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Boar freeze-dried semen evaluation using Spermac staining

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Abstract
Sperm freeze-drying is a new and alternative method to preserve male gametes in refrigeration or at room temperature. In order to protect sperm integrity special protection is required. The aim of our research was to examine the effect of vitamin C and rosmarinic acid on the spermatozoa integrity after freeze-drying the probes. We analyzed the acrosome reaction and the morphological aspects of boar spermatozoa form three different breed (Pietrain, Large White and Landrace) after rehydration. We observed that the highest percent of spermatozoa with intact acrosome and the least spermatozoa anomalies were in samples were rosmarinic acid was added. As preliminary results we could state that adding antioxidants protects spermatozoa from oxidative stress.
Key words*: dry-freezed, antioxidants,boar, Spermac*

Introduction

Usualy preservation of sperm is done in liquid nitrogen. Another recent method for preservation that enables sperm to be stored for a long time at refrigerate temperature is freeze-drying (lyophilization)(Keneko et al., 2015; Olaciregui et al., 2017).

By using this method, liquid nitrogen, safe facilities and special equipment is not needed anymore. Transportation of freeze-dryed sperm could be done at room temperature. For example, Keneko et al. (2015) freezed-dry sperm from some wild animals and after rehydration and ICSI on mouse oocytes obtained pronuclei, which mean that spermatozoa were viable; useful preliminary results for future "freeze-drying zoo" to conserve wild animals.

Boar semen has a poor capacity to be cryopreserved because is sensitive to cold shock and to peroxidative damage cause by the high content of unsaturated fatty acids in the phospholipids of the plasma membrane and the low antioxidant capacity of seminal plasma (Jeong et al., 2009). For this reason boar frozen semen is used only in research (IVF) and for conservation.

In order to combat the excess of reactive oxygen species (ROS) from ART procedures, that could be either endogenously (immature spermatozoa, leukocytes, oocyte, cumulus mass cells, follicular fluid, embryos) or exogenous environmental factors (visible light, culture media, pH, temperature, oxygen concentration, centrifugation, cryopreservation)(Mbemya et al., 2017; Agarwal et al., 2014) antioxidants are added.

Studies revealed that vitamin C (L-ascorbic acid), a water-soluble antioxidant, it is used also on assisted reproductive techniques, where has an antioxidant effect during *in vitro* maturation of oocytes (Marc et al., 2017; Sovernigo et al., 2017; Comizzoli et al. 2003; Tatemoto et al., 2001), has a beneficial role on freezing spermatozoa (Varo et al., 2014; Fanaei et al., 2014) and on improved motility and reduced DNA damage in post-thaw spermatozoa (Fanaei et al., 2014). Another antioxidant used especially in freezing extenders where improves sperm quality after cryopreservation is rosmarinic acid (Luno et al., 2014; Luno et al., 2015; Olaciregui et al., 2017).

Knowing the beneficial effects of vitamin C and rozmarinic acid on gametes cells during different assisted reproduction techniques and the promising future of semen lyophilization, in this research we want to evaluate the effect of vitamin C and rozmarinic acid on boar spermatozoa during lyophilization.

Material and methods

The research was carried out on 8 samples of diluted and refrigerated sperm obtained from boars. They belonged to four different breeds (Pietrain, Large White, Duroc and Landrace).

Sperm samples originated from Semest-BVN Targu-Mures and were transported within 24 hours under appropriate conditions (15-17^{0}C) at the CLC Assisted Reproduction Laboratory, USAMVB Timisoara. Each semen sample was divided in 3 groups: control group (M group), vitamin C group (C group) and rosmarinic acid group (RA group). In control group was no antioxidant, in group C we added 0.5 mM/L vitamin C and in group RA we added 105 µM/L rosmarinic acid. Totally we analyzed 24 samples.

The concentrations was chosen based on literature data regarding their use as antioxidants for animal semen (Olaciregui et al., 2017; Varo et al., 2014; Fanaei et al., 2014)

After lyophilization, the lyophilized sperm (on average 0.05625g/sample) was reconstituted in 500µl of DPBS (D-8662, Sigma Aldrich), then we did smears that were stained with SPERMAC dye (Minitube). SPERMAC is a diagnostic kit used to perform the morphological examination of the sperm. The aim of sperm staining is to differentiate normal sperm from abnormal sperm and to analyze the sperm acrosome integrity. In average we counted 194.55spermatozoa/sample. The percentage of tertiary anomaly, especially spermatozoa with broken tail, head detached was 11.38%, mainly this defect can be caused during reconstituition of lyophilizated spermatozoa.

Values are expressed as means ± SX. Data were analyzed by ANOVA test in order to detect statistically differences ($p<0.05$).

Results and discussions

The results obtained are presented in table 1, from which we can observe that are no significant differences between control group and experimental group (C and RA group) regarding acrosome integrity, being with 5.32% ($p\leq0.262$) and with 0.53% ($p\leq0.964$) higher in C group, respectively RA group. There were not differences between boar breeds regarding acrosome integrity. Some images with normal spermatozoa, acrosome reacted spermatozoa and spermatozoa with tertiary defects are presented in figure 1.

Table 1. Acrosome integrity of boar semen after lyophilization

	Control group (M) ±SE	**Vitamin C group (C) ±SE**	**Rosmarinic acid group (RA) ±SE**
Acrosome integrity	72.67±4.74	77.99±2.77	73.20±7.93
Acrosome defects	12.18±2.74	10.95±2.42	6.90±1.51

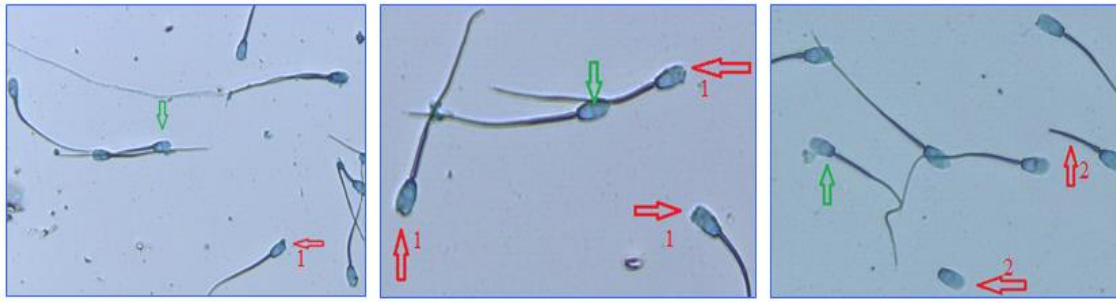

Figure 1. Boar spermatozoa stained with Spermac after rehydration (⇦ normal spermatozoa with intact acrosome, ⇦ 1 acrosome reacted spermatozoa, ⇦ 2 spermatozoa with tertiary defects)(40X)

When is an imbalance between ROS and a biological system's ability to readly detoxify the reactive intermediates or repair the resulting damage, oxidative stress apears (Roychoudhury et al., 2017). Antioxidants used during the freezing process proved beneficial to post-thaw sperm quality (Olaciregui et al., 2017; Varo et al., 2014; Fanaei et al., 2014).

From literature data we know that rosmarinic acid antioxidant effects protects ovine spermatozoa during lyophilization by maintaining the sperm DNA integrity and after reconstitution of the freeze-dryed spermatozoa, they can sustain fertilization and even embryonic development (Olaciregui et al., 2017). Also in boar semen cryopreservation rosmarinic acid it is used as an antioxidant where improves the post-thaw quality of spermatozoa and the ability to fertilize (Malo et al., 2010; Luno et al., 2014; Luno et al., 2015).

As we emphasized in introduction, preservation (biobanking) is done almost exclusively by cryopreservation, with maintenance of the samples in liquid nitrogen. Researchers turn to Nature for alternatives, where drying is a better method than water preservation via freezing (Saragusty and Loi, 2019).

Presently, freeze-drying has been applied in several mammalian species, as mouse, rat, cat, rabbit, horse, pig and monkey with different results on blastocysts % and pregnancy outcome (Patrick et al, 2017), with Wakayama and Yanagimachi (1998) being the first obtaining offspring derived from intracytoplasmic injection of freeze-dried mouse sperm.

Freeze-drying is based on the principle where frozen cells are dried by the process of sublimation of ice under vacuum conditions (Patrick et al, 2017). Spermatozoa are the most commonly dried cell type, due to their small size with little water content and highly condensed chromatin (Saragusty and Loi, 2019). Although freeze-dried spermatozoa are dead and without motility with the aid of ICSI (intracytoplasmatic sperm injection) technique, they could achive fertilization, with the main condition: no DNA damage (Patrick et al, 2017). Usualy spermatozoa DNA damage is repaired during epididymal transit, before ejaculation and is terminated as transcription and translation stop postspermatogenesis. Oocytes and early embryos can repair sperm DNA damage. (Patrick et al, 2017).DNA injures during freeze-drying and during storage with inadequate protection could be due to activation of endogenous nucleases, oxidative stresses (Shahabna et al., 2016). In order to protect especially spermatozoa nuclei, researchers are using different solution such as EGTA, EDTA, rozmarinic acid with promissing results (Shahabna et al., 2016; Olaciregui et al., 2017). Studies on human spermatozoa revealed that freeze-dried human spermatozoa could have intact structural components, but severe damage on membrane integrity and ultrastructure of sperm (Zhu et al., 2016).

Other studies reported that freeze-dried spermatozoa can induce blastocyst development following ICSI at relatively high proportion, meaning that this preservation method could be an alternative to cryopreservation (Anzalone et al., 2018; Olaciregui et al., 2017), a method that needs further development in order to be optimal.

Our preliminary results regarding acrosome integrity of lyophilized spermatozoa in medium with antioxidants needs to be continue with studies on spermatozoa DNA integrity after freeze-dry procedure, because during ICSI technique done with freeze-dried spermatozoa, DNA integrity is more important than membrane integrity.

Conclusions

- Freeze-drying method could represent an alternative to classic preservation of boar semen
- vitamin C and rosmarinic acid added on boar semen could protect spermatozoa integrity

Aknowledgments

The research was carried in the IVF (In Vitro Fertilization) laboratory from the Horia Cernescu Research Unit equipped through POSCCE 2669 program

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Antioxidant effect of vitamin C on porcine oocytes maturated *in vitro*

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Abstract
Cells are vulnerable to oxidative stress during in vitro culture systems. The objective of the present study was to determine the effect of vitamin C addition in in vitro culture media on porcine oocytes maturation rate based on morphological changes. Porcine COC's were matured according to their morphological class (class I, II and III) in two groups: control (M) and supplemented with vitamin C (0.5 mM, C) in TCM 199 HEPES (M2520) modification media with hormones (0.88UI/ml FSH, F8174) at 38.5^{0}C in 5% CO_2 humidified air atmosphere for 44h. The rates of oocytes with cumulus cells expansion were higher with addition of vitamin C as compared to control group, with 7.83% (C1), 70.59% (C2) and 6.04% (C3). It could be concluded from this preliminary study that addition of vitamin C in in vitro maturation medium has a beneficial effect on porcine oocytes especially in C2 group.
***Key words**: antioxidants, porcine oocyte, in vitro maturation*

Introduction

Assisted reproduction technique (ART) as *in vitro* fertilization (IVF) it is used in porcine reproduction research mainly for study the mammalian embryogenesis, xenotransplantation, transgenesis and genome editing (Abeydeera, 2002).

The large scale implementation of porcine IVF techniques is still poor due to polyspermy and low embryo development, even if there are studies with promissing results (Batista et al., 2016; Romar et al., 2016).

Successful ART is influenced by many factors, among which reactive oxygen species (ROS) has a significant role. Sources of ROS during ART procedures could be either endogenously (immature spermatozoa, leukocytes, oocyte, cumulus mass cells, follicular fluid, embryos) or exogenous environmental factors (visible light, culture media, pH, temperature, oxygen concentration, centrifugation, cryopreservation)(Agarwal et al., 2014).

Studies indicates that supplementing maturation media with different antioxidants such as cysteine, cystamine, β-mercaptoethanol (Mahanta et al., 2016; Mircu et al., 2015, Beheshti et al., 2011, Sadeesh et al., 2014), vitamin C (Sovernigo et al., 2017; Comizzoli et al. 2003), rosmarinic acid (Marc et al., 2017, Malo et al., 2010; Luno et al., 2014; Luno et al., 2015; Olaciregui et al., 2017) or plant antioxidants – flavonoids (Kang et al., 2016, Mbemya et al., 2017) can improve oocytes maturation based on morphological changes, nuclear changes and on gene expression.

Ascorbic acid, a powerful antioxidant, it is used in porcine somatic cell nuclear transfer to enhance the porcine embryos development, results based on increased transcript levels of reprogramming genes, such as Pou5fl, Sox, Klf (Zhao et al., 2017). In feline improves COC maturation rate, even if the COC are retrieved during non-breeding season (Comizzoli et al. 2003), in buffalo also improves the development competence of oocytes (I-Nabi et al., 2017). Tatemoto et al. (2001) suggested that a critical intracellular concentration of ascorbic acid would be necessary for normal cytoplasmic maturation of oocytes.

The purpose of this present research was to evaluate the effect of vitamin C in 0.5mM concentration on porcine oocytes maturation based on morphological changes.

Materials and methods

Sow ovaries (n=46) were collected from local slaughterhouse and transported to the laboratory in containers containing 0.9% NaCl solution supplemented with antibiotics (Pen/Strep, 17-602F, Lonza), at 37^0C within two hours. Handling medium for COC (cumulus -oocytes-complexes) was Dulbecco-PBS (D-8662) supplemented with 100 µl Pen/Strep; 3.6 mg sodium piruvate, 30 mg BSA (A9647, Sigma-Aldrich), 100 mg glucose (G7021, Sigma-Aldrich). COCs were aspirated by puncture procedure from medium to large follicles with 18G needle attached to a 5 ml syringe.

Classification of COCs based on morphological aspects was done under stereomicroscope (Stemi 2000-C, ZEISS) with hot plate (33.4^0C): *Ist class* - CI (COCs with cumulus compact and unexpanded, with full or at least 5 layers of cumulus cells, cytoplasm clearly seen, dense and homogenous, *IInd class* – CII (COCs with cumulus compact, thick, 2-4 layers of cumulus cells, covering all of zona pellucida, cytoplasm dense, with uniform granulation) and *III^{d} class* - CIII (oocytes partially denuded of cumulus cells, or with 1-2 complete layers of cumulus cells and/or with irregular shrunken cytoplasm).

The maturation culture medium, prepared in our: TCM 199 HEPES modification media, (M2520) with 10% ECS and 0.88UI/ml FSH (F8174, Sigma-Aldrich) - *group M* (control), in experimental group we added vitamin C (0.5 mM)- *group C*. Pools of 15-20 COCs were maturated in 400µl media in 4 well dishes (Nunc, Germany) covered with mineral oil at 38.5^0C in 5% CO_2 humidified air atmosphere for 44h. After 44h of culture, all COC were examined for maturation, signs as expansion and mucification of cumulus cells were observed. The COC's were maturated according to there their morphological class (M1, M2, M3, C1, C2, C3).

Results and discussions

The results of supplementation of *in vitro* media with ascorbic acid on porcine oocytes morphological aspects are presented in figure 1-3.

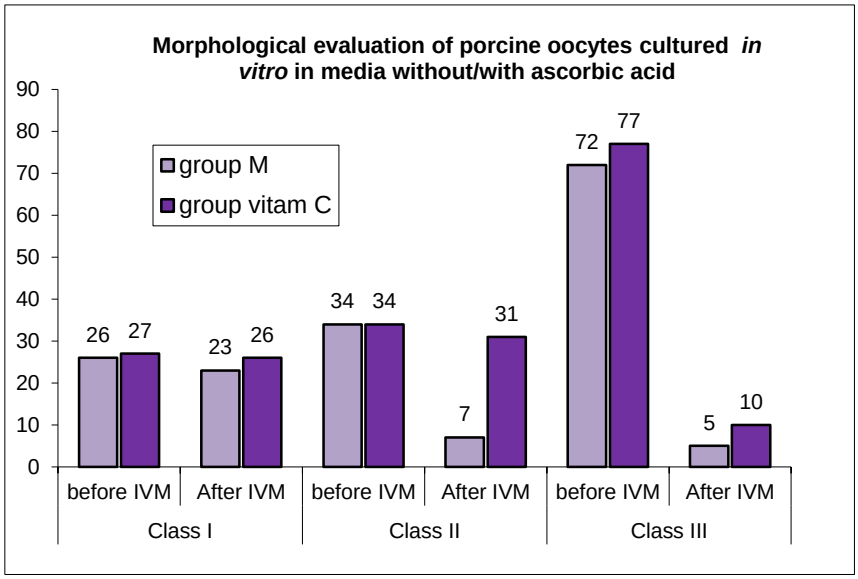

Figure 1. Morphological evaluation of bovine COC's before and after IVM

After *in vitro* maturation of porcine oocytes in the medium without antioxidant (M group) we noticed at the morphological assessment that 88.46% of class I COCs (M1), 20.58% of class COC II (M2) and 6.94% of class III COCs (M3) were matured. In group C, supplemented with vitamin C (C group), 96.29% of class I COCs (C1) were matured after 44 hours, 91.17% of class II (C2) and 12.98% of class III (C3).

Comparing the groups, relative to the number of COC's matured, a increase in their maturation sign is observed, with 7.83% % (C1), 70.59% (C2) and 6.04% (C3), respectively.

From these results we can observe that the oocyte class is associated with their capacity to mature *in vitro.* In order to improve development competence of oocytes it is not sufficient to have a proper culture media, but also to use high quality oocytes. These results are sustained also by BAX/BCL2 gene expression (unpublished data). Similar results we obtained in bovine oocytes cultured in medium supplemented with rozmarinic acid (Marc et al., 2017).

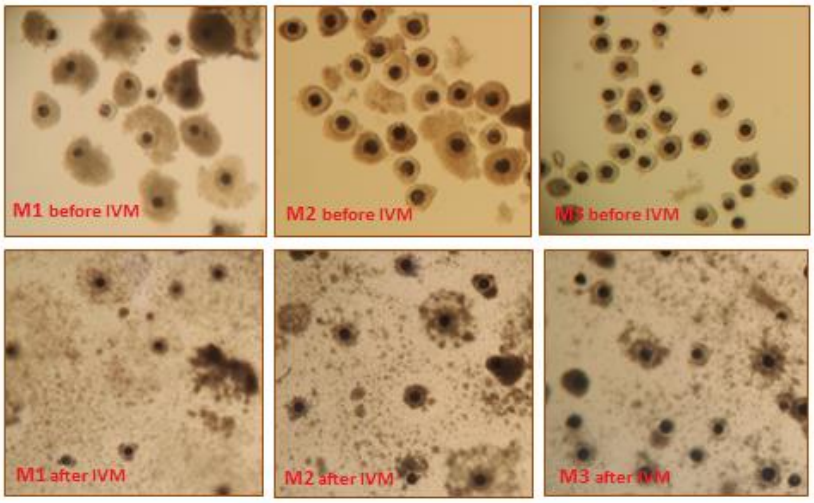

Figure 2. Aspects of porcine oocytes from control group (M)classified according to their morphological aspects before and after IVM (3.2X)

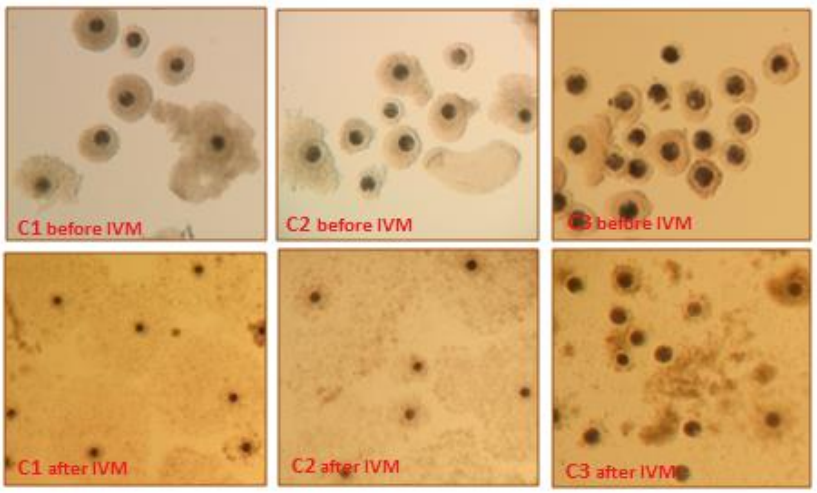

Figure 3. Aspects of porcine oocytes from vitamin C group (C) classified according to their morphological aspects before and after IVM (3.2X)

Vitamin C is no longer only an enzymatic cofactor, an antioxidant, an extracellular promoter of matrix formation by stabilizing collagen structure, but also it is studied for its potential to modulate gene expression (Belin et al., 2009; Belin et al., 2010, Ivanyuk et al., 2015, Duran et al., 2019). Belin et al. (2009) demonstrated that vitamin C stops proliferation *in vivo* and *in vitro* by down-regulation of 30 genes among these 12 belonging to families such as tRNA synthetases and translation initiation factors, involved in cell division. Other researchers demonstrated the activity of vitamin C as a signaling molecule in development and differentiation. For example Duran et al.(2019) demonstrated that ascorbic acid influence the mechanisms of fish pacu (*Piaractus mesopotamicus*) myogenesis by increasing *in vitro* the expression of *myog* and *mtor* - molecular marker of skeletal muscle myogenesis and protein synthesis respectively; probable through its role as an antioxidant agent, that decrease ROS levels and consequently induce upregulation of *igf* – molecular marker of protein synthesis. Vitamin C can enhance cardiac differentiation if applied on day 0-2 in murine CGR8 embryonic stem cell line with promising results in production of sufficient cardiomyocytes for research and potential application in regenerative medicine (Ivanyuk et al., 2015).

The reviewed literature suggest also good results of vitamin C on different animal species oocytes used in different concentration (0-25-50-100 µM)(Kere et al., 2012; I-Nabi et al., 2017), 100-250-500-750 µM (Tatemoto et al. (2001), but not in large concentration that can reduce oocyte development by inducing apoptosis. Kere et al. (2012) obtained good results during *in vitro* maturation of porcine oocytes with relatevely low concentration of vitamic C (50µg/ml), others with 50 µM in buffalo (I-Nabi, 2017) or 0.5mM in cats (Comizzoli, 2003). Our results also indicates that addition of vitamin C in 0.5mM concentration improves porcine oocytes maturation, results sustained also by BAX/BCL2 gene expression were BCL2 gene had higher levels in Ist class COC's from C1 group (unpublished data).

Our preliminary results suggests that antioxidant properties of vitamin C in 0.5mM concentration is effective on porcine oocytes *in vitro* maturation.

Conclusions

1. Supplementation of the porcine oocyte culture media with vitamin C generates a higher proportion of porcine oocytes matured *in vitro* based on morphological evaluation.
2. Quality of the COC used for *in vitro* techniques has an important role in the success of this approach.

Aknowledgments

The research was carried in the IVF (In Vitro Fertilization) laboratory from the Horia Cernescu Research Unit equipped through POSCCE 2669 program

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Morpho-topographic researches on cervico-cephalic lymphcentres at ferret

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Abstract
The muscular lymph centers from the cervico-cephalic region emerge with the muscles and in specialty literature there is little information regarding muscles at the ferret, hence why in this study we have also referred to this morphological segment. In the cephalic region, the skin muscles existing at the ferret do not present significant differences to those existing in domestic carnivores, but the masticatory muscles have some peculiarities. The temporal muscle, masseter muscle and digastric muscle are highly developed. On the surface of the masseter muscle is identified a lymphonodular group covered by the skin muscle and tegument. Due to topography, it was considered that this lymphonodular group represents the parotid lymph center, although some authors do not even mention the presence of this lymph center in the ferret. At the ventral edge of the parotid gland and at the mandibular cranio-dorsal edge there is a mandibular lymph center formed by two lymphonodular structures. The retropharyngeal lymph center is represented by a single lymph node. In the cervical region the superficial cervical lymph center was identified, formed by a single structure. Although in speciality literature the presence of the profound cervical lymph center is not signalled, the structure was identified in two from the total of four corpses used.
Keywords*: dissection, dye ink, ferret, lymph center.*

Introduction

The ferret is an animal raised for its fur, as a pet, but also as a laboratory animal. Taxonomically, the ferret belongs to the *Mammalia* class, *Carnivora* order (*Fissipeda*), *Mustelidae* family, *Putorius* species. Although they are carnivores, they exhibit anatomical particularities related to their lifestyle witch make them quite different form the other carnivores (Valentina Hrițcu & al., 2000; Coțofan V., 1975; Predoi & al., 2001).

Due to the scarce information in the literature concerning the morpho-topography of the lymph centres from the cervico-cephalic region in ferrets, our study aimed to describe the particularities of these structures in connection to the adjacent muscles (R. BARONE, 2012; G. PREDOI & al., 2002).

The study of the lymphcentres in correlation with their morpho-topography is useful for the veterinarians who can make use of the results of such investigations during their practice.

This paper is intended to be an addition to the data already present in the literature.

Materials and methods

The study material was represented by four adult ferret bodies, of different ages, without any pathological changes that would indicate the existence of any disease. The chosen method for identifying the lymphatic structures was the injection of a coloured substance – China ink dye 40%. The dye was filtered through filter paper. The filtrate was diluted 1/1 with physiological saline. Stratigraphic and regional dissections were performed up to the limit of visibility, and more detailed investigations were done by means of a SMZ-Nikon stereomicroscope. After removing the skin, the muscles, arteries and veins were dissected, revealing the lymph centres and the lymphatic vessels, preserving their relations with the adjacent formations.

The homologation of the formations was made according to Nomina Anatomica Veterinaria 2017.

Results and discussions

Compared to the size of the animal, the lymph centres have a considerable size and are able to drain lymph from an extended area.

The parotid gland has a triangular appearance, with a reduced preauricular portion and a developed retroauricular one.

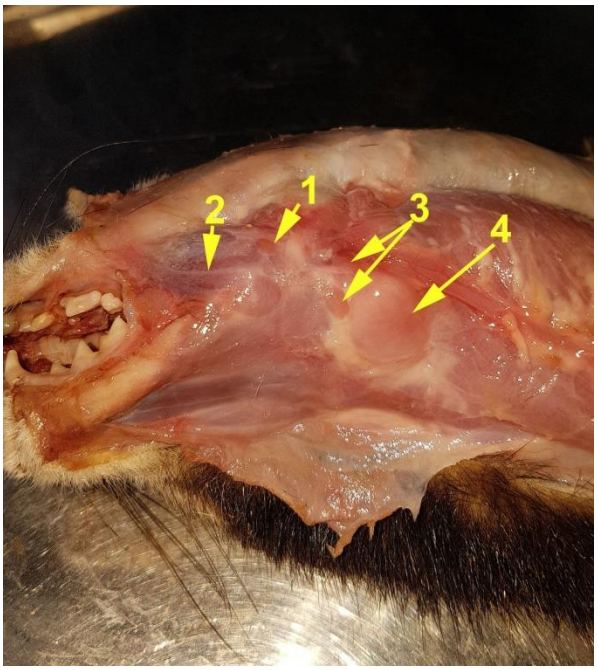

Fig. 1 Parotid and mandibular lymph centers
1-parotid lymph nodes; 2- masseter muscle; 3- mandibular lymph nodes; 4- mandibular gland.

Cranial from the ventral angle of the parotid gland, on the masseter muscle,a lymphnodular group was identified, measuring about 2 mm in diameter, and covered by the subcutaneous muscle and by the skin. Topographically, we consider that this lymph centeris the parotid lymph centre, although the literature does not mention its existence. The parotid gland duct passes below this lymphatic structure and then passes over the upper third of the lateral face of the masseter muscle.

At the ventral edge of the parotid gland and the dorso-cranial edge of the mandibular gland, two lymph nodular structures that form the mandibular lymph centre were identified.

The appearance of the mandibular lymph centre is circular with a diameter of 4 mm. A second mandibular lymph node was found cranially to the first, both superficially, covered only by the subcutaneous muscle.(Fig.1).

The lymph comes from the lips, cheeks, eyelids, salivary glands, masseter muscles, and parotid gland. The efferent vessels are tributary to the retropharyngeal lymph node.

In all studied corpses, the retropharyngeal lymph centrewasrepresented by a single lymph node, placed along the anterior third of the internal jugular vein, caudal to the larynx and on the

lateral side of the pharynx. The afferent vessels drain lymph from the tongue, oral mucosa and from the cervical muscles. The efferent vessels are drained in the tracheal trunk,near the confluence angle between the jugular veinand the axillary vein.(Fig.2).

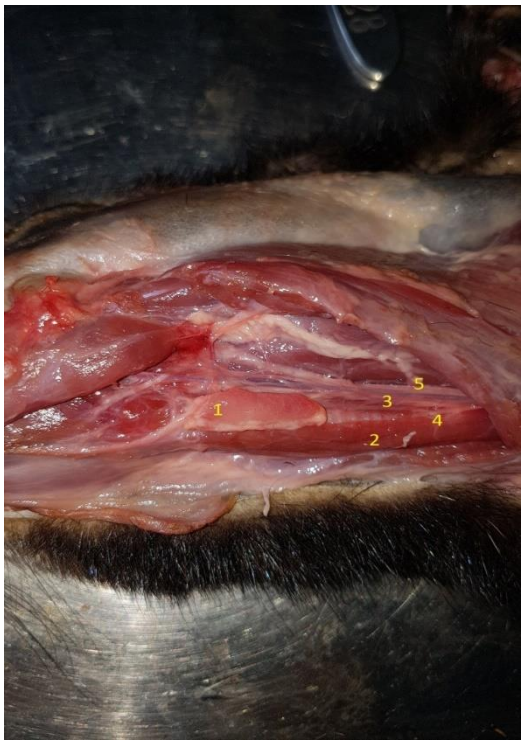

Fig. 2 Retropharyngeal lymph center
1- medial retropharyngeal lymph node; 2- sterno-hyoid and sterno-thyroid muscles; 3- internal jugular vein; 4- common carotid artery; 5- vagosympathetic trunk

In the cervical region, the cervical lymph centre is represented by a round shaped lymph node of about 4mm in diameter, disposed on the medial face of the omotransverse muscle. The afferent vessels come from the thoracic limb, from the cervical and the pectoral region. The afferent vessels are drained in the tracheal trunk.(Fig. 3).

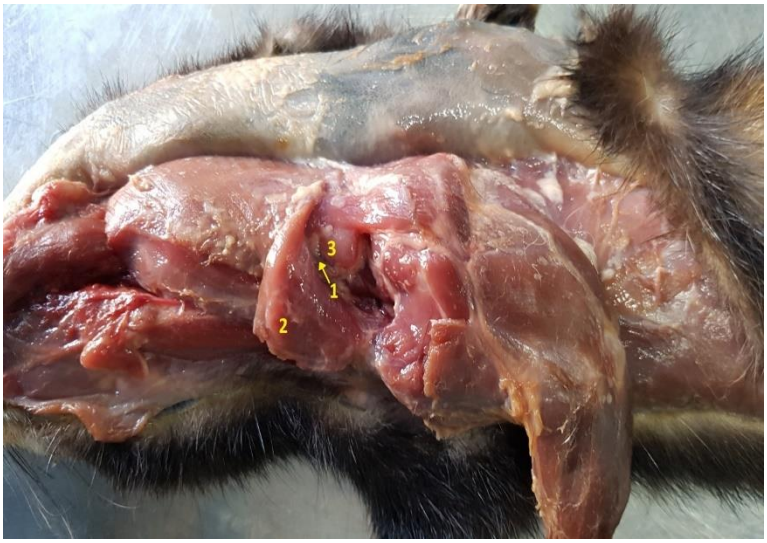

Fig. 3 Cervical lymph centre
1- deep cervical lymph node; 2-sterno-occipital muscle; 3 - superficial cervical lymph node.

The existence of the deep cervical lymph centre is not describedin the literature, but we identified it in two of the four corpses.

The deep cervical lymph centre was found on the medial face of the sternooccipital muscle. This lymph centre is represented by a rounded lymph node of about 2.5 mm. The associated vessels come from the trachea, oesophagus, and the vessels of the tracheal trunk.

Conclusions

Unlike carnivores, the lymph centresare characterized by appreciable dimensions in relation to the size and weight of the animal.

The particular morpho-topography of these lymph centresallows them to drain the lymph from a more extended area.

In the cephalic region, we identified a lymphonodular group on the surface of the masseter muscle, under the subcutaneous muscle, and covered by the skin.

Although in literature does not mention the presence of the parotid lymph nodesin the ferrets, the lymphonodular group localised on the surface of the masseter muscle may be considered as belonging to the parotid lymph centre.

Some authors do not indicate the existence of other lymph centres in the cervical region, aside from the cervical superficial lymph centre.

We have identified a single lymph node located on the medial face of the sternooccipital muscle, and topographycally it can be considered to represent the deep cervical lymph centre.

The parotid gland has a triangular aspect, with a reduced preauricular portion and another, more developed retroauricularone.

The mandibular gland is larger than the parotid gland and is located in a ventro-caudal position compared to latter.

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Morphotopographic particularities of some muscles from the trunk region of the coypu

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Abstract
The muscles from the trunk region are covered by the cutaneous trunk muscle which has in its structure two layers, a superficial and a profound one. The skin muscle of the trunk has a unitary structure, flattened appearance, inserted on the shoulder and on the base of the tail. The muscular particularitis of the trunk region in coypu consist in their appearance and development. At the level of the trapezius muscle, there is a large interstitium that separates its cervical portion from the thoracic portion. The thoracic ventral serrated muscle, the intercostal muscles and some muscles that are not described in other domestic mammals, namely, the lower ribs muscles and the thoracic square muscle, were also dissected. Among the pectoral muscles of the deep layer, the subclavicular muscle was also identified in coypu. The abdominal, dorsal and ventral muscles were also dissected. The superficial pectoral muscle showed a flattened and triangular aspect.During dissection, it was found that the superficial inguinal ring presents the muscular cruses which are generated by muscular structures.
Key words: *dissection, coypu, muscle, trunk.*

Introduction

The coypu is a semi-aquatic mammal, which lives in natural conditions over an extended geographical area. The individuals are grouped in colonies, in a vegetation- and water-rich habitat. It adapts easily to new environmental conditions, which explains the possibility of exploiting this animal in captivity.

This rodent belongs to the *Rodentia* order, *Mammalia* class, *Myocastoridae* family, *Myocastor* genus, *Coypus* species (V. Coțofan & al., 1994; H.E.Köning & al., 2004).

In captivity, the coypu is grown for fur production, but also for meat consumption. It was deemed necessary to complete the existing literature data with this study (R. Barone, 1966; H. E. Köning et al., 2004; G. Predoi ET AL., 2001).

The trunk muscles in this species have a series of particularities which do not occur in other rodents, as well as specific muscles that are absent in other domestic mammals (G. Predoi et.al., 2011).

Materials and methods

Four bodies of adult coypu without morphopathologic changes were used. Stratigraphic and regional dissection methods were realised.

The dissection was performed by maintaining the physiological relations of the adjacent structures, the vasculonervous and lymphatic formations. In the first phase of the dissection, the skin was incised; each muscle was identified following its insertions, aspect, and relationships with the surrounding formations.

The cutaneous trunk muscle was dissected by performing a vertical incision of the brachial triceps muscle. The incisions were made parallel with the muscular fibres, and the conjunctive and fatty tissues were removed. The connecting musculature between the anterior limb and trunk was dissected in order to detach the limb.

The thoracic and cervical portions of the rhomboid muscle, which are fused, were separated. Then, this muscle was sectioned at the cervical angle of the shoulder. The trapezius muscle was sectioned from its shoulder insertions and was folded dorsally. The omotransverse muscle was cut, from its shoulder insertion. The cleidobrachial muscle was dissected and the transverse scapulohumeral joint was cut. The superficial pectoral muscle was dissected and folded ventrally. The subclavicular muscle was sectioned in its middle third.

The intercostal muscles were cut longitudinally for showing their internal and external parts. As concerns the latissimus dorsi muscle, an incision was made to separatethe fleshy portion from the aponeurosis, which was then separated from the aponeurosis of the thoracic muscles.

Theserrated dorsal muscle was cutin order toreveal its insertions and interlacing with the gluteal fascia. The muscular portion of theserrated dorsal muscle was rendered evident by removing the connective tissue along the dorso cranial curvatures of the II-XII ribs. The cranial and caudal regions of this muscle intersect on the XII rib. For the dissection of the multifidus and levatores costarum muscles, two profound incisions in the large dorso ventral muscle were made. The muscular flap was suspended on the supraspinatus muscle.

The tendons and the muscles from the fourth layer of the trunk were revealed. Laparotomy was performed for the dissection of the ventrolateral abdominal muscles flank.

Results and discussions

In coypu, the superficial pectoral muscle has a flattened, triangular appearance, having its origin on the ventral face of the sternum, and the insertion on the arm and forearm fasciae (Fig. 1).

In the profound layer of the pectoral muscles, the pectoral cleidoscapular muscle, the subclavicular muscle and the pectoral ascending musclewere identified. The pectoral cleidoscapular muscle has its origin on the III-VI sternebrae and its insertion on the clavicle.The subclavicular muscle is inserted on the first rib and the clavicle. The ascending pectoral muscle has its insertion on the small tubercle of the humerus, medial scapular fascia, and large tubercle of the humerus. Caudally, it is inserted on the xiphoid appendix, on the abdominal tunic up to the umbilical scar.

The trapezius muscle has a cervical portion and a large interstitium, which separates it from the thoracic portion (Fig. 2).

The muscular portion of the trapezius muscle is inserted on the scapular spine,and the caudal aponeurosis is inserted on the spinous T13-L3 processes. The latissimus dorsi muscle presents a large muscular portion and caudally a large aponeurosis. Its insertions are on the round tubercle of the humerus, on the spinous processes T5-T11, and the aponeurosis is inserted on the rest of the thoracal, lumbar spinous processes and on the iliac angle.

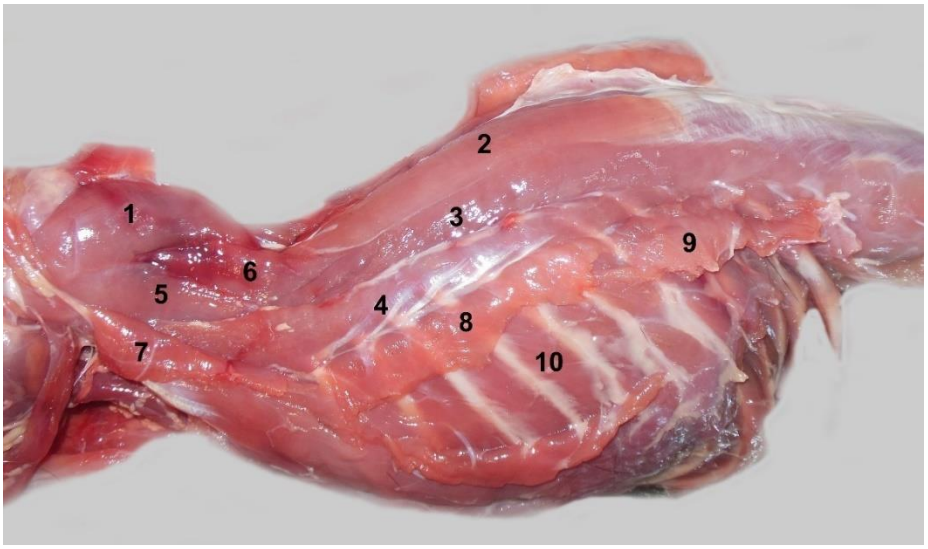

Fig. 1. Trunk muscles from the 3rd layer, after sectioning the thoracic dorsal serrated muscle in coypu (original)
1-splenius muscle; 2-thoracic spinal muscles; 3-latissimus dorsi muscle; 4-thoracic iliocostal muscle; 5-longissimus atlantismuscle; 6-biventer cervicis muscle; 7- ventral serrated cervical muscle, sectioned; 8 – ventral thoracic serrated muscle, sectioned; 9 – costal insertion of the caudal portion of the dorsal serrated thoracic muscle, sectioned.

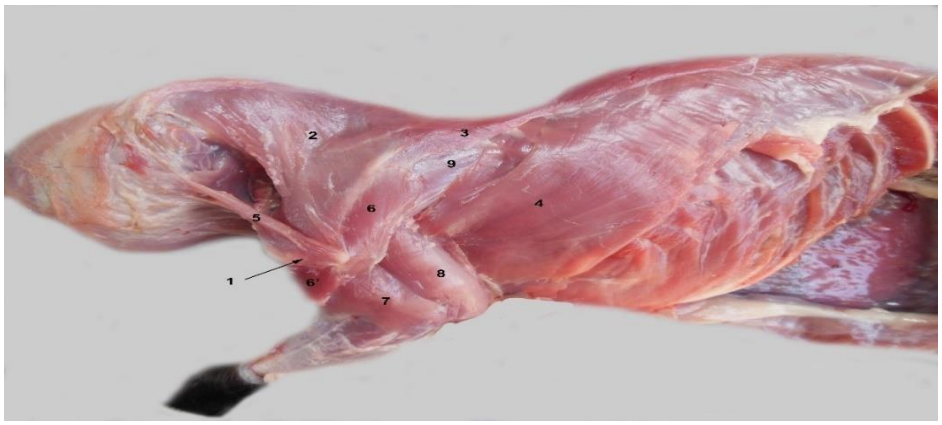

Fig. 2. The superficial trunk muscles in coypu (original)
1-clavicle; 2-cervical trapezius muscle; 3-thoracic trapezius muscle; 4-the latissimus dorsi dorsal muscle; 5-omotransverse muscle; 6-the scapular portion of the deltoid muscle; 6'-acromial portion of the deltoid muscle; 7-lateral portion of the triceps muscle; 8-long portion of the triceps muscle; 9-infraspinatus muscle partially covered by the scapular portion of the deltoid muscle

The thoracic rhomboid muscle is inserted on the tip of the spinous processes T2-T4 and on the dorsal edge of the shoulder. It has a triangular shape with the tip oriented ventro-cranially and the basedorso-caudally.

The thoracic dorsal serrated muscle presents cranial portions with insertions on the II-XII ribs and with a large caudal aponeurosis on the T2-T13spinous processes. It is noticed that the aponeurosis covers the third layer of the trunkmuscles (Fig. 3).

The thoracic ventral serrated muscle has a triangular aspect and is inserted on the lateral costal face of the III-VIII ribs. The intercostal muscles are formed of an external portion with dorsocaudally oriented fibres, and an internal portion with fibres oriented ventrocranially. The great long dorsolumbar muscle has its insertion on the spinous processes of the thoracic and lumbar vertebrae. The iliocostal muscle is inserted on the internal face of the ribs, on the iliac crest.

The multifidus muscles are disposed obliquely between the transverseapophysis of one vertebra and the transverse apophysis of the nextvertebra. The levatores costarum muscles are inserted between the transverse apophysis of one vertebra and the anterior edge of the next rib.

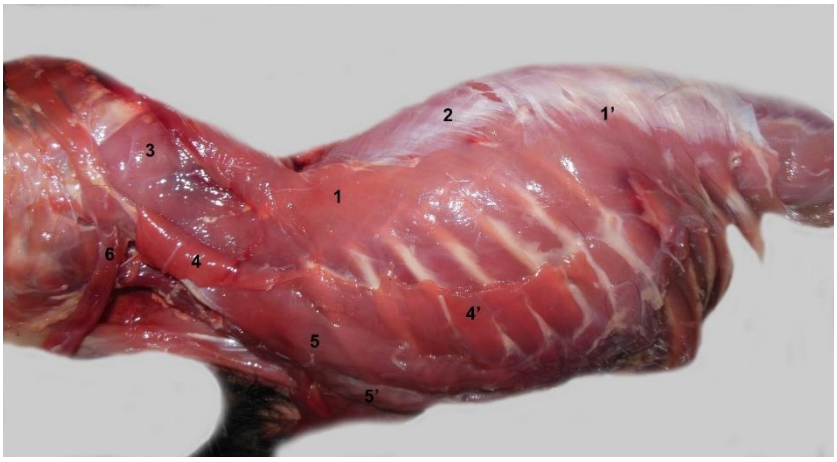

Fig. 3. Trunk muscles after removal of the anterior limb in coypu (original)
1-dorsal thoracicserrated muscle – cranial portion; 1'- dorsal thoracic muscle, caudal portion; 2 – spinal muscles from the 3rd layer covered by the dorsal thoracicserrated muscle aponeurosis; 3-splenius muscle; 4-ventral cervical serrated muscle – sectioned; 4'-thoracic ventral serrated muscle– sectioned; 5-scalen muscle, dorsal portion; 5'- scalene muscle – medial portion; 6 – omotransverse muscle.

Coypu possesses the following species-specific muscles: the descending muscles of the ribs, placedbetween the body of the thoracic vertebrae and the medial face of the ribs; thesquare thoracicmusclewith insertions between the transverse thoracic processes and proximal extremities of the ribs.

The superficial inguinal ring presents the cranial muscular crus (formed by the external oblique muscle) and the muscular caudal crus, formed by the external oblique muscle and by the straight muscle of the abdomen.

The profound inguinal ring is delimited cranially by the caudal part of the transverse muscle of the abdomen, caudolaterally by the oblique internal muscle and medially by the straight muscle of the abdomen.

Conclusions

The trunk muscles in coypu show particular interest.The latissimus dorsi muscle shows two muscular parts that emerge caudally and continue with a large aponeurosis. This aponeurosis

is inserted on the thoracic spinous processes starting with T13 and on all lumbar spinous processes, including the ilium bone edges.

The iliospinal muscle, the levato rescostarum muscles, and the iliocostal muscle are powerful extensors of the spine.

The specific muscles of the coypu (that do not appear in other species) are represented by the descending muscles of the ribs and the thoracic square muscle.

The superficial inguinal ring presents cranial,medial and caudal commissures that are generated by muscular structures.

The cutaneous trunk muscle has a unitary structure, with flattened appearance, being inserted on the shoulder and on the base of the tail.

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Gross and microscopical aspects in some vascular tumors in dogs (retrospective study 2007-2019)

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Abstract
The study was done on six dogs selected from the cadavers brought for necropsy at the Pathological Anatomy and Forensic Medicine Service, from the Faculty of Veterinary Medicine of Iași, between 2007 and 2019. The dogs had ages that ranged from 7 to 13 years, were of different breeds (3 mixed breeds, 11 Amstaff, 2 Caniche) and during the necropsic exam showed macroscopical changes of the internal organs that suggested the presence of vascular tumors. The formations that we observed were nodular in shape, of different sizes, blackish-red in color, with harder consistency than the host tissues and expressed dark red blood on the cut surface. The location of these tumors differed from one case to another, being either restricted to the liver, or disseminated in the heart (right atrium), spleen, lungs, kidneys, intestine, mesenteries, omentum and central nervous tissue. The histopathological exam showed that these structures had a trabecular pattern, with either blood filled caverns of various sizes, or numerous capillaries, of different calibers, filled or not with red blood cells. The cells that lined these structures had similar characteristics with endothelial cells, being spindle shaped, sometimes with a tendency towards a circular arrangement. The anatomo-pathological diagnostic was that of vascular tumor, with a benign or malignant character, depending on the degree of cellular proliferation and mitotic index.
Key words*: vascular tumor, hemangioma, hemangiosarcoma, dog*

Introduction

Vascular tumors are neoplasias of the blood vessels that usually have endothelial cells as an origin (but also smooth muscle fibers or pericytes).

The benigne forms are called hemangiomas, hemangioendotheliomas or pericytomas (tumor of the vascular wall). The malignant form is called hemangiosarcoma.

These tumors are generally nodular in shape, have different sizes (1 mm-20 cm), with a blackish-red color and various consistencies, depending on the internal structure (fluctuent for the cavernous type and harder for the capillary type). A differential diagnosis based on the macroscopical aspect alone should always include hematomas, red infarctus and hamartomas.

The histological aspect of benign vascular tumors is that of capillary bundles or caverns of various sizes, filled with blood and lined with a monolayer of endothelial spindle cells, usually with consistent morphological characteristics.

The malignant tumors show a much more abundant cellularity, with a reduced differentiation degree of the vascular structures and the cellular components: high variations both in the shape and size of the cell, nucleus and nucleolus and in the nucleo to cytoplasmic ratio. Numerous mitosis may be observed as well as abnormal mitotic figures (Grant, 2016).

In dogs, vascular tumors account for 2-7% of all neoplasias and between 12-21% of the mesenchimal ones (Clifford, 2000; Martins, 2013; Grant, 2016). They can be mono or multicentric, disseminated or metastasied.

The most commonly affected organs are: the spleen, heart (right atrium), liver and skin, with metastases in the lungs, central nervous system, kidneys, serosas, omentum, muscles and bones (Martins, 2013, Meuten, 2017).

Some breeds are predisposed to develope hemangiomas **(**Boxer, Airdale,Scottish terrier) or hemangiosarcomas (German shepherd, Boxer, English Setter, Pointer, Labrador Retriever,

Schnauzers) (Baba, 2002, Martins, 2013). There is also a slightly higher prevalence amongst males (Baba, 2002; Martins, 2013). The dogs affected by these tumors have ages that range from 7 to 13 years and only when genetic factors are involved the disease may present under the age of two years (Meuten, 2017).

Materials and method

The study was done on six dogs selected from the cadavers brought for necropsy at the Anatomic pathology, Forensic medicine and necropsic diagnostic Service of the Faculty of Veterinary Medicine of Iași, between 2007 and 2019. The dogs had ages from 7 to 13 years and were of different breeds (3 mixt breeds, 1 Amstaff, 2 Caniche).

All 6 individuals were submitted to necropsy and tissue samples were taken from the organs that showed macroscopical changes that would suggest the presence of a vascular tumor.

The samples for histopathology were harvested so that they would include portions of both apparently healthy and affected tissue. Fixation was done with a 10% formaldehyde solution for 24-48 hours. The techniques chosen to process the tissue samples were the paraffin embedding method, sectioning at 5 µm and the Masson trichome stain.

Microscopical images were obtained using Leica DM750 (photo camera included), with 10x, 20x, 40x și 100x objectives.

The mitotic index was calculated by counting the mitotic figures observed in 10 randomlly chosen HPF fields (40x), as indicated by the literature (Meuten, 2017).

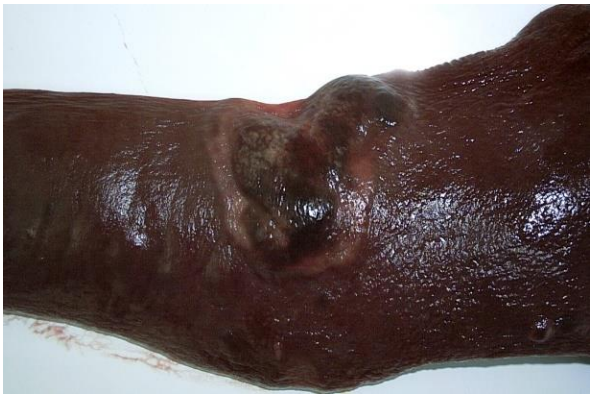
Results and discussions

Macroscopically, in all six dogs we observed nodular formations, with a blackish-red color, with sizes between 2 mm and 5 cm and a consistency different from the host tissue.
The cut surface of these formations was either dark red or bright red, shinny and expressed asphyxic blood.

The organs that were affected were the: spleen (in 4 out of 6 cases), liver (4/6), right atrium (3/6), kidneys (2/6), lungs (3/6), omentum (2/6), diaphragm (1/6), peritoneum (2/6), cerebral hemispheres (1/6).

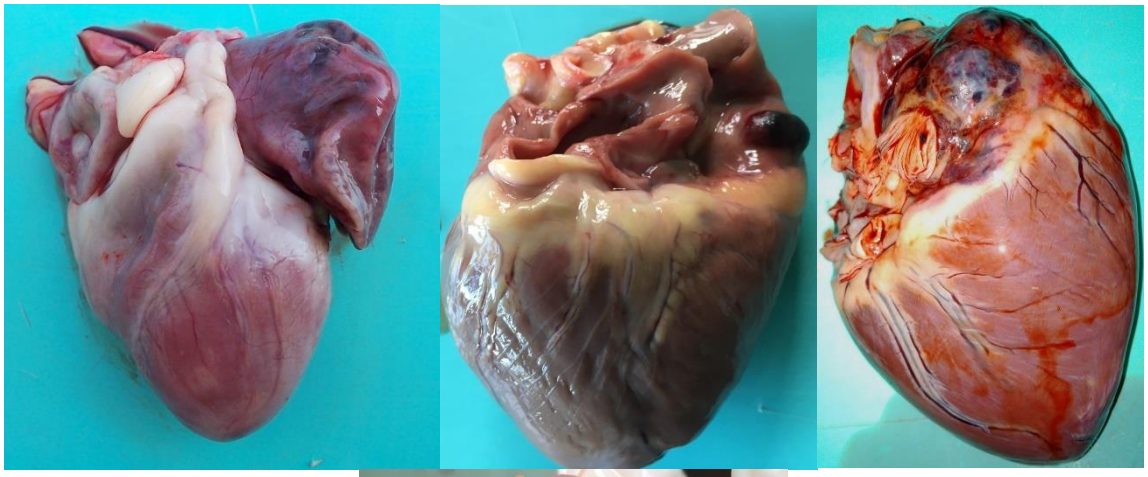
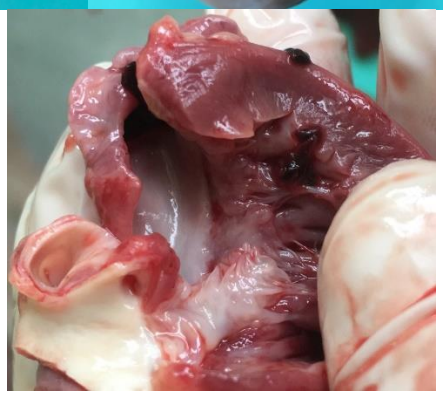
The spleen is considered the most common location for primary vascular tumors (Martins, 2013), around 28-50% of theese being found at this level rather than in other organs. Also, between 1 and 5% of splenic neoplasias are blood vessels tumors (Clifford, 2000, Meuten, 2017). Some studies mention the fact that 25% of splenic vascular tumors have a cardiac correspondent (Clifford, 2000).

From a macroscopical point of view (Picture 1, 2), splenic vascular tumors cannot be distinguished from red infarcts, hematomas or melanomas (the small sized ones).

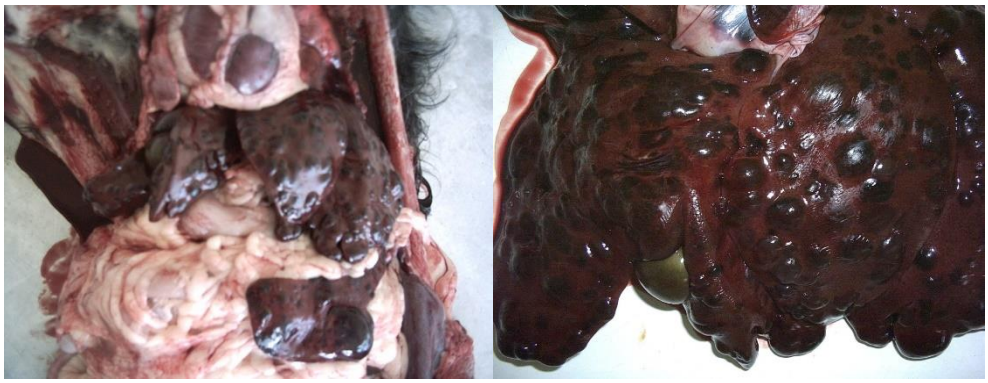
Picture 1, 2 - Gross aspect of vascular tumors in the spleen

The heart (right atrium) amounts for about 3-50% of the vascular tumors in dogs (Clifford, 2000), both primary ones and metastases (Arnesen, 2008). We identified this location in 3 out of 6 cases (Picture 3-6).

Pictures 3-6 - Vascular tumors located in the right atrium of the heart. Gross aspect

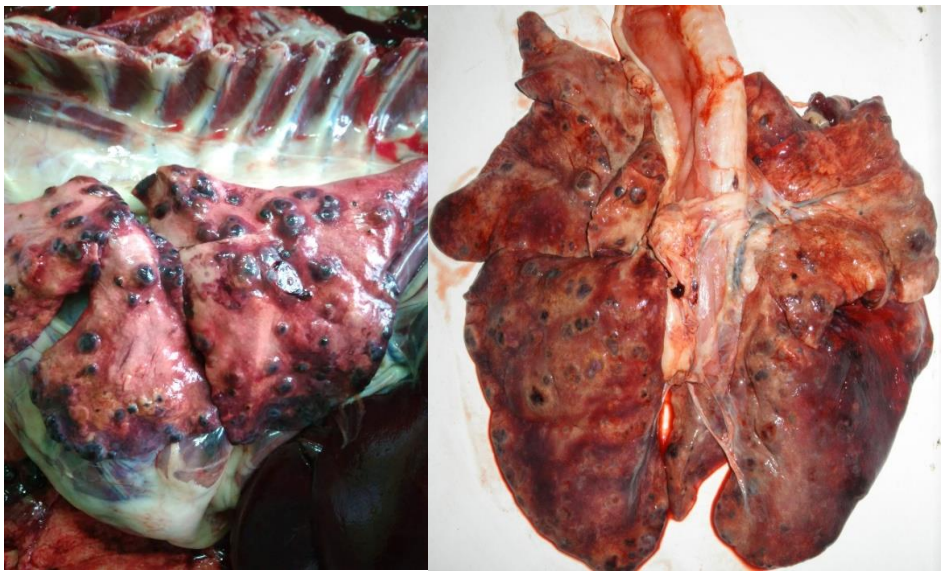
For the liver (Picture 7, 8), the literature mentions that vascular tumors amount for 4-5% of all hepatic tumors (Clifford, 2000). We found hemangiomas and hemangiosarcomas developed in the liver in 4 out of 6 cases.



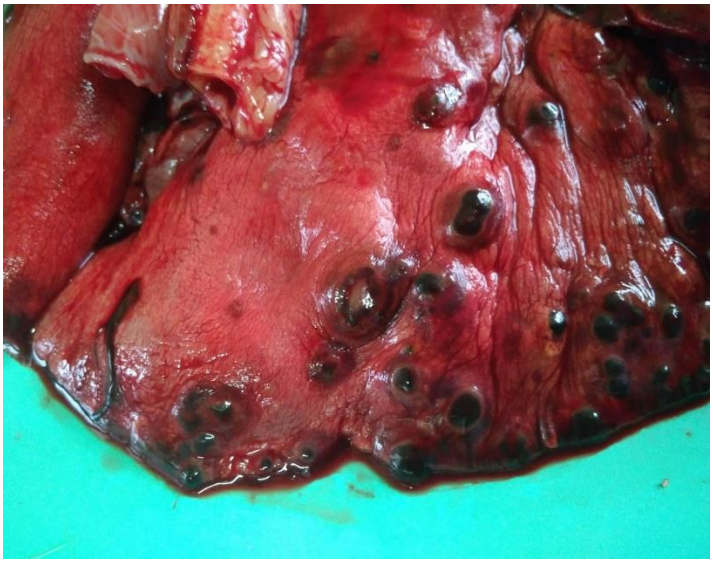
Picture 7, 8 - Multicentric vascular tumors located in the liver

Pulmonary metastases (Pictures 9, 10) were seen in 3 out of 6 cases, this being one of the most frequent locations for secondary neoplasias (Clifford, 2000).

Macroscopically (Picture 9-11), we observed the same dark red, nodular formations, of various sizes, disseminated in the entire organ mass. Histopathologically, some of them had necrotic centers, microhemorrgahes and leucocytic infiltrations. The margins were defined by the presence of compression atelectasis and compensatory emphysema. A possible complication of these tumors when located in the lungs is hemothorax (Arnessen, 2008).

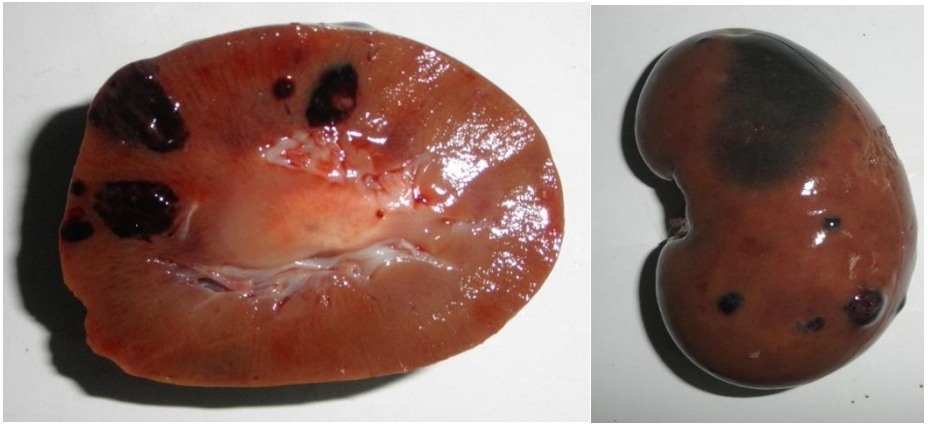


Picture 9, 10 - Metastases of hemangiomas and hemangiosarcomas located in the lungs



Picture 11 - Gross aspect of multiple metastases of vascular tumors developed in the lung

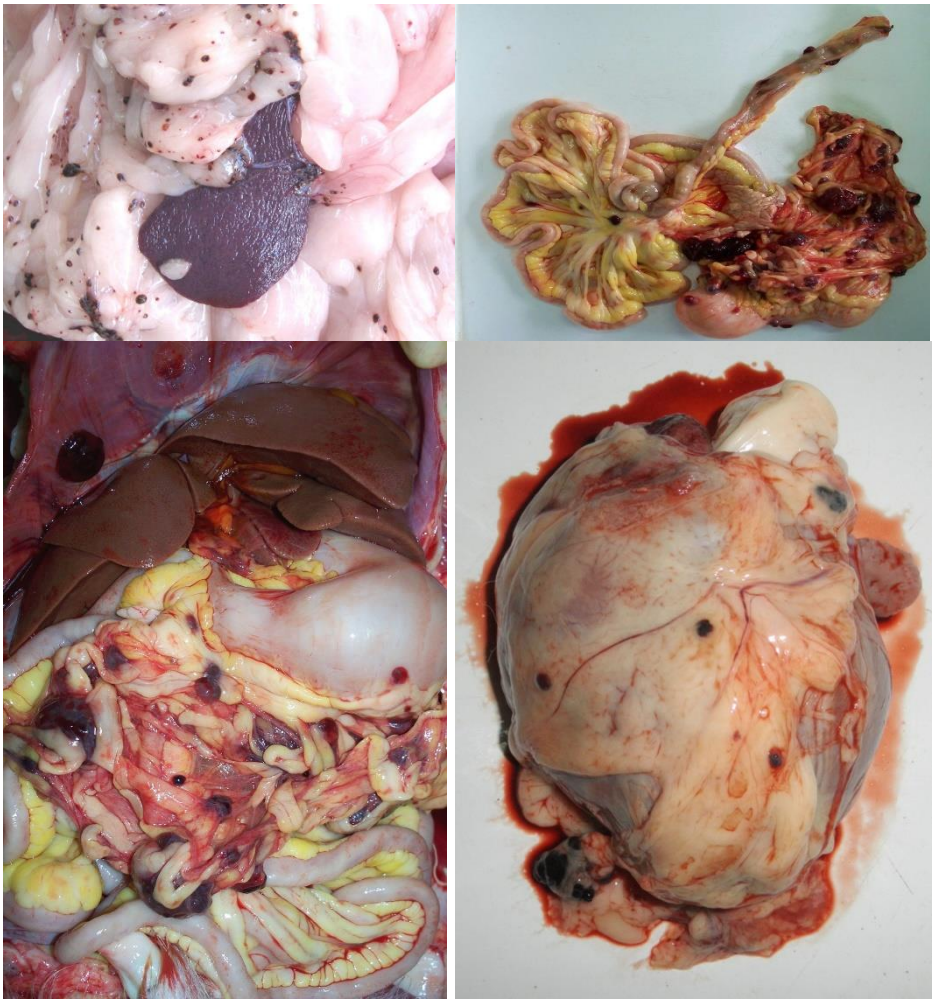
The kidneys were affected in 2 out of 6 cases and the vascular tumors located at this level would either slightly protrude or excavate the surface of the organ. Other authors (Grant, 2016) mention that this is the most frequent localizations of mesenchymal tumors in dogs (due to terminal circulation) and only rarely may it constitute the primary starting point of hemangiosarcomas (Stern, 2014). Vascular tumors may be situated in the cortical or medullar area, sometimes affecting both (Pictures 12 and 13).



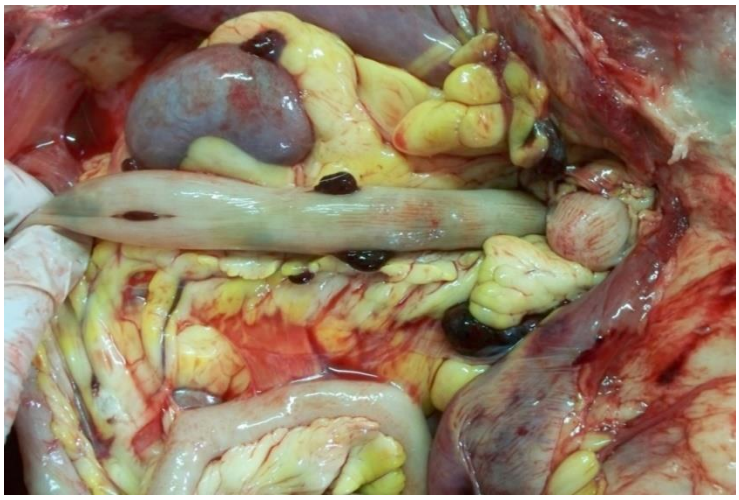
Picture 12, 13 - Vascular tumors located in the kidneys

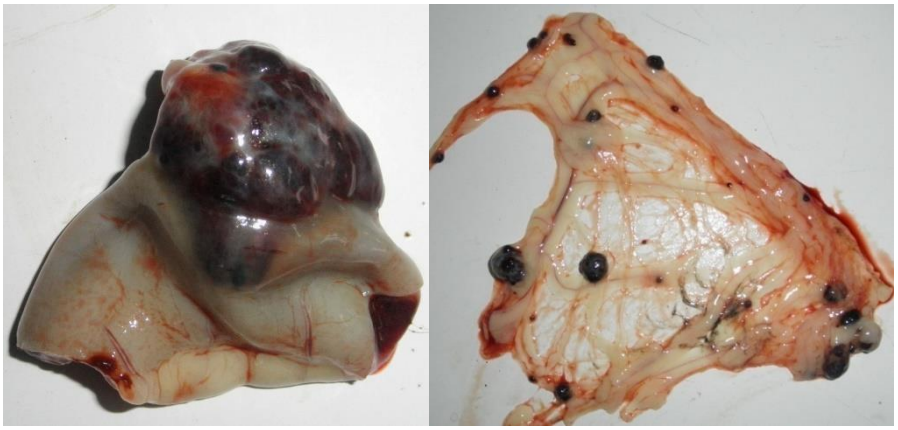
When located on the serosas, hemangiomas and hemangiosarcomas are usually metastases (Yoo, 2016), although there are authors that mention this location as a possible primary site (Massoro, 2012).

The gross aspect in this case is that of milliary multiple formations that sometimes may reach considerable sizes (Pictures 14-20). The rupture of such a tumor may cause hemoperitoneum and death by internal hemorrhage.



Pictures 14-17 - Metastases of hemangiosarcomas located on the peritoneal and pleural serosas

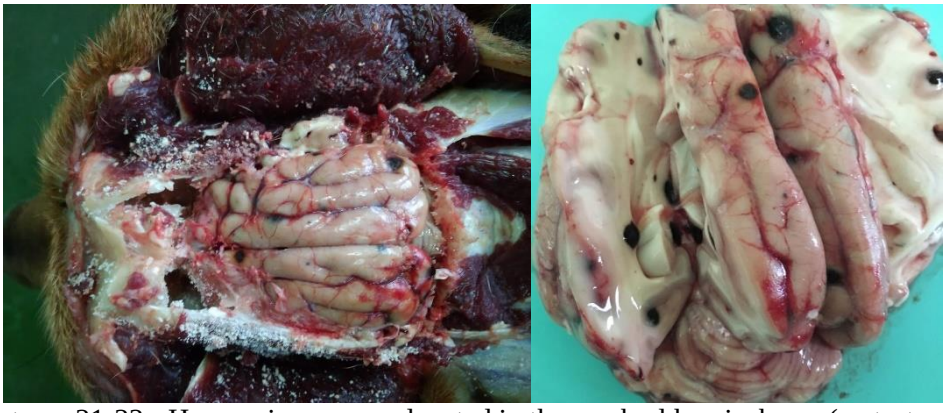




Pictures 18-20 - Metastases of vascular tumors located on the serosas of the organs situated in the peritoneal cavity

Hemangiosarcoamas give metastasis in the central nervous system in roughly 14% of cases, this type of sarcoma being the one that has the most frequent secondary locations situated intracranially (Yoo, 2016), although sometimes even primary ones have been identified at this level (Stern, 2014). The patients that already have pulmonary vascular tumors are more prone to develop metastases in the central nervous system (Stern, 2014).

In our study, we found hemangiosarcomas situated in the cerebrum in only one case (Pictures 21, 22), with the appearence of millimetrical and sub-millimetrical dark red, nodular formations, disseminated in both hemispheres.



Pictures 21-22 - Hemangiosarcomas located in the cerebral hemispheres (metastases)

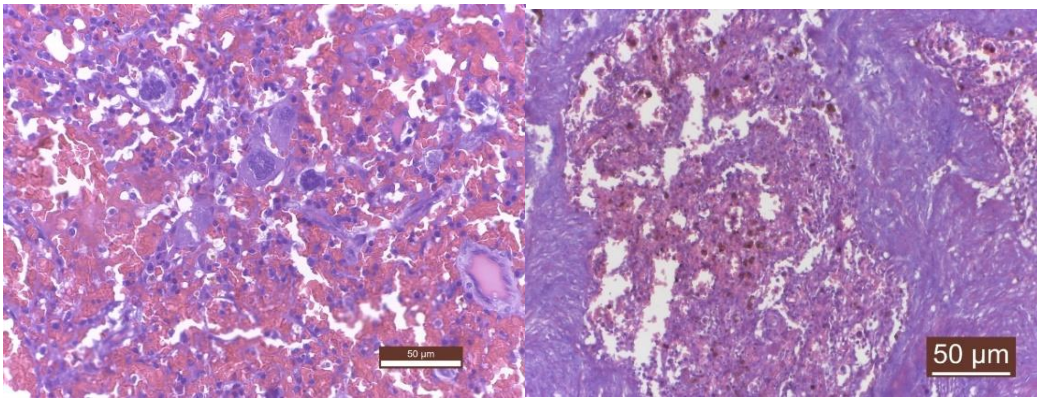
The histopathological exam showed that all the vascular tumors that we described so far from a macroscopical point of view had an internal trabecular structure. The vascular spaces that were formed inside were either caverns filled with blood or capillaries of various calibers, thus profiling the type of the tumor. A mixt pattern was also observed.

The cells that lined these structures were similar to endothelial cells, with a spindle to ovoid shape and a general circular disposition.

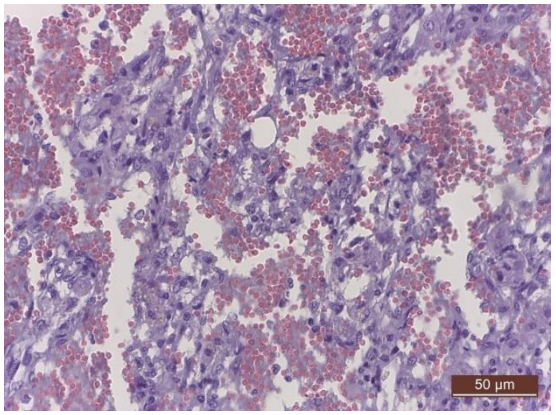
The benign or malignant character of the tumor was defined by evaluating several characteristics such as: the mitotic index, the degree of cellular differentiation (anisocytosis,

anisokaryosis, pleomorphism, variable number, shapes and sizes of nucleoli, variations in the nuclear to cytoplasmic ratio).

After taking into consideration all the above mentioned criteria we concluded that in 3 out of 6 cases the dogs were affected by hemangiomas, the benign form of the endothelial vascular tumors. These were characterized by thin conjunctive ribbons, lined with spindle or ovoid shaped endothelial cells that displayed a high degree of cellular and nuclear uniformity. Large spaces filled with blood could be observed (Pictures 23-25).

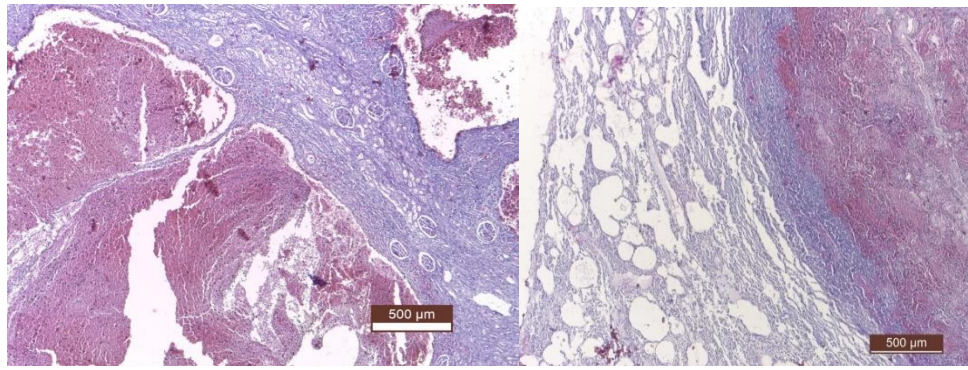


Picture 23, 24 - Spleen - vascular tumor with large spaces filled with blood and megakaryocytes present Masson's trichrome stain, x400



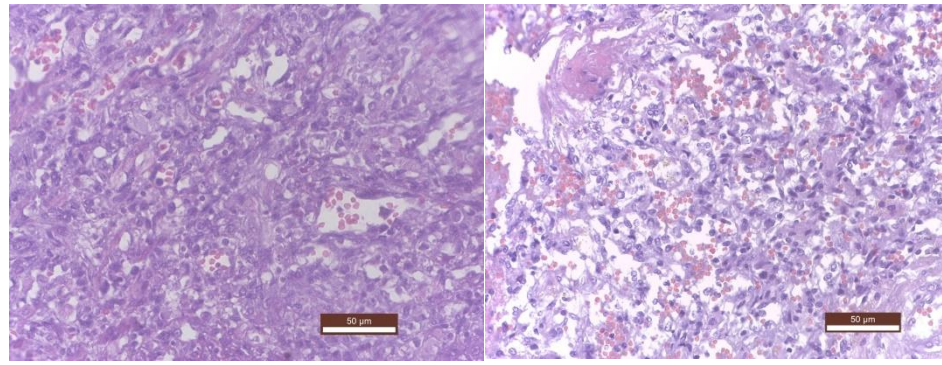
Picture 25 - Liver - vascular tumor with dilated capillaries Masson's trichrome stain, x400

The cavernous hemangioma was characterized by large spaces filled with blood and very few or lack of mitotic figures (Pictures 26, 27).



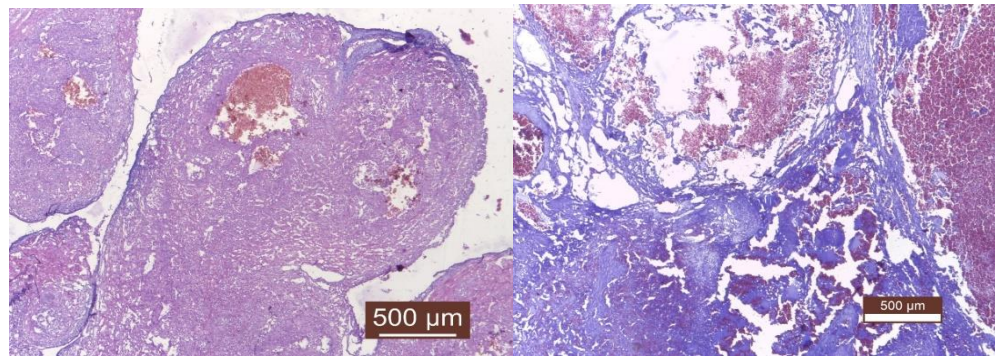
Pictures 26, 27 - Vascular tumors with a cavernous structure located in the kidneys and lungs causing atrophy of the surrounding structures Masson's trichrome stain, x40

The capillary type showed a higher cellularity, along with a stronger conjunctive support. The newly formed blood vessels varied in calibre from the size of a single red blood cell to small arterioles and venules (Pictures 28, 29).



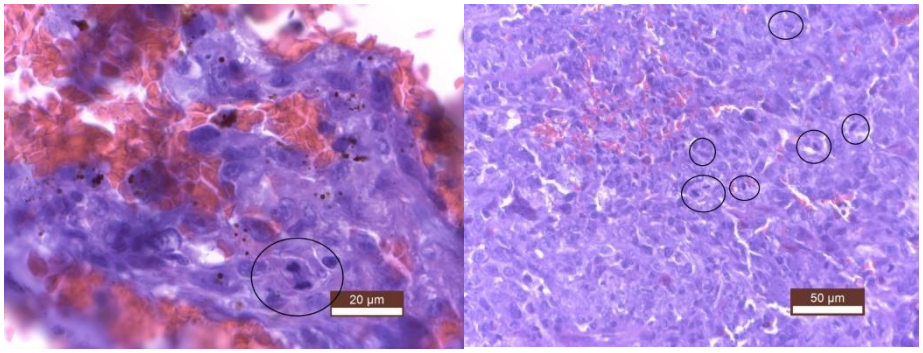
Pictures 28, 29 - Capillary type vascular tumor oocated in the heart (right atrium) and liver Masson's trichrome stain, x400

The mixt type had alternating areas of caverns filled with blood and compactly packed capillaries of small diameters (Pictures 30, 31).



Picture 30, 31 - Mixt type hemangioma and hemangiosarcoma located in the heart and spleen (both small capillaries and caverns filled with blood) Masson's trichrome stain, x40

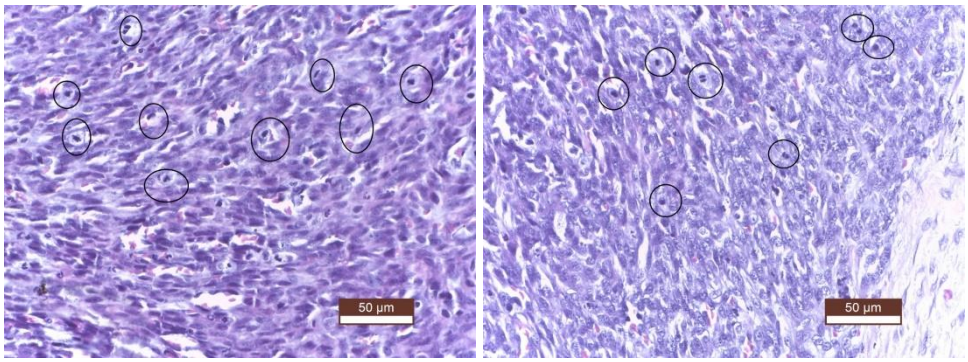
The malignant vascular tumors were diagnosed in the other 3 cases, based on the high mitotic index that varied from 1 up to 9 mitotic figures per HPF (high power field). The assesment was also based on the large number of variations in size and shape of the cells and nucleus, the nucleo to cytoplasmic ratio and characteristics of the nucleoli.



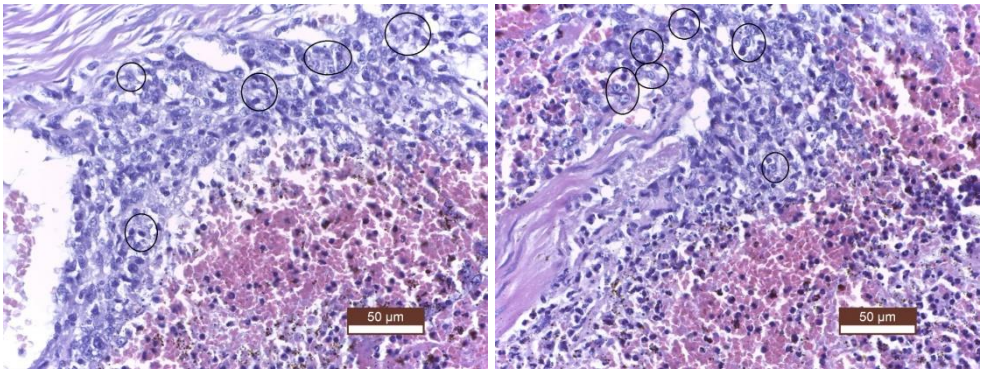
Pictures 32, 33 - Multiple mitotic figures observed in two HPF fields from a vascular tumor in the spleen Masson's trichrome stain, x1000, x400

The structure of these tumors consisted of compact areas with high cellularity, vascular spaces weakly defined or delimited, sometimes without a continuous endothelium or red blood cells inside. Apoptosis and necrotic areas were also observed.

In all three dogs we noticed an agressive and extended metastasation pattern that affected multiple organs and types of tissues (Pictures 32-37).



Picture 34, 35 - Multiple mitotic figures from a malignant hemangiosarcoma located in the right atrium Masson's trichrome stain, x400



Pictuers 36, 37 - Multiple mitotic figures from a metastatic vascular tumor located in the jejunal serosa Masson's trichrome stain, x400

Conclusions

1. The benign and malignant forms of vascular tumors cannot be distinguished based on gross morphology alone. The histopathological exam is necessary in order to establish a definitive diagnostic.
2. The incidence of vascular tumors that we observed in our case work was concurent with the one mentioned in the literature.
3. These tumors, especially the large, cavernous type, can be fatal through the hemorrhages that they cause, but also through the severe dysfunctions they generate inside the organs in which they are located.
4. The prognostic of these tumors is always severe due to the risk of internal hemorrhage.

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Biochemical profile of blood of rabbits on the dependence of the consumed fodder

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Abstract
In order to study the variation of the biochemical profile of the blood of the rabbits depending on the biochemical composition of the consumed fodder (with and without the addition of streptomycetes biomass), an experiment was carried out under laboratory conditions in which were included two lots (control and experimental) of 5 animals per lot. During the experiment, was carried out the biochemical analysis of the fodder, of the blood of the rabbits from both lots taken initially, over 15 days and the end of the experiment.As a result of the experiment, the superiority of the biochemical profile of the rabbit blood from the experimental lot was compared with that of the control lot. The quantitative increase of protein, albumin and glucose respectively by 5.34%, 25.86% and 26.32%, in the blood serum of the animals from the experimental lot compared to the control lot, was favored by the consumption, by rabbits, during 60 days, of the combined granulated fodder with the addition of 0.1% of S. levoris biomass CNMN-Ac-01 .
Keyword: *biomass, rabbit, biochemical indicators, protean metabolism*

Introduction

Rabbits, which are rodent animals, for the physiological development need nutrition rich in vitamins, proteins, calcium and minerals. It is necessary that the nutritional value of the food must cover the nutritional and energy needs in the growing phase of the young rabbits and ensure that the optimal temperature of the body is maintained (Galatanu Diana,2017; Macovschi B.,2014).

Violation of rabbit maintenance and feeding technology reduces the body's resistance to adverse environmental factors. In recent years, in the practice of veterinary medicine and the livestock sector, for the prophylaxis of some animal diseases that occur on the verge of food shortage, are used preparations with benefic micro flora (probiotics) (Nozdrin G. et al, 2012; Petrova N. et al., 2007; Titova A., 2010)

In literature sources, there is a lot of information that states that the use of probiotics contributes to the optimization of metabolic processes in the animals' body, elucidates the effectiveness in the protein amino acid status, in the modification of morphological and biochemical blood indicators (Nozdrin G. et al, 2012). In this sense, the study of the effects of probiotic preparations on the physiological state of the animal body has a theoretical and practical impact.

Feeding and maintenance conditions of rabbits influence the biochemical composition of the blood (Kotsyubenko A).

The image of the blood allows the physiological state of the animals to be evaluated and provides general information about their adaptation to environmental conditions (Petrova N. et al., 2007)

The purpose of this work was to study the effect of the combined granulated fodder with and without the addition of Streptomyces levoris biomass of CNMN-Ac-01 on the blood biochemical indicators of rabbits.

Materials and methods

To achieve the proposed goal, an experiment was conducted under laboratory conditions. Two lots of 5 rabbits were included in the experiment. Rabbits in the control lot received daily granulated combined fodder, and those in the experimental lot - combined granulated fodder with the addition of streptomycetes biomass.

The object of the research served: two types of granulated combined fodder (with and without the addition of 0.1% biomass of S. levoris CNMN-Ac-01) and blood samples collected from rabbits.

Basic research was conducted in the Disease Combat and Prophylaxis Laboratory, Biotechnologies in Embryo Reproduction and Embryo Transfer, Nutrition and Fodder Technology within SPIBZVM. The biomass of S. levoris CNMN-Ac-01 was provided by the Institute of Microbiology and Biotechnologies (National Collection of Non-pathogenic Microorganisms) of the Academy of Sciences of Moldova.

Blood samples were collected from rabbits (at the age of 60 days, 75 days, 138 days) in the morning, after the first bite, because it is forbidden to take blood from starving animals. Rabbits are very sensitive to stress. Any type of stress causes severe hyperglycemia, and flaming even in the short term leads to a severe metabolic change. If these requirements are not taken into account, the research results may be incorrect.

Biochemical blood tests (of three samples collected from rabbits in the control and experimental lots) were performed using the Stat Fax 3300 analyzer.

To perform the analyzes of the biochemical composition of the fodder, Gerhard's performance lab equipment was used.

Results and discussions

Productive parameters of domestic rabbits, such as gain weight, fecundity, prolificity, viability, etc., are ensured by protein intake in the daily ration (Bura M., 2006).

Initially, to determine if the rabbits throughout the experiment will be provided with metabolic energy, crude protein, cellulose, carotenes, some micro- and macroelements, was studied the biochemical composition of the granulated fodder intended for supplying the animals in the control and experimental lot.

Thus, as a result of the biochemical analysis of fodder, it was found that all the studied quality indicators were approximately the same in both fodder (with an insignificant deviation of crude fat, crude pulp, crude ash and carotene).

Only the content of carotene, fat and crude pulp from the compound granulated control fodder exceeded that of the experimental feed by 11.08%, 9.57% and 2.64% (Table 1).

Table 1. Biochemical composition of granulated fodder for rabbits

lots	Crude protein, %	Crude fat, %	Crude fiber, %	Crude ash, %	UN	EM, Mj/kg	Carotene, mg/kg	Ca,%	P,%
control	18.18	3.24	16.30	8.84	0.71	10.63	12.00	1.63	0.39
experimental	18.23	2.93	15.87	8.43	0.72	10.72	10.67	1,45	0.40

A primary role in the exchange of substances in the body exerts its proteins, being involved in nutrition and growth processes, tissue regeneration and immunity (Petrova N. et al., 2007).

Analyzing the results of the research it was found that during the first two weeks, the level of protein increased by 11.14% and 3.82% (Table 2) in the rabbit serum in the control and

experimental lots. Correspondingly, the albumin level was by 3.67% higher in the rabbit serum in the control lot.

Rabbit consumption for 60 days of the combined granulated fodder with the addition of 0.1% of biomass of S. levoris CNMN-Ac-01 intensified protein metabolism in the body, indicating an increase in protein and albumin synthesis in blood serum of animals in the experimental lots with 5.34% and 25.86% (P <0.001) as compared to the serum of animals of the control lot.

The quantitative importance of the blood albumin content is due to the fact that they produce a coloidosmotic blood pressure, provide the dissolution and transport of anions, carry soluble intermediate products of metabolism (Nozdrin G. et al, 2012).

Of the exposed ones, it appears that the increase in protein content in the blood plasma reflects the more intensive growth of the rabbits, and thus the increase in the muscle mass, which was proved by weekly weighing of the animals in both lots.

At the same time, it was found that protein intake in ration is directly proportional to the content of urea in the blood. Urea is a finished product of nitrogen metabolism, which is formed in the liver. Thus, as a result of the intensification of protein metabolism, it was found that in the rabbit growth period (15 days after the beginning of the experiment at the age of 60-70 days) the urea level was increased at the rabbits of the experimental lot by 22,46% compared to those in the control lot. At the end of the experiment the amount of urea was by 12.35% (P <0.001) higher in the rabbit serum in the experimental lot compared to the control and was conditioned by the level of creationin in their blood, which constituted 69.70 ± 0.36 mmol / l. Creatinine is the basic substance of skeletal muscles.

Table 2. Results of biochemical analysis of the blood of rabbits

Specification	initial	75 days		End of the experiment	
		control	experimental	control	experimental
Protein, g/l	24.17±1,08	27.20±0,47	25.13±3,01	31.40±2,83	33.17±1,94
Albumin, g/l	20.93±0,75	23.70±0,26	22.83±2,30	20.93±2,50	28.23±0,33**
Creatinine, mmol/l	69.07±2,42	33.9±4,74	90.73±31,24**	70.60±1,90	69.70±0,36
Urea, mmol/l	0.39±0,02	0.45±0,08	0.58±0,17	0.71±0,04	0.81±0,05**
Amylase, ME/l	23.77±7,90	64.33±16,55	134.67±39,21**	80.20±9,68	167.33±22,08**
Glucose, mmol/l	1.06±0,01	8.08±0,72	5.88±0,78**	4.06±0,32	5.51±0,17**
Triglycerides, mmol/l	1.29±0,15	0.69±0,01	0.40±0,13**	0.46±0,15	0.52±0,03
Cholesterol, mmol/l	193.47±31,92	67.84±6,45	53.46±13,57*	57.28±7,02	34.27±1,54**
Alkaline phosphatase, ME/l	8.07±0,44	17.37±4,13	17.47±3,4	6.80±1,63	10.59±0,59**
Ca, mmol/l	5.07±0,71	2.77±1,34	3.43±0,65	4.40±0,94	3.03±0,12**
Fe, mmol/l	0.52±0,01	0.20±0,06	0.06±0,04**	0.56±0,07	0.42±0,05**

Note: *-P<0,01;**- P<0,001

It is known that in the body of animals glucose is the main source of energy. As a result of the research it was found that in two weeks from the beginning of the experiment, the level of glucose in the blood of the animals in both lots exceeded the original one by over 80.00%. At 75

days of age in the rabbit serum of the control lot, was found by 27.23% ($P < 0.001$) more glucose compared with the experimental one. At the end of the experiment, in the rabbit serum in the experimental lot was found a quantity of 5.51 ± 0.17 mmol/l ($P < 0.001$) of glucose, which surpassed that of the control lot by 26.32%.

According to literature data, triglycerides along with total cholesterol represent the lipid profile of the body. It is known that triglycerides come from food, but a small amount is synthesized in the liver. It was found that during the experiment, cholesterol decreased significantly from 193.47 ± 31.92 mmol/l to 34.27 ± 1.54 mmol/l (by 82.29%) in the blood serum of animals in the experimental lot and by 70.39% in that of the control lot of animals.

The level of triglycerides and cholesterol in the blood of the rabbits in both lots indicates a fat intake in the food ration and the fact that there is no risk of atherosclerosis and cardiovascular disease at these animals.

Alkaline phosphatase is a tetrameric glycoprotein that is found on the surface of the osteoblast and intervenes in calcification of the bone matrix. An increase in this indicator in the blood plasma of 75-day-old rabbits can be explained by increased bone metabolism at this age when growth is more intense, and at the end of the experiment when the animals cease to grow, respectively, this indicator has diminished.

The decrease of concentration of calcium in the blood plasma of the rabbits of the experimental and control lot during the experiment is due to the fact that these animals were maintained in the rooms and as a result took place their malabsorption from the gastrointestinal tract due to the lack of vitamin D.

As regards the evaluation of iron in the blood of rabbits in the experimental lot, the dynamics of the changes signal a very varied oscillation. Thus, if at the initiation of investigations the iron content in the blood plasma constituted 0.52 ± 0.01 mmol/l, then at the 75th day in the rabbit blood plasma of the experimental lot decreased significantly by 88.46%, constituting 0.06 ± 0.04 mmol/l ($P < 0.001$). This tendency of decrease and then of increase the amount of iron at the end of the experiment was also recorded in the rabbit blood samples from the control lot.

During the experiment, the clinical condition of the rabbits was satisfactory, there were no cases of morbidity or mortality, so the low level of macro and microelements did not negatively influence the general condition of the rabbits.

Since all animals were of the same age and maintained under identical microclimate conditions, and the only difference was the composition of the fodder, it can be confirmed that the superiority of the biochemical profile of the rabbit blood in the experimental lot compared to those in the control lot is due to the addition of biomass of streptomyces in the compound granulated fodder.

Conclusions

As a result of the experiment, it was found that the feeding and maintenance conditions of the rabbits influence the biochemical profile of their blood and the addition of 0.1% of Streptomyces levoris biomass of CNMN-Ac-01 in the granulated fodder of the rabbits favored protein, lipid and carbohydrate metabolism of the growing young rabbits and did not negatively influence their body.

The quantitative increase of protein, albumin and glucose respectively by 5.34%, 25.86% ($P < 0.001$) and 26.32% ($P < 0.001$), in the blood serum of the animals from the experimental lot compared to the control lot, was favored by the consumption, by rabbits, during 60 days, of the combined granulated fodder with the addition of 0.1% of S. levoris biomass CNMN-Ac-01 .

Due to the fact that the rabbits in the experimental lot had an average daily increase of 27.67 g/head, exceeding by 10.85% that of the rabbits of the control lot we recommend streptomycete biomass as a supplement in the rabbit feed.

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Antimicrobial susceptibility of bacteria isolated from urine samples in dogs

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Abstract
Urinary tract infections are very common in dogs, while the number of antimicrobials available for therapy has decreased due to the adaptation and evolution of bacterial populations. The aim of the study was to establish the prevalence of the main etiological agents involved in the pathogenesis of urinary tract infections, antimicrobial susceptibility and treatment alternatives.For this study, 50 dogs of different breeds, both females and males, aged between 2 years and 13 years were considered. The experimental part of this study took place between February 2018 and May 2019 in the Department of Microbiology, Faculty of Veterinary Medicine, Cluj-Napoca. Samples were processed using microscopic and cultural examinations. The Petri dishes were inoculated and incubated for 24 hours at 37°C, and interpreted, analyzing the cultural and morphological characteristics of the bacterial colonies as well as the presence or absence of haemolysis areas. The identification of bacterial species was performed by microscopic examination of cells from isolated colonies and biochemical examinations. Antibiotic susceptibility testing was performed by Mueller-Hinton agar disk diffusion technique. The most frequently isolated bacterial strain was E. coli, present in 21 samples of 54 (38.8%), followed by Staphylococcus spp. in 16 (29.6%), Streptococcus spp., present in 10 samples (18.5%), Proteus spp. with 4 samples (7.4%), and Klebsiella spp. and Pseudomonas spp., each present in 3 (5.5%). Bacterial associations were only observed in 7 samples, mainly involving E. coli and Proteus spp., while 4 samples were negative for bacterial growth. Increased susceptibility was observed for enrofloxacin, doxycycline and amoxicillin with clavulanic acid, while increased resistance was recorded for cefovecin, cephalexin and trimethoprim.
Keywords: *urinary tract infections, dogs, antimicrobial susceptibility.*

Introduction

Urinary tract infections in dogs are common, with about 15% of the dogs experiencing at least one bacterial infection during their lifetime (7). Cystitis caused by bacterial infections is usually accompanied by hematuria, dysuria, pollakiuria, stranguria and discomfort. The main source of bacteria invading the lower urinary tract is the colon and skin. The proximity of the rectum to the vulva makes this more common in female dogs than in males. The most frequently bacteria isolated from urinary tract infections the dog is *E coli*, followed by *Staphylococcus* species, *Proteus*, and *Klebsiella* (2). The presence of bacterial species in the lower urinary tract is in the typical form (planktonic bacteria) and biofilm, which is also represented by bacterial cells embedded into their own gel-like secretions. The main difference is that planktonic bacteria do not easily attach to the bladder and urinary tract walls, are more susceptible to antimicrobials, while biofilm is formed on inert surfaces such as implants, stents and urinary catheters, with major importance in antimicrobial resistance and recurrent infections (2,7).

The number of antimicrobials available for therapy has decreased due to the adaptation and evolution of bacterial populations (6,8). Bacterial species have evolved and responded to antimicrobial exposure and selective pressure, exhibiting new antimicrobial resistance mechanisms (3,5). Antimicrobial resistance is important in animal care management, treatment alternatives and health complications (1,4,5). The increased resistance to antimicrobials of urinary tract pathogens

is related to amoxicillin and clavulanic acid, quinolones, and third generation cephalosporins. These are all important veterinary antimicrobial agents (6).

The aim of the study was to establish the prevalence of the main etiological agents involved in the pathogenesis of urinary tract infections, antimicrobial susceptibility and treatment alternatives.

Materials and methods

The study was carried out in Cluj-Napoca, Faculty of Veterinary Medicine Cluj-Napoca, Department of Microbiology. Dogs from different breeds (n=50), 28 females and 22 males, aged between 2 years and 13 years were evaluated between February 2018 and May 2019. The urine samples collected were initially evaluated by microscopic examination, from urine sediment, followed by the inoculation of 1 ml urine onto the surface of a blood agar and MacConkey agar Petri plates.

The Petri dishes were inoculated and incubated for 24 hours at 37°C, and interpreted, analyzing the cultural and morphological characteristics of the bacterial colonies as well as the presence or absence of haemolysis areas. The identification of bacterial species was performed by microscopic examination of cells from isolated colonies and biochemical examinations. Antibiotic susceptibility testing was performed by Mueller-Hinton agar disk diffusion technique.

The microscopic examination of the bacteria was carried out using Gram staining technique. Biochemical identification was based on API 20 Biomerieux system (Bio Mérieux, France) and Vitek 2 technique. Susceptibility testing was performed using Kirby Bauer disk diffusion method. The antibiotics included in this study were represented by amoxicillin and clavulanic acid, doxycycline, ceftiofur, enrofloxacin, penicillin, cefovecin, trimethoprim and cephalexin.

Results and discussions

The most frequently isolated bacterial strain was *E. coli*, present in 21 samples of 54 (38.8%), followed by *Staphylococcus* spp. in 16 (29.6%), *Streptococcus* spp., present in 10 samples (18.5%), *Proteus* spp. with 4 samples (7.4%), and *Klebsiella* spp. and *Pseudomonas* spp., each present in 3 (5.5%). Bacterial associations were only observed in 7 samples, mainly involving *E. coli* and *Proteus* spp., while 4 samples were negative for bacterial growth.

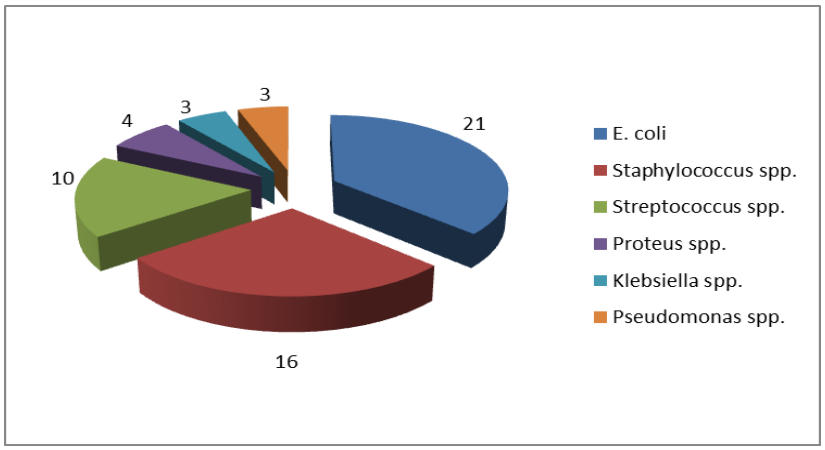

Fig. 1. Frequency of bacterial genera isolated from urine samples

Antimicrobial susceptibility performed by disk diffusion test was calculated as the average of the inhibition diameter area. The most efficient antibiotic was enrofloxacin with an average of inhibition diameter area of 15.22 mm, followed by doxycycline with 12.45 mm, amoxicillin and clavulanic acid (AMC) with 12.23 mm. For trimethoprim the inhibition diameter area was 10.9 mm, for ceftiofur 10.81 mm, for penicillin 10.5 mm, cephalexin 10.07 mm and for cefovecin 6.74 mm.

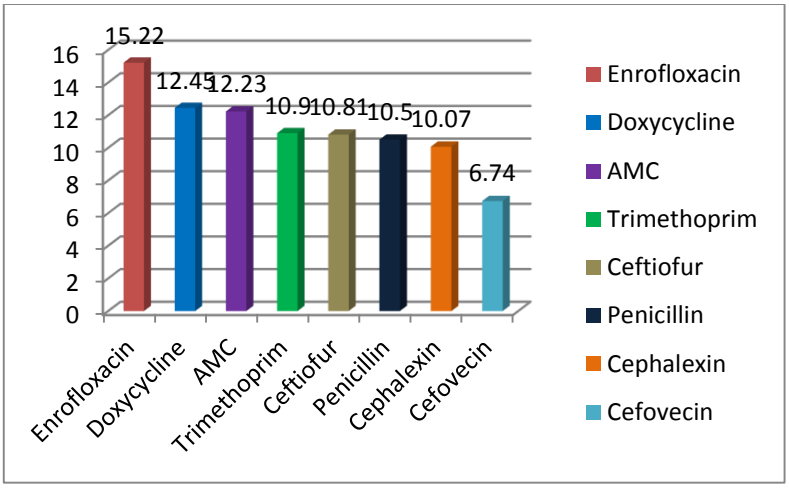

Fig. 2. The average of inhibition diameter area for the antimicrobials included in the study

The results of this study are consistent with the results of Hartmann and Thomson, with *E. coli* as the most frequently isolated pathogen, representing from 33-55% from the bacteria responsible for urinary tract, followed by *Staphylococcus* spp. and *Streptococcus* spp. (3,6). Regarding the antimicrobial susceptibility testing, past decade's trends describe an increase resistance to most antibiotics, from quinolones, third generation cephalosporins (7), doxycycline and amoxicillin-clavulanate (1).

Conclusions

The study regarding microbiological evaluation of urine samples in dogs and antimicrobial susceptibility testing of isolated strains concluded that:

- Both Gram positive and Gram negative bacteria were isolated, with a total percentage of 92.6, the rest of 7.4 samples were negative for bacterial growth;
- Gram negative species predominated, with a total of 62% from the total isolates;
- The antimicrobial profile showed moderate resistance to the antibiotics included in the study, with at least one antibiotic recommended in the treatment of each patient;
- Increased susceptibility was observed for enrofloxacin, doxycycline and amoxicillin with clavulanic acid, while increased resistance was recorded for cefovecin, cephalexin and trimethoprim.

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Evaluation of antifungal susceptibility of some *Candida* spp. strains using *Multodisc* antifungal disk system

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Abstract
The aim of this study was to evaluate the antifungal susceptibility of 33 Candida spp. isolates to classic antifungals included in Multodisc® system kits (Liofilchem – Italy). The research was conducted during January-June 2014 in the Department of Microbiology, Faculty of Veterinary Medicine, Cluj-Napoca, Romania. Identification was made from 48 hrs isolated colonies, using microscopic and biochemical methods (API Candida). From the total of 33 tested strains, 13 were represented by Candida albicans, 9 strains were identified as Candida krusei, 6 were Candida tropicalis 3 Candida catenulata and 2 were Candida parapsilosis. The antifungals included in the disk were represented by Econazole, Nystatin, Griseofulvin, Amphotericin B, Flucytosine, Miconazole, Metronidazole and Ketoconazole. The susceptibility test principle was based on the disk diffusion test, while the interpretation was performed determining the inhibition area diameter. The most efficient antifungal was represented by Ketoconazole with an average of the inhibition area of 26.4 mm followed by Miconazole with 23.4 mm and Econazole with 22.7 mm. The least efficient antifungals were represented by Metronidazole, Griseofulvin and Nystatin.
Keywords: *Candida spp., antifungals, susceptibility, Multodisc.*

Introduction

Candida yeast is a commensal micro-organism normally present in the gastrointestinal tract and an opportunistic causative agent of infections in humans and animals. The need for reproducible and clinically relevant antifungal susceptibility testing has been prompted by the increasing number of invasive fungal infections (IFIs), the expanding use of antifungal agents, and the recognition of antifungal resistance as an important clinical problem (Johnson, 2008).

In recent years, along with an increase in the incidence of candidiasis, there has been an important shift away from *Candida albicans* towards non albicans spp. The change in the epidemiology of Candida infections can be attributed to various factors like severe immunocompromised status of the host, exposure to broad spectrum antibacterial agents and empirical use of antimycotics. However, the clinical manifestations of infections caused by different non albicans Candida (NAC) spp. are usually indistinguishable from those by *C. albicans* (Deorukhkar, 2014).

In vitro antifungal susceptibility testing is now standardized internationally and is becoming essential in patient management and resistance surveillance; it remains less utilized than antibacterial testing (Deorukhkar, 2014).

Clearly, the disc diffusion method has the potential to provide a simple means of performing in vitro tests, but not all antifungal agents are available in discs. Furthermore, the discs are very expensive and their acquisition in developing countries is sometimes difficult (Magaldi, 2004).

The study was conducted on Candida isolates from animals and humans, and major objectives were to evaluate the antifungal susceptibility of 33 Candida spp. isolates to classic antifungals included in Multodisc® system kits (Liofilchem – Italy) and to recommend the best antifungal for the specific treatment.

Materials and methods

The researches of this study were conducted during January-June 2014 in the Laboratory of Microbiology, Faculty of Veterinary Medicine in Cluj-Napoca. A total of 33 Candida specimens isolated from animals with different pathologies (otitis, pharyngitis, mastitis), humans, and strains that contaminated diverse culture media, were included in this study. Candida tested strains were isolated from samples of mastitic cow milk, ear discharge from dogs with otitis, throat swabs from human subjects tonsillitis, urine samples from women with cystitis, faecal samples from hens and pigeons, ruminal fluid samples from cows, various strains that contaminated the culture media, throat swab samples from dogs and cosmetic products. Identification was made from 48 hrs isolated colonies, using microscopic and biochemical methods (API Candida).

Pure culture 48 hrs old well isolated yeast colonies were suspended in 5 ml of a sterile physiological solution until 0.5 McFarland turbidity is reached. A sterile swab was immersed in the suspension broth and then squeezed on the wall of the test tube to eliminate excess fluid. The swab was dragged along the surface of a SDA agar plate as to produce even growth. Antifungals included in the ring were represented by Econazole (ECN - 10µg), Nystatin (NY – 100 I.U.), Griseofulvin (GF - 10µg), Amphotericin B (AMB - 20µg), Flucytosine (FY - 1µg), Miconazole (MCL - 10µg), Metronidazole (MTZ - 10µg) and Ketoconazole (KCA - 10µg). The Multodisc® ring was positioned within 15 minutes from inoculation of the plates, pressing them with sterile pliers on the surface of the agar and then incubated at 35°C +/- 2° for 20-24 hours.

After incubation, the plates are examined; the inhibition halos around each disc are examined and compared with the standard inhibition haloes: in this way, the microorganisms are defined as being susceptible, intermediate or resistant to the tested antifungal agents.

Results and discussions

From the total of 33 tested strains, 13 were represented by *Candida albicans*, 9 strains were identified as *Candida krusei*, 6 were *Candida tropicalis* 3 *Candida catenulata* and 2 were *Candida parapsilosis*.

Susceptibility test principle was based on the disk diffusion test, while the interpretation was performed determining the inhibition area diameter. The interpretation was performed individually for each antifungal and yeast strain, and then the average of inhibition area was determined for each antifungal. Where total resistance was observed, the value considered for the calculation was zero. The most efficient antifungal was represented by Ketoconazole with an average of the inhibition area of 26.4 mm, followed by Miconazole with 23.4 mm and Econazole with 22.7 mm. The least efficient antifungals were represented by Metronidazole with 12.3 mm, Griseofulvin with 9.11 and Nystatin with 8.32 mm.

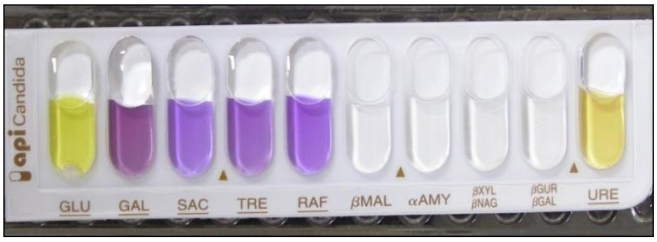

Fig. 1. Candida krusei strain identification – API Candida gallery

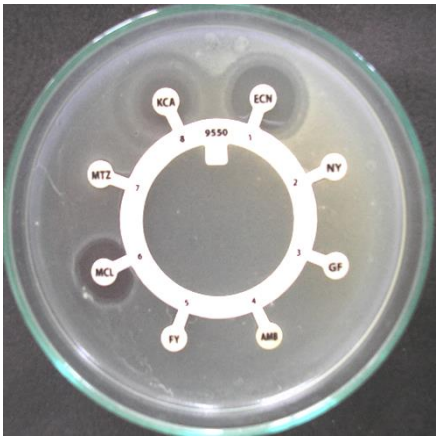

Fig. 2. Susceptibility testing for strain 23 A - *Candida albicans*

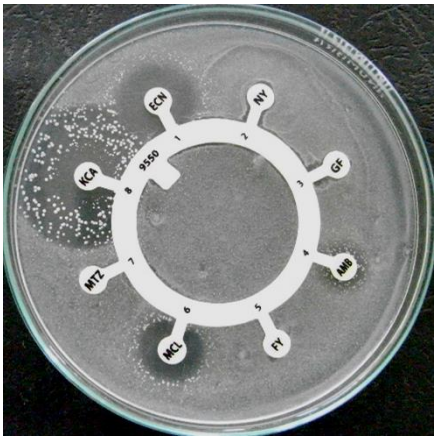

Fig. 3. Susceptibility testing for strain LM - 2 - *Candida krusei*

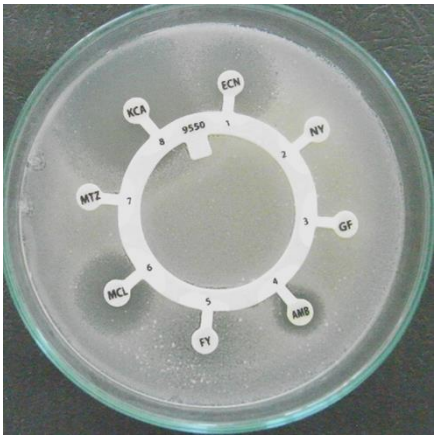

Fig. 4. Susceptibility testing for strain 171 S - *Candida tropicalis*

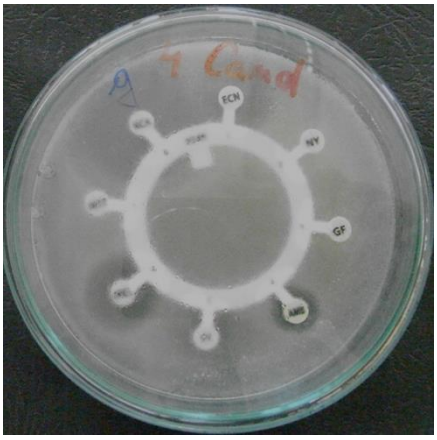

Fig. 5. Susceptibility testing for strain 4 Cand. - *Candida parapsilosis*

The results are in agreement to the studies of Giri, 2014, that on 39 Candida isolated obrained resistence patterns for fluconazole, ketoconazole and amphotericin B. The disk-diffusion method can offer a good level of sensitivity if the technique is performed by CLSI standards.

Comparing the results of Multodisc® test with the results of Etest, is observed that fluconazole is generally recommended as a therapeutic choice for systemic candidiasis, but amphotericin B does not have the same efficiency against Candida isolated in Cluj-Napoca compared to human patients with oral candidiasis (Song, 2015).

Conclusions

The researches on identification of *Candida spp*. isolates from animals and humans in Cluj county area and the sensitivity of these strains to antifungal resulted in the following conclusions:

- *Api Candida* system is a good method, easy to use and represents a relatively quick and inexpensive tool for Candida specie identification.
- *C. albicans* predominates in Cluj-Napoca area, followed by *C. krusei, C. tropicalis, C. catenulata* and *C. parapsilosis*.
- Classic antifungals tend to be less efficient in *in vitro* susceptibility testing of Candida species, due to their frequent use, except for Ketoconazole and Miconazole.
- The use of antifungals against Candida infections requires prior susceptibility testing in order to prevent antifungal resistance to these products.

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Studies on isolation and susceptibility to antibiotics of the pathogens involved in *SCUD* etiology of aquatic turtles

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Abstract
The objectives of this study were to isolate and identify the bacteria present in the shell and plastron ulcers in a group of infected turtles compared with healthy turtles, and antibiotic susceptibility testing of bacterial species identified in order to recommend the appropriate treatment. A total number of 32 red-eared slider (Trachemys scripta elegans) with specific septicemic cutaneous ulcerative disease (SCUD) lesions were sampled from the shell and plastron, compared with 8 healthy turtles. Cotton swabs were used for sampling, and the inoculation was carried out on blood agar, XLD, MacConkey and SDA plates. Biochemical characterization used API Biomerieux 20 system. Susceptibility to antibiotics was evaluated using Kirby Bauer disk diffusion method on Mueller-Hinton agar. In the samples from turtles with lesions, predominantly Gram-negative bacteria were isolated, in particular Citrobacter freundii – 16 turtles, Escherichia coli – 13 turtles, Klebsiella – 10 turtles, Serratia – 9 turtles, Shigella – 8 turtles, Salmonella – 6 turtles, plus Gram positive bacteria such as Staphylococcus – 18 turtles, Micrococcus – 11 turtles and Bacillus – 9 turtles. Regarding the group of 8 healthy turtles ratio was significantly in favor of Gram positive, with Staphylococcus – 7 turtles, Micrococcus 6 and Bacillus 4 samples. Regarding the inhibition area diameter for infected turtles, the most efficient antibiotic was Doxycycline with the average value of 15.15 mm, Enrofloxacin with 14.95 mm and Florfenicol with 14.8 mm. Lower efficiency was observed for Ceftriaxone with 4.05mm and Colistin with 7.01 mm.
Keywords: *SCUD, turtles, ulcers, antibiotic susceptibility.*

Introduction

Septicemic cutaneous ulcerative disease (SCUD) is a shell disease of aquatic turtles caused by *Citrobacter freundii*; however, various bacteria have been isolated from diseased skin and shell. Anorexia, lethargy, and petechial hemorrhages on the shell and skin are seen; liver necrosis is also common. It is more common in soft-shelled turtles (*Appalone* spp.) (Jacobson, 2007).

SCUD is viewed more as a syndrome with many bacteria such as *Citrobacter freundii, Serratia anolium, Beneckea chitonovora* and other gram negative bacteria acting together with poor husbandry, poor water quality, abrasions and invertebrate predation to culminate in SCUD (Mader, 2006).

Shell ulceration can form when there is an injury to the shell in which the damaged area becomes infected. The initial injury could be minor and not easily noticeable or could be very obvious. It may have occurred in the form of an abrasion, scratch or even a burn. If left untreated or improperly cared for, this lesion could be penetrated and lead to a number of diseases such as fungal and bacterial infections and septicemia (Joyner, 2006).

The objectives of this study were to isolate and identify the bacteria present in the shell and plastron ulcers in a group of infected turtles compared with healthy turtles, and antibiotic susceptibility testing of bacterial species identified in order to recommend the appropriate treatment.

Materials and methods

The study was conducted in the laboratory of Microbiology at the Faculty of Veterinary Medicine Cluj-Napoca, Romania, between March and July 2015. A total number of 32 red-eared slider (*Trachemys scripta elegans*) with specific SCUD lesions were sampled from the shell and plastron, compared with 8 healthy turtles. The provenience of the turtles was Târgu Mureş ZOO. Cotton swabs were used for sampling, and the inoculation was carried out on blood agar, XLD, MacConkey and SDA plates. Biochemical characterization used API Biomerieux 20 system. Susceptibility to antibiotics was evaluated using Kirby Bauer disk diffusion method on Mueller-Hinton agar.

Results and discussions

In the samples from turtles with lesions, predominantly Gram-negative bacteria were isolated, in particular *Citrobacter freundii* – 16 turtles, *Escherichia coli* – 13 turtles, *Klebsiella* – 10 turtles, *Serratia* – 9 turtles, *Shigella* – 8 turtles, *Salmonella* – 6 turtles, plus Gram positive *Staphylococcus* – 18 turtles, *Micrococcus* – 11 turtles and *Bacillus* – 9 turtles.

Fig. 1. Lesions at the level of the plastron (arrow)

Fig. 2. Lesions at the level of the shell (arrow)

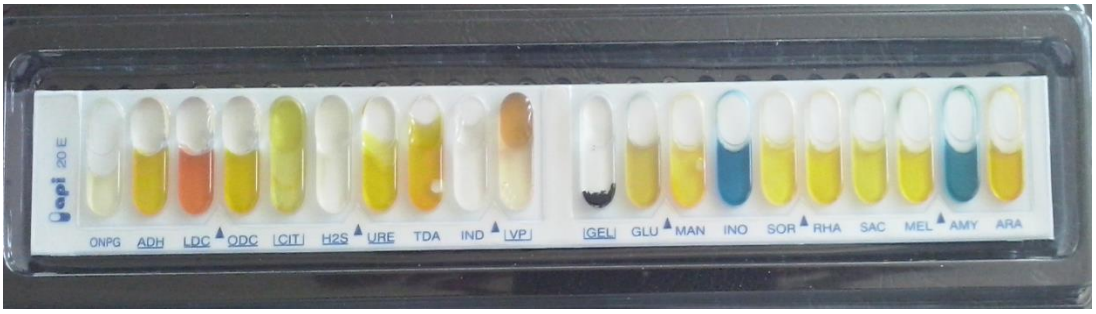

Fig.3. *Citrobacter freundii* strain identification – API 20 E gallery

Concerning the group of 8 healthy turtles ratio was significantly in favor of Gram positive, with *Staphylococcus* – 7 turtles, *Micrococcus* 6 and *Bacillus* 4 samples.

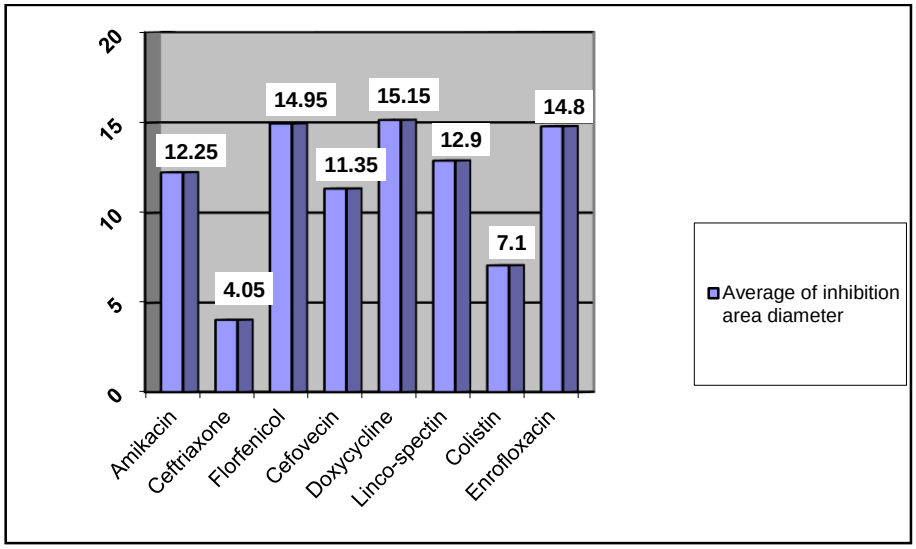

Fig.4. Average of inhibition area diameter for bacteria isolated from turtles with lesions

Regarding the inhibition area diameter for infected turtles, the most efficient antibiotic was Doxycycline with the average value of 15.15 mm, Enrofloxacin with 14.95 mm and Florfenicol with 14.8 mm. Lower efficiency was observed for Ceftriaxone with 4.05mm and Colistin with 7.01 mm.

The practice of feeding crayfish is often implicated in the ethiology of SCUD and should be discouraged.

Conclusions

1. *Citrobacter freundii* is not exclusively involved in SCUD etiology but predominates associated with other Gram negative bacteria.
2. Sanitation improvement and antibiotic administration improve turtle health status.
3. The overall susceptibility to antibiotic was only moderate probably due to previous treatments and difficult antibiotic penetration.

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Retrospective analysis on somatic cells count trend in *Staphylococcus aureus* cows' mastitis

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Abstract

The study was carried on detection of mastitis, using increasing respectively decreasing trend of somatic cells count (SCC) of two successive samplings. The analysis was performed to identify the associations between SCC trend with retrospective and transversal milk quality indicators, and mastitis. From a BIOAMR database, 28 cows with ***a posteriori*** *diagnosed mastitis with Staphylococcus aureus (4/28 cases were methicillin-resistant Staphylococcus aureus – MRSA) were sampled. The trend of SCC was Spearman's rho correlated with previous lactose (r_s = +0.785, p=0.03), pH (r_s = +0.662 at p=0.019), and current SCC measurements (r_s = +0.781, at p=0.000). Increasing trend of SCC was retrospectively associated with lactose content (Z = -2.152 at p = 0.031), pH (Z = -2.152 at p = 0.031) SCC at first measurement (Z = -1.764 at p = 0.078) and currently associated with SCC (Z = -3.316 at p = 0.001), fat content (Z = -1.88 at p = 0.060) and fat/protein ratio (Z = -1.717 at p = 0.086). The 28 samples of the study did not revealed strong association between SCC trend and type of Staphylococcus aureus (p = 0.186 by Mann-Whitney test), even if MRSA had a higher increasing trend of SCC in comparison whit S. aureus (non-MRSA) infections (1403.5 vs. 288.2 thousands somatic cells). By preliminary results the trend of somatic cells could be an indicator in detection of mastitis but more case studies are necessary.*

Keywords: *Staphylococcus aureus, somatic cell count, cow mastitis and AMR.*

Introduction

Staphylococcus aureus is one of the main contagious pathogens responsible for the intra-mammary infection in dairy cattle and mastitis is one of the most economically important health traits for milk production *(6).* Detection of mastitis is generally based upon a number of indicators of mammary gland inflammation. The detection of the inflammation is based upon the response of the body to the mammary gland infection. The aim of the study is detection - diagnosed remarks in a retrospective study of the trend of somatic cells count (SCC) as two successive measurements in association with changes in milk, in order to improve future detection and treatment patterns.

Materials and methods

Farms and animals sampling: 15 partner farms of Extension unit in three counties of West Romania were stratified sampled (5 farms for each of the counties Arad, Bihor and Timiş) in a screening for dairy mastitis infection. All farms are included in the Official Control of Milk Production managed by Breed associations *(8,9,10,11)* - the last SCC value preview farm visit was considered. The SCC trend was calculated for in 11.2 ± 1.15 days' distance between two successive measurements. From overall *Bioeconomic approach to antimicrobial agents - use and resistance (acronym BIOAMR)* database 28 cases diagnosed with *Staphylococcus aureus* mastitis were

studied. In the study, from the sampled BIOAMR database, 28 cows with *a posteriori* diagnosed mastitis with *Staphylococcus aureus* (4/28 cases were methicillin-resistant Staphylococcus aureus – MRSA) were used.

Data collection and processing: the Californian Mastitis Test (CMT) and milk samples have been taken and primary analyzed (figure 1) on the farm for all dairy cows. Only positive sample to CMT were collected for chemical milk constituents (*Funke Gerber Lactostar Dairy Analyser*) and SCC analysis (*DeLaval cell counter DCC)*. Such chemical milk analysis device features fully automatic cleaning and rinsing system and zero point calibration for fast and accurate testing. The SNF (fat free dry matter), protein, fat, lactose and minerals with reproductibility maximum ± 0.04 % were measured and freezing point and density was calculated.

Microbiology analysis. The microbiological samples were collected by *COPAN's ESwab*^{TM} liquid collection and transport system form all positive quarters. Each infected quarter was considered an individual sample and only the most affected quarter (higher number of SCC) of one animal was included in the study. The germs were isolated and later, by microbiological exam, other 28 cases were used for the retrospective study. The typifying and antibiotic resistance was done by *Walk Away System* using *MicroScan® Dried Panels.*

Figure no. 1: Collecting and primary analysis of the samples at the farms level
CMT screening of the dairy cows in the milking parlor, in order to detect mastitis (*left*). Analyzing the milk positive sample for milk constituents *(Funke Gerber Lactostar Dairy Analyser)* and content, and somatic cells count (*DeLaval cell counter DCC*) – in the Animal Production Laboratory (*right*). *Source: UEX Media, 2019*

Statistical Analysis

The trend of SCC was considered positive or negative, depending on the value difference between the SCC measurement on the day of the farm visit, and the preview measurement, usually from results of Official Control of Milk Production. *SPSS® Statistics* software for *Spearman's* correlation, *Mann-Whitney U test* and nonparametric tests were used in order to do the analyses of association, frequency and differences between SCC trend and several groups and variables of the study. Significance value was accepted to be $\alpha = 0.05$.

Results and disscution

Milk somatic cells (considered as a Somatic Cells Count) are a mix of milk-producing cells and immune cells. Various factors and management practices modulate SCC and hematological parameters in a dairy herd: udder inflammation, parity, stage of lactation, unhygienic and incomplete milking, hot-humid climate, change in housing and feed or distress increase the SCC. Otherwise, healthy udder, antioxidants, hygienic milking, proper cow therapy, selection against mastitis, lower the *SCC (3,7,12,13,14).*

Staphylococcus aureus classified as a contagious pathogen, which can efficiently adapt to the environment of the mammary gland and spread cow to cow during milking, was considered in association with SCC. Several changes occur in blood *(4),* tissues and in milk *(5),* as a reaction to infection, including infiltration of leukocytes (measured by somatic cells content - SCC) and increased vascular permeability, resulting alterations in the chemistry of the milk resulting from hydrolysis of milk proteins by hydrolytic enzymes and oxidative substances released from phagocytes, alterations in milk pH and ionic solutes, and ingestion of milk components by phagocytes.

The trend of SCC was positive *Spearman's rho* correlated with previous lactose (r_s = +0.785, p=0.03), density (r_s = +0.662 at p=0.019), and with the SCC measurements on the day of the farm visit (r_s = +0.781, at p=0.000). Increasing trend of SCC was retrospectively associated with lactose content (Z = -2.152 at p = 0.031), density (Z = -2.152 at p = 0.031) SCC at first measurement (Z = -1.764 at p = 0.078) and currently associated with SCC at farm visit time (Z = -3.316 at p = 0.001), fat content (Z = -1.88 at p = 0.060) and fat/protein ratio (Z = -1.717 at p = 0.086). The 28 samples of the study did not reveal strong association between SCC trend and type of *Staphylococcus aureus* (p = 0.186 by *Mann-Whitney* test), even if MRSA had a higher increasing trend of SCC in comparison whit *S. aureus* (non-MRSA) infections (1403.5 vs. 288.2 thousands somatic cells).

The lower SCC trend can be associated with the capacity of the body to react to infection; the higher trend can be associated with infection. In fact, the percentage of noninfectious and infectious cells from SCC is, and can be, established. The percentage of leukocytes in SCC in milk is different in healthy cows vs. infected. *Alhussien et. al. 2016 (1,2)* reported 19% vs. 75% neutrophils (diameter 12-15 μm, nucleus is multi-lobed with bridges), 66% vs. 17% macrophages (diameter 20-30 μm, the largest cell type in milk) and lymphocytes 15 vs. 8% (diameter 9-16 μm, deeply stained round nucleus with low cytoplasm).

By preliminary results and corroboration with other study *(5),* the trend of somatic cells was proven to possibly be an indicator in detection of mastitis, but more studies are necessary.

Conclusions

- The higher trend of somatic cells count was associated with persistency of mastitis caused by '*S. aureus*
- The trend of somatic cells count was retroactively and positive correlated with milk lactose content and density.
- Trend of somatic cells count is associated with SCC, fat content and fat/protein ratio, on the day of the on-farm visit.

Acknowledgments

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Research Unit in *Banat University of Agricultural Science and Veterinary Medicine "King Michael I",* Timisoara.

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Study regarding the regenerative response after induced anemia through blood collection in mouse

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Abstract
Ordinarily, animals can tolerate a loss of about 20 to 30% of the total quantity of blood without the need for transfusions or fluid therapy. Regarding mice, it is generally accepted that blood samples should be limited to 10 – 15% of total blood mass, in order to prevent the onset of anemia. For this experiment we have shown the hematological differences between the collection of 25% and 40% of total estimated blood volume. In both cases, complete blood counts were analyzed and erythropoietic response was described by means of bone marrow and spleen cytology analysis. After approximately 24 hours, both groups had increased reticulocyte counts and low hemoglobin levels. Red blood cell morphology was not altered with the exception of anisocytosis due to polychromasia.
***Keywords**: erythropoiesis, mouse, regenerative anemia*

Introduction

In the mouse, blood sample quantity should be carefully assessed. Samples larger than 10% to 20% of total blood volume may lead to the onset of anemia and can cause a series of pathophysiological effects on the animal (3) and may require fluid replacement (2, 4). Severe blood loss can cause hypovolemic shock, resulting in the death.

Acute anemia is a consequence of a loss of massive quantities of blood and is usually associated with bleeding from trauma or neoplasia, and hemolysis frequently due to toxic agents, drugs or autoimmune diseases (1).

Acute blood loss anemia is associated with a regenerative response, proportional to its severity. It is normally characterized by a decreased number of red blood cells (RBC) and packed cell volume (PCV). The anemia is usually normochromic, normocytic. Erythropoiesis in the mouse is evaluated by assessing bone marrow and spleen cellularity (6). The spleen remains a constant hematopoietic site throughout the animal's lifetime (7).

The aim of this study was to show the effects of removing 25% and 40% of total estimated blood volume by observing the regenerative response 48 hours after blood collection.

Materials and methods

Fifteen 10-week-old CD1 albino female mice were used in this experiment. The mice were quarantined for seven days for acclimatization and then were divided into three equal groups, respectively, two experimental groups and a control group.

All mice were fed a specific rodent diet and had free access to water. Evey mouse was weighed and the necesary amount of blood was gathered by the tail vessel snip techinque. Estimated total blood volume was calculated for each mouse using body weight measurements and the average blood volume in the body (8% of total body mass). The first group (Group A) was bled of 25% of total estimated blood volume and the second group (Group B) of 40% of total estimated blood volume.

After two days, the mice were anesthetized with ether, and blood samples were collected by cardiocentesis as a terminal procedure. Blood was collected in EDTA-coated microtainers.

Complete blood count (CBC) was obtained with an automated counting aparatus (Advia 2120i, Siemens).

Blood smears were made for the evaluation of peripheral blood cell cytology. Bone marrow smears were prepared from the femur using the ‚paintbrush' technique. The smears were made in the first 30 minutes after euthanasia. The myelogram was used in order to assess local cell morphology and M:E ratio (myeloid:erythroid).

Spleen impression smears were made in order to assess local cell morphology. Blood, bone marrow, and spleen smears were all stained using May-Grünwald Giemsa.

Results were statistically analysed using the GraphPad Prism 8 program. Mean and Standard deviations were calculated and the difference between the two experimental groups was shown using the paired t-Student test.

Results and discussion

The complete blood count showed modified paramethers, suggestive of regenerative anemia. Values were listed in Table 1. Both Group A and Group B had the same modified parameters.

There was a significant decrease of the number of RBCs of 6.98±0.71 $x10^6/\mu L$ for Group A, and 4.75±0.84 $x10^6/\mu L$ for group B. The amount of hemoglobin was also reduced, making the distinction of hypochromic anemia, with values of 11.3±0.4 g/dL for Group A and 6.8±0.7 g/dL for group B.

Packed cell volume was significantly modified in both groups, with mean values of 40.8±2.8% for group A and 25.5±4.5 for Group B. Slight decreases of the mean cell hemoglobin and mean cell hemoglobin concentration were noted in both groups.

Table 1. Complete blood count of the experimental and control groups

Parameter	Measuring unit	25% blood loss	40% blood loss	Control group
Red blood cells	$x10^6/\mu L$	6.98±0.71*	4.75±0.84*	9.56±0.63
Hemoglobin	g/dL	11.3±0.4*	6.8±0.7*	14.9±0.5
Packed cell volume	%	40.8±2.8*	25.5±4.5*	53.2±2.9
Mean cell volume	fL	58.6±2.8	54.5±3.2	56.2±3.7
Mean cell hemoglobin	pg	16.2±0.3	14.3±1.1	15.7±1.3
Mean cell hemoglobin concentration	g/dL	27.7±0.6	26.2±1.9	28.2±2.3
Red cell distribution width	%	17.8±1.5*	19.4±2.3*	12.7±0.3
Reticulocytes	$x10^9/L$	593.2±184	486.2±74.6	465.5±69.1
Platelets	$x10^3/\mu L$	1265±271	1471±195	1128±188
White blood cells	$x10^3/\mu L$	1.92±0.42	1.77±0.59	3.61±0.56

Data represents the mean value ± standard deviation calculated for 5 samples from each group.

**p<0.001*

There was a significant increase of the red cell distribution width values of 17.8±1.5% in group A and 19.4±2.3% in group B. There was a small increase of reticulocyte numbers in both groups, but the regenerative response is not proportional to the severity of the anemia.

At 48 hours post-bleeding, the regenerative response is visible in the complete blood count by means of reticulocyte number and red cell distribution width. Given the fact that most common hematology analyzers do not have reticulocyte counts or red cell distribution width as parameters, and the mean cell volume was not significantly modified as to provide a clear picture of cell size, the regenerative response may be easily overlooked at this stage.

Group A was characterized by mild regenerative anemia, whereas Group B had moderate regenerative anemia, but with a disproportional regenerative response at 48 hours post-bleeding.

Although we have found a study which states that it is safe to collect up to 25% of estimated total blood volume from female mice, without the onset of clinically significant anemia (5), the mice used in group A of this study have shown signs of mild regenerative anemia after 48 hours from sample collection. Although mild anemia is not life-threatening for the animal, the altered CBC could potentially influence study results.

Blood smear analysis has shown slight differences between the two groups, indicative of mild anemia, with anisocytosis (reflected in the CBC by the increase of red cell distribution width).

Group A presented mild polychromasia, erythrocytes were normocytic, normochromic without the presence of nucleated RBCs or Howell-Jolly bodies (Figure 1A). Group B has shown more marked polychromasia, with normocytic RBCs. As in the first group's case, there were no nucleated erythrocytes or Howell-Jolly bodies (Figure 1B).

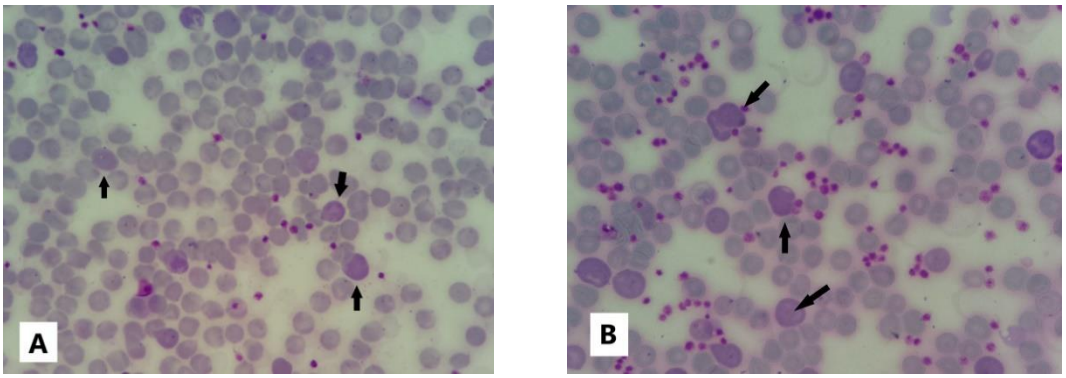

Figure 1. Blood smears images (100X). Group A with 25% blood loss shows signs of mild polychromasia with reticulocytes indicated by black arrows (A). Group B with 40% blood loss, with polychromasia and anisocytosis. Reticulocytes are indicated by black arrows. May-Grünwald Giemsa stain.
(Original images)

Bone marrow cytologic examination has shown normal cell morphology with a modified M:E ratio, due to erythroid hyperplasia, with a predominant population of rubricytes and metarubricytes (Figure 2A, 2B). The M:E ratio for the 25% blood loss group was of 1.5:1. The 40% blood loss group has shown a mean M:E ratio of 1.9:1. The control group had a mean M:E ratio of 2.1:1. Cell morphology and placement were normal.

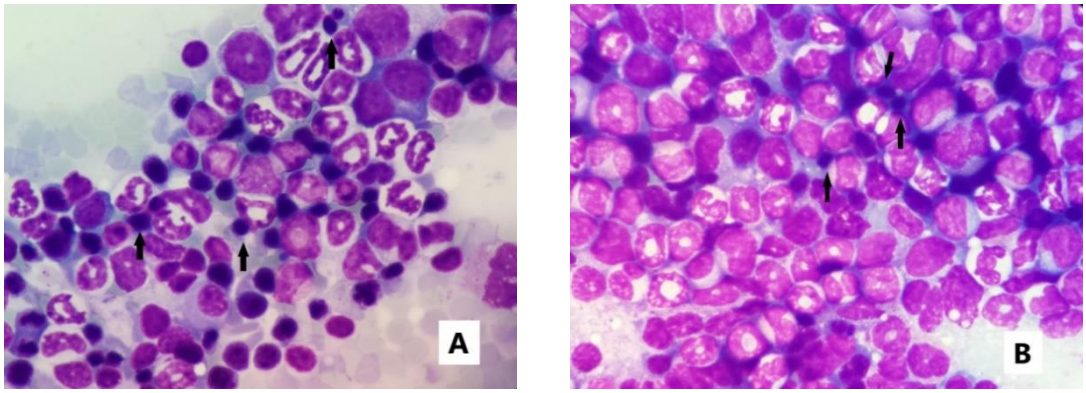

Figure 2. Bone marrow cytology (100x) with metarubricytes indicated by black arrows. A. Significant regenerative response observed in mouse group A, with the predominant cell line or late erythrocyte precursors such as rubricytes and metarubricytes. B. Bone marrow belonging to the 40 % blood loss group, with a slight regenerative response and a relatively high number of metarubricytes and rubricytes. May-Grünwald Giemsa stain.
(Original images)

Spleen macroscopic assessment has been made, and there were no visible lesions or changes in consistency and color. Organ weight was well within age and sex parameters. Spleen cytology has shown increased hematopoietic cellularity with marked erythroid hyperplasia with orderly maturation, which was more visible in Group B (Figure 3A, 3B).

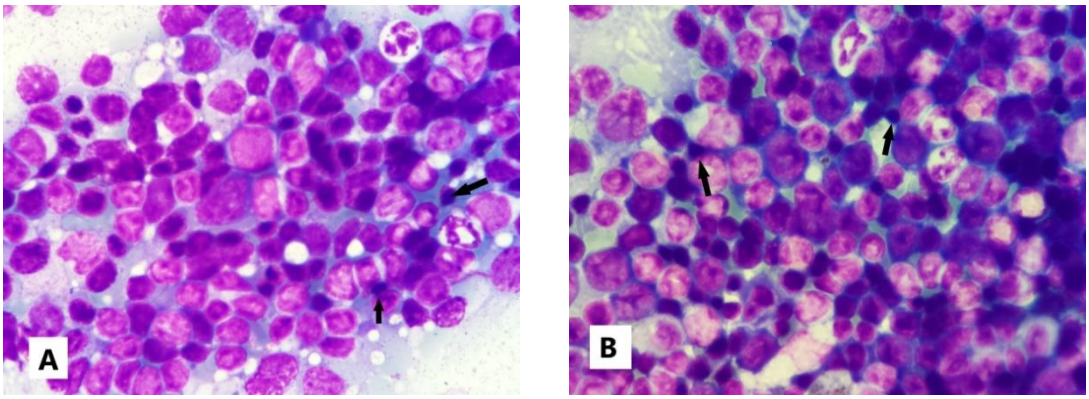

Figure 3. Spleen cytology (100x). Regenerative response observed in both experimental groups, with a predominant red blood cell progenitor population of metarubricytes (black arrows) and rubricytes. May-Grünwald Giemsa stain.
(Original images)

Bone marrow and spleen cytology confirmed the onset of a regenerative response, with a complete erythroid series, with orderly maturation. Erythroid hyperplasia was more intense in the first experimental group in the CBC results and bone marrow cytology, whereas the second experimental group had a more visible regenerative response of the splenic hematopoietic tissue.

Conclusion

During the first 48 hours after blood collection there were no major differences between the intensity of the regenerative response between the two experimental groups.

The intensity of the regenerative response was more obvious in spleen and bone marrow cytology than blood smear and hemogram.

Collecting an amount of blood of 25% or higher of estimated total blood volume leads to significant changes of the hemogram which can interfere with certain study results.

For a more clear understanding, this study will continue with observations of the regenerative response at 24 and 72 hours post-bleeding.

Acknowledgements: Preliminary results of PhD thesis.

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Implementation of a pharmacologic protocol for testing bovine colostrum nutraceutical products in Broilers

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Abstract
As antibiotic additives were deemed illegal to use in poultry feed as a growth promoter but also to prevent certain diseases such as necrotic enteritis, researches are increasingly oriented towards finding an alternative that will provide both economic gain for the farmer and safety for the end consumer. Bovine colostrum, the first milk secretion postpartum, is widely known for its beneficial properties, not only on the newborn organism, but also in the adult one. The bioactive components of the colostrum have healing properties in the gut, furthermore they help prevent bacteria from adhering to the intestinal mucosa. The biochemical constituents also have an antibacterial effect through lactoferrin and lactoperoxidase. The main objective of this paper was to establish a protocol through which to obtain consistent scientific data when researching the effects of a nutraceutical product in poultry. In order to achieve this we have conducted a study, in the University of Agricultural sciences and Veterinary medicine, Cluj-Napoca, Romania, on a population of 60 broilers that were divided into three groups: the control group and two other groups that were administered distinct nutraceutical products in their drinking water. The purpose was to evaluate the effectiveness of replacing antibiotic growth promoters and anticoccidial drugs with nutraceutical products from bovine colostrum. Various microbiological, health and productivity parameters were assessed and compared between the groups, over a period of 45 days. The immunoglobulins from the bovine colostrum, as bioactive components, will achieve its highest peak in day 14 in the blood serum of the bird. As such, blood samples were deemed best to be harvested on EDTA and Clotting agents every 14 days, as the products were administered 2 times throughout the study. Cloacal swabbing was also performed, feces samples were evaluated for microbial concentration and bacterial strain identification. A comparison was made with several other researches that performed similar clinical studies and we recommend that when administering bovine colostrum nutraceuticals, in order to obtain scientifically consistent results, a strict protocol has to be implemented, periodical evaluations have to be made according to the parameters that are assessed but also in compliance with the bioactive components of the product.
Keywords: *Broiler, colostrum nutraceutical, microbial, parameter, protocol*

Introduction

In 1999 the American Association of veterinary medicine has described the nutraceutical products as micronutrients, macronutrients and other nutritional supplements used as therapeutic agents (Pandey et al., 2011). In 2019, a veterinary nutraceutical is described, by the North American Veterinary Nutraceutical Council, as being "a substance which is produced in a purified or extracted form and administered orally to patients to provide agents for normal body structure and function and administered with the intent of improving the health and well-being of animals."(Ramesh et al., 2019).

The poultry industry is particularly influenced by the use of nutraceuticals as the sub-therapeutic use of antibiotics in their feed has been either banned or reduced in many countries worldwide (Yesuf et al., 2017, Sugiharto, 2014), as it caused antibiotic-resistant microorganisms to develop and was a danger to consumers' health. Excluding antibiotics from the feed has caused

however numerous problems in the industry as the growth performance lowered and underlying diseases developed during the rearing period (Huyghebaert et al., 2011).

A wide range of nutraceuticals were used in the poultry industry in order to improve these aspects, from fenugreek, to ginger and neem extract and sea buckthorn (Yesuf et al., 2017, Mekuriya et al., 2018, Vlaicu et al., 2017, Ramesh et al., 2019), however the main objective of this paper was to establish a protocol through which to obtain consistent scientific data when researching the effects of a bovine colostrum nutraceutical product.

The bovine colostrum is well known for its bioactive and biochemical composition as their effects have been described since the 20th century (Parrish et al., 1950). The various growth factors have healing properties in the gut, moreover studies have shown that the same bioactive components improved muscle mass in athletes using colostrum nutraceuticals (Antonio et al., 2001). Another very important bioactive component are the antibodies. Researches show that over 60% of the total proteins in the bovine colostrum is represented by immunoglobulins and 90% of these are immunoglobulins G (IgG) which are specifically responsible for binding antigens, neutralizing toxins (Alexieva, 2004).

The biochemical components with antimicrobial properties in the bovine colostrum, lactoperoxidase and lactoferrin, are proven to be effective against Escherichia Coli and Salmonella typhimurium (Freedman et al., 1998, Xu et al., 1996). Furthermore, the lysozyme is a well-known enzyme with antibacterial effect on gram-positive and gram-negative microorganisms by destroying the bacterial wall (Reiter, 1978).

The lack of reliable guidelines and results when administering nutraceuticals in poultry has reduced the trust that veterinarians put in alternative medicine. Also, some statistics claim that the results of such clinical trials are published in less publicized scientific journals (Taillon et al., 2000).

Materials and methods

This study was conducted with the approval of the bioethics committee from the University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania, where the protocol was also implemented.

The birds, obtained from a local commercial hatchery, were from the Ross308 Broiler breed. The chicks (n=60) were 1 day old since hatching and clinically healthy. They were then raised in 3 rearing pens, 20 birds in each pen, randomly selected (T1, T2 and T3), in a controlled temperature room. Male and female chickens were reared together. The provided bedding consisted of natural, untreated wood shavings; all pens were equipped with an infrared light, of adequate intensity, feeders and water troughs. Thorough cleaning was performed every week when the bedding was also replaced. All birds were given commercial starter crumbled pellets at discretion during the first 17 days of life, after which the feed was changed to small crumbles until the last day of the clinical test. Moreover, during the first 4 days of life the feed was also provided on sheets of paper placed on the bedding in order to stimulate the food intake. The water was provided at a temperature of 20℃ and was changed 3 times a day during the first 3 weeks and 2 times a day during the last 2 weeks of the study. The temperature on the ground was maintained at 32-35℃ during the first week of life, after which it was reduced by 5 degrees with each week.

The testing followed a unicentric, randomized, cross-over protocol with two treatments and a pause between them (Fig. 1). The used protocol was adapted to evaluate the immune stimulation of two nutraceutical products obtained from bovine colostrum: one of them containing a set of bioactive immunoglobulins, lactoferrin, lactoperoxidase and lysozyme (P1), while the other product though also based on bovine colostrum whey, it was devoid of proteins, with added

the added strain NCIMB 11974 of Lactobacillus plantarum (P2). The target species, according to the producer, were cattle, horses, sheep, goats, swine, canines and felines for P1 and all species of mammals, birds and fish for P2.

The protocol commenced with organizing the study (day 0), administering the products (day 1- 3), harvesting the first 15 serial blood and feces samples, 5 from each group (day 17). It is worth mentioning the fact that in accordance to the producer, the products were to be administered during the first 3 days of life and during stress phases. Moreover, as P1 did not have birds as a target species, the dose in which it was administered was the one proposed for P2: 1ml of product in 1l of drinking water. Group T1 was offered clean water with nothing added while T2 was offered water with P1 and T3 water containing P2. All chicks were kept under the same managerial, hygienic and environmental conditions, had ad libitum access to feed and water throughout and were maintained on a constant 24 hours light schedule. Also, the water was maintained at a pH of 4.4 for the duration of the study. It is important to state the fact that the chicks were vaccinated on the first day of life against the Newcastle disease and, for experimental purposes, it was decided against any other vaccination for the duration of the clinical testing.

Procedure	**Phase 1**											
Study day	0	1	2	3	4	5	6	7	9	11	14	17
Physical examination	●											
Live weighing	●							●			●	
Vital signs	●	●										
Clinical chemistry												●
Haematology												●
Microbiology												●
Serology												●
Product administration		●	●	●								●
Blood collection for drug concentration												●
Adverse events observing and recording		●	●	●	●	●	●	●	●	●	●	●
	Phase 2											
Study day	18	19	20	21	22	23	28	31	32	33	35	45
Physical examination												●
Live weighing				●			●				●	
Vital signs												●
Clinical chemistry								●				
Haematology								●				
Microbiology								●				
Serology								●				
Product administration	●	●										
Blood collection for drug concentration								●				●
Adverse events observing and recording	●	●	●	●	●	●	●	●	●	●	●	●

Fig. 1. – The schematic representation of the implemented protocol in testing two colostral nutraceutical products in Broilers

The birds were weighed every week and the mortality and morbidity were recorded if any. At the end of the clinical trial, economic calculations were made based on the feed costs and feed consumed, the cost of the administered colostrum for each group and based on the gained weight.

As blood harvesting is a well-known stress factors for birds, day 17 was also chosen for the second treatment. As such during days 17-19 the products were again administered. And on day 31, another series of 15 blood and feces samples were harvested. The final examinations were performed at the end of the second phase, including repeating the clinical and para clinical examinations (day 45).

The individual hematological samples were harvested thus: 2ml of blood on EDTA and 1ml on Heparin. In order to achieve this, we have resorted to the puncture of the brachial veins, with 24G needles which were suitable for the caliber of not only the veins but also the size of the bird erythrocytes. Each sample was clearly identified with a number, the test group, date at which it was obtained. The feces samples were also individual, they were obtained through cloacal swabbing and deposited in sterile containers until processing.

The processing and analysis of the blood samples included two sets of investigations. The first consisted of the analysis of hematological indices: blood cell count, smears, hematocrit and hemoglobin. All the samples were immediately processed without refrigeration. Once the serum and plasmas were obtained, they were however stored at -20°C for processing at a later date for the second set of investigations. The plasma was obtained by centrifuging the blood samples at 2500 rpm for 5 minutes.

Once the feces samples were collected, they were immediately processed in order to assess the total bacterial concentration and for identification of gram-negative and gram-positive strains. Furthermore, on day 17, an average sample was collected from each group so as to identify parasitic infections.

Results and discussions

According to the Committee for Medicinal Products for Veterinary use (Committee for Medicinal Products for Veterinary use, 2012) this study is considered to be an exploratory one as it has clear objectives, with no specific hypothesis, allowing data exploration during the analysis, contributing to the proof of concept yet needing more research in order to establish its efficacy. As such, though not necessarily subjected to the Good Clinical practices, it is mandatory to be pre-planned and ethical. Several studies that also researched the effectiveness of nutraceutical products in poultry integrated this step into the design of the protocol (Fatih et al., 2018, Vlaicu et al., 2017, King et al., 2005).

Even though some studies have chosen to perform the trials on either male or female chicks (Gaucher, 2015, Torok et al., 2008), randomizing the individuals is recommended and practiced by many other researchers (Fatih et al., 2018, King et al., 2005, Campbell et al., 2004). The Guidelines on statistical principles for clinical trials for veterinary medicinal products clearly state that randomization "help(s) to avoid possible bias in the selection and allocation of subjects arising from the predictability of treatment assignment".

According to Dr. Jacquie Jacob from the University of Kentucky, wood shavings is the best bedding for poultry is wooden shavings as it is nontoxic to the birds, very absorbent, has reduced thermal conductivity(Jacquie, 2015). The only downside of this bedding is the economical side as for some countries it has become expensive due to high demand. In this regard, not all researches done on Broiler chickens took this aspect into consideration (Quereshi et al., 2004, Oe et al., 1975).

Acidifying the water of birds is a well-known procedure in order to reduce the prevalence of necrotic enteritis and coccidiosis (Ayhan et al., 2019, Sugiharto, 2014, Gaucher, 2015). The feed that was provided to the chicks was mainly composed of maize and soy-beans which was formulated to meet or exceed the standards for major nutrients for broiler starters (King et al., 2005). As other researches, our feed was pressed into cold-pellets at 60°C and it was pre-conditioned at with steam addition at the temperature of 60-70°C.

The temperature maintained on the ground, along with the light intensity provided by neon lights and the infrared lights were in accordance with the Ross308 Performance Objectives. As most researchers, the light program was for 24h and the heating was maintained at 32-35℃ during the first week of life, after which it was reduced by 5 degrees with each week (Gheorghe, 2013, Insha et al., 2018).

The duration of this study was 45 days, which is the average lifespan of a Broiler chicken according to the National Chicken Council in the United States, in 2019. Similar clinical tests also conducted their research during the same amount of time (Fatih et al., 2018, Yesuf et al., 2017, Vlaicu et al., 2017). As far as the number of treatments that were conducted, each study is unique and individual, however in the current case given that fact that P1 is based on the absorption of antibodies in the bloodstream, literature shows that the highest titer of antibodies is reached around day 14 after administration (Quereshi et al., 2004). The standard brachial vein harvesting is mentioned in numerous studies, and as far as the storage is concerned, it differed depending on the upcoming tests (Kalia et al., 2018, Gheorghe, 2013). As in our case, the serum and/or plasma were kept at temperatures of -20°C until processing (Ghasemi, 2006). As far as P2 was concerned, the gut microflora can be collected at any date after the administration for of product. According to other studies which focused on this particular aspect, collected the samples around day 4, 21, 35 and in most situations, the samples were pooled into an average sample (Maciuca et al., 2015). In our case, given the limited amount of individuals it was possible to obtain both and individual and a pooled sample.

A clinical trial is not relevant unless a control group is set. As such, our research was comprised of 3 groups, T1, T2 and T3, where T1 was the control group and did not receive any additives in their diet. All the studies taken into consideration also had this group well represented in the protocol (Yesuf et al., 2017, Quereshi et al., 2004).

For the duration of this study we chose to not vaccinate the broilers unlike the standards for poultry (Yesuf et al., 2017, Gaucher, 2015). We believe that this will bring a higher value to the results obtained during this trial. It is important to mention at this point that we had no mortality for the duration of the study, with no significant morbidity to report that was relevant to the results of the study. Weighing the chicks is an important step in a protocol for animals raised for economical purposes and in our study, we performed the live weighing every week (Fatih et al., 2018, Yesuf et al., 2017). Again, this step is different among other studies where this process was done every other week.

Conclusions

We consider that, in order to obtain relevant and consistent scientific information on which the medical society can rely, protocols for testing of nutraceuticals on experimental animals or target species should be developed and even standardized. This desideratum is also essential for poultry breeding because it can significantly contribute to limiting the use of medicated feed (with sub-dosage antibiotics) for preventive purposes or as growth promoters. We also appreciate that it is very important to prove the efficacy and safety of nutraceutical products, including those from bovine colostrum, before they are placed on the market. Given that this study has conducted

extensive and up-to-date documentation on the implementation of clinical trials on poultry, we consider that this protocol is in line with current conduct and the data obtained is relevant and highly applicable.

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Epidemiology of bovine mastitis in cows in Transylvania during 2014-2019

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Abstract
Bovine mastitis is still a problem, both in terms of economic losses, animal health and welfare the increased risk of human health. Based on these affirmations, the purpose of the study was to establish the prevalence of the main etiological agents involved in the pathogenesis of bovine mastitis, while also evaluating their antimicrobial susceptibility and choosing the most effective antibiotic regarding epidemiological control. This study was based on a retrospective analysis of bovine mastitis cases registered at the Department of Microbiology, Faculty of Veterinary Medicine in Cluj-Napoca, during five years (2014 - 2019). A total of 347 mastitis milk samples from Transylvanian dairy farms and private owners were registered within the laboratory. For the total number of examined samples, a percentage of 89% were represented by Gram-positive bacteria: Staphylococcus spp., 53% (183 samples), Streptococcus spp., 24% (84 samples), Bacillus spp. 12% (42 samples) , and a percentage of 11% were represented by Gram negative bacteria E.coli 7% (23 samples) and Klebsiella spp., 4% (15 samples). After the isolation and identification of the pathogens, antibiotics susceptibility testing was performed by the Kirby Bauer disk-diffusion method, using the following antibiotics: Amoxicillin and Clavulanic Acid, Ceftiofur, Florfenicol, Mastidiscs, Enrofloxacin, Penicillin and Cloxacillin. Antimicrobial susceptibility test for the total isolates revealed good sensitivity to Florfenicol, Enrofloxacin and Mast discs. Resistance was observed for Penicillin and Cloxacillin.
Keywords: *bovine mastitis, epidemiology, etiological agents, antibiotic susceptibility.*

Introduction

Mastitis, the inflammation of the mammary gland, is one of the most serious economic animal health problems affecting the cattle industry worldwide, with incidence in cattle herds increasing since at least 3100 BC (Nemet-Nejat, 1998). There are two types of mastitis: clinical and subclinical mastitis (Mahantesh, 2014). Clinical mastitis is detected by the changes in physical appearance of milk, whereas cows with subclinical mastitis do not exhibit any major changes in milk or udder and can be detected only through laboratory tests (Reza, 2011). Different methods, based on physical and chemical changes of milk and isolation of organisms through culture are used for diagnosis of subclinical mastitis.

From the etiological point of view, the major pathogens involved in cows and other animal's mastitis are: *Staphylococcus aureus*, *Streptococcus* species and the members of the *Enterobacteriaceae* (Quinn, 2002).

In Romania, there are an increasing number of studies in the field of antimicrobial therapy using natural products (propolis or different plants extracts) (Paşca, 2017), but the common treatment is the intermammary administration of antibiotics into infected quarters of the udder.

The main objective of this paper was to evaluate the epidemiological and *in vitro* antimicrobial susceptibility of some Gram-positive and Gram-negative bacteria isolated from bovine mastitis in Transylvania over a period of five years (2014 – 2019).

Materials and methods

The study was carried out in Cluj-Napoca County, Faculty of Veterinary Medicine Cluj-Napoca, Department of Microbiology, and is based on a retrospective analysis of bovine mastitis cases. A total number of 347 mastitis milk samples from Transylvanian dairy farms and private owners were registered within the laboratory over a period of five years.

Mastitis milk samples were individually collected in separated sterile samples collected tubes. To identify the pathogens in clinical samples the material was most often cultured on blood agar and MacConkey agar plates. After 24 hour incubation in aerobic conditions, at 37ºC the identification of the isolates was performed using microscopic, cultural and biochemical methods.

The microscopic examination of the bacteria was done using Gram staining method. Biochemical identification was based on API 20 Biomerieux system (Bio Mérieux, France). For testing the sensitivity of microorganisms to antibiotic Kirby Bauer difusimetric method on Mueller Hinton agar plates, using bioMérieux disc diffusion were used; the antibiotics tested were represented by Amoxicillin and Clavulanic Acid, Ceftiofur, Florfenicol, Mastidiscs, Enrofloxacin, Penicillin and Cloxacycline.

Results and discussions:

Phenotypic identification of the pathogens. For the total number of examined samples, 89% are represented by Gram-positive bacteria: *Staphylococcus spp*., 53% (183 samples), *Streptococcus spp*., 24% (84 samples), *Bacillus spp.* 12% (42 samples), and 11% of samples examined Gram-negative bacteria: E.coli 7% (23 samples), Klebsiella spp., 4% (15 samples).

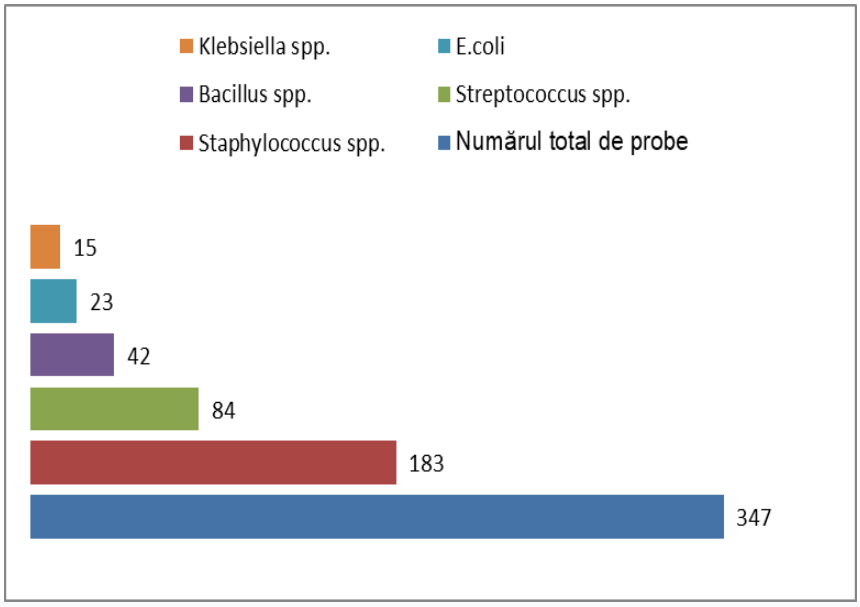


Fig. 1 Frequency of bacterial genus isolated for the total number of examined samples

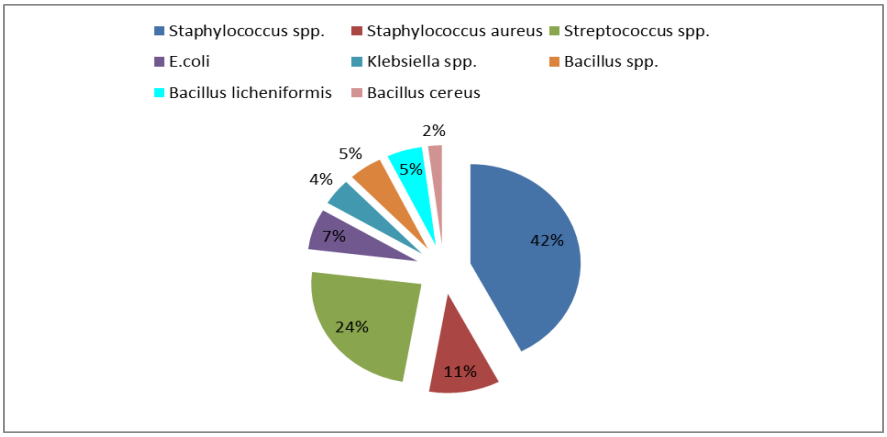
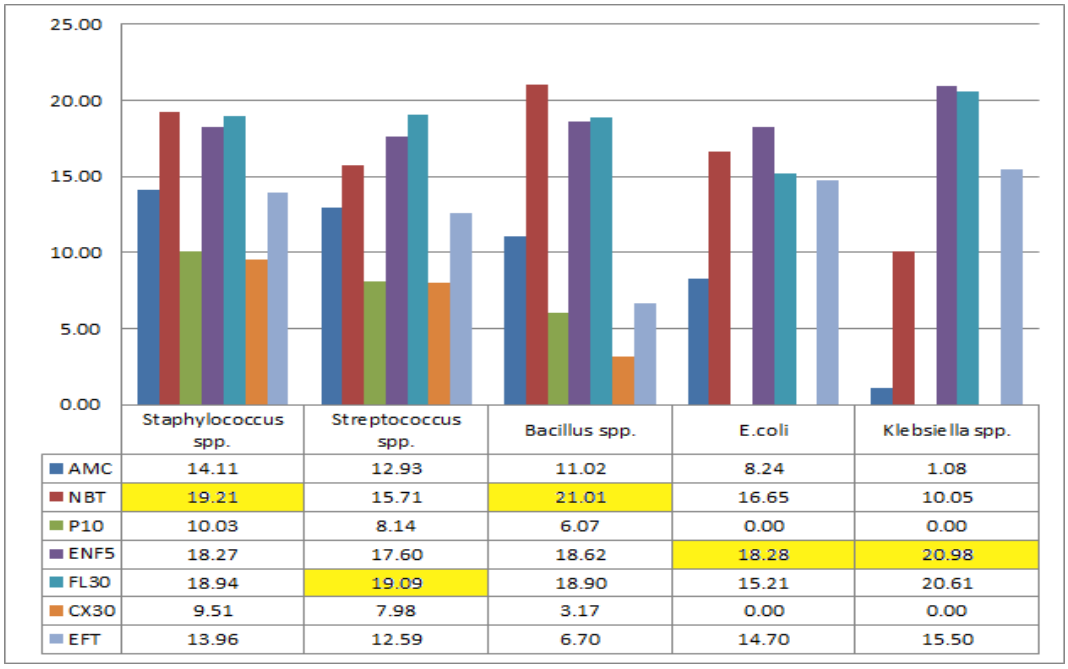


Fig.2 The aetiological agents isolated from de mastitic milk samples

Antimicrobial susceptibility. Regarding the the antimicrobials susceptibility test our data (Fig.3) showed that the most efficient antibiotics were:

- Mastidisc, follow by Florfenicol from gram positivs *Staphylococcus spp.* and *Bacillus spp.*;
- Fluorfenicol and Enrofloxacin for *Streptococcus spp.*;
- Enrofloxacin and Mastidisc for Gram negative *E.coli* strains;
- Enrofloxacin and Fluorfenicol for Gram negative *Klebsiella spp.*strains.



	Staphylococcus spp.	Streptococcus spp.	Bacillus spp.	E.coli	Klebsiella spp.
AMC	14.11	12.93	11.02	8.24	1.08
NBT	19.21	15.71	21.01	16.65	10.05
P10	10.03	8.14	6.07	0.00	0.00
ENF5	18.27	17.60	18.62	18.28	20.98
FL30	18.94	19.09	18.90	15.21	20.61
CX30	9.51	7.98	3.17	0.00	0.00
EFT	13.96	12.59	6.70	14.70	15.50

Fig. 3 Diameter of the inhibition area (mm) for the antibiotics tested for each pathogen

Legend: AMC (30µg/disc): Amoxicillin + clavulanic acid; NBT (30µg/disc): Mastidisc (neomycin, bacitracin, tetracycline); P (10µg/disc): Penicillin; ENF (5µg/disc): Enrofloxacin; FL (30µg/disc): Fluorfenicol; CX (30µg/disc): Cloxacillin; EFT (30µg/disc): Ceftiofur

Corcerning the resistance, the less efficient antibiotics were represented by:

- Cloxacilina şi Penicilina for both Gram positivs and Gram negativs bacteria;
- Ceftiofur for *Bacillus spp.*;
- Amoxicilin + clavulanic acid for Gram negativ *Klebsiella spp.*

One of the most important problems worldwide in the field of treatment is the antimicrobial resistance. Therefore, in the last period significant progress were performed and the majority of research in the field has focused on control of bovine mastitis, much efforts require developing a new and effective therapeutic alternative.

Conclusions

Our study concerning the epidemiology of bovine mastitis in Transylvania during 2014-2019 concluded that:

● For the total number of 347 examined samples a percentage of 89% were represented by Gram positives bacteria *Staphylococcus* genus, especially *Staphylococcus aureus* (184 samples), *Streptococcus* (identified in 84 samples and *Bacillus spp* (42 samples); Gram negative bacteria, represented by *E.coli* and *Klebsiella* genus were isolated in a percentage of 11%;

● Antimicrobial susceptibility test for the isolates revealed high sensitivity to Florfenicol, Enrofloxacin and Mast discs.

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The incidente of Salmonella spp. in some poultry products

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Abstract
The goal of the proposed research was to determine the presence and variation of serotypes of Salmonella spp. in poultry meat and eggs which are commercialized in various units. In particular samples were taken from the commercial units placed in the Central Agricultural Market mun. Chisinau, were the poultry products are delivered from different districts of the country. Samples were taken from the refrigerated carcasses as well as current consumption eggs placed in the marketing network. The insemination of the lavages were made on cultural artificial medium as Salmonella Shigella Agar and Bismuth sulfite agar. The bacteriological investigations results have shown that about 12% of the samples from both exanimated poultry carcasses (including the samples of the depth of the muscle) as well as from eggs, demonstrated the presence of bacterial serotypes of Salmonella spp. The serotypes prevalence was S. infantis, S. enteritidis, S. typhimurium. The results confirmed the necessity for further multilateral and depth study of Salmonella spp. spreading in conjunction with the monitoring of public health sector.
Keyword: *carcasses, microflora, serotypes, samples, colonies*

Introduction

Both domestic and wild birds may be carrying and spreading Salmonella infections manifested through variable severe clinical forms that can often be fatal. It is noted that salmonellosis is in most cases the origin of food contamination in humans, the main sources being poultry and eggs contaminated with Salmonella spp. Despite the high performance of current technologies to slaughter birds, they still do not provide germ-free products. Theoretically, a healthy and rested bird doesn't have the bacterial flora in muscles and internal organs. In practice, this condition is not achieved because the sources of contamination of poultry meat are multiple and is difficulty to remove them completely, also to obtain a sterile egg production is not possible. Most common the contamination of chicken carcasses takes place in the slaughter where man constitutes the main element, being the bearer of an important microbial flora, including salmonella (skin, hands, nose, mouth, intestine). Other important sources of microbial contamination could be water, air, bird feathers, equipment, tools, and insufficient cleaned and disinfected vehicles.

Following, are listed some pathogens that are more frequently meet on poultry: Salmonella enteritidis, Campylobacter jejuni, Yersinia enterocolitica, Clostridium perfringens, Staphylococcus aureus, Listeria monocytogenes, and some species of Bacillus. Although Salmonella is recognized as the most important pathogen associated with poultry, nobody knows the exact incidence of disease in humans associated with the consumption of poultry meat. It is estimated that of all cases of salmonellosis, approximately 20-25% of Salmonella illness occur due to poultry consumption.

The evolution of microorganisms that contaminate meat is influenced by many factors. Reducing the level of contamination with pathogens can be achieved only by respecting measures focused on the code of good working practices, using standard operating procedures for sanitation and avoiding dangerous practices that can lead to the contamination of critical points.

Analyzing the egg contamination, it was determined that they can be contaminated both internally, by the body of the bird during the formation as well as from contact with objects in the environment. Most frequently contaminations are associated with microorganisms such as Salmonella, Micrococcus, Staphylococcus, Proteus, Escherichia. Taking into account the above

mentioned, the purpose of our research was to establish the incidence of presence of bacteria of genus Salmonella spp. in poultry products (meat and eggs) used for human consumption.

Materials and methods

As research material served meat samples taken from carcasses of poultry (chickens, broiler) sold in commercial units of Central Agricultural Market in mun. Chisinau, which were delivered by poultry enterprises, from different districts of the republic, specialized in poultry meat production. In total there were 65 samples collected and examined. Simultaneously, were collected samples of table eggs (50 samples) from the units specialized in eggs production. The insemination was made on artificial culture medium as Nutrient agar, Endo Agar, SSA (Salmonella Shigella Agar), Sabouraud dextrose agar, bismuth sulfite agar. As indicators for monitoring was to establish the presence and morphological structure of the bacterial colonies grown on the culture medium. Some investigations have been carried out in the laboratory of Clinical Department II, SAUM, the subsequent investigations of serotyping of Salmonella spp. were performed in the laboratory of microbiology of Republican Veterinary Diagnostic Center.

Fig.1 Colonies of Salmonella spp. (medium BSA), insemination from the surface and depth of the sample

Fig.2 Colonies of Salmonella spp. (medium SSA), insemination from the surface and depth of the sample

Results and discution

Monitoring the presence and morphological structure of colony of Salmonella spp. was studied both on the surface of the samples (poultry carcasses) as well as their depth. Some of the results of this study are shown in figures 1-4. The cases when the inseminations were performed on bismuth sulfite agar (figure 1 and 2) in all samples were detected growth of Salmonella spp. colonies. They were often placed in the form of chain or separated in piles their number having variations within at 122 to 265 colonies. However, when the inseminations were performed on samples taken from the depth the number of colonies had variations from 10 to 66.

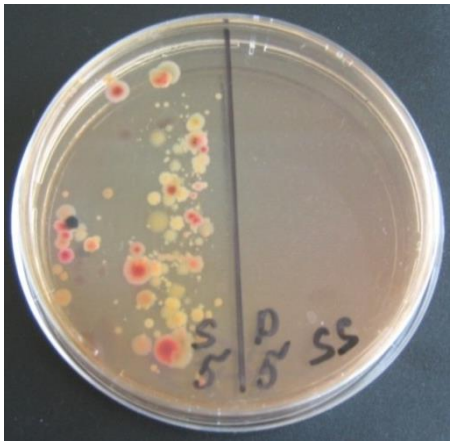

Fig.3 Colonies of Salmonella spp. (medium SSA), insemination from the surface and depth of the sample

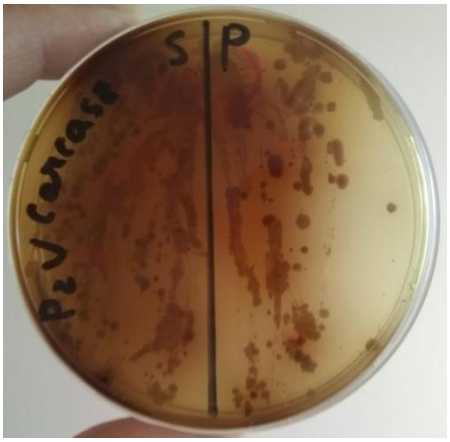

Fig.4 Colonies of Salmonella spp. (medium SSA), insemination from the surface and depth of the sample

On the Salmonella Sighella Agar (fig. 3 and 4), the number of colonies of Salmonella spp. had variation from 70 to 237 when the insemination was performed from the surface of the samples and from 0 to 82 colonies, when the insemination was performed from the depth of the sample.

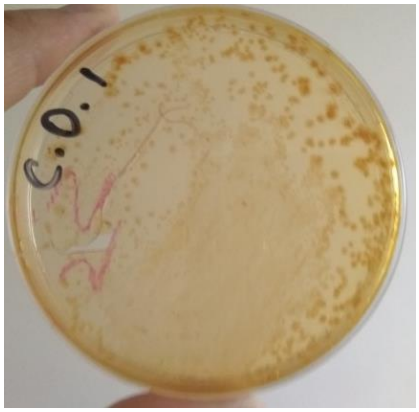

Fig. 5 Colonies of Salmonella spp. (medium SSA), insemination from the surface of the egg sample

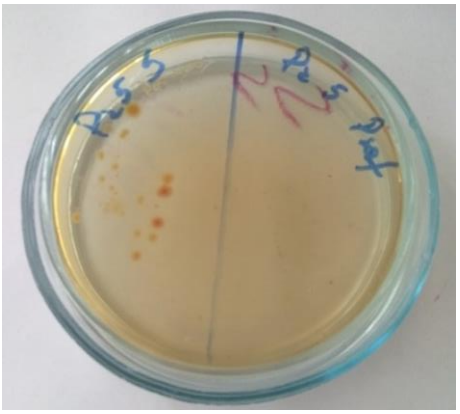

Fig.6 Colonies of Salmonella spp. (medium SSA), insemination from the surface and depth of the egg sample

Some of the researches which indicate the presence of colonies of Salmonella spp. in samples taken from the table eggs are presented in figures 5 and 6. In particular, an intensive growth of the colonies was established in the case where the insemination was performed from the lavages collected from the surface of eggs, with values varying from 44 to 315 colonies, with an evident and massive growth on Salmonella Sighiela agar medium. When the insemination was performed from the cavity of the egg, the number of colonies was less and had ranged from 0 to 25 coloni

Table 1. Serotypes of Salmonella spp. isolated from poultry productes

N. ord	Salmonella spp. serotipes	Products nime	Pozitive samples
1	Salmonella Enteritidis	Poultry meat	4
2	Salmonella Infantis	Poultry meat	10
3	Salmonella Winneba	Poultry meat	1
4	Salmonella Newport	Poultry meat	3
5	Salmonella Uppsala	Poultry ground meat	2
6	Salmonella Fillmore	Poultry meat	1
7	Salmonella typhimurium	Mechanically deboned poultry meat	1
8	Salmonella Dessau	Poultry meat	2
9	Salmonella Farsta	Mechanically deboned poultry meat	1
10	Salmonella Infantis	Meat products	5
		Total	**30**

Bacteriological research showed that in about 12% of the samples taken from poultry carcasses and eggs for consumption isolated colonies of Salmonella spp. were present.

Some results of salmonella spp. monitoring and their serotype evidence were performed in collaboration with laboratory of microbiology of Republican Veterinary Diagnostic Center. Table.1 presents data of positive samples taken from poultry carcasses and confirmed positive with Salmonella spp.

Results of table 1 show that from the total number of examined samples 30 samples were confirmed positive with Salmonella spp., specifically was detected serotypes as S. Enteritidis S. Tiphimurium that represents a danger to public health. However, the highest rate of contamination of poultry carcasses was with serotypes S. Infantis - 15 samples, that is 50% respectively of the total number of salmonella serotypes.

Conclusions

1. 1.The results of laboratory investigations confirmed that some pathogenic serotype of Salmonella spp., in particular S. enteritidis and S. typhimurium are persistent in poultry carcasses and in eggs of current consumption which represent a potential risk of birds and human contamination.
2. The microbiological tests of poultry carcasses and eggs sold in the Central agricultural market demonstrated that about 12% of the examined samples confirmed the presence of Salmonella spp., with prevailing serotype S. infantis, S. enteritidis and S. typhimurium, which confirms the need for multilateral examinations, including the public health sector.

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Evaluation of goat milk microflora in farms from Transylvania

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Abstract
Mastitis represents the inflammation of the mammary gland and is considered to be a serious disease due to the fact that it leads to the decrease of milk yield, with severe consequences on milk quality, increase of animal culling and therefore major economic losses. The aim of this study was to identify and describe the main microorganisms, as well as analyzing the Colony Forming Units in raw goat milk by collecting samples from 18 dairy goat farms from Transylvania, as follows: 6 farms from Alba County, 6 from Sălaj County and 6 farms from Cluj County. The milk samples were taken twice from each farm, gathering a total of 36 samples. The results showed that among Gram positive bacteria, 63.8% of the total number of samples were positive for Staphylococcus spp., 36.1%, for Micrococcus spp., 25% for Streptococcus spp. and in 19.4% of the milk samples was identified Bacillus spp. Other Gram positive microorganisms were isolated, but with a lower frequency: Kocuria spp. (8.33%), Aerococcus spp. (8.33%), Corynebacteruium spp. (5.55%) and Enterococcus spp. (2.77%). Results regarding the Gram negative bacteria showed that E. coli was the most frequently isolated microorganism, with a percentage of 38.9, followed by Klebsiella spp. with 25%, Hafnia spp. with 11.1%, Aeromonas spp. with 8.33% and Raoultella spp. with 5.55%. Data concerning the total number of germs showed that raw goat milk from the Transylvanian farms included in the study fulfils the hygiene criteria in the legislation.
Keywords: *milk, goat, microflora, bacteria.*

Introduction

Mastitis is defined as inflammation of the mammary gland tissue which occurs in response to various external factors such as intramammary infections, mechanical or thermal injuries. From a clinical point of view, mastitis is classified in two categories: subclinical mastitis (without visible clinical signs) and clinical mastitis (in which visible, local and even general symptoms occur, along with significant changes in milk secretion) (1). Regardless of animal species, udder diseases are considered to be limiting factors in the development of the dairy industry, which lead to major economic consequences due to significantly decreased milk production, poor milk quality and expensive veterinary treatments, severely affected animals being, most often, slaughtered (3,5). Moreover, the importance of the hygiene and safety criteria of milk and dairy products are worth to be mentioned, with the risk of illness occurring among consumers, due to the consumption of possibly contaminated products. Nevertheless, the legal aspects regarding the microbiological quality of milk should be implemented and respected accordingly (8).

An important aspect regarding the udder health is the concept of microbiota of mammary gland. In recent years, the sterility of healthy mammary tissue has been questioned and it has been disapproved by the results of several studies based on bacterial DNA. Therefore, research conducted on bovine milk confirmed the presence of an intramammary normal flora in the healthy udder composed of a variety of bacteria. Furthermore, it is stated that inflammation would be, most often, a consequence of dysbiosis of the microbiota rather than a primary infection (2,6,9). In order to confirm this hypothesis, it has been shown that the microbial flora in milk samples from healthy cows is different from that of cows diagnosed with mastitis. Moreover, the presence of several bacterial species such as *Staphylococcus aureus* and *Streptococcus uberis* in healthy bovine milk samples, with a low number of somatic cells, reinforced the idea according to which bacteria that

is normally found on the skin or intestinal mucosa are also a part of the commensal flora of the mammary gland (6). Despite the fact that numerous studies on bovine milk microflora have been published, research on goat milk is limited and sometimes contradictory. Based on these considerations, the purpose of the present study was to describe the overall goat milk microflora, but further reasearch is needed in order to fully understand and identify the bacterial population in the goat mammary gland.

Materials and methods

The study evaluated the microbiological quality of goat milk samples from 3 counties in Transylvania, with 2 successive samplings at 3 weeks interval. Bulk milk samples were evaluated from 6 farms in Sălaj County, 6 from Alba County and another 6 from Cluj County, with 18 milk samples per collection and a total of 36 included in the study. Sterile containers were used and appropriate temperature levels were conferred during the transport to the laboratory of microbiology, Faculty of Veterinary Medicine Cluj-Napoca.

The cultural examination was carried out after diluting 10 µl of milk in 990 µl of saline. One loopful of diluted milk was used to streak a blood agar Petri dish. The rest of the solution (1000 µl) was used to flood a Mueller Hinton Petri dish, removing the excess fluid. The plates were incubated for 24 hours at 37°C, examining the cultural and morphological characters of bacterial colonies and the presence of haemolysis areas.

Isolated colonies were identified by microscopic examination and biochemical characters. Gram staining technique was used for the preliminary identification of isolated colonies and biochemical characters were evaluated using API 20 Biomérieux system (Bio Mérieux, France) and Vitek 2 technique. Mueller Hinton agar plates were evaluated and colonies were counted using the automatic colony counter CC-1 and colony forming units (CFU) were calculated for each milk sample included in the study.

Results and discussions

A total number of 36 samples (100%) were positive for bacterial growth, with a higher prevalence of Gram positive species, most of these identified as cocci and rods, with Gram negative bacteria less frequently isolated. The identification of milk samples and their association with county origin and sampling interval was designated using the acronym CJ for Cluj County and 1 for the farm number for each county.

The results obtained following the analysis of cultural characteristics and the bacteriological examination showed that *Staphylococcus* spp. was the most frequently isolated Gram positive bacteria, followed by *Micrococcus* spp., *Streptococcus* spp. and *Bacillus* spp. Other Gram positive microorganisms were identified in the milk samples, but with a much lower frequency, such as: *Kocuria* spp., *Aerococcus* spp., *Corynebacterium* spp. and *Enterococcus* spp. On the other hand, *E. coli* had the highest prevalence among Gram negative isolates, followed by *Klebsiella* spp., *Hafnia* spp., *Aeromonas* spp. and *Raoultella* spp. with the lowest number of positive samples. In figure 1, the genera identified exceed 100% due to bacterial association, most frequently among *Staphylococcus* spp., *E. coli* and *Micrococcus* spp.

Table 1. The presence of Gram positive and Gram negative bacteria for each sampling, county and farm. Legend: AB – Alba County, CJ – Cluj County, SJ – Sălaj County

	Genus	Sampling nr. 1	Sampling nr. 2
Gram positive	*Staphylococcus* spp.	AB1,AB2,AB4,AB6,CJ1,CJ2, CJ3,CJ4,CJ5,SJ1,SJ2,SJ3,SJ4, SJ5	AB1,AB2,AB6,CJ4, SJ1,SJ2,SJ3,SJ5,SJ6
	Micrococcus spp.	SJ1,SJ2,SJ3,SJ4	AB3,AB6,CJ1,CJ2,CJ4,CJ5,CJ6,SJ1, SJ2
	Streptococcus spp.	AB3,CJ1,SJ2	AB1,AB2,AB3,AB4,CJ1,CJ3
	Bacillus spp.	AB1, AB5, SJ2	AB5,SJ3,SJ5,SJ6
	Kocuria spp.	SJ5	SJ3,SJ4
	Aerococcus spp.	SJ6	SJ5,SJ6
	Corynebacterium spp.	CJ1	CJ2
	Enterococcus spp.	CJ3	-
Gram negative	*E. coli*	AB3,CJ1,CJ2,CJ4	AB1,AB2,AB4,CJ1,CJ2,CJ3,CJ4,CJ6, SJ1,SJ2
	Klebsiella spp.	AB2,AB3,AB4, CJ3,CJ5,CJ6,SJ1	AB3, SJ3
	Hafnia spp.	CJ5	CJ5,SJ5,SJ6
	Aeromonas spp.	SJ3,SJ4	SJ4
	Raoultella spp.	SJ5,SJ6	-

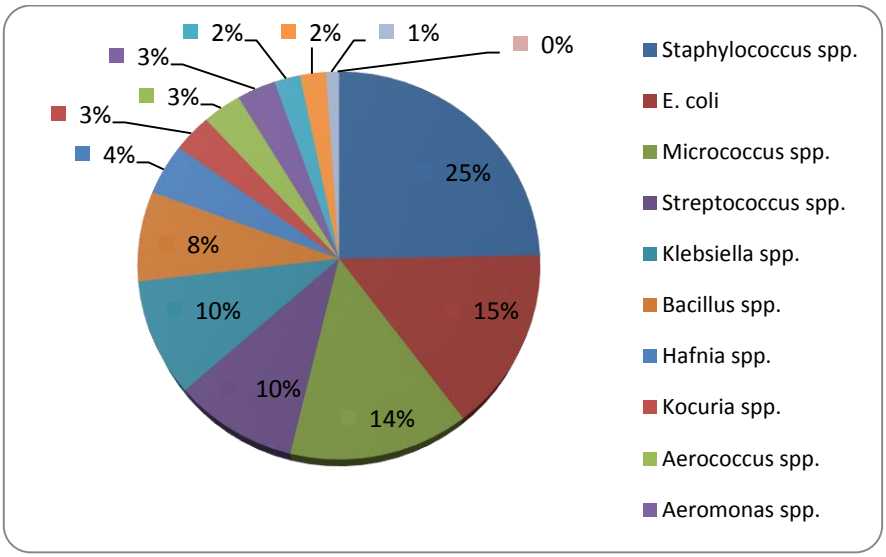

Fig.1. The overall percentage of bacterial genera identified from the total number of milk samples evaluated

Following the initial identification of bacterial colonies and the isolation of pure colonies, the biochemical characters were evaluated using the API 20 Biomérieux system (Bio Mérieux, France), as well as the Vitek 2 technique according to the protocols. Therefore, the following Gram positive species were identified: *Staphylococcus xylosus*, *Staphylococcus epidermidis*, *Kocuria varians*, *Kocuria rosea* and *Aerococcus viridans,* while the Gram negative tested strains have been identified as *Hafnia alvei*, *Raoultella planticola* and *Aeromonas sobria.*

Table 2. The average of CFU for each farm (1-6), sampling and county

Farm nr. / Average CFU/ml	1	2	3	4	5	6
Alba	27547.75	244087.1	154850.6	211411.6	282024.5	72519
Cluj	150356.5	11900	42350	139486	300713.4	194936.5
Sălaj	7050	34862.8	93038	118513.7	57434.7	289637

The results regarding CFU values in our study for the 36 milk samples had lower values compared to the accepted standard of CFU present in the European legislation for raw milk other than cow milk intended for human consumption (Reg. CE 853/2004). The proportion and genera identified in our study are consistent with the results obtained by Gelasakis et al. (3) and Marogna et al. (5). *Staphylococcus* spp. was the most frequently isolated, followed by *E. coli, Streptococcus* spp. and *Bacillus* spp., similar to the results obtained by Gosselin et al. (4).

Conclusions

The study of identification of microbial flora from raw goat milk in farms from Transylvania concluded that:

- Both Gram positive and Gram negative bacteria have been identified, with the predominance of Gram positive species;
- The milk samples from Alba County had a higher number of CFU/ml compared to the other two counties included in the study;
- Individual CFU values of the present study did not exceed the upper limit of the accepted number of CFU/ml according to the official regulation for raw milk from animal species other than bovine (Reg. CE 853/2004).

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Reactivity of mucosa-associated lymphoid tissue (MALT) in pigs that received in food black grapes seed and skin powder

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Abstract
The study was carried out on 20 pigs from the Petrain breed that received powders and skins of black grapes in 1% ratio for 3 months. At the end of the experiment, samples were taken from duodenum, jejunum, ileum, colon, mesenteric ganglia, which were fixed with 10% buffered formalin, included in paraffin, sectioned and stained with HE. Following the examination, there was a diffuse lymphoid infiltration into the lamina propria of the mucous membranes studied, agglomeration of lymph nodes in the submucosa of the jejunum, ileum and colon in pigs from experimental group (EG). lymphoid follicles from mesenteric lymph nodes are larger and more numerous in pigs from EG compared to control group (CG). Polyphenols from black grape powder in this experiment resulted in significant lymphoplasmocyte infiltration into the mucosa, digestive submucosa and mesenteric lymph nodes and increased carcass weight at slaughter by 1.08 Kg compared to LM.
Keywords*: MALT, black grapes powder, polyphenols, pigs*

Introduction

Vine by-products are a rich source of bioactive molecules, polyphenols and have a remarkable potential in the feeding of animals, being part of the class of food supplements [1]. Polyphenols are abundant in nature and extremely diverse. The term polyphenols includes over 8000 molecules which, by the presence of aromatic rings in their structure, carry one or more hydroxyl groups, with a pivotal role in mediating antioxidant properties [2], [3].

Polyphenols are normally produced by plants for their antibiotic and antifungal properties [4], [5]. Some studies have shown that a diet rich in phenolic compounds has numerous beneficial and therapeutic effects in various acute and chronic diseases [6]- [9].

In piglets, diets rich in polyphenols can cause morphological changes in the intestinal tract [10]. In particular, black grape pomace caused an increase in the size of colonic crypts suggesting better nutrient uptake and reduced activation of intestinal lymphoid tissue, with an immune-modulatory role. It has been suggested that the polyphenols from the plant products introduced into the pigs' diet improved the feed utilization ratio by modifying the intestinal microbism and anti-inflammatory effect [11].

The intestinal mucosa is a preferred gateway for the penetration of microorganisms and needs close monitoring by the immune system. The gastrointestinal tract-associated lymphoid tissue (GALT) consists of a diffuse population of lymphocytes and plasmocytes, antigen-presenting cells, present in the epithelium and the lamina propria of the mucosa, as well as agglomerations organized by lymphonodules in the small and large intestine, named Peyer patches (PP) [12]. They cooperate with a large network of lymph nodes, usually located in the mesentery, filtering the drained lymph from the intestinal wall. The protective function of GALT is extremely important for maintaining gastrointestinal tract homeostasis, in inflammatory processes, intestinal infections, ulcerative colitis etc. Experimental studies of the immune system of the intestine are of particular importance for the biomedical sciences and need a suitable model to reproduce the results used in medical applications. It seems that the best animal model for the study of the physiology and pathology of the gastrointestinal system is the pig [13], being omnivorous makes him closer to humans than to other animal species. Studies on the physiology of the immune system of the

intestine in pigs are also important for veterinary medicine, as this species is of particular economic importance, and the disorders of the gastrointestinal tract include a significant proportion of diseases in this specie.

Materials and methods

The study was conducted within a fattening farm on 20 pigs (10 males and 10 females), of which 10 animals represent the control group (LM) and 10 in the experimental group, in which 1% black grapes seed powder was administered for 3 months in the feed ration. Sampling was carried out within the slaughterhouse. After 3 months, the body weight of all pigs was registered and the pigs were slaughtered, after which blood and organs were taken. Samples from the segments of the digestive tract were taken: duodenum, jejunum, ileum and cecum. The fragments of the digestive tract were washed with a 0.9% sodium chloride solution, sectioned longitudinally, transversely, then transferred to the fixative solution (4% formaldehyde), processed by the paraffin inclusion method, sectioned at 5 μm and stained by the hematoxylin-eosin (HE) method.

Results and discussions

Histological preparations of the intestine were examined, the height of the intestinal villi (μm), the depth of the crypts (μm), the diameter of the lymph nodes in the intestinal mucosa and the mesenteric lymph nodes were measured. The results of the measurements are shown in table 1. CG duodenal villi are shorter, with a slightly increased diameter with lymphoid infiltrations in the lamina propria (Fig. 1A). Enterocytes have been surprised detaching from the tip of the villi. The villi originating from pigs of the EG are taller with smaller diameter, presenting a series of small lateral folds, compared to those of CG (Fig. 1B). Both CG and EG were found to have intraepithelial lymphocytes. The lamina propria from the periglandular area only in pigs from EG showed lympho-plasmocytic infiltration (fig. 1C, D).

Table 1. Variation of histological structures in pigs exposed to polyphenols

probes	height of the intestinal villi (μm)		depth of the Lieberkhun crypts (μm)		Lymph nodes (μm)	
	CG	EG	CG	EG	CG	EG
Duodenum	202.2±23.1	356.6±21.2	425±21.8	457,36±29.8		
Jejunum	146.4±25.1	325.3±23.4	380±24.1	445.4±27.9	300/245± 32.1/29.7	793.75/461.25± 29.2/27.2
ileum	125.2±19.1	156.4±19.2	393.4±26.8	420.6±29.1	213/156± 21.5/19.8	954.3/487.3± 32.1/28.9
mesenteric lymph nodes					200/300± 23.5/28.9	423.4/412,4± 25.1/26.7

The jejunum in pigs from EG shows large lymphoepithelial agglomerations at the level of villi (fig. 2A) in the lamina propria (fig. 2B) but also in the submucosa in the form of lymphonodule agglomerations (fig. 2C). The lymph nodes are numerically and larger in size than in the CG (Fig. 2D).

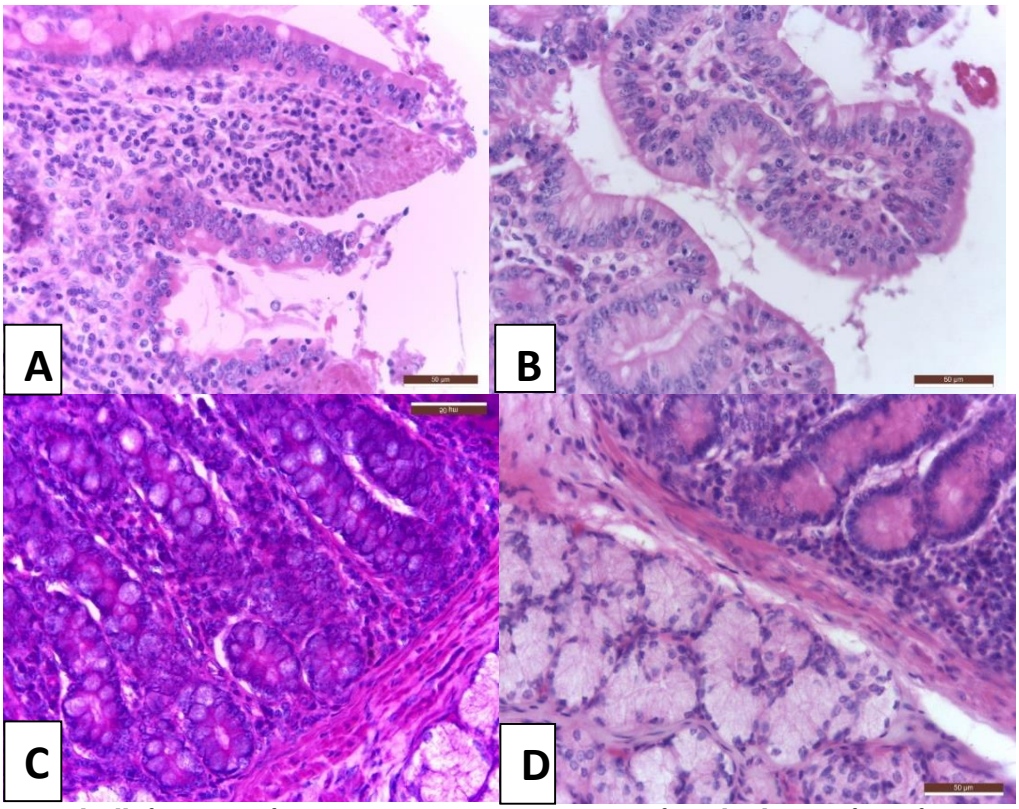
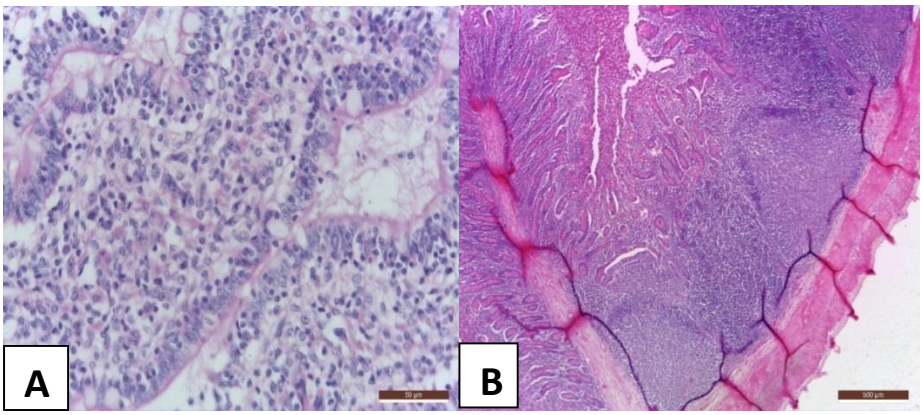


Fig. 1. Intestinal villi from pigs of A- CG; B- EG; C- Lamina propria from duodenum of pigs from CG; D- periglandular lympho-plasmocytic infiltration in EG. Col HE x100.



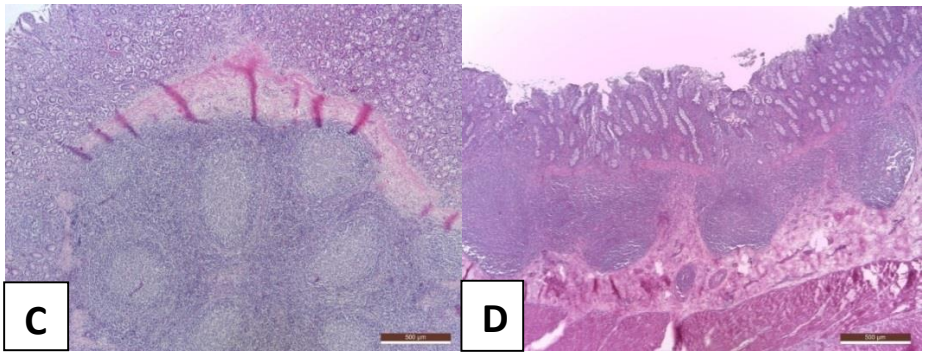


Fig. 2. Jejunum from CG and EG. A- Diffuse lymphoid infiltration into the lamina propria of intestinal villi at EG, HE x100; B- Large lymphoepithelial agglomerations in the mucosa and submucosa of the jejunum in pigs from EG, HE x40; C- Jejunum from pigs from EG with agglomerations of Peyer's patches in submucosa, HEx40; D- Peyer's patches in pigs from CG, HEx40.

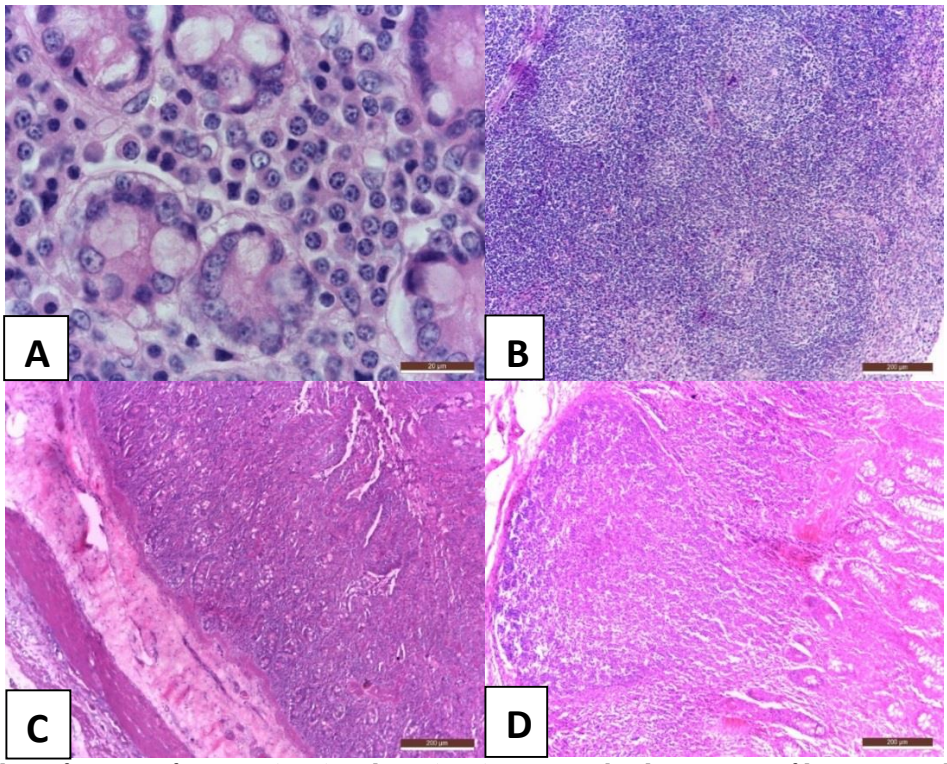


Fig. 3 Ileum from pigs from EG (A-B) and CG (C-D). A- Lymphoplasmocytic infiltration into the lamina propria, periglandular, HE x1000; B- Agglomerations of lymphatic follicles in the submucosa, HEx40; Ileum from pigs from CG. C- Mucosa, muscularis and serous submucosa, HE x40; D- Lymphoepithelial agglomeration in ileum at CG, HEx40.

The ileum from EG shows lymphoplasmocytic infiltration into the lamina propria, periglandular (Fig. 3A) and lymphatic follicles in the submucosa (Fig. 3B). At CG, smaller lymphoepithelial agglomerations were detected (Fig. 3C, 3D).

Histological examination of the colon in pigs from EG revealed a lymphoplasmocyte infiltrate appearance of the mucosa and large lymphoid agglomerations in the submucosa (Fig.

4A). In the pigs from the CG the colon in the examined sections did not presented such changes (fig. 4B).

In the mesenteric lymph nodes in the pigs from EG were surprised more lymphatic follicles and of larger dimensions (fig. 5A) compared to those from CG (fig. 5B).

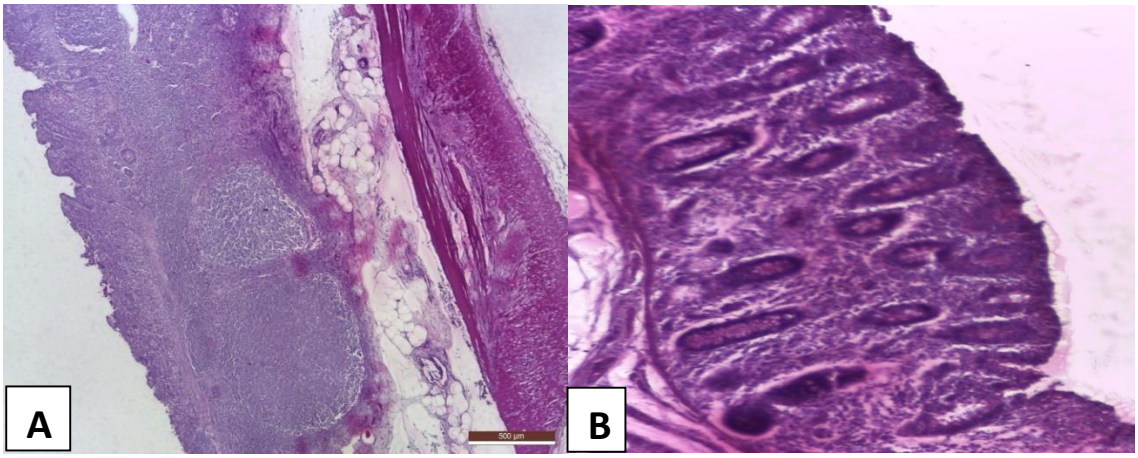


Fig. 4 Colon from pigs from LE (A) and CG (B). A- Lymphoepithelial agglomerations of the mucosa and agglomerations of lymph nodes in the submucosa, HEx40; B- Colon of pigs from CG without lymphoid agglomerations. Col HEx100.

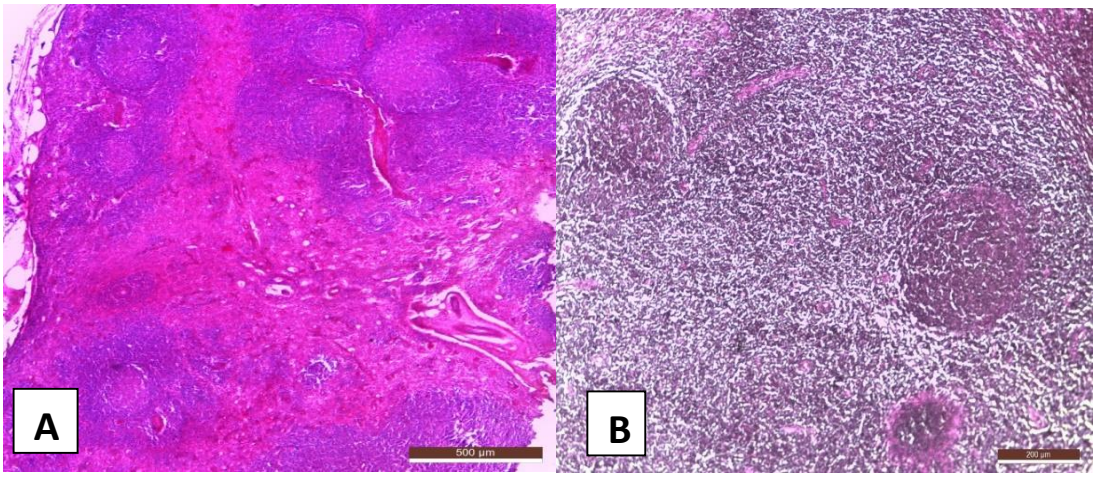


Fig. 5 Mesenteric lymph node in pigs from EG (A) and CG (B), HE x40 (A) x100 (B).

We mention that throughout the experiment the pig had no digestive and respiratory conditions. In addition the yield at slaughter was 1.08kg / individual higher at LE compared to LM.

Discusions

In this study, the inclusion of polyphenols in the diet resulted in an increase in the number of leukocytes in the lamina propria and intraepithelial in the EG. During intestinal inflammation, leukocytes are recruited to the site of infection or inflammation where, through complex interaction, contribute to attracting other immune cells and facilitates the healing of mucous membranes by releasing chemical mediators necessary for the inflammatory response. However,

reactivity can be beneficial in the case of the present experiment, although excessive recruitment and accumulation of leukocytes in the intestine under pathological conditions can be harmful [14]. It is commonly accepted that leukocytes contribute directly to the pathology of the disease when they are over-recruited and activated, leading to the release of toxic substances, massive trans epithelial migration, morphological changes of the villi and crypts and extensive mucosal lesions [14]. It is obvious that leukocytes can act as a double-edged sword that contributes to homeostasis by eliminating pathogens and participating in harmful inflammatory processes, processing and exacerbating inflammation by releasing pro-inflammatory mediators. Thus, the presence of numerous leukocytes in the lamina propria suggests that it is possible that polyphenols from grapes powder as well as those from olive mills [15] may not play a role in promoting inflammation in adult pigs. Our results are in agreement with previous research in the J774 monocyte / macrophage cell line, where different doses of hydroxyprosol have been proved capable of preventing the activation of macrophages [16]. Polyphenols exert a modulatory effect on the inflammatory response also in leukocyte [17], [18]. In fact, they are capable of deregulating the inflammatory response, keeping the tissues free of free radicals and thus preventing inflammatory cascade [19].

In pigs' jejunum, Peyer's patches are organized in the form of associated lymphoid follicles, while the ileum contains a continuous lymphoid follicle (lymphoid plaque) that extends from the distal ileum to the proximal colon [21]. The functional significance of such a lymphoid organization is not known, but it is assumed to play the barrier role of the small intestine (jejunum and ileum), in which the number of bacteria is moderate and of the large intestine where there is abundant microflora, which also contains potential pathogen microorganisms.

The functions of immune cells in the intestine are coordinated by a large network of regulatory substances, interleukins and chemokines, but they are also modulated by the enteric nervous system, which is involved in regulating inflammation and immunity during pathological processes [21-22]. Many lymphatic organs have innervation from cholinergic and adrenergic neurons [23]. Adrenergic and cholinergic nerve fibers also release neuropeptides as co-transmitters and neuromodulators that influence immune cells [24]. GALT cells express receptors for catecholamines, somatostatin, substance P, vasoactive intestinal polypeptide, galanin and neuropeptide Y, which modulate immunoglobulin activation, proliferation and / or synthesis and release [25].

Contact with invading microorganisms is crucial for the development and maturation of the immune system associated with the gut [26]. This system develops gradually in the first 6 weeks of life in several stages, depending on the diet and the environmental [27-29]. In newborns, the intestinal mucosa is very thin with rare lymphoid cells that present antigen for T cells that trigger a proper immune reaction in adults [28]. Their small intestine contains rudimentary Peyer patches (PPs), which non-specifically expand by rapid intrafollicular proliferation of B cells at two postpartum weeks [30]. Simultaneously with the significant numerical growth of B cells, the number of T cells inside the lamina propria and of the interfollicular areas is reduced [31]. The gradual emergence of conventional activated T cells is influenced by an influx of antigens that are presented by dendritic cells, together with major histocompatibility complex class II (MHC II +) and membrane markers CD45 and CD16 with which it coexpresses [28]. Between the second and fourth week of life, there is an increase in the number of mature CD4 + cells in the lamina propria, while CD8 + T cells grow significantly from 4 to 6 weeks of life [28]. Similarly, significant numbers of IgA + plasmocytes were detected after the fourth week of life [26], [30], although the occurrence of IgA-producing B cells in the lamina propria was reported on the sixth day life [30]. IgM + immunoblasts appear earlier, but exceed the number of IgA + B cells after 3 weeks [26], [32]. The maturation of immunity in the weaned pigs, as a result of the interaction with

environmental antigens and the attainment of the adult-like immunocompetence is installed from 7 to 9 weeks of life, determined either by the distribution of immune cells in the small intestine [33] or by functional in vitro analyzes [32] and by defining the role of the lamina propria as a mucosal effector site for bone vaccines [34]. Lymphocyte types vary by area and age. Thus, CD4 + lymphocytes were rare in the follicles and moderately numerous in the interfollicular area. CD8 + lymphocytes were sporadically seen only in the lymph follicles, but were numerous in the interfollicular area. CD21 + (LB) lymphocytes were very numerous in the follicles. No age-related differences were observed between the experimental groups from 3 days to 4 months [22].

To date, there is sufficient evidence to support the anti-inflammatory effect and immune stimulation activity of polyphenols [35], [36], [37]. The ability of polyphenols to act as antioxidants or free radical scavengers, as well as their ability to inhibit some enzymes involved in the generation of free radicals, such as cytochrome oxidase P450, lipoxygenases, COX, are due to the hydroxyl groups that are good hydrogen donors [38]. Polyphenols exert their multiple anti-inflammatory properties by modulating mitogen activation protein kinase and nuclear factor-j. Another way is to inhibit inflammatory cytokines and chemokines, suppressing the activity of inducible cytokine oxidase synthetase and COX [39], [40], [41]. COX-2 is an inducible enzyme that converts arachidonic acid into prostaglandins and is generally induced at the site of inflammation in response to inflammatory stimuli including pro-inflammatory cytokines such as interleukin-1 αβ / b, interferon-c, and tumor necrosis factor by inflammatory cells and tumor promoters such as tetradecanoyl phorbol acetate and Ras [42]. In the study of Varricchio et al. [15], COX-2 immunoreactivity was detected in leukocytes infiltrated into the mucosa, Peyer's patches and solitary follicles of the cecum and colon in pigs fed the control diet. In pigs fed a diet rich in polyphenol, COX-2 immunoreactivity was quite weak. The low level of COX-2 expression in intestinal immunoreactive cells in polyphenol-treated pigs may suggest a protective role of polyphenols, modulating and reducing the inflammatory response. This is in agreement with the research by Willenberg et al. [43], who reported the reduction of COX-2 expression induced by polyphenols both in vitro (HCA-7 cancer cell line and primary monocytes) and in vivo (C57BL / 6N mice). In vivo the effect of polyphenols on the morphology of intestinal villi is controversial. Fiesel et al. [11], report the absence of changes in the height of the villi and the depth of the crypts in the small intestine of the weaned pigs to which grape seeds and pomace flour extract were introduced into the feed, while Sehm et al. [10] report that in the piglets the red grape seeds had an inhibitor effect.

Conclusions

Polyphenols in seeds and black seeds powder cause increased diffuse lymphoid infiltration into the lamina propria of the intestinal mucosa. Also lymphatic follicles larger in volume and more numerous in the mesenteric lymph nodes of the pigs from EG were noted. There was also an increase in the height of intestinal villi, which explains the slightly increased sacrifice efficiency at EG compared to CG.

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The benefits of applying the microscopic examination in the analysis of meat products

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Abstract
The microscopic examination of meat products allows the identification of tissue structures and, to a certain extent, of the unauthorized content of plant and animal origin, the detection of parasites, the quality evaluation of the meat having undergone processes of freezing and thawing, the detection of muscle degeneration, the detection of foodstuffs adulteration and of the dangerous ingredients in meat products. The purpose of this study was to emphasise the importance of the results attained by microscopic evaluation of the integrity and quality of the meat used for meat products, the detection of the constituents of animal and vegetal origin, of the non-authorized tissues in the analysed products using routine staining as well as special staining. A total of 22 samples from different categories of meat products, represented by: boiled and smoked products (n = 5), cooked and double-smoked products (n = 2), raw-dried meat products (n = 6), baked and smoked meat products (n = 3), smoked meat products (n = 6), were randomly purchased from the commercial network within their validity period. All samples were processed using the routine paraffin inclusion technique, initially stained with HE (haematoxylin - eosin), special Masson's trichrome stain (connective tissue) and GMS II (Grocott Methenamine Silver, for the revealing of fungi). Microscopically, in the examined sections, there were highlighted different types of connective tissue (adipose and fibrous) in all of the samples, serous glandular tissue in baked and smoked meat products and in raw-dried meat products, visceral fragments (kidneys) in baked meat products and in raw-dried meat products, parasitic structures (Sarcocystis spp.) in baked and smoked meat products and in smoked meat products, fungal fragments in baked and smoked meat products and in smoked meat products, their presence being unauthorized. We consider that such an analysis, if appropriately performed and interpreted, can provide objective information in order to verify the compliance of these products with the legislation in force and to ensure the accurate composition and the integrity of these foods. By means of routine microscopic examination, the HE technique, non-compliant structures of animal origin were identified: visceral fragments and parasitic structures, Masson's trichrome stain method allows a better highlighting of the connective tissue, and the GMS 2 technique can be used successfully for highlighting the fungal structures in meat products..
Keywords: *microscopic techniques, quality, meat products.*

Introduction

Microscopic methods, along with chemistry, immunochemistry and molecular biology, still represent an alternative and, in some cases, a less costly alternative to food products examination and control. The history of food microscopy dates back to 1850, when Hassel used the microscope to distinguish chicory from coffee (Pospiech M, et al, 2011). In meat products, microscopic techniques have been used since 1910 in order to detect their contamination or intentional adulteration (Tremlova B, 2003). In Europe, for instance, Clinquart presented an overview of research in the same field, food analysis. 75% of the studies were performed on meat products, 25% of which were made using histology as a discipline (Clinquart et al, 2006). This topic has been addressed by many researchers such as: Aguilera: Microstructural Principles of Food Production and Analysis, Flint: Microscopy of Food, (Flint O,1994) Tremlová: Histology of Food. (Tremlová B, 2006)

Currently, various bioimaging techniques are available for the microscopic determination of food components. Commonly, the most used method is optical microscopy. This allows the identification of all structures through their morphological characteristics. In addition, special staining allows the 'selected' structures to be highlighted, with colors different from those of the other parts of the product under examination. (Pospiech M, et al., 2011).

The interest in identifying the composition has increased and many people are concerned about the meat and meat products they consume (Ballin NZ, 2010). In this respect, several studies having the microscopic examination as a method of study were published. Among these , Prayson studied the composition of hotdogs (Prayson BE, 2008a), hamburgers (Prayson B. 2008b) and other meat products (Malakauskiene S, 2016, Ghisleni G, et al., 2010). Using the same technique (Abdel Hafeez H et al, 2016) there are detected lung, ruminant stomach, large elastic blood vessels, myocardium, cartilage, spongy bone and lymphatic tissue (spleen) in meat sandwiches. Avinee et al. examined six samples of Merguez and Chipolatas sausages.

All samples contained fragments of fibrotenous tissue and parts of bone and cartilage. It is noteworthy that no nervous tissue was found in the evaluated samples (Avinee G, et al., 2010). In the same context, the microscopic examination allowed the identification of parasites in fresh fish samples, in food intended for processing (fish paste) and in finished products (smoked salmon). The method allowed the identification of two important parasites *(Anisakis simplex and Kudoa spp*). (Delphine MO.et al., 2010).

For this reason, the microscopic examination was adopted in some developed countries as a complementary method of evaluating product integrity.(Pospiech M, et al., 2011).There are many means to process and prepare samples for microscopic examinations today, along with a variety of investigative techniques. For example, in the Czech Republic the microscopic analysis of foodstuff is monitored by a team of experts from the Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno. These researchers combine microscopy methods with state-of-the-art methods from other disciplines such as immunohistochemistry, image analysis and stereology ([Pospiech M, Lukaskova ZR, Tremlova B, et al., *Microscopic methods in food analysis.* MASO Int BRNO. 2011)

Materials and methods

22 samples from the commercial network were included in the study during the period of validity and were represented by: boiled and smoked products (n = 5), boiled and double-smoked products (n = 2), raw-dried meat products, (n = 6), baked and smoked meat products (n = 3), smoked meat products (n = 6). From the microscopic examination viewpoint, the samples were subjected to the routine method by inclusion to paraffin using a histoprocessor, sectioned and stained by conventional HE (hematoxylin - eosin) technique, and special Masson's trichrome stain (connective tissue) and GMS II (Grocott Methenamine Silver for the detection of fungi). The sections were examined with the Olympus BX 41 Microscope with integrated computer photographing system.

The characteristics of the sample, including: sample type and the recipe are recorded in table1.

Table 1. Label composition of analyzed samples.

No	Sample type	Product category by processing method	Label Composition
1.	Sausage with spices	Boiled and double-smokedmeat products (n=2)	pork, salt, spice (pepper, garlic, basil) food additive, preservative, sodium nitrate, edible natural membrane
2.	Highly seasoned sausage		pork, fat, water, spices, salt, stabilizator, antioxidant, ascorbic acid, sugar, monosodium glutamate, soy vegetable protein, animal protein, preservative: sodium nitrite, carmine, edible membrane
3.	Semi-smoked pork sausage	Boiled and smoked meat products products (n=5)	minced pork meat, water, lard, vegetable protein from soy, salt, spices, antioxidant (ascorbic acid, ascorbate sodium), monosodium glutamate), acidity regulator (citric acid), extracts form spices, flavorings, preservatives (sodium nitrite),
4.	Semi-smoked pork sausage		minced pork meat, water, fat, soy protein, spices, sugar, dextrose, flavor stabilizers sodium diphosphate, sodium polyphosphates, antioxidant, sodium ascorbate, preservative: sodium nitrite,
5.	Beer-sausages		beef, pork, rind, lard, soy vegetable protein, salt,spices, preservative: sodium nitrite,dextrose.
6.	Chicken pastrami		chicken breast 91% without bone, soybean protein, potato starch, water, salt, spices, flavor, sugar, sodium phosphate stabilizers, thickener, carrageenan, anti-agglomerant (calcium phosphate), antioxidants, sodium acidity, flavor enhancers
7.	Turkey pastrami		turkey leg, marine salt, natural spices, sugar, preservative (sodium nitrite)
8.	Sibiu Salami	Raw dried products (n=6)	pork, fat, salt, sugars, spices, ascorbic acid, sodium nitrite
9.	Sibiu Salami		pork, fat, salt, sugars, spices, ascorbic acid, brandy 0.4%, sodium nitrite
10.	Ardelenesc Salami		unknown recipe
11.	Sibiu Salami		pork, fat, salt, sugars, spices, ascorbic acid, sodium nitrite, starter culture
12.	Sibiu Salami		pork, fat, salt, dextrose, spices, sodium ascorbate, sodium nitrite
13.	Sibiu Salami		pork, fat, salt, dextrose, spices, ascorbic acid, sodium ascorbate, sodium nitrite, starter culture

14.	Traditional pork sausage	Baked and smoked (n=3)	pork, sodium nitrate, water, garlic, spice extracts, sugars (dextrose), flavour enhacer (monosodium glutamate and antioxidants)
15.	Traditional pork sausage		pork, sodium nitrate, water, garlic, spice extracts,
16.	Lamb sausage		lamb sausage unknown recipe
17.	Lamb pastrami	Smoked meat products (n=6)	unknown, traditional system from small producers
18.	Beef pastrami		young beef leg, coarse salt, bay leaves, thyme, white onion, hot chili
19.	Pork Pastrami		fresh lean pork leg, salt, bay leaves, thyme, hot pepper, onion, garlic
20	Beef pastrami		beef leg, salt, natural spices, sugar, preservative (sodium nitrite)
21	Pork Pastrami		pork leg 96%, salt, pepper, thyme, hot pepper, garlic, preservative (sodium nitrite)
22	Smoked chop		Boneless loin ingredients: salt, heat treated by smoking.

Results and discussions

The tissue types identified in each sample are summarized in Table 2. The most observed tissue of connective tissue (adipose and fibrous) (n=22), was detected in all samples. Among 22 studied samples, the observed unauthorized tissues were included: organs (kidneys) (n=1), Serous glandular tissue (n=3), *Sarcocystis spp*.(n=5), lymphoid tissue (n=2), fungal structure (n=3)

Table2. Tissues detected in meat products based on microscopical examination

Sample Type	Sarcocistis spp.	Serous glandular tissue	Organs (kidneys)	Adipose Tissue	Fibrous Tissue	Lymphoid Tissue	Fungal Structure
boiled and double-smoked products (n=2)	0	0	0	2	2	0	0
boiled and smoked products (n=5)	1	1	0	5	5	2	1
smoked meat products (n=6)	3	0	1	6	6	0	2
raw-dried meat products (n=6)	0	1	0	6	6	0	0
baked and smoked meat products (n=3)	1	1	1	3	3	0	0

Microscopical Findings

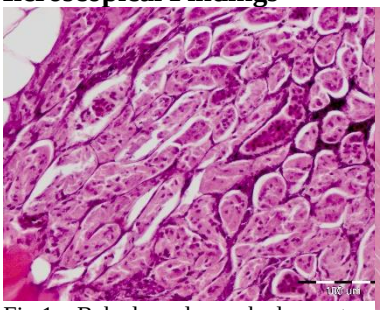

Fig.1. Baked and smoked meat products - Lamb Sausage - (kidney), (ob. 20, HE stain)

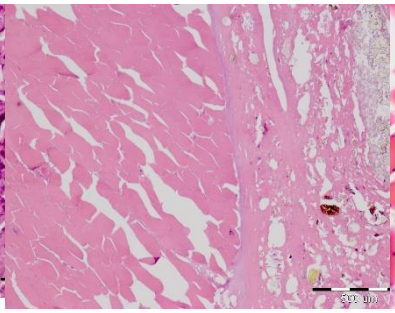

Fig.2. Boiled and smoked meat products - Turkey Pastrami – microscopical examination reveals muscle fibres with adipose tissue, and connective tissue (ob.10, HE stain)

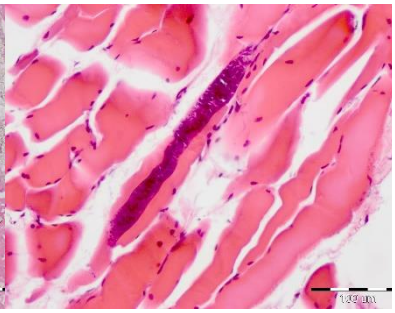

Fig.3. Baked and smoked meat products -
Lamb Sausage, intracellular parasite with a *Sarcocystis spp*. morphology, (ob.10, HE stain)

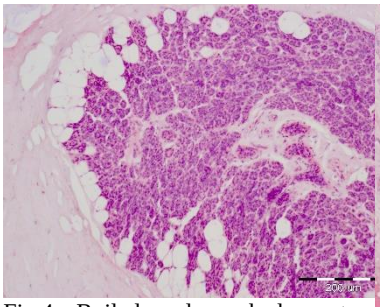

Fig.4. Boiled and smoked meat products- Pork Sausage with Serous glandular tissue, (ob. 10, HE stain)

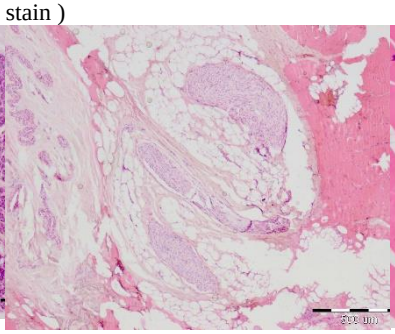

Fig.5. Raw dried products - Salami: microscopical examination reveals muscle fibers with homogenous eosinophilic to amphophilic sarcoplasm, nerve threads, conjunctive tissue and rare adipocytes, (ob.4, HE stain)

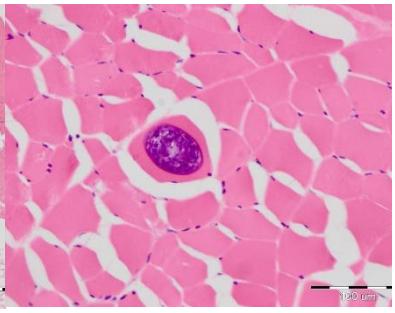

Fig.6. Smoked meat products -Beef Pastrami: in skeletal muscle the *Sarcocystis spp.* appearas basophilic bodies,round, bordered by a radial fairly thick wall (ob.20, HE stain)

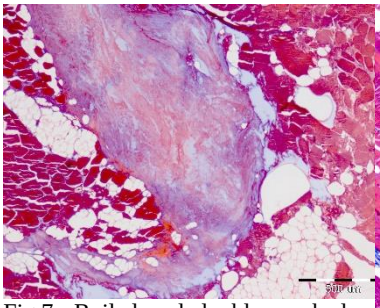

Fig.7. Boiled and double smoked meat products. Pork sausage with spices Connective tissue in abundant amounts with a collagen aspect fibres and with intense blue colour evidentiated by the special Masson's trichrome stain (ob. 10, Masson's trichrome)

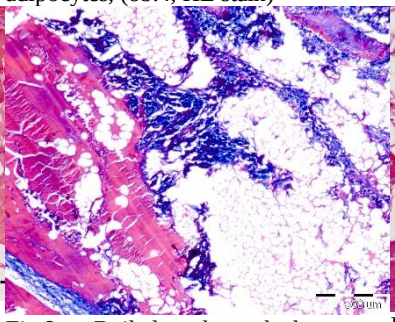

Fig.8. Boiled and smoked meat products.Pork sausage evidentiated by the special Masson's trichrome stain (ob. 10, Masson's trichrome)

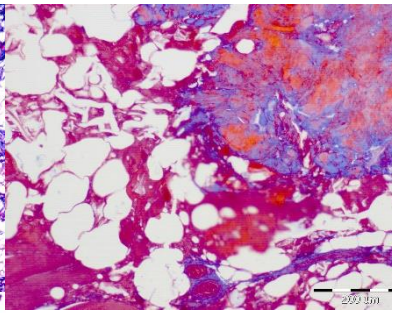

Fig.9. Baked and smoked meat products. Pork sausage connective tissue in a moderate amount, intensively colored blue areas with poorly conserved cellular histological architecture evidentiated by the special Masson's trichrome stain (ob. 10, Masson's trichrome)

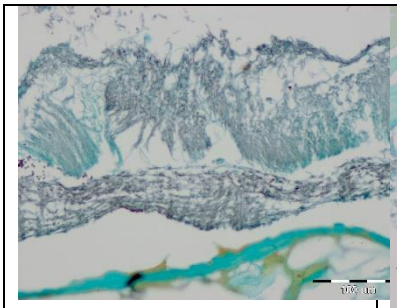

Fig.10. Baked and smoked meat products -Pork Sausage - structuri fungice localizate la periferia și în grosimea fragmentelor tisulare evidențiate prin colorația specială (ob. 20, GMS II)

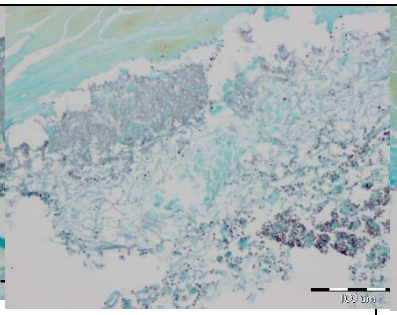

Fig.11. Boiled and smoked meat products - Chicken pastrami- the examined tissue fragments present an intensely oxyphil, filamentous mass with a tendency to penetrate the tissue (evidentiated by special staining (ob. 20, GMS II).

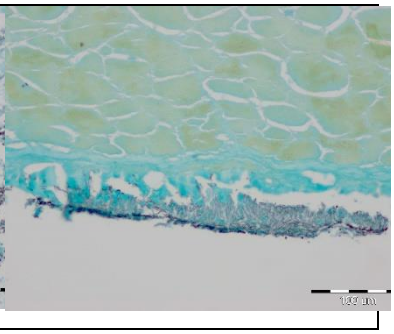

Fig.12. Smoked meat products - Beef pastrami - the periphery of the examined tissue fragments it is occupied by numerous microorganisms with fungal structures with a constant tendency to penetrate the depths of the muscular tissue, confirmed by special staining (ob. 20, GMS II)

The commonly used staining methods are hematoxylin-eosin (red shades) (fig.1, - fig.6,) normally used to highlight most morphological animal and plant components (shape, size and mutual cellular configuration, presence of crystals, granules, or other elements) in the meat products structures.

In some sections of raw dried products (salami), different tissues were evidentiated: striated muscle tissue, various types of connective tissue, adipose tissue in abundance, vascular structures and nerve fibres, in other sections of the same category of product, vegetal structures were revealed with morphology and tinctorial properties different from that of animal tissues. In sections of raw dried products stained by conventional haematoxylin-eosin, the homogeneous aspect of muscle fibers and , in an inconstant manner, their distance from the endomisium were observed, the appearance is associated with the dehydration process after treatment with salt.

The products from the meat processing industry are not exclusively composed of materials of animal origin. A simple microscopic observation with conventional H & E (hematoxylin and eosin) staining makes it easy to identify constituents of plant origin in their traditional form (Pospiech M, et al, 2011). Vanha J, et al., state that the identification of constituents in meat products, combined with an estimate of thier actual quantity, makes it possible to monitor the quality of meat products using the same staining (Vanha J, et al., 2011).

Meat products such as salami, sausages are prone to fraudulent practices. In this study in the microscopic examination of the products of the category: boiled and double-smoked products, boiled and smoked products, baked and smoked meat products, different tissues were found: connective (adipose and fibrous) in (fig.2), in (fig.5), however, a wide range of unauthorized tissue was detected including: glandular serous tissue (fig.4), lymphoid tissue, visceral fragments (kidney) in fig. 1, parasitic structures (*Sarcocystis spp.)* in (fig.3) and (fig.6).

Similarly, Atasever et al (1999) reported finding parts of organs that should not be included in the technological process of fermented sausages, according to the Regulation and Standard, such organs were found in eighteen (37%) of the fourty-eight fermented sausage samples they procured from the market . In the 50 sausage samples examined by Erdoğrul (2002), cartilage and bone tissue were found in 24%, adipose tissue in 50%, connective tissue in 10% and nerve tissue in 16%. (Erdoğrul ÖT, 2002).

In another study, 30 samples from three different types of sausages were assessed by microscopic examination. The most frequently observed additive tissues consisted of chicken skin, hyaline cartilage, peritoneal fat and kidney (Sepehri Erayi, 2008).

Sadeghi et al. used the histological method to examine 720 sausage samples in which unauthorized tissues such as adipose tissue, myocardium , cartilage, esophagus, salivary glands (Sadeghi et al., 2011) were identified.

A similar histological evaluation of hamburgesr, Kabab Loghme and minced meat , marketed in Tehran, Iran showed the presence of some unauthorized tissues such as blood vessels, nerves, cartilage, adipose and plant material (Sepehri Erayi, 2008) facts that are in accordance with our findings.

What attracted attention was the presence of round, intensely basophilic structures, identified in muscle fibers as *Sarcocystis spp*., in samples of the category of smoked meat (pastrami) and of the category of baked and smoked products. *Sarcocystis spp.* is an intracellular parasite in mammals, which represents a considerable infection rate especially in sheep and cattle. Human infection with *Sarcocystis* may be related to the consumption of raw, unprepared meat or meat products containing the enclosed/encysted parasite (Dehkordi ZS, et al., 2017).
The results showed that more than 80% of the tested samples were infected with Sarcocystis. The rate of infection in sausages and hamburger was 83.33% and 87.5%, respectively; the samples were treated with Giemsa staining and observed under the optical microscope (Guelmamene R, et al., 2018)

One disadvantage of basic staining is that the individual components are presented in different shades of a single color (Fig. 2.5.6). Also, if the product structure has been disturbed during processing, then identification can not be done with a high degree of certainty. The special staining allows highlighting the selected structure with a different color than other parts of the product. Food microscopy successfully uses a variety of staining methods. Calleja staining with green or blue Tricrom, Red alizarin can be used to detect the presence of bone fragments. Using these protocols, it is possible to monitor not only the dispersion of fat in the product, but also its formation in the layers of the outer coating, the special coloring with a Charvát modified Trichrome stain may expose other things, such as the presence of reprocessed products. (Pospiech M, et al., 2011).

The special staining used in the present study, were Masson thricrome stain, for the evidentiation of connective tissue in sausages, (fig. 7, fig. 8, fig. 9). and for the evidentiation of fungal structures in histological sections, GMS II (Grocott Methenamine Silver) staining (fig.10, fig.11, fig.12), was used.

Some studies have determined that microscopic methods are effective techniques for detecting unauthorized tissues in meat products. The organoleptic examination and the macroscopic examination, as well as the physicochemical technique, can not detect with precision the various tissue components in meat preparations such as minced meat. In this context, the microscopic examination is the only one that can detect tissue components and their position on the list of authorized and unauthorized products (Disbrey D.B. et al, 2000, Prayson BE, 2008a, Prayson BE, 2008b)

We consider that such an analysis, if properly performed and interpreted, may provide objective information as to verify the compliance of these products with the existing legislation and to ensure the composition and integrity of these foods

Conclusions

By means of routine microscopic examination, the HE technique, non-compliant structures of animal origin were identified: visceral fragments and parasitic structures, Masson's trichrome stain method allows a better highlighting of the connective tissue, and the GMS 2 technique can be used successfully for highlighting the fungal structures in meat products.

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