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A Pilot Study of Tissue Factor-Tissue Factor Pathway Inhibitor Axis and Other Selected Coagulation Parameters in Broiler Chickens Administered *in Ovo* with Selected Prebiotics*

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The tissue factor (TF) – tissue factor pathway inhibitor (TFPI) axis plays a major role in hemostasis. Disorders of the coagulation system are commonly diagnosed with the help of screening tests such as prothrombin time (PT), activated partial thromboplastin time (aPTT), and plasma fibrinogen concentration (PFC). However, the effect of prebiotics on the hemostasis system has not been characterized in poultry yet. This study was designed to determine the effect of *in ovo* administration of prebiotics on blood coagulation parameters of broiler chickens depending on their age. The study was conducted with 180 broiler chick embryos, the air cells of which were injected on day 12 of incubation with prebiotics (experimental groups: Bi^{2}tos, DiNovo® and RFO) or physiological saline solution (control group). At 1, 21 and 42 days of rearing, blood was sampled from 15 broiler chickens from each group. An enzyme immunoassay was performed to determine plasma TF and TFPI levels, and PT, aPTT and PFC were determined in the chicken blood. We demonstrated that: 1) total TF levels increased with age in the experimental groups, 2) prebiotics had no significant effect on TF levels between the groups at a particular age, 3) total TFPI levels differed between both the type of *in ovo* injected substance and the broiler chicken age, 4) in the control group, PT and aPTT were found to increase with age whilst fibrinogen concentration decreased. The main conclusion from this pilot study is that total TF and TFPI levels change with age, however no clear patterns regarding TFPI were detected yet. The levels of PT, aPTT and PFC varied with the prebiotics administered *in ovo* as well as with the age of broiler chickens.

Key words: Tissue factor, tissue factor pathway inhibitor, hemostasis, *in ovo*, prebiotic, broiler chicken.

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Hemostasis is a complex process which protects the organism from bleeding in the event of endothelial cell injury (GENTRY 2004; KIM *et al.* 2009). In general, blood coagulation occurs via the tissue factor (TF) dependent pathway (extrinsic pathway) or the contact activation pathway (intrinsic pathway). However, the presence of the intrinsic pathway in birds is still a matter of debate (DOERR *et al.* 1974; DOERR & HAMILTON 1981; LEWIS 1996; THOMSON *et al.* 2002). Accordingly, the extrinsic pathway is considered the primary coagulation process in birds. It begins with release of TF (thromboplastin, Factor III) from injured vascular endothelium and activated platelets in response to tissue damage or inflammatory stimuli (DOERR & HAMILTON 1981; THOMSON *et al.* 2002). Released TF interacts with circulating factor VII (FVII) to form the FVIIa-TF enzymatic complex, which transforms factor X into the active form, FXa (GENTRY 2004; OWENS III & MACKMAN 2010; RAO & PENDURTHI 2012). Activity of the FVIIa-TF complex is strictly regulated by tissue factor pathway inhibitor (TFPI), which demonstrates antithrombotic properties (DOOLITTLE *et al.* 2008; PONCZEK *et al.* 2008; KASTHURI *et al.* 2010; HOLROYD *et al.* 2012). Clinical studies in humans have shown that elevated TFPI levels in atherothrombotic disease are associated witch endothelial injury and platelet activation (WINCKERS *et al.* 2013). Despite the crucial role of TF and TFPI in the coagulation process, their exact functions and blood levels in poultry have never been reported. This issue may be exceptionally important in view of the fact that hemostatic disturbances are a growing and significant problem in poultry production (MURAMOTO *et al.* 2006; YEH *et al.* 2008; YEH *et al.* 2009; NAZIFI *et al.* 2010; PLISZCZAK-KROL *et al.* 2012; ZERYEHUN *et al.* 2012; WIDEMAN *et al.* 2013). Several cases of disseminated intravascular coagulation in birds caused by pathogens and tranexamic acid have been reported recently (SHIBATANI *et al.* 1997; MURAMOTO *et al.* 2006; MORIS 2015; SUBA *et al.* 2015).

In 2006, the European Union imposed a ban on antibiotic growth promoters in poultry nutrition (CASTANON 2007) and bioactive substances in more recent times, especially prebiotics, are considered as alternatives (PATTERSON & BURKHOLDER 2003; HUYGHEBAERT *et al.* 2011). Prebiotics are non-digestible food ingredients that can stimulate growth and activity of beneficial microorganisms and thus can eliminate pathogenic microbiota (GIBSON & ROBERFROID 1995). Prebiotics are most effective in poultry when administered as early in life as possible (PATTERSON & BURKHOLDER 2003; ALLOUI *et al.* 2013), for instance a single *in ovo* injection of the RFO prebiotic (raffinose family oligosaccharides) in broiler chickens increases the number of *bifidobacteria* in the caecum at hatching and ensures long-term persistence of these bacteria during rearing (VILLALUENGA *et al.* 2004; PILARSKI *et al.* 2005). The results of recent studies have shown that *in ovo* injection of prebiotics and synbiotics in broiler chickens may improve production traits (e.g. body weight and feed conversion rate) (BEDNARCZYK *et al.* 2011), meat quality (MAIORANO *et al.* 2012) and influence development of the immune system (SŁAWIŃSKA *et al.* 2014a; SŁAWIŃSKA *et al.* 2014b).

Evaluation of prebiotics for *in ovo* injection is comprised of several steps. Firstly, the oligosaccharide must exhibit complete solubility in physiological salt. Only fully dissolved prebiotics can be precisely injected *in ovo* and pass the egg membranes into the bloodstream and gut of the embryo. Secondly, the prebiotic has to be delivered *in ovo* at a specific dose rate that ensures high hatchability of the eggs and microflora development at hatching. Thirdly, the prebiotic should confer beneficial properties to the host in performance and fitness traits. There are many biologically active oligosaccharides available that could be validated for *in ovo* injection. The aim of our earlier study (BEDNARCZYK *et al.* 2016; SŁAWIŃSKA *et al.* 2016) was to evaluate the applicability of different prebiotics to *in ovo* injection. Commercially and in-house manufactured prebiotics were preliminarily evaluated based on solubility, filterability, sterility parameters and their biological properties. Three prebiotics were selected: DiNovo® (BioAtlantis Ltd., Tralee, Co., Kerry, Ireland), Bi^{2}tos, (Clasado Ltd, Sliema, Malta), and in-house manufactured (GULEWICZ *et al.* 2000) raffinose family oligosaccharides (RFO). These prebiotics were sourced from different materials, that is, marine algae (DiNovo®), cow milk (Bi^{2}tos) or plant seeds (RFO), and differed in biological composition and bioactive properties.

Disorders of the coagulation system are commonly diagnosed with the help of screening tests such as prothrombin time (PT), activated partial thromboplastin time (aPTT), and plasma fibrinogen concentration (PFC). Both prolonged and shortened blood clotting times as well as changes in fibrinogen concentration may be indicative of disorders of the hemostatic system. Effective diagnosis of hemostatic disorders in poultry remains an ongoing problem. The physiological mechanisms of hemostasis in poultry, as well as the pathological alterations that have occurred over the last years as a result of changing production conditions, are still poorly understood. The evaluation of hemostatic disorders in birds is hampered by inadequate diagnostic methods currently in use in veterinary medicine. Other important problems are the optimization of coagulometric methods

and the availability of species-specific reagents (DOERR *et al.* 1975; PLISZCZAK-KROL *et al.* 2012).

The effect of prebiotic administration on hemostasis in animals or humans is poorly understood. Therefore, the aim of the present study was to investigate the relationship between *in ovo* administration of prebiotics on selected blood coagulation parameters in growing chickens.

Material and Methods

Birds were raised as approved by the Polish Local Ethics Committee (approval no. 22/2012 of 21 June 2012) and according to the animal welfare recommendations of European Union directive 86/609/EEC, by providing good husbandry conditions with continuous monitoring of stocking density, bedding, ventilation and lighting.

In ovo injection

Hatching eggs (Ross 308) originating from a 32-week-old parent flock with an average weight of 60 g were incubated at the Drobex hatchery (Solec Kujawski, Poland) in a Petersime incubator (Petersime NV, Zulte, Belgium). On day 12 of incubation, eggs were candled to eliminate infertile eggs and those with dead embryos were excluded. A total of 180 eggs containing live embryos were included in our final analysis. Eggs were randomly allotted to four groups: control (n=45) – injected with physiological saline solution and experimental – injected with (1) Bi^{2}tos (n=45), (2) DiNovo® (n=45) and (3) RFO prebiotics (n=45). Prebiotics were administered on day 12 of incubation into the egg's air cell (Fig. 1). The *in ovo* injection procedure was performed with the use of a dedicated automatic system (BEDNARCZYK *et al.* 2011).

The optimal doses of the prebiotics injected into the egg's air cell were determined by BEDNARCZYK *et al.* (2016) based on hatching results of 13995 embryos injected with different doses of prebiotics and the analysis of the number of *Bifidobacterium* and *Lactobacillus* genera in the feces of one day old broiler chicks.

The control group was *in ovo* injected with 0.2 ml of physiological saline solution. *In ovo* injection in the three experimental groups consisted of 0.2 ml physiological saline solution containing a given dose of the prebiotic. Experimental group 1 received a commercially available prebiotic (Bi^{2}tos), which contained indigestible trans-galactooligosaccharides produced by an enzyme reaction of lactose (Clasado Ltd., Malta) at 3.50 mg prebiotic/embryo (BEDNARCZYK *et al.* 2016). Experimental group 2 received the prebiotic DiNovo®, an extract of *Laminaria* spp. containing laminarin and fucoidan (BioAtlantis Ltd., Ireland) at 0.88 mg prebiotic/embryo (BEDNARCZYK *et al.* 2016). Experimental group 3 was injected with raffinose family oligosaccharide (RFO) at 1.90 mg prebiotic/em-

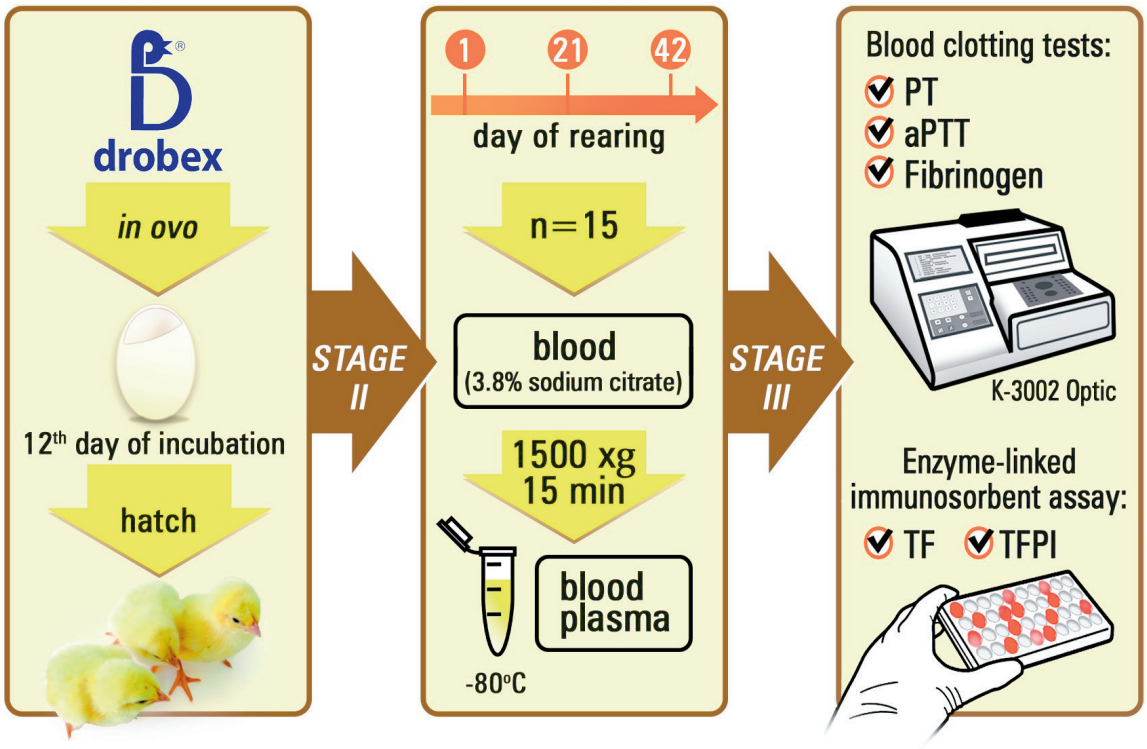

Fig. 1. Study design (see Material and Methods).

bryo (BEDNARCZYK *et al*. 2011; MAIORANO *et al*. 2012). This prebiotic was isolated and purified from lupin seeds (*Lupinus luteus* L., cv. Lord) according to the method described by GULEWICZ *et al*. (2000). RFO solution contained 6.1% saccharose, 9.4% raffinose, 65.2% stachyose, 18.0% verbascose and 1.3% other saccharides (BEDNARCZYK *et al*. 2011). Following injection, each hole was sealed and the eggs were incubated until hatching. The use of automated *in ovo* technology ensured that the bioactive substances were administered accurately and efficiently, and protected the hole in the shell after injection against moisture loss and embryo contamination.

Rearing of birds

Hatched chicks were placed in production house (Drobex, Solec Kujawski, Poland) and were reared under production conditions from February to March 2014 until 42 days of age. Standard commercial concentrate diets were fed *ad libitum* in three phases (Table 1): starter (days 1-21), grower (days 22-35), and finisher (days 36-42). Broiler chickens had constant access to water and their health was monitored throughout the experiment. Housing conditions were the same in all experimental groups.

Sampling

Control and experimental groups were divided into three subgroups containing 15 broiler chickens each, depending on the time of sampling (Fig. 1). The material for laboratory testing was blood collected at random once from each individual at 1 day of age by decapitation (HERICHOVÁ *et al*. 2001) and at 21 and 42 days of age from wing vein (DANESHYAR *et al*. 2011) into tubes containing 3.8% sodium citrate (at a blood/anticoagulant ratio of 9:1) using Vacuette® vacuum tubes (*Becton* Dickinson, Plymouth, United Kingdom). The blood

Table 1

Composition and calculated analysis of diets (g/kg as fed-basis)

Item	Starter day 1-21	Grower day 22-35	Finisher day 36-42
Ingredient			
Wheat	267.3	291.9	306.6
Maize	300.0	300.0	300.0
Extracted soybean meal	325.0	282.0	253.3
Canola	50.0	60.0	70.0
Soybeanoil	21.0	13.3	18.0
Lard	–	20.0	25.0
Feed salt	3.0	3.0	2.8
Groundlimestone	10.9	9.5	8.5
Monocalciumphosphate	11.5	9.4	6.3
DL-Methionine	2.5	1.8	1.3
L-Lysine	3.2	3.2	2.7
L-Threonine	0.6	0.9	0.5
Vitamin-mineral premix[1]	5.0	5.0	5.0
Calculated nutrient level[2]			
AME, kcal/kg	2980	3100	3200
Crude protein	220.0	205.0	195.0
Crude fat	60.9	77.0	90.4
Lysine	13.5	12.5	11.5
Methionine + Cystine	9.5	8.5	7.8
Calcium	9.0	8.0	7.0
Phosphorus	4.0	3.5	2.8
Sodium	1.4	1.4	1.3

[1] Supplied the following per kilogram of diets: Vitamin A – 12,500 IU, Vitamin D_3 – 4,500 IU, Vitamin E – 45 mg, Vitamin K_3 – 3 mg, Vitamin B_1 – 3 mg, Vitamin B_2 – 6 mg, Vitamin B_6 – 4 mg, Pantothenic acid – 14 mg, Nicotinic acid – 50 mg, Folic acid – 1.75 mg, Choline – 1.6 g, Vitamin B_{12} – 0.02 mg, biotin – 0.2 mg, Fe – 50 mg, Mn – 120 mg, Zn – 100 mg, Cu – 15 mg, I – 1.2 mg, Se – 0.3 mg, fitase – 500 FTU, diclazuril – 1 mg (only in starter and grower diets).

[2] Estimation based on the Polish feedstuff analysis tables (SMULIKOWSKA & RUTKOWSKI 2005).

samples were collected at the same hour (8:00 ± 30 min) to minimize the effect of circadian rhythm. The material obtained was centrifuged for 15 min. at 1500 g/min at 4°C. After centrifugation, plasma was carefully transferred into Eppendorf® tubes and stored at -80°C until analysis. No samples were excluded because of hemolysis, and no repeated freeze-thaw cycles were performed before analysis.

Measurement of TF and TFPI

For this study, plasma levels of chicken total tissue factor (TF) and chicken total tissue factor pathway inhibitor (TFPI) were determined. Commercially available highly sensitive enzyme-linked immunosorbent (ELISA) kits were used according to the manufacturers' instructions (BlueGene Biotech, Shanghai, China). Absorbance was read on a plate reader (Multiskan EX, Thermo Fisher Scientific Inc., Waltham, USA). The optical density of the final reaction plate was detected at 450 nm wavelength. Levels were expressed as pg/ml for TF and as ng/ml for TFPI. The estimation of TF and TFPI levels were done at the Department of Pathophysiology, Collegium Medicum of Nicolaus Copernicus University. To minimize assay variance, plasma hemostatic markers were measured in each individual on the same day.

TF and TFPI kits apply the competitive enzyme immunoassay technique utilizing a monoclonal anti-TF or anti-TFPI antibody and an TF-HRP (horseradish peroxidase) or TFPI-HRP conjugate. The plasma sample and buffer are incubated together with TF-HRP or TFPI-HRP conjugate in a pre-coated plate for one hour. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution is added to stop the reaction, which will then turn the solution yellow. The intensity of the color is inversely proportional to the TF or TFPI levels since TF or TFPI from plasma samples and TF-HRP or TFPI-HRP conjugate compete for the anti-TF or anti-TFPI antibody binding site. Since the number of sites is limited, as more sites are occupied by TF or TFPI from the sample, fewer sites are left to bind TF-HRP or TFPI-HRP conjugate. The sensitivity of the kits are 1.0 pg/ml TF and 0.1 ng/ml TFPI, respectively. The intra-assay and inter-assay variation coefficients for both tests are below 10%. These assays have high sensitivity and excellent specificity for detection of TF and TFPI. No significant cross-reactivity or interference between TF, TFPI and analogues are observed.

Determination of prothrombin time

Prothrombin time was determined by the method of QUICK (1935) using Dia-PT Liquid reagent (cat. no. 82024; lot 931206; Diagon® Ltd, Budapest, Hungary) and a K-3002 Optic semi-automated coagulometer (Kselmed s.c., Grudziądz, Poland). Each sample was determined in duplicate. The results are expressed in seconds after calculating the arithmetic mean from two determinations. The results were measured to the nearest 0.1 s, and the maximum coefficient of variation was 6%.

Determination of activated partial thromboplastin time

Activated partial thromboplastin time was determined using Dia-PTT Liquid (cat. no. 72024; lot 931005; Diagon® Ltd, Hungary, Budapest) and Dia-$CaCl_2$ reagents (cat. no. 41048; Diagon® Ltd., Budapest, Hungary) and a K-3002 Optic semi-automated coagulometer (Kselmed s.c., Grudziądz, Poland). Each sample was determined in duplicate. The results are given in seconds after calculating the arithmetic mean from two determinations. The results were measured to the nearest 0.1 s and the maximum coefficient of variation was 6%.

Determination of fibrinogen

Fibrinogen concentration was determined by the chronometric method of CLAUSS (1957) using Dia-FIB (cat. no. 61024; lot 930910; Diagon® Ltd., Budapest, Hungary) and Dia-Imidazol reagents (cat. no. 21180; Diagon® Ltd, Budapest, Hungary) and a K-3002 Optic semi-automated coagulometer (Kselmed s.c., Grudziądz, Poland). The maximum coefficient of variation was 6%. Each sample was determined in duplicate. Fibrinogen values are expressed in g/l after calculating the arithmetic mean from two determinations.

Statistical Methods

Statistical analysis was carried out using STATISTICA® 12.0 (StatSoft, Kraków, Poland). The Shapiro-Wilk test was used for testing normality of data distribution. For each dependent variable, Levene's test was used to determine homogeneity of variance. For hemostasis parameters, which demonstrated a normal distribution and homogeneity of variance, the data are expressed as mean ($\overline{X}$) and standard deviation (SD). The groups were compared using ANOVA. Significance of differences between groups was verified using Tukey's test. For hemostasis parameters, which demonstrated abnormal distribution or heterogeneity of variance, the data are expressed as a median (Me) and quartiles (Q1; Q3). The groups were compared using the Kruskal-Wallis test. Then

multiple comparisons of mean ranks for all groups were performed. A probability value of less than 0.05 was considered to be statistically significant.

Results

Total TF levels in plasma of broiler chickens at different days of age are shown in Table 2. Firstly, there were no differences in TF levels between days in the control group. In the Bi^{2}tos, DiNovo® and RFO groups, similar high TF levels were observed on days 21 and 42 and both were significantly higher compared to the 1st day of life (P=0.001; P=0.026; P=0.007, respectively) (increase in the starter phase).

The administration of prebiotics had no significant effect on total TF levels between the age groups(1, 21, 42 day of rearing) (Table 3).

Total plasma levels of TFPI in broiler chickens on consecutive days of life are given in Table 4. Surprisingly, in the control group we found a significant difference in TFPI levels (P=0.002**)**, with the lowest median on 21 day. In the Bi^{2}tos group, TFPI was absent on the first day, then its level rose significantly on the 42nd day (P=0.006). In turn, in the DiNovo® group TFPI levels were significantly higher on day 1 compared to 21 and 42 days of age

Table 2

Variability of total tissue factor (TF; pg/ml) levels in broiler chickens depending on age (N=15)

Group	Control		Bi^{2}tos		DiNovo®		RFO	
Age	$\overline{X}$	SD	$\overline{X}$	SD	Me	Q1; Q3	Me	Q1; Q3
1 day	57.03	22.32	39.21 A	9.15	29.90 a	21.01; 76.75	30.13 *a*	22.19; 40.92
21 day	82.53	26.66	77.38 B	30.76	96.85 b	90.20; 106.19	80.24 *b*	43.50; 111.10
42 day	81.15	14.28	110.08 C	29.42	89.21b	75.87; 124.20	81.42 *b*	71.26; 96.15
P value	0.072		0.001		0.026		0.007	

Me = median; Q1, Q3 = 1st and 3rd quartiles; $\overline{X}$ = arithmetic mean; SD = standard deviation.
Means/Medians with different letters differ significantly at P<0.05 (a,b,c), P<0.01 (*a,b,c*) or P<0.001 (A,B,C).

Table 3

Variability of total tissue factor (TF; pg/ml) levels in broiler chickens depending on prebiotic administration (N=15)

Age	1 day		21 day		42 day	
Group	Me	Q1; Q3	Me	Q1; Q3	$\overline{X}$	SD
Control	59.64	45.87; 65.60	97.73	57.71; 103.55	81.15	14.28
Bi^{2}tos	37.53	35.67; 40.32	74.12	57.71; 111.88	110.08	29.43
DiNovo®	29.90	21.01; 76.75	96.85	90.20; 106.19	96.75	22.89
RFO	30.13	22.19; 40.92	80.24	43.50; 111.10	81.75	16.93
P value	0.140		0.780		0.060	

Me = median; Q1, Q3 = 1st and 3rd quartiles; $\overline{X}$ = arithmetic mean; SD = standard deviation.

Table 4

Variability of total tissue factor pathway inhibitor (TFPI; ng/ml) levels in broiler chickens depending on age (N=15)

Group	Control		Bi^{2}tos		DiNovo®		RFO	
Age	Me	Q1; Q3	Me	Q1; Q3	$\overline{X}$	SD	Me	Q1; Q3
1 day	0.40 *a*	0.29; 0.60	0.00 *a*	0.00; 0.00	0.32 *a*	0.08	0.25	0.19; 0.41
21 day	0.10 *b*	0.00; 0.20	0.21*ab*	0.00; 0.36	0.13 *b*	0.11	0.21	0.04; 0.35
42 day	0.29 *c*	0.22; 0.36	0.25 *b*	0.12; 0.44	0.13 *b*	0.08	0.18	0.15; 0.29
P value	0.002		0.006		0.002		0.840	

Me = median; Q1, Q3 = 1st and 3rd quartiles; $\overline{X}$ = arithmetic mean; SD = standard deviation.
Means/Medians with different letters differ significantly at P<0.01 (*a,b,c*).

(P=0.002). We failed to find a difference in plasma TFPI levels between days of rearing in the RFO group.

Table 5 presents variation in total TFPI levels between all groups at a particular age. An interesting observation regards TFPI levels on day 1 of age, depending on in ovo prebiotic injection: in the Bi^{2}tos group TFPI was not detected in the plasma samples. In contrast, TFPI was present in other groups, in considerable amounts (0.25-0.40 ng/ml). The levels of this protein did not differ significantly between the groups at both 21 and 42 days of rearing.

Tables 6, 7 and 8 present variation in PT, aPTT, and fibrinogen concentration in the experimental groups depending on the age of broiler chickens. The observations made from hatching to 21 days of age indicate significant changes in the hemostatic system of the control chickens (P=0.01), in which PT and aPTT increased and fibrinogen concentration decreased. In the Bi^{2}tos group, PT did not change throughout rearing (Table 6). As in the control group, aPTT showed a tendency for longer duration (Table 7). Fibrinogen concentration was significantly lower (P=0.043) on day 21 of age compared to the values on days 1 and 42 of age (Table 8). On the last day of rearing in the Bi^{2}tos

Table 5

Variability of total tissue factor pathway inhibitor (TFPI; ng/ml) levels in broiler chickens depending on prebiotic administration (N=15)

Age	1 day		21 day		42 day	
Group	Me	Q1; Q3	Me	Q1; Q3	$\overline{X}$	SD
Control	0.40 A	0.29; 0.60	0.10	0.00; 0.20	0.27	0.13
Bi^{2}tos	0.00 B	0.00; 0.00	0.21	0.00; 0.36	0.27	0.14
DiNovo®	0.32 A	0.25; 0.39	0.11	0.00; 0.24	0.13	0.08
RFO	0.25 A	0.19; 0.41	0.21	0.04; 0.35	0.22	0.10
P value	0.001		0.430		0.120	

Me = median; Q1, Q3 = 1st and 3rd quartiles; $\overline{X}$ = arithmetic mean; SD = standard deviation.
Means/Medians with different letters differ significantly at P<0.001 (A,B,C).

Table 6

Variability of prothrombin time (PT; s) in broiler chickens depending on age (N=15)

Group	Control		Bi^{2}tos		DiNovo®		RFO	
Age	Me	Q1; Q3	$\overline{X}$	SD	Me	Q1; Q3	Me	Q1; Q3
1 day	52.10 A	40.10; 53.10	99.08	25.94	46.60 A	42.50; 57.40	>300 *a*	–
21 day	139.50 B	107.00; 201.30	126.98	42.81	123.70 A	114.90; 168.00	96.40 *b*	80.15; 147.95
42 day	172.80 B	140.50; 202.50	109.23	57.46	>300 B	–	146.60 *b*	107.40; 230.80
P value	0.001		0.400		0.001		0.003	

Me = median; Q1, Q3 = 1st and 3rd quartiles; $\overline{X}$ = arithmetic mean; SD = standard deviation.
Means/Medians with different letters differ significantly at P<0.01 (*a,b,c*) or P<0.001 (A,B,C).

Table 7

Variability of activated partial thromboplastin time (aPTT; s) in broiler chickens depending on age (N=15)

Group	Control		Bi^{2}tos		DiNovo®		RFO	
Age	Me	Q1; Q3	Me	Q1; Q3	Me	Q1; Q3	Me	Q1; Q3
1 day	41.85 A	31.95; 54.45	46.40 A	39.60; 51.10	47.60 A	44.00; 54.10	51.70 A	41.80; 63.50
21 day	110.50 B	76.10; 125.90	69.05 A	52.40; 100.00	121.70 B	91.70; 166.10	119.25 B	83.30; 164.40
42 day	160.60 B	131.10; 182.35	156.50 B	124.80; 171.10	166.00 B	137.00; 195.00	138.10 B	69.70; 171.10
P value	0.001		0.001		0.001		0.001	

Me = median; Q1, Q3 = 1st and 3rd quartiles.
Means/Medians with different letters differ significantly at P<0.001 (A,B,C).

Table 8

Variability of fibrinogen concentration (g/l) in broiler chickens depending on age (N=15)

Group	Control		Bi^{2}tos		DiNovo®		RFO	
Age	$\overline{X}$	SD	Me	Q1; Q3	Me	Q1; Q3	Me	Q1; Q3
1 day	4.57 a	0.74	4.66 a	4.24; 4.94	3.80	3.68; 3.90	4.56 A	4.48; 4.96
21 day	3.92 b	0.79	3.78 b	3.62; 4.72	4.00	3.60; 4.77	4.60 A	4.16; 5.50
42 day	3.94ab	0.41	4.37 a	3.92; 4.67	4.44	3.76; 4.98	3.66 B	3.62; 3.76
P value	0.011		0.043		0.080		0.001	

Me = median; Q1, Q3 = 1st and 3rd quartiles; $\overline{X}$ = arithmetic mean; SD = standard deviation.
Means/Medians with different letters differ significantly at P<0.05 (a,b,c) or P<0.001 (A,B,C).

group, aPTT values were significantly higher (P=0.001) than on days 1 and 21 (Table 7). In the DiNovo® group, PT was significantly longer (P=0.001) on day 42 of age compared to day 1 (Table 6). aPTT was longer on days 21 and 42 of age than on day 1, as in other groups (Table 7). In the DiNovo® group, fibrinogen concentration increased with the age of birds, but the differences were not significant (Table 8). In the RFO group, PT was significantly longer on the 1st day (P=0.003) than on days 21 and 42 (Table 6). On days 21 and 42 in the RFO group, the aPTT value increased with the age of birds similar to control and DiNovo® groups (Table 7). In the RFO group, fibrinogen concentration on day 42 of age was significantly lower (P= 0.001) than on days 1 and 21 (Table 8).

Tables 9, 10 and 11 show variation in PT, aPTT, and fibrinogen concentrations on different days of rearing depending on the prebiotic used. On day 1 of rearing, RFO had a significant effect (P= 0.001) on elongating PT in all individuals in this group (6 times) in relation to the same values in all indi-

Table 9

Variability of prothrombin time (PT; s) in broiler chickens depending on prebiotic administration (N=15)

Age	1 day		21 day		42 day	
Group	Me	Q1; Q3	$\overline{X}$	SD	Me	Q1; Q3
Control	52.10 A	40.10; 53.10	156.43	58.05	172.80 a	140.50; 202.50
Bi^{2}tos	98.08 A	77.30; 105.10	126.98	42.81	112.30 a	50.30; 165.10
DiNovo®	46.60 A	42.50; 57.40	128.69	39.03	>300 b	-
RFO	>300 B	–	114.05	50.47	146.60 a	107.40; 230.80
P value	0.001		0.470		0.031	

Me = median; Q1, Q3 = 1st and 3rd quartiles; $\overline{X}$ = arithmetic mean; SD = standard deviation.
Means/Medians with different letters differ significantly at P<0.05 (a,b,c) or P<0.001 (A,B,C).

Table 10

Variability of activated partial thromboplastin time (aPTT; s) in broiler chickens depending on prebiotic (N=15)

Age	1 day		21 day		42 day	
Group	Me	Q1; Q3	$\overline{X}$	SD	$\overline{X}$	SD
Control	41.85	31.95; 54.45	105.98 *ab*	39.71	158.37	40.95
Bi^{2}tos	46.40	39.60; 51.10	76.44 *a*	32.13	144.04	44.18
DiNovo®	47.60	44.00; 54.10	126.92 *b*	51.81	166.00	29.00
RFO	51.70	41.80; 63.50	124.03 *b*	45.14	126.30	51.72
P value	0.130		0.005		0.520	

Me = median; Q1, Q3 = 1st and 3rd quartiles; ‘X = arithmetic mean; SD = standard deviation.
Means/Medians with different letters differ significantly at P<0.01 (*a,b,c*) .

Table 11

Variability of fibrinogen concentration (g/l) in broiler chickens depending on prebiotic (N=15)

Age	1 day		21 day		42 day	
Group	Me	Q1; Q3	Me	Q1; Q3	Me	Q1; Q3
Control	4.46 A	3.98; 5.00	3.80 b	3.58; 4.04	3.80 AB	3.66; 4.10
Bi^{2}tos	4.66 A	4.24; 4.94	3.78 b	3.62; 4.72	4.37 A	3.92; 4.67
DiNovo®	3.80 B	3.68; 3.90	4.00 a,b	3.60; 4.77	4.44 A	3.76; 4.98
RFO	4.56 A	4.48; 4.96	4.60 a	4.16; 5.50	3.66 B	3.62; 3.76
P value	0.001		0.010		0.001	

Me = median; Q1, Q3 = 1st and 3rd quartiles.
Medians with different letters differ significantly at P<0.05 (a,b,c) or P<0.001 (A,B,C).

viduals in control and DiNovo® groups. aPTT was similar in all the groups under analysis (Table 10). Fibrinogen concentration in 1-day-old broiler chickens, which had been administered *in ovo* with DiNovo®, was significantly lower (P= 0.001) than in the control group and in other experimental groups (Table 11). On day 21 of age, PT values were similar in all the compared groups (Table 9). In turn, aPTT values in the group of chickens that received Bi^{2}tos prebiotic were significantly lower (P= 0.005) compared to other groups (Table 10). On the same day of rearing, significantly higher (P= 0.010) fibrinogen values were observed in the RFO group compared with the control and Bi^{2}tos group (Table 11). On the last day of life, PT was significantly longer (2 times; P= 0.031) in the DiNovo® group compared to other groups, whose values were comparable (Table 9). aPTT was similar in all the analyzed groups (Table 10). Significantly lower (P= 0.001) fibrinogen concentrations were noted in the RFO group compared to the Bi^{2}tos and DiNovo® groups (Table 11).

Discussion

The main conclusions from this study are as follows: (1) total TF levels increased with age in all experimental groups with prebiotics, mostly in the first stage, (2) type of prebiotic did not have a significant effect on TF levels in chickens of the same age, (3) total TFPI levels differed between both the type of *in ovo* injected substances and the age of chickens, but these differences did not form a coherent pattern, (4) in the control group, PT and aPTT increased in duration with age in the first part of the experiment (1-21 days) and fibrinogen concentration decreased, (5) the analysed parameters vary with the prebiotics administered *in ovo* as well as with the age of broiler chickens.

To the best of our knowledge, this is the first study to show the effect of *in ovo* administration of prebiotics on the plasma levels of TF and TFPI in broiler chickens. However, the relationship between prebiotics and the TF-TFPI axis in broiler chickens is still unknown. Tissue factor is released to the circulation by miscellaneous types of vessel wall cells, as in the monocytes-macrophages system. Experimental and clinical evidence strongly suggests that increased blood TF levels are associated with injury or activation of the aforementioned cell types (OWENS III & MACKMAN 2012; RAO & PENDURTHI 2012; PROCHAZKOVA *et al.* 2015). Proinflammatory cytokines, such as interleukin-6 (*IL-6*), *IL-1β*, and tumor necrosis factor alpha (*TNF-α*) are known to increase the expression of TF, leading to an activation of the coagulation system (LUTHER *et al.* 1996; PAWLINSKI *et al.* 2004; MURAMOTO *et al.* 2006). An animal study demonstrated the age-related increase in TF activity in healthy rats (HAN & RHEE 1998). Based on these observations, we speculate that the rapid growth of broiler chickens leads to the inflammatory damage of tissue cells, resulting in the elevation of total TF blood levels. Nonetheless, this conclusion should be confirmed with a larger number of animals, and by performing tests on inflammatory factors and infections in the consecutive days of age in chickens, and by purity analysis of feed.

In this study, we show that prebiotics administrated *in ovo* have differentiated effects on total TFPI levels, and these impacts may also be associated with the age of the animal. This is an important consideration as it is well known that this glycoprotein is the paramount physiological inhibitor of blood coagulation (KOTSCHY *et al.* 2010; REGLIŃSKA-MATVEYEV *et al.* 2014), however the enhancement of TFPI in some cases could be a kind of defensive response of the chicken as a result of increased TF. A particularly interesting observation concerns Bi^{2}tos prebiotic, which resulted in

no TFPI being detected in the plasma of one day old broiler chickens. According to PEDERSEN *et al.* (2005), the lack of TFPI in humans leads to unregulated activity of the TF/FVIIa complex, which results in disseminated intravascular coagulopathy and secondary bleeding.

However, in poultry this problem should be considered from a slightly different angle. Considering the quality of meat, it is rather important to avoid bleeding even by the cost of enhanced blood coagulation (KRANEN *et al.* 2000a, 2000b). Minor deficiency of TFPI would prevent hematomas in the muscles, but a lower level of TF could stimulate bleeding. Disseminated intravascular coagulopathy could possibly be a problem in the case of extreme imbalance on the side of TF/FVIIa leading to consumption of coagulation factors and bleeding, which was reported a few times in birds mostly in cases of pathogen infections, but small shifts in the hemostasis balance of chicken by lowering TFPI and enhancing of TF caused by prebiotics might have a positive effect on overall production without contributing to disseminated intravascular coagulopathy or other blood coagulation disturbances (SHIBATANI *et al.* 1997; MURAMOTO *et al*. 2006; MORIS 2015; SUBA *et al*. 2015). Notwithstanding, this observed effect of probiotics in broiler chickens still remains unclear and undoubtedly this issue must be understood.

Hemostasis research in birds is difficult due to the methodology used. In laboratory tests, the use of heterologous thromboplastins (DOERR *et al.* 1975; LEWIS 1996; PLISZCZAK-KROL *et al.* 2012) may cause PT to be longer by almost 3 times compared to homologous thromboplastin (GRIMINGER 1970; GLAZUNOVA 1972; DOERR *et al.* 1975; FERNANDEZ *et al.* 1995; LEWIS 1996; BALDIZÁN *et al.* 2010; ZERYEHUN *et al.* 2012) and this phenomenon could also be observed in some of our results. GRIMINGER *et al*. (1970) confirmed that vitamin K deficiency impairs the synthesis of plasma clotting proteins (II, VII, IX, X), which was also reflected in significantly prolonged PT. The present study demonstrated that all prebiotics caused major changes except Bi^{2}tos. Because of between-species differences in the sequences of clotting proteins, heterologous thromboplastin has lower affinity to interact with chicken factor VII to produce a reactive enzyme complex, which can initiate thrombin generation through the activation of factor X (LEWIS 1996; THOMSON *et al.* 2002). Because chicken homologous prothrombin is not commercially available, in the present study we used tissue thromboplastin from rabbit brain as a reagent. The PT values obtained in the present study are similar to the values reported by other authors (26.5-158.0 s.) who used rabbit tissue thromboplastins in poultry (DOERR *et al.* 1975; LEWIS 1996; PLISZCZAK-KROL *et al.* 2012; TAKAHIRA *et al.* 2012). Another factor potentially influencing PT value is the temperature of analysis. In animals, many physiological functions depend on species-specific body temperature. The mean temperature used to evaluate hemostatic parameters in screening tests is 37°C, but avian body temperature is close to 41.1°C. Following the observations of TAKAHIRA *et al.* (2012), who showed no effect of temperature on PT, we performed the analyses at 37°C.

Our analysis of aPTT in poultry showed that it increased during the growth of the chickens. Other authors (DOERR & HAMILTON 1981; LEWIS 1996; SHIBATANI *et al.* 1997; PLISZCZAK-KROL *et al.* 2012; ZERYEHUN *et al.* 2012) reported aPTT to vary widely (from 25 s to more than 120 s) in broiler chickens and in some of our results it was higher at 200 s, which can result from lack of coagulation factor XII and mammalian-like activation of blood coagulation by contact on a negative charged surface. Our experiment showed that aPTT in both the control group and in the prebiotic groups increased with age of the chickens. In conclusion we can state that the prebiotics used in this study had no effect on this mechanism.

A longer aPTT is associated with deficiency of blood coagulation factors (VIII, IX, XI, X and II) or can be induced by the presence of circulating antibodies against coagulation factors. The aPTT was found to increase in duration in chickens experimentally infected with bursal disease virus and in those infected with *Erysipelothrix rhusiopathiae* bacteria, which cause erysipelas in poultry (SHIBATANI *et al*. 1997; ZERYEHUN *et al.* 2012). However, the present study does not define the mechanisms involved with the significant elongation of aPTT (2-3 times).

A review of literature indicates that plasma fibrinogen concentration in poultry is 0.16-3.38 g/l (SHIBATANI *et al.* 1997; BALDIZÁN *et al.* 2010; NAZIFI *et al.* 2010; PLISZCZAK-KROL *et al.* 2012). The fibrinogen concentrations obtained in the present study fall within the upper range of values and agree with the observations of other authors (PLISZCZAK-KROL *et al.* 2012).

Interpretation of our results is hampered by the lack of detailed information concerning the composition of the prebiotics, which prevents accurate determination of whether the substances found in the prebiotic may affect hemostasis. It would also be important to keep control of chicken health parameters (bacterial or fungal infections, symptoms such as cuts and bruises, the rate of healing of injuries). In addition, we give the following recommendations: checking the feed for any contamination or harmful substances and the absorption of vitamins, as well as control of the interaction of age

and changing of feed composition. It is equally important to examine the composition and possible contamination of prebiotics (including among others the potential impact of allergenic substances).

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