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TIMIȘOARA**

FACULTATEA DE MEDICINĂ VETERINARĂ

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2021**

VECTOR-BORNE PARASITIC INFECTIONS IN DOGS IN TIMIȘ COUNTY

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Summary

Vector-borne diseases are caused by a broad spectrum of infectious agents, including bacteria, viruses and the most relevant for this paperwork: parasites (protozoa and helminths). They are transmitted by a wide range of arthropod vectors such as mosquitos, fleas and ticks. The aim of this study was to investigate the prevalence of canine vector-borne parasitosis in Timiș county, Romania, from February to November 2020. In this study were included 300 dogs of both sexes, different ages and breeds, from different regions of the county, which presented or not clinical manifestations and were tested for *Dirofilaria immitis* / *D. repens*, *Ehrlichia canis*, *Anaplasma spp.*, *Borrelia spp.* and/or *Babesia spp.* Blood samples were collected and examined by Knott modified test, specific antigen RapidCaniV-4 test (VetExpert), and by light microscopy after a Diff-Quick staining or fresh blood smear. Vector-borne pathogens were detected in 8.33% of the examined dogs. The most prevalent pathogen was *Dirofilaria immitis*, 6.33%, followed by *Babesia canis*, 3%, and *Dirofilaria repens*, 0.33%. No other vector-borne pathogens have been identified.

Keywords: dirofilariasis, babesiosis, dogs, vector-borne pathogens

Canine vector-borne diseases, including babesiosis, anaplasmosis, ehrlichiosis, dirofilariosis and many others are transmitted by a wide range of arthropod vectors such as mosquitos, fleas and ticks and they can expand through climate change, wildlife migration, and increased relocation of companion animals throughout the world (6, 9, 13, 15).

Heartworm disease is a cosmopolitan parasitic infection of domestic and wild carnivores, caused by the filarial nematode *D. immitis*, and it is commonly found in pulmonary arteries and in the right ventricle. The symptomatology of this infection includes coughing, dyspnea and weakness, unlike the infection with *D. repens* which is generally asymptomatic. Although, in case of clinical manifestation, the most common signs are pruritis, localized alopecia or ulceration of the skin, as it is a subcutaneous parasite (2, 4, 5, 9, 15, 16).

Borrelia burgdorferi, the agent of Lyme disease, is transmitted by *Ixodes* ticks during the blood meal, and it causes an infection with no clinical signs (9, 13, 15).

Anaplasma phagocytophilum is also transmitted by *Ixodes* species, and it is the causative agent of granulocytic ehrlichiosis, which leads to severe acute illness (9, 13, 15).

Canine monocytic ehrlichiosis is caused by *Ehrlichia canis* and it spreads

through *Rhipicephalus sanguineus* ticks (13, 15).

Babesia canis is an intracellular hemoprotozoan of dogs that infects the erythrocytes of hosts and cause varying degrees of hemolytic anemia, hyperthermia, hemoglobinuria and several complications (1, 3, 6, 9, 10). The parasite is transmitted by *Dermacentor reticulatus* ticks (10, 11, 12).

The aim of this study was to investigate the prevalence of canine vector-borne parasitosis in Timis county, Romania, from February to November 2020.

Materials and methods

In this study were included 300 dogs of both sexes, different ages and breeds (Table 1), from different regions of the county (Fig. 1), which presented or not clinical manifestations and were tested for *Dirofilaria immitis* / *D. repens*, *Ehrlichia canis*, *Anaplasma spp.*, *Borrelia spp.* and/or *Babesia spp.*

Blood samples (Fig. 2) were collected and examined by fresh blood smear, Knott modified test (Fig. 3), specific antigen RapidCaniV-4 test (VetExpert) (Fig. 4), and by light microscopy after a Diff-Quick staining (Fig. 5, Fig. 6).

The statistical analysis was performed by GraphPad, QuickCalcs, Fishers exact test and Office Excel 2016.

Knott's modified test detects and allows identification of microfilariae in the blood collected in an EDTA tube or heparinized syringe. Modified Knott test is more eloquent than direct smear test because it concentrates microfilaria, so they are less likely to be missed during microscopic examination (5, 9, 18).

VetExpert Rapid Test CaniV-4 is an immuno-chromatographic test based on detection of *Dirofilaria immitis* antigen and *Ehrlichia canis*, *Borrelia burgdorferi*, *Anaplasma phagocytophilum*/ *platys* antibodies, from serum, plasma or whole blood. The result can be interpreted in 5-10 minutes. The sensitivity is actually very high, but if the infection is very light or the animal is in the prepatent period of the infection, false negative results may occur (5).

Even though antigen tests are very specific, they are not always sensitive, and modified Knott test requires time and experience to interpret (5, 9, 18).

While performing the Rapid test, for a reliable confirmation of heartworm disease, a fresh blood smear is examined under the microscope, to highlight the presence of microfilaria.

Table 1

Provenience, age, breed and sex of the positive dogs from this study

Nr crt	Name	Breed	Provenience	Age	Sex	Diagnostic	
						<i>Dirofilaria spp.</i>	<i>Babesia spp.</i>
1	Becky	Beagle	Timisoara	2 years	F		✓
2	Billy	Pechinez	Timisoara	5 years	M	<i>D.immitis</i>	
3	Bobby	Metis	Timisoara	8 years	M	<i>D.immitis</i>	
4	Bruno	Viszla with short hair	Timisoara	4 years	M	<i>D.immitis</i>	✓
5	Brutus	Pitbull	Sanmihai-Timis	7 years	M	<i>D. repens+D. immitis</i>	
6	Bundi	Metis	Timisoara	7 years	M	<i>D. Immitis</i>	
7	Fluffy	Metis	Timisoara	10 years	F	<i>D.immitis</i>	
8	Giusepe	Bull Terrier	Timisoara	12 years	M	<i>D.immitis</i>	
9	Hope	Metis	Timisoara	4 years	F	<i>D. Immitis</i>	
10	Jackie	Rottweiler	Dumbravita-Timis	6 years	M	<i>D.immitis</i>	
11	Jessie	Pechinez	Dumbravita - Timis	15 years	F	<i>D. Immitis</i>	
12	Lăbuș	Metis	Timisoara	12 years	M		✓
13	Lola	Metis	Sacalaz-Timis	4 years	F	<i>D.immitis</i>	
14	Lucky	Cocker Spaniel	Becicherecu Mic-Timis	8 years	M		✓
15	Luna	Metis	Timisoara	6 years	F	<i>D.immitis</i>	
16	Max	American Staffordshire Terrier	Timisoara	5 years	M	<i>D.immitis</i>	
17	Max	Ciobănesc German	Timisoara	14 years	M	<i>D.immitis</i>	
18	Nathasa	Ciobănesc de Asia Centrală	Timisoara	2 years	F		✓
19	Neagra	Metis	Timisoara	9 years	F	<i>D. Immitis</i>	
20	Oz	Brac German	Timisoara	7 years	M	<i>D.immitis</i>	✓
21	Pufi	Metis	Timisoara	13 years	M	<i>D.immitis</i>	
22	Rita	Metis	Sacalaz-Timis	10 years	F		✓
23	Roco	Metis	Dudeștii Noi-Timis	11 years	M	<i>D.immitis</i>	✓
24	Spyke	Amstaff	Timisoara	4 years	M	<i>D.immitis</i>	
25	Tupeu	Bichon havanez	Timisoara	3 years	M		✓



Fig. 1. The provenience area of dogs



Fig. 2. Blood samples collected in EDTA tubes

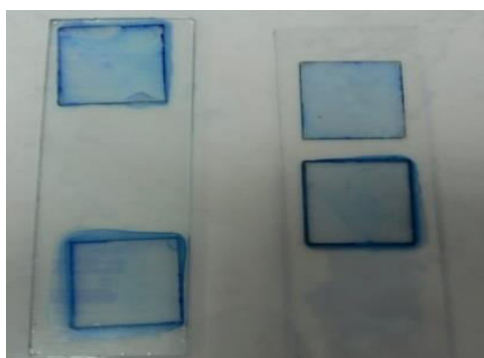


Fig. 3. Modified Knott test (left), *D. repens* tail (right)



Fig. 4. Specific antigen RapidCanIV-4 test – positive for *D. immitis*

For the diagnosis of *Babesia*, the Diff-quick smear is the most frequently used method (1, 3, 8). Examination is performed under the microscope with

immersion objective.

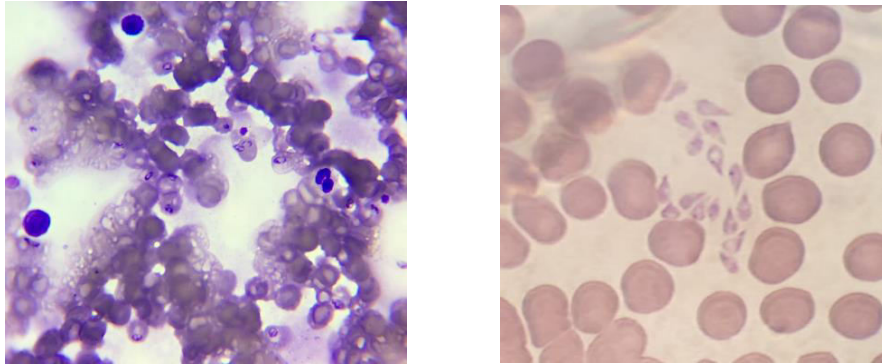


Fig. 5. *B. canis* (left and right), Diff Quick staining

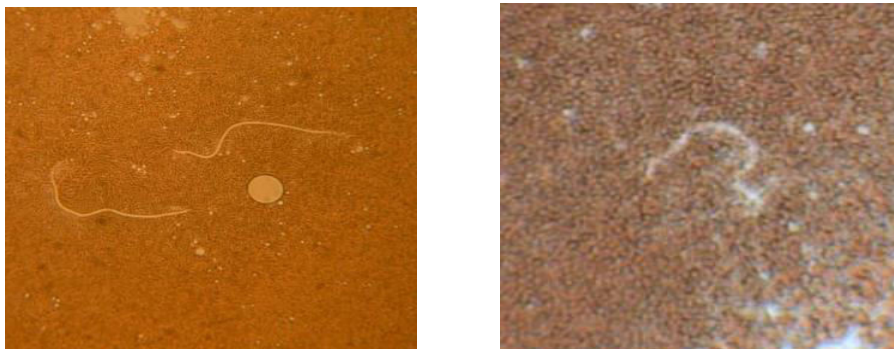


Fig. 6. *Dirofilaria* spp. (left and right), blood smear

Results and discussions

Vector-borne pathogens were detected in 8.33% (25/300) of the examined dogs. The most prevalent pathogen was *Dirofilaria immitis*, 6.33% (19/300), followed by *Babesia canis*, 3% (9/300), and *Dirofilaria repens*, 0.33% (1/300). No other vector-borne pathogens have been identified.

Statistical analysis performed by the program GraphPad, QuickCalcs, Fishers exact test showed that there were no statistically significant differences between positivity relative to the sex of animal were registered.

The prevalence values of pathogens from this study are graphically presented in Fig. 7.

On the other hand, the prevalence of pathogens in each studied locality was calculated (Fig. 8). In Timisoara, there have been 12 dogs tested positive for *D.*

immitis, 5 dogs for *B. canis* and 2 dogs with coinfection of *B. canis* and *D. immitis*, from a total of 190 dogs examined (7.37% *D. immitis*; 3.16% *B. canis*). In Dumbravita, there have been only two cases of *D. immitis* from a total number of 40 dogs examined (5%).

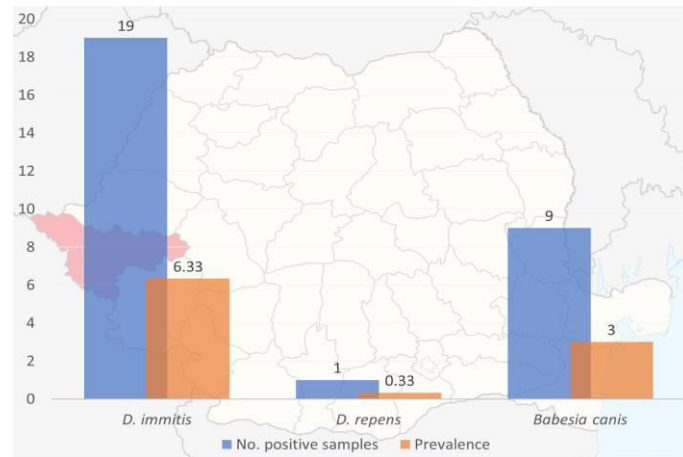


Fig. 7. The prevalence values of pathogens from this study

Sacalaz has a total number of 37 examined dogs, in which one was tested positive for *D. immitis* and one for *B. canis* (2.70% for both).

In Sanmihaiu Roman there has been identified one case in which there was a coinfection with *D. immitis* and *D. repens* from 15 examined dogs (6.67%).

Becicherecu Mic had one positive case of *B. canis* from 10 total dogs that were examined (10%).

In Dudestii Noi there was also one case, tested positive for both pathogens: *D. immitis* and *B. canis* (12.5%).

In the urban area (Fig. 8) (Timisoara) the prevalence of any infection with enumerated vector-borne parasitosis is 10% (19 positive cases out of 190), and in the rural area (Dumbravita, Sacalaz, Sanmihaiu Roman, Becicherecu Mic, Dudestii Noi) the prevalence is 6.36% (7 cases out of 110).

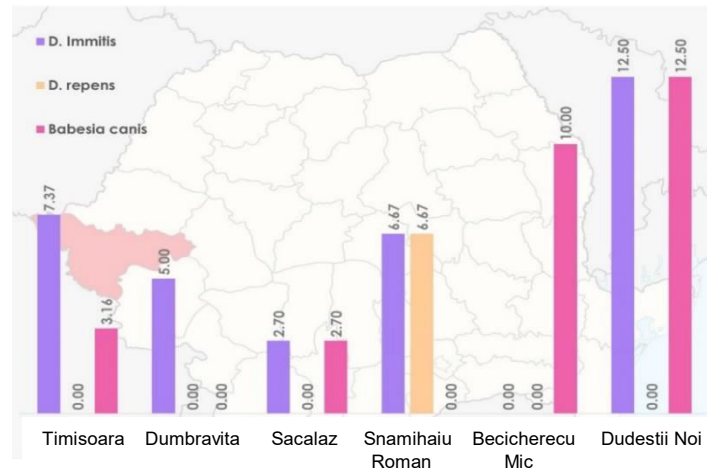


Fig. 8. Vector borne pathogens prevalence in different localities of Timis county

Considering the sex of animal as an epidemiological factor (Fig. 9), males show a higher prevalence of infection (11.85% - 16 positive cases out of 135 males) than females (5.45% - 9 cases out of 165). The dogs that were tested positive for *D. immitis* (19 dogs) had ages between 4 and 15 years old, comparing to the dogs that were tested positive for *B. canis* (9 dogs), where the age fits in a lower range of 2-12 years old. *D. repens* was diagnosed in a 7 years old dog, which had also a coinfection with *D. immitis*.

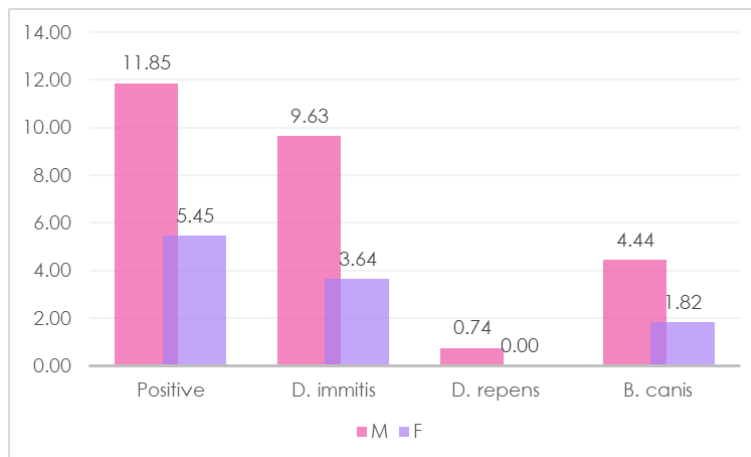


Fig. 9. Positive cases, gender repartition

Information regarding the prevalence of vector-borne pathogens in Romania was published by Ciocan et al. (2). In this study, the reported seroprevalence of dirofilariasis in Timis was 4% by modified Knott test.

According to Ilie et al. (7) the prevalence of canine babesiosis has been reported as 9.02% by direct smear, in south-western Romania.

The seroprevalence of *B. canis* was 19.8% in Banat region and it indicates a high exposure to this parasite, according to Imre et al. (10, 11). The infection was significantly higher in rural (28.4%) than in urban areas (15.4%) because of a reduced treatment for hard ticks in the countryside than in urban habitats (10, 11).

Yildirim et al. (17) and Song et al. (14) reported that the prevalence rates of *D. immitis* in males are higher than in females due to the fact that males are better propriety defendants and they are mostly kept outside, which facilitates contact between vectors and dogs.

In Cluj county, according to Mircean et al. (13) *D. immitis* prevalence was remarkably higher in dogs over the age of two years.

In Hungary, which is a neighbouring country to Timis, Farkas et al. (4) reported a prevalence of 22.4% for *D. immitis* and *D. repens*, diagnosed based on serological results. In another epidemiological study from Hungary, Hamel et al. (6) found that the dogs under investigations for *Babesia spp.*, *Dirofilaria spp.*, were positive for infections among which *B. canis* had the highest prevalence rate (43.1%).

Conclusions

The results of this study suggest the importance and widespread occurrence of these pathogens in Timis county, that have previously been confirmed by other studies from different regions of the country and the neighbouring Hungary.

Canine vector-borne diseases in Timis county have shown an alarming increase in the last years, but with an appropriate intervention like prophylactic treatment with parasiticides the level of these endemic affections can be reduced.

Acknowledgement

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THE EFFECTS OF AQUEOUS EXTRACTS OF *SILYBUM MARIANUM* AND *HYPPOPHAE RHAMNOIDES* ON RAT LIVER IN ALLOXANE-INDUCED DIABETES

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Summary

The aim of this study was to point out the hepatoprotective effect of two plants (as aqueous extracts), represented by *Silybum marianum* and *Hyppophae rhamnoides* in alloxan induce diabetes. It is well known that diabetes is a metabolic disease that affects not only humans but also many species of animals, especially pets. Today we are looking for new alternative methods, affordable and natural for the prevention and treatment of metabolic or hormonal disorders, which is why we opted to study the plants mentioned above, knowing that diabetes, in turn, induces a number of secondary diseases, indirectly affecting other organs or systems. The histological exam of the rat liver highlighted the fact that the aqueous extracts from *Silybum marianum* and *Hippophae rhamnoides* alleviate the side effects of the onset of diabetes.

Keywords: *Silybum marianum*, *Hippophae rhamnoides*, liver, histological exam

Diabetes mellitus is a common endocrine disease in humans and animals, especially in dogs, where worldwide prevalence was reported in ranges from 0.3% to 1.3% (5, 7, 8, 9). This metabolic disease starts from an absolute or relative lack of the insulin hormone; induce by many factors, which can be expressed by the loss of pancreatic beta cells. The direct factors, like immune-mediated attack on the pancreatic beta cell or vacuolar degeneration of the pancreas or pancreatitis, can decrease the circulating insulin (1, 6, 11). Also, there are risk factors, such other hormonal diseases (hypothyroidism, hyperadrenocorticism), and obesity or medications that trigger insulin-resistance (steroids or progestins), which can induce insulin-dependent diabetes mellitus (2, 10).

Based on the direct or indirect (risk) factors, the diabetes mellitus is classify in three types, but all of them are highlighted by a failure to regulate blood sugar.

Type I diabetes mellitus (insulin-dependent diabetes mellitus) results from total or near-complete destruction of the insulin-producing beta cells and the patients, human or animal, require insulin I.V. administration to stabilize blood sugar.

In type II diabetes mellitus (non-insulin-dependent diabetes mellitus), the great majority of insulin-producing cells remain, but the amount of insulin produced is insufficient, or the tissues are relatively insulin resistant. The patients with type II diabetes can often be treated with an oral medication that stimulates the remaining

functional pancreatic beta cells to produce or release insulin in an adequate amount to normalize blood sugar.

Type III diabetes results from insulin resistance caused by other hormones or hormone-secreting tumors.

Alloxan is one of the most common diabetogenic agents used to assess the antidiabetic potential of either synthetic compounds or plant extracts in studies involving diabetes.

Silymarin is a natural compound that is present in species derived from *Silybum marianum*, which is commonly known as Milk thistle. The plant contains at least seven flavolignans and the flavonoid taxifolin. The most important flavolignans present include silybin, silydianin, and silychristine. Silybin represents between 50% and 70% of the extract from silymarin. The following flavolignan isoforms are known: silbyna A, silbyna B, isosilbyna A, and isosilbyna B (13). Silymarin has been used worldwide for many years as a complementary alternative medicine because of the beneficial effects associated with the treatment of hepatic diseases. Silymarin belongs to the Aster family (Asteraceae or Compositae). The mature plant has large brilliant-purple flowers and abundant thorns. The plant grows in places with sufficient sun exposure (14). Silymarin has been reported to have antioxidant, immunomodulatory, anti-fibrotic, anti-proliferative, and antiviral properties. It also affects the synthesis of RNA and DNA. Furthermore, silymarin maintains the integrity of the hepatocyte membrane and impedes the entrance of toxic substances or xenobiotics. Due to its phenolic nature, it is capable of donating electrons to stabilize FR and reactive oxygen species (ROS). Silymarin also affects intracellular glutathione, which prevents lipoperoxidation of membranes (12).

Hippophae rhamnoides, known as sea buckthorn, belongs to the Elaeagnaceae family and includes in its composition vitamins A and C, alpha-tocopherol, large amounts of carotenoids and vitamin E, minerals (K, Na, Mg, Ca, Fe, Zn, Se), monosaccharides, amino acids, flavonoids, fatty acids, glycerolphospholipids, phytosterols, zeaxanthin esters, polyphenolic compounds. Because of its components *Hippophae rhamnoides* exhibits numerous beneficial actions: antioxidant, anti-inflammatory, antibacterial, antineoplastic, immunomodulatory, antidiabetic and hepatoprotective (4).

In animal models, it was showed that *Hippophae rhamnoides* seed oil can protect liver from damage induced by carbon tetrachloride, ethyl alcohol or acetaminophen (4).

Materials and methods

The working protocol for the study of the effects of aqueous extracts of certain medicinal plants has been described in detail in other scientific papers presented by us and collaborators. Very briefly, the rats used in the experiment were divided into the following groups:

- Group C (control group), with clinically healthy rats, which consumed

distilled water;

- Group D (diabetics), with diabetic rats injected with 2% alloxan which was administered intravenously, in the vein of the tail, at a dose of 40 mg / kg body weight, according to the protocol described by Carvalho et al., which were given distilled water until the end of the experiment;

- Group Ar (*Sylibum marianum*), with diabetic rats that received aqueous extract of milk thistle, in a concentration of 6%;

- Group Ca (*Hippophae ramnoides*), with diabetic rats that received aqueous sea buckthorn extract, in a concentration of 6%;

After completion of the experiment (7 weeks), the rats were slaughtered in accordance with the animal protection rules in force. A number of organs were collected, of which, in the present paper, we used the pancreas and liver for histological investigations. The organ fragments were fixed in 80 volumes ethyl alcohol for 7 days, after which they were washed, dehydrated and embedded in paraffin.

The sections thus prepared were processed for histological study by the usual Hematoxylin & Eosin method.

Results and discussions

Microscopic examination of the pancreas in the control group revealed its normal appearance, represented by two structural components of epithelial nature, exocrine and endocrine, included in lobules and lobes, separated by fine connective septa (Fig. 1).

By microscopic examination of the liver in the control group, the normal structure was highlighted, with hepatic lobes of polygonal shape, poorly delimited by the interstitial connective tissue, as morphological units (Fig. 2).

By microscopic examination of the pancreas in the group of rats with diabetes induced by the administration of alloxan, it was noted the installation of characteristic alterative aspects, found in our previous studies, as well as the literature, of which we mention briefly: inflammation, characterized by lymphocytic infiltration of Langerhans, which presented different dimensions (Fig. 3). In some Langerhans islands, clear, wide areas appeared, expressing oedema, as a consequence of cytolysis of pancreatic beta cells. Given that pancreatic beta cells are the most numerous, the more extensive the cytolysis lesions, the wider the areas of oedema were.

By microscopic examination of the liver in the group with diabetes induced by the administration of alloxan, the installation of congestive vascular phenomena was noticed, characterized by enlargement of the centrolubular veins and sinusoidal capillaries.

In the present study, hepatocytes showed small drops of lipids, dispersed in the cytoplasm (microvesicular steatosis), or large lipid folds, resulting from the confluence of small ones, and which pushed the nuclei eccentrically, respectively

macrovesicular steatosis.

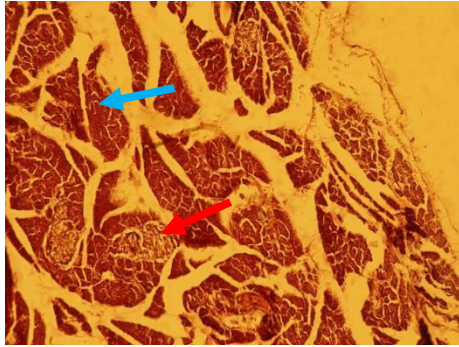


Fig. 1. Histological section performed through the pancreas in the control group: endocrine component (→) and exocrine component (→), Hematoxylin – Eosin stain, 40X

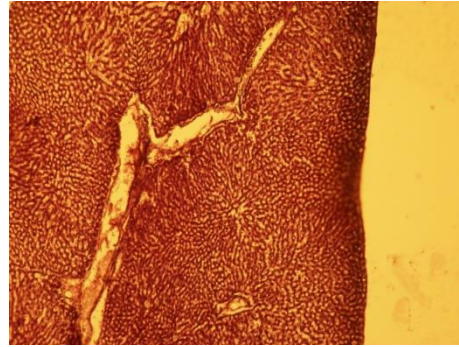


Fig. 2. Histological section of the liver in the control group: normal structural appearance, Hematoxylin-Eosin stain, 40X

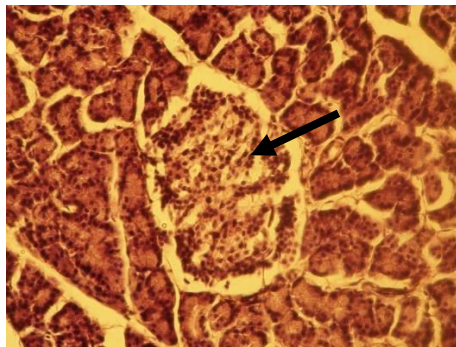


Fig. 3. Histological section performed through the pancreas in the group with diabetes induced by the administration of alloxan: insulinitis (→), Hematoxylin – Eosin stain, 400X

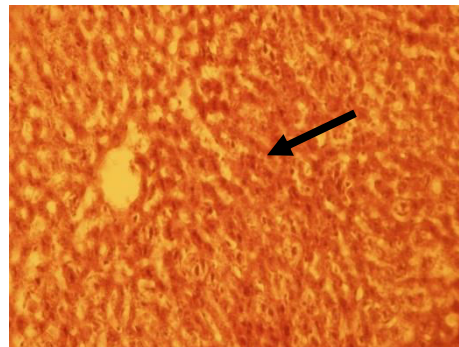


Fig. 4. Histological section performed by the liver in the group with diabetes induced by the administration of alloxan: macrovesicular steatosis (→), Hematoxylin – Eosin stain, 400X

Due to the paraffin inclusion steps, the fat droplets appeared optically hollow, but well delimited, and the hepatocytes, enlarged in volume, presented a cytoplasm with a thinner appearance (Fig. 4). We considered the degree of steatosis of being moderate because the lipid droplets were located strictly intracellularly and only in certain areas.

The increase in volume of hepatocytes, expressed by the ballooning aspect, is due to the alteration of the intermediate filaments of the cytoskeleton.

The structural changes identified in the liver are in line with those found in the literature. Thus, steatosis is the histological feature that ties together all of the various forms of Fatty Liver Disease. Usually, steatosis should involve at least 5% of hepatocytes (by visual estimation) in order to be considered clinically significant (3). True microvesicular steatosis, with its characteristic foamy cytoplasmic appearance, may be observed in single hepatocytes or in small patches, but is never diffuse. Early in the disease course, the steatosis is most prominent in zone 3, but with progression of disease or severity, the steatosis may spread evenly throughout the hepatic acinus or become irregularly distributed (15).

It seems that there is a close relationship between steatosis and diabetes, which works in both directions, respectively steatosis can induce diabetes, as well as diabetes can induce steatosis.

Microscopic examination of the liver in the group of diabetes induced by the administration of alloxan and treated with aqueous extract of *Silybum marianum* showed the maintenance of vascular congestion in the centrolobular veins, with reduction of lipid degeneration and hepatic steatosis, respectively the liver showing a relatively normal structural appearance (Fig. 5).

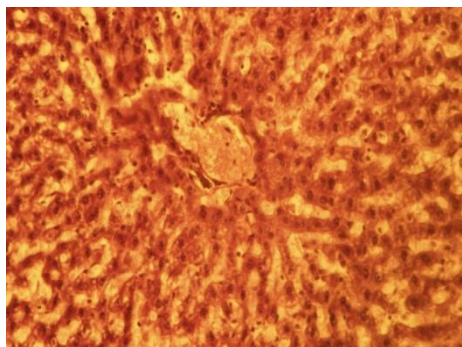


Fig. 5. Histological section performed through the liver in the group with diabetes induced by the administration of alloxan and treated with aqueous extract of *Silybum marianum*: relatively normal structural appearance, Hematoxylin – Eosin stain, 400X

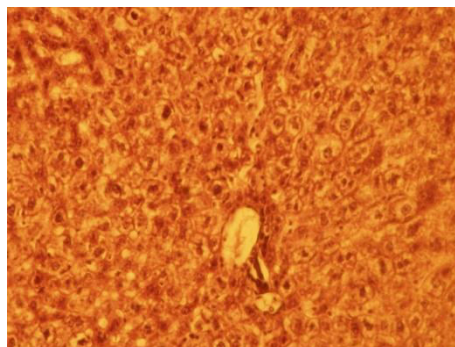


Fig. 6. Histological section performed through the liver in the group with diabetes induced by the administration of alloxan and treated with aqueous extract of *Hippophae rhamnoides*: apparent normal structural appearance, Hematoxylin – Eosin stain, 400X

The results of our study are in line with those in the literature on the liver protective effect of *Silybum marianum*, more precisely of silymarimarine. Thus, it

has been shown by numerous studies that silymarine effectively protects the liver in cases of chronic viral hepatitis, or after the administration of drugs, which have as a side effect hepatotoxicity, being used as a palliative and / or preventive medication.

Microscopic examination of the liver in the group with diabetes induced by the administration of alloxane and treated with aqueous sea buckthorn extract (*Hippophae rhamnoides*) was also noted similar to the previous group, the reduction of steatosis, the cell membranes of hepatocytes being well delimited (Fig. 6).

And in this case, sea buckthorn extract can be recommended as a liver protector in the case of diabetes, especially due to its antioxidant effect, demonstrated and exemplified in numerous studies in which hepatotoxic substances were used.

Conclusions

The alternative changes that appeared after the induction of diabetes by the administration of alloxan also have repercussions on the liver, manifested by the appearance of steatosis. The two aqueous extracts made from the studied plants can be used as hepatoprotectants, even if the lesions in the pancreas have not improved. Aqueous extracts from *Silybum marianum* and *Hippophae rhamnoides* alleviate the side effects of the onset of diabetes in the liver, and can be recommended as dietary supplements along with classic medication.

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MYCOFLORA WITH TOXIC POTENTIAL IN POULTRY FEED

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Summary

The contamination of animal feeds, with fungi that could secret mycotoxins is one of the major threats to human and animal health. In this study are presented the results obtained after feed samples from poultry were processed. Their fungal load was determined. The proportion of fungi in the genus *Aspergillus* was significantly higher compared to those in the genus *Penicillium* and *Fusarium*. Given the important role in the production of mycotoxins, some of species of the genus *Aspergillus* have been isolated and morphologically identified. The highest relative density was shown by *A. fumigatus* (33.3%), followed by *A. flavus* (24.8%). Among the isolated *Aspergillus* species, highest frequency was observed in *A. fumigatus* (91.6%), followed by *A. niger* (83.3%). The level of fungi infestation and the identified species represent important parameters that reflect the quality of the feed studied as well as of the future potential for the presence of mycotoxins.

Keywords: poultry feed, fungi, toxic potential

The harmful effects generated by microscopic fungi are particularly numerous and complex, being encountered every day. Approximately 50 genera of harmful fungi of feed and food are known, the most important species being included in the genera *Aspergillus*, *Fusarium* and *Penicillium* (8). Fungal contamination of cereals, plants and other nutrient substrates are highlighted by the loss of nutritional and energy value, decreased seed germination ability and production of toxic substances (mycotoxins) (1). It was proved that fungi have the ability to utilize the nutrients from the cereals that they contaminated and due their activity will result the loss of 5 to 100% of the nutrients in the feed (3, 7).

The relationship between the development of fungi on food and the deterioration of its quality is very difficult to establish. Fungi (mycelium or spores) are not initially visible or detectable, at the stage when the damage is already installed, when they have secreted a wide range of metabolites (enzymes, toxins) (6). In technological processes that ensure dilution, metabolites (toxins) remain and can act at very low concentrations on some components of the finished product, further deteriorating its quality. By consuming these products, toxins can accumulate in the bodies of animals and humans and can generate pathological effects on the liver, kidneys and even intoxications (6).

It is known that most toxigenic species belong to the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* (8). According to several authors mycotoxins such as aflatoxins, zearalenone, T2-toxin, deoxynivalenol, ochratoxin A, fumonisins,

and patulin can be considered the most common mycotoxins found in feed and food (4).

The identification of the contaminating mycobiota is essential because it provides data on the potential production of its mycotoxins and it can be a helpful indicator to determine feed quality (5).

Taking in consideration these aspects, the research aimed to study the microflora present in the feed used in bird feed, to establish the proportion of the dominant genera of polluting fungi and to evaluate the frequency of fungal species of the genus *Aspergillus*, with toxic potential.

Materials and methods

Total number of 16 samples of poultry feed were collected from two different farms. From each farm were taken 4 samples, one for each season. The samples were processed in the laboratory immediately after collection.

To obtain isolated fungal colonies, serial decimal dilutions (1×10^5) were made from each feed sample. For this, 10 grams were weighed, which were then mixed with 90 mL sterile water. The mixture was stirred for a perfect homogenization on a horizontal shaker for 10 minutes. From the last dilution (10^5), 1 mL was seeded in three Petri dishes. The culture medium was poured over this inoculum. The culture medium used was Potato dextrose agar (200 g peeled potato, 20 g agar and 15 g agar in 1,000 ml of distilled water) was used. The inoculated plates were incubated at $25 \pm 2^\circ\text{C}$, for 7 days. Each plate were examined visually and under a compound light microscope daily for preliminary identification of fungal genera. Colonies belonging to the genus *Aspergillus* were then sub-cultured for species identification, using the same cultivation media. Identification of fungal species was done on the basis of cultural and morphological characteristics. Macroscopically, for species differentiation was examined colony color, texture and margins. Microscopically the conidial head morphology (uniseriate, biseriate) the conidiophores and the aspect of conidia and their arrangements were examined. (6). Descriptive analysis of percent relative density and frequency for *Aspergillus* isolates were performed on the collected data.

Results and discussions

From the results obtained after examining the feed samples used in poultry farms, it resulted that they were contaminated with different fungal species belonging to the genera *Fusarium*, *Penicillium* and *Aspergillus*. It is noteworthy that the predominant species belonged to the genus *Aspergillus*. Of these, five species were identified, namely *A. fumigatus*, *A. flavus*, *A. niger*, *A. brasiliensis* and *A. orizae*.

Relative density and frequency of the different *Aspergillus* spp. found in this study are presented in Table 1. Among the isolated *Aspergillus* species, highest frequency was observed in *A. fumigatus* (91.6%), followed by *A. niger* (83.3%) and

A. flavus (75.0%). For the other identified species, the frequency was of 41.6% for *A. brasiliensis* and 16.6% for *A. orizae*. Regarding the relative density the highest value was recorded in *A. fumigatus* (33.3%) and *A. flavus* (24.8%). The species *A. niger* and *A. brasiliensis* had a moderate relative density, being 32% and respectively 25%. The lowest relative density was found in *A. orizae* (12%).

Similar results have been reported by other researchers. Most studies on different types of feed have shown the presence of species such as *Aspergillus*, *Fusarium* and *Penicillium*. However, the frequency and density of *Aspergillus* species isolated in feed differ from one study to another, depending on the type of sample, the geographical region and the storage conditions (2, 3, 7).

Table 1

Percent relative frequency and density of isolated *Aspergillus* species from hens feed samples

Specie	Relative density		Relative frequency	
	N	(%)	X= 12	(%)
<i>A. fumigatus</i>	55	33.3	11	91.6
<i>A. flavus</i>	41	24.8	9	75.0
<i>A. niger</i>	32	19.7	10	83.3
<i>A. brasiliensis</i>	25	15.1	5	41.6
<i>A. orizae</i>	12	7.3	2	16.6

X = No. of samples, N = No. of fungal isolates

Cultural and morphological characteristics examination of *Aspergillus* species isolated from poultry feed samples showed that there was variation in the colony color, margins, and texture and colony reverse colors (Fig. 1).

The morphological characteristics of *Aspergillus* species (*A. fumigatus*, *A. flavus*, *A. niger*, *A. brasiliensis* and *A. orizae*) that were isolated from poultry feed examined in this study are presented in Fig. 2.

The micotoxigenic fungi and mycotoxins in poultry feed have studied during the last years around the world. In Nigeria, Okoli et al. (7) analyzed the mycobiota of commercial poultry feed. The common fungi isolated were *Aspergillus* spp., *Penicillium* spp., *Mucor* spp., *Rhizopus* spp., *Epicoccum* spp., *Gymnoascus* spp., *Cladosporium* spp., *Mortierella* spp. A study realised in Pakistan, found *Aspergillus* species as most predominant fungi, followed by *Penicillium*, *Fusarium*, and *Alternaria* (9). Among the *Aspergillus* isolates, *A. niger* (37.74%) was the most frequently isolated species followed by *A. flavus* (22.64%), *A. ochraceus* (16.98%), *A. parasiticus* (13.21%), *A. carbonarius* (3.77%), *A. fumigatus* (3.77%), and *A. oryzae* (1.89%). In Iraq, poultry feed samples studied by Shareef (10) contained fourteen mould genera from. The most frequent fungi were *Aspergillus* followed by *Penicillium*, *Mucor*, *Rhizopus*, *Scopulariopsis*, *Alternaria*, and *Eurotium*. Study

realized on feedstuff used for poultry nutrition in Argentina revealed ten fungi genera, six of them known to be mycotoxigenic (3).

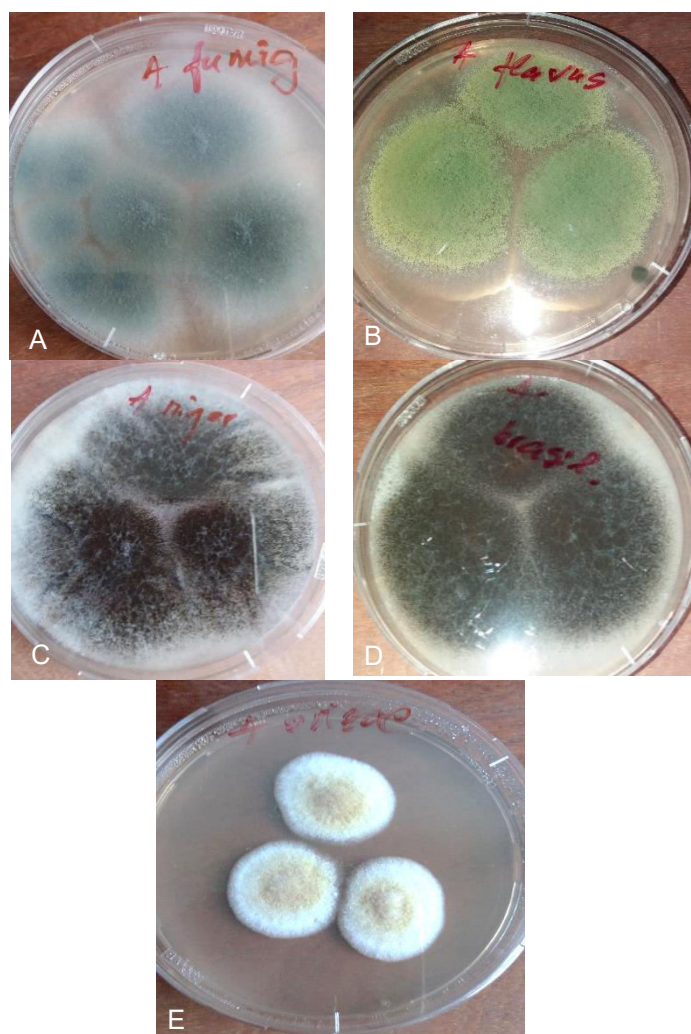


Fig. 1. Macroscopic aspect of *Aspergillus* species isolated from poultry feed (on potato dextrose agar) A: *Aspergillus fumigatus*; B: *Aspergillus flavus*; C: *Aspergillus niger*; D: *Aspergillus brasiliensis*; E: *Aspergillus oryzae*

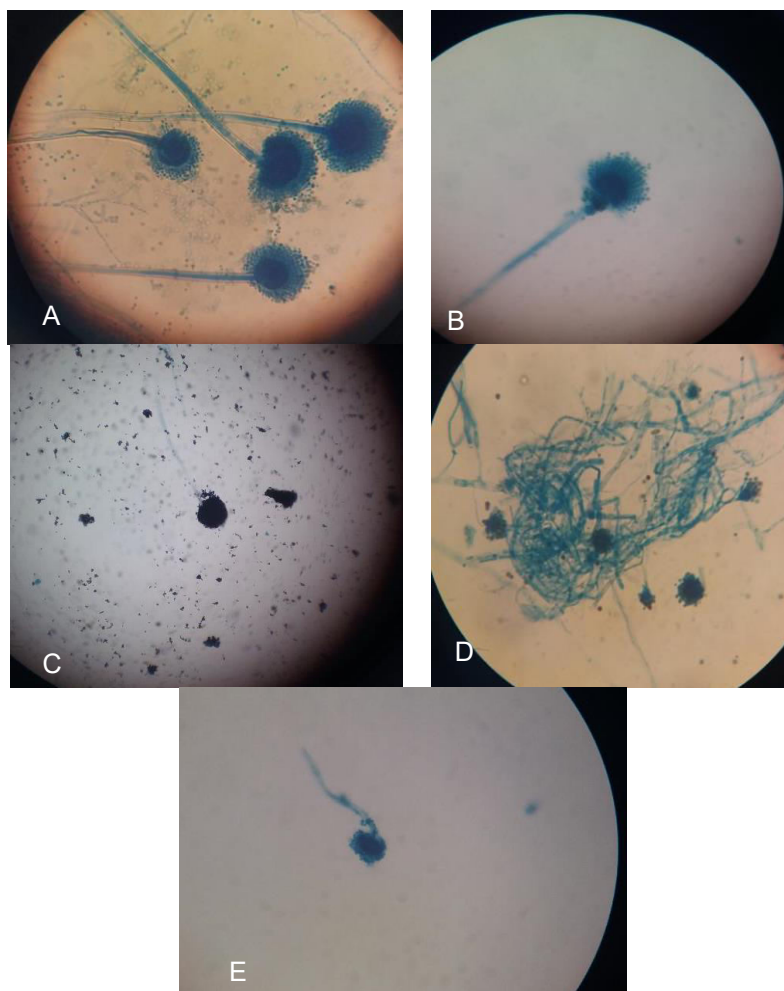


Fig. 2. Microscopic aspect of *Aspergillus* species isolated from poultry feed (on potato dextrose agar) A: *Aspergillus fumigatus*; B: *Aspergillus flavus*; C: *Aspergillus niger*; D: *Aspergillus brasiliensis*; E: *Aspergillus orizae*

Conclusions

The present study demonstrated the colonization with *Aspergillus* species of feedstuff used for poultry nutrition in two farms.

Among the isolated *Aspergillus* species, highest frequency was observed in *A. fumigatus* followed by *A. niger* and *A. flavus*.

Most of the identified species are known for their potential to produce mycotoxins.

The correct identification of fungal contaminants in poultry feed is very important to be done at early stages, to avoid their health hazardous risk.

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ANTIMICROBIAL RESISTANCE - STILL AN ACUTE GLOBAL PROBLEM

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Summary

Antimicrobial resistance is by far one of the most pressing problems worldwide. The decrease in sales of antimicrobials for veterinary use in Europe, entitles us to hope for an improvement in the situation on this issue. New European Regulations on the use of antibiotics in particular, as well as the sharing of veteran countries' own experience in the fight against antibiotic resistance, in addition to each country's own efforts and investments, could help solve this challenge. Some of these measures are available to medical practitioners, medical school teachers, state institutions and can be applied immediately. Others require time and funding.

Keywords: resistance, antimicrobials, sales, measures, regulation

The phenomenon of antimicrobial resistance is a problem that concerns all countries of the world. Globally, around 700,000 people die each year because of antimicrobial resistance, of which 25,000 are EU citizens. It is estimated that by 2050 this could cause more deaths than cancer.

Although in the last year there was a special interest for the management, respectively the improvement of the situation determined by Sars-Cov2, the European Commission as well as some national authorities organized virtual meetings in which the main discussed topic was antimicrobial resistance. In addition, Member States are preparing for applying the new Regulations starting with the end of this year, which will regulate many aspects of the use of antimicrobials in animals.

Current measures applied and results

In previous years, some measures have been taken in order to reduce the consumption of antimicrobials. Some of these measures are:

- The assessment of the efficacy of veterinary medicinal products containing antimicrobials in order to grant the marketing authorization is made in accordance with the guidelines provided by European Medicine Agency (EMA) (5);
- The use of antimicrobials to promote growth in animals has been banned in the EU since 2006 (1);
- The mode of release of veterinary medicinal products containing antimicrobials has been regulated (4);
- Compliance with waiting periods for animals of economic interest;

- The information contained in the summaries of the characteristics of veterinary medicinal products containing antimicrobials has been updated by introducing special warnings related to the correct use of antibiotics (7);
- The use of some classes of antibiotics – the 3rd and 4th generation cephalosporins (8), fluoroquinolones (6), colistin was restricted (9);
- The use of antibiotics reserved for human use was prohibited in animals (4);
- Rules have been established for the „of label” use of veterinary medicinal products (4).

The choice of the active substance for the treatment of an infection should be made taking into account the results of susceptibility tests (preferably) or epidemiological data and, if we are talking about antibiotics, depending on belonging to one of the categories of provided by the European Medicines Agency - Category A (“Avoid”), Category B (“Restricted”), Category C (“Caution”), Category D (“Caution”) (10).

Antimicrobial Advice Ad Hoc Expert Group (AMEG) recommends individual treatment (local - for the treatment of infections of the eyes, ears, mammary gland, skin, etc, parenterally - intravenously, intramuscularly, subcutaneously, orally - tablets, bolus, etc.) rather than the group medication (with injectable products, with products for oral administration by mixing with drinking water / milk / food substitute / premixes), precisely to avoid some situations favorable to the emergence of antimicrobial resistance through the phenomenon of underdosing.

Another measure taken by the European Commission in order to a better control of the use of antimicrobials in animals is the monitorisation of the volume of sales. The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project collects information on how antimicrobial medicines are used in animals across the European Union. Analysis of these data can provide information on the trend of antimicrobial consumption in Europe.

Since 2014, Romania reports annually the volume of antimicrobial sales. Data related to sales of antimicrobial class for food-producing animals, including horses in 2018, can be found in the Table 1. The overall national sales data cover sales of antimicrobial VMPs for use in food-producing animals, plus sales of tablets that are used almost solely in companion animals. As injectable dosage forms are frequently marketed for both food-producing and companion animals and their use in companion animals is minor in terms of quantity of active substance, such sales are included in the statistics for food-producing animals. Sales of tablets, and therefore use in companion animals, accounted for a minor proportion of the total sales of antimicrobial VMPs in 2018, except in Finland, Iceland, Luxembourg, Norway, Slovenia, Sweden and the United Kingdom, where they represented 10.3%, 8.4%, 5.3%, 6.9%, 5.2%, 7.2% and 5.8% of the total sales, respectively (Table 2). Overall, sales of tablets in the 31 countries represented 1.1% of the total sales in tons (3).

The charts below show the ratio and percentage of the total sales of the

amounts of antibiotics in the best-selling groups of antibiotics in 2018 (Fig. 1 and Fig. 2). The sales of the different veterinary antimicrobial classes varied between countries. These differences can be partly explained by differences in animal demographics, the selection of antimicrobial agents, dosage regimes, the type of data sources and veterinarians' prescribing habits. Across all 31 countries, the sales of tetracyclines (30.7%), penicillins (28.8%) and sulfonamides (8.4%), in mg/PCU, accounted for 67.9% of the total sales in 2018. Among the antimicrobial classes shown as 'Others', of the overall sales in the 31 countries, 0.1% was accounted for by 1st- and 2nd-generation cephalosporins, 0.2% by 3rd- and 4th-generation cephalosporins, 1.9% by amphenicols and 0.3% by other quinolones.

The proportion of the total sales of 3rd- and 4th-generation cephalosporins, fluoroquinolones, other quinolones and polymyxins for food-producing species, in mg/PCU, for 31 European countries, in 2018 (Fig. 3) in food-producing species (3). There is a worrying increase in the preference for fluoroquinolones.

As shown in Fig. 4, aggregated by the 31 countries, sales (mg/PCU) of premixes accounted for 26.9% of the overall sales, 9.0% were oral powders and 51.8% were oral solutions, i.e. 87.7% were for group treatment; 11.4% were injectable preparations, 0.6 % were intramammary preparations and 0.3 % were oral pastes, boluses and intrauterine preparations (3). Administration in the form of premix, or oral solutions in drinking water, or water-soluble powders of antimicrobials to groups of animals substantially increases the risk of developing antimicrobial risk.

In the EU the sales of veterinary antimicrobial agents, expressed as mg sold per PCU, ranged from 2.9 mg/PCU to 466.3 mg/PCU across the 31 countries between 2010-2018 in food-producing species (Table 3).

Tables 4, 5, 6, highlight the use of critically important antimicrobials with the highest priority for humans, as defined by the WHO, and antimicrobial classes belonging to the AMEG Category B (Restricted) between 2010-2018.

Table 1

Sales, in tons of active ingredient, of veterinary antimicrobial agents marketed mainly for food-producing animals, PCU and sales in mg/PCU, by country, for 2018 (3)

Country	Sales (tonnes) for food-producing animals	PCU (1,000 tonnes)	Sales in mg/PCU
Austria	48.0	957.2	50.1
Belgium	195.0	1,724.4	113.1
Bulgaria	47.8	399.9	119.6
Croatia	19.6	293.0	66.8
Cyprus	53.4	114.5	466.3
Czechia	40.2	704.6	57.0
Denmark	93.6	2,446.7	38.2
Estonia	6.1	114.0	53.3
Finland	9.3	496.8	18.7
France	456.2	7,107.0	64.2
Germany	753.1	8,517.6	88.4
Greece	113.0	1,243.9	90.9
Hungary	150.2	831.8	180.6
Iceland	0.6	116.4	4.9
Ireland	98.6	2,142.1	46.0
Italy	932.1	3,819.3	244.0
Latvia	6.0	167.3	36.1
Lithuania	10.7	323.8	33.1
Luxembourg	1.8	54.7	33.6
Malta	2.1	14.2	150.9
Netherlands	183.9	3,200.8	57.5
Norway	5.7	1,927.5	2.9
Poland	782.2	4,672.6	167.4
Portugal	191.8	1,028.1	186.6
Romania	230.7	2,788.2	82.7
Slovakia	12.1	246.6	49.3
Slovenia	7.8	179.8	43.2
Spain	1,724.1	7,865.4	219.2
Sweden	9.8	782.7	12.5
Switzerland	32.9	818.5	40.2
United Kingdom	212.9	7,215.7	29.5
Total 31 countries	6,431.4	62,315.1	103.2*

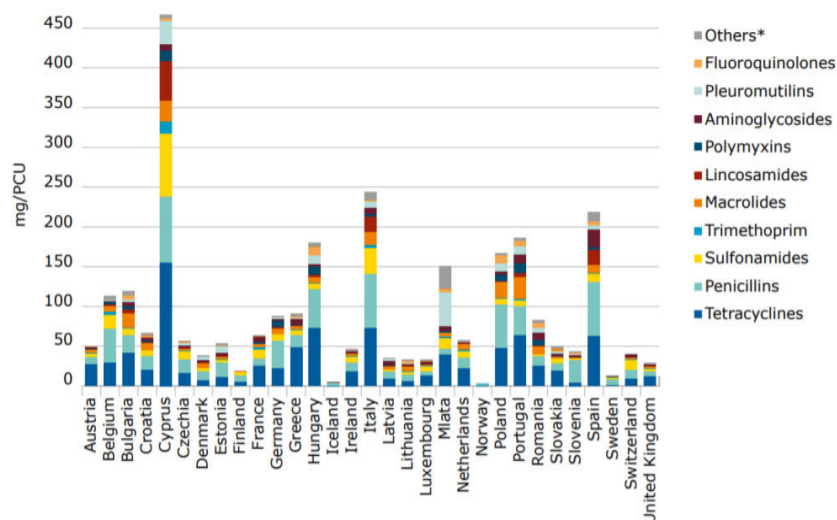


Fig. 1. Sales for food-producing species, in mg/PCU, of the various veterinary antimicrobial classes, for 31 European countries, in 2018 (3)

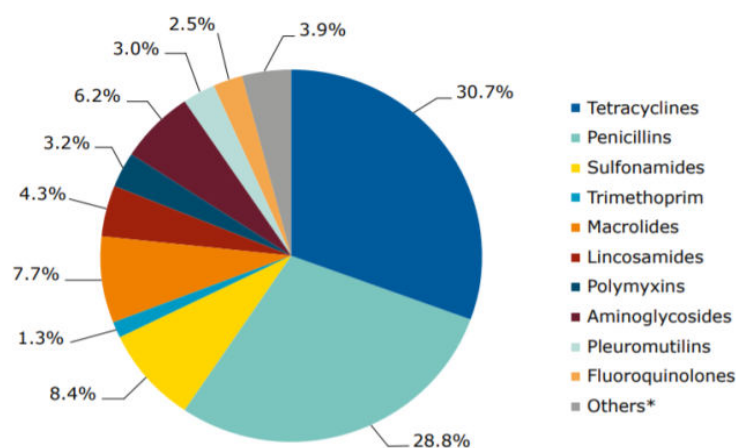


Fig. 2. Sales of antimicrobial agents by antimicrobial class as percentage of the total sales for food-producing species, in mg/PCU, aggregated by 31 European countries, for 2018 (3)

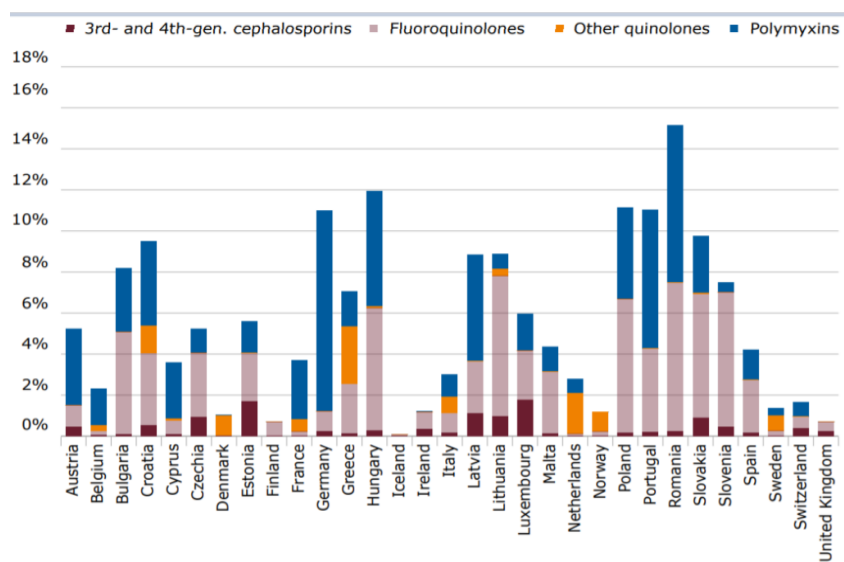


Fig. 3. Proportion of the total sales of 3rd- and 4th-generation cephalosporins, fluoroquinolones, other quinolones and polymyxins for food-producing species, in mg/PCU, for 31 European countries, in 2018 (3)

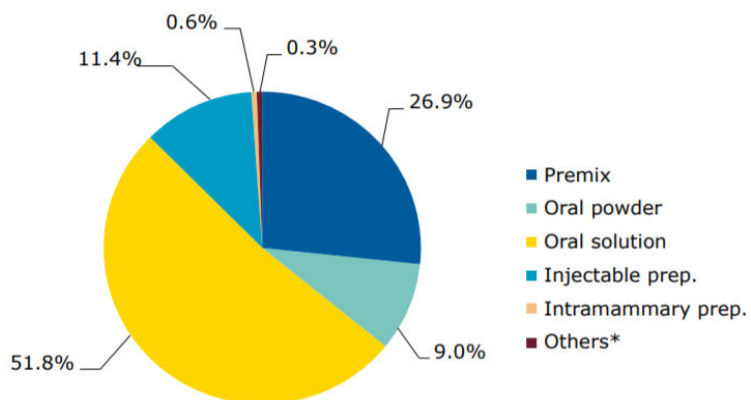


Fig. 4. Distribution of sales, in mg/PCU, of the various pharmaceutical forms of veterinary antimicrobial agents for food-producing animals, aggregated by the 31 European countries, for 2018 (3)

Table 2

Overall sales, in tons of active ingredient, split by tablets (used mainly in companion animals) and all other pharmaceutical forms (used mainly in food-producing animals), by country, in 2018 (3)

Country	Tablets		All other pharmaceutical forms		Total tonnes
	Tonnes	% of overall sales	Tonnes	% of overall sales	
Austria	0.7	1.4 %	48.0	98.6 %	48.6
Belgium	2.0	1.0 %	195.0	99.0 %	197.0
Bulgaria	0.1	0.3 %	47.8	99.7 %	48.0
Croatia	0.1	0.6 %	19.6	99.4 %	19.7
Cyprus	0.1	0.1 %	53.4	99.9 %	53.5
Czechia	1.0	2.4 %	40.2	97.6 %	41.2
Denmark	0.8	0.8 %	93.6	99.2 %	94.3
Estonia	0.1	2.3 %	6.1	97.7 %	6.2
Finland	1.1	10.3 %	9.3	89.7 %	10.3
France	15.2	3.2 %	456.2	96.8 %	471.4
Germany	9.6	1.3 %	753.1	98.7 %	762.7
Greece	0.5	0.5 %	113.0	99.5 %	113.6
Hungary	0.3	0.2 %	150.2	99.8 %	150.4
Iceland	0.1	8.4 %	0.6	91.6 %	0.6
Ireland	0.8	0.8 %	98.6	99.2 %	99.4
Italy	10.3	1.1 %	932.1	98.9 %	942.4
Latvia	0.1	1.5 %	6.0	98.5 %	6.1
Lithuania	0.1	0.8 %	10.7	99.2 %	10.8
Luxembourg	0.1	5.3 %	1.8	94.7 %	1.9
Malta	0.04	1.8 %	2.1	98.2 %	2.2
Netherlands	3.2	1.7 %	183.9	98.3 %	187.1
Norway	0.4	6.9 %	5.7	93.1 %	6.1
Poland	1.8	0.2 %	782.2	99.8 %	784.0
Portugal	1.2	0.6 %	191.8	99.4 %	193.0
Romania	3.8	1.6 %	230.7	98.4 %	234.5
Slovakia	0.3	2.3 %	12.1	97.7 %	12.4
Slovenia	0.4	5.2 %	7.8	94.8 %	8.2
Spain	0.5	<0.01 %	1,724.1	≥99 %	1,724.6
Sweden	0.8	7.2 %	9.8	92.8 %	10.6
Switzerland	0.7	2.0 %	32.9	98.0 %	33.6
United Kingdom	13.2	5.8 %	212.9	94.2 %	226.2
Total 31 countries	69.3	1.1 %	6,431.4	98.9 %	6,500.7

Table 3

Annual sales of veterinary antimicrobial agents for food-producing species, in mgPCU, by country, from 2010 to 2018 (3)

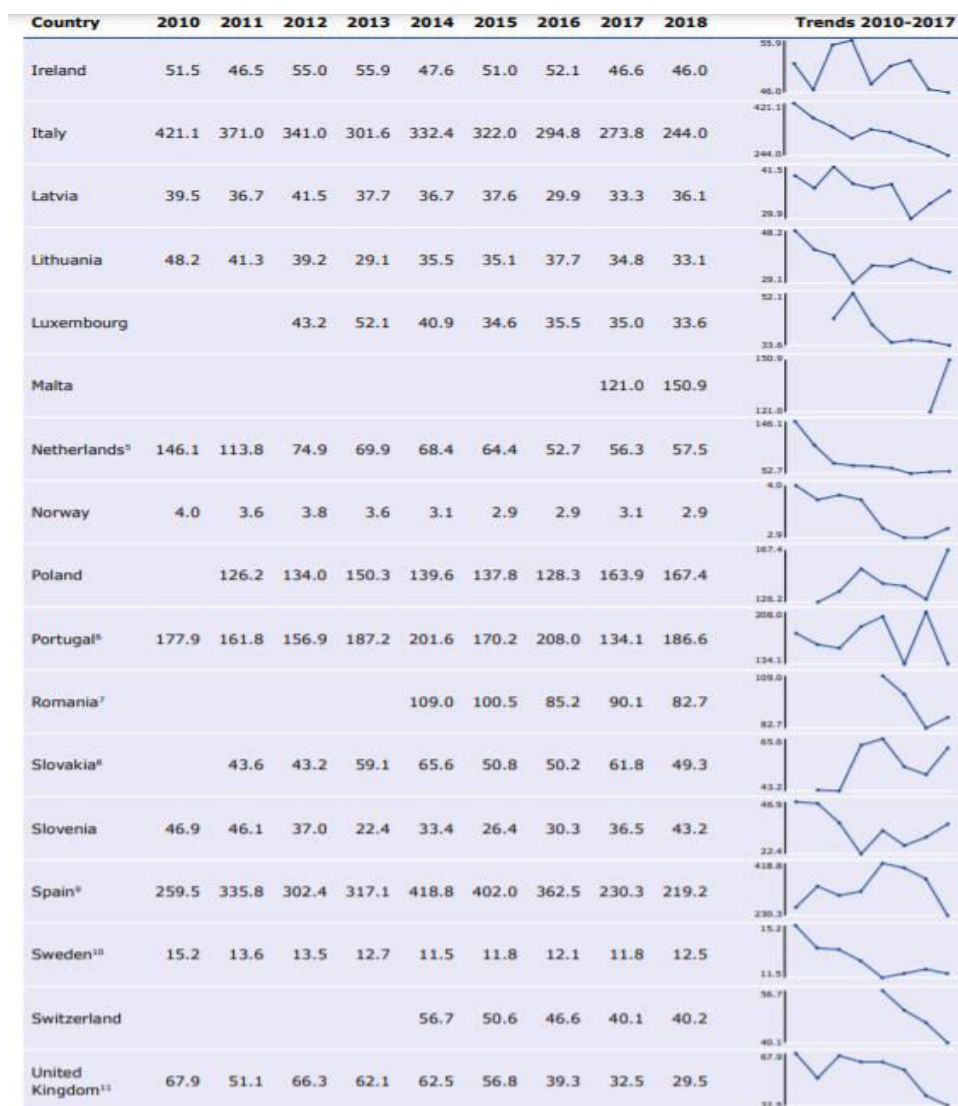


Table 4

Changes in sales of 3rd- and 4th-generation cephalosporins for food-producing species, in mg/PCU, by country, from 2010 to 2018
 Note that the scale differs between countries (3)

Country	2010	2011	2012	2013	2014	2015	2016	2017	2018	Trends 2010-2018
Austria	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	
Belgium	0.5	0.5	0.5	0.5	0.5	0.4	0.3	0.1	0.1	
Bulgaria		0.05	0.03	0.1	0.1	0.2	0.1	0.1	0.1	
Croatia					0.1	0.2	0.2	0.2	0.3	
Cyprus		0.2	0.5	0.5	0.8	0.3	0.7	0.4	0.4	
Czechia	0.4	0.3	0.3	0.4	0.4	0.4	0.4	0.5	0.5	
Denmark	0.05	0.03	0.03	0.02	0.02	<0.01	<0.01	<0.01	<0.01	
Estonia	0.4	0.5	0.6	0.7	0.6	0.6	0.7	0.8	0.9	
Finland	<0.01	0.02	0.03	0.02	0.02	0.01	<0.01	<0.01	<0.01	
France	0.3	0.3	0.3	0.3	0.3	0.2	0.1	0.02	0.02	
Germany		0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.2	
Greece						0.1	0.1	0.1	0.1	
Hungary	0.3	0.1	0.3	0.3	0.2	0.4	0.4	0.5	0.5	
Iceland	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	
Ireland	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	
Italy	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	
Latvia	0.2	0.2	0.4	0.4	0.4	0.4	0.3	0.3	0.4	
Lithuania	0.02	0.04	0.1	0.2	0.2	0.1	0.1	0.2	0.3	
Luxembourg			0.7	0.7	0.6	0.6	0.7	0.6	0.6	
Malta								0.2	0.2	
Netherlands	0.2	0.2	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	
Norway	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	
Poland		0.1	0.1	0.2	0.2	0.1	0.2	0.2	0.3	
Portugal	0.3	0.3	0.2	0.4	0.4	0.5	0.5	0.6	0.4	
Romania					0.05	0.04	0.1	0.2	0.2	
Slovakia		0.7	0.5	0.4	0.5	0.3	0.4	0.4	0.4	
Slovenia	0.1	0.1	0.2	0.1	0.1	0.2	0.2	0.2	0.2	
Spain	0.7	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.4	
Switzerland					0.2	0.2	0.2	0.2	0.2	
United Kingdom	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	

Table 5

**Changes in sales of fluoroquinolones for food-producing species, in
mg/PCU, by country, from 2010 to 2018**
Note that the scale differs between countries (3)

Country	2010	2011	2012	2013	2014	2015	2016	2017	2018	Trends 2010-2018
Austria	0.6	0.6	0.5	0.6	0.5	0.5	0.5	0.5	0.5	
Belgium	0.7	0.8	0.9	1.0	1.1	1.0	0.6	0.2	0.2	
Bulgaria		5.0	6.1	6.8	1.8	5.3	4.9	5.6	6.0	
Croatia					3.5	3.3	2.6	1.9	2.4	
Cyprus		0.5	0.8	0.9	0.9	1.1	1.6	2.4	3.1	
Czechia	1.3	1.5	1.8	1.8	1.8	1.7	1.7	1.9	1.8	
Denmark	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	
Estonia	2.5	2.3	1.1	1.7	1.6	1.8	1.3	1.3	1.2	
Finland	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	
France	0.6	0.6	0.6	0.6	0.6	0.3	0.2	0.2	0.1	
Germany		0.9	1.2	1.4	1.4	1.1	1.0	1.1	0.9	
Greece						1.7	2.2	2.7	2.2	
Hungary	8.8	6.7	11.0	9.2	9.1	9.5	9.6	8.8	10.8	
Iceland	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	
Ireland	0.4	0.4	0.6	0.5	0.4	0.4	0.5	0.4	0.4	
Italy	1.7	2.2	2.5	2.3	3.1	2.9	2.3	3.0	2.3	
Latvia	4.1	2.2	1.7	2.1	1.6	1.1	0.8	1.1	0.9	
Lithuania	0.7	0.4	0.6	0.8	3.1	1.7	1.0	0.8	2.3	
Luxembourg			0.7	0.8	0.7	0.7	0.8	0.7	0.8	
Malta								14.3	4.6	
Netherlands	0.5	0.5	0.2	0.1	0.1	0.1	0.1	0.1	0.1	
Norway	0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	
Poland		7.1	8.2	8.8	9.0	8.5	9.6	11.0	10.9	
Portugal	5.6	8.4	9.3	8.2	11.4	8.8	8.9	3.5	7.6	
Romania					5.3	6.1	3.3	4.3	6.0	
Slovakia		3.0	3.2	2.8	4.2	2.9	3.6	3.4	3.0	
Slovenia	2.6	5.9	4.1	1.8	4.0	3.0	2.9	2.9	2.8	
Spain	8.8	9.2	10.2	9.3	9.9	9.0	8.5	4.9	5.6	
Sweden	0.1	0.1	0.1	0.04	0.03	0.02	0.02	0.02	0.03	
Switzerland					0.5	0.5	0.3	0.3	0.2	
United Kingdom	0.3	0.3	0.3	0.4	0.4	0.3	0.2	0.2	0.1	

Table 6

Changes in sales of polymyxins for food-producing species, in mg/PCU, by country, from 2010 to 2018

Note that the scale differs between countries (3)

Country	2010	2011	2012	2013	2014	2015	2016	2017	2018	Trends 2010-2018
Austria	1.0	1.0	0.7	0.9	1.6	1.6	1.6	1.7	1.9	
Belgium	6.0	5.4	5.8	4.7	3.4	2.8	2.4	2.1	2.0	
Bulgaria		3.2	3.8	2.7	0.5	3.6	2.3	2.9	3.7	
Croatia					3.8	2.4	3.6	3.2	2.7	
Cyprus		8.2	8.1	8.4	11.1	12.4	11.1	10.4	12.8	
Czechia	0.9	0.6	0.9	1.1	1.0	1.0	0.8	0.6	0.7	
Denmark	0.3	0.2	0.2	0.2	0.4	0.5	0.5	0.2	<0.01	
Estonia	3.5	4.3	4.9	5.8	3.1	1.3	0.7	1.1	0.8	
France	8.6	7.7	6.7	5.9	7.0	4.0	2.8	2.2	1.8	
Germany		14.8	14.8	14.6	12.2	9.2	7.9	8.5	8.6	
Greece						3.4	1.0	1.3	1.6	
Hungary	6.9	8.9	7.8	10.0	7.1	9.6	12.2	14.9	10.1	
Italy	40.2	30.7	30.1	27.6	29.4	26.1	15.1	5.2	2.7	
Latvia	1.0	1.0	2.5	1.5	0.8	0.9	0.9	1.3	1.9	
Lithuania	1.7	1.4	1.3	0.1	0.1	0.6	1.0	0.7	0.2	
Luxembourg			1.7	3.1	2.4	1.5	1.0	1.0	0.6	
Malta								4.6	1.8	
Netherlands	2.3	1.6	1.0	0.6	0.5	0.5	0.3	0.3	0.4	
Poland		4.1	4.0	4.4	5.0	5.9	5.6	7.4	7.4	
Portugal	15.1	7.9	18.6	19.0	17.6	14.6	13.5	10.9	12.6	
Romania					6.5	7.4	5.6	4.1	6.4	
Slovakia		1.2	2.1	1.1	1.5	1.1	1.2	1.7	1.4	
Slovenia	0.1	0.1	0.1	0.04	0.1	0.1	0.1	0.1	0.2	
Spain	33.0	33.5	29.4	21.5	36.1	34.9	22.0	4.4	3.3	
Switzerland					0.9	0.6	0.5	0.4	0.3	
United Kingdom	0.1	0.1	0.1	0.1	0.1	0.1	0.02	<0.01	<0.01	

The veterinary sales of antibiotics considered critically important in human medicine is presenting a decreasing trend in the EU. Between 2011 and 2018, sales have reduced by: 24% for third and fourth generation cephalosporins; 70% for polymyxins; 4% for fluoroquinolones; 74% for other quinolones (3).

Future measures to be taken

Although at European level the situation is starting to get better in terms of sales and implicitly consumption of antimicrobials in animals, the European Commission, in order to continue this policy, has developed 2 new Regulations which provides complementary measures to be taken in order to prevent antimicrobial resistance as follows:

- Regulation (EU) 2019/4 of the European Parliament and of the Council of 11 December 2018 on the manufacture, placing on the market and use of medicated feed prohibiting the preventive and collective use of antibiotics in medicated feedingstuffs. Moreover, prescriptions for feed containing antibiotics must always be issued by veterinarians following a proper examination;
- Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products. This normative act provides regulations that have as objective the decrease of sales, respectively of the use of antimicrobials in animals and implicitly the decrease of the incidence and of the risk of spreading the antimicrobial resistance.

Considering the resistance in antimicrobials the main objectives of this regulation (EU) 2019/6 are:

- Prohibition of use of antimicrobials in groups of animals for prevention of infection;
- Prohibition of prevention by using antimicrobials administered in food;
- Restriction of the use of antimicrobials in metaphylaxis;
- Prohibition of use in animals of antimicrobials designated in the EU as medicines reserved for the human health domain;
- Extending the ban on the use of antimicrobials as promoters of growth and yield improvement in farm animals;
- Obligation to collect data on sales and use of antimicrobials in each animal species;
- Other measures: prudent and responsible use of antimicrobials (2).

Currently, in order to apply Regulation (EU) 2019/6, the General Directorate for Health and Food Safety of the European Commission is working on the elaboration / implementation of additional tertiary legislation.

An important aspect is that the new Regulation requires Member States to collect data on the use of antimicrobial veterinary medicinal products in animals - Article 57: Member States shall be authorized to apply a step-by-step approach to

the obligations laid down in this Article, so that:

- (a) within two years from 28 January 2022, data must be collected for at least the species and categories included in Commission Implementing Decision 2013/652 / EU, in its version of 11 December 2018 - monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria;
- (b) within five years from 28 January 2022, data must be collected for all food-producing animal species;
- (c) within eight years from 28 January 2022, the data must be collected for the other animals which are bred or kept (2).

Regulation also provides (in Article 55) that each the Agency shall establish and, in collaboration with the Member States, maintain, a Union database on veterinary medicinal products ('product database'). The competent authorities, the Agency and the Commission shall have full access to the information in the product database. Marketing authorization holders shall have full access to the information in the product database as regards their marketing authorizations. The public has access to the information in the product database, without having the possibility to change the information (2).

Consequently, and in relation to the Product Database, there will also be a Union Pharmacovigilance Database. Competent authorities shall have full access to the pharmacovigilance database. Holders of marketing authorizations and the public will have also access to the pharmacovigilance database.

Having the information from the two Databases, doctors will be able more easily to manage certain situations related to the inefficacy of some products, possible side effects and will have access to all the information related to available medicinal products, new active substances that appear, new combinations of active substances, new indications and target species of some active substances.

The Regulation makes new clarifications related to the method of release of veterinary medicinal products: Article 105 - Veterinary prescriptions, Article 112 Use of medicinal products outside the conditions of the marketing authorization for animal species from which no food is obtained, Article 113 Use of medicinal products outside the conditions of the marketing authorization in the case of terrestrial animal species from which food is obtained, Article 114 Use of medicinal products intended for aquatic animal species from which food is obtained (2).

The preventive (prophylactic) use of antimicrobials will be limited to individual animals and will only be allowed where there is a high risk of infection, confirmed by a veterinarian. Collective treatments (metaphylactic use) will only be allowed in cases where there are no suitable alternatives. This must be confirmed by a veterinarian (2).

Under the new regulation, veterinary medicinal products should not be used to compensate for inappropriate breeding conditions or to accelerate their growth (2).

Romania in the current context

In this general context, Romania must keep up and continue to take measures to prevent the emergence and spread of antimicrobial resistance.

What can be done at this moment?

1. The education of future veterinarians, but also of veterinarians in the spirit of the correct use of this category of active substances is essential.
2. Elaboration of a National Action Plan meant to reduce the consumption of antimicrobials.
3. Advising farmers to improve microclimate conditions, diet to reduce the risk of disease of animals, prevention of diseases through vaccination or other measures that have proven useful in other countries.

What needs to be done in the near future?

1. Continuing education is essential.
2. Counselling / helping farmers.
3. Creation of a Network system for collecting data on antimicrobial consumption.

The achievement of all these objectives, as well as the establishment and achievement of new targets can be done only through a close, non-competitive collaboration between the faculty, state authorities, practicing veterinarians, farmers.

The motto of this collaboration should be "Honesty and mutual trust".

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9. ***https://www.ema.europa.eu/en/documents/scientific-guideline/updated-advice-use-colistin-products-animals-within-european-union-development-resistance-possible_en-0.pdf
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ULTRASOUND ASSOCIATION OF THE HYPERECHOIC PORTAL AND PERIportal SPACE IN THE ABDOMINAL PATHOLOGY OF CANINES

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Summary

Portal vein and portal branch hyperechoic appearance can be seen in different circumstances described in veterinary literature as well as in human pathology, as these changes associated with the ultrasound findings are not well understood in some cases. Although periportal cuffing and the hyperechoic aspect of the perivascular portal tributaries can be connected with pancreatitis, hepatitis, cholangiohepatitis, cholecystitis, pneumobilia and inflammatory bowel disease, changes in the ultrasound appearance can be also related with other vascular and gastrointestinal disorders. This study aims to describe portal vein changes, the association between periportal cuffing, the echo-rich aspect of the portal branches and gastrointestinal changes in dogs with multiple clinical signs.

Keywords: portal vein, ultrasound, periportal cuffing, vascular, dogs

The main portal vein is formed by the confluence of the cranial and caudal mesenteric veins and the splenic vein, and therefore drains most of the blood from the abdominal organs into the liver (1, 14). The portal vein also receives a smaller tributary vein, the gastroduodenal vein, a few centimeters caudal to the hepatic entrance and connects to the right-ventral aspect of the portal vein after draining the pancreaticoduodenal and gastroepiploic veins. The splenic vein is joining the main portal vein on the left side, which follows the left limb of the pancreas after receiving the left gastric vein. The caudal extension of the portal vein is represented by the cranial mesenteric vein that receives all of the jejunal branches (3). The diameter of the portal tributaries becomes enlarged as they approach the main portal vein, and the flow is directed towards the liver, the flow being known as the hepatopetal flow (13).

Ultrasound evaluation of the portal vein and abdominal vasculature is used as a first line investigation option in case of primary pathology of the portal vein, blood flow, velocity and abnormal connections, but also in different cases of abdominal diseases which are indirectly related with the periportal space and portal vein. A Doppler ultrasound exam documents blood flow direction in both vessels as into the liver (hepatopetal). Spectral Doppler shows a low-resistance, arterial flow pattern for the hepatic artery with forward flow throughout the cardiac cycle (7). The portal vein has a reduced blood flow compared to the rest of the vascular structures,

but variations of it may be possible and are also influenced by the respiratory phase (8). The latter also influences the portal diameter, as follows: the diameter increases post-prandial, in deep inspiration; Diastolic flow velocity increases and resistance index decreases after eating. The decrease in the diameter of the portal vein is closely related to the pre-prandial period, stress, exercise (8). The portal vein shows continuous antegrade venous flow with small pulsations that mirror the cardiac cycle. Mean portal flow velocities have been reported to vary between 15 ± 3 and 18 ± 8 cm/s in normal dogs (7, 13). However, these measurements are susceptible to inaccurate estimations, especially with higher correction angles (more than 60°), and must therefore be used cautiously (8, 13).

When evaluated with ultrasound, the portal vein wall is typically seen as hyperechoic (2) over a wide range of beam-vessel angles, whereas the hepatic vein wall is hyperechoic only when the incident beam and the vessel are perpendicular (7, 13, 17). Studies from human medicine have been attributed to marked discrepancies in mural thickness, collagen content, or perivascular fat between portal and hepatic veins (17). The recent documentation that the marked differences in echogenicity between the portal vein and hepatic vein walls typically observed at ultrasonography cannot be attributed to differences in mural thickness, collagen content, or perivascular fat. Rather, the distinct composition of the hepatic vein wall renders it a specular reflector, which is hyperechoic only when the angle between the ultrasound beam and the vessel wall is close to 90° , whereas the composition of the portal vein wall enables it to appear hyperechoic at a wide range of beam-vessel angles (17).

Hyperechoic/echo-rich periportal cuffing (ErPC) is defined as an increase in echogenicity relative to the adjacent liver parenchyma (9, 11). This is represented by thick echogenic bands around the portal veins in the periportal connective tissue of the portal triad, bands mainly seen in cases of enlargement of the liver with a diffusely decreased echogenicity of the parenchyma (5, 9). This feature determines a relative increase in the echogenicity of the portal vein walls and explains the sonographic appearance of the liver in a "starry sky" or centrilobular pattern (11, 15).

This study aims to describe portal vein changes, the association between periportal cuffing, the echo-rich aspect of the portal branches and gastrointestinal changes in dogs with multiple clinical signs.

Materials and methods

Medical records were analyzed retrospectively in 30 dogs of different breeds and of both sexes, with different gastrointestinal signs, primary or associated with other diseases and presented to St. George's Veterinary Hospital (Wolverhampton, UK) between November 2020 and March 2021. The animals were clinically examined by general examination methods according to the literature (16) and a complete abdominal ultrasound was performed. For this, an ultrasound Esaote

MyLab™ Seven (Esaote, UK) device was used, with 2 transducers, convex and linear, with frequency adjusted according to the patient's size, clinical presentation and age.

The consent of the owners was obtained for each case in order to use the data in carrying out this scientific paper.

Complete abdominal ultrasound was performed on 30 dogs, weighing between 5-20 kg, the population being represented by 17 males and 13 females, aged between 1 and 8 years.

Confirmed diagnostic of the presented cases were: Pancreatitis-acute presentation, 8 cases, 1 case of necrotising pancreatitis (Fig. 1); acute hepatitis: 1 case; cholecystitis: 5 cases; 3 cases were diagnosed with multiple acquired portosystemic shunts (Fig. 2), a portocaval shunt (Fig. 3) and left gastro-portal shunt; 4 of the examined dogs have been diagnosed with portal hypertension: 1 case of posthepatic portal hypertension due to right side cardiac failure (Fig. 3), 1 case of hepatic hypertension secondary to chronic liver disease and 2 cases of prehepatic hypertension caused by neoplasia. A number of 8 cases were presented with signs of gastroenteritis, with suspicion of IBD, although a confirmation of the inflammatory bowel disease was not done, due to financial concerns. Changes of the gastrointestinal signs were associated with thickening of the mucosal layer of the proximal duodenum. White stripes striation within the muscularis layer, fluid filled distended intestinal loops with no evidence of foreign body or material were seen at the same level, extending also in the jejunal area. From the 8 dogs examined for gastrointestinal signs, only 3 of them had a follow up ultrasound examination and changes seen at the level of small intestine improved after medical management.

Following the ultrasonographic examination, in all the cases presented, the hyperechoic periportal cuffing was highlighted, represented by the increased hyperechogenicity at the walls of the portal vein, the periportal space and the adjacent structures, compared to the normal appearance of the portal vein, highlighted in Fig. 5.

Results and discussions

Periportal cuffing of the liver is an ultrasonographic sign seen mostly in cases of abdominal inflammation, in which the pathogenesis and clinical significance remain obscure (5, 12). Diseases associated with periportal cuffing are heterogeneous and most commonly arise from the liver or the gastrointestinal tract. Diseases like inflammatory bowel disease (6), appendicitis and ulcerative colitis (11) are the most frequent diagnoses associated with periportal cuffing in human medicine, although the features of ulcerative gastrointestinal disorders in relation with portal and periportal changes are not very well described in veterinary medicine.

Hyperechogenic periportal cuffings may also result from liver inflammation that occurs in inflammatory bowel disease secondary to the abnormal cell passage from the intestinal mucosa into the portal system via enterohepatic circulation, described mostly in human medicine (6, 11). Periportal hyperechogenicity can also

be seen in cases of hepatobiliary diseases and other processes commonly associated with inflammatory bowel disease and primary sclerosing cholangitis, acute cholecystitis (6), in chronic hepatitis and pancreatitis, in both humans and dogs. Also, an oedema of the gallbladder fossa with pericholecystic fluid and thickening of the venous and the falciform ligament and generalised increased echogenicity can highlight the porta hepatis and periportal connective tissue appearance as hyperechoic. Thickening in the periportal area may occur with proliferation of the bile ducts, hemorrhage, oedema, fibrosis, inflammatory changes (acute and chronic hepatitis) or a combination of these. It is important to know that a normal hepatic echotexture does not exclude the diagnosis of acute hepatitis, and in most cases the liver will have a normal sonographic appearance (8, 11, 12). In some cases of chronic hepatitis, fibrosis of the liver can cover the portal vein walls, changes seen in dogs with a heterogeneous echogenicity of the liver parenchyma, hyperechoic appearance and diffuse enlargement with ill-defined edges (5).

In human medicine, gallbladder lithiasis can be accompanied by the appearance of a periportal cuffing (6), which was not found in dogs. Instead, neoplastic infiltration of the liver parenchyma seen with lymphoma and different type of round cell tumours can cause the appearance of hyperechoic portal walls, in both humans and canines, most likely due to generalized decrease of the liver echogenicity (6, 15). Same ultrasonographic aspect of the liver can be related also with amyloidosis and hepatic congestion and has been identified in both human and canine patients (5).

In this study, portal tributaries hyperechoic appearance and periportal hyperechogenicity in canines patients, was also seen in cases of portal hypertension, hepatic, pre and post hepatic, and in cases of portosystemic shunts, congenital or acquired, these aspects not being described in the literature.

The changes are potentially related with systemic inflammation, increased pressure within the portal lumen and vasculitis. Also mild portal and periportal cuffing was seen in a dog with focal dilation of the caudal vena cava in the area of porta hepatis, with an ultrasound appearance of ying-yang sign, representing bidirectional flow, secondary to the vascular aneurysm as seen in Fig. 3.

In cases diagnosed with acute and necrotizing pancreatitis from the study, mesenteric hyperechogenicity along with steatitis and mild periportal cuffing was seen. The follow up ultrasound examination of dogs presented with acute pancreatitis after medical management showed a normal appearance of the portal and periportal space, presuming that these findings are mostly associated with the per acute and acute stage of the inflammatory process, although in a small breed dog presented for supraventricular tachycardia secondary to acute pancreatitis changes seen at the level of hepatic portal vein became obvious after 48-72 hours from presentation. These aspects can lead us to consider that the portal changes can be seen also as a delay response of the primary gastrointestinal signs (Fig. 4).

Although the hyperechoic aspect of portal vasculature can be correlated with the acute evolution of various pathological processes encountered in the abdomen,

changes in echogenicity can be highlighted in the case of vascular abnormalities and chronic diseases, described in the previous studies (4, 6, 9, 10, 11, 12). When complete examination of the portal vasculature cannot be performed, minor changes of the vascular lumen may be indicators of pathological processes in relation to the clinical presentation, ultrasound vascular examination being very important in guiding the diagnosis of certainty. Furthermore, a periportal cuffing ultrasound and a hyperechoic appearance of the portal vasculature in absence of any other major abdominal ultrasound findings, it should guide us to a different approach of the examination, portal hypertension, primary heart disease, congenital processes and vascular abnormalities being involved in the systemic changes of the portal vessels ultrasonographic aspect. Although the hyperechoic appearance of the portal vein and its tributaries was mostly related to gastrointestinal signs, these changes can also be observed in cases of vascular abnormalities secondary to systemic inflammation, increased pressure in the portal lumen and vasculitis. Moreover, the ultrasonographic features of the portal system can be useful for completing the clinical picture and evaluating the different types of pathological changes in the abdomen. The disadvantage of this study is that it was performed on a small group of animals, so detailed future studies on a larger number of animals are needed.

The article aims to present changes at the level of portal vasculature seen in dogs with abdominal pathology and the importance of periportal cuffing ultrasound sign and hyperechoic appearance of the portal vasculature.



Fig. 1. Hyperechoic/echo-rich periportal cuffing – ultrasound examination in a dog with necrotising pancreatitis

In the same dog was seen also a hypoechoic rim of the gallbladder, with hyperechoic pericholecystic area, free peritoneal fluid, mild corrugation of the small bowel and pancreatic changes. At the level of porta hepatic, the mesenteric fat was hyperechoic with increased echogenicity of the portal walls.



Fig. 2. Perivascular hyperechogenicity - seen in a dog with peritoneal collection and multiple acquired portosystemic shunts

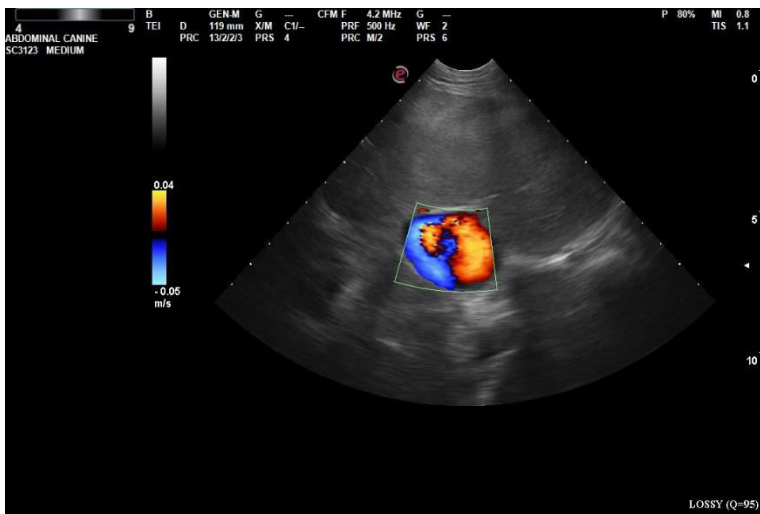


Fig. 3. Ying-Yang sign - focal dilation of the abdominal vena cava seen in a dog with hyperechoic and diffusely enlarged liver, peritoneal collection, portal hypertension and portocaval shunt



Fig. 4. Mild periportal cuffing – ultrasound examination at the level of the right liver lobes at approximate 72 h in a dog presented with supraventricular tachycardia secondary to acute onset of pancreatic signs and visceral pain. A complete ultrasound was performed after the medical stabilization of the patient

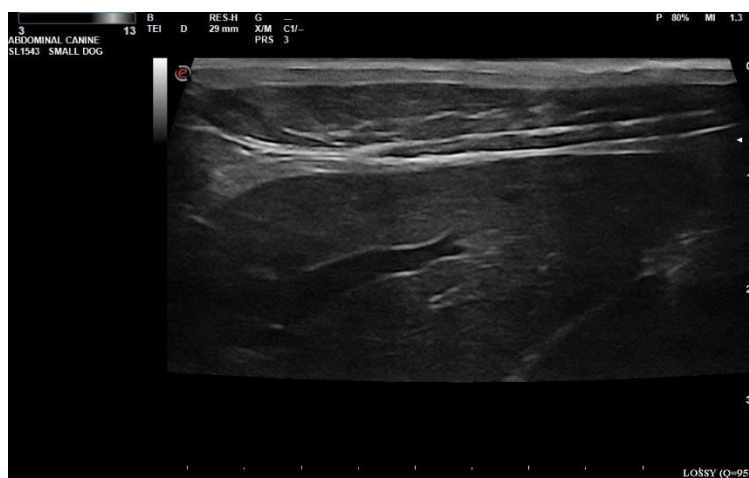


Fig. 5. Left branch of a normal portal vein. Normal appearance of the portal vein and portal branches is seen as a tubular structure with hyperechoic walls passing within the liver parenchyma

Conclusions

1. Periportal cuffing of the liver is an ultrasonographic sign seen mostly in cases of abdominal inflammation.
2. It is mostly described in relation with IBD, hepatobiliary diseases (acute and chronic cholecystitis and hepatitis) and other processes commonly associated with IBD, primary sclerosing cholangitis and pancreatitis, in both humans and dogs.
3. Echo-rich periportal cuffing can be caused by neoplastic infiltration of the liver parenchyma seen with lymphoma and different type of round cell tumors and acute hepatitis due to generalized decrease of the liver echogenicity.
4. Portal tributaries hyperechoic appearance and periportal hyperechogenicity in canines patients, can be also seen in cases of portal hypertension, hepatic, pre and post hepatic, and in cases of portosystemic shunts, congenital or acquired, being potentially related with systemic inflammation, increased pressure within the portal lumen and vasculitis.

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EFFECTIVENESS OF ONE ALTERNATIVE METHOD TO GRAM STAINING FOR BACTERIA DIFFERENTIATION

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Summary

The 3% KOH method was described in Japan by *Ryu* in 1940 and is still used in this country, but it seems that this very useful method has been applied very little in Europe. Most of the studies performed so far on the effectiveness of the KOH method compared to the Gram method are closely correlated. Researchers presented in this study aimed to assess the applicability and the effectiveness of the KOH test, compared with the Gram method, in evaluation of the Gram-negative bacteria load in a cattle shelter, in order to reduce the working time required to evaluate the microorganisms from the air. The results obtained reflected that the number of Gram-negative bacteria in one cubic meter of air determined by the 3% KOH method is very close to value determined by the Gram method. A correlation coefficient (r^2) of 0.98 was obtained between the 3% KOH test and the Gram staining method, which means a 98% correlation between these two tests. The KOH test is a rapid test that can be used to differentiate between Gram-negative and Gram-positive bacteria in current laboratory practice.

Keywords: alternative method, Gram method, bacteria

The bacterial world could be classified, using Gram staining method, into two broad categories: Gram-positive and Gram-negative bacteria based on their chemical structure of the cell wall and their affinity for basic dyes. Gram staining method is still used as bacterial diagnostic techniques, but it is known that the main disadvantage of it is the premature discoloration of some bacteria, which are mistakenly identified as Gram negative bacteria and that often leads to diagnostic errors (9).

Laboratory experts know that improper pigmentation can be influenced by many factors, such as the composition of the culture medium, the age of the culture, and antibiotic treatments on the bacterial isolate, which can allow gentian violet to wash off and the bacteria to stain differently, more precisely, some cells turn pink and others purple (3).

In most cases the interpretation of a stained smear by the Gram method is done without too many problems, but some species known to be Gram positive, especially those of the genus *Bacillus*, appear as Gram negative, when they reach a certain age, from a few hours to a few days. Moreover, strains of some species of Gram-negative bacteria belonging to the genera *Acinetobacter* and *Moraxella* have a special resistance to alcohol-acetone and its bleaching action is non-existent. Due

to these aspects, these species appear as Gram-positive bacteria, and they are known as Gram labile species. This behavior is not particularly helpful in identifying them using the Gram method (8).

As a result, over time, attempts have been made to address these shortcomings through several modifications to the Gram staining technique and several bacterial differentiation tests. Thus, tests were performed to detect aminopeptidase in Gram-negative bacteria, a constitutive enzyme found only in this type of bacteria, exposure to alkalis (KOH), as well as the susceptibility test to Vancomycin (5 µg) (1, 5, 8). Cerny (4) succeeded in differentiating Gram-negative bacteria from optional Gram-positive by analyzing aminopeptidase, a constitutive enzyme found mainly in Gram-negative bacteria.

The KOH test is based on the differences in the chemical structure of the cell wall in Gram-negative bacteria compared to Gram-positive ones. Due to this, the cell wall of Gram-negative bacteria is easily destroyed when exposed to dilute alkaline solutions. When the cell walls are broken, the suspension in KOH becomes viscous due to the release of DNA. Weak alkaline solutions have no effect on the wall of Gram-positive bacteria.

From the results presented so far, each of these tests, which were tested on collection or catalog bacterial strains, proved their effectiveness in different proportions. Furthermore, it should be emphasized that there are few studies on the practical applicability of these rapid tests, in assessing the bacterial load of some foods, beverages or milk (15). For example, in the study conducted by Harekrishna et al. (10) pathogenic-aerobic bacteria from different sample sources were investigated in and around the Bankura community. All bacterial isolates underwent Gram staining, Vancomycin susceptibility testing and KOH testing, of which 133 isolated strains, 99 (74.43%) were Gram-negative bacilli and 34 (25.56%) were Gram-positive cocci. Isolated Gram-negative bacilli were *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas spp.*, *Proteus spp.*, *Salmonella typhi*, *Citrobacter spp.*, *Enterobacter spp.*, and Gram-positive cocci were represented by *Staphylococcus spp.*, *Streptococcus spp.* and *Enterococcus*. All Gram-negative bacilli were resistant to Vancomycin and tested positive for 100% KOH. On the other hand, all Gram-positive cocci showed 98.23% sensitivity to Vancomycin, and 100% tested negative for KOH (10).

Taking in consideration these aspects, researchers presented in this study aimed to assess the applicability and the effectiveness of the KOH test, compared with the Gram method, in evaluation of the Gram-negative bacteria load in a cattle shelter, in order to reduce the working time required to assess the microorganisms from the air. It is known the importance of taking measures prompt during the evolution of bacterial diseases in farm animals. The air load in Gram negative bacteria reflects much better the sanitation of a shelter, comparatively with the total microbial load of the inside air. The large number of Gram negative bacteria in relation to the unit of volume and their repeated inhalation, together with the synergistic action of other factors (temperature, humidity), can determine the

reduction and defeat of the organisms' defense capacity. Moreover, it is known that the higher the concentration of microorganisms, the higher the risk of transmission of pathogenic microbes.

Materials and methods

The research aimed to assess the effectiveness of the fast method with the 3% KOH solution in classifying Gram negative and Gram positive bacteria, compared to the Gram method, in monitoring the total bacteria form the air in a cattle shelter. The free sedimentation method was used to assess the air microbial load (6). The examination and identification of the bacterial type was performed simultaneously by the Gram method (13) and the 3% KOH method (8).

To carry out this research, five determinations were made in a dairy cow shelter. The nutrient agar plates were exposed for 2.5 minutes and then brought to the laboratory and placed in a thermostat for incubation for 24 hours at 37°C. Each colony grown on each plate was numbered and by this mean was obtained both the number of colonies necessary to assess the bacterial load of the air in the shelter, and a clear record of the isolates that were tested by the two methods that highlight Gram-negative and Gram-positive bacteria: test with 3% KOH solution and Gram staining (Fig. 1).

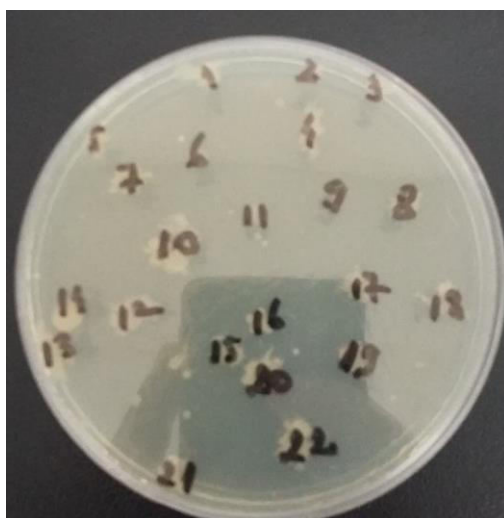


Fig. 1. Numbering of colonies on a plate with nutrient agar exposed in the shelter of dairy cows

Omeleanski's calculation formula was applied to calculate the microbial load in the shelter's air (8).

$$TNG = \frac{63662 \times n}{d^2 \times t} \text{ (cfu/m}^3\text{)}$$

TNG = total number of germs / m³ air;

n = number of colonies grown on exposed Petri plate, determined by counting all the colonies on the surface of the plate or by multiplying all the colonies counted on 1 cm², with the plate area (cm²);

63 662 = the empirical coefficient, derived from Omeleanski's work;

d = plate diameter in cm;

t = exposure time in minutes;

The Gram staining method was performed from each colony grown on the exposed plates (Fig. 2) (13).



Fig. 2. Microscopic preparations stained by the Gram method

The interpretation of this method takes into account the fact that Gram-positive bacteria which have a thick layer of peptidoglycan in their cell walls stain in violet, because they retain in the parietal structures the first dye (crystal violet solution), which is not removed even when discoloration is applied and Gram-negative bacteria, that have a thinner peptidoglycan wall will stain in red, as following the retention of the second dye – safranin (13).

The KOH test consists of depositing a drop of 3% KOH solution on a glass slide and adding or mixing a quantity of bacterial culture. For the accuracy of the operation, a quantity of 10 microliters was deposited on the slide using an automatic pipette. Then, using a sterile loop, with a loop diameter of 3 mm, in the KOH drop, a visible amount of bacterial culture (a colony) obtained on nutrient agar was transferred. With the help of the loop, the biological material was mixed with the 3% KOH solution for 60 seconds, on an area of approximately 1 cm² (Fig. 3) (8). The interpretation of the results of this method is based on the formation of a filament

adhering to the lifting of the loop, over a distance of 1 cm. So, if the suspension of bacteria and KOH becomes viscous or gelling after mixing and when lifting the loop from the drop an adherent filament is formed, which is maintained about one centimeter above the blade, the test is considered positive (Fig. 3), and the bacterial isolate is considered to be Gram negative. If no increase in viscosity and formation of the adherent filament is observed, the test is considered negative and the bacteria in that culture are considered to belong to the Gram-positive group (8).



Fig. 3. Positive reaction to 3% KOH test

The results were recorded and finally the correlation between the two methods used to determine the proportion of Gram negative and Gram positive bacteria in the shelter air was assessed.

In addition, in order to assess the applicability of the rapid KOH test, compared to the Gram method, the number of Gram-negative and Gram-positive bacteria, respectively, was calculated and reported as a percentage of the total number of aerobic mesophilic bacteria in a cubic meter of air.

Results and discussions

The results obtained after testing the bacterial isolates found in the air of a cattle shelter with the KOH method and the Gram staining are presented in Table 1.

From the total of 110 bacterial isolates (colonies) examined in the 3% KOH solution test, a positive reaction was obtained in 50 colonies, which means that they were Gram-negative bacteria. As a result, it can be stated that in the cattle shelter the proportion of Gram-negative bacteria would correspond to a value of 45.45%.

The results obtained from the examination of the 110 bacterial isolates by the Gram method show that 48 isolates consisted of Gram-negative bacteria, which

represents a proportion of 43.6% Gram-negative bacteria in a cubic meter of air in the air in the cattle shelter.

Table 1

Results of the assessment of bacterial isolates from the air of a cattle shelter by the KOH method and Gram staining

Total number of isolated colonies	Number and percentage of colonies tested by the 3% KOH method		Number and percentage of colonies tested by the Gram method	
	with positive reaction	with negative reaction	positive	negative
14	5 / 35.7	9 / 64.3	9 / 64.3	5 / 35.7
14	7 / 50.0	7 / 50.0	7 / 50.0	7 / 50.0
22	10 / 45.5	12 / 54.5	14 / 63.7	8 / 36.3
18	7 / 38.8	11 / 61.2	11 / 61.2	7 / 38.8
42	21 / 50.0	21 / 50.0	21 / 50.0	21 / 50.0
110	50 / 45.4	60 / 54.5	62 / 54.4	48 / 43.6

The difference between the proportion of Gram-negative bacteria tested by the 3% KOH method and that obtained by the Gram method was due to two isolated colonies, which consisted of both Gram-positive cocci and Gram-negative bacilli, the latter in greater proportion (Fig. 4). These two colonies were considered to have given a false negative reaction.

According to the results obtained after performing these experiments, a correlation coefficient (r^2) of 0.98 was obtained between the 3% KOH test and the Gram staining method, which means a 98% correlation between these two tests.

The 3% KOH method was described in Japan by Ryu in 1940 and is still used in this country, but it seems that this very useful method has been applied very little in Europe (8). Most of the studies performed so far on the effectiveness of the KOH method compared to the Gram method are closely correlated. The results obtained in the present study are consistent with the studies performed. The method has also been described and used to characterize 69 strains of bacteria found in veterinary microbiology clinics, proving to be 100% correlated with the Gram method (7).

The effectiveness of the KOH method was also confirmed in the study of 22 species of bacteria of medical importance found in the beer industry (12).

Out of the desire to use this rapid test for routine microbiological diagnosis performed in hospitals and to reduce costs, the Gram method and the KOH method were tested comparatively on 100 bacterial strains isolated from the same samples and proved to be 100% correlated (11).

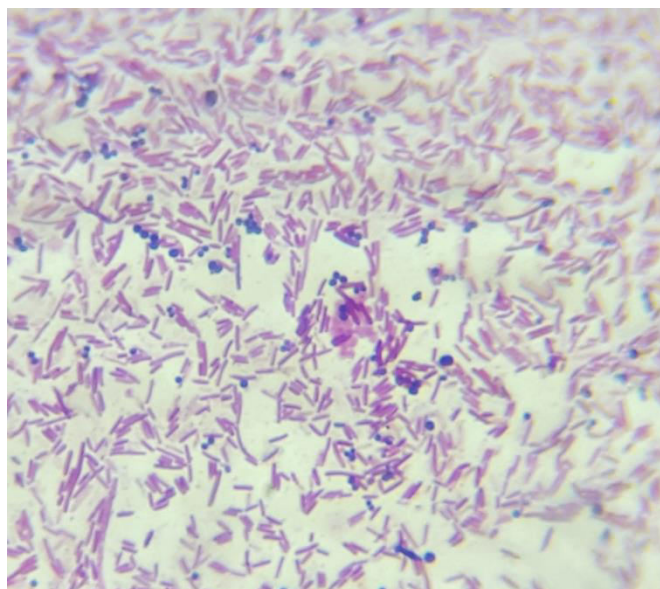


Fig. 4. Microscopic aspect of smears stained by the Gram method made from colonies that gave a negative reaction in KOH 3% (x100)

In Table 2 the microbial load of the air in the cattle shelter obtained by the free sedimentation method and the number, respectively the percentage of Gram negative bacteria appreciated by the two applied working methods is shown.

Table 2

The microbial load of the air in the cattle shelter and the number, respectively the percentage of Gram negative bacteria appreciated by the two tests

Total number of germs in the shelter air cfu/m³	The number / percentage of Gram-negative bacteria in the shelter air	
	KOH test	Gram staining method
4401.3	1571.9 / 35.7	1571.9 / 35.7
4401.3	2200.6 / 49.9	2200.6 / 49.9
6916.3	3143.3 / 45.4	2515.1 / 36.3
5658.3	2200.6 / 38.9	2200.6 / 38.9
13203.9	6601.4 / 49.9	6601.4 / 49.9

The bacterial load of the air in the cattle shelter ranged from 4401.3 cfu/m³ at the beginning of the determination (January-May) to 13203.9 cfu/m³. During this period the number of Gram-negative bacteria, determined by both methods,

increased from 1571.9 cfu/m³ to 6601.4 cfu/m³, which is a load four times higher at the end of the determinations compared to the value obtained at first determine. The difference of the load in Gram-negative bacteria of the air in the shelter, obtained by the Gram staining method and the 3% KOH test at the determination of which false negative reactions were obtained was 628 cfu/m³, which represents a value of 9%, if it refers to the final value of the total number of germs (6916.3).

As a result, it can be said that the load of Gram-negative bacterial germs in one cubic meter of air determined by the 3% KOH method is very close to their value determined by the Gram method. This could allow the recommendation to use the rapid method of highlighting Gram-negative bacteria for the assessment of microbial load in animal shelters. In this sense, research should be more extensive and to verify the correlation of this method with other methods of differentiating Gram-negative bacteria from Gram-positive ones.

The microbial load in a shelter is very varied from a systematic and quantitative point of view. An insignificant part of the shelter's microbial load comes from atmospheric air, but most microorganisms come from manure, feed, bedding and, in some cases, from the pathological excretions and secretions of animals (2).

The assessment of microbial load is made by quantitative determinations to determine the number of aerobic mesophilic germs in a cubic meter of air, and qualitative determinations, which monitor the presence of certain species of potentially pathogenic microorganisms, reflected in microbiological indicators of air contamination (14).

One of these indicators is coliform germs, in addition to staphylococci and microscopic fungi. These bacteria come mainly from animal feces and to a lesser extent from normal and pathological secretions. Increasing the proportion of coliform bacteria in the air of shelters means an increase in the risk of transmitting pathogens, which are usually eliminated by manure, which are usually Gram-negative germs. This category includes species belonging to the genera *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Morganella*, *Proteus*, *Providencia*, *Salmonella*, *Serratia*, *Shigella* (14).

In addition, Gram-negative cocci and cocobacilli such as those included in the genera *Bordetella*, *Brucella*, *Haemophilus*, *Moraxella*, *Neisseria* and *Pasteurella* can also be found in animal shelters (14).

This is why it was considered that a determination of the load in Gram-negative bacteria reflects much better the sanitation of a shelter, their particularly large number per unit volume and their repeated inhalation together with the synergistic action of other factors, they can reduce and defeat the body's ability to defend itself (14).

In some studies performed on the air microbial load of animal shelters, the following bacterial families predominated in the airborne Gram-negative bacterial flora: *Enterobacteriaceae*, *Pseudomonadaceae* and *Neisseriaceae*. Within the family *Enterobacteriaceae* the species *Escherichia coli* and *Enterobacter agglomerans* were predominant. In shelters that use straw for bedding *Enterobacter agglomerans*

was the most common, while in shelters without bedding *E. coli* was most commonly isolated. Representatives of the *Pseudomonadaceae* family were isolated in high concentrations during periods of high air humidity. Representatives of the genera *Bacillus* and *Streptomyces* were also isolated (16).

The proportion of Gram-negative and Gram-positive bacteria in animal shelters differs from study to study, with very high limits.

In experiments performed in halls with different occupancy rates (119 birds, respectively 238 birds / 100 m³ air) and with different temperatures in the hall (15.6 and 26.7°C), it was shown that a higher (238 birds / 100 m³ air) generates a larger number of airborne microorganisms. In this study 15 bacterial genera were identified. In aerobic bacteria, representatives of the genera *Bacillus*, *Micrococcus*, *Proteus*, *Pseudomonas* and *Staphylococcus* were most frequently isolated, while in anaerobic bacteria four species of the genus *Clostridium* were most often identified (14).

Some studies conducted in several types of bird shelters in terms of aeromicroflora, report that a percentage of only 0.02 - 5.2 (average 2.6%) of the total number of cultivable aerobic bacteria is Gram-negative bacteria. These values are about 10 times lower than the values mentioned above. Mandatory anaerobic Gram-negative bacteria have not been isolated (2).

Conclusions

The KOH test is a rapid test that can be used to differentiate between Gram-negative and Gram-positive bacteria in current laboratory practice.

There is a close correlation between the KOH test and the Gram staining method, as a result the two can be used and complete each other in situations where the diagnosis of bacteria is uncertain.

The obtained results allow the recommendation of the use of the fast method with KOH to highlight Gram-negative bacteria in the assessment of microbial load in animal shelters or other environments. In this sense, research should be more extensive and to verify the correlation of this method with other methods of differentiating Gram-negative bacteria from Gram-positive ones.

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PREVALENCE OF *CRYPTOSPORIDIUM* INFECTION IN CALVES, BY NON-MOLECULAR ASSAY, IN VALCEA COUNTY, ROMANIA

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Summary

Cryptosporidiosis is an important zoonotic disease caused by a apicomplexan parasites belonging to genus *Cryptosporidium*, clinically characterized by gastrointestinal disorders, pancreatic and hepatic disorders, respectively. Economic losses are reflected by growth delays, mortality and high treatment costs, respectively. The highest receptivity is found in the case of large and small ruminants, especially in calves aged between one week and three weeks. The aim of this study was to determine the prevalence of *Cryptosporidium* spp. in calves from Valcea County. To achieve this, samples were collected from several family-type microfarms. These samples were subjected to two methods of parasitological examination: direct smear and antigen rapid tests for *Cryptosporidium* spp. The overall prevalence of *Cryptosporidium* spp., in calves from Valcea County, Romania, was found to be 37.34% using direct smear and rapid antigen test.

Keywords: calves, *Cryptosporidium* spp., prevalence, direct smear, rapid antigen tests

Cryptosporidiosis is an important zoonotic disease caused by an apicomplexan parasite belonging to genus *Cryptosporidium*, clinically characterized by gastrointestinal disorders, pancreatic and hepatic disorders, respectively. Economic losses are reflected by growth delays, mortality and high treatment costs, respectively (6, 7).

From the first description, in 1907, of the species *Cryptosporidium parvum*, to the present day, more than 30 species of *Cryptosporidium* have been identified in different host species, some with a clear zoonotic potential. Cattle cryptosporidiosis has been described since the 1970s, and the identified species are considered the most important pathogens involved in the ethology of calves' neonatal diarrhoea (6, 16, 17).

The highest receptivity is found in the case of large and small ruminants, especially in calves aged between one week and three weeks.

The high prevalence, recorded in animals, may be a consequence of the fact that the diagnosis of this protozoan occupies a low percentage in the routine diagnosis of veterinarians.

The aim of this study was to determine the prevalence of *Cryptosporidium* spp. in calves from Valcea County.

Materials and methods

To achieve the proposed goal, 83 feces samples were collected, in plastic bags, from calves (Fig. 1) reared in family-type micro-farms. Micro-farms are located in localities Alunu, Copăcenii and Mateești, Valcea County in southern Romania (Fig. 2). These samples were subjected to two methods of parasitological examination: direct smear and rapid antigen tests for *Cryptosporidium* spp. Individual sheets were made for each calf, including the following details: breed, age, sex, possible digestive disorders - diarrhea and matriculation number, respectively.

The statistical analysis was performed by GraphPad, QuickCalcs, Fishers exact test and Office Excel 2016.



Fig. 1. Studied calves from Valcea County

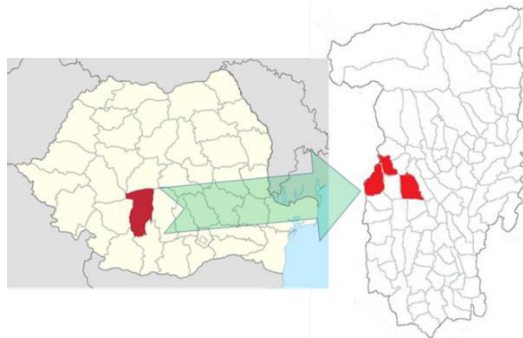


Fig. 2. The area of origin of the samples from the calves studied

Results and discussions

The 83 calves studied were grouped into 3 age categories as follows: 0-7 days (3 calves), 7-14 days (31 calves), 14-28 days (34 calves), 28-56 days (15 calves) (Fig. 3).

The overall prevalence of *Cryptosporidium* species, in calves from Valcea County, Romania, was found to be 37.34% (31/83) using direct smear and rapid antigen test (autumn period).

Out of a total of 37.34%: for the age range 0-7 days out of the total number of three calves only one was registered and being positive; the prevalence in this situation is 33.33%.

For the total number of 31 calves aged 7-14 days or recorded only 15 positive cases the prevalence being 48.38%.

In calves aged between 14 and 28 days out of a total of 34 specimens only 10 were positive for *Cryptosporidium*; prevalence with a value of 29.41%.

In the age category 28-56 days, 15 calves were examined but only five were positive the prevalence being 33.33%.

Thus, at the level of the 3 family-type microfarms, the positivity was higher in the case of the period 0-2 weeks.

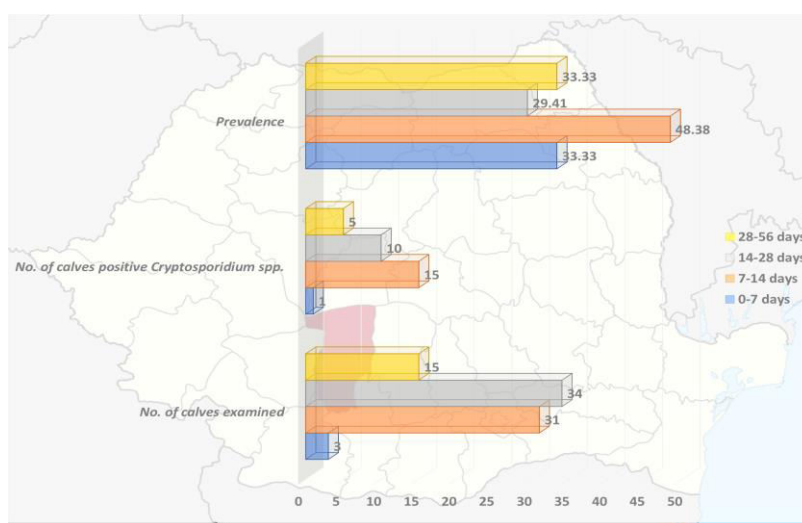


Fig. 3. The prevalence of *Cryptosporidium* spp. in calves from Valcea County

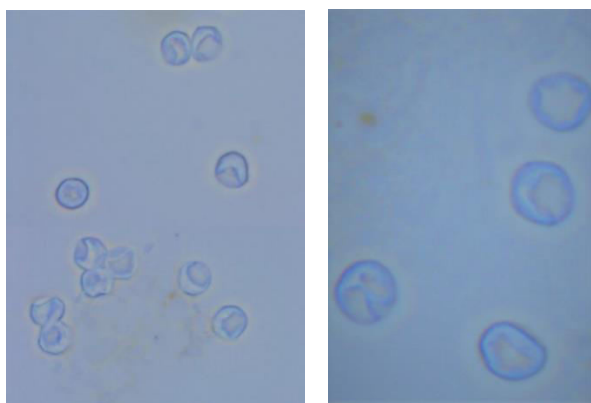


Fig. 4. *Cryptosporidium* spp. Oocysts (left and right) identified by direct smear

In Romania, studies have been performed on the presence of *Cryptosporidium* oocysts, both in animals (8, 9, 12) and in wastewater, surface water and in the main rivers (10, 11).

In cattle, in Romania, the prevalence of cryptosporidiosis shown in several studies varied between 25% - 32.3% and 41.6% (1, 4, 5) with a maximum of 100% on two farms from Arad county (13).

In the central and north-western part of Romania, respectively, the highest values of the prevalence of parasitism with *Cryptosporidium* spp. were reported in late winter- early spring: 70.21% (April), the rest of the year being recorded values of 16.80% +/- 5.78% (2).

In Switzerland, *Cryptosporidium* spp., in addition to rotaviruses, is a common pathogen, but also responsible for 50% of cases of calves a few weeks old with diarrhea. This diarrhea, it was found, persists for 4 to 6 days, with a high excretion of infectious oocysts. It was also found that it is not an exact age at which diarrhoea occurs, on average, being day 4 of life (15).

Underlying these effects is the attachment of *Cryptosporidium* spp. to the surface of intestinal epithelial cells. As immediate effects are damage to the microvilli and "joining" of the intestinal villi; all this causing significant weight loss. Other causes of diarrhoea are the presence of a thermosensitive enterotoxin in the intestinal mucosa, disturbance of the secretion-absorption system in the intestine. Another cause is the reduction of lactase activity, which causes a passive secretion of water from the mucosa to the lumen (7, 15).

Although in our study there were no statistically significant differences between the prevalence of different age groups, Bejan, 2009, noted that calves aged between 1 and 3 weeks showed a percentage of 52.06% and in second place were calves aged 3-12 weeks, with a prevalence of 39.66%.

On the other hand, it can be said that the main source of infection in newborns is the mother cow. The primary cause is the low titer of Ig G (passes from the blood into the colostrum), and the immunoreceptivity affected by advanced gestation in which the function of T cells is affected, as well as the activity of effector B cells. At the base of the immunoreceptivity alteration are plasma proteins and sex hormones (involved in gestation).

The rout of infection is oral, which is amplified by the fact that the disinfectants used in cattle farms are inadequate, or disinfection is not done correctly, sometimes it is the fact that it is not done at all (6, 7, 12, 14).

Numerous researches have shown that *Cryptosporidium* oocysts are frequently present in the drinking water of animals and humans. Even if the water is filtered and subjected to various treatments, it cannot be guaranteed that in the end, all *Cryptosporidium* oocysts will disappear (3, 10, 11).

Conclusions

The prevalence of *Cryptosporidium* spp. infection, in calves from Valcea County, was 37.34%, with a minimum value of 33.33% for the age categories of 0-7 and 28-56 days and with a maximum value of 48.38% for the age category of 7-14 days.

No statistically significant differences between positivity relative to different age groups were registered.

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RETROSPECTIVE STUDY OF THE RECTAL PROLAPSE – 53 CASES OF DOGS AND CATS

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Summary

Rectal prolapse is defined as the protrusion of the rectal mucosa from the anus. It can be classified as complete or incomplete. Rectal prolapses occur more frequently in young animals (dogs and cats) and are associated with different conditions that cause tenesmus such as endoparasites, rectal foreign bodies, tumors, urethral obstruction, prostate disease, dystocia or perineal hernia. The digital correction accompanied by suture of the structure in purse string is usually applied in cases where the integrity of the mucosa is conserved, when this one is devitalized the resection is the corrective method of choice. Recidivate prevention by colopexy may be realized by a simple suture technique or an incisional technique. In this paper a retrospective study of 53 cases of dogs and cats (clinical cases of the surgery clinic of the Faculty of Veterinary Medicine of Timisoara between 1993-2020) was reported and some considerations about aetiology, animal pathology, clinical aspects, diagnosis, therapy modalities and prolapse relapses was analyzed.

Keywords: dog, cat, colopexy, rectal prolapsed

Rectal prolapse occurs in dogs and cats, with no documented breed predisposition (6). In rectal prolapse, one or more layers of the rectum protrude through the anus. Prolapse may be classified as incomplete, in which only the rectal mucosa is everted, or complete, in which all rectal layers are protruded. Rectal prolapse (complete prolapse) is a double-layer invagination of the full thickness of the rectal tube through the anal orifice (6, 18).

Young animals more often have rectal prolapse or intussusception as a result of parasite infestation, disorders of the rectum (lacerations, diverticula, sacculations, foreign body ingestion), colitis, tumors, urethral obstruction, urolithiasis, prostate disease, dystocia or perineal hernia (2, 4, 6, 8, 16, 18, 19, 27).

The primary clinical sign, an elongated, cylindrical mass protruding through the anal orifice, is usually diagnostic. The mass must be differentiated from prolapsed ileocolic intussusception by passing a probe, blunt instrument, or finger between the prolapsed mass and the inner rectal wall. In rectal prolapse, the instrument cannot be inserted because of the presence of a fornix (6, 8). Other common signs include ulceration, inflammation, and congestion of the rectal mucosa. Shortly after the onset of a prolapse there is a short, nonulcerated, inflamed segment of rectal tissue; later, the mucosal surface darkens, and hardens

(8) Continued exposure causes excoriation, bleeding, desiccation, and necrosis (6).

The digital correction accompanied by suture of the structure in purse string (tobacco bag) is usually recommended in cases where the integrity of the mucosa is conserved, when this one is devitalized the resection is the corrective method of choice (1, 5, 6, 8, 10, 12, 14, 17, 21, 22, 23, 24, 26).

Recurrent rectal prolapse may be prevented by performing a colopexy (6, 8, 10, 11, 13, 14, 17, 20, 21, 22, 23, 24, 25, 29, 30).

Materials and methods

This retrospective study considered all cases of rectal prolapse diagnosed in the Small Animal Surgery Clinic of BUASVM "King Michael I of Romania" from Timișoara, between January 1993 and December 2020.

Data collected and analyzed included species, breed, age at the time of diagnosis, sex and reproductive status (neutered / intact), status on preventive or curative antiparasitic therapy, depending on the case, etiology, treatment method, and number of relapses. Data were inserted in Microsoft Excel spreadsheets and we calculated averages, medians and distribution of age at the time of diagnosis (for all cases and by breed), percentile distribution of breeds, reproductive status, status on preventive or curative antiparasitic therapy prior to diagnosis, main causes, treatment method used and number of relapses.

All comparative dual data were statistically reviewed using the t-test. Mann-Whitney's U test was used for rank grouped data, and to demonstrate the possible differences in species, breed, age, antiparasitic therapy, number of relapsed and results after different surgical procedures. Significance was established at $p < 0.05$.

Results and discussions

The total number of cases of rectal prolapse diagnosed in the Small Animal Surgery Clinic of BUASVM "King Michael I of Romania" from Timișoara between 1993 and 2020 was 53, of which 20 were cats and 33 were dogs.

Of the total 25852 of cases presented in our clinic between 1993 and 2020, the cases with rectal prolapse account for 0.2% (Fig. 1).

Data analysis revealed that 18 (90%) out of the 20 cats were domestic shorthair, with 1 being Persian and 1, Siamese, whilst regarding to dogs, they belonged to 12 breeds, with the mixed-breed being the most common – 9 dogs (27%).

The average age was 24 months, with a median of 11 months. The youngest patient age was 1-month-old, whilst the oldest was 144 months old.

The average age in dogs was 31 months, with a minimum of 2 months and a maximum of 144 months. Regarding the cats, the average age was 12 months, with a minimum of 1 month and an upper age of 84 months. Age distribution

revealed 2 peaks: one around 3 – 5 months, and the other 36 – 48 months. There were no statistically significant differences regarding the age peaks in dogs and cats, but 75% of cats were located within the Q3 quartile – values smaller than the median (11-months-old), whilst dogs were located in the Q1 quartile, in which 75% were older than 11 months of age.

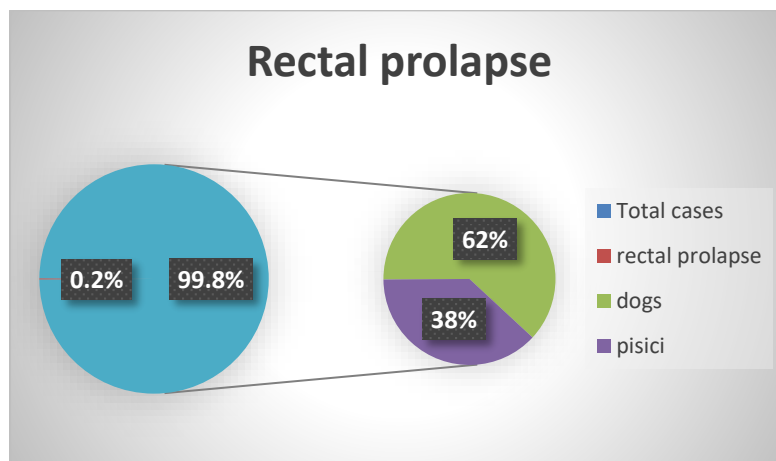


Fig.1. Prevalence of rectal prolapse in dogs and cats in the Small Animal Surgery of BUASVM 1993 – 2020

Regarding the sex, the total distribution of both dogs and cats was relatively uniform, with 47% being females and 53% being males, without any statistically significant differences regarding correlation with the breed or age.

Almost the entire casuistry – 92.5% (49 cases) consisted of intact animals. Only two dogs and two cats, all older than 3 years of age, were neutered.

Analysis of preventive antiparasitic treatments revealed that 16 cats (80%) had no registered treatment, with 12 of these (75%) being younger than 12 months of age. Regarding the dogs, 17 individuals (51%) had a single entry for antiparasitic therapy in their health books in the last 12 months, and only 4 individuals (12%) had no such entry.

As possible causes for rectal prolapse in the 33 dogs, we identified the following: parasite infestation in 6 animals (18%), diverticula or sacculation of the rectum, foreign body ingestion in 14 dogs (42%), enteritis-colitis in 5 dogs (15%), tumors in 2 dogs (6%), prostate disease in 4 dogs (12%), and dystocia in 2 bitches (6%). In cats, parasite infestation was identified in 16 animals (80%) and enteritis in 4 animals (20%).

Acute rectal prolapse at 41 cases of dogs and cats (77%) were treated by saline lavages, 2-5 minute of ice applied locally, lubrication with an antibiotic ointment, digital reduction and a placement of a purse-string suture, tight enough to

maintain prolapse reduction without interfering with passage of soft stool, and was maintained for 5-7 days (Fig. 2).



Fig. 2. Surgical treatment of the acute rectal prolapse in a dog before digital reduction (left), after placement of the purse-string suture (right)

Nonreducible or severely traumatized prolapses - 12 cases (23%) were treated by amputation and end-to-end anastomosis with simple interrupted sutures.

All 12 (23%) relapse cases were observed in the canine population. In 8 patients, treatment consisted in digital correction accompanied by suture of the anal sphincter in purse string, celiotomy and simple colopexy (4 – 5 non-perforating horizontal mattress sutures, passed up through the submucosa, placed on the antimesenteric border and anchored to the celiotomy incision) or double colopexy in 4 cases (3 – 5 cm sero-muscular linear incision in the colon and a similar incision in the peritoneum and transverse fascia of the left abdominal wall, approximately 2 cm lateral to the *linea alba*, and the edges of these incisions being sutured separately using simple interrupted sutures, thus resulting 2 different sutures).

The peak distribution at 3 – 5 months of age in cats is, most probably, due to improper preventive antiparasitic therapy, since 80% had no such therapy registered and 12 of these (75%) were younger than 12 months-of-age. Parasite infestation (2, 4, 6, 8, 16, 18, 27) represent a most common cause of rectal

prolapse in dogs and cats. With regards to dogs, 75% were older than 11 months-of-age, with 42% of them having had foreign body ingestion as an etiological factor, which might explain a peak distribution around the age of 36 – 48 months. Although the foreign body induced ileus was more frequently found in animals below 2 years of age (3, 9), foreign body prehension play-behavior doesn't end with an older age, with rectal prolapse being one of the consequences of irritating action on the mucosa of the colon in adult animals (3 – 7 years old). Constipation is one the frequently observed clinical signs in adult and geriatric dogs.

There were no relapses in any of the 12 cases in which colopexy was performed, and there were also no differences between the two techniques used, with the results being similar to those of other authors (17, 20, 23, 24). After simple colopexy, there were no complications, similar to those reported in the specialty literature (7, 15, 25), as compared to linear gastropexy in which relapses occur in 6.4% of cases (28).

Conclusions

Rectal prolapse occur more frequently in young animals, both dogs and cats, and are associated with different conditions (eg. endoparasite infestations) that cause tenesmus.

Recurrences of rectal prolapse after digital reduction treatment and placement of the purse-string suture are more common in dogs than in cats.

Colopexy was effective in preventing recurrent rectal prolapse.

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INCIDENCE AND PREVALENCE OF *SALMONELLA SPP.* ON A BIRD POPULATION FROM SOUTHERN ROMANIA

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Summary

The researches' goal was to determine the presence of *Salmonella spp.* in chicken used for production and reproduction, broilers and also avian products. A number of 5001 samples from a bird population in southern Romania were used, divided over a period of four years, according to the "Strategic program for the control of salmonellas". After the samples were examined 54 were identified as positive, mentioning that *Salmonella hadar* has been identified in a percent of 90.7%. The maximum number of positive samples, respectively 39 samples (72.2%), was identified in 2016, 12 positive samples in 2017 (22.2%) and only 3 positive samples in 2018 (5.6%). Avian salmonellosis constitutes for most of the countries with a developed intensive system, one of the most important sanitary-veterinary problem due to economical losses, as well as for the involvement of those organisms in human health by triggering food poisonings following the ingestion of contaminated products. Also, the infections with *Salmonella spp.* are responsible for the presence of an appreciably amount of acute and chronicle diseases in different species of domestic or wild birds. It is thought that over 80% of *Salmonella* strains pathogenic for mammals have been isolated from birds. The presence of the infections caused by salmonellas in private and industrial avian growing systems and the contamination of avian products constitute a clear and present danger for public health. Researches performed over the last few years have proved that over 90% of the broiler chickens, over 85% of turkeys and 45% of hens are carriers of salmonellas. The performed researches studied the incidence of salmonellas on a bird population from southern Romania, on hens both for production and reproduction, broilers and avian products, on a period of four years.

Keywords: salmonella, chicken, incidence

Salmonella spp. is considered to be one of the most important foodborne pathogens that can cause severe human infections and high economic losses worldwide in the intensive poultry farming system. The presence of *Salmonella* strains is monitored in different stages, on the production flow, within the strategic programs for control and monitoring of the presence of *Salmonella* (1, 4, 7).

Avian salmonellosis constitutes for most of the countries with a developed intensive system, one of the most important sanitary-veterinary problem due to economic losses, as well as due to the implications of those micro-organisms in

human health, by triggering food poisonings following the ingestion of contaminated products (3, 5).

Also, the infections with *Salmonella spp.* are responsible for the presence of an appreciable amount of acute and chronic diseases in different species of domestic or wild birds. It is thought that over 80% of *Salmonella* strains pathogenic for mammals have been isolated from birds, too. The presence of the infections caused by salmonellas in private and industrial avian growing systems and the contamination of avian products constitutes a clear and present danger for public health (2, 6).

Materials and methods

Fecal and organ samples were collected from chickens and hens with no clinical signs of salmonellosis. Different types of fecal samples were used, each sample consisting of fresh distinct fecal matters, with a weight of minimum one gram and collected randomly from many locations of the farm where the birds are located.

From the dead bodies were collected samples of intestinal matter and growth supports have been sawed with samples from organs and bone marrow.

In the case of avian products, the samples were collected from bird carcasses, organs (liver, spleen) and assortments resulted after butchery (back, drum sticks etc.).

The number of samples collected every year was in concordance with the "Strategic program for the control of avian salmonellas", mentioning that, when the number of samples was higher, an additional number of samples have been collected.

The collected samples have been analyzed in the bacteriology laboratory of DSVSA Dolj. Bacteriological exams were performed in order to determinate the species of *Salmonella* by observing morphological, cultural and biochemical features.

We would like to mention that, in order to identify the species, the procedures recommended by the reference community lab-SR EN ISO 6579/AC 2006 and SR EN ISO 6579/A1 2007 were used. The obtained results were processed and presented in the following tables.

Results and discussions

During the period of four years, a number of 5001 samples were examined, as follows: 710 samples in the year 2016, 843 samples in the year 2017, 2183 samples in the year 2018 and 1259 samples in the year 2019 (Table 1). The number of samples were correlated with the strategic program for each of the four years.

Analyzing the bacteriological exams, 54 out of 5001 samples, were positive, and the following serotypes were identified: *Salmonella Hadar* (30.7%), *Salmonella Glostrup* (7.4%) and *Salmonella spp.* (1.9%) (Table 2).

Table 1

**The examined samples of avian products and organs during
the period 2016-2019**

Type of sample		Year							
		2016		2017		2018		2019	
		Total samples							
		710		849		2183		1259	
		A*	I*	A	I	A	I	A	I
Hens for eggs	Total	171	-	190	-	253	-	229	-
	Growth	124	-	132	-	178	-	142	-
	Adults	47	-	58	-	75	-	87	-
Hens for reproduction	Total	88	-	73	-	84	-	94	-
	Growth	57	-	47	-	54	-	66	-
	Adults	37	-	26	-	31	-	28	-
Broilers		164	-	243	-	973	2	284	3
Poultry meat and products		287	-	873	39	343	10	652	-

Legend*: A – analyzed samples; I - isolated *Salmonella* strains

Table 2

Results of the bacteriological exam

Isolated <i>Salmonella</i> strains	Serotypes	54
	<i>Salmonella hadar</i>	49
	<i>Salmonella glostrup</i>	4
	<i>Salmonella spp.</i>	1

Analyzing the acquired results, it is ascertained that most of the positive samples have been diagnosed in the year 2017 (39), mentioning that all the samples have originated from avian meat and organs.

The results from 2017 have enforced for the year 2018, the examination a much higher number of samples (2183) in order to identify the primary contamination. It can be noticed from table 1 that the positive samples, in a much smaller number (12) originated mainly from avian meat and organs (10), although two positive samples were identified on broiler chickens. During the first three months of 2019, out of 1259 samples that have been examined, only three samples collected from broilers were diagnosed as positive.

Considering the requests of the European Commission (Directive no. 142/21 June 2006) for diminishing the prevalence of *Salmonella Enteritidis*, *Salmonella hadar*, *Salmonella infantis*, *Salmonella typhimurium* and *Salmonella virchow*, it can

be observed that, on southern Romania, out of the species mentioned above, only *Salmonella hadar* (49 out of 54) was identified.

We would like to mention, as a positive aspect, the decreasing tendency in the presence of this specie in particular, and of salmonellas in general, in the year 2019 there have been identified only 3 positive samples, simultaneously with a serious medical treatment and the carrying out of a correct disinfection and pest control.

Conclusions

Bacteriological exams performed over a period of four years (5001 samples) revealed that, the main isolated serotype was *Salmonella hadar*, which was isolated in a high percent (90.7%) and followed by *Salmonella glastrup* (7.4%) and *Salmonella map* (1.9%).

The maximum number of positive samples was identified in the year 2017 (39 positive samples), and although in 2018 the number of analyzed samples was much higher (2183 against 843), only 12 samples were identified as positive, while in the year 2019 the number dropping at only three.

The obtained results are in accordance with the request of the European Commission which requires a reduction in the prevalence of avian salmonellosis.

Acknowledgement

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PRELIMINARY STUDY IN AFRICAN HEDGEHOG (*ATELERIX ALBIVENTRIS*) REPRODUCTION

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Summary

Hedgehog domestication became popular in the 1980's. The animals were traded from central and eastern Africa, where is found their natural habitat. Despite some limitations in a few states of America, they are very famous there as pets. Lately African hedgehog's popularity as an exotic pet increased in Romania as well. The objective of the study was to describe the reproductive behavior and some reproductive parameters involving African hedgehog (*Atelerix albiventris*). The study was conducted for a period of six months on two mature African hedgehogs, male and female, aged ten and six months respectively and later on their first hoglet. The animals were kept in captivity as indoor pets in plastic storage boxes with wood shavings, natural paper bedding or cotton blankets as a substrate. In the precopulatory, copulatory and postcopulatory stages reproductive behavior was observed. During this period, the female was monitored by vaginal cytology, performed twice a day vaginal smears and for the male the morphometric analysis of the spermatozoa was carried out. The gestation was monitored through inspection and weighing of the female. It was confirmed using the ultrasonographic examination. The parturition was observed by monitoring with a surveillance system inside the wood house where the female was sleeping. The female gained one hundred ten grams at the first pregnancy and ninety-four grams at the second one. The gestation lasted thirty-five days for the first mating and thirty-four days for the second one.

Keywords: *Atelerix albiventris*, reproductive behavior, vaginal smear, sperm morphometry, gestation

African hedgehogs are small mammals whose dorsal surface are covered in a dense coat of several thousand smooth spines (3). There are 17 species in five genera of hedgehogs worldwide (11). They are native to a wide swathe of central Africa (13), their natural habitat being consisted of steppes, savanna and grassy areas (14).

Hedgehogs are nocturnal, territorial, and solitary animals, except during courtship and when raising offspring (2). The sex of the hedgehogs can be determined by their external anatomy (7). The male has a prepuce located midway along the ventral abdomen, lacking the scrotum (6). The inguinal rings are open, and the testes are located in subcutaneous para-anal recesses (7). The male's penis is mid abdomen, near the umbilicus (4), is spineless with lateral horns on either side of the meatus making it resemble a snail's head. The male hedgehog has multilobed seminal vesicles, a bilobed prostate and paired Cowper's glands as accessory sex glands (7). The accessory reproductive glands, may account for as much as 10% of

body weight during the breeding season (1). Hedgehog sperm normally have an offset or eccentrically positioned tail (7). The female urogenital system is located a couple of mm. cranially from the anus (11). The large, thin-walled vagina is located within the abdominal cavity, and is flanked on each side by large fan-shaped glands homologous to the Cowper's gland of the male. The female hedgehog has a bicornuate uterus and a single muscular cervix; there is no uterine body (7). The placenta is discoid, lies on the antimesometrial side of the uterus and is hemochorial (11). Both sexes have five pairs of teats (9).

The physiological parameters of African hedgehog are shown in Table 1.

Table 1

Physiological parameters of African hedgehog (6, 8, 16)

Average weight	250-600 g
Body length	15.2-22.9 cm
Life expectancy	4-6 years, even 8 years (in captivity)
Body temperature	36.1-37.2°C
Gastrointestinal transit	12-16 hours
Heart rate	180-280 beats/min
Respiratory rate	25-50 breaths/min
Sexual maturity	2-3 months
Breeding	Starting with 6 months
Gestation	33-42 days (usually 35)
Litter size	1-9 (average 3-4) offspring
Eyes open	14-18 days
Weaning	4-6 weeks
Ovulation	Induced (by the presence of male)

The estrus cycle for *Atelerix albiventris* lasts 3 to 17 days (10). The reproductive life of *Atelerix albiventris* lasts all life in males and just between two and three years in females. However, one wild caught female remained reproductively active for 33 months (12). Hedgehogs are polygamous and polyestrous and the mating season occurs year – round in captivity (2). In the wild, females ovulate when the conditions are right, which is typically during the rainy season when food is abundant (15). The male courtship consists of enticing the female to mate by squeaking, hissing, nudging, and running in circles around her (2). During breeding, the receptive female partially erects her spines and stands for mating. If she is not ready to mate, she will erect the spines on her forehead and butt at the male. The male has a long penis that reaches cranially to the female's vulva. If and when courtship results in copulation, the female stops and presses her belly to the ground exposing her genitalia. The male then mounts from behind using his forepaws to grasp her spines and often grasp her spines over her neck with his teeth. The male may remain mounted for 1 - 4 minutes, copulating multiple times (8, 12). Pregnancy

detection is often accomplished by monitoring body weight. Usually if a female gains more than 50 grams within 3 weeks of being with a male, then she is considered pregnant (1). Few descriptions of hedgehog births are available, and it is noted that attempts to observe parturition often result in cannibalism of the young by the female. Observations of other hedgehog species report that during birth the female may lie on her side or in sternal recumbency, or stand with hind legs apart. She licks the genital region periodically. During contractions the female strains and trembles. Birth of the entire litter may take minutes to hours (5).

Materials and methods

The study was conducted for a period of six months on two mature African hedgehogs, male and female, aged 10 and 6 months respectively and later on their first hoglet (from birth until sexual maturity). The animals were kept in captivity as indoor pets in plastic storage boxes with wood shavings, natural paper bedding or cotton blankets as a substrate. Each box has a wooden house where the hedgehogs sleep, a wheel on which they run during the night and water and food dishes. Inside the wooden box of the female a security camera (Xiaomi Mi Home Security Camera Basic 1080P) was installed (Fig. 1). Their diet consisted of commercial cat food (Royal Canin Kitten and Petkult Probiotics Kitten), cooked chicken meat, scrambled eggs, bananas and mealworms.

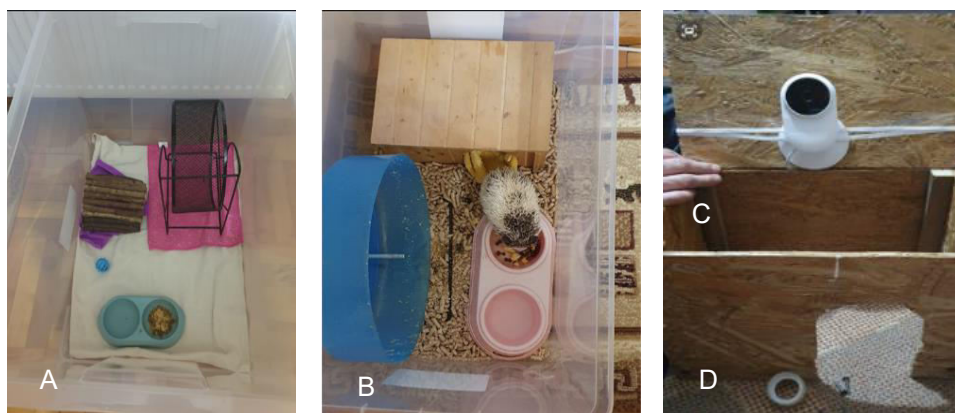


Fig. 1. The housing of hedgehogs (A-D)

The subjects were bred first time in the spring and the second time in the fall. For each breeding they were kept together for ten days. They were observed while mating for four times, so the reproductive behavior could be described later on.

Cytological examination. In females, two vaginal smears were performed daily (one in the morning and one in the night) for a period of sixteen days in order

to identify the vaginal aspects before, during and after mating. Collection of the vaginal specimen was made using cotton-tipped sticks (Fig. 2). The Diff Quick (Medion Diagnostic Switzerland) and Harris Shorr stains were used for the vaginal smears. The cells present in the vaginal smear and their distribution in the microscopic field were monitored. On Harris Shorr stained smears, the cytoplasm tinctorial affinity of vaginal epithelial cells was determined.



Fig. 2. Collection of vaginal specimens

Collection and evaluation of spermatozoa. Semen samples were collected from vaginal leakage, immediately after copulation. Three different stains were used in order to perform the morphometric analysis of spermatozoa: Diff-Quik, Eosine G2% (Minitube, Germania) and Spermac kit (Stain Enterprises, P.O. Box 152, Wellington, South Africa). Stained spermatozoa were examined by microscope, with brightfield optics at x1000 magnification, using an Olympus BX51 microscope and cell F imaging software (system Olympus Soft Imaging Solution, Munster, Germany).

Gestation was monitored by weighing the female weekly after the mating and daily since the fourth week after mating. The female was weighed with an electronic scale. After two weeks and a half since the first time they were seen mating, the female had an abdominal ultrasound examination. Five days before the expected parturition and five days after it, the female was not touched and disturbed at all in order to avoid stress. The parturition was captured by the video camera.

Results and discussions

The following were observed and noted: the reproductive behavior and specific features of mating, changes in vaginal cytology related with reproductive activity, spermatozoa dimensions, some gestation and parturition features.

Reproductive behavior of male and female and specific features of mating. The male was observed while courting the female. After being put together they started sniffing each other. The male started to chirp and the female was huffing and ran away from him. He kept following but she was not receptive. At some point

she bit his head quills. Even though she seemed unresponsive, when the male stopped running after her, the female kept circling him and got closer to him. They both urinated and defecated. After ten minutes of courtship, the female suddenly showed interest in the male. She stretched her hind limbs and also her front limbs and raised her posterior region allowing the intromission to take place. The male was holding the female with his front limbs, helped by his teeth which were holding her quills (Fig. 3).

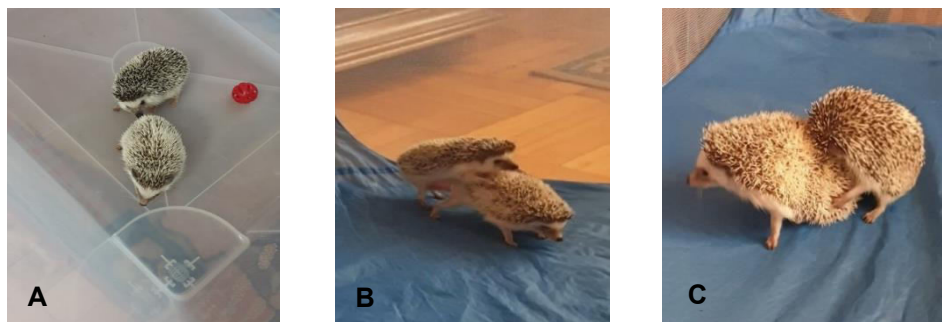


Fig. 3. Courtship (A) and copulation (B, C)

The hedgehogs were seen mating for four times. Each mating that was observed was timed. The mating process lasted minimum three minutes and a half and maximum six minutes, with an average of four minutes and a half.

Vaginal cytology. Vaginal smears made before the meeting between the female and the male are characterized by a smaller number of vaginal epithelial cells. Intermediate (oval cells with small nucleus), parabasal (round cells with large nucleus) and superficial (polygonal cells with present or absent nucleus) vaginal epithelial cells are observed. The cells are individually distributed in the microscopic field or are close together in small colonies (Fig. 4).

Immediately after the first mating, the number of vaginal epithelial cells increases significantly on the vaginal smear and cells clustered in large colonies are observed. Superficial epithelial cells are predominant on smears between first and second mating (Fig. 5). After the second mating, the intermediate cells and parabasal cells reappear on the vaginal smear (Fig. 6) and the cells containing numerous vacuoles in cytoplasm are seen.

Harris Shorr stained smears show only acidophilic cells in all collection situations: before first mating, between first and second mating and after second mating. Between first and second mating, vaginal smear show cells keratinization (Fig. 7).

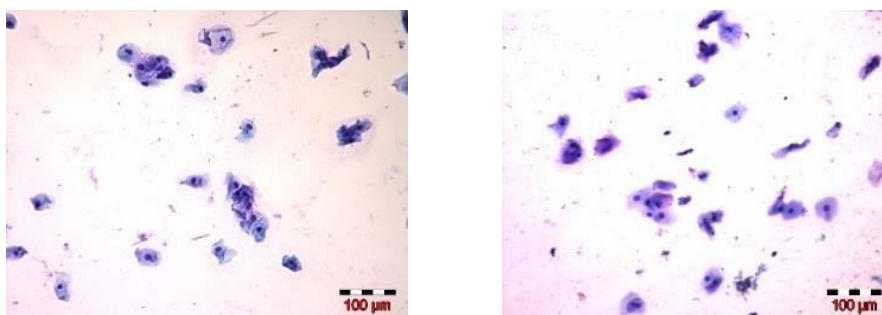


Fig. 4. Vaginal smears collected before first mating (left and right),
Diff-Quick stain

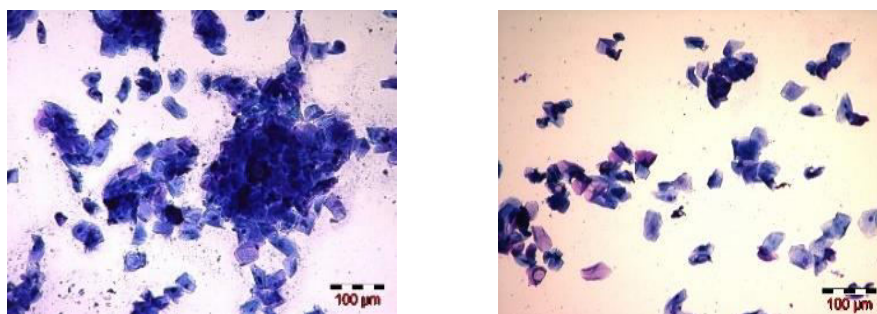


Fig. 5. Vaginal smears collected between first and second mating (left and right),
Diff-Quick stain

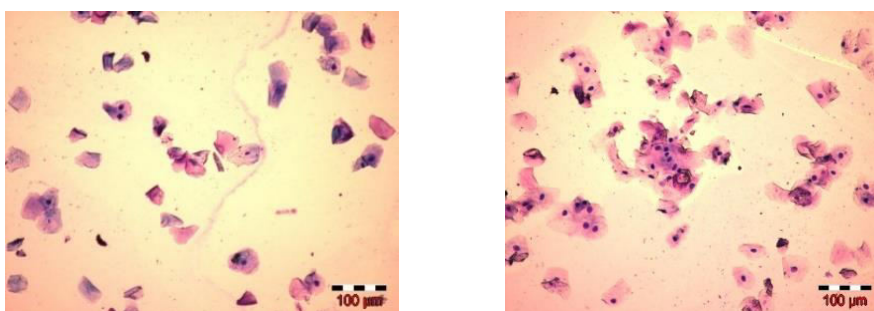


Fig. 6. Vaginal smears collected after second mating (left and right),
Diff-Quick stain

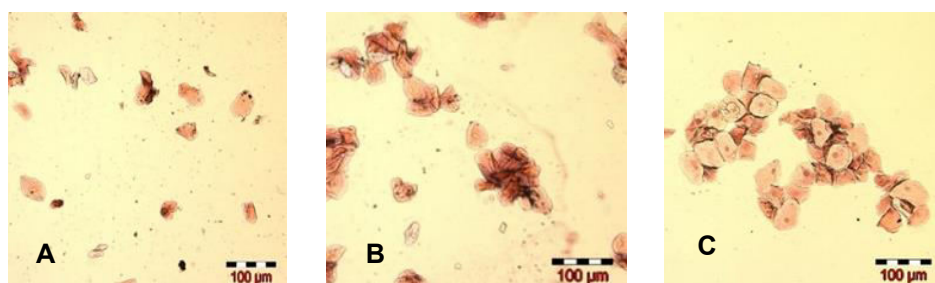


Fig. 7. Vaginal smears collected before first mating (A), between first and second mating (B) and after second mating (C), Harris-Shorr stain

Morphometric spermatozoa evaluation. All three stains used for the morphometric analysis highlight very well the structural components of the spermatozoa and abnormal spermatozoa (Fig. 8 - 11). At the morphometric analysis of the spermatozoa, the next values were obtained: $4.13 \pm 0.17 \mu\text{m}$ – average of head longitudinal axis, $3.86 \pm 0.3 \mu\text{m}$ - average of head transversal axis, $85.64 \pm 8.04 \mu\text{m}$, average of tail length and a total length of the spermatozoa of $89.77 \pm 0.21 \mu\text{m}$. Abaxial insertion of the spermatozoa tail was observed in majority of the spermatozoa.



Fig. 8. Hedgehog spermatozoa (Eosin stain)



Fig. 9. Hedgehog spermatozoa (Spermac stain)

Results of pregnancy and parturition monitoring. During the gestation period the female appetite increased and she consumed foods that she avoided before (chicken meat, bananas, asparagus, scrambled eggs).

At 19 days after the first exposure to the male an abdominal ultrasound was performed in the female using the ultrasound system X VISION MY LAB 70 VET (Esaote) and a microconvex transducer with 8.5 MHz frequency. So, the gestation was confirmed (Fig. 12).



Fig. 10. Hedgehog abnormal sperm.
(Spermac stain)



Fig. 11. Hedgehog spermatozoa
(Diff Quick stain)



Fig. 12. Ultrasonography of pregnant African Hedgehog (left and right), day 19 after first mating

The first gestation lasted 35 days and the second one 34 days.

No significant change was observed in the first two weeks, but three weeks after the second exposure to the male, a considerable increase in female weight was observed (Fig. 13). Two days before the parturition the female gained 16 g in one day and then her weight reached the plateau one day before the parturition (Fig. 14).

At the first gestation she gained 110 g (before 285 g, after 395 g) and in the second one 94 g (before 306 g, after 400 g).

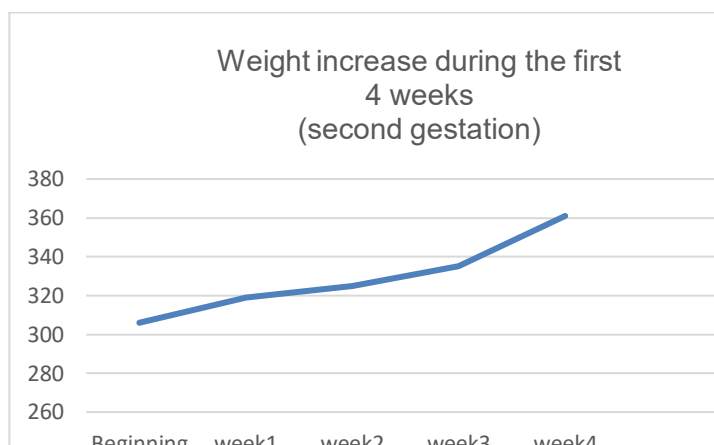


Fig. 13. Dynamics of female body weight during pregnancy

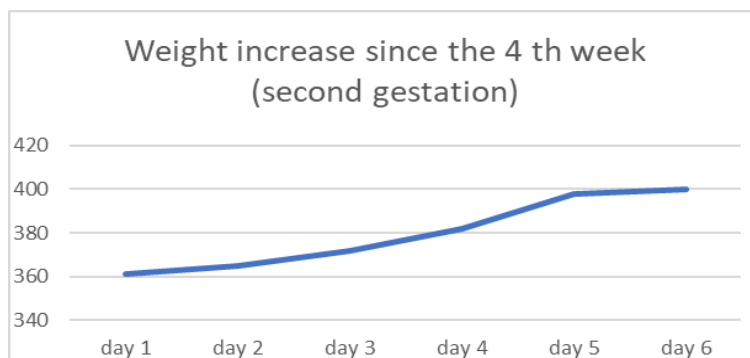


Fig. 14. Dynamics of female body weight during the 4th week of pregnancy

At the first gestation the female presented the nesting behavior three days before parturition and for the second gestation she started to add bedding to her wooden house a week or more before the parturition. With twenty-four hours before the first parturition was described the loss of appetite, which was not observed for the second parturition of the same female. One hour before the second parturition which took place in the afternoon, the female woke up and became nervous. She kept moving in her cage, she was digging in the paper bedding, and also, she was seen biting and scratching the walls of the wooden house. Then she started showing again the nesting behavior. The female was standing with her hind legs apart and was sustaining herself with the front limbs (Fig. 15).

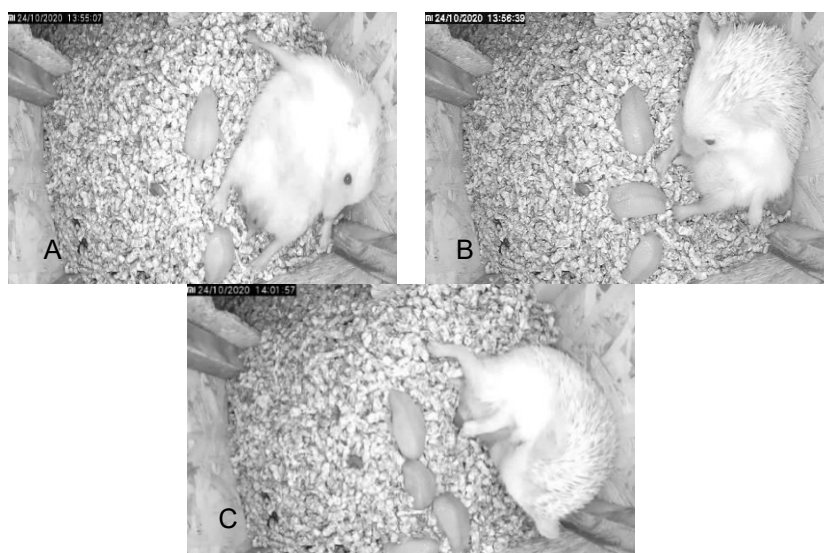


Fig. 15. Parturition in African Hedgehog (A, B, C)

She was licking her vulva periodically and also, she was trembling. The female was consuming the placenta and licking each hoglet after birth. The interval between each hoglet was of a couple of minutes. The parturition lasted approximately 30 minutes for the second gestation.

Prolificity for the first gestation was one hoglet and for the second one 6 hoglets, with a sex ratio of 5:1 (5 females to one male).

Conclusions

The duration of gestation is average 35 days. During each gestation the female gained approximately 100 grams.

The cytovaginal smears show changes during the mating stages.

Average mating time was of four and a half minutes.

The hedgehog spermatozoa present a head almost rounded and a long tail of 85 μm . Total spermatozoa length average measures 89 μm .

The prolificity registered at both of the gestations belonging to the same female was of 1 and 6 hoglets respectively.

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LUPINUS ALBUS SEEDS EXTRACTS: PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL ACTIVITY

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Summary

Lupinus albus L is an annual plant of the Fabaceae family, well known for its pharmacological properties due to its wealth of biologically active compounds. This work aims to determine the phytochemical screening of ethanolic, methanolic and aqueous extracts of *L. albus* seeds and the antibacterial activity against referenced bacteria *Micrococcus luteus* ATCC 14452, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027 and *Enterococcus faecalis* ATCC 29212, by the Disc Impregnation Method and Minimum Inhibitory Concentration by the Macrodilution Method. Phytochemical screening showed presence of flavonoids, alkaloids, cardiac glycosides and terpenoids in the ethanolic and methanolic extract of *L. albus*. Antibacterial activity was of (12.07 ± 0.2 and 9 ± 1.2 mm); (10.83 ± 1.04 and 8.21 ± 1.1 mm) and (13.39 ± 4.85 mm) ethanolic, methanolic extract on *S. aureus*, *E. coli* and *M. luteus* respectively. Minimum Inhibitory Concentration was in the range of 5-20 mg/ml.

Keywords: *Lupinus albus* L, phytochemical screening, bioactive compounds, antibacterial activity

Medicinal plants are one of the main resources of therapeutic agents. They are used by 80% of the world's population in health care (3). These plants are sources of a variety of biologically active compounds, mainly phenolic compounds, and these phytochemicals possess various biological properties such as antimicrobial properties (5).

Lupinus albus L called white lupinus is known locally as termis. It is an annual herbaceous plant grown in gardens. This plant is the most used in the traditional pharmacopoeia of the region of Tiaret in Algeria for the treatment of several diseases. Their seeds known as lupins are the only ones used in medicine, due to their richness in bioactive compounds; the latter can be an essential element for the inhibition of pathogenic bacteria (19).

In this work, we contribute to the phytochemical study of ethanolic, methanolic and aqueous extracts of *L. albus* seeds and their antibacterial effect against ATCC strains: *Micrococcus luteus*, *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* by the disc impregnation method and for the minimal inhibitory concentration by the Macrodilution method.

Materials and methods

1- Preparation of plant material and extracts

The seeds of *L. albus* are purchased from an herbalist in the Tiaret region (western Algeria); they are then cleaned, air-dried and crushed to obtain a powder.

The extracts were performed according to the protocol followed by Ghezelbash et al. (12), in different solvents (80% methanol, 80% ethanol and aqueous). The obtained mixture was thereafter filtered on filter paper and the filtrate was evaporated and dried at 40°C.

2- Phytochemical screening

Phytochemical tests were carried out on the three extracts of *L. albus* seeds following standard methods:

- a. For alkaloids, two tests were followed; Bouchardat test: two drops of Bouchardat reagent was added to two ml of each extract (29) and Wagner test: two ml extract was mixed with two ml of Wagner reagent (3). A reddish-brown precipitate indicates the presence of alkaloids for both tests.
- b. For Cardiac glycosides, 2 ml of each extract was dissolved with 2 ml of chloroform and concentrated sulfuric acid was carefully added to form a reddish layer which indicates the presence of cardiac glycosides (29).
- c. The presence of flavonoids was indicated by formation of a yellow precipitate when the extracts were treated with a few drops of lead acetate solution (30).
- d. 1ml of extract is mixed with 5 ml of absolute ethanol. The appearance of a flaky precipitate indicates the presence of mucilages (23).
- e. Salkowski Test was used to detect Terpenoids: 2 ml of chloroform and 3 ml of concentrated sulfuric acid were carefully added to 0.2 g of dry extract to form a layer. A rusty staining of the interface indicates the presence of Terpenoids (22).
- f. Saponins were reveled using test tubes with 10 ml of each extract were shaken for 15 S and then left for 15 min. The presence of saponosides was indicated by a persistent foam height, higher than 1 cm (6).
- g. Detection of tannins was assessed by adding to two ml of each extract one to two drops of ferric chloride solution (FeCl_3) diluted at 0.1%. The presence of catechic tannins was indicated by a dark green coloration and gallic tannins by a blue-green coloration (13).
- h. Presence of Anthocyanins was noted by using 3 ml of 10 % H_2SO_4 added to 1 ml of extract and then a base (1 ml of 10% NH_4OH) was added; if the coloration is accentuated by acidification and then turns blue in basic medium, test is considered positive.

3- Antibacterial activity

Antibacterial effect of ethanolic, methanolic (80%) and aqueous extracts of *L. albus* was examined against seven referenced bacteria: *Micrococcus luteus*

ATCC 14452, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027 and *Enterococcus faecalis* ATCC 29212.

This activity was achieved by the use of disc impregnation method (4) and for Minimum Inhibitory Concentration (MIC) by Macrodilution method (27).

Results and discussions

1- Phytochemical screening

Bioactive compounds evoke beneficial effects on human and animal health; physiological, behavioral and immunological. Several studies prove the chemopreventive effect of these products against several diseases such as certain forms of cancer, diabetes, obesity and Alzheimer's (10, 24).

The richness in phenolic compounds and their biological activity is influenced by several factors; the plant and climate changes, extraction time, temperature, volume and polarity of the solvent used (18, 21).

The hydro-ethanolic and hydro-methanolic extracts are the best for the extraction of bioactive compounds due to their polarity and the combination of organic solvent and water allows an extraction of soluble compounds (8).

Phytochemical screening revealed that the all extracts showed the presence of alkaloids and cardiac glycosides. Mucilages were found in the methanolic and aqueous extracts while terpenoids and saponins were only in the aqueous extract (Table 1).

Table 1

Phytochemical screening of *L. albus* extracts

Extracts	Methanolic Extract	Ethanolic Extract	Aqueous Extract
Alkaloids	+	+	+
Cardiac glycosides	+	+	+
Flavonoids	+	+	-
Mucilages	+	-	+
Terpenoids	-	-	+
Saponins	-	-	+
Tannins	-	-	-
Anthocyanins	-	-	-

+: Presence, - : Absence

These results are in line with those reported by Ibrahim et al. (15) that showed the aqueous and ethanolic extract of *L. albus* is rich in alkaloids, glycosides, flavonoids, saponins, and terpenes with absence of tannins in all extracts.

On the other hand, another study reported lupinus seeds contain appreciable concentration of bioactive compounds such as alkaloids, polyphenols,

phytosterols with antioxidant, antimicrobial, anti-inflammatory and anti-diabetic properties (19).

Sibul et al. (28) reported the richness of methanolic extract (80%) of *L. albus* herb in secondary metabolites and consequently a strong biological activity. In addition, the methanolic extract (80%) of *L. albus* grains indicates the presence of alkaloids, quinones, coumarins and an absence of flavonoids, tannins, polyphenols, saponins and terpenoids.

The flavonoids are found in all parts of plants (7) and categorized in different classes as alkaloids, terpenoids and phenolics. They are used by vegetables for their growth and defense against plaques (14).

These differences in compounds can be explained by variations in *Lupinus* varieties, the impact of climatic conditions as well as the types and extraction solvents used (1).

2- Antibacterial activity

L. albus with their richness in bioactive compounds can be essential elements for the inhibition of pathogenic bacteria and the reduction of various pathologies (17).

The bacteria inhibitory power of extracts can be explained by effect of their secondary metabolites. Several works have revealed that alkaloids, flavonoids, saponins, cardiac glycosides and terpenoids play a major role in antibacterial activity (9; 16). Bioactive compounds cause inhibitions of cell wall construction, replication of microbial genetic material, biofilm formation, attachment motility and cell communication (25).

The maximal antibacterial activity was exhibited against *S. aureus*, *E. coli* and *M. luteus* through a zone of inhibition (12.07 ± 0.2 and 9 ± 1.2 mm); (10.83 ± 1.04 and 8.21 ± 1.1 mm) and (13.39 ± 4.85 mm) ethanolic, methanolic extract respectively (Fig. 1).

Inhibition zones for *B. cereus*, *E. faecalis* and *P. aeruginosa* were 8.66 ± 1.15 ; 9.90 ± 1.43 and 10.91 ± 1.01 mm for the ethanolic extract, 7.85 ± 0.28 ; 8.03 ± 0.74 and 8.92 ± 0.81 mm for the methanolic extract and 9.38 ± 0.53 ; 9.21 ± 0.4 and 8.78 ± 0.91 mm for the aqueous extract.

These findings are similar to those reported by Abdallah et al. (1) where the methanolic extract of *L. albus* had no antibacterial activity against the Gram-positive and Gram-negative bacteria tested. In addition, other publication reported that *Lupinus* extracts have no inhibitory effect on *S. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *S. epidermidis* (2).

Lampart-Szczapa et al. (20) also pointed out the antibacterial activity of *lupinus* extracts is linked to their content in phenolic compounds.

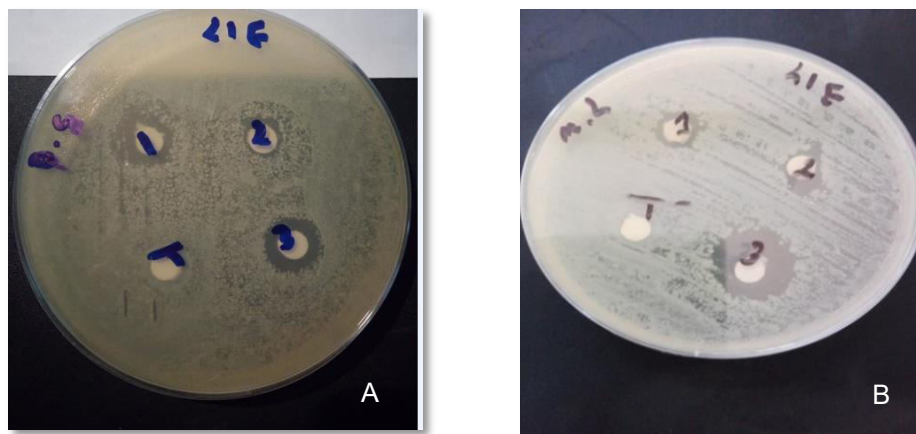


Fig. 1. Antibacterial activity of *L. albus* on *P. aeruginosa* (A) and *M. luteus* (B)

3- Minimal Inhibitory Concentration (MIC)

Lupinus albus ethanolic extracts showed a MIC of 20 mg/ml (*S. aureus*, *B. cereus*); 10 mg/ml (*E. coli*, *M. luteus*, *B. subtilis* and *E. faecalis*); 5 mg/ml (*P. aeruginosa*) whereas for the methanolic one it was of 20 mg/ml (*E. coli*, *S. aureus*, *M. luteus*, *B. subtilis*, *B. cereus* and *E. faecalis*); and 5 mg/ml (*P. aeruginosa*). The aqueous extract values were of 10 mg/ml and 5 mg/ml (*E. coli*, *M. luteus*, *B. subtilis* and *E. faecalis*) and (*B. cereus*) respectively.

This antibacterial property may be related to the presence of alkaloids in the extracts. This hypothesis was supported by the work carried out by Romeo et al. (26) with alkaloid extracts from two varieties of *Lupinus albus* (Multitalia and Calobria) on *P. aeruginosa* where a MIC of 0.128 mg/ml and 0.067 mg/ml respect was found.

Moreover, Erdemoglu et al. (11), studying the antibacterial effect of alkaloid extract from the aerial part of a plant belonging to the fabaceae on *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*, reported a MIC of 0.5; 0.125; 0.062 and 0.062 mg/ml respectively.

Conclusions

The results of this study show the richness of the ethanolic and methanolic extracts of *Lupinus albus* in bioactive compounds; flavonoids, alkaloids, cardiac glycosides and terpenoids, which play a major role in the inhibition of pathogenic bacteria and consequently the ability to be used as natural antibiotics.

Acknowledgement

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HEXAVALENT CHROMIUM IMPACT ON RATS SPERM QUALITY AFTER THREE MONTHS OF EXPOSURE

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Summary

Pollution of the environment is considered to be a major reason for the decline of human semen quality over the years. Occupational, industrial, environmental, therapeutic and dietary exposures to a wide range of chemicals and heavy metals have harmful effects on male fertility. The objective of the study was the evaluation of the potassium dichromate impact on sperm quality after three months of exposure. Three experimental and one control group, each consisting of 7 male rats, were exposed according to experimental design for three months, as follows: control group – received only distilled water, E₁ received 25 ppm Cr VI (LOAEL), E₂ received 50 ppm Cr VI (2 x LOAEL) and E₃ received 75 ppm Cr VI (3 x LOAEL) and then sperm quality markers were determined. Three months of exposure led to significant reduction in sperm number, total and progressive motility in all experimental groups. The percentage of spermatozoa with morphological abnormalities was increased after chromium treatment. The observed abnormalities were primary (flex head, detached head, head without tail, head in extension, incomplete head, unformed head) and secondary (broken tail, bent tail, curl tail in middle piece and end tail area, lysis of protoplasmic membrane indicating membrane fragility, headless tail, destruction of the membrane, of which only a few filaments remain), the secondary ones being predominant.

Keywords: hexavalent chromium, male, rat, sperm

Pollution of the environment is considered to be a major reason for the decline of human semen quality over the years. Occupational, industrial, environmental, therapeutic and dietary exposures to a wide range of chemicals and heavy metals have harmful effects on male fertility (9).

Chromium can be found in nature or it is man-made. From all oxidative states that it has, the most important ones are trivalent (Cr III) and hexavalent (Cr VI). The trivalent form is considered essential for metabolism of sugars, cholesterol and fats, being the less toxic than the hexavalent chromium. The organism is equipped with several mechanisms for reduction of chromium VI to chromium III, the process during which reactive oxygen species (ROS) are formed, responsible for oxidative stress induction. ROS production is linked to chromium toxicity expressed through cell cycle interruption, induction of apoptosis (8), dysfunction of DNA mechanisms for replication and transcription, mechanisms for DNA repair, inflammatory responses appearance (16).

Testicular tissue is a major target for metal-induced oxidative damage because of its high content of polyunsaturated membrane lipids (1).

The aim of the study was the evaluation of potassium dichromate on some biomarkers of integrity and performances of male reproductive system. The objective of the study was the evaluation of the potassium dichromate impact on sperm quality after three months of exposure.

Materials and methods

Experimental protocol was designed for twenty eight adult white Wistar male rats, which were purchased from the University of Medicine and Pharmacy „Victor Babeș” Timisoara, Romania, an authorized animal supplier. The animals were kept one week for acclimatization to the laboratory conditions (temperature $22\pm 2^{\circ}\text{C}$, relative humidity 40-60%, 12 h light/12 h dark schedule). Throughout the experiment animals were fed with Biovetimix standard diet, code 140-501 and they had free access to food and water. For animal housing standard polycarbonate cages with following dimensions l x w x h = 750 x 720 x 360 mm were used.

Approval of experimental protocol was obtained from the Ethical Committee of Banat University of Agricultural Sciences and Veterinary Medicine “King Michael I of Romania” Timisoara.

The animals were divided in three experimental and one control group and exposed according to experimental design for three months, as follows:

- Control group – received only distilled water,
- E₁ received 25 ppm Cr VI (LOAEL established by IARC, 1990 - 13);
- E₂ treated with 50 ppm Cr VI (2 x LOAEL);
- E₃ represents the group exposed to 75 ppm Cr VI (3 x LOAEL).

Animal handling during the experiment and their euthanasia were conducted in compliance with EU Directive 2010/63/EU and the NRC Guidelines (11, 15).

At the end of exposure period sperm samples were collected from cauda epididymis and diluted with Tyrode saline solution (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 1:20 dilution rate, necessary for individual sperm visualization and measurement, and then vortexed.

The computerized analyzing systems (CASA) provides the best way for semen assessment because the accuracy and precision of this systems allows detection of subtle changes in sperm motion. Using a Computer assisted sperm analysis (CASA) equipment the sperm quality markers were determined. The samples were loaded in a Leja standard count 4 chamber slide (IMV USA, Maple Grove, MN, USA) and sperm concentration, total and progressive motility were assessed using a computer assisted sperm analysis system (CASA Integrated Visual Optical System, Version 12.3 Hamilton Thorne Research, Beverly, MA, USA) with Animal Motility Software and HTB IVOS parameter settings for rat.

The percentage of abnormal sperm morphology was studied on smear made from diluted sperm and stained with Eosine. The slides were examined with 400x, 1000x magnification using Olympus BX51 microscope (Olympus, Germany),

including cell imaging software system (Olympus Soft Imaging Solutions GMBH, Münster, Germany).

The obtained results were statistically analyzed by Anova method and Student test.

Results and discussions

After three months of exposure to potassium dichromate the number of sperm count decreased significantly ($p < 0.01$) in all experimental groups comparative to control one (E_1/C : -5.07%; E_2/C : -8.79%; E_3/C : -11.96%), as presented in Table 1 and Fig. 1. By increasing the exposure level, the sperm count decreased significantly ($p < 0.01$): E_2/E_1 : -3.92%; E_3/E_2 : -3.48 %; E_3/E_1 : -7.26%).

Table 1

Mean sperm count (10^6) after three months of potassium dichromate exposure

Groups	$\bar{x} \pm S_x$	S.D.	CI 95%
C	730.61 \pm 0.29	0.77	0.89
E₁	693.59 \pm 0.51*	1.34	0.89
E₂	666.44 \pm 0.48*	1.27	0.89
E₃	643.26 \pm 0.42*	1.11	0.89

E/C: * $p < 0.01$

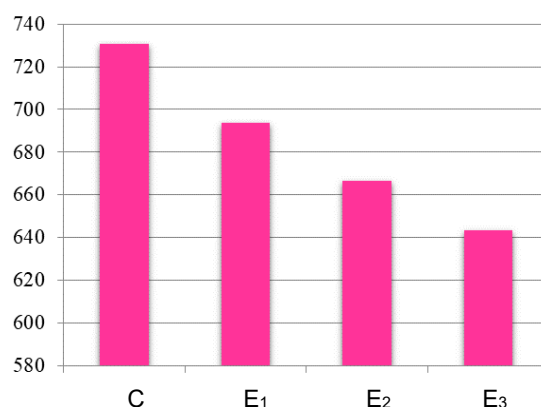


Fig. 1. Sperm count dynamics in rat after potassium dichromate exposure

Total sperm total motility decreased (Table 2 and Fig. 2), also significantly ($p < 0.01$) after chromium treatment in comparison to control group (E_1/C : -19.97%;

E₂/C: -30.49%; E₃/C: -41.53%). The lower values of sperm total motility were indirectly and significantly ($p<0.01$) correlated to chromium exposure level (E₂/E₁: -13.15%; E₃/E₂: -15.89%; E₃/E₁: -26.95%).

Regarding the progressive motility of spermatozoa in all groups exposed to hexavalent chromium it was recorded a significant ($p<0.01$) decrease (Table 2, Fig. 3) in comparison to control group (E₁/C: -44.33%; E₂/C: -58.36%; E₃/C: -69.04%). Between the exposure level to chromium and progressive motility of spermatozoa was established significant ($p<0.01$) and indirect correlation (E₂/E₁: -25.21%; E₃/E₂: -25.65%; E₃/E₁: -44.39%).

Table 2

Total and progressive motility in rat sperm after chromium exposure

Groups	Total motility			Progressive motility		
	$\bar{x}\pm Sx$	S.D.	CI 95%	$\bar{x}\pm Sx$	S.D.	CI 95%
C	80.14 \pm 0.34	0.90	0.71	64.14 \pm 0.74	1.95	0.91
E₁	64.14 \pm 0.34*	0.90	0.71	35.71 \pm 0.29*	0.76	0.91
E₂	55.71 \pm 0.36*	0.95	0.71	26.71 \pm 0.18*	0.49	0.91
E₃	46.86 \pm 0.34*	0.90	0.71	19.86 \pm 0.34*	0.90	0.91

E/C: * $p<0.01$

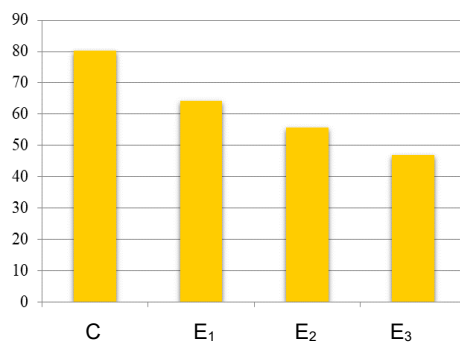


Fig. 2. Total motility dynamics in rat exposed to potassium dichromate

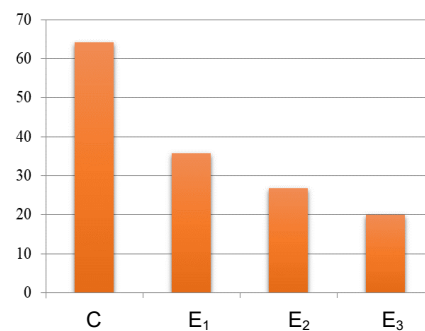


Fig. 3. Progressive motility dynamics in rat exposed to potassium dichromate

Table 3

Mean percentage of abnormal sperm

Groups	$\bar{x} \pm S_x$	S.D.	CI 95%
C	9.29 \pm 0.18	0.49	0.50
E₁	14.86 \pm 0.26*	0.69	0.50
E₂	20.43 \pm 0.20*	0.53	0.50
E₃	36.00 \pm 0.31*	0.82	0.50

E/C: * p<0.01

As presented in Table 3 and Fig. 4 percentage of abnormal sperm was significantly ($p<0.01$) higher in all experimental groups comparative to control group (E₁/C: +59.95%; E₂/C: +119.91%; E₃/C: +287.51%). Increase of spermatozoa with abnormal morphology was directly, significantly ($p<0.01$) correlated to exposure level (E₂/E₁: +37.48%; E₃/E₂: +76.21%; E₃/E₁: +142.26%).

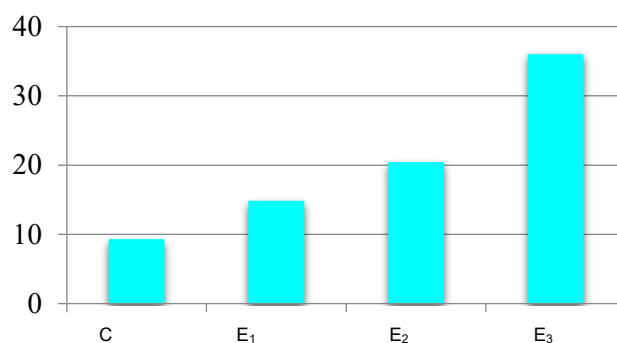


Fig. 4. Dynamics of sperm abnormalities in rat after potassium dichromate exposure

Main abnormalities found were:

- ↳ Primary: flex head, detached head, head without tail, head in extension, incomplete head, unformed head;
- ↳ Secondary: broken tail, bent tail, curl tail (in middle piece and end tail area), lysis of protoplasmatic membrane indicating membrane fragility, headless tail, destruction of the membrane, of which only a few filaments remain.

The predominant morphological abnormalities were secondary ones as presented in Fig. 5 - 10.

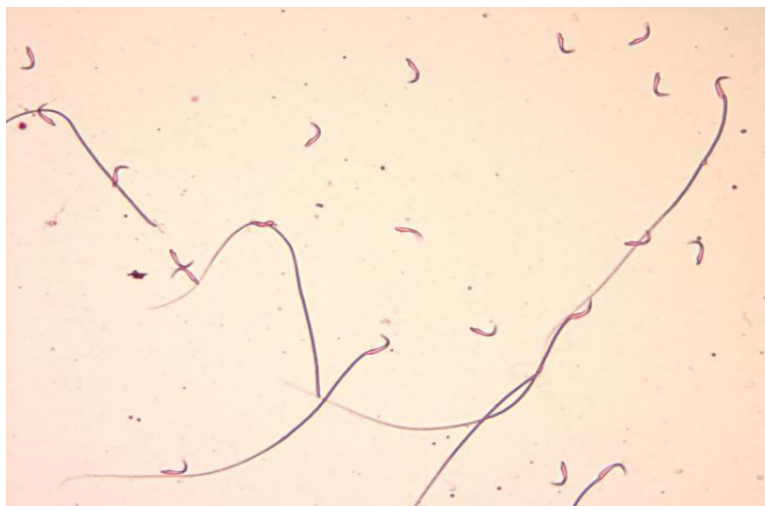


Fig. 5. Spermatozoa with anomalies: heads without tail, Eosin stain, 400X

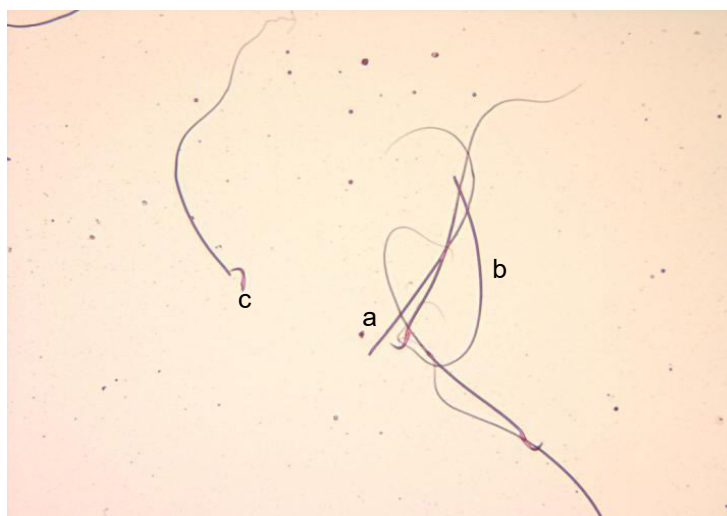


Fig. 6. Spermatozoa with anomalies: a. tail without head, b. bent tail, c. detached head, Eosin stain, 400X

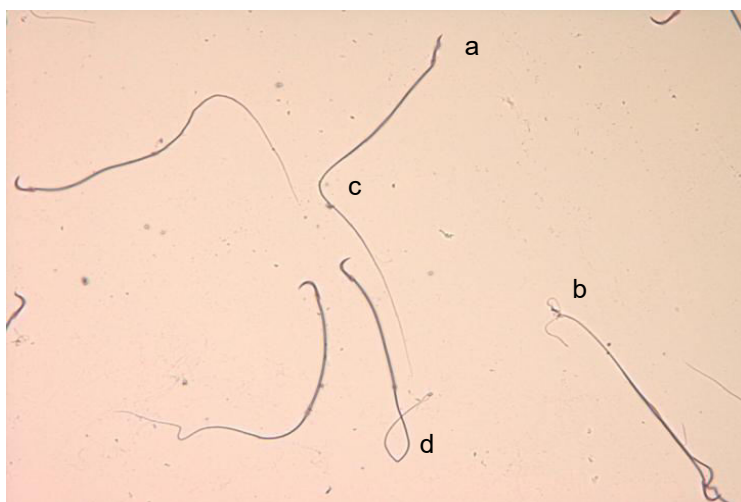


Fig. 7. Spermatozoa with anomalies: a. degenerated head, b. coiled tail, c. bent tail, d. loop-shaped tail, Eosin stain, 400X



Fig. 8. Spermatozoa with anomalies: a. membrane degeneration, b. torn tail, Eosin stain, 400X



Fig. 9. Spermatozoa membrane destruction, Eosin stain, 1000X

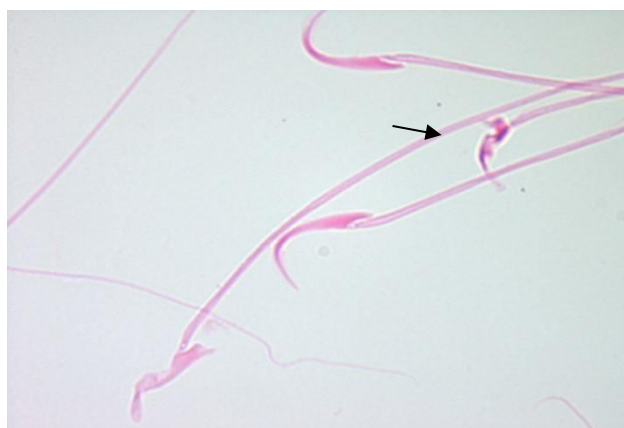


Fig. 10. Spermatozoa with anomalies: incomplete formed head, Eosin stain, 1000X

The highest percentage of abnormalities presented E₃ group, at maximum exposure level.

Impairment of spermatozoa in terms of concentration, total and progressive mobility, percentage of sperm cells with abnormalities leads to infertility and has been reported in both laboratory animals and humans exposed to hexavalent chromium (5).

The effects produced by hexavalent chromium are possible due to the fact that it enters into the cell very easily using specific transport systems (28), where it is reduced to more active intermediates and reactive oxygen species (ROS) (10). The formation of ROS and the subsequent installation of oxidative stress in the testicles, epididymis, accessory sexual glands, as well as in sperm, hypothalamus and pituitary gland due to the failure of antioxidant protection mechanisms, has been demonstrated by many authors (1, 2, 4, 6, 7, 17, 26, 27). The inefficiency of antioxidant enzymes associated with increased oxidative stress is a major risk for sperm quality deterioration (24).

The sperm cells are very susceptible to oxidative damage as well as other cells form the body (12). Oxidative stress occurs when redox processes in the cell are diverted either to the excess production of reactive oxygen species or to the deficiency of the antioxidant system (1).

Due to the lack of cytoplasm, the "home" of enzymes, sperm cells are poorly equipped to cope with ROS. Thus, haploid cells rely on the protective activities of the environment in which they are found (12).

On the other hand, the unique structure of the sperm membrane, respectively high concentrations of polyunsaturated fatty acids with double bonds, are the place of attraction for the action of reactive oxygen species (12).

The attack of ROS on sperm is followed by decreased mobility due to rapid loss of intracellular ATP, which leads to damage to axonemes (12), decreased sperm viability and increased abnormalities of the middle part, because lipid peroxidation destroys the membrane (25). All this has a disastrous effect on sperm capacity and acrosomal reaction. Lipid peroxidation in the spermatozoa membrane is the key element for the mechanism of infertility (3).

Decreased cellular ATP levels may explain the reduction in sperm motility without a corresponding change in mitochondrial function (18).

ROS can also reduce sperm motility by reducing the phosphorylation of axoneme proteins, which are essential for sperm movement (14).

The decrease in sperm count may be due to increased lipid peroxidation in the testes (27, 29). Chromium exposure is also responsible for decreasing the amount of ascorbic acid in the testes, the substance responsible for the differentiation and normal development of spermatogonia. Therefore, the decrease in the concentration of ascorbic acid in the testes of animals exposed to hexavalent chromium is followed by a drastic decrease in sperm count (2).

The morphological abnormalities of the spermatozoa are evidence of the loss of fertilization potential, reflecting the inability of the sperm cells to advance and reach the meeting point with the oocyte. Secondary anomalies, located in the tail, which were the most numerous, are the reason for the low values of total and progressive mobility.

Given that the testes and epididymis provide the necessary environment for spermatogenesis, maturation and storage of sperm, lesions induced by hexavalent chromium compounds, and described by our research team (19, 20, 21, 22, 23, 30),

are of major importance for understanding the biological effect of chromium on male reproductive function.

Conclusions

The results of our study highlighted the negative effect of hexavalent chromium on male reproductive function. Three months of exposure led to significant ($p < 0.01$) reduction in sperm number, total and progressive motility in all experimental groups.

The percentage of spermatozoa with morphological abnormalities was significantly ($p < 0.01$) increased after chromium treatment.

The observed abnormalities were primary (flex head, detached head, head without tail, head in extension, incomplete head, unformed head) and secondary (broken tail, bent tail, curl tail in middle piece and end tail area, lysis of protoplasmatic membrane indicating membrane fragility, headless tail, destruction of the membrane, of which only a few filaments remain), the secondary ones being predominant.

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INCIDENCE AND PREVALENCE OF *YERSINIA ENTEROCOLITICA* ON THE SLAUGHTERING FLOW

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Summary

During the experimental stages, the research aimed to establish the prevalence of *Yersinia enterocolitica* in pigs, on the slaughtering flow. The experiments were carried out on a large number of samples (800), collected starting from the moment of receiving the animals, until obtaining the finished product. For isolation and identification, both a modified method, proposed by the International Organization for Standardization, and CIN and SSDC culture media for isolation were used. Following the carried-out research, taking into consideration the recommendations of C.E., we can conclude that, at present, the slaughtering process allows the compliance with the hygiene and disinfection rules, in order to limit the dispersion of *Yersinia enterocolitica*, which favors the phenomenon of intercontamination.

Keywords: foodborne diseases, *Yersinia enterocolitica*, isolation, identification

The incidence of food poisoning with the species *Yersinia enterocolitica* is far from known due, first of all, to the failure of reporting all cases (2). The number of confirmed cases of food poisoning with *Yersinia enterocolitica* toxins differs from one country to another, depending on the concerns of specialists and the possibilities of isolation and identification of laboratories (3, 4). During the processes of slaughtering and processing of pork, contamination with various microorganisms can take place, which can depreciate the nutritional value of the finished products, and, in some cases, can cause disease to consumers (1, 5, 6, 7, 9).

Taking these aspects into account, it was considered important and opportune to investigate the presence of the species *Yersinia enterocolitica* during the process of slaughtering and processing of pork (8).

The main objective, during the study, was to establish the prevalence of *Yersinia enterocolitica* in swine, starting from the verification of the three major chains until obtaining the finished product (from entry into the slaughterhouse, during the technological flow of processing and in the commercial network).

Materials and methods

To evaluate the incidence of *Yersinia enterocolitica* species, on the technological slaughtering flow in pigs, 800 samples were collected and analyzed, from two units A and B, the samples being taken from animals included in 16 groups,

of ten animals each (eight lots in unit A and eight lots in unit B).

The sampling points were as follows: reception of pigs (160 samples), evisceration (160 samples), organs (liver, kidney, spleen and sanitation - 160 samples), carcasses, both before refrigeration (160 samples) and after freezing (160 probes).

In order to isolate and identify the species *Yersinia enterocolitica*, a modified method was used, proposed by the International Organization for Standardization (4).

Before slaughter, the samples were taken with the help of sterile swabs, directly from the rectum, being placed directly on the surface of the medium (agar) with the mention that on each Petri dish a total of three samples were streaked. In parallel, the bacteria were inoculated on the CIN medium, the SSDC medium and a modified medium. Incubation was performed at a thermostat at 30°C for 24-48 hours.

To perform seeding on the identification media, the samples were diluted in 10 ml of physiological serum, and after dilution and homogenization, 1 ml of this suspension was introduced into 9 ml of solution composed of 0.25% KOH and 0.85% NaCl. After stirring for 10 seconds the contents of a loop (0.1) ml were seeded on the surface of the selective isolation media (CIN, SSDC and modified).

We mention that 160 samples were collected, before slaughter, from the two units A and B, following the verification of 16 lots of 10 animals each (8 lots from unit A - 80 samples and 8 lots from unit B - 80 samples). Sampling was performed after classification in quality classes (depending on age, sex, body weight, conformation, fattening status etc.), both from groups of pigs in poor condition, with diarrhea (generally watery, without being bloody), as well as from animals that, at the veterinary sanitary examination, before slaughter, were in a good state of maintenance.

At slaughter, after evisceration, samples were collected from both the intestinal contents (160 samples) and from the organs, respectively 40 samples from the liver, tongue, kidneys and carcass (sanitation samples), collected by wiping a patterned surface of 10 cm² of the carcasses. The samples collected were seeded according to the methodology mentioned above, both on common media and on selective isolation media (CIN, SSTC and modified medium).

Also, in order to qualitatively evaluate the contamination of the carcasses, coming from the animals from the four groups, 40 samples were taken from the carcasses, 160 samples before refrigeration and 160 samples after refrigeration, respectively.

Results and discussions

Of the 160 samples (rectal swabs), following the inoculations and the bacteriological examination performed to identify the species *Yersinia enterocolitica*, five positive samples were identified, respectively three from unit A and two from unit B (Table 1).

Table 1

Overview of the results of bacteriological analysis *Y. enterocolitica* home intestinal before slaughter in units A and B

Unit	No. of lots	No. of analyzed samples	Out of which were positive			
			Lots		Samples	
			Nr.	%	Nr.	%
A	8	80	3	37.50	3	3.75
B	8	80	2	25.00	2	2.50
TOTAL	16	160	5	31.25	5	3.125

The data obtained show a higher incidence in unit A which was determined by the different origin of the animals (Fig. 1).

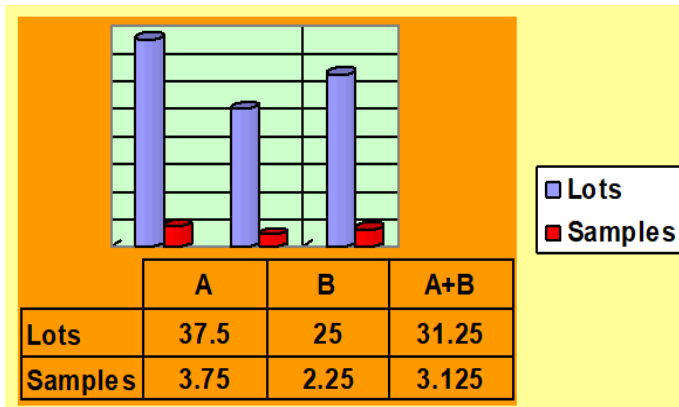


Fig. 1. Portage dynamics before slaughter

Subsequently, positive samples were subjected to biochemical testing to confirm and identify the species *Yersinia enterocolitica*. The results of the biochemical tests showed that all five isolated strains belong to the species *Yersinia enterocolitica* (Table 2).

The results obtained at the bacteriological examination of the 160 samples collected from the intestinal contents from the animals from the 16 groups showed that the incidence of *Yersinia enterocolitica* was 8.125%, but different depending on the selective medium used.

Thus, the highest number of positive samples, following the bacteriological examination, were obtained in the case of using the modified medium.

Table 2

Results of bacteriological analysis for *Y. enterocolitica* intestinal origin before slaughter in units A and B

Test	Stem				
	1	2	3	4	5
Ornithine decarboxylase	+	+	+	+	+
Urease	+	+	+	+	+
Glucose acid	+	+	+	+	+
Oxidase	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-
Rhamnosis	-	-	+	-	-
Citrate	-	-	-	-	-
Sucrose	+	+	+	+	+
Hydrogen sulfide	-	-	-	-	-

If 11 positive samples were identified on the CIN and SSTC selective media, the presence of *Yersinia enterocolitica* in 13 animals was identified on the modified medium, with the mention that the positive samples came from the animals from two groups of ten animals from each unit (Table 3).

Table 3

Results of bacteriological analysis of the *Y. enterocolitica* evisceration operation in units A and B

Culture media	Unit						Total		
	A			B			No. of analyzed samples	Out of which were positive	
	No. of analyzed samples	Out of which were positive		No. of analyzed samples	Out of which were positive			no.	%
		no.	%		no.	%			
CIN	80	7	8.75	80	4	5.00	160	11	6.875
SSTC	80	7	8.75	80	4	5.00	160	11	6.875
modified	80	8	10.00	80	5	6.25	160	13	8.125

Also, a different incidence of the presence of positive samples was found from one unit to another, but also depending on the selective environment used (Fig. 2).

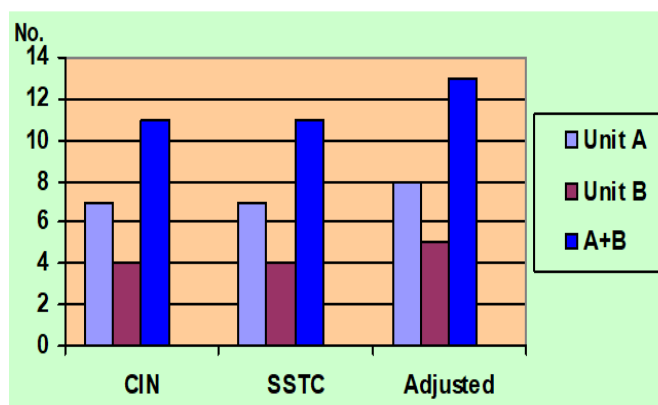


Fig. 2. Incidence of positive samples during the evisceration operation in units A and B

After testing the biochemical activity of the 13 strains isolated from the intestinal contents, it was found that all belong to the species *Yersinia enterocolitica* (Table 4).

Table 4

Results of bacteriological analysis of the germ *Y. enterocolitica* evisceration operation in units A and B

No. of strain	Test								
	Ornithine decarboxylase	Urease	Glucose acid	Oxidase	Lysine decarboxylase	Rhamnose	Citrate	Mobility at 25°C	Esculin
1	+	+	+	-	-	-	-	+	-
2	+	+	+	-	-	-	-	+	-
3	+	+	+	-	-	-	-	+	-
4	+	+	+	-	-	-	-	+	-
5	+	+	+	-	-	-	-	+	-
6	+	+	+	-	-	-	-	+	-
7	+	+	+	-	-	+	-	+	-
8	+	+	+	-	-	-	-	+	-
9	+	+	+	-	-	+	-	+	-
10	+	+	+	-	-	-	-	+	-
11	+	+	+	-	-	-	-	+	-
12	+	+	+	-	-	-	-	+	-
13	+	+	+	-	-	-	-	+	-

Regarding the results obtained from the samples collected from the organs, there was a low incidence of the presence of *Yersinia enterocolitica* in them (0.83%). We mention that among the analyzed organs, the presence of a single strain was identified, from a sample collected from the tongue (Table 5).

The results obtained from the sampling of the carcasses, before refrigeration and after storage of refrigerated meat showed that refrigeration negatively influenced the viability of *Yersinia enterocolitica* strains. Thus, the incidence of carcasses identified as positive is reduced from 5% to 3.75% (Table 6).

Table 5

Number of positive samples of germs of *Y. enterocolitica* isolated from organs after sowing directly on selective media

Organ	Unit						Total		
	A			B			No. of analyzed samples	Out of which were positive	
	No. of analyzed samples	Out of which were positive		No. of analyzed samples	Out of which were positive			No.	%
		No.	%		No.	%			
Liver	20	-	-	20	-	-	40	-	-
Tongue	20	1	5	20	-	-	40	1	2.5
Kidney	20	-	-	20	-	-	40	-	-
Carcass	20	3	10	20	1	5	40	4	10.0
TOTAL	80	4	5.0	80	2	1.25	160	5	3.125

Table 6

The results obtained after direct isolation of *Y. enterocolitica* after chilling and after storing meat chilled

Unit	Moment of sampling					
	Before refrigeration			After refrigeration		
	Carcass (no.)	Out of which were positive		Carcass (no.)	Out of which were positive	
		No.	%		Nr.	%
A	40	1	2.50	40	1	2.50
B	40	3	7.50	40	2	5.00
Total	80	4	5.00	80	3	3.75

The effect of low temperature on the viability of *Yersinia enterocolitica* was also evident in the carcass freezing process. Thus, after 24 hours from freezing the incidence of viable strains of *Yersinia enterocolitica* is reduced from 5% to 2.5% and after 72 hours to 1.25% (Table 7).

Table 7

**Qualitative assessment *Y. enterocolitica* contamination
during freezing by direct isolation**

Unit	Moment of sampling					
	After 24 h of freezing			After 72 h of freezing		
	Carcass (no.)	Out of which were positive		Carcass (no.)	Out of which were positive	
		No.	%		No.	%
A	40	1	2.50	40	-	-
B	40	2	2.50	40	1	2.50
Total	80	3	2.50	80	1	1.25

Conclusions

During the slaughter, in the first stages, there was a decrease in the presence of germs belonging to the species *Yersinia enterocolitica*.

In the conditions of a strict compliance to the hygiene rules and a correct application of the disinfecting measures, during the slaughtering process, a significant decrease of the presence of *Yersinia enterocolitica* on the technological flow is achieved.

Studies have shown a low contamination of pig carcasses both before and after refrigeration, respectively a contamination of 10% 24 hours after refrigeration and 7.5% after 72 hours after refrigeration.

Following the evisceration operation, the pig organs showed a low rate of contamination (batches found contaminated after isolation of *Yersinia enterocolitica* from rectal samples) for liver, spleen, kidney and tongue samples as well.

During evisceration, contamination can be accidental, by breaking the intestines, which favors the spread of germs on the slaughtering flow.

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COMPARATIVE STUDY REGARDING THE INFLUENCE OF TWO DIFFERENT SOLVENTS IN THE GC-MS ANALYSIS OF SOME NATURAL ESSENTIAL OILS

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Summary

The present study aims to identify and describe the possible differences that occurred during GC MS analysis from some natural essential oils, using two different solvents: hexane and methanol. Two essential oils (*Laurus nobilis*, *Salvia officinalis* and *Melaleuca alternifolia*) were studied under the same working conditions, with only one difference, respectively the type of solvent used for the dissolution of the samples. Total ion chromatograms were obtained using a 7890A Agilent Technology (Agilent Scientific, USA) Gas Chromatograph, coupled with a MSD 5975 Mass Spectrometer and equipped with a HP-5MS capillary column (30 m x 0.25 mm i.d x 0.25 µm film thickness). The NIST database was consulted for the identification of the volatile compounds. After the identification of the volatile constituents, a comparative analysis for both hexane-methanol diluted oils was performed. In principle, the main compounds were not influenced by the nature of the solvent, however, differences were observed in terms of the additional ones and the concentration value (%) from the total amount. Mainly, the analytes from the hexane dissolved oils revealed a better performance in comparison with the methanol samples, although a few compounds were favored by the methanol extraction.

Keywords: GC-MS, essential oils, hexane, methanol, sample preparation

Gas chromatography is an analytical technique used for the separation and detection of chemical components from a sample mixture, which are usually organic compounds and gases, otherwise known as analytes. Mass spectrometry can be used to identify, quantify and determine the chemical and structural properties of the molecules (16). Accuracy and precision of a gas chromatography-mass spectrometry (GC-MS) analytical method, a synergistic combination of the two powerful analytic techniques, can be affected by several factors, from the conditioning of the samples (phase, volatility, concentration), to the preparation process (extraction, solvent type, working conditions) and the column suitability regarding the nature of samples.

Essential oils (EOs) are natural, complex and volatile compounds that are produced by plants as secondary metabolites (1). The EOs possess the capacity to control pests, bacteria, fungi and viruses (7, 11, 12).

Laurel, or sweet bay (*Laurus nobilis*) of the *Lauraceae* family (4), is a plant used in the folk medicine for the treatment of different health problems, such as rheumatism and dermatitis (5, 6). Phytochemical analyses have shown the presence of compounds of volatile and non-volatile oils, flavonoids, tannins, sesquiterpenes, alcohols, alkaloids, minerals, and vitamins. The laurel essential oil yield and composition were shown to be influenced by various factors, such as growth environment, harvest season, plant parts and extraction method (6).

Salvia species have attracted researchers for their biological properties, showing strong antibacterial, antifungal, anticancer, antioxidant, anticholinesterase, and anti-inflammatory effects, as well as for the improvement of cognitive performance and mood (15). The sage essential oil has been shown to possess cytotoxic, antimutagenic (10), antimicrobial, antioxidant (3) and neuroprotective effects (8).

Melaleuca alternifolia essential oil (tea tree oil) is widely used as an ingredient in skincare products because of its recognized biological activities (2). In particular, the tea tree essential oil is used as an ingredient in formulations to treat skin diseases like acne, seborrheic dermatitis (9) and scabies (14).

Although the chemical compositions of these essential oils were studied by GC-MS in different pieces of literature, the aim of this research was to identify the possible differences that occurred during the analysis, regarding to the solvent type used for the dissolution of the oil samples.

Materials and methods

The study was conducted in the *Antioxidants Systems Research Laboratory* from *Horia Cernescu Research Unit*, Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania", Timisoara, Romania. The samples to be analyzed were represented by three natural essential oils (EOs) purchased from the Romanian market: *Laurus nobilis*, *Salvia officinalis* and *Melaleuca alternifolia*.

The sample preparation consisted of the following steps: 100 μ L of each oil were pipetted in two screw top vials (2 mL) and diluted with 500 μ L of the solvent (hexane, respectively methanol).

The analysis of the essential oils was performed on a 7890A Agilent Technology Gas Chromatograph, coupled with a MSD 5975 Mass Spectrometer and equipped with a HP-5MS capillary column (30 m x 0.25 mm i.d x 0.25 μ m film thickness). The samples were introduced into the GC-MS injection port using a 10 μ L syringe, in the split mode (the split ratio was 100:1) and a volume of 2 μ L. Helium was the carrier gas with a flow rate of 1 ml/min. The inlet pressure was 7.5622 psi, the total flow was 104 ml/min and the oven temperature was set at 50°C (3'). There were two temperature ramps: the initial temperature was increased with 4°C/min to 120°C and then with 8°C/min to 280°C (4.5'). The total run time was 45'. For the

identification of the compounds, the NIST database was used. The compounds with a concentration percent under 0.30% were not taken into consideration.

All the analytical parameters of the GC-MS method were maintained the same for both methanol and hexane dissolved samples.

Results and discussions

The first step was represented by the identification of the volatile constituents from each essential oil. The first to be analyzed was the *Laurus nobilis* oil dissolved in hexane (**L1**). The MS detected the following compounds: α -Pinene, Camphene, β -Phellandrene, β -Pinene, β -Myrcene, α -Terpinene, o-Cymene, Eucalyptol, γ -Terpinene, Linalool, Terpinen-4-ol, L- α -Terpineol, α -Terpineol acetate, Eugenol, Methyleugenol (Fig. 1, Table 1).

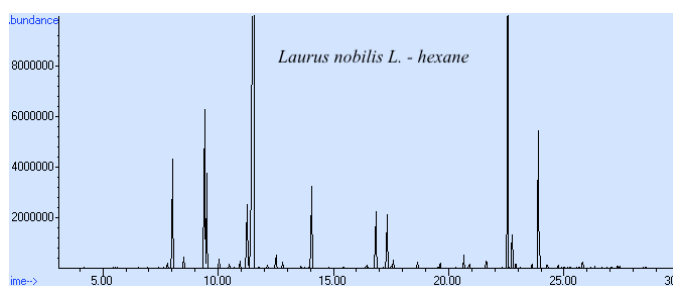


Fig. 1. The chromatogram for the *L. nobilis* oil dissolved in hexane

Table 1

The volatile constituents of the L1. EO (with hexane as solvent)

No.	RT.	Conc. (%)	Compound
1.	8.022	4.74	α -Pinene
2.	8.512	0.53	Camphene
3.	9.413	7.83	β -Phellandrene
4.	9.502	4.21	β -Pinene
5.	10.057	0.47	β -Myrcene
6.	10.954	0.33	α -Terpinene
7.	11.261	3.85	o-Cymene
8.	11.525	44.51	Eucalyptol
9.	12.515	0.63	γ -Terpinene
10.	14.064	4.07	Linalool
11.	16.850	2.91	Terpinen-4-ol
12.	17.339	2.72	L- α -Terpineol
13.	22.56	15	α -Terpineol acetate
14.	22.746	1.35	Eugenol
15.	23.892	5.32	Methyleugenol
Total: 98.47%			

In the case of **L1**, the most abundant constituent, with a concentration percent of 44.51% from the total amount was Eucalyptol (Fig. 2), followed by α -Terpineol acetate with a percent of 15% (Fig. 3) and β -Phellandrene (Fig. 4) with 7,83%.

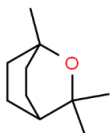


Fig. 2. Structure of *Eucalyptol*

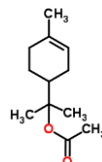


Fig. 3. Structure of α -Terpineol acetate

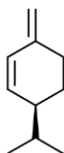


Fig. 4. Structure of β -Phellandrene

The same main constituents of the *L. nobilis* EO were also reported by Fidan et al. (4): Eucalyptol (8.10 - 48.0%) and α -terpinyl acetate (3.67 - 10.4%).

As for the same oil sample dissolved in methanol (**L2**) the detected constituents were: β -Thujene, β -Myrcene, α -Terpinene, o-Cymene, γ -Terpinene, Camphol, L- α -Terpineol, Thymol methyl ether, Isothymol methyl ether, Geraniol, Thymol, Carvacrol, α -Terpineol acetate, Caryophyllene, β -Bisabolene (Table 2).

For **L2**, the dominant volatile compound was Thymol - 38.45% (Fig. 6) followed by o-Cymene - 13.53% (Fig. 7) and α -Terpineol acetate - 8.03 (Fig. 3).

For the *Salvia officinalis* oil dissolved in hexane (**S1**) the following compounds were detected: α -Pinene, Camphene, β -Pinene, L- β -Pinene, 3-Carene, o-Cymene, Sylvestrene, Eucalyptol, γ -Terpinene, α -Terpinolen, β -Linalool, α -Thujone, β -Thujone, L-Camphor, Camphol, Terpinen-4-ol, α -Terpineol, Bornyl acetate, Caryophyllene, Humulene and Globulol (Fig. 8, Table 3).

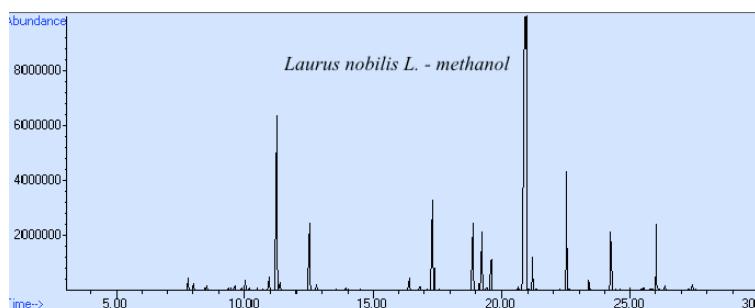


Fig. 5. The chromatogram for the *L. nobilis* oil dissolved in methanol

Table 2

The volatile constituents of the L2. EO (with methanol as solvent)

No.	RT.	Conc. (%)	Compound
1.	7.802	0.81	β -Thujene
2.	10.054	0.74	β -Myrcene
3.	10.947	1.01	α -Terpinene
4.	11.248	13.53	<i>o</i> -Cymene
5.	12.512	5.12	γ -Terpinene
6.	16.410	1.00	Camphol
7.	17.336	7.63	L- α -Terpineol
8.	18.902	5.36	Thymol methyl ether
9.	19.219	4.67	Isoshamyl methyl ether
10.	19.589	2.64	Geraniol
11.	20.934	38.45	Thymol
12.	21.189	2.42	Carvacrol
13.	22.539	8.03	α -Terpineol acetate
14.	24.253	3.64	Caryophyllene
15.	26.028	3.60	β -Bisabolene
Total: 98.65%			

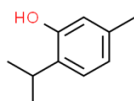


Fig. 6. Structure of *Thymol*

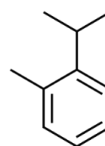


Fig. 7. Structure of *o*-Cymene

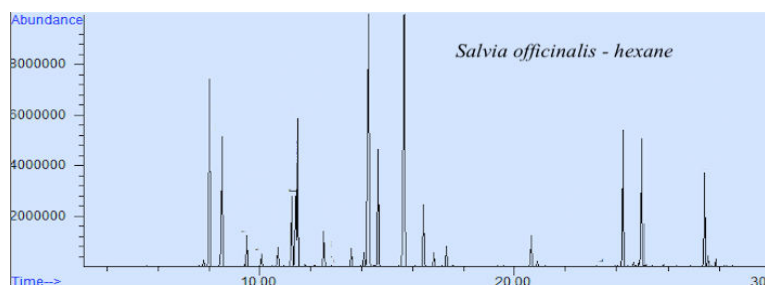
Fig. 8. The chromatogram for the *S. officinalis* oil dissolved in hexane

Table 3

The volatile constituents of the S1. EO (with hexane as solvent)

No.	RT.	Conc. (%)	Compound
1.	8.024	7.96	α -Pinene
2.	8.516	6.16	Camphene
3.	9.493	1.31	β -Pinene
4.	10.057	0.51	L- β -Pinene
5.	10.707	0.83	3-Carene
6.	11.250	3.20	o-Cymene
7.	11.401	3.33	Sylvestrene
8.	11.474	6.67	Eucalyptol
9.	12.516	1.59	γ -Terpinene
10.	13.602	0.84	α -Terpinolen
11.	14.098	0.56	β -Linalool
12.	14.289	23.00	α -Thujone
13.	14.651	5.78	β -Thujone
14.	15.669	17.99	L-Camphor
15.	16.429	3.06	Camphol
16.	16.847	0.64	Terpinen-4-ol
17.	17.334	0.93	α -Terpineol
18.	20.661	1.48	Bornyl acetate
19.	24.262	5.17	Caryophyllene
20.	24.993	4.80	Humulene
21.	27.435	3.46	Globulol
Total: 99.29%			

The main constituents for the **S1** EO were represented by α -Thujone (Fig. 9) with a concentration percent (%) equal to 23, respectively L-Camphor (Fig. 10) that indicated a concentration of 17.99% from the total amount and α -Pinene – 7.96% (Fig. 11).

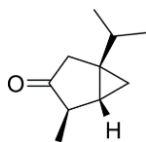


Fig. 9. Structure of α -Thujone

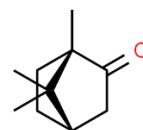


Fig. 10. Structure of L-Camphor

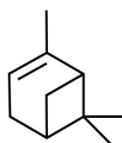


Fig. 11. Structure of α -Pinene

Ben Taarit et al. (13) reported that α -Thujone (23.43%) and Camphor (17.60%) were among the major constituents of the *S. officinalis* leaves EO, followed by Eucalyptol (13.83%), just like in the case below, where the EO has been dissolved in methanol (Table 4).

For the **S2** EO (methanol dissolved sample) the following compounds have been identified: α -Pinene, Camphene, β -Pinene, Eucalyptol, α -Thujone, β -Thujone, L-Camphor, Camphol, Linalyl anthranilate, Bornyl acetate, α -Copaene, Caryophyllene, Humulene and Globulol (Fig. 12, Table 4), the principal compounds being represented by α -Thujone - 25.47% (Fig. 9), L-Camphor - 14.60% (Fig. 10) and Eucalyptol - 10.74% (Fig. 2).

The *Melaleuca alternifolia* EO was the last one to be analyzed in this paper. For the sample dissolved in hexane (**M1**) the chromatogram from Fig. 13 was obtained, and the MS detected the following components: β -Thujene, L- α -Pinene, β -Pinene, β -Myrcene, β -Thujene, α -Terpinene, o-Cymene, Sylvestrene, Eucalyptol, γ -Terpinene, α -Terpinolene, 4-Terpineol and L- α -Terpineol (Table 5).

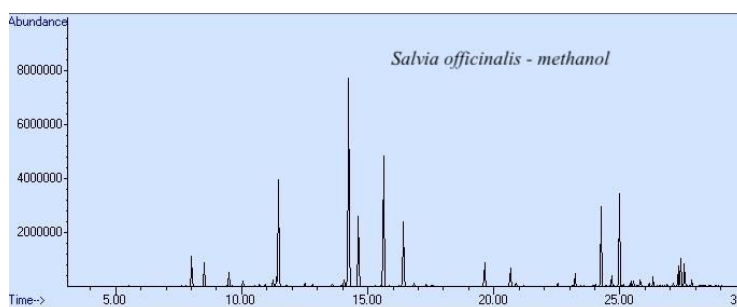


Fig. 12. The chromatogram for the *S. officinalis* oil dissolved in methanol

Table 4

The volatile constituents of the S2. EO (with methanol as solvent)

No.	RT.	Conc. (%)	Compound
1.	8.016	2.68	α -Pinene
2.	8.510	2.19	Camphene
3.	9.491	1.39	β -Pinene
4.	11.463	10.74	Eucalyptol
5.	14.251	25.47	α -Thujone
6.	14.631	7.38	β -Thujone
7.	15.629	14.60	L-Camphor
8.	16.416	7.34	Camphol
9.	19.634	2.34	Linalyl anthranilate
10.	20.655	1.96	Bornyl acetate
11.	23.222	1.19	α -Copaene
12.	24.254	6.56	Caryophyllene
13.	24.986	7.29	Humulene
14.	27.418	2.29	Globulol
Total: 93.42%			

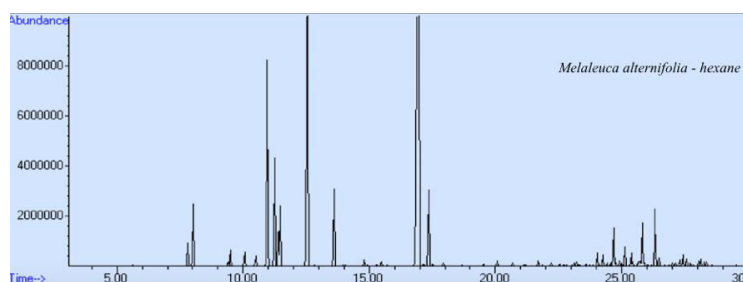
Fig. 13. The chromatogram for the *M. alternifolia* oil dissolved in hexane

Table 5

The volatile constituents of the M1. EO (with hexane as solvent)

No.	RT.	Conc. (%)	Compound
1.	7.803	0.91	β -Thujene
2.	8.018	2.47	L- α -Pinene
3.	9.492	0.67	β -Pinene
4.	10.056	0.54	β -Myrcene
6.	10.962	9.36	α -Terpinene
7.	11.250	4.80	o-Cymene
8.	11.397	1.50	Sylvestrene
9.	11.467	2.63	Eucalyptol
10.	12.549	18.38	γ -Terpinene
11.	13.305	3.52	α -Terpinolene
12.	16.961	42.19	4-Terpineol
13.	17.357	3.74	L- α -Terpineol
Total: 90.71%			

The major constituents of the **M1** EO were represented by 4-Terpineol - 42.19% (Fig. 14), γ -Terpinene - 18.34% and α -Terpinene - 9.36% (Fig. 15) in accordance with the results obtained by Capetti et al. (2).

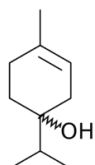
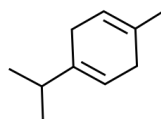


Fig. 14. Structure of 4-Terpineol

Fig. 15. Structure of γ/α -Terpinene

For the same oil sample dissolved in methanol (**M2**), no major differences have been registered, the two chromatograms being quite similar, as well as the main compounds.

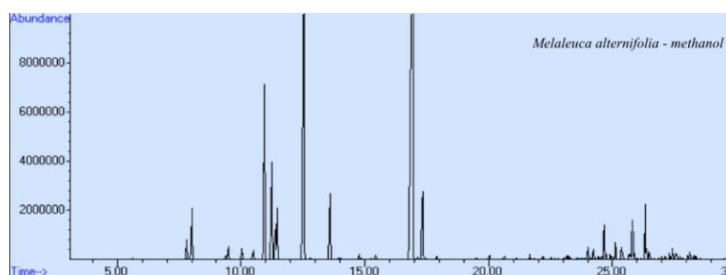
Fig. 16. The chromatogram for the *M. alternifolia* oil dissolved in methanol

Table 5

The volatile constituents of the M2. EO (with methanol as solvent)

No.	RT.	Conc. (%)	Compound
1.	7.796	0.86	β -Thujene
2.	8.010	2.35	α -Pinene
3.	9.484	0.64	β -Pinene
4.	10.046	0.52	β -Myrcene
6.	10.950	9.06	α -Terpinene
7.	11.238	5.01	o-Cymene
8.	11.386	1.47	Sylvestrene
9.	11.455	2.61	Eucalyptol
10.	12.535	17.97	γ -Terpinene
11.	13.591	3.47	α -Terpinolen
12.	16.942	42.93	4-Terpinenol
13.	17.334	3.77	L- α -Terpineol
Total: 90.66%			

Conclusions

The major differences that occurred during the GC-MS analysis of the three essential oils, regarding the solvent type used for the dissolution of the samples were obtained in the case of the *Laurus nobilis* EO. In this case, for the L1 sample, the main compound was Eucalyptol (44.51%), apparently facilitated by the presence of hexane and for L2, the dominant one was Thymol (38.45%).

In the case of the other two studied EOs, *Salvia officinalis* and *Melaleuca alternifolia* the differences consisted mainly of the concentration percentage of the volatile constituents from the total amount. However, some compounds appeared in one of the samples (for example in the hexane-dissolved sample) and were absent in the other one. As to be seen above, for the **S1 EO** (*Salvia officinalis* essential oil dissolved in hexane), 23 volatile compounds have been detected, in comparison with the **S2 EO** (*Salvia officinalis* essential oil dissolved in methanol), where only 14 constituents were obtained.

During the analysis of the **M1** and **M2 EOs** (*Melaleuca alternifolia* essential oils) the difference consisted only in the concentration percent of the compounds.

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ISSUES REGARDING FEEDING AND BEHAVIOR OF FALCO TINNUNCULUS CHICKS

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Summary

Kestrel (*Falco Tinnunculus*) is widespread in urban landscapes, growing in large numbers in the middle of many cities. The objective of this study was to follow the behavior and weight gain of two *Falco Tinnunculus* chicks in the growth period, kept in captivity, in the suburban area. The study was performed on two *Falco Tinnunculus* females chicks that fell from the nest and could not fly. They were rescued and raised freely indoors and outdoors around the house and they were fed on chicken breast and liver, ad libitum. The amounts eaten daily were weighed and the birds also weighed daily. Body weight dynamics was assessed for two months and also their behavior towards people was observed during this period.

Keywords: *Falco Tinnunculus*, behavior, ad libitum feeding, growth

The kestrel, presented in Fig. 1 is a common bird of prey, widespread in Europe, Asia, Africa and America (19).



Fig. 1. Kestrel (*Falco Tinnunculus*)

Their favorite habitat is open fields where they have open area to hunt. They live at different altitudes from 0 to 5000 m (13, 18) but they can be also found in many cities. Its wide spread in urban landscapes is due to its adaptability in terms of selection of nesting place and food type. Kestrels grow in large numbers in the

middle of cities such as Berlin or London. In some cities (like Jerusalem) they are in the middle of flower boxes in front of houses.

The species often uses tall buildings, such as industrial buildings, schools, churches, and blocks of flats as breeding grounds, which have open areas nearby with green fields for hunting.

They don't make their own nest; they use abandoned nests of other bird species (13).

Some researchers affirm that average size of the occupied territory is 5 km², but can vary from 2 to 10 km² (18). Other authors claim that the hunting area size varies between 0.8 and 25 km² (12).

In Vienna, the kestrel estimated population density in 2009 was 60.2 - 96.4 breeding pairs (bp)/100 km². The favorite areas for nesting were the central buildings with green court yards, although the number of chickens was higher in the suburban area with the larger hunting area (17). The density is lower in Berlin, Germany e.g. 22.9 - 33.3 bp/100 km² (9) or Paris, France 40 - 55 bp/km² (10). In England the estimate number of pairs was 50.000 in 2007 (4). In Spain, the breeding population has remained stable since the 1970s at 25.000 - 30.000 pairs (18). In Western Finland the population is 2 - 46 pairs per 100 km² (11).

In Romania the kestrels have a fairly wide spread, the estimated population being 20.000 - 50.000 pairs (20).

Common kestrels are migratory birds that in the autumn from August 1 to October 31 migrate between 1000 and 3000 km to warm areas. The spring migration period takes place between March 1 and April 30 (15).

Regarding diet, kestrels feed both in flight and on the ground, their prey being mainly small mammals but when they have opportunity, they hunt small birds or even lizards (5). Their diet also included grasshoppers, snakes, and rock dove chicks (8). Lizards are generally hunted between 9 and 13 o'clock, because during this time they are more active, having the right temperature to move. Instead, the little birds are hunted at lunchtime, so the kestrels are birds of prey during the day (16). Although kestrels are opportunistic hunters and hunt what is found in their area, the favorite food is short-tailed mice (6).

Kestrel reproduction occurs in the spring during the mating season, they hatch once a year in April-May. The female lays between 3 and 7 eggs which she incubates for 26 - 34 days. During hatching she spends most of time in the nest and the male hunts for her bringing her food to the nest. The chicks are fed by both parents for two months after which they become independent (1).

The reproductive success of kestrels was measured according to various studies and the conclusion was that in cities the reproductive performance is lower than villages. In large cities, on the other hand, kestrels are not threatened by other predators because predators avoid human-inhabited buildings (3).

Materials and methods

The study was conducted on two *Falco Tinnunculus* female birds, monitored two months during the growing period.

They were fed ad libitum with chicken (heart, chest and liver), without impurities such as feathers or bones, as in the case of prey.

The amount of both food and the birds' weight were measured daily. The head-to-tail length and wingspan were also measured.

Body temperature was monitored using the Thermal Imaging Camera Fluke TiS 40. Body temperature values were recorded and the maximum and minimum temperatures of the birds were identified after standing in the shade and sun. The temperatures recorded at the wings, beak and eyes were noted.

The behavior of birds in the family environment was studied. The attitude towards animals was also observed.

Results and discussions

Behavior: When the two female birds were saved, they had down and a few feathers and could not fly or eat and most of the time they slept. In time, their feathers grew and they began to clean their fluff with their beaks and began to flutter their wings and run around the house. When they started to fly they didn't know how to land and they landed hitting the objects in the house but in a week they flew quite well.

From the very beginning, the birds recognized the members of the family and let them approach and hold them. While they still did not know how to fly, they stayed with family members without any fear or stress. They were in the same place where they were placed. Both birds stayed together all the time (Fig. 2).

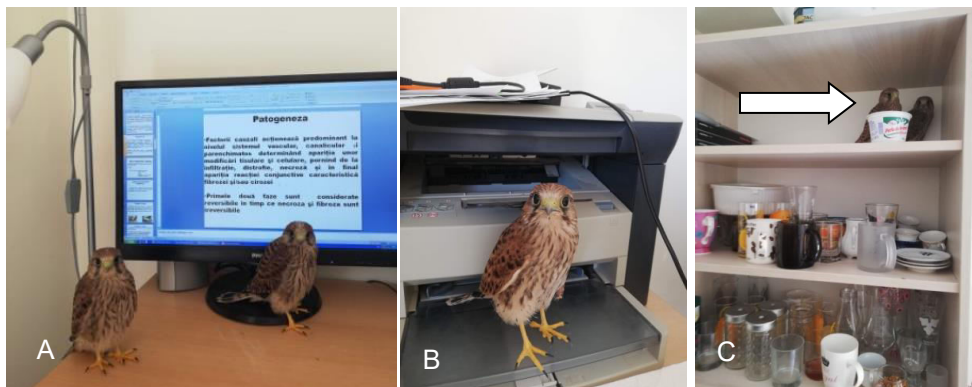


Fig. 2. *Falco Tinnunculus* in domestic environment (A-C)

Later, when they learned to fly, they chose their own place to sleep, in the

most hidden and quiet place in the house, even if they had to fly on an upper floor, (Fig. 3).

Even after they learned to fly the birds agreed to be stroked. Although they were comforted daily by all members of the family, they never showed any affection.

Towards foreigners they did not show any fear and did not run away from them but they did not let themselves be touched by strangers.



Fig. 3. The place to sleep

The attitude towards animals was the same as towards foreigners - they showed no signs of fear. On the contrary, the Alaskan Malamute mature dogs ran away when they saw the birds and the cat avoided them.

Communication: the birds were silent all the time and didn't communicate loudly with each other nor with family members, they made noises only if they felt threatened. Communication between them was done only through the movements of the head.

Daily habits: waking time was 6 in the morning when they left their sleeping place upstairs and went downstairs to where the food was. After eating about the same amount of meat every day, they flew through the living room, played with small toys that they were trying to hunt. Around 11 o'clock they were taken outside where they sat in the tree on a branch and flew around the tree exploring the environment. When they were taken out, they were tied with a long leash so that they could fly within a 20 meters distance.

On rainy days when they did not go outside, they found a place in the house and stayed quiet until evening.

At 5 o'clock they ate for the second time that day about the same amount of meat and went upstairs to sleep.

Some thermal aspects. To study the temperature variation of the birds, an infrared thermometer capable of measuring the temperature in several points at once was used. The temperature of both birds was measured both inside the house (23°C) and outside (33°C) both in the sun and in the shade. The reference points were the

eyes, the beak, respectively the hottest and coldest point of the bird. Temperatures were measured in Celsius degrees and the reference points were marked with “+” in images (Fig. 4 and Fig. 5).

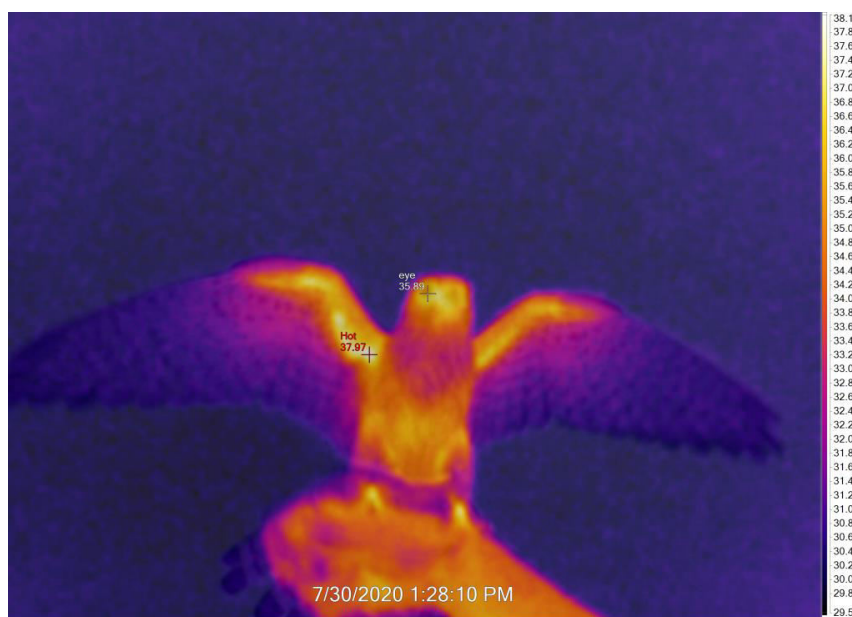


Fig. 4. Thermal image of kestrel

Seven representative images were selected which are presented in Fig. 4 and Fig. 5, where the variations of the ambient temperatures varied significantly. The temperature of the birds in the house is shown in figures 4, 5a, 5b, 5c. It is observed that at an ambient temperature of 23°C, the temperature at eye level is 35°C and the hottest point of the bird is 42.71°C, under the wing. Then, the birds were taken outside in the sun at an ambient temperature of 33°C.

After only 23 minutes the temperature of the wing outside increased from 35.72°C to 61.85°C (Fig. 5d). The eye temperature rose from 36.75°C to 38.81°C. The bird stood with its beak open and the temperature inside the beak was measured, which was 42.79°C, which means that the internal temperature of the bird did not change compared to the situation in which the bird stayed in the house at 23°C (Fig. 5e).

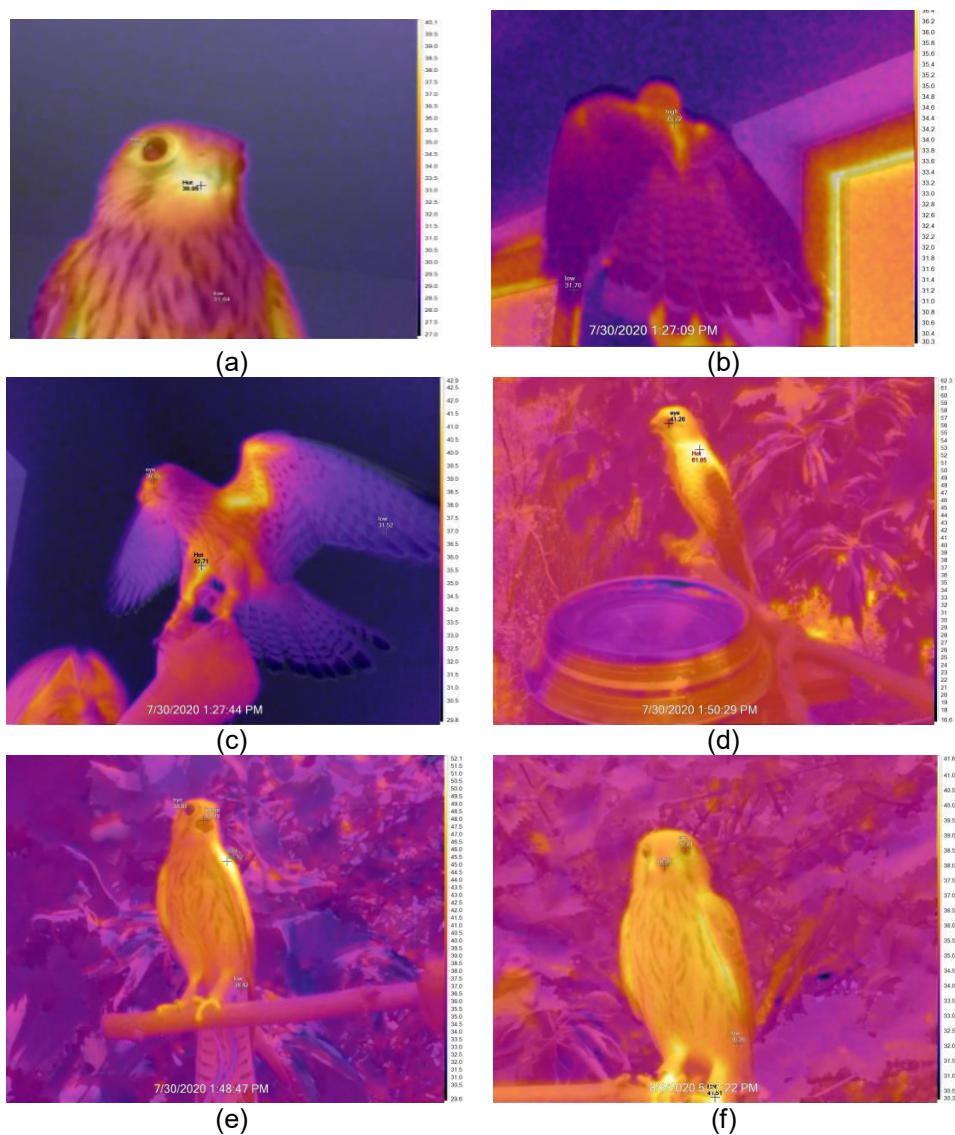


Fig. 5 Thermal image in various conditions (a-f)

The results obtained from the measurements are presented in the Table1.

Table 1

Thermal measurements

Figure	4	5.a	5.b	5.c	5.d	5.e.	5.f
Hottest point temperature	37.97	39.95	35.72	42.71	61.85	51.92	41.51
Coldest point temperature	30.25	31.64	31.76	31.52	35.89	38.42	36.26
Eye temperature	36.89	34.77	-	36.75	41.26	38.81	37.24
Beak temperature	36.6	-	-	-	38.20	42.79	36.98

Following the measurements, it was observed that although the maximum temperatures on the birds' bodies varied greatly, the internal temperature did not change much, which means that the feather layer is a very good thermal insulator.

Feeding ad libitum. The birds were monitored for two months during which they were fed ad libitum. Food was offered to them in two ways, alternately each week during two months: in the first week food was offered in large quantities throughout the day and the birds ate only the same amount daily (35 g) each. In the second week the food was offered in large quantities only in the morning and in the evening, and the birds ate only 35 grams daily each. This amount was measured by the difference between the amount offered and the amount of food left.

When they were rescued, the birds did not know how to eat alone and were fed by inserting small pieces of meat in the beak, 35 grams each, to make sure it develops normally.

After they learned to eat on their own, in the two months of monitoring, the amount of meat consumed daily was 35 grams each, regardless of the feeding method used.

They were fed raw chicken, pork and beef but they prefer chicken. From chickens they preferred dark flesh-liver and heart, then light flesh-the chest. If they had a large amount of liver, they ate only liver, leaving the other types of meat on the plate. Although they always had water, I saw them drinking water only once a week. Instead, they liked to bathe in water both indoors and outdoors. Regardless of the type of meat eaten, the feces had exactly the same consistency and color, white with a black spot in the middle.

The Table 2 shows weight gain by consuming 35 grams per day. The recordings started on June 16 because only then did they learn to eat on their own.

It was observed that the amount of food consumed under ad libitum feeding conditions was approximately 35 grams / day / bird. Although they had plenty of food at their disposal, the birds did not exceed the amount of food they would have eaten in freedom.

At the end of the monitoring period the birds weighed about 180 grams just like the birds in the wild.

Table 2

Body weight of birds

Date	Jun16	Jun26	Jul11	Jul12	Jul13	Jul15	Jul17	Jul23	Aug6
Bird 1 weight (g)	120	140	180	165	180	179	183	180	178
Bird 2 weight (g)	115	138	171	149	175	176	179	176	177

The measured dimensions were the same as those of free-ranging birds. The length measured from head to tail was 32 cm, and the wingspan was 68 cm.

According to a study about kestrels, the dimensions of a common kestrel female are: 30 - 36 cm from head to tail, with a wingspan of 70 - 80 cm. Females are larger than male. Adult male weighing 136 - 252 g, around 155 g on average; the adult female weighs 154 - 314 g, around 184 g on average (13).

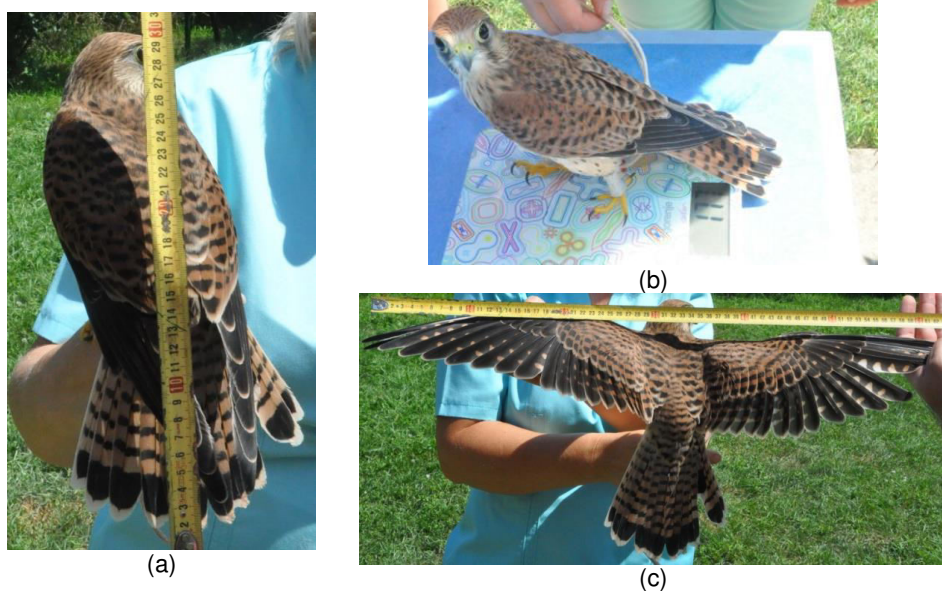


Fig. 6. Bird measurement (a-c)

Conclusions

Characteristic of birds of prey the growth was fast, they learned to eat and fly very fast, falling within the period in which free birds become independent of their parents.

Although the mode of feeding was ad libitum, they did not gain weight, and on release at the age of three months, they weighed the same as that of free-range birds.

From an early age the birds grew up with people who surrounded them with much love but they showed no signs of affection and no signs of domestication.

From the day of their release, they never returned to the yard where they grew up, although they still had water and food at the known place.

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ULTRASONOGRAPHIC FINDINGS IN A CADAVERIC FORELIMB OF A LAME HORSE

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Summary

Lameness in horses can have multiple causes starting from disorders of the bones, nerves and soft tissues that include muscles, tendons and ligaments. Ultrasonography has become one of the most useful imaging modalities for evaluating equine musculoskeletal injuries and uses high-frequency sound waves to produce images in real time. The most common affections of the soft tissue in the acropodial region in horses are represented by the suspensory desmitis, synovial effusion, tendinitis of the deep and superficial flexors and tenosynovitis of the digital sheath. A cadaveric right forelimb from a horse with palmar swelling and grade 3 of lameness was evaluated by ultrasonography after the horse was slaughtered. A transverse and longitudinal section through the metacarpal region was obtained and two pathologies of the soft tissue were detected. Ultrasonography can be used as a diagnostic method in acropodial conditions in horses.

Keywords: horse, ultrasonography, acropodium, diagnostic

The first application of ultrasonographic diagnostic in horses was done in 1970 during an echocardiography. Other applications of ultrasonography were investigated in horses after ecocardiography like rectal examination to establish the early pregnancy and the sex of the fetus. The ultrasonographic exam of the soft tissue can identify the area of injury and can be used to provide information about the prognosis to the owners and trainers (4, 11, 13).

Also, the ultrasound technique is used to examine the shoulder, hip, cervical facet and temporomandibular joints (8, 10). In bone ultrasonography the ultrasound velocity and bone mineral content can be used as accurate indicators of skeletal maturity considering increased cortical porosity and distribution of subperiosteal osteogenesis in the excessively trained horses compared to the unexercised ones (2, 7, 14).

In ultrasonography of the articular surfaces the following items need to be evaluated: the appearance and amount of synovial fluid, thickness and insertion of the joint capsule, appearance and vascularization of the capsule and synovial membrane; the appearance of the articular and periarticular ligaments (collateral ligaments, suspensory ligaments, and oblique sesamoidean ligaments); thickness and appearance of the chondral surface; appearance and regularity of the subchondral bone; and presence of osteophytes and enthesophytes (3, 8, 14, 19).

In case of tenosynovitis, ultrasonographic findings recorded different degree sheath effusion, thickening of the synovial membrane, echogenicity of the synovial fluid and presence of hyperechogenic and fibrinous spots (1, 4, 18).

Materials and methods

In the report, we evaluate the acropodial region of a lame horse that presents a swelling on the mid palmar aspect of the metacarpal right forelimb.

The horse was clinically examined in a slaughterhouse near Timisoara and presented a grade 3 of lameness (AAEP scale) (4) (Table 1), pain at mid metacarpal bone palpation and no reaction to the hoof tester for the right forelimb. After slaughter, the cadaveric right forelimb was collected and was evaluated in the Faculty of Veterinary Medicine from Timisoara.

The ultrasound exam was performed using the MyLab 70 XVG Vet device with a linear probe and a frequency between 8 and 12 MHz for evaluation. Before the exam, the hair was clipped, the skin was cleaned with alcohol and after that we applied the Eco-Gel. Transverse and longitudinal images were obtained on the palmar area of the limb and for surrounding structures including distal joints.

In order to locate the affected area we used the universal system established by David R. Hodgson, Catherine McGowan and Kenneth McKeever (2013) for anatomy of the palmar aspect of the metacarpal/ metatarsal in transverse ultrasonographic images (Fig.1) (6).

Table 1

AAEP Lameness scale

Lameness grade	Observation
0	Lameness not perceptible under any circumstances
1	Lameness is difficult to observe and is not consistently apparent, regardless of circumstances (e.g. under saddle, circling, inclines, hard surface, etc.).
2	Lameness is difficult to observe at a walk or when trotting in a straight line but consistently apparent under certain circumstances (circling, inclines, hard surface, etc.).
3	Lameness is consistently observable at a trot under all circumstances.
4	Lameness is obvious at a walk.
5	Lameness produces minimal weight bearing in motion and/or at rest or a complete inability to move.

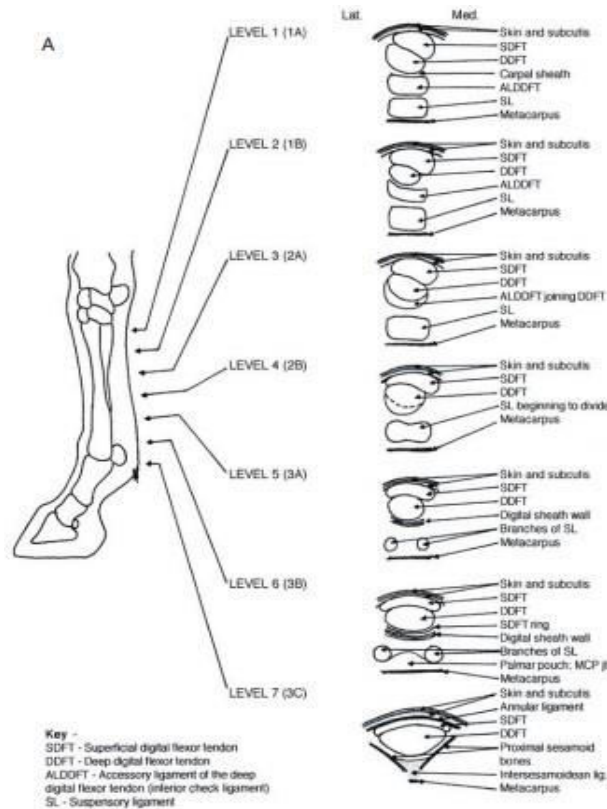


Fig. 1. Normal ultrasonographic anatomy of the palmar aspect of the metacarpus.
(A) Transverse ultrasonographic images (6)

Results and discussions

The ultrasound exam revealed a hypoechogenic area within the structure of the deep digital flexor tendon (DDFT) associated with the tendinitis of the DDFT at the level of the mid metacarpal bone corresponding to Level 3 (2A) (Fig.2).

A second pathology was identified at Level 4 (2B) which consists of accumulation of liquid and distension of the deep digital flexor tendon sheath (Fig.3). The synovial fluid was clear and anechoic.

No other reactions of the collateral ligaments, suspensory ligaments, bone surfaces and joints of the acropodial cadaveric forelimb were detected.

Injuries of the distal aspects of the deep digital flexor tendon (DDFT) are an important cause of lameness in horses. Ultrasonographic changes involving the DDFT were identified in only one of nine horses (9, 16).



Fig. 2. Transversal section of the metacarpal bone – section Level 3 (2A), hypoecogenic area (blue arrows) with tendinitis of the DDFT

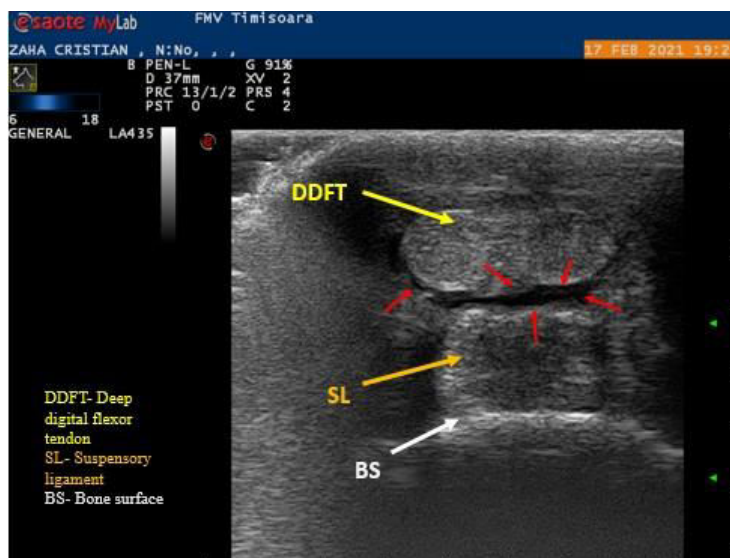


Fig. 3. Transversal section of the metacarpal bone at Level 4 (2B) –hypoecogenic area (red arrows) associated with tenosynovitis of the DDFT sheath

Out of 75 horses that presented lesions of the DDFT at the level of pastern, MRI revealed lesions in only 19 horses, and USG only in 2. DDFT injuries are not detectable using radiographic exam (Rx) (5).

Tenosynovitis of the digital sheath may result after a rupture in the deep or superficial digital flexor tendons or in other structures with synovial communication (15). Tenosynovitis of the digital sheath is a common disorder in equine and often associated with adjacent tendinitis (12).

In the nineteen horses that presented lameness and marked distension of the digital flexor tendon sheaths, ultrasonography revealed nonspecific signs of chronic tenosynovitis (18).

In septic tenosynovitis, the synovial fluid appeared more echogenic than normal and in traumatic tenosynovitis the synovial liquid is anechoic (15, 17).

Conclusions

Tenosynovitis and tendinitis of the DDFT in horses is clinically expressed by palmar distension of the metacarpal/ metatarsal palmar aspect and express different grade of lameness.

Tenosynovitis of the digital sheath is associated with tendinitis of the deep digital flexor tendon (DDFT).

The ultrasonographic lesions detected by us could be correlated with clinical symptoms.

Ultrasonography can be used as a diagnostic method in acropodial conditions in horses.

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