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Features of lung organometry in domestic animals of the Mammalian class (*Mammalia*)

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Abstract. The study of the animal body and its morphoarchitectonics is a priority area for the successful development of animal husbandry. The purpose of the study is to establish the macroscopic structure of the lungs and provide an organometric assessment of their morphological structures in domestic sexually mature animals. The study used 30 clinically healthy individuals of 6 animal species belonging to the class *Mammalia* – Mammals. According to the results of the morphometry analysis of absolute and relative lung values in domestic mammals and their right and left lobes, a pronounced asymmetry was established, the coefficient of which corresponded to: in rabbits – 1:1.30, in dogs – 1:1.33, in sheep – 1:1.37, in pigs – 1:1.34, in cattle – 1:1.37, in horses – 1:1.2. For morphological assessment of typical lung features characteristic of a particular animal species, a morphological scale of marker features of the organ is proposed. According to the results of the study, it is proposed to classify the lungs of domestic mammals, considering the structure and shape, into 4 types: 1st – expanded-shortened (lung development index (LDI) = 85–100%); 2nd –intermediate (LDI = 101–120%); 3rd – moderately elongated (LDI = 121–130%); 4th – elongated (LDI = 131–140%). Based on a detailed analysis of organometric studies, a scale for assessing marker features of the lung is proposed – in rabbits (LDI = 90 ± 1.89%) assigned to the extended-shortened type, in cattle (LDI = 117 ± 2.21%) and sheep (LDI = 114 ± 2.08%) – intermediate type, in horses (LDI = 127 ± 2.74%) – moderately elongated type, in pigs (LDI = 136 ± 3.01%) and dogs (LDI = 137 ± 2.84%) – elongated type. The obtained results of morphological studies of the lungs of the representatives of the mammalian class are of practical importance in biology and veterinary medicine since they are marker signs of their morphofunctional state and criteria for pathomorphological diagnosis of respiratory diseases

Keywords: comparative anatomy; quantitative morphology; respiratory organs; asymmetry coefficient; lung development index

Introduction

A living organism is a complex biological system built from structural elements – organs and tissues that are anatomically interconnected and form a single morphofunctional complex that directly interacts with the environment (Lyabakh, 2019). The response of the animal body to the influence of external factors occurs only during the normal morphofunctional activity of all its systems, including the respiratory apparatus, which includes the lungs (Bilash *et al.*, 2019).

An important and new area of research in morphology is the investigation of adaptive morphofunctional changes in the body of living things to the conditions of external and internal environments (Koptev, 2011; Dzubanovsky *et al.*, 2019). In addition, identifying the morphological features and functional state of organs and systems in normal conditions will allow effectively correcting vital processes of the body for pathology associated with the respiratory system. Therefore, the study of macromorphology and organometric parameters of respiratory organs, including lungs in domestic mammals, is important and is a morphological criterion for indicators of the functional state of all important systems of the animal body.

A comprehensive study of the respiratory system, including the lungs, which perform an extremely vital function in humans and animals – gas exchange, is important (Hyde *et al.*, 2009; Johnson-Delaney & Orosz, 2011).

The mammalian respiratory system should be considered as an open system of the body, providing it with several multifunctional adaptive responses to the air environment. This system is one of the most important, providing gas exchange in the mammalian body by saturating the blood with oxygen and removing carbon dioxide from it to the environment (Patwa & Shah, 2015; Samborska, 2021).

The lungs are soft, compact, parenchymal tissue. They are located in the pleural cavity within the chest (Ramchandani *et al*., 2019). The lungs in mammals are usually formed by the lobes of the lungs, which is due to the need to stretch them in different areas (Ramchandani *et al*., 2003). The typical division of the lungs into lobes is not observed in all placental animals. In primitive placental animals (most insectivores, many rodents, etc.), this division is not detected: the left lung is usually not divided into lobes and the right lung is represented by incomplete lobular division (according to the number of lobes) or indistinctly expressed lobe clippings (Ferner *et al*., 2017). The lungs of different animal species have individual morphofunctional features regarding their lobular structure (Ramchandani *et al*., 2019). Some foreign morphologists believe that in mammals, the lobular structure of the lungs is natural and has no specific features (Duncan, 2004).

The degree of morphofunctional complexity of lung organisation varies in animals: it is simpler in lower terrestrial vertebrates and becomes more complex as their overall organisation increases (Patra, 1986).

The lungs, according to many researchers, belong to the immunocompetent organs (Moyron-Quiroz *et al*., 2004). Thus, in the protection of the lungs from damage, and in the pathogenesis of many respiratory diseases, the main importance is the pulmonary part of the mononuclear phagocyte system (Hiemstra, 2016). The state of the cellular component, mobilisation ability, and functional reserves determine the resistance of lung tissue to infections, exogenous and endogenous toxins (Wright, 2004).

An urgent task of modern morphology is to examine the morphofunctional features of animal body systems and establish their adaptive capabilities, including the respiratory organs, which include the lungs. Lungs in mammals are paired parenchymal organs that perform the function of gas exchange and others: metabolic, secretory, barrier, excretory, thermoregulatory and, thereby, take part in ensuring the homeostasis of the body. The internal structural basis of the lungs consists of numerous lobes that have a cone-shaped or pyramidal shape. Moreover, indicators of morphohistological architectonics of respiratory organs are not only of cognitive importance for fundamental disciplines but also serve as a clinical basis for veterinary medicine.

Therefore, the purpose of this study was to establish the macroscopic comparative structure of the lungs and conduct an organometric assessment of their morphological structures in domestic sexually mature animals belonging to the Mammals class – *Mammalia*.

The following tasks were set to achieve the stated purpose:

- conduct organometric studies of the lungs to determine their linear (length, width, thickness) and weight (absolute and relative mass) indicators in domestic mammals;
- perform morphometric analysis of structural components to determine their linear and weight parameters of the pulmonary lobes (cranial, cardiac, caudal, additional) of the right and left lungs in domestic mammals;
- develop a morphological scale of regularities in the formation of the lung development index to determine the normal shape of the lungs.

Literature review

The parenchymal organ is a complex morphological structure that performs certain functions of interaction with other anatomically and functionally related organs, takes part in the exchange of energy and plastic substances in the animal's body during individual development. Specific features of the organ structure are not only a consequence of a high degree of adaptation of the animal body but can also be a consequence of the development of various pathological processes.

There is no doubt that studies of the morphophysiological state of the animal body always remain the most important in the morphological area in both biological and veterinary and medical studies (Kryshtoforova *et al*., 2015; Archer *et al*., 2021). In this regard, they are widely used to establish the indicators of the physiological norm in the diagnosis of diseases of various origins, in assessing the impact of conditions of keeping and feeding animals, ways to increase their productivity, etc.

A number of researchers focus on morphological research methods (Weibel, 2017; Archer *et al*., 2021). Thus, morphological indicators – the volume of the organ in general and its individual structural units, and absolute and relative mass are considered optimal parameters for macroscopic assessment, which allows reconstructing the quantitative and spatial relationships of structural elements of the animal body. Therefore, there is no doubt that an important criterion for the development of an organ is its absolute mass, which directly indicates its morphofunctional maturity. Therewith, the relative mass of the lungs directly depends on the body weight of the animal and the absolute mass of the organ.

The process of respiration is a source of energy necessary to ensure the vital activity of the body. Researchers around the world are actively engaged in respiratory research. Thus, the human and animal respiratory systems, as the entire organic nature in general, show a morphofunctional pattern of continuous unity and interdependence of anatomical structure and function (Blagojević *et al*., 2018). This is no coincidence because researchers note a clear link between the morphological structures of the respiratory system and other body systems (Johnson-Delaney & Orosz, 2011).

The lungs are constantly exposed to various environmental influences. Thus, mechanical particles, microorganisms, and gases can enter the lungs from the air, which negatively affects pulmonary homeostasis and thereby increase the vulnerability of the organ to infectious agents. The authors (Khoury *et al*., 2022) established that a substantial area of the alveolar surface of the lungs and constant contact with dangerous environmental factors create potential conditions for the development of infections. Han *et al*. (2015) note the ability of the respiratory system to remain relatively resistant to various pathogens, which is explained by an effective body defence system (Han & Mallampalli 2015). However, despite preventive measures in animal husbandry, recently, many researchers have noted a tendency to increase respiratory diseases, including lung pathology (Hiemstra, 2016; Williams & Roman, 2016). Surgical treatment, correct interpretation of research results and prevention of these diseases are impossible without knowledge of the features of comparative morphology and topography of organs in accordance with physiological characteristics.

Therefore, the study of structural and functional features of the macro- and microscopic structure of the lungs in domestic animals remains an urgent issue today of the mammalian class (*Mammalia)*: *Orictolagus cunicalus L.*, 1758 – European rabbit; *Sapis familiaris L.*, 1759 – domestic dog; *Sus scrofa, forma domestica L.*, 1758 – domestic pig; *Ovis aries L.*,

1758 – domestic ram (sheep); *Bos Taurus L.*, 1758 – domestic bull; *Equus ferus Caballus L.*, 1758 – domestic horse. Therewith, it is necessary to consider organometric studies to form morphological criteria (marker features) of the organ. The results of such studies, considering the use of morphological marker criteria developed by the authors for assessing the morphofunctional state of the lungs in clinically healthy animals, will not only have cognitive importance but will also be the basis as indicators of the norm for the prevention and diagnosis of diseases of various origins. They will also be important for the comparative anatomy of investigating the constitution of animals in zootechnics and contribute to the successful development of the livestock industry, improving the productive qualities of animals, etc.

Materials and Methods

The studies were conducted by employees of the Department of Normal and Pathological Morphology, Hygiene, and Expertise of Polissia National University during 2021-2023.

Experimental animals were selected according to the principle of analogues, breed and age. The paper uses 30 individuals of 6 species of animals belonging to the class *Mammalia* – Mammals: *Orictolagus cunicalus L.*, 1758 – European rabbit; *Sapis familiaris L.*, 1759 – domestic dog; *Sus scrofa, forma domestica L.*, 1758 – domestic pig; *Ovis aries L.*, 1758 – domestic ram (sheep); *Bos Taurus L.*, 1758 – domestic bull; *Equus ferus Caballus L.*, 1758 – domestic horse.

The fresh lungs of the examined animals were subjected to anatomical preparation. During the study, the general rules of good laboratory practice GLP (1981) and the provisions of the "Ethical Guidelines for the Use of Animals in Research", adopted by the First National Congress on Bioethics (Kyiv, 2001), were observed. The entire experimental part of the study was conducted in accordance with the requirements of the international principles of the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Strasbourg, 1986) (European Convention, 1986), "Rules for Conducting Work Using Experimental Animals", approved by the order of the Ministry of Health No. 281 of November 1, 2000 "On measures for further improvement of organisational forms of work using experimental animals" and the relevant Law of Ukraine "On the protection of animals from ill-treatment" (No. 3447-IV of 21.02.2006, Kyiv) (Law of Ukraine, 2006; Mishalov *et al.*, 2007).

Lungs from clinically healthy animals were selected for macroscopic studies and organometric analysis (n = 5). After the autopsy, the shape of the lungs and their location in the chest cavity were determined, the absolute and relative mass, length, width, and thickness of the organ lobes were identified.

The absolute mass of the lungs and their lobes was determined by weighing on a laboratory scale RADWAG PS 6000/C/2 (Poland). The relative mass of the lungs (RM) was calculated by the formula (1):

$$RM = \frac{AM}{MA} * 100\%, \quad (1)$$

where *AM* – the absolute mass of the lungs; *MA* – mass of the animal.

Determination of linear parameters of the organ (length, width, and thickness) was conducted by direct measurement with a marking ruler LR 1000 (Ukraine).

The coefficient of asymmetry (CA) of the lungs was determined by the formula (2):

$$CA = \frac{AMRL}{AMLL}, \quad (2)$$

where: *AMRL* – the absolute mass of the right lung; *AMLL* – the absolute mass of the left lung.

The lung development index (LDI) was determined by the ratio of their total length to width using the formula (3):

$$LDI = \frac{LO}{WO} * 100\%, \quad (3)$$

where: *LO* – the length of the organ; *WO* – width of the organ.

According to the indicator of the lung development index, the shape (types) of the lungs were determined (expanded-shortened, intermediate, moderately elongated, elongated): in the case of the LDI value: 85-100%, it is defined as expanded-shortened; according to the LDI indicator: 101-120% – as intermediate, according to the LDI indicator: 121-130% – moderately elongated, according to the LDI indicator: 131-140% – elongated.

Based on the outlined tasks of morphological studies, the following stages were performed: preparation of lungs; description of their shape and structure; organometry, which included determining linear measurements of the lungs (length, width, thickness); clarification of the absolute and relative mass of the lungs and their lobes (left and right); determination of absolute and relative indicators of the lungs.

The names of morphological structures of the lungs are presented in accordance with the requirements of the International Veterinary Anatomical Nomenclature (Khomych, 2005).

Digital data was processed using variational and statistical methods on a personal computer using the Microsoft Excel programme. Therewith, the arithmetic mean (M) and statistical error of the arithmetic mean (m), the probability of the difference between the arithmetic mean of two variational series were determined – according to the probability criterion (P) and the student's tables. The difference between the two values was considered substantial at $P < 0.05$; $P < 0.01$; $P < 0.001$.

Results and Discussion

The lungs of domestic mammals, according to the symmetry of their body, are divided into left and right and have a lobular structure (Autifi, *et al.*, 2009). An important criterion for the development of an organ, which directly indicates its morphofunctional maturity, is the absolute mass (Fig. 1) (Prokushenkova, 2009).

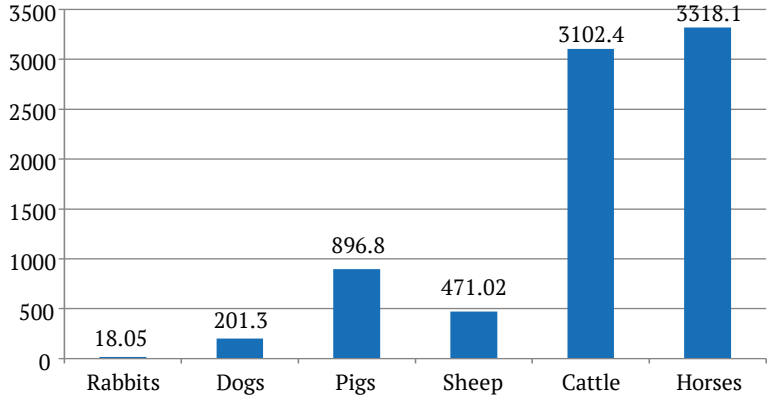

Figure 1. Specific features of the absolute lung mass of domestic mammals (g)

The relative mass of lungs in domestic mammals varies (Maina, 1988). According to the results obtained, the highest relative lung mass in dogs is 1.21 ± 0.14% (Fig. 2).

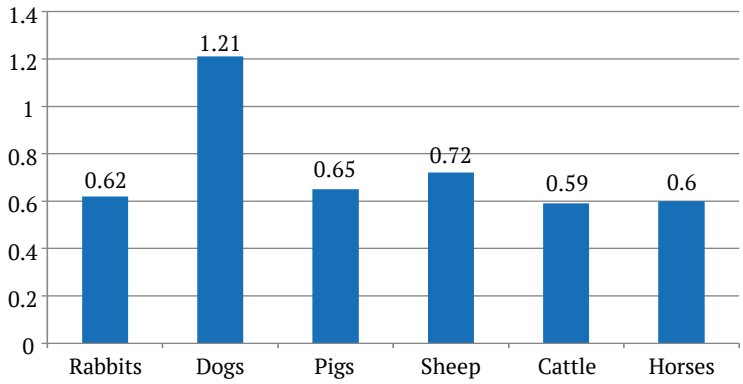

Figure 2. Specific features of the relative lung mass of domestic mammals (%)

The established feature is assumably due to the fact that this animal species has frequent and vigorous breathing, and the remaining air is used quite quickly. On average, in 1 min, depending on the age and size of the animals and at rest, the dog performs from 14 to 30 breathing movements, and during movement and under other circumstances, the intensity of breathing can increase by 2-2.5 times (Horalsky *et al.*, 2009). In the remaining animal species examined, the relative lung mass was: in cattle – 0.59 ± 0.024%, in horses – 0.60 ± 0.052%, in rabbits – 0.62 ± 0.013%, in pigs – 0.65 ± 0.018%, in sheep – 0.72 ± 0.038% (Fig. 2).

According to the classical literature on animal anatomy (Akaevsky *et al.,* 2005), the relative lung mass in horses is equal to 1.43% of the animal's body weight, which does not coincide with the results obtained (0.60 ± 0.052%) in the context of the conducted research and may be related to the breed characteristics of the examined animal species.

Thus, the absolute and relative mass of lungs in domestic animals, and their morphological structures are different and depend on the specific characteristics of animals, their body weight, and the absolute mass of the organ.

In modern morphology, using morphometric (quantitative) research methods, it is possible to establish relationships and interdependence between quantitative changes in individual structures of the animal body and their relative characteristics (individual lobes and sites, etc.) at different stages of individual and phylogenetic development. In addition to that, it is essential to consider the functional state of a particular system of the animal body, depending on their species' characteristics (Krishtoforova & Lemeshchenko, 2007; Jensen *et al.*, 2019).

It was established that the right lung in domestic mammals is larger than the left, which is manifested by signs of asymmetry, depending on the animal species (Musabayeva *et al.*, 2017; Blagojević *et al.*, 2018; Goralsky *et al.*, 2020).

Some researchers (Kling, 2011) consider the manifestation of lung asymmetry in domestic mammals to be a genetic trait. Others claim (Pantoja*, et al.,* 2020) that the asymmetry is associated with the asymmetric position of the heart and other organs in the chest cavity, which depends on the intensity of their gas exchange function, which was manifested during the evolutionary development of animals (Samborska, 2021). According to the literature data, the ratio of the size of the left to right lung is 1.21:1 in horses, 1.38:1 in cattle, 1.35:1 in pigs, and 1.32:1 in dogs (Rudik *et al.,* 2001).

According to the results of organometric studies of the authors in domestic mammals, the right lung is slightly larger than the left, since the heart is shifted to the left. Therefore, the pronounced asymmetry of the lungs occurs due to the different absolute mass of the right lung to the left, the development index of which is equal to: in rabbits – 1:1.30, in dogs – 1:1.33, in sheep – 1:1.37, in pigs – 1:1.34, in cattle – 1:1.37, in horses – 1:1.2.

In most domestic mammals, the left lung has three lobes (cranial, middle, and caudal), and the right lung has four lobes (cranial, middle, caudal, and complementary) (Kling, 2011; Prohl *et al.*, 2014).

The peculiarity of the lungs of horses is that their division into lobes is poorly expressed (the middle and caudal lobes have merged into one and form a caudal lobe), and on their sharp edge there is an inclined interlobular gap that divides each lung into cranial (smaller) and caudal (larger) lobes. The right lung also has an additional lobe. As a result of this evolutionary restructuring of the lungs, their shape and size in general, and lobes in particular, and, as a rule, the absolute and relative mass of individual lobes to the total absolute mass of the lungs, have changed. This is not accidental, since the diversity of lung shapes according to their lobular structure, which is observed in mammals, obeys certain laws of their adaptive changes in the course of evolution (Prokushenkova, 2009). Therefore, depending on the type of animal, in the process of evolutionary development, the shape of the lungs in general and the lobes in particular, their absolute and relative mass, changes.

The caudal lobes of the right lung have the highest value of the absolute mass index in experimental animals (Table 1). In the left lung, the examined indicators are slightly lower than in the right.

Table 1. Specific features of the absolute mass of lung lobes of domestic mammals (g), M ± m, n = 5

Lung lobes		Animal species					
		rabbits	**dogs**	**pigs**	**sheep**	**cattle**	**horses**
Cranial	left	0.79 ± 0.03	22.09 ± 3.01	62.59 ± 8.02	39.58 ± 4.08	259.98 ± 29.02	197.43 ± 19.24
	right	1.71 ± 0.06***	27.29 ± 3.21	82.17 ± 8.96	56.12 ± 6.04	318.31 ± 38.16	214.02 ± 24.04
Middle	left	1.49 ± 0.05	19.91 ± 2.84	72.73 ± 9.21	30.36 ± 2.31	292.25 ± 31.38	–
	right	1.98 ± 0.07***	23.65 ± 2.96***	102.72 ± 11.04	35.06 ± 3.26	402.69 ± 40.02	–
Caudal	left	5.56 ± 0.32	44,26 ± 6,02	247.88 ± 28.04	129.10 ± 10.02	756.98 ± 67.93	1308.66 ± 98.75
	right	5.63 ± 0.41	47.96 ± 6.38	26.07 ± 29.48	164.08 ± 16.17	903.78 ± 72.18	1423.8 ± 102.71
Additional	left	–	–	–	–	–	–
	right	0.89 ± 0.04	16.14 ± 2.08	61.63 ± 7.48	17.72 ± 1.72	168.41 ± 27.33	174.2 ± 16.02
In all	left	7.84 ± 0.73	86,26 ± 8,01	383,2 ± 28,75	199.04 ± 19.82	1309,21 ± 43,38	1506.1 ± 60.48
	right	10.21 ± 0.91	115.04 ± 10.14*	513.6 ± 49.88*	272.98 ± 26.91*	1793.19 ± 54.87***	1812.0 ± 62.92**
Absolute mass of both lungs		18.05 ± 1.02	201.3 ± 18.4	896.8 ± 50.66	472.02 ± 46.34	3102.4 ± 98.62	3318.1 ± 364.4
Coefficient of asymmetry (CA) of the lungs		1:1.30	1:1.33	1:1.34	1:1.37	1:1.37	1:1.2

Note: **P < 0.05;** P < 0.01; *** P < 0.001 the right lobe of the lungs compared to the left*

Therewith, in all experimental animal species, there is only a tendency to increase the absolute mass of the right lung to the left (Table 1). High values of the results of the absolute mass of the caudal lobes of the lungs in domestic mammals are associated with morphotopography of the lungs, their functional load in general, and the pulmonary lobes in particular, during pulmonary (external) respiration (Horalsky, *et al.*, 2009).

Lower morphometric values are characteristic of the cranial lobes of the lungs (Table 1). The absolute mass of the left lobe of the lungs in rabbits and sheep is substantially less by 2.2 times ($P < 0.001$) and 1.4 times ($P < 0.05$) compared to the right one. Therewith, in dogs, pigs, cattle, and horses, there is a tendency to increase the ratio of the mass of the right lung to the left (Table 1).

Therewith, the absolute mass of the middle lobes of the right and left lungs in most experimental mammals has an intermediate value compared to the same indicator of the cranial and caudal lobes (except for dogs and sheep, in which the absolute mass of such lobes is greater than that of the cranial lobes). The absolute mass of the middle lobe of the left lung almost does not change in relation to the right in all the examined animals, so there is only a tendency to reduce this indicator: in rabbits – by 1.3 times, in dogs – by 1.2 times, in pigs – by 1.4 times, in sheep – by 1.2 times, and cattle – by 1.4 times. In the lungs of horses, this middle part is absent.

Among the examined indicators (Table 1), the lowest absolute mass index is set for the additional proportion of lungs in all the examined animals.

According to the results obtained, the ratio of the relative mass of anatomical parts of the lungs (Fig. 3) (cranial, medium, caudal, additional lobes) to the average absolute mass of the lungs is directly proportional to the body mass of animals and the absolute mass of the organ and varies depending on the indicators of the absolute mass of the lobes and the absolute mass of the lungs, which also coincides with the results of other researchers (Maina & Igbokwe, 2020; Pantoja *et al.*, 2020).

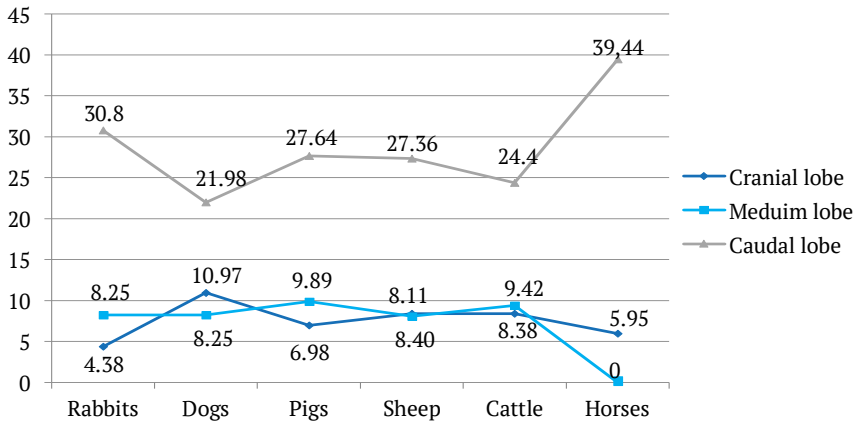

Figure 3. Specific features of the relative mass of the left lung lobes of domestic mammals (%)

According to the results of morphometric studies, the caudal lobes of the right and left lungs have the largest total mass, which depends on the absolute mass of the lobes.

In the right lung of rabbits, this indicator is 31.19 ± 1.38%, in dogs – 23.83 ± 1.82%, in pigs – 29.79 ± 1.37%, in sheep – 34.76 ± 3.04%, in cattle – 29.13 ± 2.54%, in horses – 42.91 ± 4.06%. In the left lung, the relative mass of caudal lobes in accordance with the total absolute mass of the lungs is: in rabbits – 30.8 ± 1.54%; in dogs – 21.98 ± 1.82%; in pigs – 27.64 ± 1.66%; in sheep – 27.35 ± 2.36%; in cattle – 24.4 ± 1.92%, and in horses – 39.44 ± 3.57% (Fig. 3). Therewith, the largest percentage of the relative mass of the caudal lobes of the lungs of horses (Fig. 3) can be explained by the absence of middle pulmonary lobes in the lungs.

Compared to other indicators examined, the cranial lobes of the lungs of animals have a lower relative mass. However, in the right lung, the relative mass of cranial lobes (in rabbits – 9.47 ± 0.89%, dogs – 13.56 ± 0.92%, sheep – 11.89 ± 1.07%, pigs – 11.89 ± 1.07%, cattle – 10.26 ± 0.97%, horses – 6.45 ± 0.62%) is higher relative to such indicators in the left lung (in rabbits – 4.38 ± 0.31%, dogs – 10.97 ± 0.96%, pigs – 6.98 ± 0.67%, sheep – 8.4 ± 1.04%, cattle – 8.38 ± 0.64%, and horses – 5.95 ± 0.51% (Fig. 3), which is consistent with the results of other researchers (Maina & Igbokwe, 2020; Pantoja *et al.*, 2020).

The relative mass of the middle lobes of the lungs in experimental animals varies but is lower compared to that of the caudal lobes. However, their organometric data are slightly larger than the relative mass of cranial lobes. However, in dogs, the relative mass of the middle lobes is greater than that of the cranial lobes (Fig. 4). The relative mass of the middle lobes of the right lung in rabbits is 10.97 ± 0.98%, in dogs – 11.75 ± 1.14%, in pigs – 11.45 ± 1.04%, in sheep – 7.42 ± 0.88%, and in cattle – 12.98 ± 1.32%. Therewith, the relative mass of the lobes of the left lung is lower compared to the right lung and is: in rabbits – 8.25 ± 0.81%, in dogs – 9.89 ± 0.64%, in pigs – 8.11 ± 0.72%, in sheep – 6.43 ± 0.62% and in cattle – 9.42 ± 0.88% (Fig. 4). Additional lung lobes have the lowest relative weight: rabbits – 4.93 ± 0.36%, dogs – 8.02 ± 0.48%, pigs – 6.87 ± 0.64%, sheep – 3.75 ± 0.12%, cattle – 5.42 ± 0.56%, and horses – 5.25 ± 0.68% (Fig. 4).

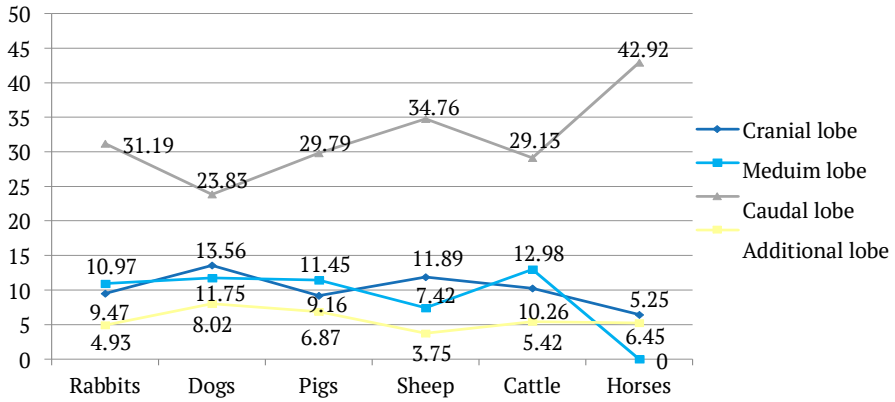

Figure 3. Specific features of the relative mass of right lung lobes of domestic mammals (%)

Thus, analysing the results of lung morphometry in domestic mammals, it was established that the absolute and relative mass of lung lobes (cranial, medium, caudal, additional) depend on the morphotopography of the lungs, functional load, and species characteristics of

experimental animals. Therewith, the lowest absolute mass of all lung lobes was established in rabbits (the smallest animals), slightly higher in dogs, then sheep, pigs, and the highest in cattle and horses, which are the largest animals in terms of body weight. Analysing the organometric linear parameters of the lungs of domestic mammals, it should be noted that the greatest total length, width and thickness of the lungs were determined in horses (Table 2).

Table 2. Specific features of linear lung measurements of domestic mammals (cm), M ± m, n = 5

Indicator		Animal species					
		rabbits	dogs	pigs	sheep	cattle	horses
Total length		7.2 ± 0.50	23.0 ± 2.42	35.3 ± 2.82	25.6 ± 2.98	55.15 ± 4.58	61.5 ± 6.32
Total width		7.98 ± 0.54	16.7 ± 1.34	25.8 ± 1.79	22.4 ± 2.90	47.08 ± 3.59	48.44 ± 4.14
Total thickness		1.52 ± 0.20	1.9 ± 0.36	3.6 ± 1.30	5.1 ± 1.12	7.68 ± 1.02	9.6 ± 1.1
Lung development index, (%)		90 ± 1.89	137 ± 2.84	136 ± 3.01	114 ± 2.08	117 ± 2.21	127 ± 2.74
Left lung	length	6.4 ± 0.45	20.2 ± 0.24	29.8 ± 1.90	21.1 ± 1.90	47.34 ± 3.46	60.35 ± 6.96
	width	3.54 ± 0.32	7.6 ± 0.54	10.5 ± 0.30	10.7 ± 1.67	21.5 ± 1.10	23.51 ± 1.44
	thickness	1.28 ± 0.38	1.5 ± 0.09	3.3 ± 1.21	5.0 ± 1.01	7.49 ± 1.32	9.1 ± 1.37
Right lung	length	7.01 ± 0.40	22.9 ± 2.18	35.2 ± 2.41	25.4 ± 2.4	55.04 ± 4.41	61.84 ± 6.39
	width	4.18 ± 0.36	8.1 ± 0.62	12.2 ± 1.34	12.1 ± 1.76	23.2 ± 1.04	23.9 ± 1.42
	thickness	1.52 ± 0.34	1.8 ± 0.11	3.6 ± 1.30	5.1 ± 1.10	7.60 ± 1.34	9.64 ± 1.48

According to Table 2, the smallest indicators of the total length, width and thickness of the lungs, in particular, the right and left lungs, were determined in rabbits. Linear lung parameters increase in accordance with the increase in body weight of animals. Therewith, the linear characteristics (length, width, thickness) of the right lung in all types of experimental animals are larger than the left, and accordingly, the development index (ratio of length to width) of the lungs in domestic animals has different values (Table 2).

Analysing the results obtained in detail, considering the macroscopic structure of the lungs, their asymmetry coefficient (the ratio of the absolute mass of the right lung to the left), a morphological scale was developed to determine the lung development index (marker signs). According to the morphological scale, the lungs of domestic mammals were classified according to their structure and shape into 4 types: 1st – expanded-shortened (LDI = 85–100%); 2nd – intermediate (LDI = 101–120%); 3rd – moderately elongated (LDI = 121–130%); 4th – elongated (LDI = 131–140%). According to the proposed scale for assessing marker signs of lungs in clinically healthy animals, their development index differs: in rabbits, the LDI is 90 ± 1.89%, so their lungs are expanded-shortened type; in cattle and sheep, this indicator is 117 ± 2.21% and 114 ± 2.08%, respectively, and their lungs are characterised as expanded-elongated type; in horses, the LDI is 127 ± 2.74%, so their lungs are moderately elongated type; in pigs and dogs, LDI, respectively, is 136 ± 3.01% and 137 ± 2.84%, and therefore their lungs are elongated.

Conclusions

According to the symmetry of the animal body, the lungs are divided into left and right and have

a typical partial structure: the left lung in all experimental animals (except horses) has three lobes – cranial, middle, caudal; and the right four – cranial, middle, caudal, and additional. In horses, the interlobular cardiac notch divides the right and left lungs into only two lobes – the cranial (much smaller) and caudal (larger), and the right lung has an additional lobe on the medial side. The left lung is much smaller than the right.

According to the results of the study, the absolute mass of lungs in representatives of domestic mammals (the smallest in rabbits – 18.05 ± 1.32 g, the largest in horses – 3318.1 ± 364.4 g) synchronously obeys the well-known and recognised facts of organ development and depends on the phylogenetic level of animal development. The higher the systematic ratio of animal species (their size, body weight, etc.), the greater the organometric parameters of the organ.

The relative lung mass in experimental mammals varies asynchronously, depending on the animals' body weight and absolute lung mass. Thus, the highest relative weight in dogs is 1.21 ± 0.14% and the lowest in cattle – 0.59 ± 0.024%. The intermediate value of the relative lung mass index is typical for horses (0.60 ± 0.052%), rabbits (0.62 ± 0.013%), pigs (0.65 ± 0.018%), and sheep (0.72 ± 0.038%).

In domestic mammals, considering the specific characteristics of animals, depending on the type of respiration characteristic of them, the lungs are different in shape and size, and therefore differ in the parameters of the development index: in rabbits (LDI = 90 ± 1.89%), the lungs are expanded-shortened type; in cattle (LDI = 117 ± 2.21%), and sheep (LDI = 114 ± 2.08%) – expanded-elongated type; in horses (LDI = 127 ± 2.74%) – moderately elongated type; in pigs (LDI = 136 ± 3.01%), and dogs (LDI = 137 ± 2.84) – elongated type.

In domestic mammals, there is a pronounced asymmetry of the lungs (the ratio of the absolute mass of the left lung to the right), the coefficient of which in rabbits is 1:1.30, dogs – 1:1.33, sheep – 1:1.37, pigs – 1:1.34, cattle – 1:1.37 and horses – 1:1.2.

The scientific originality of these studies consists in the scientific substantiation of the obtained results of morphometric studies, linear parameters, absolute and relative values of lungs, and their lobes in domestic mammals. In the future, it is planned to conduct histo- and ultramicroscopic studies of the respiratory part of the lungs of domestic animals.

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None.

Conflict of Interest

None.

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Особливості органометрії легень свійських тварин класу Ссавці (*Mammalia*)

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Анотація. Дослідження організму тварин, його морфоархітектоніки, є пріоритетним напрямом для успішного розвитку тваринництва. Мета роботи – з'ясувати макроскопічну будову легень та надати органометричну оцінку їх морфологічних структур у свійських статевозрілих тварин. У дослідженні використані 30 клінічно здорових особин 6 видів тварин, які належать до класу *Mammalia* – Ссавці. За результатами аналізу морфометрії абсолютних і відносних величин легень у свійських ссавців та їх правої і лівої часток встановлено виражену асиметрію, коефіцієнт якої відповідав: у кролів – 1:1,30, у собак – 1:1,33, у овець – 1:1,37, у свиней – 1:1,34, у великої рогатої худоби – 1:1,37, у коней – 1:1,2. Для морфологічної оцінки типових ознак легень, характерних для конкретного виду тварин, запропоновано морфологічну шкалу з маркерних ознак органа. За результатами досліджень запропоновано класифікувати легені свійських ссавців із урахуванням будови та форми за 4 типами: 1-й – розширено-вкорочений (індекс розвитку легень (ІРЛ) = 85–100%); 2-й – проміжний (ІРЛ = 101–120%); 3-й – помірно видовжений (ІРЛ = 121–130%); 4-й – видовжений (ІРЛ = 131–140%). На

основі детального аналізу органометричних досліджень запропоновано шкалу для оцінки маркерних ознак легені – у кролів (ІРЛ = 90 ± 1,89%) віднесено до розширенно-вкороченого типу, у великої рогатої худоби (ІРЛ = 117 ± 2,21%) та овець (ІРЛ = 114 ± 2,08%) – проміжного типу, в коней (ІРЛ = 127 ± 2,74%) – помірно-видовженого типу, у свиней (ІРЛ = 136 ± 3,01%) та собак (ІРЛ = 137 ± 2,84%) – видовженого типу. Отримані результати морфологічних досліджень легень представників класу Ссавці мають практичне значення у біології та ветеринарній медицині, оскільки є маркерними ознаками їх морфофункціонального стану та критеріями патоморфологічної діагностики респіраторних захворювань

Ключові слова: порівняльна анатомія; кількісна морфологія; органи дихання; коефіцієнт асиметрії; індекс розвитку легень

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Microstructural analysis of frozen and salted fish and seafood meat

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Abstract. To evaluate the quality and safety of fish and seafood meat, along with generally accepted methods, new histological research methods are used, which allow establishing microscopic changes in fresh, spoiled, and canned foods. The purpose of this study is to examine the microscopic structure of salted fish meat (Herring, Pollock) and seafood (squid, mussels) by freezing. It was confirmed that fish meat is composed of skeletal muscle, fibrous connective (endo-, perimysium) tissues with blood, lymphatic vessels, and nerves. Muscle tissue fibres have the appearance of cylindrical formations, with well-defined transverse striation and numerous nuclei. The basis of seafood meat is smooth muscle tissue with layers of loose fibrous connective tissue, blood vessels, and nerve fibres. Smooth muscle cells are fusiform in shape, without transverse striation and with a single nucleus. During freezing of fish meat at a temperature of -18ºC, ice crystals are small and well-defined in the endo- and perimysium, and at a temperature of -23ºC – in muscle fibres. When fish is re-frozen, large ice crystals form in both the muscle fibres and the endomysium and perimysium of the muscles, the muscle fibres are fragmented and have cracks. In frozen seafood meat, there is a deformation of bundles of smooth muscle cells and their fragmentation. During the salting of fish meat, in the dehydration phase, a decrease in the diameter of muscle fibres and the width of the endo- and perimysium is noted, the transverse striation and nuclei of muscle fibres are well expressed, and in the dehydration phase, the reverse processes occur. Meanwhile, the fibres become straight with cracks and crevices, and graininess is noticeable in the endomysium and perimysium. Based on the results obtained, it is possible to evaluate the microstructure of frozen and salted fish and seafood meat, which is important when monitoring the suitability of food products for consumers

Keywords: fishing industry; canning; histological methods; muscle tissue; skeletal muscles

Introduction

Fish and seafood have long been consumed by humans and used as affordable and tasty food products. Currently, the value of fish products has been substantiated based on the quantitative and qualitative evaluation of proteins, fats, carbohydrates, vitamins, microelements, and other substances contained in them, and the technologies for their production and canning have been developed to preserve natural properties and improve quality (Abraha *et al*., 2017; Alimon *et al*., 2018; Volkhova & Holembovska, 2021).

By chemical composition, muscle tissue contains a considerable amount of protein and is a source of essential fatty acids (omega-3) and minerals (phosphorus, calcium, magnesium, zinc, iron, etc.). In particular, fish protein is easily absorbed in the human body, which creates conditions for lowering cholesterol levels. Fish meat also contains vitamins (A, D, E, K, B_1, B_2) and other biologically active substances that prevent the development of metabolic disorders and the occurrence of various diseases, in particular atherosclerosis. They have a positive effect on the functioning of the brain, cardiovascular and endocrine systems (Abraha *et al.*, 2017).

Fat obtained from fish has a positive effect on the human body. In addition, sea fish contains much more fat than river fish. It contains polyunsaturated fatty acids and fat-soluble vitamins that promote the formation of prostaglandins. The latter compounds increase the body's immunity and resistance to infections and have an anti-inflammatory effect (Duarte *et al.*, 2020).

Recently, some producers often falsify fish (Semenov *et al.*, 2020). Due to the above, the fish is of improper quality and becomes a source of human diseases, in particular, salmonellosis, botulism, etc. (Pohrebnyak, 2015). Quite often, to notably increase the mass of fish and seafood, manufacturers add water during freezing and, as a result, freeze a large amount of it, and also freeze fish that has begun to spoil or is already spoiled. Moreover, fish products can be treated with a variety of stabilisers (preservatives, antibiotics), including hydrogen peroxide, to freshen the corresponding products. The consumer must be protected from counterfeiting and consume high-quality and safe products to preserve their health. Therefore, veterinary and sanitary expertise of fish and seafood meat is particularly relevant (Pohrebnyak, 2015; Semenov *et al.*, 2020).

To evaluate the quality and safety of fish meat and seafood, histological research methods based on microstructural analysis are gaining popularity (Abraha *et al.,* 2017; Popova *et al.*, 2020). They are used by veterinary medicine doctors who work in accredited laboratories and supermarkets, based on various types of food production and processing facilities and possess the technique of manufacturing histological preparations, which is one of their professional competencies. Using a light microscope, histological methods make it possible to establish microscopic changes in fresh and spoiled food products, including canned products. Histological methods allow veterinary doctors to finally confirm the suitability of meat fish and seafood for food purposes, namely, the proper quality and safety of the food product for human consumption.

The purpose of the study is to examine microscopic qualitative changes in fish and seafood meat under various canning methods (freezing and salting).

Literature Review

The fishing industry is a branch of the food industry, the enterprises of which are engaged in catching fish and seafood and producing various products from them. The latter are a source of many nutrients for humans, which are necessary for strengthening bones, increasing immunity, and preventing the development of diseases of various organ systems and apparatuses (Mahmud *et al.,* 2018; Standal *et al.*, 2018).

To prevent spoilage of fish and extend its shelf life, it is necessary to preserve products. These methods are designed to inhibit bacterial activity and metabolic changes that lead to loss of fish quality (Boziaris *et al.,* 2013; Semenov *et al., 2020*). One of the main methods of preserving the freshness of fish is its freezing (applying cold), which is used to extend the long-term storage time.

According to Kong *et al.* (2008), physical changes occur in frozen fish meat, during which frozen water in the tissue forms ice crystals, which lead to partial destruction of the sheath of muscle fibres and leakage of tissue fluids due to deformations.

Alizadeh *et al.* (2007) note that physical changes are considerably affected by the freezing rate and the condition of raw materials. In their opinion, fish should be frozen quickly and at a low temperature. During rapid freezing, small ice crystals are formed, which on histological preparations look like voids, they are evenly distributed in the tissues and do not disturb their structure. The concentration of tissue fluids almost does not change and the proteins retain the ability to swell during defrosting. Moreover, the lower the freezing temperature, the smaller the size of the crystals. When frozen slowly, large ice crystals form, mainly in fibrous connective tissue, which on histological preparations have the appearance of significant

voids, since some of the water moves there from muscle fibres. Large crystals deform muscle fibres, destroy fibrous connective tissue and during defrosting, liquid flows out of the fish's tissues, and therefore it loses its original taste and appearance.

Khomych & Bal-Prylypko (2018) indicate that fish is better preserved if it is frozen immediately after catching when the shell of the muscle fibres of its meat is elastic, has considerable elasticity, and there is no damage so that the resulting ice crystals do not destroy this shell. As the freezing point decreases, this process occurs faster and the structure of body tissues changes microscopically less.

According to Popelka *et al.* (2012), during long-term storage of fish, microscopic changes occur, which are characterised by loss of muscle fibres, swelling and destruction of their nuclei, the appearance of numerous voids that are formed by ice crystals, and loosening of the endomysium and perimysium.

In addition, another common method of preserving fish is salting, which uses salt crystals or brine Dal Bello *et al.* (2020). Sodium chloride diffuses into the muscles from the outside due to the difference in osmotic pressure between the internal environment of the muscle fibres and the brine.

According to Dal Bello *et al.* (2020) during the dry method, the moisture content in the muscles, as well as bacterial and enzymatic activity, is notably reduced. Therewith, the muscle fibres are located denser and acquire a smaller length and width. The duration of the salting period and the salt concentration depend on the expected final product.

Horner (1997) considers that the main disadvantage of the dry method of salting is the dehydration of meat products, as a result of which they become hard. In this case, the distribution of salt is quite uneven.

Materials and Methods

The study was conducted during 2020-2022 in the scientific laboratory of the Department of Anatomy, Histology, and Pathomorphology of Animals of V.G. Kasyanenko National University of Life and Environmental Sciences of Ukraine (Kyiv). Samples of frozen and salted fish and seafood meat were taken for histological studies. According to external signs and organoleptic parameters, all samples of frozen fish met the regulatory requirements of DSTU 4868:2007 (2009).

The process of making histopreparations included a series of sequential steps: sampling, fixing, washing the fixed samples with water, dehydration, sealing the samples with paraffin, making sections from the compacted material, staining the sections, and embedding the sections in balsam.

Samples were taken from fish and seafood carcasses using various canning technologies: freezing (herring, pollock, squid, mussels) and salting (herring). Two samples with a thickness of 2-3 cm were obtained from one carcass. Samples obtained from one carcass were numbered with the same number, placed in a glass jar, and fixed in a 10% neutral formalin solution. The minimum period of fixing the material was 24 hours.

The samples were washed with cold running water for several hours, after which they were dehydrated with ethyl alcohol of increasing strength (60%, 80%, 96% and absolute alcohol). Samples were kept in each of these alcohols for 12 to 24 hours. Paraffin was used to seal the dehydrated samples. Before compaction, the material was kept in two portions of chloroform for 2.5-3 hours. Samples from chloroform were transferred to a mixture of chloroform and paraffin and placed in a thermostat at 37°C for two hours. Subsequently, all samples were kept in paraffin at a temperature of 56°C. Then the paraffin samples were transferred to containers

and filled with hot paraffin. Paraffin blocks containing samples were obtained.

Subsequently, histological sections with a thickness of 6-11 microns were made on an MPS-2 microtome (Kharkiv plant "Tochmedprylad", Ukraine). Sections were stained with Karatsi's hematoxylin and eosin to establish the general microstructure of fish and seafood meat. The stained sections were placed in a Canadian balsam and examined using an Olympus CX 43 light microscope (Olympus, Japan). Individual histopreparations and their fragments were photographed with a Nikon Coolpix S3100 (Nikon, Japan).

Results and Discussion

Composition of fish and seafood meat

According to the data obtained, the composition of fish meat (herring, pollock) and seafood (squid, mussels) was not the same. The composition of fish meat included skeletal muscles, the basis of which is composed of skeletal muscle tissue, dense fibrous connective tissues, blood vessels, nerve fibres, and endings. In addition, muscle tissue was quantitatively superior to other tissues.

The skeletal muscles of fish are formed by muscle fibres, which are symplastic structures. On the longitudinal sections, they had the appearance of cylinders, the ends of which are rounded, jagged, or bevelled. The length of such fibres was 1.5-3.5 mm, and the diameter was 10-70 microns. The overall microstructure of the muscle fibre of fish meat is similar to that of mammalian meat (Stasishen, 2009; Dubinina *et al*., 2010; Khomych & Bal-Prylypko, 2018).

The fibre consisted of numerous nuclei, sarcoplasm (viscous protein solution), and sarcolemma (shells). The nuclei were rod-shaped or elongated-oval in shape and were located on the periphery of the muscle fibre, directly under the sarcolemma. There were from several dozen to several hundred of them. In the nuclei, their components were well distinguished: the nuclear envelope, chromatin (a small amount), the nucleolus, and karyoplasm (nuclear juice). A significant volume of the sarcoplasm of muscle fibres was occupied by hyaloplasm, which is a colloidal structure and, accordingly, its rarest part. The sarcoplasm contains special organelles-myofibrils, which provide contraction of muscle fibres. They were located along the muscle fibre and occupied the central part of it. Myofibrils consisted of myofilaments and had characteristic alternating dark and light stripes. As noted in the literature (Khomych *et al.,* 2013), since the light and dark stripes are located at the same level, the muscle fibre looks striated.

Individual muscle fibres were thin layers of loose connective tissue (endomysium), in which a network of hemocapillaries and nerve fibres were located. Muscle fibres were combined into bundles, between which thicker layers of loose connective tissue (perimysium) were located. Loose fibrous connective tissue consisted of cells and intercellular substance. The latter included a basic (amorphous) substance and fibrous structures. The cellular composition of this tissue was represented mainly by fibroblasts, which produce components of the intercellular substance, and in much smaller quantities, there were histiocytes, plasma cells, mast cells, etc. (Khomych & Bal-Prylypko, 2018). A considerable number of adipocytes or fat cells were found in the loose fibrous connective tissue of herring. The latter had a pear-shaped appearance and a significant diameter on histopreparations. The nucleus in such cells was shifted to the plasmolemma (shell) and had an elongated oval shape. The loose fibrous connective tissue of pollock contained notably fewer adipocytes, which were arranged in small groups.

According to the results of research, part of the fish's muscles began or ended with tendons. The latter were formed by dense fibrous connective tissue with a significant cellular content

of fibrocytes. Tendons turned into septa, which are formed by loose fibrous connective tissue (Fig. 1). Septa divided the fish muscles into myomeres, which had the appearance of concentrically arranged semicircles and circles. Fat cells were visible in individual myosepta.

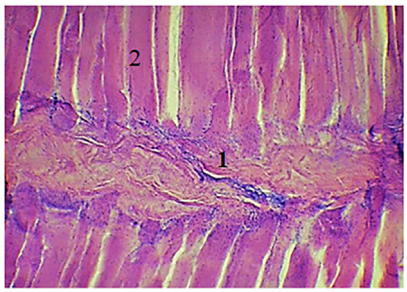

Figure 1. Skeletal muscle of fish (herring). Hematoxylin and eosin, ×90

Note: *1 – septa; 2 – muscle fibres*

Depending on the colour, fish meat is divided into white with different shades and pink-red. White muscle fibres are thicker and contain less of pigment-protein myoglobin, while red ones are the opposite (Horner, 1997; Doe, 2002). In the studied meat of herring and pollock, muscle fibres had a white colour with different shades.

According to the obtained results, the basis of the meat of invertebrate seafood is composed of smooth muscle tissue, loose fibrous connective tissue (endomysium, perimysium), vessels, nerve fibres, and endings (Fig. 2). The muscle tissue of squid was white, while mussels were white and pink in colour with different markings. It was formed by smooth muscle cells that had a microscopic structure similar to the smooth muscle tissue cells of aquatic mammals (Khomych *et al.,* 2013). These are fusiform cells with a single, centrally located oval-shaped nucleus. Their length was about 100 microns, while in aquatic mammals these cells can reach up to 200 microns (Khomych *et al.,* 2013), and the thickness is 3-10 microns. In the nucleus of smooth muscle cells, insufficient heterochromatin was noted and one, less often two nucleoli were found. There was no transverse striation in these cells, which indicates the absence of myofibrils. Smooth muscle cells were combined into bundles consisting of 8-12 cells, and the bundles formed layers.

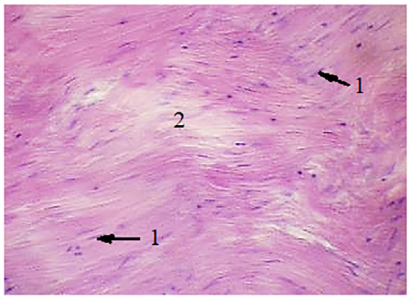

Figure 2. Smooth muscle tissue of seafood (squid). Hematoxylin and eosin, ×90

Note: *1 – smooth muscle cells; 2 – loose fibrous connective tissue*

Frozen fish meat

The microstructure of frozen fish meat depends on its condition, temperature, speed, and multiplicity of freezing. Fish is considered frozen if the temperature in its thickness is below 6°C. Usually, unassembled and disassembled fish are frozen until their posthumous conjuration. In industrial production, the freezing temperature can range from -5 to -80°C. As a rule, fish is frozen at a temperature of -5 and -20°C (Alizadeh *et al.*, 2007).

The results of the research confirmed that the tissue liquid (water) of frozen fish meat (herring and pollock) turns into ice crystals. They caused changes in the microstructure of meat. Ice crystals melted in thawed meat, and in histological preparations, their locations had the appearance of cavities of various configurations and sizes. The size of the ice crystals depended on the freezing rate of the meat.

It was confirmed by Alizadeh *et al.* (2007) and Duarte *et al.* (2010) that large ice crystals are formed during slow freezing, and much smaller crystals are formed during fast freezing. The authors believe that the size and location of ice crystals are the most important factors affecting the texture quality of frozen food. From the surface to the deeper layers, the rate of freezing of meat decreased. In addition, ice crystals were smaller in the surface areas of the meat than in the deep ones.

At a temperature of -18°C, ice crystals in frozen fish meat were found in connective tissue layers (endomysium and perimysium) (Fig. 3).

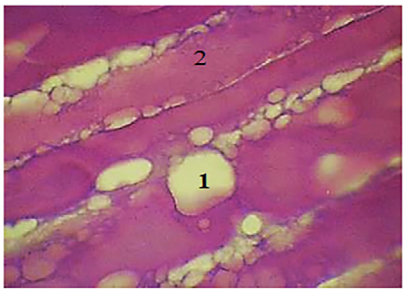

Figure 3. Ice crystals in the endomysium of herring meat. Hematoxylin and eosin, ×100
Note: *1 – ice crystals; 2 – muscle fibres*

Large ice crystals with appendages stuck into muscle fibres, leading to their destruction or deformation. The endomysium and perimysium in ice crystals had a slightly expanded appearance. Transverse striation was well expressed in the muscle fibres, and some of the nuclei in the places of their deformation were marked by destruction.

In fish frozen at -23°C or lower, ice crystals were found in the endomysium, perimysium, and muscle fibres (Fig. 4).

These crystals were small in size, oval and rounded in shape. The muscle fibres that contain ice crystals were deformed in some places, but not destroyed, with a weak transverse striation. Some of their nuclei were in a state of destruction. The endomysium and perimysium showed notable extensions.

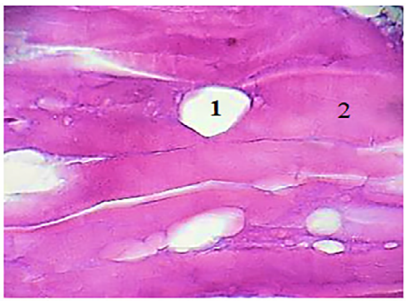

Figure 4. Ice crystals in the muscle fibres of herring meat. Hematoxylin and eosin, ×100
Note: *1 – ice crystals; 2 – muscle fibres*

Substantial destructive changes occur in fish meat when it is re-frozen (Fig. 5), manifested by the formation of large ice crystals in the endomysium, perimysium, and muscle fibres with noticeable cracks, while some of the fibres are fragmented, which is consistent with the findings of Popelka *et al.* (2012). These authors also consider unstable temperature conditions as a factor that increases lipid oxidation. Preventing temperature fluctuations during storage is important to preserve the quality of frozen fish meat.

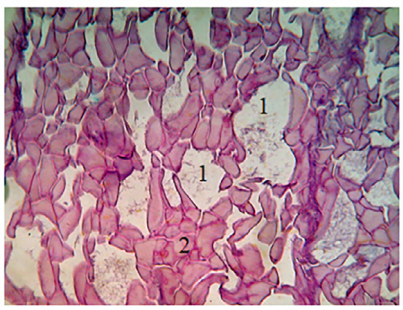

Figure 5. Muscle tissue of pollock when re-frozen. Hematoxylin and eosin, ×100
Note: *1 – ice crystals; 2 – muscle fibres*

When fish meat is re-frozen, the transverse striation of muscle fibres is almost invisible, a significant part of their nuclei is destroyed, lysed, or deformed. The endomysium and perimysium are considerably expanded and loosened. They contain a fine substance of pink colour.

Frozen seafood meat

In industrial conditions, seafood (squid and mussels) is frozen at a temperature of 30°C (shock freezing). It is also allowed to freeze these products at a temperature of -5 °C and below (Nagarajarao, 2016).

According to the results of studies, extremely pronounced destructive changes were noted in frozen squid and mussel meat. They were best expressed in perimysium, which had a loose appearance and significant expansions. Ice crystals of various shapes and sizes, noticeable pink granularity and fibrous structures were found in perimysium. Deformation of smooth muscle cell bundles was observed, which were oriented differently. In some areas, they formed grid-like formations (Fig. 6).

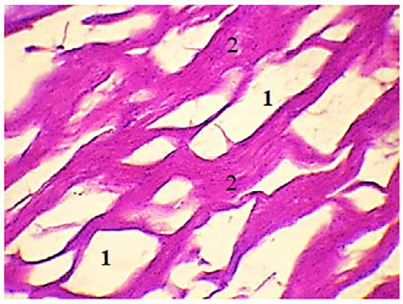

Figure 6. Locations of ice crystals in frozen squid meat. Hematoxylin and eosin, ×90

Note: *1 – ice crystals; 2 – bundles of smooth muscle cells*

The nuclei of smooth muscle cells, which are located near ice crystals, were not expressed, and the cell bodies looked fragmented and deformed. In some places, ice crystals were found in bundles of smooth muscle cells, which lead to their fragmentation and destruction. Furthermore, the destruction of individual smooth muscle cells in bundles with ice crystals was observed. Similar changes were recorded in the meat of frozen mussels (Fig. 7).

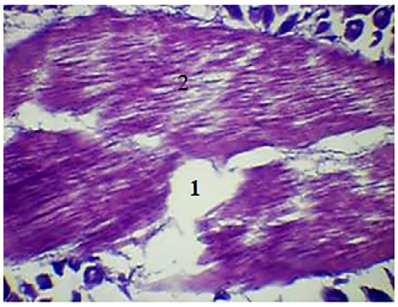

Figure 7. Location of ice crystals in frozen mussel meat. Hematoxylin and eosin, ×100

Note: *1 – ice crystals; 2 – bundles of smooth muscle cells*

Meat of salted fish

The microscopic structure of salted fish meat depends on the methods of salting and is based on the penetration of a solution of table salt into fish meat, while tissue liquid (water) and water-soluble substances are displaced from it (Kong *et al.*, 2008).

For wet salting, the duration of which is from 4 to 8 days, fish (disassembled or not disassembled) is placed in a solution (brine) containing up to 40% table salt (Varlet *et al.*, 2007). Within a few days, fish meat is considerably dehydrated (compacted), as the tissue fluid from the meat diffuses into the salty solution (dehydration phase).

According to the results of studies in the phase of dehydration of fish (herring), there was a decrease in the diameter of muscle fibres and the width of endomysium and perimysium, the fibres fit snugly together (Fig. 8).

Transverse striation and muscle fibre nuclei were well defined. The dehydration phase is quickly replaced by the dehydration phase, due

to the accumulation of salt in muscle fibres. In addition, water enters the fish meat from the brine, which led to an increase in the width of the endomysium, perimysium, and the diameter of muscle fibres (Fig. 9).

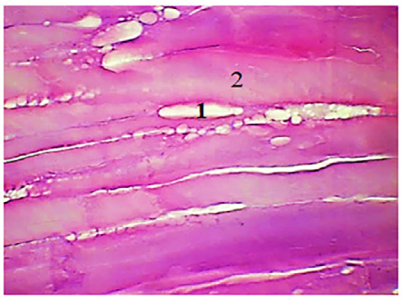

Figure 8. Dehydration phase of muscle tissue of pre-thawed salted herring. Hematoxylin and eosin, ×100

Note: *1 – ice crystals; 2 – muscle fibres*

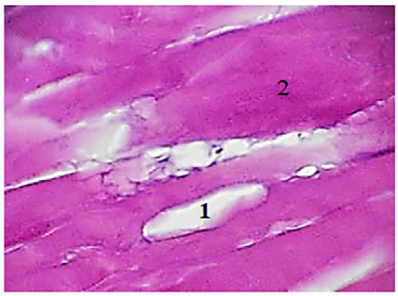

Figure 9. Hydration phase of muscle tissue of previously thawed salted herring. Hematoxylin and eosin, ×100

Note: *1 – ice crystals; 2 – muscle fibres*

Microscopic changes were observed in the muscle fibres. Some of the rectilinear fibres were fragmented and had cracks and crevices, and the striation was not visible. Some of the nuclei were destroyed. In the endomysium and perimysium, pink graininess was noted. When brine was introduced into fish meat by syringing, there was no dehydration phase for this type of wet salting. Microscopic changes in the fish meat are similar to the hydration phase, however, destruction of individual muscle fibers was noted at the sites of brine injection.

During dry salting (mixing fish with salt crystals), the duration of which is from 5 to 7 days, the fish is rubbed with salt and placed in a container in layers (rows). Layers of fish in containers were also sprinkled with salt. On the surface of the fish's body, salt dissolves in mucus, sodium ions enter the meat, and tissue fluid is released from it, that is, only dehydration of fish meat occurs, which is manifested by its compaction (Montero *et al.*, 2003). Microscopic changes in this meat were similar to the dehydration phase in the wet salting method. Thus, muscle fibers had a dense arrangement, and their length and diameter were reduced. Transverse striation and nuclei in muscle fibres were preserved. Using the mixed salting method, the fish is sprinkled with table salt and then dipped into the brine. This method of salting fish meat first dehydrates and compacts it, followed by rehydration (Goulas & Kontominas, 2005). As noted in this paper, this method is the most common and allows obtaining products of standard quality.

In the compacted fish meat, the layers of endomysium and perimysium were weakly expressed, muscle fibres acquired an elongated appearance, their diameter decreased, while nuclei and striation were preserved. Brine that penetrates into fish meat (hydration) caused reverse changes, in which the epimysium and perimysium were expanded and loosened, the diameter of muscle fibres looked enlarged, and their striation was not noted. Most of the fibres were fragmented.

Conclusions

The basis of fish meat (herring, pollack) is composed of skeletal muscles, the muscle fibres of which are symplastic structures with pronounced transverse striations and a large

content of cytoplasm and nuclei. Most skeletal muscles start and end with tendons, which are built from dense fibrous connective tissue. A characteristic feature of fish muscles is the presence of well-expressed myosepta (bridges) on histopreparations, where muscle fibres begin and end. Myosepta are loose fibrous connective tissues and are located across the muscles. They divide the fish's muscles into myomeres. Seafood meat (squid, mussels) is composed of spindle-shaped mononuclear cells of smooth muscle tissue, which do not have transverse striations. Muscle fibres of fish meat and smooth muscle cells of seafood are layers of fibrous connective tissue (endomysium and perimysium) with blood vessels and nerves.

In histopreparations made from frozen fish meat, ice crystals have the appearance of cavities of various sizes, which at a temperature of -18°C are found in layers of fibrous connective tissue and are absent in the muscle fibres, while at temperatures of -23°C – also appear in muscle fibres. In the frozen meat of invertebrate seafood, the smooth muscle cells are deformed and fragmented, while fibrous structures and a pink substance are visible in the perimysium.

During the salting of fish meat, qualitative changes in its microscopic structure occur. Thus, in the dehydration phase, fish meat is considerably compacted, and the muscle fibres retain striation and well-defined nuclei. In the rehydration phase, there is an increase in the diameter of muscle fibres and the width of endomysium and perimysium. The striation of muscle fibres disappears, cracks appear in them, and a pink substance is found in the endomysium and perimysium.

Further studies should examine the histological structure of fish meat and invertebrate seafood using other methods of preserving them (smoking, drying, frying, etc.).

Acknowledgements

None.

Conflict of Interest

None.

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Мікроструктурний аналіз замороженого і солоного м'яса риби та морепродуктів

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Анотація. Для оцінки якості та безпечності м'яса риби і морепродуктів поряд із загальноприйнятими методами використовують нові гістологічні методи досліджень, які дають можливість встановити мікроскопічні зміни у свіжих та зіпсованих харчових і консервованих продуктах. Мета цієї роботи полягала у дослідженні мікроскопічної будови м'яса соленої риби (оселедець, минтай) і морепродуктів (кальмар, мідії) за заморожування.

Підтверджено, що м'ясо риби утворене скелетною м'язовою, волокнистою сполучною (ендо-, перимізій) тканинами з кровоносними, лімфатичними судинами та нервами. Волокна м'язової тканини мають вигляд циліндричних утворень, з добре вираженою поперечною посмугованістю і численними ядрами. Основу м'яса морепродуктів формує гладка м'язова тканина з прошарками пухкої волокнистої сполучної тканини, судинами та нервовими волокнами. Гладкі м'язові клітини веретеноподібної форми, без поперечної посмугованості і з одним ядром. Під час заморожування м'яса риби за температури -18°С, кристали льоду дрібні та добре виражені у ендо- і перимізії, а за температури -23°С – є у м'язових волокнах. Під час повторного заморожування риби формуються великі кристали льоду як в м'язових волокнах, так і в ендомізії і перимізії м'язів, м'язові волокна фрагментовані та мають тріщини. У замороженому м'ясі морепродуктів спостерігається деформація пучків гладких м'язових клітин та їх фрагментація. Під час соління м'яса риби у фазі обезводнення відзначається зменшення діаметру м'язових волокон і ширини ендо- та перимізію, поперечна смугастість і ядра м'язових волокон добре виражені, а у фазі оводнення відбуваються зворотні процеси. При цьому, волокна стають прямолінійними з тріщинами і щілинами, а в ендомізії та перимізії помітна зернистість. За отриманими результатами можна оцінити мікроструктуру замороженого і солоного м'яса риби та морепродуктів, що важливо під час контролю придатності харчових продуктів для споживачів

Ключові слова: рибна промисловість; консервування; гістологічні методи; м'язова тканина; скелетні м'язи

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Safety and quality indicators of rapeseed and sunflower honey from different regions of Ukraine

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Abstract. The relevance of the study lies in the growing demand for safe and high-quality bee products, primarily honey, and the non-admission of low-quality products that can harm the health of consumers in the Ukrainian and European Union markets. In Ukraine, sunflower and rapeseed honey are in the greatest demand in export potential. The purpose of the study is to determine the compliance of rapeseed and sunflower honey obtained in Ukraine with the requirements of

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national and European Food legislation. To achieve this purpose, organoleptic methods of natural honey research were used and the diastase number, acidity, content of pollen grains, mass fraction of water, mass fraction of reducing sugars, sucrose, and hydroxymethylfurfural were determined. Analysis of rapeseed honey for the presence of genetically modified pollen was conducted using the polymerase chain reaction method in real time. It was established that prototypes of sunflower honey from Vinnytsia, Odesa, and Kyiv regions met the requirements of the national standard and European Food legislation, without signs of fraud. Currently, the use of genetically modified organisms is subject to regulation. Samples of rapeseed honey taken from the Vinnytsia, Odesa, and Kyiv regions did not contain genetically modified deoxyribonucleic acid. In terms of physical and chemical parameters, rapeseed honey met the requirements of the national standard. Honey collected in the Kyiv and Vinnytsia regions had an average water content of more than 18.5%, which meets the requirements for first-grade honey according to the national standard. Honey collected in the Odesa region corresponded to the indicators of the top-grade honey, the water content in it averaged 17.7%. According to the main physical and chemical indicators, rapeseed honey collected in Ukraine also meets the requirements of European food legislation. In addition, rapeseed and sunflower honey from the Vinnytsia, Odesa, and Kyiv regions are natural and can be sold not only in Ukraine but also on the territory of the European Union and the World Trade Organisation

Keywords: flower pollen; control; legal regulations; fraud; genetically modified organisms

Introduction

Beekeeping is one of the most promising agro-industrial sectors in Ukraine. Among all bee products consumed by the population, the first place belongs to honey. Currently, Vinnytsia, Dnipro, Zhytomyr, Mykolaiv, Zaporizhzhia, Poltava, Donetsk, and Kirovohrad regions of Ukraine are the largest producers of natural honey. These regions produce about 70 percent of national natural honey exported to different countries (Vinichenko, 2021).

Natural honey is used in the food industry, pharmacological production, cosmetology, etc. The increase in honey consumption is influenced by the culture of health-improving nutrition, the demand for organic products, and the industry's focus on natural raw materials containing natural biologically active substances that positively affect the immune system (Samarghandian *et al.,* 2017), especially during a tough epidemic situation in the world. In the context of the pandemic, the honey market continues to develop actively, and exports to the European Union are increasing (Covaci *et al.,* 2023).

By origin, honey is divided into monofloral (obtained mainly from the nectar of one plant species) and polyfloral (obtained from different types of flower nectar). Monofloral honey is in better demand and more expensive than its polyfloral counterparts, which increases the need for an appropriate analysis method that could be used to determine its origin (Voyslavov *et al.,* 2021). A set of plant species identified by pollen analysis provides information about the local or regional origin of honey. A collection of pollen samples should be available to compare the morphology of pollen grains obtained directly from flowers with those obtained during honey sediment collection. The volume of this collection depends on the studied territory and the radius of action of the bees (Almeida-Muradian, 2020).

In Ukraine, the most extensive honey collection is provided by regions with large acreage

for sunflowers; sunflower honey accounts for the largest share of domestic production (Vinichenko, 2021). Sunflower honey is a valuable therapeutic and preventive product. This honey is characterised by a high content of glucose and fructose and a low content of sucrose. Among light varieties of honey, it is distinguished by a high enzymatic activity. In the European Union, sunflower honey is one of the most expensive varieties. In addition to sunflower honey, the most common monofloral varieties in Ukraine are rapeseed, buckwheat, linden, and acacia. Honey is used for various medical purposes, and recent studies confirmed its effectiveness in the treatment of various diseases due to its components and antibacterial, anti-inflammatory, antioxidant, and antiviral properties (Fakhlaei *et al.,* 2020). Doctors recommend including honey in the diet of schoolchildren and people living in ecologically unfavourable regions and in areas of high radioactive background. The therapeutic effect of honey consumption in diseases of the gastrointestinal tract, cardiovascular system, various metabolic disorders, etc. has been identified (Fakhlaei *et al.,* 2020). Notably, the physical and chemical properties of honey are considerably influenced by natural and climatic factors, industrial characteristics of a particular region, the state of agriculture, the content of radionuclides, and so on. Therefore, it is important to examine honey samples from different areas.

One of the main problems for consumers is food fraud, since not only does the quality of food decrease, but also negative consequences for human health are possible. Food testing and counterfeit toxicology are essential to ensure that consumers are protected from fraud (Jaafar *et al.,* 2020). According to Codex Alimentarius, consumers have the right to receive truthful information about the food they consume (Codex Alimentarius…, 2019). It also notes that honey should not contain any artificially added ingredients, foreign tastes or aromas that may appear during processing and storage. It is also forbidden to heat or process honey to such an extent that its composition changes and its quality worsens.

The purpose of this study is to check the quality and safety indicators of honey varieties from different regions of Ukraine and establish their compliance with the main criteria of European and national food legislation.

Literature Review

The quality and safety of honey can be influenced by various factors, such as the period of honey collection, the terrain, the landscape of the territory, technological methods of keeping bees, feeding bees with sucrose, pumping unripe honey, unsanitary storage conditions, adding inverted sugar, etc.

To determine the safety and quality of the product, organoleptic and physico-chemical studies are conducted. In addition, an important criterion is the presence of genetically modified components in honey. Scientific data indicate that three genetically modified crops – soy, corn, and rapeseed – are most common in Ukraine (Kushnir, 2021).

It should be noted that currently, the legislation of Ukraine regulates the use of genetically modified organisms (Law of Ukraine…, 2012). A prerequisite for using such organisms in an open system is their identification. It is forbidden to release genetically modified organisms into the environment without their state registration, with the exception of testing. The legislation prohibits industrial production and introduction into circulation of genetically modified organisms, including products manufactured with their use, before official registration. Currently, not a single variety of agricultural plants is included in the state register of genetically modified organisms. Research on the impact of biotechnology products on human health and the environment still

continues. In the European Union countries, great attention is paid to food quality and biological safety of food products and a very cautious attitude towards genetically modified organisms (Regulation (EU)..., 2003; Bruetschy, 2019). Therefore, it is forbidden to sell honey without conducting an analysis of the content of genetically modified pollen. Among genetically modified plants, only rapeseed belongs to honey crops. The development of rapeseed crops is of great economic importance for Ukraine. The presence of a genetically modified rapeseed crop was recorded in the country (Oblap *et al.,* 2018). However, for the successful cultivation of this crop, various pesticides are used, which can affect bee products.

Measures are being actively implemented at the local and international levels to detect fraud (Fakhlaei *et al.,* 2020). High-quality and safe food products are the key to a healthy nation and an important link in the social and ecological well-being of the country.

Considering the economic and social conditions and the future development of beekeeping in Ukraine, it is necessary to analyse the world and European requirements for different varieties of honey. The issue of biological safety is important; therefore, in addition to the established organoleptic and physico-chemical studies of honey, including the determination of the characteristics of the pollen composition, it is planned to conduct studies on the content of genetically modified organisms and identify the competitiveness of the domestic product on the world market. The scientific originality of this study lies in the fact that for the first time, approaches to the method of pollen analysis are scientifically justified, which allows confirming the naturalness of national honey and its variety. In addition, the presented technique can be used to create an atlas of pollen of Ukraine to establish not only the variety of honey but also its geographical origin.

Materials and Methods

To conduct the study, samples of rapeseed and sunflower honey were selected, which were obtained from apiaries of different regions of Ukraine: Vinnytsia, Odesa, and Kyiv. The study was conducted during 2019-2021. A total of 60 honey samples were received for the study, indicating the date and place of selection. Organoleptic and physico-chemical parameters were determined according to the methods of the current national standard of Ukraine (National standard of Ukraine..., 2005).

The organoleptic parameters of honey and its pollen analysis were conducted based on the Department of veterinary hygiene after A. K. Skorokhodko of Life and Environmental Sciences of Ukraine 3 research groups were formed to examine sunflower honey. Experimental samples of honey were characterised by the presence of the main nectar source, which was a sunflower. Accordingly, they had similar organoleptic characteristics. Experimental samples of honey differed by the region of their collection. Considering the natural and climatic factors that affect the quality of honey, samples were taken from three regions of Ukraine for the study. The first experimental group contained 10 prototypes of honey from the Kyiv region, the second experimental group – 10 prototypes of sunflower honey from the Vinnytsia region, and the third experimental group – 10 prototypes from the Odesa region. Accordingly, three research groups were established to examine the sensory and physical-chemical parameters of rapeseed honey, with 10 samples in each group. The first experimental group included samples of rapeseed honey from the Kyiv region, the second experimental group included samples from the Vinnytsia region, and the third experimental group included samples of rapeseed honey from the Odesa region.

Determination of organoleptic parameters, namely: colour, crystallisation, and the presence

of signs of fermentation was performed visually in daylight in a laboratory transparent glass; taste – by tasting several grams of honey; two honey tastings were conducted sequentially. To determine the aroma, a sample of 30 g was taken, added to a weighing bottle, tightly closed with a lid, and heated in a water bath for 10 min, to a temperature of 45°C. After that, the lid of the weighing bottle was opened, and the aroma was examined by slowly inhaling the air. The consistency was determined at 20°C, the spatula was dipped in honey, raised and observed for the nature of its flow. The presence of mechanical impurities was examined by completely dissolving 50 g of honey in 50 mL of warm distilled water. The resulting solution was carefully poured into a laboratory glass cylinder and the degree of contamination of the product was identified. In the presence of mechanical impurities, they are found at the bottom of the cylinder or on the surface of the solution, depending on the relative density. To determine the quality, safety of the product, and the botanical composition of honey, laboratory tests are mandatory.

The following materials and reagents were used to conduct pollen analysis of honey: laboratory microscope "Granum" (China), Goryaev chamber "Sklopryad" (Ukraine); an electric centrifuge of the company "Dastan" (Kyrgyzstan) with a rotation speed of up to 5000 rpm, laboratory scales of the II accuracy class of the company "Radwag" (Poland); centrifuge tubes; water bath; laboratory mercury thermometer up to 100°C; a set of chemical glasses with a volume of 100 mL from the companies "Olis" and "Sklopryad" (Ukraine); rectified ethyl alcohol with a mass fraction of 96%; basic crystalline fuchsin, alcohol solution with a mass fraction of 10%; distilled water; acetic anhydride; glacial acetic acid; concentrated sulfuric acid, density 1.84 g/cm^3 (Ukraine, China).

To initiate the preparation for pollen analysis, a laboratory glass was utilized to weigh 20 g of honey with an accuracy of 0.01 g. Subsequently, 40 mL of distilled water was added to the measured honey. The resulting mixture was then placed in a water bath set at a temperature of 45°C. The solution was carefully heated in the water bath until the honey was completely dissolved. The resulting solution was poured into test tubes and centrifuged for 15 min at a speed of 3,000 rpm. After that, the upper layer was drained from each test tube, and 2 mL of distilled water was added to the sediment, mixed, and all solutions were poured into one test tube and centrifuged. Then the solution was drained, and 3 ml of a mixture of sulfuric acid and acetic anhydride in a ratio of 1:9 was added to the precipitate, carefully drop by drop to avoid splashing. Further, the contents of the test tube were mixed and placed in a water bath at a temperature of 80°C for two minutes. The test tube was again centrifuged for 15 min at a speed of 3,000 rpm. The sediment was washed with acetic acid (glacial), and then three times with distilled water until the vinegar smell disappeared. The resulting liquid was drained from the sediment after each washing and centrifugation for 15 min.

The sediment from the test tube was carefully poured onto filter paper to remove water. 0.1 mL of distilled water was added to the sediment, mixed, and a drop of the solution was taken and placed in a Goryaev chamber for further counting of pollen grains and determination of species composition. A suspension of pollen grains was applied to both grids of Goryaev's chamber, and a cover glass was applied so that excess liquid was removed into the grooves. At least 200 pollen grains were counted under a microscope at high magnification (X900) and their species composition was determined. The number of pollen grains of each species was calculated by the formula:

$$X = 100\ x\ a/b,$$

where *X* is the number of pollen grains of each species, %; *a* – the calculated number of pollen grains of each species, pcs; *b* – total number of counted pollen grains, pcs.

5 prototypes of sunflower and rapeseed honey were selected from each experimental group. During microscopy, the size of the pollen grain, surface features, shape, colouration, and the presence of apertures were determined. These apertures are the sites through which the pollen grains, from which the microsporangia later emerge, are released. They are characterised by the thinning of the enzyme and the formation of an intine composed of pectin cork. The latter were not clearly visualised during light microscopy. During the examination of the pollen surface, attention was paid to the presence of spikes, wrinkles, grooves, hooks, humps, etc. For better visualisation of pollen grains, the depth of field of the microscope was periodically changed. The shape of pollen grains depends on the ratio of the length of the polar axis to the equatorial diameter. Spherical, elliptical, oval, oval-oblong, triangular, hexagonal shapes of pollen grains are distinguished. During the research, pollen was distinguished by its colour as golden, yellow, dark yellow, brown, light yellow, yellow-green, grey-green. Using the gradation specified in the order on approval of requirements for honey, the botanical origin of honey was established, namely, for monofloral sunflower and rapeseed honey, the content of pollen grains of one plant species must be at least 30% (Order of the Ministry..., 2019).

An important indicator for determining the maturity and ability to store honey for a long time is the mass fraction of water. All samples with signs of crystallisation were heated in a water bath until the crystals were completely dissolved, after which a drop of honey was applied to the prism of the RL 2 refractometer (Poland). The mass fraction of the water content was found by the refractive index.

Examination of honey to establish the diastase number, hydroxymethylfurfural content, mass fraction of reducible sugars and sucrose, acidity was conducted in the Ukrainian Laboratory of Quality and Safety of Agricultural Products, Chabany, Kyiv region The sucrose content characterises the maturity of honey and is an indicator of botanical origin. An increased content may indicate fraud or insufficient maturity of honey. Determination of enzymatic activity serves as a reliable method for assessing the quality and naturalness of honey. In the countries of the European Union and according to national requirements, it is customary to characterise the enzymatic activity of honey by determining the diastase number in Goethe units. The acidity of honey was determined by the titrimetric method, and milliequivalents of sodium hydroxide solution with a concentration of 0.1 mol/dm^3 per 1 kg of honey were calculated. The content of hydroxymethylfurfural, expressed in mg per 1 kg of honey, is an indicator of the quality and safety of the product during heating.

Considering the content of pollen grains in natural honey, research was conducted on the range of genetically modified organisms in experimental samples of rapeseed honey by the polymerase chain reaction method in real-time (PRC Real-Time) on the ICycler IQ5 "Bio-Rad" amplifier (France). The research was conducted in the laboratory of molecular genetic research of the research centre for product testing of the state enterprise "Ukrmetrteststandart", which is accredited by the National Accreditation Agency of Ukraine in accordance with the requirements DSTU ISO/IEC 17025 (DSTU EN ISO..., 2019).

The grade of honey was determined in accordance with the requirements of the current national standard DSTU 4497:2005 (2005). "Natural honey. Technical specifications", the Council Directive on Honey (Council Directive 2001/110 / EC..., 2001), the criteria of the European Union (Codex Alimentarius..., 2019).

The research results were processed statistically using Microsoft Excel. Tukey test was used, which is a modified student criterion. Indices a, b indicate values that significantly differed in the same row of the table ($P < 0.05$), P – critical significance level, which is 0.05 (or 5%) and is acceptable for most biological studies.

Results and Discussion

It was established that the colour of all experimental samples of sunflower honey was amber. Bee honey shows a significant variety of aromatic shades, depending on the type of honey plants from which the nectar was collected, the conditions and shelf life, the degree of subsequent temperature treatment, and so on. The aroma of honey was pleasant and pronounced. The taste of sunflower honey is characteristic, pleasant, and has a somewhat irritating effect on the oral mucosa, there are no foreign tastes. The sweet-tart taste of honey is due to the concentration and type of sugar. The consistency of the samples in the first, second, and third groups is dense, the crystallisation is fine-grained, and the presence of crystallisation does not worsen the nutritional qualities and medicinal properties of honey. Careful studies of honey samples and their aqueous solutions did not reveal any signs of fermentation or mechanical impurities. Thus, according to organoleptic parameters, all samples of sunflower honey meet the requirements of the national standard.

The colour of rapeseed honey in the experimental samples of the first, second, and third groups was milky with a yellowish tinge. The aroma of experimental honey samples is pleasant, weak, and without foreign odours, the taste is characteristic, pleasant, somewhat irritates the oral mucosa, and no foreign flavours were detected. The consistency of samples of the first, second, and third groups of honey is viscous, crystallisation is fine-grained. During the examination of samples of rapeseed honey and their aqueous solutions, no signs of fermentation and mechanical impurities were found. Thus, according to organoleptic parameters, all prototypes of rapeseed honey meet the current requirements.

It should be noted that laboratory studies are characterised by greater objectivity in determining the honey variety compared to organoleptic parameters. The method of assessing the botanical origin of honey based on the analysis of pollen grains is essential and helps to increase the economic efficiency of the production of this product (Muhati & Warui, 2022) since monofloral honey is more valued in the world market due to its good quality and original sensory characteristics (Manolova *et al.* , 2021). In addition, the content of pollen grains serves as one of the additional methods for establishing food fraud (Pospiech *et al.,* 2021).

Natural honey must contain flower pollen, the species and quantitative composition of which depends on the species ratio of honey plants. Bee pollen contains more than 250 biologically active substances, such as amino acids, carbohydrates, lipids, enzymes and coenzymes, vitamins, and also has antioxidant activity, which is explained by the presence of phenolic acids and flavonoids (Tutun *et al.,* 2021). The largest proportion of pollen found in honey comes from the main nectar sources. Among agricultural crops, sunflower, rapeseed, buckwheat, melons, etc. are distinguished. Honey is considered monofloral in terms of a certain pollen content from one plant species, but up to 10-20 types of pollen from other plants are found in natural honey during research. The absence of pollen clearly indicates the presence of fraud. The presence of characteristic structural elements of the aperture – furrows, pores, various shapes, colour, and size, allow determining the botanical affiliation of pollen grains (Pospiech *et al.,* 2021).

According to the appearance of pollen grains contained in experimental samples of

honey, its botanical origin was established. Specially developed bases of pollen grains of plants are used to identify bee products in international practice. The territory of Ukraine is characterized by the presence of a variety of vegetation of the Carpathians, steppes, forest-steppe and Polissia, which complicates the evaluation of honey, especially in the absence of a Ukrainian pollen grain base. Moreover, products obtained from different natural and climatic zones are unique in terms of the content of minerals, vitamins, amino acids, and other useful components. The examination of these properties of honey, bee bread, and bee pollen, including their botanical origin, will create a domestic informative base.

A microscopic view of the pollen grains contained in the honey samples is shown in Fig. 1.

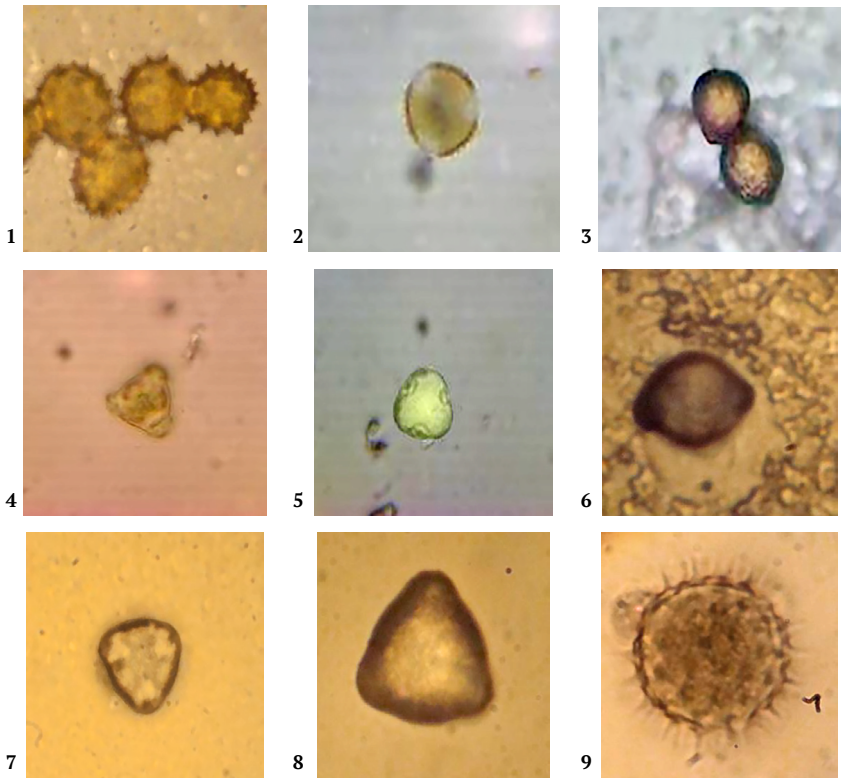

Figure 1. Morphology of pollen grains of various plant species in honey samples

Note: *1 – Common sunflower (Helianthu sannuus); 2 – Rapeseed (Brassica napus); 3 – Buckwheat (Fagopyrum esculentum); 4 – Black locust (Robinia pseudoacacia); 5 – Little-leaf linden (Tilia cordata); 6 – Red clover (Trifolium pretense); 7 – Sour cherry (Cerasus vulgaris); 8 – Apple (Malus domestica); 9 – Field sow thistle (Sonchus arvensis)*

Sunflower pollen was found in common sunflower honey (Fig. 1.1). The pollen grain is medium-sized, spherical in shape, has a golden colour and a spiky surface. The percentage of sunflower pollen grains in it averaged 52% (Table 1).

Rapeseed honey was found to contain mainly rapeseed pollen (Fig. 1.2) – pollen grain of medium size, yellow colour, rounded shape, with a reticulated surface. The percentage of rapeseed pollen grains in the product averaged 68% (Table 2).

Table 1. Pollen grain content in sunflower honey (%), x ± SD, n = 5

Plant type	Experimental group 1, Kyiv region, Ukraine	Experimental group 2, Vinnytsya region	Experimental group 3, Odesa region
Common sunflower	51.6 ± 4.9	49.2 ± 6.5	55.6 ± 42
Buckwheat	17.0 ± 3.0	19.4 ± 2.3	17.6 ± 26
Field sow thistle	11.0 ± 4.1	9.4 ± 5.5	10.4 ± 4.0
Red clover	11.2 ± 3.1	9.2 ± 3.9	8.8 ± 4.3
Cornflower	3.6 ± 1.8	4.6 ± 2.1	3.4 ± 1.5
Little-leaf linden	2.4 ± 1.5	4.8 ± 1.6	2.4 ± 1.7
Other types of plants	3.2 ± 1.8	3.4 ± 1.7	1.8 ± 0.9

Table 2. Pollen grain content in rapeseed honey (%), x ± SD, n = 5

Plant type	Experimental group 1, Kyiv region, Ukraine	Experimental group 2, Vinnytsya region	Experimental group 3, Odesa region
Rapeseed	66.4 ± 4.8	68.2 ± 3.6	68.8 ± 5.4
Black locust	12.4 ± 3.7	11.2 ± 2.5	11.8 ± 3.4
Sour cherry	7.6 ± 3.8	9.8 ± 2.8	7.4 ± 4.1
Apple	10.8 ± 4.3	8.2 ± 3.0	9.8 ± 3.8
Other types of plants	2.8 ± 1.5	2.6 ± 1.8	2.2 ± 1.3

During microscopy of experimental samples of honey, pollen grains of other plant species were found that differed in morphological characteristics, namely: buckwheat, black locust (Robinia vulgaris), field sow thistle, red clover, sour cherry, apple, blue cornflower, linden, etc. Pollen grains of buckwheat were found (Fig. 1.3), which are characterised by an average size, rounded shape, dark yellow colour, and a reticulated surface. Black locust (Fig. 1.4) is characterised by a triangular pollen grain shape, yellow colour, medium size, and fine-spotted surface. Little-leaf linden pollen (Fig. 1.5) was identified by the triangular-flattened shape of the pollen grain, which has a yellow-green colour, medium size, and fine-speckled surface. The pollen grain of red clover (Fig. 1.6) is ellipsoid, brown in colour, medium-sized, with a fine-speckled surface. In sour cherries (Fig. 1.7), the pollen grain is triangular in shape, medium in size, light yellow in colour, and has a wavy ornament on the surface. For the apple (Fig. 1.8), the triangular shape of a pollen grain, yellow colour, small-speckled surface, and medium size are characteristic. The pollen grain of field sow thistle (Fig. 1.9) is characterised by a porous surface with long spikes, rounded shape, dark yellow colour, and medium size. Pollen analysis of honey gave a positive result: pollen grains were found in all experimental samples, which is a sign of its naturalness.

An important indicator of the quality of natural honey is its water content. According to the Council Directive on honey (Council Directive 2001/110/EC..., 2001), the water content of honey should be no more than 20% (the exception is heather honey). This is an important physical and chemical parameter for the shelf life of honey. Usually, the water content in honey ranges from 13 to 25%, while the average value is about 17%. According to scientific data, the water content in mature honey ranges from 14 to 21% and is one of the essential quality indicators that affects not only product storage, but also changes in its constituent components (Almeida-Muradian *et al.,* 2020). An increase

in humidity can be observed in unripe honey, when adulterated with water or when liquid sugar syrup is added. In addition, such honey has a higher probability of fermentation during storage (Almeida-Muradian *et al.,* 2020). It should be noted that the water content may change during long-term storage, which is associated with chemical and enzymatic processes that occur immediately after pumping honey from the honeycomb. The results of studies of the physical and chemical parameters of sunflower honey are presented in Table 3.

Table 3. Physical and chemical parameters of sunflower honey, x ± SD, n = 10

Plant type	Experimental group 1, Kyiv region, Ukraine	Experimental group 2, Vinnytsya region	Experimental group 3, Odesa region
Mass fraction of water, %	18.08 ± 0.49	18.32 ± 0.46	17.91 ± 0.38
Mass fraction of reducing sugars, %	86.95 ± 1.89	85.92 ± 2.03	84.19 ± 2.48
Mass fraction ofsucrose, %	1.55 ± 0.82	1.32 ± 0.64	1.86 ± 0.95
Diastase number, Goethe units	18.90 ± 3.03	21.54 ± 3.80	20.10 ± 3.65
Content of hydroxymethylfurfural (HMF), mg per 1 kg	6.29 ± 1.72	7.04 ± 1.00	5.59 ± 1.09
Acidity, milliequivalents of sodium hydroxide per 1 kg	15.40 ± 2.29	14.41 ± 2.54	13.59 ± 1.77

The composition of the dry residue of honey includes about 300 substances and ash elements. The main ones are carbohydrates, which make up 95-99% of dry matter, among which monosaccharides – glucose and fructose-predominate. Honey also contains 11 disaccharides and 12 polysaccharides. The content of carbohydrates varies widely, depending on the botanical origin of honey, the conditions of storage and processing of nectar by bees. Despite the variety of carbohydrates contained in honey, the content of glucose, fructose, and sucrose in the product is now used for its examination. A mixture of grape and fruit sugars, namely glucose and fructose, is commonly called inverted sugar since it is obtained from nectar as a result of the breakdown of sucrose in the bee's honey chamber and in the honeycombs under the action of the invertase enzyme. Accordingly, the higher the content of inverted sugars in honey, the better its quality.

The results of the research show that the average mass fraction of water in honey collected in the Kyiv, Vinnytsia, and Odesa regions was less than 18.5%, which meets the requirements for top-grade honey.

The percentage content of reducing sugars in the honey of all experimental groups was more than 80%, which also meets the requirements for a higher grade. Honey adulterated with saccharin, glycerin, starch, gelatin or sucrose, just like a product obtained from bees that were fed sugar syrup, contains less inverted sugars. According to the national standard, the mass fraction of sucrose in flower honey normally ranges from 1-6%. The specified value is obtained during the ripening of honey, due to the work of the enzyme invertase, which breaks down sucrose to simple sugars. After pumping honey for several months at room temperature, this process continues. According to Codex Alimentarius and the Council of the European

Union, sucrose concentrations should not exceed 5 g per 100 g of honey. If the sucrose content in honey is more than 5%, this indicates its immaturity or fraud (Aykas, 2023). According to the laboratory analysis of sunflower honey samples, the mass fraction of sucrose was not more than 3.5%, which meets the requirements of the national standard for a top-grade product.

It should be noted that enzymes are biological catalysts that accelerate decomposition and synthesis reactions. Natural honey includes the following active enzymes: alpha- and beta-amylase, glucose oxidase, diastase, catalase, invertase, phosphatase, peroxidase, etc. (Puscion-Jakubik *et al.,* 2020). The amount and composition of honey enzymes depend on its botanical and regional origin, the period of nectar collection, natural and climatic conditions, primary processing, and so on. One of the most valuable and studied is the enzyme diastase, which breaks down starch into amylodextrins (glucose and maltose). The activity of this enzyme is expressed in Goethe units. It was found that heating honey to a temperature above 40°C causes partial or complete inactivation of enzymes and heating to 60°C causes their destruction (Adamchuk *et al.,* 2019). Diastase activity is also an indicator of the naturalness of honey and, according to the requirements of the national standard, should be at least 10 Goethe units for first-grade honey and 15 units for premium honey.

According to the results of studies of the enzymatic activity of diastase, presented in Table 3, prototypes of sunflower honey meet the requirements of the top grade, which indicates the proper quality and naturalness of the product.

Current DSTU 4497: 2005 "Natural honey. Technical specifications" regulates the maximum hydroxymethylfurfural content for the top-grade honey – 10 mg/kg, and for the honey of the first grade – 25 mg/kg. Notably, hydroxymethylfurfural can be formed in honey in case of violation of storage conditions, when exposed to external high temperatures, during heating. The level of toxicity of hydroxymethylfurfural for humans has not been precisely determined, as it depends on the state of health. According to scientific data, an adult can take from 30 to 150 mg of hydroxymethylfurfural daily from various food sources and it is reported that the concentration of hydroxymethylfurfural in the human body decreases to zero within 24-48 hours. Even if one consumes up to 240 mg of hydroxymethylfurfural per day (Alaerjani *et al.,* 2022). Based on the results of the conducted studies, it was established that all samples of sunflower honey had a low content of hydroxymethylfurfural, which meets the requirements of the national standard, confirms the quality of this product, the absence of exposure to high temperatures on honey, and indicates the safety for human use.

The acidity of honey depends on the type of raw material, the season of its production, and the degree of its maturity. Honey contains organic (malic, lactic, citric, formic, tartaric, acetic, oxalic, etc.) acids that have an acidic reaction which prevents the development of microorganisms and extends the shelf life of the product (Dobrinas *et al.,*, 2022). According to the current requirements for the physical and chemical parameters of natural honey, the acidity of top-grade honey should not exceed 40 meq/kg, and for the honey of the first grade – no more than 50 meq/kg. It was established that all samples of sunflower honey selected from the Kyiv, Vinnytsia, and Odesa regions met the requirements of the current DSTU for a top-grade product. In addition, an assessment of the product's compliance with the criteria of the European Union (Codex Alimentarius..., 2019) was performed according to the main physico-chemical indicators, and it is necessary to note that all experimental samples of honey meet the requirements of

European food legislation, which indicates the competitiveness of Ukrainian sunflower honey.

The main physical and chemical indicators were determined when studying samples of rapeseed honey collected in the Kyiv, Vinnytsia, and Odesa regions. It was established that the average value of the mass fraction of water in honey collected in the Odesa region was 17.73%. This corresponds to the indicators of top-grade honey. The mass fraction of water in the experimental samples of rapeseed honey of the Odesa region substantially differed from the indicators of honey obtained from the Kyiv and Vinnytsia regions, which is probably due to the natural and climatic conditions of storage at a higher average daily temperature. Rapeseed honey collected in the Kyiv and Vinnytsia regions showed an average mass fraction of water of more than 19.0%, which meets the requirements for a first-grade product and is 1.3% higher compared to honey obtained from the Odesa region (Table 4).

Table 4. Physical and chemical parameters of rapeseed honey, x ± SD, n = 10

Plant type	Experimental group 1, Kyiv region, Ukraine	Experimental group 2, Vinnytsya region	Experimental group 3, Odesa region
Mass fraction of water, %	19.50 ± 1.22[a]	19.00 ± 0.41[a]	17.73 ± 0.21[b]
Mass fraction of reducing sugars, %	89.20 ± 1.31	89.18 ± 0.84	91.07 ± 2.57
Mass fraction of sucrose, %	3.21 ± 0.19[a]	1.50 ± 0.24[b]	1.66 ± 0.90[b]
Diastase number, Goethe units	11.65 ± 1.18	12.12 ± 1.57	11.10 ± 1.42
Content of hydroxymethylfurfural (HMF), mg per 1 kg	1.74 ± 1.26	2.61 ± 1.53	1.59 ± 0.67
Acidity, milliequivalents of sodium hydroxide per 1 kg	13.6 ± 1.14	12.69 ± 0.32	13.5 ± 0.41

Note: *different superscript letters [a, b] indicate the values that considerably differed in the same row of the table (P < 0.05) based on the results of comparison using the Tukey test*

Honey with a high mass fraction of water can quickly deteriorate, so it is not subject to long-term storage.

The percentage of reducing sugars in all honey samples was at a high level, which confirms the quality of the product and meets the requirements of the top grade. According to laboratory analysis, the mass fraction of sucrose in experimental samples of rapeseed honey was below 3.5%, which indicates the maturity of honey. In the first experimental group, rapeseed honey from the Kyiv region contained 3.21% sucrose, which is on average 2 times higher than its content in the product from the Vinnytsia and Odesa regions. The result obtained may be due to the fact that honey has not fully passed the fermentation process provided by invertase. However, this type of honey meets the requirements: the maximum permissible sucrose content, according to the national standard, is 3.5% – for top-grade honey, 6% – for honey of the first grade.

The enzymatic activity of honey, which was determined by diastase activity, ranged from 10 to 14 Goethe units. The amount of hydroxymethylfurfural per 1 kg of honey was less than 10 mg, which indicates compliance with the storage conditions of the product and the absence of exposure to external high temperatures or heating.

It was found that the acidity of all samples of natural rapeseed honey selected from the Vinnytsia, Odesa, and Kyiv regions met the requirements for top-grade honey according to the current national standard. According to other physical and chemical indicators, namely, the mass fraction of water in the product samples of the Kyiv and Vinnytsia regions, rapeseed honey met the requirements of the national standard for first-grade honey. Honey collected in the Odesa region met the requirements for a top-grade product. The mass fraction of water, respectively, was below 18.5%, which is probably due to the weather conditions of the area and the storage of the product. Considerable differences in physical and chemical indicators of honey quality in national and international requirements include the following values: the content of reducing sugars according to the national standard is 10-20% higher than required by international legislation; the content of sucrose according to the requirements of DSTU 4497:2005 is 1.5% lower compared to Codex Alimentarius and Honey Directive 2001/110/EC (Council Directive..., 2001). The permissible content of hydroxymethylfurfural according to the national standard is up to 25 mg/kg, and in the EU and WTO countries (World Trade Organisation) – should not exceed 15 mg/kg. The content of the mass fraction of water must not exceed 20% according to the Honey Directive 2001/110/EC. Thus, rapeseed honey meets the requirements of Ukrainian and European food legislation.

In accordance with the standards of the European Union, quality and safety control of honey includes the establishment of organoleptic and basic physical and chemical parameters. In addition, the maximum permissible levels of residues of antibiotics, sulfonamide drugs, pesticides, heavy metals, radionuclides, and the content of pollen of genetically modified organisms are set.

Many countries have introduced state regulations on the use of genetically modified organisms and appropriate labelling of products made from raw materials of biotechnological origin. The following countries have partially or completely abandoned the use of genetically modified products: Austria, Germany, France, Great Britain, Spain, and others (Seifert, 2021). Rapeseed is one of the most common biotechnological crops. Therefore, it is important to conduct research on the presence of genetically modified rapeseed DNA in honey. It should be noted that seeds of both domestic varieties and hybrid seeds of this crop imported from abroad are used for sowing in the fields of Ukraine. The state register of plant varieties suitable for distribution in Ukraine contains 466 registered rapeseed hybrids and parental forms. Regarding the use of genetically modified rapeseed, the information is not officially confirmed (Shebanin *et al.,* 2021).

After testing prototypes of rapeseed honey from the Vinnytsia, Odesa, and Kyiv regions, it was found that there were no target sequences of the 35s promoter of the cauliflower mosaic virus *(CaMV)* and *NOS* (nopaline synthase) from *Agrobacterium tumefacient*. Thus, no genetically modified organisms were found in the experimental samples of rapeseed honey.

During organoleptic and physico-chemical examinations of sunflower and rapeseed honey, their compliance with the current national regulations was established, and the data obtained were compared with the criteria of the European Union and the requirements of Codex Alimentarius. In particular, discrepancies were found regarding the content of reducing sugars and sucrose, the permissible level of hydroxymethylfurfural, water content and, considering the regulations in different countries regarding the use of raw materials of biotechnological origin, rapeseed honey was examined for the content of genetically modified pollen.

Conclusions

The organoleptic studies and the results of pollen analysis confirmed the botanical origin of natural sunflower and rapeseed honey obtained from different regions of Ukraine: Kyiv, Vinnytsia, and Odesa. According to pollen analysis, prototypes of the product belong to monofloral honey – the content of dominant pollen was 52% in sunflower honey, 68% – in rapeseed. Sunflower honey meets the main physical and chemical national requirements for top-grade honey. The water content of sunflower honey averaged 18.1%, which meets the requirements of both the national standard and the requirements of the EU Council Directive relating to honey and Codex Alimentarius. The mass fraction of sucrose was not more than 3.5%, which corresponds to the top grade and meets the requirements of the national standard, Codex Alimentarius, and EU Council Directives (no higher than 5%). The average diastase number was 20.2 Goethe units, which considerably exceeds the requirements of Codex Alimentarius and the EU Council Directive (no higher than 8 Goethe units). Regarding the content of hydroxymethylfurfural, Codex Alimentarius and the EU Council Directive regulate no more than 40 mg/kg, which is notably higher than the obtained indicators, averaging 6.3 mg/kg. Therefore, according to the main indicators, sunflower honey meets not only national, but also international and European requirements for the quality and safety of the product, so it is in demand in many foreign countries. Rapeseed honey collected in the Kyiv and Vinnytsia regions, according to the requirements of the national standard and considering the water content in it, meets the requirements for a first-grade product, and honey from the Odesa region – top grade. Rapeseed honey produced in Ukraine also meets the main European and Codex Alimentarius requirements both in terms of organoleptic parameters and basic physical and chemical characteristics. Considering some differences in the regulations of the European Union and Codex Alimentarius on the quality and safety of natural honey, in particular, the moisture content and free acidity, and in the national standard – the water content and active acidity, it is necessary to harmonise methods for these indicators. Additionally, an examination of rapeseed honey for the presence of biotechnological pollen was conducted and the following conclusion was obtained: experimental samples of rapeseed honey do not contain genetically modified DNA. Rapeseed honey collected in the Kyiv, Vinnytsia, and Odesa regions has sufficient export potential. Comprehensive studies of sunflower and rapeseed honey have confirmed its naturalness and the absence of fraud in the product. Further studies should focus on pollen analysis and physico-chemical examination of flower and linden honey.

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None.

Conflict of Interest

None.

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Показники безпечності та якості ріпакового і соняшникового меду з різних областей України

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Анотація. Актуальність дослідження зумовлена зростанням попиту на безпечні та якісні продукти бджільництва, насамперед меду, та недопущенням на вітчизняний та ринки Європейського Союзу неякісних продуктів, які можуть завдати шкоди здоров'ю споживачів. В Україні найбільшим попитом в експортному потенціалі користується соняшниковий та ріпаковий мед. Метою досліджень було визначення відповідності ріпакового та соняшникового меду, отриманого в Україні, вимогам національного та європейського харчового законодавства. Для вирішення поставленої мети були використані органолептичні методи дослідження меду натурального та проведено визначення діастазного числа, кислотності, вмісту пилкових зерен, масової частки води, масової частки відновлювальних цукрів і сахарози та гідроксиметилфурфуролу. Методом полімеразної ланцюгової реакції в реальному часі здійснено аналіз ріпакового меду на наявність генетично модифікованого пилку. Встановлено, що дослідні зразки соняшникового меду з Вінницької, Одеської та Київської областей відповідали вимогам національного стандарту та європейського харчового законодавства, без ознак фальсифікації. Нині підлягає регулюванню використання генетично модифікованих організмів. Зразки меду ріпакового, відібрані з Вінницької, Одеської та Київської областей, не містили генетично модифікованої дезоксирибонуклеїнової кислоти. За фізико-хімічними показниками

ріпаковий мед відповідав вимогам національного стандарту. Встановлено, що мед, зібраний в Київській та Вінницькій областях мав середній показник вмісту води – вище 18,5%, що відповідає вимогам до меду першого ґатунку згідно з національним стандартом. Мед, зібраний в Одеській області, відповідав показникам меду вищого ґатунку, вміст води в ньому в середньому становив 17,7%. За основними фізико-хімічними показниками ріпаковий мед, зібраний в Україні, також відповідає вимогам європейського харчового законодавства. Також ріпаковий і соняшниковий мед із Вінницької, Одеської та Київської областей є натуральними і можуть реалізовуватися не лише в Україні, а й на території Європейського Союзу та Світової Організації Торгівлі

Ключові слова: квітковий пилок; контроль; нормативно-правові акти; фальсифікація; генетично модифіковані організми

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Clinical and morphological features of mastocyte diagnosis in dogs

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Abstract. Mastocytoma is one of the most common skin tumours in dogs. It is characterised by a specific course, unpredictable behaviour, and prognosis. This tumour is dangerous because it mimics the external signs of other neoplasms, from less-threatening lipoma to skin cancer. The purpose of the study is to conduct pathomorphological verification and classification of neoplasia in dogs diagnosed during a clinical examination and to establish the features of the morphological manifestation of the skin form of mastocytoma in them. The material for the studies was obtained by excisional biopsy during surgical interventions for skin neoplasms. The diagnosis of mastocytoma was established based on the results of a clinical examination of animals and the examination of biopsy material of neoplasms using cytological and histological research methods. It was established that the share of skin neoplasia in the structure of oncological diseases of dogs was 30.12%. Among the animals with skin neoplasia that were examined, mastocytoma was diagnosed in 17.55% of dogs aged 3 to 14 years. The highest incidence rates were established in dogs aged 7 to 12 years.

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Most often, skin mastocytoma was diagnosed in dogs of the following breeds: Shar Pei – 13.5%; Labrador – 9.5%; Boxer – 8.2%. Isolated cases of tumour formations were observed in animals of such breeds as: German shepherd, Spaniel, Central Asian Shepherd, and Pug. Depending on gender: males accounted for 52.2%, females – 47.8%. In 50% of the examined animals, mastocytomas developed with rapid dynamics, which indicates a substantial aggressiveness of tumour growth. Clinically, signs of dehydration and gastrointestinal disorders were detected in dogs, which indicates the development of paraneoplastic syndrome. According to the results of sonography, it was established that skin mastocytomas are visualised as heterogeneous hypoechoic structures with indistinct contours and uneven edges. Based on the results of the study, new information was obtained regarding the morphological features of the manifestation and development of mastocytes in dogs. Thus, the obtained results complement and expand knowledge about the pathogenesis of mastocytoma in dogs, the frequency of spread and features of the course of this oncological pathology in a separate geographical population of Ukraine

Keywords: cytological examination; histological examination; tumour; skin; metastasis

Introduction

In recent years, animal tumours have been used to test genetic engineering technologies as generally accepted models, which is important in the treatment of people with cancer (Akin, 2020). Over the past decade, there has been a growing interest in investigating neoplasia in dogs. This is due to large economic losses, which are caused by the need for examinations, diagnostics, and treatment of animals with neoplasms. Measures to prevent tumour growth also require scientific justification. The investigation of "natural models" of neoplasia in animals plays a leading role in expanding the understanding of the essence of tumour growth (Leguit *et al*., 2020).

In animals, over the past 30 years, the rate of growth of diseases for malignant tumours and mortality from them has increased (Leguit *et al*., 2020; Tamlin *et al*., 2020).

Dogs are located in the closest climatic and living conditions to humans and are also exposed to the same negative environmental factors in large and technogenically saturated cities. According to the World Health Organisation, animal tumours meet many criteria for "natural model candidates" for human tumours (Tamlin *et al*., 2020). Animal tumours have many features of the clinical course, risk factors, biochemical characteristics, and some pathogenetic and prognostic factors in common with human tumours (Willmann *et al*., 2019). Indications on the frequency of occurrence, morphology, pathogenesis, clinical course, diagnosis, and treatment of tumours in dogs are insufficient and sometimes scattered, which makes it difficult to solve a number of issues of comparative oncology (Akin, 2020; Balima & Vairamuthu, 2020).

Problems in solving most oncology issues are associated with a large histogenetic and histological specificity of malignant and benign neoplasms and are the result of versatile approaches to grouping tumours according to different classifications based on different evaluation criteria (Smiech *et al*., 2018). There are only a few banks in the world that store a substantial number of tumour samples from various tissues obtained from dogs that differed in breed, age, region, and habitat conditions. In Ukraine, almost no studies have been conducted on various

aspects of neoplasms in dogs. Therefore, there is a need to identify the regional features of the manifestation of neoplasia in dogs, their spread, and features of behaviour in the body, which is quite valuable clinical material. Conducting these studies will help to clarify some issues of carcinogenesis and contribute to obtaining new data on the role of individual environmental factors in these processes due to technogenic human activities. These data will also be of biological interest since the processes that determine the geographical accumulation of cases of neoplasms in humans and dogs are to some extent completely different and not always interdependent (Garden *et al*., 2018).

Ambiguous assessments and certain discrepancies in the diagnosis and prognosis of the biological behaviour of mastocytes indicate the relevance of conducting more in-depth and diverse studies of this neoplasia. Notably, this issue is quite important for veterinary science and practice. The study of pathomorphological and clinical-biological features of mastocytoma in different geographical populations of Ukraine is not only of scientific interest but also of great practical importance for veterinary medicine.

Considering the above, the purpose of the study was to examine the features of the clinical and morphological manifestation of the cutaneous form of mastocytoma, the frequency of spread, breed, and gender predisposition of dogs to the cutaneous form of mastocytoma in dogs under conditions of the city of Kyiv and the suburban area.

Literature review

Mastocytoma (mast cell tumour, histiocytic mastocytoma, mast cell sarcoma, *mast cell tumour*, MST, *Mastocytoma*) got its name from the type of cells from which it is formed – mastocytes (Macewen & Withrow, 2013). Mast cells are oval or round cells of mesenchymal nature, their diameter is 10-13 microns, with secretory granules that are located in the cytoplasm (Sledge *et al*., 2016; Balima & Vairamuthu, 2020). Mast cell precursors are formed in the bone marrow, then they migrate through the walls of blood vessels to the tissues, where they differentiate. Their final maturation occurs in the connective tissue. Mast cells are found in almost all body tissues, but their greatest concentration is around blood and lymphatic vessels in places of contact with the external environment (skin, respiratory tract, intestines). Mast cells are one of the main cells that perform a protective function in the animal body, and they also regulate the homeostasis of connective tissue. These cells produce biologically active substances and mediators: heparin, histamine, serotonin, tumour necrosis factor-α (TNF-α), proteases, interleukins, glycoproteins, chemotactic migration factor of eosinophils, etc. and are able to deposit and store them in cytoplasmic granules. The release of biologically active substances from mast cells occurs by exocytosis. Two types of mast cell degranulation are described: partial, which lasts the whole day, and rapid, which takes several minutes. During the activation and degranulation of mastocytes, these biologically active substances are released, which play a leading role in the course of physiological and pathological processes (Garden *et al.*, 2018).

Mast cell activation syndrome is a fairly common disorder in the pathogenesis of both rare and common diseases. Therewith, it is noted in the literature that the number of mast cells increases substantially with inflammation and immune responses (Valent *et al.*, 2012; Bautista-Quach *et al*., 2013; Akin, 2020).

An important function of these cells is their involvement in the manifestation of adaptive immunity. On their surface, mast cells carry antibodies (Class E immunoglobulins), which are released by plasma cells under the action of antigens, and in the process of cell life, they

actively bind to the surface of mast cells. There can be from 5,000 to 500,000 IgE molecules on the surface of mast cells simultaneously, which are directed against various antigens (Broudy, 1997).

The course of diseases involving activated mastocytes in animals and humans has certain differences (Willmann *et al.*, 2018; Tamlin *et al*., 2020).

Mastocytoma can also occur in other tissues of the reticuloendothelial system: in the bone marrow, kidneys, and liver, referred to by the term "systemic mastocytosis". The biological behaviour of systemic mastocytosis is very different from that of cutaneous mastocytes (Lisickaja & Sedov, 2011).

According to the medical literature (Pardanani, 2016; Frolova *et al*., 2018; Leguit *et al*., 2020), mastocytosis affects both adults and children. Adults experience systemic mastocytosis, which affects not only the skin, but also the spleen, liver, and bone marrow. A benign period is rare. The disease has an unfavourable prognosis and a very aggressive course. In children, the prognosis is more favourable and the disease occurs mainly in the skin form and in the form of pigmented urticaria.

Skin neoplasms in dogs occupy one of the first places in terms of frequency of occurrence and spread and account for 1/3 of all cancer cases (Sledge *et al.*, 2016). Most often, dogs are diagnosed with mastocytoma of the skin. This type of tumour is observed in 16-21% of cases of all skin neoplasms (Balima & Vairamuthu, 2020).

Factors that lead to the risk of skin mastocytoma in dogs remain unclear. Therewith, genetic factors play a substantial role, indicating a different frequency of neoplasia in different breeds of dogs. Another important endogenous risk factor for tumours is the age of animals. Tumours in dogs can be detected throughout their lives, but the largest proportion of animals with mastocytoma is diagnosed at the age of 7-12 years (Withrow & MacEwen's, 2013).

Other factors that may be important in the etiology of tumours in dogs are carcinogenic environmental hazards, parasitic and viral infection, and injuries (Leguit *et al.*, 2020). There is also an assumption about the viral aetiology of mastocytes (Lisickaja & Sedov, 2011). However, to date, there are no studies that would support this theory. Receptors for sex hormones (testosterone and estrogens) have been established in the cytoplasm of mast cells and, together with data on a more favourable prognosis of the disease in females, may indicate the role of hormones in tumour development (Balima & Vairamuthu, 2020).

Recently, considerable attention from researchers has been focused on finding genes that may be associated with the development of mastocytoma (Mohammad & Joanne, 2016). Mutations in the c-kit gene have recently been identified in some dog, human, rat, and mouse mastocytoma cell lines and tissue samples (Webster *et al.*, 2006). The gene encodes a receptor on the mast cell progenitor membrane in the bone marrow that binds the mast stem cell growth factor (SCF). This factor stimulates the proliferation and differentiation of mast cell progenitor cells into mature cells. Mutations in the gene cause changes in the structure of the receptors, which leads to the launch of a cascade of biochemical reactions without binding SCF itself (semke *et al.*, 2001; Jones *et al.*, 2004). Based on the results of a study in the field of humane genetics, it was established that point mutations of the gene in children are often associated with mastocytosis in adults (Jones *et al.*, 2004).

Currently, there are several types of classification of skin tumours, including cutaneous mastocytes. Classification by stages of the process is conducted according to the international clinical TNM classification (Owen, 1980), which is used to describe the anatomical spread of the tumour process and is based on three components: T – the size and spread of the primary

tumour; N – the absence or presence of metastases in regional lymph nodes; M – the absence or presence of metastases.

The figures added to these three main components indicate the spread of the process: T_0 (there is no tumour); T_1 (tumour up to 2 cm in diameter); T_2 (tumour with a diameter of 2-5 cm); T_3 (the diameter of the tumour is from 5 cm, they note germination into the subcutaneous tissue, regardless of the size); T_4 (the tumour spreads to muscles or other structures); N_0 (there are no metastases in regional lymph nodes); N_1 (there are small metastases in the nearest regional lymph nodes, but their mobility is preserved); N_2 (there are metastases in several regional lymph nodes); N_3 (damage to all regional lymph nodes, Immobile lymph nodes); M_0 (there are no metastases in other organs); M_1 (metastases were established in other organs, including the bone marrow).

In addition to the TNM classification, the WHO clinical classification is widely used, which provides for mastocyte division according to 5 stages (Table 1).

Table 1. WHO classification of skin mastocytes in dogs

0	The tumour is absent or completely removed, confirmed histologically
I	Well-limited single node without lymph node metastases
II	Well-limited single node, but with lymph node metastases
III	Single large node or multiple nodes with signs of infiltrative growth without / with lymph node metastases
IVa	A tumour with distant metastases, but no common clinical symptoms
IVb	A tumour with distant metastases with common clinical symptoms present

Source: *compiled by the author based on data from (Owen, 1980)*

According to the WHO International histological classification of canine skin tumours, mastocytomas belong to cutaneous lymphoproliferative tumours and are divided according to the degree of differentiation. According to the degree of differentiation, there is no single gradation of mastocytes. Today, three systems are used simultaneously: according to Patnaik (1984), according to Bostock (1986), and a more modern classification according to Kiupel (2011). They are all based on tumour size, cell morphology, and mitotic index, with the difference only in assigning a degree of gradation from 1 to 3 (Table 2).

Table 2. Histological classification of mastocytes according to Patnaik and Bostock

Mastocytoma	Gradation according to		Cell morphology
	Patnaik	Bostock	
Anaplastic / low-grade	3	1	A substantial number of mitotic figures; cytoplasm is poorly restricted; asymmetrical nuclei, few secretory granules.
Intermediate / medium-differentiated	2	2	A small number of mitotic figures; the ratio of the nucleus to the cytoplasm is less than the anaplastic type; the average number of secretory granules is noted
Highly differentiated	1	3	A small number of mitotic figures; large secretory granules are pronounced, the nuclei are rounded, the cytoplasm is limited

Source: *compiled by the author based on the studies by Patnaik (1984), Bostock (1986)*

According to the classification of Kiupel *et al*. (2011), mastocytomas are divided into two groups: high or low degree of differentiation and completely ignore moderately differentiated tumours. Notably, animal tumours meet similar neoplasms in humans by many criteria. The similarity in the aetiology, pathogenesis, symptoms, and morphology of tumours in dogs and humans gives researchers the opportunity to use animal models to examine the course of the tumour process and develop modern diagnostic and therapeutic measures for the treatment of humans (Garden *et al*., 2018; Gargiulo, 2018).

Materials and Methods

The study was conducted based on a veterinary clinic "Animal health" (Kyiv region, u.t.s. Hlevakha) and the laboratory of the Volodymyr Kasianenko Department of Animal Anatomy, Histology, and Pathomorphology throughout 2021-2022. During this study period, 1,205 dogs with surgical pathology were registered.

The study material included 98 dogs with skin tumours, including 9 animals with mastocytoma. The dogs were of different breeds and genders, ranging in age from 3 to 14 years. Cancer-stricken animals were examined in accordance with the criteria of clinical TNM classification of tumours using clinical and laboratory research methods (Owen, 1980).

Ultrasound examination was performed using the ESAOTE MyLab 40 device (Italy). Sonographic examination was performed on the tumour itself, surrounding tissues, and regional lymph nodes. The organs of the abdominal and pelvic cavities were examined to detect distant metastases. Sonographic images were used to analyse the echostructure of the examined objects.

Radiography of the tumour site, abdominal cavity, and chest was performed using a digital X-ray machine "ZooMax LC" (Hungary) in two standard (straight and lateral) perpendicular projections. Material for cytological examination was obtained by fine-needle puncture biopsy of neoplasia. This method is based on taking a cytological sample, by puncture, during which cells are sucked in from the neoplasm. A 10 mL plastic syringe and needle No. 22 were used to do this.

Before performing the puncture, the tumour was palpated, its interaction with surrounding tissues and blood vessels was determined, its mobility and convenient position for performing the puncture were examined. Next, the tumour was fixed with the fingers of the left hand. A needle with or without a syringe (the plunger is lowered) was passed perpendicularly through the skin directly into the tumour. Fingers checked the correctness of the puncture. If the syringe was not attached, it was attached with the plunger lowered. 2-3 sudden suction movements were performed, removing the syringe from the needle each time after lifting the plunger.

The syringe was removed along with the needle. Therewith, ensuring that the plunger of the syringe occupies a lowered position before removing the needle. After removing the needle, a syringe with a raised plunger was attached to it. The contents of the syringe were carefully squeezed out onto a slide. The distribution of cellular material on the glass surface was conducted in one direction using a needle.

The resulting smear should be oblong in shape and of medium thickness so that the cells are placed in a single layer and can be differentiated. The smear was air-dried and stained according to Poppenheim. The main criterion for mastocytoma verification is the detection of round tumour cells in cytological preparations that contain azurophilic granules in the cytoplasm.

Experiments using live animals were conducted in accordance with the recommendations of Arrive. For digital processing of the results, the statistical analysis method using Microsoft Excel 2019 and Statistica 6.0 computer programmes was used.

Results and Discussion

Among the examined animals, the average cancer rate was 12.5%. From the total number of sick animals with cancer problems (n = 295), 108 dogs were diagnosed with tumours and tumour-like skin lesions. The results of the study (Hosseini *et al.,* 2014) confirm that among animals with oncological pathologies, dogs are at risk of developing cutaneous mastocytoma, which accounts for up to 21% of all skin tumours. The aetiology of mastocytes in dogs is unknown, but assumably, is multifactorial.

According to the results of a clinical examination of animals, and based on cytological examination of biopsy punctates of neoplasia in 13 dogs, the diagnosis of mastocytoma of the skin was confirmed. In dogs, most of these tumours occur as primary skin tumours.

According to the registration data, the sick animals were of different breeds and genders, aged from 3 to 14 years. Most often, skin mastocytoma was recorded in dogs of the following breeds: Shar Pei – 3 cases, Staffordshire terrier – 2, Boxer – 2, Labrador – 3, Doberman – 1, and Rottweiler – 2 cases. German shepherd, Spaniel, Pug, Alabai, and French bulldog dogs had isolated cases (3.9%). Among animals with mastocytoma, males accounted for 52.2% and females – for 47.8%. The vast majority of animals admitted to the clinic underwent a full course of vaccinations, but the animals were also vaccinated only once in their lifetime. Not all animals were also regularly treated with antiparasitic agents. The sample includes animals of both residential and street maintenance, mainly with a mixed type of feeding.

In the age aspect, the number of cases of the disease was distributed as follows: animals aged 3 years – 1 case; 5-6 years – 2; 7-8 years – 2; 9-10 years – 4; 11-12 years – 3; 13-14 years – 1 case. The highest incidence rates were established in animals aged 7 to 13 years. It can be noted that the results of this study coincide with the data of foreign researchers (Sledge *et al.*, 2016; Smiech *et al.*, 2018). According to the literature data (Smiech *et al.*, 2018), dogs of such breeds as Boxer, American Staffordshire Terrier, Labrador Retriever, Beagle, French and English Bulldogs, Pug, and Shar Pei are susceptible to the development of the skin form of mastocytoma. It is also known that along with the above-mentioned dog breeds, high morbidity rates are noted among other breeds-these are Rottweilers, Dobermans, German Shepherds, and Cocker Spaniels (Shoop-Worrall *et al.*, 2015). Perhaps such discrepancies in the literature data can be explained by the distribution of certain breeds in different geographical populations.

In clinical practice, mastocytoma is considered a skin neoplasia with a specific clinical course, unpredictable biological behaviour and prognosis. The priority area of research in this study was to identify the prognostic factors of this disease.

According to the results of clinical examinations of dogs during the diagnostic and treatment period, a substantial polymorphism in the appearance and clinical manifestation of mastocytes was established.

The examined dogs showed neoplasia, which had the appearance of small dense nodules (Fig. 1), and nodes of 3-5 cm in size, usually similar to lipomas and giant tumours of 9-10 cm or more in size.

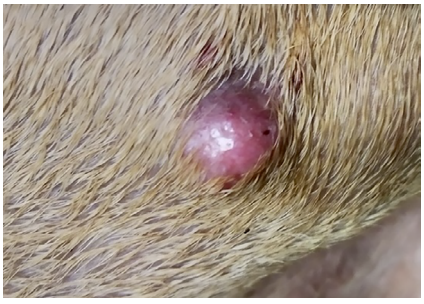

Figure 1. Mastocytoma of the body area in the form of a dense nodule

In some animals, eczema-like skin lesions were noted, which were very similar in appearance to nodular panniculitis (Fig. 2).

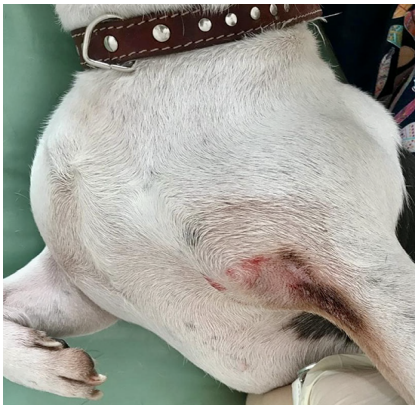

Figure 2. Eczema-like skin lesions. Boxer dog, age – 9 years

It was established that in 92.7% of dogs, mastocytomas had the appearance of single nodes and only in 7.3% – multiple (Fig. 3).

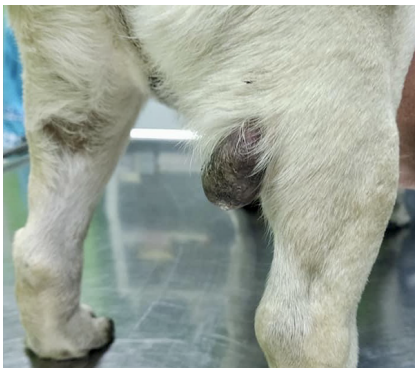

Figure 3. Mastocytoma on the pelvic limb. Pug dog, age – 7 years

Most often, mastocytomas were localised on the trunk – 44.7% and limbs – 38.5%, less often in the head and neck (Fig. 4), and inguinal and perineal-perianal areas (16.8%).

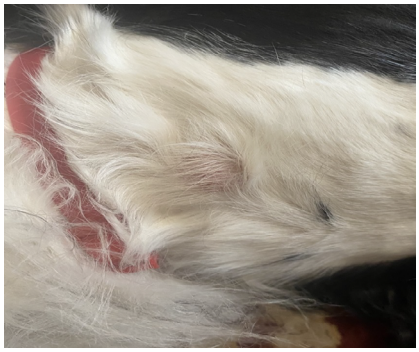

Figure 4. Mastocytoma of the neck area. Russian spaniel dog, age –10 years

Mastocytomas are located in the groin areas, especially those involving the foreskin or scrotum, and preputial mastocytomas can usually have more malignant biological behaviour.

During the studies, it was established that small neoplasms (up to 2.5-3.0 cm) were mainly located in the thickness of the skin. These neoplasms had the appearance of a node that was mobile, well delimited from the surrounding tissues, and mostly rounded or ellipsoid in shape. In some animals, at the initial stages of mastocytoma development, it manifested itself as a nodular seal in the thickness of the skin, the contours of which were almost not palpated and already based on this seal, the formation of a tumour node took place.

Based on anamnestic data and the results of the study, it was established that in seven examined animals, the rate of mastocytoma development was rapid – in two months, the tumours doubled their size. Within 8-9 weeks, small seals or nodules formed tumours with a diameter of 4-5 cm or more, indicating a substantial tumour growth aggressiveness. The growth of neoplasia was also rapid in four dogs. The time of doubling the size of the primary tumour reached an average of four months. Therewith, according to the medical history, it was established that in two dogs, the neoplasm development

period lasted about a year and a half. It is believed that one of the clinical prognostic factors in the assessment of mastocytoma is the rate of doubling of the tumour mass of neoplasia.

During the study of the local process and the results of monitoring the course of the disease, a variety in the clinical manifestation of mastocytes was noted. On palpation at the initial stage of development, the consistency of the tumours was soft, and over time they acquired a dense elastic consistency. The surface of the tumour went from smooth to bumpy. In 88% of cases, the characteristic signs of mastocytoma were swelling and inflammation. Inflammation was observed both in the tumour itself and in the surrounding tissues. Alopecia, erythema, the presence of skin ulcers, and pain on palpation, were visualised on the tumour. Notably, in addition to pain, mastocytes are characterised by itching of the skin in the tumour area, which is caused by the release of biologically active substances, in particular, histamine. Therefore, the animals constantly licked the tumour, tried to scratch and even gnaw, thereby provoking its growth. Constant irritation and trauma of the neoplasia led to the periodic exacerbation of the inflammatory process: increased oedema, ulceration, and, as a result, accelerated growth of tumours. It was established that the progressive course of the disease occurred due to the localisation of tumours in the groin, pelvic extremities, perineum, and neck.

The main indicators that indicate mastocyte malignancy are the size of neoplasms, their growth rate, the degree of germination in surrounding tissues, damage to regional lymph nodes and metastasis to internal organs (Beer *et al*., 2018). During the clinical examination of animals, the condition of the mandibular, axillary, superficial inguinal, popliteal, and other (regional) lymph nodes was considered.

According to the results of the initial examination of 13 dogs, six of them did not show any changes in the lymph nodes. They were almost not palpated, which indicates that there is no reaction to the pathological process. In five dogs, the regional lymph nodes were slightly enlarged, but did not ache, were mobile and not compacted. In two dogs with large tumours, the lymph nodes were enlarged, sedentary, and had dense consistency, which indicates the generalisation of the pathological process.

In accordance with the international clinical TNM classification of tumours, the scheme of examination of animals for oncological diseases provides for conducting a clinical study in combination with radiation diagnostics. In the available Ukrainian literature, it is not always possible to find information on the use of X-ray and ultrasonographic research methods, and their effectiveness in differential diagnosis of individual skin pathologies in dogs. Each test animal was subjected to X-ray and ultrasound examinations at the time of admission to the veterinary clinic and at certain intervals. This allowed tracing the dynamics of the pathological process and external changes in tumours. According to the results of X-ray imaging, three types of mastocyte growth were identified-limited nodular, infiltrative nodular, and diffuse infiltrative types.

Limited nodular growth of mastocytes was characterised by a homogeneous, heterogeneous radiological shadow. The tumour node was mostly oval, less often rounded in shape and was located in the thickness of the skin. Infiltrative nodular type of tumours was characterised by visualisation of the tumour shadow without clear contours, and structural changes were well expressed in the skin thickness and subcutaneous tissue. According to the diffuse infiltrative type of tumour growth, the X-ray shadow of neoplasms was compacted. Due to the swelling and thickening of the skin, it was almost impossible to establish clear boundaries of the neoplasm. In X-ray imaging, infiltrative nodular

and diffuse infiltrative types of neoplasia characterise the process of mastocyte malignancy.

During ultrasound examination, it was established that the sonographic structure of mastocytes depends on the location of the tumour, the degree of spread of the pathological process and the stage of the neoplastic process. Mostly mastocytomas localised in the thickness of the dermis were visualised as heterogeneous hypoechoic structures. These structures had uneven edges and indistinct contours. They showed signs of infiltration into the surrounding tissues, which may indicate a low ability of such tumours to form capsules. Increased peripheral vascularisation of mastocytoma and diffuse hypoechoic infiltration of surrounding tissues in such cases was a sign of inflammatory oedema. Due to sonographic studies of some mastocytes, which were characterised by prevailing inflammation, deposits of calcium salts in the thickness of the tumour were noted.

During the generalised process and the spread of mastocytoma to internal organs, the liver and spleen were primarily affected, which may indicate a preference for the hematogenic pathway of metastasis. Sonographically, such foci had similar structures and were visualised as heterogeneous dense formations with clear contours and uneven edges, with increased chaotic vascularisation.

The high variability of clinical symptoms in animals with the development of mastocytoma substantially complicates the determination of the diagnosis for a veterinarian. Therefore, determining the features of clinical changes and blood parameters in dogs with this pathology will contribute to the development of a complex of diagnostic markers.

One of the main steps in the diagnosis of mastocytes is cytological examination. Mastocytoma is characterised by heterogeneous behaviour. Highly differentiated tumours have a low metastasis potential, and the metastatic potential of low-differentiated tumours is high and amounts to up to 95% (Leguit *et al.,* 2020).

In most cases, diagnosing cutaneous mastocytoma by cytological or histological studies is not difficult, but forming an accurate prognosis is more complicated. Prognostic substantiality factors included classification (cytology and histopathology), stage (regional and distant metastases), breed, tumour location, and treatment (surgery, radiation, and chemotherapy). Cytological examination after fine needle aspiration is useful for making a diagnosis, but histopathology is necessary for evaluation. Cytological examination often helps in the diagnosis of mast cells due to the characteristic appearance of mast cells with regular staining. As with other species, Wright-Giemsa staining results in more intense granule staining in tumour mast cells. The mechanism of differences in staining is unclear. Several mast cell subtypes were identified in humans and dogs based on granule content and biological function. (Hosseini *et al.,* 2014).

In the case of the examined neoplasms, a typical cytological picture was observed. Round cells were noted against the background of a substantial number of red blood cells and eosinophils, which contained a large number of small metachromatic granules and a homogeneous nucleus in their cytoplasm, without pronounced atypia. In some cells, metachromatic granules completely covered the nucleus and made it invisible. Granules were established both in the cytoplasm and outside the cells (Fig. 5, 6).

The cells themselves were more often arranged in groups and had pronounced anisocariosis and anisocytosis. 2-3 figures of mitosis were identified on the glass. Basically, the degree of mastocytoma differentiation is determined during a histological examination, but given the individual mitosis patterns and a large number of granules, it can be assumed that all samples were highly differentiated.

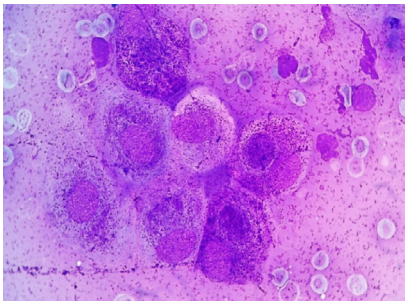

Figure 5. Highly differentiated mastocytoma. Pappenheim staining, ×100

Notes: *cells with small granules in the cytoplasm and a typical nucleus (indicated by arrows)*

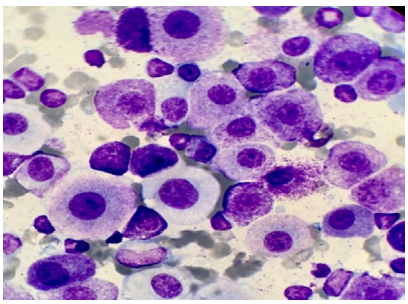

Figure 6. Highly differentiated mastocytoma. Pappenheim staining, ×100

Notes: *cells with small granules in the cytoplasm and a typical nucleus (indicated by arrows)*

According to the results of the cytological study, mastocytoma can be considered potentially aggressive, the cells of which contain few metachromatic granules and there are two or more of the following signs: the presence of multinucleated and mirror cells in the preparation; nuclear polymorphism in more than 50% of the population cells; a large number of mitoses.

Often, mastocytoma granules are not stained with Diff-Quik paint and require staining with Wright dye. During Diff-Quik cytology, if eosinophils are observed together with large round cells without granules, mastocytoma should be suspected and a different staining method should be used.

The main disadvantage of cytological examination is that it does not allow for determining the degree of cell differentiation, which is an important prognostic feature. Therefore, it is believed that in the diagnosis of mastocytes, the most accurate method is the use of histological examination.

A study (Hosseini *et al.*, 2014) clearly showed cellular infiltration of mast cells, mononuclears, and eosinophils in the thickened, hyperplastic, and hyperkeratinised epidermis. In contrast to delayed-hypersensitivity mononuclear cells, mast cells and eosinophils prevailed, indicating an immediate-type hypersensitivity reaction. Once activated, mast cells at the wound edge are known to release inflammatory mediators in the damaged tissue by degranulation. Thus, immediate hypersensitivity reactions may be responsible for the development of skin damage due to a tumour in dogs. However, the results show that intensive proliferation of mast cells after the tumour occurs mainly in the skin.

For single mastocytes, surgical excision of the tumour with a wide capture of healthy tissues is always recommended to radically remove it (Lisickaja & Sedov, 2011; Pardanani, 2016). Therewith, it is necessary to remove at least 3 cm of healthy tissue bordering the tumour. After its removal, a 0.5 × 1 cm piece of tissue is cut off from the edge of this place from 3-5 sides, placed in a 10% aqueous solution of formalin and sent for research. Histological examination of excisional mastocytoma should necessarily examine the edges of resection where it will be determined whether the tumour cells remain in the border tissue. If the result of histological examination is negative, then "clean edges" are indicated. During fine-needle aspiration of surgical excision and over-manipulation of the mastocytoma site, local bleeding often occurs, assumably due to coagulation defects caused by the release of heparin. Indeed,

there was an extended clotting time of blood taken from or around the tumour. Since bleeding can be a serious complication of surgery, doctors should anticipate local bleeding and try to avoid over-manipulating the tumour during a biopsy or during its removal.

Mastocytomas are very radiosensitive, and numerous studies show that fractionated radiation (daily or every other day for 15-18 procedures with a total dose of 46 to 54 Gray) is an effective adjunct therapy for incomplete edges after surgery) in almost 75-96% of dogs with topical treatment (no signs of re-growth 3-5 years after exposure) (Laura D Garrett, 2014). However, disagreements about what "incomplete" edges mean and how likely such dirty edges are to lead to tumour re-growth cast doubt on the need for radiation therapy in some cases. If the tumour is located in a place where only minimal resection can be obtained, then in practice the author suggests radiation therapy. Radiation therapy can also be very useful when used in weekly doses of large fractions for four to six procedures for treating bulky non-surgical mastocytes.

According to the conducted histological studies, a diverse picture was established in the samples. Cell morphology depends on the degree of differentiation, but most neoplastic cells have a clear cell membrane, a centrally located round nucleus, and a different number of cytoplasmic granules, which are coloured light grey when stained with hematoxylin and eosin, or purple with metachromatic spots when stained with toluidine blue. The connective tissue stroma varies from small to large and may look swollen or hyalinised.

However, most often indistinct boundaries of the neoplasm were determined. The surrounding area was diffusely infiltrated by tumour tissues under the skin, a small number of mitoses (no more than one in a large field of view) and massive destruction of the dermis. In some cases, the shape of the tumour cells was only round, while in others it was polygonal. The cytoplasm of cells is clearly defined and granular, in such cells, there is a separate nucleus located in the centre, with two nucleoli. Therewith, cells that had an inconspicuous cytoplasm with a dark small nucleus were observed. These cells were very similar to small lymphocytes. In the vast majority of cases, two cell types were established simultaneously in the same sample. The cells were arranged in groups, often forming nests, chains, and "paths", and were separated by bundles of collagen fibres.

On histological sections stained with hematoxylin and eosin, the nuclei of mast cells are very similar and resemble the nuclei of fibroblasts or pericytes, so special types of staining are required to establish the correct diagnosis.

The histological picture of all forms of mastocytosis is manifested by infiltrates consisting mainly of mast cells. A different number of eosinophils are either dispersed throughout the tumour mass or form aggregates. Metachromasia of mast cell granules was detected using toluidine, methylene blue, and naphthol-as-D-chloroacetate esterase, which turns the cytoplasmic granules of mast cells red.

According to the gradation of mastocytes according to Patnaik (1984), according to the degree of differentiation, all neoplasms can be attributed to the second degree. According to the Kiupel classification (2011), all neoplasms corresponded to mastocytomas with a high degree of differentiation.

A study (Hosseini *et al.,* 2014) showed cytohistopathological features of cutaneous mastocytes in dogs in the skin area. Histological features of these tumours indicate that most of them should be classified as mastocytomas with a high degree of differentiation. The use of a classification system by tumour type can be useful for differentiating this type of neoplasm from other malignancies with similar morphology. In addition, the study demonstrated that

skin mastocytomas in dogs can be used as a sensitive prognostic indicator of tumour behaviour after surgery, which is associated with other histopathological variables that are widely recognised as having prognostic value.

Conclusions

Among the dogs admitted to the veterinary clinic "Animal health" (Kyiv region, u.t.s. Hlevakha) with surgical diseases, the proportion of neoplasia was 12.5%. According to the structure of oncological diseases, skin tumour lesions in dogs accounted for 32.16%. As a result, mastocytoma was diagnosed in 13 dogs aged 3 to 14 years. The highest incidence rates were established in dogs aged 7 to 12 years. Notably, in 92.7% of dogs, mastocytomas were isolated and only in 7.3% of cases they were multiple.

The most common places of localisation of neoplasia are the trunk, limbs, less often the head and neck, and groin area. The behaviour of mastocytes is very diverse and depends on the degree of cell differentiation. Low-differentiated tumours have a high potential for metastasis and often recur. A high frequency of mastocyte recurrence after excision is noted with insufficient capture of healthy tissues, and with tumours of a low degree of differentiation. Based on the results of a comparative analysis of the clinical stages and histology of cutaneous mastocytosis, a direct correlation was established between the size of the tumour and the degree of its malignancy. A more malignant course of the disease was characterised by mastocytomas in dogs that were localised in areas of the body accessible for permanent injury (limbs, groin area, neck). According to the pathomorphological assessment of mastocytoma, it is necessary to consider the clinical stage of neoplasia to determine diagnostic accuracy.

During X-ray imaging, the infiltrative nodular and diffuse infiltrative types of mastocytes characterise the process of their malignancy. According to the gradation of mastocytes by the degree of differentiation, all the examined neoplasms can be attributed to the second degree. However, according to another classification, all neoplasms corresponded to mastocytomas of a high degree of differentiation. Prospects for further research are to examine the prognostic factors for the biological behaviour of cutaneous mastocytoma in dogs during surgery.

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None.

Conflict of Interest

None.

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Клініко-морфологічні особливості діагностики мастоцитом у собак

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Анотація. Мастоцитома є однією з найпоширеніших пухлин шкіри в собак. Вона характеризується специфічним перебігом, непередбачуваною поведінкою та прогнозом. Ця пухлина небезпечна тим, що імітує зовнішні ознаки інших новоутворень, від менш загрозливої ліпоми до раку шкіри. Мета досліджень – провести патоморфологічну верифікацію та класифікацію неоплазій у собак, які діагностовано під час клінічного обстеження, а також встановити особливості морфологічного прояву в них шкірної форми мастоцитоми. Матеріал для досліджень отримували методом ексцизійної біопсії під час хірургічних втручань з приводу новоутворення шкіри. Діагноз на мастоцитому встановлювали за результатами клінічного обстеження тварин і дослідження біопсійного матеріалу новоутворень за допомогою цитологічних і гістологічних методів дослідження. Встановлено, що частка неоплазій шкіри у структурі онкологічних захворювань собак становила – 30,12%. Серед тварин із неоплазіями шкіри, які проходили обстеження, мастоцитома була діагностована у 17,55% собак, віком від 3 до 14 років. Найвищі показники захворюваності встановлено у собак віком від 7 до 12 років. Найчастіше шкірну мастоцитому діагностували у собак породи: шарпей – 13,5%; лабрадор – 9,5%; боксер – 8,2%. Поодинокі випадки пухлинних утворень спостерігали у тварин таких порід, як: німецька вівчарка, спанієль, середньоазіатська вівчарка та мопс. Залежно від статі: кобелі становили – 52,2%, суки – 47,8%. У 50% досліджуваних тварин мастоцитоми розвивалися з стрімкою динамікою, що свідчить про значну агресивність пухлинного росту. Клінічно у собак визначали ознаки дегідратації та розлади зі сторони шлунково-кишкового тракту, що вказує на розвиток паранеопластичного синдрому. За результатами сонографії встановлено, що мастоцитоми шкіри візуалізуються як неоднорідні гіпоехогенні структури

з нечіткими контурами і нерівними краями. За результатами дослідження отримані нові відомості щодо морфологічних особливостей прояву та розвитку мастоцитом у собак. Таким чином, одержані результати доповнюють та розширюють знання щодо патогенезу мастоцитоми в собак, частоти поширення та особливості перебігу цієї онкологічної патології в окремій географічній популяції України

Ключові слова: цитологічне дослідження; гістологічне дослідження; пухлина; шкіра; метастаз

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Biochemical parameters of blood in cats with cardiogenic arterial thromboembolism and acute heart failure

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Abstract. Investigating the impact of vascular blockage on blood biochemical parameters, particularly in the context of acute heart failure, is crucial for understanding the underlying mechanisms of arterial thromboembolism in animals. The purpose of the study is to investigate the effect of vascular occlusion on the biochemical parameters of blood in cats with cardiogenic arterial thromboembolism. The study involved 12 cats and formed two groups: the first – animals with acute heart failure and the second – animals with arterial thromboembolism. Anamnesis data were collected for experimental animals, clinical examination, echocardiography, and biochemical examination of blood samples were performed. The activity of alanine aminotransferase, aspartate aminotransferase and gamma-glutamyl transpeptidase, the concentration of glucose, creatinine, urea, potassium, calcium, total and inorganic phosphorus were determined spectrophotometrically in blood serum. It was found that in cats with acute heart failure, the average value of serum aspartate aminotransferase activity increased by 2.8 times, and the creatinine concentration by 1.9 times compared to the upper limit of reference values. This indicates functional changes in the myocardium and kidneys in this pathology. In cats affected by arterial thromboembolism, the blood serum shows a significant elevation in various biochemical parameters compared to the upper limit of the corresponding reference intervals. Specifically, there is a 4.4-fold increase in alanine aminotransferase activity, a 4.3-fold increase in aspartate aminotransferase activity, a 1.4-fold increase in glucose concentration, a 1.8-fold increase in creatinine levels, a 2.0-fold increase in urea levels, and a 1.3-fold increase in inorganic phosphorus levels. The changes in biochemical parameters for feline thromboembolism may be a consequence of functional renal failure, ischemia, and muscle necrosis. Moreover, it was established that in most cats with acute heart failure, the urinary system is primarily affected, and with arterial thromboembolism, changes in muscle tissue also occur. The results obtained are of practical value for veterinary doctors when choosing a therapeutic strategy and prescribing additional diagnostic examinations

Keywords: hypertrophic cardiomyopathy; diagnosis; ischemia; pulmonary oedema; renal failure

Introduction

Among the considerable variety of heart diseases, cats are more likely to develop cardiomyopathies. Hypertrophic cardiomyopathy (HCM) ranks first in terms of prevalence, followed by dilated (DCM) and restrictive (RCM), less often arrhythmogenic (ACM) or unclassified cardiomyopathy (UCM) (Fuentes *et al.,* 2020; Tishkina *et al.,* 2022). Based on statistical data on the incidence and risk of non-cardiovascular death in clinically healthy cats and in cats with preclinical hypertrophic cardiomyopathy, Fox *et al.* (2019) found that mortality from various causes was notably higher in the group of animals with hypertrophic cardiomyopathy.

In the study by Fox *et al.* (2019), complications of cardiomyopathies include the development of heart failure (HF), arterial thromboembolism (ATE), and sudden cardiac death. The latter is not sufficiently studied among cats and is obviously more common than is possible to register. The most common structural cause of sudden death is HCM (Brugada-Terradellas *et al.,* 2021).

Acute heart failure is often the first manifestation of heart disease in cats, characterised by a rapid and severe course. Most often, hypothermia, tachycardia, auscultatory – gallop sound, and respiratory symptoms: dyspnea and tachypnea are registered. Notably, Liu *et al.*

(2020) established that animals with congestive heart failure have a substantially higher respiratory rate, a larger left atrium diameter, and a higher left atrium-to-aorta ratio compared to cats with preclinical cardiomyopathy. Kostiuk *et al.,* (2020) found that the mortality rate of cats with acute heart failure is most affected by the presence of systolic dysfunction, increased size of the left atrium, and the degree of radiological changes. According to Liu *et al.* (2020), in animals with congestive heart failure, serum concentrations of symmetric dimethylarginine (SDMA) and creatinine were elevated compared to preclinical and healthy cats.

Romito *et al.* (2022) provided a study of two cats with acute heart failure that was associated with *Toxoplasma gondii*. In one of the animals an increase in the activity of alanine aminotransferase, creatinine kinase, concentration of urea, serum amyloid A and troponin I was noted in the blood serum, while in the other – an increase in the activity of creatinine kinase and troponin I level. Studies of electrolyte abnormalities and markers of kidney damage in animals with heart failure are of great interest. A change in electrolyte balance is associated with neurohumoral activation of the renin-angiotensin-aldosterone and arginine-vasopressin systems, the use of diuretics (Roche-Catholy *et al.,* 2021; Lean *et al.,* 2022). Serum creatinine and potassium concentrations also change in both cats and dogs in response to furosemide administration (Ohad *et al.,* 2018).

The development of HF is associated with the occurrence of cardiogenic arterial thromboembolism – an acute blockage of a blood vessel that leads to ischemia of a certain area (Fox *et al.*, 2018). The prevalence of pathology is about 15% (Busato *et al.,* 2022).

The formation of a blood clot is a complex process that can have a different course, but the main elements will always be the components of Virchow's triad. The resulting embolus moves with the flow of blood and clogs a vessel smaller in diameter. Occasionally, occlusion of blood vessels of internal organs or the brain is diagnosed. The most common location for a blood clot is in the terminal branches of the aorta when both pelvic limbs are affected. With this location, the pathology is manifested by paresis or paralysis of the limbs, hypothermia, anaemia or cyanosis of non-pigmented areas of the skin of the affected limbs and vocalisation of pain (Hassan *et al.,* 2020).

Abnormally high levels of laboratory indicators are often registered in cats with ATE. Thus, in a retrospective study by Mitropoulou *et al.* (2022), in animals hospitalised for arterial thromboembolism, hyperglycemia and high serum creatine phosphokinase activity were noted as the most common abnormalities.

The purpose of this study is to determine the effect of vascular blockage on the quantitative characteristics of biochemical parameters of blood serum. Cardiomyopathy and congestive heart failure have a considerable number of similar elements. This allows separating the influence of heart pathology and investigating the effect of vascular occlusion on the body separately.

Task: to compare the values of biochemical blood parameters in cats with cardiogenic arterial thromboembolism and acute heart failure with cardiomyopathy.

Literature Review

The investigation of pathologies of the heart of cats is of great interest to researchers from all over the world. In addition to veterinary medicine research, humane doctors attach great importance to studying cardiomyopathies. Thus, the HCM phenotype in cats is very similar to the HCM phenotype in humans, but the pathology has a faster course and is a very valuable model to study (Stern *et al.,* 2019).

Today, the scientific community places significant emphasis on research areas pertaining

to arterial thromboembolism in cats, focusing on enhancing diagnostic techniques and advancing therapeutic and preventive strategies. Thus, French researchers (Eberle *et al.,* 2022) described the ultrasound characteristics of arterial thromboembolism in cats and identified a potential relationship between ultrasound results and predicting the development of the disease. The link between cardiogenic pleural effusion and a reduced risk of arterial thromboembolism in cats was found in a study by Italian researchers (Busato *et al.,* 2022). One of the latest publications on the treatment and prevention of thromboembolism in cats is a study on the effectiveness of dual therapy with clopidogrel and rivaroxaban (Lo *et al.,* 2022).

For this study, it is of interest to examine biochemical abnormalities in cats with arterial thromboembolism and acute heart failure. These pathologies are commonly mentioned in studies dealing with a broader subject – feline cardiomyopathy (Fuentes *et al.,* 2020; Kittelson *et al.,* 2021), and the highly specific studies on them are not often published. A large number of large-scale and fundamental studies on this subject with a description of clinical and laboratory indicators were published in the late 1990s and early 2000s. From modern sources, the most complete is the study of 250 cats with arterial thromboembolism in general practice (Borgeat *et al.,* 2013). However, of the biochemical parameters, only the concentrations of urea, creatinine, phosphates, and potassium are highlighted in this paper. Therefore, there was a need to refer to the study that was conducted earlier (Smith *et al.,* 2003). The features of biochemical blood analysis in cats with ATE are thoroughly described by Moise (2007).

Studies on acute heart failure in cats face a similar situation. While the topic is often discussed within broader subjects like cardiomyopathy, there is a limited amount of research specifically dedicated to this pathology as an independent focus. More often, researchers examined the effect of pathology on the concentration of electrolytes in the blood serum (Roche-Catholy *et al.,* 2021), interaction of biomarkers of kidney damage, myocardium, and inflammatory proteins (Liu *et al.,* 2020). Ohad *et al.* (2018) described changes in sodium, chloride, creatinine, and calcium concentrations in response to furosemide administration. The most extensive features of biochemical analysis of blood serum in cats with acute heart failure are described by Gountal *et al.* (2010).

Currently, cardiological pathologies of small animals have gained popularity among Ukrainian researchers. The subject of cardiogenic pulmonary oedema is covered by Zamorska *et al.* (2021). The prevalence of cardiomyopathy and arterial thromboembolism phenotypes in cats was investigated by Petrushko & Grushanska (2022). Kostiuk *et al.* (2020) devoted their study to echocardiographic parameters in cats with hypertrophic cardiomyopathy; a description of the aetiology and classification of feline cardiomyopathy are provided by Plysiuk & Tsvilikhovski (2016); report of clinical cases of hypertrophic cardiomyopathy in cats is presented by Tishkina *et al.* (2022), which expand the knowledge about this pathology.

Materials and Methods

The study was conducted based on the VetHouse veterinary centre in Vinnytsia (hereinafter VC). In the database for 2021-2022, a search for animals was conducted using keywords in the diagnoses: “pulmonary oedema”, “heart failure”, “cardiomyopathy”, and “arterial thromboembolism”. Records were analyzed, and those animals were selected that had a complete history, clinical examination, echocardiographic examination of the heart, and in which biochemical indicators of blood were examined. Cats who were first admitted with symptoms of acute congestive heart failure and

arterial thromboembolism were selected. Animals with information about previously diagnosed chronic heart failure were excluded from the study. Previous administration of drugs to stabilize the condition was not an exclusion criterion if this was the first case of heart failure.

To confirm the cardiological pathology, the animals underwent an echocardiographic examination using Esaote MyLab Gamma (Italy) or General Electric Vivid S6 ultrasound devices (USA). A 7.5 MHz phased array sensor was used. In already stable animals, the examination was performed on the right and left sides using B-mode, M-mode, and Doppler echocardiography. The right parasternal (short and long axis) and left apical positions were assessed. If it was necessary to conduct an examination before the condition stabilizes, echocardiography was performed only from the right parasternal position. Signs of left-sided congestive heart failure were considered (assessment in the right parasternal access): the size of the left atrium in the 4-chamber projection is more than 14 mm, the ratio of the left atrium to the aorta is more than 1.7 in the short projection.

Biochemical examination of cat blood serum samples was performed using a BS-3000m Sinnowa biochemical analyser (China) and SpainLab reagents (Kharkiv). Indicators studied: alanine aminotransferase (ALT; EC 2.6.1.2), aspartate aminotransferase (AST; EC 2.6.1.1), gamma-glutamyl transpeptidase (GGTP; ES 2.3.2.2), creatinine, urea, glucose, phosphorus, potassium, calcium.

The cats were divided into two groups. The group of animals with "acute heart failure" included cats admitted to the veterinary centre with respiratory signs (tachypnea, dyspnea), hypothermia, tachycardia, and echocardiographic signs of left-sided congestive heart failure. One cat had a restrictive cardiomyopathy phenotype, and five had a hypertrophic cardiomyopathy phenotype. All were males aged 1 to 9 years. They belonged to the Bengal, Himalayan, European shorthair, Sphinx, Scottish fold, and Scottish longhair breeds.

The group of cats with "cardiogenic arterial thromboembolism" included animals that were admitted to the veterinary centre in an urgent state. Owners noted a sudden deterioration in the animal's condition, vocalisation, aggression, shortness of breath, and lack of functioning of the pelvic limbs. During the clinical examination, paresis or paralysis of the pelvic limbs, anaemia or cyanosis, hypothermia, tachypnea, dyspnea, tachycardia, pain and aggression were noted. Echocardiographic examination revealed congestive heart failure. Five cats had the hypertrophic cardiomyopathy phenotype, and one had the restrictive cardiomyopathy phenotype. Two animals each belonged to the European shorthair and Mestizo breeds; one each belonged to the European long-haired and British shorthair breeds. Four of the six animals belonged to males. The animals ranged in age from 4 to 13 years.

Statistical analysis was performed using a personal computer and Microsoft Office Excel 2007 program. From the obtained data (concentration of indicators), variational series were developed. The normality of continuous data was proved according to the empirical rule: if a random variable is distributed normally, the absolute value of its deviation from the mathematical expectation does not exceed three times the mean square deviation. The statistical significance of differences between groups of cats was checked using the student's t-test and expressed using the probability of error (P). The difference between the groups was considered significant at $P < 0.05$. For descriptive analysis, the data were presented as the arithmetic mean (M; $\bar{x}$ – the sum of all fixed values of the set divided by the number of terms) and the standard error of the mean

(± m is an indicator of the accuracy of the average value of the sample).

Information on compliance with bioethical standards. The study complied with the ARRIVE guidelines and was conducted without violating the guidelines of the EU Directive 2010/63/EU on the protection of animals used for scientific purposes (Percie du Sert *et al.,* 2020; Direktive 2010/63/EU…, 2010).

Results and Discussion

Among the studied and analysed parameters of blood serum in cats with arterial thromboembolism, the average value of alanine aminotransferase, aspartate aminotransferase activity, creatinine, glucose, urea, and phosphorus concentrations are higher than the reference range and the values obtained from cats with acute heart failure (Table 1). However, the difference was not always significant.

Table 1. Biochemical parameters of cat blood serum with acute heart failure and arterial thromboembolism, M ± m

Biochemical parameters	Animals with acute heart failure		Animals with arterial thromboembolism		Reference values
	M ± m	Number of cats with a value outside the reference range (n)	M ± m	Number of cats with a value outside the reference range (n)	
ALT, U/L	51.7 ± 15.6	1	329.0 ± 59.5**	6	5.0-5.0
AST, U/L	140.9 ± 43.9	5	215.5 ± 47.7	6	5.0-50.0
GGTP, U/L	5.0 ± 1.5	0	9.5 ± 1.8	3	0.0-10.0
Creatinine, mmol/L	162.9 ± 19.0	5	251.0 ± 23.2*	6	50.0-140.0
Potassium, mmol/L	4.2 ± 0.5	1	4.8 ± 0.1	0	3.7-6.1
Calcium, mmol/L	2.2 ± 0.1	4	1.9 ± 0.1	5	2.2-2.9
Glucose, mmol/L	7.2 ± 1.8	2	9.0 ± 1.5	5	3.6-6.5
Urea, mmol/L	13.4 ± 1.9	4	22.0 ± 5.1	6	5.5-11.0
Phosphorus, mmol/L	1.6 ± 0.1	1	2.3 ± 0.4	5	0.9-1.7

Note: **P < 0.01, **P < 0.001 compared to the group of cats with acute heart failure*

In the blood serum of all animals with arterial thromboembolism, an increase in alt activity was found by 4.4 times compared to the upper limit of reference values. This intracellular enzyme is found mainly in liver and kidney cells, and in smaller amounts in heart muscle and skeletal muscle. Similar results were published earlier: out of 127 cats with arterial thromboembolism, an increase in ALT activity was observed in 72.8% of animals and a decrease in 1.2% (Smith *et al.,* 2003). This is probably due to large-scale necrosis of muscle tissue (Moise, 2007). Moreover, among cats with acute heart failure, the activity of the aforementioned enzyme increased only in one animal out of six. This is consistent with the results of a retrospective study in which 45 cats with heart failure were examined, and only 7 of them had increased activity of this indicator (Goutal *et al.,* 2010). In this study, in the group of animals with arterial thromboembolism, the average value of the indicator›s activity increased in the blood by 6.4 times compared to the group of cats with acute heart failure. The difference in indicators in the two groups is significant, so it can be argued that a high probability of an

increase in ALT activity in the blood serum is associated with muscle damage and, to a lesser extent, with myocardial damage. In addition, there is a high probability of an increase in the value of the indicator due to renal ischemia, because the concentration of urea and creatinine was also higher in cats with arterial thromboembolism. The probability of an increase in ALT activity in the liver in this case seems less likely.

Aspartate aminotransferase is one of the first cardiac biomarkers in history. It is used for the diagnosis of heart diseases, but it is of limited importance due to low sensitivity and lack of cardiac specificity. An increase in serum AST activity in animals with heart failure is expected, but it is not clear whether the development of thromboembolism will affect the activity of the indicator. In the blood serum of most of the studied animals (n = 11), except for the cat with acute heart failure, AST activity increased. The average activity value in animals with thromboembolism was higher, although the difference in AST activity between the two groups was insignificant (P < 0.2). A larger study of animals with acute heart failure showed an increase in AST activity in a smaller number of cats – in 17 out of 45 (Goutal *et al.,* 2010).

Gamma-glutamyltranspeptidase is classified as an enzyme involved in the metabolism of amino acids. This enzyme is found in the cells of the liver, bile ducts, and kidneys. All animals with acute heart failure had GGTP activity within physiological parameters, but cats with arterial thromboembolism (n = 3) had increased GGTP activity. The difference between the groups is defined as insignificant (P < 0.1). The most likely cause of the increased activity of this indicator is kidney injury.

Increased activity of AST, ALT, and GGTP is more common in liver, kidney, and heart diseases. Goutal *et al.* (2010) call increased activity of liver enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphotase) in cats with acute heart failure one of the most common abnormalities, and suggest that this is the result of low cardiac output or passive stagnation. In this study, acute heart failure was associated with the development of hypertrophic and restrictive cardiomyopathies. With these pathologies, the structure of the myocardium changes, relaxation slows down, and left ventricular stiffness increases. In this case, there is a violation of diastolic function. The chamber stretches worse, so blood enters the left ventricle under greater pressure, and its blood supply decreases. Increased diastolic pressure in the left ventricle is accompanied by increased pressure in the left atrium, pulmonary veins, and capillaries. As a result, there is stagnation of blood in the small circle of blood circulation. In the acute state, afferent vasoconstriction occurs when the neurohormonal system is activated. Because of this, liver perfusion may decrease, and systemic hypotension may occur. Such heart failure is classified as left-sided retrograde. Stagnation of blood can also be transmitted to a large circle of blood circulation, however, this is more typical for people. In cats, this is rarely recorded in the end stages of the disease. Concerning stagnation of blood, which can cause complications in the liver, it is reasonable to discuss right-sided retrograde heart failure, which is characteristic of the development of other heart pathologies and is more common in dogs (dilated cardiomyopathy, end-stage endocarditis of the mitral valve, etc.).

It is more likely that during the development of acute left-sided heart failure in cats, perfusion of organs (including kidneys and liver) was reduced, and the obtained deviations in biochemical parameters are primarily associated with renal ischemia, to a lesser extent, with damage to the myocardium and liver, and in the case of cats diagnosed with arterial thromboembolism, with muscle necrosis.

Serum creatinine concentration is considered a marker of glomerular filtration rate and is used in animals with heart failure to monitor renal function. In the blood serum of 92% of the studied cats (n = 11), the creatinine content increased and the difference in the concentration of this indicator between the groups was significant. In cats with arterial thromboembolism, the average value of the indicator increased by 1.5 times compared to animals with acute heart failure. Analyzing cases of acute heart failure in cats, Goutal *et al.* (2010) recorded an increase in creatinine concentrations relative to reference values in a smaller percentage of animals (11 out of 45 cats). The authors do not indicate that this deviation is common in cats with AHF. However, compared to healthy animals, creatinine concentrations in cats with congestive heart failure are usually higher. This is proved by the study on the cardiovascular-renal axis by Liu *et al.* (2020), during which an increase and correlation of creatinine and other renal biomarkers were observed in animals with congestive heart failure compared to healthy cats and animals with preclinical cardiomyopathy.

Borgeat *et al.* (2013) examined creatinine concentrations in only 30 of the 250 cats diagnosed with arterial thromboembolism. An increase in the value of the indicator relative to the reference intervals was found only in 26.7% of cats. In a study conducted based on the VetHouse veterinary centre (Vinnytsia), an increase in the concentration of this indicator was observed in all cats with a significant difference compared to the group of cats with AHF. The differences between the results obtained and the mentioned literature data may be explained by the smaller number of animals in the study.

The content of urea in the blood serum of all the studied animals with arterial thromboembolism increased (more than the physiological limit), and its average value is higher compared to the second group (22,05). Among animals with AHF (13,4), the urea concentration index was within the reference interval in two animals. The difference between the groups was statistically insignificant ($P < 0.1$). In most of the studied cats with AHF, the urea content in the blood increased (Goutal *et al.,* 2010), and in 68.6% of cats with arterial thromboembolism (Borgeat *et al.,* 2013). It can be stated that the concentration of urea often increases both in the blood of cats with AHF and with arterial thromboembolism.

Serum potassium concentrations in 11 cats were within physiological parameters and almost did not differ between the groups. Other authors indicate that 27.3% of 22 cats with arterial thromboembolism developed hyperkalemia (Borgeat *et al.,* 2013). Probably, such results are due to the fact that the study was conducted in the first hours after the occurrence of vascular occlusion, and hyperkalemia as a marker of reperfusion syndrome can develop at any time within three weeks after the incident of arterial thromboembolism.

In the blood serum of a cat with hypertension, a reduced potassium content was found. In a study conducted on 34 cats with AHF, 79% of the potassium content was within physiological parameters, and 21% had hypokalemia. Blood potassium concentrations did not affect the survival of cats (Roche-Catholy *et al.,* 2021). American researchers proved that the potassium content in the blood of 33 animals was within physiological limits, hypokalemia was observed in 10 and hyperkalemia in 2 (Goutal *et al.,* 2010). Hypokalemia in the blood of cats during the study was caused by the use of furosemide before blood sampling.

An increase in serum creatinine and urea concentrations in sick cats indicates changes in renal function, possibly as a result of hypoperfusion and activation of neurohumoral systems in patients with heart failure. In a study conducted by Plysiuk *et al.* (2017) on cats with

hypertrophic cardiomyopathy and symptoms of pulmonary oedema (AHF), they observed a significant increase in serum urea concentration by 2.5 times and creatinine by 2.2 times compared to clinically healthy cats. There is a strong link between the renal and cardiovascular systems. In humans, renal dysfunction is considered an important prognostic factor for heart failure Liu *et al.* (2020). In most cats with congestive heart failure, azotemia is observed and the number of cases of detection of this anomaly increases with the progression of heart pathology. According to the findings from Liu *et al.'s* (2020) study on cats with primary cardiomyopathy and cardiovascular-renal axis dysfunction, all cardiorenal biomarkers, including creatinine, exhibited a positive correlation and were higher in cats with heart failure. Additionally, elevated levels of the natriuretic hormone (B-type) N-terminal polypeptide (NT-proBNP) and symmetric dimethylarginine (SDMA) were associated with an unfavourable prognosis. Such results indicate a close functional relationship between the heart and kidneys in heart failure. In addition, congestive heart failure is an inflammatory disorder, and the prognosis, in this case, can be determined by the degree of inflammation and, possibly, the level of residual kidney function.

A systematic study of morbidity and mortality risk in cats by Fox *et al.* (2018) showed that cats with preclinical hypertrophic cardiomyopathy were slightly more likely to die from chronic kidney disease (CKD). If among clinically healthy animals the mortality rate from CKD was 6.4%, in animals with HCM, this indicator was 7.5%. This indicator is actually higher, but it differs slightly from clinically healthy animals in the control group. For comparison, the mortality rate from cardiovascular pathology in the group of cats with cardiomyopathy was 27.9%, and in the control group, only 1%. The authors note that mortality from non-cardiovascular diseases was similar between the study groups. Therefore, abnormalities in kidney function are often recorded in cats for cardiomyopathy, but such animals die from CKD no more often than animals that do not have HCM.

Some cats have arterial thromboembolism, as Borgeat *et al.,* (2013) noted, who also reported severe azotemia or hyperkalemia. Still, the incidence of acute metabolic disorders may have been underestimated due to periodic monitoring. As indicated by the authors, azotemia and hyperkalemia can be caused by reperfusion injury or acute kidney damage.

In the study, all animals involved were diagnosed with left-sided congestive heart failure, which is classified as "wet and cold" from a hemodynamic perspective. Pouchelon *et al.* (2015) established that cardiovascular and renal disorders in such cats are associated with increased venous renal pressure, decreased renal blood flow, and impaired autoregulation. As noted by Haase *et al.* (2013), in the acute state, with a significant decrease in the effective volume of circulating fluid, the neurohormonal system is activated, which leads to afferent vasoconstriction. This causes a decrease in renal blood flow and renal perfusion pressure. In such patients, low values of cardiac output and effective volume of circulating fluid may be associated with reduced systemic blood pressure. Therefore, it reduces renal perfusion pressure, even if venous renal pressure corresponded to relatively normal values since autoregulation does not always compensate for low blood pressure with a decrease in the effective circulating volume.

An additional risk factor for animals with heart failure is kidney infarction. As Hikey *et al.* (2014) established, cats with kidney infarction were 4.5 times more likely to have hypertrophic cardiomyopathy and 8 times more likely to be diagnosed with distal aortic thromboembolism compared to cats without kidney infarction.

Since ATE was cardiogenic in cats of the second group, the likelihood of developing acute kidney damage due to reduced cardiac output, activation of the neurohormonal system, and kidney infarcts in these animals is also present. In addition, cats with ATE may experience reperfusion syndrome. One of the elements of the pathogenesis of this condition is the release of a large amount of toxic substances from the area that was cut off from the blood flow. This condition occurs if the blood supply is restored more than four hours after occlusion. In this study, all cats were examined more than 6 hours after the onset of symptoms of thromboembolism, so they are likely to develop reperfusion injury.

The results of this study and the detection of a statistically significant difference in creatinine concentration in animals with acute heart failure compared to animals with arterial thromboembolism indicate more severe kidney damage in cats diagnosed with thromboembolism. Acute kidney damage in this animal species can be caused by toxic, ischemic, inflammatory, or infectious lesions. As heart failure progresses, there is inadequate perfusion of the kidneys.

The content of calcium in the blood serum of the vast majority of the studied animals (n=9) is reduced. In two animals with AHF and one with arterial thromboembolism, the value of serum calcium content corresponded to physiological limits. The quantitative indicator of calcium content in the blood serum of animals with arterial thromboembolism decreased, but the difference between the groups was insignificant ($P < 0.1$). Such changes in the concentration of calcium in the blood serum of cats may be due to hyperphosphatemia because excess phosphorus binds to calcium (Moise, 2007).

Hyperglycemia was reported in two cats with AHF and in five with arterial thromboembolism. As is known, hyperglycemia or normoglycemia is usually recorded in the blood serum of animals with arterial thromboembolism, and with damage to the extremities, the glucose concentration decreases (Klainbart *et al.,* 2014). Hyperglycemia was observed in most cats with acute heart failure (66.6%) (Goutal *et al.*, 2010). Stress-induced hyperglycemia causes an intense release of adrenergic hormones that inhibit insulin secretion. Cortisol and glucagon levels also increase in the blood. In addition, in cats with arterial thromboembolism, lactate production increases due to muscle anaerobic glycolysis, which leads to an intense increase in blood glucose concentrations (Moise, 2007). Therefore, it was expected to detect an increase in the concentration of glucose in the blood serum of cats for AHF in accordance with the norm. In fact, the average value was higher, however, the difference with the group of cats with acute heart failure was classified as insignificant ($P < 0.2$).

In a study by English researchers, an increased concentration of phosphorus in the blood is noted as the least frequent deviation among other biochemical parameters, which occurred only in 5.3% of cats (Borgeat *et al.,* 2013). However, American researcher Moise (2007) diagnosed hyperphosphatemia along with the most common biochemical abnormalities in cats with arterial thromboembolism, which may indicate kidney hypoperfusion and massive muscle necrosis. In turn, the serum phosphorus content in five of the six cats that belonged to the group with arterial thromboembolism is higher than normal. In cats with AHF, an increase in the indicator was recorded in one animal out of six. The difference in quantitative indicators between animal groups was statistically insignificant ($P < 0.1$).

Conclusions

In the blood serum of five out of the six cats with acute heart failure, an increase in aspartate aminotransferase activity and creatinine

concentration was observed (the average value was increased relative to the upper limit of the reference range by 2.8 and 1.9 times, respectively). This indicates the development of complications in the urinary system due to the progression of this cardiopathology.

In the blood serum of cats with arterial thromboembolism, the average values increased compared to the reference intervals as follows: alanine aminotransferase activity – by 4.4 times, aspartate aminotransferase – by 4.3 times, creatinine concentrations – by 1.8 times, and urea – by 2 times. Quantitative changes in these indicators compared to the reference values were recorded in all the studied animals. It was found that the difference in alanine aminotransferase activity ($P < 0.001$) and creatinine content ($P < 0.01$) was statistically significant between animals of both experimental groups.

In the blood serum of cats of the experimental groups, an increase in urea content and the development of hypocalcemia were also noted, compared with the values of the reference intervals. This indicates not only the negative impact of acute heart failure on the functional state of cats' urinary system but also structural changes in the myocardium. Moreover, an increase in gamma-glutamyltranspeptidase activity and glucose and phosphorus content was observed in animals with arterial thromboembolism compared to the values of reference intervals. The obtained regularities regarding changes in blood biochemical parameters complement the established picture of changes in the internal organs of sick animals and indicate structural and functional disorders in the kidneys, as well as ischemia and necrosis in skeletal muscles.

The observed variations in the biochemical parameters of blood serum among the experimental groups of cats can be attributed to distinct differences in the pathogenesis of the respective pathologies. However, a certain similarity in the manifestation of pathologies (cardiomyopathy, congestive heart failure) allows determining the features of the effect of blockage of a vessel on a whole organism.

In the future, studies comparing other indicators (troponin, clotting factors, etc.) in the blood of cats with acute heart failure and arterial thromboembolism will be useful.

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Conflict of Interest

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Біохімічні показники крові у котів за кардіогенної артеріальної тромбоемболії та гострої серцевої недостатності

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Анотація. З'ясування особливостей впливу закупорки судини на біохімічні показники крові, у тому числі на тлі гострої серцевої недостатності, важливі для визначення патогенезу артеріальної тромбоемболії в тварин. Мета роботи – дослідити вплив оклюзії судин на параметри біохімічних показників крові в котів за кардіогенної артеріальної тромбоемболії. У дослідження включено 12 котів і сформовано дві групи: перша – тварини за гострої серцевої недостатності а друга – тварини з артеріальною тромбоемболією. У відношенні піддослідних тварин зібрано дані анамнезу, проведено клінічний огляд, ехокардіографію та біохімічне дослідження зразків крові. В сироватці крові спектрофотометрично визначали активність аланінамінотрансферази, аспартатамінотрансферази і гамма-глутамілтранспептидази, концентрацію глюкози, креатиніну, сечовини, калію, кальцію загального і неорганічного фосфору. Встановлено, що в котів за гострої серцевої недостатності середнє значення активності аспартатамінотрансферази у сироватці крові підвищувалося в 2,8 раза, а концентрація

креатиніну в 1,9 раза порівняно з верхньою межею референтних значень. Це вказує на функціональні зміни міокарду і нирок за даної патології. За артеріальної тромбоемболії в сироватці крові хворих котів відмічали підвищення активності аланінамінотрансферази в 4,4 раза, аспартатамінотрансферази в 4,3 раза, концентрації глюкози в 1,4 раза, креатиніну – в 1,8 раза, сечовини – в 2,0 рази, неорганічного фосфору в 1,3 раза, відносно верхньої межі відповідних референтних інтервалів. Встановлені зміни біохімічних показників за тромбоемболії котів можуть бути наслідком функціонального порушення нирок, ішемії і некрозу м'язів. При цьому виявлено, що у більшості котів за гострої серцевої недостатності, насамперед, зазнає ураження сечова система, а за артеріальної тромбоемболії також відбуваються зміни у м'язовій тканині. Отримані результати становлять практичну цінність для лікарів ветеринарної медицини під час вибору терапевтичної стратегії та призначення додаткових діагностичних досліджень

Ключові слова: гіпертрофічна кардіоміопатія; діагностика; ішемія; набряк легень; ниркова недостатність

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Effect of the probiotic feed additive "Immunobacterin-D" on the productivity of black speckled cows during lactation

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Abstract. In Ukraine, as in every country in the world, an important task is to develop an effective development strategy for producing natural dairy products through probiotics. Feeding PFA to animals shows different effectiveness depending on their age, the intestinal microbiome's characteristics, the feed diet's composition and production technology. The purpose of the study was to identify the most effective period of application of PFA "Immunobacterin-D" and to select a yeast culture to increase the milk productivity of cows. The effectiveness of PFA was evaluated by the amount of milk produced, and its quality – by using an Ecomilk ultrasound analyser. The health status of cows was monitored based on the results of a spectrophotometric study of biochemical parameters of blood serum on a LabLine-010 biochemical analyser (Austria). As a result, it was determined that the optimal period of application of PFA is 30-60 days after calving. On the 13th

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day of PFA feeding, milk yields in cows of the experimental group increased by 1.2-2.5 L/day. The difference in the amount of milk produced between the animals of the experimental and control groups was 0.7-1.9 L/day. From 70 days after calving, the use of PFA in cows did not cause changes in the volume of milk yield but contributed to an increase in its fat content. By feeding cows of the experimental strain for 21 days *S. cerevisiae* as part of the PFA, the maximum increase in milk yield was obtained – by 2.13 L, and milk fat – by 0.45%. Whereas the use of PFA with a conventional strain *S. cerevisiae* AF 338 contributed to an increase in milk yield by 1.73 L, and in the control group of cows only by 1.30 L. According to the results of biochemical studies of blood serum, the positive effect of PFA "Immunobacterin-D" on the health status of cows was proved. PFA can be recommended for feeding cows in production, which will help increase milk yield and improve their health

Keywords: dairy cattle breeding; yeast; *Bacillus subtilis*; *Bacillus licheniformis*; *Saccharomyces cerevisiae*

Introduction

The United Nations has joined the food security programme to fight hunger in the world. The national approach to the transformation of food systems in Ukraine provides for three areas of action: "Healthy food for all", "Environmentally friendly production", "Resistance to market instability and availability of food products for all" (Koval, 2021).

Livestock products ensure the well-being of the population and more and more consumers want to increase the composition of dairy products in the diet to improve health. Due to military operations and the deterioration of the economic situation, Ukrainians consume 50-60% less dairy products (Koval, 2021).

Every country in the world, including Ukraine faces a difficult task – to create an effective strategy for developing the production of natural dairy products against the background of reducing greenhouse gas emissions per unit of production and improving resource efficiency (Mingmongkolchai & Panbangred, 2018; Hovhera, 2022). In dairy cattle breeding, this goal can be achieved by applying probiotics (Edmond, 2019). The sufficient effectiveness of the use of probiotics for various animal species has been proven. However, ruminants have their own characteristics in the anatomical structure and physiology of digestion (Tishkova & Krasinko, 2018).

The profitability of the dairy industry of animal husbandry depends on many factors, the main one of which is providing animals with high-quality feeding. This is not only about the quantitative and qualitative supply of feed to animals but also the ability to digest and assimilate feed with maximum efficiency in terms of growth, development and increase in milk production. The difficulty of digestion of ruminants lies in the duration of digestion of various types of feed (hay, silage, concentrated feed) (Tishkova & Krasinko, 2018), and for comparison, digestibility (%) and duration of breakdown (h) can be provided, respectively: molasses – 95 and 0.5; grains of cereals – 80 and 12-14; quality grass – 70 and 18-24; quality clover – 70 and 12-18; hay – 55 and 30-40, and straw – 40 and 45-55. The solution to this issue is facilitated by the use of probiotic feed additives to animals, which are formed from cultures of endogenous or exogenous microflora and have different effectiveness depending on the age of the animals, the growing period, the diet, production technology, and the genetic inheritance of the

body, which determines the intestinal microbiome (Ban & Guan, 2021). Given the list of exposure factors, probiotic feed additives include various types of microorganisms. The main focus of the study was on probiotic feed additives (PFA) based on bacteria of the *Bacillus* genus since the spore form of bacteria ensures good preservation and reproduction in the digestive canal of ruminants, and *Saccharomyces cerevisiae* yeast have a positive effect on digestion in the pancreas and contribute to the preservation of spore microflora by consuming oxygen.

Combination of the *Bacillus subtilis, Bacillus licheniformis* and *Saccharomyces cerevisiae* complex is used to increase the survival rate of these bacterial cultures due to the respiratory activity of yeast, which protects anaerobic bacteria from the effects of oxygen (Newbold *et al*., 2007). This is because the pre-pancreas and intestines of ruminants have their own microbiota with a numerical predominance of bacteria (1 litre of rumen fluid contains 10^{14} bacteria) and for each newly introduced bacterium there are 1 million well-adapted ones – rumen (Lemishevckyi, 2021), that is, probiotic feed additives in rumen digestion are assigned only a corrective role. The most rational application is a short-term course (Lytvynenko & Yukhymchuk, 2021), which ensures reproduction and the right concentration of probiotic cultures to correct the gut microbiome during critical or stressful periods of animal life. Short-term use of exogenous microorganisms prevents dysbiosis in emaciated animals and contributes to the improvement of the intestinal tract due to antagonism to pathogenic Salmonella and Escherichia coli (Brewer *et al*., 2014; Xiao *et al*., 2016), reducing inflammatory processes in the intestines and showing an antitoxic effect to feed toxins (Zhu *et al*., 2017; Sammes, 2022).

Global recognition of sustainable ruminant breeding through the addition of probiotics will undoubtedly ensure food security and quality food production around the world (Reuben *et al*., 2022).

The purpose of the study is to identify the most effective period of short-term use of the probiotic feed additive "Immunobacterin-D" (contains *B. subtili*, *B. licheniformis*, *S. cerevisiae*) to maintain health and increase the milk productivity of cows.

The primary task was to determine the most effective period of application of the feed additive from the beginning of lactation of cows, that is, during the milking period, in which the cow's productivity is laid during the current lactation. Secondly, to determine the most effective and stress-resistant yeast culture as part of a probiotic feed additive for cow consumption.

Literature Review

The present raises the need to solve a problematic issue – the dosage of probiotic cultures in complex use. The effectiveness of the use, cooperation, synergy of different strains of microorganisms, and the effect of their action on different age groups of animals of certain species should ensure maximum efficiency when changing the feeding diet. Even with the same type of feeding, last year's feed is changed to the current one (Podobed *et al*., 2020).

Bacteria of the *Bacillus* genus – gram-positive aerobic or facultative anaerobic microorganisms that form endospores. The ability to form spores is very useful and allows long-term preservation of the microorganism without loss of viability. Spores can withstand low pH values of gastric juice, and as a result of entering the small intestine, exhibit probiotic properties (Cutting *et al*., 2011). Therewith, they are marked by a substantial antagonistic effect with more pronounced adhesion compared to vegetative forms of bacteria (Mingmongkolchai & Panbangred, 2018).

Strains of *Bacillus* produce extracellular enzymes that promote the digestion of feed, improve the absorption of nutrients (Latorre *et al*., 2015), and also exhibit antioxidant activity (Shobharani *et al*., 2016). *B. subtilis C-3102* allows for avoiding a long-term negative energy balance of dairy cows after calving (Urakawa *et al*., 2022). Thus, influencing the microbiome of cows' pre-rumens, contributing to an increase in milk production (Sun *et al*., 2013). Treatment of cow udder teats with a bacterial preparation containing *B. subtilis C-3102* improves milk quality (Sokoliuk *et al*., 2022). In the world literature, considerable attention is paid to Synergy and potentiation in the combination of probiotic cultures *Bacillus subtilis and Bacillus licheniformis*. Their synergy and the effect of the ratio of these probiotic cultures on pigs were proved (Kim *et al*., 2022). Supplements *B. licheniformis* and *B. subtilis* in young pigs substantially improve the digestibility of feed and reduce the amount of *E. Soli* in faeces, NH_3 and H_2S emissions. Increasing *B. subtilis* coefficient in probiotic supplements reduces the smell of pus (Kim *et al*., 2022). Antimicrobial agents *B. subtilis* actively counteract gram-positive bacterial and fungal pathogens (Khochamit *et al*., 2015).

Bacillus licheniformis – gram-positive, endosporous-forming mesophilic bacterium belonging to the *Bacillaceae* family (Makowski *et al*., 2021). It is isolated from soil and feed and is resistant to antibiotics (chloramphenicol and clindamycin). Sorokulova *et al*., 2008; Muras *et al*., 2021). A combination of *B. licheniformis with B. subtilis* increases the activity of antioxidant enzymes (Salehi *et al*., 2022), and by itself, *B. licheniformis* improves innate immune function by reducing oxidative stress associated with the accumulation of ammonia in tissues and blood (Gopi *et al*., 2022), and improves liver function (Devyatkin *et al*., 2021).

The positive effect of yeast *Saccharomyces cerevisiae* on the productivity of ruminants has been known for a long time. Food additives of live yeast based on *Saccharomyces cerevisiae* improve the health and productivity of ruminants (Oeztuerk *et al*., 2009). The action of yeast stimulates the metabolism of microbial nitrogen, which leads to an increase in the supply of microbial protein to the intestine and reduces nitrogen loss (Hassan *et al*., 2019). However, the effect of yeast in the large intestine of ruminants is still unclear, there is an increase in digestibility in the rumen, which is especially pronounced in calves for improving rumen fermentation (Alugongo *et al.,* 2017).

Yeast provides the body with vitamins and growth factors for beneficial bacteria (Newbold *et al*., 1996; Poppy *et al*., 2012), protect the rumen by showing antagonism against pathogenic bacteria (Kenney *et al*., 2015). Feeding cows with *S. cerevisiae* included leads to an increase in milk yield (by 1.18 kg/day), milk fat, and protein during the first 70 days of lactation (Poppy *et al*., 2012). According to other studies, the use of yeast from 73 days of lactation lasting 70 days led to an increase in milk yield by 1.36 litres (Sun *et al*., 2021), and after 42 days of feeding yeast to cows, milk yield increased by 23% and the peak lactation lengthened by 1 week (Ayad *et al*., 2013). Other authors also report an increase in milk yield and a decrease in the percentage of protein in milk (Bakr *et al*., 2015). Especially effective for the body of cows (Maamouri *et al*., 2014) and buffaloes (Para *et al*., 2018) is the introduction of live yeast *S. cerevisiae* in a hot climate. Tishkova & Krasinko (2018), investigating the effects of the *S. cerevisiae* selenium-producing strains on the body of cows, prove that they form resistant to stress due to overheating.

The combined action of probiotic bacteria improves the productivity of not only cows but also other dairy ruminants. Thus, adding sheep to the diet *B. subtilis* and *B. licheniformis* leads to an increase in milk yield, fat, and protein

(Kritas *et al.*, 2006). A combination of *S. cerevisiae*, *B. subtilis,* and *Enterococcus faecalis* in Saanen goats improves the production and composition of milk and fecal microflora (Ma *et al.*, 2020). Based on the analysis of these literature sources, it can be concluded that depending on the duration of feeding feed additives, yeast has a positive effect of varying degrees on milk productivity at different stages of lactation of cows.

Therewith, some literature sources indicate antagonism and increased productivity of animals due to feeding probiotics. However, the value of combined probiotics with the content of *B. subtilis, B. licheniformis, S. cerevisiae* also consists in their immunostimulating effect. *Bacillus* spores can stimulate the immune system and macrophage phagocytosis (Xu *et al.*, 2012). Live *B. subtilis* bacteria, approximately at 1 in 1000 ratio, when introduced into the rumen, enter the blood and lymph, accumulate in the spleen, lymph nodes, liver, and foci of inflammation. Oral administration of spores to mice substantially increases the stimulation of antigen-presenting cells and T-lymphocytes, the induction of cytokines in the spleen and mesenteric lymph nodes. Their interaction with macrophages is similar to *B. anthracis* (Hughes *et al.*, 2005). *B. subtilis,* as a probiotic feed additive, is widely used for heterologous antigen expression and protective immunisation. It promotes an increased percentage of T cells in the mesenteric lymph nodes and spleen and higher levels of sIgA and IgG in the gut (Lin *et al.*, 2022). Administrating *B. licheniformis* to mice with water improves their condition by reducing inflammation of the colon mucosa and inhibiting the activation of inflammatory processes (Tsai *et al.*, 2022).

Combining *S. cerevisiae* and *B. licheniformis* reduces the permeability of the small intestine in pigs infected with *E. Soli* K88, showing improved intestinal integrity and barrier function (Pan *et al.*, 2017). Reducing systemic or subclinical inflammation can reduce the immune system's need for energy, which is often ignored when controlling animal productivity. The immune system of cattle and pigs has been shown to use approximately 1 kg of glucose during the first 12 hours after experimental immune infection with lipopolysaccharide (Kvidera *et al.*, 2017). The addition of yeast also stimulates the protective systems of the animal body and increases the formation of antibodies in the blood serum and the antibody titer in colostrum (Wafa *et al.*, 2020). However, the reasons for the more effective use of a complex probiotic supplement due to the activation of the immune system are not fully understood (Rychlik, 2020).

It is the immunological features of probiotic cultures that determine the name of the probiotic feed additive – Immunobacterin-D.

One of the reasons for writing this study is the papers by Ma *et al*. (2020) and Sun *et al*. (2021), where the authors highlight the lack of information on the use of yeast: firstly, on the duration of supplementation to reduce the incidence of cattle; secondly, how long the benefits of adding a yeast product persist after stopping feeding at different stages of production. Based on the results of previous studies, only partial answers to these questions have been received, and therefore the need to continue research in this area is present.

Materials and Methods

Probiotic feed supplement "Immunobacterin-D" (RP AV-05553-04-14 production of the private enterprise "Kronos Agro", Ukraine) has a powdery structure, which allows it to be used as part of mixed feeds.

Scientific and production studies on the effect of the feed additive "Immunobacterin-D" with the content of baking yeast on the milk productivity of black speckled cows were conducted in farms of the Kyiv region

during 2016-2021. Probiotic feed additives were given individually at a dose of 10 g per day to cows after calving with a body weight of 400-500 kg. Control milking operations were conducted to record the milk produced. The study was conducted in two stages: the first was in the summer period from May 27 to June 11; the second – from April 6 to May 10, during the critical period of winter stall maintenance, which allowed to get the maximum impact of natural environmental factors on the body of cows.

During the first stage, the researchers selected calved cows of black speckled breed according to the principle of pairs-analogues, in accordance with the calving date. Analogue pairs were completed from animals at the age of 10 days and with the appropriate formation of technological schemes. Control milking was performed after 3-4 days and the study lasted 13 days. Animals in the post-calving period (from 4 days after calving to 3 months of lactation) were divided into two groups – 8 in the experimental and 6 in the control. Animals of the experimental group were fed a probiotic feed supplement "Immunobacterin-D" (*B. subtilis* 12P-130, $1 \cdot 10^{10}$ CFU/g·mL, *B. licheniformis* 12P-896, $1 \cdot 10^{10}$ CFU/g·mL, *S. cerevisiae* AF 338, $1 \cdot 10^{9}$ CFU/g·mL) individually, once with mixed feed for 10 g per day. The animals in the control group remained intact.

In the second stage, for the examination to determine a more effective yeast strain, two experimental and one control group of cows from the post-calf period were formed. The cows were kept tethered, with automatic watering. Milk yield was recorded after 12 days. The experiment lasted 36 days. Cows of the first experimental group were given a probiotic feed supplement "Immunobacterin-D" (*B. subtilis* 12P-130, $1 \cdot 10^{10}$ CFU·g * mL, *B. licheniformis* 12P-896, $1 \cdot 10^{10}$ CFU·g * mL, *S. cerevisiae* AF 338, $1 \cdot 10^{9}$ CFU·g * mL (conventional); and the second – Immunobacterin-D (*B. subtilis* 12P-130, $1 \cdot 10^{10}$ CFU·g * mL, *B. licheniformis* 12P-896, $1 \cdot 10^{10}$ CFU·g * mL, *S. cerevisiae*, experimental $1 \cdot 10^{9}$ CFU·g * mL). The animals in the control group remained intact. The experiment lasted 36 days.

Studies on the assessment of milk quality indicators were conducted using an ultrasonic milk quality analyser "Ecomilk", Milkan type KAM-98-2A (Bulgaria).

The effect of the probiotic feed additive "Immunobacterin-D" on the health status of cows was assessed by biochemical parameters of animal blood serum, which were determined using an automatic biochemical analyser LabLine-010 (Austria) based on the scientific laboratory of the Department of Therapy and Clinical Diagnostics of the National University of Life and Environmental Sciences of Ukraine (Kyiv).

Experiments using live animals were conducted in accordance with the recommendations of Arrive.

The obtained study results were processed in the Microsoft Excel programme using the Student's coefficient ; $P < 0.001$; $P < 0.05$; $P < 0.01$ to compare the obtained digital values.

Results and Discussion

Based on the results of the first stage of the study, the most favourable terms for the rational use of the feed additive "Immunobacterin-D" to cows of the experimental group were determined. Thus, the average daily milk yield of cows of the experimental group before introducing the supplement was 20.5 L, in the control group – 19.7 L. During the period of feeding the probiotic feed additive (13 days), the milk yield of cows of the experimental group increased by 1.2-2.5 L per day. In the control group of cows, milk yield also increased by 0.3-1.5 L, but the difference in the amount of milk produced between the groups was 0.7-1.9 L in favour of the experimental group (Table 1).

Table 1. Effect of the feed additive "Immunobacterin-D" on the amount of milk produced, L, M ± m, n = 6

Inventory number	Name of the animal	Mass of the body 27.05	Calving date 29.05	Day of control milking before the experiment		Day of control milking during the experiment period			
				02.06	05.06	07.06	11.06		
8015	Bagheera	500	08.03	20	21	22.5	24	23	22
2734	Dymka	400	17.03	21	20	19	20	21.1	23
5638	Azalea	410	20.03	15	14	14	14	12	13.5
5645	Zorka	405	01.04	21	23	34	32.5	30	31.5
5604	Rosa	390	10.04	21	18	21.5	23	23	21.5
5607	Klumba	405	12.05	18	19	20	19	20	21.5
9085	Tryda	400	15.05	17	21	21	20	17.2	19
8968	Lypa	450	28.05	29	31	32	30	27.4	31.5
				20.3 ± 1.5	20.8 ± 1.7	23 ± 2.4	22.8 ± 2.1*	21.7 ± 2*	22.9 ± 1.1*
Increase to the average daily milk yield				20.5		+2.5	+2.3	+1.2	+2.4
8867	Mayka	390	08.03	18	20	21.5	21.5	22	22
2607	Antaliia	425	17.03	17	18	20.5	19.5	15.8	16
4545	Korona	400	01.04	18	19	19.5	20.5	19	23
2744	Niagara	405	20.04	16	15	17.5	18	16.3	19
4494	Hroza	390	12.05	18	20	19	20	20.2	15.5
5623	Ilona	410	22.05	28	29	29	28	26.8	25.5
				19.2 ± 41.7	20.2 ± 1.9	21.2 ± 1.6	21.3 ± 1.4	20 ± 1.7	20.2 ± 1.6
Increase to the average daily milk yield				19.7		+1.5	+1.6	+0.3	+0.5

Note: ****P < 0.05; **P < 0.01; *P < 0.01 compared to the control*

Based on the analysis of the results shown in Table 1, cows of the experimental group have a stable tendency to maintain an increase in the average daily milk yield. In particular, the milk yields of these cows increased by an average of 9.23%. Therewith, in animals of the control group, the increase in the average daily milk yield decreased over time and amounted to 4.65%, which is 4.58% less compared to the experimental one. During the study period, the milk productivity of cows in the experimental group increased by 4.58% compared to the control group.

The average daily milk yield of cows was compared among analog pairs grouped depending on the day of lactation for 10 days (Table 2).

The highest performance indicator compared to the amount of milk produced was observed in cows from 30 to 60 days after calving.

Comparing pairs-analogues of cows, it was established that the tendency to increase milk yields occurs as early as the 4th day of introducing a probiotic feed additive. However, based on the results of the study, there is a decrease in milk yield in cows on the 10th day of the experiment (12-25 days after calving). This is assumably due to the physiological characteristics of the cows' bodies in the post-calving period since the diet, content, and dose of probiotic feed supplement were the same for everyone.

According to the conducted studies of milk quality, an increase in the fat content of milk by 0.6-2.2% during the first 30 days of lactation was established in all cows, especially in experimental ones. With a larger difference in milk yields, an increase in density and a decrease in fat content of milk by 0.7% were observed. Feeding cows a probiotic feed supplement after 70 days of lactation affected a decrease in milk yield compared to previous values for an increase in fat content, density, and protein content in milk. According to the indicators shown in Table 3, milk yield had a maximum increase on the 30-60 days of lactation, and from the 70-90 days, the fat content, density, and protein content in milk increased when feeding cows the probiotic feed supplement "Immunobacterin-D".

Table 2. Difference in the amount of milk produced depending on the initial milk yield, L

Pairs-analogues for the period after calving (days)	Group	Inventory number	Control milkings, day of the experiment			
			4	7	10	13
Pair 1 (10)	Experiment	8968	2	0	-2.6	1.5
	Experiment	9085	2	1	-1.8	0
	Control	5623	0	1	1.2	-3.5
Pair 2 (20)	experiment	5607	1.5	0.5	1.5	3
	control	4494	3	2	-1.7	-1.5
Pair 3 (30)	experiment	5645	12	10.5	8	9.5
	control	4545	0.5	-0.5	-1.7	-3
Pair 4 (40)	experiment	5604	2	3.5	3.5	2
	control	2744	1	2	0.5	4.5
Pair 5 (70)	experiment	5638	-0.5	-0.5	-2	-1
	experiment	2734	-1.5	-0.5	-0.4	2.5
	control	2607	3	2	-2	-1.5
Pair 6 (80)	experiment	8015	2	3.5	2.5	1.5
	control	8867	2	2	2.5	2.5

Table 3. Milk quality indicators of experimental cows, M ± m, n = 6

No. п/п	Inventory number	Lactation day during sampling	Fat, %		Density, ^{0}A		Protein, %	
			before	after	before	after	before	after
1	8968	12	3.88	4.43	29.76	25.7	4.12	3.79
2	9085	25	2.83	5	30.78	26.04	4.6	3.69
3	5623	18	3.45	3.63	27.78	27.89	3.31	3.07
4	5607	30	3.72	4.26	27.84	27.12	3.35	3.07
5	4494	30	3.99	3.99	27.99	28.59	4.69	3.45
6	5645	40	3.77	2.45	27.35	28.75	3.25	3.25
7	4545	40	3.6	3.66	28.9	28.98	3.79	3.79
8	5604	60	2.1	1.75	30.03	30.46	3.21	3.21
9	2744	50	4	3.95	27.87	28.61	3.79	3.25
10	5638	80	3.23	4.36	30.16	30.58	2.88	4.41
11	2734	84	4.07	4.1	27.92	29.13	2.93	3.35
12	2607	84	3.23	3.22	30.19	30.78	2.83	3.74
13	8015	93	4.77	6.06	29.51	27.7	4.02	4.02
14	8867	93	4.2	4.05	27.72	29.06	3.16	3.94
Arithmetic mean of the experimental group			3.55 ± 0.27	4.05 ± 0.45	29.17 ± 0.42	28.19 ± 0.62	3.55 ± 0.21	3.60 ± 0.15
Arithmetic mean of the control group			3.75 ± 0.19	3.75 ± 0.16	28.41 ± 0.49	28.99 ± 0.48	3.60 ± 0.33	3.54 ± 0.17

Considering the results obtained, it is worth continuing to examine the effect of this supplement during subsequent lactation periods.

The combination of these cultures was identified to be quite successful. The results obtained coincide with those in the study by

Poppy *et al*. (2012) since the most pronounced effect of using a feed additive was manifested in the first half of lactation. According to the paper of Alugongo *et al*. (2017), the milk productivity of cows during the use of anaerobic probiotics was most pronounced in the group of cows that were fed *B. licheniformis* compared to *B. subtilis* and with a group that didn't get any supplements.

Results indicate that *B. licheniformis* is the better live feed additive compared to *B. subtilis*, which is logical for a ruminant since the advantages of *B. licheniformis* are especially pronounced for feeding coarse feed, and its optimal dose should be 0.25×10^7 CFU/500 mg of substrates (Wang *et al*., 2015).

Study results obtained by Peng *et al*. (2012) prove that the fermentation product *B. subtilis natto* was effective in increasing the lactation performance of dairy cows at the beginning of lactation. Adding *S. cerevisiae* yeast to the diet of Holstein cows increased rumen fermentation and improved serum metabolic profile, and daily milk yield increased by 4% 3, 7, 11, 15, and 19 weeks after calving (Kumprechtová *et al*., 2019).

Thus, the quantitative and qualitative indicators of milk produced depend both on the composition of the probiotic feed additive and on the lactation period of cows.

Since the interest lies in the short-term as the most rational feeding of probiotic feed additives to increase milk yield in cows, it was decided to conduct a study on the effect of various yeasts on improving their lactation in its most substantial first period – milking. Considering previous results, additional observations were made regarding the content of various yeast cultures *S. cerevisiae* in a probiotic feed supplement.

The second stage the study was conducted during the most critical winter-spring period for feeding. According to the indicators of the difference in the arithmetic mean indicators of the control milking of cows of the experimental groups, a positive effect of the probiotic feed additive "Immunobacterin-D" with the content of both conventional and experimental yeast strains was established in comparison with the control group. The maximum increase in milk yield was observed on the 24th day of the experiment in all groups: in the first group, the indicator was 23.75 ± 2.27 L; in the second – 26.88 ± 0.39 L. Figures 1 and 2 show the analysis of indicators of the difference in milk productivity between groups of animals.

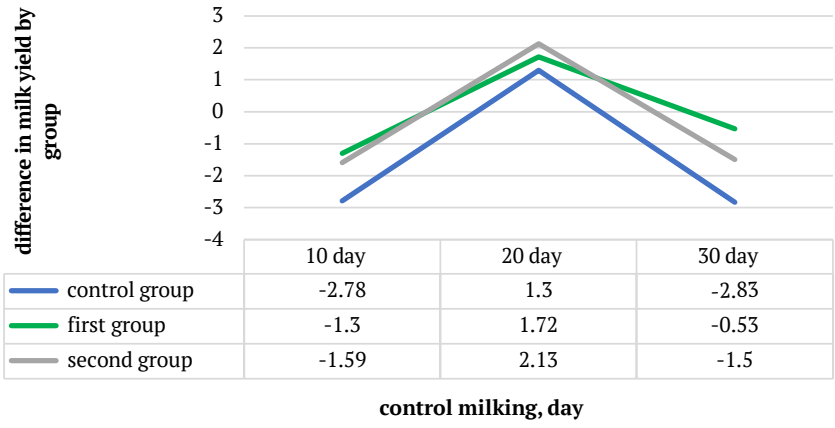

Figure 1. Analysis of milk yields (L) of cows of experimental groups

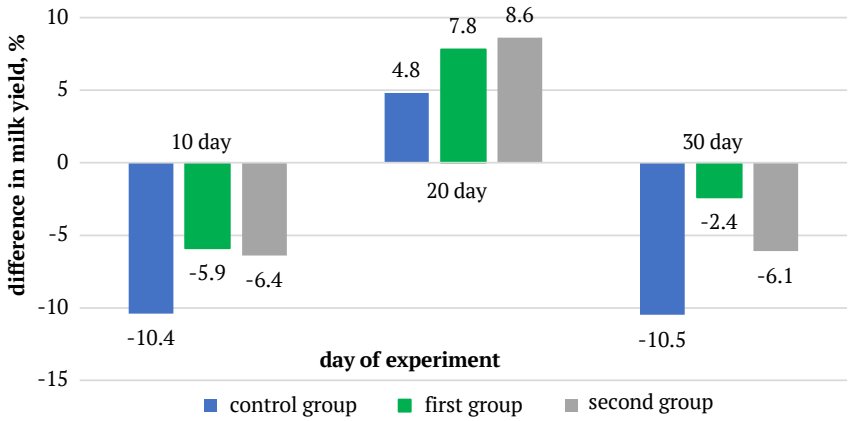

Figure 2. Indicators of the difference (%) of milk produced by cow groups

An increase in milk yield by 2.13 L compared to the initial one was observed on the 24th day of the experiment in cows of the second group, which were fed experimental yeast strains, and in cows of the first experimental group, an increase in the number of milk yields by 1.72 L was noted, in the control group – by 1.3 L. This pattern is displayed as a percentage (Fig. 2). The results of studies indicate a positive effect of the feed additive "Immunobacterin-D" on increasing milk yield, which eliminates the effect of negative factors.

The results of the study coincide with those of other authors (Poppy *et al.*, 2012; Maamouri *et al.*, 2014; Para *et al.*, 2018) on the positive effect of combined probiotic feed additives with yeast on the milk productivity of cows. As noted in the literature review, yeast supplements increase milk yield in the first half of lactation, and milk fat content increases at the end of lactation, which allows using them during different lactation periods (Ayad *et al.*, 2013; Bakr *et al.*, 2015; Sun *et al.*, 2021). However, the effectiveness of the application varies depending on the diet and strain, which is confirmed by the results obtained. Microbiological and biochemical processes in the rumen depend on the content of coarse and concentrated feed in the diet (Alugongo *et al.*, 2017). Cellulose is fermented by bacteria that are very sensitive to acidic environments. They function better at pH 6.4-7.0 which is important to know when combining feeds (Kalachniuk & Kalachniuk, 2015) to achieve optimal pH. In addition, cellulolytic microbes are sensitive to fats. If fat is more than 5% of the dry weight of the feed substance, then microorganisms sharply reduce their activity. The action of a probiotic feed additive with feed yeast is improved by its high antioxidant activity (Liu *et al.*, 2018). The supplement intake should be daily, but not permanent and not long-term to get the effect of yeast in manipulating the rumen microbiome (Ogbuewu *et al.*, 2018; Lytvynenko & Yukhymchuk, 2021).

Analysis of the effect of the probiotic feed additive "Immunobacterin-D" on milk quality was determined by comparing milk taken before and after feeding. As a result, in the first group of animals, patterns similar to the previous experiment were obtained, since milk samples were taken from cows on the 40-70 day of lactation (Table 4). In cows of the first group, the fat content of milk decreased by 0.34%, the density increased by 0.55%, and the protein content by 0.02%. Considering the indicators of the control group as physiological, then there is a tendency

to increase the percentage of fat content and reduce the density and protein content. A similar trend is observed in animals of the second experimental group, in which the fat content of milk increased by 0.45% after feeding a probiotic feed additive, and the density – by 1.4%, the protein content decreased by 0.1%. In cows of the first experimental group, the fat content of milk decreased by 0.67%, and in the second – by 0.44% ($P < 0.05$). However, the protein content in the milk of cows in the first experimental group increased by 0.06%, in the second group, it decreased by 0.06% compared to the control group. In the first group of cows, milk density increased by 0.46% ($P < 0.01$), and in the second group, on the contrary, it decreased by 0.01%.

Table 4. Influence of yeast cultures *S. cerevisiae* on cow's milk quality indicators

No.	Fat content, %			Density, °A			Protein, %		
	before conducting the experiment	after completing the experiment	difference in values	before conducting the experiment	after completing the experiment	difference in values	before conducting the experiment	after completing the experiment	difference in values
Control group									
1.	3.21	3.5	0.29	29	26.8	-2.2	3.11	2.93	-0.18
2.	3.13	3.7	0.57	29.8	27.6	-2.2	3.17	3.02	-0.15
3.	3.94	4.64	0.7	29	26.1	-2.9	3.17	2.97	-0.2
4.	4.2	3.8	-0.4	30.4	27.9	-2.5	3.42	3.06	-0.36
M ± m	3.62 ± 0.28	3.91 ± 0.23	0.29 ± 0.21	29.55 ± 0.34	27.1 ± 0.41	-2.45 ± 0.14	3.225 ± 0.06	2.99 ± 0.03	-0.2 ± 0.04
		0.29			-2.45			-0.22	
First experimental group									
5.	3.48	3.36	-0.12	28	28.4	0.4	3.04	3.06	0.02
6.	3.89	3.37	-0.52	26.7	27.8	1.1.	2.96	3.01	0.05
7.	3.98	3.3.	-0.68	27.4	29.1	1.7	3.04	3.13	0.09
8.	2.95	2.93	-0.02	29.1	28.1	-1	3.09	3	-0.09
	3.58 ± 0.23*	3.24 ± 0.1*	-0.34 ± 0.14**	27.8 ± 0.47*	28.35 ± 0.25*	0.55 ± 0.5***	3.03 ± 0.02*	3.05 ± 0.03	0.02 ± 0.03**
		-0.34			0.55			0.02	
Second experimental group									
9.	3.03	3.3.	0.27	27.3	27.8	0.5	2.94	3	0.06
10.	3.52	3.3.	-0.22	26.7	25.7	-1	2.93	2.81	-0.12
11.	2.91	3.89	0.98	28.8	27.7	-1.1	3.06	3.05	-0.01
12.	2.63	3.39	0.76	30.3	26.3	-4	3.18	2.87	-0.31
M ± m	3.02 ± 0.16*	3.47 ± 0.13**	0.45 ± 0.23	28.28 ± 0.8*	26.88 ± 0.56	-1.4 ± 0.81	3.03 ± 0.06**	2.93 ± 0.06	-0.1 ± 0.07
		0.45			-1.4			-0.1	

Note: ****P < 0.001; **P < 0.05; *P < 0.01 compared to the control group*

B. licheniformis also contributes to an increase in the protein content in milk (Qiao *et al*., 2010), in contrast to *B. subtilis,* the use of which did not affect digestion in the rumen. The results of the study prove the pronounced positive effect of yeast in the composition of the probiotic feed additive "Immunobacterin-D" on milk quality indicators and predict the prospect of further research with the *S. cerevisiae* experimental strain. Moreover, the rumen microbiota of highly productive and low-yielding cows during lactation is different and determines new strategies for improving the productivity of dairy cows.

The results of the analysis of biochemical parameters of cow blood serum are shown

in Table 5. In the blood serum of cows of the first experimental group, the total protein content increased by 4.9% (P < 0.05), in the second experimental group by – 9.0% (P < 0.01) compared to the control group. The concentration of glucose in the blood serum of cows of the first experimental group decreased by 23.3% (P < 0.01), and in the blood serum of cows of the second experimental group, on the contrary, it increased by 10.1% (P < 0.01) compared to the control group. The concentration of urea in the blood serum of cows of the second group increased by 84.8% (P < 0.05) compared to the control group.

The content of creatinine and phosphorus in the blood serum of cows of the first group increased by 18.6% (P < 0.05) and by 31.4% (P < 0.05), respectively, compared to the control group.

In the blood serum of cows of the first and second experimental groups, a decrease in the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was established compared to the control group. Thus, in the blood serum of cows of the second experimental group, the ALT content decreased by 25.6% (P < 0.05) compared to the control group. Therewith, the AST/ALT ratio in the blood serum of cows in the second study increased by 9.4% (P < 0.05) compared to the control group. No statistically substantial difference was established for the remaining indicators examined.

Table 5. Biochemical parameters of cow blood serum under the influence of *S. cerevisiae* cultures as part of the feed additive "Immunobacterin-D", G ± m, n = 6

No. п/п	Indicator	Before conducting the experiment			After completing the experiment		
		first experimental group	second experimental group	control group	first experimental group	second experimental group	control group
1.	Total Protein, g/L	76 ± 3.69	77.8 ± 4.39	82.35 ± 0.14	87.68 ± 1.79**	91.15 ± 0.67*	83.6 ± 1.62
2.	Albumins, g/L	38.28 ± 0.88	31.13 ± 1.66	31.13 ± 1.01	36.33 ± 2.50	30.27 ± 1.50	30.9 ± 0.17
3.	Glucose, mmol/L	1.07 ± 0.06	1.26 ± 0.10	1.86 ± 0.15	1.22 ± 0.08*	1.75 ± 0.12*	1.59 ± 0.03
4.	Urea, mmol/L	2.7 ± 0.08	3.0 ± 0.29	4.25 ± 0.19	2.28 ± 0.07	4.25 ± 0.19**	2.30 ± 0.08
5.	Creatinine, mmol/L	226.75 ± 9.86	168.98 ± 5.78	171.1 ± 1.57	200.3 ± 9.01**	187.63 ± 18.10	168.85 ± 1.39
6.	Calcium, mmol/L	2.62 ± 0.09	2.58 ± 0.03	2.7 ± 0.04	2.95 ± 0.18	2.58 ± 0.12	2.65 ± 0.02
7.	Phosphorus, mmol/L	1.45 ± 0.09	1.46 ± 0.06	1.9 ± 0.004	2.05 ± 0.14**	1.66 ± 0.25	1.56 ± 0.05
8.	Total bilirubin, μmol/L	2.61 ± 0.19	4.88 ± 0.53	5.97 ± 0.11	3.90 ± 0.17**	3.68 ± 0.42	4.08 ± 0.42
9.	AST, U/L	83.98 ± 6.38	79.43 ± 4.28	75.95 ± 4.74	57.93 ± 7.26	69.8 ± 10.73	77.3 ± 3.52
10.	ALT, U/L	19.25 ± 1.08	16.8 ± 1.28	25.65 ± 2.59	27.3 ± 1.72	22.77 ± 1.38**	30.6 ± 1.95
11.	Correlation of AST/ ALT	4.33 ± 0.15	4.89 ± 0.38	3.03 ± 0.12	2.27 ± 0.41	2.79 ± 0.45**	2.55 ± 0.05

Note: ****P < 0.001; **P < 0.05; *P < 0.01 compared to the control group*

The concentration of glucose in the blood serum of cows of all experimental groups indicates the occurrence of an energy imbalance at the beginning of lactation. Feeding the probiotic feed supplement "Immunobacterin-D" slightly affects the increase in the concentration of glucose in the blood serum of cows. However, it causes a likely increase in the total protein

content, but not due to the albumin fraction. Therewith, there is a substantial effect of the feed additive "Immunobacterin-D" on the value of the ratio of AST/ALT activity in the blood serum of cows of experimental groups, primarily due to a decrease in AST activity and restoration of the structural and functional state of hepatocytes, which is also confirmed by the results of the study by Kumprechtová *et al*. (2019). This is assumably due to the fact that live yeast has a positive effect on the energy balance at the cellular level and has a hepatoprotective effect. According to the results of the analysis of biochemical parameters of blood serum, an increase in the content of phosphorus (by 41%) and total bilirubin (by 49%) in cows of the first experimental group was also established. Thus, the applied symbiotic cultures contribute to improving the health of cows during lactation.

Conclusions

Application of probiotic feed additive "Immunobacterin-D" with *S. Cerevisiae, B. Subtilis, B. licheniformis* in cows provides an increase in the average daily milk yield in the post-calving period.

When feeding the probiotic feed additive "Immunobacterin-D" in cows from 4 to 90 days after calving, an increase in the value of milk yields was noted with the greatest effect of manifestation on the 30-60 days after calving. Consumption of probiotic feed additives from 70 to 90 days was accompanied by a decrease in the amount of milk produced but contributed to an increase in the fat content of milk in cows of the experimental group.

Analysing the effect of probiotic feed additives on animals of the experimental group for the entire study period, a positive effect in the increase in the average daily amount of milk from 1.2 to 2.5 L should be noted. The difference in milk yield difference with the control group of cows in favour of the experimental group is from 1.9 to 0.7 L.

The probiotic feed additive "Immunobacterin-D" in both versions during the milking period shows the best effect on the 24th day of use. The maximum result during this period was obtained with the experimental strain. In particular, the milk allowance for the cow group increased to 2.13 litres, and the milk fat content increased by 0.45%.

Application of probiotic feed additive "Immunobacterin-D" with strain *S. cerevisiae* AF 338 contributed to an increase in the amount of milk produced in cows of the experimental group by 1.73 L, while in the control group – only by 1.3 L. The milk fat content decreased by 0.34% from the previous value and by 0.67% compared to the control. Summarising the data on the total amount of milk produced for the entire study period, positive trends can be traced, mainly in cows of the first group that consumed conventional yeast and showed stress resistance to environmental factors.

The probiotic feed supplement "Immunobacterin-D" in both versions used helps to improve the health status of cows in the dairy area of productivity, as indicated by the equalisation of the ratio of AST/ALT activity in their blood serum. This has a positive effect on the processes of liver cell renewal. In addition, cows of the first group have a substantial increase in the content of phosphorus and total bilirubin in the blood serum.

If it is possible to regulate the factors of external influence on productive animals, the use of a probiotic feed additive with an experimental strain may become more promising, as indicated by obtaining more milk yield and increasing the fat content of milk.

The stidy on *Sashagomuses cerevisiae* as part of probiotic feed additives predicts the prospect of further research with an experimental *yeast* strain.

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None.

Conflict of Interest

None.

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Вплив пробіотичної кормової добавки «Імунобактерин-D» на продуктивність корів чорно-рябої породи під час лактації

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Анотація. В Україні, як і для кожної країни світу, важливим завданням є розробка ефективної стратегії розвитку з виробництва натуральних молочних продуктів шляхом використання пробіотиків. Згодовування тваринам ПКД виявляє різну ефективність залежно від їх віку, особливостей мікробіоми кишечнику, складу кормів раціону та технології виробництва. Мета дослідження полягала у з'ясуванні найефективнішого періоду застосування ПКД «Імунобактерин-D» та у виборі культури дріжджів для підвищення молочної продуктивності корів. Ефективність ПКД оцінювали за кількістю надоєного молока, а його якість – шляхом використання ультразвукового аналізатора «Екомілк». Стан здоров'я корів контролювали за результатами спектрофотометричного дослідження біохімічних показників сироватки крові на біохімічному аналізаторі LabLine-010 (Австрія). В результаті було визначено, що оптимальним періодом застосування ПКД є 30-60 доба після отелення. На 13 добу згодовування ПКД надої молока в корів дослідної групи збільшувалися на 1,2-2,5 л/добу. Різниця у кількості надоїного молока між тваринами дослідної і контрольної груп складала 0,7-1,9 л/добу. З 70 доби після отелення застосування коровам ПКД не викликало змін в обсягах надою молока, але сприяло підвищенню його жирності. Впродовж 21 доби згодування коровам експериментального штаму *S. cerevisiae* у складі ПКД відмічали максимальне збільшення надоїв молока – на 2,13 л, а молочного жиру – на 0,45%. Тоді як застосування ПКД з традиційним штамом *S. cerevisiae* AF 338 сприяло збільшенню на 1,73 л надоїв молока, а у контрольної групи корів лише на 1,30 л. За результатами біохімічних досліджень сироватки крові доведено позитивний вплив ПКД «Імунобактерин-D» на стан здоров'я корів. ПКД може бути рекомендована для згодовування коровам на виробництві, що сприятиме підвищенню надоїв молока та зміцненню стану їхнього здоров'я

Ключові слова: молочне скотарство; дріжджі; *Bacillus subtilis*; *Bacillus licheniformis*; *Saccharomyces cerevisiae*

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Infrared spectroscopy and biochemical parameters of rat tissues under heavy metal poisoning conditions

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Abstract. The increasing level of anthropogenic environmental pollution and effective means to reduce the negative impact of xenobiotics on animal and human health is an urgent problem today. Considering this, the purpose of the study is to examine the effect of heavy metals on accumulation processes under poisoning conditions, and biochemical parameters in the body of rats. Analogue groups were formed of rats of the same age, gender, and body weight to conduct the study. Rats were poisoned with solutions of copper sulfate, zinc sulfate, cadmium sulfate, and lead nitrate for 14 days. Using the method of infrared spectroscopy, substantial differences in the spatial structure of protein components in intact and poisoned animals were established. The difference between the spectral characteristics of the examined tissues is clearly demonstrated by the statistical indicators of skewness and kurtosis. It was determined that poisoning of rats with copper, zinc, cadmium, and lead ions affects the course of glycolysis reactions and the tricarboxylic acid cycle, which leads to a likely increase in serum concentrations of lactate and pyruvate, oxaloacetate and α-ketoglutarate and a decrease in Malate content compared to intact rats. It was established that under the conditions of poisoning, there is also a substantial increase ($P < 0.05$) in the content of the examined heavy metals in the blood, liver, and kidneys. In animals poisoned with heavy

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metals, a decrease in the pool of free amino acids in the kidneys is observed. In particular, the content of aspartic acid, valine, glycine, tyrosine, and cystine (more than 1.5 times) in the kidneys of such rats decreases; alanine, leucine, serine, taurine, threonine, phenylalanine (more than 2.0 times), lysine – 3.4-4.9 times. Therewith, an increase in the level of isoleucine and methionine by 1.3-1.5 times, ornithine – by 1.8-2.1 times, and glutamic acid – by 4.4-5.3 times in rats of the experimental group compared to intact ones was identified. The results of the study can be helpful in the professional activities of doctors of veterinary medicine, toxicologists, biologists, and environmentalists and used to control the quality of livestock products, conduct toxicological studies, and analyse environmental objects

Keywords: copper; zinc; cadmium; lead; blood; internal organs

Introduction

A large number of various chemical compounds, including heavy metals, that enter the environment require research to establish their impact on the biota and find ways and means to reduce the negative impact on animal and human health (Cao *et al.,* 2018; Engwa *et al.,* 2019; Djordjevic *et al.,* 2019). Balali-Mood *et al.* (2021) investigated the impact of anthropogenic environmental pollution with heavy metals, toxic to all living things, which necessitates a thorough study and establishing the biochemical effect on the body individually and under complex impact conditions. Wallace & Buha (2020) determined the complex effects of heavy metals and pesticides on the body and their probable involvement in carcinogenesis. Still, their work lacks consideration of biochemical parameters and the use of infrared spectroscopy. The body has the ability to accumulate heavy metals at the cellular, tissue, and organ levels of the organisation of living matter, which is primarily due to the specific features of metal accumulation in various tissues and organs, and the action of protective mechanisms that limit their migration (Kumar & Sharma, 2019; Ohta & Ohba, 2020; Schaefer *et al.,* 2020).

Schaefer *et al.* (2020), showed that the constant increase in anthropogenic load on environmental objects in the form of chemical, physical, and biological compounds has quite serious consequences, but they focused on toxicological aspects without investigating biochemical parameters and did not use the method of infrared spectroscopy. Targeted impact on one of the objects of the environment through a chain reaction of relationships in the biosystem can cause changes in the state of the other, which in turn is a real threat to the gene pool of all living beings and can lead to an increase in mutagenic pressure on the population of both animals and humans. Therewith, total pollution of atmospheric air, soil, drinking water, and food products with a wide range of xenobiotics can cause genetically determined pathologies manifested by congenital malformations, cytogenetic disorders in germ and somatic cells (Kumar & Sharma, 2019; Li & Saleem, 2022).

Ortiz *et al.* (2022) examined an environmental biota that is constantly in contact with a large a wide variety of chemical compounds. The authors note that not only substances that are part of food products and are contained in water and enter the air are essential for the body (proteins, lipids, carbohydrates, vitamins, macro - and microelements), but also those that do not represent nutritional value or, even in certain doses, are able to show toxic effects on the mammalian body being components of both

natural (essential oils, dyes, alkaloids, tannins) and artificial origin (preservatives, flavouring and aromatic additives, pesticides, and medicinal and hormonal preparations that were added during animal feeding), without investigating the effect on metabolic processes. Ohta & Ohba (2020) indicated that a large group of substances not involved in metabolism are called xenobiotics. Solving numerous problems due to the negative impact of xenobiotics on a living organism is extremely relevant and one of the priority tasks for humanity; however, the existing studies did not investigate the effect of heavy metals on metabolic processes and did not use the infrared spectroscopy method, as noted in the materials of this paper.

It is important to establish and examine specific molecular targets that ensure the selectivity of xenobiotics biotransformation and detoxification to understand their biological effect and the limits of the body's tolerance to them. As Stefanac *et al.* (2021) note, detoxification for organic compounds is associated with their chemical transformation, including partial oxidation of molecules, and in the case of metal ions, the neutralisation process consists in their deposition as part of specific functional groups and molecules. Aemere *et al.* (2020) report that this process occurs due to the specific features of the binding constants of metal ions to certain ligands.

Thus, the scientific literature does not fully cover the issue of animals poisoned with heavy metals, namely their effect on metabolism in tissues depending on the type of poisoning and the specific features of their effect on the content of other heavy metals, using the method of infrared spectroscopy and simultaneous monitoring of biochemical parameters.

Objective – to investigate how heavy metals affect the accumulation of substances under toxic conditions, and biochemical parameters in the body of rats.

Literature Review

Heavy metals can often interact with biological systems, losing one or more electrons and forming metal cations that are related to the nucleophilic centres of vital macromolecules. Several acute and chronic toxic effects of heavy metals affect various organs in the body. Gastrointestinal and kidney dysfunction, nervous and immune system disorders, skin damage, vascular damage, birth defects, and cancer are examples of complications of heavy metal toxicity. In particular, Kumar & Sharma (2019) indicate that simultaneous exposure to two or more metals can have a carcinogenic effect. However, they did not determine their effect on biochemical parameters and did not examine tissues using infrared spectroscopy, which is presented in this study. The fact that heavy metals are carcinogenic to mammals is an important aspect of chronic exposure. Although the exact mechanism is not fully understood, abnormal changes in the genome and gene expression are considered the main process. Carcinogenic metals such as arsenic, cadmium, and chromium can disrupt the synthesis and renewal of DNA. Kim *et al.* (2019), covered the toxic mechanism of action of heavy metals on the body, which functions in a similar way, usually through the formation of reactive oxygen species, inactivation of enzymes, and inhibition of antioxidant protection, yet infrared spectroscopy was not used.

Li *et al.* (2022) showed that some heavy metals cause toxicity according to a specific scheme and selectively bind to specific macromolecules. The involvement of metalloproteins (metal + carrier protein) by tissues is conducted by their fixing on specific membrane receptors, in combination with which they enter cells, where they are destroyed by lysosomal enzymes, and the metal is restored again and can be reused in metabolism. As is often the case, the implementation of biological functions depends on the interaction between ligand-binding residues

and metal ions, and the molecular mechanism involves the binding of metal ions to specific residues in proteins. Some of these amino acid residues are stored in the protein family, and they can create key stable interactions or play an important functional role. Each family has its own preserved residues, which are involved in protein recognition and metal ion binding. It can combine with cadmium with high affinity and play a role in detoxifying heavy metals and maintaining a stable state of major metal ions in cells. Among them are metallothioneins, which, due to their unique metal binding characteristics, can provide resistance to cadmium and contribute to the stable state of zinc.

Gazwi *et al.* (2020), investigated the formation of reactive oxygen species and release of cytochrome C, which may play a role in the hepatotoxic effects of heavy metal exposure. An analysis of male *Wistar* rats exposed to heavy metals showed intensive development of oxidative stress and activation of caspase-9, the initiator of apoptosis signalling.

Entering cells, heavy metals bind mainly to functional groups of proteins and others (for example, the formation of metallothioneins or compounds involved in biotransformation (Kim *et al.,* 2019; Aemere *et al.,* 2020; Samuel *et al.,* 2021), which may be related to detoxification mechanisms. Therewith, this is the cause of metabolic disorders and an explanation of the high toxicity of heavy metals (Saha & Paul, 2019; Proshad *et al.,* 2020). However, Kim *et al.* (2020) and Luo *et al.* (2020) note that the binding strength of heavy metal ions to the functional groups of various biopolymers may vary, which is a manifestation of the different toxicity of these toxicants.

The interaction of metals entering the cell can cause antagonism, synergy, and affect to a certain extent the content of individual metals in cells and, accordingly, in tissues. This is best demonstrated by researchers on the example of plants, when the cadmium content in the soil is too high, there is an obstacle to the supply of iron to the plant, which causes growth retardation, chlorosis, and other symptoms similar to iron deficiency (Asad *et al.,* 2019; Kashif Irshad *et al.,* 2020). The described symptoms are caused by direct or indirect interaction between cadmium and iron. The toxic effects of cadmium can cause phosphorus deficiency or reduce manganese intake and prevent the intake and transport of several essential nutrients (such as calcium, magnesium, phosphorus, and potassium) and water by plants. In addition, Kashif Irshad *et al.* (2020) note that cadmium is able to enter the cell through carriers of basic elements. Due to the lack of specificity of these vectors, it is usually assumed (Engwa *et al.,* 2019) that it is absorbed by transporters of basic elements (such as zinc, iron, and calcium). The following research expands and deepens knowledge about the effects of heavy metals on the animal body.

Materials and Methods

The research was conducted during 2020-2022 based on the National University of Life and Environmental Sciences of Ukraine. Experimental studies were accompanied by the use of three-month ols rats (males) with a similar body weight of 200 ± 15 g, kept in a vivarium under standard conditions. From animal analogues, five groups were formed: the first – control (intact), rats of the second, third, fourth, and fifth groups were orally administered solutions of copper sulfate, zinc sulfate, cadmium sulfate, and lead nitrate, respectively, in established toxicological doses. Intoxication of animals was conducted for fourteen days, then they were decapitated under ether anaesthesia, followed by the collection of blood samples and pieces of organs (liver, kidneys) for further research.

All work was conducted in accordance with the provisions of the European Convention for the protection of vertebrate animals used

for experimental and other scientific purposes (1986). The content of copper, zinc, cadmium and lead in blood, liver, and kidney samples selected for research was determined by atomic absorption spectrometric method using the absorption mode on AAS SpectrAA-55b, Varian Company (USA). Standard solutions of these metals were used as controls.

Infrared spectroscopy was used to examine the internal organs (liver, kidneys) of rats, which was performed on the infrared (IR)-Fourier spectrophotometer "Nicolet 380", manufactured by Thermo electron corporation (USA). The obtained spectra were recorded in steps of 4 cm^{-1} using a device for light reflection from the sample, the working wavelength range was 650-4000 cm^{-1}. The obtained absorption spectra were processed using specially developed programs – LabSolution IR, IR Tutor V1.1 Software Kit, MestRe-C (Smith B.C., 2016; Vinet & Anil, 2020).

Metabolites of the tricarboxylic acid cycle (malate, oxaloacetate, α-ketoglutarate) and glycolysis (lactate, pyruvate) in blood serum were determined by enzymatic methods (Vlizlo *et al*., 2012). At the beginning of the biochemical analysis, blood serum was obtained by centrifugation on an MPW-352 centrifuge (Poland) with a rotation speed of 3000 rpm, for 15 min. Specific reagents of lactate dehydrogenase (LDH, EC 1.1.1.27), malate dehydrogenase (MDG, EC 1.1.1.37) from Reanal (Hungary) and oxidised and reduced forms of pyridine nucleotides from Serva (USA) were used for the analytical study of metabolites of intermediate carbohydrate metabolism (glycolysis, tricarboxylic acid cycle). The concentration of metabolites was determined spectrophotometrically on Specord M40 UV-VIS, manufactured by Carl Zeiss (Germany) at $\lambda = 340$ nm. Determination of the content of free amino acids in the kidneys was conducted by ion exchange chromatography on an amino acid analyser model AAA t-339m of Mikrotechna (Czech Republic).

Experimental data were processed using generally accepted methods of variation statistics. Statistical data calculations were performed using Microsoft Excel 2007.

Results and Discussion

The cumulative properties of heavy metals in the body of rats were examined. Figure 1 (A) shows the copper content in the blood, liver, and kidneys of rats. It was established that during their experimental poisoning, the content of this metal increased: in the blood by 1.4 times, in the liver by 1.6 times, and in the kidneys by 1.5 times compared to animals of the control group.

For poisoning experimental rats with zinc (Fig. 1 (B), the content of the test metal increased as follows: in the blood by 1.5 times, in the liver – by 2.5 times, and in the kidneys – by 1.6 times, compared with the control group of animals. Poisoning of rats with cadmium ions led to an increase in its content: in the blood – by 27 times, in the liver – by 243, and in the kidneys – by 23 times compared to animals of the control group (Fig. 1 (B).

In addition, the mechanisms of interaction of the examined heavy metals with each other in the rat body were examined. The results of experimental studies showed that in the blood of rats poisoned with copper sulfate, cadmium increased by 7.5 times and zinc by 1.8 times, compared with intact animals. In the liver of animals poisoned with copper ions, the content of cadmium increased by 6.9 times, zinc – by 1.8 times, and lead – by 1.4 times compared to intact animals. In the kidneys, cadmium content increased by 2.2 times, zinc and lead, respectively, by 1.5 times, compared with the intact group of rats (Table 1).

Poisoning of rats with zinc sulfate resulted in an effect on the content of heavy metals in all types of biological material studied. Thus, in the blood and liver of poisoned rats, the level of cadmium increased by 5.8 and 6.5 times,

respectively, the copper content remained unchanged, and lead increased only in the liver by 1.3 times (Table 2). Therewith, the content of cadmium in the kidneys increased by 2.4 times, copper – by 1.2 and lead – by 1.7 times compared to intact animals.

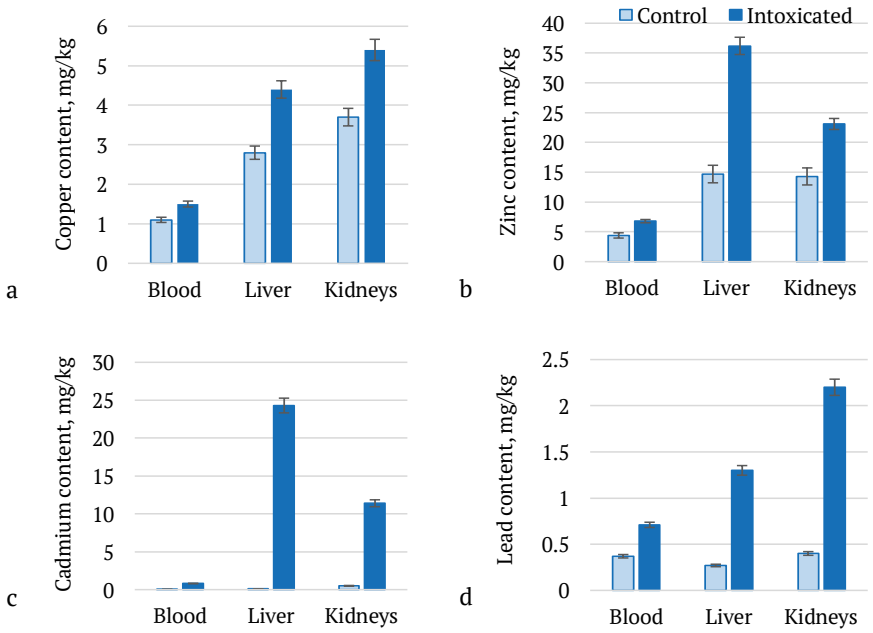

Figure 1. Content of heavy metals (a – copper, b – zinc, c – cadmium, d –lead) in the blood, liver, and kidneys of toxic rats

Notes: *(M ± m, n = 8), * P < 0.05, compared to the control*

Table 1. Content of cadmium, zinc, and lead in the blood and internal organs of rats poisoned with copper sulfate, mg/kg (M ± m, n = 8)

Metal	Intact rats	Poisoned rats
	Blood	
Cadmium	0.04 ± 0.002	0.30 ± 0.02*
Zinc	2.39 ± 0.71	4.23 ± 1.12*
Lead	0.38 ± 0.02	0.43 ± 0.03
	Liver	
Cadmium	0.14 ± 0.01	0.96 ± 0.04*
Zinc	18.74 ± 2.34	34.25 ± 3.17*
Lead	0.29 ± 0.02	0.40 ± 0.02*
	Kidneys	
Cadmium	0.52 ± 0.01	1.15 ± 0.05*
Zinc	12.73 ± 1.82	19.24 ± 2.71*
Lead	0.43 ± 0.02	0.64 ± 0.03*

Note: ** P < 0.05, compared to intact rats*

Table 2. Content of cadmium, copper, and lead in the blood and internal organs of rats with zinc sulfate poisoning, mg/kg (M ± m, n = 8)

Test metal	Intact rats	Poisoned rats
	Blood	
Cadmium	0.04 ± 0.002	0.23 ± 0.018*
Copper	1.13 ± 0.053	1.32 ± 0.064
Lead	0.38 ± 0.022	0.41 ± 0.020
	Liver	
Cadmium	0.14 ± 0.008	0.91 ± 0.049*
Copper	2.83 ± 0.082	3.29 ± 0.093
Lead	0.29 ± 0.018	0.38 ± 0.022*
	Kidneys	
Cadmium	0.52 ± 0.01	1.24 ± 0.06*
Copper	3.73 ± 0.09	4.64 ± 0.10*
Lead	0.44 ± 0.02	0.73 ± 0.04 *

Note: ** P < 0.05, compared to intact rats*

For cadmium poisoning of rats, a substantial increase in the content of heavy metals in various biological materials was also noted compared to their quantitative values in intact animals (Table 3). Thus, the content increased: in the blood, copper and zinc, respectively, by 1.6 times, lead – by 1.4 times; in the liver, copper increased by 1.5 times, zinc by 1.8, and lead by 1.2 times. Therewith, the content of copper in the kidneys increased by 5.2 times, zinc by 1.8 times, and cadmium by 1.7 times compared to intact rats.

Table 3. Content of zinc, copper, lead in the blood and internal organs of rats with cadmium sulfate poisoning, mg/kg (M ± m, n = 8)

Test metal	Intact rats	Poisoned rats
	Blood	
Zinc	2.39 ± 0.71	3.88 ± 0.93*
Copper	1.13 ± 0.05	1.80 ± 0.09*
Lead	0.38 ± 0.02	0.52 ± 0.04*
	Liver	
Zinc	18.74 ± 2.34	32.83 ± 3.67*
Copper	2.83 ± 0.08	4.25 ± 0.09*
Lead	0.29 ± 0.02	0.35 ± 0.02
	Kidneys	
Zinc	12.73 ± 1.82	23.25 ± 1.99*
Copper	3.73 ± 0.09	19.52 ± 1.79*
Lead	0.44 ± 0.02	0.75 ± 0.04*

Note: ** P < 0.05, compared to intact rats*

Under the conditions of lead poisoning of rats, substantial changes in the content of heavy metals were noted both in the blood and in internal organs (liver, kidneys). Thus, the blood content of zinc increased by 1.5 times, copper by 1.4, and cadmium – by 12.3 times, compared to intact rats. In the liver, there was an increase in the content of zinc – 1.6 times,

copper – 1.3 times, and cadmium –7.5 times compared to the control group. In the kidneys of rats, an increase in the content of all heavy metals examined was also noted: zinc by 1.7 times, copper by 4.9 times, and cadmium by 2.6 times compared to intact rats (Table 4).

Table 4. Content of zinc, copper, cadmium in the blood and internal organs of rats with lead nitrate poisoning, mg/kg (M ± m, n = 8)

Test metal	Intact rats	Poisoned rats
	Blood	
Zinc	2.39 ± 0.71	3.46 ± 0.83*
Copper	1.13 ± 0.05	1.53 ± 0.03*
Cadmium	0.04 ± 0.002	0.49 ± 0.03*
	Liver	
Zinc	18.74 ± 2.34	29.19 ± 3.22*
Copper	2.83 ± 0.08	3.78 ± 0.91*
Cadmium	0.14 ± 0.008	1.05 ± 0.05*
	Kidneys	
Zinc	12.73 ± 1.82	21.07 ± 1.95*
Copper	3.73 ± 0.09	18.25 ± 2.82*
Cadmium	0.52 ± 0.01	1.33 ± 0.06*

Note: ** P < 0.05, compared to intact rats*

The results of the examination of infrared spectra of internal organs under conditions of cadmium poisoning of rats provide a general spectrum. It is the sum of the spectra in which the absorption bands corresponding to various functional groups of organic substances and water molecules are calculated. The number of characteristic absorption bands in accordance with the groups of atoms, and the intensity and maximum positions on the obtained infrared spectra, contribute to determining the component composition of substances that are part of the macromolecules of the examined organs, and the structure of compounds and their interaction and transformation under pathological conditions. The absorption spectra are shown in Fig. 2-5.

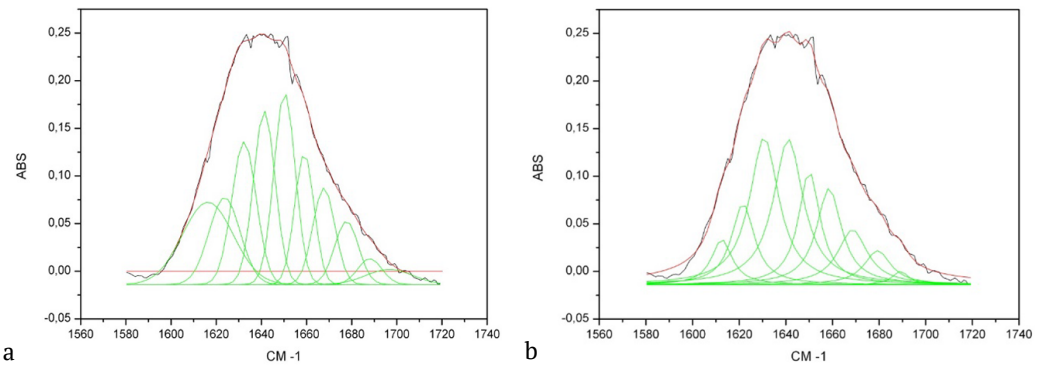

Figure 2. Infrared spectra of examined liver samples from intact rats with Gaussian (a) and Lorentz (b) distributions

Notes: *in Figs. 2-5, ABS – degree of absorption of infrared waves; cm-1 - reverse centimetres*

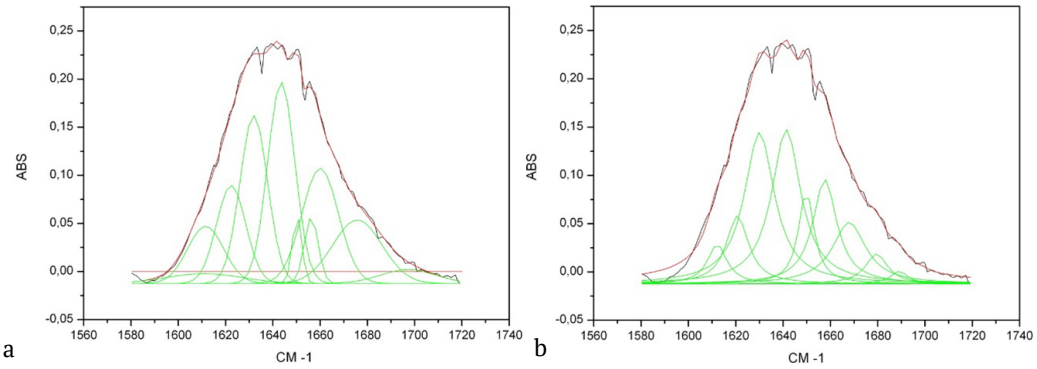

Figure 3. Infrared spectra of the examined kidney samples in intact rats with Gaussian (a) and Lorentz (b) distributions

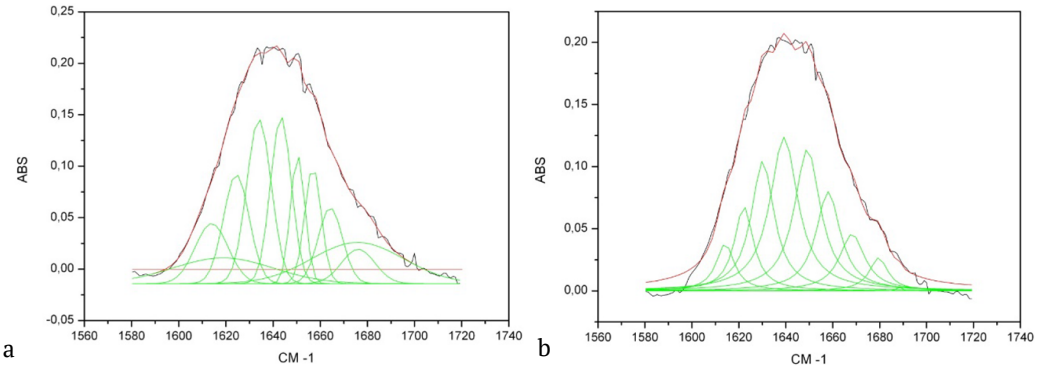

Figure 4. Infrared spectra of examined liver samples from cadmium-poisoned rats with Gaussian (a) and Lorentz (b) distributions

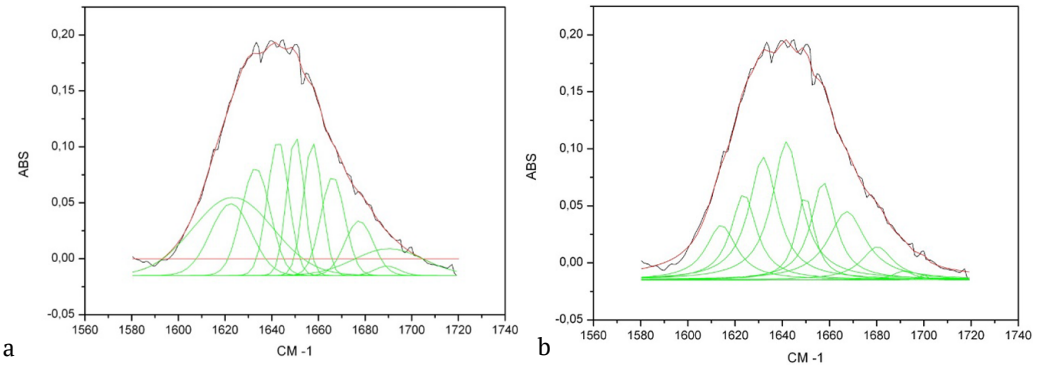

Figure 5. Infrared spectra of examined kidney samples from cadmium-poisoned rats with Gaussian (a) and Lorentz (b) distributions

Figs. 2-5 show the obtained original infrared absorption spectra of components of liver and kidney samples in intact and cadmium-poisoned animals with a Gaussian (a) and Lorentz (b) distribution. As a result of the analysis of the obtained infrared spectra, it was established that the spectrograms are represented by curves that have absorption bands with intensities of different degrees. In addition, in investigating the obtained frequency range, it was found that the maximum absorption bands at the following wavelengths: 1616 cm^{-1}, 1632 cm^{-1}, 1650 cm^{-1}, 1659 cm^{-1}, 1667 cm^{-1}, 1677 cm^{-1}, 1688 cm^{-1}, 1696 cm^{-1}. The analysis of the recorded wavelengths showed that the most intense are bands with a frequency of 1650 cm^{-1} and 1659 cm^{-1}.

Results of detailed analysis of the maximum intensity absorption bands at frequencies of 1650 cm^{-1} and 1659 cm^{-1} allow stating that these bands are characteristic of α- spiral configurations of the polypeptide chain, which indicates a predominance in the spatial structure of the liver and kidney parenchyma α- helical conformation of proteins. The data obtained are consistent with the previously published results (Brian C. Smith, 2016).

As a result of further analysis of the infrared spectra, absorption bands were detected within the wavelength range from 1640 cm^{-1} up to 1620 cm^{-1}, which is typical for β- folded protein conformations. Moreover, the maximum absorption bands with a frequency of 1622 cm^{-1}, 1627 cm^{1}, and 1633 cm^{-1} correspond to the examined liver samples of intact animals. Notably, in the mentioned frequency range (1640-1620 cm^{-1}), there is the absorption of antiparallel types β- structures (Socrates, 2004).

Therefore, the results obtained show that the proteins of liver samples in intact animals are represented by polypeptide chains β- a folded structure of the antiparallel type, which is consistent with the literature data (Smith, 2011).

When analysing the infrared spectra, the maximum absorption band at a wavelength of 1660 cm^{-1} was also established, which is caused by amorphous structures and their vibrations (Smith, 2021).

Furthermore, the presence of absorption bands in the frequency range from 1680 cm^{-1} to 1670 cm^{-1} was established. These bands are caused by fluctuations in which the bond length of carbonyl groups changes. Such fluctuations are caused by certain sites in the structures of protein molecules. The obtained spectra also show maximum absorption bands with wavelengths of 1690 and 1696 cm^{-1}. In the studied samples of liver and kidney parenchyma, absorbances were established, which indicate the presence of reversible turns in certain sections of polypeptide chains at wavelengths in the range of 1670-1688 cm^{-1} (Socrates, 2004).

The comparison of the spectrograms of intact and poisoned rats presented in Figs. 2-5 indicated that in the absorption spectra of the liver samples of animals poisoned with cadmium, there is a notable increase in the intensity of the absorption bands at the maximum frequencies of 1624 cm^{-1} and 1650 cm^{-1}. Therewith, the stability of the intensity of the absorption band at 1633 cm^{-1} was determined, which is caused by the fluctuations of flat, elongated, and twisted β-folded structures (Smith, 2016).

Using the laws of normal Gauss and Lorentz distributions, complex curves with numerical bands were decomposed and the sum of individual curves was obtained for deeper qualitative and quantitative analysis of spectrograms in experimental animals. With such a perfect analysis, substantial differences in the examined spectrograms are clearly established. The difference between the infrared spectra of the examined liver and kidney tissue samples taken from intact and cadmium-poisoned animals was demonstrated using such well-known statistical indicators as skewness and kurtosis

(Brown, 2022). In rats poisoned with heavy metals, there is a substantial increase in the content of lactate and pyruvate in the blood serum (Table 5).

Table 5. Content of glycolysis substrates and tricarboxylic acid cycle intermediates in rat blood serum under heavy metal poisoning conditions, μmol/mL (M ± m, n = 8)

Parameter	Intact rats	Poisoned rats			
		copper	zinc	cadmium	lead
Glycolysis substrates					
Lactate	3.602 ± 0.709	6.122 ± 0.918*	6.843 ± 0.955*	9.722 ± 1.056*	9.016 ± 1.012*
Pyruvate	0.113 ± 0.039	0.192 ± 0.071*	0.225 ± 0.067*	0.853 ± 0.087*	0.632 ± 0.077*
Intermediates of the tricarboxylic acid cycle					
Malat	0.033 ± 0.001	0.027 ± 0.002*	0.026 ± 0.002*	0.034 ± 0.001	0.027 ± 0.002*
Oxaloacetate	0.083 ± 0.002	0.106 ± 0.007*	0.104 ± 0.007*	0.111 ± 0.008*	0.109 ± 0.007*
α-Ketoglutarate	0.056 ± 0.001	0.076 ± 0.003*	0.075 ± 0.002*	0.082 ± 0.001*	0.079 ± 0.002*

Note: **P < 0.05, compared to intact rats*

An increase in the lactate concentration in the blood serum of rats poisoned with copper ions – 1.7 times, zinc – 1.9 times, cadmium – 2.7 times, and lead – 2.5 times compared to animals of the control group was found. The content of pyruvate in the blood serum changed towards increase as follows: for poisoning with copper ions – by 1.7 times, zinc – by 2 times, cadmium – by 7.6 times, and lead by 5.6 times compared to the control.

The examined indicators of tricarboxylic acid cycle intermediates in the blood of rats under heavy metal poisoning are presented in Table. 5.

It was established that when rats were poisoned with copper ions, the concentration of oxaloacetate and α-Ketoglutarate in the blood serum increased by 28 and 36%, respectively, compared to the control group of animals. Therewith, copper ion intoxication caused a 19% decrease in malate content compared to the intact group of rats.

When rats were poisoned with zinc ions, serum concentrations of oxaloacetate (by 25%) and alpha-ketoglutarate (by 34%) increased. Meanwhile, there was a 21% decrease in malate content compared to intact animals.

Rat poisoning with cadmium ions affected the serum intermediates of the tricarboxylic acid cycle as follows: the concentration of oxaloacetate and α-Ketoglutarate increased by 34 and 46%, respectively, and the malate content remained unchanged compared to the control.

As a result of lead ion poisoning, an increase in serum oxaloacetate (by 31%) and alpha-ketoglutarate (by 41%) and a decrease in malate (by 18%) were observed compared to intact rats.

The importance of amino acids in protein biosynthesis and highly active biological compounds has become the main prerequisite for numerous examinations of their content in body fluids and tissues both during experimental studies and during the occurrence of pathologies. In intermediate metabolism, amino acids and their derivatives play a binding role in the integration of major metabolic pathways (Kokaman, 2022).

In addition, as noted in the literature (Mo Zhou *et al.,* 2020), the pool of free amino acids is represented by metabolically and functionally interrelated compounds, the content of which is a regulatory factor in many key stages of metabolism in the mammalian body.

In this regard, the examination of the molecular mechanisms of the formation of the fund of free amino acids *in vivo* is an important part of solving numerous problems of modern biochemistry and clinical veterinary medicine, related to the purposeful regulation of metabolic processes in living organisms under the influence of biologically active natural compounds and various xenobiotics (Oluranti *et al.,* 2021).

Today, the number of pathological conditions continues to grow, in the genesis of which the violation of amino acid metabolism is given a leading place (Li & Saleem, 2022).

The ability of the kidneys to concentrate various compounds, the presence of active transport processes and systems of metabolic activation of xenobiotics allow them to be classified as target organs that affect the level of their toxic effects in the body (Engwa *et al.,* 2019).

The results of investigating the effect of heavy metals on the pool of free amino acids in rat kidneys are shown in Table. 6. Notably, among the examined amino acids, a decrease in their total amount was established in rats of all experimental groups: in rats poisoned with copper sulfate and zinc sulfate – by 1.3 times, and cadmium sulfate and lead nitrate – by 1.4 times compared to intact animals. The content of aspartic acid, valine, glycine, tyrosine, and cystine (more than 1.5 times) decreased in poisoned rats; alanine, leucine, serine, taurine, threonine, phenylalanine (more than 2 times), lysine – on average by 3.4-4.9 times compared to the control. Therewith, in rats of the experimental groups, an increase in the level of isoleucine and methionine in the kidneys was observed by an average of 1.3-1.5 times, ornithine – by an average of 1.8-2.1 times, glutamic acid – by an average of 4.4-5.3 times compared to the control.

Table 6. Free amino acid content in rat kidneys before and after heavy metal poisoning, μg/g (M ± m, n = 8)

Amino acid	Animal group				
	Intact	Copper sulfate poisoned	Zinc sulfate poisoned	Cadmium sulfate poisoned	Lead nitrate poisoned
Alanine	723.4 ± 31.14	446.4 ± 20.43*	451.2 ± 21.52*	328.3 ± 18.74*	339.3 ± 19.12*
Arginine	304.6 ± 17.21	231.2 ± 16.32*	247.6 ± 18.41*	212.2 ± 14.27*	217.4 ± 15.18*
Aspartic acid	682.7 ± 27.18	415.3 ± 24.41*	438.4 ± 27.15*	397.6 ± 20.61*	403.8 ± 22.74*
Valine	297.8 ± 22.34	164.3 ± 14.23*	161.7 ± 13.94*	149.4 ± 11.28*	158.9 ± 12.11*
Histidine	214.3 ± 19.57	169.4 ± 15.47*	171.5 ± 16.23*	151.5 ± 10.78*	156.7 ± 11.13*
Glycine	483.2 ± 27.12	339.2 ± 24.38*	344.8 ± 26.82*	318.7 ± 21.32*	321.5 ± 22.17*
Glutamic acid	182.7 ± 25.29	823.7 ± 41.25*	798.2 ± 39.18*	984.1 ± 46.27*	972.3 ± 44.16*
Isoleucine	252.4 ± 23.16	332.4 ± 26.19*	327.9 ± 27.45*	381.4 ± 30.32*	379.8 ± 29.27*
Leucine	475.8 ± 21.17	287.3 ± 19.33*	301.7 ± 20.17*	215.6 ± 16.41*	218.4 ± 17.33*
Lysine	954.5 ± 68.23	243.6 ± 18.47*	281.4 ± 19.53*	195.8 ± 15.84*	197.6 ± 16.21*
Methionine	169.8 ± 17.64	228.3 ± 17.92*	219.7 ± 18.41*	261.3 ± 20.72*	259.7 ± 19.89*
Ornithine	174.6 ± 15.31	319.8 ± 19.23*	317.1 ± 18.42*	364.2 ± 22.81*	356.4 ± 20.45*
Proline	269.3 ± 21.54	238.5 ± 20.34	241.6 ± 20.89	212.5 ± 18.92	214.8 ± 19.17
Serine	489.2 ± 27.12	234.7 ± 19.21*	245.3 ± 20.95*	218.4 ± 17.23*	221.6 ± 18.14*
Taurine	341.4 ± 19.23	182.2 ± 14.36*	194.8 ± 15.21*	145.2 ± 10.03*	148.5 ± 10.72*

Table 6, Continued

Tyrosine	294.7 ± 19.15	193.5 ± 19.32*	191.6 ± 18.93*	176.4 ± 18.27*	179.7 ± 19.44*
Threonine	357.6 ± 32.16	168.3 ± 15.64*	197.2 ±19.42*	162.8 ± 13.26*	164.6 ± 14.31*
Tryptophan	201.2 ± 21.14	227.1 ± 16.89	221.4 ± 17.23	245.3 ± 18.14	243.8 ± 17.42
Phenylalanine	598.4 ± 25.12	342.3 ± 15.43*	338.1 ± 16.25*	210.1 ± 11.47*	211.4 ± 11.53*
Cystine	187.3 ± 19.23	102.7 ± 6.02*	99.8 ± 5.19*	95.4 ± 4.23*	98.3 ± 4.71*
Sum of amino acids	7654.9	5690.2	5791.0	5426.2	5464.5

Note: ** P < 0.05, compared to the control group*

Increased methionine content, as noted in the literature (Kokaman, 2022), may indicate a violation of the transfer of sulfhydryl groups and methylation of macromolecules. Since isoleucine is utilised in the kidneys to ensure the processes of transamination and synthesis of adenosine triphosphoric acid, the increase in its content when heavy metals are introduced indicates that the affected kidneys are not able to effectively implement this process. Transamination disorders are indicated by an increase in glutamic acid (Li & Saleem, 2022). It is assumed that as a result of heavy metal poisoning, the ability to break down excess amino acids in the urea cycle is clearly impaired, as evidenced by an increase in the concentration of ornithine in the blood serum. In turn, the literature indicates (Samuel *et al.,* 2021) that a decrease in the concentration of aspartic acid can lead to a violation of the formation of oxaloacetate and the normal course of transformations in the Krebs cycle.

The data obtained, consistent with the research (Salama *et al.,* 2016), is associated with kidney damage in rats with lead poisoning, which also indicates the involvement of gamma-Glutamylcysteine in the activation of the expression of the nuclear antigen of proliferating cells, enhancing the regenerative ability of this internal organ, reducing inflammation, and regulating the apoptosis.

In addition, the results presented are the same as those of Selorm Fiat Kenston *et al.* (2018) concerning the effects of a mixture of heavy metals that reduce kidney function, increase liver damage, and disrupt electrolyte balance in rats.

Identical data were also obtained in relation to glutamate metabolism during the poisoning of rats with even one heavy metal (Li *et al.*, 2020).

The established patterns are consistent with the previous results (Flora *et al.*, 2022), which, in particular, indicate the use of chelation therapy as one of the promising methods in the treatment of heavy metal poisoning.

Thus, the poisoning of rats by the studied heavy metals is reflected in the infrared absorption spectra and characterises α spiral configurations of polypeptide chains and antiparallel types β of structures in liver and kidney tissues. Moreover, in blood serum, an increase in the concentration of lactate, pyruvate, oxaloacetate, and α-Ketoglutarate and a decrease in malate and the total content of amino acids in rat kidney tissues are observed.

Conclusions

When rats are poisoned, the copper content in the blood increases by 1.4 times, in the liver – 1.6 times, and in the kidneys – 1.5 times. Meanwhile, the zinc content in the blood of rats increases by 1.5 times, in the liver – by 2.5 times, in the kidneys – by 1.6 times; cadmium in the blood of rats increases by 27 times, in the liver – by 243 times, in the kidneys – by 23 times; lead in the blood of rats increases by 1.9 times, in

the liver – by 4.8 times, in the kidneys – by 5.5 times compared to the group of intact animals.

The obtained and analysed infrared absorption spectra showed a substantial difference in the spatial organisation of protein components in liver and kidney tissues in intact and cadmium-poisoned rats, which consists in the shift of the maximum bands, their appearance or disappearance, and increase or decrease in the integral intensity. The presence of all forms of secondary structures in the spatial structure of liver and kidney tissue proteins was established, in particular, α-spirals, β-folded structures and amorphous regions. In the case of cadmium ion poisoning, the denaturation of protein molecules and the transformation of their structure into disordered segments were recorded.

The difference between the infrared spectra of liver and kidney tissues in intact and cadmium-poisoned rats was identified and demonstrated using statistical indicators such as skewness and kurtosis.

It was established that poisoning of rats with copper sulfate, zinc sulfate, cadmium sulfate, and lead nitrate ions causes a likely increase in the concentration of lactate and pyruvate in the blood serum and leads to an increase in the content of oxaloacetate and α-Ketoglutarate and a decrease in the concentration of malate compared to intact rats. This indicates the sensitivity of glycolysis reactions and the tricarboxylic acid cycle to the intake of toxic doses of heavy metals.

Heavy metal poisoning in rats is accompanied by a decrease in the total pool of free amino acids in the kidneys. Thus, the quantitative changes in the pool of free amino acids in the kidneys of rats with the experimental introduction of toxic doses of heavy metals into the body can be an additional criterion for assessing the state of exchange of amino acids, nitrogenous compounds, proteins and nucleotides, internal reserves of the organ, the degree of disintegration of metabolism under the influence of xenobiotics and the adaptive capabilities of the body in general. The obtained research results can be used for clinical studies under the conditions of heavy metal poisoning of animals.

In the future, it is planned to expand the range of research on the effect of heavy metals on intermediate protein metabolism in the body of poisoned rats.

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None.

Conflict of Interest

None.

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Інфрачервона спектроскопія та біохімічні показники тканин щурів за умов отруєння важкими металами

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Анотація. Підвищення рівня антропогенного забруднення навколишнього середовища та пошук дієвих засобів щодо зменшення негативного впливу ксенобіотиків на здоров'я тварин і людей є актуальною проблемою сьогодення. Враховуючи це, метою роботи було вивчення впливу важких металів на процеси кумулювання за умов отруєння та біохімічні показники в організмі щурів. Для проведення дослідження сформовані групи-аналоги з щурів одного віку, статі та за масою тіла. Впродовж 14 діб здійснювали інтоксикацію щурів розчинами сульфату міді, сульфату цинку, сульфату кадмію та нітрату свинцю. Використовуючи метод інфрачервоної спектроскопії, встановлено значні відмінності в просторовій структурі білкових компонентів у інтактних та токсикованих тварин. Різницю між спектральними характеристиками досліджуваних тканин продемонстровано наочно за статистичними показниками скюнесу і куртозису. Встановлено, що отруєння щурів іонами міді, цинку, кадмію та свинцю впливає на хід реакцій гліколізу і циклу трикарбонових кислот, що призводить до вірогідного збільшення в сироватці крові концентрації лактату й пірувату, оксалоацетату і α-кетоглутарату та зменшення вмісту малату порівняно з інтактними щурами. З'ясовано, що за умов отруєння також відзначається вірогідне збільшення ($P < 0,05$) вмісту досліджуваних важких металів у крові, печінці та нирках. У токсикованих важкими металами тварин відмічається зменшення в нирках пулу вільних амінокислот. Зокрема, у нирках таких щурів знижується вміст: аспарагінової кислоти, валіну, гліцину, тирозину, цистину (більш ніж у 1,5 раза); аланіну, лейцину, серину, таурину, треоніну, фенілаланіну (більш ніж у 2,0 рази), лізину – у 3,4–4,9 раза. Разом із тим, встановлено зростання рівня ізолейцину та метіоніну в 1,3–1,5 раза, орнітину – у 1,8–2,1 раза, глутамінової кислоти – у 4,4–5,3 раза в щурів дослідної групи порівняно з інтактними. Результати дослідження можуть бути корисними у професійній діяльності лікарів ветеринарної медицини, токсикологів, біологів, екологів та використовуватися для контролю за якістю тваринницької продукції, проведення токсикологічних досліджень і аналізу об'єктів довкілля

Ключові слова: мідь; цинк; кадмій; свинець; кров; внутрішні органи

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