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Effect of *in-ovo* feeding of iron nanoparticles and methionine hydroxy analogue on broilers chickens small intestinal characteristics

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ABSTRACT. The experiment was conducted with 644 Ross fertilized egg by 7 treatments 4 replicates and 23 eggs in each. Seven treatments included two control with and without injection, iron sulfate, iron sulfate nanoparticles, Alimet, Alimet + iron sulfate, Alimet + iron sulfate nanoparticles. After hatching 2 mg iron nanoparticles were applied as new treatment. The highest increased in the intestinal relative weight ($p < 0.05$) was observed by iron+Alimet in late feeding at day old of age. Also similar trend was found in cecum and duodenum length by iron control 2 and late feeding (18 hours' after hatching). The highest cecum length was found among all treatments by *in ovo* injection of iron nanoparticles in early feeding at 21 days of age ($p < 0.05$). Significantly increased the duodenum length was found by iron sulfate in early feeding at 42 days of age ($p < 0.05$). *In ovo* injection of Alimet in late feeding was resulted in decrease jejunum crypt depth at 21 days of age ($p < 0.05$). The results of this study have shown that the highest jejunum villi width and surface area were recorded in dietary iron sulfate nanoparticles in late feeding at 21 and 42 days of age ($p < 0.05$).

Keywords: broiler chicken; in-ovo injection; iron nanoparticles; methionine; small intestine.

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Introduction

A large number of experiments have shown that deficiencies of some amino acids and micronutrients such as minerals particularly could affect not only on production but also in intestinal status (Klasing, 2007; Kogut & Klasing, 2009).

Iron-deficiency anaemia is as a public health problem (Stoltzfus, 2001). One of the strategies to overcome in this problem is add to food iron supplementary. Mineral elements such as iron are the vital components of poultry nutrition. Iron is necessary for transfer, reserve and use of oxygen (Richards, 1997). In addition, iron is a constituent of hemoglobin, transferrin, myoglobin, cytochromes, and many enzyme systems including catalase, peroxidase, and phenylalanine hydroxylase (Harvey, 2000). Hemoglobin and myoglobin are important determinant agents of the meat quality. Much of the organic iron in the body is found in the structure of hemoglobin, in muscles as myoglobin, and in liver, it is in the form of reserved ferritin and hemosiderin (Suttle, 2010). Many researches have supported the role of mineral elements in low consumption such as iron in the development of chicken embryo (McFarlane & Milne, 1934; Richards, 1997).

Sulphureted amino acids such as, methionine and cysteine, are important in the poultry diet (Grimble, 2006). The components of the poultry diet are often poor of sulphureted amino acids and, to compensate this lack, since synthetic methionine is needed to add in their diet (Al-Mayah, 2006).

The gastrointestinal tract (GIT) constitutes is the first barrier to nutrient metabolism in animals (Iji, Saki, & Tivey, 2001). The development of the digestive tract after the hatching plays crucial role in chickens in expression to the high genetic potential of growth rate. After hatching and initiation of feeding, the relative weight of the whole digestive tract increases by approximately 20% during the first 5 days, in fasting chickens there is almost no change during this time (Jin, Corless, & Sell, 1998). The rapid development of the gastrointestinal tract post hatch is necessary. Intestinal villi are small, finger-like projections that protrude from the epithelial lining of the small intestine's walls. Each villus is approximately 0.5-1.6 mm in

length, and has many microvilli projecting from the enterocytes of its epithelium which collectively form the striated or brush border. The intestinal villi are much smaller than any of the circular folds in the intestine. In parallel with these morphological changes, the ability of the intestinal tissue to digest and absorb nutrients increased steadily during in first week of post hatch (Uni, Noy, & Sklan, 1999; Uni, Tako, Gal-Garber, & Sklan, 2003). Uni, Ganot, and Sklan (1998) have reported that delaying access to feed in chickens after hatching resulted in retardation in growth of all intestinal segments. Therefore, in early growth period, nutrition and applications of feeding have increased their importance due to high relationship between nutrition and growth of the digestive tract (Uni et al., 1998).

After hatching, the gastrointestinal tract undergoes morphological and physiological changes, including increased surface area for digestion and absorption. The morphological changes involve increases in intestinal length, villus height, density and, consequently, in the number of enterocytes and goblet and enteroendocrine cells (Imondi & Bird, 1966). The presence of nutrients in the intestinal lumen is able to stimulate villus and crypt growth (Moran Junior, 1985).

It is probable that the in ovo injection of iron especially in the form of nanoparticles of iron increases the absorption and methionine in embryonic period function effectively to provide oxygen to muscles and gastrointestinal sites. Therefore, the purpose of the present study was to evaluate the effect of in ovo feeding of iron sulfate nanoparticles and methionine hydroxy analogue on small intestinal characteristics in broilers chickens.

Material and methods

Birds, housing, and diets

The field operations of the study were carried out in the hall for the broiler research in Faculty of Agricultural in, Bu Ali Sina University, Hamadan, Iran. Experimental was arranged in poultry farm and Nutrition Laboratory.

The embryo experiment was conducted with 644 Ross fertilized eggs in 7 treatments, 4 replicates and 23 eggs in each: (1) control treatment (receiving no injection); (2) second control treatment (receiving injection of physiological serum); (3) iron sulfate: 25 ppm; (4) iron sulfate nanoparticles: 25 ppm; (5) methionine hydroxy analogue: 100 ppm; (6) chelate iron sulfate + methionine hydroxy analogue: 150 ppm (7) iron sulfate + methionine hydroxy analogue: 100 ppm.

In the first day of incubation, yolk was identified through candling and 0.3 mL of solutions was injected into eggs yolk. After hatching, all chickens were transferred to the rearing hall, they were fed by the starter and grower diet up to 42 days of age.

Chickens were fed 6 and 18 hours after hatching. The post-hatch chicks were allocated to 16 treatments (8×2 factorial design with 4 replicates in each) consisted of two factors of additives: (1) control (non-injected); (2) control (injected); (3) iron sulfate; (4) iron sulfate nanoparticle; (5) methionine hydroxy analogue; (6) iron sulfate bounded to methionine hydroxy analogue; (7) iron sulfate nanoparticle; (8) iron sulfate nanoparticle in diet.

Intestinal morphology

At the one, 21 and 42 days, two birds were randomly selected per replicate and slaughtered by cervical dislocation, then relative weight of small intestine was measured. Also, about 2-3 cm segment from midpoint of duodenum, jejunum, ileum and cecum were removed. The intestinal samples were washed with phosphate buffer solution (PBS) and fixed in 10% neutral buffered formalin solution at least in 24 hours. The fixed tissue samples were processed in an automatic tissue processor (Leica TP 1020 Tissue processing) and embedded in paraffin. Embedded samples were subsequently sectioned sagittal with a Rotary Microtome at 5µm. Morphometric indices were determined using computer-aided light microscope image analysis as described by Bird et al. (1994). The tissue sections on the slides were stained using Harris's haematoxylin and eosin stains. The morphometric variables analysed included: villus height (from the tip of the villus to the villus crypt junction), crypt depth (defined as the depth of the invagination between adjacent villi), villus width, ratio of villus length to crypt depth and villus surface area. Values are means from 12 different villi and only vertically oriented villi and crypts were measured (Uni et al., 1999).

For analysis under a scanning electron microscope, the intestinal contents were removed with saline solution buffered with 0.1 M phosphate, pH 7.4, and the tissue samples were fixed in 2% glutaraldehyde in phosphate buffer for 24 h at 4° C. Subsequently, the tissue was washed in phosphate buffer and post fixed for 2 h in 1% osmium tetroxide. Next, the material was washed again with the same buffered solution and dehydrated in

increasing ethanol series (30, 50, 70, 90, and 100% for 15 min each). Samples were dried in a critical point drier with liquid carbon dioxide. The material was then placed in an appropriate specimen tray, covered with a 30-nm layer of gold, and observed under a scanning electron microscope (EM3200 model).

Statistical analyse

Data were analyzed by the GLM procedure Statistical Analysis Software (SAS, 2004). Duncan's multiple range tests was used for comparison of means ($p < 0.05$).

Results

Intestinal relative weight and length

One day-old chicken

In day-old chickens the highest increase in intestinal relative weight ($p < 0.05$) was obtained by treatment iron+Alimet with late feeding. Small intestinal length was increased by treatment control 1 and 2 ($p < 0.05$). Small intestinal and ileum length were increased by early feeding as well as increased cecum length by treatment control 2 and late feeding period ($p < 0.05$) (Tables 1, 2, 3).

Table 1. Effect of treatments on intestinal relative weight and length in one day-old chickens.

Treatments		Length (cm)				
Effects	Relative Weight (%)	Small intestinal	Duodenum	jejunum	ileum	cecum
Control 1	6.5^{ab}	44.0^{a}	8.8^{a}	17.6^{a}	17.6^{a}	8.0^{a}
Control 2	6.8^{a}	43.9^{a}	8.8^{a}	17.8^{a}	17.3^{ab}	8.0^{a}
Sulfate Fe	6.1^{bc}	42.0^{b}	8.2^{ab}	17.1^{bc}	16.8^{abc}	8.0^{a}
Iron nanoparticles	6.3^{abc}	41.9^{b}	8.2^{ab}	17.6^{a}	16.2^{cd}	7.7^{bc}
Alimet	5.9^{cd}	41.4^{b}	8.1^{bc}	16.8^{cd}	16.5^{bc}	7.9^{ab}
Iron+Alimet	6.2^{abc}	41.2^{b}	7.9^{bc}	17.0^{c}	16.3^{cd}	7.5^{cd}
Iron nanoparticles+Alimet	5.8^{cd}	40.2^{c}	7.7^{c}	16.3^{d}	16.2^{cd}	7.3^{d}
Iron nanoparticles in diet	5.5^{d}	39.7^{c}	7.9^{bc}	16.4^{d}	15.4^{d}	7.5^{cd}
P value	0.0002	<0.0001	<0.0001	<0.0001	0.0001	<0.0001
SEM	0.19	0.34	0.15	0.19	0.29	0.10
Post hatch fasting time						
6 h	5.9^{b}	42.1^{a}	8.2	17.1	16.8^{a}	7.8
18 h	6.1^{a}	41.5^{b}	8.2	17.0	16.2^{b}	7.7
P value	<0.0001	0.03	0.8	0.47	0.005	0.13
SEM	0.09	0.17	0.08	0.09	0.14	0.05

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the means.

Table 2. Effect of treatments on intestinal relative weight and length in one day-old chickens.

Treatments	Relative Weight (%)	Cecum (cm)
Control 1+6 h	6.0^{cdef}	8.0^{abc}
Control 2+6 h	6.8^{abc}	7.9^{abcd}
Sulfate Fe+6 h	6.1^{cdef}	7.9^{abc}
Iron nanoparticles+6h	5.5^{defg}	7.9^{abc}
Alimet+6 h	5.3^{fg}	7.6^{cde}
(Iron+Alimet)+6h	5.2^{g}	7.7^{abcde}
(Iron nanoparticles+Alimet) +6h	5.4^{efg}	7.6^{cde}
Iron nanoparticles in diet+6h	5.4^{efg}	7.6^{cde}
Control 1+18h	7.0^{ab}	8.0^{abc}
Control 2+18h	6.7^{abc}	8.2^{a}
Sulfate Fe+18h	6.1^{cdef}	8.2^{a}
Iron nanoparticles+18h	6.4^{abcd}	7.7^{bcde}
Alimet+18 hours	7.3^{ab}	7.9^{abcd}
(Iron+Alimet)+18h	7.2^{a}	7.3^{ef}
(Iron nanoparticles+Alimet)+18h	6.4^{abcd}	76.9^{f}
Iron nanoparticles in diet+18h	5.5^{defg}	7.4^{de}
P-value interactions	0.005	0.01
P-value treatments	<0.0001	<0.0001
SEM	0.26	0.14

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the means.

Twenty one days of age

The intestine length was decreased by control 2 compared with all other treatments ($p < 0.05$). The highest caecum length was found between all treatments by in ovo injection of iron nanoparticles in early feeding at 21 days of age ($p < 0.05$) (Tables 4, 5, 6).

Forty two days of age

Increased jejunum and intestine length were found by treatment control 1 in late feeding ($p < 0.05$). Significantly increased the duodenum length was achieved by iron sulfate with early feeding at 42 days of age ($p < 0.05$) (Tables 7, 8, 9).

Duodenum, jejunum and ileum morphology

One day-old chicken

The results in day-old chickens have indicated that no significant differences were shown with the main and interactions effects of treatments in duodenum intestinal morphology ($p < 0.05$) (Tables 10, 11). In exception of early feeding could increase the ratio of duodenum villus length to crypt depth and duodenum villus surface area ($p < 0.05$). In addition, jejunum villus length and ratio villus length to crypt depth were increased by early feeding ($p < 0.05$). In contrast the lowest crypt depth was obtained by dietary iron sulfate nanoparticles ($p < 0.05$).

Table 3. Effect of treatments on intestinal relative weight and length in one day-old chickens.

Treatments	Length (cm)			
	Small intestinal	Duodenum	jejunum	ileum
Control 1+6 h	44.2	8.9	17.5	17.8
Control 2+6 h	44.3	8.8	17.7	17.8
Sulfate Fe+6 h	44.5	8.0	17.2	17.2
Iron nanoparticles+6h	42.2	8.1	17.0	17.0
Alimet+6 h	40.7	7.9	16.7	16.1
(Iron+Alimet)+6h	40.4	7.5	16.2	16.8
(Iron nanoparticles+Alimet) +6h	39.6	8.0	16.5	15.2
Iron nanoparticles in diet+6h	39.6	8.0	16.5	15.2
Control 1+18h	43.8	8.7	17.8	17.3
Control 2+18h	43.6	8.9	18.0	16.8
Sulfate Fe+18h	41.6	8.4	16.9	16.3
Iron nanoparticles+18h	41.0	7.8	17.6	15.7
Alimet+18 hours	40.7	8.1	16.7	15.9
(Iron+Alimet)+18h	41.8	8.0	17.4	16.5
(Iron nanoparticles+Alimet)+18h	39.9	7.9	16.4	16.7
Iron nanoparticles in diet+18h	39.8	7.8	16.3	15.7
P-value interactions	0.12	0.22	0.5	0.22
P-value treatments	<0.0001	<0.0001	<0.0001	0.0002
SEM	0.49	0.22	0.27	0.41

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the means.

Table 4. Effect of treatments on intestinal relative weight and length in 21 days of age chickens.

Treatments		Length (cm)				
Effects	Relative Weight (%)	Small intestinal	Duodenum	jejunum	ileum	cecum
Control 1	14.0	124.3[a]	12.0	66.7[ab]	45.5	16.7
Control 2	13.9	112.5[b]	12.0	57.4[c]	43.1	16.7
Sulfate Fe	13.2	126.6[a]	12.4	68.7[ab]	46.2	16.8
Iron nanoparticles	13.3	125.8[a]	12.3	64.9[b]	48.7	17.0
Alimet	13.6	133.4[a]	12.9	71.6[a]	48.9	16.3
Iron+Alimet	13.1	123.3[a]	12.3	65.7[b]	45.0	16.5
Iron nanoparticles+Alimet	13.9	124.3[a]	12.4	66.0[b]	46.5	16.5
Iron nanoparticles in diet	13.2	125.6[a]	12.1	67.9[ab]	45.5	16.7
P value	0.75	0.01	0.47	0.0002	0.57	0.33
SEM	0.46	3.38	0.28	1.78	2.08	0.21
Post hatch fasting time						
6 h	14.0[a]	123.9	12.6[a]	65.7	44.8	16.6
18 h	13.1[b]	125.9	12.0[b]	66.4	47.6	16.7
P value	0.007	0.25	0.002	0.60	0.06	0.87
SEM	0.2	1.69	0.14	0.89	1.04	0.1

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the means.

Twenty one days of age

The main effects of duodenum crypt depth were increased by in ovo injection of Alimet+iron sulfate. Ratio of duodenum villus length to crypt depth was increased by control 1 and Alimet+iron sulfate nanoparticles comparison to Alimet, iron sulfate nanoparticles and Alimet+iron sulfate ($p < 0.05$). Significantly increased jejunum villus length was observed by Alimet+iron sulfate nanoparticles compared with all two controls, sulfate iron and iron sulfate nanoparticles ($p < 0.05$). Also significantly increased ratio jejunum villus lengths to crypt depth compared to all treatments were found by dietary iron nonoparticles ($p < 0.05$). The highest jejunum villi width and surface area were recorded in dietary iron nanoparticles with late feeding ($p < 0.05$). *In ovo* injection of Alimet with late feeding resulted in decrease jejunum crypt depth ($p < 0.05$) (Tables 12, 13).

Table 5. Effect of treatments on intestinal relative weight and length in 21 days of age

Treatments	Cecum (cm)
Control 1+6 h	16.7[b]
Control 2+6 h	16.7[b]
Sulfate Fe+6 h	16.9[ab]
Iron nanoparticles+6h	17.8[a]
Alimet+6 h	15.7[c]
(Iron+Alimet)+6h	16.0[bc]
(Iron nanoparticles+Alimet) +6h	16.5[bc]
Iron nanoparticles in diet+6h	16.8[ab]
Control 1+18h	16.7[b]
Control 2+18h	16.6[bc]
Sulfate Fe+18h	16.7[b]
Iron nanoparticles+18h	16.3[bc]
Alimet+18 hours	16.9[ab]
(Iron+Alimet)+18h	16.9[ab]
(Iron nanoparticles+Alimet)+18h	16.5[bc]
Iron nanoparticles in diet+18h	16.6bc
P-value interactions	0.003
P-value treatments	0.02
SEM	0.3

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the means.

Table 6. Effect of treatments on intestinal relative weight and length in 21 days of age chickens.

Treatments	Relative Weight (%)	Length (cm)			
		Small intestinal	Duodenum	jejunum	ileum
Control 1+6 h	14.9	126.0	12.1	67.4	46.5
Control 2+6 h	15.7	116.2	12.6	59.9	43.7
Sulfate Fe+6 h	14.0	125.3	12.5	68.3	44.5
Iron nanoparticles+6h	13.1	121.1	12.1	63.8	45.2
Alimet+6 h	13.9	135.4	13.6	71.3	49.6
(Iron+Alimet)+6h	13.0	116.0	12.7	61.8	41.6
(Iron nanoparticles+Alimet) +6h	14.1	122.4	13.1	65.5	43.8
Iron nanoparticles in diet+6h	13.3	122.5	12.3	66.8	43.5
Control 1+18h	13.1	122.7	11.9	66.1	44.6
Control 2+18h	12.1	108.9	11.5	54.9	42.6
Sulfate Fe+18h	12.5	127.9	12.3	67.7	47.9
Iron nanoparticles+18h	13.4	130.1	12.4	66.0	52.1
Alimet+18 hours	13.3	131.2	12.2	71.0	48.3
(Iron+Alimet)+18h	13.3	130.0	11.9	69.6	48.5
(Iron nanoparticles+Alimet)+18h	17.7	127.2	17.1	66.4	49.1
Iron nanoparticles in diet+18h	13.1	128.7	11.9	69.1	47.6
P-value interactions	0.06	0.35	0.28	0.37	0.59
P-value treatments	0.06	0.04	0.06	0.002	0.47
SEM	0.66	4.78	0.4	2.51	2.94

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the means.

Forty two days of age

No significant differences in duodenum villus length were indicated by all treatments ($p > 0.05$). In the main effects, significantly increased duodenum villus width was shown by treatment containing Alimet+iron sulfate compared to other treatments in exception of dietary iron nanoparticles and Alimet+iron sulfate nanoparticles ($p < 0.05$). Recorded lowest duodenum crypt depth and highest ratio duodenum villus length to crypt depth were shown by dietary iron sulfate and nanoparticles ($p < 0.05$). Significantly increased duodenum villus width by combination of Alimet+iron sulfate and delayed feeding. Combination of Alimet+iron sulfate nanoparticles and delayed feeding was the best treatment in regard to jejunum villus height. *In ovo* injection of Alimet with early feeding, Alimet+iron sulfate nanoparticles and delayed feeding in 42 days of age resulted the lowest jejunum crypt depth was recorded with sulfate iron and delayed feeding ($p < 0.05$) The highest jejunum villi surface area was found by dietary iron sulfate nanoparticles and late feeding ($p < 0.05$). Figures 1 shows scanning electron micrographs of the jejunum villus in 42 days of age (Tables 14, 15).

Table 7. Effect of treatments on intestinal relative weight and length in 42 days of age chickens.

Treatments	Relative Weight (%)	Length (cm)				
Effects		Small intestinal	Duodenum	jejunum	ileum	cecum
Control 1	24.0^{a}	155.3^{a}	13.9^{abc}	67.1	74.3^{a}	18.1^{a}
Control 2	23.8^{a}	150.2^{b}	14.4^{ab}	64.8	71.0^{ab}	18.1^{a}
Sulfate Fe	22.4^{a}	147.2^{bc}	14.5^{a}	64.1	68.6^{b}	17.7^{ab}
Iron nanoparticles	23.2^{ab}	147.3^{bc}	14.1^{ab}	63.7	69.5^{b}	17.6^{ab}
Alimet	22.4^{ab}	146.1^{bc}	14.2^{ab}	63.5	68.3^{b}	17.8^{ab}
Iron+Alimet	21.0^{b}	143.8^{c}	13.7^{bc}	62.6	67.4^{b}	17.3^{b}
Iron nanoparticles+Alimet	21.1^{b}	144.5^{c}	13.3^{c}	63.7	67.5^{b}	17.7^{ab}
Iron nanoparticles in diet	21.4^{b}	143.3^{c}	13.9^{abc}	62.6	66.7^{b}	17.3^{b}
P value	0.017	0.0002	0.01	0.2	0.013	0.04
SEM	1.43	3.53	0.44	2.36	2. 9	0.39
Post hatch fasting time						
6 h	22.7	148.4	14.3^{a}	56.1^{a}	69.0	17.8^{a}
18 h	22.1	145.9	13.7^{b}	62.9^{b}	69.3	17.6^{b}
P value	0.22	0.06	0.0005	0.01	0.76	0.05
SEM	0.79	1.77	0.22	1.2	1.45	0.2

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the means.

Discussion

Results have shown that, Alimet increased intestine length in 21 and 42 days of age through improving protein synthesis, which in turn, increased protein in the intestinal tissues, metabolism and growth of epithelial cells. As organic acid, Alimet can also improve intestinal tissue, since its supplementation to diets could leads to decreases pathogens (Gunal, Yayli, Kaya, Karahan, & Sulak, 2006). Changes in the empty weight of visceral organs are generally due to variation in rate of cell proliferation, cell size or protein synthesis (Iji et al., 2001). However, early feeding has influenced in this trend, because apart from Alimet, feeding in 6 hours post-hatch increased intestine length.

Small intestine serves as the primary site of nutrient absorption (Zhu, Zhong, Pandya, & Joerger, 2002). The innermost layer in small intestine is mucosa which includes absorption surface of villi. Enterocytes are the most important cells in villi which is responsible for absorption reactions. Their number is high in the villous head. Therefore, their higher number is associated with higher correlation coefficients of absorption. Bedford (1996) has reported that small intestine displayed changes in its absorptive surface in response to changes in dietary composition. Furthermore, crypt depth and villous height are effective factors determining the mucosal length and thus the nutrient absorption (Sharma, Schumacher, Ronaasen, & Coates, 1995).

Table 8. Effect of treatments on intestinal relative weight and length in 42 days of age chickens.

Treatments	Length (cm)		
	Small intestinal	jejunum	Ileum
Control 1+6 h	151.6^{abcd}	66.0^{abc}	71.1^{abc}
Control 2+6 h	150.1^{bcd}	65.6^{abcd}	70.2^{bc}
Sulfate Fe+6 h	155.8^{ab}	68.2^{a}	72.8^{bc}

Iron nanoparticles+6h	143.9^{cde}	62.9^{abcde}	66.8^{bc}
Alimet+6 h	151.9^{abc}	67.2^{ab}	69.8^{bc}
(Iron+Alimet)+6h	144.2^{cde}	67.8^{abcde}	66.8^{bc}
(Iron nanoparticles+Alimet) +6h	149.6^{bcd}	65.5^{abcd}	70.5^{bc}
Iron nanoparticles in diet+6h	140.4^{e}	61.8^{bcde}	64.2^{c}
Control 1+18h	159.0^{a}	64.1^{abcde}	77.5^{a}
Control 2+18h	150.3^{bcd}	60.0^{de}	71.9^{ab}
Sulfate Fe+18h	138.7^{e}	64.4^{abcde}	64.4^{c}
Iron nanoparticles+18h	150.7^{bcd}	59.9^{e}	72.3^{ab}
Alimet+18 hours	140.3^{e}	61.5^{cde}	66.9^{bc}
(Iron+Alimet)+18h	143.4^{de}	61.9^{bcde}	68.1^{bc}
(Iron nanoparticles+Alimet)+18h	139.4^{e}	61.9^{bcde}	64.5^{c}
Iron nanoparticles in diet+18h	146.1^{cde}	63.5^{abcde}	69.2^{bc}
P-value interactions	<0.0001	0.01	0.003
P-value treatments	<0.0001	0.008	0.002
SEM	1.2	0.83	1.02

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the means.

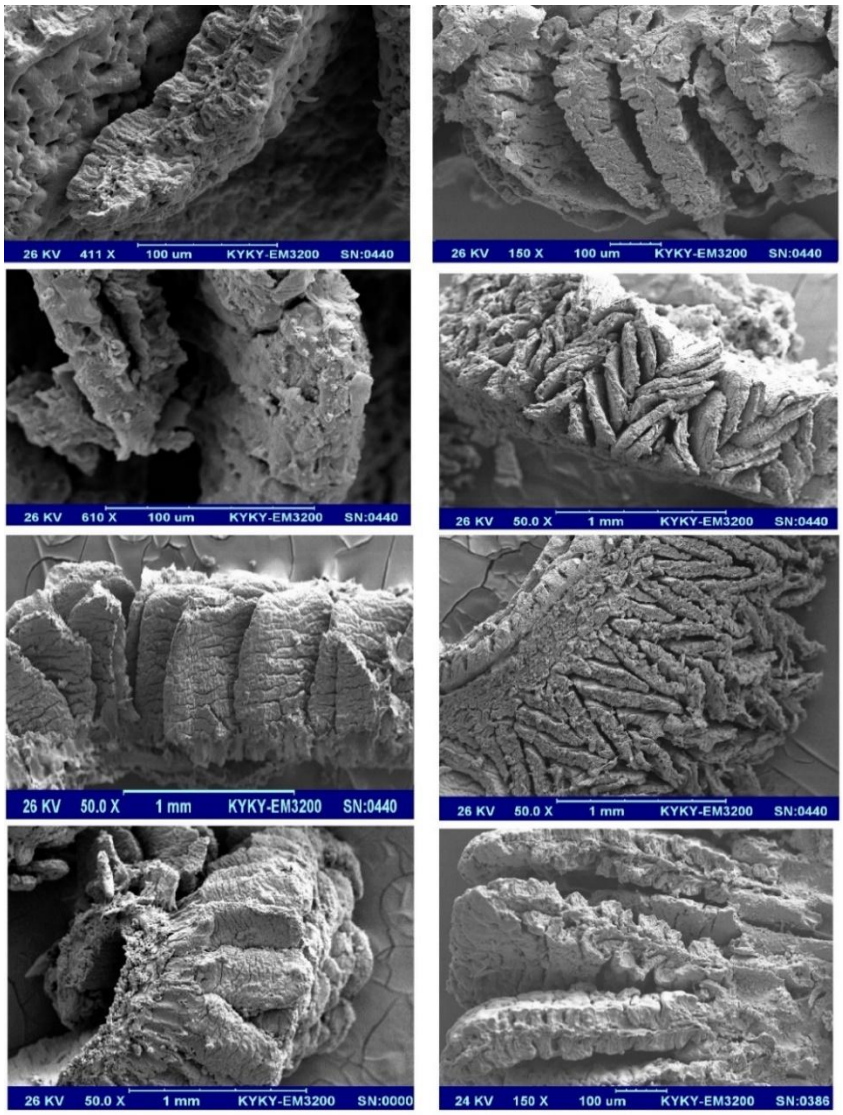

Figure 1. Scanning electron micrograph of jejunum villus.

Table 9. Effect of treatments on intestinal relative weight and length in 42 days of age chickens.

Treatments	Relative Weight (%)	Length (cm)	
		Duodenum	cecum
Control 1+6 h	24.0	14.5	18.3
Control 2+6 h	23.5	14.4	18.1

Sulfate Fe+6 h	22.9	14.7	17.7
Iron nanoparticles+6h	23.8	14.3	17.9
Alimet+6 h	21.6	14.9	17.6
(Iron+Alimet)+6h	22.2	13.6	17.7
(Iron nanoparticles+Alimet) +6h	21.8	13.6	18.5
Iron nanoparticles in diet+6h	21.8	14.4	17.3
Control 1+18h	24.0	13.3	17.9
Control 2+18h	24.1	14.4	18.1
Sulfate Fe+18h	21.8	14.3	17.7
Iron nanoparticles+18h	22.6	14.0	17.3
Alimet+18 hours	23.1	13.6	18.1
(Iron+Alimet)+18h	19.8	13.8	16.9
(Iron nanoparticles+Alimet)+18h	20.4	13.1	17.3
Iron nanoparticles in diet+18h	20.9	13.4	17.3
P-value interactions	0.63	0.16	0.24
P-value treatments	0.072	0.002	0.04
SEM	0.51	0.15	0.14

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the means.

Table 10. Effect of treatments on intestinal morphology at one day old chickens.

Effects	Duodenum					Jejunum				
	Villus length	Villus width	Crypt depth	Ratio	Villus Surface Area	Villus length	Villus width	Crypt depth	Ratio	Villus Surface Area
	μm				(mm^2)	μm				(mm^2)
Control 1	335.0	72.1	64.0	5.44	4.58	231.2	67.6	61.8^{ab}	3.79	16.0
Control 2	328.0	79.3	64.1	5.25	5.11	216.5	64.1	50.3^{bc}	4.37	13.9
Sulfate iron	350.4	79.1	67.4	5.37	5.43	235.4	66.7	51.2^{bc}	4.58	15.6
Iron nanoparticles	392.0	81.0	67.1	5.86	5.36	276.2	69.8	57.7^{abc}	4.79	19.8
Alimet	386.2	76.8	58.3	6.68	4.55	290.4	63.5	50.4^{bc}	5.94	18.4
Iron+Alimet	338.8	83.4	66.3	6.14	5.46	284.0	70.6	63.8^{a}	4.54	20.9
Iron nanoparticles+Alimet	391.5	71.3	62.0	6.85	4.46	272.9	69.9	57.2^{abc}	5.21	19.4
Iron nanoparticles in diet	401.0	71.2	64.7	6.24	4.73	287.7	71.5	47.9^{c}	6.13	20.4
P value	0.34	0.24	0.93	0.13	0.78	0.15	0.89	0.02	0.07	0.38
SEM	26.8	4.1	5.1	0.46	0.53	22.9	4.7	3.62	0.55	2.45
Post hatch fasting time										
6 h	369.6	79.1	67.7	6.39^{a}	5.38^{a}	388.2^{a}	67.3	54.3	5.36^{a}	16.2^{b}
18 h	373.7	74.2	60.7	5.56^{b}	4.58^{b}	235.3^{b}	68.6	66.0	4.48^{b}	19.9^{a}
P value	0.83	0.1	0.06	0.02	0.04	0.002	0.7	0.49	0.03	0.04
SEM	13.4	2.1	2.6	0.23	0.27	11.45	2.34	1.81	0.27	1.22

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the means.

Table 11. Effect of treatments on intestinal morphology at one day old chickens.

Treatments	Duodenum					Jejunum				
	Villus length	Villus width	Crypt depth	Ratio	Villus Surface Area	Villus length	Villus width	Crypt depth	Ratio	Villus Surface Area
	μm				(mm^2)	μm				(mm^2)
Control 1+6 h	327.7	78.6	65.6	5.39	5.15	190.5	62.1	58.3	3.46	11.6
Control 2+6 h	330.7	82.5	63.6	5.22	5.30	201.8	64.5	53.5	3.86	13.0
Sulfate Fe+6 h	346.5	86.3	70.0	4.97	6.05	203.7	67.0	49.2	4.15	13.8
Iron nanoparticles+6h	411.1	84.3	68.3	6.00	5.70	243.1	57.3	57.1	4.26	13.6
Alimet+6 h	385.1	77.8	60.4	6.33	4.80	244.3	69.2	54.1	4.55	17.4
(Iron+Alimet)+6h	368.1	83.3	74.9	4.92	6.22	254.7	68.7	57.7	4.47	18.8
(Iron nanoparticles+Alimet) +6h	370.9	72.6	72.1	5.55	5.33	276.7	69.5	60.0	5.48	19.7
Iron nanoparticles in diet+6h	411.7	67.6	66.9	6.14	4.53	263.1	80.6	47.2	5.80	21.7
Control 1+18h	337.2	63.6	62.4	5.50	4.00	271.8	73.2	65.3	4.33	20.4
Control 2+18h	326.1	63.9	64.6	5.28	4.93	231.3	63.8	47.2	4.88	14.8
Sulfate Fe+18h	354.3	76.1	64.8	5.77	4.80	267.1	66.3	55.1	5.02	17.5
Iron nanoparticles+18h	373.0	71.8	65.8	5.72	5.03	309.4	84.2	58.3	5.32	26.0
Alimet+18 hours	387.4	75.9	56.2	7.03	4.30	336.5	57.9	46.7	7.33	19.5
(Iron+Alimet)+18h	409.5	83.5	67.8	7.35	4.70	313.5	72.5	69.9	4.61	23.1
(Iron nanoparticles+Alimet)+18h	412.1	70.0	51.8	8.14	3.95	269.2	70.4	57.4	4.93	19.1

Iron nanoparticles in diet+18h	390.4	74.8	62.8	6.33	4.92	307.3	62.1	48.6	6.46	19.2
P-value interactions	0.97	0.6	0.8	0.23	0.92	0.84	0.08	0.57	0.63	0.45
P-value treatments	0.8	0.31	0.81	0.05	0.74	0.08	0.36	0. 1	0.1	0.25
SEM	38.0	5.88	7.21	0.66	0.76	32.4	6.62	5.13	0.77	3.47

[1]Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the means

As indicated by our findings *in-ovo* injection of Alimet+iron sulfate nanoparticles, sulfate iron and Alimet with early feeding and dietary iron nanoparticles had desirable performance in almost all intestinal mucosal morphology parameters of duodenum and jejunum (villous width, villous surface area, and villous height to crypt depth ratio). Changes in villous properties are associated with intestinal performance and broiler growth rate. Treatments which resulted in higher villous width also scored high in body weight gain and feed conversion ratio (FCR). Crypt is considered as the villous factory and larger crypt is associated with more rapid tissue turnover and higher demand for more tissue (Zhu et al., 2002). Thus, higher crypt depth with injected materials has higher potentials to cell proliferation (Iji et al., 2001). Rapid increases in villous surface area and height take place in broilers 1-2 days post hatch (Uni et al., 1999). Two days post hatch (Uni et al., 1998) and in 12 days of age (Geyra, Uni, & Sklan, 2001). Different structures have been reported in duodenum, jejunum, and ileum, particularly the development rate of jejunum was far more rapid than others (Iji et al., 2001). During hatching, all three parts of the intestine show equal surface area and show similar development until 3 days post hatch. Jejunum and ileum, villi surface displays an increase beginning in 4 days of age. This followed by an increase in jejunum surface area which is more pronounced than others. While duodenum and ileum showed slower rates of increase in surface area.

Table 12. Effect of treatments on intestinal morphology in 21 days of age chickens.

Effects	Duodenum					Jejunum				
	Villus length	Villus width	Crypt depth	Ratio	Villus Surface Area	Villus length	Villus width	Crypt depth	Ratio	Villus Surface Area
	μm				(mm^2)	μm				(mm^2)
Control 1	917.0	109.9^{b}	135.5^{c}	7.07^{a}	101.3	569.2^{c}	128.3^{ab}	116.5^{cd}	4.94^{bc}	73.3^{c}
Control 2	911.7	131.1^{a}	155.6^{abc}	5.97^{ab}	119.9	574.1^{c}	129.4^{ab}	108.8^{d}	5.32^{abc}	74.2^{c}
Sulfate Fe	913.3	131.4^{a}	155.2^{abc}	6.09^{ab}	120.3	569.4^{c}	137.9^{ab}	118.3^{bcd}	4.84^{bc}	78.8^{c}
Iron nanoparticles	931.5	138.5^{a}	173.2^{ab}	5.45^{bc}	129.6	600.3^{c}	134.5^{ab}	115.3^{cd}	5.24^{abc}	80.9^{c}
Alimet	900.6	135.9^{a}	164.2^{ab}	5.53^{bc}	123.4	612.9^{bc}	144.9^{a}	115.9^{cd}	5.32^{abc}	89.3^{bc}
Iron+Alimet	843.6	140.8^{a}	179.0^{a}	4.73^{c}	117.5	709.8^{a}	140.4^{a}	128.9^{b}	5.51^{ab}	100.9^{ab}
Iron nanoparticles+Alimet	981.6	138.7^{a}	147.5^{a}	6.84^{a}	136.8	704.0^{ab}	123.0^{b}	154.1^{a}	4.72^{c}	86.9^{bc}
Iron nanoparticles in diet	991.3	129.9^{a}	156.2^{abc}	6.40^{ab}	129.2	784.7^{a}	143.1^{b}	136.0^{b}	5.76^{a}	113.2^{a}
P value	0.23	0.02	0.02	0.003	0.14	<0.0001	0.045	0.0001	0.03	0.004
SEM	39.7	5.9	8.27	0.4	8.2	64.3	5.1	6.3	0.22	6.4
Post hatch fasting time										
6 h	907.8	129.3	150.0	6.25	117.7	630.4	134.3	122.8	5.17	84.6
18 h	940.0	134.7	156.7	5.77	126.8	650.7	136.0	125.4	5.24	89.8
P value	0.25	0.2	0.006	0.1	0.13	0.38	0.62	0.56	0.63	0.26
SEM	19.8	2.96	4.13	0.2	4.11	32.2	2.56	3.16	0.11	6.4

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the means.

Table 13. Effect of treatments on intestinal morphology in 21 days of age chickens.

Treatments	Duodenum					Jejunum				
	Villus length	Villus width	Crypt depth	Ratio	Villus Surface Area	Villus length	Villus width	Crypt depth	Ratio	Villus Surface Area
	μm				(mm^2)	μm				(mm^2)
Control 1+6 h	905.5	110.8	127.8	7.38	101.6	592.6	125.4^{defg}	123.5^{bcde}	4.81	74.5^{cd}
Control 2+6 h	948.7	140.1	170.2	5.65	133.4	571.3	145.2^{abcd}	105.0^{de}	5.48	83.4^{cd}
Sulfate Fe+6 h	915.3	120.4	129.6	7.13	110.3	585.5	155.4^{ab}	123.3^{bcde}	4.78	91.0^{cd}
Iron nanoparticles+6h	923.9	126.6	160.3	5.55	114.8	585.2	139.6^{abcde}	104.4^{de}	5.61	82.3^{cd}
Alimet+6 h	867.7	130.8	156.0	5.55	114.8	660.8	144.5^{abcd}	132.1^{bcd}	5.00	97.8^{bc}
(Iron+Alimet)+6h	830.4	138.9	170.8	4.89	114.1	619.9	129.9^{cdefg}	118.3^{bcde}	5.24	80.7^{cd}
(Iron nanoparticles+Alimet) +6h	943.6	145.3	137.2	7.21	137.8	682.2	109.7^{g}	141.9^{a}	4.87	74.9^{cd}
Iron nanoparticles in diet+6h	927.8	121.7	140.1	6.33	113.1	745.9	124.6^{defg}	134.1^{bcd}	5.54	80.3^{bcdef}
Control 1+18h	929.9	109.1	143.2	6.76	101.0	545.7	131.3^{cdefg}	108.8^{bcd}	5.06	72.0^{cd}
Control 2+18h	847.6	122.2	141.1	6.30	106.5	576.8	113.3^{fg}	111.9^{cde}	5.12	65.0^{d}

Sulfate Fe+18h	911.3	142.4	180.9	5.05	130.4	553.4	120.3^{efg}	113.3^{bcde}	4.09	66.6^{d}
Iron nanoparticles+18h	939.1	150.4	186.2	5.07	142.3	615.4	129.3^{cdefg}	126.2^{bcde}	4.87	79.5^{cd}
Alimet+18 hours	933.5	141.0	172.4	5.52	132.0	565.1	144.8^{abcd}	99.7^{e}	5.64	80.8^{cd}
(Iron+Alimet)+18h	856.8	142.8	187.3	4.57	121.0	799.7	150.8^{abc}	139.4^{b}	5.79	121.2^{ab}
(Iron nanoparticles+Alimet)+18h	1019.7	132.0	157.9	6.47	135.9	725.9	136.3^{bcdef}	166.3^{a}	4.56	99.0^{bc}
Iron nanoparticles in diet+18h	1054.9	138.1	164.3	6.44	145.2	823.5	161.6^{a}	138.0^{bc}	5.98	134.0^{a}
P-value interactions	0.47	0.04	0.005	0.01	0.24	0.12	<0.0001	0.024	0.31	0.0008
P-value treatments	0.87	0.12	0.11	0.4	0.26	<0.0001	0.0001	0.0006	0.013	<0.0001
SEM	56.1	8.38	11.7	0.57	11.6	22.7	7.25	8.94	0.31	9.0

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the mean

Villi surface area grows more uniformly in duodenum, while their growth in jejunum and ileum is slow after 4 days of age (Geyra et al., 2001). Study of small intestinal morphology at 0-21 days of age indicates that villous height multiplied in jejunum and duodenum, but developed slower in ileum. Villous volume and crypt depth increased but with little changes in enterocyte accumulation with the aging (Uni et al., 1998; Uni et al., 1999). Overall, there have been drastic changes in intestinal morphology with aging, which was especially well pronounced after 7 days of age (Iji et al., 2001). Bohórquez, Bohórquez, and Ferket (2011) have studied small intestinal epithelium using electron and light microscopy and reported that embryonic or early post hatch feeding chickens could bring about development in intestinal morphology and higher performance. Silva et al. (2007) have examined the surface area of the tip of the enterocytes in small intestine mucosa of Cobb broilers affected 30 per cent limitation in feeding and glutamine deprivation and found that enterocyte surface area and microvilli volume and width were improved in jejunum due to glutamine effect.

Table 14. Effect of treatments on intestinal morphology in 42 days of age chickens.

Treatments	Duodenum					Jejunum				
	Villus length	Villus width	Crypt depth	Ratio	Villus Surface Area	Villus length	Villus width	Crypt depth	Ratio	Villus Surface Area
	μm				(mm^2)	μm				(mm^2)
Control 1	946.9	130.6d	179.5ab	5.32bc	123.2d	780.9d	215.6d	179.5ab	5.32	123.2d
Control 2	952.2	142.1cd	192.8a	4.94c	135.5cd	755.6d	228.4cd	192.8a	4.94	135.5cd
Sulfate Fe	1001.1	151.1bc	197.0a	5.10c	150.7bcd	802.1cd	234.3cd	197.0a	5.10	150.7bcd
Iron nanoparticles	1014.3	154.8bc	195.1a	5.20c	157.4abc	776.1d	239.0bcd	195.1a	5.20	157.4abc
Alimet	1007.7	157.7d	178.7ab	5.75abc	160.8abc	870.4bc	262.9ab	178.8ab	5.75	160.8abc
Iron+Alimet	1005.5	178.3d	194.1a	5.21c	181.2abd	862.5bc	250.2abc	194.1a	5.21	181.2ab
Iron nanoparticles+Alimet	1089.6	170.4d	180.6ab	6.07ab	185.4a	939.8ab	225.4cd	180.6ab	6.07	185.4a
Iron nanoparticles in diet	1116.3	160.4abc	174.0b	6.46a	180.3ab	967.3a	274.3a	174.0b	6.46	180.3ab
P value	0.061	<0.0001	0.03	0.002	0.0007	<0.0001	0.0001	0.19	0.48	<0.0001
SEM	81.9	12.5	11.7	0.54	21.36	53.5	16.9	12.9	0.58	23.9
Post hatch fasting time										
6 h	1008.9	137.1b	192.8a	5.28b	138.7b	820.4b	234.5	192.8a	5.28b	138.7
18 h	1024.4	174.2a	180.9b	5.73a	179.9a	868.2a	239.0	180.9b	5.73a	179.9
P value	0.6	<0.0001	0.0009	0.02	<0.0001	0.015	0.45	0.003	0.0003	0.31
SEM	41.0	6.27	5.85	0.27	10.68	26.7	8.46	6.45	0.29	11.97

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the mean

Table 15. Effect of treatments on intestinal morphology in 42 days of age chickens.

Treatments	Duodenum					Jejunum				
	Villus length	Villus width	Crypt depth	Ratio	Villus Surface Area	Villus length	Villus width	Crypt depth	Ratio	Villus Surface Area
	μm				(mm^2)	μm				(mm^2)
Control 1+6 h	946.0	130.2d	188.0	5.13	125.5	739.6cde	226.2bcd	167.8abc	4.86	181.5def
Control 2+6 h	954.7	125.0d	200.2	4.77	119.3	764.4ef	237.7bcd	155.7abcd	5.05	182.8f
Sulfate Fe+6 h	1040.9	122.9d	204.6	5.07	128.6	800.7def	247.9bc	183.3a	4.37	198.9def
Iron nanoparticles+6h	1014.5	121.7d	204.0	4.96	123.8	746.7f	226.2bcd	164.4abc	4.58	169.2def
Alimet+6 h	981.9	130.7d	180.0	5.52	129.5	895.0abcd	287.0a	175.2abc	5.13	157.1def
(Iron+Alimet)+6h	978.3	161.4bc	196.0	5.01	170.0	747.4f	239.9bcd	153.0abcd	4.92	181.3abcdef
(Iron nanoparticles+Alimet) +6h	1059.6	162.3bc	184.7	5.74	185.4	877.2bcde	223.7bcd	156.4abcd	5.71	195.4abcde
Iron nanoparticles in diet+6h	1077.8	142.7cd	179.3	6.03	180.3	938.4abc	259.6abc	171.6abc	5.47	242.7bcdef

Control 1+18h	929.9	131.1d	171.1	5.51	123.2	768.2ef	205.1d	131.3de	5.96	181.3ef
Control 2+18h	949.6	159.2bc	185.4	5.12	135.5	746.8f	219.2cd	151.8bcd	5.03	195.4cdef
Sulfate Fe+18h	961.3	179.2ab	189.4	5.13	150.7	803.4def	220.7bcd	120.8e	6.73	242.7abcd
Iron nanoparticles+18h	1014.1	187.9ab	186.2	5.45	157.4	805.4def	251.8abc	153.7abcd	5.24	157.4abc
Alimet+18 hours	1033.5	184.7ab	177.4	5.98	160.8	845.8cdef	238.7bcd	144.7cde	5.95	164.1abc
(Iron+Alimet)+18h	1031.8	195.3a	192.3	5.41	181.2	977.7ab	260.4ab	158.7abcd	6.19	177.6ab
(Iron nanoparticles+Alimet)+18h	1119.7	178.6ab	176.5	6.39	200.7	1002.4a	227.1bcd	178.2ab	5.65	203.1abc
Iron nanoparticles in diet+18h	1154.9	178.1ab	168.7	6.90	206.9	996.2ab	289.1a	174.4abc	5.73	202.0a
P-value interactions	0.87	0.01	0.97	0.99	0.37	0.01	0.015	0.0007	0.09	0.004
P-value treatments	0.3	<0.0001	0.074	0.017	<0.0001	<0.0001	0.0001	0.0006	0.013	<0.0001
SEM	29.0	4.43	4.14	0.19	7.55	18.9	5.98	4.57	0.2	8.46

Control 1 (without injection), control 2 injected with 0.3 mL of NaCl 0/9%; a, b. Means with common superscripts in same column are not significantly different ($p < 0.05$). SEM: standard error of the mean

Conclusion

In ovo feeding can provide the embryo injection necessary to its optimum post-hatch growth. Despite the dependence of embryo growth on nutrients especially iron, data on the mineral content of the egg during incubation is limited. The injection of methionine was an important amino acid effective on performance. Iron sulfate nanoparticles alone and with methionine (Alimet) used to increase the growth during embryonic and post-hatch periods. It was concluded that *in-ovo* injection of Alimet+iron sulfate nanoparticles, sulfate iron and Alimet with early feeding and dietary iron sulfate nanoparticles had desirable performance in almost all intestinal mucosal morphology parameters of duodenum and jejunum (villous width, villous surface area, and villous height to crypt depth ratio).

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