	1.2b	1.0a	1.0a	1.0a	1.0a	1.0a	0.014
day 3	0.25a	1.66c	0.75ab	1.0b	1.0b	1.0b	1.0b	<0.001
day 21	0a	0a	0a	0a	0a	0.8b	0.4ab	<0.001

* represent Chi^2 P-value; a,b means in the same row with different letters statistically differ (P<0.05).

Due to some technical failure determination of leukocytes' ratio was not successful in Int_0 birds and the data of that treatment was discarded from statistical analysis. With blood smear evaluation moderate lymphocyte depletion and a mild increase in monocyte numbers were detected in all groups and ages (Table 10). Heterophil and eosinophil granulocyte numbers did not show remarkable alterations to dietary treatments (P>0.05).

Table 10. Effect of dietary treatment on the leukocytes* in blood smear at hatch, 48 hours post-hatch, and 21 days of age.

	Int_0	IoS_0	Int_48	IoS_48	IoT_48	Int_G48	Int_GT48	P-value
At hatch								
HE	--	0.715	0.6	0.728	0.586	0.676	0.715	0.09
LYM	-	0.216	0.316	0.198	0.305	0.241	0.216	0.13
MON	-	0.019	0.036	0.026	0.025	0.016	0.019	0.10
EOS	-	0.048	0.048	0.047	0.068	0.067	0.048	0.86
48 hours post-hatch								
HE	0.629	0.529	0.619	0.597	0.610	0.525	0.629	0.052
LYM	0.291	0.386	0.305	0.339	0.314	0.397	0.291	0.79
MON	0.035	0.023	0.028	0.025	0.034	0.028	0.035	0.90
EOS	0.045	0.038	0.048	0.039	0.042	0.050	0.045	0.05
21 days of age								
HE	0.314	0.323	0.296	0.346	0.317	0.357	0.314	0.33
LYM	0.578	0.579	0.613	0.570	0.571	0.563	0.578	0.82
MON	0.054	0.043	0.040	0.035	0.044	0.040	0.054	0.47
EOS	0.053	0.055	0.051	0.049	0.065	0.040	0.053	0.69

Leukocytes: HE= heterophils, LYM= lymphocytes, MON= monocytes, EOS= eosinophyls.

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4. Discussion

Our study aimed to evaluate the efficiency of different early feeding methods by revealing some physiological changes that might be induced by post-hatch feed deprivation. It has been investigated whether the provided early threonine supplementation either before or after hatch could alleviate the negative impact of delay in solid feed access. *In ovo* feeding was first reported several decades ago, and it is still not a common practice in hatcheries. However, an increasing number of studies evaluate the effect of specific nutrient supplementation of the poultry embryo on the growth performance of meat-type poultry and reveal the mode of action of the supplementation.

In the present study *in ovo* intervention did not reduce the hatchability of the eggs. Some studies reported a lower hatchability rate when *in ovo* feeding [28, 29,30], however, it seems that not the intervention itself but the circumstances like the amount, concentration, and osmolarity of the injected solution, etc. were responsible for the lower hatch rate in different studies [31] Statistically confirmed difference was observed between hatching weight of IoS_48 and that of the other groups, however, that result is ambiguous. The *in ovo* saline solution was used as a control of the *in ovo* intervention per se. The treatments IoS_0 and IoS_48 can be considered as identical groups at the time of emergence, since in their case *in ovo* manipulation took place on day 17 of incubation, and at hatch, even the intake of feed did not cause any difference, since the Int_0 birds were settled after the weighing. Therefore, we can conclude that eggs in groups of IoS_0 and IoS_48 were treated equally. In agreement with our results, Kadam et al., [32] confirmed in a meta-analysis that the effect of physiological saline is not consequent and the treated birds do not always have higher weight at hatch. The beneficial effects of *in-ovo* feeding on hatching weight are majorly shown when carbohydrates are used as nutrient supplementation [8, 10, 33]. In line with other results [32; 34] threonine supplementation on day 17 of incubation did not shift hatching weight in the present study. It is well documented that birds at emergence need energy and thus, carbohydrates can be promptly used as a fuel to compensate for the energy deficit at hatch. In the case of an amino acid supplementation, the aim was not to support the energy status but to provide nutrients, as building blocks for specific proteins like epithelia or functional proteins involved in defense mechanisms. We hypothesized that early Thr supplementation provided either during late embryo development or in the post-hatch period might contribute to better gut tissue development, and resulted in a better growth rate and more efficient birds. That has been at least partly confirmed. Live weight and the average daily gain of broilers were supported by early threonine supplementation when the birds had a 48-hour delay to solid feed access. However, it has to be admitted that the performance may still be slightly compromised, and the feed deprivation in the first two days of life may be hardly compensated completely by either of the early methods of Thr supplementation. Immediate post-hatch feeding is an ideal situation, but it is hard to proceed in practice. Birds get their first feed with a 36-72-hour delay very often. Therefore, in our study, the immediately-fed group represents the genetic potential of the birds. In a recent work of [34] early (within 2 or 24 hours) post-hatch feeding compared to a 48-hour delay to the first feed, the growth performance of broilers improved, most likely due to an improved hormone secretion (T3, T4, and IGF-1), and also by enhancing the intestinal health and modulating the microbiota, especially at day 21. Results of de Jong et al. [3] also confirmed that even a 24-36-hour delay in post-hatch feed access reduces the body weight of chicks to a statistically verifiable extent. It has to be noted, however, that the longer the fattening period is (e.g. 50 days), the more ability the birds have to compensate for the early perturbation.

The provision of gel supplements is the most widely used in practice among early feeding methods. Several studies report the beneficial effects of hydrogels such as hydrating the day-old-birds or adding their probiotic benefits [35, 36]. Even though it is a high supplement with high moisture content, hydrogel could not compensate for the two-day feed deprivation, as the body weight of birds was lower on day 3 than on day 1 of the trial. This result emphasizes, that the energy supply from the yolk sac is not enough even for maintenance purposes in intensive genotypes. As reviewed by Al-Huwaizi [37], the yolk sac nutrients-particularly fatty acids- can be more efficiently utilized if birds receive solid feed post-hatch. Among delayed-fed birds the early access to hydrogel,

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particularly with Thr enrichment resulted in the highest body weight at the end of the experiment numerically, but not in the starter and grower phases.

In the first 3 weeks, the *in ovo* Thr supplementation provided *in ovo* improved the growth performance like ADG and FCR of birds compared to the non-supplemented counterparts. It has been repeatedly confirmed that *in ovo* supplementation of Thr alone or in combination with carbohydrates or other amino acids like arginine can improve the growth rate of broilers [38,39,40,41,42]. Those positive results were almost completely explained by the improvements of the morphology of the gut.

It is well-documented that dietary threonine has a key role in intestinal development from both structural and functional points of view [43, 44]. Thr -being an essential amino acid-, plays a vital role in the maintenance of intestinal barrier integrity and mucin synthesis (45, 46). It is the most abundant essential amino acid in the endogenous protein secreted in the intestine, particularly in the mucins in which Thr represents 16% of total amino acids [47]. In conventional feeding trials, it has been confirmed that insufficient threonine supplementation has a negative effect on the morphological state of the intestine [48, 49]. Moreover, feeding Thr supplementation above the recommended level resulted in higher villus length, lower crypt depth, and improved VH/CD ratio in broilers, compared to the control group [43]. In contrast with Ospina-Rojas et al. [50], however, they did not experience a statistically reliable difference in gut morphology when feeding diets with an increased threonine content.

Earlier studies reported, that under commercial conditions a higher level of Thr that exceeds the current NRC recommendation [49] is required to achieve maximum immune function and health status for poultry (51, 52, 53). Thus, it is an interesting issue whether a short-term but targeted Thr supplementation has benefits for broilers.

The intestinal morphological results measured at hatch can hardly be explained by consequent dietary treatments. Overall, we could not confirm any positive effect of the provided threonine supplementation during the embryonic stage on brush border development, at hatch. However, it seems that early Thr supplementation may have a positive effect on the architecture of the intestinal epithelium, particularly on the villus height/crypt depth ratio that is correlated with the absorptive surface of the gut later on. In agreement with other studies [35, 54; 55, 56] our results show that post-hatch feed deprivation compromises the ideal trajectory of gut tissue maturation and there is a significant difference between brush border architecture when birds fed immediately or with 48 hours delay post-hatch. Moreover, data from Proszkowiec-Weglarz et al. [57] suggest, that delay in feeding may indirectly affect the gut barrier function of the small intestine as well as possibly reduce the absorption and utilization of nutrients such as carbohydrates in the intestinal tract of broilers. Those negative effects can be at least partly compensated if gel supplements or early Thr supplementation is provided, as confirmed by our results. In line with these findings, numerous studies [40, 58,] reported higher *villi* and more intensive mucin secretion in birds received *in ovo* Thr.

Periportal heterophilic infiltration in day-old chickens and the following mononuclear invasion later at the same histological sites might indicate acute inflammatory response in the young birds that developed a chronic process thereafter in the older animals [59]. The origin of acute inflammation remained unrevealed, although the nature of the lesions may indicate microbial infection before or on the occasion of hatching [60, 61]. As no significant connections were detected between the pathological alterations and the different groups, we can conclude that *in ovo* treatment neither could induce the histological changes nor had any impact on their further behavior. Hepatic lipidosis in young poultry is generally a natural process that can be connected to lipid transportation from the yolk sac and the slower lipid metabolism in day-old chickens. Usually, these processes induce only mild fat accumulation in a few days after hatching [61.], although more severe and long-lasting lesions were observed in our study. The inflammation resulting in hepatocellular damage could be in the background of prolonged and more prominent lipidosis.

The reduced lymphocyte counts together with increased monocyte numbers in blood work means the so-called stress leukogram both in mammal and bird species that is the result of glucocorticoid effects on blood cells and bone marrow [62]. Our finding indicates that chickens had

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some degree of stress during their development, although its origin was probably not the nature of experimental treatments because stress leukogram patterns could be observed in all groups and ages.

5. Conclusions

Our results confirmed that the best performance was achieved in birds without delay in feed access. Early Thr supplementation could at least partly compensate for the loss,- attributed to the 48h delay in feed access- post-hatch. The higher compensation ability can be explained by the better architecture of the gut tissue. *In ovo* Thr supplementation supported the growth and resulted in favorable villus height/crypt depth ratio in the first 3 weeks. The use of Thr fortified Hydrogel® at the post-hatch period according to the performance results, thus it can be recommended for practice for broilers with delayed feed access.

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