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DOCTORAL DISSERTATION

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Variability of secondary hemostasis in broiler chickens administered *in ovo* with selected prebiotics

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1. INTRODUCTION

Bioactive compounds such as prebiotics are currently accepted as an alternative to antibiotic growth promoters, which the European Union banned in poultry nutrition in 2006 (Castanon, 2007; Huyghebaert *et al.,* 2011; Alloui *et al.,* 2013). Prebiotics are non-digestible food ingredients that have a positive effect on birds by stimulating the growth and activity of beneficial bacteria naturally occurring in the gut and by eliminating pathogenic microorganism (Gibson and Roberfroid, 1995). *In ovo* administration of prebiotics into embryos stimulates a beneficial bacterial profile in hatched chicks (Villaluenga *et al.,* 2004; Pilarski *et al.,* 2005; Bednarczyk *et al.,* 2011; Sławińska *et al.,* 2014a, b). Additionally, *in ovo* injection of small doses of prebiotics may successfully replace antibiotics use as feed additives for broiler chickens (Bednarczyk *et al.,* 2011). The results obtained to date for *in ovo* of bioactive compounds injection in broiler chickens are promising because prebiotics and synbiotics have been shown to improve production traits (Bednarczyk *et al.,* 2011), meat quality (Maiorano *et al.,* 2012) and influence development of the immune system (Sławińska *et al.,* 2014a, b). However, there are no reports in the literature that have focused on the effect of prebiotic administration on the hemostatic system in birds.

Genetic selection has contributed to changes in the body metabolism of birds (Buzała *et al.,* 2014, 2015), but its effect on hemostatic processes remains an open issue. Hemostasis is a mechanism which protects the body from bleeding in the event of injury to blood vessel walls (Gentry, 2004). Disorders of hemostatic mechanisms are a common

pathology observed in growing poultry (Doerr *et al.*, 1976; 1981a, b; Fernandez *et al.*, 1995; Shibatani *et al.*, 1997; Thomson *et al.*, 2002; 2003; Muramoto *et al.*, 2006; Yeh *et al.*, 2008; 2009; Nazifi *et al.*, 2010; Pliszczak-Krol *et al.*, 2012; Zeryehun *et al.*, 2012; Wideman *et al.*, 2013). They are manifested by bleeding, which occurs most often in muscles, intramuscular fat, connective tissue, and internal organs (Doerr *et al.*, 1975; Skeeles *et al.*, 1980; Pisarevskaia *et al.*, 1984; Kranen *et al.*, 2000a, b; Gentry *et al.*, 2008). Carcass defects and increased bird mortality resulting from hemostatic disorders represent a problem of considerable economic importance to the poultry industry (Doerr *et al.*, 1975; Thomson *et al.*, 2002).

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. 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).

1.1. BLOOD COAGULATION PATHWAYS

The classical blood coagulation cascade includes the extrinsic (tissue factor-dependent) pathway, the intrinsic pathway, and the common pathway. In fact it is difficult to distinguish the pathways because they are closely related and usually act simultaneously. However, the cascade model is still used to describe the processes occurring during blood clotting in animals. This classical coagulation model has evolved to include three phases: initiation, amplification, and the effector phase. In accordance with the latest blood coagulation theory, the main coagulation system process is the tissue factor-dependent pathway, with the intrinsic pathway (dependent on contact factors XI, XII, plasma prekallikrein and high molecular weight kininogen) serving an ancillary role (Wheeler and Rice, 2010). In birds, the tissue factor-dependent pathway is probably essential for the thrombin formation process (Figure 1). The presence of the intrinsic factor is still a matter of debate because birds lack the contact factors XI and XII (Doerr *et al.*, 1974; Doerr and Hamilton, 1981; Thomson *et al.*, 2002; Nevill, 2009).

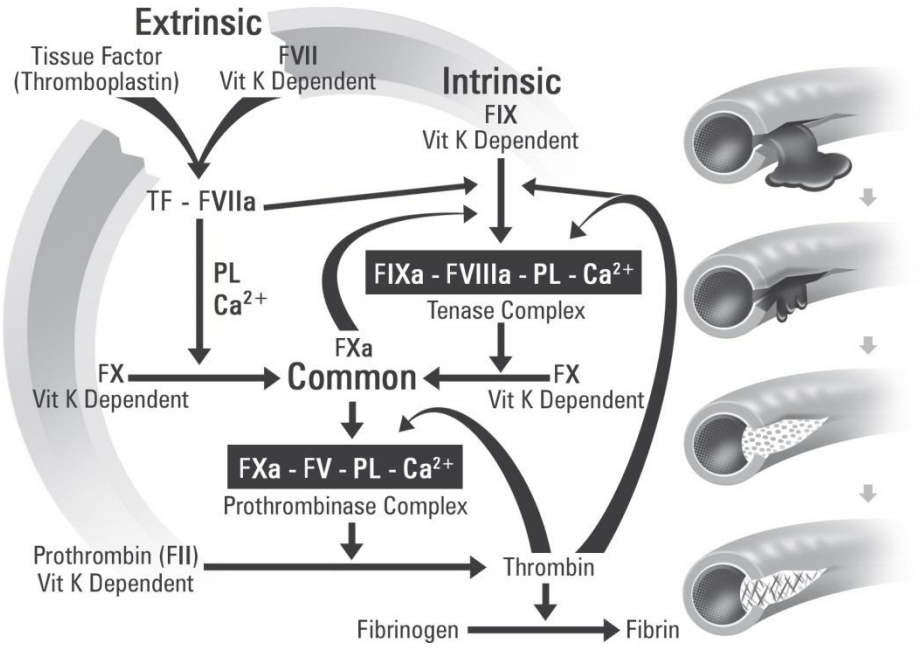

Figure 1 Blood coagulation pathway in birds (F - Factor, PL - Phospholipid)

1.1.1. Tissue factor - dependent pathway

Vascular endothelial damage due to inflammatory factors and tissue damage result in the expression on the cell membrane surface of the tissue factor (TF, thromboplastin, factor III, CD142), which is responsible for activation of factor VII (Doerr and Hamilton, 1981; Thomson *et al.*, 2002). TF is a lipid-dependent transmembrane glycoprotein essential for the development of blood vessels and maintenance of normal hemostasis (Frost *et al.*, 1999; Gentry, 2004; Takahira *et al.*, 2012). It is present mainly on the cell membrane of fibroblasts, pericytes, and blood vessels. Stimulation of vascular endothelial cells and monocytes by proinflammatory cytokines,

such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) releases TF from these cells (Muramoto *et al.,* 2006). The released TF interacts with circulating factor VII to form the enzymatically reactive TF-FVIIa complex. With the participation of phospholipids and calcium ions, this complex transforms factor X into the active form, FXa (Gentry, 2004). The activity of the TF-FVII complex is strictly regulated by TFPI (tissue factor pathway inhibitor), which inactivates the VIIa/TF complex on cell membranes (Doolittle, 2008; Ponczek *et al.,* 2008; Takahira *et al.,* 2012). The role of TFPI has been thoroughly studied in humans, but little is known about the presence and function of TFPI in the blood of domestic animals, including poultry (Gentry, 2004).

1.1.2. Intrinsic pathway

In birds, the intrinsic pathway is activated by factor IX through the factor VIIa/TF complex. On the surface of activated blood platelets, together with phospholipids, factor VIIIa and calcium ions, factor IXa forms the tenase complex (Figure 1), which activates factor X to Xa. Formation of the active tenase complex may be also facilitated by thrombin as a result of factor IX activated by feedback effect, and through activation of factor VIII (FVIIIa), which dissociates from the complex with von Willebrand's factor (vWF) (Thomson *et al.,* 2002; Gentry, 2004; Ponczek *et al.,* 2008). The binding of FVIII with factor vWF increases the half-life of factor VIII in plasma, because it protects this factor from proteolytic degradation (Gentry, 2004).

1.1.3. Common pathway

The generated FXa activates the common pathway through the tissue factor-dependent and intrinsic pathways. FXa forms the prothrombinase complex (Figure 1) by binding to phospholipids together with FVa in the presence of calcium (Gentry, 2004; Yeh *et al.,* 2008). This complex is responsible for the formation of thrombin, the main enzyme responsible for conversion of fibrinogen to fibrin, which is the principal structural component of thrombosis (Yeh *et al.,* 2008). In addition, by activating coagulation factor V, thrombin increases generation of the prothrombinase complex (Thomson *et al.,* 2002; Gentry, 2004; Yeh *et al.,* 2008).

1.2. BLOOD COAGULATION FACTORS

Biological evolution has produced considerable interspecific differences in the blood coagulation system in vertebrates (Davidson *et al.,* 2003; Ponczek *et al.,* 2008). Proteins involved in hemostasis have evolved from immune system proteins through, among others, the classical gene duplication pathway and point mutations. Prothrombin and factors VII, IX and X are homologous to the complement system proteases (C1r and C1s) and haptoglobin (Patthy, 1990; Gentry, 2004; Tentoni *et al.,* 2010). The overall domain organization of prothombin in poultry is similar to that in mammals, while the carbohydrate moiety of chicken prothrombin resembles that of ovalbumin rather than that of mammalian prothrombin (Thomson *et al.,* 2002). The large proportion of identical prothrombin and antithrombin sequences between poultry and mammals is indicative of high homology (Patthy, 1990; Frost *et al.,*

2002; Gentry, 2004). Cofactor proteins, such as factors V and VIII of the tenase and prothrombinase complex are structurally similar to ceruloplasmin (Gentry, 2004). Phylogenetic analysis shows that birds lost factor XII as a result of gene loss (Ponczek *et al.,* 2008). The other differences in coagulation system proteins may be due to genetic selection in poultry (Frost *et al.*, 2002).

There are considerable discrepancies in the literature concerning the concentration and activity of plasma-clotting proteins in poultry, among others due to the use of species-non-specific reagents. Birds have very low concentrations of major clotting proteins such as factors II, V, VII, VIII, IX and X compared to mammals, and were found to contain no contact factors (XI and XII) (Belleville *et al.*, 1982; Lewis, 1996; Frost *et al.,* 1999; Thomson *et al.*, 2002; Davidson *et al.,* 2003; Gentry, 2004; Nevill, 2009; Tentoni *et al.*, 2010). In contrast, the concentration of factor XIII in birds is similar to that in mammals. The deficiency of factor IX in birds may lead to the incidence of disturbances similar to those observed in patients with hemophilia B (Lewis, 1996; Frost *et al.*, 1999). Doerr and Hamilton (1981) postulated that unlike in humans, some clotting factors serve the same function in birds. In both birds and humans, most of the proteins involved in blood coagulation are synthesized in the liver. Because poultry are often affected with fatty liver and accumulation of abdominal fat, their body may be affected by hemorrhagic diathesis resulting from decreased synthesis of plasma clotting factors (Thomson *et al.*, 2003; Yeh *et al.*, 2008). However, recent research (Resnyk *et al.,* 2013) suggests that in addition to hepatocytes,

also adipose cells may be a source of coagulation factors in broiler chickens.

A key protein involved in blood clotting is fibrinogen (factor I), the concentration of which in birds is at least 20 times higher than for other coagulation factors. Because it is an acute phase protein, fibrinogen is used as an indicator of liver damage induced by infectious, toxic or metabolic agents, such as bacterial infections, disseminated intravascular coagulation (DIC) and other inflammatory conditions in poultry. Both the structure and concentration of fibrinogen in avian plasma (Table 1) is similar to that in mammals (Thomson *et al.*, 2002; Gentry, 2004; Tentoni *et al.*, 2010). Increased fibrinogen synthesis was observed during inflammatory reaction involving this protein in the process of fibroblast migration and proliferation, and leukocyte rolling (Shibatani *et al.*, 1997; Gentry, 2004; Tentoni *et al.*, 2010). Determination of fibrinogen concentration coupled with determination of heterophil counts in birds is a useful screening test for infection (Hawkey and Hart, 1987; Gentry *et al.*, 2008).

1.3. ROLE OF VITAMIN K IN BLOOD COAGULATION

Vitamin K is an essential nutrient needed in synthesis of prothrombin, factors VII, IX and X, and protein C and S. Birds are often used as experimental animals in vitamin K studies because they have a high requirement for this vitamin; however, it is difficult to determine the accurate nutritional requirement for vitamin K because of its short half-life (Powers, 2000; Gentry, 2004; Gentry *et al.*, 2008; Watanabe *et al.*, 2010). In poultry, which has a relatively short digestive

tract, vitamin K synthesis takes place in the intestine and fails to meet the avian requirement for this vitamin (Griminger, 1970). As a result, birds receiving low-fat feeds are often deficient in vitamin K. Vitamin K deficiency in birds is also caused by a high intake of vitamin E, which competitively inhibits vitamin K. A similar antagonism was found when sulfonamides and oxidized squalene were used. Vitamin K deficiency may also be due to antibiotic or coccidiostat treatment (Gentry, 2004). Coumarin and its derivatives found in cleavers seeds (Koreleski *et al.,* 2003; Watanabe *et al.,* 2010) as well as compounds contained in moldy legumes and grasses also act as antagonists of vitamin K and cause hemorrhagic disorders (Gentry *et al.,* 2008). Deficiency of vitamin K, which is necessary for hepatic synthesis of clotting proteins, disturbs the tissue factor-dependent pathway, which in laboratory tests translates into prolonged prothrombin time in birds (Griminger, 1970; Gentry *et al.,* 2008). In order to prevent vitamin K deficiency, poultry feed is regularly supplemented with menadione, sometimes known as vitamin K_3 (Scott *et al.,* 2005). Notwithstanding, in amounts of 10-100 mg/kg feed, menadione may act as a pro-oxidant and initiate free radicals; in high concentrations, it may cause hemolysis due to its ready solubility in water (Gentry, 2004). Blood clotting time was observed to decrease slightly in birds after administration of menadione to feed (Koreleski *et al.,* 2003).

The mode of action of vitamin K became better understood after showing that the formation of gamma-carboxyglutamic acid (GLA) residues in thrombin molecules and in other proteinases associated with blood coagulation cascade depends on vitamin K. GLA residues

are a binding site for calcium, which is necessary for activating coagulation factors. Vitamin K serves as cofactor for microsomal carboxylases, which are responsible for GLA formation (Gentry *et al.,* 2008). It should be stressed that in poultry, intensive genetic selection for high productivity has led to bone abnormalities, which may result not only from increased requirement for minerals (including calcium and phosphorus); attention should also be given to the role of vitamin K, which interacts with vitamin D in bone formation. Vitamin K contributes to the transfer of calcium from blood to bones, and interacts with vitamin D to incorporate calcium into the bones (Koreleski *et al.,* 2003; Świątkiewicz *et al.,* 2014; Buzała *et al.,* 2015). GLA residues found in bone osteoblasts (osteocalcin) and cartilage chondrocytes (Hauschka, 1989; Koreleski *et al.,* 2003) take part in regulating bone growth and formation. Administration of large amounts of vitamin K antagonists not only triggers the symptoms of hemorrhagic diathesis, but also leads to bone damage as a result of impaired bone mineralization (Suttie, 2007; Gentry *et al.,* 2008). All vitamin K-dependent proteases (factors II, VII, IX and X) require Na^+ for optimum catalytic activity. The activity and specificity of serine proteinases is controlled allosterically by the binding of Na^+ ions. However, due to the low concentration of these proteins in poultry, it is likely that vitamin K-dependent clotting factors are deprived of Na^+-dependent enhancement (Frost *et al.,* 1999).

1.4. HEMOSTATIC DISORDERS IN BIRDS

1.4.1. Disseminated intravascular coagulation

Disseminated intravascular coagulation (DIC) develops through the pathological activation of the blood coagulation system, which leads to the formation of intravascular thromboses that damage the internal organs with secondary activation of the fibrinolytic system. The primary activation of coagulation and the secondary reaction of the fibrinolytic system induce the symptoms of severe hemorrhagic diathesis (Shibatani *et al.,* 1997; Gentry *et al.,* 2008). DIC most often develops as a result of infections caused by endotoxins of Gram-negative and Gram-positive bacteria, viruses, protozoa, parasites, cancers, and injuries (Shibatani *et al.,* 1997). It is characterized by high consumption of clotting factors and thrombocytes, and the generation of fibrin and fibrinogen degradation products (FDP) (Asakura, 2014).

1.4.2. Hemostatic disturbances during bacterial infections

Birds infected with Gram-negative bacteria are often diagnosed with thrombosis complications, which are characteristic pathological changes in DIC. In chickens with acute infection of *Escherichia coli*, the presence of fibrin is often found in liver and spleen, but interestingly, not in kidneys and lungs. Damaged bursa of Fabricius and thymus were also observed in chickens infected with *E. coli*. In addition, shorter thrombin time (TT) and a decrease in fibrinogen concentration are observed in broiler chickens administered with *E. coli* lipopolysaccharide (Pliszczak-Krol *et al.,* 2012).

Additionally, after intramuscular infection with *Erysipelothrix rhusiopathiae* bacteria, broilers have enlarged and congested liver, spleen and kidney tissues as well as thromboses in liver sinuses, capillaries of renal glomeruli, and small blood vessels of lungs (Shibatani *et al.,* 1997). Furthermore, much longer PT and aPTT has been found in birds infected with this species of bacteria. In laboratory tests, longer PT and aPTT found during the course of DIC reflects the consumption of plasma clotting factors, the decreased activity of antithrombin (the main inhibitor of the blood clotting process), and the increased activity of the plasminogen activator inhibitor (PAI-1) (Shibatani *et al.,* 1997; Pliszczak-Krol *et al.,* 2012).

1.4.3. Hemostatic disturbances during viral infections

Birds are affected by many different viral diseases that induce hemostatic disorders. Infections with circovirus and polyomavirus may cause thrombocytopenia, vasculitis, and liver failure (Gentry *et al.,* 2008). Chicks infected with infectious bursal disease virus (IBDV) were found to develop significant thrombocytopenia as well as longer PT, aPTT and whole-blood recalcification time (WBRT) (Zeryehun *et al.,* 2012). Other studies reported no significant differences in fibrinogen concentration between healthy and IBDV-infected chickens, which suggests that this protein is not sensitive to infection with this virus (Nazifi *et al.,* 2010). In turn, chickens infected with IBDV showed the presence of hemorrhage in bursa of Fabricius, thigh muscles and abdominal mucosa. These observations indicate that infection with this virus may disturb the synthesis of clotting factors and megakaryopoiesis (Nazifi *et al.,* 2010; Zeryehun *et al.,* 2012).

Bleeding in many organs also occurs in chickens infected with highly pathogenic avian influenza virus of type A (HPAI). Chickens infected with this type of virus often develop DIC, which is manifested by bleeding from many tissues. It is assumed that DIC in HPAI-infected chickens is induced by damage to vascular endothelial cells caused by this virus. In addition, a significant role in the pathomechanism in chickens infected with this virus is played by proinflammatory cytokines such as IL-1β, IL-6 and TNF-α, which stimulate release of TF from monocytes/macrophages and vascular endothelial cells (Muramoto *et al.*, 2006).

1.4.4. Hemostatic disturbances during fungal infections

Aflatoxins from *Aspergillus* fungi can be found in moldy feeds and are a cause of fatty liver and biliary duct proliferation in poultry (Fernandez *et al.*, 1995; Gentry *et al.*, 2008), which may lead to blood coagulation disorders. Poultry aflatoxicosis is characterized by massive hemorrhagic lesions in the stomach, heart, intestines, lungs, kidneys and muscles resulting in death. The most sensitive indicator of aflatoxin toxicity in chickens is the measurement of PT, the elongation of which is directly proportional to aflatoxin dose and exposure time. The longer PT observed in aflatoxicosis also reflects the degree of liver damage. Therefore, PT test may be a sensitive prognostic indicator in this disease (Doerr *et al.*, 1974, 1976; Fernandez *et al.*, 1995). In addition to aflatoxins, other hemorrhage-inducing agents in broiler chickens are ochratoxin A and T2 toxin (Doerr *et al.*, 1974, 1981a, b). Research demonstrates that ochratoxin A induces hypofibrinogenemia whereas hypoprothrombinemia is observed in the case of aflatoxin infections,

both leading to the incidence of hemorrhagic diathesis (Doerr *et al.*, 1981a, b). Because mycotoxins are particularly dangerous for young chicks, a better understanding of the pathomechanisms of blood coagulation disorders in the case of fungal infections may help to prevent mycotoxin infections in the future (Doerr *et al.*, 1974).

1.4.5. Fatty liver hemorrhagic syndrome

Fatty liver hemorrhagic syndrome (FLHS) is a metabolic disorder occurring in laying hens during peak egg production (Binnington, 2000; Thomson *et al.*, 2003). This syndrome causes welfare problems and financial losses as a result of chicken mortality and decreased egg production. Etiopathogenesis of the disease is still unknown (Yeh *et al.*, 2008; 2009). Postmortem examination of chickens reveals excess abdominal and liver fat as well as liver hemorrhages of different sizes (Thomson *et al.*, 2002). In birds, FLHS also causes changes to the plasma composition of phospholipids (Thomson *et al.*, 2003; Gentry *et al.*, 2008). The type of phospholipids present in plasma may be a factor affecting the activity of clotting proteins. Birds susceptible to FLHS were observed to contain eicosatrienoic acid ($C_{20:3}$n-3) but no linolenic acid ($C_{18:3}$n-3) (Thomson *et al.*, 2002), which in chicks reduces thrombocyte aggregation (Baldizán *et al.*, 2010). In addition, the presence of arachidonic acid was found in hens susceptible to FLHS. It is essential for normal blood coagulation, because its metabolism in blood platelets leads to synthesis of thromboxane which is needed for the degradation of these cells. This acid gives rise to prostaglandins, which take part in inflammatory reactions and regulate blood vessel contractility (Thomson *et al.*, 2003; Yeh *et al.*, 2008). What is more, because lipids

are indispensable for activity of factors V, VII and X, plasma lipids in laying hens susceptible to FLHS increase the activity of factors V, VII and X, possibly leading to hemorrhages (Thomson *et al.*, 2003; Yeh *et al.*, 2008). In addition, the rate of thrombin formation in the plasma of birds susceptible to FLHS is higher than in the plasma of healthy layers, which may be associated with a different composition of phospholipids in the blood plasma (Thomson *et al.*, 2003).

1.5. SCREENING LABORATORY TEST FOR HEMOSTASIS IN BIRDS

Hemostasis research in birds is difficult for lack of species-specific tests and homologous reagents for diagnosing avian coagulopathies. Differences in the coagulation processes between birds and mammals, especially in function and structure of the proteins involved, produce considerable discrepancies in the results of coagulometric tests. The laboratory procedures used to diagnose mammals cannot always be applied for birds (Takahira *et al.*, 2012). Quantitative enzyme immunoassays, which use antibodies against species-specific clotting proteins, are increasingly used as diagnostic tools in veterinary medicine. However, coagulation system abnormalities are most often diagnosed with screening tests, which provide the general clinical picture of the birds (Gentry, 2004; Tentoni *et al.*, 2010).

Blood samples for analysing hemostatic parameters in birds should be collected using a vacuum blood collection system containing 3.8% sodium citrate (1 part of anticoagulant to 9 parts of blood), which inhibits blood clotting by binding calcium ions (Lewis, 1996; Frost

et al., 1999). Furthermore, blood before analysis should be free from erythrocyte hemolysis and damaged thrombocytes that release TF, which may significantly affect the laboratory parameters. The plasma should be analyzed immediately after collection, and the time between sample collection and analysis should be as short as possible, because freezing and thawing may be detrimental to the results obtained. Some tests allow the samples to be frozen in liquid nitrogen within 30-60 min. of collection and stored at –80°C or –20°C for no longer than a week (Bigland and Starr, 1965; Lewis, 1996; Frost *et al.,* 1999; Baldizán *et al.,* 2010). Anatomical traits of birds may prevent collecting blood samples of good quality (Tentoni *et al.,* 2010). The collection of blood from the heart and by decapitation may be accompanied by release of TF, which could shorten the clotting time (Griminger, 1970; Stopfhort, 1970), and even cause the blood sample to coagulate (Bigland and Triantaphyllopoulos, 1960). Because the clotting system may also be activated when blood is collected directly from a blood vessel (Thomson *et al.,* 2002), it is recommended to drain several drops of blood prior to collection to avoid TF contamination (Bigland and Starr, 1965). The response of birds to the loss of blood is an important factor when collecting samples of blood. In healthy birds, the amount of blood that can be collected without negative effects on health is 1-3% of body weight. When birds are weakened, 1% seems the upper safe limit for the amount of blood that can be drawn for diagnostic purposes (Lewis, 1996). In addition, many physiological functions are dependent on body temperature appropriate for a given species of animal. The mean temperature used to evaluate hemostasis parameters in screening tests is 37°C, but in the case of birds it is close

to 41.1°C, which may have an effect on the measurement results (Takahira *et al.,* 2012).

1.5.1. Prothrombin time

One-stage prothrombin time (PT), which serves to determine the tissue factor-dependent and the common blood coagulation pathway, could be most useful for evaluation of hemostatic disorders in birds. For laboratory tests, tissue thromboplastin (TF) obtained during acetone extraction from the brain of homologous animals is used (Griminger, 1970). Prothrombin time, an indicator of the activity of blood coagulation factors V, VII, IX, X, prothrombin and fibrinogen, can also serve to diagnose and monitor liver lesions in birds. To determine PT, TF is added to citrate plasma to activate factor VII. After incubation at 37°C, the time between calcium ion addition (which activates factor X in the presence of VIIa) to sample clotting is measured. To ensure reliable results, the determination should be made with fresh thromboplastin isolated from tissues on the first day after collection and used within a week (Frost *et al.,* 1999).

Because no reagents containing poultry brain thromboplastin are now available, PT results in this group of animals are highly dependent on TF source. In birds, PT should be performed with the use of homologous brain thromboplastin, because PT is around 3 times longer when heterologous thromboplastin is used (Table 1). When homologous thromboplastin is employed, poultry plasma has a fully functional tissue factor-dependent pathway (Doerr *et al.,* 1975; Shibatani *et al.,* 1997; Frost *et al.,* 1999; Thomson *et al.,* 2002; Muramoto

et al., 2006). By way of example, quail plasma shows functional activity of factors V, VII and X when quail TF compared to human TF is used as an activator (Belleville *et al.*, 1982). Today, the most common and easily available reagent is thromboplastin from rabbit tissues. These reagents are mainly used to assess abnormalities in the TF pathway, when plasma clotting time is compared with the control values for the same reagent and the same species (Gentry, 2004). Differences in the activity of heterologous tissue thromboplastins result from interspecific differences in the blood coagulation system, while chicken thromboplastin obviously shows maximum activity in homologous plasma (Glazunova, 1972). The longest clotting times are obtained for poultry plasma with TF obtained from rabbits. Although PT determination using homologous tissue thromboplastin is the best diagnostic method, researchers commonly use TF from rabbit tissues, which does not have the same activity and therefore PT is much longer (Lewis, 1996; Thomson *et al.*, 2002). Nevertheless, rabbit thromboplastin is frequently used in the tests because there is no commercially available thromboplastin from broiler chicken tissues (Belleville *et al.*, 1982; Thomson *et al.*, 2002). In order to ensure the reliability of diagnostic tests in poultry, it is necessary to develop one's own reference values. The result of PT test depends not only on the rate of thrombin formation, but also on the availability of fibrinogen as a substrate for thrombin (Thomson *et al.*, 2002). Because of the higher body temperature in birds than in humans, PT determination at 37°C and 40°C was found to have no effect on the results obtained (Takahira *et al.*, 2012). Future studies concerning the coagulation system in poultry should account for optimization of PT test to obtain accurate and repeatable results.

Table 1 The range of hemostatic parameter values reported in broiler chickens

Parameter	Range	References
Prothrombin time (PT) (homologous thromboplastin)	9.0-51.0 sek.	(Bigland and Starr, 1965; Griminger, 1970; Stopford, 1970; Glazunova, 1972; Doerr *et al.*, 1974; 1975; Fernandez *et al.*, 1995; Lewis, 1996; Baldizán *et al.*, 2010; Takahira *et al.*, 2012; Zeryehun *et al.*, 2012)
Prothrombin time (PT) (heterologous thromboplastin)	26.5-158.0 sek.	(Doerr *et al.*, 1975; Lewis, 1996; Wang *et al.*, 2008; Pliszczak-Krol *et al.*, 2012; Takahira *et al.*, 2012)
Partial thromboplastin time (PTT)	29.9-35.9 sek.	(Doerr and Hamilton, 1981)
Activated partial thromboplastin time (aPTT)	25.4->120 sek.	(Doerr and Hamilton, 1981; Lewis, 1996; Shibatani *et al.*, 1997; Wang *et al.*, 2008; Pliszczak-Krol *et al.*, 2012; Zeryehun *et al.*, 2012)
Thrombin time (TT)	16.9-161.5 sek.	(Wang *et al.*, 2008; Baldizán *et al.*, 2010; Pliszczak-Krol *et al.*, 2012)
Recalcification time (RT)	115-302 sek.	(Glazunova, 1972; Doerr *et al.*, 1974; Doerr and Hamilton, 1981)
Whole blood clotting time (WBCT)	251-592 sek.	(Doerr *et al.*, 1974; Doerr and Hamilton, 1981)
Fibrinogen	0.16-3.38 g/L	(Shibatani *et al.*, 1997; Baldizán *et al.*, 2010; Nazifi *et al.*, 2010; Pliszczak-Krol *et al.*, 2012)

1.5.2. Activated partial thromboplastin time

Assessment of the intrinsic pathway using the activated partial thromboplastin time (aPTT) test is also problematic because poultry lack contact factors. In this test, thrombin formation starts by adding a non-physiological, negatively charged compound (such as kaolin, celite or ellagic acid suspended in a mixture of phospholipids) to the platelet-poor plasma. The purpose of these substances is to activate factor XII by converting factor XI to XIa, which gives rise to the tenase complex through activation of factor IX. The lack of factors XII and XI in birds increases aPTT time in relation to mammals. It is possible that the differences in aPTT results obtained from different vertebrates are due to lack of homology in the structure of factors XII and XI between these species. aPTT value depends primarily on the activity of plasma clotting factors (XII, XI, IX and VIII), which form the intrinsic prothrombin activation system. aPTT is also dependent on thrombin formation factors (prothrombin, factors X and V) and conversion of fibrinogen to fibrin (Shibatani *et al.*, 1997; Gentry, 2004).

2. ASSUMPTIONS AND OBJECTIVE

Considering the dearth of studies concerning the effect of prebiotics on the hemostatic system in birds, the aim of the present research was to determine the relationship between *in ovo* administration of prebiotics on selected blood coagulation parameters during the growth of birds.

General objective was realized through:

- ✓ The evaluation of the TF-TFPI axis in broiler chickens in different rearing days after *in ovo* administration of prebiotics;
- ✓ Assessment of the effect of prebiotics given during embryogenesis on screening hemostatic parameters (PT, aPTT and fibrinogen concentration) of broiler chickens.

3. MATERIALS 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.

3.1. *IN OVO* INJECTION

The experiment used a total of 100 000 hatching eggs from broiler chickens (Ross 308) originating from a 32-week-old parent flock. Hatching eggs with an average weight of 60g 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 prior to *in ovo* injection. Eggs containing live embryos were randomly allotted to four groups: (1) control – injected with physiological saline solution and experimental – injected with (2) Bi^{2}tos, (3) LactoShield and (4) RFO prebiotics. Bioactive substances were administered on day 12 of incubation into the egg's air cell. 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 given to birds were determined by Bednarczyk *et al.* (2015) based on hatching results of 13995 embryos injected with different doses of bioactive substances and the analysis

of main groups of prebiotic bacteria present in the feces of one-day-old broiler chicks.

Each *in ovo* injection into the egg's air cell consisted of 0.2 ml physiological saline solution containing a given dose of the prebiotic. Experimental group 1 was injected with 0.2 ml of physiological saline solution. Experimental group 2 received a commercially available prebiotic (Bi^2tos), which contained indigestible trans-galactooligosaccharides manufactuered commercially by an enzyme reaction of lactose (Clasado Ltd., Malta) at 3.50 mg prebiotic/embryo (Bednarczyk *et al.,* 2015). Experimental group 3 received LactoShield prebiotic, a commercially available aqueous extract of β-glucans from Atlantic algae (Bioatlantis Ltd., Ireland) at 0.88 mg prebiotic/embryo (Bednarczyk *et al.,* 2015). Experimental group 4 was injected *in ovo* with raffinose family oligosaccharide (RFO) at 1.90 mg prebiotic/embryo (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 (138 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.

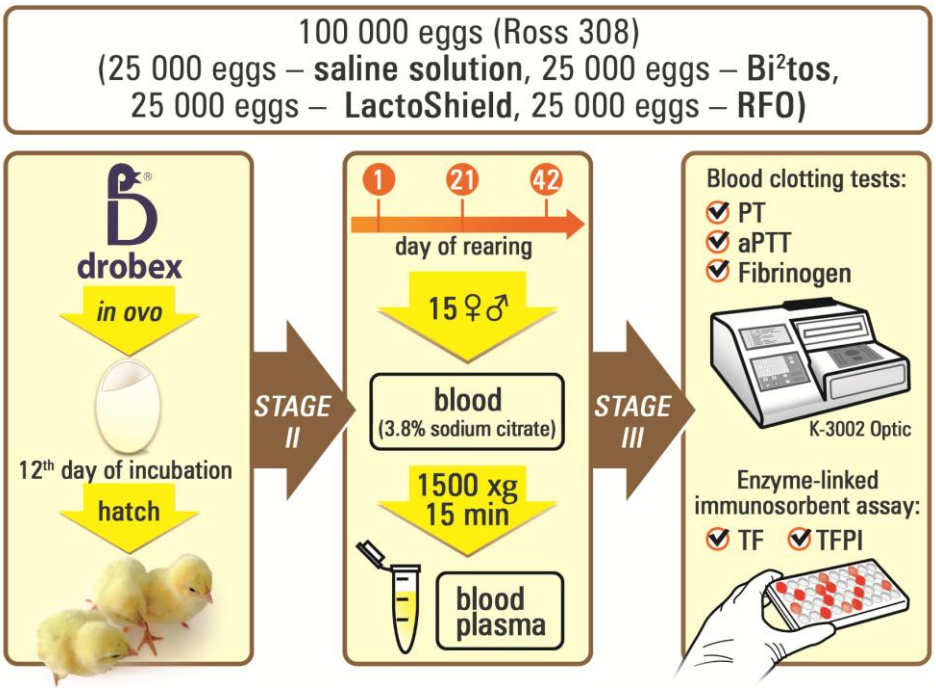

Figure 2 Study design

3.2. REARING OF BIRDS

Hatched chicks from each group were placed in 4 production houses on the same farm (Drobex, Solec Kujawski, Poland). The study was carried out on the production scale. Birds 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 2): starter (days 1-21), grower (days 22-35), and finisher (days 36-42). Birds had constant access to water. The health of birds was monitored throughout the experiment and housing conditions were the same in all the experimental groups.

Table 2 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-mieral 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 and Rutkowski, 2005).

3.3. SAMPLING

The material for laboratory testing was blood collected from 15 randomly chosen birds of each group at 1 day of age (by decapitation) and at 21 and 42 days of age (from wing vein) into tubes containing 3.8% sodium citrate (at a blood/anticoagulant ratio of 9:1) using Vacuette® vacuum tubes (Becton Dickinson, Plymouth, United Kingdom). Blood 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.

3.4. DETERMINATION OF TISSUE FACTOR AND TISSUE FACTOR PATHWAY INHIBITOR

For this study, plasma levels of total tissue factor (TF) and total tissue factor pathway inhibitor (TFPI) were determined. Commercially available highly sensitive enzyme–linked immunosorbent (ELISA) kits were used strictly 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.

Both total TF or TFPI kits are a 1.5 hour solid-phase ELISA designed for the quantitative determination of chicken TF or TFPI. TF or TFPI ELISA kit applies the competitive enzyme immunoassay technique utilizing a monoclonal anti-TF or anti-TFPI antibody and an TF-HRP or TFPI-HRP conjugate. The assay sample and buffer are incubated together with TF-HRP or TFPI-HRP conjugate in 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 color is measured spectrophotometrically at 450 nm in microplate reader. The intensity of the color is inversely proportional to the TF or TFPI concentration since TF or TFPI from 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. A standard curve is plotted relating the intensity of the color (O.D.) to the concentration of standards. The TF or TFPI concentration in each sample is interpolated from this standard curve. According to the manufacturer, coefficient of variation for both kits was below 10%. The sensitivity

of TF assay is 1.0 pg/mL and TFPI assay is 0.1 ng/mL. These assays have high sensitivity and excellent specificity for detection of TF or TFPI. No significant cross-reactivity or interference between TF or TFPI and analogues was observed.

3.5. 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). 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%.

3.6. 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). 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%.

3.7. 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%. Fibrinogen values are expressed in g/L after calculating the arithmetic mean from two determinations.

3.8. STATISTICAL METHODS

Statistical analysis was carried out using STATISTICA® 10.0 for Windows (StatSoft, Kraków, Poland). The Shapiro–Wilk test was used for testing normality. For each dependent variable, Levene's test was used to determine homogeneity of variance. For hemostasis parameters which demonstrated normal distribution and homogeneity of variance the data were expressed as means (X) and root-mean-square error (RMSE). The groups were compared using the 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 interquartile range (IQR). The groups were compared using the Kruskal–Wallis test. Then multiple comparisons of mean ranks for all groups were performed. A probability less than 0.05 was considered statistically significant ($P< 0.05$).

4. RESULTS

4.1. TISSUE FACTOR

Total TF concentration in the blood plasma of broiler chickens at different days of age is shown in Table 3. The administration of prebiotics had no significant effect on TF concentration between the groups on different days of rearing (1, 21, 42). However, TF concentration was higher in the control group on day 1 of age compared to the groups receiving prebiotics *in ovo*, but were non-significant differences. TF concentration did not differ significantly between the groups at 21 and 42 days of rearing.

Variation in total TF on different days of rearing within the studied groups is presented in Table 4. In the group of birds receiving Bi^{2}tos prebiotic, TF concentration was significantly higher between the three time points. In the group receiving LactoShield prebiotic, TF concentration was significantly higher at 21 days compared to day 1 of age. When analyzing TF concentration in the RFO group, statistically significant differences were observed in TF concentration between 1 and 21 days of age. It was also demonstrated that TF concentration at 42 days of age was significantly higher compared to day 1 of age. The physiological saline solution had no significant effect on TF concentration in the control group, despite the fact that TF concentration was higher at 21 and 42 days of rearing compared to the concentration of this protein on day 1 of age.

Table 3 Effect of age on total TF and TFPI concentration in broiler chickens

Parameter (unit)	n	Control		Bi^{2}tos		LactoShield		RFO		*P*-value
		Me/X	IQR/RMSE	Me/X	IQR/RMSE	Me/X	IQR/RMSE	Me/X	IQR/RMSE	
1 day										
TF (pg/mL)	15	59.64	45.87 - 65.60	37.53	35.67 - 40.32	29.90	21.01 - 76.75	30.13	22.19 - 40.92	0.140
TFPI (ng/mL)	15	0.40^{a}	0.29 - 0.60	0.00^{a,b,c}	0.00 - 0.00	0.32^{b}	0.25 - 0.39	0.25^{c}	0.19 - 0.41	<0.001
21 day										
TF (pg/mL)	15	97.73	57.71 - 103.55	74.12	57.71 - 111.88	96.85	90.20 - 106.19	80.24	43.50 - 111.10	0.780
TFPI (ng/mL)	15	0.10	0.00 - 0.20	0.21	0.00 - 0.36	0.11	0.00 - 0.24	0.21	0.24 - 0.35	0.430
42 day										
TF (pg/mL)	15	81.15	14.28	110.08	29.43	96.75	22.89	81.75	16.93	0.060
TFPI (ng/mL)	15	0.27	0.13	0.27	0.14	0.13	0.08	0.22	0.10	0.120

Me = median; IQR = interquartile range; n = number; *P* = probability value; TF = tissue factor; TFPI = tissue factor pathway inhibitor; X = arithmetic mean; RMSE = root-mean-square error

^{a,b,c} Means with same letters differ significantly at $P< 0.05$ (differences between Control, Bi^{2}tos, LactoShield, RFO) over successive tests.

Table 4 Effect of prebiotic on total TF and TFPI concentration in broiler chickens

Parameter (unit)	n	1 day		21 day		42 day		*P*-value
		Me/X	IQR/RMSE	Me/X	IQR/RMSE	Me/X	IQR/RMSE	
Control								
TF (pg/mL)	15	57.03	22.32	82.53	26.66	81.15	14.28	0.072
TFPI (ng/mL)	15	0.40[a]	0.29 - 0.60	0.10[a]	0.00 - 0.20	0.29[a]	0.22 - 0.36	0.002
Bi^{2}tos								
TF (pg/mL)	15	39.21[a]	9.15	77.38[a]	30.76	110.08[a]	29.42	<0.001
TFPI (ng/mL)	15	0.00[a]	0.00 - 0.00	0.21	0.00 - 0.36	0.25[a]	0.12 - 0.44	0.006
LactoShield								
TF (pg/mL)	15	29.90[a]	21.01 - 76.75	96.85[a]	90.20 - 106.19	89.21	75.87 - 124.20	0.026
TFPI (ng/mL)	15	0.32[a,b]	0.08	0.13[a]	0.11	0.13[b]	0.08	0.002
RFO								
TF (pg/mL)	15	30.13[a,b]	22.19 - 40.92	80.24[a]	43.50 - 111.10	81.42[b]	71.26 - 96.15	0.007
TFPI (ng/mL)	15	0.25	0.19 - 0.41	0.21	0.04 - 0.35	0.18	0.15 - 0.29	0.840

Me = median; IQR = interquartile range; n = number; *P* = probability value; TF = tissue factor; TFPI = tissue factor pathway inhibitor; X = arithmetic mean; RMSE = root-mean-square error

[a,b,c] Means with same letters differ significantly at $P< 0.05$ (differences between days 1, 21 and 42) over successive tests.

4.2. TISSUE FACTOR PATHWAY INHIBITOR

Total plasma concentration of TFPI in broiler chickens on different days of age is given in Table 3. An interesting observation was made when analyzing TFPI concentration on day 1 of age depending on *in ovo* prebiotic injection. The administration of Bi^{2}tos resulted in no TFPI being detected in the plasma of these birds. In contrast, TFPI was found to be present in the other groups of birds, with the highest median in the control group. TFPI concentration did not differ significantly between the groups at both 21 and 42 days of rearing. However, on these days it was interesting to note that the mean concentration of TFPI was identical in the Bi^{2}tos and RFO groups at 21 days of age, and in the control and Bi^{2}tos groups at 42 days of age.

Table 4 presents variation in total TFPI on selected days of age within the experimental groups of broilers. In the Bi^{2}tos group, TFPI concentrations were found to increase with age, which differed significantly between 1 and 42 days of rearing. In turn, in the LactoShield group TFPI concentration was significantly higher on day 1 compared to 21 and 42 days of age. In this group mean TFPI concentrations at 21 and 42 days of age were identical. RFO had no effect on TFPI, but the concentration of this natural tissue factor inhibitor decreased as broilers grew older. In the control group, physiological saline solution had a statistically significant effect on TFPI concentration on different days of growth.

4.3. PROTHROMBIN TIME, ACTIVATED PARTIAL THROMBOPLASTIN TIME AND FIBRINOGEN

Table 5 presents variations in PT, aPTT, and fibrinogen concentration in the experimental groups depending on the age of broiler chickens. On day 1 of rearing, RFO had a significant effect on increasing PT in relation to the control group and the other prebiotics. aPTT was similar in all the groups under analysis. Fibrinogen concentration in 1-day-old broiler chickens, which had been administered *in ovo* with LactoShield, was significantly lower than in the control group and in the other experimental groups. On day 21 of age, PT values were similar in all the compared groups. In turn, aPTT values in the group of chickens that received Bi^{2}tos prebiotic were significantly lower compared to the other groups. On the same day of rearing, significantly higher fibrinogen values were also observed in the RFO group. On the last day of life, PT was significantly longer in the LactoShield group compared to the other groups. aPTT was similar in all the analysed groups. Significantly lower fibrinogen concentrations were noted in the RFO group compared to the Bi^{2}tos and LactoShield groups.

Table 6 show variation in PT, aPTT, and fibrinogen concentration on different days of rearing depending on the prebiotic used. The observations made from hatching to 42 days of age indicate significant changes in the hemostatic system of control chickens, in which PT and aPTT increased and fibrinogen concentration decreased. In the Bi^{2}tos group, PT did not change throughout rearing. Like in the control group, aPTT showed a tendency to increase in duration.

Fibrinogen concentration declined significantly on day 21 of age compared to the values on days 1 and 42 of age. On the last day of rearing, aPTT values were significantly higher than on days 1 and 21. In the LactoShield group, PT was significantly longer on day 42 of age compared to day 1. On day 1 of age aPTT was longer, just like in the control and Bi^{2}tos groups. In the LactoShield group, fibrinogen concentration increased with the age of birds, but the differences were not significant. In the RFO group, PT was significantly longer, reaching values comparable to the control group values during the last days of observations. In the RFO group, similar to the other groups, aPTT value increased with the age of birds. In the RFO group, fibrinogen concentration on day 42 of age was significantly lower than on days 1 and 21, as well as compared to the Bi^{2}tos and LactoShield groups.

Table 5 Effect of age on PT, aPTT and fibrinogen concentration in broiler chickens

Parameter (unit)	n	Control		Bi^{2}tos		LactoShield		RFO		*P*-value
		Me/X	IQR/RMSE	Me/X	IQR/RMSE	Me/X	IQR/RMSE	Me/X	IQR/RMSE	
1 day										
PT (s)	15	52.10^{a}	40.10 - 53.10	98.08	77.30 - 105.10	46.60^{b}	42.50 - 57.40	>300^{a,b}	-	<0.001
aPTT (s)	15	41.85	31.95 - 54.45	46.40	39.60 - 51.10	47.60	44.00 - 54.10	51.70	41.80 - 63.50	0.130
Fibrinogen (g/L)	15	4.46^{a}	3.98 - 5.00	4.66^{b}	4.24 - 4.94	3.80^{a,b,c}	3.68 - 3.90	4.56^{c}	4.48 - 4.96	<0.001
21 day										
PT (s)	15	156.43	58.05	126.98	42.81	128.69	39.03	114.05	50.47	0.470
aPTT (s)	15	105.98	39.71	76.44^{a,b}	32.13	126.92^{a}	51.81	124.03^{b}	45.14	0.005
Fibrinogen (g/L)	15	3.80^{a}	3.58 - 4.04	3.78^{b}	3.62 - 4.72	4.00	3.60 - 4.77	4.60^{a,b}	4.16 - 5.50	0.010
42 day										
PT (s)	15	172.80^{a}	140.50 - 202.50	112.30^{b}	50.30 - 165.10	>300^{a,b,c}	-	146.60^{c}	107.40 - 230.80	0.031
aPTT (s)	15	158.37	40.95	144.04	44.18	166.00	29.00	126.30	51.72	0.520
Fibrinogen (g/L)	15	3.80	3.66 - 4.10	4.37^{a}	3.92 - 4.67	4.44^{b}	3.76 - 4.98	3.66^{a,b}	3.62 - 3.76	0.001

Me = median; IQR = interquartile range; n = number; *P* = probability value; aPTT = activated partial thromboplastin time; PT = prothrombin time; X = arithmetic mean; RMSE = root-mean-square error

^{a,b,c} Means with same letters differ significantly at $P < 0.05$ (differences between Control, Bi^{2}tos, LactoShield, RFO) over successive tests.

Table 6 Effect of prebiotic on PT, aPTT and fibrinogen concentration in broiler chickens

Parameter (unit)	n	1 day		21 day		42 day		*P*-value
		Me/X	IQR/RMSE	Me/X	IQR/RMSE	Me/X	IQR/RMSE	
Control								
PT (s)	15	52.10[a,b]	40.10 - 53.10	139.50[a]	107.00 - 201.30	172.80[b]	140.50 - 202.50	0.001
aPTT (s)	15	41.85[a,b]	31.95 - 54.45	110.50[a]	76.10 - 125.90	160.60[b]	131.10 - 182.35	<0.001
Fibrinogen (g/L)	15	4.57[a]	0.74	3.92[a]	0.79	3.94	0.41	0.011
Bi[2]tos								
PT (s)	15	99.08	25.94	126.98	42.81	109.23	57.46	0.400
aPTT (s)	15	46.40[a]	39.60 - 51.10	69.05[b]	52.40 - 100.00	156.50[a,b]	124.80 - 171.10	<0.001
Fibrinogen (g/L)	15	4.66[a]	4.24 - 4.94	3.78[a]	3.62 - 4.72	4.37	3.92 - 4.67	0.043
LactoShield								
PT (s)	15	46.60[a]	42.50 - 57.40	123.70	114.90 - 168.00	>300[a]	-	<0.001
aPTT (s)	15	47.60[a,b]	44.00 - 54.10	121.70[a]	91.70 - 166.10	166.00[b]	137.00 - 195.00	<0.001
Fibrinogen (g/L)	15	3.80	3.68 - 3.90	4.00	3.60 - 4.77	4.44	3.76 - 4.98	0.080
RFO								
PT (s)	15	>300[a,b]	-	96.40[a]	80.15 - 147.95	146.60[b]	107.40 - 230.80	0.003
aPTT (s)	15	51.70[a,b]	41.80 - 63.50	119.25[a]	83.30 - 164.40	138.10[b]	69.70 - 171.10	<0.001
Fibrinogen (g/L)	15	4.56[a]	4.48 - 4.96	4.60[b]	4.16 - 5.50	3.66[a,b]	3.62 - 3.76	<0.001

Me = median; IQR = interquartile range; n = number; *P* = probability value; aPTT = activated partial thromboplastin time; PT = prothrombin time; X = arithmetic mean; RMSE = root-mean-square error

[a,b,c] Means with same letters differ significantly at $P < 0.05$ (differences between days 1, 21 and 42) over successive tests.

5. DISCUSSION

The TF/TFPI axis plays an important role in hemostasis (Kasthuri *et al.*, 2010). TF, a 263 amino acid, 47 kDa transmembrane glycoprotein, is the cellular receptor for plasma factor VII/VIIa. It forms the TF/FVIIa complex, which is the principal initiator of *in vivo* coagulation during tissue injury, under both physiological and pathophysiological conditions (Kasthuri *et al.*, 2010; Owens III and Mackman, 2010; Rao and Pendurthi, 2012; Langer and Ruf, 2014). This complex is inhibited by TFPI, a 276 amino acid, 42 kDa glycoprotein synthesized mainly by epithelial cells (Kasthuri *et al.*, 2010; Owens III and Mackman, 2010; Holroyd *et al.*, 2012). To maintain normal hemostasis, levels of TF and TFPI need to be balanced (Kasthuri *et al.*, 2010). Maintenance of this balance can be assisted by the administration of bioactive substances, including prebiotics. This may inhibit the inflammatory state (Petkova *et al.*, 2012; Saad *et al.*, 2013), enhance the immune response and improve feed conversion and digestion (Bozkurt *et al.*, 2009). Interestingly, the effect of prebiotics on the hemostasis system has never been investigated in humans and animals. Only the influence of probiotics has been studied. The latest research with animal models demonstrated that probiotics reduce tissue injury (Zelaya *et al.*, 2012; 2013; 2014a, b), which has a marked effect on improving the general condition of animals. This being so, it seems important to investigate the TF-TFPI axis in healthy animals and following *in ovo* injection of selected prebiotics in broiler chickens.

Measurement of PT and aPTT is commonly used to evaluate the function of plasma factor-dependent hemostasis. PT is particularly sensitive to deficiencies of factors V, VII, IX, and X, prothrombin, and fibrinogen (factor I), which are mainly synthesized in the liver; thus, this parameter indirectly reflects hepatocyte function (Thomson *et al.,* 2003; Yeh *et al.,* 2008). The evaluation of aPTT in birds is problematic because they have been shown to contain no contact factors (XII and XI), which makes aPTT significantly longer than in other species of animals (Shibatani *et al.,* 1997; Gentry, 2004). aPTT is particularly sensitive to deficiencies of plasma factors II, V, VIII, IX, X, XI, XII, and fibrinogen. The determination of fibrinogen concentration is an important parameter with regard to the evaluation of prohemorrhagic tendencies. Fibrinogen is the main protein involved in blood coagulation, and its concentration in both humans and animals is many times higher compared to other coagulation factors (Thomson *et al.,* 2002; Gentry, 2004; Tentoni *et al.,* 2010). Fibrinogen is an acute phase protein, its concentration is increased by proinflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor (TNF-α) (Shibatani *et al.,* 1997; Gentry, 2004; Tentoni *et al.,* 2010). However, a recent study (Resnyk *et al.,* 2013) using transcriptome analysis in broiler chickens showed that many proteins involved in blood coagulation have a higher expression in abdominal fat, which suggests that abdominal fat may be a major site of blood coagulation factor synthesis in broiler chickens. The ban imposed by the European Union on antibiotic growth promoters has compromised the efficiency of disease prevention in poultry. As a result, an attempt was made to use prebiotics as an alternative to antibiotics. The current literature contains

no studies that analyse the effect of prebiotics on avian cogulation system. Therefore, this study was designed to determine the effect of several different prebiotics on blood clotting parameters in broiler chickens depending on their age.

The main conclusions from this study are as follows: (1) the presence of TF and TFPI has been shown for the first time in chickens; (2) significant effect on TF concentration between different days of rearing has been found in the study groups; (3) Bi^{2}tos prebiotic resulted in no TFPI being detected in the blood plasma of one-day-old broiler chickens; (4) physiological saline solution as well as prebiotics (except RFO) had a statistically significant effect on TFPI concentrations on different days of rearing; (5) in the control group, which was administered *in ovo* with a physiological saline solution, PT and aPTT were found to increase with age and fibrinogen concentration decreased; (6) the transient changes in the analysed parameters depend on the prebiotics administered *in ovo* as well as on the age of broiler chickens.

Many studies have been conducted to evaluate the hemostatic process in animals, but the function of TF and its physiological inhibitor, TFPI has not been described in birds. To the best of available knowledge, this is the first study addressing the TF-TFPI axis in broiler chickens. Understanding the role of TF and TFPI is essential for understanding basic coagulation physiology. The manufacturer of species specific immunoassays (Chicken TF and TFPI ELISA kit; BlueGene, Shanghai, China) provides no laboratory standards for total concentrations of TF and TFPI. A search of publications concerning avian hemostasis

revealed no studies on TF and TFPI concentrations. Based on the available literature, it may be compare to the values for adult broiler chickens (42 days old) with the values obtained in a group of healthy adult humans. The present findings suggest that total TF concentration in 42-day-old broiler chickens (58.61-97.34 pg/mL) is similar to the values observed in healthy adult humans (18-290 pg/mL). It should be highlighted that the current results and others have demonstrated a wide scatter of plasma concentrations of these proteins in both birds and humans. What is more, in contrast to TF concentration, much lower total TFPI concentrations were observed at 42 days of rearing in the control group (0.03-0.45 ng/mL) compared to the reference values obtained by other authors in healthy humans, which range from 75 to 125 ng/mL (Uszyński *et al.,* 2001; Parhami-Seren *et al.,* 2006; Kotschy *et al.,* 2010; Świtońska *et al.,* 2015). Both the similarities in TF concentrations and considerable differences in TFPI concentrations between poultry and humans may confirm that avian hemostasis system is distinct from that of humans. The physiological role of TF in poultry has not been studied, but in humans this glycoprotein plays a major role in the development of thrombosis (Steffel *et al.,* 2006). It is worth noting, however, that the conclusions from our study should be confirmed with a larger number of animals.

The TF-TFPI axis is strongly associated with inflammation (Cesaro *et al.,* 2011; Compare *et al.,* 2012; Chassaing *et al.,* 2014). The present work is a pilot study in which inflammatory parameters were not analyzed, but it is worth discussing these interrelationships

in the light of current research. Endotoxins and cytokines are known to increase the expression of prothrombotic TF, leading to activation of the coagulation system (Pawlinski *et al.,* 2004). Prebiotics have been shown to reduce endotoxin absorption in animals by improving the expression and activity of proteins involved in gut-barrier function (Delzenne *et al.,* 2013). This decreases plasma endotoxin concentrations, thus considerably reducing the host's susceptibility to gut infections and atopic disorders (Candela *et al.,* 2010). In addition, prebiotics used in animals may alleviate acute inflammation by reducing IL-1β production and stimulating TGF-β and IL-10 synthesis in the intestinal mucosa (Girrbach *et al.,* 2005; Hoentjen *et al.,* 2005; Osman *et al.,* 2006).

It is worthy of note that this study showed a lower TF concentration on 1 and 21 days of age in the study groups compared to the control group, however, differences were not statistically significant. Nevertheless, it may be postulated that giving prebiotics can confirm its protective function against tissue injury, because lower TF amounts are released into the blood plasma of broiler chickens. Furthermore, TF concentration tended to increase as birds grew older in both control and study groups. This phenomenon can be explained by the fact that inflammation may increase local expression of proinflammatory cytokines. Under physiological conditions, TF expression in mouse and human tissues is regulated positively by cytokines, among others (Luther *et al.,* 1996). Proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α release TF from monocytes/macrophages and epithelial cells, resulting in hemostasis disorders (Muramoto *et al.,* 2006).

It is interesting that prebiotics have different effects on plasma TFPI concentrations in broiler chickens. A particularly interesting observation concerns Bi^{2}tos prebiotic, which resulted in no TFPI being detected in the blood plasma of one-day-old broiler chickens. According to Pedersen *et al.* (2005), lack of TFPI in humans leads to unregulated activity of the TF/FVIIa complex, which results in disseminated intravascular coagulopathy and secondary bleeding. The physiological role of TFPI in poultry is not understood. In humans, this glycoprotein is the only inhibitor active in the initial phase of blood coagulation to prevent the formation of thrombin. Low blood concentration of TFPI is a risk factor for thrombosis and liver diseases (Kotschy *et al.*, 2010; Reglińska-Matveyev *et al.*, 2014). Low TFPI concentration in relation to healthy subjects is likely caused by cleavage of TFPI by inflammatory cell proteases (Asakura *et al.*, 2001; Liczko *et al.*, 2014). This phenomenon requires further study to be explained.

It is also worth stressing that the withdrawal of antibiotics from poultry production increased the incidence of necrotic diseases such as enteritis. Substitutes for antibiotics are needed to maintain poultry production in order to meet global demand. Among the feed additives evaluated to date in poultry, prebiotics are considered a favourable alternative, because they can promote competitive exclusion of pathogenic microbes and selective colonization by beneficial microbes (Janardhana *et al.*, 2009). What is more, few studies address bioactive substances administered early during embryogenesis, but the experiments performed to date confirm that *in ovo* injection has a beneficial effect on the development of the normal intestinal microbiome (Villaluenga

et al., 2004; Pilarski *et al.,* 2005; Sławińska *et al.,* 2014a, b). Recent study performed with 1.9 million broiler chickens under production conditions demonstrated that *in ovo* injection of very small prebiotic doses may successfully replace their use as feed additives for broiler chickens (Bednarczyk *et al.,* 2011). It is difficult to determine the mechanisms that integrate gut microflora after prebiotic administration with changes in hemostasis parameters. It is generally accepted that symbiotic gut bacteria in animals have a positive effect on their organism. Disturbances in the intestinal microflora may cause liver, fatty tissue, kidney and pancreas dysfunction (Tremaroli and Bäckhed, 2012). Therefore, it is of particular importance to create an adequate environment for microorganisms in the intestine following prebiotic administration (Bednarczyk *et al.,* 2011). The modes of action of bioactive substances administered *in ovo* are complex, but their positive effect on the hemostatic system still needs to be explored by researchers.

The mode of action of bioactive compounds administered *in vivo* is complex, and research concerning the effect of these substances on the hemostatic system is fragmentary. The present work is one of the first study to investigate the effect of different prebiotics on blood coagulation parameters during the growth of broiler chickens. An open issue that requires further investigation is to determine reference values for basic blood clotting parameters in broiler chickens.

Vitamin K plays a key role in maintaining hemostasis. It is an essential nutrient in the maintenance of normal hemostasis, including the synthesis of some coagulation factors (II, VII, IX, X)

in the liver. In addition, vitamin K, which is synthesized by the microbiome together with vitamin D, plays a crucial role in the incorporation of calcium into bones (Hauschka, 1989; Koreleski *et al.,* 2003; Gentry *et al.,* 2008). Modulation of the microbiome by bioactive compounds or antibiotics may alter vitamin K synthesis in birds. Griminger (1970) confirmed that vitamin K deficiency impairs the synthesis of plasma clotting proteins (II, VII, IX, X), which is reflected in significantly prolonged PT.

When analysing the effect of a prebiotic, it is necessary to account for the composition of feed that contains α-tocopherol. Vitamin E causes antagonistic inhibition of vitamin K by α-tocopherol, which may result in vitamin K deficiency in broiler chickens. Other important factors that may interfere with the hepatic synthesis of prothrombin complex are antibiotics and coccidiostats. Feeds can also contain coumarin and its derivatives (which are found, among others, in cleavers), as well as mycotoxins, which may contribute to bleeding diathesis (Koreleski *et al.,* 2003; Gentry, 2004; Gentry *et al.,* 2008).

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 increase almost 3 times compared to homologous thromboplastin (Griminger, 1970; Glazunova, 1972; Doerr *et al.,* 1975; 1976; Fernandez *et al.,* 1995; Lewis, 1996; Baldizán *et al.,* 2010; Zeryehun *et al.,* 2012). 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 has been 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 who used rabbit tissue thromboplastins in poultry (Doerr *et al.*, 1975; Lewis, 1996; Pliszczak-Krol *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.

Analysis of aPTT in poultry showed that it increased during the growth of the chickens. Other authors (Doerr *et al.*, 1981a; 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. The most characteristic change resulting from the experiment is that aPTT in both the control group and in the prebiotic groups increased together with the growth of the broilers. The increase in 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. aPTT was found to increase in chickens experimentally infected with infectious bursal disease virus and in those infected with *Erysipelothrix rhusiopathiae* bacteria, which

cause erysipelas in poultry (Shibatani *et al.,* 1997; Zeryehun *et al.,* 2012). In the current study, the increase in aPTT was most significant between 1 and 21 days of age. aPTT is particularly sensitive to deficiencies of factors VIII and IX. The present study does not allow to define the mechanisms involved in such a significant increase in aPTT. It should be taken into account, however, that prebiotic use has no effect on this mechanism, because the same trend, in which aPPT increased with age, was observed in the control and prebiotic groups.

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 (Shibatani *et al.,* 1997; Baldizán *et al.,* 2010; Nazifi *et al.,* 2010; Pliszczak-Krol *et al.,* 2012). Nazifi *et al.* (2010) showed that a viral infection (with infectious bursal disease virus) has no effect on fibrinogen concentration. Furthermore, ochratoxin A contamination of feed was found to have no influence on decreased fibrinogen synthesis, although it is a characteristic feature of ochratoxicosis (Doerr *et al.,* 1981b). In addition, experiments with broiler chickens revealed that administration of *Escherichia coli* lipopolysaccharide may cause major blood coagulation disorders. As is known the lipopolysaccharide induces an inflammatory reaction, which is associated with major disturbances in blood coagulation (Pliszczak-Krol *et al.,* 2012).

The present study suggests that with the age of broiler chickens, the hemostatic system changes through increases in PT and aPTT.

The observed changes may show the increasing tendency towards bleeding with age, which translates into the quality of the meat obtained (Kranen *et al.*, 2000a, b). The application of prebiotics does not alter this trend significantly. Changes in the blood clotting process, observed in the studied groups, are dependent on the time and type of prebiotic used. Based on the present study it cannot be determined if *in ovo* administration of prebiotics leads to major changes in the blood coagulation process. Interpretation of the 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. Another important component to explain blood clotting mechanisms in broiler chickens would be to determine the activity of different blood clotting factors and their physiological inhibitors in birds.

6. CONCLUSIONS

1. This pilot study showed the presence of TF and TFPI in the blood plasma of broiler chickens;
2. The *in ovo* injection of prebiotics and physiological saline solution to broiler chicken embryos have different effects on variation in TF and TFPI concentration during rearing;
3. Physiological saline solution as well as prebiotics (except RFO) were observed to have a statistically significant effect on TFPI concentrations on different days of growth;
4. The *in ovo* administration of prebiotics causes some changes in the basic blood coagulation parameters such as PT, aPTT, and fibrinogen concentration. However, this effect is highly variable and in some cases shows a trend similar to that in the control group.

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8. ABSTRACT

The tissue factor (TF) – tissue factor pathway inhibitor (TFPI) axis plays a major role in hemostasis, but the function of these proteins has not been characterized in birds yet. 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. Both prolonged and shortened blood clotting times as well as changes in fibrinogen concentration may be indicative of disorders of the hemostatic system. However, there are no reports in the literature concerning the effect of prebiotic administration on the hemostatic system in birds. The objective of the study was to determine blood coagulation parameters (total TF and TFPI concentrations, PT, aPTT and fibrinogen concentration) in broiler chickens depending on their age and to assess the effect of *in ovo* injection of some prebiotics on variation in hemostasis mechanisms during rearing of broiler chickens. The study was conducted with 100 000 broiler chick embryos, the air cells of which were injected at 12 days of incubation with prebiotics (Bi^2tos, LactoShield and RFO) or physiological saline solution (control group). At 1, 21 and 42 days of rearing, blood was sampled from 15 broiler chickens, randomly chosen from each group. Next, the enzyme immunoassay was performed to determine plasma TF and TFPI concentrations and PT, aPTT and fibrinogen concentration were determined in the chickens' blood. This pilot study showed the presence of TF and TFPI in the blood plasma of broiler chickens. The *in ovo* injection of prebiotics and physiological saline solution to broiler chicken

embryos have different effects on variation in TF and TFPI concentration during rearing. Physiological saline solution, as well as prebiotics (except RFO) were observed to have a statistically significant effect on TFPI concentrations on different days of growth. The *in ovo* administration of prebiotics causes some changes in the basic blood coagulation parameters such as PT, aPTT, and fibrinogen concentration. However, this effect is highly variable and in some cases shows a trend similar to that in the control group. Nevertheless, further studies are needed in order to confirm these finding.

Keywords: broiler chicken, hemostasis, *in ovo*, prebiotic

STRESZCZENIE

Oś czynnika tkankowego (TF) - inhibitora szlaku zależnego od czynnika tkankowego (TFPI) odgrywają ważną rolę w hemostazie, ale funkcja tych białek nie została scharakteryzowana u ptaków. W diagnostyce zaburzeń układu krzepnięcia najczęściej stosowane są testy przesiewowe, takie jak: czas protrombinowy (PT), czasu częściowej tromboplastyny po aktywacji (aPTT) oraz stężenia fibrynogenu w osoczu krwi. Zarówno wydłużenie jak i skrócenie czasów krzepnięcia krwi oraz zmiany w stężeniu fibrynogenu mogą wskazywać na zaburzenia układu hemostazy. W piśmiennictwie brakuje jednak doniesień dotyczących wpływu podania prebiotyku na układ hemostazy u ptaków. Celem badań było określenie parametrów krzepnięcia krwi (stężenia całkowitego TF i TFPI oraz PT, aPTT i stężenia fibrynogenu) u kurcząt brojlerów w zależności od ich wieku oraz ocena wpływu podania w wyniku iniekcji *in ovo* wybranych prebiotyków na zmienność mechanizmów układu hemostazy podczas odchowu kurcząt brojlerów. Badanie przeprowadzono na 100 000 zarodków kurcząt brojlerów, którym w 12. dobie inkubacji podawano do komory powietrznej wybrane prebiotyki (Bi^{2}tos, LactoShield i ORR) lub roztwór soli fizjologicznej (grupa kontrolna). W 1., 21. i 42. dniu odchowu od 15 losowo wybranych kurcząt brojlerów z każdej grupy pobierano krew. Następnie przeprowadzono badania immunoenzymatyczne w celu poznania stężenie TF i TFPI oraz oznaczono PT, aPTT i stężenia fibrynogenu w osoczu krwi kurcząt brojlerów. Badania pilotażowe wykazały obecność TF i TFPI w osoczu krwi kurcząt brojlerów. Podanie *in ovo* prebiotyków i roztworu soli fizjologicznej zarodkom kurcząt brojlerów

wpływało w różny sposób na stężenie TF i TFPI u kurcząt brojlerów. Zaobserwowano, że roztwór soli fizjologicznej oraz prebiotyki, z wyjątkiem ORR wpływały istotnie statystycznie na stężenie TFPI w poszczególnych dniach odchowu. Stosowanie prebiotyków *in ovo* powoduje pewne zmiany w ocenie podstawowych parametrów krzepnięcia krwi, takich jak PT, aPTT i stężenia fibrynogenu. Jednakże wpływ ten jest bardzo zróżnicowany i w niektórych przypadkach wykazuje trend podobny do grupy kontrolnej. Niemniej jednak konieczne są dalsze badania w celu potwierdzenia takiego wnioskowania.

Keywords: kurcze brojler, hemostaza, *in ovo*, prebiotyk