UNIVERSITATEA DE ȘTIINȚE AGRICOLE ȘI MEDICINĂ VETERINARĂ "ION IONESCU DE LA BRAD" IAȘI

LUCRĂRI ȘTIINȚIFICE

VOL. 63
MEDICINĂ VETERINARĂ

PARTEA 1

EDITURA "ION IONESCU DE LA BRAD" IAȘI

2020

ELECTRONIC ISSN: 2393-4603
PRINT ISSN: 1454-7406

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The management of canine transfusion reactions reported in some clinics from Transylvania

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Abstract
The transfusion of blood products is an essential and also a common therapeutic procedure used in veterinary medicine. Because blood transfusion is not a completely harmless therapeutic procedure, its usage requires a good amount of knowledge about the possible adverse effects and complications that may occur during this process. This kind of data is exactly what the present study brings to doctors attention, being based upon the management of various forms of transfusions reactions in canines which were given whole blood, erythrocyte concentrate (EC) or blood plasma (P). The main objectives were preventing, monitoring and treatment of this reaction type. The clinics included in this study reported multiple forms of transfusion reactions in canine patients, such as: severe tachycardia (no=5); passing hyperthermia (40°C) (no=5); emesis and melaena during transfusion (no=3); myoclonic head seizures and bruxism (no=1); delayed hemolytic anemia (AHI) (no=1); TRALI type respiratory syndrome (no=1). In most cases, these symptoms subsided after a few minutes from transfusion or stopped completely, except the last two cases, which presented severe reactions, without any response to treatment and resulting in death. This kind of complications resulted due to AHI condition in a patient with chronic renal failure (the diagnosis was based on pollakiuria, haematuria and BUN/creatinine ratio of 28.4) and the evolution of TRALI respiratory syndrome in another patient with malignant multicenter lymphoma (cytologically confirmed). The AHI type of post transfusion reaction diagnosis and management was done by monitoring the decreasing oscillations of the whole blood mass, after 3 transfusions with whole blood. The TRALI syndrome (Transfusion-Related Acute Lung Injury) diagnosis was based upon cytological examination and later, necropsy.
Keywords*: adverse reactions, blood products, dog, transfusion.*

Introduction

Transfusion adverse reactions are the most common complications after therapy with blood products, and in humans, they occur at least once in one hundred cases (Delaney et al., 2016; Ognean, 2017). Similar reactions with those encountered in humans have been frequently reported in pets, without a correct diagnosis and treatment in most of the cases. These kinds of reactions are commonly caused by transfusion incompatibility and sometimes by inappropriate storage or administration of blood products. The majority of the adverse reactions occur during the transfusion process or right after it and they present acute hemolytic, anaphylactic or allergic reactions. Apart from these evident reactions, there are types of complications that cannot be immediately identified, such as: delayed hemolytic reaction, immune and non-immune reactions, hypothermia, citrate intoxication or heart failure (Abrams-Ogg, 2000; Kohn et al., 2000; Hohenhaus, 2006; Ognean, 2017).

Among the principles that stand upon prevention and management of adverse reactions, we mention selection of the best compatible donor patient, proper storage and handling of blood products, patient monitoring and care during transfusion process, immediate stopping of transfusion when adverse reactions occur, immediate treatment of allergic or anaphylactic reactions with antihistaminic or cortisone products; adding adrenergic and antipyretic medication to the treatment protocol in case of fever and intravenous use of calcium in case of citrate intoxication symptoms.

Material and methods

The present paper was designed to present the management of complications and transfusion reactions occurred in canine patients transfused with whole blood, erythrocytes concentrate (EC) or plasma (P) in some veterinary clinics from Transylvania. The major purpose of this research was to evaluate the efficiency of some preventive methods and the adverse transfusion reactions treatment in dogs from clinics and veterinary practices.

The evaluation process was carried out by 3 clinics and 4 veterinary private practices, which reported 16 forms of complications/transfusion reactions, such as: severe tachycardia - 5 cases; passing hyperthermia (40°C) - 5 cases; emesis and/or melaena during transfusion - 3 cases; myoclonic head seizures and bruxism - one case; delayed hemolytic anemia (AHI) - one case; TRALI (Transfusion-Related Acute Lung Injury) type respiratory syndrome - one case. Patients evaluation and decision making of blood transfusion was based upon the correlation between the clinical, haematological and biochemical examination, with automated or semi automated hematology analyzers (Abacus Junior Vet and Idexx QBC Vetautoread) and automated analyzer for clinical chemistry (Arkray Spotchem EZ-SP-4430 Refurbished and MINDRAY BA-88[a]). In some cases of severe illness, the diagnosis confirmation was carried out at SinevoVet or other diagnostic centers.

The transfusion of blood products was made, in most of the cases, after establishing the donor-patient compatibility with blood type tests (Rapid Vet®H-DMS Laboratories and Rapid DMEVET-Alvedia) and/or Crossmatch. We observed that 4 of the patients were transfused without compatibility evaluation, the reason invoked being the emergency of the procedure and the absence of transfusion risk. The blood products were administered exclusively intravenously (cephalic vein, saphenous vein and jugular vein), by using closed circuit IV (intravenous) set tubing and catheters, which were previously selected according to patients size; in some cases, blood filters were used.

The blood product dose was set after the correlation between body weight and anemia severity and erythrocytes mass values (RBC, HCT and Hb). Some of the doctors evaluated even the lost blood volume, using the well known formula: "Given blood volume (mL) = 80 x kg x (desired Ht - receiving patients Ht)/donors Ht" (Abrams-Ogg, 2000). Taking into account the aforementioned factors, the calculated doses for whole blood and EC varied between 10 and 20 mL/kg. Two of the patients were treated with CE, diluted with saline (3:1), for the administration ease. The transfusion rate, in the first 30 minutes was of 0.3-3 mL/kg/h, upping the dose to 10 mL/kg/h. Moreover, during the entire transfusion process, some of the basic physiological parameters were monitored, namely respiratory rate and internal temperature in every patient.

The values obtained from blood chemistry tests were statically and graphically analyzed, by using GraphPad Instat, Excel, Prism 4th version and OriginLab 8.5 programs. Because most complications subsided after a few minutes of pausing the transfusion or completely stopped, we continue with two exceptions, resulting due to AHI and TRALI type transfusion reactions, characterized by severe evolution, unfavourable therapeutic response and patients death.

AHI type transfusion reaction was observed in a patient (6 years old, unneutered, female), suffering from chronic renal failure, with BUN (71 mg/dl) and creatinine values (28.4 mg/dl), complicated with severe non regenerative chronic anemia, low values of RBC (1.46 T/L), HCT (6.3%), Hb (2.1 mg/dl) and VEM (42mg/dl), critical clinical status and unfavourable prognosis. Based on the severity of the anemia, the patient received an immediate first transfusion with whole blood (one unit - 450 mg, on CPAD1), collected from a donor (male, half-breed, vaccinated and dewormed), without any blood compatibility tests done prior. This patient received in previous other 2 compatible transfusions, a unit of whole blood, in order to correct the mild increase of

erythrocyte indices, 24 hours after transfusion, and to correct the decreasing trend of these values in the next 30 days.

TRALI respiratory syndrome was diagnosed in a patient (male, Rottweiler breed, 7 years old, DEA 1.1 positive), suffering from a multicenter lymphoma, cythologically confirmed. This patient was undergoing treatment for arthritis and renal failure 1st grade, and it was brought to the clinic because he presented loss of appetite, listlessness, hypersalivation, mass weight loss and dysphagia. Clinical examination revealed fever, hypertrophy of the prescapular and popliteus lymph nodes and tonsils hypertrophy. Blood tests were performed and biopsy was performed form prescapular and popliteal lymph nodes, in order to establish a diagnosis certainty. Based upon the conducted tests and the obtained results, a complex therapeutic protocol was elaborated, focusing on chemotherapy (cyclophosphamide, doxorubicin, vincristine and prednisone), symptomatic treatment and whole blood transfusion. Unfortunately, this patient developed an extremely severe form of TRALI reaction, 72 hours after the second transfusion (with one whole blood unit), which was followed by increased values of Ht (19%) and Hb (6,9 mg/dl) and finally, a third transfusion with whole blood was necessary.

Results and disscussions

During the whole blood, EC and P transfusions, in all the canine patients subjected to this study, no severe adverse reactions, such as acute intravascular hemolysis or anaphylactic shock were observed. However, some mild intensity transfusional reactions were recorded in 5 cases, namely the increasing pulse and heart rate. The main measure chosen for subsidising these complications was to interrupt, even for a few minutes the transfusion, until the heart rate normalized - in 3 cases and in another 2 cases - the solution was to completely stop the transfusion. We have also noticed an increased sensitivity in peripheral veins, punctured for administration of whole blood and EC, characterized by patients agitation, without causing phlebitis or thrombophlebitis. In case of plasma administration, no side effects were registered. In 5 of the cases, temporary transfusion reactions were observed, such as hyperthermia (40°C), which occurred 24 hours after the transfusion, but without any complications.

The emesis and melaena symptoms during transfusion, in 3 patients with parvovirosis, were considered to be complications of this intensive treatment procedure. Another post transfusion reaction consisting of myoclonic head seizures and bruxism was present in one patient, aged 3 months, after the second transfusion with whole blood. According to this patient's anamnesis, we must recall three surgical procedures, namely an osteosynthesis for treating a fractured injury resulted after a car accident and two enterectomy procedures after an episode of recurrent intestinal volvulus. Additionally, the patient also developed a parasitic infestation which hastened its death. As expected, important increases in serum total bilirubin were reported in most patients, exceeding the maximum allowable limits (3.6 mmol/L) only in the first 24 hours after whole blood, EC and even P transfusion, but these values normalized shortly after.

Regarding the patient with AHI, we mention that 24 hours after the first transfusion with one unit of whole blood, it presented mild increases of the erythrocyte indices, which determined the requirement of another two transfusions, each of them performed with one unit of whole blood, during two months. The post- transfusion data, presented in table 1, revealed that RBC values increased the following day to 2.33 T/L and the HCT values to 13.5%; on the third day after transfusion we observed a decrease in values to 1.81 T/L, for RBC and 10.2%, for HCT. The evolution of erythrocyte indices included mild increases of RBC (4.7 T/L) and HCT (27.2%) within the 4-30 days interval, followed by an important decrease of these values, two months post transfusion, when the patient health worsened considerably. Based on this evolution, we consider

that the investigated patient developed a delayed hemolytic reaction, because the transfusion with whole blood was done without testing the donor-patient compatibility prior to transfusion and it was not followed by an important increase of erythrocyte indices, which may indicate a possible rejection of the administered red blood cells. Furthermore, the data presented in table 1 indicate that after the last transfusion, the decreasing trend of erythrocyte values amplified and the low values of RBC (1.55 T/L) and HCT (11.2%) were associated with a worsening health state. Under these conditions, intensive care measures were taken, with hydration intravenous (IV) infusion and glucocorticoids to lower creatinine levels. Furthermore, it is noteworthy to mention the severe chronic renal failure evolution, which also led to important changes in blood leukocyte and biochemical indices. Therefore, we can only mention the very high values of leukocytosis (21-42 G/L) and granulocytosis (78.8-84.4%), maintained in the first 30 days of the survival period.

Equally important were metabolic indices changes, such as blood sugar fluctuations (101-228 mg / dL), associated with increased values of BUN (40-155 mg / dL) and creatinine (2.5-11.2 mg / dL) (Table 1). Finally, all the undergoing measures proved to be inefficient, because the patient went into cardio respiratory arrest and died.

Table 1.
Haemato-biochemical parameters evolution in a patient with AHI condition

Parameter/Day	References	1	2	3	4	5	6	8	12	33	00	660
RBC (T/L)	5-7.9	1.49	2.33	1.81	2.08	2.2	2.5	3.18	4.8	4.7	1.55	
HCT (%)	35-57	6.3	13.5	10.2	11.02	11.9	13.7	17.8	21.7	27.2	11.2	
HGB (g/dL)	12-19	2.1	4.7	3.7	3.9	4	4.6	6.2	6.7	9.6	4.4	
MCV (µm3)	66-77	42	58	56	54	54	55	56	53	58	72	
MCH (pg)	21-26.2	13.9	20.1	20.5	18.9	18.4	18.3	19.5	16.4	20.5	28.2	
MCHC (g/dL)	32-36.3	32.9	34.6	36.5	34.8	34	33.4	34.9	30.9	35.5	38.9	
RDW (%)	14-17	17.9	22.4	22.2	24.7	25.6	24.4	22.5	23.4	21.9	13	
WBC (G/L)	5-14.1	33.8	33.9	30.1	25.4	28.9	39.6	-	42	29.5	9	
GR(%)	58-88	71.7	84.2	84.4	82.1	83	80.6	-	78.8	84.2	87.4	
LYM (%)	8-21	21	10.5	10.5	14	13.2	14	-	13.4	11.3	8.2	
MONO (%)	2-10	7.3	5.3	5.1	3.9	3.8	5.4	-	7.8	4.5	4.4	
PLT (x10^9/L)	211-621	280	355	250	460	697	675	642	571	616	425	
Glu. (mg/dL)	76-119	76-119	228	114	152	101	118	141	115	115	144	
BUN (mg/dL)	8-28	8-28	71	55	57	45	40	49	53	43	155	
Tbili. (mg/dL)	0-0.3	0-0.3	0.3	0.5	-	-	1.1	1.2	1.0	0.9	-	
Ca (mg/dL)	9.1-11.7	9.1-11	10.5	-	-	-	-	-	12.7	-	-	
Tprot. (g/dL)	6.0-7.5	5.4-7.5	7.9	8.1	8.6	8.6	9	8.5	8.9	7.3	6.3	
Alb. (g/dL)	2.3-3.1	2.3-3.1	2.1	2.2	-	-	-	-	2.6	-	1.9	
ALT (UI/L)	22-47	10-109	26	-	11	11	15	8	6	6	118	
ALP (UI/L)	1-114	1-114	108	146	204	190	224	218	216	190	77	
Crea. (mg/dL)	0.5-1.7	0.5-1.7	2.5	2.9	4.7	4.7	4.4	3.9	3.1	2.5	11.2	

Legend: **RBC**-Red blood cells; **HCT**-Haematocrit; **HGB**-Haemoglobin; **MCV**-Mean corpuscular volume; **MCH**-Mean corpuscular hemoglobin; **MCHC**-Mean corpuscular hemoglobin concentration; **RDW**-Red cell distribution width; **WBC**-White blood cells; **GR**-Granulocytes; **LYM-**Lympocytes; **MONO**-Monocytes; **PLT**-Platelets; **Glu**-Glucose; **BUN**- Blood urea nitrogen; **Tbili**.-Total bilirubin; **Ca**-Calcium; **Tprot**.- Total serum protein; **Alb**.- Albumin; **ALT**-Alanine aminotransferase; **ALP**-Alkaline phosphatase; **Crea**.-Creatinine.

Regarding TRALI respiratory syndrome evolution, we have observed that this post transfusion reaction manifested in an extremely severe form, although the patient presented slight

improvements of the health state and hematologic parameters right after the transfusion with whole blood (Table 2). 72 hours after the second transfusion, with one unit of whole blood, the values of HTC (19%) and Hb (6,9 mg/dl) decreased (Table 2), which determined the transfusion of another unit of whole blood. 24 hours after the last transfusion, the health state worsened, the patient presenting emesis, severe dyspnea, fever, pale mucosa, tachycardia, decubitus position. Under these conditions, the emergency therapeutic protocol was supplemented with oxygen therapy, intravenous rehydration, vitamins, furosemid, antacids, antiemetics, and liver protection. Despite the implemented measures, the onset of decompensated shock was inevitable, with cardio-circulatory arrest and patient death.

The necropsy examination emphasized specific changes for TRALI syndrome, due to the presence of a foamy fluid in the trachea and in the entire bronchial tree, and dense formations the size of a millet, onto the entire surface of the lung.

Most of the adverse transfusion reactions reported in this study are undesirable metabolic or immunological disturbances that may occur frequently during or after administration of blood products (Mcdevitt et al., 2011; Ognean, 2017). Moreover, they were of mild intensity and did not present any threat to the patients life.

Table 2.
Main haematological parameters evolution in a patient with TRALI syndrome

Parameter	Evolution of haematological parameters prior and post transfusion						References
	First transfusion		Second transfusion		Third transfusion		
	Prior	Post	Prior	Post	Prior	Post	
HTC (%)	21.5	21.9	20.8	24.3	19	23.2	37-55
HGB (g/dL)	7	7.9	6.9	8.3	6.9	8.3	12-18
WBC (G/L)	26	26.7	17.7	16.1	24.1	11.5	6-16.9
GR (G/L)	25.5	25.1	16.3	14.1	22.7	7.9	3.3-12
PLT ($x10^9$/L)	476	323	547	694	272	170	175-500

Legend: **HCT**-Haematocrit; **HGB**-Haemoglobin; **WBC**-White blood cells; **GR**-Granulocytes; **PLT**-Platelets.

The early administration of citrate, in an weakened patient, with an underlying condition of hypocalcemia, can result in the so called citrate intoxication (Lucas et al., 2004; Ognean, 2017); therein, we can explain the existence of myoclonic seizures and bruxism in one case. Other signs that can occur in these situations are tetany, hyperreflexia, epileptic form seizures, laryngeal spasms and even respiratory arrest (Giger et al., 1990). None of the patients presented any clinical signs of an acute hemolytic reaction.

Generally, in the case of an acute hemolytic reaction, serum and urinary levels of hemoglobin increase in a matter of minutes after transfusion and the incompatible cells are removed from the circulatory system flow in less than 2 hours (Capon et al., 1995). Concerning the investigated patients included in this study, the post transfusion decrease of the total serum bilirubin levels sustained the positive evolution and not at all the onset of intravascular hemolysis, which had to be represented by hyperbilirubinemia (Weingart et al., 2004). The existence of emesis and melaena symptoms in 3 cases, could be attributed to parvovirosis and not to transfusion complications, although some authors claim that acute hemolytic reaction may go undetected or even falsely attributed to an underlying disease (Kessler et al., 2010). Hyperthermia presented by

some patients receiving compatible blood, was due to the platelets or leukocytes amount brought by the administered blood. We mention that the non hemolytic fever is frequently associated with increased anti leukocytes antibodies to receptors. Moreover, one analysis based upon 348 transfusion cases in dogs at the Berlin small animals clinic described the evolution of some transfusion reactions caused by the administration of CE, in 4 patients and the administration of P in 2 patients, these aspects representing only 1.7% of the cases (Kohn et al., 2000). Reitemeyer et al. (2000) identified 2.2% of temporary transfusion reactions, during the procedure or immediately after the administration of red blood cells products in 186 dog patients. In general, the frequency of transfusion reactions, detected in this study, as well as by other researchers in the field is decreased, these being controlled by pre-transfusion testing of partner compatibility. On the other hand, we mention that only a small percentage of patients needed to repeat the transfusion with one of the blood products, which means that the number of previously sensitized dogs that had the opportunity to show an undesirable post-transfusion reaction was low. However, special attention should be paid to the identification of compatible blood when repeating the transfusion in any canine patient (Ognean, 2015). Concerning the evolution of the delayed hemolytic reaction, we remind that the symptoms have developed only after 9 days from the first incompatible transfusion, linked with increased levels of antibodies. Such a transfusion incompatibility can be caused by red blood cells bearing DEA 3, 5 and 7 antigens observed with a frequency of 10% in dogs that are DEA 3 negative and 20% in dogs that are DEA 5 negative (Ognean, 2017). Furthermore, AHI type reactions have been reported in DEA 7 negative dogs, after transfusion with DEA 7 positive blood (Ognean, 2017). It is also important to highlight the general tendency of labeling any undesirable effect of blood products transfusion as an immunological or non immunological reaction, with immediate response or later onset. In this regard, a major importance must be attributed to preventive measures designed to decrease the risk of developing any post transfusion reactions, with closer monitoring of the donor, the conditions in which the blood is collected, prepared, stored and administered.

It is well known that the most worrying form of hemolytic transfusion reaction is the acute one, observed in canine patients DEA 1.1 negative that received DEA 1.1 positive blood, that were sensitized prior with red blood cells carrying DEA 1.1 antigen. The symptoms in this acute hemolytic reaction are fever, tachycardia, dyspnea, muscular tremor, emesis, apathy, low levels of hemoglobin and hemoglobinuria. As opposed to the acute form, the delayed hemolytic reaction has an extravascular form, with similar symptoms, but not with the same severity. This kind of reaction may occur from day two until the 21th day after transfusion.

TRALI syndrome is one of the most severe forms of post transfusion reactions, due to its high rate of morbidity and mortality (Kopko et al., 1999; Toy et al., 2005). Confusions may sometimes appear because this syndrome has been known under different names, such as pulmonary hypersensitivity reaction, allergic pulmonary oedema, non cardiogenic oedema. In addition to this, it is unanimously accepted that this pathological entity is still hard to recognize, because it is not yet completely understood and described, due to diagnostic errors and due to lack of awareness about its importance (http://www.mymed.ro/injuria-pulmonara-acuta-post-transfuzionala-trali.html).

Regarding the evolution of the patients subjected to the present study, we regard the TRALI syndrome as the main factor that caused the patient death. The early onset of pulmonary symptoms, occurring during the transfusion, followed by late symptoms after transfusion (dyspnea, cyanosis, fever) described a characteristic clinical picture of this respiratory syndrome. This was well argued by morphopathological changes, focused on the predominance of tracheal and bronchial infiltrate. It should be noted, however, that the patient did not show symptoms of Acute

Pulmonary Injury prior to transfusion. Another observation that could support the existence of TRALI in this patient is the persistence of thrombocytosis (Kohn et al., 2006). Platelets are thought to secrete numerous proinflammatory factors, often involved in the mechanism of TRALI syndrome, such as chemokines, which attract and activate neutrophils causing endothelial layer permeability. (Toy et al., 2005; Marik et al., 2008).

Conclusions

Most of the reported transfusion reactions had a minor clinical impact and ensured a high level of recovery of the transfused patients, which was also supported by the implementation of adequate measures for the preparation and monitoring the transfusion therapy. However, we also encountered severe forms of transfusion reactions, which progressively worsened, ending with the patients death. In this regard, we attributed major clinical interest to delayed hemolytic anemia and TRALI respiratory syndrome, which caused serious complications in two patients, transfused 3 times with whole blood, as palliative treatment in a severe form of chronic renal failure and malignant lymphoma, respectively. We consider that the patient with chronic renal failure developed an AHI-type transfusion reaction, because it was transfused with large volumes of whole blood, without prior testing of patient-donor compatibility, which did not cause a rapid increase in erythrocyte indices, but their significant decrease in the first three days, with a slight remission at 30 days, followed by a decreasing trend. The results of the three blood transfusions indicated a possible rejection of red blood cells administered to this patient. The evolution of TRALI syndrome in a patient suffering from malignant lymphoma was based on the major risk conferred by repeated transfusions and the relevant changes detected at necropsy.

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Comparative research regarding the hematological and biochemical blood profile in hypovolemic and toxicoseptic shock conditions, in dogs

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Abstract

The purpose of this paper is to make a comparative study between two evolution forms of shock with different etiologies, namely hypovolemic shock and toxicoseptic shock in dogs. This comparison took into account two groups of parameters, the hematological and the biochemical blood values. In order to carry out our study we took for analysis 15 dogs, of different breeds and aged between 1 and 12 years, diagnosed with shock. Depending on the etiology of the shock, the cases were grouped into two categories: animals in hypovolemic shock (n = 10) and animals in toxicoseptic shock (n = 5). The animals in the first category were grouped into two groups: dogs in post-hemorrhagic hypovolemic shock (n = 5) and dogs in post-dehydration hypovolemic shock (n = 5). Blood samples were used from the animals studied to determine: plasma calcium, lactic acid, plasma albumin, total plasma proteins. leukocyte count, hematocrit, hemoglobin and plasma fibrinogen. Hematocrit and hemoglobin were elevated in animals with post-dehydration shock. Hematocrit and hemoglobin were low in dogs with posthemorrhagic shock. Leukocytes were increased in animals with toxicoseptic shock. Fibrinogen showed elevated levels in dogs with toxicoseptic shock and those with post-dehydration shock. Lactic acid was recorded with high values, which exceed the reference values, in the case of all three experimental groups. Calcium, plasma albumin and plasma protein levels were decreased in all three experimental groups.

Key words: *hypovolemic shock, toxicoseptic shock, leukocyte, hematocrit, hemoglobin.*

Introduction

The purpose of this paper is to make a comparative study between two evolution forms of shock with different etiologies, namely hypovolemic shock and toxicoseptic shock in dogs. This comparison took into account two groups of parameters, the hematological and the biochemical blood values.

Materials and methods

In order to carry out our study we took for analysis 15 dogs, of different breeds and aged between 1 and 12 years.

These dogs were analysed by physicians, who indicated the diagnosis of shock, based on the evaluation of the Shock Index (SI). This parameter results from the ratio of Heart Rate (Fc) to Systolic Pressure (Ps), according to the formula: SI = Fc / Ps.

According to Peterson (2013) and Porter (2013), the classification of cases according to the Shock Index is done as follows:

-Dog outside the shock stage: SI <0.6.

-Dog in light shock: SI> 0.6 and <1.0;

-Dog with moderate shock: SI> 1.0 and <1.4;

-Dog with severe shock: SI> 1.4.

Depending on the etiology of the shock, the cases were grouped into two categories: animals in hypovolemic shock (n = 10) and animals in toxicoseptic shock (n = 5).

The animals in the first category were grouped into two groups: dogs in post-hemorrhagic hypovolemic shock (n = 5) and dogs in post-dehydration hypovolemic shock (n = 5).

During the clinical examinations and the therapeutic maneuvers, blood samples were taken from each animal studied, which were preserved and used to determine hematological and biochemical parameters.

Blood samples were analyzed for the purpose of assessing blood biochemical parameters, such as:

-plasma calcium;
-lactic acid;
-plasma albumin;
-total plasma proteins.

These parameters were determined in the veterinary clinic where the study was performed. An IDEXX VetTest Chemistry Analyzer was used for this purpose.

We also took into account the level of hematological parameters, such as:

-number of leukocytes;
-hematocrit;
-hemoglobin;
-plasma fibrinogen.

Hematological parameters were determined in the same veterinary clinic. An IDEXX VetAutoread ™ Hematology Analyzer was used for this purpose.

The data obtained were statistically analyzed calculating the average, but also the significance of the differences between the groups through the Student test.

Results and discussions

The results obtained in order to evaluate the targeted hematological parameters are presented in table 1.

Table 1

Average levels of hematological parameters in the case of the three experimental groups

	Lot number		
	1 Post-hemorrhagic shock	**2 Post-dehydration hypovolemic shock**	**3 Toxicoseptic shock**
Hematocrit (%)	**33,2**	**60,2**	**47,4**
Hemoglobin (g/dl)	**10,7**	**19,94**	**17,46**
Leukocyte (10^3 x µL)	**4,28**	**12,0**	**17,12**
Fibrinogen (g/l)	**1,9**	**2,2**	**5,11**

The differences between group 1 (posthemorrhagic shock) and group 2 (hypovolemic shock after dehydration) in terms of hematocrit and hemoglobin were statistically significant (P <0.05). The values of these parameters were 81.32% higher in the case of group 2 compared to group 1, in the case of hematocrit and by 86.35% higher in the case of group 2 compared to group 1, in the case of hemoglobin.

The differences between group 1 (posthemorrhagic shock) and group 3 (septic shock), in terms of hematocrit and hemoglobin, were statistically significant (P <0.05). The values of these parameters were 45.77% higher in the case of group 3 compared to group 1, in the case of hematocrit and by 63.17% higher in the case of group 3 compared to group 1, in the case of hemoglobin.

The differences between group 2 (post-dehydration hypovolemic shock) and group 3 (septic shock), in terms of hematocrit and hemoglobin were statistically significant (P <0.05), the values of these parameters being 27% higher in in the case of group 2 compared to group 3, in the case of hematocrit and 14.20% higher in the case of group 2 compared to group 3, in the case of hemoglobin.

The results obtained in the case of group 3 fall within the limits of the reference values (36-55%), being a sign that in the conditions we provided in our study neither the hematocrit, nor the hemoglobin levels were affected during the septic shock.

From the results obtained we can see an obvious posthemorrhagic anemia in the case of the animals from group 1. However, in the case of the dogs from group 2 we found a hemoconcentration explainable by the severe dehydration to which the animals from this group were subjected.

The differences between group 1 (posthemorrhagic shock) and group 2 (hypovolemic shock after dehydration) in terms of leukocyte count and fibrinogen were statistically significant (P <0.05), the values of these parameters being 180% higher in the case of group 2 compared to group 1, in the case of leukocytes and by 15.78% higher in the case of group 2 compared to group 1, in the case of fibrinogen.

The differences between group 1 (posthemorrhagic shock) and group 3 (septic shock), in terms of leukocyte count and fibrinogen, were statistically significant (P <0.05), the value of these parameters being 300.0 % higher in case of group 3 compared to group 1, in the case of leukocytes and by 168.0% higher in case of group 3 compared to group 1, in case of fibrinogen.

The differences between group 2 (post-dehydration hypovolemic shock) and group 3 (septic shock), in terms of leukocyte count and fibrinogen were statistically significant (P <0.05), the values of these parameters being 42.66 % higher in the case of group 3 compared to group 2, in the case of leukocytes and by 132.0% higher in the case of group 3 compared to group 2, in the case of fibrinogen.

From these results we can observe a value placed at the minimum level of the leukocytes normal limits in the case of animals from group 1 (4-15 103 x µL). This modest level of the leukocyte parameter could be explained as being a consequence of the posthemorrhagic condition to which the animals were subjected. Instead, in the case of the dogs from group 2, we found values placed between the physiological limits of the species.

The results obtained by us in the case of lot 3, regarding the number of leukocytes, are placed above the limits of the reference values (4-15 103 x µL), indicating a natural reaction to the septic conditions that led to the installation of shock for the animals in this category.

From the results obtained we can observe a value placed at the minimum limit (1-4 g / l) for fibrinogen in the case of animals from group 1. Instead in the case of dogs from group 2 we found higher values, but placed between the physiological limits of the species.

The increased level of statistical significance in the differences between group 1 and group 2, regarding fibrinogen, could be explained by the specific conditions of dehydration, which through its effects can induce intense cytolysis in various tissue areas, resulting in inflammatory mediators release, leading to an amplification of the fibrinogen levels.

In group 3, the fibrinogen level results are above the reference values, indicating a natural reaction to the septic conditions that induce the amplification of the fibrinogen level, known as a positive acute phase protein effect.

The results obtained by us for the purpose of evaluating plasma calcium in the case of the three experimental groups are presented in table 2.

Table 2

Average levels of blood biochemical parameters in the case of the three experimental groups

	Lot number		
	1 Post-hemorrhagic shock	**2 Post-dehydration hypovolemic shock**	**3 Toxicoseptic shock**
Calcium (mg/dl)	**8,12**	**8,1**	**8,34**
Lactic acid (mmol/L)	**4,1**	**4,16**	**4,36**
Albumin (g/dL)	**2,16**	**2,26**	**2,11**
Protein (g/dL)	**4,22**	**3,98**	**3,92**

Regarding the level of plasma calcium, the differences were statistically insignificant (P> 0.05) in the case of comparisons made between:

-lot 1 (posthemorrhagic shock) and lot 2 (hypovolemic shock after dehydration);

-lot 1 (posthemorrhagic shock) and lot 3 (septic shock);

-lot 2 (Post-dehydration hypovolemic shock) and lot 3 (Septic shock).

All the values obtained in the case of the three experimental groups were placed below the lower limit of the physiological range (8.9-11.4 mg / dl) in which this parameter is placed. These values coincide with those obtained by other authors (9), who indicated that during hypovolemic and septic shock the value of plasma ionic calcium decreases. These values are consistent with those related to the evolution of plasma albumin in the context of our experiment, which can be explained by the fact that plasma albumin also has the function of transporting calcium ions.

Regarding the level of lactic acid, the differences were statistically insignificant (P> 0.05) in the case of comparisons made between:

-lot 1 (posthemorrhagic shock) and lot 2 (hypovolemic shock after dehydration);

-lot 1 (posthemorrhagic shock) and lot 3 (septic shock);

-lot 2 (Post-dehydration hypovolemic shock) and lot 3 (Septic shock).

However, all the values obtained in the case of the three experimental groups were placed above the upper limit of the physiological range in which this parameter is placed. These values coincide with those obtained by other authors (9,10), who indicated that during the hypovolemic and septic shock the value of lactic acid increases, as a result of the intense anaerobic glycolysis carried out during the shock evolution phases.

Regarding the level of plasma albumin, the differences were statistically insignificant (P> 0.05) in the case of comparisons made between:

-lot 1 (posthemorrhagic shock) and lot 2 (hypovolemic shock after dehydration);

-lot 1 (posthemorrhagic shock) and lot 3 (septic shock);

-lot 2 (Post-dehydration hypovolemic shock) and lot 3 (Septic shock).

However, all the values obtained in the case of the three experimental groups were lower than the lower limit of the physiological range (2.7-4.4 g / dL) in which this parameter is placed. The values obtained by us coincide with those obtained by other authors (9,10), who showed that during hypovolemic and septic shock the value of plasma albumin decreases, these being recognized as negative acute phase protein effect.

Regarding the level of plasma proteins, the differences were statistically insignificant (P> 0.05) in the case of comparisons made between:

-lot 1 (posthemorrhagic shock) and lot 2 (hypovolemic shock after dehydration);

-lot 1 (posthemorrhagic shock) and lot 3 (septic shock);

-lot 2 (Post-dehydration hypovolemic shock) and lot 3 (Septic shock).

However, all the values obtained in the case of the three experimental groups were lower than the lower limit of the physiological range (5.5-7.5 g / dL) in which this parameter is placed. The values obtained by us coincide with those obtained by other authors (9), who showed that during hypovolemic and septic shock the level of plasma proteins decreases. This decrease is mainly due to plasma albumin which, as can be seen in our experiment, shows obvious decreases during shock.

Conclusions

Following our research, we drew the following conclusions:

1. Hematocrit and hemoglobin tests recorded values higher than the maximum limit of the reference interval in the case of animals with post-dehydration shock;

2. Hematocrit and hemoglobin recorded values lower than the minimum limit of the reference interval in the case of dogs with posthemorrhagic shock;

3. The leukocytes recorded values higher than the maximum limit of the physiological reference interval in the case of animals with toxicoseptic shock.

4. Fibrinogen showed levels higher than the maximum limit of the reference interval in the case of dogs with toxicoseptic shock and those with post-dehydration shock.

5. Lactic acid recorded high values, which exceed the reference values, in the case of all three experimental groups.

6. Levels of Calcium, plasma albumin and plasma proteins were lower than the minimum levels of the reference intervals for all three experimental groups.

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Pelophylax ridibundus (Amphibia: ranidae) as paratenic host of *Spirocerca lupi* species (Secernentea: spirocercidae) in the Republic of Moldova

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Abstract
The paper presents data on the identification of the helminth fauna structure of Pelophylax ridibundus species and the determination of its role as intermediate and paratenic host for various groups of vertebrate helminths. 84 specimens of Pelophylax ridibundus (26 - males, 31 - females, 27 - juveniles) from the Dniester River, border area of Talmaza village, Ştefan Vodă district, were helminthologically investigated. As result of helminthological investigations, it was established that the structure of the helminth fauna of Pelophylax ridibundus species is characterized by 13 species of helminths (Haematoloechus variegatus Rudolphi, 1819; Codonocephalus urniger Rudolphi, 1819; Opisthioglyphe ranae Froelich, 1791; Paralepoderma brumpti Buttner, 1951; Prosotocus confusus Looss, 1894; Tylodelphys excavata Rudolphi, 1803; Diplodiscus subclavatus Pallas, 1760; Parastrigea robusta Szidat, 1928, Strigea falconis Szidat, 1928; Cosmocerca ornata Dujardin, 1845; Oswaldocruzia filiformis Goeze, 1782; Icosiella neglecta Diesing, 1851; Spirocerca lupi Rudolphi, 1809), from 13 genera, 11 families (Omphalometridae, Haematoloechidae, Plagiorchiidae, Lecithodendriidae, Diplodiscidae, Diplostomatidae, Strigeidae, Cosmocercidae, Molineidae, Onchocercidae, Spirocercidae), 6 orders (Plagiorchiida, Echinostomida, Diplostomida, Ascaridida, Strongylida, Spirurida), 2 classes (Trematoda, Secernentea) and 2 phylums (Plathelminthes, Nematoda). Of the 13 helminth species detected, a special importance is attributed to the nematode *Spirocerca lupi Rudolphi, 1809, for which the taxonomic classification, synonims, hosts, organic specificity and biological cycle are described, for which the Pelophylax ridibundus species is as paratenic host. The diversity and intensity of helminth infestation of Pelophylax ridibundus has been established*
Keywords: *Pelophylax ridibundus, Spirocerca lupi, paratenic host.*

Introduction

Helminthological research is more frequently focused on the study of the degree of helminth infection in domestic, wild, pets' animals and human. Currently, in different regions of the world several taxa of animals species are insufficiently studied helminthologically, including amphibians.

Amphibians are the most primitive terrestrial vertebrate animals, which has preserved the aquatic and terrestrial way of life, being vectors of various parasitic agents. In some cases, amphibians participate not only at the contamination of domestic and wild animals, but are an important factor in maintaining their circulation in the nature and actively participate in the formation of parasitic zoonoses [1].

In the foreground, the study of helminth fauna in amphibians is also interesting in terms of knowledge of fauna.

The study of amphibian helminth fauna, the specificity of the circulation in the natural and anthropized biotopes and their contact with the host, allow the establishment of the parasitological situation, some characteristics in the pathogenesis of the formation of outbreaks of parasitic agents and the elaboration of measures with epizootic and epidemiological impact.

In addition to the faunal importance of research, anurans are definitive hosts for several classes of helminths, including Cestoda, Monogenea, Trematoda, Secernentea and Palaeacanthocephala [1, 3-6, 8-11, 13-16, 18-20]. They also serve as intermediate hosts [7, 11, 12, 17, 19, 20] or as paratenic hosts [12, 8-10, 13, 14, 16] for a wide variety of helminths specific to the vertebrates.

It is known that the wild animals are an important source of parasites for humans and domestic animals. According to researchers from the Republic of Moldova, it was found that out

of 178 parasitic agents established in wild animals, 20 species were recorded in humans and domestic animals [21, 29].

The parasitic diseases of domestic and wild vertebrates include spirocercosis, which is caused by the nematode *Spirocerca lupi* Rudolphi, 1809. This disease is spread all over the world and in the Republic of Moldova the nematode, which causes the disease, was detected for the first time.

In the context of determining the sources of the spread of parasitosis in domestic animals, pets and humans in the Republic of Moldova, it is necessary to conduct an in-depth study of helminth fauna, especially in amphibians *Pelophylax ridibundus*, and identify its role as a paratenic host for various groups of parasitic agents.

Materials and methods

The investigations regarding the study of the helminth fauna, the determination of the degree infestation by helminth of *Pelophylax ridibundus* species were carried in the laboratory of Parasitology and Helminthology of the Institute of Zoology.

A total of 84 amphibian specimens (males - 26, females - 31, juveniles - 27) collected from the Dniester River, Talmaza village area in the Stefan-Voda district during the years 2019-2020 were helminthologically investigated.

The amphibians were determined by external characters [23].

The helminthological analysis of biological samples was performed according to the standard method proposed by K.I. Skrjabin, which involves the examination of all the internal organs of the animal [27]. Helminthological research of the parenchymal organs was performed with the help of compressors, and the digestive tract - by successive washes. The collection, fixing, determination and processing of the helminthological material was carried after the methods proposed by various authors [22, 23, 24, 25, 26, 28]. The determination of the helminthological material was performed after standard methods [25].

To quantify the contamination characteristic by helminthes, the Intensity indice was calculated (*II, exemplars*) – the minimum and maximum number of parasites of a species and the extensivity of invasion (*EI, %*) – the percentage of host contamination by a species of parasite.

Laboratory helminthological investigations of biological samples of Peloplylax ridibundus to the presence of helminths or helminthic elements (eggs, larvae), allowed to obtain data of special value in order to determine the importance of amphibians in the formation and maintenance of outbreaks of common parasitic organisms in wild animals, pets and human.

Results and discussions

Unlike other species of green and brown frogs, *Pelophilax ridibundus*, due to its ecological plasticity is able to populate a wide range of aquatic habitats, from natural (permanent or temporary, with standing or flowing water) to moderately-polluted in lowland areas [23].

The study of helminth fauna in amphibians, the specificity of circulation in natural and anthropized biotopes and their contact with the host, allow establishing the parasitological situation, some characteristics in the pathogenesis of parasitic agent outbreaks and development of measures with epizootic and epidemiological impact.

According to the helminthological investigations performed on *Pelophylax ridibundus* from Talmaza village, Ștefan-Vodă district, the presence of 13 helminths species was established: *Haematoloechus variegatus* Rudolphi, 1819; *Codonocephalus urniger* Rudolphi, 1819; *Opisthioglyphe ranae* Froelich, 1791; *Paralepoderma brumpti* Buttner, 1951; *Prosotocus confusus* Looss, 1894; *Tylodelphys excavata* Rudolphi, 1803; *Diplodiscus subclavatus* Pallas, 1760;

Parastrigea robusta Szidat, 1928, *Strigea falconis* Szidat, 1928; *Cosmocerca ornata* Dujardin, 1845; *Oswaldocruzia filiformis* Goeze, 1782; *Icosiella neglecta* Diesing, 1851; *Spirocerca lupi* Rudolphi, 1809, which from a taxonomic point of view fall into 2 classes (Trematoda, Secernentea), 6 orders (Plagiorchiida, Echinostomida, Diplostomida, Ascaridida, Strongylida, Spirurida), 11 families (Omphalometridae, Haematoloechidae, Plagiorchiidae, Lecithodendriidae, Diplodiscidae, Diplostomatidae, Strigeidae, Cosmocercidae, Molineidae, Onchocercidae, Spirocercidae) and 13 genera (*Haematoloechus,Codonocephalus,Opisthioglyphe, Paralepoderma, Prosotocus, Tylodelphys, Diplodiscus, Parastrigea, Strigea, Cosmocerca, Oswaldocruzia, Icosiella,Spirocerca*).

Unlike previous helminthological research conducted on the amphibians in the Central and Northern areas of the Republic of Moldova, in the southern area a differentiated helminth fauna structure was established. Thus, it has been found the presence of a new species of nematodes *Spirocerca lupi* Rudolphi, 1809 with faunal, bioindicative and veterinary medical importance Fig. 1.

This species of nematode forms spirocercosis in carnivores (dog, fox, wolf), and accidentally in goats, horses, cattle, pigs, etc., it is located in the esophagus, clinically characterized by digestive, cardiovascular and general disorders [2].

The development of *Spirocerca lupi* species is heteroxenous. The coprophage beetles (Geotrupes, Scarabaeus) serve as intermediate hosts. They become infected through the ingestion of parasite eggs, and in their body the L_1 larvae hatch, which suffer two moults and become L_3 infestants after they are encapsulated. Infested beetles are ingested by paratenic hosts - amphibians, reptiles, birds, in whose body the nematode encapsulates again, and the infestation of the definitive hosts occurs through the consumption of paratenic hosts [2].

Fig. 1. *Spirocerca lupi* Rudolphi, 1809. Original

Spirocercosis evolves in countries with warm climates, and in our country the nematode that causes this disease - *Spirocerca lupi* was detected for the first time. For this disease the amphibians serve as a sure source of transmission, representing true reservoirs of infesting larvae.

One of the main factors that determine the infection with parasitic agents of animals is the type of biotope. Thus, the succession of climatic and anthropogenic transformations in biotopes can lead to the interruption of the biological cycles of parasitic agents and respectively to the disappearance of historically formed parasitic systems, or (rarely) can influence to the increase of helminth diversity.

Quantitative analysis of parasitological indices in *Pelophylax ridibundus* demonstrate that infestation with the trematode species *Opisthioglyphe ranae* was recorded in 69.0% of cases (*II* – 8-86 exemplars), with *Haematoloechus variegatus* in 38.1% of cases (*II* -1-10 exemplars), with *Codonocephalus urniger* in 47.6% of cases (*II* -1-30 exemplars), with *Prosotocus confusus* in 22.6% of cases (*II* -1-32 exemplars), *Diplodiscus subclavatus*în 10.9% of cases (*II* - 4-9 exemplars), with *Paralepoderma brumpti* in 39.3% of cases (*II* -1-9 exemplars), with *Tylodelphys excavata* in 36.9% of cases (*II* – 8-96 exemplars), with *Parastrigea robusta* in 7.1% of cases (*II* – 3 exemplars), with *Strigea falconis* in 3.6% of cases (*II* – 150 exemplars), with *Oswaldocruzia filiformis* in 1.2%

of cases (*II* – 1 exemplar), with *Cosmocerca ornata* in 19,0% of cases (*II* – 4-40 exemplars), with *Icosiella neglecta* in 26.2% of cases (*II* – 1-14 exemplars), but with *Spirocerca lupi* species the infestation was recorded in 21.4% of cases (*II* – 2-98 exemplars) (*Table 1*).

When evaluating the parasitological indices obtained, it has been established that the highest degree of infestation was with the species of trematode *Opisthioglyphe ranae,* and among nematodes - with *Icosiella neglecta* and *Spirocerca lupi* species.

Helminthological investigations depending of the host sex performed on 57 mature individuals (males - 26, females - 31) of *Pelophylax ridibundus* s demonstrated that the infestation degree by helminthes depends on the helminth species and the host sex. Although, the males of the species are the first individuals to come out of hibernation and have a longer contact with the environment, they were characterized by a simpler helminth structure being infested with only 7 species of helminths, of which 6 species of trematodes (*Haematoloechus variegatus*,*Codonocephalus urniger*, *Opisthioglyphe ranae, Paralepoderma brumpti, Prosotocus confusus*, *Tylodelphys excavata*) and one species of nematode (*Icosiella neglecta*) *Table 2*.

Table 1.

Parasitological indices of *Pelophylax ridibundus* species (n = 84)from the studied ecosystems

No	Invasion	*EI - %*	*II, ex.* min-max
TREMATODA			
1	*Opisthioglyphe ranae*	69.0	8-86
2	*Haematoloechus variegatus*	38.1	1-10
3	*Codonocephalus urniger*	47.6	1-30
4	*Prosotocus confusus*	22.6	1-32
5	*Diplodiscus subclavatus*	10.9	4-9
6	*Paralepoderma brumpti*	39.3	1-9
7	*Tylodelphys excavata*	36.9	8-96
8	*Parastrigea robusta*	7.1	3
9	*Strigea falconis*	3.6	150
SECERNENTEA			
10	*Oswaldocruzia filiformis*	1.2	1
11	*Cosmocerca ornata*	19.0	4-40
12	*Icosiella neglecta*	26.2	1-14
13	*Spirocerca lupi*	21.4	2-98

Unlike males, the females of *Pelophylax ridibundus* were infected by 13 species of helminthes, of which 9 species of trematodes (*Haematoloechus variegatus, Codonocephalus urniger, Opisthioglyphe ranae, Paralepoderma brumpti, Prosotocus confusus, Tylodelphys excavata,Diplodiscus subclavatus, Parastrigea robusta, Strigea falconis,*) and 4 species of nematode (*Cosmocerca ornata, Oswaldocruzia filiformis, Icosiella neglecta, Spirocerca lupi*), whose degree of infestation differs from one species to another (*Table 2*).

Another question frequently addressed in the literature is the study of helminth fauna depending on the age of host. Various authors (69, 79, 98, 128 - teza) affirm that the degree of helminth infestation increases with the age of host, but according to our investigations in the juveniles of *P. ridibundus* collected from the southern part of the republic, their infestation with 5 species of trematodes was established: (*Haematoloechus variegatus, Codonocephalus urniger,*

Opisthioglyphe ranae, Paralepoderma brumpti, Tylodelphys excavata,) whose extensivity overrun that of adults (*Table 2*).

Table 2.

Parasitological indices depending of the sex and age of host *Pelophylax ridibundus* from the studied ecosystems

No	Invasion	Sex				Age			
		♂, n=26		♀, n=31		Adults, n=57		Juveniles, n= 27	
		EI -%	*II, ex.*	*EI - %*	*II, ex.*	*EI - %*	*II, ex.*	*EI - %*	*II, ex.*
TREMATODA									
1	*O. ranae*	76.9	4-180	45.2	38-86	59.6	4-180	88.9	8-41
2	*H. variegatus*	50.0	1-30	38.7	1-4	43.9	1-30	29.6	3
3	*C. urniger*	38.5	1-30	83.9	2-11	63.2	1-30	14.8	1
4	*P. confusus*	38.5	1-14	29.0	1-32	33.3	1-32	-	-
5	*D.subclavatus*	-	-	29.0	4-9	15.8	4-9	-	-
6	*P. brumpti*	61.5	1-8	29.0	10-26	43.9	1-26	29.6	3-9
7	*T. excavata*	57.9	26-49	38.7	9-96	47.4	9-96	14.8	8
8	*P. robusta*	-	-	19.4	3	10.5	3	-	-
9	*S. falconis*	-	-	9.7	150	5.3	150	-	-
SECERNENTEA									
10	*O. filiformis*	-	-	19.4	1	10.5	1	-	-
11	*C. ornata*	-	-	38.7	6-10	21.1	6-10	-	-
12	*I. neglecta*	19.2	1-3	29.0	1-14	24.6	1-14	-	-
13	*S. lupi*	-	-	58.1	2-98	31.6	2-98	-	-

Therefore, the results of helminthological research revealed that the amphibians species *Pelophylax ridibundus* from the Ranidae family in the natural ecosystems of lower Dniester River is infested with 13 species of helminthes (trematodes - 9, nematodes - 4), as well as the identification of the nematode species *Spirocerca lupi* show the role of amphibians in maintaining and forming the foci of parasitic agents specific to vertebrates.

Conclusions

1. It has been studied the helminth fauna of 84 specimens (males - 26, females - 31, juveniles - 27) of Pelophylax ridibundus from the natural ecosystem - the Dniester River, from the village of Talmaza from the Stefan-Voda district.
2. The structure of the helminth fauna of the host species was established - Pelophylax ridibundus has been represented by 13 species of helminthes: *Haematoloechus variegatus* Rudolphi, 1819; *Codonocephalus urniger* Rudolphi, 1819; *Opisthioglyphe ranae* Froelich, 1791; *Paralepoderma brumpti* Buttner, 1951; *Prosotocus confusus* Looss, 1894; *Tylodelphys excavata* Rudolphi, 1803; *Diplodiscus subclavatus* Pallas, 1760; *Parastrigea robusta* Szidat, 1928, *Strigea falconis* Szidat, 1928; *Cosmocerca ornata* Dujardin, 1845; *Oswaldocruzia filiformis* Goeze, 1782; *Icosiella neglecta* Diesing, 1851; *Spirocerca lupi* Rudolphi, 1809, from 2 classes, 6 orders, 11 families and 13 genera.
3. The degree of helminth infestation of the species *Pelophylax ridibundus* increases with age, because in juveniles the infestation has been established with 5 from 13 species of helminths detected.

4. It was estimated that the structure of the helminth fauna in *Pelophylax ridibundus* depends on the helminth species and of the host sex, thus in males the presence of 7 from 13 species of helminths was established the, and in females - all 13 species of helminths were found.
5. It was determined the role of amphibian species *Pelophylax ridibundus* in maintaining and forming parasitic foci of *Spirocerva lupi*, which causes Spirocercosis in vertebrates, and for which the amphibians serve as a paratene hosts.

Acknowledgement: The study was performed in the frame of institutional project 20.80009.7007.12 and with support World Federation of Scientists

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Effects of food supplemented with ZooBioR product in young chickens on the functional state of the liver

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Abstract
Cyanobacteria Spirulina platensis is widely used as a biotransformer of bioelements and as a producer of biologically active substances with a wide spectrum of use. The current study is aimed at objectively examining the impact of the product ZooBioR (obtained from Spirulina platensis) on health, and especially on the marker parameters of the functional state of the liver in hens, in the first technological period of laying. The experiment was performed on 5 groups of birds (of 14 heads/group). In 4 groups out of 5, the food was supplemented with the remedy ZooBioR in different doses (5.0; 10.0; 15.0; 20.0 mg active substance/kg of fodder). It has been established that the tested product improves the health of laying hens, in the first technological period of laying, significantly contributes to improving the metabolic processes in the body, especially the functional state of the liver
Key words: *Young laying hens; ZooBioR Remedy; Liver; ALT and AST transaminases; Bilirubin and its fractions.*

Introduction

In recent years, in the Republic of Moldova, as well as in other countries worldwide, there is a growing interest in aviculture, which has certain advantages over other branches of modern animal husbandry due to its intensification and functionality (Macari V., Putin V., Gudumac V., 2009; Macari V. et al., 2014; Van Il., Marin Gh., 2016; Zoltan P. et al., 2011; Фисинин В. И., 2012). Birds` breeding and exploitation, especially using intensive breeding systems, is difficult, very even often compromised by the technological stress, of different origin and intensity. These factors indisputably influence in a negative way the birds` productivity and health. Also, according to literature data, in the process of breeding and intensive exploitation of birds, the most requested and the most affected organ is the liver (decrease of ALT transaminase and increase of AST, decreasing tendency in the first stage of research of total bilirubin and its fractions, as well as an increasing tendency at the end of research of total bilirubin and its fractions, alkaline phosphatase and its fractions decreased activity in the blood serum during research), the positive impact being oriented towards the optimization of metabolism in situations of higher metabolic loads, leading as well to higher egg production. (Macari V. et al., 2014; Putin V., 2012 ; Pavlicenco N., 2019; Rotaru A., 2016; Кольберг Н. А., Садовников Н. В., 2010).

Therefore, it is necessary a profound literature review of the various biologically active remedies action on the functional state of the liver, up to date studies regarding the long-term action of harmful external and internal factors on the animal body, on the digestive system, and especially on the liver (Macari V. et al., 2014; Maţencu D., 2019). Besides that, in recent years, there has been an increase in interest in growth stimulants, especially natural ones. Out of a great range of growth stimulators of different origin and categories, with varoius properties, the ones of natural origin, especially of plant origin, are considered to be the best ones, as they are harmless, and have good usage potential (Macari V. et al., 2014; Maţencu D., 2019; Pavlicenco N., 2019; Rotaru A., 2016; Moostan KM, 2011; Nickolova M., Penkov D., 2010; Offor CE, Aja PM, 2014).

As for these reasons, we have decided to evaluate the impact of the ZooBioR spirulina product, administered with food to young laying hens, on the liver functional state, studying the tolerance of this medicinal product, and shaping of the optimal dose of this remedy.

Material and method

The experiment was carried out at the avicol factory within the ***"Acustic Tehnologic"*** LLC, Floreni village, Republic of Moldova. The objective of the research was focused both, on the study of the new medicinal product - ZooBioR, as well as on the influence of this remedy on young laying hens. The product ***ZooBioR***, tested by us, is a complex natural remedy containing biologically active compounds derived from the cyanobacterium *Spirulina (Arthrospira) platensis*. ***ZooBioR - 2plus*** contains: amino acids, including free immunoactive ones and as component parts of peptides and proteins; polysaccharides; sulphated polysaccharides; phospholipids and the trace elements **zinc** and **selenium**.

The research was carried out on a number of 70 hens belonging to the Braun-Nic hybrid, divided into 5 groups, 14 heads per each. The birds included in the research were analogous in terms of age, physiological condition, origin, body weight, being accommodated in the same hall, with the same environmental conditions, and veterinary care. During the experiment the birds were monitored and examined for health assessment.

At the same time, the object of the research was the local product ***ZooBioR - 2plus***, administered to birds, in different doses, according to the experimental scheme, from table 1.

Birds` groups	No of birds	Administration route	Dose, mg active substance/kg of fodder	Administration regimen
Control	14	-	-	
Experimental 1	14	per os with food	5.0	daily
Experimental 2	14		10.0	
Experimental 3	14		15.0	
Experimental 4	14		20.0	

In order to assess the state of health, at the beginning of the experiment, and later on, the birds were examined, and in 5 laying hens, from each group, the body temperature and respiratory movements had been determined in one minute.

For laboratory investigations, blood samples were taken in three stages, in standard test tubes: at the beginning of the experiment, until the administration of the ZooBioR remedy, from 5 random hens; during the study, from 5 birds in each group – at about 1 month after the beginning of the study, as well as at the end of the experiment, which coincided with the 129th day of research.

The functional status of liver in birds was assessed by determining the activity of ALT and AST transaminases, alkaline phosphatase and its fractions, as well as bilirubin level and its fractions. The analyses were performed in blood serum on PowerWave HT plate spectrophotometric reader, BioTek, USA. The statistical results of the clinical and hematological indices were made using the parametric criterion Student`s t-distribution with a veracity of less than 0.05 ($P < 0.05$).

Results and discussions

During the experiment, for a period of more than 4 months, the ZooBioR product, tested on young laying hens, in the first technological laying period, in avicola factory conditions, did not cause side effects or other deviations in the development, productivity or health of birds. Regarding the morphopathological aspect, following the control sacrifices (5 hens from each group), no significant changes were found in the carcass and organs of the thoraco-abdominal cavity or the

brain. The study reveals that, in general terms, the tested product improves the birds` health status, presenting anti-stress and adaptive properties, through lower values of body temperature and respiratory movements as well. Besides other parameters investigated in this research, the most accurate and conclusive marker of liver function state is the activity of ALT and AST transaminases, which are presented in the statistics in table 2.

Table 2.

Transaminases and total bilirubin and its fractions values in blood serum in laying hens

Signification	Onset	Birds` Groups				
		CG	EG 1	EG 2	EG 3	EG 4
ALT, u/l						
1 sampling	13,41±1,4	16,97±2,35	14,67±1,78	15,30±1,68	19,49±0,88	15,29±2,39
2 sampling		18,02±1,14	14,25±1,09*	17,18±1,60	16,34±1,42	14,88±1,83
AST, u/l						
1 sampling	45,68±2,9	45,47±3,18	52,59±1,79	52,38±2,57	61,33±2,51***	53,43±2,77
2 sampling		59,92±3,15*	52,80±2,84	70,40±8,36	62,44±7,11	61,81±4,32
Total Bilirubin µmol/l	60,93±1,6					
1 sampling		63,88±3,21	69,37±2,97	56,28±1,32	60,93±4,15	56,99±2,71
2 sampling		59,09±3,25	60,32±4,36	67,56±7,68	71,05±5,48	65,42±3,34
Direct Bilirubin, µmol/l	31,42±1,1					
1 sampling		34,66±2,35	42,79±4,40	31,03±1,70	37,89±3,89	33,63±3,52
2 sampling		32,53±2,09	33,23±3,49	38,69±4,57	45,00±6,70	38,62±3,90
Indirect Bilirubin, µmol/l	29,51±0,60					
1 sampling		29,22±1,85	26,58±2,79	25,26±1,67	23,03±2,24	23,36±2,61
2 sampling		26,56±1,55	27,09±1,27	28,87±3,55	26,05±1,88	26,80±1,94

Note: * – P<0,05; *** – P<0,001; 1st sampling – approx. 1 month from the study onset; 2nd sampling – the end of the study, at the 129th day from the onset.

The results obtained (table 2) reveal that in the blood serum, at the first research term, the AST transaminase activity shows a marked growth tendency, which in the intact birds from the control group is 16.97 ± 2.35 u/l, representing an increase of 26.5%, compared to the background values. At this stage, the studied product exerts a dose-dependent action on the activity of the AST enzyme. Thus, the activity of this biochemical parameter decreased in three experimental groups (EG 1, 2 and 4), by 9.8-13.6% compared to the control group. At the same time, the parameter investigated in hens from EG 3, on the contrary, increased by 14.8% compared to the control values. The dynamics of ALT enzyme activity, at the end of the study, shows an upward tendenct of its value in the birds from the CG (+ 6.2%) compared to the previous level, while this upward tendency in EG 2 is 12.3%. The study shows that the ZooBioR product contributes to the decrease of AST transaminase activity, its value decreasing by 4.7-20.9% compared to the control group (p <0.05, for EG 1), good results that show the positive action of the tested product on the functional state of the liver. Similar results were obtained by other authors who administered to animals different biologically active remedies (Balanescu S., Voiniţchi E. et al., 2014; 2019; Macari V., Putin V., Gudumac V., 2009; Caradailî D., Manastîrli T., Roşca I., 2018).

The results estimating the activity of ALT (table 2) reveal that, at the first research, there were no changes in the control group, while the value investigated in the experimental groups showed a clear increase tendency of 15.2-34.9% in relation to the control group ($p < 0.001$, for EG 3). Similar results were found in quails, also used for egg production, and treated intramuscularly with the BioR remedy (Macari V. et al., 2014; Pavlicenco N., 2019), as well as in broilers, which benefited from other bioactive remedies. (Balanescu S., Voinițchi E. et al., 2019; Falcă C., Mocofan E., Morar D., 2009). In this context, the justification of the obtained results is revealed at the end of the experiment, when in the birds from the CG was found an increase of 31.8%, of ALT enzyme activity, compared to previous values ($p < 0.05$), repeating the dynamics of this enzyme, previously reported in the birds from the experimental groups. Therefore, the increased activity of this enzyme (AST) in the blood serum could be physiological, specific to the laying cycle of hens. At this last experimental stage, the serum activity of the ALT enzyme in 3 EG, except for EG 1 (minimum dose of ZooBioR), showed higher levels, of 3.2-17.5% in relation to the reference values. Based on the results obtained, a possible action mechanism of the test product could be the improvement of the proteosynthetic function of the liver, the reduction of hepatocyte alteration, as well as the improvement of protein metabolism in general.

In order to assess the evolution of liver metabolic processes in intact hens, as well as under the action of the studied remedy, we considered as important to assess the total bilirubin content and its fractions in the blood serum (table 2). Based on our study, we found out that at the first research term, serum bilirubin, in the birds from the reference group, showed a growth tendency of 4.8%, such a tendency being also reported in EG 1, of 8.6% compared to the group of control. The administration of the tested bioremedy, with food, to the birds from EG 2, 3 and 4, in periods of high metabolic load, such as the first laying period, managed either to maintain at the initial level, or to reduce the concentration of total bilirubin in blood by 10.8-11.9% compared to the control group. The decrease could be due to the involvement of the ZooBioR product in the metabolic processes that take place in the body of hens, and especially in the liver. Those specified are certified at the end of the study when there is a delayed tendency of serum bilirubin decrease in the CG by 7.5%, and in EG 1 by 13.0% compared to previous values. At the same time, in hens, whose food had been supplemented with the tested remedy, there was a slight serum bilirubin increase tendency, in general depending on the dose of the administered product, of 2.1-20.2% compared to the reference data. Similar results were obtained by other authors who administered to pregnant dogs the BioR remedy (Caradailî D., Manastîrli T., Roşca I., 2018), as well as to the rabbits, also the BioR remedy (Maţencu D., 2019).

The content of direct bilirubin (conjugated, bound) in serum, in intact hens, at the 1st research, has an increasing tendency of 10.3%, as compared to the background values, a phenomenon that can be attributed to the intensification of the physiological-metabolic processes that take place in the body of birds in the first intensive laying period. At this research term, the tested product did not unequivocally influence the investigated parameter, inducing in the birds from EG 1 and 3 an incresing tendency, of 9.3-23.5% compared to the reference indices. The study shows that the usage of the studied phytoproduct, at a dose of 10.0 mg active substance/kg fodder determines the maintenance of the investigated biochemical indicator at background levels, while the average value of direct bilirubin in EG 2 being 31.03±1.70 µmol/l, a decrease of 10.5% compared to the control group, and of 7.7-27.5% compared to the other three experimental groups ($p < 0.05$, compared to EG 1), results that will be taken into consideration as to determine the optimal dose of the product.

The study reveals that, at the last stage of research, the value of the parameter investigated in birds, from the CG, decreased, reaching an average of 32.53±2.09 µmol/l, the decrease being of

6.1%. Higher decreased values had been also reported in EG 1, birds in which the food was supplemented with ZooBioR in the lowest dose, the decrease being of 22.3% compared to previous values. At this last stage, the tested remedy induced, in all bird from all EGs, higher values of direct serum bilirubin, of 2.2-38.3% compared to the control group. These results can be explained by the intensification of the body metabolic processes, especially in the liver. Similar results regarding the increase of the serum level of direct bilirubin in rabbits, during the reproductive cycle, physiological, but stressful periods for the animals, have been obtained following the use of another biologically active remedy - BioR (Maţencu D., 2019).

An important biochemical criterion in assessing the functional state of the liver - indirect bilirubin (free, unconjugated) presented in the first research a serum stability in young intact hens, located at the background level, results that sum up several factors, reflected in birds` good health state. Indirect bilirubin shows a marked decrease tendency in hens from the groups supplemented with ZooBioR. While in the control group, the value of indirect bilirubin was 29.22 µmol/l, in birds from the EG it was 26.58-23.03 µmol/l, the decrease being 9.0-21.2%, an undeniably positive phenomenon, which probably denotes the intensification of the physiological-metabolic processes in the liver, as well as the improvement of erythrocytes function. Indirect bilirubin in the birds from the CG, at the end of the study, showed a decreasing tendency, of 9.1% compared to previous values, a delayed manifestation, which occurred in birds from EGs, during the first research. Meanwhile, in the hens from the EGs there was a slight upward tendency, of 1.9-14.7% compared to previous values, obviously in those groups. At the same time, much lower increased values had been reported in the birds from EG 1, 2 and 4 compared to the control group, the increase being of 0.9-8.7%. Similar direct bilirubin dynamics had been reported in animals by other authors, following the administration of other biologically active remedies. This phenomenon could be explained by the improvement of the liver function duet o these remedies (Maţencu D., 2019).

The evaluation results of the alkaline phosphatase and its fractions dynamics, in blood serum, in young laying hens, are shown in table 3.

Tabel 3.

Alkaline phosphatase and its fractions vaues, in blood serum, in young laying hens

Signification	Onset	Birds` Groups				
		CG	EG 1	EG 2	EG 3	EG 4
Total alkaline phosphatase, u/l	729,72± 64,11					
1 sampling		579,56±111,21	710,01±88,69	604,02±88,66	571,41±78,65	673,33±110,62
2 sampling		527,92±108,11	377,77±20,33**	455,22±65,71	580,92±125,71	383,20±29,45*
Termostable alkaline phosphatase, u/l	519,09± 87,60					
1 sampling		350,59±66,80	505,50±54,00	401,55±80,49	366,90±45,56	425,33±73,79
2 sampling		347,87±87,12	235,09±32,55	307,11±48,10	364,18±127,89	242,56±14,04
Termolabile alkaline phosphatase, u/l	210,63± 25,25					
1 sampling		228,97±47,74	204,51±47,52	202,47±32,82	204,51±37,09	248,00±47,42
2 sampling		235,77±52,80	142,68±24,64	148,12±21,54	216,74±67,55	140,64±26,03

Note: * – P<0,05; ** – P<0,01

The general analysis of the results (tabel 3) allows to highlight in birds a unique tendency of the total alkaline phosphatase decrease at the first research, this parameter reaching in the CG, the 579.56±111.21 u/l value, the decrease being of 20,6%, compared to the background values. In the case of EG 1, 2 and 4, the ZooBioR product stopped the decline of this enzyme by 4.2-22.5%, compared to the control group. Similar tendencies of ALP increase, in broilers, have been obtained by other authors who administered with food selenium (Falcă C., Mocofan E., Morar D., 2009).

We have noticed that, in the second research, the analyzed enzyme undergoes relevant changes, in the birds from the CG, decreasing by 8.9%, compared to the first research, a marked reduction tendency reported as well in EG 1, 2 and 4, its value decreasing by 1.3-1.9 times, compared to the previous values ($p < 0.05$, EG 4; $p < 0.01$, EG 1). We would like to highlight the fact that the parameter in the birds from the EG 3, proved to be higher, the increase being of 10.0% compared to the control group. Meanwhile, in the EGs, with the exception of EG 3, the total alkaline phosphatase (ALP) value shows a marked tendency of decrease compared the values of the control group, a decrease of 1.2-1.4 times, a phenomenon that could be considered positive, showing the anti-stress and hepatoprotective action of the ZooBioR product.

Studies have shown that the serum activity of thermostable ALP (liver fraction), in the 1st research, in birds from the CG, has a marked decreasing tendency, of 32.5%, compared to the initial level. It has been established that the usage of the tested product induces a statistically significant increase in the level of this enzyme, in the blood serum, which exceeded by 4.7-44.2% the control values. The results of the studies indicate that the serum concentration of the investigated enzyme, at the end of the experiment, remains practically at the same level (+ 0.80%), which invokes a unique health state of the intact birds throughout the whole experiment. The decreasing tendency of the thermostable ALP enzyme persists in birds from EG 1, 2 and 4, in which the investigated enzyme value is also lower than the control group values, the decrease being 11.7-32.4%. Similar results, regarding the possibility of decreasing the serum level of this enzyme, in rabbits, especially in the 45th postpartum day, as well as in broilers, at the end of the experiment, have been obtained by other authors, following the usage of certain bioactive remedies (Maţencu D., 2019; Putin V., 2014).

The data obtained (table 2) show that the activity of the thermolabile ALP enzyme (bone fraction) in intact birds, in the first research stage, has a growth tendency (+ 8.7%), an increase that persists in birds in EG 4, whose food was supplemented with the maximum dose of ZooBioR, the increase being of 17.7%, compared to the background values. In the other three EGs, the tested bioactive compound reduces the activity of the studied parameter, in which the functional capacity is 2.9-3.9% lower than the background, results that can be considered positive. At the end of the research, in birds from the reference group and EG 3, in serum had been attested a weak positive dynamics of the investigated enzyme activity, of 3.0% and 6.0%, respectively, compared to the previous values. At the same time, the investigated parameter in EG 1, 2 and 4 shows a marked decreasing tendency, of 1.4-1.8 times compared to the previous values, in the respective groups.

At the same time, in birds from all groups whose food was supplemented with the tested product, the investigated parameter has a marked decreasing tendency compared to the control values, by 1.1-1.7 times, a phenomenon that can probably be explained by the intensification of metabolism in general, and mineral metabolism in particular, including the processes related to eggshell formation. In the analyzed context, we would like to specify that animal production, including egg production, are accurate indicators of animal health. The study reveals that the egg laying intensity, at the beginning of the study, was 64.3-71.4% in all 5 groups of hens. It is important that, on the last day of study, this zootechnical parameter in the CG, constituted an average of 91.67%, compared to 100% in the EGs, resulting an increase of 8.3%.

The obtained results attest show that the ZooBioR product administered for a long period of time to laying hens was well tolerated. In addition, the changes of the enzymes` activity, namely the hepatic ones, probably represent one of the body's adaptation reactions, aimed at optimizing metabolism in situations of high metabolic load, such as intensive hens` exploitation, especially during the first technological period of laying.

Conclusions

1. The ZooBioR product, obtained from Spirulina platensis, administered with food, to young laying hens, for a period of approximately 4 months, was well tolerated and did not induce adverse reactions.

2. During the oral administration of the ZooBioR remedy to young laying hens, bred under physiological conditions of avicol factory, has been established a decreasing tendency of the ALT enzyme and an increasing tendency of the AST enzyme. ZooBioR has also induced, in the first research, a tendency of decrease of the total bilirubin and its fractions in blood serum, and an increase tendency of these parameters, at the end of the experiment, dynamics that reveal the functional state of the liver.

3. The study of the ZooBioR action on the activity of the ALP enzyme and its fractions revealed the property) of this local product to stop the decline of the ALP enzyme and its thermostable fraction, at the first experimental stage, of high metabolic load, while at the end of the experiment, a decreasing tendency of these parameters persisted, especially in hens treated with lower doses of the tested remedy.

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Monitoring of the epidemiological situation of avian salmonellosis in poultry marketing units

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Abstract
The aim of the proposed investigation was to establish the bacterial microflora present on poultry carcasses and eggs sold in the food network of the central agricultural market from Chisinau; especially, bacteria of the genus Salmonella spp. During the investigation was studied the type and number of bacterial colonies and was serotyped the bacteria of the genus Salmonella spp. At the same time was performed the sensitivity of the isolated microflora to some more frequently antibiotics used in poultry farms. The research samples were taken from refrigerated carcasses of broilers chikens and eggs from poultry farms from the republic. The investigation's result confirmed the presence of an associated microflora with Srteptoccocus, Staphylococcus, E. Coli and bacteria of the genus Salmonella spp. in poultry carcasses and eggs of current consumption. From the total number of samples taken, in 12% of them was detected the bacteria of the genus Salmonella spp. The serotyping confirmed the presence of the following Salmonella spp. serotypes: S. Infantis, S. Enteritidis and S. Typhimurium. The antibiotic resistance tests confirmed a low sensitivity of the isolated microflora to some of the most common antibiotics used in birds' raising. The area of inhibition ranged from 8mm to 0 mm in most tested antibiotics; the most sensitive antibiotic proved to be florfinicol with a maximum inhibition area of 18 mm. The obtained results demonstrate the presence of some pathogenic serotypes of Salmonella spp. which can have a major risk for public health.
Key words: *chickens, carcasses, antibiotic resistance, contamination, samples, serotyping.*

Introduction

Chicken meat is one of the highest consumed meat across the globe. Global Livestock Counts report that there are almost 19 billion chickens in the world, making it the most common species of birds. Nowadays, worldwide, poultry meat occupies an important place in human nutrition due to its benefits in terms of biological components. Compared to meat produced by other domestic animals, poultry meat has varios advantageous aspects which will be further discussed. Chickens have a low body weight which allows to obtin fresh meat production in a short timeframe. Poultry meat and organs represent a rich source of mineral salts and vitamins. From biochemical aspect, this type of meat contains all the essential amino acids necessary for the human nutrition [7,10].

However, there are also potential risks associated with the consumption of poultry meat because it can be contaminated with various conditionally pathogenic or pathogenic micro-organisms. These micro-organisms may be present in the finished products, meat or eggs, following the contamination during the growing chain or slaughter, storage and marketing as well as during preparation. Compliance with all veterinary health rules, technological maintenance and feeding indices are the main criteria in maintaining the health of poultry flocks as well as reducing the risk of transmitting communicable diseases to humans, either through direct contact with poultry flocks or through contaminated poultry products (meat and eggs). Currently, more than 200 diseases are included in the category of zoonoses of which over 60% are diseases with infectious etiology [2,6].

Protecting poultry from contamination with unwanted microorganisms is an essential component of the poultry industry. The application of daily biosecurity procedures represent the best management practices on poultry farms as they contribute significantly to reduce the possibility of contacting zoonotic microbiological infections such as *Salmonella* and

C*ampylobacter*, as well as other infectious diseases such as avian influenza, newcastle disease, Gumboro diseaseand et all [5,9].

Taking a retrospective of bacterial diseases, in the group with a major risk to the poultry health, but especially to human health, bacteria of the genus *Salmonella spp*. represents up to 25% of foodborne infections causes in humans due to poultry contaminated with this genus of bacteria. Although it is known that there are currently about 2500 different *Salmonella* serotypes, only about 200 serotypes are associated with foodborne infections in humans. Vertical transmission of Salmonella serotypes from breeding flocks to commercial poultry flocks was analyzed for two of the most important serotypes, *Salmonella Enteritidis* and *Salmonella Typhimurium*. However, recently, disimination of these two serotypes have been reduced in many countries due to the introduction of strict biosecurity measures, effective surveillance, but also because vaccinations has been introduced; which, usually causes some difficulties in serologic monitoring system of salmonellosis. General veterinary sanitary measures are a good start, but they may not be enough to completely eliminate the infection in most situations. Still, a major importance of avian salmonellosis is to prevent the contamination of poultry products with this type of bacteria through the prism of systematic bacteriological investigations on poultry meat and the exclusion of its penetration into the public alimentation [1,8].

Objectives

In this context, our scientific research activities have focused on monitoring the epidemiological situation of avian salmonellosis and contamination of poultry products with bacteria of the genus *Salmonella spp*. and establishing the presence and diversity of pathogenic serotypes of the genus *Salmonella spp* [3,4].

Materials and methods

Samples were taken from the carcasses of birds sold in the Central Agricultural Market of mun. Chisinau and from eggs for current consumption. In total, were collected 80 samples from the poultry carcasses and 40 from eggs of current consumption. The samples from the poultry carcasses were delivered from the following poultry units: “Valcovschii Iurii”, Ialoveni district, v. Dmbreni; GŢ “Marandici Serghei”, Teleneşti district, v. Mîndreşti; SRL “Dobrocolischii”, mun. Chisinau, v. Singera; GŢ “Goroşenco Angela”, Anenii Noi district, v. Chetrosu; SRL "Viamar", Teleneşti district, v. Mîndreşti; II ”Gachiuţa Elena, Ialoveni district, v. Ulmu; SRL ”Lutam Com”, SRL “Primantol Grup”, SRL “Genevis Grup”, SA “Floreni”, SRL “Margaritar Impex”, ÎI Poperecnaea Elena ”, SRL“ Procolnis ”. The examinations were performed in the laboratory of microbiology of the faculty of Veterinary Medicine, SAUM and in the laboratory of microbiology of the Animal Health and Welfare Department of the Republican Center for Veterinary Diagnosis. In laboratory conditions the inoculations were performed on culture media as: Nutrient agar, Endo Agar, Salmonella Shigella Agar (SSA), Sabouraud Dextrose Agar, Bismuth sulfite agar (BSA). The presence and morphological structure of bacterial colonies grown on culture mediaserved as monitoring indicators. Subsequently from the bacterial colonies were prepared smears that were stained using the Gram method and examined under a biological microscope, 10x100 objective. Serotyping of bacteria of the genus *Salmonella spp*. was performed in the Republican Center for Veterinary Diagnosis.

The washes from the consumer eggs were taken from the units specialized in the eggs production and sold in the Central Agricultural Market of mun. Chisinau, delivered from poultry companies as: SRL “Intervetcom”, Cimişliea district, SRL Redi Agro, Donduşeni district,

v.Tîrnova, SRL Dant Agro, Ungheni district, v. Pîrliţa, SRL "Solar Nord", Edineţ diastrict, v.Gordineşti, SRL Avicola Rişcani, v. Corlăteni, SRL Pasărea Silver ", mun. Chisina, v. Ciorescu.

Results

The laboratory investigations were focused on the microbiological investigations done in order to determine the level of contamination of bird carcasses and eggs with the bacterial flora. These investigations were afterwards combined with the microscopic investigations to establish the identity and association of bacterial forms isolated from examinated samples, and performe antibiogamas of the isolated microflora to more common antibiotics used in birds grows. Some of the of laboratory research results are presented in figures 1-9. Figures 1-4 show some of the colonies of microorganisms that have predominantly grown on culture media.

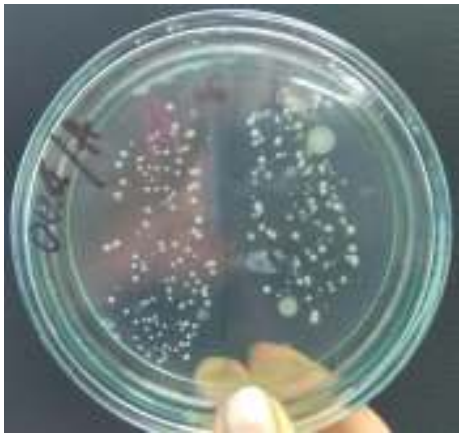

Fig. 1 Associated colonies of *Strepto* and *Staphylococcus* (the peptone agar medium)

Fig. 2 *E. Coli* colonies on the Endo medium

As a result of bacteriological investigations was established that from all samples taken from poultry carcasses and the current consumption eggshells the isolated microflora was associated with predominance of bacterial forms as *E. coli, Salmonella spp., Streptococcus* and *Staphylococcus.* Figure 1 shows the colonies of *Streptococcus* and *Staphylococcus* that have a round or oval shape, white-gray color, placed separately or in piles, being spread over the entire surface of the Petri dish, with predominantly central localisation. Their number ranged from 105 to 215 colonies. An intensive growth of *E. coli* colonies was present simultaneously in all samples examined (Fig. 2), the color of the colonies varying from red with intensity up to burgundy, with metallic luster, placed on the entire surface of the Petri dish, numerically they constituted from 78 to 155 colonies.

Fig. 3 Colonies of *Salmonella spp*. on the SSA medium (pure culture)

Fig. 4 Colonies of *Salmonella spp*. on the BSA medium

In the examined samples *Salmonella spp*. colonies increased from moderate to intensive growth. Some of the results of Salmonella spp. colonies are shown in Figures 3 and 4. On the Salmonella Sigiella Agar medium, *Salmonella spp*. colonies have a dark brown color, with a more intense center and a lighter periphery, placed evenly on the surface of the Petri dishes, with a numerical variation from 168 to 374 colonies.

At the same time, were prepared the smears from the colonies of microorganisms and examined under a biological microscope. The results of this study are shown on figures 5-8. Figure 5 and 6 show bacterial forms as *Streptococcus* and *Staphylococcus* isolated from the eggshells and from chicken carcasses. They were dispersed almost uniformly throughout the microscope field, placed in a chain, separated one by one, in groups of two or in piles in different shapes, having a spherical shape and blue color.

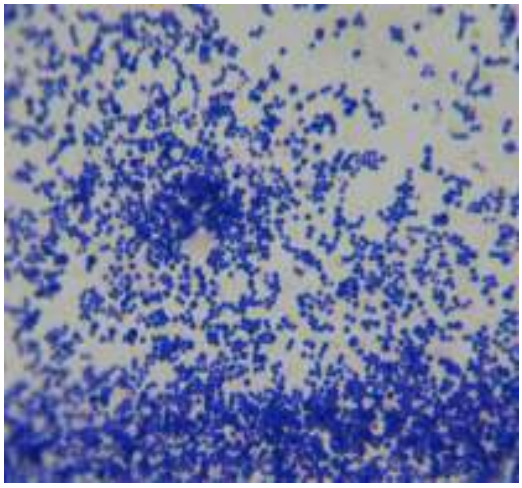

Fig.5 *Streptococcus* and *Staphylococcus* (colonies on nutrient agar, ob. 10x80)

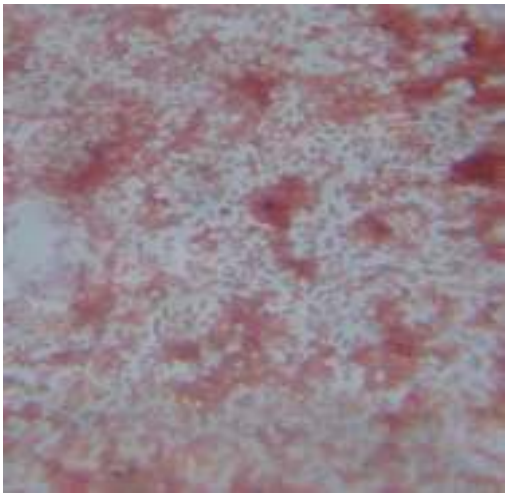

Fig. 6 *Salmonella spp* (colonies on the medium SSA, ob. 10x80)

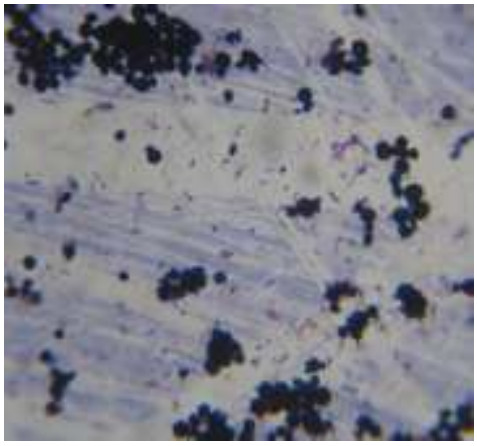

Fig.7 Forms of *Streptococcus*, *Salmonella spp*., (colonies on SSA medium, ob. 10x80)

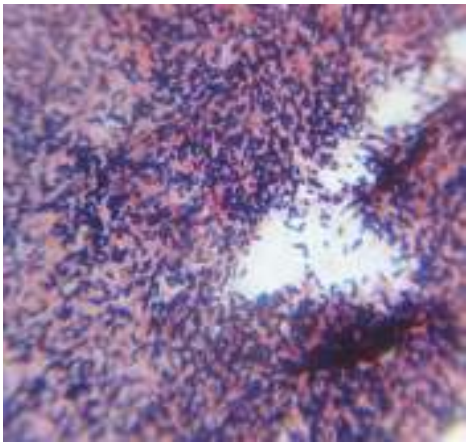

Fig. 8 Fungal and yeasts (colonies on *E.coli*, Sabouraud Dextrose Agar, ob. 10x80)

When the smears were prepared from microbial colonies wich have grown on the Salmonella Shigella agar environment (Fig. 6), the microorganism colonies were associated with bacteria of the genus *Salmonella spp*., *E coli*, which was shaped like sticks, with pink color and oval form. Figure 7 and 8 show some forms of fungi and yeasts in association with *Streptococcus* bacteria. In this case, the smears were prepared from the colonies that have grown on the SSA and Sabouraud Dextroze Agar.

Microbiological investigations performed on poultry carcasses and eggs sold in the Central Agricultural Market of mun. Chisinau show that from the total number of samples taken, 12% of the samples demonstarte the presence of colonies of *Salmonella spp*. Serotyping of *Salmonella spp*. cultures confirmed the predominant presence of serotypes *S. Infantis, S. Enteritidis, S. Typhimurium*.

Subsequently, the isolated bacterial flora was tested for sensitivity to some antibiotics that are more commonly used in poultry farming.

a

b

Fig. 9 a) and b) Antibiogram (area of inhibition of microorganism colony growth)

As a result of this study has been established a moderate sensitivity of the isolated microflora to some antibiotics that have the following parameters: Eurosol 5mg - 4 mm, Florfenicol

30mg - 18 mm, Oxytetracycline 30mg - 8mm, Genta plus 10mg - 3mm, Tilmivap 15mg - 0 mm. The results of this study is represented in figures 9 a) and b. The most sensitive antibiotic against the isolated microflora proved to be Florfinicol, where the inhibition zone of bacterial colonies was 18 mm, and the lowest action was obtained using Tilmivap having an index of 0 mm.

Conclusions

1. The results of the research demonstrate the presence of pathogenic serotypes of *Salmonella spp*. in poultry products; therefore, confirming the existence of risks of contamination of poultry products at some stages of production, processing or marketing, which thus favors the occurrence of toxin infections in humans.
2. The bacteriological investigations of poultry carcasses and eggs of current consumption indicate the presence of an associated microflora which was represented by bacterial forms as: *Srteptococcus, Stafilococcus, E. Coli* and bacteria from genus *Salmonella spp*.
3. From the total number of examined samples, in 12% of them was isolated the bacteria of genus *Salmonella spp.* and the serotiping procedure confirmed the presence of serotipes as: *S. Infantis, S. Enteritidis and S. Typhimurium.*
4. The bacterial forms isolated from the poultry carcasses as well as the common consumption eggs have shown a reduced sensitivity to some antibiotics commonly used in poultry grows, however a higher sensitivity was determined using Florfinicol which demonstrated a maximum inhibition area of 18 mm.

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Passive immunity stimulated by vaccination of dry cows with a trivalent vaccine against neonatal calf diarrhea

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Abstract
Passive transfer of colostrum immunoglobulins from cow to newborn is extremely important because calves under 5 weeks of age do not have active immunity and colostrum antibodies are the only source of immunoglobulins to protect calves from infectious diseases immediately after birth. One of the most common causes of calf death is acute neonatal diarrhea caused by pathogens such as rotavirus, coronavirus and Escherichia coli. In the first weeks of life, calves acquire maternal antibodies from colostrum and milk can have a local protective effect against intestinal enteropathogens. Vaccination of pregnant cows reduces the morbidity and mortality rates of the calf. Vaccination of cows even in the early stages of pregnancy (6 months before calving) can provide passive protection in newborn calves against etiological agents such as enterotoxigenic Escherichia coli. Previous studies have shown that continuous administration of colostrum from immunized cows prevents severe diarrhea and mortality in calves. In North America and Europe, various vaccines against neonatal calf diarrhea have been developed to increase antibody titers in colostrum and cow's milk. However, there are not many studies on maternal vaccination to protect against diarrhea in calves in Romania. In this article we have detailed the results of a field study on improving passive immunity in calves by administering a multipurpose maternal vaccine available in Europe.
***Key words**: calf, colostrum, neonatal calf diarrhoea, passive immunity, vaccination.*

Introduction

Neonatal diarrhea mainly affects calves under the age of 4 weeks. It is characterized by diarrhea that leads to dehydration and acidosis, which can have systemic consequences and can lead to death [5]. More than 50% of all cases of neonatal diarrhea occur in the first week of life and only 15% occur after the second week of life [4]. Calf loss, disease management and health consequences, growth rate and reproductive potential of surviving calves can lead to significant economic loss [1, 5].

The main infectious agents causing diarrhea, especially in calves under 12 days of age, are bovine rotavirus (BoRV), bovine coronavirus and enterotoxigenic Escherichia coli (ETEC), but other pathogens may also be responsible for this disease [1 , 4, 5]. The good part is that vaccines against some of the most common pathogens responsible for this disease are available on the market. [2. 3]

The aim of this study was to evaluate the titer of specific BoRV, BoCV and E.coli F5 (K99) antibodies in the serum of pregnant cows, in colostrum and in the serum of newborn calves in order to highlight the immune response in cows and the passive transfer in calves.

Material and methods

In order to carry out the study, 2 experimental groups were established: the control group (M), to which no specific prophylaxis method against bovine rotavirus (BoRV), bovine coronavirus and *Escherichia coli* was applied and the experimental group (E) which was immunized with a trivalent Rotavec ™ Corona vaccine, MSD Animal Health, within 220-230 days of gestation. Each group consisted of 20 pairs of cow-calves from which 104 serum samples and 40 colostrum samples were collected between November 2019 - December 2019. In group E, 2 twin calvings were registered. Blood samples were collected from cows at 21 days post-vaccination, respectively between 241-251 days of gestation, and colostrum samples were collected immediately after calving. Calves received a dose of 4 liters of fresh colostrum harvested from their mothers in the

first hour of life and blood samples were taken before an after colostrum consumption at 24 hours of age.

Specific antibodies were detected using the competition enzyme immunoassay kits ELISA Bovine Rotavirus BIO-X Diagnostics, ELISA Coronavirus BIO-X Diagnostics, and ELISA blocking E. coli F5 (K99) BIO-X Diagnostics. The analyzes were performed in the Immunology laboratory of FMV Iaşi and the descriptive statistical data and intra-group comparisons were performed using the IBM SPSS Statistics Subscription program.

Results and discussions

In the Figure 1 are represented graphically the mean values of bovine anti-rotavirus antibodies (BoRV), expressed as percent inhibition (PI%). It can be seen that the PI% of cow serum antibodies - colostrum antibodies - calf serum antibodies have higher values in group E. The level of anti-rotavirus antibodies in the serum of pregnant cows in group E shows a significant difference compared to group M, in group E registering an average of 77% PI, compared to 41.5% PI in group M (p value <0.001). Increased serum antibody titer in group E cows resulted in a higher synthesis of specific antibodies in colostrum (79.3% PI). In the serum of calves from group E there is an average of 94.4% PI and in the group M there was an average of 59%. The difference between the averages from the 2 groups is statistically significant with a p value <0.001.

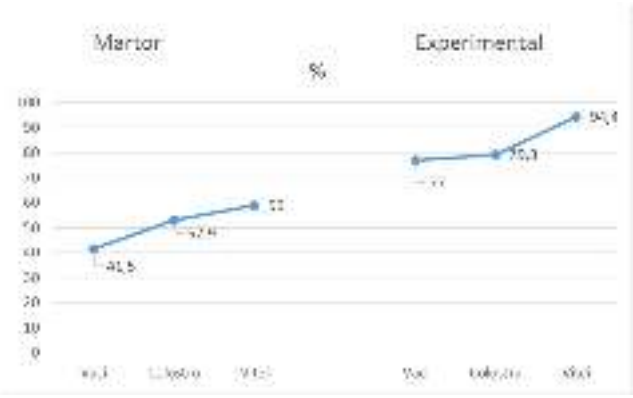

Fig. 1 – Graph of mean values of BoR expressed as percent inhibition measured with competitive ELISA from pregnant cow sera, colostrum and calf sera

In Figure 2 are represented graphically the mean values of bovine anti-coronavirus antibodies (BoRV), expressed as percent inhibition (PI%). As in the case of the analysis of anti-rotavirus antibodies, it can be observed that in group E the level of anti-coronavirus antibodies in the serum of pregnant cows increased considerably compared to group M, in group E registering an average of 96.2% PI, compared to of 32.6% PI in group M (p value <0.001). Elevated serum antibody levels in group E cows resulted in a high level of synthesis of specific antibodies in colostrum (98.9% PI). An average of 97.3% PI was recorded in the serum of calves from group E, and an average of 32.6% PI was recorded in the group M. The difference between the averages of the 2 groups is statistically significant with a p value <0.001.

Fig. 2 – Graph of mean values of BoCV expressed as inhibition percentages measured by competitive ELISA from pregnant cow sera, colostrum and calf sera

The values of anti-E. coli F5 (K99) antibodies, expressed as percent inhibition measured with ELISA blocking in the sera of pregnant cows and the sera of calves are plotted in Figure 3. In group M, both in the serum of pregnant cows and in the serum of calves, a minimal presence of anti-E.coli F5 (K99) antibodies was detected, with an average of 0.3% PI cows and 0.2% PI calves, respectively. The differences between group E and group M are statistically significant ($p < 0.001$), in group E registering an average of 90.9% PI in the case of pregnant cows serum and 97.4% PI in the case of calves serum.

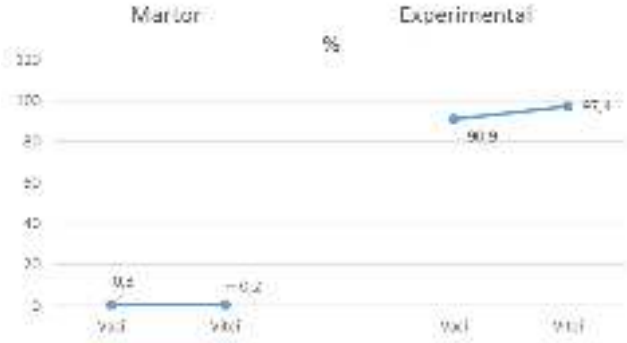

Fig. 3 – Graph of mean values of anti-E.coli F5 antibodies (K99), expressed as percentages of inhibition measured by competitive ELISA in pregnant cows and calves

The calf has an essential feature in terms of immunology: the titer of antibodies at birth is zero, during intrauterine life the transfer of antibodies by transplacental route from mother to fetus is zero. Consequently, it is born without adequate humoral immunity and is totally dependent on passive transfer via colostrum. To test this hypothesis, blood samples were collected from calves in group E before colostrum administration and 24 hours after administration to show the level of anti-rotavirus, anti-coronavirus and anti-E.coli antibodies. F5 (K99). The results are shown in Table 1.

Table 1.

Specific antibodies in calf serum before and after colostrum, expressed as% inhibition measured by competitive ELISA

Statistical parameters	Antibody values expressed in PI%							
	BoRV, strain UK-Compton, serotype G6 P5 (inactivated)			BoCV strain Mebus (inactivated)			E. coli F5 (K99) agglutinate	
	Calves serum before colostrum intake	Colostrum	Calves serum after colostrum intake (24 h life)	Calves serum before colostrum intake	Colostrum	Calves serum after colostrum intake (24 h life)	Calves serum before colostrum intake	Calves serum after colostrum intake (24 h life)
$\bar{X}$	0	79,3	94,4	0	96,4	97,3	0	97,4
SE	0	6,4	1,1	0	1,8	1,0	0	0,9
Median	0	89,2	95,1	0	98,9	99,1	0	98,7
SD	0	20,3	3,7	0	5,8	3,5	0	3,2
Min.	0	35,0	88,3	0	80,2	89,7	0	88,5
Max.	0	97,9	98,5	0	99,1	99,2	0	99,1
CI 95%	0	14,5	2,3	0	4,2	2,2	0	2

The presence of bovine anti-rotavirus, anti-bovine coronavirus or anti-E.coli F5 (K99) antibodies was not detected in the 22 serum samples collected before colostrum, which highlights the importance of colostrum administration in newborn calves for passive immune transfer of anti-infective specific immunoglobulins.

Conclusion

Because rotavirus and coronavirus infections are endemic in cattle, most adult cows have been infected naturally. The sustained response of antibodies found in the serum and colostrum of cows in group M is most likely due to such infectious exposure. Also, the immune response of group E cows may be due to a primary infectious exposure prior to systemic vaccination, but vaccination causes a marked increase in specific serum antibody levels. If in the case of rotavirus and bovine coronavirus were detected after analysis titers of maternal antibodies synthesized following active infections (group M), in the case of anti-E.coli F5 antibodies (K99) in the study group they were almost nonexistent. The obtained results showed that by the active immunization of cows in the last gestation period the specific antibody titers increased significantly in group E compared to group M, in all 3 infectious strains. This proves the effectiveness of vaccination in the herd.

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The influence of colostrum consumption on serum lactoferrin in newborn calves

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Abstract
Lactoferrin is a glycoprotein from the transferrin family, proteins capable of binding and transferring Fe^{3+} ions. Lactoferrin from bovine colostrum and milk has become increasingly important due to its wide range of biological properties. Colostrum intake leads to increased serum lactoferrin levels in calves [11], foals [2] and piglets [5]. Together with IgG, it is transferred from the intestinal tract into the systemic circulation through passive absorption in the case of newborns. Fewer studies have been performed in animals, but it has been shown to reduce morbidity and improve the growth rate of calves [9]. Studies have also indicated that lactoferrin can lead to the elimination of pathogens and therefore to the reduction of the incidence of neonatal diseases through the mechanism of iron binding, inhibition of bacterial growth and proteolytic activity [1].
Key words*: calf, colostrum, lactoferrin, passive transfer.*

Introduction

Lactoferrin was first isolated from Sorensen's bovine milk in 1939. It is the largest protein fraction in whey and is present in various physiological fluids, including plasma, tears, saliva, vaginal fluid, semen, nasal, lacrimal and bronchial secretions, biliary secretion, gastrointestinal fluid, urine, synovial and amniotic fluid, plasma and neutrophil granules. Lactoferrin has multiple properties, among the most important being antimicrobial, immunomodulatory and anti-inflammatory. Lactoferrin has been extensively researched in humans, primarily in infants and young children, and there is evidence that lactoferrin reduces the severity and longitudinal prevalence of diarrhea [8].

Some authors reported a mean lactoferrin concentrations of 1.96 ± 0.27 mg / ml in the colostrum of 45 Holstein cows [12], while others reported an average of 0.34 ± 0.23 mg / ml for 6 cows by chromatography [13]. High amounts of lactoferrin, ranging from 1 to 5 mg / ml, are found in bovine colostrum [10]. Lactoferrin is significantly influenced by the number of lactations ($r = 0.555$) and daily milk production ($r = –0.472$) [3]. As an antimicrobial protein, lactoferrin produced by the mammary gland can have a dual role, protecting both the mammary gland as well as the intestine of newborn calves.

Some researchers have suggested that there was no evidence between colostrum absorption and serum lactoferrin levels [6], while others reported that they found serum lactoferrin levels before colostrum absorption and that they are about 5 times lower than colostrum levels measured at 24 h in calf serum [4]. Moreover, these researchers showed a progressive decrease in serum lactoferrin levels from the first day until the end of the first month of calf life.

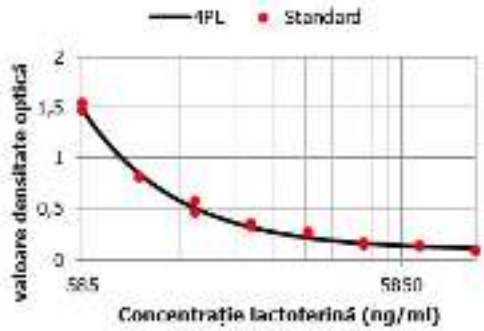
The aim of this study was to observe variations in serum lactoferrin concentrations following the intake of freshly harvested colostrum and administered to calves in the first hour of life.

Material and methods

In order to conduct the study, between November 2019 and January 2020, 42 samples of calf serum were collected and tested immediately after birth and 42 serum samples every 24 hours after colostrum administration. Calves were given freshly harvested colostrum from their mothers immediately after calving.

For the analysis of serum and colostrum lactoferrin, the Lactoferrin Bovine Bio-X Diagnostics Competition Elisa kit was used, which uses microplates sensitized with bovine lactoferrin-specific polyclonal antibodies. The samples are incubated concomitantly with a specific enzymatic conjugate consisting of chemically peroxidase-bound purified bovine lactoferrin. For the quantitative evaluation of lactoferrin in the samples a calibration curve obtained using dilutions from a known concentration of bovine lactoferrin using the Four Parameter Logistic Curve program (log-log) was used (figure 1).

The testing of the samples was performed within the FMV Iaşi immunology laboratory. Descriptive statistics and intra-group comparisons were performed using IBM SPSS Statistics Subscription and Microsoft Office Excel.



a	-0.1589
b	0.5852
c	660.5
d	5.945
MSE	0.006227
R^2	0.9885
SS	0.04359
SYX	0.1205

Fig. 1 – Standard calibration curve for the calculation of lactoferrin concentrations

Results and discussions

The statistical results of the analyzes performed are shown in Table 1.

Table 1.

Lactoferrin concentrations following colostrum consumption in newborn calves

	Colostrum (mg/ml)	Calf serum day 0 (µg/ml)	Calf serum day 1 (µg/ml)
$\bar{X}$±SD	0,67 ± 0,42	2,68 ± 1,05	5,24 ± 1,87
SE	0,10	0,30	0,42
Minim	0,15	0,25	2,70
Maxim	1,40	3,97	9,05
CI 95%	0,22	0,67	0,87
p value	calf serum day 0 – calf serum day 1: p< 0,0001.		

In the 40 colostrum samples, the mean value of colostrum lactoferrin was 0.67 ± 0.42 mg / ml, with a maximum value of 1.4 mg / ml and a minimum of 0.15 mg / ml (Table 1). The mean serum lactoferrin concentration on day 0 (2.68 µg / ml) increased approximately 2 times a day after colostrum intake (5.24 µg / ml, Table 3.4). Similarly, some authors showed low serum lactoferrin (1.09 µg / ml) in newborn calves immediately after birth, which increased approximately 10-fold to 8 hours after the first colostrum and thereafter it gradually decreased until the second day of life [7]. These authors suggested that the tendency to increase serum lactoferrin concentrations was

most likely caused by its absorption from colostrum. A relatively low concentration of lactoferrin in the blood plasma of calves at birth (0.20 µg / ml) increased approximately 10-fold at 6 hours after the first colostrum intake, but these higher concentrations were sustained only in the first 12 hours of life [11]. The increasing trend of bovine serum lactoferrin concentration observed in the present study one day after lower colostrum intake compared to the results of the authors mentioned above, could be influenced by the biological activity of absorbed colostrum lactoferrin, which is rapidly metabolized in the blood of calves approximately 12 hours after colostrum intake [7].

The concentrations of lactoferrin found in the blood of newborns are dependent on the concentration of lactoferrin in the ingested colostrum and the amount of colostrum administered [3]. To test this hypothesis, we set up 2 experimental groups, depending on the averages of the colostrum lactoferrin concentrations obtained, namely:

- Group 1 - colostrum lactoferrin concentrations between 0.15-0.2 mg / ml, with an average of 0.4 mg / ml, 28 cows, 30 calves;
- Group 2 - colostrum lactoferrin concentrations between 1.1-1.4 mg / ml with an average of 1.18 mg / ml, 12 cows, 12 calves.

The mean serum lactoferrin concentration in group 2 was significantly higher than in group 1 ($p < 0.03$, figure 2). This result confirms that lactoferrin in the blood of newborns is dependent on the concentration of lactoferrin in ingested colostrum, as as some authors suggest [3].

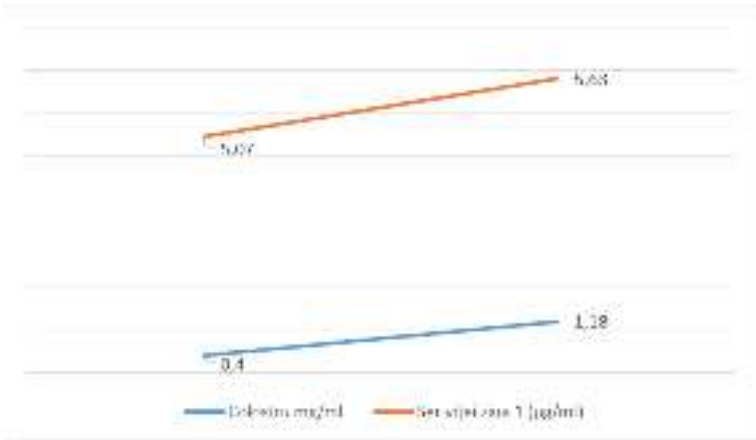


Fig. 2 - Influence of colostrum lactoferrin concentrations on serum lactoferrin in newborn calves

Conclusion

The obtained results showed that following the administration of colostrum, the serum lactoferrin concentration increased discreetly but significantly (calf serum day 0 - serum calf day 1 $p < 0.0001$). We also found that a higher concentration of colostrum lactoferrin causes a higher concentration of serum lactoferrin in calves 24 hours after colostrum administration. In conclusion, the results obtained showed significant changes in serum lactoferrin concentrations which reflects the calves' response to colostrum intake.

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Genetic diversity in *Babesia canis* and associated comorbidities can be fatal in dogs` babesiosis – a case study

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Abstract
The aim of this paper is to briefly present some aspects of Babesia spp. taxonomy, incidence, clinical signs of their infection, and possibilities of prevention, as an introduction to a case study of canine babesiosis presentation. A 7-year-old Malinois dog was presented in August 2020 with signs of generalized icterus, high body temperature and mustard urine, all of them indicating babesiosis. Cytological examination confirmed the large Babesia canis spp., and the biochemical investigations revealed renal and hepatic failure. Although the therapeutic protocol included the specific antidote, imidocarb dipropionate – Imizol® (0.5 ml/10 kg body weight, in a single dose), fluid therapy, vitamin therapy, an antiemetic drug, and supplements for renal and hepatic functions sustaining, the investigated dog died. The post-mortem investigation revealed generalized icterus. We consider the delaying of dog presentation at vet an important factor of this outcome; although an infection with various subspecies of Babesia canis was not excluded, the therapeutic intervention would have been the same.
Keywords: *Intra erythrocyte parasites, jaundice, anemia, antidote*

Introduction

Babesiosis is a tick-borne disease caused by various species of *Babesia* genus parasite. This disease is particularly common in dogs, its worldwide distribution drawing a special attention (Solano-Gallego and Baneth, 2011; Sudhakara Reddy et al., 2014). Infecting *Babesia* species in dogs were classified in two main groups, large forms being a part of *Babesia canis* group while small species were generally considered as *Babesia gibsoni* [Fabisiak et al., 2010; Solano-Gallego and Baneth, 2011; Zahler et al., 2000(a)].

The infection with *Babesia canis* is attributed to several species of ticks, such as *Dermacentor reticulatus* (Fabisiak et al., 2010; Födvári et al., 2005), while an additional transmission path via blood transfusion was reported in dogs for *Babesia gibsoni* (Stegeman et al., 2003) or from dog to dog during their fights (Solano-Gallego and Baneth, 2011).

The diagnosis of babesiosis in dogs is basically done by clinical examination and parasite` identification on May-Grünwald Giemsa stained peripheral blood smears (Fabisiak et al., 2010). Clinical signs of *B. canis* infection often include dehydratation, apathy, anorexia or decrease appetite, fever (Furlanello et al., 2005; Solano-Gallego et al., 2008); associated anemia has a multifactorial component based on plasma volume increasing, erythrocyte retention in the spleen, erythrocyte destruction partly due to parasite proliferation (Schetters et al., 1997). Other clinical signs may include jaundice or icterus, congested conjunctiva, tachycardia, tachypnea, lymphadenopathy, haemoglobinuria, bilirubinuria, with a general condition of lethargy or dullness (Fabisiak et al., 2010; Sudhakara Reddy et al., 2014). Splenomegaly, hemolytic anemia, and thrombocytopenia were reported in infections with *B. gibsoni* (Stegeman et al., 2003).

The diagnosis in peripheral blood smears take into account the size and morphological appearance of the intraerythrocytic parasites (Födvári et al., 2005; Muhlnickel et al., 2008). Unfortunately, such an examination only provides a presumptive diagnosis of the two groups of *Babesia,* the large and small ones. Different subspecies, although morphologically identical, are important to be independently diagnosed due to the difference in their clinical signs (Solano-Gallego and Baneth, 2011).

B. gibsoni can be difficult to be detected in red blood cells (RBCs) on peripheral blood smear examination, and serologic investigations using fluorescent antibody tests give unsatisfactory results in young dogs (1-3 months old) and due to serologic cross-reactivity among *Babesia spp.* (Farwell et al., 1982; Stegeman et al., 2003). Therefore, molecular investigations using Polymerase Chain Reaction (PCR) represent the best method to distinct various subspecies of this parasite (Cacciò et al., 2002). For example, the results obtained on pairwise identities, distance, parsimony, and maximum likelihood analyses of the 18S rRNA gene provided valuable information about genetic phylogeny of various types of *Babesia* [Zahler et al., 2000(a), (b)]. Subsequent sequencing of PCR products allows identity to be established by comparison with nucleotide sequences already known and recorded in GenBank®, as in the case of *B. canis canis,* accession numbers AY611731.1; AY611732.1; AY611733.1 (Adaszek and Winiarczyck, 2008; Födvári et al., 2005).

For this purpose of taxonomic identification, Cacciò et al. (2002) and Fabisiak et al. (2010) reviewed three distinct but morphologically identical subspecies of *Babesia canis*, such as *B. canis canis, B. canis rossi,* and *B. canis vogeli,* isolated from dogs in Europe, Africa, and US, respectively. The presence of *B. canis canis* in European isolates and of *B. canis rossi* in South-African isolates was confirmed by Schetters et al. (1997b), while *B. canis vogeli* was detected in dogs from Croatia, along with other infectious species such as *B. canis canis, B. gibsoni, Theileria annae* and, surprisingly, with two parasites usually found in horses, *Babesia caballi* and *Theileria equi* (Beck et al., 2009). In 2004, Matjila et al. confirmed for the first time *B. canis vogeli* in domestic dogs in South Africa; they also reported the presence of *B. canis rossi* but none of the investigated dogs was a carrier of both subspecies together. Solano-Gallego et al. (2008) reported the vast majority of *B. canis canis* infections in Northern Italy, while *B. canis vogeli* was mainly detected in Central and Southern Italy. In 2012, the presence of *Babesia vogeli* in a clinically normal dog in Romania was confirmed for the first time. It was part of a group of five asymptomatic dogs whose blood was comparatively tested by PCR technique with that of 11 other dogs with symptoms of babesiosis. Investigations of the 18S rRNA segment showed that all dogs with symptoms of babesiois were infected with *Babesia canis* (Ionita et al., 2012). Although the authors did not explicitly specify the identified subspecies of *Babesia canis*, it is well-understood the presence of *Babesia canis canis,* considering the type of vector predominantly found on the Romanian territory *(Dermacentor reticulatus).* They revised the transmission of *Babesia vogeli* by *Rhipicephalus sanguineus,* commonly called the brown dog tick, while the other *Babesia canis* subspecies, *Babesia rossi* is transmitted by *Haemaphysalis elliptica*, with eastern and southern Africa location (Beugnet et al., 2019; Fourie et al., 2019).

Various isolates from infected dogs demonstrated close phylogenetic relationships with other *Babesia* species, such as *B. microti, B. rodhaini, B. conradae, B. bigemina, B. divergens,* and *B. odocoilei* [Birkenheuer et al., 2004; Camacho et al., 2001; Solano-Gallego and Baneth, 2011; Zahler et al., 2000(a), (b)]. In 2008, Muhlnickel et al. firstly identified *B. gibsoni* in dogs from Australia, and in 2009, Beck et al. reviewed that some of *Babesia* small species appear to be closely related to *Theileria* genus. However, several classical differences, such as the absence of extra-erythrocytic multiplication (schizogony) in *Babesia,* or the forming of two daughter cells (merozoites) in *Babesia* comparing to four in *Theileria,* may contribute to their individualization; however, recent opinions do not consider *Babesia microti* as a *Babesia, Babesia equi* being already designated as *Theileria equi* (Uilenberg, 2006).

In conclusion to this introductive review, there is a need for molecular characterization of each isolated *Babesia* parasite in order to perform an appropriate treatment. Large *Babesia* species are commonly treated with imidocarb dipropionate at 5 mg/kg body weight, given intramuscularly

as a single dose, associated with fluid therapy and even blood transfusion for a good clinical response (Irwin and Hutchinson, 1991; Solano-Gallego and Baneth, 2011). Vercammen et al. (1995) reported the use of imidocarb dipropionate at 6 mg/kg b.w. They also reported a dog who became subclinical chronic carrier of *B. canis* as a result of a treatment with long acting oxytetracycline (20 mg/kg b.w.). Sudhakara Reddy et al. (2014) reported the use of diminazene aceturate, 5 mg/kg b.w., single dose, along with supportive and symptomatic therapy in *Babesia* infection of dogs. Both diminazene aceturate and imidocarb dipropionate drugs were reported safe to use for haemoprotozoan diseases in the same dosage (Olukunle et al., 2018).

Small *Babesia spp.* are considered more resistant to anti parasitic drugs, an incomplete elimination of *B. gibsoni* being translated in chronic carrier survivors (Solano-Gallego and Baneth, 2011; Stegeman et al., 2003). A treatment with clindamycin (25 mg/kg b.w., per os, at 12 hours, for 14 days) of 10 experimentally infected dogs with *B. gibsoni* was reported to reduce parasitemia levels as a result of parasite degeneration, with the improvement of clinical signs of the disease (Wulansari et al., 2003).

Vaccination of dogs against *B. canis* using soluble parasite antigens (SPA) has been reported as a limiting factor in splenomegaly development and an improvement in the immune response at 6 days post-vaccination (Schetters et al., 1997).

The aim of this paper is to review some taxonomic and incidence aspects of *Babesia spp.* and to present a case study of *Babesia* in dogs whose unfortunate ending shows the importance of fast therapeutic intervention until various comorbidities develop.

Material and methods

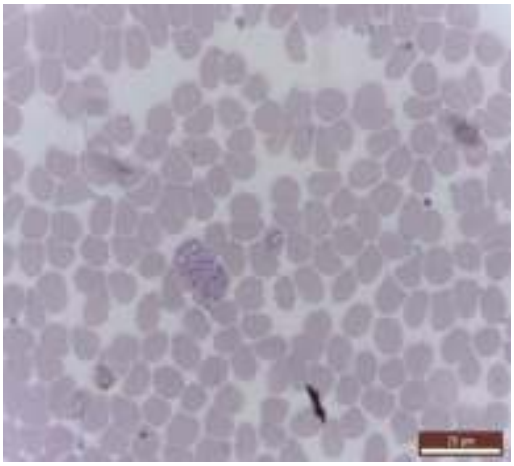
Some aspects reported in medical science about *Babesia spp.* infection, taxonomic including, clinical evolution, treatment and prevention were debated basing on 27 scientific papers studying. All these were used to provide an overview of a case study of babesiosis in dog, the diagnosis of which was established on the basis of usual clinical examination together with cytological investigations of May-Grünwald Giemsa stained peripheral blood smear. Blood samples were biochemically investigated for the following parameters: serum creatinine, gamma glutamyl transferase (GGT), aspartate aminotransferase (ASAT) or (serum) glutamic-oxaloacetic transaminase (GOT), alanine aminotransferase (ALAT) or (serum) glutamic-pyruvic transaminase (GPT), serum urea [all of these by the method of spectrophotometry], serum potassium and serum sodium [both by indirect Ion Selective Electrode (ISE) method]. Post-mortem, a necropsy examination was performed in order to observe changes in the organs.

Results and discussions

In august 2020, a 7-year-old Malinois dog with signs of babesiosis was presented for investigations at the Medical Clinic of Veterinary Medicine Faculty of Iași, Romania. Increased body temperature (40.6^0C) and general jaundice, at which mustard urine was associated, firstly drew the attention at the clinical examination (Fig. 1).

Fig. 1. Various clinical aspects in a 7-years-old Malinois dog babesiosis

As a result of cytological examination, *Babesia canis* was confirmed as parasiting some of RBCs (Fig. 2).



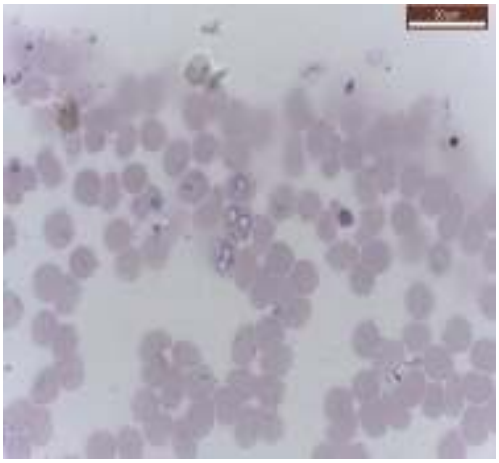


Fig. 2. Intra erythrocyte *Babesia canis.* May-Grünwald Giemsa

The therapeutic intervention was prompt, with imidocarb dipropionate - Imizol® specific antidote, in a single dose (5 mg/kg b.w. or 0.5ml/10 kg solution 8.5%), together with fluid and vitamin therapy. Two days after specific antidote administration, the dog showed polydipsia and polyuria.

Meanwhile, biochemical analyses were performed and their results showed normal values but at the lower limit only for serum potassium and serum sodium (3.7 mmol/L and 140 mmol/L, respectively). The other investigated parameters exceeded the upper reference limit, such as: serum creatinine 2.52 mg/dL vs. 1.8; GGT 147 U/L vs. 10; ASAT 286 U/L vs. 50; ALAT 714 U/L vs. 40; serum urea 151.9 mg/dL vs. 26.

After 5 days, although the clinical evolution was better and the dog started to eat, he died.

On necropsy, jaundice was generalized, including important organs, such as kidneys, heart, and liver (Figure 3).

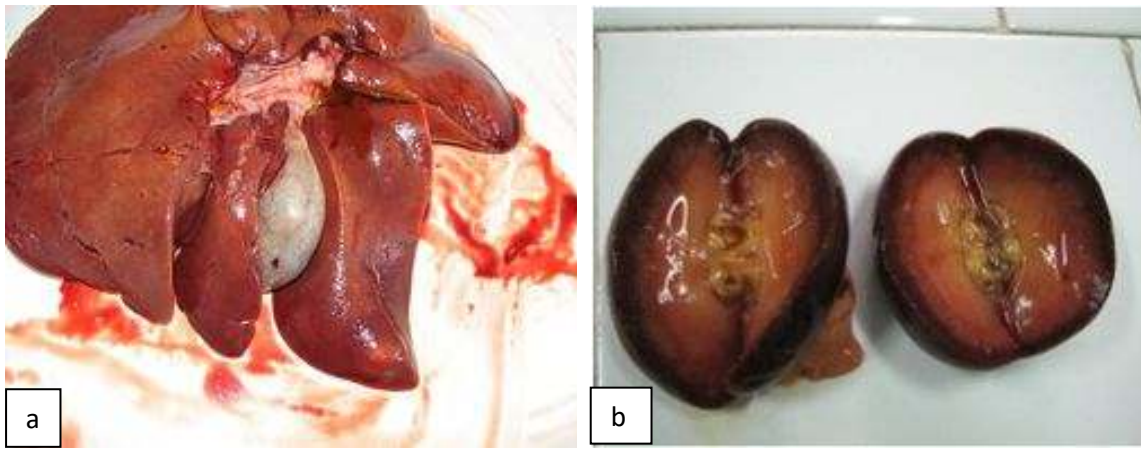


Fig. 3. Aspect of liver (a) and kidneys (b) on necropsy

From our point of view, the established therapeutic intervention was medically correct. The specific antidote for *B. canis* was chosen, which was confirmed as safe by medical studies. The first signs of polydipsia and polyuria led us to think of renal failure, along with that of liver, both confirmed by the results of laboratory tests. In order to dilute urea, a sustained fluid therapy was performed. A probiotic supplement with *S. thermophilus, L. acidophilus,* and *B. longum* was administered to support the renal function, as well as a dietary supplement in food, containing chitosan. Liver function was sustained with a phospholipid-based supplement, and a maropitant-based drug (Cerenia®) was administered as antiemetic, the dose of which was correlated with the animal`s weight. Therefore, the therapeutic intervention was correctly performed and in a sustained way for the healing of the dog. Its dead may be firstly justified by comorbidities developed (such as liver and renal failure) in the context of its delayed presentation to the doctor. Although we were not able to diagnose subspecies of *B. canis,* the established treatment is considered effective for all large *Babesia spp.* However, a precise taxonomic classification would have been helpful in justifying the clinical evolution.

Conclusions

Babesia genus includes two main groups, of large and small species, worldwide distributed and with a variety of clinical signs in their infection. A 7-year-old dog infected with large *Babesia canis* died although its clinical evolution after a specific antidote improved. Although therapeutic protocol was right instituted, comorbidities installed (kidney and liver failure) as a result of its delaying to vet consult decided this outcome.

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Inula helenium: A literature review on ethnomedical uses, bioactive compounds and pharmacological activities

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Abstract
Dynamic growth of antimicrobial and anthelmintic resistance throughout the years has caused increased interest in natural alternatives to synthetic drugs. Elecampane (Inula helenium L.), a widely distributed herbaceous plant, is one of the most researched and well-known member of the genus Inula, family Compositae. I. helenium has been included in the Chinese Pharmacopeia, Russian Pharmacopeia and Pharmacopeias of some European countries. This review is an up-to-date summary of the existing knowledge on Inula helenium's ethnomedicinal uses, secondary metabolites and pharmacological activities. Initially used in the treatment of respiratory and digestive diseases in both humans and animals, the roots of elecampane have been also proven to possess a cytotoxic and antiproliferative effect on cancer cell lines, as well as anti-inflammatory, antioxidant, antibacterial, antifungal and anthelmintic activities. The main bioactive compounds isolated from elecampane roots known to be responsible for their pharmacological activities are inulin, sesquiterpene lactones such as alantolactone and isoalantolactone, thymol derivatives, phenolic acids and flavonoids. This review suggests that I. helenium's secondary metabolites have a strong therapeutic potential. However, further in vitro and in vivo studies of isolated I. helenium bioactive compounds are required in order to understand their mechanism of action, pharmacokinetics and potential adverse effects.
Keywords*: Inula helenium, Chemical composition, Pharmacological activity*

Introduction

Inula helenium L., commonly known as elecampane or horseheal, is an herbaceous perennial that belongs to the family Asteraceae (Compositae), genus *Inula*. Elecampane is believed to be native to West and Central Asia but spread throughout Europe during the Bronze Age, and only recently introduced and naturalized in United States and Canada (Preston et al., 2004; ITIS, 2020).). *I. helenium* grows well in temperate regions with dump, well-drained soil, in full sun or semi-shade habitats, located on the fields and hills near river or forest (Bojor, 2003). Due to its wide distribution and ethnomedicinal uses, elecampane has been included in various pharmacopeias, including the Chinese Pharmacopeia, Ayurvedic Pharmacopeia of India, the State Pharmacopeia of the Russian Federation, the British Herbal Pharmacopeia, and Pharmacopeias of some European countries (Zhao et al., 2015; Stojakowska et al., 2016; Shikov et al., 2017).
Since ancient times, dried roots and rhizome of *I. helenium* have been used for the treatment of respiratory and digestive diseases, but also as anthelmintic, antimicrobial and general tonic herb in both humans and animals (Seca et al., 2014; Shikov et al., 2017). Over the past decades the rapid spreading of antimicrobial resistance, together with continuous development of medical technology, have encouraged the researchers to study bioactive potential of plants and their secondary metabolites, including elecampane. Isolated and purified bioactive compounds as well as the extract of *I. helenium* root have been investigated *in vitro* and *in vivo* for their potential anti-inflammatory, antioxidant, antibacterial, antifungal, anthelmintic, antiproliferative and cytotoxic activity. Most of these biological activities are explained by the presence of sesquiterpene lactones (alantolactone and isoalantolactone), thymol derivatives, polysaccharide inulin, flavonoids (quercetin, kaempferol, catechin gallate and epicatechin) and phenolic acids (chlorogenic, caffeic, hydroxibenzoic) (Yan et al., 2012; Spiridon et al., 2013; Zlatić et al., 2019). In this review, we have compiled the existing data on the ethnomedicinal and modern uses of *I. helenium*, bioactive compounds and their mechanism of action, providing a baseline for the future studies.

Ethnomedical Uses of *Inula helenium*

The information about medicinal properties of *I. helenium* dates back to the ancient Romans and Greeks. It has been described by the ancient herbalist Pedianos Dioscorides in the first Pharmacopoeia entitled *De materia medica* as *oinos nektaries*, used for the treatment of stomach and chest pain, and as a diuretic (Dioscorides et al., 2000). Galen recommended the root of *I. helenium* for alleviation of sciatica symptoms (Castleman, 2017).

According to the Traditional Chinese Medicine (TCM) web platform, *I. helenium* roots (Tu Mu Xiang) shows a tropism for liver, lung and spleen tissues, relieving abdominal pain, treating diarrhoea, emesis and preventing spontaneous abortion (TCM, 2020). In the Chinese Pharmacopeia, dry roots of *I. helenium* are also mentioned as stomachic medicine (Tang and Eisenbrand, 1992). In Tibetan medicine, the roots of elecampane ("Ma Na Ba Zha") are used for the treatment of digestive and circulatory system diseases, and as an expectorant (Pasang, 1999). During the Middle Ages the roots of *I. helenium* were used in preparation of the medieval digestive wine, known as Potio Sancti Pauli (the drink of Saint Paul). In veterinary medicine, elecampane was used in the treatment of psoroptic mange in sheep and for lung disorders in horses, from where the name horseheal and scabwort originated (Wynn and Fougère, 2006).

In Romanian folk medicine, *I. helenium* was used mainly for the cleaning and treatment of wounds (Grințescu, 1945). The rhizome was also used in the mixture with honey for the treatment of tuberculosis; the tea from rhizome was used for the asthma treatment (Grigore, 2008). In Oltenian region, elecampane decoction it is used for its expectorant, anthelmintic and anti-inflammatory properties (Tita et al., 2009). In the folk medicine of Republic of Moldova, *I. helenium* was collected during vernal equinox or summer solstice, when it was considered the most effective, and used to wash the body and especially hair, believed to promote the hair growth (Grădinaru, 2005).

In Hungarian folk medicine, essential oil of elecampane is used in the treatment of digestive and respiratory diseases (Babulka, 2011). In Bulgaria, the infusion of elecampane rhizomes is used as anti-ascaridic, antitussive and in the treatment of bronchial and throat affection (Ivancheva and Stantcheva, 2000). In Montenegro, the roots of *I. helenium* are used in the external treatment of psoriasis (Menkovic et al., 2011). In Serbian folk medicine, the tea of leaves and flowers of *I. helenium* are used internally for the cold treatment, as expectorant and for blood detoxification; and externally as tincture for rheumatism and sciatica pain (Jarić et al., 2015). In Italian traditional medicine, *I. helenium* roots are used internally as infusion and decoction for the treatment of bronchitis, catarrh and coughs; as bitter tonic, diuretic and choleretic. Externally it is applied for pruritus (Leporatti and Ivancheva, 2003). In Moroccan traditional medicine, decoction of leaves and roots of *I. helenium* are used in the treatment of hypertension, and the decoction of leaves of *I. helenium* in combination with *Inula conyza* and *Inula viscosa* is used in diabetes mellitus (Eddouks et al., 2002; Eddouks et al., 2007).

Bioactive compounds isolated from *Inula helenium*

Identification and isolation of bioactive compounds from *I. helenium* roots and rhizomes play an important role in understanding their biosynthetic pathway, biological properties, mechanism of action and potential toxicity. Table 1 summarizes the secondary metabolites identified from the roots of *I. helenium*.

Table 1.
Bioactive compounds isolated from *I. helenium*

Secondary metabolite classification and name	Parts of plant used	References
Terpene compounds:		
Thymol derivatives (Monoterpenes)		
10-isobutyryloxy8,9-epoxy-thymol isobutyrate	Roots	Stojakowska et al. (2006)
10-isobutyryloxy6-methoxy-8,9-epoxy-thymol isobutyrate	Roots	Stojakowska et al. (2006)
10-(2-methylbutyryloxy)-8,9-epoxy-thymol isobutyrate	Roots	Stojakowska et al. (2006)
Eudesmanolides (Sesquiterpenes)		
Alantolactone		
Isoalantolactone	Roots/roots essential oil	Bourrel et al. (1993), Yan et al. (2012)
11α,13-Dihydroalantolactone	Roots/roots essential oil	Bourrel et al. (1993), Yan et al. (2012)
11α*H*,13-Dihydroisoalantolactone	Roots	Konishi et al. (2002)
4α,5α-Epoxyalantolactone	Roots	Yan et al. (2012)
Diplophyllin	Roots	Jiang et al. (2011), Yan et al. (2012)
5α-Epoxyalantolactone	Roots essential oil	Stojanović-Radić et al. (2012)
Macrophyllilactone E	Roots	Konishi et al. (2002)
5α,6α–Epoxyalantolactone	Roots	Yan et al. (2012)
3-Oxo-4(5),11-eudesmadien-8,12-olide	Roots	Yan et al. (2012)
Spiroalanpyrroid A	Roots	Yan et al. (2012)
Spiroalanpyrroid B	Roots	Cai et al. (2020)
Helenalanproline A	Roots	Cai et al. (2020)
Helenalanproline B	Roots	Cai et al. (2020)
	Roots	Cai et al. (2020)
Elemanolides (Sesquiterpenes)		
Elema-1,3,11(13)-trien-12,8b-olide		
	Roots	Konishi et al. (2002)
Germacranolides (Sesquiterpenes)		
Isocostunolide		
	Roots	Chen et al. (2007)
Phenolic compounds:		
Phenolic acid		
Gallic acid	Roots	Spiridon et al. (2013), Petkova et al. (2017)
2-Hydroxy benzoic acid	Roots	Spiridon et al. (2013), Petkova et al. (2017)
Chlorogenic acid	Roots	Spiridon et al. (2013), Petkova et al. (2017)
Neochlorogenic acid	Roots	Petkova et al. (2017)
Caffeic acid	Roots	Spiridon et al. (2013), Petkova et al. (2017)
p-Coumaric acid	Roots	Petkova et al. (2017)
Sinapic acid	Roots	Petkova et al. (2017)
Ferulic acid	Roots	Spiridon et al. (2013), Petkova et al. (2017)
3,4-Dihydroxybenzoic acid	Roots	Spiridon et al. (2013), Petkova et al. (2017)
Vanillic acid	Roots	Petkova et al. (2017)
Cinnamic acid	Roots	Spiridon et al. (2013), Petkova et al. (2017)
Flavonoids		
Quercetin	Roots	Spiridon et al. (2013), Petkova et al. (2017)
Kaempferol	Roots	Spiridon et al. (2013), Petkova et al. (2017)
Myricetin	Roots	Petkova et al. (2017)
Catechin	Roots	Spiridon et al. (2013), Petkova et al. (2017)
Epichatechin	Roots	Spiridon et al. (2013), Petkova et al. (2017)
Quercetin-3-*O*-β-glucopyranoside	Roots	Spiridon et al. (2013), Petkova et al. (2017)
Polysaccharide		
Inulin	Roots	Petkova et al. (2017)

Pharmacological activities of *Inula helenium*

Several *in vitro* and *in vivo* studies (Table 2) have shown that elecampane extracts possess a wide range of pharmacological activities including anti-inflammatory, antioxidant, neuroprotective, anti-proliferative effects on cancer cell lines, antibacterial, antifungal, anthelmintic, insecticidal, prebiotic etc. These activities may be attributed to the main bioactive compounds isolated from *I. helenium*, mainly sesquiterpene lactones (alantolactone, isoalantolactone) and total phenolic compounds (Wang et al., 2015; Peng et al., 2016). In the Table 2 we have included some of the recent studies on elecampane biological activities.

Tabel 2.

Biological activity studies of *I. helenium* extracts

Evaluated pharmacological activity	Plant parts used	Extraction method	Results	References
Anti-inflammatory (*in vitro*)	Roots	Ethanolic extract 60% (v/v, 1:10)	Ethanolic extract of *I. helenium* (20-100 µg/mL) suppressed the neutrophil binding to the epithelium surface (A549), decreasing the expression of β-integrin on neutrophil membrane. It also inhibited IL-8, IL-1β and TNF-α production in lipopolysaccharide-stimulated A549 cells.	Gierlikowska et al. (2020)
Antioxidant and neuroprotective (*in vitro*)	Not identified	Ethanol extract – Total phenolic compounds	Total phenolic compounds (0.5-5 μ g/mL) isolated from *I. helenium* blocked ROS production, inhibited the reduction of SOD, reversed the changes in MMP and increased ATP production.	Wang et al. (2015)
Anti-proliferative (*in vitro*)	Rhizome and roots	Ethyl acetate extract	Ethyl acetate extract of *I. helenium* produced mitochondria-dependent apoptosis of CFPAC-1 cells, IC-50 value being of 4.3 µg/mL.	Zhang et al. (2018)
Antibacterial (*in vitro*)	Not identified	Aqueous extracts	Aqueous extract of *I. helenium* showed antibacterial activity against *Bacillus mycoides* (MIC 5 mg/mL) and synergism with sodium nitrite and potasium sorbate against *Bacillus subtilis* and *Pseudomonas fluorescens*.	Stanojević et al.(2010)
Antimicrobial (*in vitro*)	Roots	Supercritical fluid extraction (SFE)/ Hydrodistilled essential oil (HD)	HD and SFE oil manifested strong antifungal activity against *Candida* spp., MIC values being 0.009-0.6 mg/mL and 0.07-0.12 mg/mL, respectively. HD oil was more active against *Acinetobacter baumannii* (MIC 0.017 mg/mL), both oils showed strong bactericidal effect against *Enterococcus faecium* (MIC 0.12 mg/mL).	Deriu et al. (2008)
Antimicrobial (*in vitro*)	Roots	Ethanolic extracts (50% and 70%)	Extracts exhibited moderate to high antibacterial activity on *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus* and *Bacillus subtilis*. In addition, low to moderate antifungal activity on *Candida albicans*, *C. parapsilosis* and *C. lipolytica*.	Diguță et al. (2014)
Antistaphylococcal (*in vitro*)	Rhizome and roots	Hydroethanolic extract 50%	The extract was 100% effective against 200 clinically isolated antibiotic-resistant	O'Shea et al. (2009)

			and -sensitive strains of *S. aureus*¸ at concentrations between 0.9-9.0 mg/mL.	
Antistaphylococcal (*in vitro*)	Roots	Hydrodistilled essential oil	Manifested a potent but retard antistapylococcal activity, causing membrane damage. The MIC and MBC values were 0.013 µg/mL and 0.02 µg/mL, repectively.	Stojanović-Radić et al. (2012)
Anticandidal (*in vitro*)	Roots	Hydrodistilled essential oil	Showed medium to high anticandidal activity against clinical isolated of *C. albicans* and *C. cruset*, MICs varying between 0.009-0.312 mg/mL.	Stojanović-Radić et al. (2020)
Anthelmintic (*in vitro*)	Rhizome and roots	80% Hydroethanolic extract	Manifested a higher larvicidal effect against *Trichostrongylus colubriformis* in comparison with albendazole (Zentel).	Urban et al. (2008)
Anthelmintic (*in vitro*)	Rhizome and roots	Aqueous extract	Exhibited strong ovicidal and larvicidal activity against donkey strongyles, with LC-50 values of 0.041 mg/mL and 0.41 mg/mL, respectively.	Buza et al. (2020)
Insecticidal (*in vitro*)	Roots	Acetone/methanol 2:1	Reduced the fecundity of *Oncopeltus fasciatus*, had a strong juvenoid and antifeedant effect. Caused shortening of antennae and proboscis.	Alexenizer and Dorn (2007)
Prebiotic and antioxidant (*in vivo*)	Rhizome	Ethanol extract (EE)	1000 mg/kg of EE increased body weight gain and feed conversion ratio of broiler chickens. Decreased *E. coli* and *Clostridium* spp. population, and increased the count of *Lactobacillus* spp. Increased the activity of antioxidant enzymes in jejunal and ileal mucosa.	Abolfathi et al. (2019)

LC-50 - median lethal concentration
MBC - minimum bactericidal concentration
MICs - minimal inhibitory concentrations
MMP – mitochondrial membrane potential
ROS - reactive oxygen species
SOD - superoxide dismutase

Conclusions

We can conclude that *I. helenium* is a promising medicinal plant for both human and veterinary medicine. However, further *in vivo* studies of *I. helenium* extracts and isolated secondary metabolites are needed to prove their safety, bioavailability, pharmacokinetics, potential sites and mechanism of action.

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Macroendoscopical and histopathological aspects in *Helicobacter pylori* gastroenteritis in dogs– case report

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Abstract
In current veterinary medical practice, more clinical and imaging investigations are needed to fully assess a patient with digestive symptoms. In this case, the anamnesis and patient history are the starting points in staging a diagnosis. Questions with uncertain answers lead to laboratory tests – blood count and blood biochemistry. The etiologic diversity in digestive pathologies implies these blood tests to exclude some of the diseases. X-ray and ultrasound examinations are the next steps to follow; these are diagnosis methods that precede the endoscopic examination. After the macroendoscopic evaluation of the digestive tract, biopsy samples must be taken for the Helicobacter pylori quick test (Figure 1). Histological examination of the biopsy samples taken from canine patients, which were positive for Helicobacter pylori, can reveal another lesions in the digestive tract; in this moment a treatment can be initiated to reduce or even to abolish the simptomatology.
Key words: *digestive pathologies, endoscopy, Helicobacter pyolri, histological examination*

Introduction

Digestive pathology is most common in the veterinary practice in pets. Extensive etiology implies multiple investigations which lead to a diagnosis in reducing or even abolish digestive symptoms. In most cases, no results are obtained with symptomatic treatment and the patient's condition is gradually degraded.

Most of the time, a possible complicated bacterial gastritis or a gastritis with etiological agent bacteria of *Helicobacter spp* is excluded from the treatment scheme. It scales in gastric and sometimes duodenal mucosa, causing even neoplastic lesions [Figure4, Figure 8]. Quick tests from gastric or duodenal mucosa taken endoscopically are carried out to confirm this type of gastritis in order to complete the histological examination to fill their margin of error. The studies carried out so far on animals have detected several subspecies of *Helicobacter canis, Helicobacter felis, Helicobacter heilmannii, Helicobacter bizzonii,* requiring complex typing examinations (immunohistochemistry, PCR). [1, 2, 3]

The dual antibiotherapy treatment scheme (methronidazole, amoxicillin/claritromycin), gastric protector (omeprazole) and probiotic administration are the first steps to be taken in improving symptomatology.

After assessing blood and biochemical parameters, ultrasound and radiological examinations, the present case shows the importance of some diagnostic methods: the endoscopic method and the histological examination. The endoscopic examination is a certain and minimum invasive diagnosis method, it helps visualizing surface lesions in the digestive tract mucosa. Multiple biopsy samples can also be taken; one biopsy sample is used for the quick test for *Helicobacter pylori* presence, and at least three biopsy samples are used for histopathological examination.

Materials and methods

This case report includes a male dog, mixed breed, neutered, 11 years old, 32 kg, both internal and external anti-parasite treatment given regularly; he was clinicaly and imagistic evaluated because of digestive signs: vomit, capricous appetite, weight loss. The patient was being treated with metronidazole (7.5 mg / kg twice daily) and probiotics for gastroenteritis. Following

the blood examination (hematological and biochemical profile), certain digestive pathologies (uremic gastroenteritis, enteropathies with protein loss) were excluded, and the imaging examination (ultrasound, radiography) showed the narrowing of the gastric mucosa and normal intestinal transit.

A basic preanesthetic cardiological consultation (electrocardiogram and blood pressure) was performed, which showed no changes. The endoscopic intervention was decided, under inhalatory anesthesia, to evaluate the esophagus, stomach and duodenum.

Pre-anesthesia consisted of intramuscular administration of medetomidine hydrochloride (0.025 mg / kg) and butorphanol (0.1 mg / kg); for induction, propofol (3mg / kg) was administered slowly, intravenously, and maintenance was performed with isoflurane (5 minutes 3%, then reduced to 1.5%).

Biopsy samples were taken from the duodenal mucosa for histological examination, and biopsy samples were taken from the gastric mucosa for both rapid testing for *Helicobacter pylori* (Figure 1) and histological examination.

Rapid tests in the gastric mucosa detect the presence of urease eliminated by the bacterium, but the specificity is reduced due to the administration of metronidazole.

Fig. 1. Rapid test to detect urease activity eliminated by *Helicobacter pylori*

Endoscopic sampling involves the collection of biopsy samples, 1-3 mm, from the gastric and / or intestinal mucosa. It is the least invasive method of tissue sampling for histological examination.

Immediately after harvesting, tissue biopsy samples were fixed in 10% formaldehyde buffered solution for 48 hours, processed by paraffin inclusion method, sectioned and trichromic Masson stained.

Histological preparations were examined with a Leica DM 750 optical microscope, and images were captured with a Leica ICC 50 HD histological camera (3mpx) and LAS software version 4.2 (2012).

Results

Following the endoscopic examination, areas of moderate congestion were observed in the pyloric area (Figure 2), the retroflexion did not show neoplasms on the region of the small curvature (here gastric tumor formations are usually hidden) (Figure 3); In the first duodenal part, the mucosa had a proliferative, degenerative appearance (Figure 4), in order to highlight a less modified mucosa macroendoscopically (Figure 5).

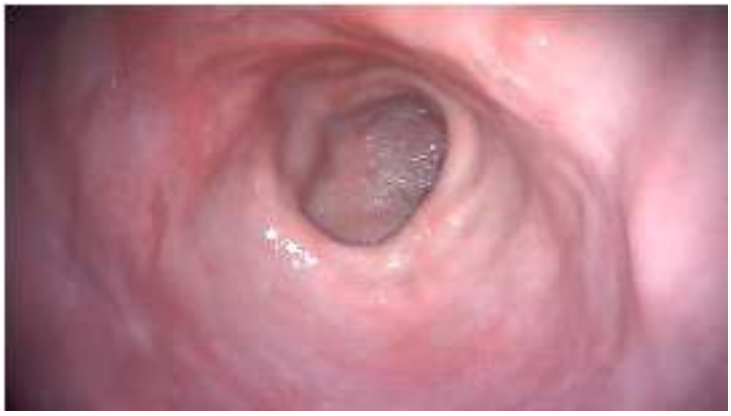

Fig. 2 - Pyloric orifice with slightly modified mucosa - reddish areas appear, with an inflammatory appearance (congestion)

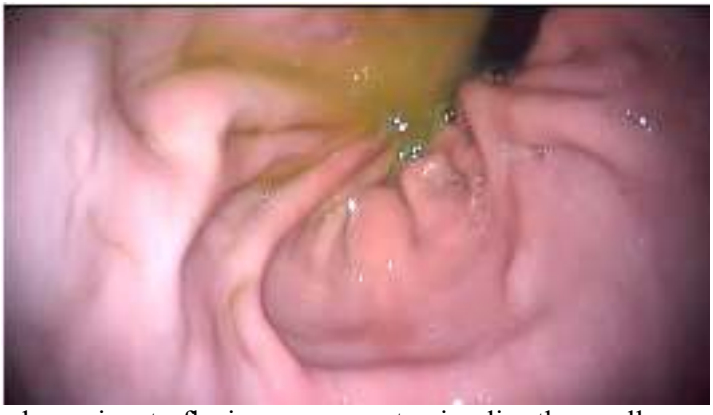

Fig. 3 - Endoscopic retroflexion maneuver to visualize the small curve - normal appearance

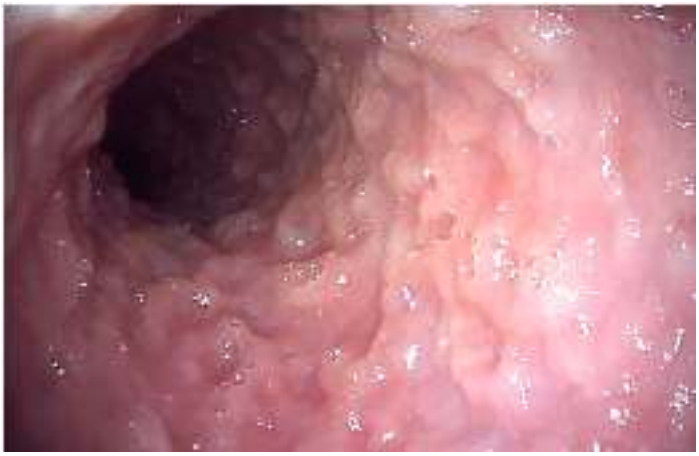

Fig. 4 Endoscopic image of the first duodenal third - proliferative pathological aspect of the mucosa, with ulcers and the absence of the intestinal cilia.

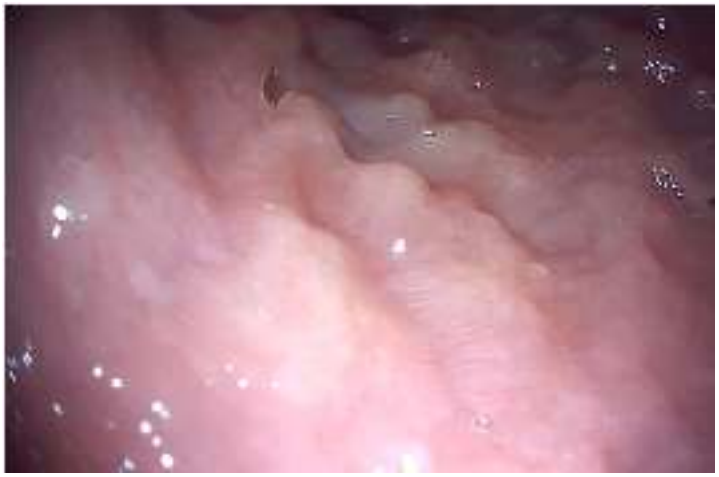

Fig. 5 Endoscopic image of the duodenal mucosa less affected - areas with cilia are observed.

Rapid testing directly from the gastric mucosa for the detection of *Helicobacter pylori* had a false negative result, as histological examination revealed helical bacteria in the duodenal (Figure 6) and gastric (Figure 7) submucosa.

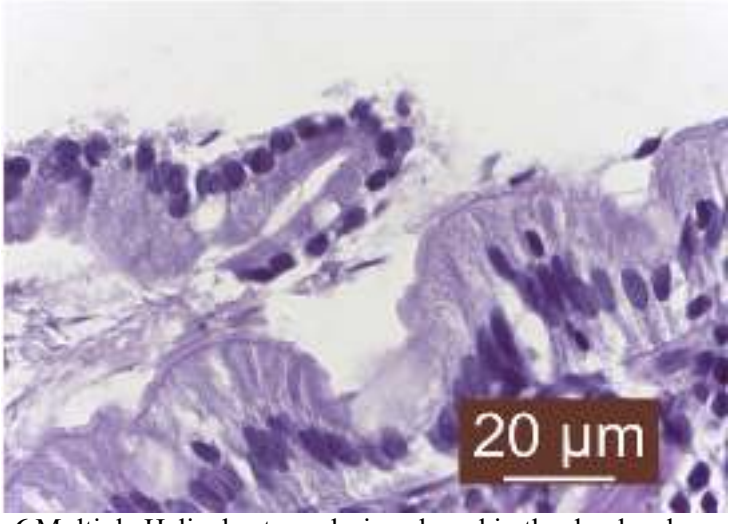

Fig. 6 Multiple Helicobacter pylori anchored in the duodenal mucosa

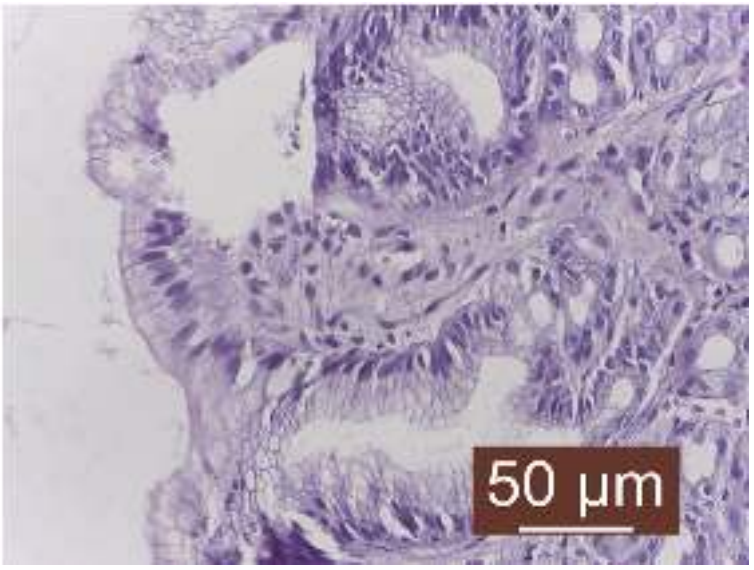

Fig. 7 Hyperplastic gastritis with evidence of *Helicobacter pylori*

Fig. 8 Epithelial dysplasia (preneoplastic lesion) of the mucosa (intestinal epithelial cells are unorganized, with loss of polarity and arranged in several layers)

Discussions

In the case of diseases of the digestive tract, if the symptomatic treatment does not obtain positive results of the patient's condition, endoscopy is a minimally invasive method of diagnosis. Endoscopy can assess the appearance, integrity of the lining of the esophagus, stomach and intestines and biopsy can be taken for histological examination. Given the histological protocol, rapid tests in the gastric mucosa to detect the presence of *Helicobacter pylori* offer the possibility of instituting treatment to eliminate the bacterium and to partially remit the symptoms of the infection.

Although the rapid test had a false negative result, it was decided to continue treatment with metronidazole (7.5 mg / kg twice daily), with additional amoxicillin (12.5 mg / kg twice daily), omeprazole (1 mg / kg, twice daily before meals) and probiotics for 21 days (standard treatment). In this case report, the histological result was conclusive for *Helicobacter pylori* infection, with helical bacteria present in the gastric and duodenal epithelium, along with other lesions (areas of focal pyloric congestion, proliferative appearance of the duodenal mucosa, lack or very low number of intestinal cilia).

Conlusions

Any digestive disorder requires thorough investigation to have a definite diagnosis. Blood tests, ultrasound and radiological examination, pre-anesthetic consultat (EKG, tensiometry) are essential for endoscopic examination. Vomiting, loss of appetite and progressive weight loss are symptoms that require endoscopic evaluation and mandatory biopsy. It is not examined only macroendoscopically, as most of the time the lesions are deep, and the histological examination is meant to confirm a suspicion of digestive pathology. It should be noted that some patients with helicobacterial gastritis may have false negative tests. It is not excluded that patients with rapid positive tests and histologically missing helical bacteria; in this case the degree of infestation and the place of sampling of the biopsy are important. Therefore, investigations should be continued through detailed histological analysis.

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Comparative analysis of hematological parameters and blood compatibility in different bird species

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Abstract

Birds seem to survive and recover much faster than mammals after acute blood loss. Their ability to tolerate severe bleeding may be due to the lack of automatic responses to irreversible shocks, faster transfer of extracellular fluids into the vascular space and faster mobilization (at 12 hours) of a large number of immature erythrocytes after significant blood loss; for example, pigeons return to normal hematocrit only 7 days after 60% of the blood has been drawn, without any clear clinical signs. In this study our objectives were the comparative analysis of hematological parameters in samples from domestic birds of different species and the investigation of the possible compatibility between the blood of chickens and palmipeds. The investigations were carried out between October 2018 - May 2019 in the Physiology Laboratory of the Faculty of Veterinary Medicine, CLUJ-NAPOCA, on blood samples collected from a batch of 23 birds, composed of: 9 chickens, 5 turkeys, 4 ducks, 2 geese, 1 quail, 1 pheasant, 1 pigeon.. Blood samples were taken from the basilic vein of each bird and collected on an EDTA tube. Comparative hematological analyzes included the determination of the erythrogram parameters (total number of erythrocytes, hematocrit, hemoglobin concentration and mean erythrocyte constants) and leukogram parameters (total number of leukocytes and proportions of leukocyte subpopulations). In order to evaluate and analyze the intra- and interspecific blood compatibility, 110 Crossmatch reactions were performed, 55 for the major test and 55 for the minor test.. The hematological results, interpreted comparatively on batches and species, showed significant variations at both individual and species level. We also noticed a major inconsistency between the values of the mean erythrocyte constants and the main hematological parameters. The results regarding the evolution of intra- and interspecific blood compatibility revealed the absence of preformed isoantibodies against the various antigens of the blood group systems of the tested species. In conclusion, 14.54% positivity proportion outlined the level of the incompatibility rate for heterologous combinations.

Keywords: *birds, hematology, blood compatibility.*

Introduction

Blood groups are the result of gene expression by the presence of certain substances on the surface of erythrocytes. These substances are called erythrocyte antigens.

The existence of different erythrocyte antigens in chickens was discovered in 1924 by Landsteiner and Miller (Walford et al., 1964).

In 1930 and 1931 Todd performed isoimmunizations among the chickens in his study and obtained such a multitude of antibodies that he concluded that every chicken had a different erythrocyte antigen except close relatives (Walford et al., 1964). In addition, he proved that an individual had the same erythrocyte antigen as at least one of his parents, which means that this antigen is inherited as a dominant trait.

The first two blood group systems in chickens were discovered in 1948 by Briles, McGibbon and Irwin and were referred to as group A and B in 1950. Two other blood group systems referred to as C and D were discovered by Briles and Quinsenberry and 1951. Finally a fifth blood group system, E, was discovered by Briles (1958); C. Briles, McGibbon and Irwin (1959) (Walford et al., 1964). Independently D. G. Gilmour discovered in 1959 the systems of groups A, B, C and E but also two other systems which he calls L and N (Walford et al., 1964; Schiermann and Nordskog, 1965).

Although the majority of the 47 domestic turkeys (*Meleagris gallopavo*) were refractory to the production of detectable isoagglutinins, the isoimmunization experiment of these 47 specimens produced 18 blood typing reagents that define individual differences in the antigenic structure of turkey RBC. Corresponding to the reactions obtained with these reagents, the antigenic factors A (subtypes A1 and A2), B (B1, B2 and B3), C (C1 and C2), D, E, F (F1 and F2), G, H. I, J, K and L have been named (Law et al., 1964).

Homologous transfusions (between individuals of the same species) are strongly recommended in birds (Lichtenberger Marla, 2004). If homologous transfusion is not possible, heterologous transfusion (between individuals of different species) should be taken into consideration. In both cases, minor and major compatibility tests must be performed. Since birds do not have pre-formed antibodies against blood groups, the first heterologous transfusion is usually safe. (Morrisey, 1999; Marla Lichtenberger, 2004; Matos and Morrisey, 2005). However, it has been shown that the mean half-life of donor erythrocytes is reduced by at least half in heterologous transfusions compared to homologous transfusions. For this reason it is more beneficial that the heterologous transfusion is performed between two individuals of related species (same Genus) or as a last resort between two individuals of the same Order (*Psittaciformes, Falconiformes, Columbiformes,* etc.) (Martinho, 2012).

Materials and methods

The research was carried out in the period October 2018 - May 2019 in the Animal Physiology laboratory within the Faculty of Veterinary Medicine, CLUJ-NAPOCA. The main objectives of our study were the comparative analysis of haematological parameters of different species of domestic birds, investigation of blood compatibility in the population of studied birds and establishment of the proportion of positivity in the blood compatibility test of the sample analyzed.

To conduct the investigations of our study, we collected blood from a total of 23 different specimens (n=23). The percentage distribution of the different species illustrates that the most representative species in terms of the number of subjects is the domestic hen *(Gallus domesticus)* with a percentage of 40%, which represents 9 individuals out of 23. In contrast, the pheasant (*Phasianus colchicus),* the pigeon *(Columba livia)* and the domestic quail (*Numida meleagris)* each represent only 4%; that is, one subject out of the 23 totals. The origin of the birds was different and the study was carried out in four distinct stages (Table 1). The birds introduced in this study were divided into different categories according to their species (Table 1).

Table 1.

The species and number of birds introduced into the study

Stage	Total number of specimens	Number of specimens per species	Species
1	**5**	**5**	*Gallus domesticus* (domestic hen)
2	**6**	**2**	*Meleagris gallopavo* (turkey)
		2	*Anas platyrhynchos domesticus* (duck)
		2	*Gallus domesticus* (domestic hen)
3	**7**	**3**	*Meleagris gallopavo* (turkey)
		1	*Anas platyrhynchos domesticus* (duck)
		2	*Anser cygnoides* (goose)
		1	*Gallus Domesticus* (domestic hen)

4	**5**	**1**	*Numida meleagris* (domestic quail)
		1	*Columba livia* (pigeon)
		1	*Anas platyrhynchos domesticus* (duck)
		1	*Phasianus colchicus* (pheasant)
		1	*Gallus Domesticus* (domestic hen)

Haematological examinations. For all birds introduced into our study, blood samples were collected by venipuncture from the basilic vein on an EDTA (approximately 2 ml of blood for each bird).

Determination of hematocrit (Ht). Hematocrit represents the volume, expressed as a percentage, occupied by circulating red blood cells in circulating whole blood. It is a ratio between the total volume of RBCs and the total blood volume. In birds, HT is estimated using the microhematocrit method (Ghergariu et al., 2000; Ognean and Cernea, 2006; 2011). An HT between 35% and 55% is considered physiological in most adult birds. However, an interpretation of HT should be made in comparison with the physiological norms of the species and the breed. An increase in HT may be absolute with a cellular origin or relative with a plasma origin. A decrease in HT reflects a state of anemia.

Hemoglobin dosage (Hb). This determination is difficult due to the presence of the RBC nucleus in the case of blood samples from birds (Hawkey and Samour, 1988; Samour, 2006; Campbell et al., 2007). We used the semi-automatic spectrophotometric method (Ognean and Cernea, 2011). In most vertebrates, hemoglobin is made up of four subunits each with an oxygen binding site. However, there are significant differences between the Hb of birds and that of other vertebrates. There are two types of Hb in adult birds, Hb A and Hb D. Hb A is most prevalent in bird populations and its affinity for oxygen is lower than the Hb D. This lower affinity allows easier dissociation of oxygen.

Determination of the total number of red blood cells (RBC) and leukocytes (WBC). The count of RBC in s shows some differences from the count of RBC in mammals. Prochaska-modified Natt-Herrik dilution fluid is used, which protects all the figured blood elements (erythrocytes, leukocytes, platelets), being considered the standard method (Ognean and Cernea, 2011; Pierson, 2000; Campbell et al., 2007).

The determination of the erythrocyte constants was based on the use of known in the field calculation formulas (Ghergariu et al., 2000; Samour, 2006; Ognean and Cernea, 2011), these indices being important for birds, especially for the detection of nutritional origin stress.

Leukogram determination. Coloring of bird blood smears is based on the use of most Romanovsky-type stains used in mammalian smears (Wright, Gimsa, Wright-Gimsa, Leishman, Wright-Leishman, May-Grunnwald, May-Gundwald-Gimsa, DiaPanoptic etc.). For fastness reasons, in this study we used DiaQuick Panoptic staining, based on the use of 2 dyes (acidophilic and basophilic) and a fixative containing absolute methyl alcohol (Campbell, 1994).

Cross matching: cross compatibility test. This is a method of ensuring blood compatibility between the donor and the recipient by quantitatively detecting the serum level of antibodies against erythrocyte antigens. It is a reliable, fast and inexpensive method that can detect incompatibility and thus prevent transfusion accidents.

*The major cross-compatibility test***.** Major cross-compatibility represents the compatibility between the donor's red blood cell concentrate (RBC) with the serum or plasma of the recipient patient. It therefore assesses the effect of the recipient's serum antibodies on the donor's erythrocytes. The major cross-match test detects the presence of antibodies in the recipient's plasma

that could cause a hemolytic reaction upon transfusion of the donor's RBC. The major cross-compatibility test therefore consists of combining the donor RBC and the recipient's plasma (1: 4).

The minor cross-compatibility test. Minor cross-compatibility is the reverse of major cross-compatibility; it represents the compatibility between the recipient's red blood cells and the donor's plasma. It therefore assesses the effect of the donor's serum antibodies on the recipient's cells. This test is very important in the event that the donor has been previously transfused as anti-red blood cell antibodies from the recipient may be present in the donor's plasma. The minor cross-compatibility test therefore consists of combining the donor's plasma with the recipient's RBC (1: 4).

	Major compatibility test	**Minor compatibility test**
Material	• RBC from the donor • Plasma of the recipient	• GR of the recipient • Plasma of donor
Working technique	• On the slide, we put 3 µL of RBC + 12 µL of plasma • We waited 1 minute • We examined macroscopically for the absence or presence of agglutination	
Results and Interpretation	• Agglutination or hemolysis is considered a positive reaction and demonstrates blood incompatibility (Figure 1). • The absence of agglutination or hemolysis is considered a negative reaction and demonstrates blood count (Figure 1).	

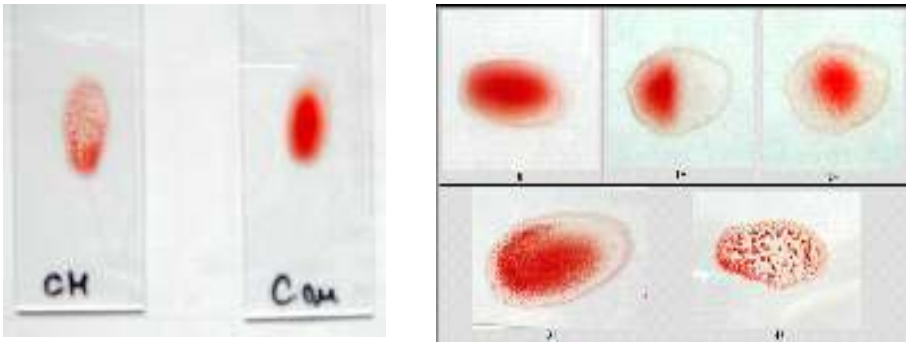


Fig. 1. Cross-match: Macroscopic aspects (Ognean and Cernea, 2011)

The verification pattern for the cross-match test was different depending on the stage of our study.

For step 1, we examined the blood compatibility between the 5 hens (Table 2). For this check, we made 20 combinations as follows: 10 combinations for major compatibility and 10 combinations for minor compatibility.

Table 2.
Cross reactions embodiment of the step 1

		Recipients				
	Identification	**1**	**2**	**3**	**4**	**5**
Donors	**1**		X	X	X	X

	2			X	X	X
	3				X	X
	4					X
	5					

For step 2, we examined the blood compatibility between the two turkeys, between the two turkeys and the two ducks, between turkey 1 and the two hens (Table 3). For this assay, we made 14 combinations as follows: 7 combinations for major compatibility and 7 combinations for minor compatibility. The amount of blood that we collected from all the individuals was insufficient; thus we could not verify the compatibility between turkey 2 and the two hens, duck 1 & 2 and the two hens and also between the two hens.

Table 3.

Cross reactions embodiment of the step 2

		Recipients					
	Identification	**Turkey 1**	**Turkey 2**	**Duck 1**	**Duck 2**	**Hen 1**	**Hen 2**
Donors	**Turkey 1**		x	x	x	x	x
	Turkey 2			x	x		
	Duck 1						
	Duck 2						
	Hen 1						
	Hen 2						

For step 3, we investigated the blood compatibility between the two turkeys, between the two turkeys and the two ducks and between turkey 1 and the two hens (Table 4).

For this check, we made 36 combinations as follows: 18 combinations for major compatibility and 18 combinations for minor compatibility. For step 3, we did not check the compatibility between the two geese, between goose 1 and the hen and finally between the goose 2 and the hen.

Table 4.

Cross reactions embodiment of the step 3

		Recipients						
	Identification	**Turkey1**	**Turkey2**	**Turkey3**	**Goose1 (m)**	**Goose 1 (f)**	**Duck**	**Hen**
Donors	**Turkey 1**		X	X	X	X		X
	Turkey 2			X	X	X		X
	Turkey 3				X	X		X
	Goose 1 (m)							
	Goose 1 (f)							
	Duck	X	X	X	X	X		X
	Hen							

*For step 4***,** we investigated all possible combinations of blood compatibility between quail, pigeon, duck, pheasant and hen (Table 5). For this study, we made 40 combinations as follows: 20 combinations for major compatibility and 20 combinations for minor compatibility.

Table 5.
Cross reactions embodiment of the step 4

		Recipients				
	Identification	**Quail**	**Pigeon**	**Duck**	**Pheasant**	**Hen**
Donors	Quail		X	X	X	X
	Pigeon	X		X	X	X
	Duck	X	X		X	X
	Pheasant	X	X	X		X
	Hen	X	X	X	X	

Within our study, the total number of combinations that we made between the individuals in our study is 55, which means we performed a total of 110 cross-match reactions (major compatibility and minor compatibility).

Results and discutions

Comparative analysis of haematological parameters During our study we observed significant variations in HT between our birds. Stage 1 hens have an Ht that falls within the physiological values for the species; while that of the hens in step 2 is lower than normal. The HT for all stage 2 birds is below standard and has much lower values than for the other two stages. Within step 3, there are also significant variations between the HT of gallinaceae and that of palmipeds.

Comparative analysis of Hb (g / dl) reveals values within broadly similar margins for all gallinaceans among themselves and for all palmipeds among themselves. The palmipeds introduced in our study nevertheless have higher Hb values than those of the gallinacea. The Hb values of all the hens in the study are physiological. For all the ducks in our study, Hb had values above the physiological upper limit.

The total RBC count (10^{12} / L) of stage 1 birds was found to be significantly higher overall than that of stage 2 and stage 3 birds. Goose 1 from stage 3 was the individual with the lowest total RBC count in our entire study, while hen 1 from stage 1 is the individual with the lowest total RBC count. The total RBC count of all palmipeds in our study was below physiological norms and was found to be lower than that of the gallinaceae in our study; With the exception of turkey 1 from step 2. Hens 1 and 5 from step 1 are the only gallinacea with a total number of RBCs within physiological standards. Finally, it appears that overall all the individuals in our study have a total number of RBCs lower than normal.

The comparative analysis of MCV (fl) shows large variations within stage 3 birds. Indeed, step 3 illustrates that palmipeds have a significantly higher MCV than gallinacea.

Stage 3 birds, except Turkey 1 and Turkey 2, all have higher than normal MCV. On the other hand, the differences in MCV values are less marked between gallinacea and palmipeds for stage 2. turkey 1 from stage 3 is the gallinaceous with the highest MCV while the duck from stage 3 is the palmiped with the highest MCV. The MCH (pg) values of almost all the birds introduced in our study are above the physiological values of the species. Only hen 1 from stage 1 and all turkeys in our study had physiological MCH values. Male goose and all ducks have significantly higher MCH values than other specimens. Stage 2 turkeys 1 and 2 had a higher MCH than all the hens in our study. With the exception of turkey 1 and turkey 3 from step 3, all MCHC (g / L) values were found to be greater than physiological values. The comparative analysis of MCHC values shows much higher values for stage 2 birds than for stage 1 and 3 birds; these values being clearly higher than the physiological values. In human literature (Healthline Media UK Ltd, Brighton, UK.), elevated MCHC is often associated with conditions where Hb is more concentrated in red

blood cells or with conditions where red cells are more fragile. This increased fragility of the red blood cells leads to vascular hemolysis with dissemination of the Hb outside the RBCs (Reavill and Joseph, 2002).

The comparative analysis of total leukocytes reveals very varied values between the different stages of our study but also between different individuals and species. Overall all the individuals presented leukocytosis with the exception of the hen from stage 3. We observed an excessively higher level of leukocytes than the other individuals for duck 1 from stage 2 and for turkey 1 from stage 3. Stage 1 hens have a physiological heterophile percentage, as do the geese from stage 3. Thus there is no significantly different distribution in heterophile percentage between gallinacea and palmipeds. The percentage of heterophiles of the birds in stage 2 is generally within the same range of values and reveals an overall heterophilia of the group. Analysis of the percentage of eosinophil reveals values that are well above the standards for stage 2 ducks. By comparing the values of step 1 (normal) with those of geese in step 3 we notice that there is no difference between the standards of gallinacea and those of palmipeds. In our study, we observed that only one basophil in all of our 23 blood smears. The comparative analysis of the lymphocyte level reveals very low values for all the individuals from stage 2 but also for the geese from stage 3. Stage 1 hens and stage 3 chickens show similar variations. The greatest difference is observed for the values of ducks between those of step 1 and that of step 3. Analysis of the percentage of monocyte revealed that all of the individuals introduced into our study presented monocytosis. However, compared to other individuals, hen 2 from stage 1 and ducks from stage 2 show a more moderate increase.

Analysis of blood compatibility. The cross-compatibilities reactions performed in step 1 between the hens at the university did not show any agglutination and therefore were all found to be negative.

Recipient hens do not have pre-formed circulating antibodies directed against RBCs of donor hens and the donors do not have pre-formed circulating antibodies against RBCs of recipient hens. In other words, the chickens at our university all exhibit blood compatibility with each other.

The combinations made within step 2 showed the highest percentage of positive reactions each in a major cross match. Of the 7 combinations investigated, 5 came out positive (positivity of 71.42%).

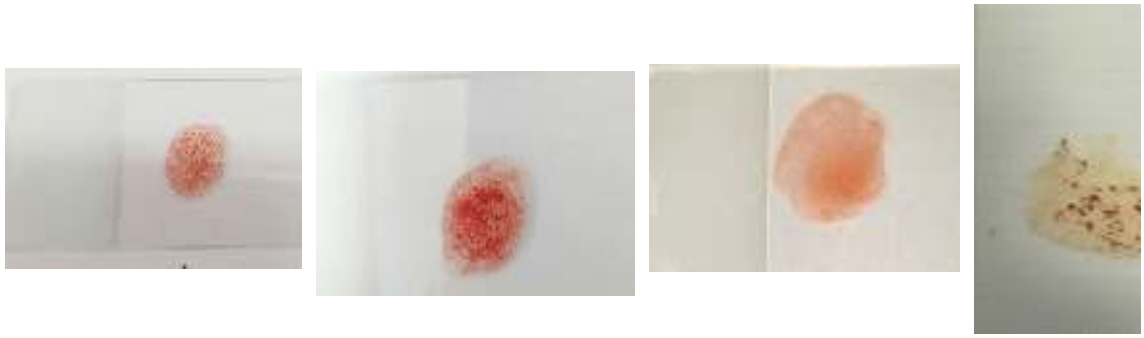
When combining turkey 1 / duck 2, we obtained a positive reaction with moderate agglutination during the major cross match. This shows the existence of anti-turkey RBC antibodies in the circulating blood of the duck. In contrast, the absence of agglutination during the minor cross-match demonstrates the absence of serum antibodies in turkey plasma to duck RBC. When combining turkey 1 / hens (1 and 2), we obtained a positive reaction with strong agglutination during the major cross match. This shows the existence in the circulating blood of the hens of anti-RBC antibodies in turkeys. In contrast, the absence of agglutination during the minor cross-match demonstrates the absence of serum antibodies in the turkey plasma directed against the RBC of the hens. When combining turkey 2 / ducks (1 and 2), we obtained a positive reaction with moderate agglutination during the major cross match. This shows the existence in the circulating blood of ducks of anti-turkey RBC antibodies. In contrast, the absence of agglutination during the minor cross match demonstrates the absence of serum antibodies in turkey plasma directed against duck RBC.

The combinations made in step 3 revealed two positive reactions. Of the 18 combinations investigated, 2 combinations came out positive in the major cross match (positivity of 11.11%). When combining turkey 1 / hen, we obtained a positive reaction with moderate agglutination during the major cross match. This shows the existence in the circulating blood of the hen of anti-turkey

RBC antibodies. In contrast, the absence of agglutination during the minor cross-match demonstrates the absence of serum antibodies in the turkey plasma directed against the RBC of the hen. In the duck / hen combination, we obtained a positive reaction with strong agglutination during the major cross match. This shows the existence in the circulating blood of the hen of anti-duck RBC antibodies. In contrast, the absence of agglutination during the minor cross-match demonstrates the absence of serum antibodies in the duck plasma directed against the RBC of the hen.

The combinations made in step 4 revealed a positive reaction (with agglutination) during a major cross-match. This represents a positivity of 5% out of the 20 combinations investigated. In the quail / pigeon combination we got a positive reaction with very strong clumping during the major cross-match. This shows the existence in the circulating blood of the pigeon of anti-quail RBC antibodies. In contrast, the lack of agglutination during the minor cross match demonstrates the absence of serum antibodies in the quail plasma to the pigeon's RBC.

At the level of all the combinations performed in our study (n = 55), we had only 8 positive combinations (14.54%).We observed that all the positive reactions took place during the major cross- match but with different agglutination intensity (Figure 2).



A **B** **C** **D**

Fig. 2 - Different agglutination intensity: A – turkey – hen; B – duck – hen; C- turkey – duck; D – quail - pigeon

Conclusions

1. The comparative analysis of haematological parameters revealed variations for each parameter; both individually and in terms of belonging to a phyllogenetic group (ducks showed the highest values for erythrocyte parameters overall).
2. Despite erythrocyte and leukocyte parameters generally outside physiological ranges, clinical examination of each animal did not suggest anything abnormal. Thus, the birds appear clinically resistant and the clinical manifestation of the hematological changes therefore implies extremely reduced and / or increased values.
3. The homologous combinations made between the hens at our university (step 1) did not reveal any incompatibility; just like the homologous combinations of turkeys (stages 2 and 3). It can thus be assumed that individuals of the same species do not have performed antibodies to antigens of

different blood groups of that species. In other words, regardless of their belonging to a blood group system, individuals of the same species should not be incompatible within the first transfusion.
4. There is no positive correlation between blood compatibility and belonging to the same order (here *Gallinaceae* and *palmipeds*). Indeed, we obtained compatibility between turkeys and geese; while the turkey / duck, turkey / hen, and duck / hen combinations have demonstrated varying degrees of incompatibility.

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Comparative ionogram assessment before and after probiotic treatment for healthy dogs and dogs with apparent dysbiosis

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Abstract
Probiotics are considered live formulas composed by microorganisms that, when administered in appropriate amounts, produce a beneficial effect to the host. The effect of probiotics is present both in the gastrointestinal tract and systemically. For this reason, a noteworthy aspect is the impact that these formulas have on commonly systemic investigated parameters. Of these, the main ions are dosed in order to clarify various aspects, being used as a marker in various pathologies. The aim of the present study was to make a comparison between the values of the main ions (calcium, phosphorus, potassium and sodium) obtained before and after a 30-day probiotic treatment. The study population was represented by two groups of dogs: group 1- healthy dogs (n = 5) and group 2- dogs with apparent dysbiosis (n = 6). The treatment was performed with a probiotic product consisting of Bacillus subtilis, Bacillus licheniformis and Pediococcus acidilactici, for 30 days. The analyzed samples were blood serum samples obtained by centrifugation and separation from blood samples collected on anticoagulant on day 0 and day 31 of the study, respectively. Analyzes were performed by dry biochemistry methods using the VetScan biochemistry analyzer. The results obtained by ionograms suggest that probiotic treatment does not have a direct influence on the values of the main ions, neither in the group of healthy dogs nor in the group of dogs with apparent dysbiosis. Variations in ion values were considered physiological, and could not be directly attributed to the treatment performed. In conclusion, the probiotic composed of B. subtilis, B. licheniformis and P. acidilactici does not directly influence the values of the main ions, and can be considered safe for administration in both healthy dogs and dogs with gastrointestinal manifestations.
***Key words**: ions, probiotics, dogs*

Introduction

Probiotics are complex formulas composed from different microorganisms (bacteria, arcanobacteria, fungi, viruses) that are able to confer a benefic effect to the host when it is administered in adequate amounts (Sauter et al., 2006; Barko et al., 2018).

For dog use, most probiotics available at the present moment are composed by lactic acid bacteria like *Lactobacillus* and *Bifidobacterium* (Barko et al., 2018; Lucena et al., 2019). One of the most important characteristics for probiotics in order to provide an effect to the host is their ability to remain viable in unfriendly conditions like the ones meet in gastro-intestinal (GI) tract. This is why it is important for the probiotic bacterial strains to survive on low pH created by the gastric juice and bile acids (Barko et al., 2018).

Sporulated bacteria have a plus compared with lactic acid bacteria because they are more resistant in hard environment conditions (Biourge și colab., 1998; Schmitz and Suchodolski, 2016). Bacteria from genus Bacillus are sporulated bacteria and this is why they are considered to be more resistant in GI tract. At the origins, bacteria from Bacillus genus was considered to have their origin in soil. After few years this concept was unvalidated based on some studies that demonstrated that those bacteria are GI tract commensals (Cutting, 2011).

From the mechanism of action point of view, probiotics are considered to have the capacity to improve the intestinal mucosa health status using different ways of action, synergic or alone. From those mechanisms of actions, the following are considered the principal ones: pathogenic bacteria replacement (Lee et al., 2003), antimicrobial substances production (Jones et Versalovic, 2009), increasing of immune response (Paganini et al., 2010) and metabolites regulation (Soo et al., 2008). Because those mechanisms of action are complex, the probiotics are considered to have a systemic impact on the host. In this way, some usually assessed parameters can be influenced by

a probiotic treatment. Thus, it is important to know if those parameters variations are dependent or independent to the probiotic treatment.

Our aim was to assess the potential effect of a probiotic formula containing *Bacillus subtilis, Bacillus licheniformis* and *Pediococcus acidilactici* (Fidospore®, Microbiome Labs, LLC) on the serum activity of the main ions when administered 30 days consecutively on healthy dogs and dogs with apparent GI dysbiosis. Moreover, we wanted to find out if this treatment may influence the ions, in order to establish if a confusion can be made when the ions are dosed in different pathologies.

Materials and method

Study design

The study was conducted at the Faculty of Veterinary Medicine Cluj-Napoca in the departments of animal physiology and internal medicine.

The cases were taken from the clinics and were represented by real clinical cases. 11 dogs of different ages were enrolled in the study based on inclusion and exclusion criteria. The study population was divided in 2 groups: group 1 (n=5) - healthy dogs and group 2 (n=6)- dogs with GI manifestations. All dogs were enrolled after the owners were fully informed about all the procedures and voluntarily signed an informed consent. The study was approved by the Institutional Bioethics committee (decision no 130/December 2018).

On day 0 and day 31 blood samples without coagulant were collected in order to separate blood serum. Between day 1 and 30 the probiotic product was administered to the dogs by the owners.

Study population

11 dogs, divided in 2 groups formed the study population. They were enrolled in the study after a full clinical exam performed in order to establish if the inclusion/exclusion criteria are met for each group (Table 1).

Table 1.
Inclusion and Exclusion criteria for study population groups

	Inclusion criteria	**Exclusion criteria**
Group 1- Healthy dogs	Absence of GI manifestation (vomiturition/diarrhea) No antibiotic treatment in the last 14 days Current on vaccination and deworming Clinically healthy One meal per day	Diarrhea Vomiturition Current on antibiotic treatment
Group 2- Dogs with GI manifestations	One meal per day Diarrhea Vomiturition Antibiotic treatment	Acute/Chronic renal failure Acute/Chronic hepatic failure Intestinal parasites

Treatment

Each dog received the probiotic treatment for 30 consecutively days. The product was administered by the owner, together with dog's regular food. The probiotic product was composed by two bacterial strains: *Bacillus subtilis, Bacillus licheniformis* and *Pediococcus acidilactici* (Fidospore®, Microbiome Labs, LLC).

Ions assessment

The ions were assessed using VetScan machine, Comprehensive test kit. The samples used were represented by serum samples (Table 2).

Table 2.
Assessed ions

No	Parameter	Indication	Principle of method
1.	**Calcium**	Parathyroid, bone and chronic renal disease; tetany	Calcium in sample binds with arsenazo III to form a calcium-dye complex. Absorbance is measured.
2.	**Phosphorus**	Kidney disease, hypoparathyroidism and nutritional disorders.	The method uses sucrose phosphorylase (SP) coupled with the phosphoglucomutase (PGM) and glucose- 6-phosphate dehydrogenase (G-6-PDH) reactions. Absorbance is measured.
3.	**Potassium**	Malnutrition and renal disease. This electrolyte is used to diagnose the causes of vomiting, diarrhea and cardiac symptoms.	Enzymatic method is based on the activation of pyruvate kinase (PK) with potassium. Absorbance is measured.
4.	**Sodium**	Dehydration, and diabetes. This electrolyte is used to diagnose the causes of vomiting, diarrhea and cardiac symptoms.	β-galactosidase is activated by the sodium in the sample. The activated enzyme catalyzes the reaction of o-nitrophenyl-β-D-galactopyranoside (ONPG) to o-nitrophenol and galactose. Absorbance is measured.

Statistics

Statistical analysis was performed using GraphPad Prism 8 software. Statistics tests realized were represented by descriptive statistics and T test for comparation between results pre-administration and after probiotic administration, $p<0.05$.

Results and discussion

The results obtained show a physiological variance of values before and after the treatment (Table 3, Table 4, Figure 1).

Table 3.
Descriptive statistics of ions results before and after the probiotic treatment for Group 1 - healthy dogs

Group 1	Descriprive statistics						T test	
Parameter	Min	Max	Mean	St Dev	St error of mean	CV (%)	p value	Sign
Ca pre-A	2.33	2.64	2.482	0.1219	0.0545	4.913	0.3074	ns
Ca post-A	2.03	2.65	2.404	0.2543	0.1137	10.580		
P pre-A	1.20	2.10	1.612	0.3560	0.1592	22.090	0.9512	ns
P post-A	1.18	1.99	1.618	0.2988	0.1336	18.470		

K pre-A	4.60	8.50	5.540	1.6710	0.7474	30.170	0.3151	ns
K post-A	4.70	12.00	6.340	3.1720	1.4190	50.040		
Na pre-A	145.00	149.00	146.400	1.6730	0.7483	1.143	0.0647	ns
Na post-A	139.00	146.00	142.400	2.5100	1.1220	1.763		

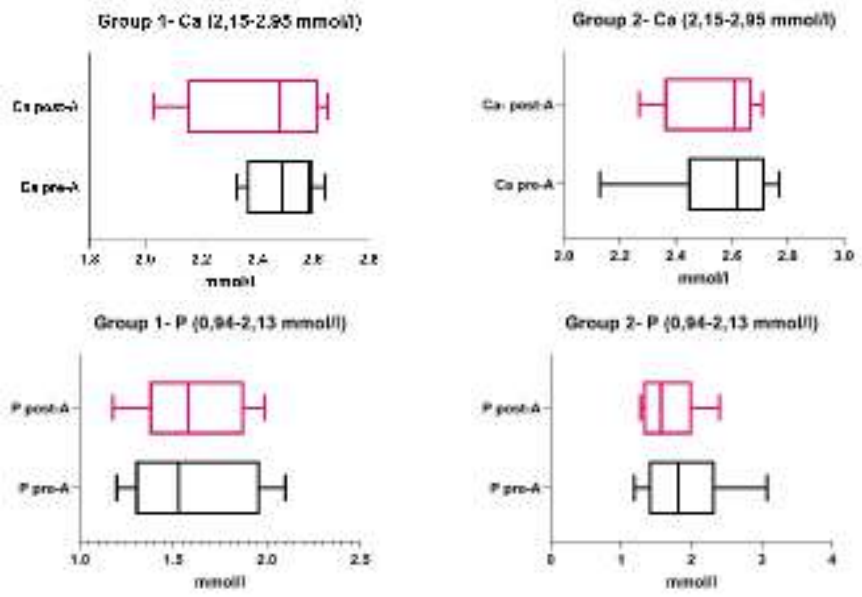
Ca- calcium; P- phosphorus; K- potassium; Na- natrium; pre-A- measurement before treatment (day 0); post-A- measurement after treatment (day 31); ns- without statistical significance

Table 4.

Descriptive statistics of ions results before and after the probiotic treatment for Group 2- dogs with GI manifestations

Group 2	Descriprive statistics						T test	
Parameter	Min	Max	Mean	St Dev	St error of mean	CV (%)	p value	Sign
Ca pre-A	2.13	2.77	2.565	0.2284	0.0932	8.903	0.6211	ns
Ca post-A	2.27	2.71	2.542	0.1728	0.0705	6.798		
P pre-A	1.19	3.08	1.908	0.6555	0.2676	34.350	0.0800	ns
P post-A	1.28	2.40	1.672	0.4347	0.1775	26.000		
K pre-A	3.90	4.90	4.650	0.3886	0.1586	8.357	0.0648	ns
K post-A	4.00	4.70	4.417	0.2927	0.1195	6.627		
Na pre-A	144.00	149.00	145.800	1.7220	0.7032	1.181	0.6203	ns
Na post-A	141.00	149.00	145.000	2.8280	1.1550	1.951		

Ca- calcium; P- phosphorus; K- potassium; Na- natrium; pre-A- measurement before treatment (day 0); post-A- measurement after treatment (day 31); ns- without statistical significance



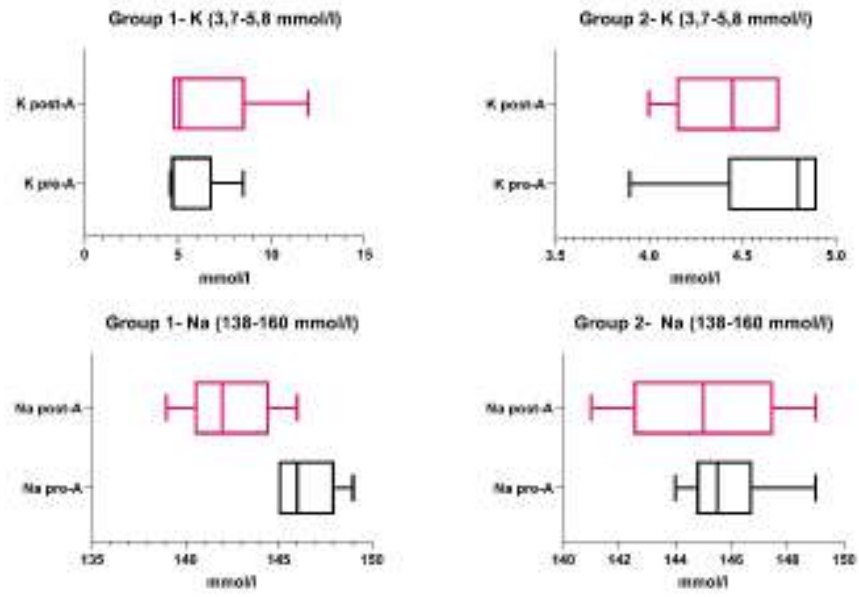


Fig. 1. Graphical representations of ions results before and after the treatment

The probiotic potential of *Bacillus* spp. was discovered starting with 1958, being a component of a therapeutic formula used as nutritive supplement in Italy. However, the real potential of those bacteria was discovered only in the last 20 years (Cutting, 2011). Studies show that those type of bacteria are able to survive into canine GI tract, regardless the hard environmental conditions, even if they are not present anymore in feces after 3 days from the end of the treatment (German et al., 2000).

B. subtilis, B. licheniformis and *P. acidilactici* combination did not induce any significant change in calcium, phosphorus, potassium or natrium serum values. Moreover, this combination had no clinically observable adverse effects neither on the healthy dogs' group, nor in the group of dogs with GI manifestations.

Ions values on the first assessment showed normal values for all the parameters. At the second examination, normal variations were registered, but all the values remained in the physiological interval. This variation can be considered normal and linked with every individual, being a physiological observation. Moreover, this variance between the values cannot be attributed to the probiotic treatment as long as there is no evidence on the literature that can prove that a probiotic treatment is able to influence one way or another the serum ions values.

The electrolyte and ions equilibrium are affected by a several number of conditions. Every pathology that involve water loss or a heavy loss of body fluids this equilibrium is evidentially affected. GI manifestations like diarrhea and vomiturition are two conditions that evolve with the loss of water and other body fluids. As a mechanism of action, if the electrolyte balance is affected, this fact can be revealed as clinical signs (i.e. dehydration) and also on ions profiles (Wirth, 1967).

On the other hand, ions values are modified in other important pathologies, thus they can be used as an important indicator. Calcium as an assessed parameter usually is representative for tumoral process or endocrinological diseases. Phosphorus can be modified in some endocrinal diseases, bones diseases or renal diseases. Potassium in physiological limits translate a good

functioning of the heart and muscle. Sodium is representative of the kidney function (Poli G., 2016). Because all those parameters have an important clinical significance, all the factors that are able to influence their values, regardless the disease, should be known as much as possible. This pilot study suggests that *B. subtillis, B. licheniformis* and *P. acidilactici* are safe to use when the ions are assessed, without any significant influence on their values.

Conclusion

The present pilot study suggests that the probiotic combination of *B. subtilis, B. licheniformis* and *P. acidilactici* administered to healthy dogs and dogs with GI manifestation for 30 days induced no significant variation in serum ions. In conclusion, this combination is safe to use in conditions when serum ions are assessed, without inducing any bias in the interpretation of results.

ACKNOWLEDGMENT: This work was supported by 1 funding source:
MICROBIOME LABS PHYSICIANS EXCLUSIVE, LLC, Agreement no 30130/18 December 2018.

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Quality assessment of the feed in dairy cows diet from a Bucovina Farm

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Abstract
The study was conducted on a cattle farm with a number of 200 cows located in Bucovina County. With the help of the information received from the staff employed on the farm and through the visual examination, assessments were made on the technology of raising dairy cattle, the hygiene and welfare conditions on the farm and on the animal feed. A number of 4 feed samples were collected, representing all the feed used in the feed of these categories of dairy cows, both components and mixtures administered to animals. Sampling was done individually and was conditioned shortly after harvest by relative drying and grinding. The organoleptic examination was performed, the crude chemical composition was determined, the mycotoxicological examination was performed and the aflatoxin and zearalenone content was determined. The results obtained indicated an adequate quality of the fodder from the organoleptic point of view and of the crude chemical composition. Following the mycotoxicological examination, it was found that the fodder was contaminated with aflatoxin B1 in all the samples examined, but no sample exceeded the maximum permitted values, and with regard to the zearalenone values in the samples we analyzed, we found that the values recorded in lucerne hay, natural hay and concentrate for dairy cows were below the maximum limits imposed, instead the corn silo showed values that exceed twice the maximum values allowed by regulations for calves and dairy cows, respectively, we recorder a value of 1098,41 µg/kg.
Keywords: *cattle, gross chemical composition, mycotoxins, aflatoxin B1, zearalenone.*

Introduction

Diseases caused by the consumption of contaminated feed and food are becoming an increasingly important problem worldwide. The most important factor involved in food and feed contamination is represented by microorganisms and among them, especially fungi. They make secondary metabolites with toxic properties called mycotoxins. Most mycotoxins are ubiquitously encountered and can cause serious health disorders (such as neurological disorders and even tumours) in both animals and humans. That is why their study, information and public awareness, as well as the development and implementation of effective methods of detection and inactivation is a matter of utmost importance for veterinary public health (Bhat et al., 2010).

The indispensable nutritional factors, which the animals provide in a minimum dose, in the conditions of free access to food, cannot be ensured without a rigorous scientific control to the animals raised in artificial conditions.

In principle, increasing the economic efficiency of raising different categories of animals cannot be achieved without knowing the level of food and the quality of feed used and these goals also require continuous assessment of the quality of food used.

The quality of fodder is determined by a lot of factors related to soil and plant, as well as all the conditions of production, transport, storage and handling.

The quality assessment allows the knowledge of the measures to be taken in the technological process of animal feeding.

Materials and methods

A number of 4 fodder samples that made up the diet of 200 dairy cows were analyzed, assessing the organoleptic characteristics, raw chemical composition and mycotoxicological examination of the fodder samples. The method of collecting and forming the laboratory sample corresponded to the regulations in force. Thus, from the elementary samples collected from different points, general well-homogenized samples were constituted. The laboratory sample was

extracted from the general sample. The method of successive reduction of the general sample was used, up to the weight of 1-3 kg.

To determine aflatoxin B1 in feed, we used the Ridacascreen®Aflatoxin B1 kit, which is an ELISA kit based on the competition method for the quantitative detection of aflatoxin B1 in cereals and feed. The test is based on the antigen-antibody immuno-enzymatic reaction. The wells of the plate are labeled with anti-aflatoxin B1 antibodies. All reagents required for enzymatic determination (including standards) were included in the kit. The detection limit is 1 ppb (1µg / kg) and the reproducibility of the test is 80-100%.

In performing mycotoxicological examinations we also used the RIDASCREEN® Zearalenone test, which is based on competitive enzyme-linked immunosorbent assays in order to determine the quantitative determination of zearalenone in cereals, feed, beer, serum and urine. All reagents required for enzyme immunoassays are continuous in the Test Kit. The test is based on the antigen-antibody reaction. The microtiter wells are coated with capture antibodies, directed against anti-mycotoxin antibodies. Enzyme conjugate and anti - mycotoxin antibodies are added to each well for both standard and sample. The free mycotoxin and the enzyme conjugate compete for the binding sites of the well-covering antibodies (competitive enzyme-linked immunosorbent assay). The unbound enzyme conjugate is removed in the washing phase. Add substrate / chromogen, observing the color change from red to blue. The addition of the reaction stop reagent causes the blue to yellow to change color. The samples are read at 450 nm. The absorbance is inversely proportional to the concentration of mycotoxin in the sample.

Results and discussions

Following the analysis of the chemical composition of the alfalfa hay sample, the percentage of protein is above average, being 21.95%, while an average value of this type of feed is 17%. Low values were obtained in fat 1.66%, the average value being 2.3% (Table 1).

Feeds had values of humidity within normal limits, and their composition in protein, fat, cellulose, ash and non-nitrogenous extractive substances are close in value to those found in the literature (Preston, 2013).

Regarding the values of zearalenone in the 4 samples analysed, we found that the values recorded for alfalfa hay, natural hay and dairy cow concentrate were below the maximum limits of 500µg/kg imposed by the European Commission. Instead, the corn silo presented values that exceed twice the maximum values allowed by regulations for calves and dairy cows, namely we recorded a value of 1098.41µg/kg (Table 2). We replaced this fodder assortment with farm animal feed to avoid reproductive problems such as ovarian cysts, placental retention or premature abortions that could be caused by elevated zearalenone levels in the ration.

Table 1.

Crude chemical composition (%)

Sample No.	Sample type	D.M.*	Crude protein	Crude fat	Crude celulose	N.F.E.**	Crude ash
1.	Corn silage	35,39	3,60	0,86	5,91	23,67	1,35
		100	10,17	2,43	16,70	66,88	3,82
2.	Alfalfa hay	89,56	21,95	1,66	21,94	39,78	4,23
		100	24,51	1,85	24,50	44,42	4,72
3.	Natural hay	90,22	11,76	2,15	22,43	46,05	7,83
		100	13,04	2,38	24,86	51,04	8,68

4.	Dairy cow concentrate	88,49	21,04	2,57	6,44	52,96	5,48
		100	23,78	2,90	7,28	59,85	6,19

D.M.*- dry matter, N.F.E.** - nitrogen free extract

Aflatoxin B1 was detected in all examined samples, but none of them exceeded the maximum values of 20 µg/kg allowed in the feeding of dairy cows (Table 2). The values of total aflatoxins in the 4 analyzed samples were: 13.97 µg / kg - corn silage; 9.41 µg / kg - alfalfa hay; 12.28 µg / kg - natural hay; 17.32 ppb - concentrated dairy cows.

Table 2.

Zearalenone and Aflatoxin B1 values

Number of samples	4	
	Zearalenone (µg/kg)	Aflatoxin B1 (µg/kg)
% positive samples	100	100
Interval	180,96-1098,41	9,41-17,32
Average	443,8	13,245
Median	247,915	13,125
Standard deviation	437,8444	3,305032

The low values of Aflatoxin B1 in the samples analyzed by us were also confirmed by the fact that Aflatoxin M1 from milk coming from farm cows, no problems were reported regarding the maximum level allowed by European legislation for Aflatoxin M1.

In 2015, Milăşan determined zearalenone in several types of bread such as: white bread, intermediate bread, black bread, which were sold in several regions of Romania. The limit value allowed according to Regulation (EC) no. 856/2005 is 50 µg / kg.

Conclusions

Aflatoxin B1 was detected in all analyzed samples, but none of the samples analyzed did exceed the maximum values allowed for this mycotoxin. The values of zearalenone recorded in the samples of natural hay, alfalfa hay and concentrate for dairy cows were within normal limits, and however in the corn silage sample was recorded a value that exceeded twice the maximum value allowed by European legislation.

We proposed to eliminate from the ratio the assortment of corn silage contaminated with zearalenone due to the fact that the high values recorded for this mycotoxin could lead to reproductive disorders in heifers and dairy cows.

We recommend to the farmer the use of mycotoxin inhibitors in the ratio of dairy cows in order to avoid the problems caused by the accidental consumption of mycotoxins in fodder.

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Determination of total aflatoxins and aflatoxin B1 content in oleaginous seeds and dried fruits coming from supermarkets and small shops

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Abstract
A very important category of mycotoxins are aflatoxins, made by Aspergillus fungi (A. flavus, A. parasiticus and A. nominus). From all aflatoxins, the following stand out: aflatoxin B1 (AFB1) - most toxic one, aflatoxin B2, G1 and G2 Study was conducted on 25 samples of fruit and seeds originating from supermarkets and small shops around Transylvania. Organoleptic and mycotoxicological tests were performed for all samples. For mycotoxicological testing, RIDASCREEN®FAST Aflatoxin was used, an immunoenzymatic competitive test for quantitative determination of aflatoxins from aliments and cereals and RIDASCREEN AFLATOXIN B1, ELISA test for quantitative determination of Aflatoxin B1 from cereals and fodders. Upon organoleptic testing, 2 samples were noticed to have modified parameters. Mycotoxicological testing revealed 6 samples with higher than normal total aflatoxin content, and 1 sample contained aflatoxin B1 above the upper limit established by the European legislation. The highest total aflatoxin and aflatoxin B1 levels were found in roasted corn (46.5 ppb for total aflatoxins, respectively 4.01ppb for Aflatoxin B1).
Keywords: *fungi, mycotoxins, total aflatoxin, aflatoxin B1, oleagineous seeds*

Introduction

The impact of mycotoxins in food chain is a major global problem, they represent a real danger. Mycotoxins are secondary products of fungal metabolism that can be identified in both food and animal feed. It is known that in small quantities mycotoxins are not dangerous, but public authorities from several countries have set legal limits regarding the presence of mycotoxins in food, in order to prevent the risk of higher contamination. Diseases caused by mycotoxins are called mycotoxicosis and can be acute, chronic or sub chronic. A very important category of mycotoxins are aflatoxins, produced mainly by fungi of the genus *Aspergillus,* the most important species being represented by *A. flavus*, *A. parasiticus* and *A. nominus* and are considered to have a carcinogenic potential in the liver, being also a teratogen and mutagen in both humans and animals. They can enter the body directly through cereals, seeds, spices, fruits and other plant products and indirectly through food products from animals whose contaminated food has led to residues in meat, milk, eggs. and their derivatives (Galvano, 2001). From the category of aflatoxins, the most important are: aflatoxin B1 (AFB1), the most toxic, contributes to the inhibition of messenger RNA synthesis and leads to the formation of liver tumors (hepatocellular carcinoma), aflatoxin B2, G1 and G2 (Masoero, 2009).

Over the past decade, preventing mold contamination in some foods, especially nuts and nuts, has become a real health problem. For prophylactic purposes, the implementation of Good Agricultural Practices (GAP) on regulation in the cultivation, harvesting and storage of fruit and oilseeds is recommended (Yazdanpanah, 2005).

Materials and methods

A number of 25 samples of oleaginous and dehydrated fruits were collected. The samples were collected from hypermarkets, supermarkets and small shops in Cluj and Alba county. Sampling and preparation was done according to the standardized methodology. Oilseeds and fruits were stored either in bulk or in bags. If the samples were collected in bulk, the working method was as it follows: an imaginary division of the lot into an approximately equal number of parts was

made. Randomly, was selected a number of parts, corresponding to the number of partial or elementary samples and was taken at least one sample from each part.

From the mixing and homogenization of the partial (elementary) samples, the general sample was obtained, and from this, using the reduction, by the method of quarters, the average sample was obtained, with a mass of about 100 grams for laboratory analyzes. The samples were packed in new nylon bags, then labeled and sent to the laboratory, where they were recorded and subjected to organoleptic examination.

To perform laboratory tests, was used the RIDASCREEN®FAST Aflatoxin test, a competitive enzyme-linked immunosorbent assay for the quantitative determination of aflatoxins in cereals and food. The test is based on the antigen-antibody reaction. The wells of the plate were labeled with antibodies anti-aflatoxin. Aflatoxin or sample standards, enzyme-aflatoxin conjugate and anti-aflatoxin antibodies were added. Free aflatoxin and enzyme-aflatoxin conjugate compete for antibody binding sites (competitive enzyme-linked immunosorbent assay). At the same time, anti-aflatoxin antibodies are also bound by immobilization of capture antibodies. The unbound conjugate is removed in the washing step. The substrate-chromogen mixture is added to the wells and the bound enzymatic conjugate converts the chromogen into a blue product. Adding the stop solution changes the color from blue to yellow. The measurement is made by spectrophotometry at a wavelength of 450 nm. The absorbance is inversely proportional to the concentration of aflatoxin in the sample.

The samples were processed and analyzed in strict hygienic conditions, respecting the legislation in force so that the results are as conclusive as possible.

Results and discussions

Following the organoleptic examination, changes in appearance, consistency and odor were found in a number of 2 samples: a sample of roasted almonds in which a gray-whitish color was observed on the surface, as well as odor and rancid taste; a mixed fruit sample in which changes in consistency were observed, which were strong and had an aging smell. Following the mycotoxicological examination, it was found that the mixed fruit sample showed an increased level of total aflatoxins, 12.46 ppb, above the maximum limit of 10 ppb allowed by European legislation (EC Reg. No. 1881/2006 and No. 1126/2007).

Out of the total of 25 samples analyzed (Table 1) for the total aflatoxin content, 21 (84%) samples were positive, and out of the total positive samples 24% exceeded the maximum values allowed by European legislation.

Following the analyzes for the content in total aflatoxins, it was found that from the total samples from supermarkets, 9.09% of the samples exceeded the maximum level allowed by European regulations, and in the case of small shops the percentage was 35.71%.

Table 1.

Aflatoxin content in dried fruits and oleaginous seeds (µg/kg)

Specification	Total Aflatoxins - all samples	Total Aflatoxin small shops	Total Aflatoxins supermarket	Aflatoxin B1 - all samples
Nr. of samples	25	14	11	10
% positive samples	84	92.857	72.72	20
Min. and max. of positive samples	1.70 - 46.50	1.70-46.50	1.70- 7.81	2.79 – 4.02

Mean	6.362	8.865	3.176	0.68
Median	4.080	4.430	2.550	0.00
Std. Dev.	9.924	12.664	2.812	1.46
p	≤0.005	≤0.05	≤0.005	≤0.5

Most elevated values of total aflatoxins were identified in the category of roasted peanut-corn-cashew. The maximum value allowed by European legislation for this category is 4 ppb. Out of the total of the 12 samples analyzed in this category, a number of 5 samples exceeded the maximum allowed limits.

Regarding the aflatoxin B1 content, out of the total of the 10 samples examined, only one sample of roasted maize exceeded the maximum level of 2 ppb, allowed by the legislation in force, with a value of 4.01 ppb.

According to a report published by the Iranian Ministry of Health, 7926 pistachio samples were analyzed using the HPLC method from March 2001 to March 2002 in food and drug control laboratories in Iran. The results indicated that 5290 samples (68%) were not contaminated (values <detection limit, LOD) with Aflatoxin B1, 1324 samples (17%) contained Aflatoxin B1 with detection limit 2 µg / kg, 541 samples (5 %) contain AFB1 with values between 2 and 10 µg / kg. According to the report made by the Ministry of Health of Japan, out of 2422 samples analyzed, those between 1972-1989 and sample 2339 (97%) were not contaminated (<LOD), 35 samples (1%) were contaminated with AFB1 with LOD values -10µg / kg, 48 samples (2%) contained AFB1 in an amount greater than 10µg / kg (Yazdanpanah, 2005).

Conclusions

Comparing the samples from supermarkets and small shops, it was found that 9.09% of the total samples from supermarkets respectively 35, 71% of the samples collected from small shops, exceeded the maximum values allowed by European legislation for total aflatoxins;

A contamination with total aflatoxins and aflatoxin B1 was observed both in the non-heat treated and in the processed samples (roasted, seasoned), the thermal factor not decisively influencing the decrease of the aflatoxin content in the analyzed samples.

The study concluded that the level of total aflatoxins was much lower in samples from supermarkets than those from small shops, due to both the origin and storage conditions.

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Deoxynivalenol and T2 toxin content in wheat and bread from different Transilvania Region

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Abstract
The toxins produced by Fusarium fungi that contaminate cereals are a serious concern. The most important and broad family of fusariotoxins are trichothechenes, which comprise several components divided into 4 groups, with types A and B being the most significant. In order to determine the level of DON and T2 toxin, we collected a number of 25 samples, of which 10 were bread and 15 wheat, all of them were collected from the Transylvania region. In wheat samples, DON was identified in all 15 samples analyzed, with values between 33-2225 µg / kg, with an average value of 672.6 µg / kg and with a median of 372 µg / kg. Of these, 4 samples exceeded the maximum limit imposed by European legislation of 1250 µg / kg for DON, the maximum value being 2225 µg / kg. In the bread samples, the presence of DON was found in 8 out of 10 samples analyzed (80%) with values not exceeding the limits of European legislation of 500 µg / kg, between 0-321 µg / kg. Regarding the T2 toxin content, it was identified in only 2 of the samples analyzed (8%), a wheat sample and a bread sample, with values of 7 µg / kg and 5 µg / kg, these values not exceeding European standards. The results obtained by us show a high DON contamination at the level of wheat grains harvested from different counties of Transylvania, some of the values not respecting the European standards, but with low values recorded in bread samples. Regarding T2 mycotoxin, this was detected only in 2 samples, with low values and for this reason do not consist a high risk for human health.
Keywords*: wheat, bread, deoxynivalenol, T_2 toxin*

Introduction

There are a number of mycotoxins that belong to the trichothecene chemical compound family. Most of these (T2 toxin, deoxynivalenol) are derived from one or more species of *Fusarium*.

One of the most studied trichothecenes, T2 toxin, apparently binds to cell membrane receptors, decreases both RNA and DNA production, and interferes with protein synthesis by blocking the initiation of translation. The chemical effects of this toxicosis are epithelial necrosis – both dermal and mucosal - severe enteritis, vomiting in some animals, coagulopathy, hematopoietic depression and leukopenia with secondary septic status.

According to the FAO, an allowable limit of 1 µg / kg / body weight and 0.06 µg / kg / body weight was set for T2 toxin and HT2 toxin. It should be noted that these data are valid only for European regions, so that more information and analytical methods are needed for other parts of the world (Van der Westhuizen et al, 2002). Following a study in the Netherlands, a concentration limit of 129 µg / kg DON was proposed in wheat-based products for children as the maximum dose limit. The researchers also pointed out that between September 1998 and January 2000, due to increased concentrations of DON in wheat, the dietary intake of DON has exceeded the permitted level, which leads to the negative effects on health.

Despite data on lack of consumption in some countries and little information on children and baby food, the researchers pointed out that among cereals, maize has the highest level of trichothecene contamination, and wheat and wheat products (bread and pasta) is the major source of contamination.

Materials and methods

For the determinations we collected a number of 25 samples, of which 10 samples of bread and 15 samples of wheat. All analyzed samples were purchased from Transylvania region.

Sampling and preparation were performed according to the standardized methodology. As for the wheat, it was stored either in bulk or in sacks. For sampling the bulk wheat samples, the following method was used: an imaginary division of the lot into an approximately equal number of parts was made. A certain number of parts were randomly selected; corresponding to the number of partial or elementary samples, at least one sample was taken from each part. The next step was to mix and homogenize the partial (elementary) samples, thus obtaining the general sample, and from this, using the reduction, by the method of quarters, an average sample was obtained, with a mass of about 100 grams for laboratory analysis. The samples were packed in new nylon bags, followed by labeling and delivery to the laboratory for recordings and the actual examinations.

For laboratory tests we used the RIDASCREEN®FAST Mycotoxin test, a competitive enzyme-linked immunosorbent assay, based on the antigen-antibody reaction, for the quantitative determination of mycotoxins in bread and cereals. An ELISA photometer was for data interpretation.

The samples were kept in a cool place, protected from light. For T2 toxin determination, were weighed 5 g of ground sample into a vessel and dissolve in 25 ml of methanol / distilled water (70/30), then mix using the magnetic stirrer for 10 minutes. The extract was filtered with a filter paper. 50 µl of the filtered sample was diluted with 300 µl of buffer for diluting the sample 1: 7 (1 + 6), dilution factor 35. From the mixture obtained, 50 µl per well was used. A required number of wells for standards and samples were inserted into the support and then 50 µl of standard or sample was added. 50 µl of conjugate was added to each well followed by 50 µl of anti-toxin T2 antibody for each well. It was stirred by rotating the plate and left in the incubator for 1 hour at room temperature in the dark. After the time expired, the liquid was discarded, tapping the plate face down on absorbent paper to remove traces of liquid, and 250 µl of distilled water was added, discarding the liquid again. This washing step was repeated twice according to the protocol requirement. Further, 50 µl of substrate and 50 µl of chromogen were added to each well and mixed by rotating the plate, then incubated for 30 minutes at room temperature (20-25 ° C) in the dark. 100 µl of stop solution was added to each well, stirring gently by rotating the plate and measured at 450 nm from the air blank. It is important that the measurement does not exceed 10 minutes after the addition of the stop solution. The minimum limit of detection is 0.1 µg / kg (ppb). According to the implementation of the test, the detection limit is 3.5 µg / kg (ppb) T2 toxin in the grain or hayfield sample.

For DON determination, were weighed 5 g of ground sample into a pot with a lid and added 25 ml of distilled water to each of them and stirred vigorously for 3 minutes. The extract was filtered with a Whatman filter paper no. 1. A dilution was made for 100 µl of filtrate. From the mixture obtained, 50 µl per well was used. A required number of wells for standards and samples were inserted into the support and then 50 µl of standard or sample was added. 50 µl of enzymatic conjugate was added to each well followed by 50 µl of antibody to each well. It was mixed by hand by gently rotating the plate and left to incubate for 30 minutes at room temperature (20-25 ° C) after which the liquid was discarded and beaten vigorously face down on an absorbent paper to remove traces of liquid. 250 µl of distilled water was added and the liquid was discarded again. The washing step was repeated twice. 100 µl (2 drops) of substrate / chromogen was added to each well. Mixed by rotating the plate and incubated for 15 minutes at room temperature (20-25 ° C). 100 µl of stop solution was added to each well. It was mixed gently by rotating the plate and measured at 450nm from the air blank.

Results and discussions

In the wheat samples, DON was identified in all 15 samples analyzed (100%), with an average value of 672.6 µg / kg and with a median value of 372 µg / kg (table 1). Regarding the bread samples, out of the total of the 10 analyzed samples, the presence of DON was found in 8 samples (80%), average of 106.2 µg / kg, median of 82 µg/kg (table 1).

Out of the total of the 15 wheat samples, a number of 4 samples exceeded the values imposed by the European legislation of 1250 µg / kg for DON, the registered values being of 1530, 1650, 1485, respectively 2225 µg / kg.

Regarding the T2 toxin content, it was identified in only 2 of the analyzed samples (8%), respectively a wheat sample and a bread sample, with values of 7 µg/kg and 5 µg/kg, respectively. These values did not exceeding European standards (EC Reg. No. 1881/2006 and No. 1126/2007).

Table 1.

DON values found in wheat and bread samples

Sample type	Number of analyzed samples	Detected mycotoxin	Number of pozitive samples	Range	Average	Median
				µg/kg	µg/kg	µg/kg
Wheat	15	DON	15	33-2225	672.6	372
Bread	10	DON	8	0-321	106.2	82

Compared to other countries, following a study on bread and pasta in Spain in 2010, the occurrence of mycotoxins in bread was 28.0% and 2.6% for deoxynivalenol and T2 toxin, whereas in pasta, the occurrence for both toxins was higher, ranging from 9.3% to 62.7%. The average content of DON (42.5 µg / kg) in bread was lower than the content of mycotoxin T2 (68.37% µg / kg), while in pasta the content of DON (137.1% µg / kg) was higher (Gonzáles-Osnaya, 2011). A study from 2012 shows the presence of DON in wheat in Parana, Brazil. The occurrence of this mycotoxin was evaluated in a study conducted on 113 wheat samples from the northern and central / southwestern regions of the state of Parana, during the growing seasons of 2008-2009. DON was detected in 66.4% of samples at levels ranging from 206.3 to 4732.3µg / kg, many of the samples exceeding the estimated daily dose of 1,250µg / kg (Sifuentes dos Santos, 2013).

The presence of 7 major mycotoxins in wheat flour, purchased from supermarkets in Novi Sad, a Serbian city in the capital of Vojvodina, was determined in a study from May 2012. DON was the predominant toxin in all samples analyzed, followed by zearalenone (ZON), and T2 toxin, with a frequency of 33.3% and 26.7%, respectively. All samples complied with European / Serbian legislation, except for one sample that exceeded the maximum permitted DON level of 750µg / kg. However, DON doses were assessed to be close to the tolerated daily dose level for adults (Biljana, 2012).

Conclusions

The results obtained by us show a high DON contamination at the level of wheat grains harvested from different counties of Transylvania, with some values that exceed European standards, but with low values recorded in bread samples. Regarding T2 mycotoxin, this was detected only in 2 samples, with low values and for this reason do not consist a high risk for human health.

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The importance of balanced diets administration and vitamin-mineral supplements in puppies and adult dogs

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Abstract
Balanced is a term that may be applied to a diet, ration, or feed having all the required nutrients in proper amount and proportion based upon recommendations of recognized authorities in the field of animal nutrition, such as the NRC, for a given set of physiological requirements. Growth is one period where concerns are frequently reported, as this is one of themost nutritionally demanding life stages, and deficiencies and excesses are manifested quite rapidly. Minerals and vitamins are part of the diet, providing the energy for growth and activity. Individual variation, increased growth rate and/or level of activity may lead to exceeding the daily requirements of minerals and vitamins and thus to disturbance of skeletal growth as a cause of frequently diagnosed orthopedic diseases. Growth rates of young dogs are affected by the nutrient density of the food and the amount of food fed. It is important that puppies be fed to grow at an optimal rate for bone development and body condition rather than at a maximal rate. This study includes 8 dogs all different breeds. The study covers the correction of bone deformities when dogs are given a balanced diet along with vitamin mineral supplements. To obtain the proper information, correct diagnosis and a treatment plan a clinical exam was given to each dog. Radiology reports/images were obtained, as well as blood tests. Once results were collected it is clear supermarket puppy food that does not contain minimum 1.2 - 1.8% CA and 0.8 -1.6% P can lead to bone deformities such as "rickets". Premium dog food and vitamin supplements should be given to small to medium breeds four months to one year old, and larger breeds one and a half to two years old.
Keywords: *diet, vitamins, minerals, bone deformities, puppies.*

Introduction

Most problems with the bone system occur in animals during the breeding season and that is why it is necessary to advise animal owners on a balanced diet of puppies and cats as soon as possible. Today, a healthy animal should receive all the vitamins and minerals in an adequate proportion if it is fed a balanced and appropriate diet for its species, age and lifestyle (Agar, 2001). The purpose of a diet plan for young animals is to create a healthy adult. The goals are to achieve healthy growth, optimize the immune system and minimize the chances of becoming obese or developing orthopedic conditions. The most common orthopedic conditions due to nutrition are hip dysplasia and osteochondrosis (Wortinger and Burns 2015). Many nutritionists say it is in the animal's best interest to consume commercial food as much as possible. An important aspect to remember is that home-prepared diets have not been analyzed in laboratories to confirm that they are adapted to the stage of life for which they were prepared. However, there are a number of medical reasons why a home-cooked diet may be recommended, such as food allergies (Fascetti and Delaney, 2012). Supplements are usually given to add vitamins and minerals to an animal's diet in the form of tablets, liquid or powder. Today, a healthy animal should receive all the vitamins and minerals in an adequate proportion if it is fed a balanced and appropriate diet for its species, age and lifestyle. These nutrients are needed in very small amounts and supplementing them does not necessarily mean a good thing. For example, the administration of mineral supplements to puppies of large breeds to encourage the growth of the bone system is not necessarily beneficial (Agar, 2001). The growing period is one of the most demanding stages of life from a nutritional point of view and deficiencies or excesses manifest quickly. (Tomsa et al., 1999; McMillan et al. 2006). Both deficiencies and excess minerals (calcium, phosphorus) and vitamins (D and A) can be factors that cause orthopedic conditions including fractures, rickets, osteochondrosis, panosteitis, hip dysplasia.

Minerals and vitamins are part of the diet providing energy for growth and activity. Depending on the individual, a rapid growth rate or an increased level of activity may exceed the daily need for minerals or vitamins affecting skeletal growth and leading to orthopedic conditions (Fascetti and Delaney, 2012). Adult dogs food for maintenance contains a dangerous concentration of calcium for young dogs in the growth phase. The influence of nutrition during different stages of development can be well illustrated by the results of a study done on Great Dane dogs fed a diet containing 3.3% calcium compared to other dogs fed a diet with 1.1% calcium. Administered in 3-17 week old puppies leads to hypophosphatemic rickets and in 6-21 week old puppies osteochondrosis (Hazewinkel, 1999; Fascetti and Delaney, 2012).

Materials and methods

This study aimed to correct some bone deformities by administering balanced diets and vitamin-mineral supplements on a number of 8 dogs of different breeds. The objectives were to perform a clinical examination of the animals, radiographs, blood tests necessary to be able to make a correct diagnosis and appropriate treatment. The clinical examination was accompanied by clinical observation sheets focusing in particular on the anamnesis and food history of the animals. The inspection was done both in the rest and on the movement state, assessing any defective aplomb, limb deformities, limping, possible fractures or asymmetries between limbs or various body regions. The palpation was directed especially towards the deformed regions and towards the level of the costochondral joints. For the biochemical examination, the values of some essential parameters in the diagnosis of rickets were followed, namely alkaline phosphatase, calcium and phosphorus. Blood was collected in the morning and the dogs were not fed before sampling. Radiological examination is recommended because it provides valuable data for confirming or refuting the diagnosis in rickets. On radiological examination, the specific change in rickets is osteopenia, when bone density is obviously low compared to normal being a sign of bone demineralization. Bone deformities can often be observed, more obvious in the long bones, the growth cartilages are modified compared to normal being thickened and the epiphyseal extremities are widened with the appearance of a suction cup. To correct bone deformities diagnosed after clinical examination, radiography and blood tests, 3 vitamin-mineral supplements were used and 5 premium diets available on the market.

Results and discussions

For case 1 (female, Cane Corso, 8 months), at the radiographic examination accentuated radiolucency was observed. Bone deformities with curvature of the femur were present. The distal epiphyses of the femur were enlarged in volume and showed areas of uneven mineralization at this level. The treatment was based on the transition to a balanced diet represented by: premium food 3 approximately 300 g/day; administration of injectable vitamin AD_3E; nutritional supplements No. 1, 1 tablet of 8 g/day. The daily intake of calcium through food and supplements was 4.54g (1.47%) and that of phosphorus was 3.45g (1.12%), the calcium-phosphorus ratio being 1.24: 1. On radiographic examination of the case 2 (female, mixed breed, 6 months), increased radiolucency was observed. At the level of the distal epiphysis of the radius, it is increased in volume with uneven mineralization. The treatment was based on the administration of: 2 ml of vitamin AD_3E at 14 days; administration of vitamin-mineral supplements 2, 2 tablets/day for 6 weeks after which 2 tablets/day for maintenance; premium food 1, about 260 g/day. The daily intake of calcium through food and supplements was 4.84g (1.84%) and that of phosphorus 3.2g (1.21%), the ratio of calcium to phosphorus being 1.52: 1. Regarding case 3 (female, mixed breed, 7 months), the radiographic examination showed an increase in the volume of the distal epiphyses of the radius and ulna

(arrows) with a typical widened appearance, more obvious in the left anterior limb. Treatment consisted in administration of: injectable vitamin AD_3E 2 ml every 14 days; vitamin-mineral supplements 2, 2 tablets/day; premium food 1, about 280 g/day. The daily intake of calcium through food and supplements was 5.12g (1.81%) and that of phosphorus was 3.4g (1.20%), the calcium-phosphorus ratio being 1.54: 1. For case 4 (male, mixed breed, 10 months) on radiographic examination, radiolucency and asymmetric mineralization between different areas of the bone were observed. There are islands of demineralization at the level of the ulna and bone deformities. The epiphyses appear widened, enlarged in volume. The treatment consisted in the administration of: vitamin AD_3E 2 ml every 14 days 1 tablet/day; vitamin-mineral supplement 1, 1 tablet / day; 300 g / day from premium food 3. The daily intake of calcium through food and supplements was 4.54g (1.47%) and that of phosphorus 3.45g (1.12%), the ratio of calcium to phosphorus being 1.3:1. For case 5 (female, mixed breed, 6.5 months) bone deformation and radiolucency were observed on radiographic examination. The distal epiphyses of the radius and ulna have a widened appearance and are enlarged in volume. The treatment was based on: vitamin AD3E 2 ml every 14 days; approximately 220 g / day of premium food 1; 2 tablets / day of vitamin-mineral supplement 2. The daily intake of calcium through food and supplements was 4.28g (1.92%) and that of phosphorus was 2.8g (1.21%), the ratio of calcium to phosphorus being 1 52: 1. For case 6 (male, Pinscher, 1.5 years) the radiographic examination showed increased radiopacity and the existence of mineral deposits in the distal epiphyses of the tibia and tibia-tarsus-metatarsal joint. The owner administered food for the growing period of an adult dog - premium food 4 and a vitamin-mineral supplement 2. After calculating the daily intake of calcium and phosphorus in the food showed an excess of minerals especially calcium 2.03% (compared to 1.8% maximum recommended). It was thus recommended to switch to a food for small adult dogs, premium food 5 and stop supplementing the food with calcium and phosphorus. For case 7 (female, mixed breed, 7 months) radio transparency was observed on radiographic examination. At the level of the radius and the ulna, bone deformation was observed and at the level of the humerus, areas of bone demineralization. The treatment consisted in the administration of: vitamin AD_3E 1ml every 14 days; vitamin-mineral supplements 1, 1 tablet / day for 6 weeks; premium food 2, about 140 g / day. The daily intake of calcium through food and supplements was 2.56g (1.81%) and that of phosphorus was 1.7g (1.2%), the calcium-phosphorus ratio being 1.5: 1. For case 8 (female, Labrador mixed breed, 2 years) on examination at the rest, a deviation of the distal region of the forelimbs to the side was observed. The metacarpal sesamoid-phalangeal joints appeared thickened. Left hind limb deviated to the side, in the metatarsus-sesamoid-phalangeal region. On palpation of the thorax, the presence of costal rosaries was detected, both on the right and on the left. The treatment consisted of the administration of: vitamin AD_3E 2 ml every 14 days; premium food 1, about 280 g / day; 4 tablets of vitamin-mineral supplement 2. The daily calcium intake was 1.77% and the phosphorus intake of 1.42%, the calcium-phosphorus ratio being 1.24:1.

As shown in Figures 1, alkaline phosphatase was increased in all patients studied with a minimum of 223, 8 U / I and a maximum of 438 U / l, calcium was below the normal limit of 11 mg/dl in 7 of the patients with a minimum of 6.5 mg/dl and a maximum of 8.9 mg/dl (Figure 2). One case, no. 6 showed a value higher than 12.8. This patient was 1.5 years old and was still fed premium chicken feed and was receiving vitamin and mineral supplements.

Phosphorus values were below the normal limit in 4 of the patients (case 1,2,4,5) with a minimum of 2.1 mg / dl and a maximum of 2.5 mg / l. Case 3 and 6 recorded values above the maximum allowed limit and case 8 had a value of 3.1 mg / l within the normal limits (Figure 3).

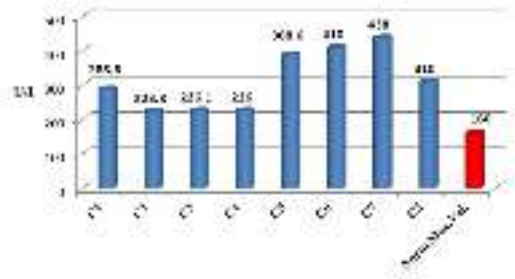

Fig.1. Alkaline phosphatase values

Fig.2. Calcium values

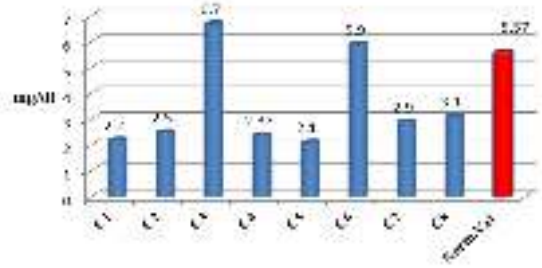

Fig.3. Phosphorus values

The use of supermarket food in the diet of growing puppies or other diets that do not contain 1.2-1.8% Ca as well as 0.8-1.6% P in their structure, as seen in the cases investigated by us, can lead to the appearance of typical bone changes for rickets.

In a study conducted in 1992 by Roudebush and Cowell, they showed that 89 and 93% of home-cooked diets used in dogs and cats were not complete and balanced).

Another study in which owners administered home-prepared diets for their animals for a period of 30 days demonstrated the following: home-prepared diets were evaluated and compared with recommendations for nutritional profiles given by AAFCO (the Association of American Feed Control Officials) (Streiff et al., 2002). 35 of the diets had inadequate levels of calcium, phosphorus, potassium, zinc, copper and vitamin A and E compared to AAFCO recommendations (Fascetti Delaney J. S., 2012). In 2005, Lauten et al. evaluated 49 maintenance diets and 36 for breeding in dogs and cats. These were prepared according to books that veterinarians often recommend. Comparing the AAFCO standards for the animal's life stage, 55% had inadequate amounts of protein, 64% of vitamins and 86% of minerals. In 2012, Fascetti and Delaney also showed in a study how 4 cats of close age who received colostrum and commercial milk replacement formula and after weaning received a homemade diet based on 60% minced beef, chicken and turkey and 40% vegetables (carrots, green leaves, broccoli, turnip, celery roots). From time to time they also received boiled bones, showing signs of lameness from 2.5 months. Cats had a normal appetite and were energetic. On blood tests they came out normal except for the alkaline phosphatase which was high. The radiographs showed a severe bone rarefaction with decreased

bone opacity and fractures in the femur, ilium and the possibility of compression in the sacral vertebrae. At the gastrointestinal level, bone fragments were found.

Conclusions

The diet used to feed the investigated animals was either unknown or came from supermarkets, and on their label was not found the level of calcium and phosphorus contained. It is obvious that these products do not comply with the Ca / P ratio necessary for the development of the dog's bone system, and the clinical manifestations, as well as the paraclinical examinations performed, confirm the nutritional imbalances. It is also good to recommend a calculation of the total amount of calcium administered daily when using the diet in the supermarket (which has noted on the packaging especially the calcium and phosphorus content) or premium category, to avoid excess Ca and P , which in turn can lead to ectopic calcium deposits in the body.

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