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THE IMPACT OF PLANT CRYOEXTRACT ON PRODUCTIVITY AND FACTORS OF INNATE IMMUNITY OF POND FISH AGAINST THE BACKGROUND OF STRESS IN THE EXPERIMENT

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Summary. The search for effective and environmentally friendly means to increase the productivity and natural resistance of animals, especially in aquaculture, remains an urgent socio-economic task. This article presents the results of the study of the effect of the cryobiological supplement 'Immunolife-Fish', based on medicinal plants, on the weight gain of juvenile common carp (*Cyprinus carpio*) and indicators of innate immunity of two-year-old common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*) under the influence of thermal stress factor in aquarium conditions. It was found that the weight gain of juvenile carp treated with the cryobiological supplement 'Immunolife-Fish' was higher than that of the control group at all stages of the study. The increase was most pronounced during the first 42 days of feeding. A significant increase in the number of leukocytes was found in the blood of fish (common carp and silver carp) exposed to a heat stressor compared to control values. In fish that received a supplement of the herbal preparation during stress, this indicator only tended to increase. The effect of the stressor in fish of both species is manifested by a significant increase in the leukocyte content, a decrease in the indicators characterizing the functional state of cellular immunity — phagocytic activity and number of phagocytes, as well as a tendency to decrease the phagocytic index of neutrophils in the blood. The use of the cryobiological supplement 'Immunolife-Fish' in fish exposed to stress not only prevented the decrease in the phagocytic activity of neutrophils but also contributed to a slight increase in their activity in comparison with the control. A significant decrease in the activity of lysozyme and the level of circulating immune complexes, as well as an increase in the content of seromucoids, was found in the blood serum of common and silver carp under the influence of the stress factor. The use of the drug against the background of stress leveled the negative changes in the above indicators characterizing the state of the humoral link of innate immunity. In addition, a mild immune stimulating effect of the cryobiological supplement on fish of both species, which were kept in optimal conditions, was noted. The research is aimed at the development and testing of organic, environmentally friendly anti-stress and immunostimulant agents in commercial fish farming

Keywords: common carp, silver carp, thermal stress, medicinal plants

Introduction. Today, global aquaculture provides half of the world's fish stocks and is one of the fastest-growing food production sectors. It is believed that the development of environmentally and socially sustainable aquaculture is based on three main principles: first, aquaculture should take into account the full range of ecosystem functions and services; second, aquaculture should contribute to the well-being of people representing all stakeholders; and third, aquaculture should be developed taking into account other sectors, policies and their goals (Vdovenko, 2013; Prysiazhniuk et al., 2019). Success in achieving the above objectives requires, on the one hand, proper management of aquaculture development at the institutional level and, on the other hand, social acceptance of the measures taken. Pond carp farming is a type of aquaculture that can meet all of the above criteria (Zhu, 2020). Other steps to improve the environmental performance of aquaculture include the development of 'multitrophic aquaculture' using nutrient-rich by-products; 'polyculture' (e.g., a combination of carps with different feeding niches in one pond) (DG MARE, 2021).

Carp is the main fish species growing well and raised in slow-flowing or stagnant water ponds. In particular, the Ukrainian common carp (*Cyprinus carpio*) grows better than other carp subspecies, is 17–20% more viable, and effectively utilizes the natural food base of ponds. Carp is an omnivorous fish: at a young age, it consumes

zooplankton, at an older age — benthos, it eats well artificially produced feed, grain waste, weed seeds, etc. Herbivorous fish, such as silver carp (*Hypophthalmichthys molitrix*), are often grown in polyculture with carp, as the period of its cultivation to marketable weight is the same as for carp (two to three years) (Pustova N., Pustova Z. and Balickiy, 2023).

In conditions of intensive fish farming, where the natural feed base has an insignificant share in fish feeding or is absent, the use of artificial feeds, especially with the use of various biogenic elements, the so-called functional feeds, plays an important role. They can be used to increase growth, and natural resistance and optimize the antioxidant status of fish and other aquaculture objects (Pacitti et al., 2015, 2016; Oleshko et al., 2021; Prysiazhniuk et al., 2023). The main factors of fish diseases and mortality against the background of reduced resistance are the effects of stress factors and pathogens of infectious and invasive diseases (Horchanok and Prysiazhniuk, 2020). At the same time, the constant use of various agents for disease control can lead to many problems: increased resistance of pathogens, pollution of water bodies, and loss of phyto- and zooplankton. Therefore, it is recommended not to use drugs or chemicals unless absolutely necessary. An alternative can be the use of biological control methods and environmentally friendly measures to prevent fish diseases and increase their natural resistance (Abdel-Ghany, El-Sisy and Salem, 2023).

As shown in numerous literature sources (Ekasari et al., 2014; Ibrahim et al., 2021; Novitskyi and Horchanok, 2022), increasing the resistance of fish to both pathogens and abiotic environmental factors is one of the modern and important problems of pond fish farming. The immune system of fish is self-regulated by direct interconnection of cells (macrophages, neutrophils, etc.), as well as with the participation of humoral defense factors.

The main components of the nonspecific (innate) immune system of fish are macrophages, granulocytes, monocytes, and humoral elements such as lysozyme (Saurabh and Sahoo, 2008). Bioactive compounds act as immunostimulants to enhance the immune response of fish (Salinas, Zhang and Sunyer, 2011; Mohammadi et al., 2020; Effendi et al., 2022), namely lysozyme, phagocytosis, complement system, reactive oxygen and nitrogen species, antiproteases, glutathione peroxidase, against bacterial, fungal, viral and parasitic diseases (Oleksiuk and Yanovych, 2010; Effendi et al., 2022; Janssens et al., 2000). The immune system of fish is a combination of cellular and humoral immunity factors, which are respectively realized by cells of the lymphoid-macrophage complex and humoral components (Kovalchuk, 2011; Mohammadi et al., 2020; Bavia, 2022). Cellular elements of the immune system are organized into tissue and organ structures (Rubio-Godoy, 2010; Salinas, Zhang and Sunyer, 2011). A significant part of immunocompetent cells is a component of blood and lymph. The immune system of fish differs from that of higher vertebrates in the absence of lymph nodes, bone marrow, and the pharyngeal bursa in birds; immunoglobulins in fish are represented only by IgM-like antibodies (Ibrahim et al., 2021).

The natural resistance of pond fish is influenced by many factors: housing conditions, adequate feeding, a hydrochemical regime in the reservoir, the content of toxic substances of various kinds in the water, etc. This is evidenced by a decrease in the resistance of fish to pathogens under the influence of negative factors. In pond fish farming, a variety of drugs and supplements are actively used to prevent the emergence and spread of pathogens, as well as immunostimulants that activate the system of non-specific resistance factors in fish (Ekasari et al., 2014). Phytotherapy is one of the modern ways to increase the natural resistance of animals.

The mechanisms and processes related to the use of medicinal plants in the world of fisheries are not well understood. Generally, people's attention is focused on mice, chickens, and humans (Mohammadi et al., 2020). Medicinal plants can be administered as parts or whole plants (seeds, leaves, roots, bark, or fruits) or complex extracts, as a feed supplement, through a water regimen, alone or as a combination of extract compounds, or even as a mixture with pro- and prebiotics (Effendi et al., 2022).

Plants and their extracts have been reported to have various effects, such as antistress, appetite and growth stimulation, and immunostimulation in various aquaculture targets, due to their various active components, such as alkaloids, terpenoids, tannins, saponins, polysaccharides, and flavonoids (Mohammadi et al., 2020). This makes the plants suitable for the treatment of multifactorial diseases, and their use can be an alternative to antibiotics with a low risk of developing resistance (Reverter et al., 2017).

One of the modern technologies for processing medicinal plants is cryoextraction (or cryoextension). The method is based on the obtaining of extracts from plant materials using low temperatures to isolate biologically active compounds. It is believed that the cryoextraction system preserves the active essence of plants, allows the use of plants that act in synergy, and their biological activity is 5–10 times higher than that of dried plant extracts. This method allows the preservation of the biological activity of substances and can be particularly effective for obtaining extracts from plants containing thermolabile compounds (Verkin, Zinov'ev and Povstyanyy, 1985). The low temperature stops the action of oxidizing enzymes and the oxidation process. The bonds of biologically active compounds with large protein molecules are broken, which increases their digestibility. This contributes to the 'cryoactivation' of

plant materials and increases the bioavailability of substances many times due to their transition to a state unrelated to proteins (Verkin, Dmitriev and Pavlyuk, 1986).

According to the literature, plant species with the greatest potential for use in aquaculture include garlic (*Allium sativum*), pomegranate (*Punica granatum*), Bermuda grass (*Cynodon dactylon*), ashwagandha (*Withania somnifera*), and ginger (*Zingiber officinalis*) (Reverter et al., 2017).

The study aimed to investigate the effect of the cryobiological supplement 'Immunolife-Fish' on the productivity of juvenile common carp (*Cyprinus carpio*) and innate immunity of two-year-old pond fish (common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*)) under the influence of heat stress in the experiment.

Materials and methods. To perform the tasks under aquarium conditions, two experiments were carried out using the cryobiological supplement 'Immunolife-Fish', produced by cryo-grinding technology. For this purpose, a composition of medicinal plants was used: golden root (*Rhodiola rosea*) roots and rhizomes, hawthorn (*Crataegus*) flowers, licorice (*Glycyrrhiza glabra*) roots, pine (*Pinus*) needles, and purple coneflower (*Echinacea purpurea*) roots in equal proportions. The drug was mixed with standard feed (feed concentrate Multigain™) in a final concentration of 1.5%. The experimental sample of the cryobiological additive was produced by the pharmaceutical company 'AIM' (Kharkiv, Ukraine).

The experiment to study the effect of cryobiological supplementation on fish productivity was carried out using two aquariums with a capacity of 100 liters, in which 20 individuals of juvenile common carp with an initial weight of 20.4 ± 0.25 g were kept. The water temperature was 24–25 °C, and the average oxygen concentration during the experiment was 5.2 mg/l. The fish of the experimental group were fed with the cryobiological additive 'Immunolife-Fish' together with the food, and the fish of the control group were fed only with the standard food. The feed was given 6 times a day in equal parts in the amount of 15% of the fish's weight. The duration of the experiment was 8 weeks.

Every 14 days, the fish were weighed on electronic scales to determine the dynamics of growth and development of the fish. Growth intensity was evaluated by absolute, relative, and average daily growth (Korol-Bezpala, 2020).

An experiment to study the effect of the cryobiological supplement 'Immunolife-Fish' on the state of innate immunity was conducted on two-year-old common carp and silver carp with an average weight of 450 ± 3.5 g and 570 ± 4.6 g, respectively, of which 4 groups (3 experimental and 1 control) were formed on the principle of analogs, 5 individuals per group. The fish were kept in aquariums with a capacity of 150 liters. Fish were fed in the morning and evening in equal parts in the amount of 15% of the fish weight per day. Fish of groups I and III of both species were exposed to a stress factor, which was modeled by increasing the water temperature in the aquarium to 26–27 °C, fish of groups II and III of both species received the cryobiological supplement 'Immunolife-Fish' with food.

The material for the study was blood taken from the heart of fish on 11th day of the experiment using a Pasteur pipette after anesthesia with essential oil of clove (*Eugenia caryophyllus*). In the heparin-stabilized blood, the number of leukocytes and the effectiveness of phagocytosis, namely phagocytic activity, phagocytic number, and phagocyte index, were determined (Vlizlo, 2016; Stegniy et al., 2013). In fish blood serum, lysozyme activity, seromucoid level, and circulating immune complexes level were studied according to generally accepted methods using standard reagent kits of PJSC 'Reagent'.

The studies were conducted in the Laboratory of Toxicological Monitoring, Clinical Biochemistry, Quality and Safety of Agricultural Products of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' of the National Academy of Agrarian Sciences of Ukraine (Kharkiv, Ukraine).

Experiments on animals were conducted following the recommendations of the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), and under Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006) and basic bioethical principles (Simmonds, 2017). Under the current procedure, the research program was reviewed and approved by the Bioethics Committee of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'.

Statistical analysis. The results are presented as the mean ± standard deviation (x ± SD). Analysis of variance (ANOVA) was used to compare the mean difference between experimental and control groups. The p-value $p < 0.05$ was considered a significant value.

Results. It was found that the weight gain of juvenile common carp treated with the cryobiological supplement 'Immunolife-Fish' was higher than that of the control group at all times during the study, reaching a maximum of 17.7% on 42nd day of the experiment and finally 5.2% (Table 1).

According to the results of determining the calculated indicators of productivity of common carp in the dynamics of the experiment, it was found that the most pronounced effect of the drug on the absolute, average daily, and relative growth occurred on 29th–42nd days of

feeding, when the indicators of the experimental group maximally exceeded the control ones by 29.2%, 28.3%, and 10.6%, respectively (Table 2).

In the blood of common carp and silver carp of the first experimental groups exposed to the stress factor, there was a significant ($p < 0.05$) increase in the number of leukocytes by 22.2% and 19.6%, respectively, compared to the control values. In the fish of the third group, which received a plant supplement during the stress, this indicator only tended to increase, reaching 12.2% and 11.3%, respectively. A slight stimulating effect of the cryobiological supplement was also observed in the fish of the second group, which were kept in optimal conditions (Table 3).

Table 1 — Body weight (g) dynamics of common carp under the influence of cryobiological supplement 'Immunolife-Fish' ($n = 20$, $x \pm SD$)

Group	Day of the experiment				
	1	14	28	42	56
Control	20.3 ± 0.18	87.60 ± 0.23	173.6 ± 1.74	247.6 ± 1.96	329.0 ± 2.03
Experimental	20.5 ± 0.08	93.68 ± 0.18	195.5 ± 1.22	291.1 ± 1.54	389.2 ± 1.85

Table 2 — Calculated average group productivity indicators of common carp under the influence of cryobiological supplement 'Immunolife-Fish'

Group	Experimental period, day			
	1–14	15–28	29–42	43–56
Absolute weight gain, g				
Control	67.3	86.0	74.0	81.4
Experimental	73.2	101.8	95.6	98.1
Average daily weight gain, g				
Control	4.8	6.1	5.3	5.8
Experimental	5.2	7.3	6.8	6.4
Relative weight gain of fish, %				
Control	331.5	98.2	42.6	32.9
Experimental	357.0	108.7	48.9	34.6

Table 3 — The level of leukocytes ($\times 10^9$/dm^3) in the blood of pond fish under the influence of stress factor and application of cryobiological supplement 'Immunolife-Fish' ($n = 5$, $x \pm SD$)

Fish species	Group			
	Control	**I** (stress)	**II** (supplement)	**III** (stress + supplement)
Common carp	19.41 ± 0.48	23.72 ± 0.76*	20.38 ± 0.57	21.77 ± 0.93
Silver carp	22.32 ± 0.33	26.69 ± 0.51*	23.43 ± 0.71	24.84 ± 0.56

Note. * — $p < 0.05$ relative to control.

The study of the functional state of neutrophils in the blood of fish of groups I and II of both species revealed a decrease in their phagocytic activity by 29.4% ($p < 0.05$) and 22.8% ($p < 0.05$), phagocytic index by 23.8% and 20.9%, phagocytic number by 24.2% ($p < 0.05$) and 22.6% ($p < 0.05$), respectively. In fish of groups II and III, which received the cryobiological supplement 'Immunolife-Fish', an increase in phagocytic activity by 14.8% and 12.1%, 11.6% and 9.4%; phagocytic index by 14.1% and 11.3%, 10.8 and 10.2%; phagocytic number by 13.2% and 10.8%, 11.0%, and 9.6%, respectively, was observed compared to the control (Table 4).

The study of the indicators characterizing the state of the humoral link of the innate immunity of fish organisms revealed a decrease in the activity of lysozyme in the blood serum of the groups I of common carp and silver carp by 32.4% ($p < 0.05$) and 29.8% ($p < 0.05$), as well as a decrease in the level of circulating immune complexes by 26.8% and 27.4%, respectively. On the contrary, the level of seromucoid in fish of these groups increased by 43.2% ($p < 0.05$) and 39.6% ($p < 0.05$). In the blood serum of common carp and silver carp of the groups II, an increase in serum lysozyme activity by 16.3% and 17.8%, as well as an increase in circulating immune complexes' levels by 15.5 % and 14.6 %, respectively, was recorded. In the third experimental group of both fish species, serum lysozyme activity tended to increase (Table 5).

Table 4 — Indicators of the functional state of the cellular link of nonspecific resistance of pond fish under the influence of stress factor and the use of cryobiological supplement 'Immunolife-Fish' (n = 5, x ± SD)

Group	Phagocytic activity, %		Phagocytic index, units		Phagocytic number, units	
	Common carp	Silver carp	Common carp	Silver carp	Common carp	Silver carp
Control	26.34 ± 1.02	31.22 ± 0.94	3.18 ± 0.26	4.12 ± 0.28	5.36 ± 0.19	6.11 ± 0.18
I (stress)	18.59 ± 0.38*	24.10 ± 0.67*	2.42 ± 0.14	3.25 ± 0.18	4.06 ± 0.16*	4.73 ± 0.13*
II (supplement)	30.24 ± 1.38	34.99 ± 1.12	3.63 ± 0.11	4.58 ± 0.21	6.06 ± 0.21	6.77 ± 0.17
III (stress + supplement)	29.39 ± 0.84	34.15 ± 1.08	3.52 ± 0.16	4.54 ± 0.11	5.94 ± 0.14	6.69 ± 0.12

Note. * — $p < 0.05$ relative to control.

Table 5 — Indicators of the humoral link of the innate immunity of pond fish under the influence of stress factors and application of cryobiological supplement 'Immunolife-Fish'

Group	Serum lysozyme activity, %		Seromucoids, mg/cm^3		Circulating immune complexes, mg/cm^3	
	Common carp	Silver carp	Common carp	Silver carp	Common carp	Silver carp
Control	29.78 ± 1.68	31.36 ± 1.84	0.180 ± 0.010	0.170 ± 0.020	0.330 ± 0.018	0.360 ± 0.024
I (stress)	20.13 ± 1.13*	22.01 ± 1.63*	0.257 ± 0.017*	0.237 ± 0.013*	0.241 ± 0.011*	0.261 ± 0.018*
II (supplement)	34.63 ± 2.06	36.94 ± 2.16	0.185 ± 0.014	0.178 ± 0.011	0.381 ± 0.017	0.412 ± 0.013
III (stress + supplement)	31.27 ± 1.74	32.92 ± 1.48	0.188 ± 0.016	0.179 ± 0.015	0.316 ± 0.015	0.344 ± 0.012

Note. * — $p < 0.05$ relative to control.

Discussion. One of the areas of further intensification in aquaculture is to increase the natural resistance of fish to biotic and abiotic factors (Rudenko, Vishchur and Kovalenko, 2016; Hrytsyniak and Gurbyk, 2016; Solopova, 2020), particularly through the use of phyto-preparations that have appetite-stimulating, anti-stress, and immunostimulating effects. The aquaculture industry is increasingly favoring phytomedicine-based methods/compounds to develop resistance in farmed fish because they are inexpensive and safe for the environment (Semwal, Kumar A. and Kumar N., 2023).

Our studies have proven the positive effect of using 'Immunolife-Fish', produced by the technology of cryo-grinding a mixture of medicinal plants: golden root (*Rhodiola rosea*) roots and rhizomes, hawthorn (*Crataegus*) flowers, licorice (*Glycyrrhiza glabra*) roots, pine (*Pinus*) needles, and purple coneflower (*Echinacea purpurea*) roots on the growth of live weight of young common carp. Absolute growth rates, in a certain sense, reflect the growth rate of animals and are of great practical importance, as they allow us to compare actual results with planned ones and control the fulfillment of tasks. A

t the same time, juvenile fish grow unevenly, so that absolute weight gain does not accurately reflect the intensity of actual growth processes, namely the ratio between body weight gain and growth rates. Therefore, relative growth rates were determined. The analysis of the calculated data allows us to state the positive effect of the use of the cryobiological supplement 'Immunolife-Fish' on the productivity of juvenile common carp, which is most pronounced in the first 42 days of feeding.

According to Effendi et al. (2022), the greasy grouper (*Epinephelus tauvina*) fed a diet supplemented with a mixture of methanolic herbal extracts (*Cynodon dactylon*, *Piper longum*, *Phyllanthus niruri*, *Tridax procumbens*, and *Zingiber officinalis*) had 41% more weight than control fish. The increase in weight gain is attributed to improved digestibility and availability of nutrients with the introduction of plant extracts, which leads to increased feed conversion and increased protein synthesis in fish (Mohammadi et al., 2020). It has been proven that medicinal plants activate digestive enzymes, thereby increasing the growth of fish and animals. For example, an artemium-rich herbal diet improves the survival and growth rate of the Indian prawn, *Fenneropenaeus indicus* (Zhu, 2020). The herbal components of traditional Chinese medicine have a beneficial effect on carp growth, in particular, feed supplemented with herbs increases the efficiency of fish digestion (Reverter et al., 2017). The activation of metabolic processes can also contribute to the growth of fish weight (Deren, 2009).

As mentioned earlier, medicinal plants also have antibacterial, antifungal, and immunostimulating properties (Korylyak, 2015; Mohammadi et al., 2020). The analysis of our data obtained in the study of the effect of the cryobiological supplement 'Immunolife-Fish' on the organism of pond fish of the carp family allows us to assert that a compensatory increase in the number of leukocytes was found in the blood of common carp and

silver carp of the experimental groups I exposed to the stress factor. At the same time, in fish that received a supplement of the herbal preparation during stress, this indicator only tended to increase. In addition, a mild stimulating effect of the cryobiological supplement on fish of the second group, which was kept in optimal conditions, was recorded.

Leukocytes are the main protective elements and a key part of immunity, and their increase in fish can indicate both the development of inflammatory processes against the background of stress (groups I of common carp and silver carp) and an increase in the activity of non-specific immunity (groups II and III). This fact is supported by data on the activity of phagocytosis, which is the main protective function of leukocytes (Honcharov, 2019).

We found that the phagocytic activity of blood leukocytes of common carp and silver carp treated with the drug (groups II and III) was higher than that of fish in the control groups. At the same time, the phagocytic number, which indicates the average number of microorganisms per active phagocyte, and the phagocytosis index, which characterizes the number of microorganisms captured by an active phagocyte, were also increased in the blood of both fish species of groups II and III. These data indicate an increase in the activity of the cellular link of non-specific resistance in the blood of these fish. On the contrary, the data on phagocytosis activity in the body of common carp and silver carp of group I indicate a suppression of the functional capacity of the cellular link of non-specific resistance, which can negatively affect the condition of fish and lead to the development of pathological processes.

According to the literature, *Astragalus* and *Lonicera* extracts increased the production of intracellular superoxide anion by tilapia leukocytes (Ardó et al., 2000). *Lonicera* flower extract contains many different active components, one of them, chlorogenic acid, can activate macrophages through the calcineurin pathway (Effendi et al., 2022) and act as a macrophage activator *in vivo* (Mohammadi et al., 2020) and thereby activate the cellular link of fish innate immunity.

The application of herbal products also stimulates humoral factors of non-specific immunity (Mohammadi et al., 2020; Ibrahim et al., 2021). It should be noted that lysozyme activity is an integral factor of the body's natural resistance of the humoral type, which indicates the blood's ability to self-purify (Pukalo et al., 2008). The formation of circulating immune complexes is a natural defense mechanism of the body; it promotes the rapid elimination of endogenous and exogenous antigens through phagocytosis and the reticuloendothelial system. The study of the level of immune complexes in blood serum is important for the diagnosis of acute inflammatory processes and type 3 allergic reactions when the level of circulating immune complexes can increase, as well as for determining the effectiveness of treatment (Solopova and Vishchur, 2020).

The increase in serum lysozyme activity and circulating immune complexes in the organism of common carp and silver carp treated with the cryobiological supplement 'Immunolife-Fish' indicates the self-regulation of the immune system against the background of the use of a preparation rich in biologically active substances and a significant increase in the factors of nonspecific resistance of the experimental fish, especially against the background of the negative effect of the stress factor. Similar results were obtained by other researchers. For example, in the Nile tilapia (*Oreochromis niloticus*), which was fed with mistletoe (*Viscum album coloratum*) for 80 days, an increase in lysozyme and complement activity, phagocytic activity was recorded, which provided a 42% increase in survival when infected with the bacterial pathogen *Aeromonas hydrophila* (Park and Chai, 2012), and a similar effect was observed with the use of the Asteraceae plant *Eclipta alba* in the Mozambique tilapia (*Oreochromis mossambicus*) (Christybapita, Divyagnaneswari and Dinakaran Michael, 2007).

In addition, fish treated with herbal medicine, in particular, an alcoholic tincture of *Echinacea purpurea*, showed higher levels of red blood cells, lymphocytes, monocytes, and hemoglobin (Reverter et al., 2017; Deren, 2009). The study of the action of pure garlic components allicin and ajoene in aquaculture demonstrated their immunostimulatory ability and effectiveness against the pathogenic protozoa *Spironucleus vortens, Ichthyophthirius multifiliis* and the bacterium *Aeromonas hydrophila* (Millet et al., 2011; Nya et al., 2010; Tanekhy and Fall, 2015).

Ethanol extract from green tea added to the diet also improves lysozyme activity, lipid utilization, and recovery from stress, and reduces total cholesterol levels in black rockfish, *Sebastes schlegeli* (Hwang et al., 2013). *Astragalus* extract, the main active ingredients of which are polysaccharides, as an immunostimulant increased plasma lysozyme activity (Hanif et al., 2005; Kim and Austin, 2006).

Conclusions. It has been established that the cryobiological supplement 'Immunolife-Fish', produced using the technology of cryo-grinding of a composition of medicinal plants: golden root (*Rhodiola rosea*) roots and rhizomes, hawthorn (*Crataegus*) flowers, licorice (*Glycyrrhiza glabra*) roots, pine (*Pinus*) needles, and purple coneflower (*Echinacea purpurea*) roots in the amount of 1.5% of the main feed for 8 weeks increased the live weight gain of juvenile common carps by 5.2% in comparison with the control values.

Feeding the cryobiological supplement 'Immunolife-Fish' for 10 days against the background of heat stress to two-year-old common carp and silver carp leads to an

increase in the number of leukocytes by 22.2% and 19.6%, respectively, and activation of the phagocytic activity of leukocytes by 14.8% and 12.1%, respectively, compared to the control values.

The use of the supplement contributed to an increase in serum lysozyme activity by 16.3–17.8% and the level of circulating immune complexes by 14.6–15.5%. A mild stimulating effect of the cryobiological supplement was also observed in fish kept under optimal conditions.

Thus, the herbal food supplement 'Immunolife-Fish', produced using cryo-grinding technology, helps to increase the productivity and non-specific resistance of the organism and to adapt the fish body to the stress factor.

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INFECTIOUS DISEASES OF BEES AND THEIR IMPACT ON THE VITAL ACTIVITY AND HONEY PRODUCTIVITY OF HONEY BEE COLONIES IN UKRAINE

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Summary. The article presents the results of the study of the mass mortality of honey bee colonies in different apiaries from different regions of Ukraine. The epizootic status of 102 honey bee colonies was studied and 607 samples of pathological material were analyzed in 2021–2023. According to the results of the monitoring of the epizootic situation in Ukrainian beekeeping it was found that the share of parasitic diseases (54.4%, 74.0%, 69.3%) constantly prevails over infectious diseases. It was noted that the incidence of varroosis in honey bees (34.4%, 71.4%, 41.47%) remains the highest among other diseases. Against the background of severe damage to honey bee colonies by the *Varroa* mite, infectious diseases began to appear in an atypical form, which significantly complicates their differential diagnosis. A probable increase in the incidence of nosemosis in adult bees and the detection of *Nosema* cysts in the intestines of bees, both in spring and summer, and the detection of cysts in honey indicate the spread of another pathogen, *Nosema cerana*, which causes nosemosis in the summer season. The reason for the periodic mass death of bees in Ukraine is the combination of a high number of parasitic *Varroa* mites in the honey bee colony with the presence of bee infection with microsporidia *Nosema* spp. These pathogens negatively affect the immunity of the honey bee colony, and cause exacerbation of latent infections in bees, which leads to a decrease in the number of honey bee colonies, weakening their viability and reducing the quality of honey products

Keywords: parasitic diseases, mites, microsporidia, fungi, viruses, bacteria

Introduction. The health and viability of the honey bee colony should be the basis of beekeeping because bee pollination is the main guarantee of maintaining the biodiversity of flora and fauna (Galatyuk, 2010).

However, studies by US scientists show that the benefits of bee pollination of entomophilous crops are 150 times higher than the cost of beekeeping products (Klatt et al., 2013; Traynor et al., 2016).

Without bee pollination, there would be no livestock production, as it would be impossible to obtain animal feed. Improving the food security level in Ukraine requires increased production, processing, and storage of agricultural products and the safety of their consumption.

This fully applies to the beekeeping industry, which provides: pollination of entomophilous crops, contributing to their yield; produces raw materials for many industries and exclusive bee products for human consumption (Polishchuk and Haidar, 2008; Ratnieks and Carreck, 2010; Zakhariya, Davydova and Gotska, 2020).

However, beekeeping is suffering significant losses due to several negative factors. The biggest problem is the periodic mass death of bees — a phenomenon known worldwide as 'colony collapse disorder' (Arnauta and Kalachniuk, 2017).

In Ukraine, in 2014–2015, about 40% of honey bee colonies died (Efimenko, Halat and Odnosum, 2014), and in 2020, 2022 and 2023, massive bee mortality was observed. The causes of death were various pesticide poisonings, consumption of honeydew honey during hibernation, and infectious and invasive diseases (Yefimenko and Odnosum, 2017). Laboratory tests of dead colonies revealed pathogens of invasive diseases in 60% of them.

The study aimed to find out what caused the massive loss of honey bee colonies in different apiaries from different regions of Ukraine.

Materials and methods. The study was conducted from 2021 to 2023 in the Laboratory of Veterinary Sanitation, Parasitology and Honey Bee Diseases of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine). In 2021 the pathological material of 60 apiaries from 17 regions of Ukraine was examined, a total of 372 samples of pathological material were examined, 278 samples of dead adult bees, and 94 samples of brood. In 2022, 23 apiaries in 5 regions were inspected and 140 samples of pathological material were examined. In 2023, 19 private apiaries in Kharkiv, Poltava, and Sumy regions of Ukraine were inspected. A total of 95 samples of pathological material were examined.

Pathological material was taken from 10% of honey bee colonies. The material was examined by group and individual methods. Microbiological, mycological, and parasitological studies were carried out according to existing methods (Fasulati, 1971).

Bee corpses were taken from the middle layer of dead bees formed at the bottom of the hive. Live bees were taken from the upper bar of the frames. Adults and brood

were examined for the presence of ectoparasites (detection and counting of *Varroa destructor* mites). To detect acarapidosis, 200 g of garbage from the bottom of the hive, a bee brood on the honeycomb from the bottom edge of 3–15 cm, and 100 live bees were selected. Bees were dissected and tracheal microscopy was performed for the presence of *Acarapis woodi* mites; microscopic examination of the intestinal contents for the presence of *Nosema* spp. spores and *Malpighamoeba mellificae* cysts.

Microscopy of the intestinal contents of the flight bee for the presence of pathogenic microflora and inoculation into nutrient media.

Laboratory diagnosis of the affected brood included:

— smear microscopy;

— cultivation of the pathogen on nutrient media and determination of its culture properties.

To exclude foulbrood diseases, honeycombs of 10×15 cm with brood were selected. In case of suspicion of membranous brood, 10×15 cm honeycombs were preserved in 50% glycerin solution. In case of suspicion of septicemia, paratyphoid, gafniosis, and polybacteriosis, 50 live bees were taken from the hive. For microscopy, smears were made from the larval suspension and stained with Gram stain to detect the vegetative form of the pathogen. The vegetative form is Gram-positive (appears blue or purple), and to detect spores, the smears were stained with a 2% alcohol solution of carbol fuchsin. Brownian motion is characteristic of *Paenibacillus larvae* spores. To detect Brownian motion, a carbol fuchsin-stained smear from larval suspensions was washed off with water and covered with a layer of immersion oil while still wet. After the smear was dried, it was examined under a microscope. The Brownian motion of spores was observed in the field of view in the water droplets in the immersion oil.

The diagnosis of bee virus diseases was made using PCR, 50 bees preserved in a 50% glycerol solution were examined.

Results and discussion. In 2021, we examined the pathological material from 60 apiaries from 17 regions of Ukraine, a total of 372 samples of pathological material were examined, of which 278 samples were dead adult bees and 94 samples were brood, the data are presented in Table 1.

Table 1 — Share of bee diseases in Ukrainian apiaries in 2021

No.	Region	The number of apiaries	The number of samples	Number of positive samples for pathogens			
				viral	bacterial	mycoses	parasitic
1	Kharkiv	20	92	6	20	5	25
2	Poltava	5	20	6	2	12	15
3	Sumy	5	30	6	5	–	17
4	Dnipropetrovsk	3	18	6	4	–	15
5	Donetsk	2	12	5	2	–	6
6	Luhansk	2	12	5	2	–	8
7	Lviv	2	12	1	2	6	7
8	Kyiv	2	18	1	2	–	15
9	Odesa	4	24	1	3	12	14
10	Kirovohrad	1	10	1	6	–	5
11	Mykolaiv	1	10	3	6	–	6
12	Ternopil	1	10	3	6	–	4
13	Chernihiv	3	24	4	–	–	18
14	Zhytomyr	2	18	1	2	8	14
15	Ivano-Frankivsk	3	24	1	–	–	15
16	Rivne	2	20	1	9	–	14
17	Zaporizhzhia	2	18	1	8	–	14
Total		60	372	52	79	38	202
				Share of bee diseases, %			
				13.9	21.3	10.4	54.4

According to the results of epizootological survey of apiaries and laboratory examination of pathological material in 2021, it was found that the proportion of parasitic diseases was significantly higher than bacterial diseases — 33.1%, viral diseases — 40.5%, and mycoses — 44.0%. The following bacterial pathogens were isolated from the dead bees and affected brood: American foulbrood pathogen *Paenibacillus larvae* — 4.3%; European foulbrood pathogen *Melissococcus pluton* — 15.3%, and *Paenibacillus alvei* — 1.7%. Our research is close to the results obtained by Stupak and Masliy (2009).

Among viruses, PCR was used to diagnose chronic bee paralysis virus (CBPV) isolated in 13.2% of samples,

and deformed wing virus (DWV) in 0.7% of samples. Among the mycoses, the pathogen of chalkbrood (*Ascosphera apis*) was recorded in Kharkiv, Lviv, Poltava, Zhytomyr, and Odesa regions, and the pathogen of aspergillosis (*Penicillum fusarium*) in Poltava Region.

In 2021, varroosis was detected in 34.4% of samples. Mite infestation in colonies averaged 0.91 ± 0.36%, which did not exceed the conditional level of colony well-being.

Thus, in Kharkiv Region, the average infection of a colony was 2.17 ± 1.42%, in Poltava Region — 1.00 ± 0.71%, in Ivano-Frankivsk Region — 0.15 ± 0.05%, in Odesa Region — 0.73 ± 0.16%, in Kyiv Region — 0.28 ± 0.06%, in Rivne Region — 0.11 ± 0.01%. Differences in bee infection by *Varroa* mites depended on many factors, including abiotic factors: a long bribe-free period in bees, significant changes in the thermo-hydro regime in spring and summer, and biotic factors: bee breeds, diversity of the nectar base. However, the main influence was exerted by the anthropogenic factor, in particular, non-compliance with the recommendations for veterinary and sanitary measures at apiaries during the year (Arnauta and Kalachniuk, 2017).

The share of nosemosis cases amounted to 47.9%. The disease was recorded in all regions. Nosemosis (*Nosema* spp.) was detected in high degree '++++' (more than 1,000 spores in the field of view of the microscope) in 14.6% of samples, average '+++' — 30.1%, weak '++' — 24.0%, and single spores '+' — 31.3% of samples. Infection with *Nosema* spp. spores were recorded in not only autumn and spring but also in summer, which gave us reason to suspect the presence of another pathogen, *Nosema ceranae*, which causes nosemosis in the summer. Characteristic clinical signs confirmed the presence of the disease: a significant decrease in the number of adult bees in the hive, a reduction in flight activity and honey productivity, and, as a result, a substantial weakening of the strength of the honey bee colony as a whole. Our assumptions coincide with the results obtained by other scientists (Efimenko, Halat and Odnosum, 2014; Odnosum, Yefimenko and Soroka, 2018; Yefimenko et al., 2014).

Most bee infections by pathogens of contagious diseases were detected in apiaries whose technological direction is honey collection (Kisil and Fotina, 2018; Sklyar, Gerasymova and Shkromada, 2017), especially during the nomadism period in areas with insufficient sown areas of entomophilic crops. Violation of veterinary and sanitary measures, such as the density of honey bee colonies per unit area, as well as the transportation of apiaries during the season to different regions without certification and laboratory diagnostic tests for the presence of pathogens in the colonies are the main factors in the spread of pathogens.

In 2022, 23 apiaries in 5 regions were inspected, and 140 samples of the pathological material were analyzed. According to the results of the epizootic survey of apiaries and laboratory examination of pathological material in 2022, it was found that the proportion of infectious diseases (26%) is significantly lower than parasitic diseases (74%).

Varroosis was diagnosed in 100 samples of pathological material, which amounted to 71.4% compared to other diseases. The prevalence of mite infection averaged 0.95 ± 0.34 %, which does not exceed the conditional level of colony well-being. Thus, in Kharkiv Region, the average prevalence was 1.17 ± 1.12%, in Poltava Region — 1.00 ± 0.25%, in Zaporizhzhia Region — 0.25 ± 0.05%, in Ternopil Region — 0.83 ± 0.11%, in Sumy Region — 1.25 ± 0.05%. In 2022, bee infection with *Varroa* mites depended mostly on anthropogenic factors, which were associated with military operations.

Nosema was diagnosed in 68 samples. The disease rate was 48.6%. The disease was found everywhere. Nosemosis was detected in high degree '++++' in 14.8% of samples, average '+++' — 17.6%, weak '++' — 17.6%, and single spores '+' — 50.6%. The average and slight degree of damage decreased slightly, and the number of single cysts increased by 19.9%.

The mixed course of bee diseases was detected in 8 apiaries, which represented 35% of the examined apiaries, the proportion of diseases was 57.14%. Thus, a mixed course of varroosis with non-communicable bee diseases (chemical toxicosis, pollen toxicosis) (10%), varroosis with nosemosis (46%), and varroosis with chalkbrood (1.14%) were detected. Musiienko, Kirik and Tsyt (2018) obtained similar results and developed methods of combating mixed forms of infectious diseases in honey bees.

In 2023, surveys were conducted in 19 private apiaries in Kharkiv, Poltava, and Sumy regions of Ukraine. A total of 95 samples of pathological material were examined. According to the results of the study of the epizootic situation in 2023 in the apiaries of Kharkiv, Poltava, and Sumy regions of Ukraine, it was found that the proportion of invasive diseases averaged 79.33% and was significantly higher than infectious diseases (20.67%). The share of varroosis was 41.47%, nosemosis — 27.86%, foulbrood — 16.85%, mycosis — 14.34%, and viruses — 3.48%. According to the results of the research, out of 19 private apiaries, five were healthy in Kharkiv Region, seven in Poltava Region, and one in Sumy Region, which is 73.7% of the total number of the examined apiaries.

Additionally, honey samples were tested for the presence of pathogens according to DSTU 8684:2016 (SE 'UkrNDNC', 2016). The results of the study of honey samples from beekeeping farms of different forms of ownership for contamination with pathogens of invasive bee diseases: nosemosis (*Nosema* spp.), amoebiasis (*Malpighamoeba mellificae*), varroosis (*Varroa destructor*), tracheal mites (*Acarapis woodi*), mermitidosis (nematodes) (*Mermis*), and braulosis (*Braula coeca*, *B. smitzi*, *B. orientalis*) are presented in Table 2.

Table 2 — Contamination of honey harvested in 2022 with pathogens of invasive bee diseases

No.	Region	Botanical origin	*Nosema apis*	*Nosema cerenae*	*Malpighamoeba mellificae*	*Varroa destructor*	*Acarapis woodi*	*Mermis*	*Braula coeca*, *B. smitzi*, *B. orientalis*
1	Kharkiv	sunflower	+	–	–	+	–	–	–
2		various herbs	+	–	–	–	–	–	–
3	Zaporizhzhia	various herbs	+	–	+	+	–	–	–
4	Poltava	various herbs	+	+	–	–	+	–	–
5	Ternopil	various herbs	+	–	–	+	–	+	–
6	Sumy	sunflower	+	–	–	+	–	–	–

The causative agent of nosemosis (*Nosema apis*) was identified in honey; microscopy revealed mature oval, ovoid cysts of the parasite measuring 4.5–7.5×2–3.5 μm with a smooth, three-layer shell 0.2–0.3 μm thick. The pathogen *Nozema cerenae* was identified in only one sample. It was also possible to identify the causative agent of amoebiasis (*Malpighamoeba mellificae*) in honey in the form of oval cysts 5–8 μm in size, with a smooth, dense shell (up to 1 μm thick) in Gram-stained smears.

Larvae of *Mermis albicans*, the causative agent of nematodosis, were identified in honey by their size length of 0.74 mm, and diameter of 0.034 mm. The pathogens of varroosis (*Varroa destructor*) and tracheal mites (*Acarapis woodi*) were detected in individual cases.

During the study the pathogens of invasive diseases were identified in honey samples from unfavorable apiaries (Table 3).

Table 3 — Contamination of honey harvested in 2023 with pathogens of invasive bee diseases

No.	Region	Botanical origin	*Nosema apis*	*Nosema cerenae*	*Malpighamoeba mellificae*	*Varroa destructor*	*Acarapis woodi*	*Mermis*	*Braula coeca*, *B. smitzi*, *B. orientalis*
1	Kharkiv	sunflower	+	+	–	+	–	–	–
2		various herbs	+	–	–	+	–	–	–
3	Poltava	sunflower	+	+	–	+	+	–	–
4		various herbs	+	–	–	–	–	–	–
5	Sumy	sunflower	+	–	–	+	–	–	–
6		various herbs	+	–	–	–	–	–	–

According to the results of the research, *Nosema apis* was identified in honey samples from apiaries unfavorable for diseases — 5 samples, *Varroa destructor* — in 4 samples, *Acarapis woodi* — in one sample. *Nosema cerenae* was identified in two samples from Kharkiv and Poltava regions. In honey samples where *Nosema apis* were detected, the mass fraction of water was higher (18.61%), while the average mass fraction of water was 17.59%. In the samples where *Varroa destructor* was detected, the diastase number was 11.20 Gothe units, while the average was 18.24 Gothe units. In samples of honey from unfavorable farms where *Ascosphaera apis* were detected, the mass fraction of water was 1.5% higher than the average.

Conclusions: According to the results of monitoring the epizootic situation in Ukrainian apiaries, it was found that the share of parasitic diseases (54.4%, 74,0%, 69.3%) constantly exceeds the infectious diseases.

It was noted that the incidence of varroosis in bees (34.4%, 71.4%, 41.47%) remains the highest among other diseases. Against the background of severe damage to honey bee colonies by the *Varroa* mite, infectious diseases began to appear in an atypical form, which significantly complicates their differential diagnosis. Thus, from an epizootiological point of view, varroosis should be considered as one of the main factors reducing the overall resistance of the entire honey bee colony as an integral biological organism.

A significant increase in the incidence of nosemosis in adult bees and the detection of *Nosema* cysts in the intestines of bees, both in spring and summer, as well as the detection of cysts in honey, indicates the spread of another pathogen — *Nosema cerana*, which causes nosemosis in the summer season. This leads to a significant decrease in the number of adult bees in the hive, a decrease in honey productivity, a significant weakening of the strength of the honey bee colony in the summer, and the departure or death in the fall.

The reason for the periodic mass death of bees in Ukraine is the combination of a high number of parasitic

mites *Varroa* in a honey bee colony with the presence of bee infection with microsporidia *Nosema* spp. These two pathogens negatively affect the immunity of the honey bee colony and cause exacerbation of latent infections in bees, which leads to a reduction in the number of honey bee colonies, weakening their viability and lowering the quality of honey products.

Prospects for further research. More research is needed to determine the impact of *Nosema cerana* and other pathogens on the quality of honey produced by bees.

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ANALYSIS OF KEY INDICATORS OF CHRONIC STRESS IN CATS AND DOGS

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Summary. Stress is an integral part of the life of every organism. This issue has become especially important now, during wartime, when stress affects both humans and animals. Military events have led to unprecedented changes in the lives of both humans and animals, affecting their daily routines, social interactions, and stress levels. The study was conducted on 12 dogs and 14 cats. The effects of stressors on cortisol, glucose, total leukocytes, and eosinophils levels were shown. The study's results show that during chronic stress, the studied animals showed an increase in cortisol and glucose levels, indicating increased stress in these animals in response to changes in their daily lives. Total leukocyte counts in dogs and cats were also higher than reference levels, indicating an immediate activation of the immune system in response to stressors. The differential response of eosinophils in animals underscores the complexity of the immune system's response to stress. Dogs, as social animals, may experience more pronounced immune modulation in response to stressors, potentially making them more sensitive to fluctuations in immune cell numbers. The study revealed important behavioral changes in dogs and cats. Behavioral manifestations are the most visible indicators of an animal's emotional well-being. Changes in behavior, including anxiety, hiding, vocalization, and altered social interactions, may reflect the emotional and psychological effects of stress. Our findings underscore the importance of considering individualized strategies for managing animal welfare in emergencies

Keywords: cortisol, glucose, leukocytes, war zone

Introduction. Animal welfare is becoming increasingly important as society becomes more aware of the ethical and humane aspects of animal treatment. An animal is in a state of well-being when it is healthy, comfortable, well-nourished, safe, able to behave naturally, and free from unpleasant conditions such as pain, fear, and suffering (as demonstrated by scientific evidence) (Nedosiekov et al., 2021). Animal welfare requires disease prevention and veterinary care, proper housing, maintenance, nutrition, and humane treatment. Ensuring comfortable conditions and respecting the physiological and psychological needs of animals affects their health (Amat, Camps and Manteca, 2016). Today's realities: the war in Ukraine, determines the need to bring Ukrainian veterinary medicine to a new level, focusing on the decent treatment of animals and their welfare.

Stress is an integral part of the life of every organism. This topic is especially relevant now, during war, when both humans and animals suffer from stress. Animals in war zones may suffer even more than humans because they do not understand what is happening and often live in a state of chronic stress. Stress as a complex and often destructive factor has long been studied in the context of animal health and welfare. In dogs and cats, stress can manifest itself in physiological and behavioral changes that often mirror human responses to environmental factors or situations. These responses, including changes in cortisol levels, immune system dynamics, and behavioral displays, provide insight into the welfare and adaptability of these animals during stressful situations. That is, stress can be considered an adaptive syndrome (Stella and Croney, 2016). The factors that cause stress can be physical, emotional, or social. Physical, when the animal feels uncomfortable due to environmental factors. For example, changes in temperature, pain, hunger and thirst, noise, or vibration. Emotional factors may include loneliness or separation from the owner. Social factors include competition or aggression from other animals (Grigg and Kogan, 2019).

Behavior is often the most visible indicator of an animal's emotional well-being. Changes in behavior, including anxiety, hiding, vocalization, and changes in social interactions, can reflect the emotional and psychological effects of stress (Beerda et al., 1999).

When an animal is under stress, all body systems begin to work more intensively. For a stress response to occur, it is important that the activity of the endocrine glands, especially the hypothalamus-anterior pituitary-adrenal cortex system, be intensified. Depending on the duration and intensity of the stressor, stress can be subtle, leading to adaptation, or develop into pathological stress that can cause illness or even death in animals (Part et al., 2014). Like any other response of the body, stress has several stages.

The first stage of stress called the anxiety or mobilization stage is a general activation of the body to combat negative external influences. This stage is characterized by the activation of the sympathetic nervous system and the release of stress hormones, including adrenaline and norepinephrine. Adrenaline stimulates the breakdown of glycogen in the liver and muscles, leading to an increase in blood glucose levels. This gives the muscles extra energy to respond quickly to

stress. Activation of the sympathetic nervous system also stimulates the breakdown of fats (lipolysis) in adipose tissue, resulting in the release of free fatty acids into the bloodstream. These can be used as a source of energy for muscles and other tissues (Pizzino et al., 2017). In general, the overall metabolic rate of the body increases during this phase. This increases the availability of energy for the animal, allowing it to act more efficiently in the face of a threat.

The immune system also plays a critical role in the body's response to stress. During stressors, the immune system may become activated, leading to changes in the number of different immune cells, including white blood cells. An increase in the total number of white blood cells can indicate the immune system's response to stress. Eosinophils are involved in the body's immune response and can be affected by stress (Nagaraja et al., 2016).

The second stage called the resistance or adaptation stage develops with prolonged exposure to a stressor and is characterized by a significant increase in the size and activity of the adrenal glands, as well as an increase in the body's general and specific resistance. At this stage, there is an increased release of corticotropin from the pituitary gland, which stimulates the adrenal glands to increase production of glucocorticoids such as cortisol. Cortisol is known to help regulate stress and the body's response to danger, but this is only one, albeit important, function (Kooriyama and Ogata, 2021).

Cortisol, a steroid hormone produced by the adrenal glands, is a well-established marker of stress in both humans and animals. Stressful situations trigger the release of cortisol, which often leads to an increase in blood levels. The physiological response of dogs and cats to stress is assessed by monitoring cortisol levels (Nenadovic et al., 2017).

Cortisol plays a key role in maintaining blood glucose levels and adapting the body to prolonged stress. Under the influence of cortisol, gluconeogenesis is stimulated. This provides the body with energy under conditions of prolonged stress when glycogen stores can be depleted. To support the process of gluconeogenesis, cortisol stimulates the breakdown of proteins (proteolysis) in the muscles. The amino acids released from this process are used to synthesize glucose in the liver. Although this helps maintain energy balance, prolonged proteolysis can lead to muscle atrophy and a decrease in overall muscle mass (Markovszky et al., 2020). Cortisol also promotes lipolysis, the breakdown of fats, which leads to the release of free fatty acids that can be used as a source of energy.

One marker of acute stress in dogs is salivary cortisol. However, its use has some drawbacks that can lead to misinterpretation of the data. The key aspect is a standardized sampling method and subsequent processing before immunoassay. In addition, circadian changes and individual variability in cortisol levels should be consistently taken into account in the preparation of the experimental design, statistical data processing, and subsequent interpretation of the measurements (Chmelíková et al., 2020).

The stage of resistance can last for a long time, but if the stressor continues to act, the body may become exhausted, leading to the transition to the third stage of stress — the stage of exhaustion.

The third stage, known as the exhaustion stage, occurs when the body is exposed to a stressor for an extended period. At this stage, the adaptive functions of the adrenal glands, despite their hypertrophy and other body systems decline, leading to exhaustion. Despite the initial hypertrophy of the adrenal glands, their functional capacity gradually decreases. This leads to a decrease in the production of glucocorticoids, especially cortisol, which makes it impossible to further support stress adaptation (Viena et al., 2012). A decrease in cortisol levels means that the body can no longer maintain the required level of gluconeogenesis and energy mobilization. Lack of sufficient cortisol and depletion of glycogen stores leads to a decrease in blood glucose levels, which can lead to hypoglycemia. This makes it difficult to provide energy to cells, especially neurons, which depend on a stable supply of glucose.

Due to the prolonged protein breakdown during the previous phase, amino acid stores are depleted, leading to significant catabolism of muscle tissue and other protein structures. Muscle atrophy becomes pronounced, reducing physical strength and recovery.

Lipolysis may be impaired at this stage and the free fatty acids released may not be used effectively due to reduced metabolic activity. This can lead to an accumulation of lipids in the blood and impaired cell membrane function.

Prolonged exposure to cortisol in the previous stages suppresses the immune system. In the exhaustion stage, the immune system is further weakened, making the body susceptible to infections and other diseases. The body's specific and non-specific resistance is significantly reduced. The body can no longer effectively use energy reserves, leading to general exhaustion, weakness, weight loss, and organ failure. The ability to regenerate tissues is significantly reduced, making it difficult to recover from injury or illness. This can contribute to the development of chronic diseases and irreversible changes in organs and tissues (Feldsien, 2010).

Chronic stress can cause serious damage to the lives of animals, affecting their health, behavior, and overall well-being, underscoring the importance of creating conditions that minimize stressors and promote adaptation and recovery. Animals, especially dogs and cats, in human care should experience as little stress as possible, so it is necessary to measure and quantify stress levels. Stress parameters that can be measured non-invasively can help identify poor animal welfare.

The study aims to quantify stress levels and identify effective methods to help animals cope with stressful conditions.

Materials and methods. The study was conducted on 12 dogs and 14 cats evacuated from the combat zone. Inclusion criteria included animals without visible injuries. The examination of the animals began with the study of the general clinical condition of the dogs and cats by determining the TPR indicators, and general condition of the animals.

Blood samples were collected for biochemical and hematological studies; to assess any changes related to blood components.

Biochemical analysis was performed in the laboratory to determine serum cortisol and glucose levels. Blood samples were collected from the jugular vein or saphenous vein (external for dogs and medial for cats) into vacuum blood collection tubes (Vacusera) with a clotting activator inside using a sterile needle and syringe. The animals were handled carefully and without excessive stress during collection. The collected blood samples were allowed to clot and then centrifuged to separate the serum from the blood cells.

Hematologic analysis was performed on an automatic HTI microCC-20Plus analyzer with the determination of the total number of leukocytes and eosinophils as the main indicators of stress in animals. Cortisol levels were determined by a Bionote Vcheck V200 fluorimeter, and glucose levels by an HTI BioChem SA biochemical analyzer.

Statistical processing of the results was performed using the Statgraphics software package (Manugistic Inc.; STATistical GRAPHICsystem, USA). Data were presented as M ± SE (mean ± standard error); $p < 0.05$ was considered statistically significant.

Results and discussion. Stress is a complex physiological response to an anticipated threat or challenge, and it can have several negative effects on the physiology and behavior of pets. Stress can weaken the immune system, making animals more vulnerable to disease (Rom and Reznick, 2015).

The data obtained in the course of the studies allow us to conclude that there was a significant increase in serum cortisol and glucose levels observed in dogs and cats (Table 1).

Table 1 — Cortisol and glucose levels in blood serum of dogs and cats

Indicator	Dogs		Cats	
	Result obtained	Reference limits	Result obtained	Reference limits
Cortisol, nmol/l	241.9 ± 16.5	28–170	276.5 ± 17.2	28–140
Glucose, mmol/l	11.8 ± 1.21	4.3–6.6	15.2 ± 2.11	3.2–7.9

The results show significant statistical changes in the values of cortisol and glucose in the blood serum during chronic stress in which the animals were exposed compared to the reference values ($p < 0.05$).

The increase in cortisol levels indicates increased stress in these animals in response to changes in their daily lives. Cortisol is a well-established biomarker of stress response in both humans and animals, and its increased secretion is a physiological response to stressors. Our results are in line with studies that emphasize the role of cortisol as a sensitive indicator of stress in the study of biological mechanisms of animal behavioral laterality (Salgirli Demirbas et al., 2023).

The increase in cortisol levels during prolonged stressors (under combat conditions) indicates that the dogs and cats perceived changes in the environment as stressful. These changes included increased noise levels, frequent explosions, vibrations, changes in living conditions, lack of familiar routines and social contact, and possible difficulty in accessing food and water. The fact that both species (dogs and cats) showed this response underscores the impact of chronic stress on the physiological functions and behavior of companion animals.

Based on our observations, high cortisol levels cause increased anxiety, fear, and nervousness in animals. This manifests itself as avoidance, trembling, or hiding. Animals may also react aggressively to normal stimuli. In general, prolonged elevation of cortisol levels due to chronic stress can significantly impair the quality of life of dogs and cats, which underscores the importance of creating favorable conditions to reduce the effects of stress.

An increase in glucose levels by an average of three times (compared to reference values) may indicate an increase in the process of gluconeogenesis stimulated by excess cortisol (Table 1).

Stress hyperglycemia (also called physiological hyperglycemia) is the most common condition observed in cats. An increase in blood glucose levels occurs due to prolonged exposure to stressors. Chronic stress leads to constant activation of the sympathetic nervous system and the adrenal glands, which secrete stress hormones, especially cortisol. This hormone promotes the breakdown of glycogen in the liver and the release of glucose into the bloodstream to provide the body with energy.

Prolonged hyperglycemia due to chronic stress can have negative consequences: consistently elevated glucose levels lead to the development of insulin resistance, which increases the risk of diabetes; chronic stress and concomitant hyperglycemia can reduce the effectiveness of the immune response, making animals more susceptible to infection and disease.

Hematological studies of serum have also shown an increase in the total number of leukocytes in both dogs

and cats during chronic stress, indicating an increased immune system response to stressors. An increase in the number of leukocytes is consistent with the idea that stress can trigger an immune response as the body prepares to defend itself against potential threats (Beerda et al., 1999). As shown in Table 2, an increase in neutrophil count is an indicator of a non-specific defense response in dogs and cats.

Table 2 — Total and differential leukocyte counts in the dogs and cats studied

Indicator	Dogs		Cats	
	Result obtained	Reference limits	Result obtained	Reference limits
Leukocytes, $\times 10^3$/mm^3	18.17 ± 2.12	5.0–14.1	22.21 ± 3.38	5.5–19.5
Neutrophils, %	85.90 ± 9.35	61–88	70.01 ± 11.12	47–66
Eosinophils, %	0.10 ± 0.12	0–9	0.50 ± 0.15	0–4
Basophils, %	0.20 ± 0.10	0–1	0.15 ± 0.08	0–1
Monocytes, %	6.75 ± 1.32	2–10	3.91 ± 0.42	0–5
Lymphocytes, %	7.05 ± 0.91	8–21	25.43 ± 5.94	27–36

The number of eosinophils, a component of the immune system, showed species-specific variation in response to stressors. A significant decrease in the number of eosinophils was observed in dogs, which may indicate suppression of immunity in response to acute stress.

The differential response of eosinophils in dogs and cats underscores the complexity of the immune system's response to stress. Dogs, as social animals, may experience more pronounced immune modulation in response to stressors, potentially making them more sensitive to fluctuations in immune cell numbers (Salgirli Demirbas et al., 2023). Cats, with their more solitary and independent nature, may maintain a more stable immune response under similar circumstances (Westropp, Kass and Buffington, 2006).

The results of the study show that during chronic stress, the dogs studied had an increase in the total number of leukocytes, indicating an immediate activation of the immune system in response to stressors. This observation is consistent with the idea that dogs are highly sensitive to the emotional state of their owners and may respond more dynamically to changes in human behavior (Brooks et al., 2018). In contrast, cats may have a somewhat delayed white blood cell response. This delay is indicative of the more self-sufficient nature of cats and their ability to cope with stress independently (Amat, Camps and Manteca, 2016).

Behavior is often the most visible indicator of an animal's emotional well-being. Changes in behavior, including anxiety, hiding, vocalization, and altered social interaction, can reflect the emotional and psychological effects of stress (Alho, Pontes and Pomba, 2016). Thus, several changes were observed in animals under chronic stress, the most common of which was increased anxiety (55.9%) and attention-seeking behavior (44.1%) in dogs. Cats, on the other hand, hid more (55.2%), groomed less (48.3%), and became more territorial (41.4%).

Conclusions. 1. Chronic stress has created unique stressors for dogs and cats, with both species experiencing elevated cortisol levels and behavioral changes.

2. The difference in eosinophil counts between these two species highlights their different immune responses to stressors during prolonged stress.

3. This study highlights the importance of understanding the specific needs of dogs and cats in emergencies and adapting interventions to reduce stress and promote well-being.

4. Further research is needed to elucidate the underlying mechanisms that drive these species-specific stress responses.

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ON THE ISSUE OF PREVENTION AND ERADICATION OF MINOR VIRAL BOVINE DISEASES IN UKRAINE

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Summary. The study aimed to evaluate the epizootic status of livestock in Ukraine concerning the prevalence of bovine immunodeficiency virus and bovine foamy virus infections. A literature review was conducted to analyze the epizootic status of livestock farming in various countries regarding bovine immunodeficiency and spumavirus infections. To investigate this issue in Ukrainian livestock, blood samples were collected from 10–15 cows with further DNA extraction and studies via PCR, according to the developers' recommendations. The biological characteristics of bovine foamy virus and bovine immunodeficiency virus were studied by infecting bovine fetal lung (LEK) and calf coronary vessels (KST) cell cultures, with each passage being visually monitored and examined through light microscopy. PCR was performed on the third and fifth passages to detect the genetic material. The genetic material of bovine leukemia virus, bovine immunodeficiency virus, and bovine foamy virus was confirmed in 12 farms across 8 regions of Ukraine. It was demonstrated that bovine immunodeficiency virus and bovine foamy virus can integrate into homologous cell cultures derived from cattle. The immunosuppressive effects of bovine foamy virus and its capability to inhibit components of the non-specific immune system were established on laboratory animal models. Emphasis is placed on the necessity to develop domestic tools for the retrospective diagnosis of bovine immunodeficiency and spumavirus infections and to implement a national anti-epizootic program

Keywords: bovine leukosis, bovine immunodeficiency, bovine spumavirus infection, PCR, immunosuppression, national anti-epizootic program

Introduction. The stability and profitability of the livestock sector in collective farms depend on many factors. Primarily, these include the genetically embedded potential capabilities of the livestock population concerning milk or meat productivity. These potentials, in turn, are realized when the animals are provided with a balanced diet that meets the needs of each age group and productivity direction. An important element ensuring the profitability of livestock farming is the management of animal housing, specifically: the microclimate of livestock buildings, the organization of the work schedule depending on the season, physiological state of individual animals and groups. Perhaps the most critical factor in maintaining a stable and profitable operation in the livestock sector is the health of the herd concerning infectious diseases.

While preventive measures have been developed and implemented against the most particularly dangerous infectious diseases of cattle, there are no specific means developed for minor viral diseases. These minor diseases include *bovine leukosis* (caused by the bovine leukemia virus —BLV), *bovine immunodeficiency* (caused by the bovine immunodeficiency virus — BIV), and *bovine spumavirus infection* (caused by the bovine foamy virus — BFV). Therefore, the system of preventive health measures is limited to general veterinary-sanitary approaches.

A unifying factor for these minor infections in cattle is not only the affiliation of the pathogens to the same Retroviridae family but also the pathological changes caused by their persistence in the bodies of infected animals. Concerning bovine leukosis, it is worth noting that the persistence of the pathogen in the herd and the disease caused by it leads to the loss of the gene pool due to the early culling of valuable breeding and commercial young animals, as well as adult animals. Another significant aspect of the losses is related to the quality deterioration of livestock products: in meat and milk, the protein-fat balance is disrupted. Milk obtained from leukemic animals is prohibited for consumption without prior thermal decontamination.

Special requirements apply to the use of milk from cows with clinical leukosis: according to Ukrainian legislation, such milk, even after thermal treatment, cannot be used for human or animal consumption due to the accumulation of tryptophan metabolites in the product, which are carcinogenic. This milk must be mixed with a disinfectant and then disposed of (SCVMU, 2007).

Another critical element of loss in the livestock sector is the immunosuppressive condition of animals in the stage of infection and clinical manifestation of diseases caused by the pathogens of minor viral infections. It should be noted that the immunosuppressive state hinders the expected immune response of animals to the administration of specific preventive agents, as well as antibiotic and stimulatory treatment methods.

Despite significant costs associated with the use of high-cost preventive and therapeutic measures, their effectiveness in the bodies of animals infected with the pathogens of minor infections decreases by several orders

of magnitude (Gorbatenko et al., 2009; Willems et al., 1993; WOAH, 2018).

It is also important to note that the retrovirus family of pathogens poses a potential medical and social threat because they are structurally similar to the pathogens that cause AIDS and human T-cell leukemia.

If bovine leukemia, which had pandemic characteristics in the recent past, has been reduced to isolated cases of infected animals due to the introduction of diagnostic tools and government programs in most developed countries of the world, particularly in Europe, the eradication program for bovine immunodeficiency and spumavirus infection has not yet achieved the expected results, even though 20–45% of cattle are infected in some countries (Constable et al., 2017; Nuotio et al., 2003; Pinheiro De Oliveira et al., 2013).

The study **aimed** to evaluate the epizootic status of livestock in Ukraine concerning the prevalence of bovine immunodeficiency virus and bovine foamy virus infections.

Materials and methods. A review of literature reports on the epizootic state of animal husbandry in various countries regarding bovine immunodeficiency and spumavirus infections has been conducted. To study a similar issue in Ukrainian livestock sector, blood samples stabilized with anticoagulant (EDTA) were selectively taken from 10–15 cows from livestock farms in the central-eastern region of Ukraine. Molecular genetic methods, specifically polymerase chain reaction (PCR), were used to isolate the genetic material of BFV and BIV. For the detection of BFV proviral DNA, the *Int1-Int2* primer system (external pair, amplified product length of 430 base pairs (bp)) and *Int3-Int4* (internal pair, amplified product length of 221 bp) were used through the 'nested' PCR variant, following the developers' recommendations (Materniak-Kornas et al., 2017). For the detection of BIV proviral DNA, the *RT_+(-)* primer pair was used, flanking a conserved domain of reverse transcriptase (PCR product length 495 bp), as well as the *BIV_Pol_+(-)* primer pair, flanking the *pol* gene of BIV (PCR product length 235 bp). Amplification was carried out using the standard PCR method according to the developers' recommendations (Moody et al., 2002). To detect proviral DNA of BLV, the *BLV-env_3-4* primer pair was used under WOAH recommendations (Fechner et al., 1996), flanking a fragment of the *env* gene of BLV with a length of 444 bp. Reverse transcription and the creation of cDNA were performed using MMLV reverse transcriptase following the manufacturer's instructions. Amplification was performed on a Biometra thermocycler (USA). PCR analysis results were visualized by horizontal gel electrophoresis in a 1.5–2.0% agarose gel.

The biological properties of BFV and BIV were studied using genetic material by infecting two transplanted cell cultures: bovine fetal lung (LEK) and calf coronary vessels (KST). Each passage was monitored daily visually and using light microscopy. In the third and fifth passages, samples were examined using PCR to detect the genetic material of the pathogens.

A study on the biological properties of BFV was conducted using a model of laboratory animals. For this purpose, the experimental group of rabbits (5 individuals) received a single subcutaneous injection of 1 cm^3 of native blood from a donor animal that had confirmed the presence of BFV genetic material. The second group served as the control group. The condition of the experimental rabbits was monitored through visual observation of the animals' viability after infection, as well as conducting hematological, biochemical, and molecular-genetic analyses of blood samples. Blood samples were taken and subjected to analysis every 15 days.

Results. BIV has been identified in many countries worldwide, and co-infection with two or even three minor disease pathogens is common. It is noteworthy that serological studies on bovine immunodeficiency in different countries, based on several scientific publications, reveal a significant prevalence of the disease in the livestock sector globally. For instance, seropositivity in the United States was observed at 4%, in the Netherlands at 1.4%, in Canada at 5.5%, in Germany at 6.6%, and in France at 4%. Immunodeficiency, according to laboratory studies, has also been confirmed in the United Kingdom, Sweden, Costa Rica, Venezuela, New Zealand, and Australia. The percentage of seropositive cattle compared to healthy animals generally ranged between 1–7%. However, in some herds with chronic disease (epizootic persistence), the infection rate reached up to 50%. Among 64% of BIV-seropositive animals with lymphosarcoma, lymphadenopathy, and other disorders, 74% were infected with the immunodeficiency virus (Meas et al., 2002; Rua and Gessain, 2015; Rethwilm and Lindeman, 2013; Rethwilm and Bodem, 2013).

Regarding spumavirus infection, the literature indicates that 30% to 45% of cattle are seropositive for BFV, and the infection caused by it is widespread globally (Materniak et al., 2010, 2013).

According to the results of molecular-genetic studies conducted at the Molecular Diagnostics Laboratory of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine), it was established that in 12 livestock farms across 8 regions, where blood samples from a limited number of animals (10–15 individuals) were selectively examined mostly in farms where anti-leukemia health measures were in their final stages there was evidence of the circulation of BFV, BIV, and associations between the pathogens of spumavirus infection, immunodeficiency, and bovine leukemia. Moreover, in each case, the genetic material of the pathogens, often in associated form, was detected in 20–35% of the samples tested (Fig. 1).

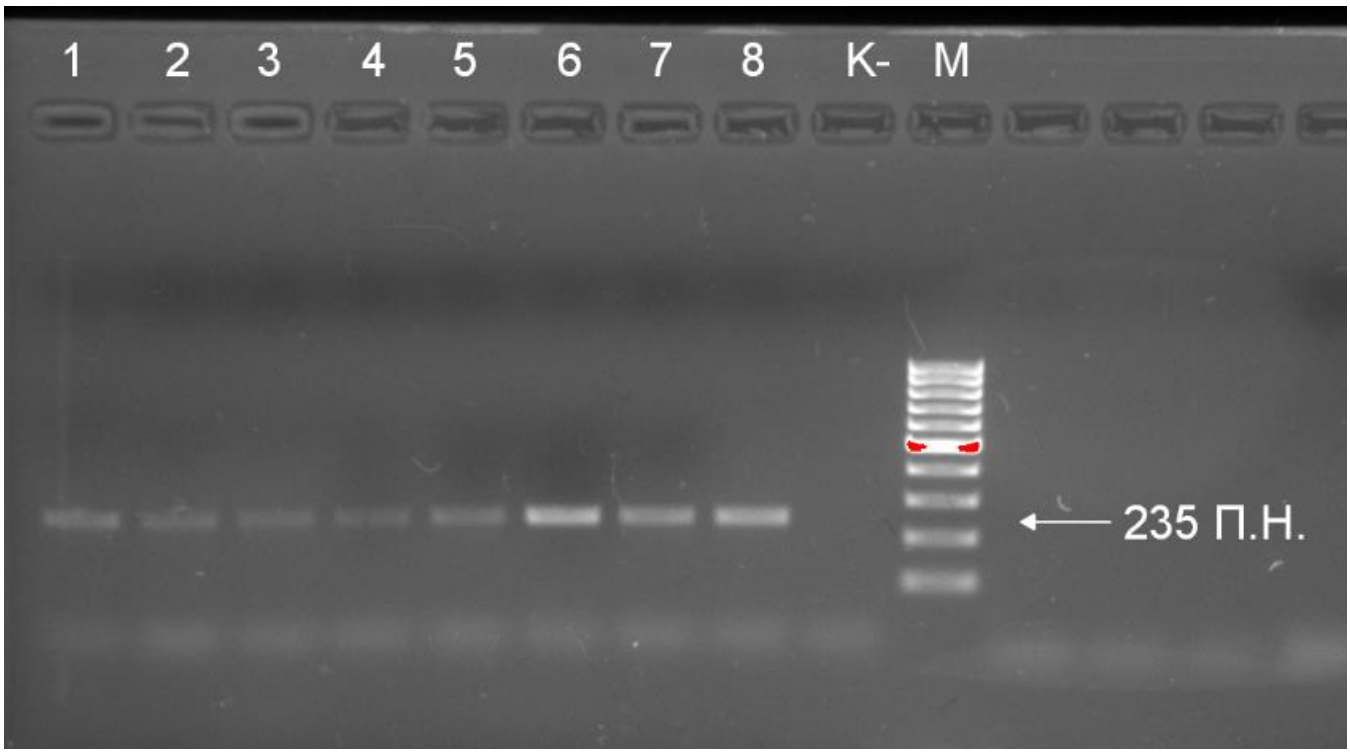

Figure 1. Gel-electrophoresis results of PCR products are as follows: 1–8 — positive clinical samples from cattle infected with BIV; K- — negative control; M — 100 bp molecular weight marker.

Based on the results of testing a limited number of blood samples from each farm's herd, it would be risky to draw conclusions about the infection intensity of the entire herd, taking into account different age groups. From our research, we can only confirm the circulation of slow virus pathogens, namely BLV, BIV, and BFV, in the livestock sector of the examined region of Ukraine.

This data emphasizes the need for further studies aimed at determining the prevalence of these diseases to develop targeted measures for minimizing the damage caused by these diseases to the livestock industry.

Microscopic examinations of LEK and KST cell cultures, conducted after infection, showed that the addition of short-term cultivated lymphocytes did not cause destructive changes in the morphology of both cell lines.

The monolayer cells were densely packed with clearly defined boundaries, and the cytoplasm exhibited a minimal number of vacuoles, while the nuclei retained their typical oval shape (Fig. 2).

Observations of the state of monolayer cell cultures (LEK+BIV) and (LEK+BFV) at 1st, 2nd, and 3rd passages revealed satisfactory coverage of the monolayer. Morphologically, the cells in the experimental cultures were similar to the control cells. PCR results in 3rd passage indicated the presence of genetic material from BIV and BFV in the cells of the monolayer. In 4th to 6th passages, the experimental cell cultures exhibited morphological destruction with signs of syncytium formation — enlarged cells with two or three nuclei were observed. It became more challenging to detach the monolayer cells from the glass using trypsin-versen solution (Fig. 3).

In 7th and 8th passages, the condition of the monolayer remained similar, with a significant increase in the number of dead cells in the culture medium (Fig. 4).

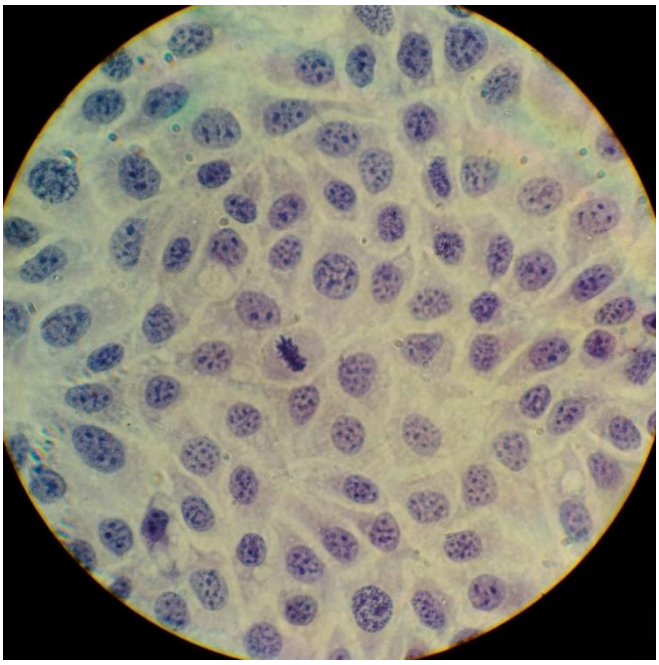

Figure 2. Normal monolayer of LEK cell culture.

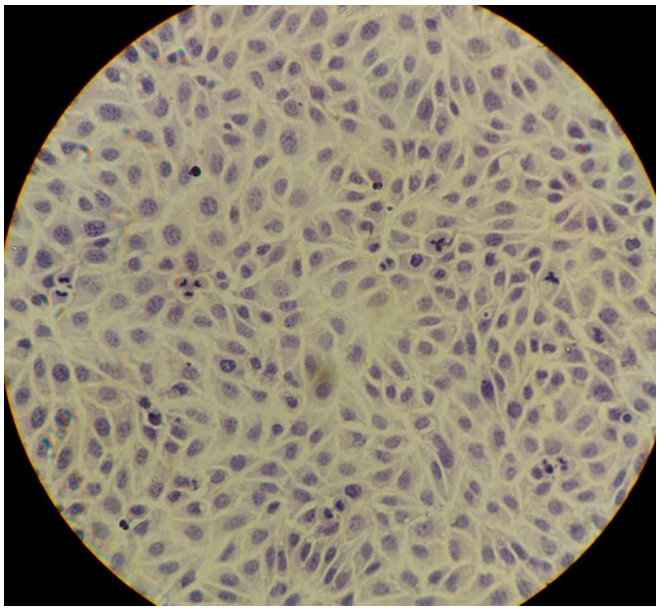

Figure 3. Syncytium formation in the monolayer culture of LEK cells at 4th–6th passages after infection with virus-containing material.

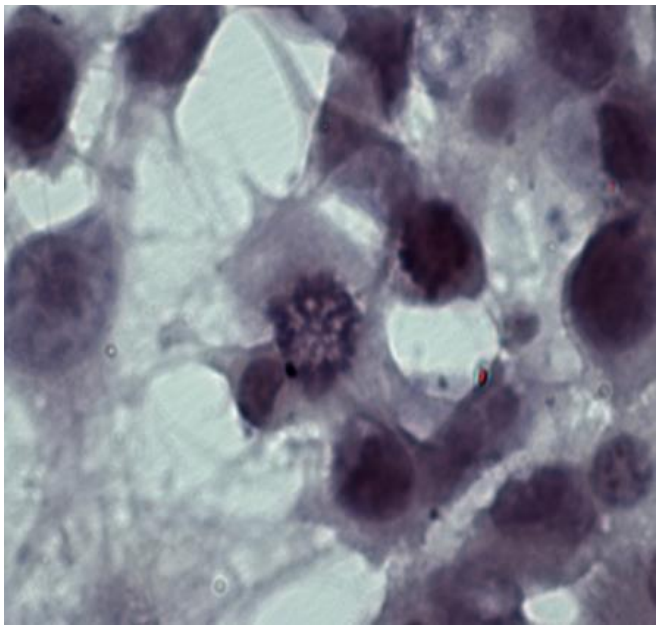

Figure 4. Cell death in the LEK monolayer at 7th–8th passages.

A total of 15 passages were performed for both cultures (LEK+BIV) and (LEK+BFV). The genetic material of BIV and BFV was also detected in the material isolated from the 10th passage. According to the PCR results, the genetic material of the above-mentioned viruses was not detected in the DNA from the cell culture at 13th and 15th passages. The study on the potential integration of field strains of BIV and BFV into the transferrable cell culture of calf coronary vessels (KST) showed a lower sensitivity of this culture to viruses of the Retroviridae family compared to the LEK cell culture.

A total of 7 passages were conducted. At the 5th passage, PCR results still indicated the presence of genetic material from the retroviral pathogens, while the material from the 7th passage yielded a negative result.

Regarding the study of the biological properties of BFV in laboratory animals, it should be noted that molecular-genetic analysis of blood samples from the experimental group of rabbits revealed the presence of BFV genetic material in four out of five rabbits fifteen days after inoculation. After 30 days, positive results for the presence of the genetic material of the mentioned pathogen were found in two rabbits. A third analysis, conducted 45 days after the start of the experiment, yielded similar results as the second analysis, with the genetic material being detected in the blood samples of two experimental animals. By the second month after inoculation (fourth analysis), the genetic material of BFV was found in only one rabbit. It was established that the inoculation of rabbits with the genetic material of BFV causes a short-term persistence of up to 60 days according to molecular-genetic research data. The persistence of BFV virus in rabbits does not cause significant hematological changes, although the redistribution of the leukocyte fraction towards a pronounced lymphocytosis indicates the development of an immunosuppressive state. Experimental infection of rabbits with BFV causes a minor activation of the immune system 30 days after infection, which is followed by pronounced suppression of both functional arms of the nonspecific immune response. The genetic material of BFV causes the manifestation of immunosuppression in rabbits post-inoculation, characterized by leukocytosis and redistribution of the leukocyte fraction towards significant (80–88%) lymphocytosis, a decrease in the concentration of circulating immune complexes, a reduction in globulins, and an increase in serum mucoproteins (Tables 1 and 2).

Discussion. The results of the monitoring studies on the epizootiological situation in Ukrainian livestock concerning minor infections, specifically bovine spumavirus infection and bovine immunodeficiency, conducted using molecular-genetic methodologies, only indicate the presence of issues related to these minor infections without any recommendations for addressing the epizootiological status. Research has widely demonstrated the global presence of BLV in dairy cattle, with significant variation in prevalence depending on geographic region and control measures. For instance, Yang et al. (2019) investigated BLV in Chinese dairy cattle, confirming the circulation of genotype 4 and underscoring the virus's significant impact on herd health due to its association with bovine leukosis, a cancer-causing condition. Studies such as this align with our findings, emphasizing the need for more comprehensive surveillance to control viral infections in livestock.

Table 1 — Dynamics of white blood cell's level (%)

Days of the experiment	Segmented neutrophils		Band neutrophils		Basophils		Lymphocytes	
	Experiment	Control	Experiment	Control	Experiment	Control	Experiment	Control
15	29.4 ± 2.6	25.4 ± 2.6	4.5 ± 1.2	5.6 ± 1.2	1.4 ± 0.5	1.3 ± 0.2	48.3 ± 4	47.6 ± 4
30	26.6 ± 3.3	23.4 ± 3.3	3.6 ± 1.4	3.8 ± 1.5	0.8 ± 0.4	2.2 ± 0.3	59.1 ± 3	52.2 ± 3
45	19.4 ± 2.6	27.1 ± 1.9	4.5 ± 1.1	4.1 ± 0.7	0.5 ± 0.3	3.2 ± 0.6	67.3 ± 4	49.4 ± 5
60	18.7 ± 2.2	24.6 ± 2.4	5.8 ± 1.4	4.3 ± 1.1	0.8 ± 0.4	2.6 ± 0.5	85.2 ± 5	51.3 ± 4
75	21.2 ± 3.5	26.4 ± 3.7	3.8 ± 1.5	3.2 ± 0.2	0.9 ± 0.4	1.8 ± 0.3	80.6 ± 3	47.6 ± 3
90	23.7 ± 2.3	30.3 ± 2.1	3.3 ± 1.2	4.4 ± 1.2	1.6 ± 0.2	1.6 ± 0.2	81.8 ± 4	49.1 ± 5
105	21.9 ± 3.9	32.5 ± 2.9	3.9 ± 0.7	5.5 ± 1.3	0.8 ± 0.3	1.8 ± 0.3	88.4 ± 3	50.6 ± 2
120	20.8 ± 2.7	33.4 ± 2.7	4.1 ± 1.1	5.9 ± 1.3	1.2 ± 0.4	1.2 ± 0.1	67.2 ± 5	49.4 ± 6

Table 2 — Biochemical parameters of rabbit serum

No.	Total protein, g/L	Albumin, g/L	Globulin, g/L	Circulating immune complexes, mg/mL	Seromucoids, mg/mL
Before the infection					
Experiment					
1	83.1	59.3	23.8	0.15	0.22
2	59.6	44.8	14.8	0.13	0.22
3	76.9	52.4	24.5	0.13	0.25
4	74.8	49.7	25.1	0.11	0.26
5	72.9	51.1	21.8	0.12	0.22
M ± m	73.5 ± 4.7	49.5 ± 4.9	24.0 ± 0.7	0.13 ± 0.01	0.23 ± 0.008
Control					
6	71.2	55.2	16.0	0.11	0.22
7	65.3	45.5	19.8	0.12	0.22
8	75.8	53.1	22.7	0.10	0.23
9	67.0	48.3	18.7	0.10	0.23
10	70.6	54.5	16.1	0.11	0.22
M ± m	69.9 ± 1.2	51.3 ± 1.9	18.7 ± 1.3	0.11 ± 0.004	0.22 ± 0.002
105 days after infection					
Experiment					
1	64.2	45.9	18.3	0.11	0.24
2	61.7	45.3	16.4	0.12	0.30
3	71.2	47.0	24.2	0.11	0.29
4	75.8	50.0	25.8	0.14	0.32
5	70.0	45.3	24.7	0.14	0.32
M ± m	68.6 ± 2.8	46.7 ± 0.9	21.9 ± 1.9	0.124 ± 0.006	0.294 ± 0.016
Control					
6	64.2	45.3	18.9	0.16	0.32
7	79.3	47.0	32.3	0.15	0.26
8	63.2	45.3	17.9	0.18	0.22
9	75.8	46.5	29.3	0.16	0.24
10	78.7	48.2	30.5	0.14	0.21
M ± m	72.2 ± 3.2	46.5 ± 0.6	25.8 ± 2.5	0.158 ± 0.008	0.250 ± 0.022

As for BIV, similar to our findings, this virus has been detected in several cattle populations worldwide. Although BIV is not as thoroughly researched as BLV, its role in immunosuppression and potential to exacerbate other infections is well-established. Bhatia, Patil and Sood (2013) identified BIV's immunosuppressive effects, which contribute to reduced immune responses and increased vulnerability to secondary infections. This echoes our results where BIV's presence correlates with immunosuppressive conditions in cattle.

Bao et al. (2015) studied BFV in cell cultures, particularly its effects on cell morphology and replication dynamics. Similar to our results, BFV was found to cause syncytium formation and other morphological changes in infected cell cultures. BFV, although generally non-pathogenic, has been implicated in the modulation of immune responses in infected animals, a conclusion supported by the observations of immune suppression in our study.

The experimental inoculation of rabbits with BFV in our study aligns with other research investigating retroviral infections in laboratory animals. Rethwilm (2010) examined the persistence of BFV in laboratory animals, demonstrating its short-term persistence and its effects on immune function, including lymphocytosis and leukocytosis. These findings are consistent with those reported in Ukraine, where BFV caused a temporary immune response followed by immunosuppression.

The presence of these minor infections in cattle herds causes significant damage to livestock both directly, by reducing the volume and quality of production, and indirectly, by decreasing the effectiveness of preventive and therapeutic measures due to the immunosuppressive state of infected animals.

A logical task for the near future is to develop a domestic method for retrospective diagnosis of livestock herds to obtain information on the level of infection and develop measures to control and eradicate the epizootic situation. According to the vision of researchers concerned with minor infections, there is a need to accumulate viral material of BFV and BIV and to develop an antigen for the serological identification of infected animals. The molecular-genetic methodology used in our studies does not allow for the examination of the entire herd due to insufficient funding for a comprehensive survey of livestock and monitoring support during the implementation of health programs.

The development of a national serological diagnostic tool for minor viral diseases of cattle, specifically immunodeficiency and spumavirus infections, will enable the creation and implementation of guidelines for their diagnosis and prevention. This will include considerations of transmission pathways, barriers to infection of susceptible individuals, ensuring the quality of livestock products, environmental sanitation, and strategies for preventive and therapeutic programs.

Conclusions. Minor viral diseases of cattle, such as bovine immunodeficiency and spumavirus infections, are widespread in livestock operations worldwide. Selective blood tests from 12 farms in 8 regions of Central and Eastern Ukraine have confirmed the presence of genetic material from BLV, BIV, and BFV viruses, with instances of co-infection. The ability to integrate these pathogens into homologous cell cultures for cattle and the potential for virus mass accumulation have been established. Laboratory animal models have demonstrated that inoculation with spumavirus genetic material leads to immunosuppressive effects and suppression of both branches of nonspecific immunity. Addressing preventive measures and eradicating these viral slow infections, specifically bovine immunodeficiency and spumavirus infections requires the development of domestic retrospective diagnostic tools and the implementation of a national anti-epizootic program.

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Part 2. Biosafety

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SEROLOGICAL MONITORING OF INFLUENZA A AMONG WILD AND DOMESTIC UNGULATES IN UKRAINE

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Summary. The article provides a brief historical background of equine influenza, the spread of this disease worldwide, and the current epizootic situation. The results of serological monitoring by ELISA of wild and domestic ungulates from different farms and regions of Ukraine for the presence of antibodies to influenza A viruses are presented. Blood serum samples from 372 domestic horses and 32 wild ungulates were tested. Samples from animals collected in 2023 and 2024 and archival blood serum samples from 2021 were used and tested according to the manufacturer's instructions using ELISA test systems manufactured by IDEXX, INGEZIM, and IDVet. The data obtained indicate a fairly active circulation of influenza A viruses in populations of unvaccinated domestic horses. The circulation was established not only in recent years (2023–2024, seroprevalence from 10% to 100%), but was observed earlier, as evidenced by the detection of 60.9% of positive samples in samples collected in 2021. In addition, two out of three positive samples were found in wild horses from Kherson Region, which indicates the circulation of influenza A virus among wild animals and requires further investigation. The results correlate with the worsening of the epidemiological situation regarding influenza in animals in Europe. The subsequent phase of the research is serotyping, which involves determining the presence of antibodies to specific virus subtypes by hemagglutinin

Keywords: seroprevalence, equine influenza, enzyme-linked immunosorbent assay

Introduction. Equine influenza (EI) is an acute, highly contagious viral disease of horses, donkeys, mules, and other ungulates that is associated with severe respiratory disorders in animals. Today, the disease is widespread in Europe and North America, and sporadic outbreaks of equine influenza have been reported in Africa, Asia, Australia, and South America (Bryant et al., 2011; Chambers, 2022). From an economic standpoint, equine influenza is regarded as one of the most significant respiratory diseases affecting horses and other ungulates (Olguin-Perglione and Barrandeguy, 2021). At the same time, the disease should be considered a potential threat to human and other animal health. The causative agent of EI, the equine influenza virus (EIV), is an RNA-containing virus belonging to the Orthomyxoviridae family and is a typical representative of the influenza type A virus (Singh, 1994; Bryant et al., 2009; Zhang et al., 2021). Influenza A viruses are currently the most common influenza viruses, primarily infecting humans, horses, birds, and pigs (Cauldwell et al., 2014). They therefore pose a significant threat to both animal and human health. This is particularly important in the context of the worsening epizootic situation with avian influenza, including highly pathogenic influenza, and its ability to cross the interspecies barrier. Cases of infection with avian influenza virus in domestic and wild carnivorous mammals (cats, dogs, foxes, fur-bearing animals) recorded in many European countries (Poland, Finland, etc.) in 2022–2024 (Domańska-Blicharz et al., 2023; WHO, 2023; Lindh et al., 2023; Tammiranta et al., 2023), as well as recent cases in the United States of cows infected with avian influenza virus (highly pathogenic avian influenza virus of subtype H5N1) with subsequent spread in several states and human infection from sick cows (Burki, 2024; Oguzie et al., 2024; Eisfeld et al., 2024), have increased concerns about the next pandemic, which could potentially be caused by a zoonotic influenza virus. It is also necessary to remember that new types of influenza viruses are emerging, the role of which as a dangerous pathogen for human health is still unclear. For example, a new type of influenza virus is the influenza D virus, which has recently been detected in farm animals — cattle, pigs, and horses in different parts of the world (Yu, Li and Wang, 2021; Skelton and Huber, 2022; Nedland et al., 2018).

Concerning the peculiarities of equine influenza as an infectious disease, the spread of EI is facilitated by the

uncontrolled movement of animals from their places of residence, stress factors in the keeping of horses at show or sale grounds, and the absence of quarantine measures and preventive vaccination against influenza. Equine influenza is highly seasonal with a peak in the winter and spring (Pusterla et al., 2011, 2015; Vaala et al., 2019). However, it should be remembered that EI can occur in any month of the year (Chappell et al., 2023). At high incidence rates (up to 100%), mortality can be as high as 2% (Bryant et al., 2011; Chambers, 2022; Pusterla et al., 2015; Chappell et al., 2023; Rodriguez et al., 2018; Sack et al., 2019; Oladunni et al., 2021; Paillot et al., 2016), except in donkeys, which can have high mortality rates (up to 30%) at high incidence rates (up to 100%) (Chambers, 2022; Waghmare et al., 2010). The severity of the disease depends on the pathogenicity of the EI virus, the immune status of the animals, and their age — although EI is usually described as a disease of young horses and donkeys, the highest rate of positive PCR results is observed in the age groups 1–4 years and 5–9 years (Chappell et al., 2023; Landolt, 2014; Paillot, 2014). It should also be noted that in adults, death is usually a consequence of the general condition of the body and/or secondary bacterial infection, leading to complications and the development of pleurisy and pneumonia (Sarasola et al., 1992; Liu, 1993; Kästner et al., 1999; Anzai et al., 2000; Muranaka et al., 2012). The animal's sex also plays a role — females are more likely to be infected than males (Chappell et al., 2023).

Historically, the first documented clinical case of EI was reported in the United States in 1872. High incidence (100%) and low mortality (up to 2%) were observed in horses. The second fact is the report that this pathogen could cause influenza infection in poultry in the United States in 1874 (Morens and Taubenberger, 2010). In fact, the first characterized EI virus was isolated in Czechoslovakia in 1956 (A/equine/1/Prague/56, H7N7, equi-1). It did not have highly pathogenic properties, but an outbreak caused by this virus was reported in Italy 13 years later. Subsequently, the virus of this subtype was isolated in India in 1987, in Egypt in 1989, and in Mongolia in 2011 (Singh, 1994; Ismail et al., 1990; Yondon et al., 2013). Another subtype of influenza virus that causes disease in horses is the H3N8, equi-2 virus. This virus was first detected in 1963 during a large epizootic of equine respiratory disease in Miami, Florida (USA), and since then this subtype has circulated continuously in equine populations causing outbreaks of EI worldwide (Waddell, Teigland and Sigel, 1963; Cullinane and Newton, 2013; Daly et al., 2011; Singh et al., 2018; Scholtens et al., 1964).

In the context of the One Health concept and the risks of new zoonotic influenza viruses emerging, it is important to note the detection of viruses in horses that had connections with viruses of other subtypes obtained from different animals, as well as instances of the influenza virus crossing the interspecies barrier. In China, a significant outbreak of H3N8 influenza in horses and donkeys in 1989 was caused by the 'avian-origin' virus (A/equine/Jilin/89) (Webster and Yuanji, 1991; Murcia, Wood and Holmes, 2011). This outbreak led to the infection of 20,000 horses and the death of 400 animals. Similarly, in Mongolia, it was reported that 97 out of 585 tested horses were seropositive for the H3N8 avian influenza virus (Zhu et al., 2019). Additionally, a highly pathogenic avian influenza virus, H5N1, was isolated from clinically ill donkeys in Egypt (Abdel-Moneim, Abdel-Ghany and Shany, 2010).

The authors suggest that avian influenza may be more prevalent among horses and not cause clinical signs, which makes the disease more difficult to detect. However, in the majority of cases, interspecies transmission of this virus typically results in limited further transmission to a new host. Chinese scientists reported that during the surveillance of pigs in 2005 and 2006, they isolated viruses from them. The closest neighbors of these viruses in all eight gene segments were EI viruses from Europe, isolated in 1991–1993 (Tu et al., 2009). Furthermore, H3N8 equine influenza has been isolated from camels, pigs, and infected cats in experimental conditions (Yondon et al., 2014; Su et al., 2014; Tu et al., 2009).

It is also important to note the role of horses in maintaining existing and forming new reservoirs of influenza A viruses, as evidenced by the interspecies transmission of the H3N8 subtype H3N8 virus to dogs in the late 1990s. This resulted in the formation of a separate canine influenza cluster responsible for several outbreaks of acute respiratory infections in dogs. For several years, there was persistent circulation of this virus in dog populations in the United States and sporadic cases of infection among dogs in the United Kingdom and Australia (Parrish, Murcia, and Holmes, 2015; Gibbs and Anderson, 2010; Crawford et al., 2005; Daly et al., 2008; Crispe et al., 2011; Newton et al., 2007).

It is important to consider the potential for human infection with equine influenza. The first documented outbreak of EI in a population of people in contact with horses was reported in 1957 in Kharkiv (Ukraine), and the diagnosis was confirmed serologically (Gaidamaka et al., 1959; Xie et al., 2016), indicating a significant risk of virus exchange between horses and humans.

Regarding the current epizootic situation in the world. Over the past decade (2010–2021), numerous outbreaks of EIV infection have been reported in many countries on different continents. The increase in EIV outbreaks has been reported not only in North America, especially in the United States, where the disease is endemic, but also in Europe, Africa, Asia, and South America (Oladunni et al., 2021). In the United States, outbreaks were reported in 23 states in 2015, 16 states in 2016, 22 states in 2017, and 33 states in 2018–2019 (Sack

et al., 2019; OIE, 2020). It should also be noted that EIV is not a notifiable disease in the United States, so the true picture may be much worse. Europe and the United Kingdom experienced particularly severe outbreaks of EI in 1989, 2003, and 2018–2019. Each outbreak affected approximately 5,000 to 10,000 horses. In Europe in particular, outbreaks have been reported for many years in France, Germany, Ireland, Sweden, and the United Kingdom (Sack et al., 2019). One of the most recent large outbreaks of EI in Europe was reported in 2018–2019, with 228 horses in the United Kingdom, 80 — in Ireland, and 60 — in France (OIE, 2020). These outbreaks occurred in both vaccinated and unvaccinated horses. Until the mid-2000s, EIV outbreaks were rarely reported in Africa, but in 2018–2019 they were reported in many African countries, affecting both horses and donkeys (OIE, 2020; Shittu et al., 2020; Diallo et al., 2021). As a result, more than 66,000 horses and donkeys died in Burkina Faso, Chad, Cameroon, Gambia, Ghana, Mali, Niger, Nigeria, and Senegal. Outbreaks of EIV have also been reported in Asia, South America, and the Middle East (Sack et al., 2019; Oladunni et al., 2021; Motoshima et al., 2011).

The spread of EI among humans is supported by recent serologic studies in Australia, Mongolia, and the United States. Antibodies to H3N8 EIV were detected in 3–36% of human participants in these studies (Burnell et al., 2014; Khurelbaatar et al., 2014; Larson et al., 2015).

As far as the equine influenza epidemic in Ukraine is concerned, the disease is not officially registered today, but there is no data on the surveillance of horses or other animals.

Thus, the above-mentioned facts about the circulation of equine influenza viruses in the world, the concern about the possible emergence of a new pandemic zoonotic virus due to reassortment and crossing of the interspecies barrier, as well as the lack of up-to-date information about the epizootic situation regarding equine influenza in Ukraine, prompted us to start research on the circulation of influenza A viruses among mammals, including horses, in Ukraine.

The first stage of our research aimed to conduct serological monitoring of wild and domestic ungulates from different farms and different regions of Ukraine for the presence of antibodies to influenza A viruses.

Materials and methods. The study was conducted in the Department of Poultry Diseases and Molecular Diagnostics of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine).

Sampling and preparation of blood sera from animals were performed according to classical methods. Blood serum samples from 372 domestic horses and 32 wild ungulates were tested. The study used samples from animals collected in 2023 and 2024, as well as archival blood serum samples from 2021. The list of samples used in the study is shown in Table 1.

Table 1 — List of selected blood serum samples from ungulates

Year of sampling	Animal species	Region	Number of surveyed farms	Total number of samples collected
2021 (archive samples)	Horse (*Equus ferus caballus*)	Poltava	1	23
	Sika deer (*Cervus nippon*)	Kherson	1	7
	Przewalski's horse (*Equus ferus przewalskii*)		1	3
	Saiga (*Saiga tatarica*)		1	2
2023	Horse	Kharkiv	6	56
		Vinnytsia	1	20
		Dnipropetrovsk	1	10
		Zaporizhzhia	1	20
		Sumy	1	20
		Lviv	2	30
		Volyn	1	20
		Ternopil	1	20
		Ivano-Frankivsk	1	13
2024	Horse	Cherkasy	1	10
		Chernihiv	1	10
		Chernivtsi	1	10
		Zhytomyr	1	10
		Odesa	1	10
		Dnipropetrovsk	1	10
		Rivne	1	10
		Zakarpattia	1	10

Table 1 — continuation

Year of sampling	Animal species	Region	Number of surveyed farms	Total number of samples collected
2024	Horse	Khmelnytskyi	1	10
		Kharkiv	1	10
		Kherson	1	10
		Kirovohrad	1	10
		Kyiv	1	10
		Poltava	1	10
	Mouflon (*Ovis gmelini*)	Lviv	1	20

Blood sera from ungulates were tested for the presence of antibodies to Influenza A virus by ELISA using the following test systems: IDEXX Influenza A Ab Test ELISA (USA), INGEZIM Influenza A, manufactured by Ingenasa (Spain), and IDVet ID Screen Influenza A Antibody Competition Multi-species-FLUACA (France). The ELISA was performed, the reaction was recorded, and the results were interpreted according to the instructions of the test system manufacturers. All studies were performed following good laboratory practice and in compliance with all biosafety and biosecurity requirements.

Results. The results of serologic studies in domestic horses are shown in Tables 2 and 3.

Table 2 — Results of the ELISA test for the presence of antibodies to influenza A in horses from farms in different regions of Ukraine in 2021 and 2023

No. of the farm	Number of samples			Seropre-valence, %
	total	positive	negative	
Archive (2021), Poltava Region				
1	23	14	9	60.9
2023, Kharkiv Region				
2	10	6	4	60.0
3	10	4	6	40.0
4	10	0	10	0.0
5	10	0	10	0.0
6	10	7	3	70.0
7	6	4	2	66.6
2023, Vinnytsia Region				
8	20	2	18	10.0
2023, Dnipripetrovsk Region				
9	10	10	0	100.0
2023, Zaporizhzhia Region				
10	20	0	20	0.0
2023, Sumy Region				
11	20	0	20	0.0
2023, Lviv Region				
12	20	0	20	0.0
13	10	10	0	100.0
2023, Volyn Region				
14	20	4	16	20.0
2023, Ternopil Region				
15	20	4	16	20.0
2023, Ivano-Frankivsk Region				
16	13	2	11	15.4
Total	232	67	165	2.9

Table 3 — Results of the ELISA test for the presence of antibodies to influenza A in horses from farms in different regions of Ukraine in 2024

No. of the farm	Number of samples			Seropre-valence, %
	total	positive	negative	
Cherkasy Region				
1	10	1	9	10.0
Chernihiv Region				
2	10	10	0	100.0
Chernivtsi Region				
3	10	0	10	0.0
Zhytomyr Region				
4	10	0	10	0.0
Odesa Region				
5	10	2	8	20.0
Dnipropetrovsk Region				
6	10	2	8	20.0
Rivne Region				
7	10	1	9	10.0
Zakarpattia Region				
8	10	5	5	50.0
Khmelnytskyi Region				
9	10	2	8	20.0
Kharkiv Region				
10	10	8	2	80.0
Kherson Region				
11	10	1	9	10.0
Kirovohrad Region				
12	10	0	10	0.0
Kyiv Region				
13	10	3	7	30.0
Poltava Region				
14	10	4	6	40.0
Total	140	39	101	27.9

Studies on the presence of antibodies to the influenza A virus in horses were conducted in 16 farms in different regions of Ukraine, all samples were taken from animals older than one year of working productivity. Table 2 illustrates that the percentage of positive samples in horses from different farms ranged from 10–20% (4 farms) to 40–100% (7 farms) in 2021 and 2023. Antibodies were not detected in horses from 5 of the 16 farms studied.

In 2024 antibodies to influenza A were not detected only in animals from three farms in Zhytomyr, Kirovohrad and Chernivtsi regions, in all other farms the percentage of positive samples ranged from 10 to 100%.

Blood sera from wild ungulates of the following species were also tested: sika deer, Przewalski's horse, saiga, and mouflon. All animals were over one year old. The results are shown in Table 4.

Table 4 — Results of the ELISA test for the presence of antibodies to influenza A in wild ungulates

Animal species	Number of samples			Seropre-valence, %
	total	positive	negative	
2021, Kherson Region				
Sika deer	7	0	7	0.0
Przewalski's horse	3	2	1	66.6
Saiga	2	0	2	0.0
2024, Lviv Region				
Mouflon	20	0	20	0.0

As illustrated in Table 4, the analysis revealed the presence of antibodies to the influenza A virus in two out of three samples from Przewalski's horses (Kherson Region).

Discussion. Previously, the issue of equine influenza was regarded as an economic concern. For many years, two subtypes of the influenza A virus (H7N7 and H3N8) have been closely associated with respiratory diseases in horses, resulting in significant economic losses due to the treatment and prevention of the disease (Singh, 1994; Waddell, Teigland and Sigel, 1963). Given the recent instances of interspecies transmission, monitoring the circulation of influenza viruses among mammalian hosts, including horses, has become a priority for the early detection of new reassortant viruses.

Monitoring studies, particularly serological studies, are an effective tool for controlling the epizootic status of equine influenza in a specific animal population. The results of serological studies can provide up-to-date information on the potential circulation of the virus in a herd of animals or establish the strength of group immunity following preventive vaccinations. In light of the ongoing uncertainty regarding the equine influenza situation in Ukraine, our initial research phase entailed conducting serological monitoring of domestic, wild horses, and other ungulates to ascertain the presence of specific antibodies to influenza A viruses.

Serological surveillance in Ukraine confirmed the circulation of influenza A viruses among unvaccinated domestic horses. According to the ELISA results, the percentage of positive horses ranged from 10% to 100% in different farms. Such a high seroprevalence is not surprising, although it may vary in different countries and regions. Numerous studies have been conducted worldwide on the seroprevalence of influenza in horses and donkeys, and this figure is very variable (Baydar et al., 2023). For example, the seropositivity of horses to influenza is 38% in Mexico (Blitvich et al., 2010), 11% in Pakistan (Sajid et al., 2013), and 44.7% in Brazil (Daly et al., 2021).

On the other hand, the seroprevalence in five different regions of Turkey in a study of more than 600 horses was 31% (Ataseven and Daly, 2007), and according to the results of other researchers from the same country, it was 3.03%.

Only New Zealand and Iceland, with their large horse populations, have remained EIV-free. Some countries, including Australia and South Africa, have eradicated EIV after past outbreaks. However, EIV is generally considered an enzootic in Europe, the Americas, and Asia (Lim et al., 2023). With its almost worldwide distribution, this highly contagious infection can lead to infection through direct and indirect contact and sometimes have a subclinical course.

Conclusions. Our data in Ukraine indicate that influenza A viruses are currently circulating among unvaccinated domestic horses. This circulation has been observed not only in recent years (2023–2024), but also earlier, as evidenced by the detection of 60.9% of positive samples in samples collected in 2021. Furthermore, two out of three tested samples from wild horses in Kherson Region were positive, indicating the potential circulation of the influenza A virus among wild animals. Further investigation is required to confirm this. The data obtained correlate with the worsening of the epizootic situation regarding influenza in Europe among animals. The detection of specific antibodies to the influenza virus in wild ungulates is a notable finding that requires further investigation. Additionally, the subsequent phase of research is serotyping, which involves determining the presence of antibodies to specific virus subtypes by hemagglutinin.

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MICROBIAL LOAD OF FACILITIES FOR KEEPING PIGS OF DIFFERENT PRODUCTION GROUPS

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Summary. The study analyzed the microbial load of objects in the facilities where pigs of different production groups were kept at the final stage of production cycles, immediately before disinfection measures. The study found that the number of mesophilic aerobic and facultative anaerobic microorganisms (MAFAnM) in the swabs from the surfaces of the studied objects varied from 5.00 to 6.88 log CFU/cm³. The lowest quantity of bacteria was found on drinkers and feeders, while the highest quantity was on the facilities' floor. The average level of microbial load in the facilities for keeping sows, farrowing, and growing piglets ranged from 5.91 to 6.07 log CFU/cm³. The highest values were observed for the study of swabs taken in the piglet-rearing facility. The proportion of field isolates of the rod, cocci, and spiral shapes of microorganisms in the rearing facility was 62.1%, 28.8%, and 9.1%, respectively, in the farrowing facility — 63.9%, 29.2%, and 6.9%, and in the sow housing facility — 66.2%, 26%, and 7.8%. *Escherichia coli* was dominant in the rearing facility — 13.9% of isolates, *Proteus mirabilis*, *Bacillus subtilis*, and *Campylobacter jejuni* — 9.7% each, and *Citrobacter freundii*, *Enterococcus faecalis*, and *Enterococcus faecium* — 8.3% each. In farrowing facilities, the proportion of *E. coli* isolates was 16.6%. 7.5% fewer isolates belonged to *B. subtilis*, *Streptococcus salivarius*, and *C. jejuni*, and 9% fewer isolates belonged to *Klebsiella pneumoniae*, *P. mirabilis*, *E. faecalis*, and *E. faecium*. In the sow housing facility, the proportion of *E. coli* isolates was 12.9%, the number of *P. mirabilis* isolates was 1.2% less, and *C. freundii* was 3.8% less

Keywords: MAFAnM, contamination, disinfection

Introduction. Pig farming is one of the most important livestock industries and plays an important role in meat production. For the efficient production of pork, it is important to establish and maintain proper sanitary and hygienic conditions in pig housing facilities. Among these conditions, the microbial load of livestock facilities deserves special attention as it has a major impact on animal health, productivity, and product quality (Haidukevych and Semenova, 2023; Kot et al., 2019; Myronchuk and Peleno, 2023).

It is known that the microbiocenosis of surfaces with which animals come into contact influences the development of infectious diseases, the state of the animals' immune system, and their general physiological condition (Rudenko et al., 2021).

Exceeding permissible standards for the number of microorganisms in the air is often an etiological factor in developing respiratory diseases, which are one of the most common problems in pig farms (Bolibrukh and Rublenko, 2023; Luyckx et al., 2016).

Changes in the microbial load of facilities can affect the metabolism of pigs and the occurrence of infectious diseases, resulting in decreased weight gain, increased production costs, and deterioration of meat quality (Trinh et al., 2018).

According to Wen et al. (2021), the species and quantitative composition of the indoor microflora can vary significantly at different stages of the production cycle. The factors that cause these changes can be the number of animals kept in a given facility, their density, temperature, humidity, disinfection quality, ventilation, etc. (Buoio et al., 2023; Wen et al., 2021).

In modern pig production, it is important to identify and eliminate potential risks and implement effective measures to minimize the microbial load of the facilities. These tasks are usually accomplished through regular monitoring of the microbial load of the air in the facilities where the animals are kept and of the surfaces with which they come into contact, as well as through high-quality disinfection, the introduction of modern ventilation systems on the farms, temperature, and humidity control, etc. (Luiken et al., 2020).

Since the creation of optimal conditions for the keeping of pigs, taking into account the microbial load of the facilities for their keeping reduces the risks of occurrence and development of diseases, improves the general physiological condition of animals, increases their productivity and economic performance of enterprises, the planned research is relevant.

The study **aimed** to investigate the total microbial load and the species composition of the microflora of farrowing, piglets rearing, and sow housing facilities at the end of each production cycle.

Materials and methods. The experiments were conducted at the LLC 'Eco Meat', which was established in October 2013 with the support of Polish partners. It is located in the village of Batiatychi, Lviv District, Lviv Region. The farm has a total capacity of 3,200 sows. The animals are kept in two farrowing facilities, six piglet-rearing facilities, two rooms for growing animals, and

two rooms for keeping single and farrowing sows. The piglets are kept in the farrowing room until they are 28 days old, and in the rearing room from 28 to 63 days old, after which they are sold to other farms. At the end of each stage, the farm is disinfected by spraying with 'Vulkan Max' (Huvepharma, France).

The material for the study were swabs taken at the end of the production cycle, immediately before disinfection, from the floor, feeders, drinkers, walls, and cage partitions in the piglet rearing, sow housing, and farrowing rooms, five samples from each facility. Sampling to determine the type and total contamination of livestock facilities with mesophilic aerobic and optionally anaerobic microorganisms (MAFAnM) was performed according to the 'Recommendations for the Sanitary and Microbiological Examination of Swabs from the Surfaces of Test Objects and Objects of Veterinary Surveillance and Control' (Yakubchak et al., 2005). The total contamination was determined by the amount of MAFAnM in the swabs and expressed as log CFU/cm^3.

Special and selective media were used to cultivate field isolates. Microorganisms of the Enterobacteriaceae family were cultured on Endo agar (HiMedia, Germany), *Pseudomonas aeruginosa* on Cetrimide Agar (Merck, Germany). For *Staphylococcus aureus*, salt agar for the isolation of staphylococci (Farmaktiv, Ukraine), *Streptococcus salivarius* — blood agar (Merck, Germany), and for *Enterococcus faecalis* and *Enterococcus faecium* — Enterococcus agar (Farmaktiv, Ukraine) were used. Campylobacter selective agar (HiMedia, Germany) was used for *Campylobacter jejuni*. The spore-forming microorganisms *Bacillus subtilis* and *Bacillus megaterium* were cultured on nutrient agar with subsequent identification by the ability to hydrolyze pectin. *Clostridium perfringens* was cultured under anaerobic conditions using Kitt-Tarozzi medium (Conda, Spain) (Scully and Orlygsson, 2023).

Field isolates were identified based on the study results of morphological, tinctorial, cultural, and biochemical properties under the following regulatory documents: ISO 10272-1:2017 Microbiology of the Food Chain — Horizontal Method for Detection and Enumeration of *Campylobacter* spp. — Part 1: Detection Method (ISO, 2017a), ISO 21528-1:2017 Microbiology of the Food Chain — Horizontal Method for the Detection and Enumeration of Enterobacteriaceae — Part 1: Detection of Enterobacteriaceae (ISO, 2017b), ISO 15213-2:2023 Microbiology of the Food Chain — Horizontal Method for the Detection and Enumeration of *Clostridium* spp. — Part 2: Enumeration of *Clostridium perfringens* by Colony-count Technique (ISO, 2023), ISO 7932:2004 Microbiology of Food and Animal Feeding Stuffs — Horizontal Method for the Enumeration of Presumptive *Bacillus cereus* — Colony-count Technique at 30 Degrees C (ISO, 2004), ISO 13720:2010 Meat and Meat Products — Enumeration of Presumptive *Pseudomonas* spp. (ISO, 2010), ISO 16266:2006 Water Quality — Detection and Enumeration of *Pseudomonas aeruginosa* — Method by Membrane Filtration (ISO, 2006), and Bergey's Manual of Systematic Bacteriology (Garrity et al., 2005a, 2005b).

The obtained numerical values were statistically processed using the program Statistica ver. 10.0 (StatSoft, USA) with the determination of the arithmetic mean (M) and its error (m). The reliability of the results was assessed by the Student's test.

Results and discussion. Determination of the microbial load of objects before disinfection allows us to estimate the level of contamination and to choose the most effective disinfection measures. From the results presented in Fig. 1, it can be seen that after the technological process was completed, the amount of MAFAnM in the farrowing, piglet rearing, and sows' housing facilities ranged from 5.00 to 6.88 log CFU/cm^3 of the swab.

The lowest number of bacteria was on the surface of drinkers and feeders and ranged from 5.00 to 5.20 and 5.28 to 5.65 log CFU/cm^3 of the swab, respectively. On the walls of the facilities, the number of mesophilic aerobic and facultative anaerobic microorganisms ranged from 6.10 to 6.24 log CFU/cm^3 of swab, and on plastic partitions — from 6.23 to 6.41 log CFU/cm^3 of swab. The highest level of bacteria was recorded in the swabs taken from the floor — from 6.78 to 6.88 log CFU/cm^3 of the swab.

Comparing the microbial contamination of the studied objects, it was found that drinking bowls and feeders were the least loaded with microorganisms in the facility intended for farrowing sows, and the most — for rearing piglets. The number of MAFAnMs on their surfaces was 5.00 and 5.16 and 5.20 and 5.65 log CFU/cm^3, respectively. On the walls and plastic intercellular partitions, the lowest number of bacteria, 6.10 and 6.23 log CFU/cm^3 of the swab was in the sow housing facility, and the highest number, 6.24 and 6.41 log CFU/cm^3 of the swab was in the piglet rearing facility. The floor, drinking bowls, and feeders were the least contaminated with microflora in the piglet rearing facility, and the most — in the sow housing facility, and the number of MAFAnMs was 6.78 and 6.88 log CFU/cm^3 of the swab, respectively.

Compared to the least microbially loaded objects, which in all studied facilities were drinkers, on the surface of feeders, walls plastic partitions, and floors, the number of MAFAnMs in the farrowing facility was 5.6%, 23.4%, 26.4%, and 35.6% higher, respectively, in the piglet rearing facility by 8.6%, 20.1%, 23.3%, and 31.3%, and in the sow housing facility by 3.3%, 18.2%, 20.7%, and 33.3%.

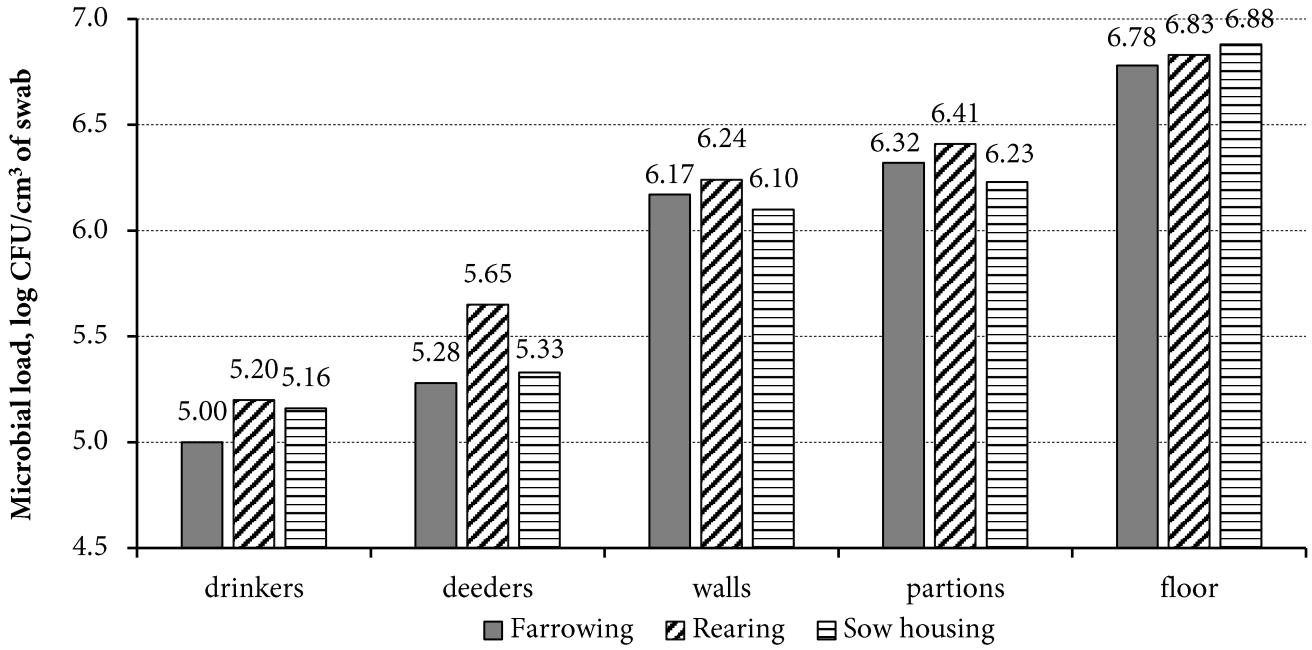

Figure 1. Microbial load of farrowing, piglet rearing, and sow housing facilities after the end of the production cycle.

From the data shown in Fig. 2, it can be seen that the lowest average level of microbial load was observed in the farrowing facility and amounted to 5.91 log CFU/cm³ of the swab, slightly higher in the sow housing facility (5.94 log CFU/cm³ of the swab), and the highest in the growing facility (6.07 log CFU/cm³). The data obtained are consistent with the results obtained by other researchers (Shkromada, 2014).

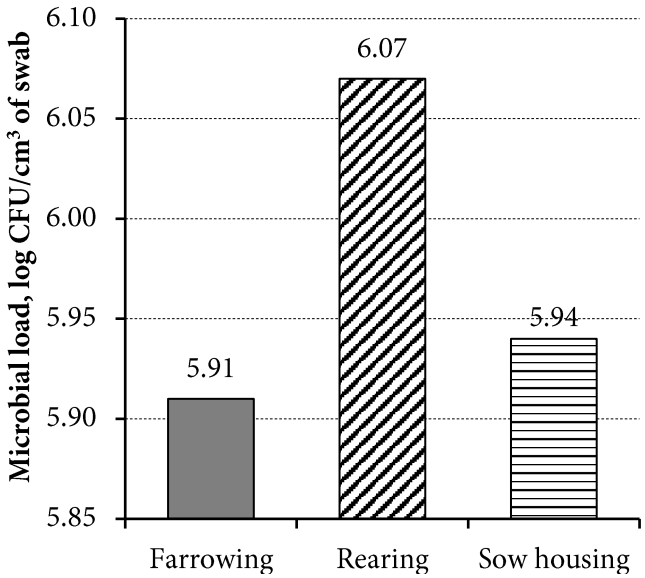

Figure 2. Average level of microbial load in farrowing, rearing, and sow housing facilities.

Analyzing the species composition of microorganisms isolated from the swabs taken from the facilities for keeping and farrowing sows and rearing piglets (Table 1), it was found that the microbiocenosis was formed by field isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Bacillus subtilis*, *Bacillus megaterium*, which have a rod-shaped form, *Staphylococcus aureus*, *Streptococcus salivarius*, *Enterococcus faecalis*, *Enterococcus faecium*, which belong to cocci, and *Campylobacter jejuni*, which belong to the spiral shape.

Table 1 — Characteristics of the species composition of microflora of facilities for keeping pigs of different production groups (n = 215)

Species of micro-organisms	Number of isolates					
	rearing		farrowing		sow housing	
	Abs.	%	Abs.	%	Abs.	%
E. coli	10	13.9	11	16.6	10	12.9
K. pneumoniae	5	6.9	5	7.6	4	5.2
C. freundii	6	8.3	4	6.1	7	9.1
P. mirabilis	7	9.7	5	7.6	9	11.7
P. aeruginosa	4	5.7	3	4.5	5	6.5
C. perfringens	5	6.9	3	4.5	5	6.5
B. subtilis	7	9.7	6	9.1	6	7.8
B. megaterium	2	2.8	4	6.1	5	6.5
S. aureus	5	6.9	3	4.5	6	7.8
S. salivarius	4	5.7	6	9.1	3	3.9
E. faecalis	6	8.3	5	7.6	6	7.8
E. faecium	6	8.3	5	7.6	5	6.5
C. jejuni	5	6.9	6	9.1	6	7.8

The lowest number of microbial isolates was identified in the farrowing facility. Of the 66 field isolates, 41 (or 62.1%) were rod-shaped, 19 (or 28.8%) were spherical, and 6 (or 9.1%) were spiral. A total of 72 field isolates were isolated in the piglet-rearing facility. Of these, 46 (63.9%) were rod-shaped (*E. coli*, *K. pneumoniae*, *C. freundii*, *P. mirabilis*, *P. aeruginosa*, *C. perfringens*, *B. subtilis*, *B. megaterium*), 21 (29.2%) were spherical (*S. aureus*, *S. salivarius*, *E. faecalis*, *E. faecium*), and 5 (6.9%) were spiral (*C. jejuni*).

The largest number of microorganisms (77) was isolated from the swabs taken from the sow housing facility. This number was 6.9% and 11.7% higher than the number of isolates from the piglet-rearing and farrowing facilities. At the same time, rod-shaped microorganisms (*E. coli*, *K. pneumoniae*, *C. freundii*, *P. mirabilis*, *P. aeruginosa*, *C. perfringens*, *B. subtilis*, *B. megaterium*) were represented by 51 field isolates, which amounted to 66.2%, spherical (*S. aureus*, *S. salivarius*, *E. faecalis*, *E. faecium*) — 20 field isolates (26.0%), and spiral (*C. jejuni*) — 6 field isolates (7.8%).

In all the studied facilities, the dominant number of field isolates was identified as *E. coli*. Their number in the farrowing facility was 16.6%, in the piglet rearing facility — 16.9% and in the sow housing facility — 12.9%. In the piglet rearing facility, the number of field isolates of *P. mirabilis*, *B. subtilis*, and *C. jejuni* was 4.2% less than *E. coli; C. freundii*, *E. faecalis*, and *E. faecium* — 5.6 %; *K. pneumoniae*, *C. perfringens*, and *S. aureus* — 7.0%; *P. aeruginosa* and *S. salivarius* — 8.2%; and *B. megaterium* — 11.1%. In the farrowing facility 9.1% of field isolates belonged to each of *B. subtilis*, *S. salivarius*, and *C. jejuni*; 7.6% to each of *K. pneumoniae*, *P. mirabilis*, *E. faecalis*, and *E. faecium;* 6.1% to each of *C. freundii* and *B. megaterium;* 4.5% to each of *P. aeruginosa*, *C. perfringens*, and *S. aureus.*

The difference compared to *E. coli* was 7.5%, 9.0%, 10.5%, and 12.1%, respectively. In the sow housing facility, as well as in the piglet-rearing facility, the largest number of field isolates, after *E. coli*, belonged to *P. mirabilis*, and the difference was only 1.2%. The third, by the number of field isolates, was *C. freundii*, the fourth — *B. subtilis*, *S. aureus*, *E. faecalis*, and *C. jejuni*, the fifth — *P. aeruginosa*, *C. perfringens*, *B. megaterium*, and *E. faecium*, the sixth — *K. pneumoniae* and the seventh — *S. salivarius*, which accounted for 9.1%, 7.8%, 6.5%, 5.2%, and 3.9% of field isolates, respectively, and the difference compared to *E. coli* was 3.8%, 5.1%, 6.4%, 7.7%, and 9.0%.

Thus, the study of the microbial load of objects in the facilities for keeping pigs of different production groups showed that the number of MAFAnM on the floor, partitions, walls, feeders, and drinkers ranged from 5.00 to 6.88 log CFU/cm^3 of the swab. The highest microbial load was in the piglet rearing room and amounted to 6.07 log CFU/cm^3 of the swab. Similar results were obtained by Scicchitano et al. (2024), who studied the spread of antimicrobial-resistant bacteria on pig farms and in the environment, and Luyckx et al. (2016) when studying the bacterial load in pig nurseries where the objects for research were synthetic mesh, concrete wall, synthetic wall, drinkers, and feeders.

The established values of the microbial load meet the sanitary and hygienic requirements for livestock premises (Nebylytsia et al., 2023), and the microbial load from 6.78 to 6.88 log CFU/cm^3 of the swab on the floor of the studied facilities indicates the need for increased attention to the sanitation of floors and surfaces in such facilities (MHU, 2023).

As a result of identification of 215 field isolates by morphological, tinctorial, cultural, and biochemical properties, 138 of them were rod-shaped (*E. coli*, *K. pneumoniae*, *C. freundii*, *P. mirabilis*, *P. aeruginosa*, *C. perfringens*, *B. subtilis*, *B. megaterium*), 60 spherical (*S. aureus*, *S. salivarius*, *E. faecalis*, *E. faecium*), and 17 spiral (*C. jejuni*). Similar microorganisms have been isolated from the pig pen premises (Shkromada, 2014; Shkromada and Hrek, 2022). These data emphasize the importance of regularly monitoring microbial forms for rapid response in case of detection of pathogenic microorganisms.

The division of field isolates into Gram-positive and Gram-negative microorganisms is justified because gram-positive microorganisms have a thick layer of peptidoglycan in the peptide wall, which provides them with additional protection against physical and chemical factors and, in addition, they can produce special cryptoproteins that help to withstand environmental stresses (Xue, 2020).

The microorganisms we isolated belonged to aerobes (86.05%), anaerobes (6.05%), and 7.90% to microaerophiles, which were represented by 17 field isolates of *C. jejuni*, which is consistent with the studies of Zhu et al. (2019).

Other researchers (Ferone et al., 2020; Fischer et al., 2016) have isolated microorganisms similar to those we identified in terms of cultural and biochemical properties in swabs taken from the facilities of a pig farm.

Thus, before the sanitation measures, the pig housing facilities were contaminated with *E. coli*, *K. pneumoniae*, *C. freundii*, *P. mirabilis*, *P. aeruginosa*, *C. perfringens*, *B. subtilis*, *B. megaterium*, *S. aureus*, *S. salivarius*, *E. faecalis*, *E. faecium*, *C. jejuni*, which poses a risk to animal health and economic efficiency of production, requiring regular microbial monitoring and controlled disinfection.

Conclusions. 1. After completion of the technological process, the number of MAFAnM on the floor, partitions, walls, feeders, and drinkers in the farrowing facility was in the range of 5.00 to 6.78 log CFU/cm^3 of the swab, in the piglet rearing facility — from 5.20 to 6.83 log CFU/cm^3

of the swab and in the sow housing facility — from 5.16 to 6.88 log CFU/cm^3 of the swab.

2. The highest average microbial load was found in the piglet rearing facility (6.07 log CFU/cm^3 of the swab), while it was 2.14% and 2.64% lower in the sow housing and farrowing facilities, respectively.

3. The lowest number of bacteria was on the surface of drinkers and feeders (5.00–5.65 log CFU/cm^3 of the swab), the average number was on the walls of the facilities and plastic partitions (6.10–6.41 log CFU/cm^3 of the swab), and the highest number was in the swabs taken from the floor (6.78–6.88 log CFU/cm^3 of the swab).

4. The microbiocenosis of the studied facilities was formed by rod-shaped forms (*E. coli*, *K. pneumoniae*, *C. freundii*, *P. mirabilis*, *P. aeruginosa*, *C. perfringens*, *B. subtilis*, *B. megaterium*), spherical forms (*S. aureus*, *S. salivarius*, *E. faecalis*, *E. faecium*) and spiral forms (*C. jejuni*), the proportion of which in the facility for rearing young animals was 62.1%, 28.8%, and 9.1%, in the farrowing facility — 63.9%, 29.2%, and 6.9%, and in the sow housing facility — 66.2%, 26.0%, and 7.8%, respectively. The dominant species in the piglet rearing facility were *E. coli* — 13.9% of field isolates, *P. mirabilis*, *B. subtilis* and *C. jejuni* — 9.7%, and *C. freundii*, *E. faecalis*, and *E. faecium* — 8.3%. In farrowing facilities, the number of isolated *E. coli* was 16.6%, which is 7.5% fewer than the number of isolates belonging to *B. subtilis*, *S. salivarius*, and *C. jejuni*, and 9.0% fewer than *K. pneumoniae*, *P. mirabilis*, *E. faecalis* and *E. faecium*. In the sow housing facility, the number of *E. coli* isolates was 12.9%, the number of *P. mirabilis* isolates was 1.2% lower, and the number of *C. freundii* isolates was 3.8% lower.

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Part 1. Veterinary medicine

Part 2. Biosafety